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Mucosal Humoral Immune Response to SIVmac239Δnef Vaccination and Vaginal Challenge

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R. Paul Johnson,¶5 and Ashley T. Haase*5

Live attenuated vaccines such as SIV with a deleted nef gene have provided the most robust protection against subsequent vaginal challenge with wild-type (WT) SIV in the SIV–rhesus macaque model of HIV-1 transmission to women. Hence, identifying correlates of this protection could enable design of an effective HIV-1 vaccine. One such prechallenge correlate of protection from vaginal challenge has recently been identified as a system with three components: 1) IgG Abs reacting with the viral envelope glycoprotein trimeric gp41; 2) produced by plasma cells in the submucosa and ectopic tertiary lymphoid follicles in the ectocervix and vagina; and 3) concentrated on the path of virus entry by the neonatal FcR in the overlying epithelium. We now examine the mucosal production of the Ab component of this system after vaginal challenge. We show that vaginal challenge immediately elicits striking increases in plasma cells not only in the female reproductive tract but also at other mucosal sites, and that these increases correlate with low but persistent replication at mucosal sites. We describe vaginal ectopic follicles that are structurally and functionally organized similar to follicles in secondary lymphoid organs, and we provide inferential evidence for a key role of the female reproductive tract epithelium in facilitating Ab production, affinity maturation, and class switch recombination. Vaccination thus accesses an epithelial–immune system axis in the female reproductive tract to respond to exposure to mucosal pathogens. Designing strategies to mimic this system could advance development of an effective HIV-1 vaccine.

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The great advances in treating HIV-1 infections have reduced the morbidity and mortality HIV-1 infection causes, but there remains an urgent and continuing need to develop an effective vaccine to halt the progress of the epidemic, especially to stop transmission of HIV-1 to women who bear the brunt of infection in the pandemic’s epicenter in Africa (1, 2). Toward that goal, we have been seeking correlates of the robust protection conferred by the live attenuated vaccine, SIV with a deleted nef gene (SIVmac239Δnef) (3–5), because these correlates could provide design principles for development of an effective HIV-1 vaccine. In these studies in the SIV–rhesus macaque model of HIV-1 transmission to women, we recently identified (6) IgG Abs to trimeric gp41 (gp41t) prior to vaginal challenge as one correlate of the temporal maturation of protection (7) between 5 and 20 wk post vaccination, times, respectively, when animals are not or are protected against high-dose vaginal challenge with wild-type (WT) SIV. We showed that these Abs are locally produced by plasma cells and ectopic lymphoid follicles in the cervix and vagina and are concentrated by the neonatal FcR (FcRn) (8) in the overlying epithelium, thus providing a mechanism for the Abs to react with virus on the path of entry and thereby inhibit transmission. The interaction just described was only one of the interactions identified at mucosal frontlines that point to a mucosal epithelium–immune system axis. Vaccination also induced expression of CXCL10 in the female reproductive tract (FRT) epithelium as a chemotactic mechanism to recruit CXCR3+ IgG+ plasma cells to the underlying submucosa (6), as well as expression of the inhibitory FcgRIIB inhibitory receptor in cervical epithelium to interact with immune complexes formed following vaginal challenge. This interaction then induced an inhibitory program (9) preventing the recruitment of CD4+ T cell targets that fuel local expansion in unvaccinated animals (10). In this study, we describe further evidence of a mucosal epithelial–immune system axis to facilitate Ab production, affinity maturation, and class switch recombination (CSR) after vaginal challenge. This in situ rapid
recall and sustained humoral immune response literally generates a wall of IgG Abs at mucosal frontlines as defenses against exposure to mucosal pathogens as one concept and design principle for developing effective vaccines against HIV-1.

