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Mucosa-Associated Epithelial Chemokine/CCL28 Expression in the Uterus Attracts CCR10+ IgA Plasma Cells following Mucosal Vaccination via Estrogen Control

Hye-Ran Cha,*† Hyun-Jeong Ko,*,‡ Eun-Do Kim,* Sun-Young Chang,* Sang-Uk Seo,* Nicolas Cuburu,* Sangyelo Ryu,‡ Sunhoon Kim,§ and Mi-Na Kweon*‡

Previous studies demonstrated cross talk between mucosal and reproductive organs during secretory IgA Ab induction. In this study, we aimed to clarify the underlying mechanisms of this cross talk. We found significantly higher titers of Ag-specific secretory IgA Ab in the vaginal wash after mucosal vaccination by both the intranasal (i.n.) and the intravaginal routes but not by the s.c. route. Interestingly, Ag-specific IgA Ab-secreting cells (ASCs) were found mainly in the uterus but not in the cervix and vaginal canal after i.n. vaccination. The fact that most Ag-specific IgA ASCs isolated from the uteri of vaccinated mice migrated toward mucosa-associated epithelial chemokine (MEC)/CCL28 suggests dominant expression of CCR10 on the IgA ASCs. Further, IgA ASCs in the uteri of vaccinated mice were reduced drastically in mice treated with neutralizing anti-MEC/CCL28 Ab. Most intriguingly, the female sex hormone estrogen directly regulated MEC/CCL28 expression and was augmented by i.n. vaccination with cholera toxin or stimulators for innate immunity. Further, blockade of estrogen function in the uterus by oral administration of the estrogen antagonist raloxifene significantly inhibited migration of Ag-specific IgA ASCs after i.n. vaccination with OVA plus cholera toxin. Taken together, these data strongly suggest that CCR10+ IgA ASCs induced by mucosal vaccination via the i.n. route migrate into the uterus in a MEC/CCL28-dependent manner and that estrogen might have a crucial role in the protection against genital infection by regulating MEC/CCL28 expression in the uterus. The Journal of Immunology, 2011, 187: 3044–3052.

The immune system of the female reproductive tract is similar to that of other mucosal sites, such as the gastrointestinal and respiratory tracts, but is compartmentalized in several critical aspects (1–3). Differences include the commensal microbiota, IgG mainly from the circulation, and hormonal fluctuations that can modify genital immunity (4). Also, female reproductive tissues must tolerate sperm and developing fetuses and respond to sexually transmitted bacterial and viral pathogens (5). Naturally occurring Abs in uterine luminal fluid might help to protect the endometrium against microbial infections (6). Several previous studies suggested that epithelial cells in the genital tract have a key role in Ag presentation and immune responses, although there are no distinct lymphoid structures in the genital tract (5, 7). It is critical to understand the female genital tract immune system to develop strategies against such sexually transmitted infections as HSV-2, Chlamydia trachomatis, human papillomavirus, and HIV (8–11).

Expression of chemokines and chemokine receptors contributes to specific accumulation of leukocyte subsets in various tissues (12). Previous studies found that chemokines and tissue-specific adhesion molecules are involved in the migration of Ab-secreting cells (ASCs) (13). Of these, CCR9 and CCR10 enable plasma B cells to migrate into mucosal tissues (14, 15). Mucosa-associated epithelial chemokine (MEC/CCL28), a well-known ligand for CCR10, is commonly produced by epithelial cells at several mucosal sites and attracts IgA- but not IgG- or IgM-producing ASCs into the intestines, lungs, and oral cavity (14). In addition, a previous study revealed that MEC/CCL28 attracts IgA ASC migration to the lactating mammary gland and supports IgA Ab transfer to the neonate (15). In CCR10−/− mice, accumulation of IgA ASCs in the gastrointestinal tract is minimally affected, whereas IgA ASCs in the lactating mammary gland are entirely absent, indicating that reliance on CCR10-mediated recruitment of IgA ASCs varies within mucosal tissues (16). These findings suggest a broad and unifying role for the interaction of CCR10 and MEC/CCL28 in mucosal IgA induction.

