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Secreted heat shock protein gp96-Ig: next-generation vaccines for cancer and infectious diseases

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Abstract Over the past decade, our laboratory has developed a secreted heat shock protein (HSP), chaperone gp96, cell-based vaccine that generates effective anti-tumor and anti-infectious immunity in vivo. Gp96-peptide complexes were identified as an extremely efficient stimulator of MHC I-mediated antigen cross-presentation, generating CD8 cytotoxic T-lymphocyte responses detectable in blood, spleen, gut and reproductive tract to femto-molar concentrations of antigen. These studies provided the first evidence that cell-based gp96-Ig-secreting vaccines may serve as a potent modality to induce both systemic and mucosal immunity. This approach takes advantage of the combined adjuvant and antigen delivery capacity of gp96 for the generation of cytotoxic immunity against a wide range of antigens in both anti-vial and anti-cancer vaccination. Here, we review the vaccine design that utilizes the unique property/ability of endoplasmic HSP gp96 to bind antigenic peptides and deliver them to antigen-presenting cells.

Keywords Heat shock proteins · Gp96 · Vaccine · Cancer · HIV · Immunotherapy

Introduction

Existing vaccines for infectious disease have been developed mostly against pathogens that show no or limited antigenic variation and that can be controlled by neutralizing serum antibodies. In contrast, the conquest of pathogens that display more variable antigens (*HIV*, *M. Tuberculosis*, *P. falciparum*) and require T-cell immunity remains elusive. The vaccine principles necessary for the generation of appropriately activated cellular immunity mediated by CD8+ cytotoxic T cells (CTL) for infectious diseases also apply to therapeutic cancer vaccines. Effective cancer immunotherapy is widely believed to originate with appropriately activated CD8+ CTL to tumor antigens displayed on MHC I; however, the vast majority of cancer vaccine approaches in development lead to preferential display of vaccine antigen (either purified or cell based) on MHC II following macrophage-mediated phagocytosis of vaccine cells or protein. The innovative approach taken by our laboratory, the secreted gp96-Ig vaccine principle, relies on secreted gp96-Ig chaperoning infectious or tumor antigenic proteins that are efficiently taken up by activated antigen-presenting cells (APCs) and cross-presented via MHC I to CD8 CTL, thereby stimulating an avid, antigen-specific, cytotoxic CD8 T-cell response. This vaccine principle has been used successfully in murine models of cancer, in non-human primates for SIV prophylaxis and in clinical trials for the treatment of non-small-cell lung cancer patients.

Heat shock proteins (HSP) are highly conserved and abundant molecules that are constitutively expressed in all eukaryotic and prokaryotic species, making up to 5–10 % of the total protein content in most cell types. Their

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intracellular concentrations can be increased two to three times by insults that induce protein unfolding, misfolding or aggregation and a flux of newly synthesized non-native proteins [1–4]. In physiological conditions, some of these proteins function as intracellular molecular chaperones or proteases. Chaperones take part in the assembly, stabilization, folding and translocation of oligomeric proteins, whereas proteases, such as the ubiquitin-dependent proteasome, mediate the degradation of damaged proteins [5, 6]. The term HSP is something of a relic to the initial identification of HSP as response elements to elevated incubation temperatures for drosophila. It is now well understood that the class of 'heat shock proteins' is more broadly defined as cellular stress proteins, since in addition to raised temperature, exposure to oxidative stress, nutritional deficiencies, ultraviolet irradiation, chemicals, ethanol, viral infection and ischemia–reperfusion injury can also induce the expression of these proteins.

Gp96, also known as glucose-regulated protein (grp) 94, endoplasmin and 99-kDa endoplasmic reticulum protein (ERp99), is a 94–96-kDa member of the HSP90 family of molecular chaperones/stress proteins that resides within the lumen of the endoplasmic reticulum [7–9]. Among HSP, the unique cellular localization of gp96 to the ER is maintained via recognition of a C-terminal KDEL domain in gp96 by the COP transporter system, which provides recycling of gp96 through the ER–Golgi network. Gp96 was initially discovered through a series of meticulous fractionation studies from murine sarcoma cell lysates in which the tumor-protective immunogenicity of these lysates was traced to the 96-kDa molecular weight fraction. Although the immunogenicity of gp96 was initially proposed to be secondary to unique mutations acquired during tumorigenesis, considerable immunological and structural evidence now supports the notion that gp96 as well as hsp90, hsp70, calreticulin, hsp 110 and grp170 are peptide- and protein-binding proteins and are associated with antigenic epitopes [10–14]. Gp96 is a dimer, able to exist in an open and closed conformation, apparently providing a protected cavity for protein folding and peptide binding [15, 16]. Removal of peptides chaperoned by HSPs abrogates the immunogenicity of HSP preparations [17, 18]. The peptide-binding properties of gp96 have been investigated by several groups and found to be both unusually promiscuous as well as highly stable [19–21]. There is disagreement about the location of the peptide-binding domain which has been located to both the C-terminal portion of the protein near the dimerization domain [22, 23] and to the N-terminal domain [24–26]. In contrast to MHC I, gp96 is capable of binding peptides with variable length and composition. In addition to binding potentially antigenic peptides, gp96 has a protein-binding domain which is involved in chaperoning the folding of newly synthesized proteins, IgGs, some integrins and all of Toll-like receptors [27–29].

The properties and location of gp96 in the ER place it into a strategic position to come into contact with virtually all proteins and peptides that are present in a cell and are used for MHC I loading. If the cell is infected by viruses or other intracellular parasites, gp96 also serves as chaperone for viral and other pathogen proteins [30]. Similarly, gp96 serves as chaperone for tumor-associated antigens expressed by tumor cells [31].

