

Therapeutic vaccination immunomodulation: forming the basis of all cancer immunotherapy

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Abstract: Recent immunotherapy advances have convincingly demonstrated complete tumour removal with long-term survival. These impressive clinical responses have rekindled enthusiasm towards immunotherapy and tumour antigen vaccination providing ‘cures’ for melanoma and other cancers. However, many patients still do not benefit; sometimes harmed by severe autoimmune toxicity. Checkpoint inhibitors (anti-CTLA4; anti-PD-1) and interleukin-2 (IL-2) are ‘pure immune drivers’ of pre-existing immune responses and can induce either desirable effector-stimulatory or undesirable inhibitory-regulatory responses. Why some patients respond well, while others do not, is presently unknown, but might be related to the cellular populations being ‘driven’ at the time of dosing, dictating the resulting immune response. Vaccination is in-vivo immunotherapy requiring an active host response. Vaccination for cancer treatment has been skeptically viewed, arising partially from difficulty demonstrating clear, consistent clinical responses. However, this article puts forward accumulating evidence that ‘vaccination’ immunomodulation constitutes the fundamental, central, intrinsic property associated with antigen exposure not only from exogenous antigen (allogeneic or autologous) administration, but also from endogenous release of tumour antigen (autologous) from in-vivo tumour-cell damage and lysis. Many ‘standard’ cancer therapies (chemotherapy, radiotherapy etc.) create waves of tumour-cell damage, lysis and antigen release, thus constituting ‘in-vivo vaccination’ events. In essence, whenever tumour cells are killed, antigen release can provide in-vivo repeated vaccination events. Effective anti-tumour immune responses require antigen release/supply; immune recognition, and immune responsiveness. With better appreciation of endogenous vaccination and immunomodulation, more refined approaches can be engineered with prospect of higher success rates from cancer therapy, including complete responses and better survival rates.

Keywords: cancer, immunotherapy, *in vivo* vaccination, melanoma, survival, vaccine

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Introduction

The consideration of vaccines being applied therapeutically to treat cancer dates back over 100 years or more and constitutes the first form of immunotherapy.^{1–26} Coley’s toxins could produce complete responses, and induced fever from a nonspecific innate immune response which most likely augmented an existing adaptive immune response to the tumour. The mechanism remains unclear but may have provided ‘danger signals’ through costimulatory pathways to remove regulatory adaptive T-cell

suppression. Immune stimulation for cancer therapy has a long and chequered history pre-dating knowledge of an ‘immune system’ and possibly alchemy as a primitive medical science, since many early herbal remedies often had assumed ‘tonic’ elements of inflammatory stimulation or suppression by which they modulated diseases, which is only now being more scientifically realized. This ‘folklore’ history has not helped the modern understanding or acceptance of the immune system’s involvement in cancer therapy. Indeed, as recently as

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less than a decade ago, many medical oncologists and cancer therapists (now unbelievably) did not seriously consider that the immune system was involved 'at all' in cancer or cancer treatment, and that vaccines or vaccination had no role. Historically, vaccination was ascribed a 'passive', protective role against diseases, usually infective in origin. However, vaccination is almost always a truly 'active' process where the immune system is specifically responding to antigenic signals that stimulate B-cell (plasma) or T-cell responsiveness, and often both. As a consequence, either antibody is produced, or T cells are activated, for example, by production of cytokines or cytotoxicity. The nonspecific or innate immune system is not exempt from this process either, occupying a pivotal role in immune stimulation during vaccination events.

When the term 'immunotherapy' is considered, it often has the connotation of a more active exogenous form of treatment where the immune system is overtly stimulated and an effect is observable, compared with 'vaccination'. However, the two are almost indecipherable in real, practical terms, because the immune system is activated in both, and both influence a wide range of diseases or disorders, not exclusively of infective origin.

Only now is vaccination technology and consideration of 'vaccines' for clinical treatment of cancer being seriously reconsidered in light of the recent immunotherapy successes and understanding. Indeed, vaccines may be essential for improving checkpoint-immunotherapy clinical response rates. This article revives understanding about vaccines and vaccination for cancer therapy in this new light, placing it within our contemporary understanding of recent immunotherapies and how they appear to be working for the benefit of the cancer patient.

Melanoma, for which most information exists, has been used as a model for immunotherapies, including therapeutic vaccines, and gradually, many principles of melanoma therapy are being demonstrated for other cancer types. In this review, the recent advances in melanoma, where immunotherapies including cancer vaccine therapies, have been explored to demonstrate technical approaches that have advanced cancer immunotherapy more generally. This presents the case for

in vivo vaccination as the basis for most, if not all, cancer (immuno) therapies. Recent knowledge suggests that many of these approaches can be extrapolated and applied to other cancers, including lung, bladder, renal, Merkel cell, colon, haematological, head and neck squamous cell carcinoma (SCC), gastric and breast cancers, revealing common underlying immunotherapy mechanisms between different cancer types, and therefore suggesting that *in vivo* vaccination is a generalized phenomenon which needs unlocking for great benefit.

Cancer vaccine therapy

Cancer vaccines of various types have been devised and utilized for over 100 years with variable success, but notably with numerous reports of complete clinical responses (CR) with regression of all measurable disease in patients with advanced cancers of different types through immunomodulation.¹⁻³⁸ Such CRs are highly significant events because they often underpin long-term durable survival periods of decades or more, effectively amounting to clinical cancer 'cure'.²³⁻²⁶ The problem has been the generally low and unpredictable rate of CR induction, or even partial response (PR) or stable disease (SD) outcomes. However, the very fact that these types of clinical useful responses occur is of great importance because they demonstrate convincing evidence that cancer vaccines offer an effective therapeutic approach which requires more intensive investigation and development.^{23,25} The lack of consistency of results across many cancer vaccine platforms has unfortunately biased the view of many influential commentators against the 'vaccine' approach, perhaps understandably. Moreover, largely because of the lack of understanding of the mechanisms of action of cancer vaccines, and the paucity of information on the immune-system functions in general over the years, the pharmaceutical world has tended to favour simpler commercial-scale molecular synthetic options for production and marketing with the security of more financial return, such as cytotoxic chemotherapies and more recently, monoclonal antibodies. However, it should be recognized that monoclonal antibodies were recently considered relatively commercially unfavourable, too, until these mechanisms of action and production were better understood and when enough clinical trials demonstrated efficacy.

Many of the vaccine studies have been performed in the adjuvant setting following surgical removal, for example, higher-risk resected stage II and III patients rendered no evidence of disease.^{33–38} That assumption undoubtedly arose from the tenet that ‘vaccination’ would be more effective for treating residual low-volume microscopic metastases by ‘preventing’ tumour recurrence, in a similar manner to the way vaccines have been utilized in preventing infection. Interestingly, the highest clinical complete response rates from vaccination have been obtained in surgically unresectable stage IV melanoma patients, indicating that the strength of the active antigenic signal might be of central importance.^{23,25}

Clinical efficacy has been another issue with immunotherapies, including therapeutic vaccines. The ‘clinical effectiveness’ of a treatment is where a clinical response occurs to prolong survival. That is, induction of a CR, PR or SD associated with that treatment, especially where it is the only therapy administered, or where prolonged survival results. The strength of the association of a drug with a useful clinical response makes further use of that agent more compelling for an observer. However, for the specific patients concerned who are successfully treated with resulting CRs, the treatment has been 100% effective. This aspect has made evaluation and comparison of many agents problematic, as most therapies are only ‘clinically effective’ in a small or moderate cohort of patients (even many newer therapies), and selection of responders at the outset is often difficult. For vaccine immunotherapy (and indeed, all immunotherapy until recently), the relative clinical efficacy of inducing CR, PR, SD or prolonged survival has been low, and this has tempered acceptance of immunotherapy in general terms.

Another issue increasingly being recognized is the fact that immune responses are variable in their speed of onset, intensity and duration. Some clinical responses to vaccine therapy for advanced malignancy can be rapid, occurring within a few weeks, with immediate noticeable regression of cancer deposits, although this speed of onset is not commonplace for most vaccine/immunotherapy regimens, it has been observed. Usually, vaccine responses are slower and can take many months to develop. This has been a real issue arising chiefly from our contemporary

understanding of chemotherapy and radiotherapy clinical responses which are usually considered to be more immediate, gauged in terms of 2–3 months to observe an effect. With vaccines, and with multiple other forms of immunotherapy, it is being increasingly appreciated that responses take longer, sometimes up to 6–12 months, where partial responses and stable disease can sometimes gradually convert to a complete clinical response. Pseudo progression can also occur where the tumour enlarges due to inflammatory swelling. Therefore, the classical World Health Organization and RECIST criteria perhaps need to be considered and applied differently, or modified, which has led to the newer immunological response criteria,^{39–41} which is gradually gaining acceptance, including by the US Food and Drug Administration (FDA). Future refinements of the currently available methods of measuring successful responses to immunotherapy might include better volumetric assessments, such as utilization of three-dimensional computed tomography scanning, positron emission tomographic volume scanning, heat mapping or magnetic resonance spectroscopic analyses. Part of the remaining issue with all of these methods has been establishing techniques that are time effective in more accurately assessing changes in multiple metastases, because most current evaluation tools for tumour size assessment only provide approximations for the response to therapies but are rapid, practically. It should be noted, however, that CRs are essentially detectable by most methods, although detection of small tumour volume (<4 mm diameter) remains an issue by any method except pathological sampling.