Previous results showed that induction of Ag-specific Ab levels and protective immunity in the genital tract is greatly enhanced after intranasal (i.n.) vaccination compared with systemic vaccination including s.c. vaccination (17–20). For instance, i.n. immunization using recombinant chlamydial protease-like activity factor plus IL-12 resulted in IgG2a and IgA Abs in bronchoalveolar lavage and vaginal fluids and significantly reduced bacterial shedding upon genital Chlamydia muridarum challenge in BALB/c mice (18). Predominant levels of human papillomavirus 16 L1-specific IgA Ab and CTL responses were induced in
the vaginal lumen by i.n. immunization with human papilloma-virus-like particle (19). In addition, i.n. immunization of mice with HSV-1 glycoprotein B and CpG oligodeoxynucleotide (ODN) resulted in significantly elevated levels of glycoprotein B-specific IgA Abs in vaginal wash samples and protective immunity in the genital tract against genital HSV-2 challenge (20). Further, our previous data demonstrated that not only i.n. vaccination but also other sublingual or transcutanous mucosal routes were highly effective in inducing Ag-specific IgA Abs in the vaginal wash (21, 22). Thus, there might be cross talk between mucosal and female reproductive tissue; however, the underlying mechanisms are not well defined.

In this study, we found that substantial numbers of Ag-specific (i.e., OVA and cholera toxin [CT] B subunit [CTB]) IgA+ ASCs were in the uteri of mice after i.n. vaccination with OVA plus CT. MEC/CCL28 expression was enhanced significantly in the uterus but not in the vaginal canal within 24 h of i.n. vaccination. Further, Ag-specific IgA ASCs failed to migrate into the uteri of i.n. vaccinated mice when MEC/CCL28 was blocked by neutralizing Abs. Of note, the blocking of estrogen function in the uterus abrogated expression of MEC/CCL28 in the uterus and hence blocked migration of Ag-specific IgA ASCs into the uterus by i.n. vaccination. Our findings strongly indicate that estrogen regulates MEC/CCL28 expression in the uterus and is crucial for IgA ASCs to migrate into the uterus after mucosal vaccination.

Materials and Methods
Mice and virus strain
BALB/c and C57BL/6 mice were purchased from Charles River Laboratories (Orient Bio, Sungnam, South Korea). All of the mice were maintained under specific pathogen-free conditions in the experimental facility at the International Vaccine Institute (Seoul, South Korea), where they received sterilized food and water ad libitum. All of the animal experiments described were approved by Institutional Animal Care and Use committees. Wild-type HSV-2 186 syn and HSV-2 186 syn ΔTK were kindly provided by Dr. David Knipe (Harvard Medical School, Boston, MA) (23). Both wild-type and ΔTK of HSV-2 186 strain were propagated in Vero cells (24), and viral stocks were prepared using the serum-free media VP-SFM (Invitrogen, Carlsbad, CA) and titered in Vero cells.

Immunization
Mice were immunized i.n. with 20 μg OVA plus 0.5 μg CT (List Biological Laboratories, Campbell, CA) or immunized s.c. or intravaginally (i.vag.) with 100 μg OVA plus 2 μg CT. In different experiments, female BALB/c mice were immunized i.n. with HSV-2 ΔTK (1 × 10⁸ PFU) or CpG (5 μg/ml). To determine the hormone effect, mice were injected with tamoxifen (1 mg per mouse; MP Biomedicals, Solon, OH) i.p., raloxifene (2 mg per mouse; Sigma-Aldrich, St. Louis, MO) orally, or medroxypregesterone (2 mg per mouse; Sigma-Aldrich) s.c. every day beginning on day 2 before i.n. immunization. Mice were anesthetized with a ketamine/xylazine mixture before i.n. or i.vag. challenge.

Cell isolation
For mononuclear cell (MNC) isolation, each uterus and cervico-vaginal canal was removed and cut into 0.5-cm pieces that were then rinsed with Ca/Mg-free HBSS. The tissues then were incubated in a mixture of 5 nM EDTA and Ca/Mg-free HBSS at 37°C for 30 min with gentle stirring. Tissue then was incubated with RPMI 1640 containing 10% bovine calf serum, antibiotics (penicillin and streptomycin), 0.1% mg/ml DNase I (Sigma-Aldrich), and 2 mg/ml collagenase/dispass (Roche Diagnostics, Mannheim, Germany) and incubated at 37°C with stirring for 30 min three times. The isolated cells were pooled and separated on a 40/75% discontinuous Percoll gradient (Pharmacia, Piscataway, NJ) and placed in a centrifuge for 20 min at 600 × g at 25°C. Cell layers for MNCs were recovered and suspended in complete RPMI 1640 at 4°C until use. Human endometrium tissues were taken from cervical cancer patients. This study was approved by, and all samples were obtained in accordance with, the Institutional Review Board of the Yonsei University College of Medicine.

