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A crucial role for plasmacytoid dendritic cells in antiviral protection by CpG ODN–based vaginal microbicide

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Topical microbicides represent a promising new approach to preventing HIV and other sexually transmitted infections. TLR agonists are ideal candidates for microbicides, as they trigger a multitude of antiviral genes effective against a broad range of viruses. Although vaginal application of CpG oligodeoxynucleotides (ODNs) and poly I:C has been shown to protect mice from genital herpes infection, the mechanism by which these agents provide protection remains unclear. Here, we show that plasmacytoid DCs (pDCs) are required for CpG ODN–mediated protection against lethal vaginal challenge with herpes simplex virus type 2 (HSV-2). Moreover, we demonstrate that cells of both the hematoepoietic and stromal compartments must respond to CpG ODN via TLR9 and to type I IFNs through IFN-αβ receptor (IFN-αβR) for protection. Thus, crosstalk between pDCs and vaginal stromal cells provides for optimal microbicide efficacy. Our results imply that temporally and spatially controlled targeting of CpG ODN to pDCs and epithelial cells can potentially maximize their effectiveness as microbicides while minimizing the associated inflammatory responses.

Introduction

Sexually transmitted infections (STIs) such as HIV-1 and herpes simplex virus type 2 (HSV-2) represent a major public health concern. Currently, there are no effective vaccines against STI causative agents. In the absence of effective vaccines, the development of a safe and effective topical microbicide to prevent the sexual transmission of STI could play a major role in world-wide reduction of STI transmission and potentially save millions of lives lost to HIV-1 (1). A promising topical microbial approach is the use of agents to trigger a nonspecific broad antiviral state in the genital mucosa prior to encounter with sexually transmittable viruses. In this regard, the TLR agonists represent an ideal candidate that can induce an antiviral state. The innate immune system has evolved to recognize pathogens that enter through a variety of mucosal surfaces and to induce a rapid cascade of events leading to antimicrobial defense (2). Various TLRs are expressed by a variety of cell types present in the genital mucosa (3, 4). Agonists of TLR9 (CpG oligodeoxynucleotides [ODNs]) (5–7) and TLR3 (poly I:C) (4) have been used successfully to provide complete protection against HSV-2 in the mouse model of genital herpes.

Intravaginal (ivag) inoculation of CpG ODN has been shown to induce several changes in the vaginal mucosa. First, CpG ODN induces active proliferation of the genital epithelium (5). Second, a variety of inflammatory cells have been shown to accumulate in vaginal mucosa, including NK cells, CD11b+ F4/80+ myeloid cells, and CD11b+ CD11c+ DCs (5, 8). There are conflicting observations regarding the roles of T cells and IFN-γ. A previous study has shown that the protection requires IFN-γ, IL-12, IL-18, and T cells (6) while others have demonstrated that complete protection is conferred by CpG ODN in the absence of any lymphocytes (RAG2−/−γc−/−) (9) or IFN-γ (5). However, the precise cellular and molecular mechanism by which CpG ODN provides protection against HSV-2 challenge is unclear, which may hinder the clinical application of CpG ODN as a microbicide.

In this study, we aim to address several key questions with respect to the mechanism of CpG ODN–mediated antiviral protection. First, what are the critical cell types that recognize CpG ODN and mediate its ability to induce an antiviral state, keratinocytes or hematopoietic cells? The first cells to come in contact with CpG ODN are the keratinocytes lining the vaginal cavity. The importance of direct recognition of CpG ODN by the vaginal keratinocytes in vivo is unknown. In addition, it is not clear whether recognition of CpG ODN by hematopoietic cells is required for protection. Second, what is the significance of the recruitment of various DC types to the vaginal mucosa in providing protection against HSV-2? Third, are type I IFNs, the most potent set of antiviral cytokines, required for CpG ODN–mediated protection against HSV-2? Fourth, if type I IFNs are involved in generating an antiviral state in the host, do they act on the keratinocytes, which are the primary target of HSV-2 infection (10), or do they act on hematopoietic cells and induce their antiviral activity?

