Immunization with adenovirus at the large intestinal mucosa as an effective vaccination strategy against sexually transmitted viral infection

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The large intestinal mucosa contains immunological structures that may potentially serve as a site for induction of mucosal immunity against infections. Adenovirus (Ad), which is effective in gene transfer to epithelia, may be an ideal antigen delivery system for vaccination at the large intestinal mucosa. To investigate this potential, we immunized mice with recombinant replication-deficient Ad through a single intracolorectal (ICR) administration. Effective transfer of encoded genes was found in both the epithelial layer and lamina propria of the colorectal mucosa. Dendritic cells were able to transfer antigen to the draining lymph nodes, where antigen-specific CD8$^+$ T cells were primed. Functional antigen-specific CD8$^+$ T cells and IgA-specific antibodies were detected during the effector phase in the large intestine. Compared to other immunization routes (intranasal, subcutaneous), ICR immunization induced stronger colorectal immune responses and more potent protection against rectal challenge with pathogenic viruses. Further, this immunization strategy provided vaginal protection, more potent than that induced by vaccination in the nose or skin. Therefore, large intestine mucosal immunization using Ad represents an effective vaccination strategy against virus infection at both rectal and vaginal mucosal tissue sites.

INTRODUCTION

Many infections occur primarily through entry via mucosal surfaces. Sexually transmitted infections, such as human immunodeficiency virus or herpes simplex virus type 2 (HSV-2), are usually transmitted through genital mucosa, but can also occur through the large intestine via the rectal route. The large intestinal epithelium is made up of a monolayer of epithelial cells, which physically isolates lumenal microorganisms from the underlying lamina propria (LP), representing the first line of host defense.[1,2] However, pathogens can enter the mucosa by infecting intestinal epithelial cells (IECs) or through an abraded, lacerated epithelium during sexual contact.[2,3] Along the lining of the gastrointestinal tract, the mucosal immune system is made up of secretory antibodies, especially IgA, that can prevent the cellular entry of pathogens[3] and T cells, especially CD8$^+$ T cells, that can play major roles in eradicating intracellular pathogens and eventually control the infection that has occurred.[4,5] Thus, the generation of immunological resistance at the mucosal surface may effectively protect the host against pathogen invasion via the mucosal surface.

A number of studies have shown that owing to the existence of the common mucosal immune system, immunization at one mucosal site provides better mucosal immune responses at a distant mucosal site than equivalent immunization through a systemic route.[6–9] Immunization at the nose can induce mucosal responses at distal mucosae such as the vaginal tract.[10–14] However, some data also suggest that nasal immunization may not be as effective as needed at these genitourinary mucosal sites.[6,7,9,15] Indeed, priming of immune cells locally in the draining lymph node (LN) at a mucosal infection site is likely to provide strong local mucosal immunity.[8] Mucosal dendritic cells (DCs) beneath the epithelium were shown to acquire pathogen-derived antigens and present them to mucosal lymphocytes in draining LNs and stimulate these cells to become effector cells specific for these antigens.[16–18] From a practical and clinical perspective, mucosal immunization through the large intestine, for example through the use of a suppository, may be ideal for local priming of this tissue. Although there has been an increasing interest in exploring immunization at the large intestinal mucosa, this approach is hampered owing to the lack of an

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effective means for antigen delivery and potent immunological adjuvants that can be used.[19]

Adenovirus (Ad) is a non-enveloped, double-stranded DNA virus. *In vivo*, gene transfer mediated by adenoviral vectors expressing heterologous antigen genes has proved to be one of the most successful ways to trigger or modulate immune responses.[20,21] Moreover, administration of Ad vectors to mice through the colonic mucosa was able to transfer therapeutic levels of cytokine genes for the experimental treatment of colitis.[22–24] On the basis of these previous observations, in this study we employed Ad vectors as vaccines to immunize mice at the large intestinal mucosa by intracolorectal (ICR) administration and demonstrated that antigen-specific immune responses, including both cellular and humoral responses, were induced with a single dose of the vaccine. We also developed a novel mouse model for virus challenge at the large intestine using a highly pathogenic virus strain, HSV-2, to test the efficacy of the vaccination strategy. Importantly, ICR immunization induced more potent protection against colorectal challenge of HSV-2 compared to immunization by other routes. It is intriguing that this immunization strategy also conferred better protection at the vaginal mucosa.

RESULTS

Gene transfer to the large intestine by Ads

We first administered adenoviral vectors expressing the β-galactosidase (β-gal) gene (Adβ-gal) by ICR delivery to evaluate gene transfer to the colorectal mucosa of mice. Cell-associated β-gal expression was seen in the epithelium, extending from mid-colon to the anal end (**Figure 1a**), and also beneath the epithelium, in the LP (**Figure 1b**). There were more gene expression clusters in the epithelium than in the LP (**Figure 1c**).