Neutralizing of MEC/CCL28 in vivo
To neutralize MEC/CCL28, mice were injected i.p. every other day beginning 1 d before i.n. immunization with 50 μg anti-MEC/CCL28 Ab (134306; R&D Systems, Minneapolis, MN) until sacrifice (25).

ELISA
Serum and vaginal wash samples were obtained, and Ag-specific Ab titers were determined by ELISA as described elsewhere (26). The plates (BD Falcon, Mississauga, ON, Canada) were coated with 10 μg/ml OVA or 2 μg/ml CTB (List Biological Laboratories) in PBS and incubated overnight at 4°C. After a blocking step, serial dilutions of serum or vaginal washes were added in duplicate, and the plates were incubated for 2 h at 37°C. HRP-conjugated goat anti-mouse IgG or IgA Abs (Southern Bio-technology Associates, Birmingham, AL) were added to each well at 37°C. Goat anti-mouse polyclonumoglobulin receptor Ab (R&D Systems) and HRP-conjugated rabbit anti-goat IgG Ab (Southern Biotechnology Associates) were used to measure secretory IgA in vaginal wash samples. For color development, the substance solution (tetramethylbenzidine; Moss, Pasadena, MD) was added and stopped by adding 0.5 N HCl. The color development was measured at 450 nm by ELISA reader (Molecular Devices, Sunnyvale, CA). End point titers were expressed as the reciprocal log₂ of the last dilution giving an OD at 450 nm of 0.1 greater than that of the background.

ELISPOT
Ag-specific ASCs were determined by ELISPOT as described previously (27). The 96-well nitrocellulose microplates (Millipore, Bedford, MA) were coated with 5 μg/ml goat F(ab)’; anti-mouse Ig (Southern Bio-technology Associates) in PBS to determine total ASCs and 10 μg/ml OVA or 2 μg/ml CTB for Ag-specific ASCs. After being blocked with RPMI Fulo (Life Technologies, Carlsbad, CA) supplemented with 10% FBS (Life Technologies), serially diluted MNCs were plated and incubated for 4 h at 37°C in a 5% CO₂ incubator. For color development, peroxidase substrates (3-amin-9-ethylcarbazole kit; Meso) were adopted, and ASCs were counted with the aid of a stereomicroscope (SZ2-ILST; Olympus, Tokyo, Japan). To visualize spots, ELISPOT plates were photographed with an ImmunoSpot analyzer (Cellular Technology, Shaker Heights, OH).

Chemotaxis assay
To evaluate the expression of chemokine receptors on Ag-specific ASCs, both chemotaxis and ELISPOT assay were combined (22, 28). Briefly, 5-μm Transwell inserts (Corning Costar, Cambridge, MA) containing 1 × 10⁶ MNCs were placed on a 24-well plate so as to make contact with 600 μl of the medium alone (basal) or with one of the following chemokines as well: 100 nM SDF-1α/CXCL12 (R&D Systems), 100 nM MIP-3α/CCL20 (R&D Systems), 100 nM SLC/CCL21 (PeproTech, Rocky Hill, NJ), or 250 nM MEC/CCL28 (R&D Systems). After 2 h, the inserted membrane was removed, and the migrated cell population in the bottom well was recovered and added to the ELISPOT plates for the measurement of the number of Ag-specific IgA ASCs.

cDNA synthesis and real-time quantitative PCR
After a wash with nuclease-free water, we homogenized the whole uterus and cervico-vaginal canal samples at different time points after i.n., i.vag., and s.c. immunization with CT (2 μg), HSV-2 ΔTK (1 × 10⁸ PFU), or CpG (5 μg). Total RNA was extracted using TRIzol (Invitrogen), and cDNA was synthesized by Superscript II reverse transcriptase with oligo(dT) primer (Invitrogen). The products were used as a template for each chemokine-specific real-time PCR set for the amplification of chemokines specific for MEC/CCL28. TaqMan primer–probe sets for each molecule were designed for gene expression assays (Applied Biosystems, Carlsbad, CA). The amplification reactions were performed with 300 ng cDNA (TaqMan Universal PCR Master Mix; Applied Biosystems) and each of the designated probes (Applied Biosystems). Gene expression quantification then was performed using an ABI PRISM Sequence Detection System (Applied Biosystems). The levels of mRNA expression were displayed as the expression units of each target gene relative to the expression units of GAPDH.

