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Cutting Edge: Novel Vaccination Modality Provides Significant Protection against Mucosal Infection by Highly Pathogenic Simian Immunodeficiency Virus

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Vaccine-induced protection against infection by HIV or highly pathogenic and virulent SIV strains has been limited. In a proof-of-concept study, we show that a novel vaccine approach significantly protects rhesus macaques from mucosal infection by the highly pathogenic strain SIVmac251. We vaccinated three cohorts of 12 macaques each with live, irradiated vaccine cells secreting the modified endoplasmic reticulum chaperone gp96-Ig. Cohort 1 was vaccinated with cells secreting gp96SIV Ig carrying SIV peptides. In addition, Cohort 2 received recombinant envelope protein SIV-gp120. Cohort 3 was injected with cells secreting gp96-Ig (no SIV Ags) vaccines. Cohort 2 was protected from infection. After seven rectal challenges with highly pathogenic SIVmac251, the hazard ratio was 0.27, corresponding to a highly significant, 73% reduced risk for viral acquisition. The apparent success of the novel vaccine modality recommends further study. The Journal of Immunology, 2013, 190:000–000.

Gp96 is a dominant endoplasmic reticulum (ER) chaperone and a danger-associated molecular pattern. In its chaperone function, gp96 in the ER receives all cellular peptides generated by the proteasome from endogenous proteins that are translocated by the transporter associated with Ag processing into the ER for subsequent selection and trimming for MHC class I loading. When released from necrotic cells, gp96 functions as a danger associated molecular pattern serving as an adjuvant to activate dendritic cells (DCs) via TLR2 and TLR4 (1) and, by being endocytosed by CD91, as an Ag carrier for Ag cross-presentation to CD8 T cells (2–4). By replacing gp96’s ER retention sequence with the hinge and Fc domain of IgG1, we generated a secreted chaperone, gp96-Ig, which optimally cross-primes Ag-specific CD8 T cells at 10⁻¹⁵ M peptide concentration (5, 6). Because gp96-Ig carries all peptides of a cell that will be selected in the recipient/vaccinee for MHC class I loading, including transfected or infected Ags, it has the broadest, theoretically possible antigenic epitope spectrum for cross-priming of CD8 T cells by any MHC class I type. In addition, gp96-activated DCs can take up antigenic proteins and, after processing, present their epitopes via MHC class II, thereby promoting Ab production by B cells. Thus, gp96 is a powerful Th1 adjuvant for CTL priming and for stimulation of Th1-type Abs that are of isotype IgG2a and IgG2b, in mice (N. Srtsbo, unpublished observations).

Protection from HIV infection requires mucosal immunity. Comparison of gp96SIV Ig vaccination in mice and of gp96SIV Ig vaccines in macaques by the s.c., intrarectal, intravaginal, or i.p. route demonstrated that i.p. vaccination generates a stronger mucosal CTL response in mucosal intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) than ever reported (7, 8). Therefore, the i.p. route was chosen for this study to determine the protective efficiency against mucosal SIV challenge in a proof-of-principle study.

Materials and Methods

Animals and vaccine cells

Indian-origin, outbred, young adult, male and female, specific-pathogen-free rhesus monkeys (Macaca mulatta, n = 36 animals) were housed and handled in accordance with the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care International at Rockville Advanced Bioscience Laboratories (Rockville, MD). Groups were balanced for Mamu-A*01 (three in each group) and Mamu-B*08 (one in each group), as well as for susceptible and resistant TRIM5α alleles. There were no Mamu-B*17” animals. Gp96SIV Ig vaccine cell were generated by transfection of 293 cells with plasmids encoding gp96-Ig, SIVmac251 rev-tar-nef, Gag, and gp160, as described previously (8). Macaques were injected i.p. with 10⁷ irradiated gp96SIV Ig vaccine cells, which secrete 10 µg/24 h gp96SIV Ig, in HBSS. In one group of macaques, 100 µg rSIVgp120 protein (Advanced Bioscience Laboratories) was added to the vaccine cells. Mock controls received 293-gp96 Ig not transfected with SIV Ags.