Molecular mechanism of HSP gp96 immunogenicity

Heat shock proteins/chaperones and their chaperoned peptides are released from cells primarily upon necrotic cell death that may be caused by infection, trauma, nutrient deprivation, extreme cell stress or by tumor necrosis [32] (Fig. 1). The immune system has evolved to recognize free gp96-peptide complexes and other chaperones and uses chaperoned antigenic peptides to activate CD8+ CTL through the process known as antigen cross-presentation (Fig. 1). First described in 1976 by Michael Bevan [33–35], cross-priming is a process that leads to the presentation of exogenous (extracellular) antigens on MHC class I molecules, which are otherwise destined for presentation on MHC II and resulting in a CD8+ T-cell response. There are thought to be two main pathways that lead to antigen cross-priming: the cytosolic and vacuolar pathways. The first is similar to the classic MHC I pathway, with the exception that exogenous antigens are taken up by endosomes prior to ER localization via the TAP system. The second is more similar to the MHC II pathway, wherein exogenous antigen is taken up by endosomes, degraded by peptidases such as cathepsin S, loaded onto recycled MHC I molecules and transported to the extracellular membrane without entering the ER or interacting with TAP [36]. It is unclear whether endosomal acidification is required in this pathway [36, 37]. The ability of tissues to sense molecular chaperone-based alarm signals predicts the existence of HSP receptors. The first evidence suggesting the existence of a receptor came from binding and competition studies wherein it was found that the interaction of HSPs with APCs was specific for macrophages and dendritic cells and could only compete with HSPs and not control proteins [38–41]. Chromatographic and cross-linking studies identified the scavenger receptor, CD91, as a receptor for gp96 [42] and later for hsp90, hsp70 and calreticulin [43]. Subsequent studies demonstrated that blocking CD91 in vivo abrogates the ability of tumor-derived gp96 to confer protection to immunized mice given a live tumor challenge. In addition, although germ-line deletion of CD91 is lethal, conditional deletion of CD91 in macrophages and treating cells with anti-CD91 antibodies (or

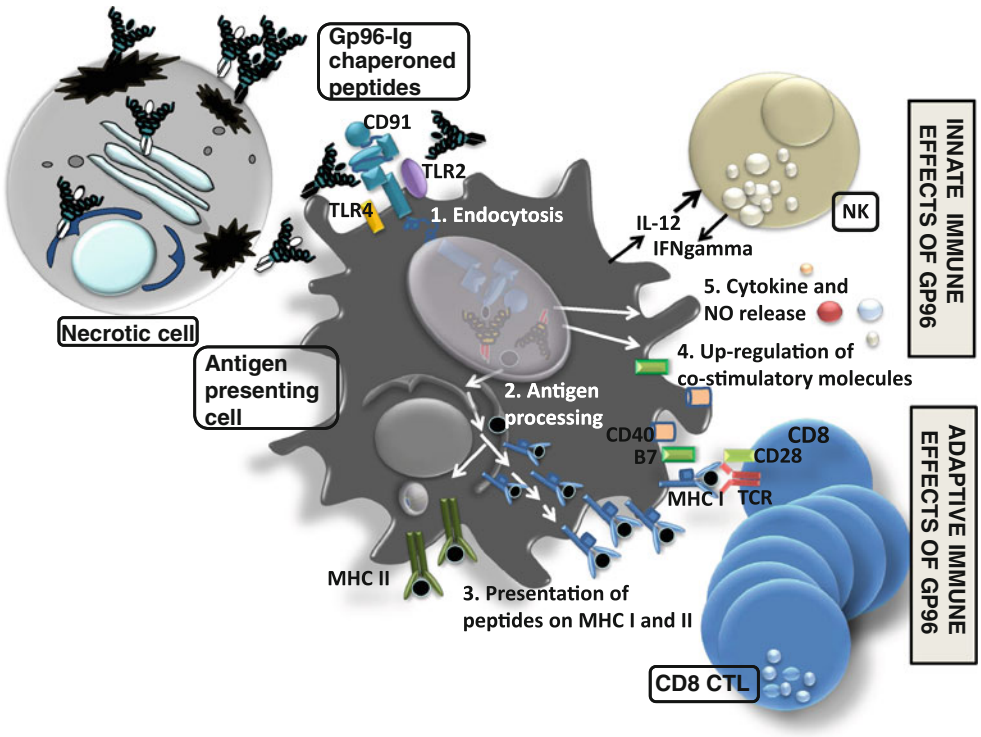

Fig. 1 The effect of gp96-peptide complex on the antigen-presenting cell (*APC*). Gp96-chaperoned peptides are released from cells only upon necrotic cell death. Gp96-peptide complexes are internalized via receptor-mediated endocytosis (CD91, TLR2/4 and SRA, LOX-1). The peptides enter the MHC pathway of antigen processing and presentation. Ultimately, the peptides chaperoned by gp96 are presented on MHC I and MHC II molecules (adaptive effects). Gp96 also trigger signaling receptors to activate NF-kb. APCs mature and up-regulate costimulatory molecules and release cytokines, chemokines and NO (innate effects)

competing ligands) prevent those cells from internalizing gp96 and the chaperoned peptides from presentation by MHC I [44, 45].

Dendritic cells (DC) recognize gp96 primarily via CD91 receptor [42] and other salvage receptors SRA [46], Lox-1 [47], CD36 [48] and endocytose gp96 along with its chaperoned peptides [40, 49]. Gp96 also interacts with the Toll-like receptors TLR2 and TLR4 (Fig. 1) [50, 51]. Internalization of gp96-peptide complex by APCs involve intracellular processing by proteasomal processing of the peptide, transport into the endoplasmic reticulum by transporter associated with antigen processing (TAP), loading onto the MHC heavy chains, and transport of peptide-loaded MHC to the cell surface [40, 43]. Following endocytosis, dissociation of the peptide from gp96 occurred rapidly within a few minutes. Dissociation reflects changes in pH or activity of compartment-specific enzymes [52]. Alternatively, a recently identified endosome-specific 'unfolding' mechanism could cause the gp96 to release the chaperoned peptide [53]. Consistent with this unfolding mechanism, gp96 was observed to translocate to the cytosol rapidly and earlier than the peptide [52]. This observation is also consistent with the observation that endocytosed gp96 is excluded from lysosomes [52], and it is degraded in the cytosol [54]. In addition, the delay in peptide translocation to the cytosol might explain how some of these HSP-chaperoned peptides are presented by MHC II [55]. Thus, peptide-containing vesicles can fuse with MHC II-loading compartments before peptide can be translocated to the cytosol. This concerted processing event might explain how gp96-chaperoned peptides are presented on MHC I or MHC II by the same APC [53, 54, 56, 57] (Fig. 1).

A second fundamental property of gp96 is an inherent adjuvanticity, which is secondary to gp96's interaction with Toll-like receptor-2 and receptor-4. Gp96 binding to TLR2 and TLR4 on dendritic cells and macrophages leads up-regulation of costimulatory molecules B7-1, B7-2, CD40, MHC II and the release of cytokines and chemokines IL-12, IL-1β, TNF-α, RANTES, MCP-1 and nitric oxide [32, 49, 58–60]. These outcomes involve the stimulation of the central signaling molecule NF-κb and its translocation into the nucleus [32]. In vivo, APCs are also stimulated to migrate to regional lymph nodes [61].