FTY 720 treatment
To induce lymphocyte retention in secondary lymphoid organs, mice received FTY 720 (1 mg/kg body weight; Cayman Chemical, Ann Arbor, MI) by i.p. injection every other day (29). The effect of FTY 720 treatment was monitored by regular analysis of the PBL and tissue lymphocyte count.
Western blot analysis

Approximately 30–40 mg of the uterus and vaginal tissues of naive and i.n. immunized mice were homogenized in 400 μl PRO-PREP solution (iNtRON, Sungnam, South Korea) in ice, and lysis is induced by shaking the incubator for 30 min at 4˚C. The supernatants were placed in a centrifuge at 16,000 × g at 4˚C for 5 min to remove the insoluble materials, and the supernatant was transferred to a fresh 1.5-ml tube. Protein concentrations of the supernatant were measured using a bicinchoninic acid assay kit (Pierce, Rockford, IL). The proteins in the supernatants were separated by 12% SDS-PAGE and transferred to 0.45-μm polyvinylidene fluoride membrane (Millipore). After being blocked for 1 h at room temperature with 10 mM Tris, 150 mM NaCl, and 0.05% Tween 20 (pH 7.6) containing 5% nonfat milk, the membranes were incubated with goat anti-mouse CCL28 polyclonal Ab (R&D Systems) and HRP-conjugated anti-goat IgG Ab (R&D Systems) or HRP-linked anti-β-actin Ab (Santa Cruz Biotechnology, Santa Cruz, CA). The proteins were visualized by ECL (Thermo, Rockford, IL.). Densitometric analysis of the bands was performed using Gel Doc XR (Bio-Rad, Milan, Italy) and analyzed by Quantity One software (Bio-Rad).

Statistical analysis

Data are expressed as the mean ± SD. Statistical comparison between experimental groups was performed using Student t test and ANOVA (Prism; GraphPad, La Jolla, CA)

Results

High levels of Ag-specific ASCs were detected in the uterus after i.n. immunization with OVA plus CT

As the first step in investigation of the relationship between i.n. vaccination and immune responses in the female genital mucosa, groups of female BALB/c mice were immunized i.n., i.vag., or s.c. with OVA plus CT twice at 2 wk intervals. At 7 d after booster vaccination, we collected serum and vaginal wash samples from each immunized mouse and after sacrifice obtained cells from several tissues including spleen, cervical lymph node (LN), iliac LN, vaginal canal, and uterus. Mice vaccinated by the i.n. and i.vag. routes showed significantly higher levels of OVA-specific IgA Ab in the vaginal wash than those vaccinated s.c. showed (Fig. 1A). In contrast, all of the vaccinated groups had similar levels of IgG and IgA Ab in serum samples and IgG Ab in vaginal wash samples (Fig. 1A). To further investigate whether IgA Ab in the vaginal wash is in secretory form, we used ELISA to assess secretory component levels. As shown in Fig. 1B, there were significantly higher levels of secretory component in vaginal wash specimens from mice given i.vag. or i.n. OVA plus CT than from those mice given s.c. OVA plus CT. We used the ELISPOT method to study the induction of Ag-specific ASCs in the draining LNs and effector tissues after the vaccination. Results showed significantly more OVA-specific IgA ASCs in the cervical LNs and uterus but not in the iliac LNs and vaginal passage (including cervical and vaginal canal) after i.n. vaccination (Fig. 1C, 1D). Ag-specific IgG ASCs increased in both the vaginal passage and the uterus after i.n. vaccination, although total IgG ASC numbers were relatively lower than those of IgA ASCs (Fig. 1D). Ag-specific IgA ASCs after i.vag. vaccination were detected in the iliac LNs, vaginal canal, and uterus, but IgA ASC numbers were significantly lower than those after i.n. vaccination (Fig. 1C).

We next assessed pregnant mice to determine the effectiveness of i.n. vaccination during pregnancy. After becoming pregnant, mice

![FIGURE 1](http://www.jimmunol.org/)

