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A Neonatal Fc Receptor-Targeted Mucosal Vaccine Strategy Effectively Induces HIV-1 Antigen-Specific Immunity to Genital Infection▽

Li Lu,[1] Senthilkumar Palaniyandi,[1] Rongyu Zeng,[1] Yu Bai,[1] Xindong Liu,[1] Yunsheng Wang,[2] C. David Pauza,[3] Derry C. Roopenian,[4] and Xiaoping Zhu[1,5]*

Laboratory of Immunology,[1] Virginia-Maryland Regional College of Veterinary Medicine,[2] and Maryland Pathogen Research Institute,[5] University of Maryland, College Park, Maryland 20742; The Jackson Laboratory, 600 Main Street, Bar Harbor, Maine 04609[4]; and Institute of Human Virology, University of Maryland School of Medicine, Baltimore, Maryland 21201[3]

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Strategies to prevent the sexual transmission of HIV include vaccines that elicit durable, protective mucosal immune responses. A key to effective mucosal immunity is the capacity for antigens administered locally to cross epithelial barriers. Given the role of neonatal Fc receptor (FcRn) in transferring IgG across polarized epithelial cells which line mucosal surfaces, FcRn might be useful for delivering HIV vaccine antigens across mucosal epithelial barriers to the underlying antigen-presenting cells. Chimeric proteins composed of HIV Gag (p24) fused to the Fc region of IgG (Gag-Fc) bind efficiently to airway mucosa and are transported across this epithelial surface. Mice immunized intranasally with Gag-Fc plus CpG adjuvant developed local and systemic immunity, including durable B and T cell memory. Gag-specific immunity was sufficiently potent to protect against an intravaginal challenge with recombinant vaccinia virus expressing the HIV Gag protein. Intranasal administration of a Gag-Fc/CpG vaccine protected at a distal mucosal site. Our data suggest that targeting of FcRn with chimeric immunogens may be an important strategy for mucosal immunization and should be considered a new approach for preventive HIV vaccines.

The majority of human immunodeficiency virus type 1 (HIV-1) infections are acquired by mucosal exposure. HIV-1 penetrates the mucosal epithelium by infecting epithelial or dendritic cells (DCs) (18). Mucosal tissues are the predominant sites for HIV-1 replication before systemic spread. The time gap between original viral contact and plasma viremia is 7 to 21 days in macaque models (6, 18), which may be a window of opportunity for local immunity to prevent systemic infection. However, parenteral immunization and systemic immunity have not been able to produce potent sterilizing immunity to HIV (6, 9, 18). Poor mucosal immune responses are due partly to the physical properties of this epithelial barrier. Formed by tight junctions among polarized epithelial cells, mucosal epithelium reduces the chances for lumenal or external antigens to contact immune effector cells, including T or B cells and antigen-presenting cells within the lamina propria (35). Hence, native HIV proteins cross the mucosal barrier inefficiently and are poor immunogens for eliciting protective responses (36). Mucosal immune responses might be improved by engineering antigens for efficient mucosal delivery.

Some approaches have already been explored for vaccine antigen delivery across mucosal barriers. One example is antigen targeting to differentiated microfold (M) cells that normally pass antigens along to underlying DCs and macrophages within mucosal tissues (33, 37). Unfortunately, M cells are relatively uncommon compared with the number of less-differentiated columnar epithelial cells that constitute the majority of mucosal surfaces. It is important to explore alternate vaccine delivery strategies that target immunogens to a majority of mucosal epithelial cells for HIV vaccine antigens.

A more promising strategy focuses on the neonatal Fc receptor (FcRn) for IgG, a major histocompatibility complex (MHC) class I-related molecule (7) first identified in intestinal epithelial cells of a suckling rodent, where it is expressed at high levels. We now know that FcRn is expressed in a variety of cells and tissues, including mucosal epithelial cells of adult animals and humans (42, 48). A normal function of FcRn is to transfer maternal IgG across polarized placental epithelial cells (48), which delivers maternal IgG to the fetus and provides pathogen immunity before the neonatal immune system develops. FcRn also transports IgG across polarized epithelial cells lining mucosal surfaces (10, 26). In addition to its function as a transporter, FcRn extends the half-life of IgG antibodies by recycling them through gut intestinal and other types of cells, such as endothelial cells (16, 21, 22). The capacity to transfer IgG and extend the half-life of these antibodies is based on the abilities of FcRn to bind the Fc-region of IgG at acidic pH (6.0 to 6.5) and to release IgG at neutral or higher pH (48). In mice, amino acids I253, H310, and H433 are located at the interface between the CH2 and CH3 domains of IgG and are particularly important for pH-dependent binding to FcRn in acidified early endosomal vesicles (24). In that subcellular compartment, FcRn binds IgG that has entered by pinocytosis or endocytosis. Subsequently, FcRn rescues the IgG from lysosomal degradation by transporting it to the opposite surface of polarized cells, where the ex-

* Corresponding author. Mailing address: VA-MD Regional College of Veterinary Medicine, University of Maryland, 8075 Greenmead Drive, College Park, MD 20742. Phone: (301) 314-6814. Fax: (301) 314-6855. E-mail: xzhu1@umd.edu.

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tracellular pH causes IgG release from FcRn. IgG which does not bind intracellular FcRn traffics to the lysosome, where it is degraded (48).

The major goal of mucosal immunization is to provide protection against pathogens which cross epithelial barriers in mucosal tissues. Our understanding of FcRn-mediated IgG transport across mucosal epithelial barriers suggests that IgG Fc-fused antigens will be transported from the lumenal surface, through the mucosal epithelium, to underlying antigen-presenting cells. Mucosal immune responses will be more effective against ingested or inhaled vaccine antigens if they gain access to mucosal lymphoid tissues (2, 36).

Recent studies show that the targeting of HIV antigens to FcRn is feasible and might improve mucosal immune responses. In the rodent, IgG-mediated immune complexes are transported from the mucosal lumen (41, 52) to encounter underlying DCs (52). In our recent study, FcRn efficiently transported IgG Fc-fused herpesvirus antigen and induced protective immunity to a viral challenge (30, 50). Here we fused the p24 protein from HIV Gag with IgG heavy chain (Gag-Fc). We then determined the ability of FcRn to deliver this Gag-Fc fusion protein across mucosal barriers and defined protective immune responses to Gag-Fc when combined with the Toll-like receptor 9 agonist CpG to stimulate innate cells, including antigen-presenting cells. We found that FcRn-targeted mucosal immunization was effective at inducing Gag-specific antibody responses in serum or mucosal secretions, and high levels of stable immune memory were obtained. Memory responses were dominated by antibody-secreting plasma cells and gamma interferon (IFN-γ)-producing T cells. These immune responses conferred resistance to viral replication after a vaginal challenge of immune mice with vaccinia virus (VV) expressing HIV-1 Gag. Our results demonstrate that the FcRn-dependent IgG transcytotic pathway is a mechanism for delivering HIV to the mucosal immune system and may elicit protective responses to virus exposure at mucosal surfaces.

MATERIALS AND METHODS

Mice, cells, antibodies, and viruses. Six- to 8-week-old female inbred C57BL/6 mice were purchased from the National Cancer Institute or Charles River. FcRn knockout (KO) mice on a C57BL/6 background (43) were from the Jackson Laboratory. All mice were bred and maintained in HEPA-filtered caging units. Animal experiments were approved by the Animal Care and Use Committee at the University of Maryland.

Madin-Darby canine kidney (MDCK) cells expressing rat FcRn were obtained from Pamela Bjorkman at the California Institute of Technology. Vero and Chinese hamster ovary (CHO) cells were purchased from the American Tissue Culture Collection. MDCK, Vero, and CHO cells were maintained in complete Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies) supplemented with 10 mM HEPES, 10% fetal bovine serum, 2 mM L-glutamine, nonessential amino acids, penicillin (0.1 μg/ml), and streptomycin (0.292 μg/ml). Recombinant MDCK and CHO cells were also grown under 400 μg/ml G418 if necessary. Spleen or bone marrow cells were grown in complete RPMI 1640 medium. All cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. Affinity-purified antibody for mouse FcRn was produced as previously described (51). Purified mouse IgG and chicken IgY were from Rockland Laboratories (Gilbertsville, PA). Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit or rabbit anti-mouse antibody was purchased from Pierce (Rockland, IL); HRP-conjugated goat anti-mouse IgG1, IgG2a, and IgG3 were from Southern Biotech. The purified recombinant HIV Gag p24 proteins were from Meridian Life Science (Cincinnati, OH). Mouse anti-Gag p24 hybridoma, a plasmid encoding HIV-1 BH10 Gag, and a recombinant VV expressing HIV-1 BH10 Gag (rVV-Gag, vP1287) were acquired from the NIH AIDS Research and Reference Reagent Program.