Materials and Methods

Animals, vaccination, and vaginal challenge

We examined tissues from 10 naïve animals archived from previously described studies (11) of transmission following high-dose inoculation of WT SIV, as well as tissues from 23 SIVmac239Δnef-vaccinated female rhesus macaque monkeys (Macaca mulatta) at days 4 (n = 1), 7 (n = 1), 11 (n = 1), and 14 d (n = 1) after vaccination, at 5 (n = 4) and 20 wk (n = 4) after vaccination, and at days 4 and 5 (n = 5), 7 (n = 3), and 14 (n = 3) after high-dose vaginal challenge at 20 wk postvaccination (6). The animals had been housed in accordance with the regulations of the American Association for the Accreditation of Laboratory Animal Care and the standards of the Association for the Assessment and Accreditation of Laboratory Animal Care International at the New England and California Primate Centers.

Tissue collection and processing

At the time of euthanasia, lung, jejunum, colon, cervix, vagina, spleen, and various peripheral lymph nodes and other tissues were collected and fixed in 4% paraformaldehyde or SafeFix II and embedded in paraffin for later analyses, as described (6). Portions were also snap frozen for later extraction of RNA and DNA.

FRT mucosal epithelial in vitro model

The HEC-1A uterine epithelial cell culture system was used to examine responses to SIV, as described in Li et al. (6).

Immunohistochemistry and immunofluorescence

These methods were performed as described in Li et al. (6) and Zeng et al. (12). Tissue sections (10 μm) mounted on glass slides were deparaffinized and rehydrated in deionized water. Heat-induced epitope retrieval was performed using a water bath (98˚C) in EDTA Decloaker (Biocare Medical), followed by cooling to room temperature. Tissue sections were then blocked with Sniper blocking reagent (Biocare Medical) for 30 min at room temperature. Primary Abs were diluted in TNB (0.1 M Tris-HCl [pH 7.5], 0.15 M NaCl, and 0.05% Tween 20, and then incubated with biotin-conjugated secondary Abs in TNB for 2 h at room temperature, washed with PBS with 0.1% Tween 20, and then incubated with anti-biotin Ab conjugated to alkaline phosphatase in TNB for 2 h at room temperature. After the incubation of anti-biotin Ab, the sections were washed with PBS with 0.1% Tween 20. Signal was detected with a Warp red kit (Biocare Medical). The sections were counterstained with Harris hematoxylin (Surgipath), dehydrated in rapidly in gradient ethanols, and mounted with Permount (Fisher Scientific). Stained sections were examined by light microscopy at ambient temperatures. Light micrographs were taken using an Olympus BX60 upright microscope with the following objectives: ×10 (0.3 numerical aperture), ×20 (0.5 numerical aperture), and ×40 (0.75 numerical aperture); images were acquired using a SPOT color mosaic camera (model 11.2; Diagnostic Instruments) and SPOT acquisition software (version 4.5.9; Diagnostic Instruments). Anti-biotin Ab conjugated to alkaline phosphatase was used as negative control Abs in all instances and yielded negative staining results. For immunofluorescent staining, after the primary Ab incubation, sections were washed with PBS and then incubated with biotin-conjugated secondary Abs in TNB for 2 h at room temperature; washed with PBS with 0.1% Tween 20, and then incubated with anti-biotin Ab conjugated to alkaline phosphatase in TNB for 2 h at room temperature. After the incubation of anti-biotin Ab, the sections were washed with PBS with 0.1% Tween 20. Signal was detected with a Warp red kit (Biocare Medical). The sections were counterstained with Harris hematoxylin (Surgipath), dehydrated in rapidly in gradient ethanols, and mounted with Permount (Fisher Scientific). Stained sections were examined by light microscopy at ambient temperatures. Light micrographs were taken using an Olympus BX61 FluoView confocal microscope with the following objectives: ×20 (0.75 numerical aperture), ×40 (0.75 numerical aperture), and ×60 (1.42 numerical aperture); images were acquired and mean fluorescence intensity was analyzed by using Olympus FluoView software (version 1.7a). Positive stained cells or follicles were enumerated in 20 randomly acquired, high-powered images (×200 or ×400 magnification) by manual counting in each image.

RNA extraction

Frozen tissue specimens were homogenized with a power homogenizer in TRIzol without thawing. Total RNA was isolated according to the manufacturer’s protocol and further purified with an RNeasy mini kit.

