Published in final edited form as:

Am J Reprod Immunol. 2015 October ; 74(4): 333–344. doi:10.1111/aji.12409.

Effect of mucosal cytokine administration on selective expansion of vaginal dendritic cells to support nanoparticle transport

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Abstract

Problem—The capacity of antigen-carrying vaccine nanoparticles administered vaginally to stimulate local immune responses may be limited by the relatively low numbers of antigen-presenting cells (APCs) in the genital mucosa. Because inflammation is associated with increased susceptibility to sexually transmitted infections, we sought to increase APC numbers without causing inflammation.

Method of Study—In this study, we evaluated intravaginal delivery of chemokines, growth factors, or synthetic adjuvants to expand APCs in reproductive tissues.

Results—We found that granulocyte macrophage-colony stimulating factor (GM-CSF) stimulated expansion of CD11b+ dendritic cells within 24 h of intravaginal administration, with no effect on Langerhans cells or macrophages. Expansion of the CD11b+ DC population was not associated with increased inflammatory cytokine production, and these cells retained phagocytic function.

Conclusion—Our data suggest that non-inflammatory expansion of mucosal APCs by intravaginal GM-CSF could be used as an adjuvanting strategy to potentiate the genital immune response to nanoparticulate mucosal vaccines.

Keywords

dendritic cells; GM-CSF; chemokines; cellular expansion; reproductive mucosa

Introduction

Overcoming the tightly down-regulated immune environment at mucosal sites has significant implications for the design of mucosal vaccines[1]. An additional challenge in the

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lower female reproductive tract is the tissue's unique anatomy, including the limited network of lymphatic structures and secondary lymphoid structures, which restricts migration of immune cells in the absence of inflammation[2-6]. In general, this predisposes the female reproductive tract to weak immune responses to intravaginal vaccination.

Antigen presenting cells (APCs), specifically dendritic cells (DCs), are important for maintaining the balance between tolerance and pathogen-induced inflammation. A critical role for DCs in shaping genital immunity is suggested by the observation that strong adjuvants are required to boost genital immune responses to non-replicating subunit vaccines[7,8]. Since DCs are not a homogeneous population, targeting vaccines to specific DC subsets could allow effector T cell functions to be tailored to the relevant pathogen. However, targeting DCs is challenging given that they constitute a small fraction of cells in the vaginal mucosa, typically 5-10% of the cells in the basal vaginal epithelium and lamina propria of women without an active infection[9]. As such, strategies that recruit or expand mucosal DC subsets *in situ* may enhance immunity to vaginally administered vaccines.

Several approaches have been investigated to modulate DC numbers and activation state at immunization sites. Molecular adjuvants, such as the TLR9 agonist CpG, upregulate expression of co-stimulatory molecules[10-13]. Mucosal vaccines delivered in the presence of CpG induced cytokine secretion and increased infiltration of activated CD8+ T-cells[14]. Furthermore, intravaginally-delivered CpG transiently recruited MHC II+ CD11b+ antigen presenting cells to the vaginal submucosal[15]. However, molecular adjuvants like CpG cause strong, non-specific immune stimulation[16,17]. Such generalized inflammation can break down the natural mucosal barrier and recruit immune cells that serve as targets for mucosal pathogen transmission[18,19].

In addition to synthetic adjuvants, cytokines or growth factors can be used to enhance DC populations in the mucosa. This strategy minimizes non-specific inflammation by specifically recruiting immature or precursor APCs. Chemokines, such as MIP-3α, can promote chemotaxis of APCs to the site of administration[20-23]. Growth factors, such as granulocyte-macrophage stimulating factor (GM-CSF), may specifically stimulate the differentiation of DCs from local undifferentiated monocytes[24,25]. In fact, recombinant GM-CSF (Leukine®) is used in the clinic to reconstitute myeloid cell populations in blood after chemotherapy[26,27].

In vaccination studies, GM-CSF alone and in combination with other cytokines has been shown to differentiate monocytes into fully functional DCs that can stimulate T cell immune responses. For example, *in vivo* transfection with a GM-CSF encoded plasmid successfully expanded liver CD11c+ dendritic cells that were highly efficient in priming T cells[28]. In CSF2-null mice, which cannot make GM-CSF, DCs and macrophages in the murine uterus express less MHC class II on their surface and are less effective in priming antigen-specific CD4+ and CD8+ T cells[29]. Cultures of PBMC with GM-CSF and other cytokines yields DCs that show high expression of HLA-DR and co-stimulatory molecules that efficiently present antigen and stimulate CD4+ T cell responses[30-32]. These results suggest that GM-CSF is important for DC function.

While many studies have evaluated the use of chemokines and growth factors in the context of systemic injections, few studies have evaluated their use for recruiting cells into the vaginal mucosa following topical administration. The need for mucosal adjuvants that modulate immunity in the reproductive mucosa motivates the discovery and application of agents that can expand key immune cell populations without causing concomitant local inflammation. Our goal is an intravaginal administration strategy focused on chemokine- or growth factor-mediated expansion of mucosal DCs. Here, we evaluate the numbers and phenotypes of mucosal DCs that arise from topical administration of a synthetic adjuvant (CpG) or a growth factor (GM-CSF), either alone or in combination with the chemokine MIP-3α, to the vaginal mucosa. Our results demonstrate that low doses of GM-CSF expanded *in situ* a functionally phagocytic mucosal DC population without eliciting inflammatory cytokine production. Furthermore, we show that GM-CSF specifically expanded CD11b+ dendritic cells but not Langerhans cells. Fluorescent nanoparticles administered intravaginally were phagocytosed by this mucosal DC population. Therefore, expanding functional DC subsets by topical administration of GM-CSF may be a promising strategy to potentiate protective immune responses to mucosal vaccines.

