

Delivery of antigen to nasal-associated lymphoid tissue microfold cells through secretory IgA targeting local dendritic cells confers protective immunity

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Background: Transmission of mucosal pathogens relies on their ability to bind to the surfaces of epithelial cells, to cross this thin barrier, and to gain access to target cells and tissues, leading to systemic infection. This implies that pathogen-specific immunity at mucosal sites is critical for the control of infectious agents using these routes to enter the body. Although mucosal delivery would ensure the best onset of protective immunity, most of the candidate vaccines are administered through the parenteral route.

Objective: The present study evaluates the feasibility of delivering the chemically bound p24gag (referred to as p24 in the text) HIV antigen through secretory IgA (SIgA) in nasal mucosae in mice.

Results: We show that SIgA interacts specifically with mucosal microfold cells present in the nasal-associated lymphoid tissue. p24-SIgA complexes are quickly taken up in the nasal cavity and selectively engulfed by mucosal dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin-positive dendritic cells. Nasal immunization with p24-SIgA elicits both a strong humoral and cellular immune response against p24 at the systemic and mucosal levels. This ensures effective protection against intranasal challenge with recombinant vaccinia virus encoding p24.

Conclusion: This study represents the first example that underscores the remarkable potential of SIgA to serve as a carrier for a protein antigen in a mucosal vaccine approach targeting the nasal environment. (*J Allergy Clin Immunol* 2015;■■■:■■■-■■■.)

Key words: Nasal vaccine strategy, secretory IgA, reverse transcytosis, microfold cells, nasal-associated lymphoid tissue, protection

Abbreviations used

DC:	Dendritic cell
DC-SIGN:	Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin
HLT:	Heat-labile toxin
KO:	Knockout
M cell:	Microfold cell
MCP-1:	Monocyte chemoattractant protein 1
MIP:	Macrophage inflammatory protein
NALT:	Nasal-associated lymphoid tissue
PLA:	Polylactic acid
SC:	Secretory component
SIgA:	Secretory IgA
UEA-1:	<i>Ulex europaeus</i> agglutinin 1

The emergence of new diseases and the lack of efficient vaccines against numerous resistant or nontreatable pathogens require development of novel vaccination strategies. Safe, effective, and affordable vaccines represent the best hope for restraining the spread and consequences on health of numerous mucosal pathogens.¹⁻³ Mucosal vaccination can potentially stimulate immune responses in both the mucosal and systemic compartments and has the advantage of being a noninvasive procedure avoiding any injection. However, limited uptake across intrinsically tight epithelial barriers lining the mucosa is a limitation that often prevents efficient delivery at these sites. Other drawbacks include poor antigen stability, altered immunogenicity, and safety concerns related to the use of some mucosal adjuvants in human subjects.³

A promising approach to address inefficient transepithelial vaccine uptake consists of targeting mucosal microfold cells (M cells) intrinsically endowed for antigen sampling.^{4,5} In intestinal Peyer patches secretory IgA (SIgA) binds to M cells^{6,7} and can enhance the transcytosis of bound antigens across the follicle-associated epithelium through Dectin-1 and Siglec-5.⁸ Passage of SIgA-antigen complexes in gut-associated lymphoid tissue was followed by association with dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN)-expressing dendritic cells (DCs) in the subepithelial dome region.⁸ On oral administration, a complex made of a protein antigen and SIgA was shown to elicit antigen-specific immune responses in the mucosal and systemic compartments and ensured protection against a recombinant vaccinia virus expressing the antigen.⁹

In nasal-associated lymphoid tissue (NALT) DCs are separated from the nasal cavity by an epithelium containing M cells, thus similarly allowing the transfer of antigens to underlying lymphoid

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tissues.¹⁰⁻¹² This anatomic similarity with the gastrointestinal tract makes intranasal antigen administration an attractive and valid alternative to conventional vaccines. Indeed, the nasal mucosa is a practical site for vaccine administration because of the absence of an acidic environment, the lack of abundant secreted enzymes, and small mucosal surface areas, features resulting in a lower dose requirement of antigen than the digestive tract. Moreover, nasally administered adjuvanted vaccines can induce both mucosal and systemic immune responses, as has been exemplified after nasal immunization of both mice and human subjects against diphtheria, tetanus,¹³ influenza,¹⁴ and infection with *Streptococcus mutans*.¹⁵ In addition, it has been described in animal studies that potent responses in the respiratory and genital tracts could be induced by intranasal immunization in the context of the common mucosal immune system.¹⁶⁻¹⁸

The purpose of the current study was to evaluate the feasibility of delivering the model antigen p24 from HIV through SIgA into the nasal mucosae. At the mechanistic level, we first establish *in situ* that after nasal immunization, p24-SIgA is quickly taken up by M cells through the Dectin-1 receptor and transmitted to mucosal DC-SIGN⁺ DCs. Nasal immunization with p24-SIgA induces strong humoral and cellular immune responses against p24 both at the systemic and mucosal levels. Challenge with a recombinant vaccinia virus expressing p24 provides the demonstration that SIgA-mediated M cell-targeted mucosal immunization elicits protective immunity against the inducing antigen. This suggests that mucosal delivery of antigen-SIgA complexes could be an attractive approach for both prophylactic and therapeutic vaccination against a variety of mucosal infectious agents but also against mucosally derived pathologies.

METHODS

Mice

Mouse strains used in this study consisted of 8-week-old female BALB/c mice (Charles River, Wilmington, Mass) and 8-week-old female Dectin-1 knockout (KO) mice. All animals were housed at the Unité hospitalo-universitaire d'expérimentation animale (Saint-Etienne, France). The protocol was approved by the Ethics Committee of CREEA (permit no. 69387487).

Production of p24-SIgA complexes

Polymeric IgA (pIgA) from the hybridoma clone IgAC5 specific for *Shigella flexneri* serotype 5a LPS was obtained and purified, as previously described.¹⁹ Purified free human secretory component (SC) was produced in Chinese hamster ovary cells.²⁰ SIgA were obtained by combining pIgA molecules in PBS with a 2-fold excess of human SC for 1 hour at room temperature, as previously described.²¹ IgA complexes containing SC were then purified before being covalently linked to the model antigen p24gag (referred to as p24 in the text) at an equimolar ratio. p24 was produced in CHO cells (Px Therapeutics, Lyon, France). Covalent association between p24 and SIgA was carried out with the heterobifunctional cross-linker Sulfo-KMUS, according to the protocol provided by the manufacturer (Thermo Scientific, Waltham, Mass).

NALT dissection and immunolabeling

NALT dissection was done, as previously described.²² Processed tissue was embedded in OCT solution and placed on microscope slides. Seven-micrometer sections (Leica cryostat model CM1950; Leica Microsystems, Wetzlar, Germany) were placed on Ultra+ Superfrost microscope slides (VWR International, Radnor, Pa). Slides were stained with hematoxylin and eosin by using a standard protocol. Slides were washed in PBS to eliminate

residual OCT and blocked with PBS containing 5% FBS for 30 minutes at room temperature. Immunolabeling was performed with the following primary antibodies (1:200 dilution): anti-human DC-SIGN, anti-mouse Dectin-1, anti-mouse glycoprotein 2 (GP2), or anti-*Ulex europaeus* agglutinin 1 (UEA-1), all of which were fluorescein isothiocyanate conjugated, and serum from an HIV⁺ patient recognizing the p24 molecule in free and bound form. Anti-HIV antibodies were detected with Cy3-conjugated anti-human IgG (1:400 dilution). Slides were examined by means of immunofluorescence microscopy (Eclipse; Nikon, Tokyo, Japan).

