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ProtEx™ technology for the generation of novel therapeutic cancer vaccines

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Abstract

Therapeutic vaccines present an attractive alternative to conventional treatments for cancer. However, tumors have evolved various immune evasion mechanisms to modulate innate, adaptive, and regulatory immunity for survival. Therefore, successful vaccine formulations may require a non-toxic immunomodulator or adjuvant that not only induces/stimulates innate and adaptive tumor-specific immune responses, but also overcome immune evasion mechanisms. Given the paramount role costimulation plays in modulating innate, adaptive, and regulatory immune responses, costimulatory ligands may serve as effective immunomodulating components of therapeutic cancer vaccines. Our laboratory has developed a novel technology designated as ProtEx™ that allows for the generation of recombinant costimulatory ligands with potent immunomodulatory activities and the display of these molecules on the cell surface in a rapid and efficient manner as a practical and safe alternative to gene therapy for immunomodulation. Importantly, the costimulatory ligands not only function when displayed on tumor cells, but also as soluble proteins that can be used as immunomodulatory components of conventional vaccine formulations containing tumor-associated antigens (TAAs). We herein discuss the application of the ProtEx™ technology to the development of effective cell-based as well as cell-free conventional therapeutic cancer vaccines.

Current treatment of primary tumors usually involves a combination of surgery, radiotherapy, and chemotherapy. These treatment modalities often are associated with adverse side effects arising from lack of specificity for tumors and, importantly, frequently fail to eliminate residual and micrometastatic tumors, which can lead to recurrences (Kasimir-Bauer, 2001). It is therefore vital to develop tumor-specific therapies that not only eliminate primary tumors but also micrometastatic tumor cells, and prevent recurrences. In this context, cancer vaccines may serve an ideal treatment due to their specificity for tumor cells and long lasting immunological memory that may safeguard against recurrences.

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The notion that the immune system can recognize and mount a response against tumors was postulated in the late nineteenth century by Coley (Coley, 1928) who demonstrated that attenuated bacteria or bacterial products injected into tumor-bearing patients stimulated tumor necrosis factor (TNF) production, and in some cases resulted in tumor regression (O'Malley *et al.*, 1963; Old, 1985). Nearly a century later, it was demonstrated that immunization of mice with mutated tumor cells could induce a protective anti-tumor immune response against non-immunogenic tumor (Van and Boon, 1982). Together, these studies set a foundation for cancer immunotherapy research and demonstrated the therapeutic potential of strategies targeting immune modulation for tumor eradication and protection against tumor recurrences. Therefore, the development of cancer vaccines capable of generating an active tumor-specific immune response serves as a promising venue for cancer therapy. However, despite demonstrated efficacy in various murine models, cancer vaccines have found little success in the clinic. Although several factors may contribute to the failure of therapeutic cancer vaccines in the clinic, the most important ones are i) the weak immunogenicity of TAAs, ii) central and peripheral immune tolerance to self TAAs, and iii) various immune evasion mechanisms employed by the progressing tumor. Therefore, the success of therapeutic cancer vaccines may require formulations that induce potent immune responses that overcome immune tolerance to TAAs as well as reverse or inhibit tumor-mediated immune evasion mechanisms.

Cancer and the immune system

Although cancer is widely regarded as a cell-autonomous disease, there is compelling evidence that the immune system can recognize and mount an effective response against tumors. The rejection of tumors requires a complicated and orchestrated attack by the immune system that involves innate (DCs, macrophages, and NK cells) and adaptive cellular ($CD4^+$ and $CD8^+$ T cells) and humoral (B cells) responses. Tumors are targeted by the immune system because they express either mutated or over/aberrantly expressed self-proteins, termed TAAs, or proteins derived from oncogenic viruses unique to the tumor, termed tumor-specific antigens (TSAs). Tumor-specific immunity in mice was first established using chemically-induced sarcomas in the 1950's. Subsequently, many tumor antigens have been identified and shown to elicit tumor-specific $CD8^+$ cytotoxic lymphocytes (CTLs). Under physiological conditions, tumor antigens are picked up by DCs, carried to peripheral lymphoid organs, and presented to naïve T cells under immunogenic conditions allowing for their activation and differentiation into T effector (T_{eff}) cells. These activated T_{eff} cells then traffic to tumor sites and generate anti-tumor responses for tumor eradication. While activated $CD8^+$ T cells directly kill target tumor cells, activated $CD4^+$ T cells can promote inflammation, cooperate in the induction of $CD8^+$ T effector and memory cells, and provide help for B cells to produce destructive anti-tumor antibodies.

Tumors escape from the immune system through both selection of non-immunogenic tumor cells (immunoediting) and/or active suppression of the immune response (immunosubversion). Immunoediting and immunosubversion are thought to be required for cancer establishment, survival, and ability to kill the host (Dunn *et al.*, 2004). As such, avoidance of immunosurveillance has been proposed recently as the seventh hallmark of cancer (Dunn *et al.*, 2004; Smyth *et al.*, 2006). Tumor immunosurveillance is supported by studies demonstrating that mice lacking critical components of the immune system have increased development of both spontaneous and chemically-induced tumors. For example, mice knockout for recombination-activating gene 2, and as a result lack T cells, B cells, and natural killer T cells, are more vulnerable to both spontaneous and chemically induced tumors (Dighe *et al.*, 1994; Kaplan *et al.*, 1998; Shankaran *et al.*, 2001). Similarly, mice deficient for $\alpha\beta$ and $\gamma\delta$ T cells (Gao *et al.*, 2003; Girardi *et al.*, 2001) or anti-tumor effector molecules, such as perforin (Smyth *et al.*, 2000) or tumor necrosis factor-related apoptosis-