Tumour antigens and mutational load or burden

Tumours are genetically and antigenically heterogeneous.^{42,43} A wide range of internal and externally expressed antigens are present within tumour cells, including peptides and glycolipids. Most of the focus has been on peptide antigens and major histocompatibility complex (MHC) antigen presentation; however, glycolipids and CD1 presentation of these has been relatively ignored.⁴⁴ Moreover, refined selected peptide antigens appear less capable of inducing effective immune responses against tumour than mixed multiple antigens which can provide a stronger

signal to the immune system.^{22,25,32} There is evidence that intense selection pressure is applied from the tumour microenvironment in early clinical untreated non-small-cell lung cancers (NSCLCs) by the immune system which produces multiple routes to immune evasion, leading to different levels of mutational burden, clinical behaviour and disease outcomes.⁴⁵ Indeed, neo-antigen depletion appears to occur in many tumours that have become ‘unresponsive’ to therapies of different types, including inhibitory-checkpoint blockade. This is likely a mechanism for evasion of antitumour immune responses and resistance to therapies. Moreover, the immune microenvironment is highly variable between tumours, patients, and between metastases, and even within the same tumour deposit within patients with almost 30% of tumours showing diverse levels of immune infiltration. This implies that different metastases within the same individual and even parts of the same tumour mass will demonstrate differential susceptibilities to many therapies, including checkpoint blockade, due to their relative expression of tumour antigens, and thus proportionate T-cell stimulatory capacity, leading either to immune evasion (low mutation/antigen expression) or an effective response (higher mutation/antigen expression).⁴⁵

Higher tumour mutational burden across a number of tumour types showed an association with improved survival. Patients treated with immune-checkpoint inhibitors showed an improved survival among those with a higher mutational burden, although the degree of mutational load required to confer the improved survival varied with tumour type.⁴⁶ This infers that the greater the mutational load, the greater the antigenic signal and therefore the stronger the *in vivo* (vaccine) immune response to the tumour. When high-mutational-burden tumour cells are killed, multiple tumour-associated antigens are released and further (potentially stronger) T-cell-mediated (*in vivo* vaccine) immune responses can proceed.

In patients with NSCLC treated with first-line nivolumab plus ipilimumab, higher tumour mutational burden was associated with significantly longer progression-free survival than for chemotherapy-treated patients, regardless of the degree of programmed cell-death ligand 1 (PD-L1) expression,⁴⁷ suggesting tumour mutational burden as a potential independent biomarker for

response to checkpoint agents. Transfer of *ex vivo*-cultured autologous tumour-infiltrating lymphocyte (TIL) has been associated with higher CR rates of around 20%, and Prickett and coworkers have also demonstrated that these autologous TILs recognize a range of distinct mutated gene products so that the TILs represent an enriched population of T cells capable of recognizing autologous tumour antigens but are inhibited, so that responsiveness is reduced; however, in the right circumstances (autologous transfer), can elicit a powerful response inducing a CR.⁴⁸

Balance between T-stimulatory and T-regulatory responses

Most tumours reaching clinical diagnosis appear to exist because of relative immunological tolerance, although the degree and character of the inflammatory infiltration has been observed to vary considerably between different tumours and even among different metastases in the same patient. We do know from experience with the interleukin 2 (IL-2) and checkpoint therapies that induction of ‘overdrive’ of the immune response (as evidenced by autoimmunity which is also induced and generally correlates with an effective antitumour clinical response, e.g. vitiligo) can overcome the tolerant or regulatory state to tip the balance of immune responsiveness towards producing successful clinical responses to a pre-existing *in vivo* immune response. Higher regulatory T-cell (T-reg) levels and suppressor macrophage levels have been associated with more advanced cancers and poorer overall clinical outcomes.⁴⁹ However, recent evidence suggests that the Treg population is nonhomogeneous with different functions that may influence antitumour immune responses and clinical outcomes.

Only some 60% of advanced melanomas respond to checkpoint-blockade agents, suggesting that even with efficient immune-driver therapies, the nonresponsiveness cannot be overcome to induce effective clinical responses by those methods. This immunological ‘nonresponsive state’ also encumbers vaccine therapies to cancer, but exactly whether the patient group unresponsive to checkpoint therapies is identical to those not responsive to vaccine therapies is not yet known. Therapies designed to reduce T-reg effects in the tumour microenvironment are currently being explored; however, in about 20% of patients, the

balance towards effective antitumour therapy has successfully been achieved by vaccines alone or checkpoint agents alone, to achieve long-term survival.

Higher levels of macrophage density within many types of solid tumours (tumour-associated macrophages; TAMs) with an immunosuppressive phenotype have been correlated with poorer outcome. The mechanisms reported are enhanced angiogenesis, immunosuppression, and inflammation, to facilitate cancer growth and a relatively immunologically tolerant state. However, it is not clear-cut, as macrophages can act in either a stimulatory or inhibitory capacity on other immune cells, and thereby on the overall immune response to influence outcome. TAMs are differentiated from maturation of myeloid-derived suppressor cells (MDSCs), which in turn are derived from circulating monocytes. TAMs then undergo further differentiation into functional subtypes. For example, granulocyte-macrophage colony-stimulating factor (GM-CSF) or cytosine-phosphate-guanine (CpG) can induce toll-like receptor (TLR) expression by TAMs to reduce cancer growth and metastasis; while IL-10 can upregulate PD-L1 on TAM and monocytes causing programmed cell-death 1 (PD-1)-induced immunosuppression.⁵⁰

MDSCs are immature myeloid cells capable of stimulation of Tregs and strong inhibition of T and natural killer (NK) cells to reduce antitumour responses, and appear to have a role in immunosuppression in many cancers. MDSC levels in patients have been used as a biomarker for resistance to immune-checkpoint-inhibition therapies.⁵¹

Therapies aimed at reducing TAMs and MDSCs in the tumour microenvironment are currently being explored to enhance checkpoint and other therapies.

***In vivo* and *in situ* vaccination**

If the hypothesis is correct that tumour-cell death or injury leads to tumour antigen release, and further, that antigen release and immune stimulation is necessary for an effective antitumour immune response, then it follows that *many* forms of local (*in situ*) injury or damage to, or lysis of, the cancer cell '*in situ*' or '*in vivo*' (in the patient) can result in multivalent antigen stimulation of the immune

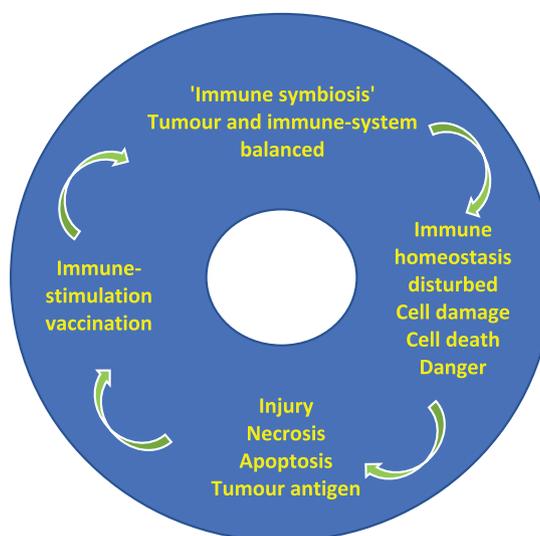


Figure 1. Cancer immunomodulation immunotherapy vaccination cycle.

system. However, 'immune symbiosis' or 'immune homeostasis' is what probably prevents elimination of cancers *in vivo* unless enough immune perturbation occurs and sufficient immunomodulatory stimulation can then reject the cancer (Figure 1). Clearly, cytotoxic therapies such as chemotherapy and radiation are directly aimed at damaging tumour cells. Complete surgical resection of metastases is reported with over 20% of patients surviving 4 years or more, some with durable longer-term survival periods.^{42,52} It is currently unclear whether this 20% of survivors is similar to the 20% longer-term survival mentioned above for immunotherapy and vaccine therapies. Surgery itself is associated with local and systemic inflammatory responses and in some cases, tumour-cell lysis. Therefore, **many** forms of cytotoxicity or cellular injury through the spectrum of available and utilized cancer therapies, including traditional chemotherapy, radiation therapy and surgery, or any of the newer therapies, such as inhibitors, receptor blockers or immunotherapies, can exert this effect of antigen release and immune stimulation.²⁴ This means that many of the available cancer therapies are in fact capable of exerting a vaccine effect, intentionally or otherwise. The mechanisms of this vaccination effect through repeated waves of release/exposure of tumour antigen *in vivo* is not widely appreciated, although it has been observed that tumour antigens are released when cells are lysed or when irradiated, intact, whole tumour cells have been used as

vaccines. Further, T-cell antigens specific for the respective tumour have been extracted (eluted) from TIL and these antigens have been used either directly or synthesized from their sequences in peptide- or glycolipid-based vaccines, and combination with the recent immunotherapeutic monoclonal antibody agents is being explored in trials. The processes of antigen spreading, immunoeediting, tumour antigen identification and sequencing, the presence of mutations within the tumour and the extent of these (mutational burden), antigenic immune selection with neoantigen emergence, tumour-antigen heterogeneity and multicloneality, the role of pluripotential stem cells, host tumour microenvironment, tumour extracellular matrix and vascular properties, proteomic and metabolic properties and pathways in tumours, mitochondrial function, and tumour microenvironmental (and systemic) regulatory immunomodulation through lymphocytic, dendritic cellular and macrophage pathways are being actively explored. All of these facets are pertinent to the discussions and understanding of tumour immunology and therapeutic vaccine design, and although germane to the argument of *in vivo* vaccination, some are beyond the scope of this review and are discussed elsewhere.

