Expert Opinion

Delivery strategies of melanoma vaccines: an overview

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For many years, various cancer vaccines have been widely evaluated, however clinical responses remain rare. In this review, we attempt to address the question of which delivery strategies and platforms are feasible to produce clinical response and define the characteristics of the strategy that will induce long-lasting antitumor response. We limit our analysis and discussion to microparticles/nanoparticles, liposomes, heat-shock proteins, viral vectors and different types of adjuvants. This review aims to provide an overview of the specific characteristics, strengths and limitations of these delivery systems, focusing on their impacts on the development of melanoma vaccine. To datze, only adoptive T-cell transfer has shown promising clinical outcomes compared to other treatments.

Keywords: adjuvant, liposomes, melanoma, microparticles, vaccine viral vector

Expert Opin. Drug Deliv. (2008) 5(9):979-1001

1. Introduction

Vaccine has played a vital role in the prevention of infectious diseases worldwide ever since its discovery in the 1960s; however cancer vaccine has yet to live up to expectations in a prophylactic as well as a therapeutic setting. A large number of human clinical trials have been initiated, mostly in stage III and intravenous (i.v.) melanoma patients using different categories of antigens (Ags), including whole tumor cells or tumor cell lysate (autologous or allogeneic), DNA vaccines (syngeneic or xenogeneic), recombinant peptides and viral vector encoding genes for tumor-associated antigens (TAA) or immunostimulatory cytokines, along with different types of adjuvants including incomplete Freud's adjuvant (IFA), alum, Bacillus Calmette-Guérin (BCG) and cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-α (IFN-α), interleukin-2 (IL-2) and IL-12 [1,2]. These clinical trials showed various degrees of success based on different end points defined by the researchers, therefore it is difficult to compare these results. However, the common trend observed through these trials was that the induction of T-cell response failed to correlate with the clinical outcome. Clinical response was poor; partial and complete response was relatively rare in these clinical trials. To date there has been no significant objective response observed in clinical trials and currently no melanoma vaccine is approved by the FDA and none is available on the market.

The poor clinical response in human trials has highlighted the issues and barriers faced by cancer vaccine in the therapeutic setting. The distinction between prophylactic and therapeutic vaccine is lost in the translation from animal trials to human clinical trials. In general, prophylactic vaccine confers long immunity by the induction of both humoral and cellular immune response and is given to healthy patients for disease prevention. Therapeutic vaccine, in contrast, is given to patients to cure their diseases. Therefore, in a therapeutic setting, an immediate or rapid immune response is required to slow and halt the growth of tumors via the induction of cytotoxic T lymphocytes. This immune response is usually short-lived,

10.1517/17425240802333322 © 2008 Informa UK Ltd ISSN 1742-5247

due to the failure to induce $CD4^+$ T cells, the immunosuppressive microenvironment created by the tumors and the weakened immune system of the patient due to chemotherapy, radiotherapy and antineoplastic drugs [3]. The ill correlation between clinical outcome and animal trials indicated that alternatives to murine models – such as dogs and cats – may be closer models to humans, as the biology of their tumors possess great similarities to humans and because they developed these tumors naturally and live in similar environments to humans. The first xenogeneic DNA vaccine was approved by the USDA in 2006 to treat malignant melanoma in dogs [4-6]. The success and the findings of cancer vaccines in the veterinary world should help to shed light on our efforts to create effective cancer vaccines.

In addition, there are other barriers faced in the quest to create a cancer vaccine including, but not limited to, priming of naïve T cells, migration of activated T cells to tumor sites and T-cell effector function in the tumor environment, delivery vehicles, route of administration, rapid tumor cell mutation and clone selection and tumor burden [7,8]. In order for T cells to kill the tumor cells and eventually reject the tumors, first the T cells need to be activated by antigen-presenting cells (APC) cells, mature and proliferate. However, this can be inhibited by various factors including CTLA-4, immunosuppressive cytokines such as tumor growth factor beta (TGB-β) and IL-10, the lack of toll-like receptors (TLRs) signals and γ-chain cytokines that promote T-cell growth and proliferation. Second, the activated T cells face some obstacles in migration to tumor sites such as lack of inflammatory signals and chemokines expression on tumor cells and also lack of appropriate chemokine receptors on the activated T-cells. Finally, even if the activated T cells reach the tumor sites, tumors have evolved multiple strategies to escape T-cell recognition, such as the presence of regulatory T cells, coinhibitory B7 molecules and enzymes [8]. Moreover, the traditional delivery platform using injection via intraperitoneal (i.p.), subcutaneous, i.v. and intra-muscular routes was used in most of the clinical trials of cancer vaccine, either with or without adjuvants, as mentioned above. While a traditional delivery platform has proved to be effective in vaccination for various infectious diseases, it is less than ideal for cancer vaccines. A cancer vaccine should ideally be able to discriminate between cancerous cells and normal cells, be able to kill tumor cells and prevent the recurrence of tumors, whereas the delivery vehicle should be able to provide protection for the Ags from degradation *in vivo*, prolong the residence time of the Ags, be able to induce both Th1 and Th2 immune responses, and act as adjuvant at the same time. Advances in delivery platforms have created various delivery systems and new adjuvants in an attempt to improve vaccine delivery and more importantly to produce a better clinical response in humans. The delivery systems, vehicles and adjuvants are important components of a vaccine formulation beside the Ags, therefore in this review we discuss the delivery systems and adjuvants, as different types of melanoma vaccines have been discussed elsewhere [2,9].

2. Particle vehicles

It is well documented that particulates are taken up by immune cells such as macrophages, dendritic cells (DCs) and M cells [10-21] and they also function as an adjuvant [22,23] as a result of their particulate nature. The fate of these particulates and the immune response induced depends on their characteristics, particularly their size and processing pathways after being taken up [24-26]. However, the mechanisms behind the processing pathways which are responsible for either Th1 or Th2 responses remained controversial. To date, few mechanisms have been proposed to explain the intricate relationship between the particulates' characteristics and the induction of different arms of immune responses. For example, it was proposed that to induce a cytotoxic response, the particulates have to escape endosomes and be available in the cytoplasm to be processed, bound to major histocompatibility complex (MHC) I and presented by APCs to the $CD8^+$ T-cells [25]. In contrast, some of the particulates were trapped in the endosomes, processed and bound to MHC II to be presented to $CD4^+$ T-cells, which would led to antibody production. These two arms of immune response (cellular and humoral) are critical to tumor rejection and their effector functions may be synergistic, although conflicting reports suggest that only the cytotoxic response is important to the success of tumor rejection [7,27]. Rarely does a delivery vehicle possess the ability of inducing both arms of immune response, therefore particulates hold great promise as a delivery vehicle for tumor vaccines.

2.1 Microparticles and nanoparticles

Microparticles and nanoparticles are particulate matters which are made of synthetic or natural polymers such as poly (D,L-lactic-co-glycolic acid) copolymers (PLGA), polylactic acid, alginate, albumin, chitosan, polystyrene, gold, silica, etc. These particulates are mostly spherical or sometimes irregular in shape and possess different characteristics such as porosity, hydrophobicity, surface charge and size. These characteristics can be altered to produce carriers with desirable attributes such as the controlled release of encapsulated drugs, acid resistance, charged surface and antibody or ligand conjugation. Moreover, proteins and drugs can be adsorbed, conjugated or encapsulated in these particulates and have the flexibility of tailoring their characteristics, making them a versatile platform for vaccine delivery. Furthermore, micro or nanoparticles can be conjugated with ligand or antibodies that target the surface molecules or receptors overexpressed by tumor cells, thereby reducing systemic adverse effects and damage to normal cells.

The most widely used polymers for micro and nanoparticle fabrication are biodegradable and biocompatible polymers such as PLGA. This is an important requirement for any

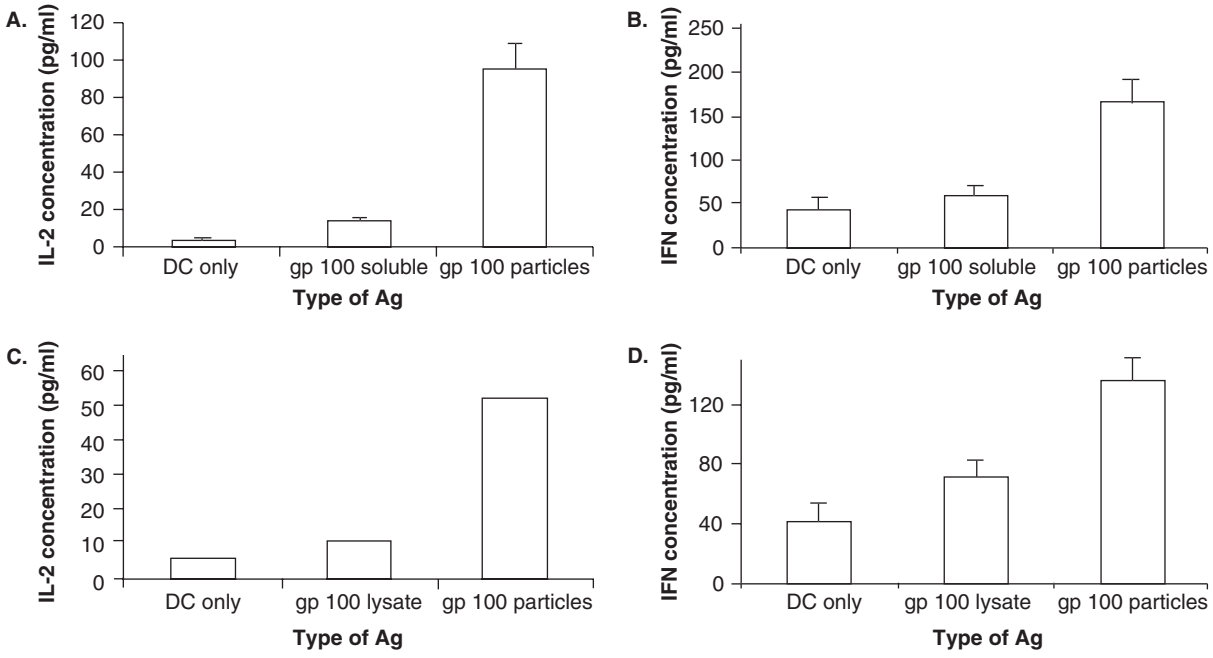

Figure 1. Enhanced stimulation of naïve gp100-specific CD8⁺ T-cells after presentation of nanoparticle-encapsulated Ag by DC.
Experiments were performed with gp100 protein (**A** and **B**) and gp100 protein within a murine B16 melanoma cell lysate (**C** and **D**). T-cells were evaluated for secretion of IL-2 (**A** and **C**) and IFN-ç (**B** and **D**).
Reprint with permission from Solbrig *et al.* Polymer nanoparticles for immunotherapy from encapsulated tumor-associated antigens and whole tumor cells. Mol Pharm 2006;4(1):47-57. Copyright (2006), American Chemical Society.

