Published in final edited form as:

Vaccine. 2011 March 21; 29(14): 2619–2625. doi:10.1016/j.vaccine.2011.01.044.

Gp96^{SIV}Ig immunization induces potent polyepitope specific, multifunctional memory responses in rectal and vaginal mucosa

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Abstract

The ER-resident chaperone gp96, when released by cell lysis, induces an immunogenic chemokine signature and causes innate immune activation of DC and NK cells. Here we show that intraperitoneal immunization with a genetically engineered, secreted form of gp96, gp96-Ig chaperoning SIV antigens, induces high levels of antigen specific CD8 CTL in the rectal and vaginal mucosa of Rhesus macaques. The frequency of SIV Gag- and SIV Tat-tetramer positive CD8 CTL in the intestinal mucosa reached 30-50% after the third immunization. Tetramer positive CD8 CTL expressed appropriate functional (granzyme B) and migration markers (CD103). The polyepitope specificity of the mucosal CD8 and CD4 response is evident from a strong, multifunctional cytokine response upon stimulation with peptides covering the gag, tat and env proteins. Induction of powerful mucosal effector CD8 CTL responses by cell-based gp96^{SIV}-Ig immunization may provide a pathway to the development of safe and effective SIV/HIV vaccines.

Keywords

gp96-chaperone; mucosa; non-human primate; rectum; vagina

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Disclosures

Dr. Podack has financial interest in the technology reported.

1. Introduction

Many pathogens including HIV gain entry into the body through mucosal membranes in rectum and vagina. To protect against invasion it is necessary to define pathways that lead to the efficient induction of high levels of mucosal immunity. Subsequent challenge studies then can be carried out to determine whether the type of mucosal immune response that has been generated is protective and to what degree.

Here we report a pathway of generating extraordinarily strong antigen-polyepitope specific and multifunctional mucosal CTL responses in macaques in the rectal lamina propria, rectal intraepithelial cells and at other mucosal sites with the aid of a novel xenogeneic cell based vaccine secreting the ER-chaperone heat shock protein gp96-Ig carrying SIV-derived peptides.

Heat shock proteins are peptide- and protein-chaperones crucial for normal protein function and turnover [1]. As initially described by Srivastava's group [2] and later confirmed by other laboratories, heat shock protein gp96 binds to and activates dendritic cells (DC) via CD91, TLR2 and TLR4; gp96 then is endocytosed and the chaperoned peptides are cross presented by MHC I of the DC to cognate CD8 T cells [3-5]. Chaperones normally function only inside the cell. However upon necrotic or traumatic cell death they are released and taken up by DC and prime powerful CTL responses [6-8].

Gp96 is highly conserved phylogenetically. We have modified the human endoplasmic reticulum chaperone gp96 by replacing its KDEL retention sequence with the Fc portion of mouse and human IgG1 [8]. Gp96-Ig transfected into tumor cells is secreted together with its chaperoned clients that include tumor antigens. Gp96-Ig secreting tumor cells when injected into mice induce tumor specific CTL expansion that can reject parental tumors that do not secrete gp96-Ig [9,10].

Maximal cross priming of CD8 T cells by gp96-Ig requires only femto-molar amounts of antigen [11,12]. This efficiency of gp96 is due to its strong adjuvanticity via binding to TLR2 and TLR4 on DC and its function as antigen carrier, uptake via CD91 and powerful antigen cross priming of CD8 CTL, obviating the need for CD4 help via CD40-L [11]. Moreover, gp96-Ig recruits and activates DC, NK and CD8 T cells to the site of its secretion resulting in local expansion in the absence of lymph nodes [11].