***In vivo* natural tumour oncolysis**

During the growth of cancers, small, early, cancer-deposit expansion of cancer cells initially keeps pace with the vascularity, so that nutrition is adequately provided to the cancer cells in the growing mass of tumour tissue. However, it is apparent that many cancer cells are not entirely immortal and a proportion of the population of cells within the cancer die through the natural mechanisms of apoptosis and necrosis. This process becomes accentuated when the growth of cancer cells outstrips the vascular supply to the mass of tumour tissue, leading to death of cells that do not receive adequate nutrition. 'Central necrosis' of the tumour is a commonly observed phenomenon which is most associated, although not always, with larger tumour masses. It has been assumed that the reason necrosis occurs centrally rather than elsewhere is the progressive loss of vascularity in the deepest and most remote parts of the tumour mass away from the outer vascular shell, leading to effective devascularization of deeper parts. This assumption arises largely because the main vascular supply derives

from outside the tumour mass, often with the peritumoural blood supply being observably relatively rich at the tumour surface, with communicating vessels into and out of the tumour, typically becoming progressively less prolific deeper into the tumour mass. However, it is also well recognized, that for some tumours, the cut *in vivo* tumour surface can bleed freely, demonstrative of high internal vascularity, so there are other mechanisms that produce the necrosis, such as tumour necrosis factor alpha (TNF- α) and other cytokines. Importantly though, the natural cell death typically results in a combination of apoptosis and necrosis, and these processes serve to release tumour antigens and cause *in vivo* vaccination. Repeated cycles of therapy can produce waves of repeating *in vivo* vaccination.

Oncolytic viruses

Another wave of intense interest has developed relatively recently concerning viruses, and modifications of them, for treatment of cancer. Virus therapy for cancer, also termed virotherapy, falls into two basic groups: (a) viruses that infect cancer cells and induce expression of viral antigens on the surface of cancer cells, making them more visible to the immune system through improved antigen recognition, resulting in better stimulation of the immune response, thereby eliciting a more effective anticancer response, together with enhanced stimulation of the production of interferons and other cytokines by adjacent immunological and stromal cells (innate immunity activation); and (b) oncolytic viruses, which are termed 'oncolytic' because they are capable of lysing tumour cells (more) selectively over normal cells, again with direct *in situ* stimulation of the immune response and cytokine cascades. The term 'oncolytic' has been used less precisely than desirable for viral therapies that induce immunogenic apoptosis rather than true 'oncolysis' (bursting) of cancer cells *via* the necrotic death pathways. Indeed, there is becoming much imprecision in the terminology that is used. This 'looseness of terminology' has, and continues to, hamper the proper definitions in the immunological therapeutic virotherapy field. Immunogenic apoptotic-cell death probably should properly be termed 'oncoptosis' rather than oncolysis, because no bursting lysis occurs; rather, the cells involute and shrivel. The use of oncological virotherapy has a long history, much greater than many people may think,

dating back to the early 1930s, with the first larger-scale research trials being performed and reported over the 1960s and into the 1980s.^{33–38} Many types of animal and human viruses have been tested, including adenovirus, vaccinia, Newcastle disease virus, reovirus, measles, herpes simplex and zoster, parvo, and ECHO-7, principally as a consequence of their ability to infect and cause selective lysis of tumour cells over normal cells, and thus act as a vaccine. The action of oncolytic viruses can be harnessed either through systemic infection of the patient and *in situ* oncolysis *in vivo*, or through *ex vivo* means first lysing the cultured tumour cells *in vitro* external to the patient, and then administering the lysate in *in vivo* doses. The principal effect of oncolytic viruses is typically natural oncolysis of abnormal cell membranes, usually on malignant cells, but oncolytic actions on normal cells can occur as well, for example the vesicular eruptions of normal tissues seen with the cutaneous manifestations of measles or pox-virus infections, as in herpes simplex (coldsore or shingles) or small pox.

Most routes of administration have been used, intravenous, intramuscular, inhaled, oral, subcutaneous and intratumoural, but perhaps the most clinically effective route has been intradermal, probably due to the abundance of dendritic and other antigen-presenting cells in the skin with relatively rapid drainage *via* lymphatic channels to regional lymph nodes. The most recent addition to the entourage of oncolytic viral agent therapies is T-Vec (talimogene laherparepvec; Imlygic[®], Amgen, Thousand Oaks, California, USA) which is a genetically engineered herpes virus with two genes removed and a gene for GM-CSF being added.⁵³ Injection of T-Vec into tumour deposits has been associated with necrosis/apoptosis of the tumour cells with observable clinical responses in the injected tumour deposits, with reported bystander responses in adjacent lesions, and also some systemic responses indicative of a systemic immune response.^{44,54,55} T-vec has been combined recently with anti-PD-1 therapy with some improvement in the response rates, but the full results are currently not available yet,^{56–59} and combined with ipilimumab, showing an objective response rate (ORR) of 39% *versus* 18% with ipilimumab alone.⁵⁸ Interestingly, there was a doubling of the visceral response rate in the combined *versus* single-agent ipilimumab arm, a phenomenon (systemic;

abscopal) also discussed later, and CRs with combined radiotherapy.^{58–60} A Coxsackie-virus-based vaccine therapy has been reported and is being commercialized with similar application, injected into cutaneous and subcutaneous melanoma deposits with observable responses in the injected and adjacent non-injected masses.^{56,61,62}

Vaccination from cytotoxic chemotherapy and chemical oncolysis

Most current cytotoxic chemotherapy kills a percentage of cells in the tumour which releases tumour antigens. Cytotoxic chemotherapy primarily interferes with cell division through interrupting nucleic acid replication and processing. The interaction of cytotoxic agents with the deoxyribonucleic acid (DNA), ribonucleic acid (RNA), proteins or cell cytoskeletal functions (mitotic spindle) is generally well understood, but the effects leading to cell death and consequently on cancer growth is perhaps less clearly determined. Further, the effects of cytotoxic chemotherapy on the cell membrane and antigens is currently quite poorly understood. Classical cytotoxic chemotherapy induced cell death is chiefly *via* an apoptotic pathway by acceleration of senescent cell death pathways (apoptosis). This itself can have immunogenic consequences arising from macrophage and dendritic-cell engulfment and presentation of tumour antigens to the immune system *in vivo*. However, the resulting onco-apoptosis can follow the usual line of normal senescent cell death with low or little stimulation of host immune mechanisms (even immune-response regulation or suppression), the so-called ‘nonimmunogenic’ apoptotic pathways. This constitutes a type of involitional cell death. The distinction between induction of nonimmunogenic *versus* an immunogenic route of apoptotic-cell handling is not clearly understood at this time, but the presence of danger signals appears to be important in determining whether the immune system responds and the strength of that response to apoptotic end products of cell death. Consequently, inflammation and immune responses can arise from vaccination by apoptotic cancer-cell bodies as a result of cytotoxic chemotherapy cell death. The critical problem, as far as cancer destruction is concerned, is that apoptosis does not usually act as sufficiently strong a stimulus for inflammation, nor engender an active immune response, in most cases.

However, it is less well appreciated that cytotoxic chemotherapy can damage the cancer-cell membranes and that this can give rise to a different type of immune response.⁶³ When the cancer cell membrane is damaged, and this can occur through a variety of mechanisms related to the membrane structure or function, the cell can no longer maintain its integrity to the degree that the cell lyses or bursts. This process is chemical oncolysis, and necrotic cell death occurs (rather than apoptosis). Lysis of cancer cells produces release of membrane fragments and antigenic molecules associated with the cancer-cell membrane. Not only are a variety of cancer antigens released, but danger signals are typically present as well. Tumours can become erythematous, hot and inflamed, sometimes associated with fever.

Chemical damage to the cancer-cell membrane from cytotoxins can be sufficient to release antigens from the cell surface but without killing the cancer cell, thus liberating antigens that effectively act as an *in vivo* vaccine to stimulate the immune response.

Classical chemical cell death occurs when damage to the cell membrane, nuclear material or cytosol is sufficiently great to lead to rupture of the cancer cells (oncolysis or necrosis). Cytotoxic chemotherapeutic agents create chemical injury that may damage the nuclear material (e.g. mustard compounds), cytosolic elements (e.g. antibiotic agents like dactinomycin), or the cell membrane structurally or functionally. The intensity of the damage and the capacity of repair mechanisms to correct the injuries determines the ability of the cell to survive or if sufficiently deleterious, causes the death of the cell. For example, if the membrane is damaged such that the sodium/potassium adenosine triphosphate (ATP) pump is affected then the cell swells and bursts, which effectively constitutes lysis and an *in vivo* vaccine event.

A multitude of mechanisms exist which result in cellular damage to the cancer cell, altering, for example, functional capacity and survival, with the outcomes not always being predictable due to the inherent cellular heterogeneity that exists within most tumours.

Other chemical agents have been reported to act directly on cancer cells to induce cell killing,

such as the pink dye Rose Bengal (also known as PV-10; Provectus, Tennessee, USA).^{64,65} This agent selectively penetrates the cancer-cell membrane, but not the cell membranes of normal cells, to act internally at the lysosomal level, leading to cell death. The cell death appears to be apoptotic, leading to autophagy. PV-10 is injected directly into cutaneous and subcutaneous melanoma masses causing lysis and necrosis of tumour, inducing local inflammatory responses in the injected lesions. Interestingly, 'bystander' responses are reported in non-injected lesions and some systemic responses were also noted, indicating generation of a systemic immune response. Although the cellular response to PV-10 is reported to be involutinal and apoptotic, clinically, the injected tumour masses undergo overt ulceration more indicative of necrosis. It is therefore likely that the response to direct injection of PV-10 into tumours is a mixed necrotic and apoptotic process. This would explain the observed clinical responses of bystander and systemic responses, which come from induction of local, regional and systemic immune responses, most likely as a result of immunogenic danger signals arising from necrosis. Indeed, this model probably serves as a generalizable model for many agents that locally damage the tumour, causing an element of tumour necrosis, for instance T-Vec, Cavatak[®] Merck, New Jersey, USA (Coxsackie virus) and radiation therapy.⁵³⁻⁶⁶