Immunization with p24-SIgA complexes

Mouse intranasal immunizations were performed by applying 50 μ L of the vaccine and controls into the nasal cavity after achievement of short light anesthesia with isoflurane. The immunization procedure is based on 3 administrations of 50 μ L at 1-week intervals, followed by a last booster immunization 3 weeks later. Groups of 5 mice were immunized with either PBS, 50 μ g of p24, 50 μ g of p24 plus 2.5 μ g of HLT,²³ 50 μ g of p24-SIgA, or 50 μ g of p24-SIgA plus 2.5 μ g of HLT at each administration. HLT was a generous gift from Dr J. Clements.²³ Two additional control groups were immunized with either p24-poly(lactic acid) (PLA) through the nasal route or p24-alum through the subcutaneous route. p24-coated PLA nanoparticles and p24-alum were prepared, as previously described.²⁴ Subcutaneous administrations of 100 μ L of p24-alum were performed in the leg at days 0, 14, and 28. Inject Alum (Pierce/Thermo Scientific) was used to adsorb p24 at a 1:1 volume ratio according to the manufacturer's instructions.

Viral challenge was performed with the vDK1 recombinant vaccinia virus expressing HIV-1 p24gag (this reagent was obtained through the National Institutes of Health AIDS Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health: vDK1 was from Dr Daniel R. Kuritzkes). A dose of 4×10^7 plaque-forming units of the virus in 100 μ L was administered through the intranasal route 1 week after the last immunization. Mice were then weighed daily for up to 8 days or killed prematurely if their weight loss was more than 20%. Lungs were recovered at death or at the end of the experiment. Tissues were washed and homogenized in PBS with an ultrasonic homogenizer (Omni Ruptor 250; Omno International, Kennesaw, Ga). Plaque-forming assays were performed, as previously described, on BHK-21 cells for 48 hours followed by counterstaining with 5% (wt/vol) crystal violet in water.⁹ Viral titers were expressed as log₁₀ plaque-forming units.

Sample, tissue, and fluid collection

Biological fluids were recovered before and 1 week after each immunization to examine their antibody content. Sera were obtained from whole blood recovered by means of retro-orbital collection followed by centrifugation at 16,000g and stored at -20°C until use. Vaginal lavage samples were taken after injection of 50 μ L of PBS with adapted tips (CP100; Gilson, Middleton, Wis). Five fresh feces samples from separate stools were collected from each animal. Saliva samples were collected from the mouths of mice and diluted with 100 μ L of PBS. Feces, vaginal lavage, and saliva samples were stabilized with Halt protease inhibitor cocktail (Thermo Scientific), centrifuged at 16,000g, and stored at -20°C until use.

The mouse genital tract was dissected from the animal and placed in cold complete medium (RPMI-1640 supplemented with 10% FBS, 2 mmol/L glutamine, 10 mmol/L HEPES, 100 IU/mL penicillin, 100 μ g/mL streptomycin, and 10 μ g/mL gentamycin). The tissue was finely cut with a scalpel, washed with complete medium, and digested at 37°C for 1 hour with shaking in 5 mL of serum-free RPMI-1640 medium that contained 2 mg/mL Collagenase Dispase and 0.1 mg/mL DNase I (Roche Diagnostics, Mannheim, Germany). The digested tissue was spun, and the cell pellet was washed twice. The lymphoid cell population was separated from the stromal cells by means of density gradient centrifugation (Lympholyte; Cedarlane Laboratories, Burlington, Ontario, Canada).

Splenocytes were isolated by means of mechanical dissociation through a sterile nylon mesh, followed by red blood cell lysis.

Quantification of p24-specific IgG, IgA, and IgG₁/IgG_{2a} antibodies in biological fluids

p24-specific IgG, IgA, and IgG₁/IgG_{2a} in serum (1:100 dilution) and vaginal lavage (1:20 dilution), feces (1:2 dilution), and saliva (1:10 dilution) samples were measured by means of ELISA. Ninety-six-well Maxisorp plates were coated with 50 μ L of p24 antigen solution (5 μ g/mL in sterile PBS). Bound or captured immunoglobulins were detected by means of incubation (for 1 hour at 37°C, 1:4000 dilution) with horseradish peroxidase-conjugated goat anti-mouse IgG, IgG₁, IgG_{2a}, and IgA. Results are presented as mean $\pm$ SEM concentrations, with subtraction of background antibody concentration measured in preimmune samples. The limit of detection corresponds to 1 ng/mL for all ELISAs.

Detection of p24-specific immune cells by means of ELISpot in the spleen and vagina

Mice were killed by means of cervical dislocation after achievement of anesthesia 1 week after the last immunization. ELISpot assays were performed with cells isolated from the spleen and genital tract, as previously described.^{25,26} Cells (3×10^5 per well) were directly plated and cultured for 24 hours with or without stimulation with p24 antigen. The plates were assayed for IgG, IgA, or IFN- γ , according to the procedure provided by the manufacturer (Mabtech, Cincinatti, Ohio). ELISpot assays for IgG and IgA antibody-secreting cells were performed by coating plates with p24 antigen, whereas IFN- γ -secreting T-cell ELISpot assays were performed by means of stimulation with soluble p24 antigen. Spot-forming cells were counted on an ELISpot reader (BIOREADER 3000; BIO-SYS, Karben, Germany). The results are expressed as the number of spots per 3×10^5 cells after subtracting the number of spots in unstimulated wells.

Cytokines and chemokines

The evaluation of multiple cytokines/chemokines was performed with a Luminex 100 instrument (Luminex, Austin, Tex) in combination with the Bio-Plex mouse cytokine 23-plex panel and Bio-Plex mouse cytokine T_H17 panel B 8-Plex Group III (Bio-Rad Laboratories, Hercules, Calif). Biological samples were recovered 1 week after the last immunization. The intestinal cell lysate was prepared with the Bio-Plex cell lysis kit (Bio-Rad Laboratories), as specified by the manufacturer. Cytokine and chemokine concentrations were determined as the mean of 3 replicates per time point.

Statistical analysis

All statistical analyses were performed with InStat software (version 5.02; GraphPad Software, La Jolla, Calif). A nonparametric Mann-Whitney *U* test or 1-way ANOVA followed by the Bonferroni *post hoc* test was used where appropriate.⁹ *P* values of less than .05, less than .01, and less than .005 were considered significant. Statistically significant differences between groups are emphasized by bars connecting the relevant columns.

