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## Extracellular Matrix Adjuvant for Vaccines

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### 1. Introduction

In 1796, Edward Jenner first used the term, “vaccination,” to describe his studies which used poxvirus derived from lesions in cows to protect humans against infection with smallpox (Barquet & Domingo, 1997). Later, Louis Pasteur demonstrated that animals and people could be protected against disease when administered microbes that had been attenuated to reduce pathogenicity. From this early work, it became evident that stimulation of the immune system by exposure to specific antigens associated with pathogens could lead to a response that would protect individuals from infection and, of paramount importance, disease associated with infection.

Since the times of Jenner and Pasteur, vaccination has proved over time to be one of the most cost-effective means to control infectious disease (Kaufmann, 2007). For example, in humans smallpox has been eradicated, and the incidence of polio has been greatly reduced; and in cattle, rinderpest has been eradicated (Kieny & Girard, 2005; Normile, 2008). Indeed, stimulation of the immune system by vaccination has proved to be a cornerstone of preventive medical strategies for several decades in both human and veterinary medicine.

The targets within a vaccine, and against which the immune response is directed, are referred to as “antigens.” Often, antigens consist of proteins, though some vaccines utilize polysaccharides, nucleic acids, toxoids, peptides, and inactivated whole or fractions of microorganisms or cells as antigens (Liljeqvist & Ståhl, 1999). Great effort has been given to identification and production of purified recombinant protein subunit vaccines as a way to drive the immune system to target specific antigens key to the colonization, survival, and pathogenesis of infectious agents. Similar work has recently extended to the use of vaccination as an approach to cancer treatment, with vaccines based upon antigens ranging from recombinant subunit proteins to whole, inactivated cancer cells being evaluated in preclinical models and, in some cases, clinical trials (Buonaguro et al., 2011; Melenhorst & Barrett, 2011). In spite of significant progress in identification and production of vaccine antigens, many antigens stimulate only a weak immune response insufficient to offer protection to the patient.

### 2. Vaccine adjuvants

Adjuvants are compounds added to vaccines to improve the immune response to vaccine antigens. Indeed, the word ‘adjuvant’ derives from the Latin ‘adjuvans’ which means ‘to help.’ Adjuvants exert their action in a number of different ways, such as by increasing the

immunogenicity of weak antigens; strengthening the immune responses of individuals with weak immune systems; reducing the amount of antigen needed in the vaccine, thus reducing the cost; extending the duration of the immune response; modulating antibody avidity, specificity, or isotype distribution; and promoting specific forms of immunity, such as humoral, cell-mediated, or mucosal immunity.

### 2.1 Types of vaccine adjuvants

A variety of compounds have been investigated for potential use as vaccine adjuvants. For example, immunostimulatory molecules such as the cytokines IL-2, IL-12, gamma-interferon, and granulocyte-macrophage colony-stimulating factor (GM-CSF) have all been shown to enhance aspects of the immune response following vaccination (Coffman et al., 2010). However, because these molecules are proteins that are relatively expensive, have short half-lives *in vivo*, and may exert a variety of other, often unpredictable systemic effects, their practical application as vaccine adjuvants is unlikely.

Compounds which lack the challenges that restrict the application of cytokines, but which in turn enhance immunostimulatory cytokines, have also been studied. Monophosphoryl lipid A (MPL), a molecule derived from bacterial lipopolysaccharide (LPS), stimulates the release of cytokines, likely through interaction with toll-like receptor 4. MPL induces the synthesis and release of IL-2 and gamma-interferon (Gustafson & Rhodes, 1992; Ulrich & Myers, 1995). In contrast, saponins are derived from the bark of a Chilean tree, *Quillaja saponaria*. The saponin QS21 is a potent adjuvant for IL-2 and gamma-interferon; and it appears to act by intercalating into cell membranes through interaction with structurally similar cholesterol. This interaction results in formation of pores in the cell membrane, and it is thought that this may allow antigens a direct pathway for presentation to the immune system (Glaueri et al., 1962). QS21 has been associated with pain on injection and local reactions, and the balance between potency and adverse events is an important consideration (Kensil & Kamer, 1998). More recently, unmethylated CpG dinucleotides have been evaluated for potential use as vaccine adjuvants. Such CpG molecules are common in bacterial DNA, but not in vertebrate DNA, and it is thought that the interaction of the immune system with these moieties is related to an evolutionary characteristic of vertebrate defense against microbial infection (McKluskie & Krieg, 2006). In this way, then, it may be that cells of the immune system are particularly adept at recognizing antigens conjugated to CpG DNA. Responses to CpG DNA are mediated by binding to toll-like receptor 9 (Hemmi et al., 2000). Further, it is believed that CpG conjugates are taken up by non-specific endocytosis and that endosomal maturation is necessary for cell activation and release of pro-inflammatory cytokines (Sparwasser, et al., 1998).

One approach toward vaccine adjuvants has been to focus on delivery of antigens to the immune system. In this regard, a variety of methods for delivery of antigens have been investigated, including oil-in-water liposomes, alginate microparticles, and inclusion of antigens in live viral or bacterial vectors. For example, the MF59 squalene oil-in-water emulsion has shown an adjuvant effect in animal models for an influenza vaccine (Cataldo & Van Nest, 1997) and hepatitis B vaccine (Traquina et al., 1996). Microparticles produced by polymerization of alginate by divalent cations have been used to vaccinate animals against bacterial respiratory pathogens (Suckow et al., 1999). Alginate microparticles enhance antibody responses when administered with incorporated antigens by either the subcutaneous or mucosal (intra-nasal or peroral) routes. Adjuvants based upon delivery of antigens via live viral or bacterial vectors have gained recent interest, particularly for

stimulation of immunity at mucosal surfaces. For example, modified intestinal pathogens such as *Salmonella typhimurium*, *S. choleraesuis*, *Listeria monocytogenes*, and *Escherichia coli* have all been used to deliver antigens, most often through vaccination at mucosal surfaces (Becker et al., 2008). Similarly, poxvirus and adenovirus have been used as a means of delivering antigens for vaccination purposes (Liu, 2010). A primary advantage of vaccination using a live vector is that the replication of the vector within the host can lead to a heightened and sustained immune response to the vectored antigens.