Vaccination from physical oncolysis

Radiation therapy causes both apoptosis and necrosis in tumour cells exposed to sufficient levels of radiation to damage DNA. The resulting cell-membrane damage and lysis releases tumour antigens from the malignant cells, producing *in vivo* vaccination events as each cell is damaged or dies. Radiation-induced antitumour immunity has been previously described and can act as an immune adjuvant by exerting systemic immunological effects beyond the local radiation field of delivery.⁶⁶⁻⁷¹ In another study following radiation exposure in murine and human melanoma, DNA damage-response-marker expression was upregulated days prior to immune-marker expression of death receptors and T-cell costimulatory/co-inhibitory ligand changes indicating a time-dependent effect.⁶⁹ Complete regression of established murine

tumours in most animals using combined local radiation and intratumourally injected IL-2-linked tumour-specific antibody immunocytokine and regression of large tumours was reported with the addition of systemic anti-CTLA-4 T-cell-checkpoint blockade.⁶⁹ The ‘abscopal effect’ of radiation therapy where tumour regresses outside of the field of radiation has been well recognized both clinically and in animal models, where locally administered radiation can cause systemic immune response against remote tumours not receiving radiation exposure.⁷¹ Indeed, MacManus and colleagues showed that 1.1% of 2337 patients with NSCLC receiving localized palliative radiation therapy survived 5 years or more after therapy, indicating that radiation could induce systemic responses with improved survival.⁷²

Radiation and other forms of physical cellular damage induces damage (or danger)-associated molecular patterns (DAMPs), intrinsic danger signals (also known as alarmin) that are capable of eliciting an inflammatory response in a non-infectious situation. These are a diverse variety of predominantly intracellular molecules that are not usually revealed at the external aspect of the cell membrane but can become expressed on the cell surface of damaged cells or are released when cells are lysed when the contents are released into the surrounding tissues and vessels. DAMPs are released in response to trauma or injury to tissues, for example, and when cancer cells are injured from a variety of mechanisms, the DAMP molecules are released into the tissues and are capable of inducing an inflammatory response.

DAMPs are tissue dependent and include intracellular proteins, for example, heat-shock proteins (HSPs), or the chromatin-associated lysosomal protein high-mobility group box 1 (HMGB1), and also include extracellular-matrix-associated stromal proteins, such as hyaluronan fragments. DAMPs also include nonprotein molecules like DNA, adenosine, ATP, uric acid, and heparan sulfate. Calcium regulatory proteins such as S-100 are also able to act as DAMPs in some situations, and even calcium itself, depending on the concentration and location. DAMPs appear to be concentration and location dependent in terms of how great their stimulatory capacity is on inciting danger signals in the immune system in a particular situation.

DAMPs perform a similar function to pathogen-associated molecular patterns (PAMPs) which are a variety of molecules associated with (extrinsic) pathogens usually recognized by the innate immune system through TLRs and other pattern-recognition receptors in plants and animals. Although there are many PAMPs, examples include glycans, bacterial lipopolysaccharides, glycoconjugates, endotoxins, flagellin, lipoteichoic acid, peptidoglycan, viral nucleic acids [e.g. double-stranded ribonucleic acid (dsRNA)] and CpG. Many microbes contain natural danger signals capable of inciting an immune response upon exposure to the animal and human immune system, as an evolutionary adaptation. Such responses have been selected for over millions of years.⁷³ Cancer cells, at least within established tumours, are not only of ‘self’ origin, but are usually lacking the necessary danger signals for eliciting an effective immune response, which forms part of the observed ‘nonresponsiveness’, or natural ‘tolerance’ of tumours in the host. When danger signals are present together with tumour antigens then an immune response can proceed, and this may explain some of the ‘spontaneous regressions’ of cancer observed following an infective or traumatic episode. Many of the current cancer immunotherapy trials are now utilizing adjuvants or danger signals with vaccination, or checkpoint-agent therapy on this basis. Examples are heat-killed vaccines and adjuvants, such as Coley vaccines, site-specific immunomodulators, and systemic immune modulators. Imodulon (IMM-101, Immodulon Therapeutics, Uxbridge, UK) contains heat-killed *Mycobacterium obuense* and is capable of modulation of the innate and adaptive immune systems in response to cancer, through interaction with a number of receptors (PAMPs) and $\gamma\delta$ T cells, granulocytes, and antigen-presenting cells, to downregulate a type 2 (Th2) response bias, in favour of a type 1 (Th1) response.^{74–79} Radioprotective effects can also occur in tissues through TLRs; either arising from DAMPs or through secondary infection (PAMPs) or when TLR agonists are administered with radiotherapy.⁸⁰ In essence, radiation therapy can act to cause tumour cell death with *in vivo* vaccination events through release of antigen in waves with each successive treatment cycle stimulating the immune system; or produce radioprotective responses in target tumour cells, the balance of which can determine the clinical response.

Topical and systemic sensitizing agents

Topical sensitizing agents have been used for many decades as mechanisms for adjuvant boosting for the immune system and have been shown to induce regression of cancer due to stimulation of a pre-existing *in vivo* immune response. These agents are usually administered percutaneously by painting them onto the skin overlying the tumour deposit such that local absorption of the agent influences the local tumour microenvironment; however, bystander responses have been observed where nontreated metastases also can regress, indicative of enhancement or development of a systemic immune response.^{81–83}

Such immunomodulating agents can act as effective therapies, even after standard therapies have failed, although the response is unpredictable. The agent 2, 4-dinitrochlorobenzene (DNCB) was one of the early agents demonstrating effective clinical responses. Using intralesional DNCB for treating in-transit metastases, CRs occurred in about 60% of patients.⁸¹ DNCB has also been used topically.^{82–84}

Diphencyprone (DPCP) is another topically administered agent that has been used more recently to induce immune responses in patients with surgically nonoperable deposits, especially in difficult areas, and has shown remarkable results in some patients with high rates of complete (46%) and partial (38%) regressions being recorded.^{85–87} DPCP immunotherapy has been used to treat cutaneous warts and alopecia areata, and more recently, has been used as a single agent to successfully treat radiotherapy-resistant extensive, multiple scalp-melanoma metastatic confluent nodules not amenable to surgery.^{85,86} The fact that complete responses have been reported in locally advanced melanoma, with bystander effects in nontreated lesions and regression of visceral metastases, are highly significant observations.⁸⁷ Treatment was well tolerated with daily topical applications, was non-invasive and very cost effective.

Both DNCB and DPCP have been utilized combined with other therapies, notably radiation therapy and chemotherapy, and appear to augment these in some situations.⁸⁸

Dinitrophenyl (DNP) is another agent, used as a hapten, to bind to tumour-cell surface *ex vivo* (*in vitro* culture) and are then injected back into the

patient to form a melanoma vaccine.^{89–93} Multiple intradermal injections of DNP-modified autologous tumour cells mixed with *Bacillus Calmette–Guérin* (BCG) acting as an immunological adjuvant/stimulant were used to induce and enhance local inflammation in the metastatic melanoma deposits to incite a systemic immune response against the patient's tumour.⁹⁰ Delayed-type hypersensitivity (DTH) developed in almost all patients against autologous DNP-modified melanoma cells and in about 50% of patients exposed to autologous, unmodified tumour cells. Development of DTH was associated with significantly longer survival. The 5-year overall survival (OS) rate in 214 stage III patients was 44%, compared with a survival of 20–25% after stage IV surgical metastasectomy.⁹² DNP autologous vaccine treatment following low-dose cyclophosphamide was also used for 97 (83 evaluable) unresectable stage IV melanoma patients with 11 (13%) clinical responses, 2 (2.5%) CRs, 4 (4.5%) PRs and 5 (6%) mixed responses. Survival was prolonged for the responders, and those with DTH to unmodified autologous tumour cells. Regression of tumour required at least 4 months, with some cases not achieving maximum regression until up to 1 year after commencing treatment. Long survivals beyond 2 years and 5 years occurred in some patients developing CR and PR.^{90–92}

Interestingly, antibody responses to DNP have been shown to directly correlate to better outcome. One primary mechanism of action of these agents appears to be haptentation of cell surface and cytoplasmic proteins, which can induce CD8(+) T-lymphocyte-mediated allergic-contact-hypersensitivity responses.

Haptens are small molecules acting as incomplete antigens, often without their own intrinsic activity, but when bound to another, often larger protein, can act with this carrier to generate stronger immune responses. The response strength is determined by several host factors that can determine immunological responses, such as nutrition, tumour burden, the pre-existing immune status and immunosuppression. Haptens can also produce autoimmunity, which is of considerable interest, since the clinical efficacy of checkpoint-inhibitory agents are directly associated with the generation of autoimmune side effects. Haptens can augment or create an effective or partially