Prominent Ag-specific IgA ASCs detected in the uteri of mice immunized i.n. Female BALB/c mice were immunized i.n., i.vag., or s.c. with 100 μg OVA plus 2 μg CT twice 2 wk apart. A, At 7 d after booster vaccination, levels of OVA-specific IgG or IgA Abs were determined in serum and vaginal wash samples. Results are representative of three repetitive experiments. B, OVA-specific polyimmunoglobulin receptor levels were detected in serum and vaginal wash samples after i.n. or i.vag. vaccination. C, Numbers of OVA- or CTB-specific IgA ASCs in spleen (SP), cervical lymph node (CLN), iliac lymph node (ILN), cervico-vaginal canal, and uterus after i.n., i.vag., or s.c. immunization. At 7 d after booster vaccination, numbers of OVA- or CTB-specific IgA ASCs in the cervico-vaginal canal and uterus were significantly lower than those after i.n. vaccination (Fig. 1C).
were immunized i.n. twice with OVA plus CT. After mothers gave birth, MNCs from their uteri were isolated, and OVA-specific IgA ASCs were detected. As with nonpregnant mice, the increased numbers of OVA-specific IgA ASCs in the uterus indicated that i.n. vaccination for the induction of Ag-specific Abs in the uterus is effective (Fig. 1E). Birth rates did not differ for nonvaccinated and vaccinated mice (data not shown). To address induction of protective immunity in female reproductive tissues, groups of mice were immunized i.n. or i.vag. with HSV-2ΔTK and challenged with wild-type HSV (Fig. 1F). All of the mice survived, indicating the effectiveness of the i.n. vaccination routes against viral infections in the female reproductive tract. When all of the results were considered, i.n. vaccination resulted in protective IgG and IgA Abs in the genital tract; Ag-specific IgA ASCs predominantly accumulated in the uterus not in the vaginal canal.

Upregulation of MEC/CCL28 mRNA in the uterus after i.n. administration

To determine which chemokine attracted IgA⁺ plasma cells after i.n. immunization, we assessed expression of various chemokines after the administration of CT by way of s.c., i.vag., and i.n. routes. mRNA levels from the uterus and vaginal canal were assessed by real-time PCR. mRNA expression of MEC/CCL28 was increased drastically in the uterus 24 h after i.n. or i.vag. vaccination (Fig. 2A). The upregulated levels of MEC/CCL28 in the uterus gradually decreased in a time-dependent manner (Fig. 2A). However, no significant changes in mRNA expression levels of thymus-expressed chemokine (TECK/CCL25) and cutaneous T cell-attracting chemokine (CTACK/CCL27) in the uterus followed vaccination by any method (Fig. 2A). Unlike the expression patterns in the uterus, there were no changes in chemokine expression in the vaginal canal after i.n. vaccination with CT when compared with those at steady state (Fig. 2B). To assess the effect of other stimulators, mice were challenged with HSV-2ΔTK (1 × 10⁶ PFU per head) or CpG ODN (5 μg per head) via the i.n. route, and MEC/CCL28 expression in the uterus was determined in a time-dependent manner. Similar to CT, mRNA expression of MEC/CCL28 in the uterus was increased greatly and peaked 48 h after i.n. HSV-2ΔTK challenge and at 24 h after CpG ODN challenge (Fig. 2C). However, mRNA expression of MEC/CCL28 did not increase significantly in the vaginal canal after CpG ODN challenge (Fig. 2D). This remarkable upregulation of MEC/CCL28 in the uterus, but not in the cervix and vaginal canal, might correlate with the recruitment of CCR10 (chemokine receptor for MEC/CCL28) IgA⁺ plasma cells into the uterus after i.n. vaccination.

IgA ASCs in the uterus migrated to MEC/CCL28 at steady state and after i.n. immunization

We next asked whether IgA ASCs from the uterus can respond to MEC/CCL28 in an in vitro chemotaxis assay. First, MNCs were isolated from the uteri of naive mice, and the migratory characteristics of Ag-specific IgA ASCs toward SDF-1α/CXCL12, MIP-3α/CCL20, SLC/CCL21, or MEC/CCL28 were evaluated. As shown in Fig. 3A, total IgA ASCs in the uterus of naive mice migrated into MEC/CCL28 but not into SDF-1α/CXCL12, MIP-3α/CCL20, or SLC/CCL21 (Fig. 3B). As in murine cells, total IgA⁺ plasma cells of human uterus had migrated to MEC/CCL28 but not to SDF-1α/CXCL12, MIP-3α/CCL20, or SLC/CCL21 (Fig. 3B). We then studied the migration patterns of Ag-specific IgA ASCs after i.n. vaccination with OVA plus CT. Both...
OVA-specific (Fig. 3C, left panel) and CTB-specific (Fig. 3C, right panel) IgA ASCs migrated to MEC/CCL28 specifically but not to SDF-1α/CXCL12, MIP-3α/CCL20, or SLC/CCL21. Moreover, OVA-specific IgA ASCs from iliac LNs failed to migrate to candidate chemokines, whereas OVA-specific IgA ASCs from cervical LNs migrated in response to MEC/CCL28 (Fig. 3D). These results imply that Ag-specific IgA ASCs in the uterus might migrate from cervical LNs, the draining LNs of i.n. challenge. When we examined chemoattractant patterns after i.vag. immunization, vaginal IgA plasma cells specifically migrated toward SDF-1α/CXCL12 and MEC/CCL28 (Fig. 3E, left panel). Further, IgA plasma cells in iliac LN but not cervical LN migrated toward SDF-1α/CXCL12, MIP-3α/CCL20, and MEC/CCL28 (Fig. 3E, right panel). We could not find any specific migration of uterus IgA plasma cells toward tested chemokines. Thus, vaginal IgA plasma cells induced by the i.vag. route could express CCR10 partially. These findings suggest that i.vag. immunization primarily induces IgA plasma cells in the vagina and that i.n. immunization elicits IgA plasma cells in the uterus in a MEC/CCL28-dependent manner. Taken together, these results indicate that MEC/CCL28 has a critical role in the selective homing of total and Ag-specific IgA ASCs from cervical LN into uteri in both mice and humans.