inducing ligand (Cretney *et al.*, 2002), are more susceptible to tumor development. These animal data are consistent with studies demonstrating higher incidence of tumors in immunocompromised humans, such as graft recipients kept under chronic immune suppression (Hollenbeak *et al.*, 2005; Penn, 2000). Moreover, evidence of an anti-tumor immune response is often a positive prognostic marker in cancer patients. For example, increased tumor infiltration by lymphocytes is a positive prognostic marker in patients with melanoma, ovarian, and gastric cancers (Haanen *et al.*, 2006; Pages *et al.*, 2005; Zhang *et al.*, 2003). In patients with ovarian and gastric cancers, increased levels of antibodies against the tumor suppressor protein p53 correlate with patient survival (Goodell *et al.*, 2006; Mattioni *et al.*, 2007; Zhang *et al.*, 2003). Conversely, high percentage of CD4⁺CD25⁺FoxP3⁺ T regulatory (T_{reg}) cells, capable of suppressing anti-tumor immune responses, is associated with poor prognosis of patient survival with ovarian cancer (Curiel *et al.*, 2004; Sato *et al.*, 2005).

Although evidence in both mice and humans suggests that the immune system has an important role in tumor suppression, often the immune response fails to eliminate the tumor. This is largely due to tumors having developed various immune evasion mechanisms to modulate innate, adaptive, and regulatory immunity for survival. Tumors can avoid detection from T cells by disrupting signal 1 (antigen recognition) and/or signal 2 (costimulation), both necessary for proper T cell activation. Lack of signal 1 can result from defects in transduction of this signal or inefficient display of MHC/tumor antigen complexes on tumor cells by improper antigen processing and presentation, including downregulation of transporter associated with antigen processing 1 and loss of HLA class I, as demonstrated in patients with melanomas and lung cancer (Atkins *et al.*, 2004; Seliger *et al.*, 2000; So *et al.*, 2005). Absence of signal 2 can be due to the lack of costimulatory molecules and/or expression of coinhibitory molecules or decoy receptors on tumor cells, leading to T cell anergy. In addition tumors have evolved mechanisms to directly suppress immune responses and avoid destruction by tumor-specific T cells through the secretion of immune-subversive cytokines such as IL-10 (Salazar-Onfray, 1999) and TGF- β (Li *et al.*, 2006; Liu *et al.*, 2007). Tumors can also physically eliminate T_{eff} cells *via* increased expression of apoptotic molecules such as FasL (Igney and Krammer, 2005). Certain B cell lymphomas and ovarian carcinoma directly recruit T_{reg} cells capable of suppressing the function of T_{eff} cells, B cells, NK cells, and other immune cells, through the production of chemokine ligand 22 (Curiel *et al.*, 2004; Yang *et al.*, 2006). Tumors also can induce the differentiation of T_{reg} cells through various mechanisms including the production of TGF- β (Liu *et al.*, 2007). Direct evidence for the dominant role of T_{reg} cells in immune evasion was verified in various experimental tumor models, where physical depletion of these cells or modulation of their function demonstrated therapeutic efficacy (Elpek *et al.*, 2007a; Knutson *et al.*, 2006; Yu *et al.*, 2005). Tumors also can recruit myeloid suppressor cells and prevent the differentiation into mature DCs (Huang *et al.*, 2006; Zou, 2005). In addition, these myeloid suppressor cells produce nitric-oxide synthase that can inhibit tumor-specific T cells (Zou, 2005). Accumulating evidence suggests that many of these immune evasion mechanisms operate simultaneously in patients with large tumor burdens (Elpek *et al.*, 2007a). Therefore effective anti-tumor therapeutic vaccines must not only generate/boost tumor destructive responses, but also reverse existing tumor-derived immune evasion mechanisms.

Approaches to cancer vaccines

Recent advances in tumor immunology, genetic tools, and identification of TAAs and TSAs have led to increased efforts to develop vaccination strategies to treat cancer and/or prevent cancer relapse. Cancer vaccines are designed to either prevent (prophylactic) or treat established cancer (therapeutic). Prophylactic vaccines are administered to healthy individuals having a normal, functional immune response, and are designed to generate

neutralizing humoral immunity targeting either cancer-causing viruses or target components of malignant cells, such as TAAs. Therapeutic vaccines are administered to cancer patients and are designed to induce/strengthen immune defense against developed tumors, commonly by targeting the generation of tumor-specific T cell responses. Ideal cancer vaccines must discriminate between tumorigenic and non-tumorigenic cells, eradicate both primary as well as residual or micrometastatic tumor cells, and develop long-lasting immunological memory to safeguard against recurrences with tolerable adverse side effects.

Current vaccine strategies against cancer include the use of whole tumor cells/cell lysates, tumor cells genetically modified to express costimulatory molecules, cytokines, chemokines, gene therapy by intratumoral injection of a range of vectors encoding various immunostimulatory molecules, DCs pulsed with tumor antigens or transfected with tumor RNA, and vaccination with TAAs in conjunction with appropriate immunomodulators.

Whole tumor cells as vaccine

In principle, autologous whole tumor cells contain all relevant TAAs and, as such, have the potential to elicit an effective anti-tumor immune response when administered in conjunction with an appropriate immunomodulator. Vaccination with whole tumor cells was shown to stimulate the immune response through both direct tumor-antigen presentation as well as prolonged release of TAAs that allows for sufficient tumor-antigen uptake by host APCs and subsequent activation of immune effector cells (Ward *et al.*, 2002; Yang *et al.*, 2008). Mice vaccinated with irradiated whole tumor cells in combination with an adjuvant, and subsequently challenged with non-irradiated tumor cells, were found to efficiently generate anti-tumor immune recall responses (Webster *et al.*, 2007). However, despite containing all relevant TAAs and thus avoiding the problem of identifying appropriate tumor antigens, the use of autologous tumor cells for cancer vaccines faces major limitations (de Gruijl *et al.*, 2008). In addition to the weak immunogenic nature of unmodified autologous tumor cell-based vaccines, the lack of sufficient tumor mass for vaccine preparation due to early detection as well as the inability to surgically access tumor cells for certain cancers represent other limitations. Although tumor cells may be cultured for an extended time period to generate enough cells for vaccine preparation, the inability of certain tumor cells to grow under culture conditions poses another problem. As an alternative, allogeneic tumor cells have been used as a source of TAAs, which ideally would be taken up by APCs and presented to autologous immune effector cells. Potentially, this would provide a limitless source of TAAs through well established cell lines. Although allogeneic tumor cell lines allow for standardized and large scale production of potential cancer vaccines, such vaccines were demonstrated to direct the majority of the immune response towards the alloantigens, and not TAAs, due to dominant antigenic competition, leading to inefficient anti-tumor immune responses (de Gruijl *et al.*, 2008).