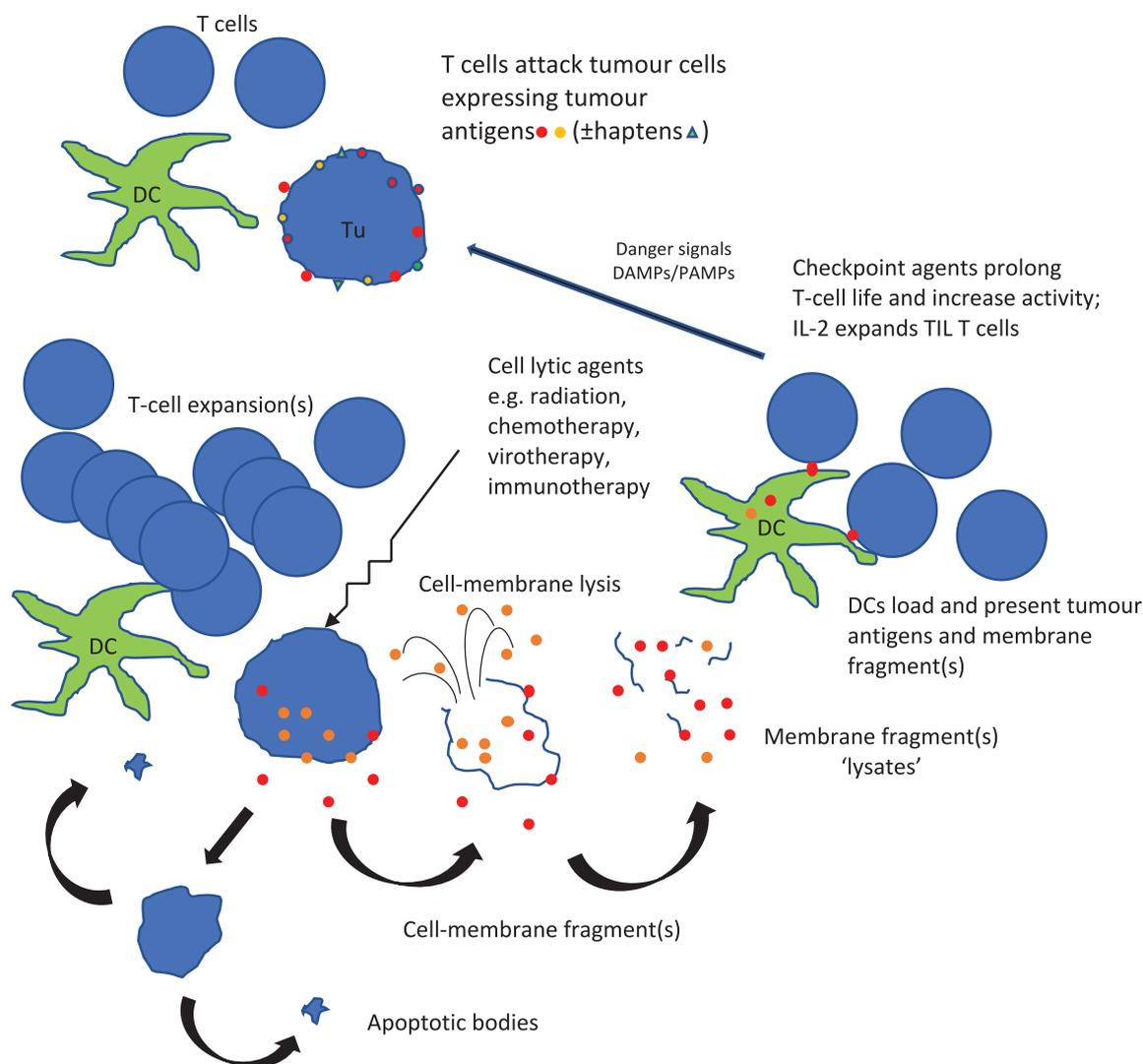


Figure 2. Mechanism of *in vivo* cancer vaccination from multiple divergent therapies. DAMP, danger-associated molecular patterns; PAMP, pathogen-associated molecular patterns; IL, interleukin; TIL, tumour-infiltrating lymphocyte.

effective antitumour immune response when bound to a carrier protein that is a suitable tumour antigen⁹³ Another immune stimulant, imiquimod, stimulates TLR 7/8 and has demonstrable antitumour activity, especially against skin cancers [basal cell carcinoma (BCC), SCC and melanomas] which is addressed later⁹⁴ (Figure 2).

Systemic and local intratumoural therapies

Bacillus Calmette-Guérin

Another agent with impressive long-term survival in a notable proportion of treated cases is intradermal and intravesical BCG therapy. In

the late 1920s, it was noted that bladder cancer rates were lower in patients dying of tuberculosis, which prompted the initial trials of BCG as a therapy for superficial bladder cancer.⁹⁵

Intralesional *Mycobacterium bovis* BCG has been used for local immunotherapy for melanoma nodules, with local complete responses occurring in the directly injected lesions of about 90% of injected lesions and bystander responses in 17% of non-injected, with 25% of patients rendered disease free and alive at 1–6 years postinjection.^{96,97}

In a study of a multivalent melanoma vaccine used systemically, delivered intradermally, together

with BCG *versus* BCG alone as the control group, in resected stage III and stage IV melanoma, the BCG arm performed better than historical controls from a previous study with no therapy, indicating that the therapy BCG had significant systemic efficacy of its own in slowing or preventing recurrence.^{32,97,98} BCG vaccination therapy has been previously reported as exerting long-term survival effects in melanoma patients.^{97–100} The role of BCG in bladder cancer treatment has become well established.^{101,102} Although the precise mechanism of action remains unclear, proposed to be through induction of TRAIL (TNF-related apoptosis-inducing ligand), IL-2, IL-8, IL-18, IL-12, interferon (IFN)- γ , and TNF, NK cells, macrophages, $\gamma\delta$ -T cells, augmentation of specific antigen-primed T cells and direct actions of BCG itself.^{95,100–102} BCG acts as a powerful TLR 2/4 agonist causing immune stimulation, but can also downregulate the immune response. In a placebo-controlled, randomized phase III trial of 254 resected stage II/III colon cancer patients vaccinated with BCG with autologous tumour cells and BCG in the adjuvant setting for all patients, recurrence rates were statistically reduced (44%; $p=0.023$) and there was longer recurrence-free survival (42%; $p=0.032$) with a trend towards improved OS in the vaccine arm that led to regulatory approval in The Netherlands. However, the benefit resided for the stage II patients rather than the resected stage III patients, where no statistical benefit was demonstrable.¹⁰³

Interleukin-2

IL-2 has been used for over 20 years, chiefly at high dose for systemic treatment of advanced renal cell carcinoma (RCC),¹⁰⁴ metastatic melanoma and a range of other advanced cancers.^{104–106} It was the first immunotherapy approved by the FDA for advanced RCC and soon after, for advanced melanoma therapy, and has stood the test of time with a small (about 4%), but significant, rate of CRs and PRs. Most notably, when a CR or prolonged PR occurs (and interestingly, like checkpoint agents, some PRs develop into CRs over time), then almost universally, the response leads to long-term survival and in the case of CRs, effective 5- or 10-year (or longer) ‘cure’.^{104–106} There is some evidence that lower doses of IL-2 (low and intermediate dosing) are also effective in augmenting antitumour immune responses with lower incidence of toxic side effects.¹⁰⁴

IL-2 has also been used for intralesional injection into superficial melanoma deposits with good efficacy showing an ORR of 82%, a CR rate of 51% and PR rate of 31%, and an intralesional CR rate of 76%, with a significant in-transit free and OS in partial responders.^{107–109} Similar results were reported in a systematic analysis of over 2000 patients.¹⁰⁹ IL-2 has been combined with a range of other therapies including imiquimod, with a reported 100% intralesional CR rate¹¹⁰ and anti-PD-1 therapy.¹¹¹ The highest CR rate of 5.0% was associated with IL-2 combined with vaccine therapy in RCC.¹⁰⁴ In a separate study, topical daily application of 5% imiquimod cream (Aldara, Bausch, Quebec, Canada) was undertaken for 4 weeks to superficial melanoma lesions, followed by IL-2 injected either intralesionally, subcutaneously or a combination of both, according to three regimens. In six of the eight patients treated where Peripheral Blood Mononuclear Cells (PBMC) could be obtained, the Th1/Th2 balance appeared to be tipped in favour of Th1 cells evidenced by an increased CD4+CD25+ population of PBMC shown to be activated T cells and not Tregs (using CTLA-4, GITR, γ -IFN, and Foxp3). In these studies, the baseline starting point of mean percentage of CD4+CD25+ cells in cancer patients was observed to be lower than for normal controls.^{112,113} Although IL-2 acts as a pure immune driver augmenting a pre-existing immune response against the active tumour within the patient, when T cells kill tumour cells, antigen release occurs to create another wave of *in vivo* vaccination.

Electrochemotherapy

A range of cancers, including breast cancer, melanoma, SCC, BCC, sarcoma and lung adenocarcinoma, have been treated with short, high-voltage electric pulse electroporation to aid permeabilization into the tumours of chemotherapeutic agents administered topically or systemically.¹¹⁴ The reported results show induction of CRs and PRs, averaging a CR rate of 52% (range 11–80%) and PR rate of 25% (range 11–49%), with no reported systemic side effects.^{115,116} Most of the tumours treated are more easily accessible local cutaneous and superficial metastases and in-transit deposits treated with intravenous bleomycin and electroporation, but deeper liver tumours, for example, hepatocellular and colorectal carcinoma, have been treated also using electrochemotherapy.

However, there is increasing interest in combining these approaches with systemic-checkpoint immunotherapies.¹¹⁷ Although the cellular responses are not particularly clear, cuffing of chronic inflammatory infiltrates consisting of lymphocytes and plasma cells was observed at the outer edge of the fibrous tissue of the treated colorectal liver metastases, similar to that seen with BRAF inhibitors (below).¹¹⁸ As electroporation kills tumour cells, antigen is released, with each treatment resulting in *in vivo* vaccination.

OK-432

OK-432 is a vaccine comprising group A *Streptococcus pyogenes* of human origin that was approved for clinical use in Japan in 1975 for treatment of gastric, primary lung, head and neck, and thyroid cancers, especially when resistant to other chemotherapies. In a recent meta-analysis of 14 trials for patients with stage III or stage IV gastric cancer after curative resection, OK-432 was used in an adjuvant setting against standard chemotherapy controls. A 12% reduction in the risk of recurrence was found with OK-432 treatment with an overall hazard ratio (HR) of 0.88 [95% confidence interval (CI) 0.77–1.00, $p=0.050$]. In previous studies, the infiltration of Langerhan's cells into the tumour after endoscopic OK-432 vaccine injection into stage III gastric cancers prior to resection, was associated with a better clinical response.¹¹⁹ These observations would indicate that injection of OK-432 possibly provides PAMPs to activate innate immunity and thereby stimulate an adaptive T-cell-mediated response, akin to Coley's and oncolytic viral approaches, modifying a pre-existing *in vivo* antitumour immune response in the patient.