Based on these data, we reasoned that gp96-Ig secreting cells carrying viral antigens will be able to induce virus specific CD8 CTL activation and clonal expansion. As test system we used HEK 293 cells transfected with cDNAs encoding gp96-Ig and the SIV antigens gag [13], retanef (a fusion protein of rev, tat and nef) [14] and gp160 [15] (293-SIV[gag,retanef,gp160]-gp96-Ig). The cells secrete gp96-Ig together with its client peptides including those derived from SIV antigens, collectively referred to here as gp96^{SIV}-Ig. Maximal mucosal immune responses in mice were achieved by intraperitoneal injection of gp96-Ig secreting cells [16]. We hypothesized that intraperitoneal injection it would have similar effects on mucosal immunity in non-human primates.

2. Materials and Methods

2.1. 293-gp96^{SIV}Ig Cells

Human embryonic kidney (HEK)-293 cells, obtained from the American Tissue Culture Collection (ATCC), were transfected with plasmids encoding gp96-Ig and SIV rev-tat-nef (rtu) [14] (B45-gp96-Ig-rtu), Gag (pCMV-SIVGag)[13], and gp160 (pCMV-SIVgp160) [15] using Effectene (QIAGEN, Valencia, CA) following the manufacturers' protocols. Cells

were irradiated with 120 Gy in a Co irradiator and stored frozen with 10% DMSO until use as immunogen.

2.2. Western Blotting and ELISA

Protein expression was verified by SDS-page and Western blotting using rat anti-Grp94, mouse anti-SIV-Gag, mouse anti-SIV-Nef, mouse anti-SIV-gp120 and peroxidase-conjugated goat-anti-rat/mouse antibodies. Gp96 and SIV antigens were visualized by an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ) (Fig. 1 a). One million cells were plated in 1 ml for 24 h and gp96-Ig production was determined in the supernatant by ELISA using anti-human IgG antibody for detection and human IgG1 as a standard (Fig. 1 b).

2.3. Animals and vaccination schedule

All animals used in this study were colony-bred rhesus macaques (*Macaca mulatta*) obtained from Covance Research Products (Alice, TX). The animals were housed and handled in accordance with the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care International.

A total of 7 Mamu-A*01⁺, naïve females macaques were enrolled in the study (Fig. 1C). Macaques in groups I, II and III were immunized at week 0, 4 and 25 with intraperitoneal inoculations of 10⁶, 5×10⁶ or 50×10⁶ irradiated 293-gp96-Ig-SIV cells that secrete 1, 5 or 50 µg of gp96-Ig/24h, respectively. In control animal, irradiated 293 cells (50×10⁶) were injected.

2.4. Isolation of lymphocytes from blood and tissues

Mononuclear cells from blood and LNs were isolated by density-gradient centrifugation on Ficoll. Rectal and vaginal pinches were treated with 1 mM of Ultra Pure Dithiothreitol in calcium/magnesium-free Hank's balanced salt solution. Following the removal of epithelium and intraepithelial lymphocytes, the tissues were incubated with collagenase D (3.7U/ml; Boehringer Mannheim, Mannheim, Germany) and DNase (1mg/ml; Invitrogen) for 1-2h at 37 ° C. The dissociated mononuclear cells were then placed over 42 % Percoll and lamina propria lymphocytes were collected from the cell pellet.

2.5. Intracellular cytokine staining

SIV-specific CD4 and CD8 T cell responses were stimulated using pools of 15-meric peptides overlapping by 11 amino acids covering the entire Gag, Env, and Tat proteins of SIVmac239. For intracellular staining of IL-2 and IFN-γ in LPL and IEL, specific peptide pools (1µg/ml of each peptide), anti-CD28, anti-CD49d (BD Pharmingen, San Diego, CA) and Brefeldine A (GolgiPlug; BD Bioscience) were added to the culture for 5 h before flow cytometric analysis. The results are calculated as the total number of cytokine-positive cells with background subtracted.