Thus, gp96 is believed to have evolved to serve as a molecular warning signal for necrotic cell death. The unique localization of gp96 to the ER, and local exposure to all peptides destined for presentation on MHC I, likely

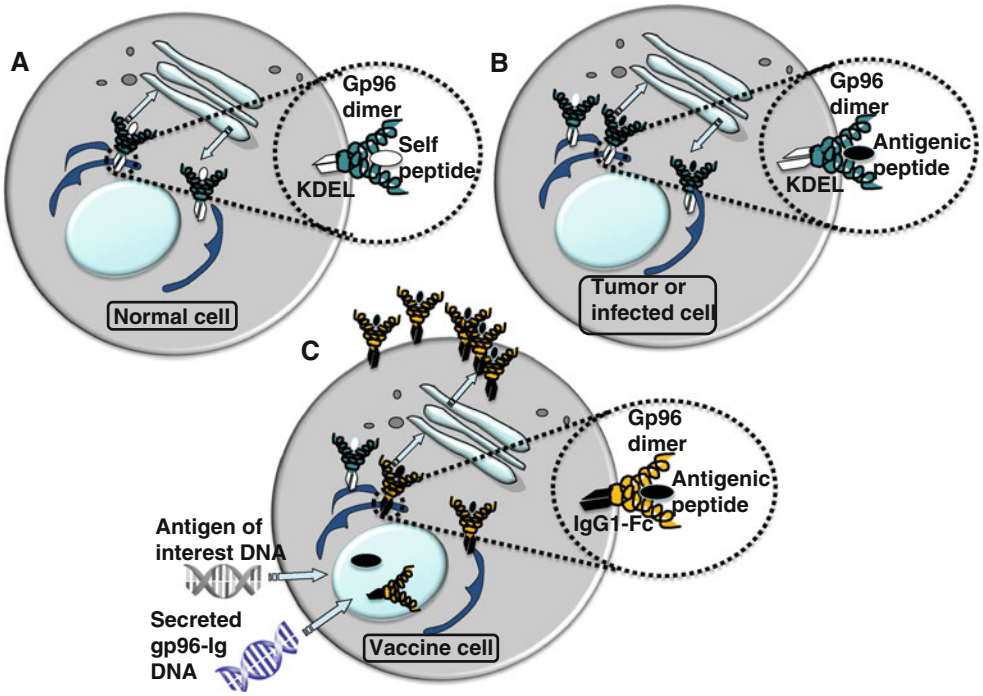

Fig. 2 Secreted gp96-Ig. Gp96 is a naturally occurring protein that stays within all normal and tumor/infected cells (**a**, **b**). The reason gp96 cannot leave living cells is because it contains a retention signal, KDEL Gp96 containing KDEL sequence is retained in ER after sorting in the Golgi apparatus by retrograde transport (**a**, **b**). We developed genetically modified gp96 by replacing the retention signal with a secretion signal, IgG1-Fc (**c**). Gp96-Ig is exported together with other secreted proteins. Cells that secret gp96-Ig generate a very powerful immune response against its target

influenced the acquisition of both the dual antigen delivery and antigen-presenting cell adjuvant properties of gp96. These properties make gp96 one of the few endogenous signals that can both activate and deliver antigen to APCs (Fig. 1). Further, the specific transfer of gp96-chaperoned antigens by antigen-presenting cells to MHC I through the cross-presentation pathway endow gp96 with truly unique characteristics as a basis for a CD8+ T-cell-specific vaccine protein.

Secreted gp96 vaccines

To take advantage of this unique adjuvant effect and ability to transport relevant peptides, we have made a secretable form of gp96, gp96-Ig [62–64] (Fig. 2). We set up a model system that imitates necrotic cell death with regard to the release of HSP. This system allowed us to analyze the immunological effects of HSP in vivo independent of infectious agents and cell death.

The bulk of proteins in the ER are destined for secretion or for insertion into the plasma membrane or membranes of other cellular organelles. Proteins residing permanently in the ER, to which group gp96 belongs, are retained there by the KDEL retention signal usually located at the C-terminus of the protein (Fig. 2a, b). We replaced the KDEL sequence with the Fc-domain of IgG1 and transfected the gp96-Ig and antigen of interest (ovalbumin, SIV/HIV gag, retanef, gp120) cDNA into different cell lines (293, 3T3, EG7, LLC) (Fig. 2c) [62, 63]. The transfected tumor cells indeed secreted gp96 and, when transplanted into syngeneic mice, were rejected by the immune system. The untransfected parental tumor cells, in contrast, grew and killed the mice. Immune rejection was dependent on CD8 but independent of CD4 cells and CD40 ligand. Several lines of evidence suggested that immunological memory against several different natural and surrogate tumor antigens could be generated in this way [62, 63].

Induction of mucosal immune response by gp96-Ig vaccines

In model systems in mice we have shown that gp96-Ig transfected, antigen expressing tumor cells secrete gp96-Ig in vivo and stimulate cognate systemic cellular CD8 CTL immune responses and generate specific CD8 memory independent of CD4 help, CD40L and in the absence of lymph nodes [65–68]. There is evidence that the memory phenotype is coupled to anatomic location and that memory CD8 T cells residing within the intestinal mucosa differ from their clonotypic counterparts within the spleen with regard to phenotype and function [69]. T effector memory (TEM) cells that circulate and localize to mucosal sites of

infection are likely to be crucial as a first line of defense [70–74]. We have recently demonstrated that gp96-Ig vaccination induces expression of essential intestinal homing receptors on antigen-specific CTL [75]. Given that quantity, quality and location of memory CD8 T cells comprise critical determinants of mucosal pathogen protection [76, 77], vaccine strategies that can elicit high frequencies of mucosal effector CD8+ T-cell populations have the greatest potential for success. Findings from our studies strongly indicate that the gp96-Ig vaccine approach induces a long-lasting memory in gut mucosal sites with the ability to rapidly undergo multiple rounds of proliferation in response to antigen, a hallmark of memory cells [63, 64, 75].