## 2.2 Aluminum salt adjuvants

The use of aluminum salts (alum) as vaccine adjuvants to boost the immune response to vaccine antigens has a long record of success over the past 70 years (Clapp et al., 2011). In this regard, both aluminum phosphate and aluminum hydroxide have been used, in varying ratios and concentrations specific to different vaccines. Typically, the antigen is adsorbed to alum through electrostatic charge, and the degree of antigen adsorption by aluminum-containing adjuvants is generally considered to be an important characteristic of vaccines that is related to immunopotentiality by the adjuvant. In this regard, the World Health Organization (WHO) requires that at least 80% of the antigens in alum-precipitated diphtheria and tetanus toxoid vaccines be adsorbed (Clapp et al., 2011); however, others have found that there appears to be no correlation between the percentage of antigen adsorbed and subsequent antibody production following vaccination (Clausi et al., 2008). Further, antigen presenting cells have been demonstrated to take up desorbed antigen from the interstitial fluid as well as antigen adsorbed to aluminum-containing adjuvants (Iver et al., 2003); and aluminum-containing adjuvants potentiate the immune response to non-adsorbed protein antigens (Romero-Mendez et al., 2007), suggesting that adsorption may not be a requirement for adjuvancy by alum.

The most widely accepted theory for the mechanism of alum adjuvancy is the repository effect, whereby the antigen adsorbed by the aluminum-containing adjuvant is slowly released after intramuscular or subcutaneous administration (Seeber, et al., 1991). However, it has been demonstrated using  $^{28}\text{Al}$  in rabbits that aluminum quickly appears in the interstitial fluid and is rapidly eliminated from the body (Flarend et al., 1997). The best information regarding the mechanism of action for alum suggests that alum activates a complex of proteins, termed the inflammasome, in lymphocytes and that these proteins initiate a complex cascade of actions which result in an enhanced immune response to antigens (Eisenbarth et al., 2008).

At present, aluminum salts are the only adjuvants that are currently approved for use in human vaccines by the United States Food and Drug Administration (FDA). The FDA limits the amount of aluminum in biological products, including vaccines, to 0.85 mg/dose. However, in spite of this regulatory oversight and a widespread acceptance of safety, aluminum adjuvants have been associated with severe local reactions such as erythema, induration, formation of subcutaneous granulomas, and pain. A further major limitation of aluminum adjuvants is their inability to elicit cell-mediated Th1 responses that are required to control most intracellular pathogens such as those that cause tuberculosis, malaria, leishmaniasis, leprosy, and AIDS ; and to elicit Th1 responses typical of immunity to cancer. Additionally, failure of alum to augment the humoral antibody immune response in some circumstances, such as with influenza immunization in the elderly (Parodi et al., 2011), clearly illustrates the need for additional safe and effective vaccine adjuvants.

### 3. Extracellular matrix

Extracellular matrix (ECM) is a network of macromolecules, largely proteins and polysaccharides, that aggregate to form a complex meshwork that acts as a both physical lattice and a biologically active promoter for the cellular component of tissue. Proteins typically found in ECM include collagen, elastin, fibronectin, and laminin all of which contribute structural strength to the ECM and adhesive attraction for the cells. Complementing these proteins are glycosaminoglycans, a class of polysaccharide which are often found covalently linked to proteins to constitute proteoglycans. The proteoglycans exist as a highly hydrated, gel-like ground substance in which the proteins are embedded. The proteoglycan gel allows diffusion of nutrients and molecular signals important for cell survival and growth. Several glycosaminoglycans are typically present in ECM, including hyaluronan; chondroitin sulfate and dermatan sulfate; heparan sulfate; and keratan sulfate. The glycosaminoglycans are active participants in the maintenance and survival of cells and exhibit a variety of biological functions. For example, hyaluronan is thought to facilitate migration of cells during the process of tissue repair. Further, proteoglycans are believed to have a major role in chemical signaling between cells. An example is heparan sulfate which binds to fibroblast growth factors which subsequently stimulates proliferation by a variety of cells. Heparan sulfate proteoglycans immobilize secreted chemotactic attractants called chemokines on the endothelial surfaces of blood vessels at sites of inflammation. In this way, the chemokines remain there for a prolonged period, stimulating white blood cells to leave the bloodstream and migrate into the inflamed tissue. In short, the extracellular matrix is an active participant in the dynamic process of tissue function.

#### 3.1 Small intestinal submucosa

Small intestinal submucosa (SIS) is a natural, bioactive extracellular matrix that has proven successful as a tissue graft material in a variety of clinical applications related to tissue repair (Ellis, 2007; Mostow et al., 2005). The material is processed, using porcine intestine as a raw material, into an acellular, multilaminar medical-grade material that serves as a bioscaffold for in-growth of, and subsequent incorporation into, normal, repaired tissue (Fig. 1).

SIS has been found to be a suitable material for tissue engineering applications at varied sites, including the lower urinary tract, the body wall, tendon, ligament, flat bone, cutaneous wounds and blood vessels. One common cause of tissue defects that might benefit from augmentation is tumor resection. It is interesting that, in spite of its tissue growth promoting characteristics, SIS has been shown not to promote re-growth of tumors when added to the remaining tumor bed following surgical tumor resection in a pre-clinical model (Hodde et al., 2004).