2.6. Flow cytometry

For the detection of SIV-specific CD8 T cells, cells were labeled with CD8β-PE (Beckman Coulter), CD28-FITC, CD95-PECy5, CD103-FITC and with allophycocyanin (APC)-conjugated Gag 181 – 189 CM9 (p11C) (CTPYDINQM)-Mamu-A*01 and Tat 8–35 SL8 (TTPESANL)-Mamu-A*01 tetrameric complexes (Beckman Coulter). Unless otherwise indicated, all antibodies and reagents were obtained from BD Pharmingen, San Diego, CA. Intracellular staining for granzyme B was performed using the Cytotfix/cytoperm kit and anti-human granzyme B-PE (Caltag). A total of 100,000 events were collected in the

lymphocyte region (R1) and analyzed with CellQuest software (BD Biosciences) and FlowJo.

2.7. ELISPOT

Peripheral blood mononuclear cells (1×10^5 /well) were stimulated with and without SIV-specific peptides (2 μ g/ml) (PBMC) and cultured overnight in triplicate, in 96-well plate coated with anti macaque IFN- γ antibody. ELISPOT was performed according to the manufacturer's guidelines (U-Cytech, Utrecht, The Netherlands).

2.8. Statistical analysis

Statistical analyses were performed using unpaired two-tailed Student t test. *P* values less than 0.05 were considered to indicate statistical significance.

3. Results

3.1. Generation of Gp96^{SIV}Ig vaccine cells

Gp96^{SIV}Ig vaccine cells were generated by transfection of human embryonic kidney (HEK) 293 cells with plasmids encoding gp96-Ig and the SIV antigens retanef (Rev-Tat-Nef fusion protein) [14], Gag [13] and gp160 [15]. 293- gp96^{SIV}Ig vaccine cells expressed endogenous gp96 in addition to its secreted form, gp96-Ig. The latter is detected with anti gp94 as a higher molecular weight, 125kDa band (Fig. 1a). The SIV antigens retanef (55kD), Gag (55kD) and Env (120kD) are seen in comparable amounts in Western blots (Fig. 1 a). Gp96-Ig was secreted equally from irradiated and non-irradiated cells at a rate of 1000 ng/24h by 10^6 cells (Fig. 1 b). We have shown previously that in tissue culture, irradiated gp96-Ig-transfected cells were unable to form colonies, indicating their inability to replicate, but still secrete gp96-Ig [8,17,18]. Six macaques were vaccinated intraperitoneally in groups of 2 with the number of irradiated cells (1, 5, or 50×10^6) that secreted 1, 5 or 50 μ g gp96-Ig within 24 hours. Control animal received untransfected irradiated 293 cells (50×10^6). Macaques were immunized three times, week 0, 4 and 25 and analyzed 4 times, week 1, 5, 20, and 26 (Fig. 1c).

3.2. Strong mucosal memory response after gp96^{SIV}Ig immunization

To assess SIV-specific T-cell responses in the intestinal tract of macaques, intraepithelial and lamina propria lymphocytes were isolated from rectal pinch biopsies. Gag-CM9 and Tat-SL8 tetramer-specific CD8⁺ T cells were detected already 5 days after the first vaccination (2 animals had more than 1% SIV-specific CD8⁺ T cells) (Fig. 2 a). A vaccine boost, at week 4 did not induce significant increases in SIV-specific CD8⁺ T cells, however a third vaccination at week 25 resulted in powerful expansion of Gag-specific and Tat-specific CD8 CTL cells of up to 20% frequency in the lamina propria (LPL) of the rectal mucosa (Fig. 2a), and up to 35% in rectal intraepithelial cells (IEL) (Fig. 3a). A mucosal immune response to gp96^{SIV}Ig was observed in the rectal mucosa, in jejunum, ileum and colon (Fig. 2d and Supplementary Fig 1). In the jejunum gag and tat specific CD8 CTL frequencies exceeded 40% in some macaques (Fig 2d). In ileum and rectum 5 and 50 μ g secreted gp96^{SIV}Ig gave equivalent responses however in jejunum 50 μ g was required for full response. It has been described before [19,20] that antigen specific responses elicited by gp96 show a dose-restriction. For all animals, the lowest frequency of SIV-specific CD8 T cells was always observed in the ileum.