Materials and Methods

Materials

Recombinant murine MIP-3α was purchased from Peprotech (Rocky Hill, NJ) and GM-CSF from Cell Sciences (Canton, MA). Chemokines and growth factors were resuspended in sterile Dulbecco's Phosphate-Buffered Saline (DPBS) to a concentration of 1 mg/ml. Murine TLR9 ligand CpG ODN 1826, a Class B CpG oligonucleotide, was purchased from Invivogen (San Diego, CA). CpG was formulated in endotoxin-free water at 1 mg/ml. Calginate swabs used to remove mucus from mouse vaginal tracts were obtained from Fisher Scientific (Waltham, MA). Medroxyprogesterone acetate was obtained through the University of Washington pharmacy from Greenstone LLC (Peapack, NJ). Murine TNF-α and IL-1β Standard ELISA kits were purchased from Peprotech. Fluorescent yellow/green 200 nm nanoparticles (FluoSpheres) 505/515 nm (excitation/emission) were purchased from Invitrogen.

Animals and intravaginal delivery of materials

Female C57Bl/6J mice (8-12 weeks old) were purchased from Jackson Laboratories. Prior to intravaginal administration, mice were subcutaneously administered 2 mg of medroxyprogesterone acetate (Depo-Provera®) formulated in sterile DPBS to reduce variability resulting from differences in stage of estrus cycle. All mice receiving treatments were anesthetized with isoflurane in an induction chamber. Genital tracts were flushed out three times with 50 μl sterile DPBS and vaginal lumens were swabbed with Calginate swabs to remove mucus. Mice were intravaginally administered 10 μg of chemokines, growth factors, adjuvants, or sterile DPBS (negative control) in 10 μl using a micropipette. Mice were transferred back to the induction chamber and hung upside down for 10 min to improve vaginal retention of materials. Animals were euthanized 24 h after treatments by carbon dioxide followed by cervical dislocation. Treatment groups, dosing, and animal experiment timelines are described in Figure 1a. All animal studies were approved by and in

compliance with guidelines set by the University of Washington Institutional Animal Care and Use Committee.

Antibodies

All antibodies were purchased from BD Biosciences (Franklin Lakes, NJ) unless otherwise indicated. Primary antibody-fluorochrome pairs used for staining and identifying cell populations included: APC anti-mouse CD11c, PE anti-mouse I-Ad/I-Ed (MHC II), PerCP-Cy5.5 anti-mouse CD45, APC-Cy7 anti-mouse CD11b, PE-Cy7 anti-mouse CD8, and FITC anti-mouse F4/80 were purchased from eBioscience. Isotype antibodies used were APC Hamster IgG1 λ1, PE Rat IgG2b κ, APC-Cy7 Rat IgG2b κ PerCP-Cy5.5 Rat IgG2b κ FITC Rat IgG2a κ and PE-Cy7 IgG2a κ. LIVE/DEAD Fixable Green dead cell stain kit was purchased from Molecular Probes (Eugene, OR). For nanoparticle uptake studies, the following additional stains were used: Brilliant Violet-421 anti-mouse CD86 (BioLegend) and LIVE/DEAD Fixable Near-IR dead cell stain kit. Purified rat anti-mouse CD16/CD32 was used to block Fc receptors (Fc block) on cells prior to cell surface staining. One Comp eBeads were purchased from eBioscience for antibody compensation and ArC amine reactive compensation bead kit was purchased from Molecular Probes for live/dead compensation.

Isolation of single cells from vaginal tissue and staining

Single cell suspensions were prepared from excised spleens and vaginal tissues using established techniques. Splenocytes were isolated by mechanical digestion of spleens with a syringe plunger in a 70 μm cell strainer on a petri dish with 5 ml of cRPMI (complete RPMI – RPMI 1640 + 10% heat inactivated FBS). Suspensions were restrained through a second 70 μm filter and centrifuged in cRPMI at 1200 rpm for 5 min. Cells were subsequently incubated for 5 min with red blood cell (RBC) lysis buffer and further washed by centrifugation in cRPMI. Vaginal tracts including cervix, but not uterine horns, were dissected, placed in sterile DPBS on ice, and cut into small pieces (~2 mm diameter). Organs within each group (n = 3-6) were pooled to reduce variability. Cell isolation from vaginal tissues was adapted from previously published protocols[33]. Vaginal tissues were placed in 3 ml of digestion media, made with a 1:1 ratio of PBS and R15 (RPMI 1640 with FBS, 1% Penicillin Streptomycin) at a final collagenase from clostridium histolyticum (Sigma-Aldrich) concentration of 1 mg/ml. 3 μl of DNAse at 1 Unit/ml was added to tissue digestion preparations. Tissues were agitated for 30 min on an orbital shaker (New Brunswick Scientific, Incubator Shaker Series). Cell isolation suspensions were subject to further mechanical digestion through a blunt-end, 16-gauge needle and filtered through a 70-μm cell strainer. Vaginal tissues were subjected to two rounds of chemical and mechanical digestion.