It is known that bioactive growth factors are retained in the SIS following lyophilization and sterilization. These growth factors include transforming growth factor-beta (TGF- $\beta$ ), which is important in wound healing, and the highly angiogenic growth factor, basic fibroblast growth factor (FGF-2). In addition, it has been demonstrated that SIS contains glycosaminoglycans such as hyaluronic acid, dermatan sulfate, chondroitin sulfate A, and heparan sulfate (Hodde et al., 1996). Because some of the glycosaminoglycans endogenous to SIS assist chemotactic cytokines, it is not surprising that antigen-processing cells such as macrophages are drawn to sites where SIS has been implanted (Badylak et al., 2008).



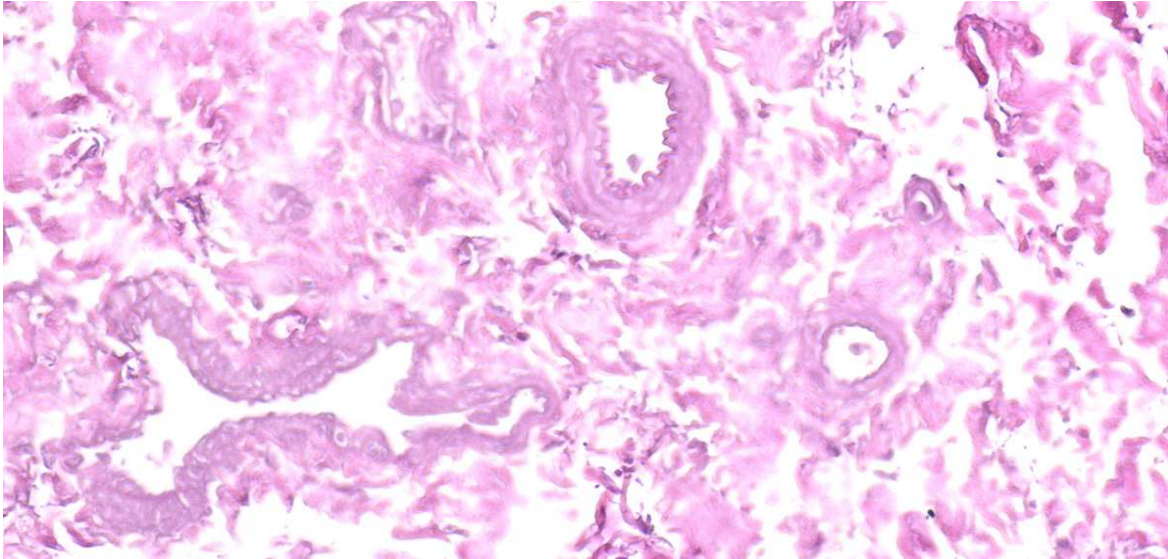


Fig. 1. Photomicrograph of small intestinal submucosa, demonstrating lack of cellular components but with remnant acellular matrix and vascular structures.

#### 4. Use of SIS as a vaccine adjuvant and immune modulator

Because small intestinal submucosa has biological activities that are known to include attraction of antigen-presenting cells, such as macrophages, to sites of implantation, the potential application of the material as a vaccine adjuvant is obvious. That SIS is available as a medical-grade material in sheet form (Fig. 2) and has been safely used in a variety of applications in a large number of patients supports the idea that it could be safely applied as a vaccine adjuvant as well.

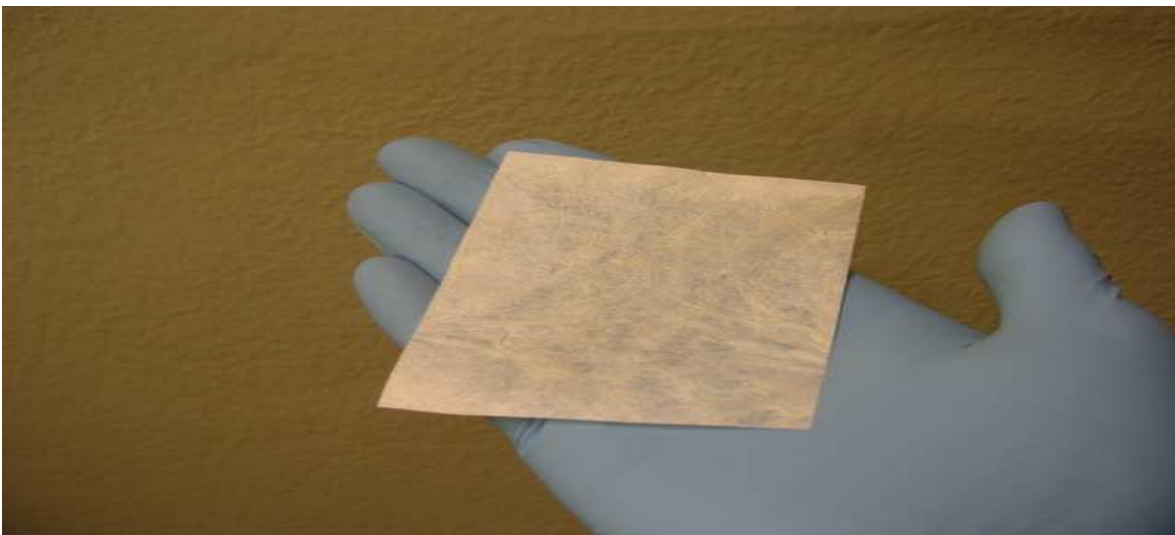


Fig. 2. A sheet of medical-grade SIS.

##### 4.1 SIS enhances the performance of whole cell cancer vaccines

The idea that cancer is a disease that can be treated by vaccination is relatively new, and great effort has been given to development of therapeutic, and in some cases prophylactic,

vaccines. For example, the Provenge® dendritic cell-based vaccine for treatment of prostate cancer was recently approved by the U. S. Food and Drug Administration (Madan & Gulley, 2011); and the Gardasil® and Cervarix® vaccines for prevention of cervical cancer are approved as well (Harper, 2009). Indeed, it is believed by many that the age of cancer immunotherapy has dawned and offers entirely new possibilities for the clinical approach to cancer.