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Abbreviations used in this article: ASC, Ab-secreting cell; CI, confidence interval; DC, dendritic cell; ER, endoplasmic reticulum; HR, hazard ratio; IEL, intraepithelial lymphocyte; LPL, lamina propria lymphocyte; TCID50, tissue culture infective dose 50; TEM, effector memory T.
Macaques were primed at week 0 with vaccine or mock cells alone without gp120-addition and boosted at weeks 6 and 25, adding gp120 to one group. Beginning at week 33, all monkeys were challenged weekly by up to seven intrarectal instillations of 120 tissue culture infective dose 50 (TCID50) highly pathogenic SIV mac251 swarm virus (not cloned; National Institutes of Health challenge stock, provided by Dr. Nancy Miller, National Institutes of Health, Bethesda, MD; virus was propagated in macaque’s PBMCs), which generates three or four founder viruses in mock-infected controls. Viral loads were determined weekly by nucleic acid sequence–based amplification (BIOQUAL, Rockville, MD), and challenge was discontinued when positive. Animals were euthanized at week 52. In a parallel study, 24 animals received 100 μg gp120/alum or alum alone at weeks 12 and 24. All animals were challenged with the same virus stock (provided by Dr. Nancy Miller), and at the same dose as described above, at week 28. All animal studies were approved by the University of Miami Miller School of Medicine Institutional Animal Care and Use Committee.

Tissue preparation, flow cytometry, and SIV gp120 Abs in serum
Mononuclear cells were isolated from blood and rectal tissue pre- and post-vaccination, as described (8). SIV-specific cellular immune responses were assessed by multiparameter intracellular cytokine staining assay. Humoral immune responses were measured by gp120 ELISA and ELISPOT for Ab-secreting cells (ASCs), as well as by flow cytometric analysis of the plasmablast frequency in peripheral blood.

Statistical analysis
Analyses of virological and immunological data were performed using Wilcoxon rank-sum tests, and analyses of survival were assessed by log-rank tests. For these tests, p < 0.05 was considered significant, and two-tailed tests were performed. Hazard ratios (HRs) calculated by the Gehan–Wilcoxon test do not require a consistent HR, but they do require a consistently higher risk for one group. HRs were also calculated by proportional hazards regression analysis, with exact resolution of ties computed in SAS 9.2. Immunological correlates were evaluated using both parametric and nonparametric correlation tests. Graphical analysis was performed using the GraphPad Prism software package (GraphPad).

Results and Discussion
Gp96SIV Ig vaccines induce cellular and humoral immune responses
Some SIV vaccine concepts showed postinfection virological control (9–11), whereas other vaccine studies in humans (12) and macaques (13) reported significant protection against acquisition of SIV/HIV infection that appears to require specific cellular and humoral responses (14, 15).