To address these issues, using irradiation-induced BM chimeric mice, we examined the importance of various cell types and their responsiveness to CpG ODN in conferring an antiviral state in the host. Further, we demonstrate that type I IFNs, but not type II IFN or IL-12, play a critical role in CpG ODN–mediated protection and that both stromal and hematopoietic compartments must respond to type I IFNs for this process. We show that depletion of individual lymphocyte subsets (CD4, CD8, or NK cells) prior to CpG ODN treatment does not compromise protection. Finally, we demonstrate that plasmacytoid DCs (pDCs) are recruited to the site of CpG ODN inoculation and that they play a critical role in CpG ODN–mediated protection. These results imply that pDCs might be exploited to confer a general state of antiviral innate immunity against a variety of STIs within the genital mucosa.

Nonstandard abbreviations used: HSV-2, herpes simplex virus type 2; IFN-αβR, IFN-αβ receptor; ivag, intravaginally; ODN, oligodeoxynucleotide; pDC, plasmacytoid DC; STI, sexually transmitted infection.

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Results

TLR9 and MyD88 are required for antiviral protection conferred by vaginal CpG ODN. To examine the importance of TLR9 signaling in antiviral protection by CpG ODN, mice deficient for either TLR9 or MyD88 were inoculated ivag with CpG ODN and, 24 hours later, challenged with a lethal dose (10⁴ PFU) of HSV-2. As expected, both TLR9 and the obligate adapter MyD88 (11) were required to confer protection upon CpG ODN inoculation (Figure 1). Thus, TLR9−/− and MyD88−/− mice failed to control local viral replication (Figure 1C), consequently developing rapid pathology (Figure 1B) and succumbing to death (Figure 1A). As reported previously (5–7), WT mice were completely protected from challenge by pretreatment with CpG ODN. These data provided us with the basis from which to examine the importance of different cell types in conferring CpG ODN–induced innate protection against HSV-2.

Antiviral protection requires both hematopoietic and stromal cell responses to CpG ODN. It is known that cells found within the vaginal epithelial layer express TLR9 (3, 4). In addition, in purified vaginal keratinocytes, TLR9 was expressed at a low level, which was upregulated upon CpG ODN stimulation (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI28681DS1). To elucidate the cell types involved in the CpG ODN–mediated innate protection against HSV-2, 2 sets of irradiation-induced BM chimeras were generated. To examine the importance of CpG ODN recognition by the hematopoietic cells in providing protection, the BM from TLR9−/− mice was used to reconstitute irradiated WT mice (TLR9−/−→WT). Upon vaginal delivery of CpG ODN, these mice succumbed to viral infection and death, with similar kinetics seen in the TLR9−/− mice (Figure 2). These results indicate that hematopoietic cells must respond through their TLR9 in order to provide antiviral protection against HSV-2 following CpG ODN microbicidal treatment. Next, the role of CpG ODN recognition by the radioresistant stromal cells was examined by generating WT→TLR9−/− chimeras. Interestingly, these mice also succumbed to HSV-2–induced disease, albeit with delayed kinetics compared with the TLR9−/−→WT chimeric mice (Figure 2), indicating that stromal cell responsiveness to TLR9 is important in CpG ODN microbicidal activity. This delay correlated with reduced viral titers found at days 2 and 3 after infection in the vaginal washes of WT→TLR9−/− chimera compared with those of TLR9−/−→WT chimeric mice (P < 0.05, Figure 2C). Thus, these results indicate that both hematopoietic and stromal compartments must respond to CpG ODN in order to confer antiviral protection against HSV-2 challenge and that the effect was more significant when hematopoietic cells’ responsiveness to CpG ODN was ablated compared with that of the stromal cells.

pDCs are required for CpG ODN protection against HSV-2. It is known that a variety of cell types of the lymphoid and myeloid lineages are recruited to the vaginal mucosa following CpG ODN inoculation.
The type I IFNs are the best characterized antiviral cytokines capable of inhibiting replication of most viruses (14, 15). Our data on the variety of viral and synthetic stimuli, including CpG ODN (12, 13), revealed that CD4+ T cells or CD8+ T cells contribute minimally to protection against genital herpes (Figure 3). While there was a modest increase in the viral titers and pathology in mice depleted of NK cells following CpG ODN microbicide delivery, the majority of NK-depleted mice were still protected from viral challenge (Figure 3A). All of these depletion protocols resulted in more than 90% depletion of the targeted cell subset (Supplemental Figure 2).