Cell suspensions were stained with a LIVE/DEAD Fixable Cell stain kit, washed, incubated with Fc block at 4°C for 15 min, and stained with antibody cocktails before washing and fixation with 1% paraformaldehyde. Samples were acquired on a FACSCanto2 with a 405-nm violet laser, a 488-nm blue laser, and a 633-nm red laser.

Tissue homogenization and cytokine measurements

The vaginal tract, including cervix, but not uterine horns was dissected 24 h after delivery of chemokines, growth factors, or adjuvants for tissue homogenization. Tissues were frozen at −80°C until use. Vaginal tracts were massed and placed in 0.5 ml of homogenization buffer (0.05% Triton X-100 in Hank's Balanced Salt Solution - HBSS buffer) and 2 ml of protease inhibitor. Tissues were fully homogenized using a Tissue Tearor (Biospec Products, Inc). Murine standard ELISA development kits for TNF-α and IL-1β (Peprotech Rocky Hill, NJ) were used to determine cytokine levels in tissue homogenates using standard procedures provided by the vendor.

Identification of draining lymph nodes

Draining lymph nodes, including the inguinal and iliac lymph nodes (Figure 1b,c), were identified by dye-guided lymph node mapping. Briefly, mice were subcutaneously injected in the footpad with a 5% (w/v) solution of Evans Blue prepared in sterile PBS. Mice were euthanized by CO_2 and cervical dislocation after 15 minutes, to allow for lymphatic trafficking of the dye. As described by Ruddell et al., draining inguinal and iliac lymph nodes were identified visually[34].

FluoSphere preparation and quantification

FluoSpheres (supplied at 2%, w/w) were buffer exchanged to remove sodium azide. Briefly, nanoparticles were washed three times with 70% EtOH in Amicon filters (10 KDa molecular weight cutoff) and twice with endotoxin-free water by centrifugation at 4,000 × g for 20 min. FluoSpheres were collected and the concentration was determined using a TECAN fluorescence microplate reader. All particles were resuspended in endotoxin-free water to a final concentration of 0.5% (w/w) for intravaginal administration.

Intravaginal FluoSphere administration and imaging

Depo-provera treated mice were administered DPBS, GM-CSF, or CpG 24 h prior to intravaginal administration of nanoparticles. Mice were anesthetized using isoflurane in an induction chamber and intravaginally administered 10 μl of 0.5% (w/w) FluoSpheres. After 24 h, mice were euthanized by CO_2 and cervical dislocation. Vaginal tracts and the iliac and inguinal draining lymph nodes were dissected and placed on ice for imaging and flow cytometry analysis. A xenogen *in vivo* imaging system (iVis) was used to measure fluorescence at 505/515 nm (ex/em) for evaluating nanoparticle distribution in the vaginal tract, and in iliac and inguinal lymph nodes. Samples were acquired on a FACSCanto2. Samples were subsequently processed using an ImagestreamX Mark II imaging cytometer (Amnis, Seattle, WA) to visualize and evaluate cell internalization of nanoparticles.

Results

Mucosal APC subsets at homeostasis in the genital tract of female mice

Female mice treated with progesterone were maintained in the diestrous phase of the menstrual cycle. During diestrous, histological analysis shows that the murine lower female genital tract is comprised of a thinned epithelium overlying the lamina propria (Figure 1d).

Phenotypic analysis of murine vaginal cells by flow cytometry revealed that leukocytes (CD45+) accounted for up to 15% (11.03 ± 5.07 / mean ± S.D.) of the total cell population. Total mucosal DCs (CD45+MHCII+CD11c+) accounted for <1% (0.95 ± 1.16) of the total cells but up to 5% (3.93 ± 1.54) of total CD45+ leukocytes (Supplementary Fig. 1). Of this mucosal DC population, we observed that ~75% (75.33 ± 7.77) expressed the CD11b+ marker typical of subepithelial DCs whereas ~25% (24.68 ± 7.73) were CD11b negative (Supplementary Fig. 1), which is characteristic of Langerhans cells (LCs)[35] ($p < 0.0001$, Student's t-test). Macrophages (CD45+MHCII+CD11c-CD11b+F4/80+) accounted for ~1% (0.67 ± 0.71) of the total cells and ~5% (5.78 ± 3.97) of the CD45+ cell population. In summary, we found that macrophages and dendritic cells have similar abundance of total mucosal leukocytes in the lower genital tract of female mice during diestrous.

GM-CSF treatment selectively enriches for CD11b+ dendritic cells

We tested the effect of delivering different agents topically to the vaginal mucosa to enrich for mucosal APCs. MIP-3α and GM-CSF were administered alone or in combination and compared to CpG, a synthetic adjuvant that recruits DCs by triggering local inflammation[19] (Figure 1a). Flow cytometry was used to measure the quantity and phenotype of different APC populations amongst total leukocyte cells (CD45+) collected from the vaginal tissue at 24 h post-administration. Live, CD45+ cells were gated on MHC II, CD11c, CD11b, and F4/80 cell surface markers as described in Supplementary Figure 2.