delivery vehicles intended for human administration and seeking approval by the FDA because safety is a major concern. Using interleukin-1 receptor antagonist (IL-1Ra) encapsulated PLGA microparticles, Lavi and colleagues were able to significantly prolong the survival of treated mice compared to empty microparticles and a non-treated group with $p < 0.0005$ in B16 murine melanoma model [28]. In addition, they also demonstrated that IL-1Ra microparticles treatment was able to reduce the B16 melanoma lung metastases by 70% after removal of the primary tumor. This study also highlighted one of the advantages of using microparticles/nanoparticles as a delivery vehicle, which was the ability of these carriers to sustain the release of IL-1Ra for at least 7 days. Another interesting study was performed by Solbrig and colleagues using PLGA nanoparticles [29]. They displayed the flexibility of modifying the characteristics of PLGA nanoparticles to fulfil the requirements as an efficient delivery vehicle. For example, to modify the release profile and encapsulation efficiency, different molecular weights of PLGA, different amounts of stabilizer and protein loading was used. In the *in vitro* study performed using gp100-specific T-cells, they found that gp100 encapsulated in PLGA nanoparticles showed 10-fold and 3-fold enhanced stimulation of IL-2 and IFN-γ respectively, indicating increased levels of Th1-associated cytokine release (**Figure 1**). Further, a DC-based challenge study was performed in C57BL/6 mice. It found that vaccination with DCs loaded with B16 cell lysate nanoparticles showed the greatest protection against tumor growth with the smallest mean tumor volume (mm^2). However, surprisingly they also found that animals vaccinated with B16 lysate nanoparticles only showed the greatest tumor volume, which the authors suggested was due to tolerance induced by the nanoparticles. This observation needs to be further investigated; it may be related to the frequency and interval of vaccinations, as well as to the dose and numbers of nanoparticles injected. Although a number of researchers have shown that microparticles and nanoparticles were able to be presented to APCs at least 10^2- to 10^4-fold more efficiently than soluble proteins, the mechanism of the processing and presentation of exogenous Ag via MHC I presentation pathway remains controversial [30,31]. Recently, a study by Shen and co-workers has once again demonstrated that Ag encapsulated in PLGA nanoparticles were able to enhance and prolong cross-presentation of exogenous Ag *in vitro* [32]. They showed not only that ovalbumin (OVA) encapsulated in PLGA nanoparticles required 1000-fold less Ag to achieve a comparable level of Ag presentation compared to soluble OVA, but also that nanoparticles were able to enhance the stimulation of $CD8^+$ T-hybridoma cells. They further demonstrated that Ag delivered using PLGA nanoparticles increased the amount of Ag escaped from endosomes into cytoplasm via the extraction of cytosol and highlighted the prolonged cross-presentation of Ag as a result of the sustained release of Ag from the

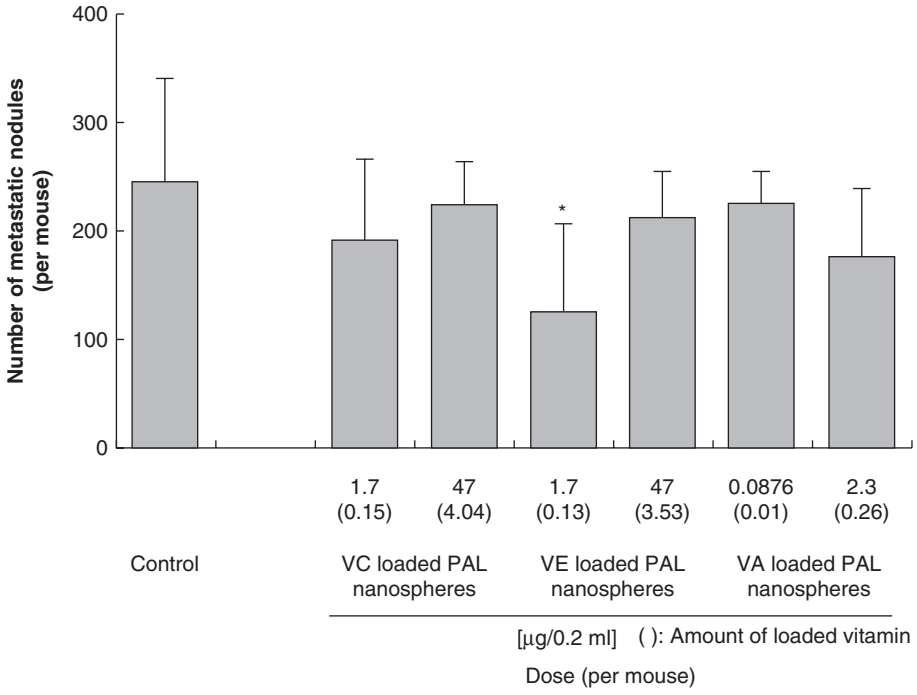

Figure 2. The number of metastatic nodules on the lung intravenously administered with VC, VE, and VA loaded PAL nanospheres.

*$p < 0.02$.

Reprint from Hara K *et al.* The effect of poly (aspartic acid-co-lactic acid) nanospheres on the lung metastasis of B16BL6 melanoma cells by intravenous administration. Oncol Rep 2006;16:1215-20. Copyright (2006), with permission from Spandidos Publications.

PAL: Poly(aspartic acid-co-lactic acid); VA: Retinol palmitate (pro-vitamin A derivative); VC: Ascorbyl tetraisopalmitate (pro-vitamin C derivative); VE:DL-alfa-tocophenyl acecatet (pro-vitamin E derivative).

PLGA nanoparticles. These observations were in agreement with the prior hypothesis that one of the mechanisms of exogenous presentation was via the escape of Ag into cytosol to be processed for the presentation by MHC I molecules [25,31-33,34]. In addition, PLGA microparticles encapsulated within synthetic peptides also demonstrated cytotoxic T-cell response following immunization via i.p. and oral routes [35-37].

In addition to PLGA, other polymers have also been evaluated for the fabrication of microparticles and nanoparticles for the delivery of various therapeutic agents such as antineoplastic agents, vaccines, DNA, proteins and peptides. One of the new omers is poly-(aspartic acid-co-lactic acid) (PLA), which is a biodegradable, bioadsorbable polymer developed over the past few years [38]. Hara and colleagues have produced PLA nanoparticles encapsulated with pro-vitamin A, C and E. They have shown that PLA nanoparticles encapsulated with pro-vitamin E injected intravenously was able to significantly reduce the metastatic foci count compared to control after challenge with B16BL6 melanoma cells (**Figure 2**). More importantly, they concluded that the PLA nanoparticles were safe to be administered intravenously from the perspective of cancer metastasis [39]. However, to serve as drug delivery carrier, further investigations need to be conducted to evaluate the effects of PLA, including immunogenicity and toxicity. Other biodegradable polymers such as gelatin, chitosan and alginate have long been used to fabricate microparticles and nanoparticles. For instance, gelatin in combination with chondroitin 6-sulfate was used to encapsulate and deliver IL-2 in malignant glioma in rat brains [40]. Alginate is another biodegradable polymer which also possesses muco-adhesive properties and has been used to encapsulate vaccines such as formalin-killed *Aeromonas sobria* (MVC). Sun and colleagues reported that the serum agglutinating antibody and the phagocytic activity of the blood monocytes of mice immunized orally with MVC were comparable to those of i.p. injection with formalin-killed *A. sobria*, significantly higher than the control and achieved the relative percentage survival of 87.5% [41]. Prior to that, alginate was used to encapsulate various vaccines administered via different routes and was shown to confer protective immunity in animal models [42-44]. Alginate microparticles were also bound to antibodies specific to the surface markers of murine macrophages, DCs and B cells. Brubaker *et al.* found that these antibody-coated microparticles bound specifically to APC *in vitro* [45]. This observation implied that theoretically these alginate microparticles can be used to target APC, enhance the uptake and translocation of these microparticles. Furthermore, according to the authors, protease and

hydrolase resistance properties of these microparticles might prolong the loading of Ags into class I or II MHC molecules and deliver a greater amount of Ags to the cellular compartment of APC. In addition, chitosan has also been evaluated as a drug carrier for various vaccines, DNA and therapeutic proteins with varying degree of success [46-51]. For melanoma vaccine, gold particles coated with gp100 was coadministered with GM-CSF using a burst of helium gas to accelerate the gold particles into the targeted cells [52,53]. The authors found that this epidermal particle delivery system resulted in significant protection from subsequent tumor challenge and coadministration with GM-CSF resulted in greater protection in a C57BL/6 mice model. Following that, a human clinical trial was initiated and although only a modest immune response was observed, this regimen yielded biologically active gene expression and greater infiltration of DCs into treated sites when compared to the administration of gp100 alone. The authors concluded that further investigations are needed to best utilize this epidermal particle delivery system to produce greater antimelanoma response.