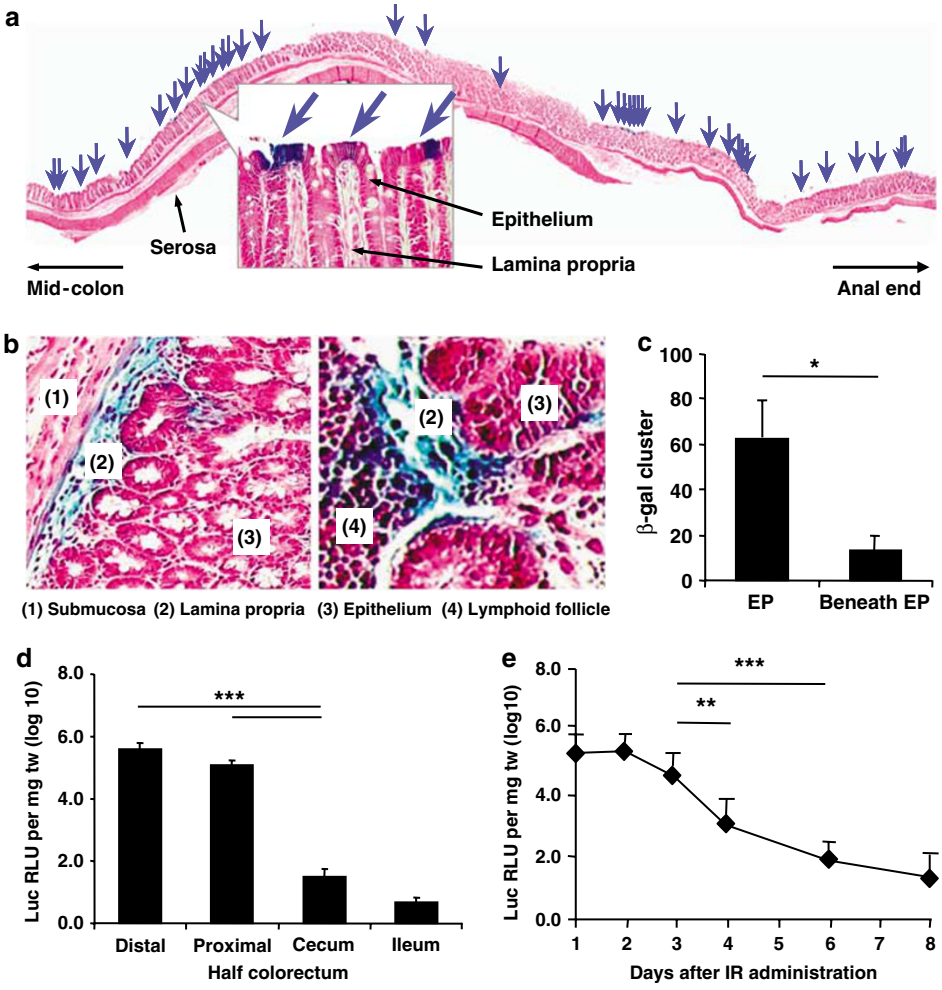

Figure 1 Adenovirus (Ad) delivered intracolorectally (ICR) transfers gene to the colorectal mucosa. (**a**) Paraffin-embedded sections of the colorectum were stained with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) 2 days after ICR administration of 2×10^9 PFU Adβ-gal. Cell-associated positively stained blue clusters along the epithelium are indicated with arrows. Microscopy images with ×200 magnification were connected end-to-end using Adobe Photoshop to reveal 2-cm-long distal-half colorectums (photo reduced). (**b**) Paraffin-embedded sections with β-gal staining in LP (left panel) or lymphoid follicles (right panel) at day 2. Original magnification ×400 and ×800, respectively. (**c**) β-Gal-expressing cells were enumerated at day 2. The number is averaged from longitudinal tissue sections of three different whole colorectum. (**d**, **e**) Luciferase (Luc) enzymatic activity was measured at 2 days or as indicated after ICR administration of AdLuc. Luc activity determined by an increase in relative light unit (RLU) compared to background is expressed as mean±s.e.m. from three mice in each group. *$P<0.05$, **$P<0.01$, ***$P<0.001$. Results are representative of at least two independent experiments. β-gal, β-galactosidase; PFU, plaque-forming unit.

By using Ad expressing luciferase (AdLuc) for quantification of gene expression, we showed that genes were predominantly transferred and expressed in the colorectum, primarily in the distal half, and minimally in the cecum and ileum (**Figure 1d**). In the colorectum, gene expression was abundant from day 1 through day 3 (**Figure 1e**). Thus, a significant amount of antigen can be transferred to the colorectal mucosa for mucosal immunization.

Transport of gene to draining LNs by intestinal DCs

We investigated whether large intestinal DCs are involved in processing Ad-delivered genes and inducing antigen-specific immune responses. Forty-eight hours after ICR administration of Ad expressing green fluorescent protein (GFP) (AdGFP), GFP-expressing cells were detected both in the large intestinal LP and iliac LNs (ILNs), the draining LNs for the large intestine (**Figure 2a**, upper panel), but not in mesenteric LNs (data not shown). DCs double positive for major histocompatibility complex (MHC) class II^+ and $CD11c^+$ resided in the large intestine (**Figure 2a**, lower panel). Within the GFP^+ cell population in LP, only a small proportion was MHC class II^+CD11c^+ DCs, whereas in the ILNs, a majority of the GFP^+ cells were DCs (**Figure 2a**, lower panel). To test whether gene-carrying DCs can migrate from the large intestine to the ILNs and subsequently induce specific immune responses, we labeled bone marrow-derived DCs with carboxyfluorescein diacetate succinidyl ester (CFSE) and injected these cells intermucosally into colorectal mucosa. Two days after injection, double-positive CFSE-DCs were recovered in the ILNs and this appeared to be a dose-dependent process (**Figure 2b**). When DCs that had been transfected *in vitro* with Ad expressing ovalbumin (AdOVA) were injected into the colorectal mucosa, cytotoxic T lymphocytes (CTLs) against the OVA CTL epitope SIINFEKL were detected in the ILN, as assessed at day 5 after administration (**Figure 2c**). Therefore, DCs can transport antigen from the large intestine to ILNs and effectively prime antigen-specific T cells.

Induction of large intestinal immune responses by Ad ICR immunization

Antigen-specific $CD8^+$ T-effector cells are essential for eliminating virally infected cells[25,26] and produce interferon-γ (IFN-γ)