Intraperitoneal gp96-Ig immunization up-regulates CD103 (αEβ7) on peritoneal dendritic cells and efficiently induces CCR9 on responding T cells

In order to activate CD8 and NK cells, secreted gp96-peptide complexes need to be taken up by dendritic cells or peritoneal macrophages [32, 65]. It has been reported that CD103+ dendritic cells are important for generation of gut-tropic CD8 effector cells [78] as well as T regulatory cells (Treg) [79]. We analyzed $CD11c^{high}$ MHC class II^{high} dendritic cells isolated from the peritoneal cavity of control (PBS) or vaccinated (3T3-OVA and 3T3-OVA-gp96) mice for their CD103 expression. Gp96-Ig immunization induced a dramatic increase in CD11c+MHC class II+CD103+ cells compared to control mice ($p < 0.001$) [75]. Both subsets CD103+ and CD103− DC from vaccinated and control animals induced proliferation of OT-I cells, but only CD103+ DCs induced CCR9 on responding OT-I cells [75]. By day 4, gp96-dependent proliferation by CD8 cells in the PC was very pronounced. Using the transgenic CD11c− DTR/GFP mouse system that allows conditional depletion of CD11c+ DC in vivo through administration of diphtheria toxin, we have shown that CD11c+ DC are absolutely required for gp96-Ig-mediated cross-presentation of antigen to antigen-specific CD8 T cells. Our data suggest that secreted gp96-Ig immunization stimulates expansion of mucosa-homing CD8 effector cells (CCR9+) through the up-regulation of CD103 on DC. These activities mediated by gp96 in the peritoneal space enhance mucosal immunity by providing excellent OVA-specific mucosal immune responses in the lamina propria and intraepithelial compartments. Intraperitoneal (IP) vaccination in these studies provides proof of principle. It is clear that IP immunization is not a practical method of vaccination in humans and that the target population may not accept such a procedure. In murine studies, we have found that intravaginal and intrarectal delivery of cell-based gp96-Ig vaccines establishes excellent mucosal immunity with little systemic immunity. Subcutaneous immunization also generates a good degree of mucosal immunity in mice and non-human primates ([75] and Strbo N unpublished observation).

High level of rectal and vaginal mucosal immunity by gp96-lg vaccines

There are strong indications from animal models that HIV/SIV may be most vulnerable to elimination by CTL during the first few days of infection, prior to the initial infectious burst and spread to other tissues [77]. Establishing HIV-specific memory CD8 T cells at sites of transmission, where they may respond immediately upon infection, is potentially a critical component for a successful CTL vaccine. We have shown that $gp96^{OVA}$lg provides excellent mucosal immune responses in the lamina propria and intraepithelial compartments in mice [75]. Since mucosal immunity is crucial for protection from mucosal HIV and SIV infection, we examined mucosal responses to $gp96^{SIV}$Ig in macaques [63]. Our data demonstrate for the first time that IP immunization with $gp96^{SIV}$Ig can overcome systemic immune compartmentalization and generate spectacular frequencies of SIV-specific CD8 T cells (up to 40 %) localized throughout the intestinal (jejunum, ileum, colon and rectum) and vaginal mucosa, where they appear to maintain their lytic activity. To our knowledge, this degree of mucosal immunization, poly-epitope specificity and multi-functionality has not been reported previously. Importantly, the boosting response of the mucosal response after 24 weeks greatly exceeded systemic boosting response, the latter averaging around 0.5–1 % of tetramer-specific CD8 cells.

Cancer: a challenge for immunotherapy

Immunotherapy has been evaluated for prevention and treatment of different types of cancer with varying results. It is well known that many tumors, such as sarcomas and carcinomas, express tumor-specific antigens that can serve as targets for the immune system [80]. However, overall immune surveillance against tumors is frequently subverted through the acquisition of both directly immunosuppressive and immune evasive characteristics in established tumors. Cancer cells develop several strategies to escape the immune system by escaping immune cells and suppressing immune reactions including recruitment of regulatory T cells and myeloid-derived suppressor cells, down-regulation of MHC molecules or specific tumor antigens as well as expression of molecules including CTLA-4, PD-L1, IDO and TGF-β. This development ends in the conversion of immune cells into supporters of tumor

growth during disease progression [81–87]. This established tumor microenvironment poses a challenge for a successful immunotherapy [86]. Furthermore, tumor heterogeneity [88, 89] and the lack of a tumor-associated antigen universally expressed within each cancer type pose additional challenges, contributing to the low efficacy of single epitope approaches. Hence, for a successful immunotherapy, a potent polyepitope cytotoxic approach is warranted.

Gp96 and cancer immunotherapy

The potential role for HSP in the immune response to cancer was recognized by Srivastava and colleagues, who demonstrated that HSP complexed with antigenic peptides, released from tumor cells (or virus-infected cells) in vivo during lysis, were taken up by APC and subsequently cross-presented to stimulate potent CD8-mediated antitumor immunity [90, 91]. Heat shock protein-based vaccines have been shown to activate tumor-specific immunity, triggering the proliferation and cytotoxic capabilities of cancer-specific CD8+ T cells, inhibiting tumor growth [91]. In addition, HSP also activates natural killer cells to impart anti-tumor responses [92]. Exogenous antigens chaperoned by HSP are presented by MHC class I molecules and recognized by CD8+ T lymphocytes offering one mechanism for the classical phenomenon of cross-presentation as well as offering a role within the immune danger theory [30, 93]. Lysates from heat-shocked tumor cells provide an optimal source of tumor antigens, generating DC with improved cross-presentation capacity [94].

Gp96 has been evaluated in several approaches for immunotherapy, including Gp96 antigen linkage as adjuvant, the use of autologous gp96 peptide complexes and allogeneic gp96 tumor cell vaccines. We will discuss these strategies, their strengths and limitations.

Gp96 antigen linkage

It has been shown that linkage of antigens to gp96 as an adjuvant represents a potential approach for increasing the vaccine potency [95–97]. Gp96, when complexed with virus- or tumor-derived antigens, induced a MHC class-I-restricted cytotoxic T-lymphocyte (CTL) response by cross-presentation of antigenic peptides to MHC class I molecules [98–100]. This makes gp96 a powerful adjuvant for generation of CD8+ responses. Other groups have evaluated the adjuvant activity of the N-/C-terminal domains of gp96 with varying results. Promising results have been shown by cross-linking N-/C-terminal (NT/CT) domain of gp96 with human papilloma virus (HPV) 16 E7 oncoprotein. Daemi et al. [101] evaluated the effect of linkage of HPV16 E7 to the N-terminal and/or C-terminal domain of gp96 on the potency of E7-specific immunity generated by DNA vaccines in mice. They found that E7-CT (gp96) DNA vaccination resulted in significant TC-1 tumor regression and survival rates in comparison with control groups. Immunization with E7-CT (gp96) DNA vaccine significantly retarded the tumor growth rate, leading to an increase in survival rate compared to survival rates observed in E7/or E7-NT (gp96)-immunized mice between 41 and 50 days. Their results indicate that structural domains of immune chaperones show potential of generating effective immune responses against cancer. Mohit et al. [102] evaluated the use of adjuvant-free recombinant (r) HPV 16 E7-NT-gp96 fusion protein vs rE7 alone in a tumor mice model (C5 BL/6 mice). They found that vaccination with rE7-NT-gp96 protein delayed the tumor occurrence and growth as compared to rE7 protein alone. Their results suggest that linkage of NT-gp96 to E7 could enhance protective anti-tumor immunity. Also, it was demonstrated that the Gp96 N-terminal domain has potent adjuvant activity toward hepatitis B surface antigen [95, 97]. In contrast, studies by Pakravan et al. [103] showed that treatment with Her2/neu fused to gp96 N-terminal domain resulted in tumor progression compared to groups vaccinated with Her2 linked to C-terminal.