Western blotting and SDS-PAGE. Purified proteins or cell lysates were resolved by 12% SDS-PAGE under reducing or nonreducing conditions. Proteins were transferred to a nitrocellulose membranes (Schleicher & Schuell); membranes were blocked with 5% nonfat milk, probed separately with primary antibodies for 1 h, and then incubated with HRP-conjugated rabbit anti-mouse or donkey anti-rabbit antibody for 1 h. Proteins were visualized by the ECL method (Pierce, Rockland, IL).

Expression of Gag-Fc fusion proteins. The cDNA encoding Gag p24 from HIV-1 isolate BH10 was amplified by PCR primers (5′-CTGGTCGCTTCCGTGCTACCTAGAACTTTAAATGCATG-3′, 5′-AGATCCCGAGCCACCTCCTCCGGACCCACCCCCGCCTGATCCCAAAACTCTTGCCTTATG-3′) from plasmid pBKBH10S, which was provided by the NIH AIDS Reference Reagent Program. The antisense primer introduces an extension with 12 codons for glycine and serine residues (single-letter code, GSSGGGSSGGSSS). The Fc fragment of mouse IgG2a, containing the hinge, CH2, and CH3 domains, was amplified by reverse transcription-PCR from the OKT3 hybridoma. Similarly, the forward primer for IgG2a Fc has complementary glycine and serine codons for Gag. A mutant Fc (HQ310 and HN433) was made by oligonucleotide site-directed mutagenesis (Clontech, Mountain View, CA) as described previously (24, 50) and designated Fc/mut. Oligonucleotide site-directed mutagenesis was used to replace the C1q binding motif consisting of Glu318, Lys320, and Lys322 with Ala residues in the Fc fragment (12). Fusions were then performed by a PCR-based gene assembly approach by mixing the cDNA for Gag and the Fc fragments. All of these DNA fragments were digested with BamHI and EcoRI and then ligated into an engineered pCDNA3 vector carrying a CD5 protein secretion signal sequence. Each construct was verified by DNA sequencing.

Plasmids containing the chimeric wild-type Gag-Fc/wt or mutant Gag-Fc/mut fragment were transfected into CHO cells with Effectene (Qiagen, Valencia, CA). G418-resistant clones were selected and tested for the secretion of Gag-Fc fusion proteins. SDS-PAGE and Western blotting were performed to assess the recombinant fusion proteins in serum-free medium (Invitrogen, Carlsbad, CA) using HRP-conjugated rabbit anti-mouse IgG or anti-Gag p24 antibody. Proteins were made from CHO cell supernatants first cleaned by ultrafiltration and then further purified by affinity chromatography using protein A-Sepharose 4 Fast Flow (Amersham Pharmacia, Piscataway, NJ) for Gag-Fc/wt or a goat anti-mouse IgG affinity column (Rockland) for Gag-Fc/mut proteins. Protein concentrations were measured with Bradford protein assay kits (Pierce) using mouse IgG2a as the standard.

In vitro and *in vivo* transcytosis. The *in vitro* IgG transport assay was performed as a modification of previously described methods (28, 45). MDCK cells expressing rat FcRn (45) were grown on Transwell filter inserts (Corning, Lowell, MA) to form a monolayer exhibiting a transepithelial electrical resistance of 300 Ω cm^2, as measured with planar electrodes (World Precision Instruments). Monolayers were equilibrated in serum-free medium for 3 h. Fusion proteins at a final concentration of 0.1 mg/ml were applied to the apical compartment and incubated for 1 h at 37°C with DMEM with or without 1 mg/ml chicken IgY as a competitor. Transported proteins were sampled from the basolateral chamber and analyzed by reducing SDS-PAGE and Western blotting–enhanced-chemiluminescence (ECL) assay. For *in vivo* transport assays, 20 μg of Gag-Fc fusion protein or Gag alone in 20 μl of phosphate-buffered saline (PBS) was administered intranasally (i.n.) to wild-type or FcRn KO mice after anesthesia with 100 μl of avertin (40 mg/ml). Transported proteins in serum were determined by HIV p24 antigen enzyme-linked immunosorbent assay (ELISA; Zeptomatrix Corporation, Buffalo, NY).

Mouse immunization and virus challenge. Groups of 5 mice were immunized i.n. with 20 μg Gag-Fc/wt, Gag-Fc/mut, or Gag protein in combination with 20 μg CpG ODN1826 (5′-TCCATGACGTTCCTGACGTT-3′; Invivogen, San Diego, CA) at weeks 0 and 2, respectively. An additional group of 5 mice was immunized with PBS. For i.n. inoculation, 20 μl protein or PBS was applied to each nostril of mice anesthetized with 100 μl of avertin (40 mg/ml; Sigma). Mice were kept on their backs under anesthesia to allow the inoculums to be taken up.

Mice were challenged with viruses by intravaginal inoculation as described previously (17). Five days before each inoculation, mice were treated subcutaneously with 2 mg of depot medroxyprogesterone acetate (Depo-Provera). Mice were anesthetized with avertin (40 mg/ml; Sigma) and exposed intravaginally to rVV-Gag (5×10^7 PFU) in 30 μl of PBS. Mice were kept on their backs under anesthesia for 1 h. Five days after the challenge, mice were sacrificed. Paired ovary tissues were removed and homogenized in nylon mesh. For virus titration, serially diluted ovary samples were inoculated into Vero cells and incubated for 45 min at 37°C. The cells were washed, and DMEM containing 0.8% methylcellulose and 2% fetal bovine serum was overlaid on the cells. The cells were

cultured for 3 days, the overlay was removed, and the cells were fixed with 3.7% formaldehyde for 1 h and stained with 1% crystal violet. Clear plaques were counted to determine virus titers in terms of plaque-forming units.

ELISA and enzyme-linked immunospot (ELISPOT) assay. HIV Gag p24-specific antibodies were detected in serum, bronchoalveolar lavage (BAL) fluid, and vaginal fluid. High-binding ELISA plates (Maxisorp; Nunc) were coated with purified p24 protein (1 μg/well) in PBS. The remaining ELISA procedures were performed as described previously (50). Antibody titers represent the highest dilution of samples showing a 2-fold increase in optical density at 450 nm over controls. The mean log of the endpoint dilutions was determined and used to calculate the average endpoint titer. Each assay was done in triplicate. The mouse cytokines IFN-γ, interleukin-2 (IL-2), and IL-4 from the cell culture supernatant were analyzed with ELISA kits (BD Biosciences).

For the measurement of HIV Gag-specific antibody-producing plasma cells, 96-well ELISPOT assay plates (Millipore) were coated with 5 μg/ml Gag and blocked with 5% fetal calf serum (Invitrogen). Serial dilutions of single-cell bone marrow suspensions were prepared in RPMI 1640 medium and incubated in the coated wells for 24 h at 37°C in 5% CO_2. Cells were removed; the plates were washed 5 times with 0.1% Tween 20 in PBS and then incubated with biotin-labeled goat anti-mouse IgG-specific antibody (Sigma). After washing, avidin-conjugated HRP (Vector Laboratories) was incubated and developed with the AEC kit (BD Biosciences). Spots were counted with an ELISPOT Reader.

Preparation of single-cell suspensions from spleen and vaginal tissues. Spleens were made into single-cell suspensions by passage through a sterile mesh screen as described previously (50). Vaginal cells were isolated from tissue that was excised, cut longitudinally, and minced with a sterile scalpel in complete RPMI 1640 culture medium. Minced tissues (epithelium and lamina propria) were digested in complete medium with sterile 0.25% collagenase D at 37°C for 30 min. After collagenase treatment, tissues and cells were filtered through a sterile gauze mesh and washed with RPMI 1640 medium and additional tissue debris was excluded by slow-speed centrifugation for 1 min. Cells were collected from the supernatant by centrifugation and resuspended in Hanks' balanced salt solution (HBSS), and viable cells were counted by trypan blue dye exclusion.

Flow cytometry. Flow cytometry was performed as described previously (50). In brief, cells were preincubated with an Fc block (monoclonal antibody [MAb] 2.4G2 to CD16-CD32; PharMingen, San Diego, CA) and washed in fluorescence-activated cell sorting (FACS) buffer (HBSS, 2% bovine serum albumin, 0.01% sodium azide). Blocked cells were incubated with specific antibody directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), cyanine dye Cy7, and peridinin-chlorophyll proteins and then washed, transferred to FACS buffer, and analyzed using a FACSAira (Becton Dickinson, Mountain View, CA) and FlowJo software (Tree Star). The MAbs (PharMingen) we used were as follows: anti-CD3ε, 500A2; anti-CD4, RM4-5; anti-CD8, 53-6.7; anti-IFN-γ, XMG1.2; anti-B220, RA3-6B2; anti-FAS, Jo2. Peanut agglutinin (PNA)-FITC was from Sigma. Purified HIV Gag proteins were labeled with the Alexa Fluor 647 protein labeling kit (Invitrogen). The isotype control antibodies included in each experiment were considered to represent the true baseline fluorescence used to evaluate and illustrate the results for cell-specific antigen markers.