2.2 Liposomes

Liposomes are phospholipid, either single or multi-lamellar layer vesicles, which have long been evaluated for the delivery of various agents including DNA, therapeutic proteins, antineoplastic agents, bacterial and viral components. As liposomes are also particulate in nature, they possess some similar characteristics to microparticles and nanoparticles. Therefore, they are capable of serving as adjuvant and enhancing the uptake of APCs, as well as inducing both humoral and cellular immune response via MHC class I and II pathways [54-56]. The ability of liposomes to present exogenous Ags was found to be similar to that of viruses. Yewdell and colleagues found that this ability depended on the fusion of viral or cellular membrane and the delivery of viral proteins and Ag to the cytosol [57]. Therefore, one way to introduce exogenous Ags to cytoplasm is using the fusogenic properties of acid-sensitive liposomes [58-60]. Yoshikawa and colleagues have encapsulated B16 melanoma cell lysate in pH sensitive liposomes and showed that *in vivo* and *ex vivo* immunization induced anti-B16 melanoma prophylactic effects [61]. In the *in vivo* direct immunization and *ex vivo* DCs pulsed manner, fusogenic liposome encapsulated with tumor cell lysate delayed the tumor growth compared to the control. The authors explained that the enhancement of Ag presentation to APC was resulted from the rapid fusion of plasma membrane and direct delivery of the encapsulated Ags into cytoplasm. Despite the ability of the fusogenic liposome to delay tumor growth, the use of Sendai accessory proteins would be a concern from a safety standpoint in a clinical setting. Besides the fusogenic liposome, Jerome and co-workers have shown that administration of cationic liposomes in combination with CpG oligonucleotides (CpG ODN) adjuvant led to the induction of tumor cell specific immune response [62]. They have shown that the cationic liposome, but not neutral liposome, selectively bound to DCs but not T-cells and the encapsulation of tyrosinase-related protein 2 (TRP2) peptide into these cationic liposome achieved similar T-cell response with 10-fold less TRP2. This observation once again highlighted the fact that encapsulation is one of the prerequisites for improved immune response. In addition, the authors demonstrated that low dose liposomal TRP2 induced high avidity of T-cells, which has been shown to be important in antitumor response and tumor rejection [63,64]. These cationic liposomes encapsulated with TRP2 were shown to form an Ag depot up to 7 days and were delivered to the lymph nodes. More importantly, they showed that the TRP2 liposome induced a protective immune response against B16 melanoma tumor challenge *in vivo* in both prophylactic and therapeutic models. However, tumor rejection was only observed in 20% of the vaccinated mice.

Liposomes fabricated to target APCs, particularly DCs and macrophages, have also been explored by various researchers. Lu and co-workers have fabricated cationic liposomes using (Man-C4-Chol) ,which was aimed to target mannose receptor on APCs and also provide an amino group for pDNA binding [65]. They found that man liposomes enhanced the delivery of pUb-M into peritoneal macrophages approximately five times compared to the unmannosylated liposomes. Different routes of administration for liposomes were also investigated and they found that the i.p. route resulted in significantly higher cytotoxic lymphocyte activity compared to intramuscular, intradermal or subcutaneous administration. Moreover, they demonstrated that unmannosylated liposome enhanced gene delivery approximately 50 times into splenic DCs and 15 times into macrophages, as compared to naked gene delivery. In addition, the mannosylated liposomes demonstrated significant gene delivery enhancement into splenic DCs, but not macrophages, when compared to the unmannosylated liposomes. This showed that both the unmannosylated and mannoasylated cationic liposomes significantly enhance gene delivery into splenic DC and macrophages compared to naked gene delivery without vector. More importantly, mice innoculated with the mannosylated liposomes produced significantly higher IFN-γ, B16BL6-specific lymphocyte proliferation, blocked melanoma propagation and prolonged the survival of immunized mice (**Figure 3**). Taken together, these observations demonstrated that the effectiveness of cationic liposomes can be further enhanced by modifying the liposomes to target APCs. Optimization in size, charge ratio, spacer length of mannosylated liposomes and phospholipids used may also be explored to enhance the delivery efficiency of liposomes.

Another approach to targeting APCs that been explored is using TLR3 and TLR9 agonists (polyinosinic–polycytidylic [poly IC], plasmid DNA, CpG oligonucleotides) complexed with liposome. This complex has also been shown to act as adjuvant. Zaks and colleagues found that TLR3 and TLR9 agonist complexed liposome and OVA elicited strong $CD4^+$

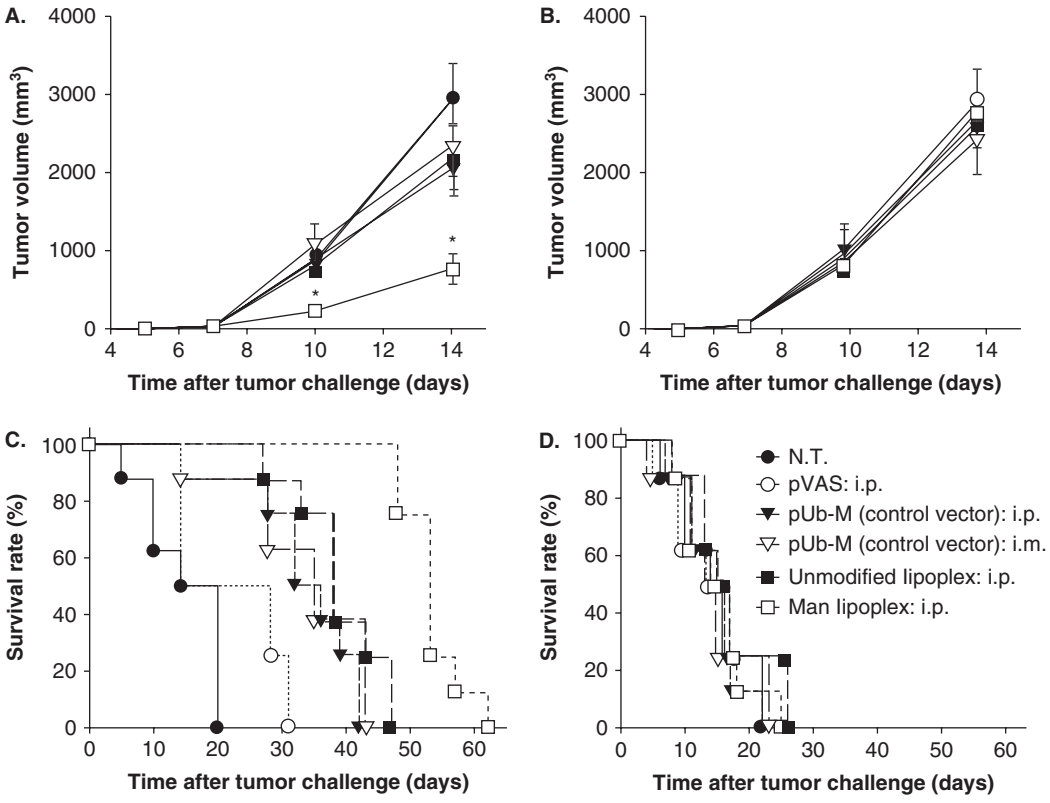

Figure 3. Induction of tumor-protective immunity. Mice were inoculated with 50 mg pUb-M as naked or Man lipoplex three times at 2-week intervals. Two weeks after the last immunization, B16BL6 cells or EL4 cells (1×10^5 cells) were inoculated subcutaneously into the back of the mice. The tumor volume (**A** and **B**) was evaluated (each value represents the mean ± SD) and the survival (**C** and **D**) of the mice was monitored up to 100 days after the tumor challenge (n = 8). Differences in B16BL6-specific immunity between experimental groups treated with i.p. administration of Man lipoplex and other groups were statistically significant.
*$p < 0.01$.
Reprint from Lu *et al.* Development of an antigen-presenting cell-targeted DNA vaccine against melanoma by mannosylated liposomes. Biomaterials 2007;28:3255-62. Copyright (2007), with permission from Elsevier.

and CD8+ T-cell responses even at a dose as low as 1 µg. They also found that the i.p. route was the most efficient route of administration for these liposome complexes. The induction of strong CD4+ and CD8+ T-cell responses was attributed to the physical association of DNA and Ag to liposome and this effect disappeared when only two components of this complex was inoculated. More importantly, they also demonstrated that T cells induced by this complex via immunization were functionally active and long-lived. Large numbers of T memory cells were also detected in the lung 3 months after the first immunization in mice. They further showed that immunization with TRP 2 peptide and the complexed liposome in B16 melanoma model managed to control the growth of established tumors [66].

As mentioned above, CpG, a TRL9 agonist, is widely used as adjuvant in vaccines to enhance CD8+ T-cell response [67-73]. It has also been found in various studies that a requirement for its adjuvancity is physical association with the Ags usually achieved by chemical conjugation [74-76]. Co-encapsulation of CpG ODN with Ag in liposome was also shown to magnify the immune response [77,78]. Li and co-workers demonstrated the important of association of CpG ODN with Ag via co-encapsulation as a prerequisite for CpG to demonstrate its adjuvancity using p63 – 71 as Ag. They found that co-encapsulation of p63 – 71 with CpG in the same liposome induced an approximately 140-fold higher frequency of IFN-γ producing cells and a significant higher immune response compared to p63 – 71 alone (**Figure 4**) [79].