Furthermore, some studies have demonstrated that C-terminal domain has adjuvant activity [96], while others report it has no adjuvant activity [95]. Fusion of the C-terminal domain to Her2/neu has been shown to inhibit tumor growth [104]. All together, these results suggest that the adjuvant activity and the resultant immune response of gp96 terminal domains may be directed by the antigen of interest [101]. This is one of the limitations of the cross-linking approach, given it cannot be generalized to other antigens. Also, this approach relies on targeting a single antigen, which as previously stated will limit efficacy in other tumor types due to tumor heterogeneity and lack of knowledge of universally expressed tumor-associated antigens (TAA).

Autologous gp96 peptide complexes

In attempts to avoid the need of specific TAA identification and use of a potent multiepitope approach, the use of autologous gp96 peptide complexes for vaccination has been evaluated. In mice, autologous gp96 peptide complex, isolated from tumor tissues, has been shown to elicit potent activation of innate and adaptive immunity and generate antitumor response in both poorly immunogenic and immunogenic tumor models [10, 11]. These and other studies in animal models indicate that vaccination with autologous tumor-derived HSP results in prophylactic and therapeutic antitumor activity without the need to identify tumor-specific antigenic epitopes [11, 17]. The most potent

antitumor activity of HSP was observed in animals rendered disease-free by surgery, but at high risk of recurrence of metastatic cancer [11].

Autologous gp96-peptide complexes have been studied as therapeutic vaccines for a range of tumors in several clinical trials, including phase I/II and III trials. Vitespen is the trade name of an autologous tumor-derived gp96-peptide complex preparation, previously designated HSPPC-96 or Oncophage (Agenus). For this strategy, tumor material is obtained by surgical excision and shipped on dry ice to a good manufacturing practice-compliant facility where gp96 is isolated through sequential chromatography [105, 106]. Frozen vials with the purified gp96-peptide complexes are then shipped to the hospital or cancer center where the respective patient will be treated with Vitespen vaccination. So far, clinical trials indicate safety and feasibility of this autologous approach and generation of MHC class-1 restricted tumor-specific CTL response; however, the need of viable tumor for vaccine generation has been a limitation.

While this approach has been associated with very limited and weak side effects, minimal clinical benefits have been reported [107–118]. The majority of patients neither experienced tumor regression nor showed prolonged OS. There were technical difficulties with the approach, which limited feasibility for many patients. Evidence suggests that multiple independent injections of autologous vaccine might be necessary to achieve clinical benefit. Hence, the need of viable tumor for vaccine production is an obstacle. This was specially reported in a phase I trial for treatment of pancreatic adenocarcinoma, where it was possible to generate HSPPC-96 from 5 of 16 subjects with adenocarcinoma identifiable by pathology review [115]. In this study, they modified the protocol for handling of the tumor specimen, but even after the change, 2 out of 7 patients with adenocarcinoma in the specimen did not have a successful preparation of their HSPPC-96 vaccine. Other studies have the same limitation due to tissue requirements for vaccine production, as seen in the study conducted by Testori et al. [116]. They conducted a phase III comparison of Vitespen with physician's choice (PC) of treatment for stage IV melanoma. The PC consisted of alkylating agents, IL-2 or complete tumor resection. Gp96 was isolated and used for therapy in 133 patients. Tumor-derived HSP-peptide vaccine could only be prepared from 61.9 % of the initially assigned 215 patients. The intention-to-treat analysis did not show a statistically significant difference between groups in overall survival (OS). However, statistically significant benefit in OS was observed in patients who received 10 or more vaccinations, as compared to those in PC arm, when focused on patients with earlier stage IV disease (M1a and M1b). Even though results were promising, 38.1 % of the patients were not able to receive vaccination due to technical difficulties limiting its feasibility and applicability.

The most recent study utilizing this approach for glioblastoma multiforme is promising, but also requires sufficient viable tumor for vaccine generation, which limits its availability. Crane et al. [119] evaluated the use of autologous tumor-derived peptides bound to gp96 in the treatment of recurrent glioblastoma multiforme (GBM). In this study, 9 out of 28 patients had minimal viable tumor for vaccine and therefore were inadequate for vaccine production. Only 12 met the final inclusion criteria for treatment and safety assessment. No toxicity attributable to HSPPC-96 was observed in any of the 12 patients treated, with exception of mild injection site erythema and/or induration. Eleven of the 12 patients had a significant response to HSPPC-96. These patients had a significant increase in IFNγ production following restimulation. In addition, an increase in IFNγ-producing T cells suggests that peptides chaperoned by gp96 induced peptide-specific T-cell expansion following repeated vaccination. Brain biopsies of immune responders after vaccination revealed focal CD4, CD8 and CD56 INFγ-positive cell infiltrates, consistent with tumor site-specific immune responses. Immune responders had a median survival of 47 weeks after surgery and vaccination, compared with 16 weeks for the single non-responder. Again, the results are promising but limited by the vaccine construction requirements. Hence, an approach utilizing gp96 without the need of viable autologous tumor would be beneficial.