At the doses used in our studies, the CpG and MIP-3α treatment groups did not significantly affect the magnitudes of any of the APC populations compared to the PBS controls (Figure 2). Mucosal macrophages (CD45+MHC II+CD11c-CD11b+F4/80+) did not change in response to any of the intravaginal administrations (Figure 2a). In contrast, we found that the total mucosal DC (CD45+/MHCII+/CD11c+) population doubled to nearly 10% of the CD45+ leukocyte population upon administration of GM-CSF alone or in combination with the MIP-3α chemokine compared to the MIP-3α treatment group (Figure 2b). Surprisingly, we found that GM-CSF significantly expanded the CD11b+ DC populations compared to the synthetic adjuvant CpG used at an equivalent dose. In particular, CD11b+ mucosal dendritic cells (CD45+MHC II+CD11c+CD11b+F4/80−) were expanded an average of two fold by GM-CSF as compared to the PBS treatment group, and constituted up to 5.5% of the CD45+ leukocyte population (Figure 2c). We did not observe significant changes in the magnitude of mucosal Langerhans cells (CD45+/MHC II+/CD11c+/CD11b−) (Figure 2d). Although total tissue leukocytes (CD45+) appeared to increase in GM-CSF-treated mice, the difference was not statistically significant (Figure 2e).

Chemokines and growth factors do not impact systemic cell populations or vaginal cytokines

The reproductive mucosal tissues are highly vascularized, allowing for systemic distribution of intravaginally delivered agents. To understand if intravaginal administration of MIP-3α, GM-CSF, or CpG impacts systemic APC populations, we measured the magnitude of APC populations in the spleen by flow cytometry. At the doses used in our studies, we did not observe a significant difference in the total number of leukocytes or DCs (CD45+ MHC II+ CD11c+) in the spleen between any of our treatment groups (Figure 3a,b). Leukocytes

constituted about 50% of the total splenic cells and DCs represented only 1-3% abundance of these cells. Subpopulations of cells expressing CD11b and F4/80 were not observed in splenocyte-derived dendritic cells. These results suggest that the delivered factors did not impact systemic levels of leukocytes and dendritic cells.

During mucosal infections, expansion of APCs also results in production of inflammatory signals to modulate local immune responses[36-38]. We measured TNF-α and IL-1β cytokine levels in the vaginal tract 24-h post-topical administration of GM-CSF and CpG to evaluate if delivery of these agents and the resulting changes in the local immune cell populations caused inflammation. Our treatment groups show concentrations of TNF-α at ~1 pg/ml/mg and of IL-1β at ~5 pg/ml/mg, expressed per mass of vaginal tissue Figure 4). A trend towards increased IL-1β levels was observed in the CpG treatment group. We found no significant differences in the levels of these pro-inflammatory cytokines between mice that received GM-CSF compared to control mice treated with PBS. Therefore, despite significant enrichment of DCs and CD11b+ DCs up to 2-3 fold, we did not observe evidence of pro-inflammatory cytokine secretion.

Fluorescent nanoparticles distribute in the vaginal tract and accumulate in draining lymph nodes

To evaluate if APCs retained their functional capacity to associate with or phagocytose nanoparticles, we measured uptake and trafficking of 200 nm fluorescent nanoparticles 24 hours after vaginal tissues were administered with PBS, GM-CSF, or CpG. Fluorescence in the vaginal tract and iliac lymph nodes was quantified using LivingImage Software, and autofluorescence was normalized to the respective tissues from control mice. We observed high fluorescence intensity in the vaginal tract for all mice receiving nanoparticle administrations (Figure 5a). Quantification of fluorescence in the vaginal tracts demonstrated significant retention of intravaginally delivered nanoparticles in the genital lumen after 24 hours, with a trend towards CpG-treated mice retaining the most fluorescent nanoparticles (Figure 5c). We also analyzed for the presence of fluorescent nanoparticles in the iliac and inguinal lymph nodes, which drain the lower female genital tract. No fluorescence was observed in inguinal lymph nodes of mice from any treatment group (data not shown). However, we found fluorescent nanoparticles in the iliac lymph nodes of mice treated with PBS and GM-CSF but not CpG (Figure 5b). Although the mean fluorescence in the iliac lymph nodes was higher for GM-CSF treated mice, we observed no significant difference between our treatment groups (Figure 5d). In summary, nanoparticles delivered into the vagina were well retained for at least 24 hours and distributed to the iliac but not inguinal lymph nodes.

Dendritic cells internalize nanoparticles

To evaluate the function and phagocytic capacity of the total mucosal DC (CD45+/MHC II +/CD11c+) population that was expanded in the vaginal tract by GM-CSF treatment, we assessed accumulation of fluorescent nanoparticles in these cells by flow cytometry. Fluorescent nanoparticles (10 μL of 0.5%, w/w) were administered intravaginally at 24h following initial treatment with PBS, CpG, or GM-CSF. After allowing an additional 24h for nanoparticle trafficking, the mice were sacrificed and total mucosal dendritic cells (MHC

II+ CD11c+) were quantified for NP fluorescence. In comparison to mice that did not receive NPs, we found that ~30% of DCs were NP+ (Figure 6a). At 48h post-administration of growth factors or adjuvants, total mucosal DCs remained elevated in GM-CSF treated mice compared to mice administered PBS or CpG (Figure 6b). We did not observe a difference in phagocytosis of NPs by mucosal DCs between treatment groups, indicating that treatment with GM-CSF did not stimulate enhanced uptake of particulates (Figure 6c).