In the industry setting, liposome-based platform technology such as VacciMax (VM) developed by Immuno Vaccine Technologies (IVT; Halifax, Nova Scotia, Canada) showed promising results in inducing an effective cytotoxic response to multiple tumor-associated Ags (TRP2 and mutated p53) and the rejection of 6-day-old tumor in B16-F10 melanoma mice model after a single injection of VM [80]. In agreement with previous reports, they demonstrated that encapsulation of multiple Ags in liposomes resulted in a better immune response than single Ag [81]. In addition, they found that co-encapsulation of CpG oligonucleotide with Ags in liposomes significantly

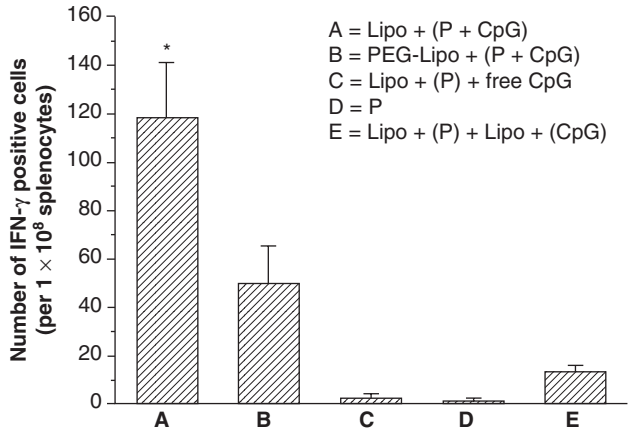

Figure 4. Co-encapsulation of CpG ODN with p63 – 71 within the same liposomes are more effective to activate cellular immune response. BALB/c mice were immunized once (day 0) via the i.v. route with p63 – 71 and CpG ODN co-encapsulated in plain liposomes (**A**) or PEGylated liposomes (**B**), p63 – 71 encapsulated in liposomes plus free CpG ODN (**C**), p63 – 71 alone (**D**) or p63 – 71 and CpG ODN encapsulated in separate liposomes (**E**). On day 8, mice were terminated to monitor the IFN response of isolated splenocytes by ELISPOT. Isolated splenocytes were cultured for 24 h with and without p63 – 71 in the ELISPOT plate, as described in Section 2, above. The p63 – 71-specific response was obtained by subtracting the frequency of IFN-positive cells obtained without p63 – 71 stimulation as background. Data presented are averaged results obtained from four mice ± SEM. One-way ANOVA was performed for statistical analysis. $p < 0.05$ was considered significant.

*Denotes significantly different from all other groups.

Reprint from Li *et al.* Effective induction of CD8+ T-cell response using CpG oligodeoxynucleotides and HER-2/neu-derived peptide co-encapsulated in liposome. Vaccine 2003;21:3319-29. Copyright (2003), with permission from Elsevier.

increased the number of Ag-specific splenocytes, which is in concert with previous studies [62]. Moreover, the VM was shown to overcome the immuno-dominancy by one Ag over other Ags *in vivo*. The stabilization of liposome formulation in water-in-oil emulsion is one of the advantages of this platform compared to other liposome formulations. The presence of oil in this formulation may also contribute to the potent adjuvancity and effectiveness of this formulation in the rejection of melanoma tumors in mice, as water-in-oil emulsion such as Freud's complete and incomplete adjuvant have long been used as adjuvant in vaccines, although their use in humans is banned in the US [82,83]. However, the recurrence of tumor in the mice that had been rendered tumor-free shows that there is still room for optimization in terms of immunization schedule and frequency, dose and liposome formulation. To date, this is a promising vaccine delivery platform for cancer vaccines and it is hoped that its efficacy *in vivo* can be replicated in human clinical trials in the near future.

3. Heat-shock protein

Heat-shock proteins (HSPs) are induced when normal cells are stressed by heat, cold, oxygen and other key elements for survival. HSPs serve as chaperones in helping misfold proteins to fold into the right conformation for them to be functional. They also deliver proteins to different cell compartments and, more importantly, loading proteins on MHC molecules in order for them to be recognized by circulating immune cells. As a result of these functions, HSPs are usually bound with a wide array of proteins inside the cells. HSPs frequently transport abnormal peptides to molecules that are responsible for the presentation of these peptides on the cell surface, which are recognized by immune cells. In cancerous cells, when necrosis occurs, HSPs bound with tumor Ags are released into the extracellular fluid and provide the danger signal for the induction of a vigorous immune response. The HSP carrying library of tumor Ags attract the infiltration of APCs such as DCs and secrete cytokines that prompt APCs to present and deliver tumor Ags that result in the induction of cellular immunity. Thus, the role of HSPs in cancerous cells makes them an ideal carrier for tumor-associated Ags and a good candidate for cancer vaccines. Furthermore, it has been reported that HSP gp96 binds to receptors such as CD91, which is expressed primarily on DCs [84]. Other receptors for HSP are also found on macrophages and platelets.

Across the spectrum of cancers, rapid changes of Ag expression on tumor cells is one of the tactics adopted by tumor cells to evade the immune surveillance of the immune system. This is also a stumbling block for most cancer vaccines that target a single or a combination of few Ags which may be successful at the beginning of the treatment, but the tumor cells will downregulate the Ags that are recognizable by immune cells and upregulate new Ags. In addition, different cancer patients demonstrate different sets of tumor-associated Ags. Therefore, attempts have been made to use HSPs that are bound with a library of tumor-associated Ags as cancer vaccines.

An investigational, patient-specific cancer vaccine called Oncophage® has recently been granted fast track and orphan drug designation by the FDA for the treatment of metastatic melanoma and kidney cancer. Phase III clinical trials for the treatment of metastatic melanoma have been conducted and they found that patients who received ≥ 10 doses of vaccine survived longer than those who received the physician's choice of treatment, such as IL-2 and/or dacarbazine/temozolomide and/or tumor resection (377 vs 478 d; $p = 0.072$) and it conferred a qualitative survival benefit over the physician's choice for M1a melanoma patients but not M1b and M1c patients [85]. However, a similar success has yet to be repeated in wider patient populations and an objective response has yet to be seen.

Besides HSP96 (gp96), Udono and Srivastava have found that HSP70 extracted from tumor cells can act as a tumor

Ag carrier and elicited antitumor immunity [86]. Recently, Geng and co-workers have shown that the blockade of B7-H1 could reverse tumor resistance and enhance the therapeutic efficacy of melanoma vaccine in a pulmonary metastatic melanoma model. Their findings suggested that the blockade of tumor B7-H1 using sPD-1 following HSP70 vaccination may provide a promising approach for tumor immunotherapy [87]. This is a step forward in designing cancer vaccines, and other mechanisms of tumor escape should be explored to effectively block them in order to produce an effective cancer vaccine.

Several key elements of HSP have been identified as requirements for HSP to induce antitumor immunity. Park and co-workers have found that grp170 is responsible for the immunoadjuvancity of HSP70 and a direct correlation between chaperone function, binding to APCs and antitumor immunity was observed [88]. Interestingly, they also found that chaperone function, but not the sequence of HSPs, is crucial to the APC binding, Ag presentation and induction of antitumor activity. To avoid the tedious procedure of extracting HSP from tumor cells, attempts have been made to produce recombinant HSP. Zhang and co-workers have successfully produced a recombinant ATPase domain of HSP which has been coupled with TRP2. These vaccines exhibited ATPase activity, managed to elicit Ag-specific cytotoxic T lymphocyte (CTL) *in vivo* and delayed tumor growth on B16 melanoma mice model [89]. However, this approach suffers from the aforementioned rapid changes in Ag expression on tumor cells as a mechanism for tumor cells to evade immunosurveillance.

From clinical trials performed using HSPs, it seems that understanding of the complex tumor escape mechanism is key to designing cancer vaccines that are capable of rejecting tumors, avoiding remission and prolonging the survival of cancer patients. Thus far, none of the investigative approaches stands out as highly effective in treating melanoma cancer and objective response and disease-free state is rare among patients. However, they all hold promise and in light of the advances made in understanding the escape mechanisms of tumors, it is hoped that this approach will become one of the platforms of cancer vaccine.

4. Adjuvant

Adjuvant is a substance that is used to induce vigorous immune response. Adjuvant helps to augment immune response by forming a depot and therefore enabling the sustained release of adjuvant that helps to prolong the immune response. It also helps to deliver Ags efficiently to APCs or act as Ag carriers, resulting in recognition by immune cells. The particulate nature of the adjuvants also promotes the efficient delivery of Ags to APCs for presentation to MHC molecules. In addition, the induction of innate immune response via TLRs is also another mechanism being explored to induce adjuvant effects. A current hypothesis suggested that the toxicity of adjuvant may be caused by the necrosis of the APCs which ingested the adjuvants coupled with Ags. When necrosis occurs, the cell membrane is compromised and the adjuvant–Ags and inflammation mediators are released into cytoplasm inducing vigorous immune response. At present, alum is the only approved adjuvant for human use in the US, whereas incomplete Freud's adjuvant (IFA) is used in humans in some countries. Different categories of adjuvant have been developed over the years which are used in combination with cancer vaccines; they include incomplete alum, IIFA, BCG, monophosphoryl lipid A (MPL), purified saponin like QS21, water-in-oil emulsion such as Montanide ISA51 and oil-in-water emulsion such as MF59, immunostimulating complexes (ISCOMS), TLR agonists such as CpG oligonucleotides and cytokines such as GM-CSF, IL-12 and IL-2. The role of adjuvant is even more important in melanoma vaccine as melanoma Ags are poorly immunologic [90,91].

Different types of adjuvant elicit their effects via different mechanisms and currently some mechanisms remain unclear. Some adjuvant bias towards the induction of CTL response such as CpG, IL-12 and live vector, whereas other adjuvants promote Th2 response such as alum, heat labile enterotoxin (LT) and chlorella toxin (CT) [92]. Therefore the choice of adjuvant is an important part in the process of designing a cancer vaccine. The adjuvants that have been used in melanoma vaccine clinical trials are depicted in **Table 1**.

4.1 Toll-like receptors

One category of adjuvant being explored is TLR agonists such as CpG, lipopolysaccaride (LPS) and imidazoquinolines (**Table 2**). These adjuvants activated APCs by binding to the TLRs expressed on the surface APCs. One adjuvant from this category is CpG. CpG derived from the unmethylated CpG motifs from bacteria plasmid DNA and the synthetic form of CpG oligonucleotides (CpG-ODN) has widely been used as adjuvant in vaccines. CpG is agonist for TLR9, which is expressed on B cells and DCs in human. It exerts its adjuvancity by triggering the release of inflammatory cytokines such as IL-12, TNF-α and IFN-γ and steers the immune response towards Th1 response and induction of CTL. As a result of these properties, CpG has been widely used in trials for cancer vaccines such as melanoma vaccine. Spieser and co-workers have shown that CpG 7909 administered monthly with Melan-A and IFA for four vaccinations elicited a significantly higher frequency of Melan-A-specific $CD8^+$ T-cells compared to a control group without CpG. They also demonstrated that the CpG treated group showed a mean of 43-fold higher Melan-A-specific $CD8^+$ T-cells frequencies before vaccination versus only 1.9-fold for the group without CpG [93].