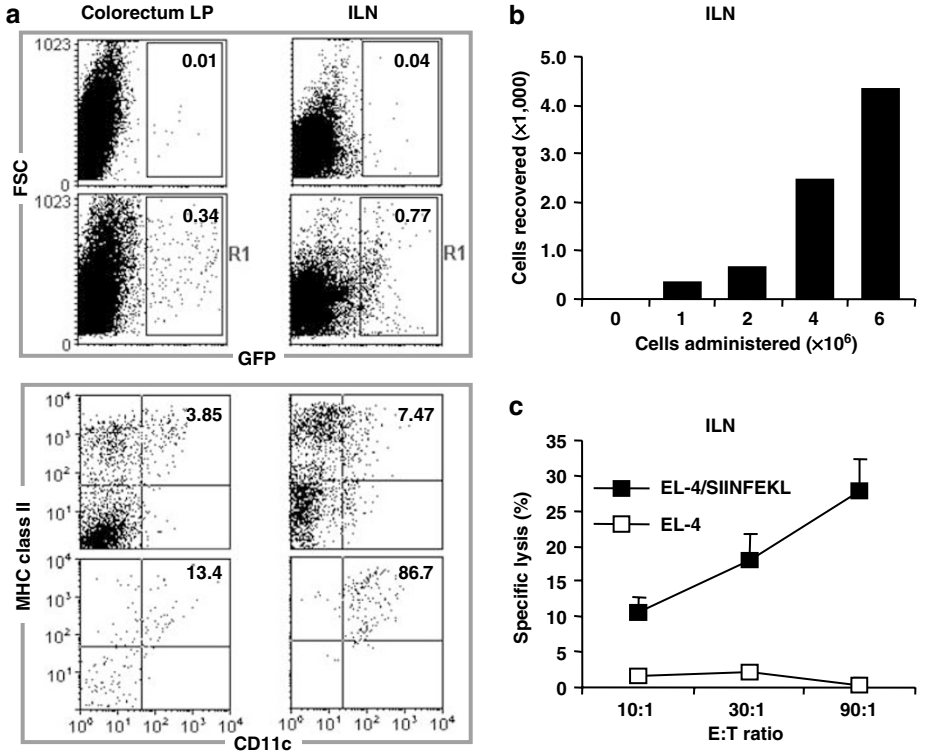

Figure 2 Colorectal DCs can acquire and transport Ad-encoded gene to ILNs. (**a**) Two days after ICR administration of 2×10^9 PFU of AdGFP, both colorectal LP and ILNs were isolated to determine GFP^+ cells by flow cytometry (upper panel/top row: AdBHG control vector lacking GFP; bottom row/AdGFP: numbers indicate percentage of total cells recovered). LP and ILN cells were stained for MHC class II and CD11c (lower panel/top tow; numbers indicate percentage of total regardless of GFP^+ or GFP^-). Gated on the GFP^+ cell population (R1), cells double positive for MHC class II and CD11c were evaluated for gene expression (lower panel/bottom row; numbers indicate percentage of GFP^+ cells). (**b**) CFSE-labeled, BM-derived DCs at various doses were injected into the colorectal mucosa, and ILN cells were recovered 5 days later and pooled from two mice. $CFSE^+$ cells were analyzed by flow cytometry. (**c**) BM-derived DCs were transfected with AdOVA overnight and injected into the colorectal mucosa. Five days after immunization, ILN cells were cultured without peptide stimulation for 3 days. Specific killings of ILN effector cells on EL-4/SIINFEKL target cells at indicated ratios (E:T) were assayed by Cr release assay. The results are representative of at least two independent experiments. Ad, adenovirus; DC, dendritic cell; ILN, iliac lymph node; AdGFP, Ad expressing green fluorescent protein; CFSE, carboxyfluorescein diacetate succinidyl ester; LP, lamina propria; PFU, plaque-forming unit; MHC, major histocompatibility complex; BM, bone marrow; AdOVA, Ad expressing ovalbumin.

to clear virus-infected cells.[27] Ad vectors have been shown to be capable of activating DCs and stimulating T cells to become cytotoxic effector cells and produce immune factors such as IFN-γ.[28] To determine whether Ad vector immunization by ICR administration can induce antigen-specific immune responses, ILN cells, colorectal LP lymphocytes (LPLs), and splenocytes were recovered after immunization with AdOVA. OVA-specific IFN-γ-secreting cells were detected in all the three tissues of immunized animals at days 4 and 6 (**Figure 3a**). At a later time, day 14, IFN-γ-secreting cells in ILNs became undetectable, in contrast to IFN-γ-secreting cells in the colorectum and spleen, which were increased. Thus, Ad vector immunization by ICR administration effectively induced cellular immune responses in the large intestine. We found that $CD8^+$ T cells were the primary cells that produce IFN-γ by this immunization protocol (**Figure 3b**) and as tested in the secondary lymphoid organs, the cells exhibited cytotoxic activities (**Figure 3c**) that were primarily $CD8^+$ T-cell-mediated (**Figure 3d**).

As Ad vectors are effective in inducing antibody responses,[29] we next measured antigen-specific antibodies after AdOVA ICR. Significant levels of OVA-specific mucosal IgA antibodies were detected in the homogenates of colorectal tissue (**Table 1**). Also, serum OVA-specific IgG2a was present at levels greater than IgG1 (**Table 1**). Thus, Ad vector immunization by ICR administration can effectively induce both antigen-specific cellular and humoral immune responses at the large intestinal mucosa.

Vaccination at the large intestine against local virus challenge

We next addressed the relevant question as to whether Ad immunization via the large intestinal mucosa can provide effective protection against infection in the large intestine. We evaluated the effectiveness of this novel immunization strategy in comparison with nasal (intranasal, IN) or systemic (subcutaneous, SC) immunization. Doses for ICR and IN were determined on the basis of equivalent antigen expression levels in the corresponding local tissue as assessed using transfer of AdLuc. We found that 2×10^9 PFU (plaque-forming unit) of Ad given ICR

Table 1 Antigen-specific antibodies induced 3 weeks after ICR immunization with adenovirus (AdOVA)

	Antibody titers (log10)		
Group	Serum IgG2a[a**]	Serum IgG1[a**]	Colon IgA[b]
Immunized	2.94±0.09***	2.24±0.14***	2.92±0.06***
Unimmunized	0.48±0.17	0.53±0.51	0.88±0.42

AdOVA, Ad expressing ovalbumin; ICR, intracolorectal.
[a]Inverse dilution geometric mean±s.d. of IgG subclass from serum. N=3 per group.
[b]Inverse dilution geometric mean±s.d. of IgA from colon homogenates. N=3 per group.
**P < 0.01, significance between IgG1 and IgG2a.
***P < 0.001, significance between immunized and unimmunized.