Real-time RT-PCR to determine SIVmac239Δnef viral loads in tissues and plasma

RT-PCR assays were performed using primers specific for SIVmac239Δnef to determine the levels of SIVmac239Δnef in tissues and plasma, but with modifications for tissue RNA to accommodate the higher amounts and complexity of input RNA (13).

Soluble gp41t and reverse immunohistochemistry

Cells with Abs to gp41t were stained by reverse immunohistochemistry (RIHC), as described in Li et al. (6).

Statistical analyses

Data represent means ± SEM in all graphs depicting error bars. The statistical significance of differences between experimental groups was
determined using GraphPad Prism 6 and the indicated statistical tests. A \( p \) value <0.05 was considered significant.

**Results**

**Increased IgG\(^+\) plasma cells and ectopic follicles throughout the mucosal immune system**

SIVmac239\(\Delta\)nef vaccination has previously been shown to elicit increased numbers of IgG\(^+\) plasma cells and ectopic lymphoid follicles beneath the cervical vaginal epithelium accompanying the maturation of protection between 5 and 20 wk postvaccination (6). These increases, we now show, were not confined to the FRT, but were general throughout the mucosal immune system, including the lung and gut-associated lymphoid tissue (Fig. 1).

**SIVmac239\(\Delta\)nef Ag exposure in the mucosal immune system**

What might drive the increases in plasma cells and ectopic follicles producing IgG Ab at mucosal sites? One possibility we examined is that SIVmac239\(\Delta\)nef replicates at these sites to induce the mucosal IgG production. Although this initially seemed unlikely, as we found and as others have shown for SIV and HIV (14–16), SIVmac239\(\Delta\)nef replicates predominantly in T follicular helper (TFH) cells (Fig. 2) and was barely detectable at mucosal sites in the first 2 wk following i.v. infection, and it was undetectable in cervix and vagina at 5 and 20 wk postvaccination by assays available at the time (not shown). However, when there was sufficient tissue available for contemporary assays (13) of viral RNA in \(10^8\) cell equivalents, we found that although vRNA was most abundant in acute infection throughout lymphoid tissues, there

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**Table I. SIV\(\Delta\)nef RNA in mucosal sites and lymphoid tissues**

<table>
<thead>
<tr>
<th>Days after vaccination</th>
<th>Number of SIV RNA Copies per (10^8) CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pelvic LN</td>
<td>(5.8 \times 10^6)</td>
</tr>
<tr>
<td>Inguinal LN</td>
<td>(7.0 \times 10^6)</td>
</tr>
<tr>
<td>Mesenteric LN</td>
<td>(2.0 \times 10^6)</td>
</tr>
<tr>
<td>Axillary LN</td>
<td>(2.0 \times 10^6)</td>
</tr>
<tr>
<td>GALT</td>
<td>(1.7 \times 10^6)</td>
</tr>
<tr>
<td>Spleen</td>
<td>(6.8 \times 10^3)</td>
</tr>
<tr>
<td>Lung</td>
<td>(1.5 \times 10^6)</td>
</tr>
<tr>
<td>Cervix</td>
<td>(9.3 \times 10^6)</td>
</tr>
<tr>
<td>Vagina</td>
<td>(7.9 \times 10^3)</td>
</tr>
<tr>
<td>Weeks after vaccination</td>
<td></td>
</tr>
<tr>
<td>Cervix</td>
<td>(2.0 \times 10^6)</td>
</tr>
<tr>
<td>Vagina</td>
<td>(7.9 \times 10^6)</td>
</tr>
<tr>
<td>Weeks after vaccination</td>
<td></td>
</tr>
<tr>
<td>Cervix</td>
<td>(6.4 \times 10^2)</td>
</tr>
<tr>
<td>Vagina</td>
<td>(5.8 \times 10^2)</td>
</tr>
</tbody>
</table>

SIV RNA copies per \(10^8\) cell equivalents (CE) are shown. When sufficient tissue was available for the assay, RNA was extracted from single animals from the tissue sites and on days shown following SIV\(\Delta\)nef i.v. infection. There were sufficient cervical and vaginal tissues available from three animals each at 5 and 20 wk for the assay. Copy numbers in extracted tissue RNA were determined by RT-PCR using specific primers and are expressed as number of copies of SIV\(\Delta\)nef RNA per \(10^8\) CE. The values shown for GALT are the highest, respectively, for jejunum, ileum, colon, and rectum.