In Figure 2c the frequency of SIV-Gag⁺ and SIV-Tat⁺ cells induced by Gp96^{SIV}Ig vaccine is compared in the rectal lamina propria versus that in inguinal lymph nodes. The immune response of CD8 CTL in inguinal lymph nodes (Fig. 2c) was modest compared to rectal LPL. We estimate that approximately 1 out of 1000 secreted gp96-Ig molecules is loaded

with a SIV-derived peptide. The average length of gp96 chaperoned peptides is 20 aminoacids (~2kD). The secretion within 24h of 5µg gp96-Ig thus corresponds to 80ng client peptides, 80pg (160fmole) of which may be derived from SIV. This estimate demonstrates the extraordinary efficiency and sensitivity gp96-Ig mediated antigen cross priming of CD8 CTL in the mucosa. Furthermore, robust expansion of SIV-specific CD8 T cells in the rectal mucosal tissue as compared to modest expansion in inguinal lymph nodes (Fig. 2C) indicates difference in organ specific memory turnover and longer retention in mucosal tissues.

Intraperitoneal gp96^{SIV}-Ig vaccination resulted in remarkable migratory and phenotypic plasticity of SIV-specific CD8 T lymphocytes: all Gag-specific CD8 T cells in the rectal and vaginal intraepithelial compartment express CD103 (integrin αE/β7) which interacts with its epithelial ligand E-cadherin, to maintain T cells within epithelial tissues (Fig. 3 b and c). Furthermore, Gp96^{SIV}-induced SIV-specific CD8 T cells in the intraepithelial compartment and lamina propria express granzyme B (Fig. 3 b and Supplementary Fig 2). As shown in Fig. 2 c, ~5% gag-specific CD8 T cells were detected in vaginal intraepithelial compartment 5 days after the last immunization.

3.3. SIV-specific mucosal response is polyspecific and multifunctional

The multifunctional profile of the gp96^{SIV}-Ig induced immune response in the gut lamina propria and intraepithelial compartment was assessed by intracellular staining for IFN-γ and IL-2 after *in vitro* stimulation with overlapping 15mer peptide pools covering the entire SIV gag, tat and env antigens. Data on Fig 3d showing the overall polyfunctionality of SIV-specific CD8+ and CD4+ T-cell responses in freshly isolated rectal lamina propria and intraepithelial cell samples from Mamu-A*01-positive animal M943. 17% of all T cells (6.5% CD8 and 10.5% CD4) in the rectal lamina propria, and 27.6% of rectal IEL are SIV-gag, tat or env specific as indicated by IL-2 and IFN-γ secretion. As expected, SIV-Gag and SIV-Env elicited the major response, but considerable activity was seen also for tat peptide specific CD4 and CD8 T cells in the gut lamina propria and IEL (Fig. 3d). SIV-specific CD4 cells in the lamina propria were almost exclusive producers of IL-2 with 90% of the Gag-specific, 93% of tat-specific and 97% of env specific CD4 T cells expressing only IL-2 which strongly supports helper function. It has been shown by Matsutake et al [21] that antigen-specific CD4+ T cells can be elicited by immunization with gp96 preparations chaperoning miniscule amounts of peptides. SIV-specific CD8 LPL in contrast were producing IFN-γ alone or together with IL-2 in 18% of gag, 59% of tat and 45% of env specific cells. (Fig. 3 d). In the intraepithelial compartment over 25% of the CD8 cells respond with cytokine secretion to SIV peptide stimulation (Fig. 3 d). Importantly, the SIV-specific CD8 T cell response in the intraepithelial compartment was highly multifunctional: more than 25% of gag-specific, 22% of tat specific and 30% of env-specific CD8 T cells co-expressed IL-2 and IFN-γ.