Having ruled out the role of these lymphocytes in critically providing CpG ODN–mediated innate protection, we focused on the role of pDCs. The pDCs express TLR9 and respond to CpG ODN and are potent secretors of the antiviral cytokines type I IFNs (12, 13). First, we examined the presence of pDCs in the vaginal mucosa prior to and following CpG ODN treatment. pDCs were found in small numbers in the vaginal mucosa at steady state and were recruited rapidly following ivag CpG ODN delivery (Figure 4).

Next, to examine the importance of pDCs in the context of CpG ODN–based microbicide, mice were depleted of pDCs by Abs prior to and following inoculation of CpG ODN, and these mice were challenged with a lethal dose of HSV-2. Mice depleted of pDCs were remarkably susceptible to viral infection and disease (Figure 5). These mice harbored high viral titers in the vagina, even more so than the WT mice not treated with CpG ODN (Figure 5C), suggesting that pDCs present in WT mice are able to limit viral replication even in the absence of CpG ODN priming. Further, pDC-depleted mice suffered from disease almost comparable to that of non–CpG ODN–treated WT mice (Figure 5B). As a consequence, most of the pDC-depleted mice succumbed to death (Figure 5A) albeit with delayed kinetics compared with the TLR9−/− WT chimeric mice (Figure 2A). The fact that some pDC-depleted mice survived may reflect incomplete pDC depletion (>90% depletion; Supplemental Figure 2) or indicate that a hematopoietic, non-pDC cell type contributes to the residual protection against viral infection.

The importance of type I IFNs in CpG ODN–induced antiviral state. pDCs represent the most potent secretors of type I IFNs in response to a variety of viral and synthetic stimuli, including CpG ODN (12, 13). The type I IFNs are the best characterized antiviral cytokines capable of inhibiting replication of most viruses (14, 15). Our data on the requirement for pDCs in protection offered by the CpG ODN–based microbicide suggest the importance of type I IFNs. To examine this possibility, mice deficient in IFN-αβ receptor (IFN-αβR) were inoculated vaginally with CpG ODN and, 24 hours later, challenged with the lethal dose of HSV-2. This analysis showed that CpG ODN completely failed to provide protection from HSV-2 challenge in IFNαβR−/− mice (Figure 6). Thus, IFN-αβ is required to confer protection following vaginal CpG ODN treatment. In contrast, mice deficient in other key antiviral cytokines, such as IL-12 and IL-23 (IL-12p40−/− mice or IFN-γR−/− mice), were completely protected from HSV-2 challenge following CpG ODN treatment (Figures 7 and 8). Collectively, our data thus far suggest that pDCs are required to secrete type I IFNs at the site of infection and prevent replication of the virus in the vaginal epithelial cells via IFN-αβR, which is the main target of primary HSV-2 infection and replication (16).

Both stromal and hematopoietic cell responsiveness to type I IFNs are required for protection. To elucidate the importance of IFN responsiveness by the vaginal keratinocytes, we generated irradiation-induced...
chimeric mice that lack IFN-γR on the radioresistant stromal cells but retain intact IFN-γR expression on the hematopoietic cells (WT→IFNγR−/−). Upon vaginal treatment with CpG ODN, these mice were challenged with HSV-2, and disease progression was monitored. To our surprise, the lack of IFN-γR on stromal cells, including vaginal epithelial cells, did not severely compromise these mice from CpG ODN–mediated protection against HSV-2. Thus, improved survival (Figure 6A) and pathology (Figure 6B) were observed in the CpG ODN–treated WT→IFNγR−/− mice compared with the IFNγR−/− mice. Further, control of local viral replication remained intact when the hematopoietic cells, including pDCs, expressed IFN-γR (Figure 6C). These results led us to examine the importance of IFN responsiveness in the hematopoietic cells in CpG ODN–mediated protection. To address this issue, BM cells of the IFNγR−/− mice were used to reconstitute lethally irradiated WT mice (IFNγR−/−→WT). Upon reconstitution, these chimeric mice were treated with CpG ODN and challenged with HSV-2. IFN responsiveness by the hematopoietic cells was also found to be required for CpG ODN–mediated protection against HSV-2 (Figure 6). These data indicate that stromal cell responsiveness to type I IFNs was not sufficient and that BM-derived cells must also respond to IFN-γR in order for CpG ODN–based microbicide to provide antiviral protection. In fact, IFN responsiveness by the hematopoietic cells appeared to be slightly more important than that of the stromal cells in providing control of viral replication (Figure 6C) and subsequent protection from disease (Figure 6, A and B).