T cell proliferation assay. Carboxyfluorescein diacetate succinimidyl ester (CFSE, 5 mM stock; Invitrogen) dilution was used to assess T cell proliferation in response to Gag antigen. CFSE was added to splenocyte suspensions of 10^7/ml in prewarmed PBS–0.1% bovine serum albumin to a final concentration of 2 μM; reaction mixtures were incubated for 10 min at 37°C. After labeling with CFSE, splenic T cells were added in the presence of HIV Gag p24 protein (20 μg/ml), medium alone, or anti-CD3 (0.1 μg/ml) plus anti-CD28 (2 μg/ml) as a positive control. Cells (5×10^5) were cultured for 4 days. The cells were then harvested and used in flow cytometry assays.

Intracellular cytokine staining. Intracellular IFN-γ production by primed $CD4^+$ and $CD8^+$ T cells was evaluated using bulk splenocytes or isolated vaginal infiltrating lymphocytes incubated for 12 h with the purified Gag protein at 20 μg/ml or medium alone. Cells were incubated for 6 h with 10 μg/ml brefeldin A (Sigma). Cells were then incubated with the 2.4G2 MAb and stained with PE-conjugated anti-mouse CD3ε FITC-conjugated anti-CD4 and APC-Cy7-conjugated anti-mouse CD8 antibodies for 30 min at 4°C. The cells were fixed, permeabilized (Cytofix/Cytoperm Plus; BD Biosciences), and stained with APC–anti-IFN-γ MAb (XMG1.2; BD Biosciences) for 30 min at 4°C. Cells were analyzed by flow cytometry.

Histological analysis. Immunohistochemical staining of mouse FcRn was performed using an affinity-purified rabbit anti-mouse FcRn antibody (51). Briefly, the lungs and trachea were excised and embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN). Thin sections cut with a cryostat were transferred to glass slides and stored at −80°C. Before staining, sections were fixed in ice-cold acetone for 10 min. After extensive washes in PBS and blocking buffer (PBS–2% bovine serum albumin–10% normal goat serum) for 1 h, sections were incubated with affinity-purified rabbit anti-mouse FcRn antibody, followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG. Tissues were washed at least three times with 0.1% Tween 20 in PBS. Nuclei were then labeled with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min. Coverslips were mounted on slides with the ProLong antifade kit (Molecular Probes) and examined using a Zeiss LSM 510 confocal fluorescence microscope. Images were handled in Adobe Photoshop 7.0.

Statistics analysis. Antibody titers, serum Gag concentrations, cytokine concentrations, and virus titers were assessed with unpaired two-tailed *t* tests. GraphPad Prism 5 was the software used for statistical analyses.

RESULTS

Production and transcytosis of HIV Gag-Fc fusion proteins. To determine whether HIV antigens targeted to FcRn would elicit antibody and cellular immune responses, we first generated the fusion protein HIV Gag-Fc/wt by cloning HIV Gag in frame with the carboxyl terminus of the heavy chain of mouse IgG2a antibody (Fig. 1A). We used the mouse IgG2a Fc fragment, since mouse IgG2a, but not IgG1, is capable of binding mouse FcγRI in antigen-presenting cells. We also generated a Gag-Fc mutant version that does not bind FcRn by creating point mutations (HQ310 and HN431) known to prevent FcRn binding to the Fc domain. These same Fc mutations in IgG1 Fc are known to exhibit a 100-fold reduction in binding to FcRn (24). In all of the constructs, constant regions of mouse IgG2a were also modified to remove the complement C1q-binding motif (12) and produce nonlytic fusion proteins.

The fusion proteins were synthesized in CHO cells transfected with the Gag-Fc constructs. Secreted Gag-Fc fusion proteins formed monomers under reducing conditions but were disulfide-linked homodimers under nonreducing conditions in Western blotting using both the affinity-purified anti-Gag (Fig. 1B, top) and anti-mouse IgG Fc antibodies (Fig. 1B, bottom). Functional testing of the Fc domain was confirmed by precipitating Gag-Fc/wt, but not Gag-Fc/mut, protein with staphylococcal protein A on beads. It has been shown that protein A and FcRn recognize overlapping amino acids of IgG Fc and mutations in this region can affect both properties. As a result, protein A effectively and competitively inhibits IgG binding to FcRn (unpublished results). This implies that Fc portions of IgG in Gag-Fc/wt maintain all of the structures necessary for the binding of FcRn.

To ascertain whether the Gag-Fc/wt, but not the Gag-Fc/mut, fusion protein is transported by FcRn, we used an MDCK-FcRn cell line to transport Gag-Fc fusion proteins. MDCK cells expressing rat FcRn and β_2-microglobulin have been shown to specifically transport murine IgG *in vitro* (45). Hence, FcRn-dependent transcytosis of purified Gag-Fc/wt protein applied to the apical reservoir was transported to the basolateral reservoir (Fig. 1C, lanes 4 and 5) and detected by Western blotting. In contrast, the Gag-Fc/mut (Fig. 1C, lanes 2 and 3) and chicken IgY (lanes 2 to 5) proteins were not transported across the MDCK-FcRn monolayer, suggesting that Gag-Fc/wt was transported specifically after binding FcRn. Transport was not inhibited by an excessive amount of chicken IgY, which does not bind FcRn (Fig. 1C, lanes 4 and 5).

We then asked whether Gag-Fc could be detected in serum after i.n. inoculation. Expression of murine FcRn in the lungs (44) was verified in epithelial cells of the trachea and lungs, but not of the intestines, of adult mice and compared