The use of whole tumor cells as vaccine components allows a greatly increased menu of antigens to be presented to the immune system. Although many antigenic moieties in such vaccine preparations may be unidentified, it can be presumed that the rich choice of antigenic targets facilitates the likelihood of a successful immune response. As an example, allogeneic (from the same species) human prostate cancer cells have been examined for potential use in vaccination therapy of prostate cancer patients. The basic reasoning for this approach is that because tumor antigens are often conserved between tumors, allogeneic vaccines might stimulate cross-protective immunity. To test this idea, monthly intradermal administrations of a vaccine composed of three inactivated (irradiated) allogeneic prostate cancer cell lines were given for 1 year to patients having progressive disease as defined by two consecutive increases in prostate-specific antigen (PSA). The treatment did not produce any evidence of toxicity and resulted in decreased PSA velocity, as well as a cytokine response profile consistent with a Th1 immune response (Simons et al., 1999). In addition, median time to disease progression was 58 weeks in vaccinated patients compared with 28 weeks for historical controls.

Tissue vaccines represent an expansion on the idea of whole cell vaccines. Tissue vaccines are produced directly from harvested tumor material and do not undergo any *in vitro* culture (Suckow et al., 2007a). In this regard, tissue vaccines include an enormous menu of antigen targets composed of various stages of an evolving, growing population of cancer cells; antigens associated with an evolving and expanding extracellular matrix; and antigens expressed uniquely *in vivo* but not *in vitro*. In the Lobund-Wistar rat model of hormone-refractory prostate cancer, a tissue vaccine was demonstrated to reduce the incidence of autochthonous prostate cancer by 90% (Suckow et al., 2005); reduce metastasis to the lungs by 70% (Suckow et al., 2008a); and augment tumor reduction by external beam irradiation by approximately 50% (Suckow et al., 2008b). Further, a xenogeneic tissue vaccine was demonstrated to reduce growth by 70% of tumors associated with human prostate cancer cells xenotransplanted in immunodeficient mice (Suckow et al., 2007b).

#### **4.1.1 SIS as an adjuvant for prostate cancer whole cell vaccines**

Melanoma is a cancer of pigment-producing skin cells referred to as melanocytes. Often aggressive with metastasis to the lymph nodes, lungs, liver, and brain, melanoma is usually first treated by surgical resection of the primary tumor. Though often curative, return of the tumor is not infrequent and can result in a particularly aggressive form of cancer.

To evaluate the ability of SIS to enhance the protective effect conferred by vaccination with a whole cell melanoma vaccine, C57Bl6/J mice were administered B16 mouse melanoma cells to produce subcutaneous tumors. When the tumors were palpable they were harvested, dissociated with a 80-mesh stainless steel screen and allowed to incubate on 2 × 2 cm sections of sterile SIS for three days. Sections of SIS with cells were then treated with 2.5% glutaraldehyde to produce a tissue vaccine as previously described (Suckow et al., 2008c). A separate portion of cells was used to make a tissue vaccine without added SIS, and separate

sections of SIS were treated with glutaraldehyde and washed to produce an SIS control treatment.

Groups of naïve mice were administered B16 cells to produce subcutaneous melanoma tumors. Fourteen days after administration of cells, all mice had palpable tumors and were prepared for aseptic surgical debulking of the tumor masses. Mice were anesthetized with a mixture of ketamine hydrochloride, acepromazine maleate, and xylazine; the hair overlying the tumor mass was shaved; and the skin scrubbed with an iodophore. Following incision of the skin, tumors were carefully dissected free of attachments except for a residual portion of the underlying tumor bed. Groups of mice then either received no further treatment other than wound closure and routine post-surgical care; direct administration onto the tumor bed of  $1 \times 10^6$  glutaraldehyde-fixed tumor (GFT) cells; application onto the tumor bed of glutaraldehyde-fixed SIS (ECM) with no added cells; or application onto the tumor bed of glutaraldehyde-fixed SIS on which tumor cells had been grown and then fixed (GFT/ECM). Fourteen days after surgery, mice were euthanized and necropsied to assess the re-growth of tumors. As demonstrated in Fig. 3, the combination of GFT/ECM reduced the mass of re-grown tumors by approximately 70% compared to all other treatment groups.

### Vaccination After Melanoma Resection

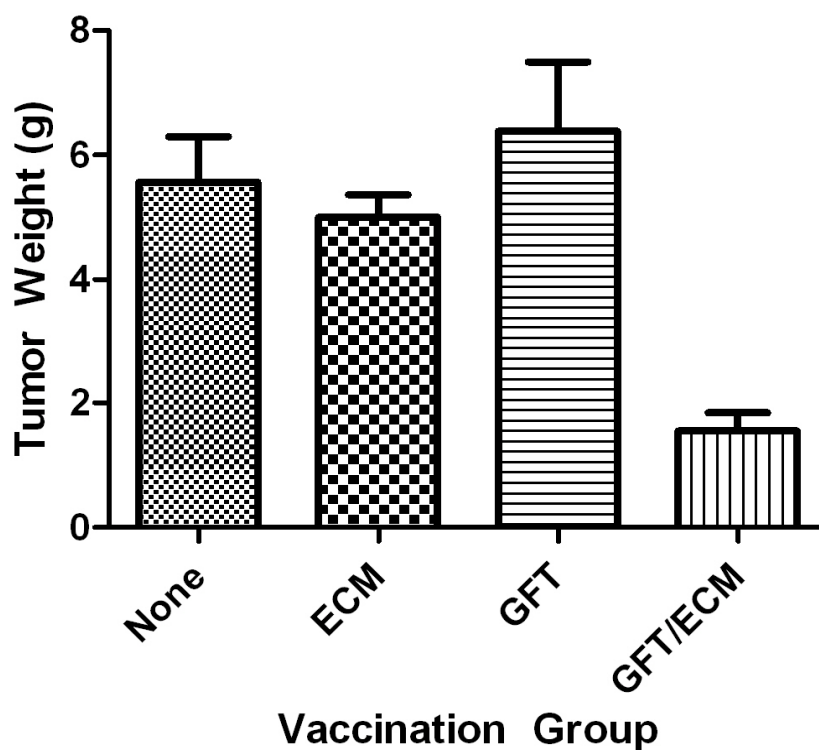


Fig. 3. Neither a melanoma tissue vaccine (GFT) nor SIS (ECM) alone reduced the size of re-grown melanoma tumors compared to controls following surgical resection, however the combination of the tissue vaccine with SIS (GFT/ECM) led to an approximately 70% reduction in tumor mass.