Genetically modified tumor cells as vaccine

In an effort to increase the efficacy of whole cell-based vaccines, tumor cells were genetically modified to express costimulatory molecules. Vaccination with such modified cells was shown to induce effective anti-tumor responses by direct antigen presentation (Townsend and Allison, 1993). For example, vaccination of mice with cervical cancer cells transfected with the costimulatory molecule B7 was demonstrated to induce antitumor immune protection in host mice upon rechallenge with U14 tumor cells (Tao *et al.*, 2001). Over expression of CC chemokine ligand 5 intratumorally delayed tumor growth and increased tumor cell infiltration in mouse model (Lavergne *et al.*, 2004). Similarly in humans, cancer cells engineered to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) using *ex vivo* gene transfer as vaccines for the treatment of genitourinary

malignancies have demonstrated both safety and bioactivity as assessed by the generation of anticancer immune responses (Nelson *et al.*, 2000).

Cancer gene therapy also was tested as a more practical alternative to genetically-modified tumor cell vaccines. Genetically-modified viruses carrying TAAs and/or immunostimulatory genes were employed as attractive vehicles for cancer gene therapy due to high efficiency of gene delivery and ability to mediate long-term gene expression in both dividing and non-dividing cells. As such, a number of gene therapy strategies using a range of viral vectors in both animal models and patients have demonstrated the therapeutic potential of viral vectors to introduce genes of interest into cancer cells and subsequently induce anti-tumor immune responses. For example, a recent phase I clinical trial using intratumoral cytokine administration of ALVAC GM-CSF or ALVAC IL-2 in skin metastases of melanoma or leiomyosarcoma revealed that the transgene determines the composition of the inflammatory infiltrate. GM-CSF induced monocyte and macrophage enrichment of the peritumoral inflammatory infiltrate, whereas IL-2 increased local T lymphocytes. IL-2, but not GM-CSF, led to partial regression of some tumors with acceptable safety profile (Hofbauer *et al.*, 2008). However, virus vector systems have various limitations, such as requiring mitotic cell division for transduction, low maintenance of transgene expression, and immune elimination of infected cells that limits gene expression *in vivo*. In addition, there are safety concerns arising from mutations in the transferred viral genome leading to virulent forms (Mancheno-Corvo and Martin-Duque, 2006).

Naked DNA-based vaccines formulated to encode TAAs and/or immunostimulatory molecules to elicit an effective anti-tumor immune response have emerged as a safer and advantageous alternative to gene therapy using viral vectors. For example, intradermal injection of mice transgenic for rat neu and HER-2/neu homologue with rat neu cDNA in combination with plasmids encoding costimulatory molecules CD80/CD86 and 4-1BBL resulted in the induction of both cellular and humoral immune responses (Disis *et al.*, 2003). In a recent phase I study, it was demonstrated that intranodal infusion of a Melan-A/MART-1 DNA plasmid vaccine in patients with stage IV melanoma was well tolerated and induced an immune response. However, the vaccine did not induce regression of established disease (Weber *et al.*, 2008). Despite pre-clinical success, the development of clinically successful naked DNA cancer vaccines faces major hurdles, such as the need for high doses of plasmid DNA, inefficient delivery of the gene of interest, low expression efficiency of the introduced gene, lack of vaccine efficacy, and finally safety concerns.

Dendritic cell-based cancer vaccines

Antigen uptake and presentation by DCs are critical to the generation of effective adaptive immune responses. As such, DC-based vaccines have gained intense interest and shown significant promise in preclinical studies as a potential treatment for malignant diseases. Pulsing of autologous DCs with TAAs or whole tumor cell lysates, transfection of DCs with RNA isolated from tumor cells, or fusion of tumor cells with DCs are some approaches used to generate effective immune responses against tumors.

A series of studies demonstrated the efficacy of autologous DCs pulsed with defined TAAs as therapeutic cancer vaccines in preclinical models (Sas *et al.*, 2008; Zhang *et al.*, 2008). Although not as effective as in preclinical models, DCs pulsed with TAAs were shown to induce durable anti-tumor responses with demonstrable clinical efficacy. For example, vaccination with MUC1 peptide-pulsed DCs induced both immunologic as well as clinical responses without severe side effects in a limited number of patients with metastatic renal cell carcinoma (Wierecky *et al.*, 2006). DCs fused to tumor cells were used as vaccines in an effort to broaden the repertoire of presented TAAs for better efficacy. Vaccination with DCs

fused to tumor cells resulted in the induction of immunological and clinical antitumor responses without detectable toxicity in patients with metastatic breast and renal cancer (Avigan *et al.*, 2004). Similarly, vaccination of patients with stage IV renal cell carcinoma with allogeneic DC/autologous tumor cell fusion was well tolerated and resulted in immunologic and clinical responses in a limited number of patients in a phase I/II study (Avigan *et al.*, 2007). Although they have shown efficacy in the clinic, DC-based vaccines are time and labor intensive, costly, and most importantly are patient-customized which severely limits their broad clinical application.