3.4. Gp96^{SIV}Ig induces systemic SIV specific CD8 T cell response

Five days after primary immunization, circulating SIV-specific CD8 T cells were detected in the blood of 3 of 6 vaccinated macaques. An early boost 4 weeks later did not induce further expansion of tetramer-specific CD8 T cells and at week 20 a complete contraction of the peripheral CD8 response was observed. The third immunization at week 25 induced rapid expansion of gag and tat-tetramer specific CD8 CTL in all immunized macaques (Fig. 4a). Blood mononuclear cells to secrete IFN-γ measured by ELISPOT following stimulation with gag, tat and env peptide pools is shown in Figure 4b. Primary immunization induced little response but substantial reactivity was induced by boosting at 4 weeks especially at the higher vaccine doses (Fig. 4b). Without additional vaccination the gag and tat specific ELISPOT response remained stable or slightly increased by week 20. In contrast the env-

specific response collapsed by week 20. All specificities were boosted by the third immunization in week 25.

Memory CD8 T cells in rhesus macaques have been designated as T_{CM} ($CD95^{+}CD28^{+}$), T_{EM} ($CD95^{+}CD28^{-}$) and naïve ($CD95^{low}CD28^{int}$) [22-25]. SIV-Gag tetramer specific CD8 T cells in the peripheral blood of macaque (M943) who received $5\mu g$ of gp96^{SIV}-Ig (group II) showed a 63% T_{CM} and 31% T_{EM} phenotype one week after priming (Fig. 4 c). The boosted response at week 26 shifted to a predominant T_{EM} phenotype (76% T_{EM}). The results indicate that 293-gp96^{SIV}-Ig immunization can elicit both, T_{CM} and T_{EM} -based T cell immunity in blood and, importantly, maintain SIV-specific T cells that are skewed toward T_{EM} differentiation. SIV-specific CD8 cells in lymph nodes of all primed monkeys exhibited the T_{CM} phenotype after the first immunization and shifted towards T_{EM} after the third immunization (Supplementary Fig 3). A single administration of gp96^{SIV}-Ig initially induced SIV-specific CD8 T_{CM} cell responses systemically, which upon tertiary immunization differentiated to T_{EM} in large numbers in the peripheral blood. We did not phenotypically characterize SIV specific CD8 T cell responses in the gut mucosa, but from our previous findings in mice we know that the gp96 vaccine generates high frequencies of T_{EM} cells at gut mucosal sites [16].

4. Discussion

Given that quantity, quality and location of memory CD8 T cells may comprise critical determinants for protection [26,27], strategies that can elicit high frequencies of mucosal effector CD8 T cell populations are likely to have the greatest potential to eliminate pathogens at their port of entry. Findings from this study indicate for the first time that the gp96^{SIV}Ig 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 (Fig. 2). SIV-Gag and SIV-Tat specific CD8 T cell responses in the rectal lamina propria and in the intraepithelial compartment (Fig. 2 and Fig. 3) were potently boosted after the 3rd immunization. From our previous work in mice, we know that gp96 predominantly induces memory precursor cells ($CD127^{High}$, $KLRG1^{Low}$) that survive and further differentiate into the pool of long-lived memory cells [28] (data not shown).