**MEC/CCL28-dependent homing of Ag-specific IgA ASC into the uterus**

We next sought to determine the organ for class switch recombination of IgA ASCs that accumulated in female reproductive tissues after i.n. vaccination. Mouse groups were treated with FTY 720, which interferes with the egress of B lymphocytes as well as T lymphocytes from secondary lymphoid organs (30), during i.n. vaccination with OVA plus CT. We found that FTY 720 treatment dramatically reduced OVA- and CTB-specific IgA ASCs in the uteri of treated mice compared with those in the untreated control group (Fig. 4). In contrast, identical or slightly decreased numbers of Ag-specific IgA ASCs were shown in cervical LNs in control and FTY 720-treated mice (Fig. 4). These results indicate that the IgA ASCs in the uterus after i.n. vaccination might have migrated from secondary lymphoid organs but were not class-switched in the uterus. To further ascertain the role of MEC/CCL28 in the selective migration of IgA ASCs to the uterus by i.n. immunization, mice were injected i.p. every other day with anti-MEC/CCL28 Abs to neutralize MEC/CCL28 activity. Mice treated with anti-MEC/CCL28 Abs had significantly fewer Ag-specific IgA ASCs in the uterus than control Ab-treated mice (Fig. 5A). However, migration of IgG ASCs in the uterus after i.n. vaccination did not seem to be affected (Fig. 5B). Collectively, these results indicate that MEC/CCL28 has a critical role in the selective homing of IgA ASCs in the uterus.

**Estrogen plays a crucial role in MEC/CCL28 expression in the uterus**

To synchronize the estrus cycle, mice were treated s.c. with 2 mg medroxyprogesterone. At days 6 to 11 after the synchronization, whole uteri were isolated from naive and i.n. vaccinated mice, and the mRNA expression of MEC/CCL28 was determined by real-time PCR. The mRNA expression levels of MEC/CCL28 in the uteri of naive and i.n. vaccinated mice were increased significantly in a time-dependent manner (Fig. 6A). We suspected that estrogen secretion might increase with time after medroxyprogesterone treatment and that it could affect expression of MEC/CCL28 in the uteri of naive and i.n. vaccinated mice. Indeed, we found levels of MEC/CCL28 in the uteri of i.n. vaccinated mice to be relatively higher than those of naive mice (Fig. 6A). In addition, protein levels of MEC/CCL28 in the uteri of i.n. vaccinated mice also were enhanced in a time-dependent manner (Fig. 6B).

To see whether estrogen was involved in MEC/CCL28 expression in the uterus, mice were treated with estrogen agonists (i.e., tamoxifen) or antagonists (i.e., raloxifene) for 2 d before i.n. immunization with CT. Tamoxifen was developed as an estrogen...
antagonist for breast cancer treatment, but it works as an agonist in the uterus (31). Tamoxifen treatment accelerated expression levels of MEC/CCL28 in the uterus after i.n. vaccination, yet raloxifene did not (Fig. 6C). We further investigated whether blockade of estrogen function can inhibit migration of Ag-specific IgA ASCs into uterine tissue after i.n. immunization with OVA plus CT. Mice were fed raloxifene orally from 5 d before i.n. immunization until 7 d after booster vaccination. Numbers of OVA- or CTB-specific IgA ASCs in the uteri of raloxifene-treated mice were significantly lower than those in the uteri of PBS-treated mice, although there were no significant changes in IgA ASCs in inductive tissue after i.n. immunization (Fig. 6D). Overall, our findings suggest that female sex hormones (e.g., estrogen) are indispensable in the expression of MEC/CCL28 and subsequent migration of IgA ASCs to the uterus after i.n. immunization.