Conventional, cell-free cancer vaccines

Conventional vaccines based on defined TAAs or TSAs have significant potential as preventive and therapeutic cancer vaccines because of they are cost effective and their ease of preparation, transport, and applicability to a wide variety of tumors in a broad patient population. During the last decade, a large number of TAAs and TSAs recognized by T cells have been identified. This has led to renewed interest in the use of active immunization as a modality for the prevention and treatment of cancer. Vaccination with human papillomavirus-like particles has been shown to effectively control cervical cancer in preclinical as well as clinical settings (Govan, 2008), providing strong rationale for the development of preventive vaccines against other bacterially and virally caused cancers as well as spontaneous cancers with well-defined TAAs without microbial infection etiology.

Unlike preventive vaccines, the efficacy of therapeutic conventional vaccines based on TAA or TSAs have been limited. Vaccination with whole NY-ESO-1 protein, a recombinant TAA formulated with cholesterol-bearing hydrophobized pullulan, generated both humoral and adaptive T cell responses with limited clinical response in advanced esophageal cancer patients (Wada *et al.*, 2008). Similarly, women with high-grade cervical intraepithelial neoplasia treated with HPV-16 Hsp-E7 fusion protein resulted in lesion regression in a phase II study (Roman *et al.*, 2007). However, antigen processing and presentation were found to limit the generation of an effective immune response and better vaccine efficacy. The use of complete TAAs does not require HLA typing and may generate effective immune responses due to the potential of harboring epitopes for both CD4⁺ and CD8⁺ T cells. However, the oncogenic nature of certain TAAs, such as E7, as well as their mass production using conventional recombinant DNA techniques, present safety as well as technical issues that can be circumvented by using synthetic peptides representing the immunodominant epitopes. Synthetic peptides are easy to produce and store, are safe, and can be readily presented to T cells by APCs in the context of MHC molecules for robust generation of effective immune responses against cancer cells, including development of CTLs that can recognize and directly lyse tumor cells. For example, patients with resected HPV16-positive cervical cancer vaccinated with an overlapping set of long peptides comprising the sequences of the HPV16 E6 and E7 oncoproteins emulsified in Montanide ISA-51 resulted in the generation of HPV16-specific CD4⁺ and CD8⁺ T cell immune responses to a broad array of epitopes (Welters *et al.*, 2008). In addition, a phase I immunotherapeutic trial in end-stage cervical cancer patients using long peptides spanning the E6 and E7 sequences demonstrated low toxicity and robust immunogenicity (Kenter *et al.*, 2008).

Unlike viral-derived TSAs, therapeutic vaccines based on self-modified or overexpressed TAAs have shown limited efficacy in various clinical trials. For example, multiple vaccination using 5 class I HLA-restricted synthetic peptides derived from multiple ovarian cancer-associated proteins and a T helper epitope derived from tetanus toxoid protein in conjunction with GM-CSF in Montanide ISA-51 adjuvant resulted in T cell responses in patients with advanced stage ovarian cancer with limited clinical response (Chianese-Bullock *et al.*, 2008). Similarly, an immunodominant HER-2/neu-derived HLA-A2 peptide

with GM-CSF as adjuvant induced HER-2/neu peptide-specific IFN- γ -producing CD8⁺ T cells. However, the magnitude of the responses was low as well as short-lived (Knutson *et al.*, 2002). Vaccination with a recombinant carcinoembryonic antigen (CEA) as a TAA generated durable CEA-specific humoral and cellular immune responses in colorectal carcinoma patients when used with GM-CSF in a phase I clinical trial (Ullenhag *et al.*, 2004). The prognostic effect of the CEA-induced immune response was correlated with the clinical outcome. In another phase I clinical study for patients with advanced or recurrent breast cancer, vaccination with the survivin-2B peptide mixed with incomplete Freund's adjuvant (IFA) increased the frequency of peptide-specific CTL, but neither survivin-2B peptide alone or in combination with IFA could induce efficient clinical responses (Tsuruma *et al.*, 2008). In a pilot phase III trial in early-stage breast cancer patients using oxidized mannan-MUC1 as vaccine, potent humoral and moderate T cell responses to MUC1 were detected over an extended period of time. Importantly, as compared to placebo where the recurrence rate was 27%, the expected rate of recurrence in stage II breast cancer, none of the vaccine group scored positive for recurrences, demonstrating the efficacy of the vaccine (Apostolopoulos *et al.*, 2006). Taken together, these clinical trials demonstrate the utility of TAA-based vaccines in inducing tumor-specific responses and in some cases measurable clinical response. However, the overall therapeutic efficacy of the vaccines remains minimal.

Why vaccines fail

Although the aforementioned vaccine approaches have demonstrated efficacy in various murine cancer models, they have been either ineffective or demonstrated limited efficacy in clinical settings. Obstacles for clinical success may include immune tolerance to TAAs, the weak antigenic nature of TAAs, and active immune evasion mechanisms employed by progressing tumors. These obstacles may be overcome by using adjuvants/immunomodulators that not only boost the existing immune responses to the tumor in patients, but also generate new immune responses and, most importantly, overcome the immune evasion mechanisms. In this context, the choice of adjuvants is critical. Adjuvants can affect the nature of the elicited immune responses, such as Th1 vs Th2 responses, T cell vs B cell responses, generation and maintenance of immunological memory, and reversal of the immunoregulatory mechanisms, such as T_{reg} cell mediated suppression and T cell anergy. As such, adjuvants that modulate innate, adaptive, and regulatory immunity in favor of the generation of effective anti-tumor immune responses may have the best efficacy. Of critical importance is the development of adjuvants with safety profiles in humans. Alum, an aluminum compound, is presently the only FDA approved adjuvant employed in a variety of human vaccines. However, the application of alum as adjuvant for cancer vaccines is limited (McKee *et al.*, 2007). Alum-based vaccines primarily induce effective Th2 responses (Grun and Maurer, 1989) with minimal efficacy in eliciting Th1 immunity (Bomford, 1980), which is necessary for the eradication of tumors. Therefore, the development of effective therapeutic cancer vaccines for the clinic will require adjuvants/immunomodulators that can efficiently stimulate innate and adaptive immune responses as well as reverse/inhibit tumor-mediated immune suppressive mechanisms.