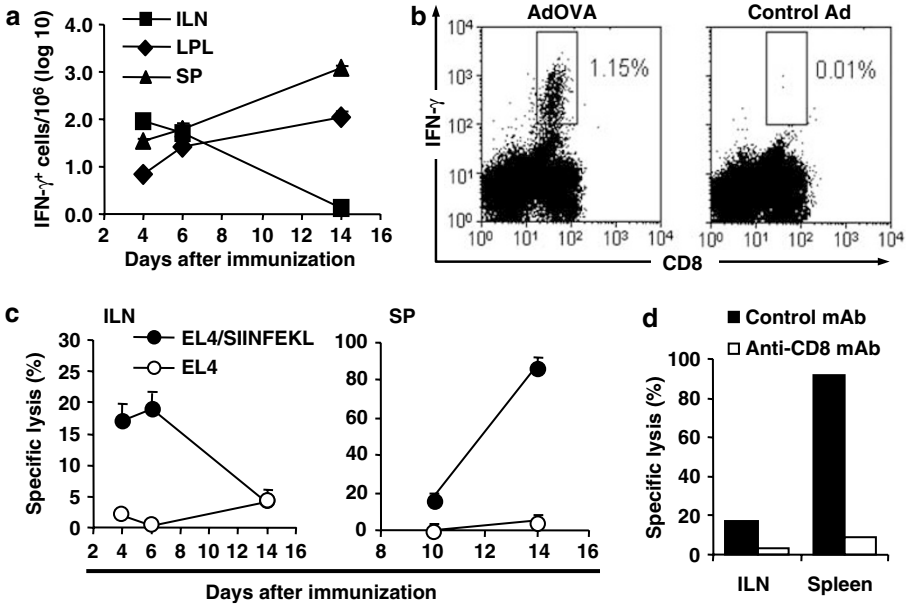

Figure 3 ICR immunization with Ad induces CD8-mediated antigen-specific cellular immune response. (**a**) At days 4, 6, and 14 after ICR immunization with 2×10^9 PFU AdOVA, colorectal LPLs, splenocytes, and ILN cells were isolated and examined by ELISPOT for IFN-γ secretion during 24 h of incubation in the presence of OVA SIINFEKL peptides. A control vector without OVA gene did not generate specific responses (data not shown). (**b**) Splenocytes isolated from immunized mice (left panel) or control vector immunized mice (right panel) 14 days after immunization were stained for intracellular IFN-γ production after 4 h of *in vitro* stimulation with SIINFEKL in the presence of brefeldin A. (**c**) After AdOVA ICR immunization with 2×10^9 PFU AdOVA, ILN cells were isolated at days 4, 6, and 14, and splenocytes were isolated at days 10 and 14. ILN cells were cultured without restimulation (for primary CTL response), whereas splenocytes were restimulated with EL-4/SIINFEKL (for recall response; no response tested without restimulation). Three days later, Cr release assay was conducted on EL-4/SIINFEKL as targets. (**d**) The assay was conducted as described in (**c**). Anti-CD8 blocking mAbs or isotype control IgG2a were added to T cells before the addition of target cells in the Cr release assay. The results represent one of two independent experiments and E:T ratio at 90:1 is shown. ICR, intracolorectal; Ad, adenovirus; PFU, plaque-forming unit; AdOVA, Ad expressing ovalbumin; LPL, lamina propria lymphocyte; ILN, iliac lymph node; IFN-γ, interferon-γ; OVA, ovalbumin; CTL, cytotoxic T lymphocyte; mAb, monoclonal antibody.

and 5×10^8 PFU IN resulted in comparable levels of transgene expression in the colorectum and paired lungs, respectively, at 48 h after administration (**Supplementary Figure S1** online). The higher dose needed when administered ICR than IN may be partially explained through delivery loss such as absorption by feces, leaks, and so on. For SC, there is no equivalent method to measure gene expression; the dose was set at 5×10^8 PFU, which is not less than the IN dose, but not as high as the ICR dose. This dose is one that is known to induce a strong systemic immune response in the rodent.

Firstly, compared to IN and SC routes of immunization, ICR immunization with AdOVA induced antigen-specific IFN-γ-secreting colorectal LPLs at levels eightfold higher and a colorectal IgA content that is threefold higher (**Figure 4a**). When mice were challenged with recombinant vaccinia virus expressing OVA (VV-OVA) following AdOVA ICR immunization, no replicable viruses were detected in either the large intestine or ovaries (indicating no dissemination from the mucosa) (**Figure 4b**, left panel). In contrast, both SC and IN immunization with AdOVA showed only marginal protection, with significant virus dissemination (**Figure 4b**, left panel).

To further assess the Ad ICR immunization strategy, we established a murine lethal rectal challenge model with HSV-2 (**Supplementary Figure S2** online). HSV-2 is a highly pathogenic virus, and infection with HSV-2 has often been associated with human immunodeficiency virus transmission as a cofactor.[30–32] After ICR immunization with Ad encoding HSV glycoprotein B (AdgB), a similar level of antigen-specific IFN-γ-secreting $CD8^+$ T cells was elicited as we had previously seen for ICR immunization with AdOVA, representing an approximately ninefold increase compared to IN or SC immunization (data not shown). We found that ICR immunized animals showed almost full protection from lethal challenge with HSV-2, as assessed by pathology score, virus shedding in the rectal lumen and mortality (**Figure 5a** and **Supplementary Figure S3** online). However, IN and SC immunized animals were significantly less protected (**Figure 5a**). It is worth noting that both $CD8^+$ T cells, IFN-γ, and a Th1 type response are essential for such mucosal protection because mice deficient in CD8 ($CD8^{-/-}$), IFN-γ (IFN-$\gamma^{-/-}$), or interleukin (IL)-12 (IL-$12^{-/-}$) were almost completely unprotected (**Supplementary Figure S4** online). We also found that it is important to include priming immunization at the large intestine to induce full colorectal protection, as a prime–boost protocol at the nose (IN/IN) was still found to be not as effective as ICR priming with IN boost (**Supplementary Figure S5A** online). These data indicated that ICR immunization with Ad can induce strong mucosal immunity and local protection against infectious agents.

Vaccination at the large intestine against vaginal virus challenge

Finally, we asked whether this Ad large intestinal immunization strategy can provide effective protection against viral infection at the vaginal mucosa, as there are limitations in immunizing via the genital tract. We found that delivering Ad directly to