Imiquimod is another adjuvant that has been used in melanoma vaccine and belongs to the imidazoquinolines family, which is an agonist for TLR7. A study by Prins and colleagues in CNS tumor-bearing mice model showed that

Table 1. Clinical trials for melanoma vaccines using adjuvants.

Disease stage	Vaccine	Adjuvant	Ref.
II, III, IV	MART-1	IFA	[191]
III, IV	Canvaxin	BCG	[192]
IV	Tyrosinase	GM-CSF	[193]
IV	NY-ESO	GM-CSF	[194]
III	Canvaxin	BCG	[195]
IV	Canvaxin	BCG	[196]
IV	Melacine™	DETOX	[197]
IV	Tyrosinase, gp-100	GM-SCF, IFA	[198]
III, IV	Melan-A (MART-1)	IL-12	[199]
IV	HLA matched peptides	IFA	[200]
IV	MAGE-A12:170 – 178	IFA	[201]
Not reported	Fowlpox viruses encoding gp-100	IL-2	[202]
III, IV	NY-ESO-1	ISOMATRIX	[203]
III	Hapten modified vaccine	BCG	[204]
IV	NY-ESO-1	IFA	[205]
II-IV	gp-100 and tyrosinase peptides	IL-2	[206]
IV	Melan-1/MART-1, gp-100	Interferon-α	[207]
II, III, IV	Tyrosinase, MART-1, gp-100	IFA, IL-2, alum, GM-CSF	[208]

Table 2. Toll-like receptor ligands.

Human TLR	Ligand
TLR3	dsRNA, polyinosinic-polycytidylic (PolyIC)
TLR4	LPS, MPL, AGP (aminoalkyl glucosaminide
TLR5	4-phosphate), HSP, fibrinogen
TLR7/8	Flagellin
TLR9	Imidazoquinolines e.g., Imiquimod, ssRNA, CpG

5% of imiquimod in combination with melanoma-associated Ag (MAA) peptide-pulsed DC vaccination resulted in significantly greater protection against brain tumors [94]. However, the decrease in tumor growth was accompanied by an increase in inflammation-induced mortality, implying that the dose and timing of administration is key to the successful use of imiquimod as adjuvant in cancer vaccines. However, TLR agonists suffer from reactogenicity due to the activation of inflammatory cytokines such as TNF-α and induction of autoimmunity in susceptible individuals.

Agonists for TLR4 such as LPS and monophosphoryl lipid A (MPL) is also a potent immunostimulant. LPS is derived from bacteria and contains lipid A moiety, which is a potent immunostimulating agent but very toxic. MPL was derived from *Salmonella minnesota* and was developed to replace LPS with reducing toxicity and reactogenicity. MPL binds to TLR on macrophages and DCs and caused the release of pro-inflammatory cytokines such as TNF-α, IFN-γ and IL-2 leading to the induction of Th1 response. MPL has been used in cancer and infectious diseases vaccines including Melacine® (Corixa Corporation, Seattle, Washington), a melanoma vaccine, HPV, malaria, hepatitis B, genital herpes vaccines and tuberculosis [95-100]. It has been shown to have lower toxicity compared to alum and IFA, potentiated humoral response, but had no effect on the delayed type hypersensitivity (DTH) response to melanoma vaccine, failed to improve disease-free survival (DFS) and did not potentiate a tumor-protective immunity to melanoma compared to alum or IFA [101,102].

Besides TLR agonists, cytokines such as IL-12, IL-2, GM-CSF and IFN-γ is another category of adjuvant that is widely used in cancer vaccines. To prolong the circulating half-life and intensity of immune response, plasmid DNA encoding cytokines are used in place of direct injection of cytokines. Ferrone *et al.* reported that DNA encoding cytokines such as IL-2, IL-12, IL-15, IL-18, CCL-21 and GM-CSF fused to Fc domain of immunoglobin are capable of increasing the $CD8^+$ T cells response towards gp100 Ag. They also demonstrated that the most effective cytokine in improving tumor-free survival are IL-12, IL-15, IL-21, CCL-21 and GM-CSF, whereas the combination of IL-2/Ig + IL-12/Ig, IL-2/Ig + IL-15/Ig, IL12/Ig + IL-15/Ig, IL12/Ig + CCL-21/Ig are most effective in eliciting their adjuvancity, therefore antitumor effect without significant induction of autoimmunity [103]. As expected, they found that the timing of cytokine administration relative to Ag vaccination is key in determining the magnitude of immune response and ultimately the clinical outcome. Another recent study by Hamid *et al.* suggested that IL-12 in combination with alum managed to increase the immune response rate to gp100 and MART-1 compared with the combination of IL-12 and GM-CSF. The authors concluded that IL-12/alum augment the immune response that is associated with prolonged relapse-free survival to melanoma Ags versus IL-12/GM-CSF [104]. Another study by Overwijk *et al.* demonstrated that IL-23 vaccinated with gp100 greatly increased the numbers of $CD8^+$ T cells and enhanced their effector function at the tumor site, as well as causing a depletion of bystander $CD8^+$ cells [105]. Although these trials have shown the effectiveness of cytokines as adjuvant, the benefits need to be weighed against their adverse effects, such as the induction of autoimmunity. Therefore, the dose, timing and route of administration need to be carefully selected to bias towards antitumor response in place of autoimmunity.

4.2 Bacillus Calmette-Guérin

BCG has been accepted worldwide as an effective vaccine for the prevention of tuberculosis and attempts have been

made to use BCG as adjuvant in cancer vaccines [106-109]. Some early reports have shown that BCG may be used as cancer vaccine as a result of its ability to suppress tumor growth. In addition, clinical trials involving Canvaxin for stage III and i.v. melanoma patients also used BCG as adjuvant as its benefits have been shown by numerous researchers including Barth *et al.* and Cascinelli *et al.* [110-114]. There are other studies that showed the benefits of BCG such as a study by Duda *et al.* [115] which showed that both BCG and BCG encoding IL-2 DNA demonstrated potent antitumor and immunomodulatory properties. However, in 1984, Patterson *et al.* showed that BCG as a vaccine showed no benefits in stage I melanoma patients after 2 years of oral and transdermal administration [114]. In 2004, Argawala *et al.* reported that in the clinical trial involving 734 stage I – III (AJCC) melanoma patients, BCG showed no significant improvement in disease-free survival (DFS) and overall survival (OS) compared to the control group [115]. The authors concluded that there is no benefit for BCG in an adjuvant setting and this result further confirms the negative results obtained for using BCG as adjuvant in other clinical trials [116].

4.3 Emulsions

Water-in-oil and oil-in-water emulsions such as Montanide, MF59 and Lipovant are another category of adjuvant that have been used in vaccine trials. Oil-in-water consisting of submicron particles are irritants, and therefore induced the infiltration of macrophages to the injection site. These particles, along with the Ags, are rapidly phagocytosed by macrophages and moved to draining lymph nodes, inducing a potent immune response. MF59, which contains varying amounts of squalene and non-ionic surfactants administered with or without muramyl tripeptides, has been recently developed by Chiron to replace complete Freud's adjuvant (CFA). It has been shown to induce a better immune response in humans than alum [117-119]. Numerous clinical trials have used IFA in an adjuvant setting for melanoma clinical trials, however, this adjuvant suffers from its reactogenicity and toxicity, and therefore is not suitable for use in the prophylactic setting [120-123].

5. Viral delivery

An ideal viral delivery carrier for the treatment of cancer should be effective in delivering vaccines, protein and DNA/RNA to multiple sites within the tumor, evading and invoking immune responses, producing rapid viral replication and spreading within the tumor. Vesicular stomatitis virus (VSV), which is a member of the family Rhabdoviridae, genus *Vesiculovirus*, has been shown to be an effective oncolytic virus in a variety of tumor models. The application of viral fusogenic membrane glycoproteins (FMGs) was viewed as a new class of therapeutic genes for the control of tumor growth. Fusogenic membrane glycoproteins, such as the vesicular stomatitis virus G glycoprotein (VSV-G), can cause cytotoxic fusion when expressed on tumor cells. FMGs kill cells by fusing them into large multinucleated syncytia, which die by sequestration of cell nuclei and subsequent nuclear fusion by a mechanism that is non-apoptotic. FMG-mediated tumor cell death can stimulate antitumor immunity since FMG gene expression is associated with increased immunostimulatory signals and could act as a potent immunogen. Syncytial formation is accompanied by the induction of immunostimulatory HSPs, and tumor-associated FMG expression in immunocompetent animals generated specific antitumor immunity [124].

FMG-expressing allogeneic tumor cells were investigated as a novel platform for *ex vivo* and *in situ* vaccination. Murine B16 melanoma-derived cell lines expressing autologous or allogeneic MHC class I, expressing fusogenic or non-fusogenic VSV-G, were used to vaccinate mice *in vivo* against a live tumor challenge. Expression of fusogenic VSV-G enhanced the immunogenicity of an allogeneic cellular vaccine. Intratumoral injection of FMG-expressing allogeneic cells led to significant tumor regression using both fusogenic or nonfusogenic VSV-G. FMG-expressing allogeneic tumor cells served as a potent source of anti-tumor vaccines. Exosome-like vesicles released by fusing allogeneic cells (syncitiosomes) and intratumoral injection of fusing vaccines showed potential for their antitumor effects. Syncitiosomes given with adjuvant and intratumoral injection of fused cells indicated new approaches for cancer therapy [125].