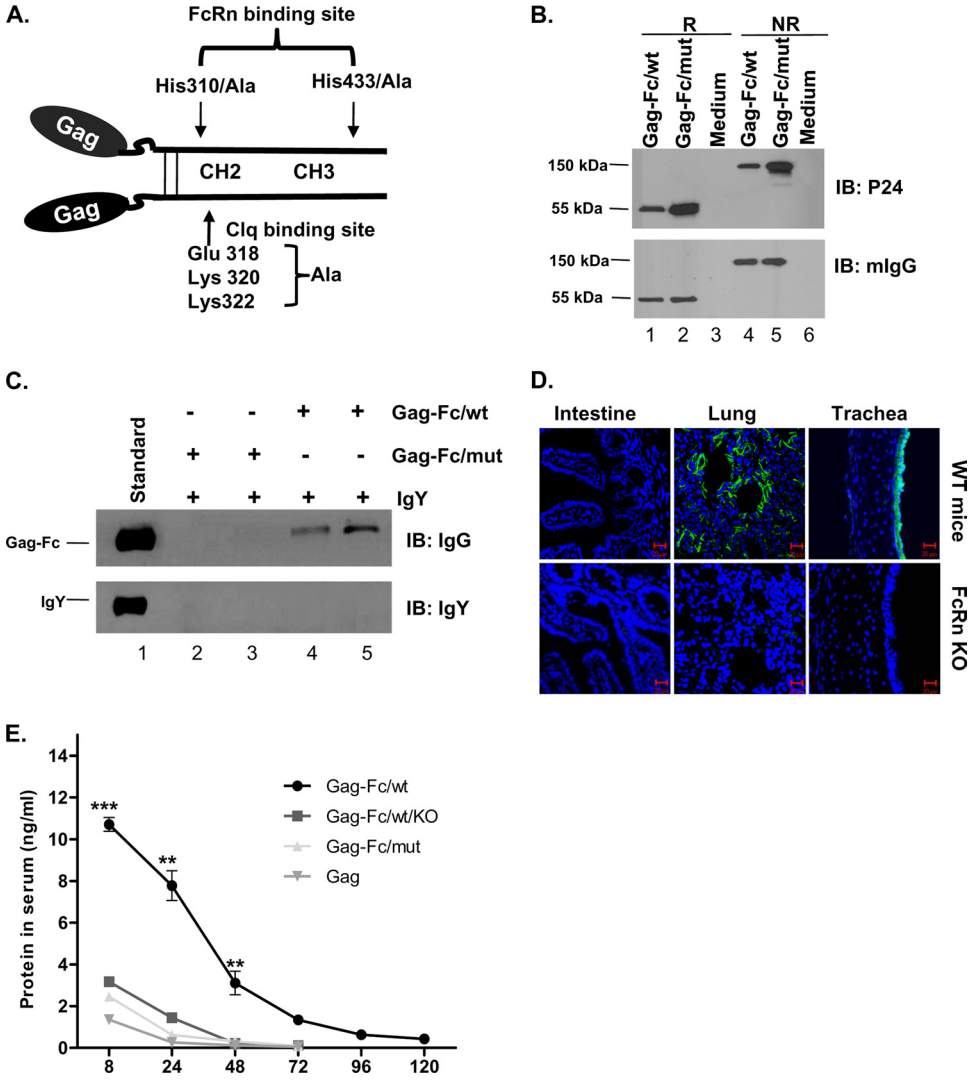

FIG. 1. Design and characterization of HIV-1 Gag fused to IgG Fc fragment and FcRn-dependent transcytosis of Gag-Fc/wt. (A) Schematic illustration of the genetic fusion of HIV Gag and murine Fcγ2a cDNA to create a Gag-Fc fusion gene. Mutations were made in the CH2 domain of the Fcγ2a fragment by using site-directed mutagenesis to replace Glu318, Lys320, and Lys322 with Ala residues to remove the complement C1q binding site and to replace His 310 and His 433 with Ala residues to eliminate FcRn binding sites. (B) The Gag-Fc fusion proteins were secreted by CHO cells. Gag-Fc was recognized by either an anti-Gag MAb (top) or rabbit anti-mouse IgG (bottom). The fusion protein appeared as a dimer under nonreducing (NR) conditions or as a monomer under reducing (R) conditions. IB, immunoblot. (C) Transport of Gag-Fc/wt fusion protein in MDCK-FcRn cells. MDCK-FcRn cells were plated onto 24-mm Transwell filter inserts and grown for 3 to 6 days to allow the formation of a polarized monolayer with a transepithelial electrical resistance of greater than 300 Ω cm^2. Purified Gag-Fc/wt (100 μg/ml) and chicken IgY were applied to the apical reservoir, and transcytosis was allowed to proceed for 2 h. The proteins were collected from the basolateral reservoir and blotted with anti-Gag or anti-IgY antibody under reducing conditions. The Gag-Fc/wt fusion protein (lanes 4 and 5, top), but not Gag-Fc/mut (lanes 2 and 3, top) or IgY (bottom), was detected by Western blotting. Lane 1, representing Gag-Fc/wt or IgY protein, was used as a positive control. (D) Expression of mouse FcRn in the tracheas and lungs of adult mice. Frozen sections of tissue samples obtained from wild-type or FcRn KO mice were stained with affinity-purified rabbit anti-FcRn antibody, followed by Alexa Fluor 488-conjugated IgG (green). FcRn staining was not observed in the presence of normal rabbit IgG. The nucleus is stained with DAPI (blue). The data are representative of sections from at least three independent mice. Images were originally obtained at ×40 magnification. Scale bars represent 20 μm. WT, wild type. (E) Transport of the Gag-Fc/wt protein across the mucosal barrier. Purified Gag, Gag-Fc/wt, and Gag-Fc/mut proteins (20 μg) were i.n. inoculated into wild-type and FcRn KO mice under the indicated inoculation conditions. Mouse sera were collected at the times indicated (in hours) on the *x* axis. The protein concentrations in the blood circulation after the transfer were measured by ELISA. ***, $P < 0.001$; **, $P < 0.01$.

with that in FcRn KO mice (43) by immunofluorescence staining using a mouse FcRn-specific antibody (Fig. 1D). To determine whether Gag-Fc appears in the circulation after i.n. inoculation, 20 μg of the Gag-Fc/wt, Gag-Fc/mut, or Gag protein was administered i.n. and measured in the blood 8 h later using ELISA. As shown in Fig. 1E, Gag-Fc/wt was detected readily in the serum of wild-type mice but not in that of FcRn KO animals. In addition, Gag-Fc/mut or Gag protein alone was transported poorly, indicating that i.n. administered Gag-Fc/wt efficiently crossed the mucosal barrier. Transported Gag-Fc/wt protein entered the circulation and persisted for 5 days, much longer than the

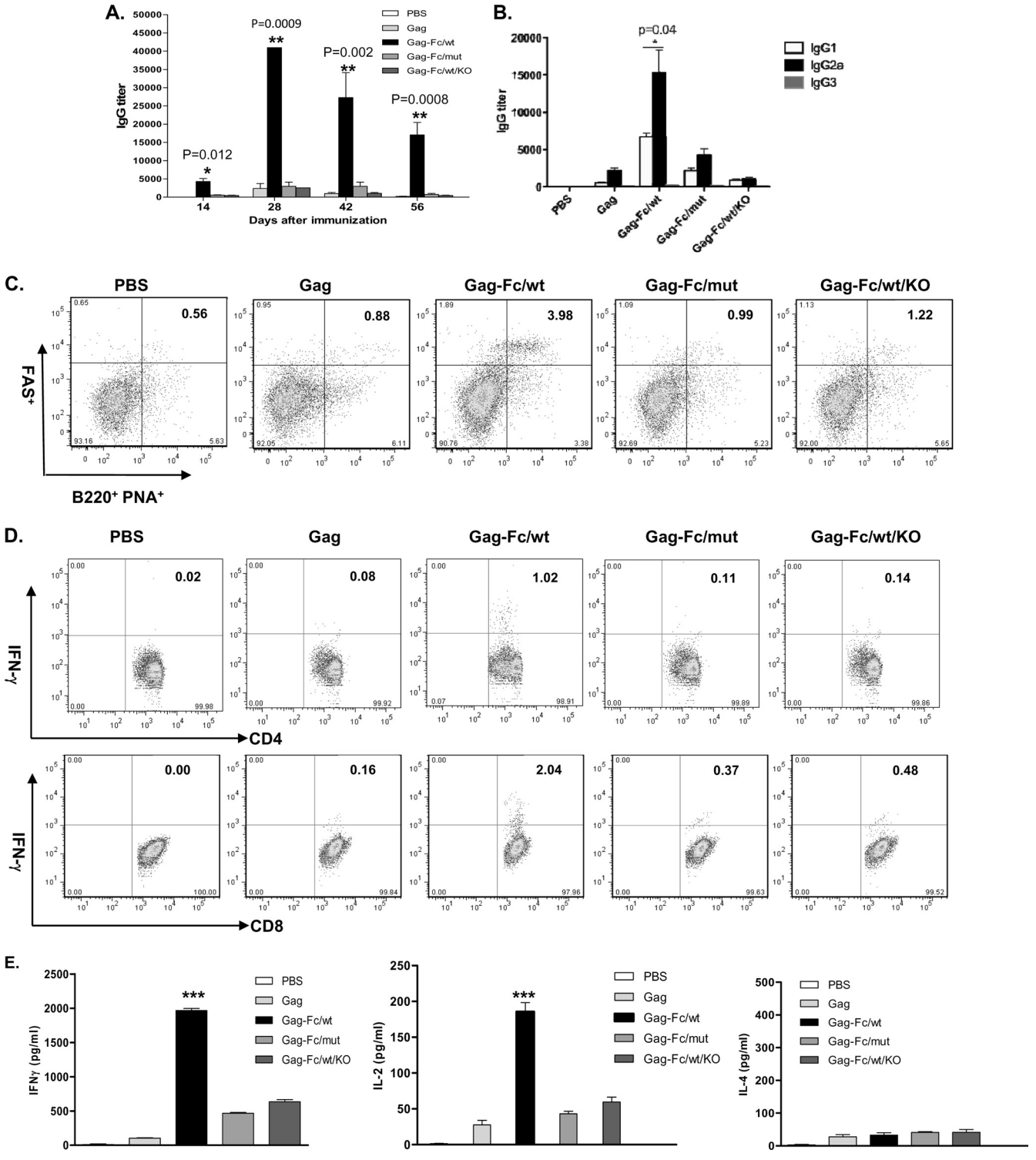

FIG. 2. FcRn-targeted mucosal vaccination induces enhanced Gag-specific antibody and T cell immune responses. A 20-μg sample of Gag-Fc/wt, Gag-Fc/mut, or Gag and 20 μg of CpG were i.n. administered to wild-type or FcRn KO mice at day 1, and the mice were given a booster immunization at day 14. (A) Measurement of anti-HIV Gag-specific IgG antibody titers in serum before and after booster immunization. The HIV Gag-specific IgG antibody titer in serum was measured by ELISA after the indicated number of days. Immunization conditions are displayed at the right. Asterisks denote significant differences as indicated. (B) Measurement of HIV Gag-specific IgG isotype titers in serum. Blood samples were taken from mice by tail bleeding. HIV Gag-specific IgG isotype titers in serum at 28 days were measured by ELISA. Immunization conditions are displayed at the bottom. (C) Detection of activated B cells in the GC of immunized mice by flow cytometry. Representative flow cytometric analyses of GC B cells among $B220^+$ B cells in the spleen at 10 days after booster immunization. Values are the percentages of activated GC B cells (PNA^+ FAS^+) among the gated $B220^+$ cells. (D) Percentages of IFN-γ-producing T cells in the spleen 7 days after booster immunization. Splenocytes from immunized mice were stimulated for 18 h with purified Gag or a medium control. Lymphocytes were gated by forward and side

other proteins tested (Fig. 1E), although it was difficult to determine the half-life of the other proteins accurately because they were transported so poorly. Taking these results together, we conclude that rodent FcRn can transport the Gag-Fc/wt fusion protein across the polarized epithelial cell monolayer lining the airway in an FcRn-dependent manner.