and CD4$^+$ T cells secrete TNF-$\alpha$, IFN-$\gamma$, and IL-2 upon SIV-specific peptide stimulation. SIV-specific CD8$^+$ T cell responses at week 26 were detected, using pools of 15-meric peptides overlapping by 11 aa covering the entire Gag, Nef, and Env proteins, by multiparameter intracellular cytokine staining assay. Intracellular staining for TNF-$\alpha$, IFN-$\gamma$, and IL-2 was performed on freshly isolated rectal lamina propria mononuclear cells from rectal pinch biopsies stimulated for 5 h with overlapping SIV peptides in the presence of monensin and brefeldin A. After gating on live CD3$^+$CD8$^+$ or CD3$^+$CD4$^+$ T cells, the frequency of cytokine-positive cells was determined. (C) Vaccination induces gag- and tat-specific CD8$^+$ T cells in lamina propria and intraepithelial compartment of rectal mucosa. Pinch biopsies from the rectal mucosa at weeks 7 and 26 (5 d after third and third vaccinations) 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. (D) Phenotype analysis of CD8$^+$ SIV-gag$^+$ T cells in lamina propria and intraepithelial compartment. The markers CD28 and CD95 define the central memory (T_CM), transitional memory (T_TM), and T_EM among rhesus macaque T cells. T_CM, T_TM, and T_EM cells express CD28/CD95$, CD28$^+$/CD95$, and CD28$^+$CD95$^+$ phenotypes, respectively.
293-gp96SIV Ig cells were created by permanent transfection of HEK293 cells (not containing T Ag) with plasmids encoding gp96-Ig, SIV rev, nef tat (as fusion protein), gag, and gp120, as described (8). Intraperitoneal injection of 293-gp96SIV Ig generated extraordinary mucosal, rectal, and vaginal frequencies of polypeptide-specific MHC-restricted CTLs in LPL and IEL for SIV Gag, Tat, Nef, and gp120, secreting IFN-γ and IL-2 upon Ag stimulation (8). In this study, we determined the protective activity of the gp96SIV Ig vaccine strategy in 36 Indian-origin rhesus macaques (M. mulatta) divided into three groups of 12 and balanced by gender, MHC type, and TRIM5α expression. Group I received 293-gp9 SIVIg to generate CTL; SIVmac251 gp120 protein was added in group II to generate CTL and Ab; and group III was the control group, receiving 293-gp96-Ig not containing SIV Ags (Fig. 1A). A protein-only group, gp120/alum done in a parallel study (G. Franchini, unpublished observations), is included for comparison.

Vaccination was administered in weeks 0, 6, and 25, and the immune response was determined in weeks 7 and 26. Potent MHC-restricted CTLs in IEL and LPL secreting multiple cytokines were generated in groups I and II but not in controls (Fig. 1B, 1C) (16, 17). The gp96SIV Ig vaccine in rhesus macaques resulted in the preferential development of effector memory T (TEm) cells in the lamina propria and epithelial layer (Fig. 1D), in agreement with our previous findings (7). SIV-specific CD4 responses were also detected in gut lamina propria. Importantly, we observed an increase in the frequency of envelope-specific CD4 responses (Fig. 1B) only in the animals vaccinated with gp96SIV Ig + gp120, indicating that MHC class II presentation of gp120-derived peptides by DCs required addition of the gp120 protein (Fig. 1B).

Elevated humoral immune responses were found only in group II, as measured by ELISA for gp120-specific IgG and IgA Abs (Fig. 2A) (18), by ELISPOT assay for gp120-specific ASCs (Fig. 2B) (19), and by multiparameter staining for plasmablasts (data not shown) (20).

**Protective efficacy of gp96SIV Ig vaccines**

To evaluate the protective power of the immune response induced by gp96SIV Ig vaccines, all 36 macaques were challenged starting at week 33 (8 wk after the last vaccination) with up to seven weekly intrarectal instillations of SIVmac251 swarm virus, 120 TCID50 (provided by Dr. Nancy Miller). Challenge of individual macaques was discontinued when they had positive virus titers in blood, assessed 5 d after each challenge. Intrarectal inoculation of 120 TCID50 SIVmac251 generates three or four founder viruses in control, unvaccinated monkeys (G. Franchini, unpublished observations). Gp96SIV Ig + gp120 vaccination (group II) induced statistically significant (p = 0.01) protection against SIV acquisition. After seven rectal challenges, the HR was 0.27 [95% confidence interval (CI): 0.09–0.79 calculated with the GraphPad/Prism statistics package] or HR = 0.32 (95% CI: 0.13–0.80 computed with exact resolution of ties in SAS 9.2) (Fig. 3A), corresponding to a vaccine efficacy of 73 or 68% (vaccine efficacy = 100 × [1 − HR]). Protection was completely unaffected by the presence of TRIM5α or restrictive MHC alleles (data not shown), confirming a previous report (21). In contrast, 50% of mock-control macaques (group III) became infected after the first challenge compared with only 8.3% of the gp96SIV Ig group (group I) and 0% of the combined-vaccine group (group II); for 50% infection, macaques in...
group I required two challenges, whereas those in group II required three challenges (Fig. 3D).