The idea that cancer immunotherapy might be enhanced by inclusion of SIS in the vaccine preparation needed further validation in additional models of cancer. In this regard, prostate cancer was viewed as a likely system for validation, as it is a common cancer and one which has been shown to be responsive to immunotherapy as demonstrated by the Provenge® vaccine. The Lobund-Wistar (LW) rat model of prostate cancer closely replicates the disease in man in that it progresses to a hormone-refractory cancer and readily metastasizes through the circulatory system (Pollard & Suckow, 2005). The LW rat develops autochthonous tumors, and a transplantable cell line (PAIII cells) has been isolated and characterized. When transplanted subcutaneously, PAIII cells rapidly produce aggressive tumors that metastasize to the lungs. Following surgical resection, PAIII tumors become exceptionally aggressive and there is little that can be done to ameliorate consequent growth and spread of the tumor.

As in the mouse melanoma model, subcutaneous tumors were produced as a source of vaccine material. PAIII cells administered subcutaneously rapidly grew into palpable tumors that were harvested 14 days after administration of cells. Following similar procedures as for the melanoma tissue vaccine, harvested tumor cells were added to 2 x 2 cm sections of SIS and allowed to grow for three days at 37° C, followed by glutaraldehyde fixation and extensive washing. Separate groups of rats which were administered PAIII cells 14 days earlier underwent surgical debulking of subcutaneous PAIII tumors then received no treatment other than standard peri-operative care; direct administration onto the tumor bed of  $1 \times 10^6$  glutaraldehyde-fixed tumor (GFT) cells; application onto the tumor bed of glutaraldehyde-fixed SIS (ECM) with no added cells; or application onto the tumor bed of glutaraldehyde-fixed SIS on which tumor cells had been grown and then fixed (GFT + ECM). Histological examination of a section of the GFT + ECM vaccine material shows that cells readily grow along the edge of SIS (Fig. 4) and within the substance of the SIS (Fig. 5). Rats were euthanized 28 days later and tumors weighed to assess the effect of vaccination on tumor re-growth.

Microscopic examination of SIS upon which harvested tumor tissue was cultured demonstrated robust growth of tumor cells. Cells readily grew along the margin of SIS as shown in Fig. 4 and were arranged as a monolayer along the edge.

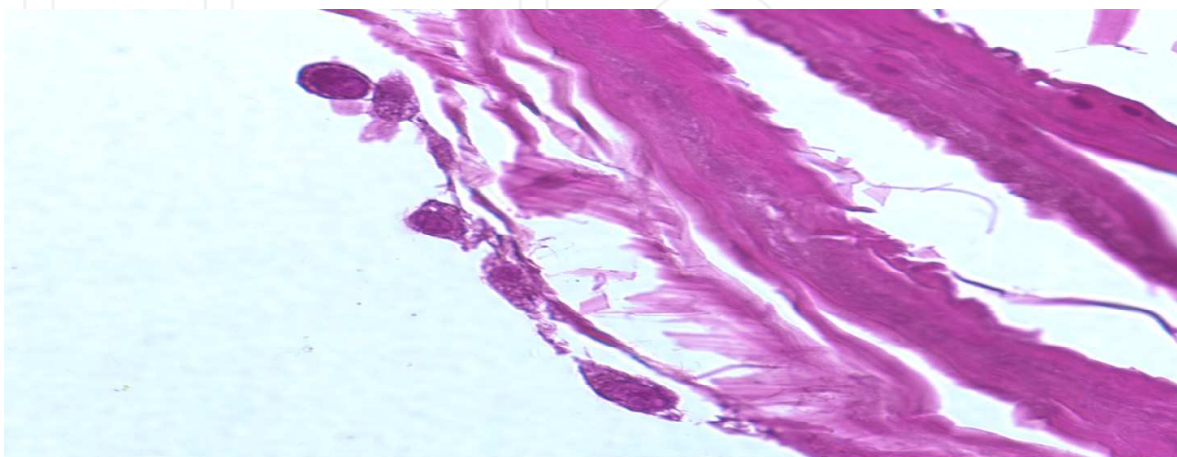


Fig. 4. Photomicrograph demonstrating growth of harvested PAIII tumor cells along the edge of SIS.

In contrast, within the substance of the SIS, cells were present as islands of piled-up cells (Fig. 5). Some islands of cells formed what appeared to be primitive vascular structures, consistent with the highly angiogenic character of PAIII tumors.

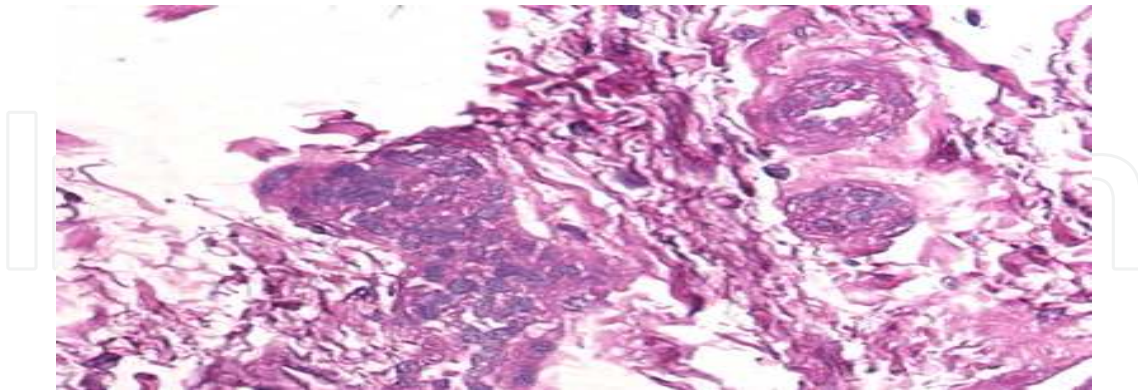


Fig. 5. Photomicrograph demonstrating growth of harvested PAIII tumor cells within the substance of SIS.