Strong anti-Gag antibody and T cell responses after FcRn-targeted mucosal immunization. To test whether FcRn-dependent transport across mucosal surfaces generates immune responses against HIV vaccine antigens, wild-type mice were immunized i.n. with Gag-Fc or Gag protein in combination with CpG and boosted 2 weeks later. Use of Gag alone allowed us to assess the efficiency of Gag-Fc/wt transport by FcRn *in vivo* and to determine the magnitude of increased immune responses to Gag-Fc/wt. DC capture antigen in mucosa-associated lymphoid tissues or in lymph nodes primes T cells that home to the mucosa (52). To overcome the normal tolerogenic function of at least some immature DCs *in vivo*, we included immunostimulatory DNA rich in CG motifs (CpG), an agonist for Toll-like receptor 9 (47). CpG is potent, since mice immunized i.n. with Gag-Fc/wt fusion protein in the presence of CpG developed IgG concentrations in their serum that were much higher than those of mice immunized with Gag-Fc/wt protein alone (unpublished data). First, we determined whether antigen targeted to FcRn by the Gag-Fc proteins elicits antibodies specific to HIV Gag protein. Antibody responses among immunized animals, including PBS control mice, were assessed at various time points up to 56 days after the primary immunization by measuring Gag-specific serum IgG. As shown in Fig. 2A, significantly higher titers of IgG, mainly restricted to the IgG2a subclass (Fig. 2B), were seen in Gag-Fc/wt-immunized mice than in the other groups. The antibody response was further associated with the germinal centers (GC) in the spleens of immunized mice. Spleens were removed 10 days after booster immunization. Splenocytes were gated on $B220^+$ cells and stained for the presence of PNA and FAS-positive B cells. Mice immunized with Gag-Fc/wt, but not those immunized with Gag-Fc/mut or the Gag protein alone, developed significant numbers (3.98% of the total isotype-switched B cells) of FAS^+ PNA^+ $B220^+$ B cells, suggesting that GC were formed after immunization (20, 34) (Fig. 2C). In addition, FcRn KO mice immunized with Gag-Fc/wt failed to show comparable numbers of FAS^+ PNA^+ $B220^+$ B cells. These results indicate that FcRn-targeted mucosal delivery of the Gag-Fc/wt results in an effective B cell response.

An effective immune response would also involve T cell activation (5). Seven days after boosting, splenocytes were harvested from immunized mice and pulsed with purified Gag; IFN-γ-producing T cells were measured by flow cytometry. We detected significant numbers of IFN-γ-producing $CD4^+$ (Fig. 2D, top) and $CD8^+$ (Fig. 2D, bottom) T cells in response to Gag in mice immunized with Gag-Fc/wt but not in any of the other groups, including mice immunized with the Gag-Fc/mut protein or FcRn KO mice immunized with the Gag-Fc/wt protein. In wild-type mice immunized with the Gag-Fc/wt protein, about 1% of the $CD4^+$ and 2% of the $CD8^+$ T cells responded to Gag stimulation. Thus, targeting of Gag-Fc/wt to FcRn via mucosal administration was at least 10 to 15 times as effective at initiating IFN-γ-producing $CD4^+$ and $CD8^+$ T cell immunity as in Gag-Fc/mut- or Gag-immunized mice. Cytokine responses were mainly IFN-γ and IL-2; little IL-4 was seen in cultures pulsed with antigen (Fig. 2E). Mucosal immunization with Gag-Fc/wt proteins therefore induced strong $CD4^+$ and $CD8^+$ T cell responses, whereas immunization with Gag-Fc/mut or Gag-Fc/wt in FcRn KO mice did not. We concluded that immunizing by targeting HIV antigens to FcRn together with CpG produces a strong response to HIV Gag, including both B and T cell immunity.

FcRn-targeted mucosal immunization significantly reduced viral replication after a challenge. To determine whether FcRn-mediated mucosal vaccine delivery could protect against viral infection at a distant mucosal site, we intravaginally challenged immune mice with 5×10^7 PFU of virulent rVV-Gag at 4 weeks after booster immunization. Ovary tissues were harvested at the peak of infection, day 5. Control (PBS-treated) mice had the highest titers of rVV-Gag in their ovaries after the virus challenge (Fig. 3A). Mice immunized with Gag-Fc/mut or FcRn KO mice immunized with Gag-Fc/wt also had high titers of rVV-Gag in their ovaries. In marked contrast to the virus titers of these control mice, the virus titers measured in the ovary tissues of wild-type mice immunized with the Gag-Fc/wt protein showed significantly lower levels of virus by day 5 after the challenge (Fig. 3A). Compared with the uterine sizes of Gag-Fc/mut-immunized wild-type mice or Gag-Fc/wt-immunized FcRn KO mice, those of mice immunized with Gag-Fc/wt protein after infection with rVV-Gag were much smaller, presumably because of reduced edema, hemorrhage, and inflammation (Fig. 3B). Control (uninfected) mice sampled at day 0 showed normal uterus morphology. The amounts of virus detected were consistent with the gross changes in uteri among groups of animals. Overall, these results demonstrate that i.n. administration of Gag-Fc/wt protein to wild-type mice efficiently induced protective immunity. These results suggest a significant role for the immune responses from FcRn-dependent mucosal immunization in the control of viral infection.

Induction of local mucosal immune responses. Sexually transmitted HIV enters through mucosal sites and spreads rapidly to distant mucosal and systemic lymphoid tissues. Local

scatter and T cells labeled with anti-CD3 and identified by their respective surface markers (CD4 and CD8) and intracellular IFN-γ staining. Immunization conditions are displayed at the top. The values in the quadrants are percentages of IFN-γ^+ $CD3^+$ $CD4^+$ (top) or IFN-γ^+ $CD3^+$ $CD8^+$ (bottom) T cells. Isotype controls included FITC-conjugated mouse IgG1 and show the baseline response. (E) Cytokine secretions from stimulated spleen T cells. Splenocytes were collected from three immunized mice per group on day 7 after booster immunization and pooled. Cells were stimulated *in vitro* specifically with purified Gag for 24 h. Cytokines IFN-γ, IL-2, and IL-4 in the culture supernatant were detected by ELISA. They are presented as picograms/ml of culture supernatant. The data shown are representative of three experiments with three mice pooled in each experiment. ***, $P < 0.001$.

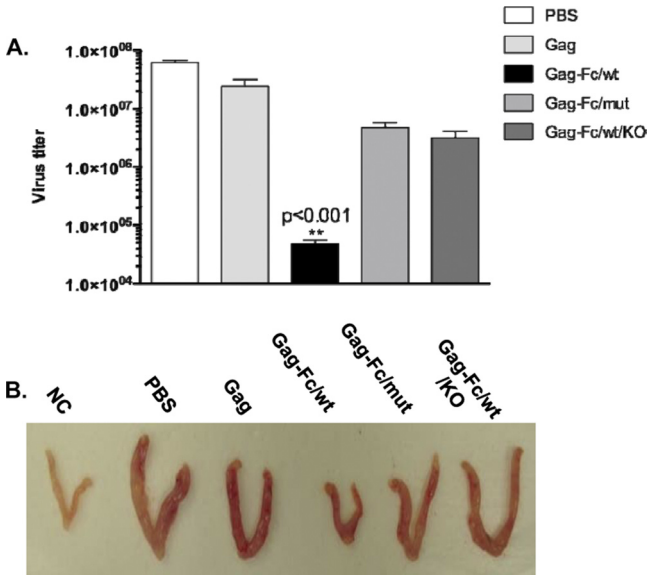

FIG. 3. FcRn-targeted mucosal immunization engenders protective immunity to an intravaginal viral challenge. (A) Mean viral titers in ovaries following an intravaginal challenge with VV expressing HIV-1 Gag (rVV-Gag). Four weeks after a booster immunization, groups of five mice were intravaginally challenged with 5×10^7 PFU of rVV-Gag. Mice were sacrificed 5 days after infection, and paired ovaries were collected. Ovaries were homogenized, and viral titers were determined by standard plaque assay on Vero cell monolayers. The data represent three similar experiments. (B) Macroscopic pictures of uteri from normal mice or immunized mice challenged with rVV-Gag. Immunization conditions are displayed at the top. NC, normal control.

immune responses and protection against virus dissemination from mucosal tissues are important factors for vaccine development. Local mucosal immunization confers maximum protection against a mucosal challenge (2, 15, 36). Mediastinal lymph nodes (MeLN) are the sites where mucosal immune responses to vaccine antigens that reach the lung after i.n. immunization are initiated. We looked at changes in MeLN GC after the FcRn-targeted mucosal delivery of Gag-Fc/wt. As shown in Fig. 4A, i.n. immunization with Gag-Fc/wt efficiently induced a significantly increased frequency (3.93%) of FAS^+ PNA^+ $B220^+$ B cells in the MeLN, compared to a 0.51 to 0.98% frequency of FAS^+ PNA^+ $B220^+$ cells in the other groups by 10 days after booster immunization. Therefore, FcRn-targeted HIV Gag mucosal immunization induced the formation of GC in draining MeLN.