Vesicular stomatitis virus G glycoprotein generates large multinucleated syncytia in infected tumor cells. The syncytia development and death provide an effective pathway for the presentation of tumor Ag to the host immune system. Vaccines consisting of a 1:1 mix of fusing allogeneic and autologous cells led to dramatic increases in survival of mice in both prophylactic and therapeutic animal models. Macrophages activated by syncytia and cell death mediated by fusogenic membrane glycoprotein efficiently promoted the cross-priming of immature DCs within B16 cell established murine tumor model [126].

Additional viral vectors have been investigated serving as Ag delivery vehicles. Poliovirus, a human enterovirus and member of the family of Picornaviridae, is the causative agent of poliomyelitis. Poliovirus is a well-characterized virus, and has become a useful model system for understanding the biology of RNA viruses. Recombinant polioviruses expressing foreign Ags may provide a convenient vaccine vector system to induce protective immunity against diverse pathogens. Advantages of the life-attenuated poliovirus vaccine (Sabin strains) include its extensive use in humans, its safety and its ability to induce long-lasting protective immunity. Poliovirus vaccines are easy to administer by the oral route, low in cost to deliver in the developing world and induce both systemic humoral immunity and local intestinal mucosal resistance to poliovirus infection; that mucosal immunity is thought to be important to protect

against pathogens that cause disease at the mucosal surfaces [127]. Replication-competent chimeric viruses can be constructed by inserting foreign antigenic sequences within the poliovirus proteins. Exogenous Ags can be expressed along with mature and functional viral proteins by properly constructed recombinant poliovirus. A recombinant poliovirus to induce CTL responses was developed containing a segment of the chicken OVA gene, which contains a CTL epitope SIINFEKL. This recombinant virus replicated with near wild-type efficiency in culture and stably expressed high levels of the OVA Ag. Inoculation of mice with recombinant poliovirus that expresses OVA elicits an effective specific CTL response. Vaccination with these recombinant polioviruses induced protective immunity against challenge with lethal doses of a malignant melanoma cell line expressing OVA [128].

Another interesting candidate for viral vector delivery of tumor vaccines is alphavirus. alphavirus belongs to the group IV Togaviridae family of viruses and it has a positive sense single-stranded RNA (ssRNA) genome. The potential application of alphavirus replicon-based vectors serving as vaccine vectors was developed because the production of double-stranded RNA (dsRNA) intermediates can induce a strong adaptive immune response which will trigger the innate immunity. The immunogenicity and efficacy of nucleic acid vaccines can be greatly enhanced when Ag production is under the control of an alphaviral replicase enzyme. Replicase-based nucleic acid vaccines encode the replicase enzyme complex together with the gene encoding Ag. The replicase acts as an RNA polymerase, amplifying mRNA that encodes the Ag. The generation of dsRNA species resulting from the RNA amplification can trigger antiviral defense pathways in viral infection. Compared to conventional DNA plasmids, the replicase-based constructs were very immunogenic. Viral infections can trigger innate immune responses and subsequent production of interferons (IFNs) will increase surrounding cells' resistance to viral infection. Cells transfected with replicase-based plasmids produce increased amounts of IFN-α and IFN-β, compared to cells transfected with a conventional DNA plasmid *in vitro*. Alphavirus replicase-based DNA vaccines may serve as a new strategy eliciting strong immune responses for a possible melanoma vaccine, involving Type I IFN by enhancing vaccine capacity to trigger innate antiviral defense pathways [129].

The induction of apoptotic cell death of transfected cells using alphaviral replicons *in vivo* increased the effectiveness of replicase-based vaccines. Propagation-incompetent alphavirus vectors (virus-like replicon particles, VRP) encoding melanoma Ag tyrosinase served as vehicle expressing tyrosinase, which was investigated for its vaccine potential. VRP encoding either mouse or human tyrosinase was shown to induce immune responses and tumor protection when administered. Antitumor T-cell responses were observed and tumor growth was delayed *in vivo*. Evaluation of a heterologous vaccine regimen using DNA prime and VRP boost showed a markedly stronger immune response than DNA vaccination alone. This heterologous vaccine regimen combining DNA prime and VRP boost serves as a unique strategy for viral vaccine clinical trials [130].

The induction of cell apoptosis *in vivo* can increase the effectiveness of replicase-based vaccines. This interesting observation provided us with the additional understanding that replicase-based DNA vaccines are much more immunogenic than conventional constructs despite reduced Ag production [131]. Replicase-based DNA vaccine can break tolerance and provide immunity to melanoma, compared to conventional DNA vaccines. The vaccine mediates production of dsRNA, as evidenced by the autophosphorylation of dsRNA-dependent protein kinase R (PKR). Double-stranded RNA can activate the innate immune system via TLRs, potentially improving the efficacy of immunization with the replicase-based DNA vaccine [132-133].

Adenoviruses are non-enveloped icosahedral viruses containing double-stranded DNA. In C57BL/6 mice, recombinant adenovirus vaccine encoding xenogeneic human tyrosinase-related protein 2 (Ad-hTRP2) induces protective but not therapeutic cellular immunity against the growth of transplanted B16 melanoma cells. Adenoviral vaccination in combination with peritumoral injections of CpG DNA and synthetic dsRNA, TLR ligand adjuvants, can reject the established B16 melanoma in the skin. Administration of Ad-hTRP2 adenoviral vaccine followed by injections of TLR ligands resulted in the delayed growth of autochthonous primary melanomas in the skin and reduction in the number of spontaneous lung metastases [134].

The tumor Ag-specific engineered T cells demonstrated Ag-specific, non-MHC-restricted cytolysis of h5T4-positive B16 and CT26 tumor cells *in vitro* by cytotoxicity assay and antitumor activity *in vivo*, providing support for T-cell immune response in antitumor immunity [135]. In-depth analysis of the components of immune responses in the antitumor activity induced by cancer vaccines provides us with understanding of the tumor immunotherapeutic mechanisms. The recombinant vaccines, based on melanoma Ag tyrosinase-related protein (TRP)-2, induced Ag-specific CD8(+) T lymphocytes in a murine animal model. The tetramer(+) CD8(+) T lymphocytes were expanded *in vitro* with immunization of the immunodominant TRP-2 Ag and involved in antitumor immunity in animal models [134-136].

Dendritic cells are important APCs. Dendritic cells present processed Ags to $CD4^+$ T cells in order to induce immune response against specific Ags. By infecting DCs using viral vector to deliver Ags directly to the APCs, the expectation is that DCs can serve as Ag delivery systems to induce stronger immune responses. When DCs were infected with an adenovirus (Ad) expressing human gp100 (hgp100), these DCs could serve as a vaccine providing the greatest protection against challenge with B16F10 melanoma. Both Ad and DC/Ad vaccines elicited CD8(+) CTL activities against human gp100 and provided protection against

B16F10 engineered to express human gp100, generating protective CD8(+) T-cell mediated immunity [137]. Mutated tumor Ags can be immunogenic. HSPs have the promiscuous ability to chaperone and present a broad repertoire of tumor Ags to APCs such as DCs. A novel HSP-mediated oncolytic tumor vaccine, referred to as HOT vaccine, was developed to overexpress HSPs to chaperone Agic peptides with the oncolytic activity of viruses. This HOT vaccine, a recombinant replicative adenovirus overexpressing HSP proteins, was designed to cause oncolytic activity to solid tumor cells and release tumor Ags complexed with HSPs. The HSP-mediated immune responses may present a broad array of mutated tumor Ags, servicing as a potent vaccine against metastatic tumor. The adenovirus vaccine overexpression HSPs should elicit individual tumor-specific immune responses against solid tumors. Intratumor vaccination with a recombinant oncolytic adenovirus overexpressing the HSP70 protein established strong immunity against primary tumors, inhibiting the growth of established metastatic tumors in mice. HSP-mediated oncolytic tumor vaccine has the potential to become a personalized vaccine against various solid tumor with broadly applicable potentials [138].

Antigen delivery using genetic immunization through *ex vivo* transduction of DCs is supposed to enhance the induction of antitumor responses in humans by activating a broad range of peptide-specific $CD8^+$ T-cells. Adenoviral (Ad)-transduced as well as peptide-pulsed DC were evaluated to induce melanoma Ag-specific T-cell responses *in vitro*. The glycoprotein 100 (gp100) peptide-pulsed DC vaccine induced long-lasting specific $CD8^+$ T-cell responses against single Ag peptides, whereas Ad-transduced DC induced broad and strong specific immunity against various peptides of the gp100-Ag. The antiadenoviral T-cell responses provided an 'adjuvant' effect by inducing an early release of high amounts of IL-2/IFN-gamma, enhancing CTL induction in the initiation phase. A prime/boost vaccination strategy in melanoma patients, combining the use of Ad-DC and peptide-pulsed DC, obtained efficient and long-term antitumor T-cell responses, producing better antitumor immunity. Also, genetic immunization through *ex vivo* transduction of DCs has been suggested as an effective approach to enhance antitumor immunity by activating both $CD4^+$ and $CD8^+$ T-cells. Melanoma peptide-specific $CD4^+$ T-cells recognized human leukocyte Ag (HLA)-DRbeta1 * 0401 + tumor cells that constitutively expressed the MAGE-6 protein. MAGE-6-derived epitopes could induce an immune-monitoring index of clinically important Th1-type immunity in patients [139,140].

The DCs cancer vaccine, transduced with an adenovirus expressing the human melanoma Ag glycoprotein 100 (DCAdhgp100), provided protective immunity and potent CTL response against melanomas expressing murine glycoprotein 100 *in vivo*. The potency of the DCAdhgp100 vaccine appears to be a result of its ability to directly prime autoreactive $CD4^+$ cells. As a key component of the protective immunity, $CD4^+$ cells are thought to play an vital role in tumor rejection [141]. Intratumoral coinjection of recombinant adenoviruses (Ads) expressing CD40 ligand (CD40L) as a potent costimulator of immune system, and IL-2 leads to strong antitumor immunity and induces efficient protective immunity as a vaccine *in vivo* [142].