Allogeneic gp96 vaccine

The use of purified gp96 from autologous tumor biopsies as autologous tumor vaccine given as a bolus injection has shown encouraging results, as previously discussed. However, the need of viable tumor for vaccine generation has been a limitation and the clinical results have been modest. In order to bypass the need of viable autologous tumor for vaccine generation and to make a vaccine that can be applied to all patients with the same type of cancer, our group developed a novel gp96-Ig-secreting and allogeneic tumor cell-based vaccine [62]. As described previously, this vaccine strategy stimulates the generation of potent, polyepitope-specific, multi-cytokine-secreting CTL responses against all antigenic tumor epitopes present in the tumor vaccine cell. We showed that allogeneic tumor cells transfected with and secreting gp96-Ig work equally well as autologous vaccine cells. Using irradiated allogeneic gp96-Ig transfected tumor cells as vaccine, the vaccine cells are still alive but replication incompetent and will survive for several days in the vaccinated patient. The live vaccine cells continue to secrete gp96-Ig and stimulate CD8 CTL responses. The continuous release of gp96 is a

more appropriate and stronger stimulus for CD8 priming than a bolus of gp96 [65]. In addition, the use of allogeneic tumor cells overcomes the need for tumor from each patient and makes a universal vaccine. Finally, the work from our laboratory [67, 85] showing that frequent immunizations with gp96-Ig dampened the growth of certain experimental tumors suggested that such a protocol restored anti-tumor immunity and overcame tumor-induced immunosuppression.

The use of allogeneic tumor cells as a source of tumor antigens chaperoned by secreted gp96-Ig and cross-presented via MHC I to patient CD8 T cells is based on the hypothesis that allogeneic tumors have an overlapping repertoire of similar tumor antigens analogous to allogeneic melanomas and allogeneic small-cell lung cancers [62, 120, 121]. Gene array analyses of many tumor types, including NSCLC tumors [122], support the notion of sharing tumor-associated antigens.

Our group conducted a phase I trial in stage IIIB/IV non-small-cell lung cancer (NSCLC) to evaluate the safety and feasibility of this approach. We also evaluated for the first time in patients the method of frequent vaccination. Patients were vaccinated with a gp96-Ig-secreting allogeneic NSCLC line (AD100-gp96-Ig), irrespective of their HLA type. Vaccination was administered intracutaneously (5×0.1 ml, forearms or thighs, rotating) in one of the three schedules: 9 vaccinations with 5×10^7 cells each, every 2 weeks (DS-1); 18 vaccinations with 2.5×10^7 cells each, every week (DS-2) and 36 vaccinations with 1.25×10^7 cells each, twice a week (DS-3).

Nineteen NSCLC patients (IIIB/IV) with measurable disease who had failed two or more (average 5.3) lines of therapy were vaccinated in three 6-week courses over an 18-week period, with the above-mentioned schedule. All patients enrolled had progressive disease at the time of enrollment. Blood samples for immunological evaluation were obtained before the initial vaccination and on the last day of each course. Of the nineteen patients enrolled, one patient was never treated due to early disease progression and clinical deterioration. Eleven patients were treated in DS-1, four in DS-2 and three in DS-3. There were no treatment-related serious adverse events (SAE) or immune-related events (IRE). Most of the AEs were grade 1 or 2, such as erythema and skin induration, which were transitory and usually resolved in 1–2 weeks. There were no complete or partial clinical responses. Stable disease was achieved in seven of 18 patients (39 %; 95 % CI 17.3–64.3 %), which is not common among patients with NSCLC who have received multiple lines of therapies. Overall, a total of 15 patients have died and three surviving patients have been followed for 12.2, 21.0 and 38.8 months. The Kaplan–Meier estimate of median survival was 8.1 months (95 % CI 6.7–18.2), and the 1-year, 2-year and 3-year rates were 44.4 % (95 % CI 21.6–65.1 %), 19.0 % (95 % CI 4.8–40.3 %) and 9.5 % (95 % CI 0.8–32.1 %), respectively. As shown in Fig. 3, survival was higher in those patients who developed a CD8 CTL response (16.5 months; 95 % CI 7.1–20.0), evidenced by increased frequency of IFN-γ CD8 T cells re-stimulated with vaccine cells in vitro. Among the 11 patients who developed a CD8 CTL response, 3 were alive at the last follow-up of 12.2, 21.0 and 38.8 months. Patients who had no CD8 response had shorter survival times (2.1, 2.3 and 6.7 months). This finding supported progression to phase II clinical trials (NCT015044542) which are currently ongoing.

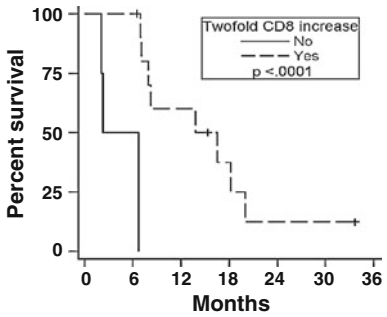

Fig. 3 Survival of stage IIIB/IV NSCLC patients in response to vaccination with irradiated, allogeneic tumor cells (AD100-gp96-Ig) secreting gp96-Ig. Twofold CD8 increased as a measure of response to vaccination. Survival was higher among patients who developed a CD8 CTL response (16.5 months; 95 % CI 7.1–20.0). Among the 11 patients who developed a CD8 CTL response, 3 were alive at the last follow-up of 12.2, 21.0 and 38.8 months. Patients who had no CD8 response had shorter survival times (2.1, 2.3 and 6.7 months)

Although the study is limited in lacking a control arm and in having been closed prematurely by the institution for reasons entirely unrelated to the study, it offers extremely interesting insights into the effects of therapeutic vaccine immunotherapy. Our method of preparing vaccines from established allogeneic tumor cell lines by transfection with gp96-Ig provides a relatively simple and inexpensive way to conduct tumor vaccine immunotherapy. Off-the-shelf allogeneic vaccines are of great advantage as therapeutic option. Furthermore, in this single institution study of 18 patients, the tumor cell-based gp96-Ig-secreting AD100-gp96-Ig vaccine was found to have an acceptable safety profile, achieving a significant disease control rate in a heavily pretreated population and a substantial CD8 CTL response. Our data indicates that CTL responses are required to obtain a clinical benefit, but that in the majority of patients, the tumor burden was too extensive to achieve complete stop or reverse tumor progression with the gp96-Ig-induced CTL response. Several groups have shown a correlation with tumor burden and elevated level of regulatory T cells (Treg) in circulation, which in turn act to suppress immunity [123, 124]. It is believed now that

patients with minimal tumor burden are the best candidates for immunotherapy [125]. Hence, further evaluation in a phase II trial, both in NSCLC and other tumor types, in the patients with minimal tumor burden is warranted.