Antibodies, in particular, secretory IgA and IgG, represent a first line of defense on mucosal surfaces. To assess the ability of FcRn-targeted immunization to induce a Gag-specific antibody in mucosal secretions, BAL fluid specimens were collected 10 days following the booster immunization and tested for Gag-specific IgG and IgA by ELISA. Furthermore, in order to determine if the antibody responses induced by i.n. immunization were disseminated to remote mucosal sites, vaginal wash fluids were collected for antibody analyses. The Gag-specific IgG levels were increased significantly in lung lavage fluids by 10 days after booster immunization (Fig. 4B) and in vaginal wash fluids by 2 weeks after booster immunization (Fig. 4C) among the Gag-Fc/wt-immunized mice. Low levels of Gag-specific IgG were detected in the BAL and vaginal wash fluids of mice immunized with Gag-Fc/mut or Gag alone. Only wild-type, and not FcRn KO, mice that received Gag-Fc/wt had the highest levels of Gag-specific IgG antibodies in their BAL and vaginal wash fluids, suggesting that the appearance of mucosal IgG is FcRn dependent. In contrast, we detected only a small amount of IgA in all BAL and vaginal wash fluids (unpublished results).

To address whether FcRn-targeted delivery of mucosal vaccine can induce T cell immune responses in vaginal tissues, infiltrating vaginal lymphocytes in single-cell suspensions isolated from challenged mice were pulsed with purified Gag; specific IFN-γ-producing T cells were measured by flow cytometry. We detected significant numbers of IFN-γ-producing $CD4^+$ (Fig. 4D, top) and $CD8^+$ (Fig. 4D, bottom) T cells in response to Gag in mice immunized with Gag-Fc/wt in comparison with those in the other groups, including the mice immunized with Gag-Fc/mut and the FcRn KO mice immunized with Gag-Fc/wt protein. Immunization with the Gag-Fc/wt fusion protein induced strong IFN-γ-producing $CD4^+$ and $CD8^+$ T cell responses, whereas immunization with Gag-Fc/mut or immunization of FcRn KO mice with the Gag-Fc/wt protein did not.

FcRn-targeted mucosal immunization elicits long-term humoral and T cell immune responses. Activated B cells can differentiate to plasma cells which secrete antibodies at a high rate and reside in niches in the bone marrow, or they become memory B cells capable of responding rapidly to antigen exposure. Memory B cells contribute to the plasma cell pool serum antibody levels over prolonged intervals (3). To determine whether antigen targeting to the FcRn-mediated IgG transfer pathway leads to long-lasting memory B cell immune responses, splenocytes were isolated 4 months after a booster immunization and restimulated with Gag protein. Memory B cells were barely present after immunization with control Gag but were increased by Gag-Fc/wt (Fig. 5A). Differences between Gag-Fc/wt-immunized mice and Gag-Fc/wt-immunized FcRn KO or Gag-Fc/mut-immunized mice were significant. To determine whether antigen targeting to FcRn also elicited plasma cells that secreted Gag-specific antibodies, the number of IgG-secreting plasma cells in bone marrow were measured by ELISPOT assay. Higher numbers of Gag-specific IgG-secreting cells were present in the bone marrow of mice immunized with Gag-Fc/wt than in that of the other groups (Fig. 5B). To show whether increased memory B cells and antibody-secreting plasma cells correspond to increased IgG production and antibody half-life, IgG antibody titers in serum were measured 4 months after a booster immunization (the latest time point we tested). High titers of Gag-specific IgG antibodies were maintained in mice immunized with Gag-Fc/wt but not in mice immunized with Gag-Fc/mut or Gag alone (Fig. 5C). Immunization with Gag-Fc/wt was about 20-fold more effective, respectively, than immunization with Gag-Fc/mut or HIV Gag alone, indicating that Gag-specific antibody persisted much longer after FcRn-targeted mucosal immunization.

An important feature of memory T cells is their proliferative response upon antigen restimulation. To test if memory T cells could be detected 4 months following FcRn-targeted mucosal immunization, we measured $CD4^+$ and $CD8^+$ T cell proliferation in response to HIV Gag antigen restimulation. Spleno-

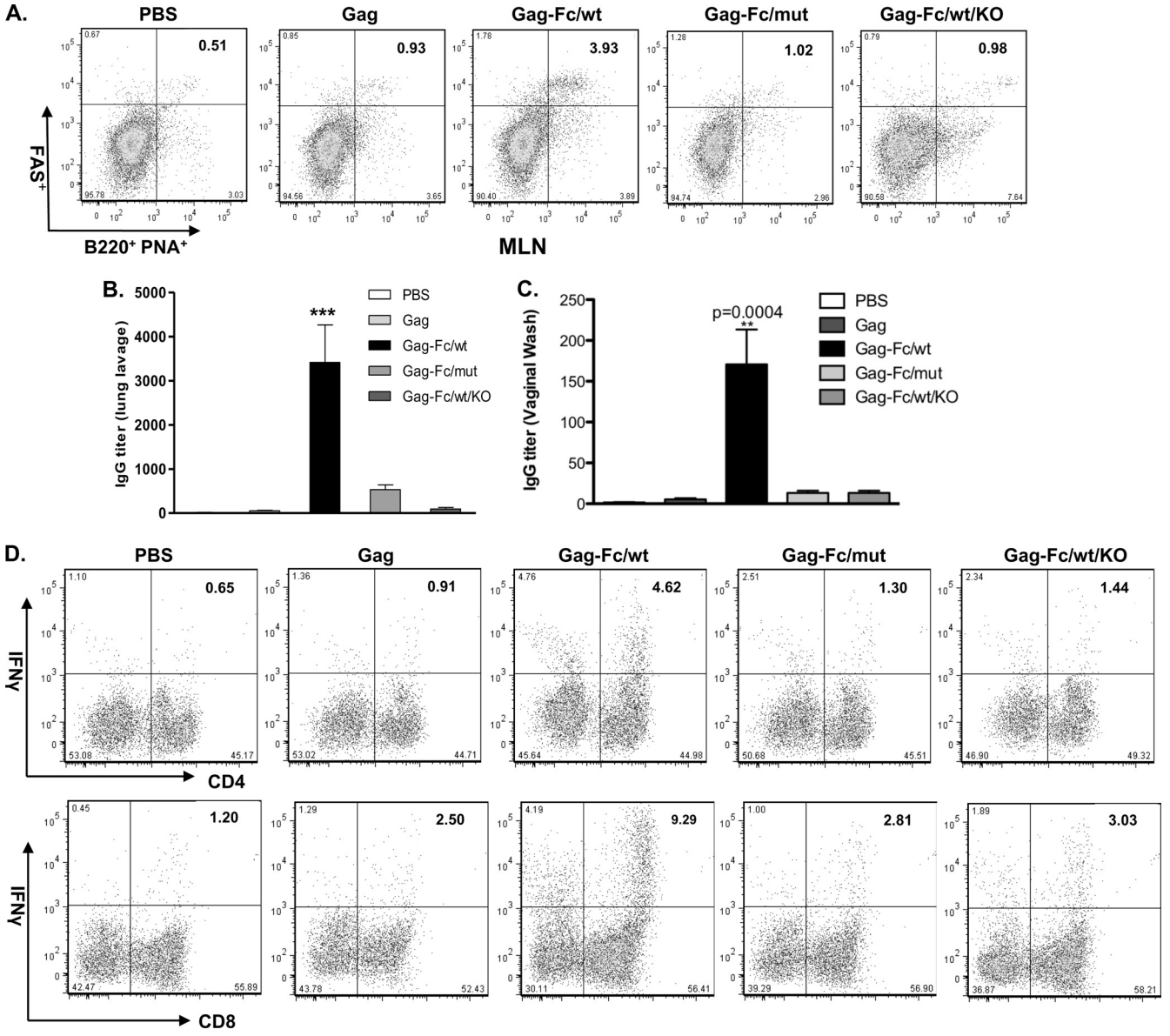

FIG. 4. Local immune responses induced by FcRn-targeted mucosal immunization. (A) Detection of activated B cells in the GC of immunized mice by flow cytometry. Representative flow cytometric analyses of GC B cells among the $B220^+$ B cells in the MeLN 10 days after a booster immunization. Values are the percentages of activated GC B cells (PNA^+ FAS^+) among the gated $B220^+$ cells. (B and C) HIV Gag-specific antibody responses in BAL fluid and vaginal secretions following immunization. BAL fluid (B) and vaginal wash fluid (C) were obtained from mice 10 days after a booster immunization, and Gag-specific IgG titers were determined by ELISA. Antibody titers for 3 mice from a representative experiment were quantified by endpoint titer. Titers of HIV Gag-specific IgG antibody in BAL and vaginal wash fluids of naive mice always fell below the limit of detection and were omitted for clarity. The data shown are representative of three independent experiments. ***, significant difference among groups ($P < 0.001$). (D) Increased presence of HIV Gag-specific T lymphocytes in the vaginal epithelia after a challenge. Lymphocytes were harvested from collagenase-treated vaginal tissues 5 days after the intravaginal inoculation of rVV-Gag. Intracellular staining for IFN-γ expression on $CD4^+$ and $CD8^+$ T cells was analyzed after gating on viable $CD3^+$ lymphocytes. The values in each column show the percentages of IFN-γ-positive T lymphocytes among the gated $CD4^+$ or $CD8^+$ T cells. Isotype controls included FITC-conjugated mouse-IgG1 and show the baseline response. The data shown are from a representative of three experiments using 3 mice per experiment.