To ascertain if the cell-associated fluorescence in our nanoparticle uptake studies was due to internalized or cell surface-associated nanoparticles, we analyzed samples from each treatment group on an Amnis ImageStream imaging flow cytometer. In these experiments, after nanoparticle treatment and cell isolation, cells were stained for viability and MHC II+ expression. Intracellular total fluorescence intensity was normalized to the total cell fluorescence to calculate an internalization score using the Amnis software. Cell surface intensity was defined by PE fluorescence, indicating MHC II+ antigen presenting cells. We calculated internalization scores of 1.7 for PBS, and 1.9 for GM-CSF and CpG treatment groups. These values indicate that approximately 65% of nanoparticle fluorescence was due to internal fluorescence in MHC II+ cells. The internalized particles were observed as punctate fluorescence in MHC II+ cells captured by the imaging flow cytometer (Figure 6d).

Discussion

In this study, we examined if intravaginal delivery of DC chemokines, growth factors, or synthetic adjuvants could expand mucosal dendritic cells and facilitate nanoparticle biodistribution to distal sites. We found that a low dose of GM-CSF doubled the frequency of total mucosal DCs (CD45+ MHC II+ CD11c+) to 10% of the leukocyte population in the vaginal mucosa. Furthermore, we observed a specific enrichment of subepithelial dendritic cells, which were identified by the presence of the CD11b surface marker from ~2% to ~5% of leukocytes. No change was observed in mucosal F4/80+ macrophages or CD11b- mucosal Langerhans cells. Overall, we observed a trend towards increased CD45+ leukocytes in GM-CSF-treated mice, but the difference was not statistically significant. Because DCs constitute only 2-5% of the CD45+ cell population, even a doubling of DCs would cause only a small change in the CD45+ leukocyte population, explaining why total leukocytes increased only marginally. Given what is known and described in the literature about GM-CSF, it is likely that increased proliferation or differentiation of local DCs and their precursors contributed to the expanded CD11b+ DC population, rather than recruitment from the periphery. However, our data do not distinguish between these possibilities, and future work will need to elucidate if GM-CSF acts strictly on mucosal cells to influence cellular infiltration of immune cells into the vagina.

While the exact function of each DC subset remains controversial, CD11b+ DCs have been previously demonstrated to be important for priming protective CD4+ T responses to vaginal infection by HSV-2[39]. In this study by Zhao et al., mucosal Langerhans cells did not exhibit the ability to migrate to draining lymph nodes and prime T cells, implicating a potential tolerogenic role for these cells in the reproductive tissues. Evidence has also showed that GM-CSF-induced dendritic cells are monocyte-derived and are generated under inflammatory conditions. Unlike steady-state dendritic cells, which are CD11b-, these

inflammatory dendritic cells are CD11b+ and play an important role in shaping immunity[40,41]. Staining for other DC subset surface markers, such as CD14, may help further elucidate the specific polarization of these expanded DCs towards Th1 versus Th2[42]. Thus, the GM-CSF-expanded population of CD11b+ dendritic cells in our study may play a role in enriching a cell population that has phagocytic and migratory capacity. As such, our results demonstrate that GM-CSF can be used to expand the local mucosal dendritic cell population in the vaginal tract within a 24-hour window.

Reproductive tissues are highly vascularized, potentially allowing for systemic delivery from topical administrations. To evaluate if vaginal administration of chemokines, growth factors, and adjuvants at the doses used in our animal models modulated immune cell populations in the periphery, we analyzed cell populations in the spleen. We found no statistically significant difference in levels of leukocytes and dendritic cells in the spleen between all treatment groups, suggested that intravaginally delivered agents acted locally and did not influence systemic immune cell populations.

Although MIP-3α is a well-defined DC chemoattractant, we found no significant increase in the total mucosal DC population in response to this chemokine, but this may be due to the low dose used in our studies. To our surprise we also found that the CpG-ODN 1826, a synthetic adjuvant that has been previously described to expand MHC II+ CD11b+ cells after intravaginal delivery, had no significant effect on the antigen presenting cell population in our study. This is likely a consequence of the lower dose used in our study as compared to other studies that deliver CpG at 20-100 μg[15,43,44]. That CpG was likely dosed too low in our study is also reflected by the finding that it did not stimulate TNF-α and IL-1β.

In general, the levels of TNF-α and IL-1βwere consistent with published data measuring these cytokines in the mouse reproductive mucosa at steady-state after Depo-Provera administration[45]. We did not observe an increase in TNF-α and IL-1β upon GM-CSF treatment, suggesting that while GM-CSF was able to expand the CD11b+ DC population, it did not stimulate increased cytokine secretion. This does not rule out, however, that increased dosing or co-delivery of GM-CSF and other maturation agents would induce cytokine secretion[46,47].