Toll-like receptor agonists as potential adjuvants in therapeutic cancer vaccines

The importance of Toll-like receptor (TLR) signaling in innate, adaptive, and regulatory immunity presents TLR agonists as ideal adjuvants for cancer immunotherapy. TLRs are the largest and most well characterized family of a diverse set of germ line-encoded receptors, termed pattern recognition receptors (PRRs), which recognize broad classes of conserved molecular structures common to groups of microorganisms (Akira *et al.*, 2006; Janeway, Jr.

and Medzhitov, 2002; Sansonetti, 2006). Recognition of microbial components by TLRs (or other PRRs) allows for the sensing and detection of foreign pathogens and the stimulation of immediate immune responses (Akira *et al.*, 2006; Janeway, Jr. and Medzhitov, 2002; Sansonetti, 2006). As such, TLR agonists, such as unmethylated-CpG-motifs (CpG) and lipopolysaccharide (LPS) derivative monophosphoryl lipid A (MPL), have recently dominated the adjuvant field. MPL already has been approved in Europe as an immunomodulatory component for various vaccines.

TLR agonists are currently being developed as immunomodulators/adjuvants for TAA-based cancer vaccines. These agonists have been demonstrated to generate anti-tumor immunity in preclinical studies in mice by enhancing innate immunity through the activation of DCs, NK cells, monocytes, and macrophages and induction of cytokines with both direct and indirect anti-tumor activity. Engagement of TLRs on APCs, such as DCs, results in their maturation and migration to lymph nodes where they initiate adaptive immune responses and generate long-lasting memory against tumors. TLR-activated APCs upregulate MHC and costimulatory molecules, allowing for enhanced ability to present antigens to naïve T cells. Activated APCs also secrete cytokines that augment CTL proliferation and development into effector cells that are able to directly kill tumor cells. Limited clinical investigations using TLR agonists as vaccine components have demonstrated efficacy against cancers in selected settings. A melanoma antigen peptide-based vaccine containing CpG was evaluated in patients with melanoma. Compared to historical control groups, patients receiving the vaccine with CpG had statistically improved T cell responses, including the generation of T effector memory cells with the ability to express perforin and granzyme, secrete IFN- γ , and lyse syngeneic melanoma cells in an antigen-specific manner (Speiser *et al.*, 2005).

However, there are several concerns that need to be addressed for effective and safe use of TLR agonists as adjuvants for therapeutic cancer vaccines. The use of TLR agonists as components of vaccines is often associated with severe toxicity, resulting from non-specific activation of lymphocytes and plausibly from signaling into non-immune cells (Akira and Takeda, 2004; den Haan *et al.*, 2007; Krieg, 2007). Also, despite demonstrated efficacy as adjuvants for therapeutic cancer vaccines in mice and in limited clinical settings, the importance of TLR signaling for the induction of adaptive immunity, which is critical to the establishment of long-term immunological memory and prevention of tumor recurrences, has been challenged recently by several studies (Gavin *et al.*, 2006; Ishii and Akira, 2007; Krieg, 2007; Meyer-Bahlburg *et al.*, 2007). In addition, the cancer milieu, with respect to cytokines and functional status of existing immune cells, can determine the outcome of TLR signaling *vis-à-vis* generation of tumor destructive *vs.* immune evasive mechanisms. For example, TLRs can generate regulatory immunity that may counterbalance productive immunity against cancer. Signaling through TLR4 has been shown to expand T_{reg} cells *ex vivo* and induce IL-10 producing CD4⁺ T_{reg} cells *in vivo* (den Haan *et al.*, 2007). Similarly, CpG was demonstrated to endow plasmacytoid DCs with the ability to convert CD4⁺ T_{eff} cells into T_{reg} cells based on the cytokine milieu (Moseman *et al.*, 2004), and induce CD19⁺ dendritic cells to acquire potent T cell suppressive functions through the production of indoleamine 2,3-dioxygenase (Mellor *et al.*, 2005). Therefore, the discovery and development of alternative adjuvants with safety profiles and potent immunomodulatory activities on cells of innate, adaptive, and regulatory immunity, without significant toxicity at therapeutic doses, is of vital importance in the field of cancer immunotherapy.

Costimulatory molecules as potent immunomodulators for cancer vaccines: ProtEx™ technology

Costimulatory molecules of the CD28 and TNFR superfamilies play critical roles in modulating innate, adaptive, and regulatory immune responses. As such, agonistic ligands to

these costimulatory receptors have the potential to serve as effective immunomodulatory components of therapeutic cancer vaccines. For example, lack of costimulation by tumor cells serves as an effective mechanism of immune evasion. Direct recognition of tumor cells in the absence of proper costimulatory signals limits the magnitude of primary T cell activation, leading to T cell anergy (Cuenca *et al.*, 2003). Therefore, introduction of costimulatory molecules into tumor cells *ex vivo* or *in vivo* using gene therapy led to the generation of effective anti-tumor immune responses with preventive and therapeutic efficacy in various preclinical tumor models (Guckel *et al.*, 2005; Singh *et al.*, 2003). Routine application of cancer gene therapy to the clinic, however, faces several hurdles (Anderson, 1998) that include insufficient cancer tissue mass for *ex vivo* preparation of vaccine due to early detection, surgically inaccessible tumors, inefficient delivery and targeting methods for the transfer of gene of interest into primary cancer cells *in vivo*, low efficiency of expression of the introduced gene, and safety concerns associated with the introduction of exogenous foreign DNA into patients. Therefore, innovation of novel alternative approaches to gene therapy remains of paramount interest in the field of cancer immunotherapy.