RESULTS

Specific uptake and transport of p24-SIgA across murine NALT

To validate our working hypothesis, we first aimed to assess whether association of p24 with SIgA can improve the delivery of antigen across the NALT (Fig 1, A) and then whether SIgA-mediated DC targeting was ensured in this tissue. Administration into the mouse nasal cavity confirmed the presence of p24-SIgA in association with M cells identified by the expression of UEA-1 (Fig 1, B), GP2 (Fig 1, C), and the Dectin-1 receptor (Fig 1, D). Subsequent dissemination in NALT is observed in Fig 1, B (white arrows). No p24 administered alone could be detected in the NALT tissue (Fig 1, E-G). Moreover, p24-SIgA complexes transported in the NALT were found

in close association with DC-SIGN⁺ DCs (Fig 1, H) present locally, as illustrated by the appearance of yellow dots. In contrast, green DCs within the tissue and red p24 in the nasal cavity only were detected when the antigen was delivered alone (Fig 1, I). Such sampling and spreading characteristics prompted us to investigate whether nasal immunization with p24-SIgA complexes can induce subsequent p24-specific immune responses.

p24-specific antibody responses after nasal administration of p24-SIgA complexes

BALB/c mice were immunized as described in the Methods section. Mucosal and systemic p24-specific humoral immune responses were assessed by means of ELISA in serum and different mucosal compartments, including feces, vaginal lavage, and saliva samples collected 1 week after the last immunization (Fig 2, A). Antibody responses measured at earlier time points (during the first 3 weeks) led to no significant increase compared with preimmune sera (data not shown). Controls included administration of free p24 combined or not with heat-labile toxin (HLT) as a mucosal adjuvant,²⁷ as well as PBS buffer. Mice exhibited no abnormal clinical signs, such as diarrhea or weight loss, after HLT administration.

Fig 2, A (left and middle columns), shows that the group of mice immunized with p24-SIgA (group 4) exhibited significantly higher p24-specific IgG titers in serum, vaginal lavage samples, and saliva than mice immunized with p24 alone (group 2). This held true for IgA in serum and vaginal lavage samples. P24+HLT (group 3) yielded similar or lower (IgG in serum and IgG and IgA in vaginal washes) antibody titers when compared with p24-SIgA, suggesting that SIgA-mediated M cell targeting of the antigen ensured stimulation resembling that of HLT. In comparison with p24 alone, adjuvantization of p24-SIgA with HLT (group 5) induced the highest production of both IgA and IgG in all samples tested. Subcutaneous administration of PLA nanoparticles (group 6)²⁴ or p24-alum (group 7)²⁸ was used as a control of vaccine efficacy.⁹ P24-PLA nanoparticles did not induce humoral responses in the systemic and mucosal compartments. Consistent with its well-known properties, the p24-alum formulation was able to induce systemic humoral responses only. This allowed us to conclude that with the notable exception of serum antibody titers in the case of p24-alum, the SIgA-based formulations turned out to be superior to nanoparticle- and alum-based antigen preparations for triggering antigen-specific antibody responses. IgG_{2a}/IgG₁ ratios (Fig 2, A, right panel) calculated for each mouse revealed that responding groups elicited a higher IgG_{2a} than IgG₁ response in serum, feces, and vaginal lavage samples, suggesting a T_H1-skewed type of response.

Next, cells recovered from the spleen and vagina were isolated to analyze both p24-specific IgG- and IgA-secreting plasma cells by using the ELISpot assay (Fig 2, B and C). Only splenic cells recovered from mice immunized with p24-SIgA and p24-SIgA+HLT had significantly higher levels of IgA- and IgG-secreting plasma cells than control samples, indicating specific activation and migration of locally primed cells to distant effector sites.

To confirm the necessary role of the antibody in ensuring the transport of the p24 antigen through the Dectin-1 receptor present on the surfaces of NALT M cells, Dectin-1 KO mice were immunized with p24 alone or p24-SIgA. Although, as expected,

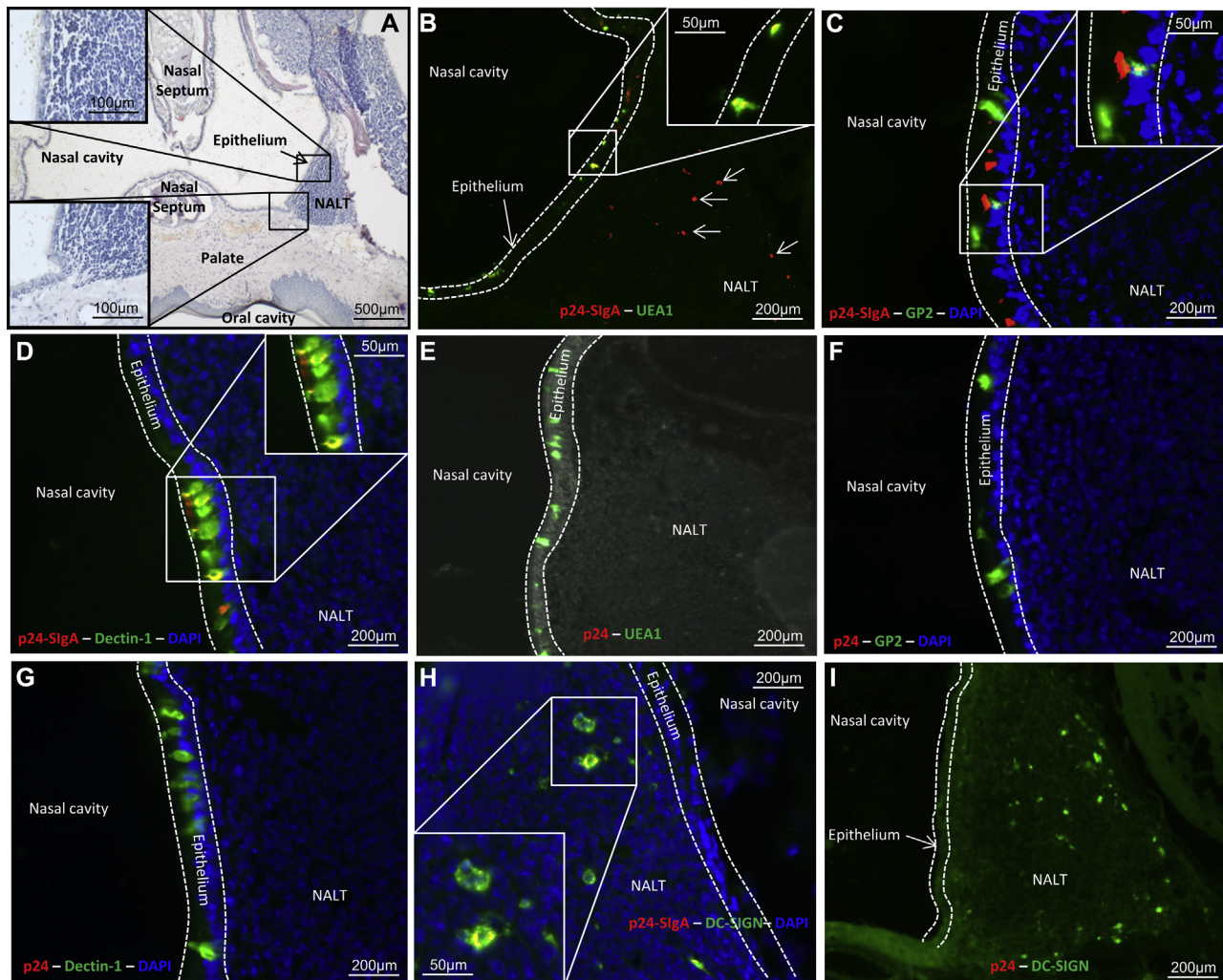


FIG 1. Specific uptake and transport of p24-SIgA across the murine NALT. **A**, NALT staining with hematoxylin and eosin. **B-I**, p24-SIgA (Fig 1, **B**, **C**, **D**, and **H**) or p24 alone (Fig 1, **E**, **F**, **G**, and **I**) was intranasally administered and incubated for 60 minutes in the nasal cavity. p24 staining was performed with an HIV-1⁺ serum. Anti-HIV antibodies were detected with Cy3-conjugated anti-human IgG. Cell staining was performed with anti-human DC-SIGN, anti-mouse Dectin-1, anti-mouse glycoprotein 2 (GP2), or UEA-1, all conjugated with fluorescein isothiocyanate. In contrast to p24 alone showing luminal red spots, interaction between p24-SIgA and GP2⁺, Dectin-1⁺, or UEA-1⁺ M cells or DC-SIGN⁺ DCs resulted in yellow dots on green-labeled cells. Images were obtained from single optical sections acquired by means of confocal microscopy. On all panels, *dotted lines* delineate the interface between the nasal cavity and tissue (side view). Experiments have been repeated twice showing similar results.