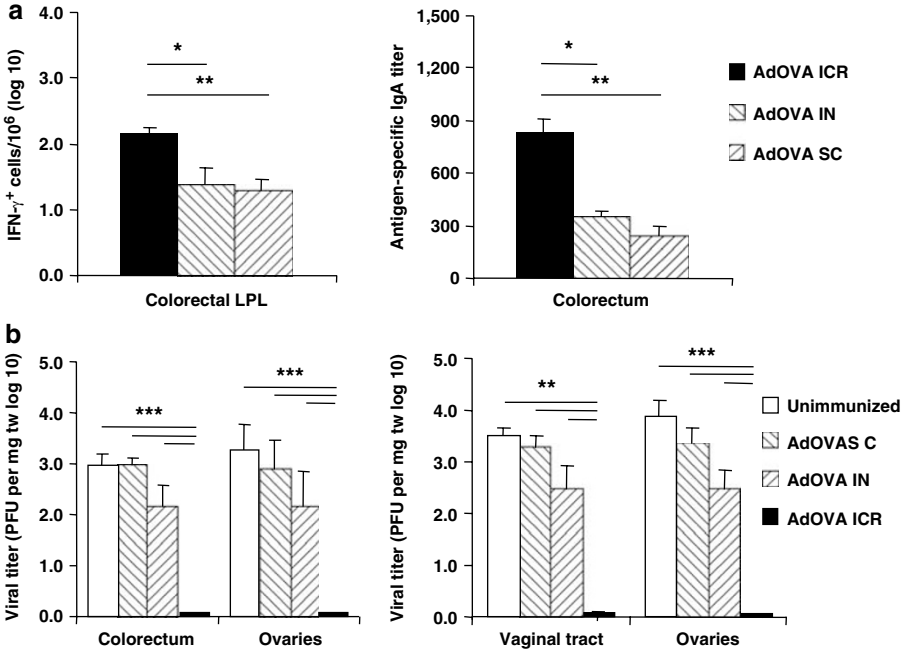

Figure 4 ICR immunization induces effective local mucosal immunity at both colorectal and vaginal mucosa. (**a**) Mice were immunized with AdOVA by ICR (2×10^9 PFU), IN (5×10^8 PFU), or SC (5×10^8 PFU). Both OVA-specific IFN-γ-secreting LPLs (left panel) and local IgA production in colorectal homogenates (right panel) were compared after 2 weeks. (**b**) Animals were challenged rectally (left panel) with 5×10^7 PFU or vaginally (right panel) with 2×10^7 PFU of VV-OVA. Six days later, local tissue and paired ovaries were examined for infectious viral particles by plaque assays. Results represent one of two independent experiments and are expressed as mean±s.e.m. from three mice in each group. *, **, and *** indicate $P<0.05$, 0.01, and 0.001, respectively. AdOVA, Ad expressing ovalbumin; ICR, intracolorectal; PFU, plaque-forming unit; IN, intranasal; SC, subcutaneous; IFN-γ, interferon-γ; LPL, lamina propria lymphocyte; VV-OVA, vaccinia virus expressing OVA.

the vaginal mucosa (intravaginal, IVAG) could not lead to comparable antigen expression unless animals were pretreated with Depo-Provera (**Supplementary Figure 1** online). However, no protection against either vaginal or rectal challenge with HSV-2 was seen after IVAG administration of AdgB, regardless of earlier Depo-Provera treatment (**Supplementary Figure 6** online). In contrast, AdOVA ICR immunized mice had nearly undetectable levels of infectious vaccinia virus recovered from either vaginal tissue or ovaries after vaginal challenge with VV-OVA (**Figure 4b**, right panel). After SC or IN immunization with AdOVA, however, no animals were protected from this vaginal challenge (**Figure 4b**, right panel). In the herpes challenge model, Ad ICR immunized mice were effectively protected from vaginal challenge with a lethal dose of HSV-2, whereas IN and SC immunized animals were marginally protected (**Figure 5b**). In addition, as described above, it is important to have the primary immunization at the large intestine to induce full vaginal protection, as IN/IN prime–boost immunization was still found to be not as effective as ICR/IN prime–boost immunization (**Supplementary Figure S5b** online). Thus, our results suggest that primary vaccination at the large intestinal mucosa represents a more effective way to achieve vaginal protection in addition to rectal protection against infectious viral challenge.

DISCUSSION

In addition to transmission through the genitourinary tract, rectal transmission is a major mode of infection adding to the development of widespread sexually transmitted infections,[33,34] in apparent pandemic proportions for decades. Developing a vaccination strategy that can effectively control either route of infection has been challenging. The development of protective immunity against these infections requires an effective approach with a powerful antigen delivery system and an appropriate route for vaccination. Using a highly pathogenic HSV-2 lethal challenge model, we show here that primary immunization at the large intestinal mucosa with Ad expressing glycoprotein B induced almost complete protection against subsequent rectal challenge of HSV-2. Additionally, such immunization conferred efficient protection at the vaginal mucosa against HSV-2 challenge. We reasoned that using viral vectors as vaccines would be suitable for induction of antiviral infection immunity because these vectors target the same specific arms of the immune system as do viruses in nature. However, more importantly, priming at the large intestinal mucosa should be advantageous in protecting against sexually transmitted infections because the most effective protection is induced at the site of immunization and the neighboring vaginal mucosa.