We recently pioneered the ProtEx™ technology as a safe, efficient, and practical approach to gene therapy for immunomodulation (Yolcu *et al.*, 2002). This technology involves the generation of novel recombinant chimeric molecules composed of the extracellular domains of immunological ligands of interest and a modified form of streptavidin, biotinylation of biological surfaces, such as cells, tissues, or organs, and the display of chimeric proteins on biotin-modified surfaces taking advantage of high affinity interaction ($K_d = 10^{-15}$ M) between biotin and streptavidin (Fig. 1) (Singh *et al.*, 2005). The fusion partner streptavidin exists as stable tetramers and oligomers under physiological conditions (Reznik *et al.*, 1998; Green, 1990), and therefore not only allows for the durable display of chimeric immunological ligands on the surface of biotinylated cells, but also for the generation of immunological ligands with potent activities as soluble proteins or displayed on the cell surface as compared with their native counterparts. The better immunological function of the chimeric ligands is plausibly due to their ability to crosslink their respective receptors on target immune cells for the transduction of potent signals (Grakoui *et al.*, 1999).

Biotin persists on the cell surface for weeks *in vivo*, thereby providing a platform to display exogenous proteins with extended cell surface kinetics. Chimeric proteins also persist on the surface of biotinylated cells for extended periods of time *in vitro* and *in vivo* varying from days to weeks. However, the kinetics of exogenous protein turnover on the cell surface were dependent on the protein and the cell type displaying the protein (Singh *et al.*, 2003; Yolcu *et al.*, 2002). For example, cells that are metabolically active have faster protein turnover kinetics as compared with those that were metabolically less active. Inasmuch as important immune decisions are made *via* the interaction of cell surface receptors and ligands and these interactions are short in duration requiring minutes to hours, the transient persistence of chimeric immunological ligands for days on the cell surface suffices the time requirement for effective transduction of immunological signals. Importantly, the transient display of immunological ligands also may obviate the undesired effects arising from persistent, long-term expression of immunological ligands with pleiotropic effects using gene therapy.

The display of SA-FasL protein on cells, tissues, and organs as an effective alternative to gene therapy for immunomodulation

To demonstrate the utility of ProtEx™ technology for immunomodulation, we generated a chimeric molecule containing the extracellular functional domain of rat FasL with a modified form of core streptavidin (SA-FasL). The chimeric protein has potent apoptotic activity in soluble form or when displayed on the cell surface against Fas⁺ (Askenasy *et al.*,

2003b; Yolcu *et al.*, 2002). Importantly, immunomodulation of allogeneic recipients with donor cells engineered with SA-FasL effectively blocked primary as well as secondary alloreactive responses by causing the apoptosis of T cells responding to alloantigens. Graft recipients systemically treated with donor splenocytes achieved tolerance to cardiac and pancreatic islet allografts in the absence of chronic immunosuppression (Askenasy *et al.*, 2003b; Askenasy *et al.*, 2005; Franke *et al.*, 2007; Yolcu *et al.*, 2002; Yolcu *et al.*, 2008). Tolerance was mediated by physical depletion of alloreactive T cells followed by induction/expansion of T_{reg} cells that maintained tolerance. Importantly, ProtEx™ technology was effective not only in engineering cells, but also organs such as the heart. Perfusion of the heart with biotin followed by SA-FasL under extracorporeal conditions used for clinical transplantation resulted in effective display of SA-FasL on heart vasculature without a significant effect on the survival of engineered heart in syngeneic recipients. Importantly, SA-FasL-engineered hearts overcame acute rejection when transplanted into allogeneic hosts (Askenasy *et al.*, 2003a). Taken together, these data demonstrated the utility of ProtEx™ technology as a practical, safe, and effective alternative to DNA-based gene therapy for engineering cells, tissues, and organs for immunomodulation with demonstrated efficacy and potential application to the treatment of various acquired immune disorders and graft rejection.

CD80 engineered tumor cells as effective cancer vaccines

Several studies demonstrated the utility of tumor cells genetically modified to express costimulatory molecules as cancer vaccines. We hypothesized that the transient display of costimulatory ligands on tumor cells may convert them into professional APCs for the generation of effective anti-tumor immune responses with preventive and therapeutic potential. To test this notion, a chimeric protein composed of the extracellular portion of CD80 costimulatory molecule and core streptavidin (CD80-SA) was generated and efficiently displayed on the surface of various primary and established cell lines. Chimeric CD80-SA persisted on the surface of established or primary tumor and nontumor cells for weeks *in vitro* and *in vivo*. Tumor cells engineered with the chimeric protein served as APCs for the generation of effective antitumor immune responses *ex vivo*, and vaccination with the engineered tumor cells prevented tumor growth in an aggressive model of mouse lymphoma (Singh *et al.*, 2003). Efficacy of the vaccine correlated with the generation of tumor specific CTLs and long-term immune memory.

With demonstrated efficacy in mice, we next tested whether primary tumor cells resected from cancer patients could be engineered with chimeric CD80-SA and whether such cells serve as APCs to generate autologous T cell responses *ex vivo*. Tumors and peripheral blood lymphocytes were collected from 14 lung, 9 colon, and 2 breast “treatment-naïve” cancer patients with various clinical stages of disease. Tumors engineered with CD80-SA generated significant proliferation of autologous T cells from 9 of 16 evaluable patients. Importantly, CD80-SA engineered tumors generated specific CTL responses against autologous tumors in 15 of 15 evaluable patients. Eleven out of 15 CTL responses were significant, and killing of autologous tumor cells ranged from 5% to 70% (Singh *et al.*, 2006). Taken together, these preclinical and *ex vivo* clinical studies demonstrate that primary tumor cells can be effectively engineered with recombinant costimulatory ligands, and that such cells may serve as potent APCs to generate autologous antitumor T cell responses.