Discussion

In this study, we show that CCR10+ IgA ASCs induced by i.n. immunization can migrate into genital mucosa, especially in the uterus but not in the cervico-vaginal mucosa, in a MEC/CCL28-dependent manner. Intranasal immunization with adjuvant or virus itself accelerated MEC/CCL28 expression in the uterus but not in the cervico-vaginal mucosa. These findings expand those of our earlier study in which there were high levels of IgA Ab in the vaginal wash and predominant numbers of IgA ASCs in the genital tract, including the uterus and cervico-vaginal mucosa, after i.n. immunization (21). Thus, IgA ASCs specifically migrate into the uterus rather than the cervico-vaginal tract, and IgA Abs in the vaginal wash originate from plasma cells in the uterus. The female sex hormone (i.e., estrogen) is indispensable in the homing of IgA ASCs to the uterus via the acceleration of MEC/CCL28 expression.

In the current study, i.n. immunization resulted in increased numbers of Ag-specific IgA ASCs in cervical LNs but not in ilioc LNs (Fig. 1C). Ag-specific IgA ASCs isolated from cervical LNs specifically migrated in response to MEC/CCL28, indicating CCR10 expression on their cell surfaces after i.n. immunization with OVA plus CT (Fig. 3D). After the treatment with FTY 720, CCR10+ IgA ASCs in the uterus were decreased drastically, but there were no significant changes in cervical LNs (Fig. 4). It was suggested previously that CCR10 is expressed selectively by IgA ASCs, including circulating IgA+ plasmablasts, and by most IgA+ plasma cells in the salivary glands, small and large intestines, appendix, and tonsils (14–16, 32). Our results suggest that CCR10+ IgA ASCs in the uterus might migrate from cervical LNs but not from ilioc LNs that directly drain LNs of reproductive
tissues. Also, unlike other mucosal tissues, female reproductive tracts are devoid of MALT, which could induce T and B cell activation (1, 33).

Although Ag-specific IgG ASCs also were induced in both the uterus and the vaginal canal after i.n. administration, total numbers of IgG ASCs were much lower than those of IgA ASCs (Fig. 1D).

In addition, blockade of MEC/CCL28 markedly inhibited migration of uterus IgA ASCs but had negligible effects on IgG ASCs in the genital mucosa (Fig. 5B). This likely is caused by different chemokine expression patterns on both IgA and IgG plasma cells. We have tried to identify specific chemokine receptor expression of the IgG plasma cells in the cervical LN that mainly migrate to systemic tissues and to mucosal compartments as well. Interestingly, chemokine receptors (e.g., CCR6, CCR7, CCR9, and CCR10) that were involved mainly in mucosal homing were not defined on the IgG plasma cells (data not shown). It is plausible that IgG ASCs in genital mucosa including the uterus and vaginal canal migrate from cervical or other systemic sites independent of MEC/CCL28.

One unique finding in this study was that i.n. challenge with CT, HSV-2, or CpG ODN accelerated MEC/CCL28 expression in the uterus but not in the cervico-vaginal tract (Fig. 2). Others report that MEC/CCL28 is expressed by epithelia in diverse mucosal tissues (14, 34, 35). Thus, MEC/CCL28 expression could vary depending on the type of epithelial cell in the uterus and vaginal tract. The uterine epithelium is a type I mucosa that is covered by simple single-cell epithelia, whereas the lower genital tract including the vaginal canal is type II mucosa covered by stratified squamous epithelia, which share many features with the skin (36, 37). MEC/CCL28 is expressed in type I mucosa, including the large intestinal epithelial cells and lactating mammary gland (14, 15). Of note, total and Ag-specific IgA plasma cells isolated from lactating mammary glands were attracted specifically toward the MEC/CCL28 after i.n. vaccination, whereas the percentage of cells migrating from lactating mammary glands was significantly lower than the percentage from the uterus (Supplemental Fig. 1).

We therefore speculate that type I mucosal epithelia of the uterus is more susceptible to expressing chemokines such as MEC/
CCL28 against several danger signals than the stratified squamous type II mucosa of the cervico-vaginal tract.