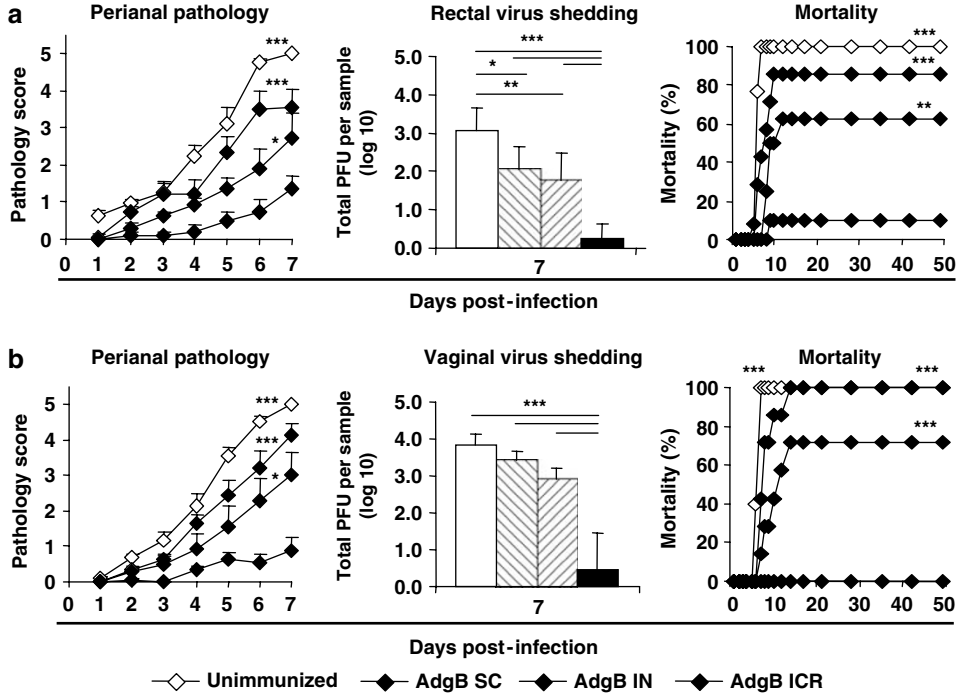

Figure 5 ICR immunization provides comparably effective mucosal protection against HSV-2 transmitted through either rectal or vaginal mucosa. Mice were immunized by ICR (2×10^9 PFU), IN (5×10^8 PFU), SC (5×10^8 PFU), or unimmunized before HSV-2 challenge. Pathology score (left panels), virus replication (middle panels), and mortality (right panels) were examined after either rectal or vaginal challenge. (**a**) Mice were challenged rectally with 2×10^6 PFU of HSV-2 3 weeks after immunization by ICR ($N=10$), IN ($N=8$), SC ($N=7$), or without immunization ($N=13$). No differences in pathology score or viral shedding were found between IN and SC, but both groups were statistically different from the unimmunized group (left panel, $P<0.05$ and middle panel). IN differed from unimmunized in mortality (right panel, $P<0.01$). (**b**) Mice were challenged vaginally with 2×10^5 PFU of HSV-2 3 weeks after immunization by ICR ($N=9$), IN ($N=7$), SC ($N=7$), or without immunization ($N=10$). A significant difference in mortality was also found between IN and SC in vaccinated animals (right panel, $P<0.05$), but not in viral shedding or pathology score. IN, but not SC, was statistically different from unimmunized in both pathology score (left panel, $P<0.01$) and mortality (right panel, $P<0.05$). Asterisks of pathology score and mortality indicate a statistical difference of the group in comparison with the ICR immunization group. *, **, and *** indicate $P<0.05$, 0.01, and 0.001, respectively. ICR, intracolorectal; HSV-2, herpes simplex virus type 2; PFU, plaque-forming unit; IN, intranasal; SC, subcutaneous.

We showed effective gene transfer to the epithelium and LP of the large intestine, where the transgene was highly expressed for 3 days. This should give resident DCs in the large intestine sufficient time to acquire expressed antigens. Adenoviral vectors have been shown to be capable of activating DCs and stimulating T cells to become cytotoxic effector cells and produce cytokines such as IFN-γ on antigen exposure.[28] We found that MHC II$^+$CD11c$^+$ DCs were positive for Ad-expressed antigens in the LP. Although these cells accounted for only a small proportion of total antigen-expressing cells in the large intestinal mucosa, almost all antigen-expressing cells in the ILNs were DCs. It is likely that only DCs migrated from the mucosal tissue to the draining LNs for induction of antigen-specific immune responses. Alternatively, as effective gene transfer was also found in the epithelium of the colorectal mucosa, immunogenicity in our system could be due to IECs directly presenting antigen to effector cells. Mayer and Shlien[35] initially showed that IECs express MHC class II and can take up and present antigens to T cells. More recently, a polarized epithelial cell line transfected to express HLA-DR was shown to present soluble antigen to restricted CD4 T cells.[36] The contact of IECs with underlying immune cells in the LP may be facilitated owing to the presence of fenestrations or pores, 0.5–5 μm in diameter, in the basement membrane immediately below the intestinal epithelium, allowing immune cells to migrate between compartments and also IECs to project basal protrusions into the LP.[37,38] Together, these studies raise important questions concerning how antigen is transferred to effector cells in the intestinal mucosa.

There were opposing changes over time in the frequencies of functional antigen-specific T cells found in different tissues, a decrease in the ILNs but an increase in both spleen and LP, and kinetic changes in CTL presence are mirrored by IFN-γ ELISPOT assessments. These discrepant kinetics might be due to the fact that antigen-specific CD8$^+$ T cells emigrate from the ILNs after clonal expansion[39,40] and ultimately return to the local mucosal tissues via the bloodstream.[18] Thus, ILNs might represent the major induction site for the large intestinal mucosa, where priming of antigen-specific CD8$^+$ T cells occurs during days 4–6 after immunization, and this immunization can induce antigen-specific T cells that are able to home to the site of immunization.

After Ad ICR immunization, we detected a significant production of antigen-specific colorectal IgA antibodies, in keeping with previous studies.[29,41] It is worth noting that mice protected from lethal outcome by ICR immunization demonstrated virus replication and local inflammation after HSV challenge, indicating that mucosal cells were infected immediately post-challenge. As protection was completely abrogated in CD8 and IFN-γ knockout mice, it appears that T-cell immunity plays an essential role in providing protection at the large intestinal mucosa and presumably at the vaginal mucosa as well, in addition to humoral immunity having an early role in immunological resistance.[42]

We noticed that IVAG immunization with Ad did not generate adequate protective vaginal immunity. Our previous studies[43] showed protection against vaginal challenge after IVAG immunization, but with a 20-fold lower dose of HSV-2 at challenge; however, evaluation was not performed at higher doses as used here. Moreover, others have shown that the vaginal mucosa was not effective in antigen transfer and in inducing vaginal antibody responses.[44–46] This may be due to the lack of inductive structures in the vaginal mucosa or difficulty with antigen penetration into the vaginal epithelium. However, our results showed that large intestine immunization provided effective vaginal protection against HSV-2. As ILNs drain both the rectum and female urogenital mucosae,[8] immunization at the large intestine also resulted in priming of T cells derived from vaginal mucosa through the lymphatic system. These primed cells may be eventually destined to home to vaginal mucosa. Thus, vaginal mucosa probably acts more as an effector site than as an inductive site, and large intestinal immunization with Ad is a promising strategy for vaginal protection against viral infection.

It was previously shown that immunization through the nose confers immunity and protection at the vaginal tissue against a low-dose herpes challenge.[14,47] However, we show here that mucosal immunization by the IN route is only marginally more effective than systemic immunization, when challenged with a high lethal dose of infectious HSV-2 virus, even though measurable immune responses can be detected. Even after using higher doses of AdgB for IN immunization, the animals showed no improvement in protection (data not shown). We reasoned that the use of a higher virus dose for lethal challenge in this study accounts for the differences seen from our earlier findings. In fact, we found that IN immunization, similar to SC, induced substantially lower ILN responses compared to ICR. This may be due to the sub-compartmentalized immunologic networks within the common mucosal immunologic system as suggested.[48] It is probable that entry of lymphocytes into the large intestine and vagina requires a specific homing mechanism that is restricted to those cells that are activated through specific mucosal sub-compartments. Therefore, the insufficient accumulation of activated local mucosal lymphocytes plus local mucosal antibodies after IN or SC immunization could result in the poor effector function seen at either colorectal or vaginal mucosae.