The efficacy of adenovirus-mediated gene therapy for the treatment of metastatic B16 melanomas was assessed via an *ex vivo* cytokine vaccine approach in syngeneic C57BL/6 mice with established tumors. Combination cytokine/herpes simplex virus–thymidine kinase (HSV-tk) suicide gene delivery and treatment with ganciclovir (GCV) were evaluated in an animal model so that these suicide gene-transduced tumor cells could serve as vaccines, along with adjuvant cytokines. The B16 melanoma cells were evaluated as potential tumor vaccines. The B16 cells were transduced *in vitro* by adenovirus containing either IL-2, GM-CSF, or tumor necrosis factor-alpha cytokine genes. Then the Ad-transduced B16 melanoma cells were subcutaneously injected into the flank as vaccine. Unmodified B16 melanoma cells were administrated by subcutaneous challenge injection performed 15 days later. Significant reductions in challenge tumor volume were observed in the IL-2 group and the GM-CSF group. Challenge tumor growth was reduced by 56% for the IL-2/tk/adv/GCV treatment ($p = 0.041$) and by 77% for the GM-CSF/IL-2/tk/adv/GCV treatment ($p = 0.037$), in comparison with the beta-gal/tk/GCV control group. These data may hold significant promise for the development of effective *ex vivo* and *in vivo* gene therapy modalities to counter the highly metastatic nature of human melanoma. The disadvantages of adenovirus vaccines are population immunogenicity to some strains of the virus and certain types of human adenoviruses can be oncogenic in animal models, raising potential concerns of oncogenicity issues [143-145].

In clinical studies, patients received escalating doses (between $10 - 7$ and $10 - 11$ plaque-forming units) of recombinant adenovirus encoding either MART-1 or gp100 melanoma Ag administered either alone or followed by the administration of IL-2. Recombinant adenoviruses expressing MART-1 or gp100 were safely administered. One of 16 patients with metastatic melanoma receiving the recombinant adenovirus MART-1 alone experienced a complete response. Other patients achieved objective responses but they had received IL-2 along with an adenovirus, and their responses could be attributed to the cytokine. Immunologic assays showed no consistent immunization to the MART-1 or gp100 transgenes expressed by the recombinant adenoviruses. High levels of neutralizing antibody were found in the pretreatment sera of the patients. High doses of recombinant adenoviruses could be safely administered to cancer patients. High levels of pre-existing antiadenovirus neutralizing antibody may have impaired the ability of these adenovirus vaccines to immunize patients against melanoma Ags. In other studies, vaccines were successfully manufactured for 34 (97%) of 35 patients. Vaccination elicited DC, macrophage, granulocyte and

lymphocyte infiltrates at the injection sites in 19 of 26 assessable patients. Vaccination with irradiated, autologous melanoma cells engineered to secrete GM-CSF by adenoviral-mediated gene transfer augments antitumor immunity in patients with metastatic melanoma [146,147].

Poxviruses (members of the family Poxviridae) viral particles (virions) are generally enveloped (external enveloped virion [EEV]). Vaccinia virus (VACV or VV) is a large, complex, enveloped virus belonging to the poxvirus family. Immunotherapy could be combined with conventional chemotherapeutic modalities aimed at reducing tumor burden. Such combination therapy may potentially help to avoid some of the immunosuppressive effects of these drugs [148]. Five mouse melanocyte differentiation Ags, gp100, MART-1, tyrosinase and tyrosinase-related proteins (TRP)-1 and TRP-2 were used to induce immune responses in a melanoma animal model. The recombinant viral vector expressing TRP-1 elicited strong antitumor immunity and $CD4^+$ T lymphocytes was described as an integral part of this process [149]. Tumor-specific CD8 CTL is considered to be an important factor in epitope-based vaccination for cancer immunotherapy. A recombinant poxvirus melanoma vaccine encodes for 10 HLA-A2-restricted epitopes derived from five melanoma Ags. Target cells infected with the melanoma polytope vaccinia were recognized by three different epitope-specific CTL lines derived from HLA-A2 melanoma patients, and CTL responses to seven of the epitopes were generated in at least one of six HLA-A2 transgenic mice immunized with the construct [150]. A few patients with stage IV melanoma were immunized with autologous CD34-derived DCs transduced with a modified vaccinia Ankara virus encoding human tyrosinase gene (MVA-hTyr). The vaccination with MVA-hTyr-transduced DCs was well tolerated, and the initial data suggested the potential of the MVA vector for transducing tumor-associated Ags to DCs for tumor immunotherapy [151].

Patients with advanced metastatic melanoma were recruited for a clinical trial, utilizing prime/boost vaccination with recombinant vaccinia and fowlpox vectors encoding tyrosinase as vaccines was explored alone, or concurrently with low-dose or high-dose IL-2. While prime/boost immunization with recombinant vaccinia and fowlpox viruses enhanced antityrosinase immunity in some patients with metastatic melanoma, generally the prime/boost combination immunization was ineffective on its own in mediating clinical benefit, and in combination with IL-2 did not mediate clinical benefit significantly different from that expected from treatment with IL-2 alone [152].

Also, the immunologic effects of direct lymph node (LN) injections of canarypox virus (ALVAC) vectors expressing the melanoma Ags were studied in high-risk HLA-A * 0201 positive patients, with the intention of optimizing CD8(+) cytotoxic T lymphocyte (CTL) responses. Melanoma Ags included gp100 and immunogenic gp100 peptides, and the vaccines were co-administered with the helper adjuvant, tetanus toxoid. Increased gp100-reactive CTL frequencies and ALVAC antibody levels were observed when viruses were injected directly into LNs. Intranodal (i.n.) injections of ALVAC(2)-gp100m viruses are feasible, safe and may be a superior method of vaccination in humans. However, tetanus toxoid failed to improve CTL responses to this vaccine [153]. In other studies, fowlpox vaccines encoding the gp100 melanoma Ag in patients with metastatic melanoma were evaluated for its immune responses and therapeutic effectiveness. Although the data supported the concept of modifying anchor residues of nonmutated self-Ag peptides to generate cellular immune responses, the result did not show a significant improvement of recombinant fowlpox viruses encoding modified epitopes [154].

In more clinical studies, repeated vaccination with ALVAC miniMAGE-1/3 is associated with tumor regression and with a detectable CTL response in a minority of melanoma patients. The correlation between tumor regression and CTL response is significant [155-158]. In other studies, ALVAC encoding the melanoma-associated Ag, Melan-A/MART-1 (MART-1), served as vaccines in cancer immunotherapy, using a DC-based approach. ALVAC MART-1-infected DC express tumor Ag coded by the viral vector. Dendritic cells infected with recombinant canarypox viruses could serve as an efficient presentation platform for tumor Ags [159,160]. The disadvantages of poxvirus vaccines are they have large genome and complex construction, their vaccine genome constructions may not be always stable and can be expensive for production. Further, the weakness of poxvirus-based viral vaccine remains in possible host immunity against vaccinia virus, which could limit the effectiveness of poxviruses in recombinant vaccination strategies [161]. Vaccinia melanoma cell lysates (VMCL), live vaccinia virus-augmented allogeneic polyvalent melanoma cell lysate, or vaccinia melanoma oncolysate (VMO) failed to elicit strong tumor-specific immune response for clinical benefits [162,163].

6. Plasmid DNA

Plasmid DNAs encoding cytokines enhance immune responses to vaccination in models of infectious diseases and cancer. These plasmid DNA vaccines produced cytokines as serving adjuvants have been compared for their ability to enhance immunity against a poorly immunogenic self-Ag expressed by cancer. DNAs encoding cytokines that affect T-cells, such as IL-2, IL-12, IL-15, IL-18, IL-21 and the chemokine CCL21 and Ag-presenting cells, such as GM-CSF, were compared in mouse models as adjuvants to enhance $CD8^+$ T-cell responses and tumor immunity. A DNA vaccine against a self-Ag, gp100, expressed by melanoma, was used in combination with DNA encoding cytokines and cytokines fused to the Fc domain of mouse IgG1 (Ig). The potential of cytokine DNA adjuvants for immunization against self-Ags expressed by cancer emphasized the importance of timing and the enhancement of immune responses by

Fc domains through mechanisms unrelated to increased half-life. Targeting Ags to FcgammaRs (FcRs) for IgG on DCs has been demonstrated to enhance Ag presentation. Secondary lymphoid tissue chemokine (SLC) has been shown to increase immune response not only by promoting co-clustering of T cells and DCs in the lymph nodes and spleen, but also by regulating their immunogenic potential for the induction of T-cell responses. DNA vaccine constructed by the fusion of SLC and IgG Fc fragment genes to Ag-coding gene is an effective approach to induce potent antitumor immune response via both $CD4^+$ and $CD8^+$ T-cell dependent pathways. Reported data from depletion experiments suggested that CD8+ T lymphocytes were crucial for the anti-tumor activity of vaccines, as well as $CD4^+$ lymphocytes [139,156,174,180,194,203]. Adjuvant activity of CD40 agonists seems to be involved in enhancing the CD8(+) T-cell-dependent response in tumor bearing hosts, suggesting that sustaining tumor-specific T lymphocyte in subjects undergoing vaccination may be key in successful vaccination with melanoma tumor Ags [174,180,194]. Collectively, genetic vaccination can result in a potent antitumor response *in vivo* and constitutes a potential immunotherapeutic strategy to fight cancer. However, the weakness of plasmid DNA vaccines is that they are generally less immunogenic than viral vector vaccines anticipated in humans. Plasmid DNA vaccines usually need dose increase, adjuvants, electroporation/delivery technology, or the addition of heterologous modality boost to be sufficient in order to elicit a tumor-specific immune response in patients [66,164-175].