Discussion

The innate immune system is designed to detect a variety of pathogens through multiple pattern recognition receptors and to induce optimal sets of antiviral or antibacterial genes to provide immediate defense against invading pathogens (17, 18). By engaging the appropriate TLRs, a broad antiviral state can be achieved in the host. In this study, we dissected the mechanisms by which the vaginal inoculation of CpG ODN provides protection against subsequent challenge by HSV-2. We showed that CpG ODN responsiveness via TLR9 is required by both the hematopoietic and stromal compartments for protection. We demonstrated that pDCs, but not NK cells, CD4 T cells, or CD8 T cells, are required for CpG ODN to protect the host from HSV-2. Further, type I IFNs were required for protection. The type I IFNs induced as a result of vaginal CpG ODN inoculation must act on both the stromal and hematopoietic compartments to suppress viral replication and prevent disease. The mice lacking IFN-γR from the hematopoietic cells had

Figure 5

pDCs are critical in CpG ODN–mediated protection from genital HSV-2 challenge. WT mice were treated with anti–mPDCA-1 or isotype control on −1 and 1 day of CpG ODN treatment and were challenged ivag with 10⁴ PFU of HSV-2 24 hours following CpG ODN inoculation. (A) Survival rates, (B) genital mean pathology scores, and (C) virus titers in vaginal washes of mice were monitored. Values represent mean ± SEM (n = 5/group). Results are representative of 2 separate experiments. *P < 0.05 between PBS-treated (open squares) and pDC-depleted, CpG ODN–treated (open triangles) groups.

Figure 6

Responsiveness to type I IFNs is required in both the stromal and hematopoietic cells for complete protection from lethal challenge of genital HSV-2 following CpG ODN treatment. (A) Survival rates, (B) genital mean pathology scores, and (C) virus titers in vaginal washes were monitored in IFNαβR−/− mice, WT (129 background) mice, and IFNαβR−/→WT or WT→IFNαβR−/− chimeric mice following CpG ODN ivag inoculation and HSV-2 challenge (10⁴ PFU). Values represent mean ± SEM (n = 4/group).
a more severe phenotype than those lacking IFN-αβR from the stromal cells, including the vaginal keratinocytes.

We showed that CpG ODN–mediated protection did not require T or NK cells, IFN-γR, or IL-12p40. These data differ from the findings of a previous study that demonstrated that SCID and nude mice succumbed to HSV-2 challenge despite CpG ODN treatment (6). Although we do not know the reasons for this discordance, it may relate to the use of (a) high HSV-2 challenge dose and (b) the 48-hour time point used for viral challenge in the study by Harandi et al. (6). Further, our finding that individual lymphocyte subsets and IFN-γR are dispensable for CpG ODN–mediated protection against HSV-2 is consistent with previous reports showing that RAG2−/− (lacking B and T cells), RAG2−/−γc−/− (lacking B, T, NK, and NKT cells) (9), and IFN-γ−/− (5) mice were all protected from HSV-2 challenge following CpG ODN inoculation.

One potential complication of interpreting data from the irradiation-induced BM chimera stems from the radioresistant nature of the Langerhans cells (19). If Langerhans cells in the vaginal epithelia were repopulated from radioresistant host precursors, our interpretation must consider the contributions of Langerhans cells reflecting the host genotype. However, unlike the Langerhans cells that reside in the skin epidermis, vaginal Langerhans cells are repopulated entirely from BM precursors upon reconstitution of the irradiation-induced BM chimera (N. Iijima, M. Linehan, and A. Iwasaki, unpublished observations). Thus, in the WT→TLR9−/+ mice, the vaginal Langerhans cells are of WT origin (TLR9−/+), and the reduction in the viral titers in these mice compared with the TLR9−/+ mice reflects the contribution of the hematopoietic cells, including the vaginal Langerhans cells in their response to CpG ODN. In a similar vein, the observation that IFNαβR−/→WT mice succumbed to more severe disease compared with the WT→IFNαβR−/+ mice following CpG ODN treatment indicates the importance of the hematopoietic cells, including vaginal Langerhans cells, in responding to type I IFNs and providing antiviral protection in situ.