4-1BBL and LIGHT co-engineered tumor cells as effective cancer vaccines

One attractive aspect of the ProtEx™ technology is that several proteins with synergistic functions can be displayed on the cell surface with equal efficiencies (Fig. 2). Therefore, we tested whether tumor cells engineered to co-display two costimulatory molecules have better

efficacy than those displaying individual molecules in a cervical cancer mouse model. Given the paramount importance of TNF family member costimulatory molecules in regulating innate, adaptive and regulatory immunity, we generated two chimeric forms of costimulatory molecules, SA-4-1BBL and SA-LIGHT. The choice of 4-1BBL stems from its potent immunostimulatory activity on both innate and adaptive immune responses as well as its ability to curb various immunoregulatory mechanisms, such as reversal of T cell anergy and T_{reg} functions (Elpek *et al.*, 2007b; Melero *et al.*, 2008; Schabowsky *et al.*, 2007). The choice of LIGHT was due to its critical role on stromal cells for secretion of various chemokines, resulting in infiltration of lymphocytes and disruption of immunosuppressive environment of tumor stroma (Granger and Rickert, 2003; Yu and Fu, 2008). Using the HPV E7 expressing TC-1 cells as a cervical cancer animal model, we demonstrated that TC-1 cells can effectively be engineered to codisplay SA-4-1BBL and SA-LIGHT on their surface, and that such engineered cells served as an effective vaccine with both preventive and therapeutic efficacies. The efficacy of tumor cells co-displaying both molecules was better than cells displaying individual molecules as vaccine with respect to the generation of antigen-specific primary T cell and B cell responses to E7 as well as prevention and treatment of established tumors (Sharma *et al.*, manuscript in preparation). In conclusion, rapid and durable display of recombinant costimulatory molecules on tumor cells possess the simplicity, safety, and efficacy required to make it a clinically relevant alternative to gene transfer approaches in the treatment of a broad spectrum of immune-based disorders, including cancer.

ProtEx™ technology as a platform for generation of soluble vaccines

Although tumor cells engineered using ProtEx™ technology to display on their surface costimulatory molecules as autologous vaccines harbor significant potential, the application of this approach to the clinic may face various challenges. First, this approach is patient-customized as it requires the extraction of primary tumors for autologous vaccine preparation. Second, the unavailability of sufficient tumor mass from patients or surgically inaccessible tumors for vaccine preparation represent another limitation for the widespread application of autologous tumor cell vaccines in the clinic. As an alternative, we developed a classic vaccine approach consisting of defined TAA/TSAs and soluble recombinant costimulatory molecules as immunomodulators. This approach was designed based on our observations that chimeric costimulatory molecules have potent activities in soluble forms (Elpek *et al.*, 2007b; Kilinc *et al.*, 2006). This is because streptavidin in chimeric proteins exists as tetramers and oligomers, thereby allowing the costimulatory ligands to crosslink their respective receptors on immune cells for transduction of potent immunostimulatory signals (Kilinc *et al.*, 2006).

Given the paramount role costimulation plays in modulating immune responses (Dawicki *et al.*, 2004; Uno *et al.*, 2006; Wilcox *et al.*, 2002a; Zheng *et al.*, 2004), we tested the efficacy of 4-1BBL costimulatory protein as a component of various vaccine formulations. 4-1BBL, a member of the tumor necrosis factor receptor family, is an attractive target for immunomodulation for several reasons (Watts, 2005). 4-1BBL is expressed on activated B cells, macrophages, and DCs (Futagawa *et al.*, 2002; Lee *et al.*, 2003; Zhou *et al.*, 1995). The receptor, 4-1BB, is constitutively expressed on the surface of T_{reg} cells, a sub-population of DCs, and neutrophils, and inducibly expressed on activated CD4⁺ and CD8⁺ T cells, NK cells, and monocytes (Futagawa *et al.*, 2002; Kienle and von Kempis, 2000; Pauly *et al.*, 2002; Wilcox *et al.*, 2002b; Choi *et al.*, 2004; McHugh *et al.*, 2002). 4-1BB/4-1BBL interaction is important for the activation of monocytes and DCs and their synthesis of cytokines. 4-1BB signaling into DCs was demonstrated to induce production of IL-6 and IL-12, and *in vivo* treatment of *Rag*^{-/-} mice led to an increase in DC activity in *ex vivo* T cell proliferation assays (Futagawa *et al.*, 2002). 4-1BB signaling into T cells allows for CD4⁺

and CD8⁺ T cell expansion, cytokine production, development of CTL effector function, and prevention of apoptotic cell death by upregulating anti-apoptotic Bcl-xL and Bcl-2 molecules. 4-1BB/4-1BBL signaling has been shown to selectively promote type 1 cytokines, such as IL-2, IFN- γ , and TNF- α , which are critical for anti-tumor immune responses (Cannons *et al.*, 2001; Watts, 2005). 4-1BB/4-1BBL interaction also results in the establishment of long-term immunological memory, allowing for protection against tumor recurrences (Miller *et al.*, 2002; Myers *et al.*, 2006; Waller *et al.*, 2007). Most importantly, we recently demonstrated that 4-1BB signaling into T cells renders them refractive to the suppressive function of T_{reg} cells (Elpek *et al.*, 2007b). In conclusion, signaling via 4-1BB has pleiotropic effects on cells of innate, adaptive, and regulatory immunity, and as such possesses significant potential as the immunomodulatory component of therapeutic cancer vaccines.