When we searched the genetic structure of MEC/CCL28 using a computational application (i.e., MAPPER) (38, 39), the estrogen receptor (ER) binding site was expressed frequently in the promoter regions of MEC/CCL28 (Supplemental Fig. 2A). In contrast, there was no ER binding site in the promotor regions of other chemokines such as TECK/CCL25 and CTACK/CCL27 (data not shown). Further, treatment with an estrogen agonist (i.e., tamoxifen) accelerated expression levels of MEC/CCL28 in the uterine epithelium after i.n. vaccination, whereas an estrogen antagonist (i.e., raloxifene) inhibited their expression in the uterus (Fig. 6C). The mucosal immune system of the female reproductive tract is regulated precisely by sex hormones to protect against potential pathogens without compromising fetal survival (1). The female genital tract is unique in that epithelial cells are responsive to sex hormones (40). Both estradiol and progesterone secreted during menstrual cycles act directly and indirectly on epithelial cells in the female reproductive tract to modify acquired and innate immune responses (37). In addition, Sentman et al. (41) demonstrated that estradiol treatment enhances mRNA expression of CXCL10 and CXCL11, which is necessary for NK cell migration in human endometrium. A recent study also revealed that SDF-1/CXCL12 gene expression in rat uterine cells is induced by estrogen and reduced by estrogen antagonists (42). Thus, our findings and those of others suggest that immunization via mucosal routes (i.e., i.n.) might accelerate female sex hormone secretion and thereby activate expression of MEC/CCL28 through ER binding in its promotor region in the uterine epithelium. It seems likely that female hormones did not directly influence the immune cells but rather acted on stromal and epithelial cells to produce chemokines to recruit immune cells. Although it is still unclear whether immune modulators (i.e., adjuvants) directly stimulate estrogen secretion or whether some signaling activated by adjuvants affects estrogen release, our findings clearly demonstrated that MEC/CCL28 expression by the uterus after i.n. immunization is regulated by female sex hormone (i.e., estrogen).

The uterine immune system is under the control of estradiol, which acts to increase the levels of both IgA and secretory component in uterine secretions (37); however, progesterone has a direct role in suppressing production and release of secretory component (43, 44). Previous studies demonstrated that sex hormones regulate migration of circulating IgA ASCs into the genital tract (45). Moreover, both lgs and lactoferrin levels are suppressed in women given oral contraceptives (37). Postmenopausal women with estrogen deprivation have decreased CD4 T and B lymphocytes and less cytotoxic NK cell activity (46). We found significantly fewer Ag-specific IgA ASCs in the uterus after mice were fed raloxifene to block estrogen, mimicking the effect of oral contraceptives (Fig. 6D). Taken together, female sex hormone (i.e., estrogen) controls recruitment of CCR10+ IgA ASCs via MEC/CCL28 expression in uterine mucosa.

In addition to the location of ER in the promoter region of MEC/CCL28, we found innate immune-related genes such as IFN regulatory factor (Supplemental Fig. 2A). Also, i.n. challenge with CT could not accelerate MEC/CCL28 expression in the uteri of mice with innate immunity deficiency (i.e., MyD88/TRIF−/− and Ifnar−/−) unlike wild-type mice with innate immunity (Supplemental Fig. 2B). Several recent studies demonstrate that the innate immune responses in female reproductive organs are controlled by hormones and vary with menstrual cycle stage (37, 47). For instance, production of proinflammatory cytokines and antimicrobials by human uterine epithelial cells are regulated differently by estradiol (47). ERα interacts with target gene promoters either directly, through specific estrogen response elements, or indirectly, through contacts with other DNA-bound transcription cofactors (coactivator or corepressor) (48). It seems plausible that certain levels of estrogen can activate innate immune responses, which subsequently modulate activation of MEC/CCL28 expression in the uterus; however, as of yet, we have not been able to verify this hypothesis. Further clarification of how i.n. immunization increases estrogen release and how estrogen affects MEC/CCL28 production in the uterus is being addressed currently.

Our findings suggest that Ag-specific IgA ASCs are mainly in the uterus and not in the cervix and vaginal canal after i.n. vaccination. These IgA ASCs in the uteri of vaccinated mice express high levels of CCR10, the counterpart of MEC/CCL28. Further, IgA ASCs in the uteri of vaccinated mice migrate to MEC/CCL28 and are reduced in mice treated with neutralizing anti-MEC/CCL28 Ab. Estrogen regulates MEC/CCL28 expression in the uterus both at steady state and after i.n. vaccination. Therefore, our results strongly suggest that a mucosal vaccination regimen targeting a combination of CCR10-expressing IgA ASCs in the cervical LN and MEC/CCL28 activation in the uterus could be useful for preventing infectious diseases in the reproductive organs. Further, physiologically, maintaining high concentrations of estrogen and estradiol during pregnancy could protect the fetus by providing Ag-specific IgA secretion in the uterus that protects against pathogenic infection.

Disclosures
The authors have no financial conflicts of interest.

References