Indoleamine 2,3-dioxygenase inhibitors

Indoleamine inhibitors block the key enzyme indoleamine 2,3-dioxygenase (IDO) which catabolizes tryptophan to kynurenine which causes immunosuppressive effects. IDO upregulation in multiple cancer types has been associated with poorer survival. IDO inhibitors have been shown in preclinical models and clinical trials to directly and indirectly block IDO. Therefore, blocking immune regulation *via* the IDO pathway is currently being explored alone and combined with other agents, principally chemotherapy and

immunotherapy in a variety of cancers. A proposed mechanism of action of IDO inhibitors is increased production of IL-2 by TIL CD8 T cells to reactivate suppressed TIL.¹²⁰ IDO inhibitors are capable of reprogramming immunologically 'cold' tumours into 'hot' tumours, and appear to be well tolerated, but early results are mixed.^{121–123}

Small-molecule inhibitors

BRAF therapies, often combined with MEK inhibitors, have been used in the approximately 40–50% of melanoma patients who have BRAF-mutation-positive tumours. CR rates are variably reported between 0.9 and 6.25%.²⁶ The median OS was 26.1 months for combined dabrafenib plus trametinib therapy, while in the group treated with the combination of vemurafenib and cobimetinib, the median OS was 22.3 months, while vemurafenib alone was 17.8 months (HR=0.68). While 3-year OS was 45% and 31%, respectively, for patients treated with vemurafenib and cobimetinib, or vemurafenib alone.

Vemurafenib-alone therapy *versus* dacarbazine was trialled in 675 patients in the BRIM-3 study and showed a median OS of 13.3 months *versus* 10 months for V600E-mutant advanced melanoma, respectively.^{124,125} In the final results, 84 dacarbazine-group patients crossed over to vemurafenib, and the OS rates for vemurafenib *versus* dacarbazine at 4 years were 17% *versus* 16%, and at 1, 2, 3 years were 56% *versus* 46%, 30% *versus* 24%, 21% *versus* 19%, respectively, by Kaplan–Meier estimates.¹²⁶

The action of BRAF inhibitory agents is to block the BRAF pathway, reducing cancer-cell proliferation and inducing cell death. Spectacular reductions in active metabolizing melanoma metastases have been demonstrated, although gradual development of resistance to the agents is commonplace over time with a median duration of effect of about 11 months before resistance to the therapy develops.¹²⁵

The induction of cancer-cell death releases tumour antigens that act also as repeated vaccine events, promoting an immune response. Recent reports indicate that clinical efficacy is associated with infiltration of lymphocytes around metastatic deposits, and removal of lymphocytes close

to the tumour results in loss of effectiveness, indicating that these agents are operating through immune mechanisms as well.^{127–129}

Interestingly, breaks in BRAF treatment with later reinstatement, and combination with other immunotherapies, have been associated with durable CRs in patients with BRAF-mutated metastatic melanoma after initial failure of sequential immunotherapies (high-dose IL-2 followed by ipilimumab with or without concurrent radiation therapy).¹²⁹ The presence of non-Treg, CD4-positive effector-phenotype T cells in these patients was associated with the durable responses. Synergism between conventional or targeted cytotoxic therapy and immunotherapy in cancer treatment is suggested.¹²⁹ Rechallenge after a break in failed BRAF inhibitor therapy has been demonstrated in several studies to induce clinically useful responses, including PRs and CRs.^{130–132}

The above local and systemic therapies are capable of killing tumour cells, causing release of tumour antigens *in vivo*, thus creating local vaccination events, with systemic manifestations. Most therapies rely on repeated cycles of treatment for their clinical effects, thus repeated waves of tumour cell death and repeated vaccine events. The immunotherapies are next addressed with similar consideration due to their ability to induce T-cell-mediated tumour-cell killing and *in vivo* antigen release.

Recent immunotherapies

Checkpoint inhibitors

The literature on the recently approved checkpoint-immunotherapy agents has become extensive and complex, creating many controversies, so this discussion will be abbreviated. The main difficulties with comparisons between treatments rests in the difference in doses, regimens, combinations, sequences, crossovers, pretreatments, biomarkers and staging subgroups used in the various studies which hamper clear interpretation. Many results are also being reported as meeting abstracts rather than peer-reviewed published papers with supplementary information, as the field is advancing so rapidly, so that the detailed data are not always available for full evaluation and comparison. Some of the data is also

held commercial in-confidence for a period of time. Indeed, previous evidence shows that many conference abstracts do not reach publication for a variety of reasons.^{133–137}

The inhibitory checkpoint monoclonal antibodies, anti-CTLA-4 and anti-PD-1, operate by taking the brake off of the immune system and by reducing apoptotic lymphocyte death rates, respectively, and have been demonstrated to improve immune responses against the tumour in the patient, resulting in about 10% CR rates and improved disease-free and OS durations.^{26,138–142} These agents improve survival in about 20–30% of treated patients as monotherapies, and about 30–50% when combined. The main message is that the predominant action of these agents is driving forward pre-existing endogenous immune responses occurring *in situ*, locally, at the tumour site(s), regionally and systemically within the patients. Although these agents do not supply antigen directly or act as ‘vaccines’ of themselves, their action in causing cancer-cell killing produces tumour antigen release and therefore creates repeated vaccination events. In essence, by inciting immune mechanisms, immunotherapies act in a positive-feedback loop where the immune response is augmenting itself, with immunotherapies causing tumour-cell death, causing subsequent antigen release, inducing stronger immune reactivity, thus producing more tumour-cell death and more immune reactivity in a feed-forward mechanism. Immune responses are not always activatory, however, but can be inhibitory and induce immunological tolerance. The containment of these immune responses remains a significant issue too due to promulgation of autoimmunity, which sometimes has toxicity-related treatment-limiting effects, including a low incidence of treatment-related deaths, as can be seen below.

Anti-CTLA-4 therapy: ipilimumab

Hodi and colleagues reported a median OS of 10 months for ipilimumab plus gp100-treated advanced-melanoma patients, compared with 6.4 months for gp100 alone (HR for death, 0.68; $p < 0.001$) and 10.1 months for ipilimumab alone. No difference in median OS was detected between the ipilimumab groups with or without gp100.

Serious immune-related adverse events (grade 3 or 4) were reported in 10–15% of patients with

ipilimumab treatment, but 3% treated with gp100 alone, with 14 deaths related to the study drugs (2.2%), most being autoimmunity related.¹³⁹ The OS at 3 years for ipilimumab alone was 34% in the Wolchok and coworkers¹⁴⁰ study; the ORR was 10.8% (4/37) in the ipilimumab-alone group ($p < 0.001$), with a CR rate of 0 in the ipilimumab-alone group.¹⁴¹

Anti-PD-1 therapies

Nivolumab

In the CheckMate 066 study, the nivolumab-alone response rate was higher than chemotherapy in first-line BRAF-wt advanced-melanoma treatment, 40% *versus* 13.9%¹⁰⁸ and 2-year OS was higher at 57.7% *versus* control 26.7%; and 3-year OS rates were 51.2% and 21.6%, respectively. The CR and PR rates were 19.0% (40 of 210) and 23.8% (50 of 210) for the nivolumab group compared with 1.4% (3 of 208) and 13.0% (27 of 208) in the dacarbazine group, respectively.^{138,142,143} As an indicator of long-term outcome, the phase I study (CheckMate 003) tested various doses of nivolumab (0.3–10 mg/kg) in 107 patients showing a 5-year OS in 34% of patients, with an apparent plateau at about 2 years, and a median OS in all treated patients of 17.3 months, and 20.3 months (for a 3 mg/kg dose of nivolumab).¹³⁸

Pembrolizumab

Pembrolizumab was evaluated in the KEYNOTE 001 study in various doses for 655 advanced-melanoma patients with a median OS of 23.5 (2 mg/kg dose), 22.9 (10 mg/kg 2 weekly), and 25.9 months (10 mg/kg 3 weekly). The median OS was 20 months across all doses in patients previously treated with ipilimumab and 28 months in ipilimumab-naïve patients with identical 3-year OS for both groups at 41%. However, in completely treatment-naïve patients, the median OS was 32 months with a 3-year OS of 45%.¹³⁸ The objective response rate ranged from 8 to 53% being directly related to the degree of programmed death ligand 1 (PD-L1) expression in the pretreatment tumour biopsies¹⁴⁴ and baseline tumour size (BTS) was related to OS so that a BTS of <10.2 cm (additive longest dimensions of all target lesions monitored) had a better OS than >10.2 cm (HR 0.38; $p < 0.001$) and ORR of 44% *versus* 23%, respectively.¹⁴⁵ Durable CRs have

been reported in about 16% of patients (105 of 655) in the Keynote-001 study, with about 10% of those patients relapsing after discontinuation of the agent.¹⁴⁶

In a pooled analysis of 1012 patients from KEYNOTE-001 and KEYNOTE-002, three dose schedules of pembrolizumab (2 mg/kg every 3 weeks, 10 mg/kg every 2, and 10 mg/kg every 3 weeks) were compared, similar toxicity showing treatment-related adverse events (AE) in 75–83% of patients; most being graded 1 and 2, but grade 3 and 4 AEs were noted in 13.5% of patients [hypothyroidism (7.4%), pneumonitis (2.6%), and hyperthyroidism (2.4%)], and colitis, hypophysitis, nephritis, hepatitis and death in less than 2% of patients.^{138,147,148} Similar findings were noted in ‘real-world’ settings external to clinical trials.¹⁴⁹

Combined CTLA-4 and PD-1 therapy

In the CheckMate 067 trial, untreated advanced-melanoma patients were randomized 1:1:1 to receive nivolumab (1 mg/kg) plus ipilimumab (3 mg/kg) followed by nivolumab (3 mg/kg); nivolumab (3 mg/kg) plus placebo; or ipilimumab (3 mg/kg) plus placebo, until progression or unacceptable toxicity or withdrawal for other reasons. Stratification was for PD-L1 status, BRAF mutation status, and metastasis stage.