The requirement for delivery of nutrients and removal of waste metabolic products characteristic of a rapidly dividing and growing tissue is what drives the need for an expanded blood supply; thus, it is not surprising that cells from a harvested tumor would have an angiogenic phenotype. Indeed, vascular endothelial growth factor (VEGF) has been implicated in a number of aspects of cancer growth, including angiogenesis, remodeling of the ECM, generation of inflammatory cytokines, and hematopoietic stem cell development. Rats vaccinated with the GFT vaccine to which SIS was added had tumors that had a mean weight of 3.91 g, while tumors from rats that had not been vaccinated had a mean weight of 11.63 g (Fig. 6). Similarly, tumors from rats vaccinated with SIS only had a mean weight of 13.04 g and those from rats vaccinated with the GFT vaccine only had a mean weight of 9.96 g. While there was a significant ( $P < 0.01$ ) reduction in tumor weight of rats vaccinated with GFT plus SIS compared to all other groups, tumors from rats of all other treatment groups did not significantly differ in this very aggressive model of cancer (Suckow et al., 2008c).

#### Adjuvancy of SIS on Post-Resection Vaccines

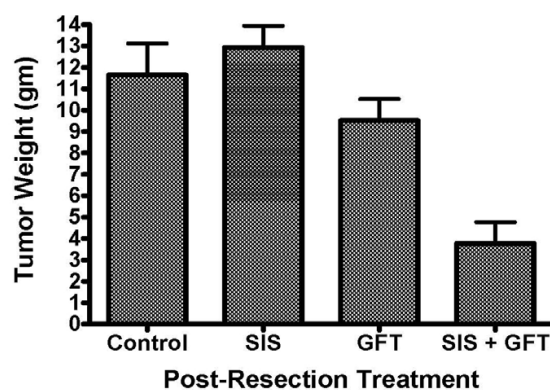


Fig. 6. Neither a PAIII prostate cancer tissue vaccine (GFT) nor SIS (ECM) alone reduced the size of re-grown prostate tumors compared to controls following surgical resection, however the combination of the tissue vaccine with SIS (GFT/ECM) led to an approximately 65% reduction in tumor mass.

Vaccination also reduced the number of rats which had foci of metastasis in the lungs. Specifically, only 40% of rats vaccinated with SIS + GFT had pulmonary metastatic foci, compared to 70% of rats immunized with the GFT vaccine and 100% of rats which were administered only SIS or those receiving no treatment (Fig. 7).

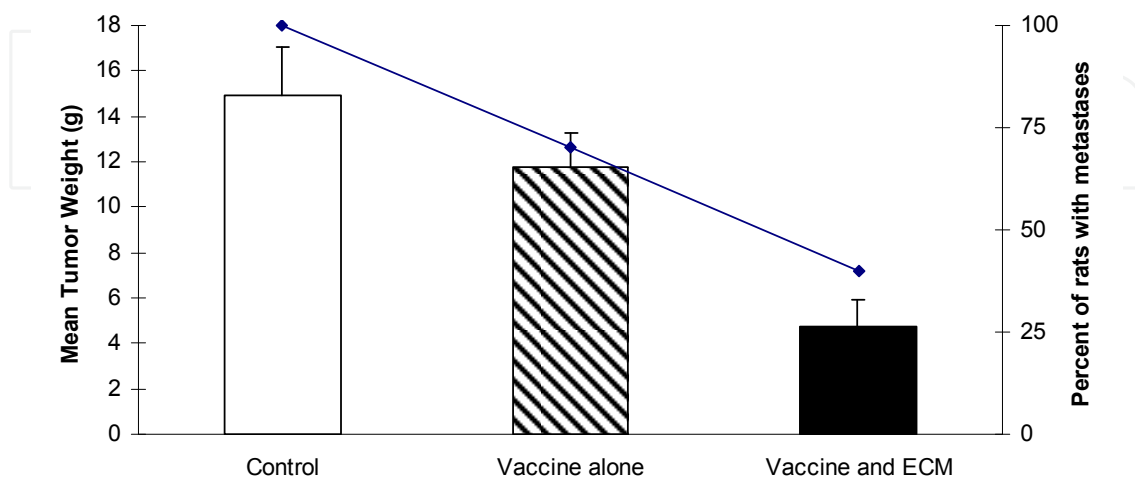


Fig. 7. Reduction in the number of PIII prostate tumor-bearing rats having pulmonary metastasis (right X-axis). For reference, the mean tumor weight is indicated on the left X-axis.

#### 4.1.2 Gel SIS exhibits adjuvancy for whole cell prostate cancer vaccine

Though vaccines produced from growth of cells directly on SIS extracellular matrix demonstrated remarkable stimulation of anti-tumor responses, it requires surgical implantation. The advantages of a similar vaccine that could be easily and quickly administered are clear. In particular, multiple doses could be given to patients without the requirement for repeated surgery.

Vaccination involving injection into the subcutaneous or intramuscular tissues is rapid and has relatively few adverse outcomes other than acute discomfort at the site of injection. Compounds administered in this way must be of a size and consistency that allow passage through a relatively narrow needle, typically 21-gauge or smaller. Obviously, a sheet of SIS is not amenable to administration in this manner. In this regard, a gel form of SIS, produced by chemical digestion, was developed and evaluated for vaccine adjuvancy. Of note, this same gel preparation has been found to be safe and effective for myocardial infarct repair in rats (Okada et al., 2010).