In contrast to the robust SIV-specific T cell responses in the rectal mucosa, SIV-gp96 vaccination elicited a significantly lower systemic response (Fig. 2 and Fig. 4). It is well appreciated that the site of immunization directs the imprinting of the ensuing T cell response and controls the expression of trafficking molecules [29,30]. The intraperitoneal route of vaccination appears to be optimal for the induction of mucosal CD8 CTL immunity by the gp96 vaccine [16]. The omentum has recently been recognized as a unique secondary lymphoid organ that samples antigens from the peritoneal cavity and promotes local immune responses [31,32] and therefore may direct mucosal immune responses. Gp96-Ig immunization administered intraperitoneally increases the frequency of $CD11c^{high}MHCII^{high}CD103^{+}$ cells [16] that cross-present exogenous antigen for class I MHC-restricted T cell responses [33]. The same DCs are also required for the induction of gut-homing molecules (CCR9 and $\alpha 4\beta 7$) on activated antigen specific CD8 T cells. Our data demonstrate for the first time that intraperitoneal immunization with gp96^{SIV}-Ig can overcome systemic immune compartmentalization and generate significant frequencies of SIV specific CD8 T cells localized throughout the intestinal and vaginal mucosa, where they appear to maintain their lytic activity (Fig.3.b and c). We therefore propose peritoneal vaccination as a unique and optimal route of vaccine delivery to generate high frequency, functional intestinal and vaginal mucosal immune memory. A mucosal immune response to gp96^{SIV}-Ig was observed in the rectal mucosa, in jejunum, ileum and colon (Fig. 2 d). The frequency of SIV-specific CD8 T cells in the jejunum was dramatically increased in the

animal that received the highest dose (group III, M945) (Fig. 2 d). For all animals, the lowest frequency of SIV-specific CD8 T cells was always observed in the ileum. Selective expression of CCL25 in the proximal parts of the small bowel [34] underlies the homing of CCR9⁺ intestinal memory T cells to the jejunum rather than to the ileum and could explain the predominant distribution of gp96^{SIV} induced CD8 T cells in the jejunal lamina propria. Unlike in the small intestine, CCL25 is not expressed at significant levels in the colon [34], and *in vivo*-activated CCR9^{-/-} CD8 T cells are equally efficient at gaining access to the colonic mucosa as their WT counterparts [35]. Gp96 up-regulates expression of CCR9 and as well as CXCR3 on CD8 T cells (unpublished observation) that could explain homing of SIV specific CD8 T cells to the colonic mucosa [36,37].

The gp96 vaccine elicited polyepitope specific cellular immune responses. In the blood, IFN γ - ELISPOT responses to overlapping Gag, Tat and Env peptides were observed in all vaccinees (Fig 4b). When stimulated *in vitro* with SIV peptides, rectal lamina propria CD8⁺ T-cells produce IFN γ and IL-2 cytokines (Fig 3d). SIV-Gag and SIV-Env encompassed the predominant responses of CD4 and CD8 T cells. The potential utility of vaccine-elicited mucosal CD8 T lymphocytes could be counterbalanced by the induction of activated CD4 T lymphocytes that would increase the number of target cells available for SIV infection in the mucosa. We were unable to perform detailed analysis of the phenotype of gp96-induced SIV specific CD4 T lymphocytes, but we did observe that SIV-specific CD4 cells in lamina propria were almost exclusive producers of IL-2 (90% of the total Gag-specific CD4 T cells express IL-2). Further studies will therefore be required to evaluate gag-specific CD4 T lymphocyte responses induced by gp96 in mucosal tissues.

Altogether, our data suggest that after single dose of gp96^{SIV}-Ig immunization there is preferential compartmentalization of T_{CM} and T_{EM} immune responses. The boosting strategy resulted in the development of T_{EM} that were abundant in mucosal tissues. If CD8⁺ T_{CM} cells can be thought as a long-lasting immunological reserve that requires time to be fully deployed upon the reencounter with the pathogen, and that T_{EM}, constitute the frontline defense within the epithelial layer and the lamina propria of mucosal sites which promptly recognizes and kills infected cells, then the gp96^{SIV}-Ig provides an ideally balanced generation of both arms, T_{CM} and T_{EM}, of the memory response. The presence of T_{CM} in the draining LN and a balance between these cells and T_{EM} CD8⁺ T cells in the mucosa may be required to maintain effective, homeostatic anti-SIV T-cell activity.