Our data demonstrate the importance of the responsiveness to type I IFNs by both the stromal and hematopoietic cells in providing CpG ODN–mediated antiviral protection in the genital mucosa. The requirement for IFN-αβR on the vaginal epithelial cells, the target of HSV-2 (16), was not surprising since signals transduced by the engagement of this receptor via type I IFNs result in the expression of hundreds of antiviral genes necessary to inhibit viral replication and spreading (14, 15). It is interesting to note that the level of viral suppression in the vaginal mucosa achieved in CpG ODN–treated WT→IFNαβR−/+ chimeric mice was comparable to that of CpG ODN–treated WT mice (Figure 6C). These data indicate that pDCs might provide type I IFN–independent antiviral activities for vaginal keratinocytes. However, the mechanism still remains unclear. It may involve other antiviral cytokines, NK-mediated lysis of infected cells, or direct lysis of the infected cells by pDCs, as shown by a recent study demonstrating that pDCs

Figure 7
IL-12 and IL-23 are not required for CpG ODN–mediated protection against genital HSV-2 challenge. (A) Survival rates, (B) genital mean pathology scores, and (C) virus titers in vaginal washes of WT (C57BL/6) and IL-12p40−/− (C57BL/6 background) mice were monitored following CpG ODN ivag inoculation and HSV-2 challenge (104 PFU). Values represent mean ± SEM (n = 5/group). Data are representative of 2 separate experiments.

Figure 8
IFN-γ responsiveness is not required for CpG ODN–mediated protection against genital HSV-2 challenge. (A) Survival rates, (B) genital mean pathology scores, and (C) virus titers in vaginal washes of WT (129 background) and IFN-γR−/− (129 background) mice were monitored following CpG ODN ivag inoculation and HSV-2 challenge (104 PFU). Values represent mean ± SEM (n = 5/group). Data are representative of 3 independent experiments.
acquire cytolytic activities through expression of TNF-related apoptosis-inducing ligand upon CpG ODN stimulation (20).

The basis for the requirement for IFN responsiveness by the hematopoietic cells in providing this innate protection was less obvious. Among other possible explanations, it is important to note that IFNγR−/− pDCs fail to secrete robust type I IFNs (ref. 21 and data not shown). Like many other cell types, pDCs require positive feedback through the IFN-α/βR (22) for production of high levels of type I IFNs, owing to the requirement for the IFN-induced IFN regulatory factor 7 (IRF-7) in this process (21). Thus, in the IFNγR−/−→WT chimera, one of the major defects in CpG ODN–mediated antiviral protection is the lack of pDC secretion of type I IFNs. In addition, type I IFNs are a potent activator of NK cytotoxicity (23). Combined with our data indicating that NK depletion had a moderate defect in viral control by CpG ODN treatment (Figure 3), the lack of protection seen in the IFNγR−/−→WT chimera may also reflect reduced NK cell activities.

Collectively, our results contribute to the understanding of several key mechanisms of CpG ODN–based treatment in providing an innate antiviral immune state. These findings suggest that CpG ODN–based microbialidal approaches may benefit from proper targeting of CpG ODN to the pDCs and to the vaginal epithelial cells. Such selective targeting of CpG ODN is expected to reduce the amount of CpG ODN required for effective microbialidal activity and also diminish inflammation that is associated with general application of TLR agonists (24) without compromising the effectiveness of CpG ODN as a microbiocide. Finally, this study reveals the importance of the intricate crosstalk between pDCs and vaginal stromal cells in orchestrating innate antiviral protection upon local delivery of CpG ODN through type I IFNs.

Methods

**Virus and CpG ODN.** HSV-2 186 syn (25) was propagated and titrated as described previously (16). CpG 1826 ODN (5’-TCCATGACGTTCTCCTGACGTT-3’) with phosphorothioate backbone (26) was synthesized and HPLC-purified by TriLink BioTechnologies.