CE, cell equivalent; LN, lymph node; NA, tissue samples of sufficient size for the assay were not available.
were lower levels of in the FRT, lung, and gut (Table I). In the limited number of samples available for assay in the FRT at weeks 5 and 20 postvaccination, SIVmac239Δnef RNA was still detectable, albeit at even lower levels, through week 20 (Table I). Thus, persistent exposure to virus at mucosal sites, low level inflammation, and expression of IFN-γ and type I IFN (17, 18) could have generated expression of chemokines to recruit cells and induce ectopic follicles at mucosal surfaces.

Vaginal plasma cell and the IgG wall in vaccinated animals following challenge

Vaginal challenge increased the number of IgG+ plasma cells and ectopic follicles over the already increased number present pre-challenge in the FRT of vaccinated animals compared with unvaccinated controls (Fig. 3). By day 4 following high-dose WT SIV vaginal challenge, the increased density of IgG+ plasma cells in the vagina far exceeded the modest increases in unvaccinated animals inoculated vaginally with the same high doses of WT SIV (Fig. 3A, 3B), and these increases continued so that by day 14 nearly 6-fold as many IgG+ cells were found in the submucosa of vaccinated animals as in the naive controls. At this time, the conjunction of IgG+ plasma cells and IgG concentrated in the overlying FcRn+ basal vaginal epithelium (6) imparted the appearance of an IgG wall (Fig. 3C). As surmised above, increased epithelial expression of CXCL9 (Fig. 3D) and CXCL10 (previously shown in reference Ref. 6) in the vaccinated

![Image](http://www.jimmunol.org/DownloadedFrom)
animals provides a mechanism for the continued recruitment of plasma cells into the FRT after vaginal challenge.

**Follicle structural and functional organization for Ab production**

In addition to the increased numbers of submucosal plasma cells for IgG production after vaginal challenge, there were also more ectopic follicles, structurally and functionally organized for Ab production, affinity maturation, and CSR. The remarkable structural similarities that the ectopic follicles in the vagina and ectocervix share with the mature follicles and germinal centers characteristic of secondary lymphoid organs (19) included a CD35 + follicular dendritic cell (FDC) network in the center of the follicle (Fig. 4A); IgG + plasma cells, CD20 + B cells, and PD-1 + CD4 + TFH cells in and around the follicle center, surrounded by CD3 + T cells at the periphery (Fig. 4A–C); and CD31 + high endothelial venules (Fig. 4D).