Whole animal fluorescent imaging allowed us to macroscopically visualize biodistribution of fluorescent nanoparticles in the vaginal tract and lymph node tissues. Polystyrene nanoparticles were retained in the vaginal tract for at least 24-h post-topical administration. Furthermore, fluorescence imaging demonstrated that nanoparticles distributed to the iliac lymph nodes. As the iliac lymph nodes are one of the major draining lymph nodes from the reproductive tissues, we expect intravaginally administered materials to reach these tissues either by cell-associated or cell-free trafficking. Previous studies evaluating biodistribution of quantum dots demonstrated cell-free trafficking to the lumbar lymph nodes after intravaginal delivery[48]. Other studies have shown that nanoparticles in the range of 20-200 nm are able to drain to lymph nodes where they are taken up by cells whereas NPs larger than 500nm were associated with tissue resident DCs originating from the site of injection[49]. In our study, we did not distinguish between cell-free and cell-associated trafficking of the nanoparticles. Future work may focus on differentiating between NP fluorescence in lymph

node resident immune cells versus migratory immune cells to elucidate modes of NP trafficking from the genital mucosa.

By analyzing the cell populations that showed nanoparticle fluorescence, we found that within all treatment groups about 30% of mucosal dendritic cells (MHC II+ CD11c+) associated with NPs. We observed no statistically significant difference in NP association with cells between treatment groups, indicating that phagocytic function of mucosal DC populations was not altered by any of the pre-treatments. We employed the Amnis ImageStream X imaging flow cytometer to ascertain if nanoparticle fluorescence in antigen presenting cells (MHC II+) was surface associated or intracellular. Our analysis showed that ~65% of nanoparticle fluorescence was localized inside MHC class II+ cell populations for all treatment groups. This indicates that 24 hours after intravaginal administration, nanoparticles were still localized inside MHC II+ APCs. Punctate nanoparticle fluorescence in MHC II+ cells suggests that nanoparticles were likely sequestered in intracellular vesicles. Although we observed fluorescence in lymph nodes by Xenogen imaging, we were unable to detect nanoparticles in lymph node preparations after staining for mucosal DCs by conventional flow cytometry (data not shown). Given the low percentage of mucosal DCs in lymph nodes, it is likely that cell-associated nanoparticles were lost during sample staining and washing. Other groups that have attempted to detect particulates by flow cytometry after intravaginal administration of materials have also noted limited success identifying cell populations with particles in draining lymph nodes[48]. In conclusion, GM-CSF induced a greater number of DCs in the vaginal mucosa, but it did not alter their individual phagocytic capabilities. Thus, intravaginal administration of GM-CSF can be used to increase the number of nanoparticle-processing DCs in the vagina, which may be a useful strategy to improve the presentation and immunogenicity of topically delivered particulate vaccine antigens.

Conclusion

Our study aimed to describe the effect of exogenously delivered chemokines and growth factors on the local immune cell populations of the murine genital tract. We found that GM-CSF increased the total mucosal DC population, specifically CD11b+ dendritic cells. Further, we found that the effects of GM-CSF were localized to the vaginal tract and did not impact systemic leukocyte levels. Finally, our results demonstrate that the enriched APC population in the vaginal tract is functional and able to phagocytose nanoparticulate materials. Extensions of our work will aim to optimize the *in situ* expansion of DCs using GM-CSF as a function of dose and administration schedule, and establish that its co-delivery with an antigen improves immune responses to a mucosal vaccine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work is funded by an NIH Director's New Investigator Award to K.A.W. (1DP2HD075703) and with the support of a UW STD/AIDS Training Fellowship to R.R. (NIHT32AI07140).

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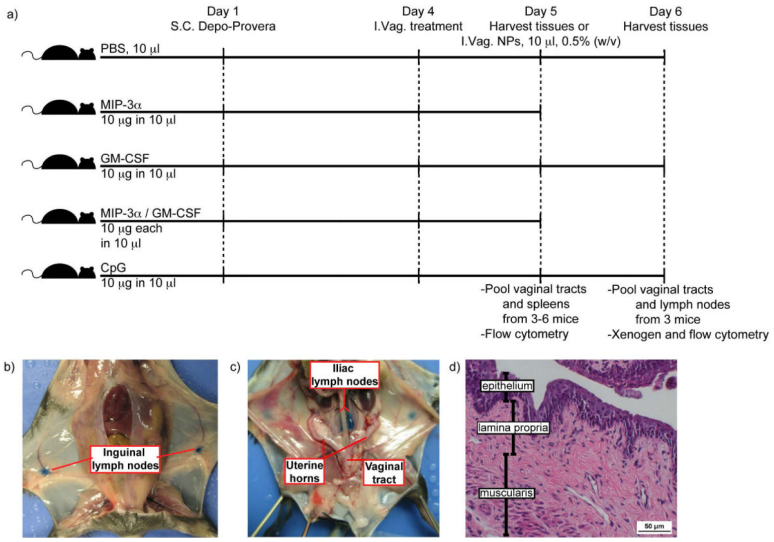

Figure 1. Administration timeline, lymph node mapping, and histologic features

Mice were cycled with Depo-Provera on Day 1 and intravaginally administered chemokines, growth factors, and adjuvants on Day 4. On day 5, tissues were harvested for cell analysis from all treatment groups; Nanoparticles (NPs) were administered to indicated mouse groups (continuing lines), which were further followed by fluorescence imaging to detect nanoparticle distribution and tissue processed for flow cytometry analysis on Day 6 (a). Evans Blue was subcutaneously injected in the footpad to map the inguinal (b) and iliac (c) lymph nodes. The uterine horns and vaginal tract are identified upon necropsy (c). Photomicrograph of H&E-stained mouse vaginal mucosa during progesterone-induced diestrous shows a thinned epithelium (d).