**Mice and virus infection.** Six to eight-week-old female C57BL/6 and 129 mice were purchased from the National Cancer Institute, and IL-12p40−/− mice on C57BL/6 background were purchased from Jackson Laboratory. MyD88−/− mice (27) and TLR9−/− mice (28) backcrossed onto a C57BL/6 background and IFNαR−/− mice (29) and IFNγR−/− mice (30) backcrossed onto a 129 background were used. For virus infection, mice were injected s.c. in the neck ruff with Depo-Provera (Pfizer) at 2 mg per mouse in a 100 μl volume 5 days before infection, swabbed with calcium alginate, and inoculated ivag with 104 PFU of HSV-2 strain 186 syn in a 10 μl volume by a blunt-ended micropipette tip. For CpG ODN treatment, mice were inoculated with 100 μg of CpG ODN in 10 μl volume ivag 24 hours before viral infection. This procedure was carried out according to a previous report demonstrating that the optimal period of protection was from −4 to 4 hours after CpG ODN treatment (6, 31).

The severity of disease was scored (32) as follows: 0, no sign; 1, slight genital erythema and edema; 2, moderate genital inflammation; 3, purulent genital lesions; 4, hind limb paralysis; 5, premonitory. Due to humane concerns, the animals were euthanized prior to reaching moribund state. All procedures used in this study complied with federal guidelines and were approved by the Yale University Institutional Animal Care and Use Committee.

**Construction of BM chimeras.** BM chimeric mice were constructed by a standard method described previously (3). In brief, recipient mice were lethally irradiated with 9 Gy of total-body irradiation by Cs-iradiator (Yale Cancer Center). BM was obtained from the femur and tibia of donor mice and collected in sterile PBS. The cells were subsequently washed twice, counted, and resuspended in sterile PBS at a concentration of 5.0 × 10⁷ cells/ml. Irradiated recipient mice were reconstituted with 1.0–1.5 × 10⁶ cells by means of tail vein injection. The transplanted mice were treated with antibiotics and maintained in a clean facility and used for experiments upon complete reconstitution (>8 weeks). BM reconstitution was confirmed as previously described (3).

**Collection of vaginal secretions and virus titration.** Vaginal secretions were collected from −1 to 5 days after infection by pipetting a total volume of 50 μl of PBS in and out of the vagina 20 times. Then the vaginal surface was swabbed twice. The vaginal swabs were left in the vaginal washes. Virus titers in the vaginal washes were determined by infecting confluent Vero cells for 2 to 3 days in triplicate by a standard method (16).

**pDC, NK cell, and T lymphocyte depletion.** pDCs were depleted at 24 hours before and 24 hours after the CpG ODN treatment by i.p. injection of 500 μg anti–mPDCA-1 in a volume of 500 μl PBS (rat IgG2b; Miltenyi Biotec) according to the manufacturer’s instructions. NK cells were depleted 3 times at −3, −1, and 1 day after infection by i.p. injection of 200 μg in 200 μl of anti-NK.1.1 (Mouse IgG2a). CD4 and CD8 T cells were depleted at −3, −1, and 1, and 3, and 5 days after infection by i.p. injection of 400 μg anti-CD4 (GK.1.5; rat IgG2b) and anti-CD8 (53–67.2; rat IgG2a). Abs. Mice injected with rat IgG (500 μg) or mouse IgG (200 μg) at −3, −1, and 1 day after infection were used as controls. All depletion procedures followed a standard protocol, and in vivo depletion was confirmed by FACS analysis of the splenocytes from the Ab-treated mice.

**Isolation of cells from vaginal tissues and FACS analysis.** Vaginal tissues from mice treated with PBS or CpG ODN were excised and washed with PBS once. Tissues were incubated with 1 ml of 2.0 U/ml Dispase II (Roche Diagnostics) PBS solution at 37°C for 1 hour. After removal of Dispase II solution, tissues were minced and incubated with 5 ml of 0.05% Trypsin-EDTA PBS solution at 37°C for 1 hour. After incubation, tissues were washed 3 times with 5 ml/vagina of PBS solution containing 0.05% FBS. Cells were stained with PE-conjugated anti–mPDCA-1 in a volume of 500 μl PBS and anti-CD4/CD8–PE/FITC/Fram (Miltenyi Biotec) according to the manufacturer’s instructions. Cells were collected and washed 3 times with 5 ml/vagina of PBS solution containing 0.05% FBS. Cells were stained with PE-conjugated anti–mPDCA-1 Ab (or isotype control) and 7-AAD (BD Biosciences), and live cells were gated and analyzed by FACS.

**Statistics.** Statistical differences of the viral titers were determined by the ANOVA method. A P value of less than 0.05 was considered statistically significant.

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