The ectopic follicles were also functionally organized to support proliferation and survival of B cells and plasma blasts, as well as affinity maturation and CSR of Abs: 1) In naive animals, there is little expression of Ki67 in the lamina propria in FRT tissues (most Ki67+ cells in Fig. 5A are epithelial cells), whereas at 20 wk postvaccination, although there are Ki67+ proliferating IgG + cells, most are Ki67 terminally differentiated plasma cells (Fig. 5A). Following vaginal challenge, the number of both Ki67 + and Ki67 − IgG + cells significantly increased 4 d after vaginal challenge, in parallel with enlargement of the ectopic follicles in the vaccinated animals (Fig. 5B). 2) Many of the IgG + cells in the ectopic follicles are CD27 + CD38 − CD20 + surface IgG + memory B cells (20) or early plasmablasts (Fig. 5C). 3) The expression of activation-induced cytidine deaminase (AID), a DNA-editing enzyme essential for Ig gene diversification by somatic hypermutation, and for CSR (21–24) also increased following vaginal challenge (Fig. 5D).
The vaginal epithelium not only can actively recruit plasma cells via CXCL9 and CXCL10 expression, but it also expresses cytokines that create a favorable niche for the B cells and plasma cells to proliferate, survive, and differentiate. Because of the importance of BAFF in B and plasma cell survival and proliferation, Ab production, maturation, and CSR (25–33), we stained sections to determine whether there might be a source of BAFF to induce the changes in the follicles just described. We indeed found that the epithelium could be one source of BAFF after vaginal challenge, because there were striking increases in BAFF staining in vaginal epithelium after challenge (Fig. 6A). One hypothetical mechanism driving increased vaginal epithelial expression of BAFF could be exposure to SIV in the inoculum, and we did find that exposing an FRT epithelial cell line, HEC-1A cells, to WT SIV significantly increased BAFF expression (Fig. 6B), consistent with this hypothesis. We have also previously shown that SIV in immune complexes is taken up by epithelium and interacts with the Fcg2Rb inhibitory receptor to block CD4 T cell recruitment following vaginal exposure, and in future work we plan to determine whether viral binding might intersect with additional signaling pathways to elicit increased expression in BAFF.

We also documented potential functional interactions between BAFF and one of its receptors, B cell maturation Ag (BCMA) (29, 30), within ectopic follicles and plasma cells in the lamina propria (Fig. 6C, 6D), and that two key mole-

**FIGURE 5.** Rapid induction of SIV-specific humoral immune response within vaginal ectopic follicles after WT SIVmac251 challenge. (A) Increased red-stained IgG^+^ cells and follicles at 20 wk in vaccinated compared with unvaccinated animals. Most of the IgG^+^ cells are Ki67^+^ (the green-stained Ki67^+^ cells are primarily epithelial cells). Following vaginal challenge, Ki67^+^IgG^+^ cells increase beneath epithelium and particularly in the ectopic follicles. Representative images are from n = 4 for SIV animals, n = 4 for 20 wk and 3 for day 4 after 20 wk (D4p20w) animals. Scale bars, 20 μm. (B) Quantification of increases in Ki67^-^cells at 20 wk and day 4 after 20 wk (D4p20w). *p ≤ 0.05. (C) Phenotypic analysis of the IgG^+^ cells within ectopic follicles shows that they are CD38^+^CD27^+^CD20^+^ surface IgG^+^ plasmablasts. Scale bars, 20 μm. (D) Fluorescence staining shows increased expression of AID in the ectopic follicles after WT SIVmac251 challenge. Scale bar, 10 μm. Representative images were taken from animals at day 4 after 20 wk vaccination.
molecules involved in BCMA signaling, phosphorylated JNK and phosphorylated SAPK/ERK1 (SEK1) (34–36), were also upregulated within follicles in the vaccinated animals (Fig. 6E). Thus, there is a spatially proximate axis between the vaginal epithelium and underlying plasma cells and ectopic follicles to facilitate Ab production and maturation in response to vaginal challenge.

**Production of SIV-specific Abs in ectopic follicles and vaginal submucosa**

We have thus far visualized and characterized a general system for production of IgG Abs in the FRT in response to vaginal challenge, and we now show that the B cells and plasma cells in the submucosa and ectopic follicles were producing SIV-specific IgG Abs to gp41t, which we had previously shown to be one correlate of the matu-
ration of SIVmac239Δnef-mediated protection against vaginal challenge (6). We detected these Abs by RIHC using a labeled soluble gp41t construct (6). Four and 5 d following vaginal challenge, we found more trimeric gp41t+ plasma cells in the submucosa and ectopic follicles, consistent with expression of Ab specific for this Ag (Fig. 7A, 7B). Some of the IgG+ cells in the follicles at day 4 after challenge were also Ki67+ (Fig. 7B), consistent with gp41t-specific plasmablasts generated in situ in response to vaginal exposure to cognate Ag. The RIHC staining was specific (Fig. 7C), and the ~4-fold increases in gp41t+ cells in the ectopic follicles was significant, with a p value of <0.01 (Fig. 7D).