Infectious diseases

From Edward Jenner to the present day, vaccines have delivered and continue to deliver significant improvements to global health. Smallpox is eradicated, polio has been controlled and the frequency of childhood diseases such as measles has been reduced. However, the most successful vaccines have been against diseases where the causal pathogen does not have major anti-immune defense mechanisms. Many pathogens, including hepatitis C virus (HCV) and human immunodeficiency viruses (HIV), *M. tuberculosis* (TB) and Plasmodium falciparum have evolved complex immune evasion strategies and require a high level of effector T-cell activation for their eradication. So far, these pathogens have proved intractable to existing vaccination strategies. Heat shock proteins possess significant properties that support their inclusion and testing in the next generation of infectious disease vaccines.

Secreted SIV/HIV gp96-Ig vaccine

The use of viruses and bacteria or viral vectors or attenuated viruses for induction and analysis of the immune response relies to a large extent on the ability of viral or bacterial components to activate the immune system (e.g., via pattern recognition receptors). Live-attenuated vaccines against smallpox and yellow fever elicit brisk, polyepitope-specific, polyfunctional CD8+ T-cell responses that contribute to protection [70, 126]. Similarly, in live-attenuated SHIV-immunized macaques, polyfunctional T-cell responses are associated with a better control of challenge virus replication [127, 128]. The cell-based secreted gp96-Ig vaccines, by prolonged in vivo secretion of immunogenic gp96-Ig-peptide complexes, resemble viral replication and contribute to the cytotoxic response by providing immune stimuli comparable to attenuated viruses. Although non-viral and non-bacterial in nature, gp96-mediated CD8 CTL responses bear the hallmarks of memory responses characteristically seen after viral or bacterial infections. We attribute this observation to the adjuvanticity of gp96 which is specifically directed toward cross-priming, cytotoxic CD8 CTL responses [129].

Our vaccine, denoted as 293-gp96^{SIV}Ig, was made by transfecting 293 cells with gp96-lg and plasmids encoding SIV-retanef, SIV gag and SIV-gp120 (retanef is a fusion protein of rev nef and tat) provided by Drs. Franchini, Felber and Pavlakis (NIH) in collaboration. The significance of the cell (HEK-293 cells, not containing T antigen) is that it acts as a 'pump,' continuously secreting gp96-Ig over 3–4 days and activating immune responses until the cells are rejected by allo- or anti-SIV responses. In effect, continuous secretion of gp96-Ig provides a continuous stimulus quite similar to replicating attenuated viruses, which provide excellent protection when used as vaccines. Thus, when cells containing SIV antigens and secreting gp96^{SIV}lg are injected into recipients, secreted gp96^{SIV}lg induces a SIV-specific CD8− CTL response (Fig. 4). As described above, gp96 is a potent Th1 adjuvant by activating APCs and NK cells (Fig. 1). In addition, gp96-Ig chaperones client peptides derived from SIV antigens (Figs. 4, 5). Gp96-Ig is endocytosed by CD91 on the activated APCs, and the client peptides are cross-presented by MHC I, priming antigen-specific CD8 T cells. Since adjuvant and antigenic peptide (epitope) are part of the same molecular complex, cross-presentation and priming of antigen-specific CD8 T cells is extraordinary efficient requiring only femto-molar (10^{-15} M) antigenic peptide [65]. Polyepitope specificity is achieved because gp96-Ig carries all client peptides generated from the transfected SIV antigens by the proteasome and translocated by TAP into the ER of the host DC. The client peptides are further trimmed and selected for MHC I presentation by the host DC. Thus, any T-cell epitope present in the transfected SIV antigen will be cross-presented by the host MHC I and primes corresponding antigen-specific CD8 T cells. This principle provides the largest degree of poly-epitope specificity possible to be presented by any MHC I type. The strong adjuvant and Th1 activity of gp96-Ig provides for multi-cytokine CTL responses [63, 65, 75]. Importantly, self-peptides do not generate CD8 CTL responses due to normal tolerance mechanisms. We have not observed any signs of autoimmunity in mice, macaques or humans (in vaccine trials for lung cancer) in any of our gp96-lg-based vaccine studies.

Gp96^{SIV}Ig as a novel adjuvant for antibody production

Gp96-Ig-chaperoned peptides are cross-presented primarily by MHC I. Accordingly, gp96-Ig-based immunization generates powerful CD8 CTL responses but little antibody [63, 64, 130]. Since gp96-Ig is a potent adjuvant for DC activation, addition of protein antigen, gp120, resulted in uptake by DC via classical endo- or pinocytosis and presentation of processed antigen by MHC II to generate help for B-cell antibody production (Figs. 4, 5) [64]. The gp96-Ig induced-Th1 environment results in isotype switching of B cells to generate IgG1 and IgG3 antibodies that bind to Fc receptors on macrophages and NK cells and activate complement and may contribute to SIV virus neutralization.