ICR vaccination may be considered as "regional" or "local" immunization for both the large intestine and vaginal tracts, as they are geographically neighboring within the same sub-compartmentalized immune network in the mouse and a similar genitorectal lymphatic system is found in humans.[49] In previous studies mainly using cholera toxin for vaccines, limited effects at the vaginal mucosa were shown not only in mice [13,50–52] and monkeys[12,51] but also in humans.[10,11,53] In addition, effects at other mucosal sites such as lung, nose, and saliva were similarly minimal.[52] These studies indicate that mucosal immunization with cholera toxin and protein vaccines provides immunity primarily at the site of immunization. Thus, both animal and human studies have encountered difficulties in generating vaginal immunity through vaccination by the rectal route. However, our data shown here demonstrate in principle that ICR vaccination with Ad-expressed antigen gene leads

to beneficial local protection in the mouse. We reasoned that the effective vaginal protection induced by rectal immunization was due to the use of Ad as a powerful system of antigen delivery and immune activation, compared to some others. Ads have no significant side effects if administered at appropriate low doses,[54,55] and their mucosal epithelial tropism maximizes immunological consequences at the intestinal mucosa.[56–59] Comparison studies by Shiver *et al.*[60] showed that although Ad and poxvirus were almost equally effective when used for boosting, priming with poxvirus was ineffective, as opposed to Ad, regardless of boosting agents. Interestingly, Ad efficiently promotes human DC maturation and production of inflammatory cytokines,[61,62] whereas poxvirus may inhibit DC maturation and antigen presentation for T-cell priming.[63–65] The differences between two vectors in DC activation and antigen presentation may contribute to the difference in the results of our studies and the monkey trials as well as recently published clinical outcomes.[66,67] Similarly, in spite of its strong immune stimulation, cholera toxin as a vaccine adjuvant has been shown to be a potent inhibitor of both mitogen- and antigen-induced T-cell activation[68,69] and mediate apoptosis of CTL.[70,71] Whether this activity was responsible for the ineffectiveness of vaginal immunity induced through gut immunization remains to be determined. Therefore, as suggested earlier, choosing the right venue and a suitable and potent antigen delivery and immune activation system for vaccination is extremely important.[53] As the nature of the vaccine will most likely influence the outcome, our strategy of using Ad to deliver antigen genes to the colorectal tissue may provide benefits that are unique in overcoming apparent limitations of the mucosal immune system and provide protection to the vaginal mucosa, suggesting that testing this concept in humans may be worthwhile.

Although the details of this experimental immunization with Ad at the colorectal mucosa remain to be determined for direct translation to humans, using suppositories to administer vaccines would seem to be a logical progression from our studies. Potentially, applying ICR vaccination has many advantages in humans over other vaccination routes: (1) it is less invasive and highly applicable using suppository form; (2) it avoids menstrual cycles, which affect vaginal delivery of vaccines; (3) no vital organs such as the respiratory system and brain are jeopardized, as sometimes happens following IN immunization; (4) it bypasses the small intestine where tolerance may be induced after oral delivery[72,73]; and (5) if proven to be effective in males, ICR vaccination using Ad or similar viral delivery systems may benefit the other half of the population in the world.

In conclusion, our results indicate that large intestine immunization with Ad represents an effective vaccination strategy against viral infections transmitted through the rectal tissue, and at least in the mouse, provides functional protection at the vaginal mucosa. Using an animal model of genital HSV-2 infection, which is relevant to human sexually transmitted infections, we provide an innovative vaccination strategy for humans to defend against HSV exposure and could also be potentially used to immunize against human immunodeficiency virus.

METHODS

Animals, cells, and viruses. Female C57BL/6 mice (6–8 weeks) were purchased from Harlan (Indianapolis, IN) and housed in pathogen-free conditions at the Central Animal Facility at McMaster University. IFN-$\gamma^{-/-}$ mice[74] were purchased from the Jackson Laboratory (Bar Harbor, ME). $\beta 2m^{-/-}$/$CD8^{-/-}$ mice were kindly provided by Dr J Magram (Hoffmann-La Roche, Nutley, NJ). All animal experiments were approved by the Animal Ethics Research Board of McMaster University and conducted according to the regulations of the Canadian Council on Animal Care.

EL-4, CV-1, and Vero cell lines (American Type Culture Collection (ATCC), Manassas, VA, USA) were cultured in complete RMPI medium. Bone marrow cells were cultured in the presence of 10 ng ml^{-1} of granulocyte macrophage colony-stimulating factor and 10 ng ml^{-1} of interleukin-4 for generation of DCs as described previously.[75] At day 6, cells were infected with Ad 1 day before administration. VV-OVA was recovered from tissues 6 days after challenge by homogenization.[9] HSV-2 was recovered from the vaginal tract[76] or the rectal lumen by swabbing. Plaque-forming assays were performed with CV-1 or Vero cells for 48 h, followed by counterstaining with 5% w/v crystal violet. Virus presence was expressed as total PFUs ($\log_{10}$ PFUs)/organ or fluid sample.

Replication-deficient Ad expressing chicken OVA was constructed as described below. Total RNA was isolated from fresh oviduct tissue of *Gallus gallus* (chicken), and the first strand was synthesized with Superscript RT and oligo dT primers (Invitrogen Corp, Carlsbad, CA). PCR was performed with Vent Polymerase (Invitrogen Corp) using primers (forward: 5′-GTTCACCATGGGCTCCATCGGCGCAGCAAGCATGG-3′; and reverse: 5′-CTACAGTGCTCTGGGTCTTGTTGGAAGGGAC-3′). *Sma*I (T4 ligase, New England Biolabs, Beverly, MA) was used to digest pDC104. Purified OVA PCR fragment was cloned into a vector as pDC104ova6. The pDC104ova6 and pBHG10-CRE plasmids were co-transfected into 293 cells, as described previously,[77] and the product was confirmed with Southern and western blot, respectively. The $OVA_{257–264}$ epitope SIINFEKL for H2-K^b was confirmed by sequence analysis. Immunogenicity of AdOVA was demonstrated by the ability to induce significant primary CTL responses in the popliteal LN following footpad injection. AdOVA were propagated in 293 cells and purified by cesium chloride gradient centrifugation.[77] Recombinant AdLuc, Adβ-gal, AdGFP (huAd5), and AdBHG contain firefly luciferase, *Escherichia coli* β-gal, GFP, and no heterologous gene, respectively.[78] All virus stocks were aliquoted and stored at −70 °C before use.