At 36-months minimum follow up, the median OS was not yet reached in the nivolumab-plus-ipilimumab group and was 37.6 months in the nivolumab group and 19.9 months in the ipilimumab group. The OS at 3 years was 58% (53% at 4 years) for nivolumab-plus-ipilimumab therapy and 52% (46% at 4 years) for nivolumab alone, and 34% (30% at 4-years) for ipilimumab alone. The 2-year OS was 64%, 59% and 45%, respectively. In subgroup analysis, the survivals at 4 years were about 5–13% higher for BRAF-mutant *versus* BRAF-wild-type melanomas; with a similar differential for PD-L1-positive *versus* -negative melanomas. AEs related to treatment occurred in 96% of the combination group, 86% of the nivolumab-alone group, while grade 3 or 4 treatment-related AEs were reported in 59% of the nivolumab-plus-ipilimumab group, 21% of the nivolumab group and 28% of the ipilimumab group. At 4-year analysis, four treatment-related deaths were reported; two in the combined group and one each in the single-agent groups, from

cardiomyopathy, liver necrosis, neutropenia and colon perforation, respectively.^{146–150}

In a double-blind study of 142 metastatic melanoma treatment-naïve patients randomized 2:1 between ipilimumab (3 mg/kg) plus nivolumab 1 mg/kg or placebo; followed by nivolumab or placebo, the ORR was 61.1% (44/72) in the combined nivolumab and ipilimumab group *versus* 10.8% (4/37) in the ipilimumab-alone group ($p < 0.001$), with a CR rate of 16 (22.2%) for the combination group and none in the ipilimumab group. The combination group showed 54.3% grade 3–4 drug-related AEs compared with 23.9% in the ipilimumab-monotherapy group; results supported by other studies.^{141,151,152}

Checkpoints combined with other agents

Following the response rate of 84% using topical DPCP for cutaneous melanoma metastases in a 50-patient case series with nodal or visceral metastasis regression in four of the patients,⁸⁷ DPCP was combined with the PD-1 inhibitor nivolumab treatment which showed internal metastasis regression.^{153,154}

In a small phase Ib clinical trial of 21 stage III/IV patients, 6 (43%) of the 14 patients with visceral metastatic melanoma had complete regression reported following combined intralesional T-Vec and anti-PD-1 therapy.⁶⁰ The ability of combined intralesional T-Vec and anti-PD-1 therapies to induce complete regression of distant metastases was also demonstrated in two patients with lung metastases in a 10-patient stage III/IV study.⁵⁷ These are further examples of a local therapy combined with a systemic therapy to induce ‘off-target’ systemic responses. The overall response in **injected lesions** was reported as 90% with 6 (60%) of the 10 patients achieving lesional CRs.

Other immune-checkpoint protein-modulating agents

A number of clinical trials are currently investigating drugs that target other checkpoint-control proteins such as OX40, B7-H3, and LAG3.¹⁵⁵

OX40

OX40 (CD134) is a TNF-receptor superfamily member (TNFRSF-4) appearing on CD4+ cells,

and to a lesser degree, on CD8+ cells after activation, and transmits a potent costimulatory signal when engaged. OX40 is minimally expressed on circulating T-reg cells in humans, but is upregulated in inflammation, including in cancer. T-receptor binding to antigen transiently upregulates OX40 on activated T cells about 24–72 h after stimulation (early-to-intermediate activation marker), notably on TILs. The ligand for OX40 (OX40L) is expressed on activated antigen-presenting cells including dendritic cells, B cells, macrophages, and endothelial cells, but also by activated T cells. When OX40 receptors on T cells are bound by OX40L, apoptosis is delayed and cytokine production increases to maintain the cells beyond the initial stimulation to prolong immune response. OX40 induces *in vivo* T-cell survival and memory functions of both Th1- and Th2-mediated reactions.¹⁵⁶ Excessive effects of OX40 have been associated with cytokine storm and autoimmunity. Clinical trials of OX40 agonist antibodies such as GSK3174998, which binds specifically to OX40 to activate TIL, are in progress alone or with pembrolizumab for advanced NSCLC, SCC of the head and neck, RCC, melanoma, bladder, soft tissue sarcoma, triple-negative breast cancer, and colorectal cancer [ClinicalTrials.gov identifier: NCT02528357]. OX40-blocking agents are also being explored to reduce inflammation, for example, in autoimmunity and transplant rejection.

LAG-3

LAG-3 (CD223) is a surface molecule identifying an immune checkpoint physically closely associated with CD4, but having only less than 20% amino-acid homology with CD4; and like CD4, binds to MHC-II as the main ligand on antigen-presenting cells, but with higher affinity. LAG-3 is a member of the immunoglobulin superfamily expressed on TILs, activated CD4+ and CD8+ T cells, as well as Tregs, NK cells, B cells and dendritic cells (DCs). LAG-3 acts to inhibit cellular proliferation and activation of T cells similarly to CTLA-4 and PD-1 and reduces cytokine production. Inhibitory antibodies to LAG-3 take the brakes off the anticancer immune response. Over 20 studies (e.g. anti-LAG3 monoclonal relatlimab; BMS-986016) [ClinicalTrials.gov identifier: NCT02966548] have entered phase II/III trials, and combination trials with LAG-3 and CTLA-4 or PD-1 antibodies are in progress for

advanced melanoma, breast, colon, lung, brain, lung, gastric, haematological and renal cancer.¹⁵⁷

TLR and STING agonists

The innate immune response is important for anticancer immune responses and for initiating and augmenting adaptive immune responses. Therefore, innate and adaptive immunity are often proceeding synchronously to generate effective immune responses against cancer, leading to tumour regression. Unpicking the precise roles of these responses in antitumour immunity is complex and proving immensely challenging. Many tumours do not demonstrate immunological infiltrates or show low-grade activity and have been termed 'cold' tumours. However, initiating innate immune responses can offer a route to activating adaptive T-cell immunity to turn 'cold' tumours into 'hot' tumours with significant T-cell infiltration. In the process of natural exposure to exogenous antigens, the nonspecific innate immune response provides rapid recognition of danger signals from foreign pathogens such as viruses, bacteria, and parasites. It is this process, detection of foreign antigens through innate immune responses that appears to be the basis of 'infection-associated' immune responses to cancer, as discussed above for BCG-, virus- and bacterial-based therapies. Innate immune cellular reactions include macrophages, fixed-tissue histiocytes, NK cells and dendritic cells. Other parts of the innate immune system include basophils, mast cells, eosinophils and neutrophils (granulocytes), all of which can incite immune responses in different settings and have been associated with antitumour immunity. Many of the innate immune cells are responsible for either direct or indirect liberation of cytokines including the ILs. TLRs are proteins comprising at least 10 groups found on the surface of macrophages that can recognize the presence of foreign DNA sequences or small fragments (PAMPs) or aberrant DNA within the cell cytoplasm or external to cells. TLRs can recognize viral proteins and DNA, which appears to be a fundamental evolutionary adaptation for rapid, early detection of danger from viral infection.

TLR9 recognizes the short DNA sequence CpG that is more frequent within bacterial DNA compared with human DNA. With enough CpG, an immune response can be triggered, and incite

both an innate immune response through macrophages and an adaptive response by activating T cells.

Levy and colleagues showed that *in situ* vaccination with a TLR9 ligand (CpG oligodeoxynucleotide) could induce OX40 expression on intratumoural CD4 T cells in spontaneous murine breast cancers, and that administration of an agonist anti-OX40 antibody induced regression of distant metastases in that model.^{158,159} Furthermore, the *in situ* vaccination with CpG and anti-OX40 not only caused tumour regression, but offered a protective effect for mice genetically prone to spontaneous breast cancers, and also, those cancer-prone mice showed increased survival.^{158,159} These studies also demonstrated tumour specificity for these effects, showing protection against the same tumour, but not different tumours. Interestingly, TLR7/8 could replace TLR9, but anti-PD-1 could not replace anti-OX40 to reproduce the effect in those studies. An equivalent CpG agent, SD-101, has entered preliminary clinical trials as monotherapy or in combination with other therapies [ClinicalTrials.gov identifiers: NCT02927964, NCT02266147, NCT01745354, NCT02254772, and NCT02521870]. Anti-OX40 antibody is also under investigation in phase I clinical trials [ClinicalTrials.gov identifiers: NCT02559024, NCT01644968, NCT02221960, NCT02318394, NCT02274155, NCT01862900, NCT01303705 and NCT02205333]. Previously, CpG oligodeoxynucleotide (PF-3512676), in two dose sizes, was tested alone or with dacarbazine for 184 advanced-melanoma patients; however, no CRs were seen and ORRs were low in all groups, indicating that the type of CpG and choice of combination appears critical.¹⁶⁰ Other TLR9 agonists in clinical trials are mostly in combination with immune checkpoint drugs: IMO-2125, MGN1703 (lefitolimod) and DV281. Activators of TLR7/8, including NKTR-262 and MEDI9197, have also entered trial phases.