Consistent with the notion of using costimulatory agonists as soluble components of cancer vaccines is the demonstrated therapeutic efficacy of agonistic Abs to 4-1BB in various preclinical cancer and viral infection models (Miller *et al.*, 2002; Myers *et al.*, 2006; Waller *et al.*, 2007). These Abs are assumed to co-stimulate DC-primed T cells that have upregulated 4-1BB after tumor-antigen recognition. Agonistic Ab-mediated anti-tumor activity was demonstrated to be mediated by CD8⁺ T cells, NK cells, and CD4⁺ T cells in selected settings. Due to preclinical success, humanized agonistic 4-1BB mAbs have been developed and are presently being tested in various phase I clinical trials (National Institutes of Health Clinical trials database NCT00309023). However, the use of agonistic antibodies to costimulatory molecules is often associated with severe toxicity arising from nonspecific, systemic activation of lymphocytes (Ferlin *et al.*, 1998; Hixon *et al.*, 2001; Hixon *et al.*, 2002; van Mierlo *et al.*, 2002; Vonderheide *et al.*, 2007). For example, a single intravenous dose of a super agonist anti-CD28 monoclonal Ab in 6 healthy volunteers resulted in life threatening toxicity due to systemic inflammatory responses in a phase I clinical trial (Suntharalingam *et al.*, 2006). Importantly, it was recently demonstrated that multiple injections of anti-4-1BB mAbs in mice resulted in cytokine-mediated disruption of lymphocyte trafficking, lymphadenopathy, splenomegaly, and multifocal hepatitis (Niu *et al.*, 2007). Therefore, the use of costimulatory agonists as immunomodulatory components of therapeutic vaccines in humans requires the generation of agonists that transduce appropriate stimulatory signals without severe toxic side effects.

We hypothesized that signaling by natural ligands may be qualitatively and quantitatively different from that transduced by agonistic Abs, and thus results in superior therapeutic efficacy and safety. Inasmuch as the natural 4-1BBL functions as a cell membrane-bound protein and has no activity in soluble form (Rabu *et al.*, 2005), we generated the chimeric SA-4-1BBL protein and tested its immune stimulatory function in soluble form. SA-4-1BBL forms tetramers/oligomers and has potent costimulatory activity in soluble form on CD4⁺ T cells (Elpek *et al.*, 2007b). Because the 4-1BB receptor is constitutively expressed on a subpopulation of immature DCs and CD4⁺CD25⁺FoxP3⁺ T_{reg} cells (Elpek *et al.*, 2007a; Elpek *et al.*, 2007b; Zheng *et al.*, 2004), and inducibly expressed on activated T_{eff} cells (Bukczynski *et al.*, 2004; Cannons *et al.*, 2001), we tested the immunomodulatory function of SA-4-1BBL on each cell population. Vaccination with SA-4-1BBL in combination with a synthetic peptide (E7₄₉₋₅₇) representing the dominant CD8⁺ T cell epitope for E7 resulted in the generation of robust antitumor CTL responses and impressive therapeutic efficacy in a mouse cervical cancer model. SA-4-1BBL also demonstrated better efficacy than TLR agonists LPS, MPL, and CpG for immune activation and eradication of established TC-1 tumors (Sharma *et al.*, manuscript under revision). SA-4-1BBL was more effective than an agonistic 4-1BB Ab in generating immune responses, and did so without Ab-associated severe toxicity (Schabowsky *et al.*, manuscript in preparation). Potent immunomodulatory activity combined with lack of toxicity rationalizes the use of this novel SA-4-1BBL

molecule as a platform for the development of therapeutic vaccines against cancer and chronic infections.

Conclusions and future prospects

Conventional vaccines based on TAA/TSAs present an attractive choice for the prevention and treatment of cancer due to their cost-effectiveness, ease of production, storage, distribution, and broad applicability to a wide range of tumor types. However, the efficacy of therapeutic cancer vaccines will depend on not only their ability to boost the existing immune responses in cancer patients, but also to generate new immune responses and most importantly overcome various immune evasion mechanisms that allow tumor growth to begin with. This will require vaccine formulations that modulate innate, adaptive, and regulatory immunity that impact tumor progression vs elimination. In this context, the development/discovery of immune modulators with pleiotropic effects on a broad range of immune cells with defined mechanisms of action and lack of toxicity in humans will be an important step forward. Novel costimulatory molecules, such as streptavidin chimeric proteins described herein, and TLR agonists are such promising immunomodulatory candidates. Moreover, the next generation vaccines may benefit from the targeted delivery of TAA/TSAs into DCs given the importance of these cells in the initiation of immune responses to tumors. In particular, therapeutic cancer vaccine formulations employing immunomodulators that not only regulate the function of DCs, T cells, and other critical cells of innate and regulatory immunity, but also serve as vehicles to specifically deliver TAA/TSAs to DCs *in vivo* may have potential to succeed in the clinic.

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Abbreviations

DCs	dendritic cells
NK cells	Natural Killer cells
APCs	antigen-presenting cells
CC	(chemokine ligand 5...
CTLs	cytotoxic T lymphocytes
CEA	carcinoembryonic antigen
TLRs	Toll-like receptors
PRRs	pattern recognition receptors
LPS	lipopolysaccharide
MPL	monophosphoryl lipid A

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Figure 1.

ProtEx™ technology generates novel recombinant chimeric proteins consisting of core streptavidin that allows for the formation of functional tetramers/oligomers. These novel chimeric proteins can be displayed on the cell surface of biotinylated cells for the use of whole cell tumor vaccines (A) or used in soluble form as an immunomodulator in combination with any TAA/TSAs for use in classical TAA-based cancer vaccines.

Figure 2. Co-display of exogenous proteins on the cell surface. Confocal microscopy pictures of bone marrow cells (left) and tumor cells (right) engineered to co-display on their surface streptavidin in combination with SA-FasL (left) or CD80-SA (right).