The skin represents an excellent site for vaccine inoculation due to its natural role as a first-line of contact with foreign pathogens and the high local frequency of Ag-presenting cells. To facilitate skin-directed immunization, a new technique has been developed (termed microporation) whereby a vaporization process is used to remove tiny areas of the stratum corneum, creating microscopic pores that allow access to the underlying viable epidermis. Microporation vaccination enabled delivery of an adenovirus vaccine carrying a relevant melanoma Ag, resulting in the development of autoimmune vitiligo and tumor protection. The microporation technology has proven to be a reliable and easy method to enable skin-directed vaccination [176]. Transcutaneous immunization strategies were extended to stimulate systemic cytotoxic T lymphocyte responses for generating antitumor or antimicrobial immunity. Topical application of vaccines consisting of synthetic peptides formulated with adjuvant imiquimod (TLR agonist 7) generates strong T-cell responses against tumor cells in a murine melanoma animal model. These results provide animal data to use the peptide-based transcutaneous vaccines as a noninvasive and effective strategy for antitumor immunotherapy. The safety and immunologic effects of particle-mediated epidermal delivery (PMED) of gp100 and GM-CSF genes were assessed in melanoma patients. Exploratory immunologic monitoring suggested modest activation of an antimelanoma response. PMED with cDNAs for gp100 alone or in combination with GM-CSF is well tolerated by patients with melanoma [53,177].

The potential of GM-CSF-based melanoma cell vaccines was investigated in an allogeneic setting, with subcutaneous injection of GM-CSF-expressing cancer cells *in vivo*. Trinitrophenyl-derivatized bone marrow-derived DCs were found to elicit a contact hypersensitivity response in syngeneic but not in allogeneic recipients, compatible with their expected mode of direct Ag presentation. However, GM-CSF expressing haptenized M3 melanoma cells was able to induce a contact hypersensitivity response but, in contrast to bone marrow-derived DCs, not only in syngeneic but also in allogeneic recipients. These experiments provide a rational basis for the use of GM-CSF-based melanoma cell vaccines. Additionally, the feasibility, safety and immunogenicity of mature, peptide-pulsed DC vaccines were evaluated by different routes of administration. A randomized clinical trial of dose-escalation study evaluated four autologous peptide-pulsed DC vaccinations in patients with metastatic melanoma. Patients were randomly assigned to an i.v., i.n., or intradermal (i.d.) route of administration. Administration of this peptide-pulsed mature DC vaccine by i.n., i.v., or i.d. routes is feasible and safe. Intravenous administration seems to result in superior T-cell sensitization as measured by *de novo* target-cell recognition and DTH priming, indicating that i.n. administration of peptide-pulsed mature dendritic cell vaccines could be the preferred route of administration (ROA) for mature DC vaccination. [178-180].

7. Conclusion

Although multiple clinical trials on melanoma vaccine have been conducted over the years, reliable and reproducible tumor rejection has yet to be achieved. Therefore, any future melanoma vaccine needs to overcome the difficulties and obstacles faced by the current cancer vaccines, including rare and short-lived clinical response, adverse effects and self-tolerance. Careful consideration of the factors involved in future vaccine design such as the ease and cost of manufacturing, selection of adjuvants, patient populations, dose, schedule and route of administration also needs to take place. A multimodal strategy involving the selection of patients with minimal tumor burden, providing the 'danger signals' needed to activate the immune cells by using potent adjuvants, vaccination with polyclonal vaccines or combinations of multiple vaccines to reduce the down-regulation of targeted Ag and generation of Ag variants, overcoming self-tolerance by blocking the immunosuppressive microenvironment created by tumors, as well as the negative costimulatory molecules. In addition, discrepancies between the measured immune response and clinical response raised the question of the reliability of the current immunomonitoring method as an indicator of clinical response after vaccination. Thus, new strategies are required to better predict the outcome of vaccination.

8. Expert opinion

Jenner's effort of inoculating with cowpox to prevent smallpox two centuries ago marked the beginning of vaccinology. Through the history of vaccination, vaccines have proven their efficacy in preventing many infectious diseases, but this success fails to translate into the field of cancer prevention and treatment. The limitation of vaccines in cancer prevention or treatment stems from the challenges faced by cancer immunotherapy, which include but are not limited to multiple mechanisms of tumor escape, tumor-induced immunosuppression, difficulties in inducing activation, maturation and migration of T-cell antitumor function in tumor microenvironment. The lack of effective and safe vaccine delivery strategies for humans is one of the major challenges.

Although it accounts for only 3% of skin cancer, melanoma cases are on the rise in the US. According to American Cancer Society, there were approximately 59,940 new cases of melanoma diagnosed in 2007 and about 8,110 people will die of this disease this year. Early stage melanoma is curable, but it is very lethal once it becomes metastatic. Surgery, chemotherapy, radiation and immunotherapy are the standard treatments currently available to melanoma patients. Nevertheless, only cytokines (IFN-α, IL-2) were administered and to date no melanoma vaccine has been approved by the FDA for the treatment of melanoma in the US.

To date, prophylaxis against tumors can be achieved by many vaccines, but the rejection of tumors can only be achieved by a combination of adoptive T-cell transfer and vaccination with immunoregulatory molecules such as IL-2 in an animal model. Nevertheless, these results have yet to be replicated in human clinical trials. Currently, many melanoma vaccines have entered Phase I, II and/or III clinical trials, which includes M-Vax (Avax Technologies, Inc., Philadelphia, PA, USA), Canvaxin (CancerVax Corporation, Carlsbad, CA, USA), Allovectin-7 (Vical, San Diego, CA, USA), Polynoma-1 (Vaccinoma Inc., Sydney, Australia), Oncophage® (Antigenics Inc., New York, NY, USA), GMK (Progenics Pharmaceuticals Inc., Tarrytown, NY, USA), MDX-010 (Medarex, Inc., Princeton, NJ, USA) and Melacine® (Corixa Corporation, Seattle, WA, USA), which is only approved in Canada, not the US. Encouraging results with varying degrees of efficacy were obtained for Phase II trails for both Canvaxin [111] and Melacine [181].

However, upon close scrutiny, Melacine failed to show benefits in disease-free survival compared to control in patients with no or minimal residual disease, whereas the Phase III clinical trial of Canvaxin ended early due to the indication that no benefit of Canvaxin coupled with BCG would be obtained compared with the placebo group. Although clinical trials and experiments in animal models have improved our understanding of the complex interaction between our immune system and melanoma, our understanding of the important mechanisms underlying a successful melanoma vaccine is still lacking, such as different carriers, immune response variability to melanoma vaccine, tumor escape mechanisms, roles of adjuvant, and immunoregulatory molecule functions. Therefore, there is a need for studies addressing these specific mechanisms and vaccine delivery strategies.

This can be achieved with the identification of more robust adjuvant and effective delivery carriers such as microparticles, nanoparticles, liposomes, HSPs and virosomes/virus-like particles. Microparticles and nanoparticles were shown to enhance the delivery of melanoma vaccine *in vitro* [31,39,182] and also via the epidermal delivery pathway using helium pulsed dermal injector [53,183]. In addition, liposomes also demonstrated more potent antitumor effects compared to control [53,60,62,65,183,184]. These particular vaccines managed to target APCs directly via binding to surface molecules of DC or indirectly via transcytosis and endosomal uptake, such as phagocytosis and pinocytosis [185]. It is also well documented that because of the particulate nature of these carrier systems, they also act as adjuvant [22,23]. The microparticles, nanoparticles and liposomes offers more stability and flexibility in formulation and Ag selections compared to other delivery systems. They have also been shown to protect the vaccine from intracellular degradation by APCs, controlled release properties and ease of preparation compared to virus-like particles and virosomes. However, liposomes and ISCOMs suffer from low encapsulation efficiency and lower stability compared to microparticles and nanoparticles. Despite these shortcomings, they still remain an attractive platform for the delivery of melanoma vaccine.

Another delivery system, Melan-A virus-like particles vaccine, has been evaluated in a Phase II clinical trial for its safety and immunogenicity in advanced stage melanoma patients (Cytos Biotechnology, AG Schlieren, Switzerland). Also, virosomes technology has been used to deliver encapsulated Melan-A peptide to a plasmacytoid DC line for generating Melan-A specific T-cells [186]. Vaccines made from an Ag combined with a modified virus serving as a delivery vector may prompt the body to kill tumor cells, in addition to inducing both the innate and adaptive immune responses.

Clinical trials have been conducted to evaluate viral vector delivery systems as melanoma vaccines, such as recombinant fowlpox virus encoding the gp100 melanoma Ag (Aldesleukin) with/or without IL-2 in metastatic melanoma patients and recombinant fowlpox virus vector encoding a triad of costimulatory molecules (B7-1, ICAM-1 and LFA-3) (TRICOM) enhancing DCs Ag presentation to T-cells. In other trials, matured DCs treated with melanoma Ags were evaluated as vaccines for metastatic melanoma patients [187]. Beside recombinant fowlpox virus, ALVAC encoding the melanoma-associated Ag, Melan-A/MART-1 was evaluated to enhance DC Ag presentation potential [160] and alphavirus replicon particle vectors encoding the melanoma Ag

tyrosinase were used as delivery system to induce immune responses and tumor protection in animal model [130].

There have been significant efforts in developing vaccines to treat or prevent cancer, particularly for melanoma. Most recently, two groups of researchers, namely the Rosenberg and the Yee groups, have achieved encouraging results in the adoptive transfer of T-cells in clinical trials, which is a leap forward despite the mixed results reported so far for melanoma vaccine [188-190]. A better understanding of basic immunologic principles and more efficient immunization strategies against melanoma are needed to design a more efficacious vaccine. Future strategies include exploring new vaccine delivery technologies, inducing a constant and potent T-cell response and downregulation of regulatory T-cells/inhibitory cytokines secreted by melanoma cells. The combinational strategy could serve us well to improve the clinical efficacy of melanoma treatment, and melanoma immunotherapy will eventually play a significant role in melanoma combinational therapies along with chemotherapy or surgery.

Declaration of interest

The authors state no conflict of interests and have received no payment in the preparation of this manuscript.

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