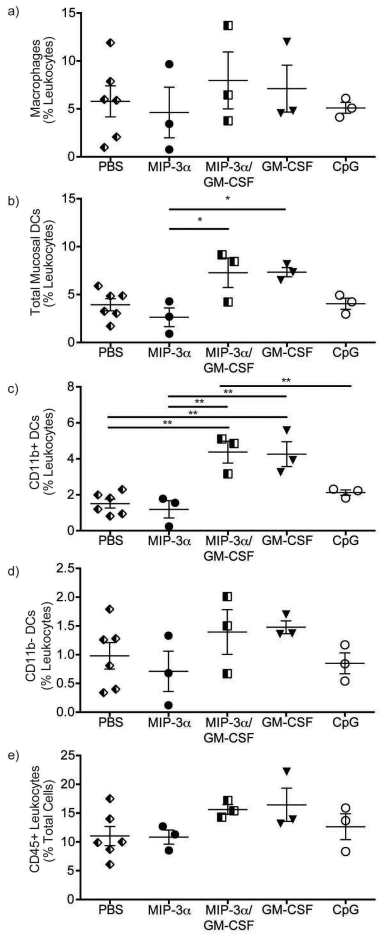

Figure 2. Identification of expanded immune cell populations in the vagina

Murine vaginal cells were isolated and stained to identify cell populations 24 hours post-vaginal administration of PBS, MIP-3α, GM-CSF, MIP-3α and GM-CSF, or CpG-ODN 1826. Each symbol represents vaginal tracts pooled from 3-6 mice to reduce mouse-to-mouse variability, and at least 3 independent experiments were performed per treatment. Effects of treatments are described on macrophages (a), total mucosal DCs (b), CD11b+ DCs (c), CD11b- DCs (d), and CD45+ leukocytes (e). Data were analyzed for statistical significance by a one-way ANOVA followed by a Bonferroni post-test (* $p<0.05$, ** $p<0.01$) and are displayed as mean ± S.E.M. Asterisks above lines indicate statistical significance between treatment groups.

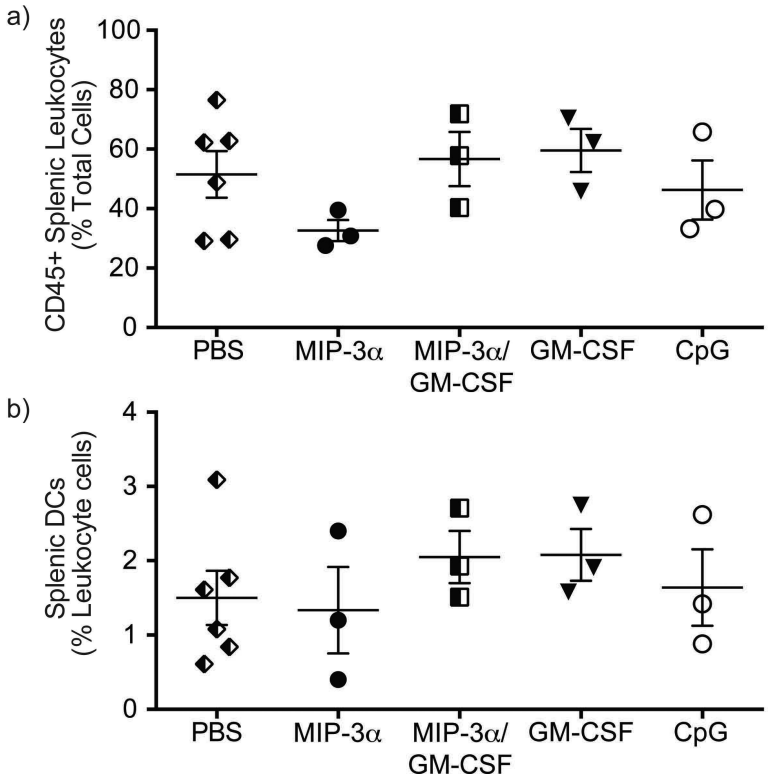

Figure 3. Spleen immune cell populations

Murine splenocytes were isolated and stained to identify immune cell populations following vaginal administration of PBS, MIP-3α, GM-CSF, MIP-3α and GM-CSF, or CpG-ODN 1826. Each symbol represents spleens pooled from 3-6 mice to reduce mouse-to-mouse variability, and 3 or more independent experiments were performed per group. Effects of treatments are described on leukocytes (CD45+) (a) and splenic DCs (MHC II+ CD11c+) (b). Data were analyzed for statistical significance by a one-way ANOVA followed by a Bonferroni post-test and are displayed as mean ± S.E.M. No significant differences between any groups were found.