cytes isolated 4 months after a booster immunization were stimulated *in vitro* with HIV Gag (Fig. 5D). After 4 days of incubation, the CFSE profiles on CD4- or CD8-gated T cells were read and subsequently analyzed by flow cytometry. We detected significant $CD4^+$ (Fig. 5D, top) and $CD8^+$ (Fig. 5D, bottom) memory T cell proliferation in response to Gag restimulation in mice immunized with Gag-Fc/wt in comparison with that in mice immunized with Gag-Fc/mut or FcRn KO mice immunized with the Gag-Fc/wt protein. The Gag-specific T cell response to Gag-Fc/wt in wild-type mice included a substantial memory component. Recall IL-2 and IFN-γ cytokine responses were also detected within 12 to 48 h of Gag restimulation among mice immunized with Gag-Fc/wt but not in the other groups. Collectively, these results show that mu-

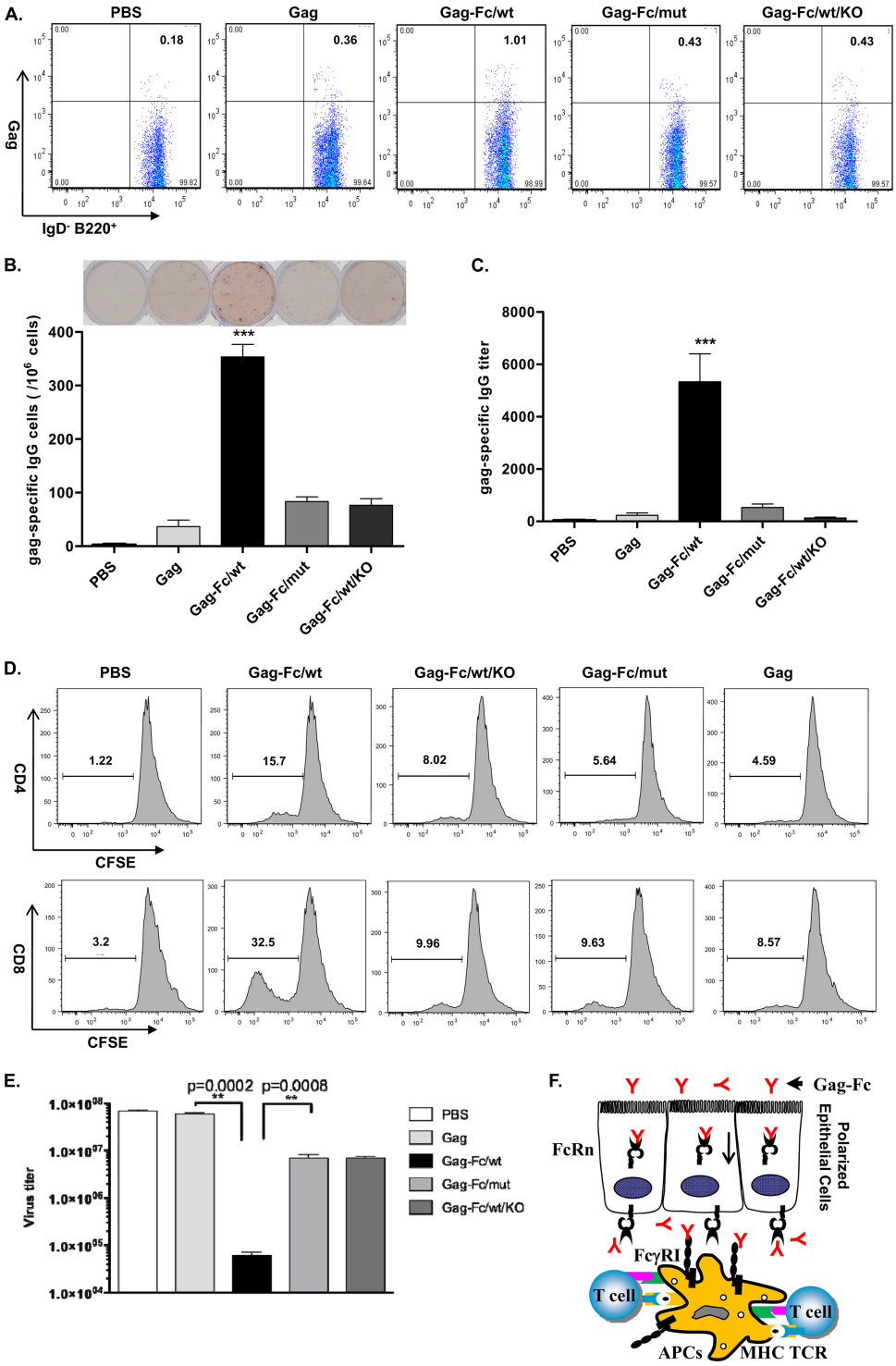

FIG. 5. Increased memory immune response after FcRn-targeted mucosal immunization. (A) Induction of Gag-specific memory B cells in the spleen. The frequency of Gag-specific memory B cells was assessed 4 months after a booster immunization. Gag-specific memory B cells, defined as $B220^{+}$ IgG^{+} IgD^{-}, were analyzed by FACS 4 months after booster immunization. Purified HIV Gag proteins were labeled with Alexa Fluor 647. Splenocytes (2×10^6) were incubated with 1 μg Alexa Fluor 647-labeled Gag protein and B220 antibody. The values in the quadrants are percentages of HIV Gag-specific memory B lymphocytes. (B) Long-lived HIV Gag-specific antibody-secreting cells in bone marrow. Bone marrow cells removed 4 months after booster immunization were placed on Gag-coated plates and quantified by ELISPOT analysis of IgG-secreting plasma cells. Data from three separate experiments with three mice in each experiment were pooled. The graphs were plotted based on the average ELISPOT assay results for replicate wells (top). Values for the Gag-Fc/wt fusion protein-immunized mice (marked with asterisks) are significantly greater ($P < 0.001$) than those for the other groups (indicated at the bottom). (C) Durability of HIV Gag-specific serum IgG response. In two separate experiments, the HIV Gag-specific IgG endpoint titer in serum was quantified by ELISA for three mice in three similar experiments at 4 months after booster immunization. HIV-specific IgG antibody was not detected in naive mice. ***, $P < 0.001$. (D) Long-lived HIV Gag-specific

cosal immunization by antigen targeting to FcRn was effective in eliciting long-term memory T cell immune responses to HIV Gag antigen.

To test whether memory immune responses elicited from FcRn-targeted mucosal immunization are functional for resisting virus, we challenged the immunized mice intravaginally with rVV-Gag (5×10^7 PFU) at 4 months after booster immunization. Ovary tissues were harvested 5 days after the challenge, and virus titers were measured. Mice immunized with Gag-Fc/mut or Gag alone and FcRn KO mice immunized with Gag-Fc/wt failed to control viral replication (Fig. 5E). In contrast, the virus titers in ovary tissues from wild-type mice immunized with Gag-Fc/wt were significantly lower at 5 days after the challenge (Fig. 5E). The virus titer was significantly higher in either Gag-Fc/mut-immunized wild-type mice or Gag-Fc/wt-immunized FcRn KO mice, demonstrating that the protection observed was from HIV Gag-specific memory immune responses.

DISCUSSION

A chimeric fusion protein composed of HIV Gag protein and a modified murine Fc portion from IgG was transported efficiently across the mucosal epithelium in mice. Transported Gag-Fc persisted in serum, which is consistent with the fact that FcRn protects IgG from degradation (48). When Gag-Fc fusion protein was used for i.n. immunization, mice developed strong T cell ($CD4^+$ and $CD8^+$) and B cell responses, including persistent memory T cells. By introducing genetically modified Fc fragments into the HIV Gag protein or using FcRn KO mice, we show that the capacity for transepithelial transport and that for eliciting strong immune responses both depended on the intact Fc sequence in the fusion protein and FcRn expression on murine cells. Finally, immune responses elicited by i.n. immunization were sufficiently potent to protect mice from infection at a remote mucosal site. The properties of antigen transport, antigen persistence in blood, B and T cell immune responses, and protection from a virus challenge all required intact Fc sequences in the fusion protein and FcRn expression in the mouse.