A tissue vaccine produced from harvested LW rat PIII prostate tumors, as described earlier, was mixed with SIS gel such that each dose contained  $1 \times 10^6$  GFT cells. In this case, groups of naïve LW rats were vaccinated with either saline; GFT cells; SIS gel; or GFT cell plus SIS gel. Vaccines were given subcutaneously once before challenge with live PIII cells, and weekly for two doses afterward. The ability of vaccination to prevent growth of PIII tumors was assessed by weighing tumors 28 days following PIII cell challenge. As shown in Fig. 8, neither saline, GFT, nor SIS gel alone demonstrated a protective effect against development of PIII prostate tumors; however, rats vaccinated with the GFT vaccine combined with SIS gel had tumors which were approximately 75% smaller than those in rats from other vaccination groups.

Prevention of Tumors with Vaccine and SIS Gel Adjuvant

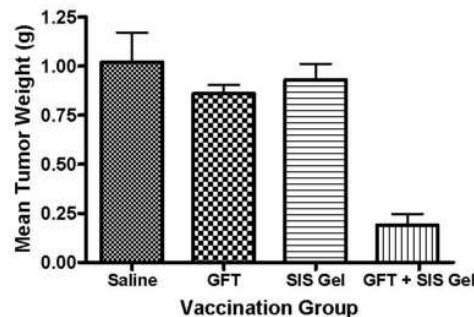


Fig. 8. SIS gel has an adjuvant effect on a tissue vaccine used to prevent prostate cancer.

An obvious concern with use of SIS as an adjuvant for cancer vaccines is whether a material that is known to promote tissue growth, and is used clinically for such applications, would also promote the growth of cancer tissue. Interestingly, when PAIII cells were administered directly onto implanted SIS sections in LW rats, no difference in tumor growth or metastasis was noted compared to control rats that did not have SIS implants (Hodde et al., 2004). This result demonstrates that the SIS extracellular matrix material is capable of promoting growth of normal tissue, but not neoplastic tissue. It may be that the mild inflammatory process which accompanies implantation of SIS facilitates growth of normal tissue, but recognizes foreign material or abnormal cells which happen to be in the vicinity of SIS, thereby alerting the immune system and stimulating an immune response to combat the challenge presented by tumor cells or other pathogenic agents.

#### 4.2 SIS enhances the performance of vaccines for diseases associated with infectious agents

While the idea of vaccination to prevent or treat cancer is relatively new, the concept of vaccination for prevention of diseases associated with infectious agents is not. In 1796, Edward Jenner inoculated an eight-year-old boy with material taken from an active cowpox lesion on another individual and discovered that this strategy could be used to confer protection to smallpox (Barquet & Domingo, 1997). Since that time, vaccines have been developed for a variety of diseases related to infectious agents, including polio, tetanus, influenza, whooping cough (pertussis), measles, diphtheria, and hepatitis (Stern & Markel, 2005).

In spite of the many successes with vaccination, there are some infectious agents for which successful vaccination has proved elusive. For example, malaria and HIV are both important diseases caused by infectious agents and for which there are a lack of viable vaccination strategies. Because SIS proved to be a powerful adjuvant for vaccines designed to protect against cancer, it was reasoned that the material might also have value as an adjuvant for vaccines designed to protect against disease associated with infectious agents.

##### 4.2.1 Particulate SIS vaccine adjuvant

Although sheet SIS and gel SIS were effective as adjuvants for cancer vaccines, it was reasoned that an adjuvant composed of particulate SIS would allow greater surface area of SIS to be exposed and interact with the cellular immune system. This was seen as an advantage because many antigens associated with vaccines for infectious diseases are composed of proteins or sub-units that are more rapidly degraded and processed than



whole, inactivated cells (as with whole cell cancer vaccines). Thus, a more rapid and robust adjuvant stimulation was expected to offer greater efficacy for infectious disease vaccines. Particulate SIS was produced by mechanical grinding of medical grade sheet SIS. Sterility of the preparation was maintained and final particulate size was limited to 150  $\mu\text{m}$ . The resulting product was a fine, white dust-like particulate (Fig. 9).



Fig. 9. Particulate SIS. Individual particles in this preparation are no greater than 150  $\mu\text{m}$  in diameter.

#### 4.2.2 Particulate SIS is an effective adjuvant for tetanus vaccine

Though relatively rare in the developed world, tetanus remains an important cause of death worldwide, with up to 1 million deaths estimated annually (Dietz et al., 1996). The disease, characterized by cardiovascular complications which result from autonomic dysfunction, is caused by a Gram-positive bacillus, *Clostridium tetani*. This microorganism is ubiquitous in soil; and under the anaerobic conditions found in necrotic tissue, the bacterium secretes two toxins: tetanolysin, which damages local tissues and optimizes conditions for further bacterial multiplication; and tetanospasmin, which is responsible for development of the clinical disease. Tetanospasmin, also commonly referred to as tetanus toxin, is a two-chain polypeptide of 150,000 Da which, when cleaved by tissue proteases, yields a light chain that acts pre-synaptically to prevent neurotransmitter release (Wright et al., 1989).

Vaccination for the prevention of tetanus has been available since 1923, with routine vaccination beginning later, mostly during the 1950s and 1960s. The vaccine is most commonly a suspension of alum-precipitated (aluminum potassium sulfate) tetanus toxoid. Though vaccination stimulates strong protective immunity in most cases, serological surveys have demonstrated an increasing proportion of patients with inadequate immunity with advancing age (Cook et al., 2001). Though some of these individuals were never vaccinated, a substantial number simply lost immunity over time (Prospero, et al., 1998).