In summary, our results indicate that the gp96^{SIV}-Ig induces polyepitope specific multifunctional T cell responses mucosally and systemically. Importantly, the elicited response in peripheral blood is highly skewed toward T_{EM} differentiation after boosting. Together these data suggest that cell based gp96-Ig vaccines may constitute a profoundly important advancement in prophylactic vaccine design.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank James L. Phillips from Flow Cytometry Core Facility, Sylvester Cancer Center, University of Miami for expert help. We thank Phillip Markham, Jim Treece and Deborah Weiss at Advanced BioScience Laboratories, Kensington, MD for help with the animal study.

This research was supported by Public Health Service Grants R21 AIO68515, R21/R33 AI073234, CA109094, by a Grant from ACGT (Alliance for Cancer Gene Therapy), Developmental Center for AIDS Research (DCFAR) University of Miami and by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

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FIGURE 1. Expression of gp96-Ig and SIV antigens in transfected 293 cells and secretion of gp96-Ig

Antigen expression: 293 cells were transfected with gp96-Ig and SIV antigens as described and the cells selected with G418. Expression of gp96-Ig, Nef, Gag and Env was determined by Western blotting. (a) Lane 1- 293, 2- 293-gp96^{SIV}-Ig₁ 3- irradiated 293-gp96^{SIV}-Ig₁ cells. (b) **Gp96-Ig secretion:** One million 293-SIV-gp96-Ig cells were plated in 1 ml for 24 h and gp96-Ig production in the supernatant was determined by ELISA using anti-human IgG antibody for detection with human IgG₁ as a standard. (c) **Immunization schedule:** Seven naïve female Rhesus macaques (M620, M940, M943, M944, M945, M947, M964), that carry the MHC class I Mamu-A*01 molecule were enrolled in the study and divided into four groups. The schedule of immunization is shown in black arrows. Macaques in groups I, II and III were immunized at weeks 0, 4 and 25 with intraperitoneal inoculations of 10⁶, 5x10⁶ or 50x10⁶ irradiated 293-gp96^{SIV}-Ig cells that secrete 1, 5 or 50μg of gp96-Ig respectively in 24h. Irradiated 293 cells (50x10⁶) were injected in the control animal (mock-control). Dashed arrows (5 days after each vaccination) represent time of the collection of PBL, lymph nodes, rectal and vaginal pinch biopsies.

FIGURE 2. SIV-gp96 immunization induces SIV-Gag and SIV-Tat specific CD8 T cells in the lamina propria of rectal mucosa

Seven Rhesus macaques were immunized with gp96-SIV by the intra-peritoneal route with cells secreting the quantity of gp96-Ig within 24h as indicated. Immunization was administered 3 times, at 0, 4 and 25 weeks, and one animal received 293 cells alone. Samples were harvested from the rectal mucosa 5 days after every immunization in addition to samples at week 20 (without prior immunization). (a) **Tetramer specific, mucosal CD8 cells.** SIV-Gag- and Tat-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. In addition, cells were stained with monoclonal antibody to CD8 β and analyzed by flow cytometry. After gating on the CD8 β ⁺ population, the percentage of tetramer-positive cells was determined at each time point. The individual data for each monkey have been plotted. (b) **Rectal immune response:** Dot plots from a representative macaque, M944, immunized with 5 μ g of Gp96-Ig-SIV. Numbers represent % of tetramer+ cells within the CD8 gate. (c) Rectal response to gp96^{SIV}-Ig significantly exceeds peripheral response. Frequency of Gag CM9 tetramer + (right) and Tat SL8 tetramer+ (left) CD8 T cells in the rectal lamina propria and inguinal lymph nodes from macaques that received cells secreting 5 μ g gp96^{SIV}-Ig/24h (Group II) and control monkey at week 26. Bars represent mean $\pm$ SE of the percentage of tetramer+ cells (n=2) (d) Differences in regional intestinal response to gp96^{SIV}-Ig Frequency of tetramer+ cells (Gag shown in black and tat shown in white) within CD8 gate. Bars represent mean $\pm$ SE of the percentage tetramer+ cells (n=2) ND-not determined