p24 alone led to antibody background levels in the 2 types of mice (Fig 3), the absence of a humoral response in serum of Dectin-1 KO mice compared with WT mice immunized with p24-SIgA demonstrated the importance of this receptor in the SIgA-mediated antigen-sampling process.

p24-specific cellular responses after nasal administration of p24-SIgA complexes

In addition to local antibody production, the contribution of IFN- γ -secreting T cells in the process of fighting mucosal infections, in particular viral infections, is highly relevant. Nasal immunizations with p24-SIgA or p24-SIgA+HLT, but not with the other vaccine formulations, promoted the production of IFN- γ by CD4⁺ and CD8⁺ T cells in response to p24 stimulation in ELISpot assays (Fig 4, A). Similar results were obtained with

resident vaginal T lymphocytes (Fig 4, A). Because the local tissue-dependent nature of immune responses relies highly on the controlled production of cytokines and chemokines, a selection of these was examined in serum and mucosal compartments, including feces, vaginal lavage samples, and intestinal cell lysates obtained 24 hours after the last immunization. In comparison with p24+HLT, p24-SIgA and p24-SIgA+HLT administered through the nasal route were potent inducers of a large cytokine and chemokine panel, generating a pattern close to that seen with p24-SIgA+HLT. Control groups given PBS, SIgA, and p24 alone led to the same or a slightly reduced central polygon area as seen with p24+HLT.

To determine the ability of the vaccine to promote chemotaxis of immune effector cells, such as antigen-presenting cells and T and B lymphocytes, chemokine levels in different compartments were analyzed (Fig 4, B, and see Table E1 in this article's Online

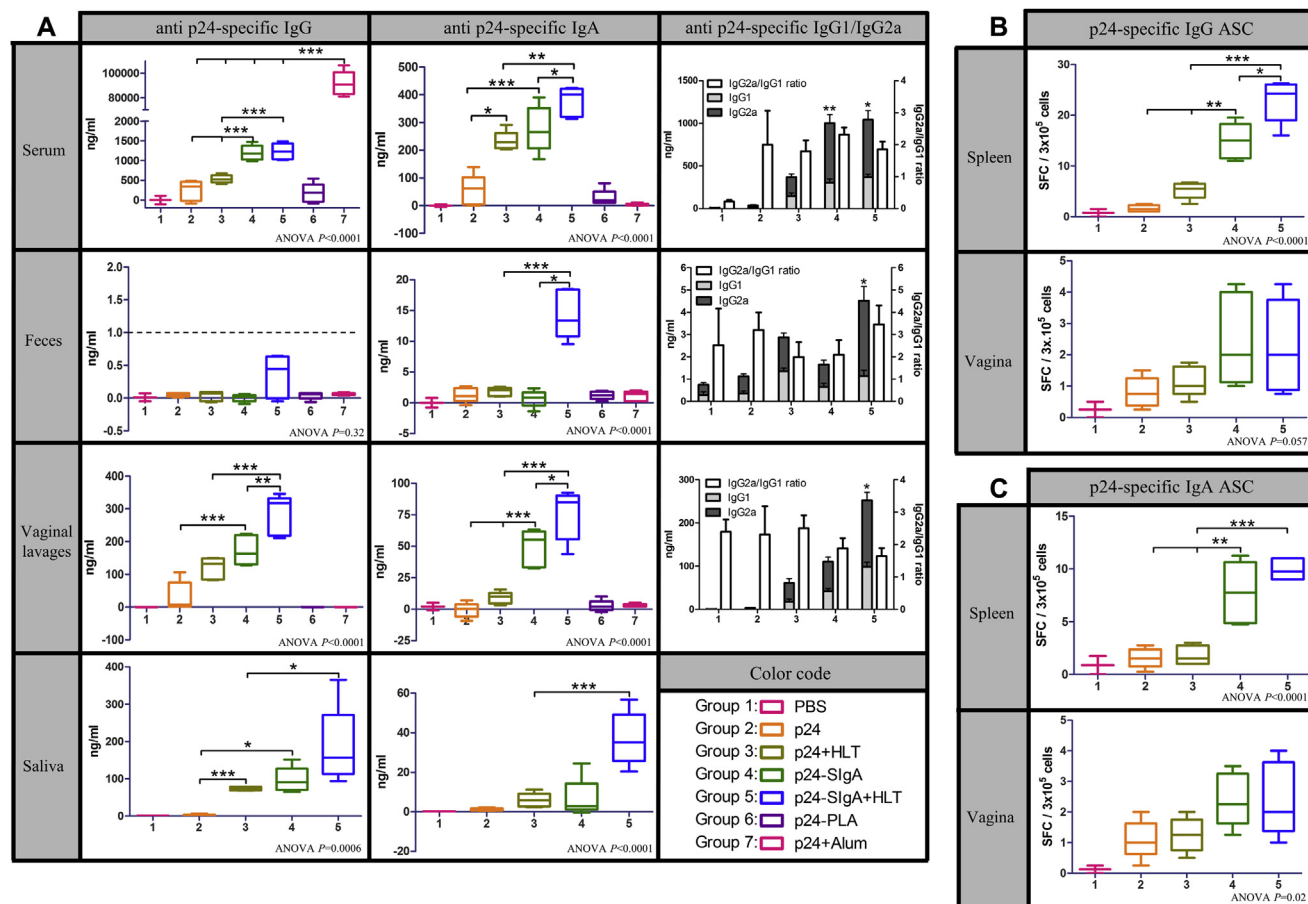


FIG 2. p24-specific IgA, IgG, IgG₁, and IgG_{2a} responses after nasal administration of p24-SIgA complexes. **A-C.** Mice were immunized intranasally with PBS, p24, p24+HLT, p24-SIgA, p24-SIgA+HLT, and p24-PLA nanoparticles and immunized through the subcutaneous route with p24-alum. Fig 2, A, Serum, feces, vaginal lavage samples, and saliva were isolated 1 week after the last immunization. Data are shown as medians $\pm$ 95% intervals, and the minimum and maximum are indicated (left and middle columns) or shown as means $\pm$ SEMs (right column). Dotted lines indicate the limit of detection (Fig 2, B and C). Spleens and intestines were isolated after 41 days, and ELISpot assays were performed on IgG and IgA antibody-secreting cells (ASC). Data are shown as medians $\pm$ 95% intervals, and the minimum and maximum are indicated. ANOVA followed by the Bonferroni *post hoc* test (left and middle columns) and the Student *t* test (right column, comparison between IgG₁ and IgG_{2a} within the same experimental group) were performed. * $P < .05$, ** $P < .01$, and *** $P < .005$. Data from 2 independent experiments consisting of 5 mice per group showing very close results have been pooled (total $n = 10$).