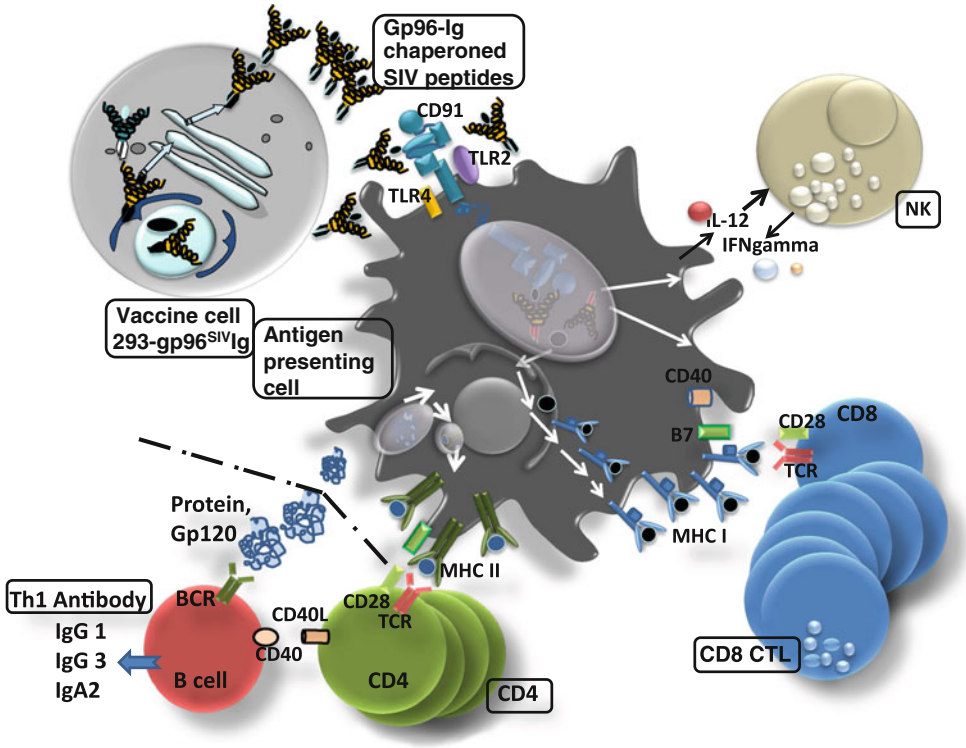

Fig. 4 Principle of Gp96^{SIV}Ig-mediated CD8 CTL, NK and B-cell activation. Transfected gp96-Ig serves as chaperone for SIV-derived antigens transfected into 293 cells. Gp96^{SIV}Ig secreted from 293 activates DC via CD91, TLR2/4, up-regulates B7.1 and B7.2 and is endocytosed by CD91. Activated DCs secrete IL-12 and activate NK which secrete IFN-gamma to stimulate DC and create a Th1 environment. Gp96-Ig-chaperoned SIV peptides are MHC I cross-presented and prime CD8 T cells. Protein antigen, gp120, resulted in uptake by DC via classical endo- or pinocytosis and presentation of processed antigen by MHC II to generate CD4 help for B-cell antibody production. In addition, Th1 environment induced isotype switching of B cells to generate IgG1, IgG3 and IgA2 antibodies

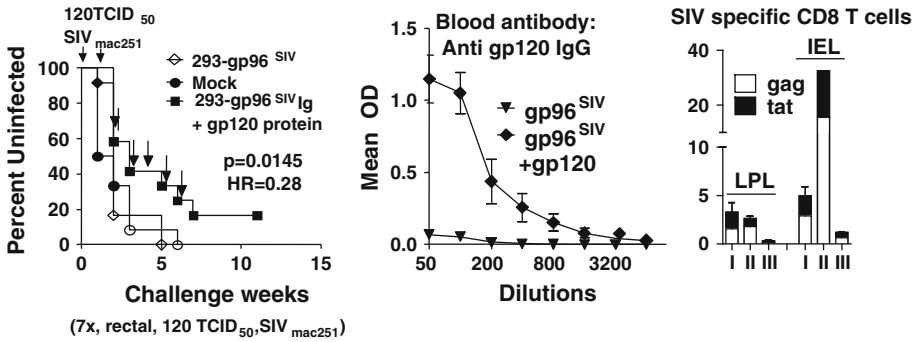

Fig. 5 Protection of macaques vaccinated with 293-gp96^{SIV}Ig + gp120 from SIV infection. *Left panel arrows* indicate time of rectal challenge with 120 $TCID_{50}$ units of SIVmac251 followed by analysis 5 days later. *Middle panel* antibody levels in blood of gp96^{SIV}Ig + gp120 vaccinated macaques at week 30. *Right panel* Vaccination-induced SIV gag- and tat-specific CD8+ T cells in lamina propria (*LPL*) and intraepithelial compartment (*IEL*) of rectal mucosa. Pinch biopsies from the rectal mucosa at week 26 (5 days after third vaccination) were analyzed. SIV-specific CD8 T cells were detected by Mamu-A*01/Gag181–189 CM9 (CTPYDINQM; Gag-CM9) and Tat 28–35 SL8 (TTPESANL; Tat-SL8) tetramer staining. After gating on the CD8+ population, the percentage of tetramer-positive cells was determined; *I* gp96^{SIV}Ig, *II* gp96^{SIV}Ig + gp120, *III* gp96^{Mock}Ig

Protection from mucosal SIVmac251 infection requires strong mucosal CTL and antibody responses

Using the novel principle for generating SIV-specific Th1 CTL and Th1 antibodies described above, we have achieved for the first time significant protection (73 %) against infection by up to 7 weekly rectal challenges with highly pathogenic SIVmac251 (Fig. 5) [64]. Unlike SIV-mac239 which is a cloned virus, SIVmac251 is not cloned and has considerable sequence diversity. In our study, the combination of gp96^{SIV}Ig with gp120 protein was required for protection. CTL alone or antibody alone did not provide

protection [64]. 73 % immunization efficiency is an encouraging starting point for further development of the immunization strategy and to understand which immune responses are required for better protection from SIVmac251. We hypothesize that mucosal antibody in the mucus of rectum and vagina will trap the virus in the mucus antibody network, preventing contact with mucosal cells and hence preventing infection. The viruses that penetrate the barrier reaching and infecting cells require CTL or NK or other cytotoxic responses to eliminate infected cells to prevent viral replication and spreading.

Secreted gp96-Ig and other infectious diseases

Malaria infects nearly 250 million people annually and causes almost 1 million deaths. An effective vaccine against malaria would be a valuable public health tool, complementing anti-malaria drugs, vector control and environmental modification. Despite intensive research, no malaria vaccine is commercially yet available. The vaccine farthest along in field testing is based on a single malaria antigen (circumsporozoite protein, CSP) and is not as effective as experimental radiation-attenuated whole parasite vaccines. When immune responses to the protective irradiated parasite vaccines are analyzed, no single target antigen has been identified that explains the full extent of host immunity. Protection is thought to be strongly associated with interferon gamma (IFN-γ) secretion by CD8+ T-cell immunity during the liver stage of infection. This suggests that protective vaccines should be designed that are specifically capable of stimulating malaria antigen-specific CD8+ T-cell responses. Our approach to vaccine development is to develop a multi-antigen malaria vaccine, which is specifically designed to generate high levels of antigen-specific CD8 CTL that localize to the liver. Our current malaria vaccine studies are designed in such a way that will provide a head-to-head comparison to another promising malaria vaccine candidate and may immediately influence the priority of future malaria vaccine development.

Summary

Secreted heat shock protein gp96-Ig possesses significant properties that support its inclusion in the next generation of cancer and infectious diseases vaccines: First, gp96-Ig is an excellent cellular and humoral Th1 adjuvant and second, gp96-Ig delivers broad antigenic peptide fingerprint that can induce adaptive immune responses to provide broad coverage against pathogens and effective cancer therapy. Secreted gp96-Ig vaccines provide an exciting and innovative strategy for the development of much needed vaccines; data from clinical trials are now needed to confirm that gp96-Ig vaccines provide an effective new approach in man.

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Conflict of interest Dr. E. R. Podack and the University of Miami have financial interest and hold equity in a commercial enterprise developing this vaccine technology.

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