FcRn-targeted immunization induced strong antibody and cellular immune responses to HIV Gag at mucosal and systemic sites. The Gag-Fc/wt fusion protein induced strong IFN-γ-producing $CD8^+$ and $CD4^+$ T cell responses relative to the Gag-Fc/mut or HIV Gag protein. The mucosal immunization of FcRn KO mice demonstrated that FcRn was essential for mucosal immunization in this system. Gag antigen targeted to FcRn increased the efficiency with which HIV Gag antigens engendered strong T cell immunity when given together with CpG stimuli to promote DC maturation. We also noticed that the FcRn-targeted subunit vaccine induced high levels of serum IgG with a preference for IgG2a as the major isotype. This is not surprising, because the type 1 cytokine IFN-γ is associated with production of IgG2a, whereas the type 2 cytokine IL-4 helps switching to IgG1. However, we did not distinguish between the effects of mucosal targeting and CpG in our study.

An effective vaccine to block the sexual transmission of HIV must be capable of eliciting protective mucosal immune responses to stall initial virus replication, slow $CD4^+$ depletion, and inhibit the rapid dissemination of virus from the mucosa into systemic lymphoid tissues (2, 6). Our strategy of FcRn-targeted mucosal delivery for HIV Gag antigen engendered strong mucosal immune responses. We observed IgG in lung and vaginal wash fluids and cytokine-producing T cells in vaginal tissues of immunized mice. Of note is the observation that Gag-binding IgG levels in BAL fluid or vaginal wash fluids were much higher than the specific IgA levels detected. Indeed, IgG is a major isotype of immunoglobulin in the lower respiratory and reproductive tracts (31, 39). Thus, IgG antibodies detected in BAL and vaginal wash fluids may be produced locally or come from the circulation, but it was clear that HIV antigen targeted to FcRn plus adjuvant produced strong humoral and T cell mucosal immune responses.

Perhaps our most compelling finding was that FcRn-targeted i.n. immunization protected against an intravaginal virus challenge. Viral replication in ovary tissues was reduced in immune mice, and there was less evidence of gross pathology in the uterus. I.n. immunization targets cells in the nasal lymphoid tissue and its draining lymph nodes. Antibody-secreting cells and IFN-γ-producing T cells likely migrate from the airway to the genital tract (38, 49). We used this system to test whether the humoral and T cell immune responses elicited by the i.n. delivery of FcRn-targeted HIV Gag protected the distant vaginal mucosa. We know that protective responses depended on intact Fc and FcRn expression, and both T and B cell responses were detected. Several immune mechanisms may account for the protection seen. T lymphocytes were present in the vaginal epithelium of rVV-Gag-infected mice at times coinciding with virus clearance. IFN-γ-producing $CD4^+$ and $CD8^+$ T cells were also present in vaginal tissues of immune mice. These strong T cell responses may also promote the direct lysis of MHC class I- or II-bearing infected cells. The effector/memory $CD4^+$ and $CD8^+$ T cells at mucosal effector sites (lamina propria) are crucial for containing initial HIV

T cell memory response to FcRn-targeted mucosal vaccination. Splenocytes were isolated 4 months after booster immunization, stained with CFSE, and stimulated *in vitro* with 20 μg/ml purified Gag for 4 days. The data shown are expressed in CFSE histograms of fluorescence intensity versus the number of fluorescing cells, indicating the percentage of the cell population positive for the CD4 or CD8 antigen. The values in the figure are percentages of $CD4^+$ and $CD8^+$ proliferating T cells. The data are representative flow cytometry profiles of three similar experiments with three mice per group. The immunization conditions are displayed at the top. (E) Mean viral titers in ovaries following a vaginal challenge with rVV-Gag. Four months after booster immunization, groups of five mice were intravaginally challenged with 5×10^7 PFU of rVV-Gag and sacrificed 5 days after the challenge. The ovaries were collected from each mouse, and viral titers were measured by a plaque assay. The data represented three similar experiments. (F) Proposed model of FcRn-mediated mucosal vaccine delivery. Fc-fused HIV Gag antigens are transported by FcRn across the epithelial mucosal barrier and targeted to mucosal antigen-presenting cells (APCs) such as DCs. Antigen is taken up by pinocytosis or FcγRI-mediated endocytosis in antigen-presenting cells and then processed and presented or cross presented to T cells. TCR, T cell receptor.

replication and subsequent virus dissemination. Consistent with these data, previous studies reported that the breadth of Gag-specific T cell responses correlated with viral load control in HIV-1-infected humans (23) and SIV (simian immunodeficiency virus)-infected rhesus macaques (27). It may be argued that the local T cell responses observed after a challenge with rVV-Gag were due to nonspecific inflammatory responses against the challenge virus. However, the responses were Gag specific and occurred only in wild-type mice immunized with Gag-Fc/wt. It is important to note that the antigens used in this study contained only a single HIV Gag antigen and did not include a homologous Env gp120 antigen. The observed protection was likely provided by Gag-specific cellular immune responses, since it is unlikely that Gag-specific antibodies offered substantial protection in blocking viral attachment to and penetration of target cells. This may explain why Gag-Fc/wt-immunized mice failed to clear the virus completely after infection; however, we cannot exclude a protective role for antibody-dependent cell-mediated cytotoxicity (8, 13, 14). In our study, FcRn-targeted mucosal vaccination induced significant amounts of Gag-specific IgG in serum, BAL fluid, and vaginal secretions. IgG is a major protective antibody in vaginal secretions after immunization (26, 31, 39). The potential roles for antibody produced by FcRn-targeted HIV gp120 antigen immunization may be more important for full protection in humans. Additional studies to test this idea are ongoing.

FcRn-targeted mucosal immunization produced durable memory immune responses. Immunological memory is exemplified by increased levels of effector T and B cells and, functionally, by the ability to respond faster and more vigorously to a second encounter with the vaccine antigens (1). Hence, another criterion for successful HIV vaccines is the ability to generate durable memory responses that maintain strong immune surveillance over lengthy intervals. These effector memory responses might improve vaccine efficacy by impairing viral replication at its earliest stage or at viral entry sites (19). An obstacle to the successful implementation of a HIV mucosal vaccine is the production and maintenance of a pool of memory lymphocytes. It has been difficult to implement an HIV subunit vaccine strategy via mucosal surfaces because these approaches have resulted in inefficient immune responses that waned rapidly. However, the most striking finding in this study is that the FcRn-targeted mucosal delivery of HIV antigen sustained high levels of HIV Gag-specific IgG-secreting plasma cells and memory B and T cells. The presence of Gag-specific memory B cells in the spleen and long-lived plasma cells in the bone marrow may explain the high levels of IgG antibody seen in serum. T cell memory was also long-lived in our model. The reason for generating potent memory T cell activity is not completely clear, although IL-2-producing T cells were generated and may be important for supporting long-lived memory T cells (11).

FcRn might contribute to increased immunity in two ways: by efficiently transporting Gag-Fc and by protecting it from degradation. It is generally believed that slow release of vaccine antigen over a prolonged time can facilitate long-term memory immune responses. As a result, long-term retention of Gag-specific plasma cells and memory lymphocytes might be important for resistance to HIV replication and transmission. Indeed, this conclusion was strongly supported by the observation that protective immune responses were still present at 4 months after immunization (Fig. 5E). FcRn is expressed in both the upper and central airways in nonhuman primates, as well as in humans. Additionally, FcRn can mediate the pulmonary delivery of an erythropoietin Fc fusion protein in nonhuman primates (4). Therefore, it will be of interest to determine whether FcRn-targeted mucosal immunization is capable of eliciting long-term protective memory immune responses by modulating the replication and transmission of SIV in a rhesus macaque model.

In conclusion, our study demonstrates that a subunit vaccine based on HIV Gag-Fc fusion protein targets the antigen to FcRn and induces long-term immune memory and protection against a mucosal virus challenge (Fig. 5F [modified from Fig. 4f in reference 50]). Robust durable immune responses with protection against a virus challenge document the potential for this approach in developing vaccines against mucosal HIV exposure. From this study, we deduce that an FcRn-targeted HIV subunit vaccine delivers soluble antigens to mucosal DCs and gives rise to long-lived T cell help for antibody responses (5, 25, 46). FcRn-targeted mucosal immunizations may be further benefited by an additional function for FcRn in antigen presentation (29, 32, 40). Additional studies are required to study memory and secondary responses after the targeted delivery of antigens to DCs via FcRn-mediated transport. Nonmucosal immunization with the Gag-Fc/wt or Gag-Fc/mut fusion protein, as described by Mi et al. (32), can also be performed to measure the immune responses and examine the efficacy of protection from a challenge. Our next goals are to target multiple HIV antigens to FcRn and test these antigens in nonhuman primate models for mucosal exposure to SIV. Future studies on HIV vaccination should also address this possibility and evaluate protective efficacy against heterologous virus challenges that model the natural exposure to virus among human beings. Together with results from ongoing work in our laboratory, knowledge gained from this study will be useful in the development of effective mucosal vaccine strategies for HIV and other pathogens that invade mucosal surfaces.

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