Because immunization with alum-adsorbed tetanus toxoid is very effective at stimulating protective antibody titers, it represents a good model against which to compare other vaccine adjuvants. In this regard, we undertook studies to evaluate the ability of ECM (SIS) adjuvant to stimulate anti-tetanus toxin antibody and protective responses in a mouse model.

Groups of 15 adult female balb/c mice were vaccinated subcutaneously with 0.03  $\mu\text{g}$ /dose of tetanus toxoid (TT) in sterile saline either with no adjuvant; with 300  $\mu\text{g}$  of alum



(Alhydrogel®); or with 300 µg of particulate ECM. In addition, an untreated control group was included in these studies. Mice were vaccinated initially and received booster vaccinations five weeks later. In addition, serum samples for evaluation of antibody responses were obtained immediately prior to challenge with tetanus toxin, 10 weeks after initial vaccination.

Serum IgG anti-tetanus toxin antibody responses were evaluated by enzyme-linked immunosorbent assay (ELISA). While control animals demonstrated no appreciable anti-tetanus toxin antibody titers, samples from mice vaccinated with alum-adjuvanted tetanus toxoid had measureable antibody levels. In contrast, serum samples from mice vaccinated with tetanus toxoid that was adjuvanted with ECM had five-fold higher titers compared to samples from mice vaccinated with alum-adjuvanted tetanus toxoid (Fig. 10)

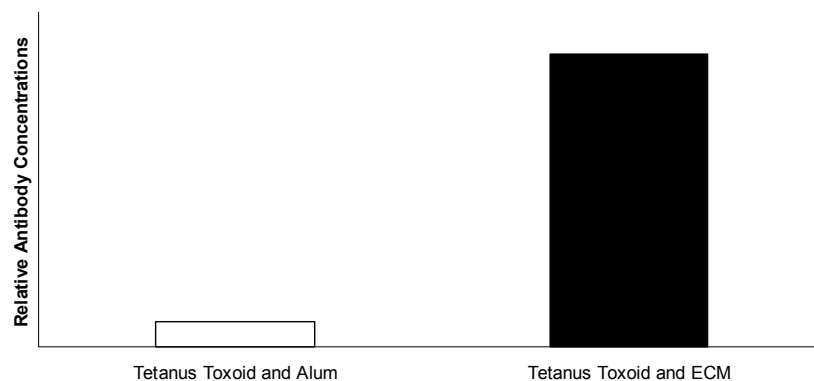


Fig. 10. Relative antibody concentrations in mice immunized with the tetanus toxoid combined with alum or ECM adjuvant.

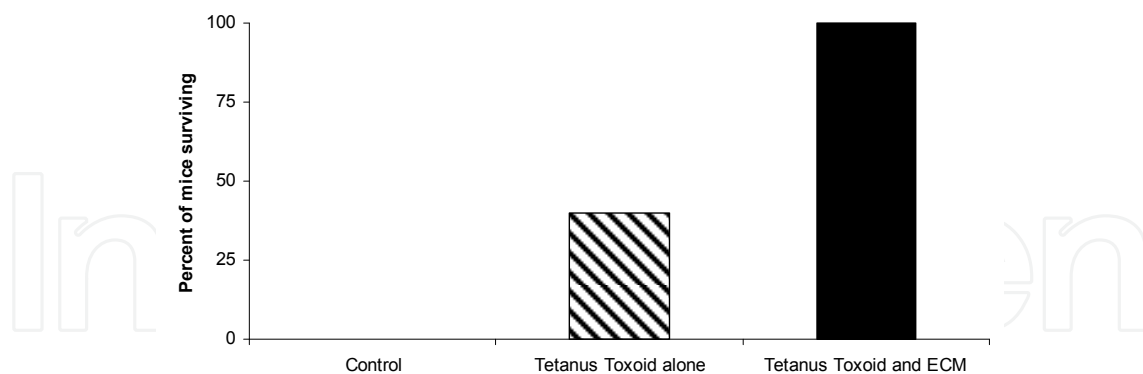


Fig. 11. Survival of mice following challenge with 1 ng/mouse of tetanus toxin

The strength of the anti-tetanus toxin serum antibody response is a reasonable measure of the likely protective effect; however, to assure that vaccination correlated with protection, vaccinated mice were challenged with 1 ng of tetanus toxin administered intraperitoneally. Mice were then observed over the next 96 hours and the number of surviving mice recorded for each group. All mice vaccinated with tetanus toxoid in alhydrogel or ECM survived challenge, while only one-third of mice vaccinated with tetanus toxoid alone, and no untreated control mice survived. Figure 11 shows the percent of surviving mice in mice

vaccinated with tetanus toxoid and ECM compared to the non-vaccinated and non-adjuvanted tetanus toxoid-vaccinated control mice.

## 5. Conclusion

Vaccination has greatly reduced the incidence of many infectious diseases. Currently, a variety of new vaccines based on nucleic acids or other subunits are in development. Further, great promise is offered by vaccination for the treatment and prevention of cancer. With these new vaccines comes a need for improved adjuvants which are safe and effective. Though aluminum salts have been used for adjuvant purposes with great success, there are some vaccines and some patient populations for which alum is not effective.

We have demonstrated the powerful adjuvant effect of SIS, a medical grade ECM. SIS demonstrated a powerful adjuvant effect for vaccines against two types of cancer, melanoma and prostate cancer, and we believe that it will likely prove efficacious with vaccines for other cancers. We further used SIS adjuvant with tetanus toxoid vaccine as a model for evaluation in a vaccine for disease associated with infectious pathogens. We found that the ECM-adjuvanted vaccine stimulated markedly higher anti-tetanus toxin antibody levels than, and offered clinical protection that was at least as good as that conferred by, the alum-adjuvanted vaccine.

In summary, the ECM material, SIS, is an effective vaccine adjuvant and offers an outstanding alternative to the current standard, alum. SIS has a proven safety record when used in humans for a variety of other applications, and we believe that it has unlimited potential for use as a vaccine adjuvant.

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