Discussion
In vaccines evaluated in NHP models, SIVmac239Δnef and other live-attenuated vaccines have consistently provided the most robust protection against acquisition on subsequent WT SIV challenge by parenteral and mucosal routes (3–6), and thus such studies provide an opportunity to identify correlates of protection and design principles for developing an effective HIV-1 vaccine. This approach has thus far been identified as one correlate of protection prior to vaginal challenge, that is, IgG Abs reactive with trimeric gp41 (6, 9). These Abs are produced locally as well as systemically and are concentrated by the FcRn receptor in cervical reserve and basal vaginal epithelium, thereby optimally localizing the Abs to intercept virus and block access to target cells. In this way, they could prevent establishment and local expansion of infected founder populations at the portal of entry. The concentrated Abs also play a critical role in the second correlate of protection by generating immune complexes generated after vaginal challenge. These immune complexes interact with the inhibitory FcγRIIb receptor in cervical epithelium to suppress recruitment of CD4 T cell targets to thereby prevent local expansion of infection (9).

In this study, we characterize the response after vaginal challenge in SIVmac239Δnef-vaccinated animals. We first show that vaccination induces general increases in plasma cells and ectopic follicles at mucosal sites that are not principal sites of viral replication. SIVmac239Δnef, other live-attenuated SIV strains, WT SIV, and HIV infect TFH and other CD4+ T cells in lymphoid

**FIGURE 7.** Increased gp41t Ab-producing plasma cells and ectopic follicles after vaginal challenge shown by RIHC. (A) RIHC staining shows increased gp41t+IgG+ (red-brown) plasma cells in the vaginal submucosa 5 d after vaginal challenge at 20 wk. (B) Some of the gp41t+ cells (green) in follicles are also Ki67+ (red) and have a white color in the merged confocal micrograph of follicles after vaginal challenge at 20 wk, consistent with rapid proliferative response following vaginal exposure. (C) Representative image of RIHC control staining in an SIV- uninfected, unvaccinated animal. Scale bars for (A)–(C), 20 μm. (D) Quantification of increased gp41t+ cells in follicles after vaginal challenge. **p ≤ 0.01.
tissues (13–16). Nonetheless, we document low levels of replication at mucosal sites and speculate that it is the persistent low levels of SIVmac239Δnef replication and expression of IFNs (17, 18) that induce expression of CXCL9 and CXCL10 responsible for the influx of B cells and plasma cells and induction of ectopic lymphoid follicles at mucosal sites prior to challenge. In the FRT, we speculate that vaginal exposure to WT SIVmac251 might act as a pull, as has been described for T cells (37) to increase epithelial expression of these chemokines and rapidly augment the recruitment of plasma cells over and above the larger number already recruited there by 20 wk compared with 5 wk postvaccination (6). These plasma cells and ectopic follicles then produce a wall of IgG in vaginal epithelium as striking evidence of the recall response in the FRT.

The ectopic follicles are structurally and functionally organized in a remarkably similar way to follicles in secondary lymphoid organs (19). The common features include follicles with an FDC network and high endothelial venules in germinal centers with the necessary cellular constituents of B cells, plasma cells, and TFF for Ab production, as well as cells expressing AID and other proteins that mediate B cell proliferation and affinity maturation and CSR.

Our findings provide further evidence of a mucosal epithelium–immune system axis and a role for this axis in the response to vaginal challenge. The epithelial components of this axis include: 1) expression of CCL20 in cervical epithelium associated with recruitment of CD4+ T cells to fuel local expansion of small founder populations of infected cells following vaginal challenge (10); 2) cervical vaginal epithelial expression of CXCL10 and FcRn to recruit plasma cells to produce and concentrate IgG Abs at mucosal frontlines to intercept virus at entry (6); and epithelial expression of FcγRIIb, SPRED1, COMMD1, and RORA to block T cell depletion in HIV-1 and SIV infections. J. Clin. Invest. 121: 998–1008.