FIGURE 3. Gp96-Ig-SIV vaccine induces polyfunctional SIV-specific CD8 T cells in the rectal and vaginal intraepithelial compartment and lamina propria
 At week 26 (5 days after 3rd immunization) intraepithelial lymphocytes (IEL) were harvested from all macaques. (a) **Rectal IEL memory response.** SIV-Gag- specific CD8 T cells were detected by Mamu-A*01/Gag181-189 CM9 (CTPYDINQM; Gag_CM9) and SIV-Tat-specific CD8 T cells by Mamu-A*01/Tat 28–35 SL8 (TTPESANL; Tat SL8) tetramer and CD8 β and analyzed by flow cytometry. Numbers represent % of tetramer⁺ cells within the CD8 gate. The individual data for each monkey have been plotted. (b) **SIV specific, rectal IEL and** (c) vaginal mucosal cells express CD103 and granzyme B. Dot plots from representative monkeys (M945 and M964) demonstrating binding of SIV-Gag tetramer on CD8 T cells from the intraepithelial compartment (rectal mucosa and vagina). Numbers represent percent of Gag-CM9-tetramer⁺ cells within CD8 gate or percent of CD103⁺ and granzyme B⁺ cells within tetramer gate. Frequency of Gag tetramer⁺ cells (SIV-gp96-Ig shown in black and 293 shown in white) within CD8 gate. Bars represent mean $\pm$ SE of the percentage tetramer⁺ cells (n=2) (d) Polyepitope specific rectal LPL and IEL secrete IL-2 and IFN- γ upon peptide stimulation. SIV-specific CD4 and CD8 T cell responses were detected using pools of 15-meric peptides overlapping by 11 amino acids covering the entire Gag, Env, and Tat proteins. The overall polyfunctionality of SIV-specific CD8⁺ and CD4⁺ T-cell responses in freshly isolated rectal lamina propria and intraepithelial cell samples from Mamu-A*01-positive animal M943 is shown as a pie chart. Each colored piece of a pie chart represents ratio of IFN- γ , IL-2 and IFN- γ + IL-2 secreting cells within the total number of responding cells, set to 100%. The number in the center of each pie represents the total percentage of CD8 or CD4 cells responding to Gag, Tat or Env stimulation.

FIGURE 4. Gp96^{SIV}-Ig stimulates SIV-specific CD8 T cells in the blood

Seven Rhesus macaques were sorted into 4 groups and immunized as in Fig. 2. Five days after every immunization, blood was collected, and PBMC obtained by density-gradient centrifugation. (a) **Frequency of SIV tetramer⁺ CD8 CTL in blood.** SIV-Gag- and Tat-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. In addition, cells were stained with monoclonal antibody to CD8 β and analyzed by flow cytometry. After gating on the CD8 β ⁺ population, the percentage of tetramer-positive cells was determined at each time point (vaccine induces SIV-Gag- and tat-specific CD8 T cells in the peripheral blood). (b) **IFN- γ spot forming cells in blood** Frequency of Gag- tat- and Env-specific cells in PBMC of vaccinated macaques as measured by IFN γ - ELISPOT assay following the gp96-Ig-SIV vaccination. The y-axis represents IFN γ -spot- forming cells per 2x10⁵ cells; and the x-axis time (weeks). PBMC were incubated on ELISPOT plates in the presence of peptides pools of 15-meric peptides overlapping by 11 amino acids covering the entire Gag, tat and Env proteins. Control include animal M694 that received only 293 cells. (c) Frequency of gag specific CD8 memory cells in blood Representative FACS plots for monkey M943 (group II) showing expression of CD28 and CD95 on gated CD8 T cells (gray contour plot) and Gag CM9+ CD8 T cells (red contour plot) in the blood. Numbers on the FACS plots represent frequency of central memory (T_{CM}) and effector memory (T_{EM})-positive cells as a percent of total Gag CM9+ cells as defined by the expression of CD28 and CD95 (T_{CM} CD28+CD95+ and T_{EM} CD28-CD95+).