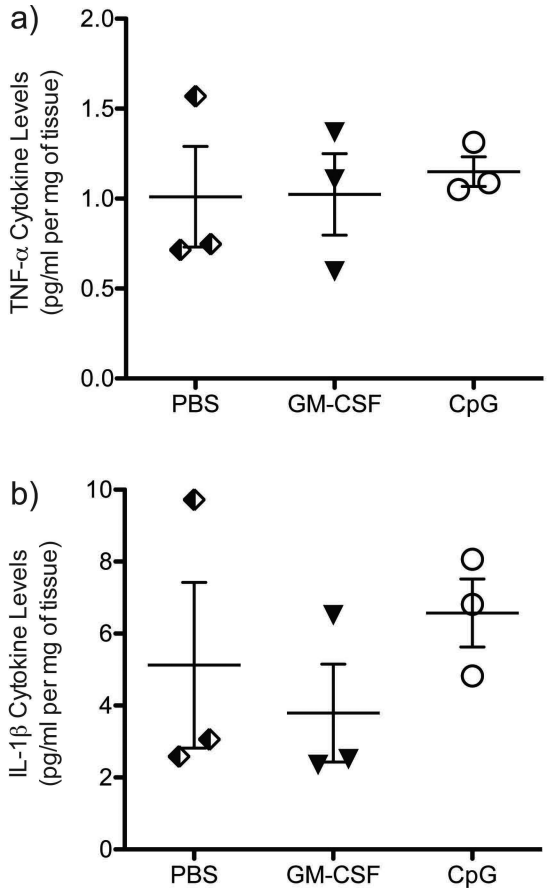

Figure 4. Inflammatory cytokine levels in vaginal tissues

TNF-α and IL-1β cytokine production was measured in reproductive tissues using an ELISA. Tissues were homogenized and quantified by comparison to a standard, and normalized to tissue mass. No significant differences were observed in TNF-α (a) or IL-1β (b) cytokine levels between PBS, GM-CSF, and CpG topical administrations. Data were analyzed for statistical significance by a one-way ANOVA followed by a Bonferroni post-test and are displayed as mean ± S.E.M.

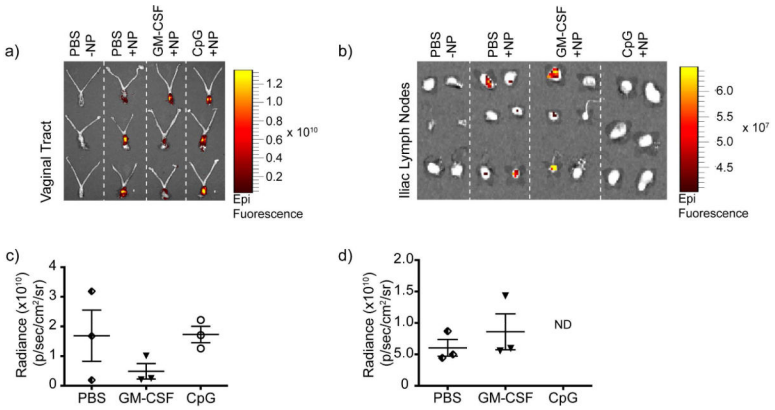

Figure 5. Xenogen imaging and fluorescence quantification

Vaginal tracts and draining lymph nodes were dissected 24-h after nanoparticle administration of mice pre-treated with PBS, GM-CSF, and CpG (n=3 per group). Tissues were imaged using a Xenogen *in vivo* imaging system (iVIS). Fluorescence was observed in the vaginal tract in all treatment groups (a) and in the iliac lymph nodes (b) in PBS and GM-CSF treated animals. Fluorescence in the vaginal tract and iliac lymph nodes was quantified using the LivingImage Software (c,d). ND indicates that fluorescence was “not detected.” Data are displayed as mean ± S.E.M.

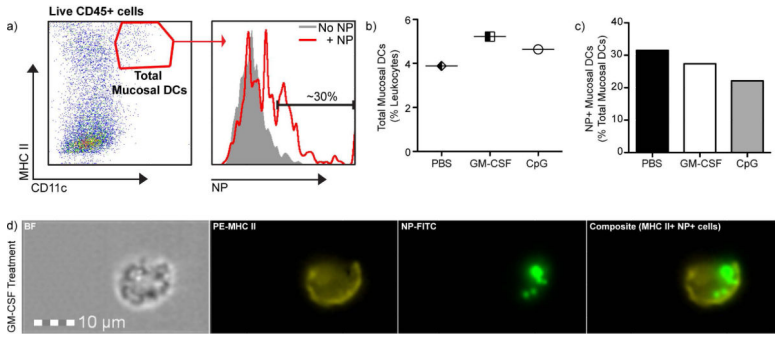

Figure 6. Nanoparticle uptake in vaginal mucosal DCs

Total mucosal DCs were identified and nanoparticle uptake by these cells was quantified (a). Vaginal tracts from 3 mice were pooled within each group. GM-CSF treated animals displayed increased levels of total mucosal DCs (MHC II+ CD11c+) (b), but no significant difference was observed in NP phagocytosis by mucosal DCs between treatment groups (c). ImageStream imaging flow cytometry was performed to evaluate if nanoparticles were internalized in mucosal MHC II+ cells. A representative image of nanoparticle internalization in MHC II+ cells is shown (d). Bright field (BF), PE, and FITC channels show live cells, MHC II cell surface staining, and internal NP fluorescence, respectively. The composite image shows punctate NPs in MHC II+ cells.