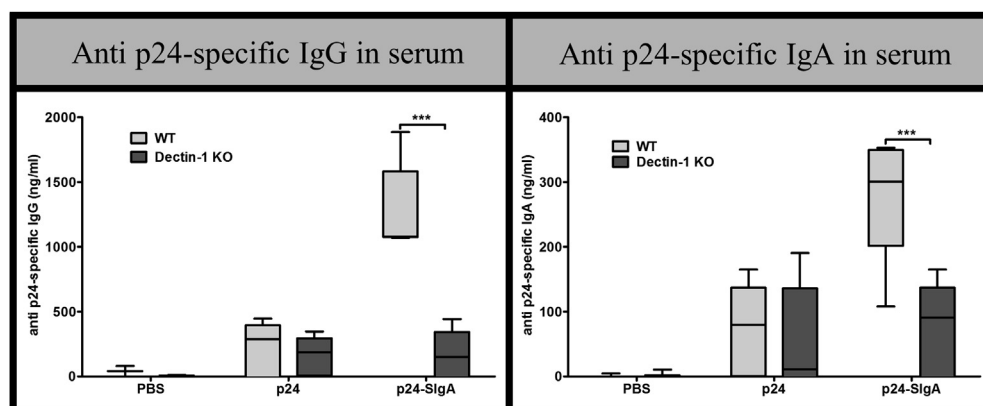


FIG 3. p24-specific IgA and IgG responses after nasal administration of p24-SIgA complexes in Dectin-1 KO mice. p24-specific IgA and IgG responses were measured in serum after nasal administration of p24-SIgA complexes in Dectin-1 KO mice versus wild-type (WT) mice. Column content and statistical analyses are as explained in the legend of Fig 2. *** $P < .005$.

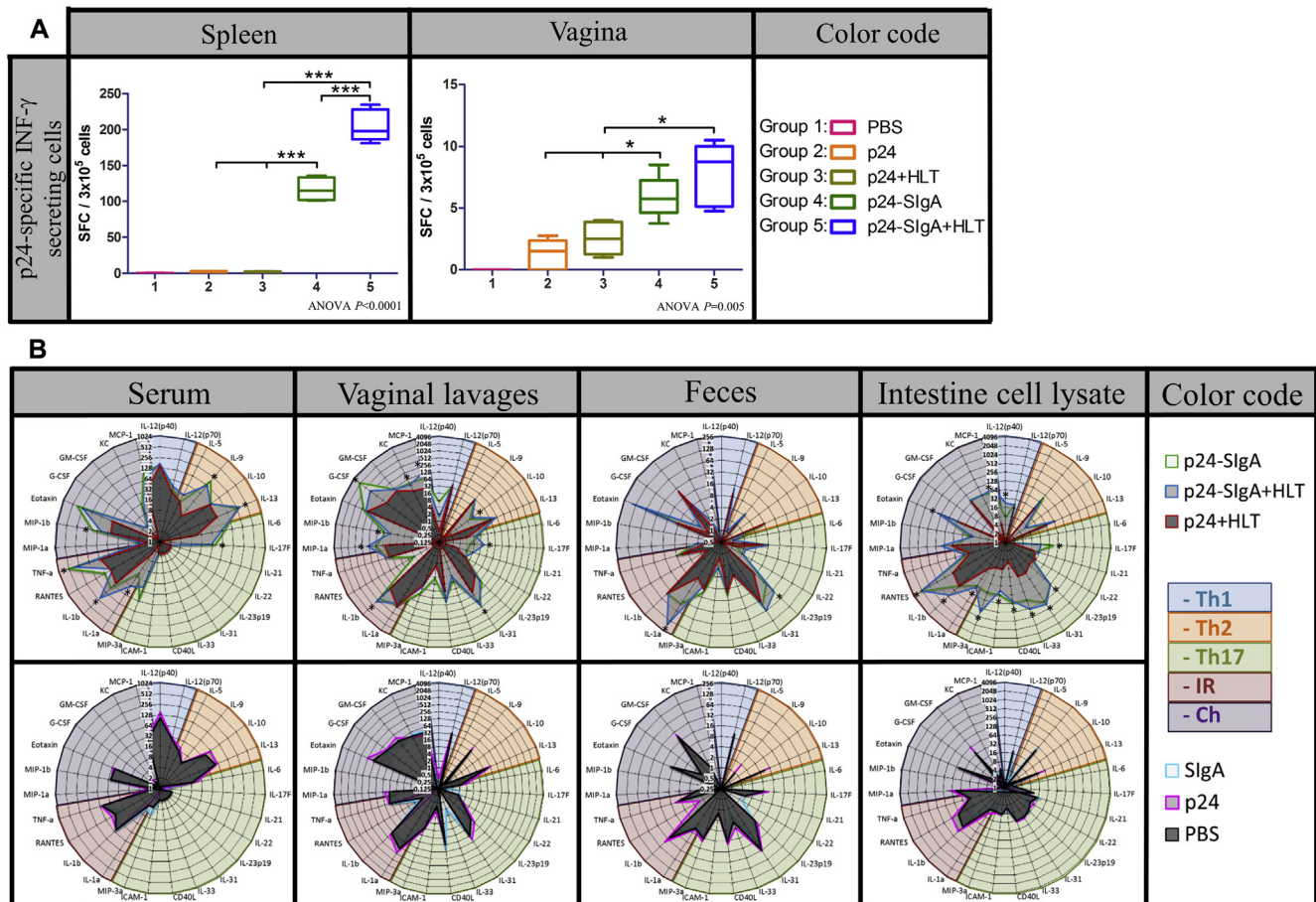


FIG 4. p24-specific cellular responses after nasal administration of p24-SIgA complexes. **A**, Mice were immunized with PBS, p24, p24+HLT, p24-SIgA, and p24-SIgA+HLT. Splenocytes and vaginal cells were evaluated by using ELISpot assays for IFN- γ -producing cells. Data are shown as medians $\pm$ 95% intervals, and the minimum and maximum are indicated. Data were pooled from 2 separate experiments, consisting of 5 mice per group (total $n = 10$). ANOVA followed by the Bonferroni *post hoc* test was performed. $*P < .05$ and $***P < .005$. SFC, Spot-forming cells. **B**, Cytokine concentrations were determined in triplicate by using the Luminex cytokine assay. Cytokine profiles are shown as radar charts; each axis displays the mean quantity (in picograms per milliliter) of each cytokine/chemokine after immunization with p24-SIgA, p24-SIgA+HLT, and p24+HLT (upper panel) and SlgA, p24, and PBS (lower panel). The values of each axis were joined to form the central polygon area, which represents the overall cytokine balance. Increasing or decreasing central polygon areas reflect higher or lower expression of the protein under analysis. The experiment was repeated twice with 5 mice for each group showing similar results. P values have been calculated by comparing the p24-SIgA group with the p24+HLT group. $*P < .05$. Ch, Chemokines; IR, mediator of the inflammatory response.