TLRs recognize distinct PAMPs and DAMPs. For example, TLR2 recognizes lipoproteins and peptidoglycans, TLR3 viral dsRNA/viral RNA analogue polyinosinic-polycytidylic acid; TLR4 recognizes lipopolysaccharides, TLR5 bacterial flagellin, TLR7/8 single-stranded RNA; and TLR9 detects CpG-containing oligodeoxynucleotides. TLR2/6, TLR2 and TLR4 recognize

endogenous matrix and HSPs. TLRs approved for cancer therapy are the TLR2/4 agonist BCG, TLR4 agonist monophosphoryl lipid A and TLR7 agonist imiquimod. The TLR5 agonist flagellin-derived CBLB502 (entolimod) has entered phase II study in patients with advanced solid tumours.¹⁶¹ As mentioned above, imiquimod stimulates TLR7/8, and when driven by IL-2, can induce CRs (and PRs; SDs) through both local and systemic immune response activation, indicating that innate immune response activation can, in certain circumstances, drive antigen-mediated adaptive systemic vaccine responses.¹¹³

Stimulator of interferon genes [STING; also transmembrane protein 173 (TMEM173)], is a protein that is part of the innate immune system specifically capable of responding to intracellular pathogen infection (PAMPs). On exposure to, for example, intracellular viruses, mycobacteria and intracellular parasites, STING induces production of type-I IFNs (IFN- α and IFN- β) that have a cell protective effect for the infected and locally surrounding cells to limit and reduce further infection. DCs are activated by STING which is a mechanism for activation of T-cell adaptive immunity. Agonists based on the STING structure are currently in clinical trials (e.g. ADU-S100/MIW815 and MK-1454) where the agents are being injected intratumorally to stimulate T-cell activation and tumour-cell destruction (DAMP and antigen release). The systemic side effects of STING agonists can be significant with pyrexia, systemic inflammatory responses and autoimmunity.^{155,162}

Tim-3

Another immune checkpoint is T-cell immunoglobulin and mucin domain-3 (Tim-3), which acts as a negative regulatory molecule to induce immunological tolerance and T-cell exhaustion. Tim-3 is expressed on T-effector and regulatory cells, dendritic cells, B cells, macrophages, NK cells and mast cells, and also on a wide range of tumour cells. Tim-3 can reduce antitumour immunity through blocking γ -IFN and IL-2 production, as well as Th1-cell depletion. Tim-3 expression has been related to survival in colorectal, lung, renal, prostate, and cervical cancer, and increased Tim-3 expression has been related to anti-PD-1 resistance to therapy. Anti-Tim-3 antibodies are being

clinically trialled in combination with anti-PD-1 antibodies and other agents.¹⁶³

Numerous other checkpoint molecules and agents are further reviewed in greater detail elsewhere,¹⁵⁵ together with how they might influence acquired resistance to the antitumour immune response and immunotherapies.¹⁶⁴

Cellular therapies

These include treatments under the categories lymphokine-activated killer cell, TIL, adoptive cell therapy, T-cell receptor TIL therapy, autologous circulating T cells targeting either unspecified tumour-associated antigen or a tumour-specific antigen, chimeric antigen-receptor T-cell (CAR T) therapies, other experimental T-cell therapies based on pluripotent stem cells, CRISPR or $\gamma\delta$ -T cells, or cell therapies based on DCs, NK or NK T cells, macrophages or other cell types. Some of these therapies have been associated with a variety of lymphodepletive and other preconditioning regimens such as marrow ablation, using cytarabine and intensive whole-body radiation. The fact that lymphocytes can be cultured or engineered and reinfused to produce homing and specific tumour killing demonstrates the ability of the immune system to treat cancer, and reinforces the *in vivo* actions of anticancer vaccines to produce effective lymphocyte clones. Combined therapies, including cellular therapies as a component are being trialled clinically. The field is broad and emerging, even though some aspects have been decades in development, and many of the therapies are currently heavily individualized and costly. Toxicity, including death, has been a problem in some studies leading to holds placed on some trials, but the technique looks promising with future refinement and generalization potentially making it more cost effective on a larger scale, especially if allogeneic methods are developed. Cellular therapies have been reviewed in a number of articles and are not detailed further here.¹⁶⁵⁻¹⁷⁵

Concluding remarks: vaccination as the basis of immunotherapy

What is so remarkable is that many vastly different treatment modalities for cancer can all induce a definite, but often small, rate of complete clinical regression responses. This fact is highly indicative that a common underlying mechanism is

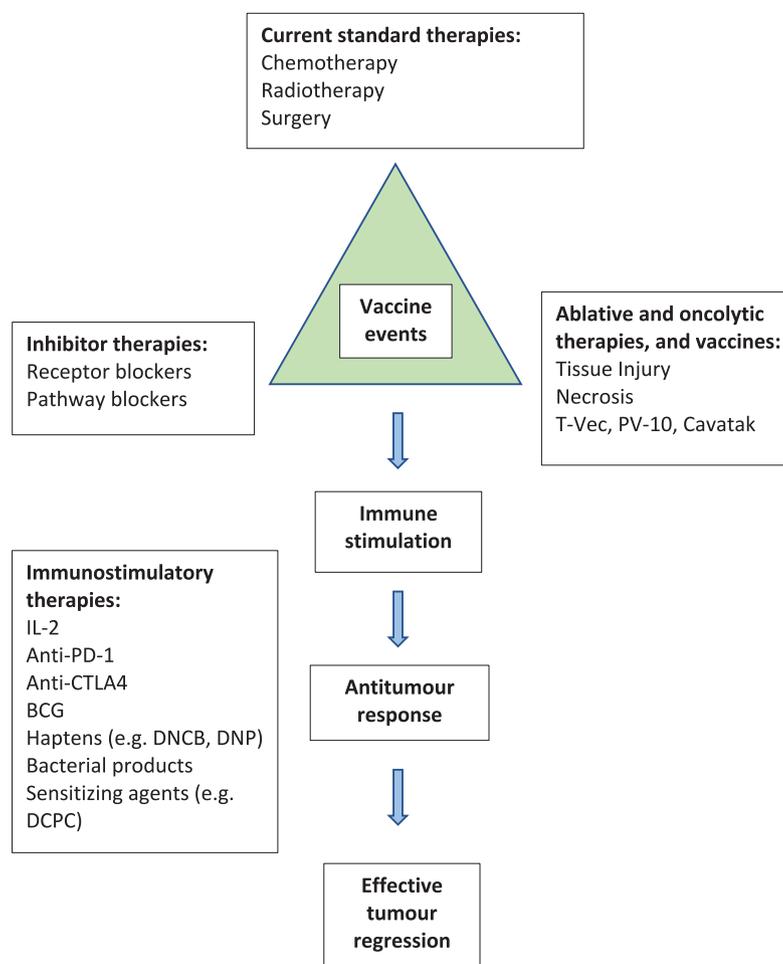


Figure 3. All cancer therapies can lead to *in vivo* vaccination events.

T-Vec, talimogene laherparepvec; PV-10, Rose Bengal pink dye; IL, interleukin; PD-1, programmed cell-death 1; CTLA, cytotoxic T-lymphocyte antibody; BCG, *Bacillus Calmette-Guérin*; DNCB, 2, 4-dinitrochlorobenzene; DNP, dinitrophenyl; DCPC, diphencyprone.

likely responsible and is operating to cause this effect and observation. Moreover, it is rapidly emerging that for almost all cancer therapies, the immune system is either directly or indirectly implicated. For example, small-molecule BRAF inhibitory therapies appear dependent on an immune infiltrate into the tumour occurring and are severely reduced or abrogated when the immune system is depleted. Clearly, the checkpoint inhibitors and IL-2, IFNs, T-Vec and vaccines, all being immune stimulants, function through the immune system for their activity. It is rapidly emerging that radiation therapy, chemotherapy, electrochemotherapy, oncolytics, cell-pathway inhibitors and receptor-blocking agents have an immunomodulatory role too, and that due to the ability of these agents to damage or lyse

and kill cancer cells, those apparently cytotoxic therapies can provide release of tumour antigens which constitute active waves of *in situ*, *in vivo* vaccine events; in essence, they are performing as vaccines to cause their effects (Figure 3).

The tumour microenvironment (TME) comprises tumour cells, cancer-associated fibroblasts, endothelial cells, myelomonocytic cells, MDSCs, TILs, extracellular matrix, and vessels (arterial, venous and lymphatic), nerves, and a variety of peritumoural stromal vascular and leukocytic cells (some normal and some tumour influenced). This level of complexity has made understanding of the interaction between all of these elements in successful and unsuccessful regression of cancer enigmatic.

The phenomenon of peritumoural cuffing of lymphocytic infiltrates has been observed in a number of therapeutic approaches and has been related to successful outcomes. Indeed, the relationship between lymphocytic infiltration and survival has been historically noted for a long time in breast^{176, 177} and many other malignancies. A possible future direction would be more detailed studies of the TME across many different modalities of therapy (and tumours) in order to investigate for possible common features or differences that might more clearly indicate similar underlying mechanisms associated with response or failure. For example, the state of the TME before therapy, such as the activation status of the T cells prior to any therapy, has emerged as pivotal. In general terms, tumours that are ‘hot’ and have higher levels of inflammation with activated T cells and low regulatory-cell densities appear to be better positioned to respond to immunotherapies, while tumours that are ‘cold’ and are in a suppressed state with a low infiltration of T cells respond less well to immunotherapies. One of the key approaches is therefore to modulate ‘cold’ tumours into ‘hot’ ones to prepare the TME for more successful immunotherapeutic intervention.

As we understand more about the immunological mechanisms associated with the therapies above capable of causing tumour regression, that is, antigen release, neoantigen unmasking/exposure, the control of antigen recognition, immune responsiveness, immunosuppressive T-reg responses to inhibit TIL activation, suppressor macrophages, augmentation of initially weak antitumour responses into effective tumour killing, how innate immunity interacts with adaptive responses and how these relate to natural vaccination responses that continually occur to a vast range of exogenous and endogenous antigens throughout life, the prospect of *in vivo* vaccination and immunomodulation will become clearer. To generate an effective antitumour immune response, antigen must be released/supplied, immune recognition must occur and immune responsiveness proceed.

In essence, whenever tumour cells are killed, antigen release can provide repeated *in vivo* **vaccination events**. Most of the treatments used for cancer outlined above require **repetitive cycles of therapy** to achieve cancer regression when it occurs. This aspect is now emerging as possibly the most important mechanism by which

perhaps the entire range of standard and newer cancer therapies are exerting their anticancer effects, by causing waves of antigen release and **repetitive** *in vivo* vaccination events.

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