Administration route and animal models. Mice were anesthetized during the following procedures. For ICR administration, virus in 100 µl phosphate-buffered saline was pipetted into the colorectum shortly after 50% ethanol pretreatment.[79] The anal opening was sealed for 4–5 h with Dermabond (Ethicon, Somerville, NJ) to prevent leakage. For IVAG administration, mice were given subcutaneously 2 mg Depo-Provera (medroxyprogesterone acetate; Pharmacia & Upjohn, Kalamazoo, MI) 5 days before immunization to thin the lining of the vaginal wall.[76, 80] Virus (in 10 µl aliquots) was pipetted into the lumen followed by Dermabond at the vaginal opening. For intermucosal administration, DCs were injected into the rectal mucosal from the anus using a 0.3 ml insulin syringe with 30G×3/8″ needle (Terumo, Somerset, NJ). Two animal models were used: an OVA model using AdOVA for immunization and recombinant VV-OVA for challenge,[9] and an HSV model using AdgB for immunization and HSV-2 for challenge. After HSV challenge, perianal inflammation and hind limb paralysis were clinically scored to evaluate infection status (**Table 2**).

Cell isolation and tissue homogenates. Colorectum was removed and fragmented into a few pieces and stirred for 30 min at 37 °C with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution containing 10% fetal bovine serum, 15 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 5 mM EDTA, 0.014% w/v dithiothreitol, and 100 µg ml^{-1} gentamycin to isolate epithelial cells and intraepithelial lymphocytes, which were then discarded by filtering. The remaining fragments

Table 2 Pathology score to assess severity of rectal HSV-2 infection in mice

Point	Manifestations	Location	Location
		Rectal challenge	Vaginal challenge
0	No sign of disease	Anal opening	External vagina
1	Redness	Anal/perianal area	External vaginal area
2	Swelling	Perianal	External vaginal area
3	Hair loss, severe redness	Perineum	Perineum
4	Ulcerative lesion	Perianal/perineum	External vaginal area/perineum
5[a]	Paralysis (or moribund)	Hind limbs	Hind limbs

[a]Experimental end point.

were further cut into smaller pieces and incubated with complete RPMI 1640 medium containing 250 U ml^{-1} collagenase VIII (Sigma Chemical, Somerville, NJ). LPLs were purified from liberated cells by a 30%/75% gradient of Percoll (Sigma Chemical). To prepare tissue homogenates, tissues were removed and homogenized in phosphate-buffered saline with a homogenizer (POLYTRON, Kinematica, Cincinnati, OH).

β-gal staining and luciferase assay. Fresh colorectal tissue was placed in tissue fixative (2% formaldehyde, 0.05% glutaradehyde, 1.6 sodium cacodylate, and 0.025% calcium chloride) for 30 min on ice and microwaved for 5–8 s at ~45 °C and immediately put back on ice. The samples were overlain with 1 mg ml^{-1} X-gal (5-bromo-4-chloro-3-indolyl β-D-galactoside, Sigma Chemical) at ~37 °C overnight in staining buffer. Samples were washed with phosphate-buffered saline, fixed in 10% formalin for 48 h, paraffin-embedded, and cut into 4–5 μm-sections and counterstained with eosin and nuclear fast red. Cell-based blue staining clusters were enumerated under the microscope. Luciferase activity was measured using the Luciferase Assay System Kit (Promega, Madison, WI) according to the manufacturer's instructions.

Flow cytometry and ^{51}Cr release assay. Antibodies were purchased from BD Biosciences Pharmingen (Mississauga, ON). Splenocytes were stimulated for 4–6 h at 37 °C with 2 μM SIINFEKL (Sigma Genosys, Woodlands, TX) in the presence of brefeldin A. Following surface staining, cells were fixed and permeabilized before intracellular cytokine staining. Samples were analyzed with a FACScan (Becton Dickinson, Sunnyvale, CA) and WinMDI 2.8 software (The Scripps Research Institute, La Jolla, CA). Dead cells were excluded using forward and side scatter measurements. For Cr release assay, EL-4 cells pulsed with 1 μM SIINFEKL were labeled with sodium chromate as target cells and incubated for 4 h at 37 °C with CD8$^+$ effectors. Supernatants were harvested and measured for radioactivity.[75] To block CD8-mediated CTLs, 10 μl of anti-CD8 blocking antibody (53-6.72, ATCC) or isoytpe control IgG2a antibody was added to T cells 30 min before the assay.

ELISPOT and ELISA. Mouse IFN-γ ELISpot kit (R&D Systems, Minneapolis, MN) was used according to the manufacturer's directions. In short, freshly isolated cells were seeded in an IFN-γ antibody-precoated 96-well filter plate and cultured with 1 μM SIINFEKL at 37 °C for 24 h. Captured IFN-γ was assayed by using biotinylated IFN-γ detection antibodies in conjunction with Strep-AP/BCIP-NBT. Spot numbers twofold higher than the background were considered as positive. For enzyme-linked immunosorbent assay, colorectal homogenates or sera were serially diluted and incubated in OVA protein-precoated plates for 2 h. The plates were then incubated with alkaline phosphatase-conjugated anti-mouse IgA, IgG1, or IgG2a detection antibodies for 2 h, and color was developed by incubating *p*-nitrophenyl phosphate for 30 min in darkness before reading optical density. Antibody titers were derived from the inverse dilution at which the sample yielded an optical density twice that of the background of control specimens from non-immunized mice.

Statistical analysis. Comparisons between groups were analyzed by Student's *t*-test. Comparisons among means of more than two groups were determined by one-way analysis of variance *post hoc* with Bonferroni correction. Analyses were performed with SPSS for Windows (SPSS, Chicago, IL). *P*-values < 0.05 were considered statistically significant.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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DISCLOSURE

The authors declared no conflict of interest.

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