Repository at www.jacionline.org). p24-SIgA with or without HLT administered through the nasal route was a potent inducer of chemokines, including eotaxin, macrophage inflammatory protein (MIP) 1 α , and monocyte chemoattractant protein 1 (MCP-1) in serum; MIP-1 α , MIP-1 β , granulocyte colony-stimulating factor, keratinocyte chemoattractant, and MCP-1 in vaginal lavage samples; and eotaxin, keratinocyte chemoattractant, and MCP-1 in intestinal cell lysates. Compared with the negative controls, there was little or no significant change in the other cytokines or chemokines named in the Methods section in these tissues.

Next, to analyze the capacity of p24-SIgA to induce T-cell activation systemically but also locally, the T_H1/T_H17 profile in immunized mice was measured (Fig 4, B, and see Table E1). The increase in IL-17F (serum, intestine, and vaginal lavage samples), IL-23p19 (feces and intestines), and IL-31, IL-33, CD40

ligand, intercellular adhesion molecule 1, and MIP-3 α (intestine) levels shows that administration of p24-SIgA through the nasal route contributes to local and systemic T_H17 stimulation *in vivo*. A specific increase of IL-12p40 levels in the intestine was also found, which might indicate an accompanying mucosal T_H1 -type of response. Furthermore, induction of mediators of the inflammatory response can be detected after nasal immunization with p24-SIgA, as revealed by increasing levels of IL-1 α (serum, feces, and intestine), IL-1 β (serum, vaginal lavage samples, and intestine), TNF- α (serum), and RANTES (intestine).

Because an effective nasal vaccine should induce antibody production at mucosal sites, we have analyzed the T_H2 profile in vaccinated mice (Fig 4, B, and see Table E1). After nasal immunization, the increase in IL-9 and IL-13 (serum) and IL-10 (vaginal lavages) levels indicates a prototype T_H2 type of immune response²⁹ elicited by p24-SIgA with or without HLT.

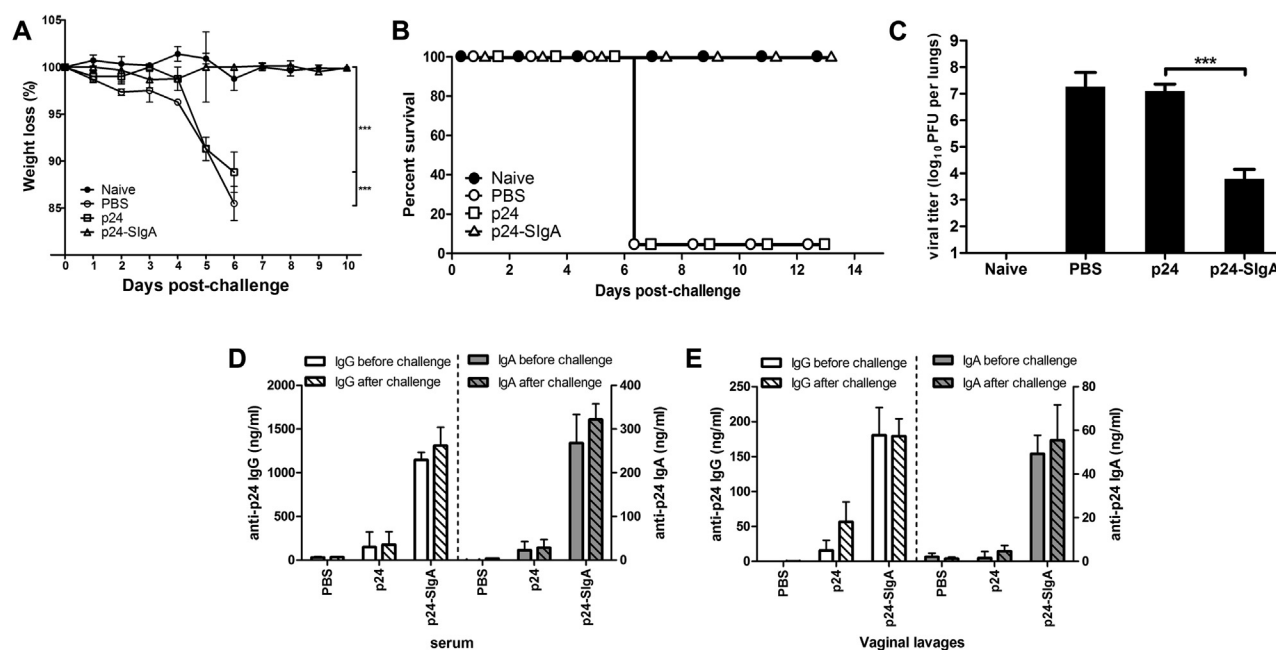


FIG 5. Challenge with recombinant vaccinia virus expressing p24. Mice were challenged intranasally with p24-expressing recombinant vaccinia virus (4×10^7 plaque-forming units) 1 week after the last immunization with PBS, p24, or p24-SIgA. Naive, Nonimmunized noninfected mice. Protection of mice was assessed based on weight loss (A), survival rate (B), and lung viral load (C). Diagrams show means $\pm$ SEMs of 5 mice for each group. ANOVA followed by the Bonferroni *post hoc* test was performed. *** $P < .005$ indicates the difference between the p24-SIgA and p24 groups. p24-specific antibody responses were measured before (day 41) and after (day 47) the challenge at systemic (D) and mucosal (vaginal; E) levels.

IL-2, IL-3, IL-4, and IFN- γ levels were less than the limit of detection for all analyses performed at this time point.

Together, these data indicate that the vaccine strategy reported herein is able to stimulate immune effector cells in mucosal compartments, eliciting both a strong humoral and a mixed $T_H1/T_H2/T_H17$ cellular immune response specific for the model antigen p24 both in the systemic and mucosal compartments.

Mice vaccinated intranasally with p24-SIgA resist challenge with a recombinant vaccinia virus expressing p24

Based on the antibody and cell profiles identified when dissecting the immune response elicited by the vaccine formulation, we sought to determine whether protection at a mucosal site would occur. We focused on the preparation devoid of HLT because this is believed to represent the most appropriate approach when thinking in terms of human administration. Mice immunized with p24-SIgA or p24 as a control were challenged by means of intranasal administration of a recombinant vaccinia virus expressing the p24 model antigen. In comparison with mice of the PBS and p24 groups, p24-SIgA-immunized animals did not experience any significant change in their initial weight on challenge (Fig 5, A). The absence of symptoms in vaccinees correlated with a 100% survival rate (Fig 5, B). Protection was accompanied by a significant decrease in viral load, as exemplified by a 1000-fold reduction in lung viral plaque-forming units of p24-SIgA-immunized mice compared with those seen in control groups (Fig 5, C). p24-specific antibody responses were measured before and after the challenge at systemic (Fig 5, D) and mucosal (Fig 5, E) levels, and we did

not see any significant differences in the humoral immune response. These challenge experiments demonstrate that SIgA-mediated M cell-targeted nasal immunization elicits protective immunity against the inducing antigen.