We observed that some animals had very high plasma virus titers on the first day of testing postchallenge. For those with virus loads exceeding $10^6$ RNA copies/ml plasma (eight in group I, five in group II, and two in group III), it is possible that the animal had already been infected 1 wk earlier but the virus was not yet detectable in blood. Rescoring data under this conservative assumption gave results indicating that significant protection was also conferred in group II (HR $= 0.31$; 95% CI: 0.1–0.95, $p = 0.041$, Mantel–Cox test; Supplemental Fig. 1C).

Gp96SIV Ig alone (group I) did not provide significant protection (Fig. 3B). Likewise, adjuvanted gp120 alone is not protective (G. Franchini, unpublished observations, Fig. 3C). Although infection occurred in most macaques vaccinated with the combined vaccine (group II) (Fig. 3B), viral acquisition required significantly more challenges than in the other groups (compare Fig. 3B with 3A), indicating a substantial degree of immunity. Gp120 protein in the vaccine mixture was essential for the generation of Ab, ASCs, and plasmablasts in blood (Fig. 2).

Infected macaques showed peak plasma virus loads on day 14 following infection (Supplemental Fig. 1B), followed by a relatively stable state of virus replication. Macaques vaccinated with gp96SIV Ig + gp120 had a 1-log reduction in the mean peak virus load compared with mock controls at week 3 ($p = 0.048$, Wilcoxon rank-sum test). Overall, however, vaccinated groups did not show significant virological control once infected (Supplemental Fig. 1A).

Correlates of protection against acquisition of infection with gp96SIV Ig vaccines

Our data show that vaccination with gp96SIV Ig alone is not protective although it provides for potent Ag cross-presentation of SIV Ags generating CD8 CTLs and little or no Ab (Fig 3A). Likewise, gp120 protein vaccination alone does not provide protection, although it generates Ab and few CTLs (Fig. 3C). Because the combined vaccine provides significant protection (Fig. 3B) and generates both CTLs and Ab, it is inescapable that both cellular and humoral immunity are required for protection. The data indicate that gp96-Ig serves as an MHC class II adjuvant for gp120 (Fig. 1B). Because immunization takes place in a gp96-Ig–created Th1 environment, Ab responses are likely to be polarized to IgG3 and IgG1. Isotyping of the Ab response in a protected macaque (Supplemental Fig. 2D) showed predominant IgG3 and IgG1 isotypes.

Analysis of the correlation of protection with a mixed CTL/Ab response (Supplemental Fig. 2A–C) is likely to reveal the effector component that limits the degree of protection. In this case, it appears that Ab is limiting relative to CTL activity.

The primary goal of current efforts in HIV/SIV is to find a modality of vaccination that provides immunity from infection by subsequent viral challenge. This goal has been elusive.

To our knowledge, in this first test of the novel modality of cell-secreted gp96-Ig vaccination, we achieved a significant degree of protection in a highly pathogenic SIV model that has not been seen in any previous study and is matched by only one recent report (22) using traditional vaccines.

Our next challenge is to improve the degree of protection near 100%. The correlative analysis suggests that the CTL response to our vaccine is necessary and sufficient, whereas the Ab response limits the degree of protection. This result provides a clear path for further development.

We are using the i.p. route because it gives the highest degree of mucosal CD8 CTL-based immunity compared with other routes (7) and, therefore, is the best basis for proof-of-concept studies. Upon achieving full protection in macaques, the final challenge will be to adapt the methodology to routes of vaccination that achieve comparable mucosal immunity and protection but are more suitable for human use. The applicability of our results using i.p. immunization to vaccine efficacy using clinically relevant routes of administration remains to be determined.

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Disclosures

E.R.P. and the University of Miami have a financial interest in the commercial development of gp96-Ig–based vaccines. The other authors have no financial conflicts of interest.

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