DISCUSSION

In this study we investigated the potential of targeting SIgA-based vaccines to M cells distributed along the nasal cavities of mice. We found that binding of SIgA to M cells involved Dectin-1 and that transepithelial transport of SIgA-bound antigen into the NALT targeted DC-SIGN⁺ DCs. Next, we showed that the use of p24-SIgA complexes *per se* or in association with HLT stimulated important parameters of the immune response, such as antigen-specific antibody production and cell reactivity, as well as cytokine/chemokine secretion, in various biological fluids and tissues. Indeed, we found that mice nasally immunized with p24-SIgA had significantly higher levels of antibody titers in both the systemic and mucosal compartments in comparison with mice immunized with p24 alone. The induction of $T_H1/T_H2/T_H17$ p24-specific cellular responses, which are critical components of protection against several bacterial and viral mucosal pathogens,³⁰ was also measured in the spleen and vaginal mucosa. In protection assays we established that the comprehensive immune response induced by p24-SIgA was sufficient to markedly limit infection by a recombinant vaccinia virus expressing p24.

Our results support previous findings that inserting a small antigenic peptide epitope within the SIgA structure¹⁹ to the validation of coupling a whole protein to ensure its nasal delivery to the site of sampling in the NALT, thus permitting the onset of

both mucosal and systemic immune responses through preferential targeting to DCs. Furthermore, it can be envisioned that coupling of more than 1 antigen molecule or engineered molecular motifs on the SIgA carrier might lead to the induction of antibodies and cellular responses with a broad spectrum of action.³¹ Moreover, in addition to ensuring stability, combination with SIgA might allow administration of a reduced dose of antigen, increase antigen uptake by the NALT, and potentially impart an adjuvant effect to the antigen without any toxic effect.³ An increase in affinity for specific receptors of SIgA-based immune complexes compared with naked SIgA has been described to structural changes in the antigen-loaded antibody.³² Surprisingly, it appears that covalent coupling of p24 leads to similar changes that ensure selective, yet not overwhelming, entry into the NALT. This opens the way to coupling other antigens, including more potent ones, when searching for efficacious protection against HIV infection. Another interesting and valuable feature associated with the ability of SIgA to transport antigens across M cells is its capacity to promote the induction of antigen-specific immune responses in the absence of a mucosal adjuvant. The expected booster effect of combining an adjuvant was noticed on immunization with p24-SIgA+HLT. p24-SIgA led to similar and occasionally better antibody responses compared with p24+HLT, further suggesting that coupling to SIgA positively changed the antigenicity of the p24 antigen.

Examples of the clinical relevance of nasal immunization are documented in the field and in the recent literature. This includes the approval in 2009 of the intranasal vaccine Flumist for the H1N1 influenza virus in the United States.³³ A promising mucosal candidate vaccine against HIV has shown that immunization of female nonhuman primates through the intranasal route with gp41 subunit antigens grafted on virosomes elicited full protection against repeated SHIV-SF162P3 vaginal challenges, a protection that relied on gp41-specific cervicovaginal SIgA and IgG antibodies.³⁴ Recently, other studies have established that mice had local and systemic immunity against HIV strains after intranasal immunization.³⁵⁻³⁷ From a more general perspective, the use of antigenized SIgA as a potent system for addressing nasal vaccination might deserve further research for other diseases involving infectious agents *per se* or in cancer conditions in which there are specific causative agents, such as human papilloma virus and *Helicobacter pylori*. The current work could contribute to the development of novel and safer delivery vehicles, together with identification of surface receptors that can be exploited for cell targeting and subsequent immune responses, features instrumental for the design of more effective intranasal vaccines against mucosal pathogens and pathologies.

Key messages

- Intranasal administration of the model antigen HIV p24gag coupled to SIgA ensure Dectin-1-dependent sampling across M cells targets DC-SIGN⁺ DCs located in the nasal cavity.
- Two-tiered mucosal and systemic antibody and cellular immune responses are elicited after 4 prime-boost immunizations.
- Protection is conferred in vaccinated mice challenged with a recombinant vaccinia virus expressing p24gag.

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TABLE E1. Cytokine profiles after nasal administration of the p24-SIgA complex

Cytokines	Serum						Vaginal lavage samples			
	p24+HLT	p24-SIgA	p24-SIgA+HLT	SIgA	p24	PBS	p24+HLT	p24-SIgA	p24-SIgA+HLT	SIgA
T_H1										
IL-12p40	152 ± 101	158 ± 62	172 ± 14	59 ± 11	106 ± 12	112 ± 66	<1.53	7 ± 4	2 ± 4	<1.53
IL-12p70	30 ± 7	39 ± 14	42 ± 12	26 ± 6	28 ± 3	32 ± 3	37 ± 11	37 ± 8	45 ± 24	33 ± 2
T_H2										
IL-5	19 ± 22	18 ± 27	31 ± 7	12 ± 1	15 ± 5	18 ± 5	<3.57	<3.57	<3.57	<3.57
IL-9	9 ± 13	184 ± 6	126 ± 15	8 ± 5	9 ± 4	8 ± 1	25 ± 33	31 ± 9	30 ± 41	12 ± 4
IL-10	58 ± 0	65 ± 8	63 ± 24	23 ± 17	41 ± 8	48 ± 25	<2.95	5 ± 0	8 ± 8	<2.95
IL-13	58 ± 1	252 ± 58	313 ± 11	47 ± 11	52 ± 3	50 ± 8	33 ± 25	39 ± 1	55 ± 44	<47.2
T_H17										
IL-6	12 ± 11	76 ± 64	62 ± 66	5 ± 9	9 ± 2	7 ± 4	2 ± 3	6 ± 11	5 ± 9	<0.74
IL-17F	<1	38 ± 10	30 ± 28	<1	<1	<1	<1	3 ± 0	11 ± 14	<1
IL-21	<6	<6	<6	<6	<6	<6	<6	<6	<6	<6
IL-22	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	2 ± 0	3 ± 1	2 ± 1	1 ± 0
IL-23p19	<3.4	<3.4	<3.4	<3.4	<3.4	<3.4	16 ± 32	41 ± 22	31 ± 15	<3.4
IL-31	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	49 ± 12	156 ± 19	221 ± 41	16 ± 9
IL-33	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5	3 ± 4	7 ± 0	5 ± 1	1 ± 1
CD40L	<3.6	<3.6	<3.6	<3.6	<3.6	<3.6	17 ± 12	39 ± 5	46 ± 12	49 ± 4
ICAM-1	<2.9	<2.9	<2.9	<2.9	<2.9	<2.9	<2.9	4 ± 4	<2.9	<2.9
MIP-3α	1 ± 0	56 ± 5	24 ± 29	6 ± 7	4 ± 1	4 ± 4	10 ± 14	25 ± 11	13 ± 0	5 ± 6
IR										
IL-1α	3 ± 2	12 ± 1	33 ± 48	5 ± 0	4 ± 0	5 ± 2	231 ± 11	207 ± 123	165 ± 99	54 ± 4
IL-1β	54 ± 85	84 ± 121	218 ± 46	56 ± 63	55 ± 28	57 ± 2	93 ± 10	425 ± 322	655 ± 764	55 ± 9
RANTES	41 ± 20	64 ± 11	35 ± 14	20 ± 30	31 ± 2	35 ± 15	<2.78	<2.78	<2.78	<2.78
TNF-α	57 ± 68	512 ± 58	347 ± 61	52 ± 56	55 ± 24	45 ± 4	21 ± 14	26 ± 1	26 ± 8	18 ± 28
Ch										
MIP-1α	<36	<36	<36	<36	<36	<36	<36	64 ± 43	148 ± 198	<36
MIP-1β	26 ± 21	89 ± 15	50 ± 15	29 ± 14	28 ± 8	25 ± 2	<3.3	27 ± 22	17 ± 26	<3.3
Eotaxin	<257	364 ± 216	246 ± 63	<257	<257	<257	270 ± 170	404 ± 23	329 ± 241	<257
G-CSF	<5.1	6 ± 3	6 ± 0	<5.1	<5.1	<5.1	81 ± 61	2240 ± 1479	615 ± 83	63 ± 87
GM-CSF	<21.2	<21.2	<21.2	<21.2	<21.2	<21.2	89 ± 47	114 ± 3	113 ± 24	26 ± 22
KC	<3.2	14 ± 9	11 ± 13	<3.2	<3.2	<3.2	36 ± 16	86 ± 19	73 ± 8	49 ± 53
MCP-1	34 ± 46	106 ± 33	70 ± 28	27 ± 37	30 ± 17	31 ± 5	29 ± 40	79 ± 74	140 ± 196	47 ± 69

Cytokine concentrations were determined in triplicate by using a Luminex assay with 5 mice per 2 independent groups. Each value shows the mean quantity (in picograms per milliliter) of each cytokine, followed by the range.

<, Less than the detection limit; *CD40L*, CD40 ligand; *Ch*, chemokines; *G-CSF*, granulocyte colony-stimulating factor; *ICAM-1*, intercellular adhesion molecule 1; *IR*, mediator of the inflammatory response; *KC*, keratinocyte chemoattractant.

TABLE E1. (Continued)

Vaginal lavage samples		Feces						Intestinal cell lysate					
p24	PBS	p24+HLT	p24-SIgA	p24-SIgA+ HLT	SIgA	p24	PBS	p24+HLT	p24-SIgA	p24-SIgA+ HLT	SIgA	p24	PBS
<1.53	<1.53	<1.53	<1.53	<1.53	<1.53	<1.53	<1.53	<1.53	7 ± 1	23 ± 5	<1.53	<1.53	<1.53
35 ± 5	34 ± 3	10 ± 2	6 ± 7	13 ± 1	11 ± 6	10 ± 0	9 ± 1	19 ± 1	23 ± 1	21 ± 4	19 ± 9	19 ± 1	12 ± 0
<3.57	<3.57	<3.57	<3.57	<3.57	<3.57	<3.57	<3.57	<3.57	<3.57	<3.57	<3.57	<3.57	<3.57
19 ± 8	17 ± 9	<6.89	<6.89	<6.89	<6.89	<6.89	<6.89	34 ± 18	101 ± 24	73 ± 58	59 ± 82	47 ± 0	43 ± 18
<2.95	<2.95	<2.95	<2.95	<2.95	<2.95	<2.95	<2.95	<2.95	7 ± 2	7 ± 4	<2.95	<2.95	<2.95
<47.2	<47.2	<47.2	<47.2	<47.2	<47.2	<47.2	<47.2	<47.2	48 ± 22	72 ± 14	<47.2	<47.2	<47.2
<0.74	<0.74	<0.74	<0.74	<0.74	<0.74	<0.74	<0.74	<0.74	<0.74	<0.74	<0.74	<0.74	<0.74
<1	<1	8 ± 11	8 ± 2	8 ± 1	2 ± 0	4 ± 5	4 ± 2	2 ± 6	44 ± 24	16 ± 18	<1	<1	<1
<6	<6	<6	<6	<6	<6	<6	<6	10 ± 23	12 ± 11	15 ± 20	19 ± 25	14 ± 6	19 ± 6
2 ± 0	2 ± 1	<0.5	2 ± 0	2 ± 0	1 ± 0	<0.5	1 ± 1	14 ± 19	8 ± 5	10 ± 11	6 ± 7	10 ± 8	12 ± 6
10 ± 14	8 ± 9	7 ± 2	19 ± 2	36 ± 14	4 ± 1	5 ± 1	5 ± 2	13 ± 12	129 ± 9	121 ± 120	15 ± 20	14 ± 5	11 ± 2
33 ± 12	32 ± 23	34 ± 18	41 ± 5	63 ± 22	26 ± 4	30 ± 4	27 ± 6	18 ± 26	282 ± 83	350 ± 338	14 ± 18	16 ± 8	13 ± 3
<0.5	<0.5	4 ± 3	2 ± 0	2 ± 0	1 ± 0	3 ± 1	2 ± 2	18 ± 22	127 ± 171	271 ± 33	15 ± 19	16 ± 8	12 ± 2
33 ± 4	38 ± 22	10 ± 11	12 ± 0	10 ± 0	7 ± 2	8 ± 4	8 ± 2	5 ± 1	120 ± 40	107 ± 131	6 ± 6	5 ± 1	5 ± 1
<2.9	<2.9	<2.9	<2.9	<2.9	<2.9	<2.9	<2.9	9 ± 10	65 ± 19	46 ± 64	8 ± 10	8 ± 4	8 ± 0
7 ± 6	7 ± 3	10 ± 9	12 ± 2	6 ± 1	6 ± 5	8 ± 4	7 ± 2	8 ± 13	68 ± 60	353 ± 468	7 ± 8	8 ± 5	8 ± 1
143 ± 45	109 ± 125	8 ± 11	39 ± 26	175 ± 29	5 ± 4	6 ± 5	6 ± 1	12 ± 3	59 ± 4	70 ± 6	4 ± 7	8 ± 3	7 ± 6
74 ± 5	70 ± 26	42 ± 23	41 ± 4	37 ± 7	13 ± 16	27 ± 14	20 ± 20	156 ± 105	143 ± 67	433 ± 151	29 ± 42	92 ± 65	82 ± 90
<2.78	<2.78	<2.78	<2.78	<2.78	<2.78	<2.78	<2.78	119 ± 28	2340 ± 3310	2104 ± 2912	16 ± 24	67 ± 34	45 ± 73
20 ± 4	21 ± 2	6 ± 4	8 ± 7	5 ± 2	<5.8	<5.8	<5.8	19 ± 15	24 ± 4	29 ± 6	16 ± 12	17 ± 6	14 ± 2
<36	<36	<36	<36	<36	<36	<36	<36	71 ± 84	50 ± 69	158 ± 79	<36	<36	<36
<3.3	<3.3	<3.3	<3.3	<3.3	<3.3	<3.3	<3.3	<3.3	18 ± 2	14 ± 21	<3.3	<3.3	<3.3
<257	<257	<257	<257	<257	<257	<257	<257	<257	260 ± 72	348 ± 61	<257	<257	<257
72 ± 25	70 ± 13	<5.1	<5.1	<5.1	<5.1	<5.1	<5.1	<5.1	<5.1	<5.1	<5.1	<5.1	<5.1
58 ± 1	45 ± 44	26 ± 21	14 ± 19	16 ± 21	18 ± 14	22 ± 9	20 ± 6	68 ± 4	77 ± 0	74 ± 24	<21.2	37 ± 16	31 ± 44
42 ± 8	47 ± 9	<3.2	<3.2	<3.2	<3.2	<3.2	<3.2	<3.2	66 ± 91	64 ± 58	<3.2	<3.2	<3.2
38 ± 17	35 ± 13	<22.4	<22.4	<22.4	<22.4	<22.4	<22.4	<22.4	54 ± 10	77 ± 21	<22.4	<22.4	<22.4