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Title

Modified Vaccinia virus Ankara vector induces specific cellular and humoral responses in the female reproductive tract, the main HIV portal of entry

Running Head

Mucosal vaccine responses in female genital tract

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Abstract

The female reproductive tract is one of the major mucosal invasion site of HIV-1. This site has been neglected in previous HIV-1 vaccine studies. Immune responses in the female reproductive tract after systemic vaccination remain to be characterized. Using a modified vaccinia virus Ankara (MVA) as a vaccine model, we characterized specific immune responses in all compartments of the female reproductive tract (FRT) of non-human primates after systemic vaccination. Memory T cells were preferentially found in the lower tract (vagina and cervix), whereas antigen-presenting cells and innate lymphoid cells were mainly located in the upper tract (uterus and fallopian tubes). This compartmentalization of immune cells in the FRT was supported by transcriptomic analyses and correlation network. Polyfunctional MVA-specific CD8^+ T cells were detected in the blood, lymph nodes, vagina, cervix, uterus and fallopian tubes. Anti-MVA IgG and IgA were detected in cervicovaginal fluid after a second vaccine dose. Systemic vaccination with an MVA vector thus elicits cellular and antibody responses in the female reproductive tract.
**Introduction**

Heterosexual intercourse is the major route of HIV-1 transmission\(^{(1)}\), and viral entry occurs mainly via the female reproductive tract (FRT) mucosae. One of the attempts to prevent this transmission should focus on inducing mucosal immune responses.

The FRT contains two types of mucosae. The type I mucosal surface is found in the upper genital tract (endocervix, uterus and Fallopian tubes), and is covered by a monolayer of columnar epithelial cells with tight junctions. The type II mucosal surface is found in the lower genital tract (vagina and ectocervix), and is lined with a stratified squamous epithelium. The boundary between the type I and II mucosae is called the cervical transformation zone. The transformation zone is considered to be more vulnerable to HIV-1 infection\(^{(2, 3)}\), owing to the abundance of immune target cells (CD\(^{4+}\) T cells, macrophages and dendritic cells), and the transition of the epithelial phenotype. Macrophages and T cells from the vagina and cervix are permissive to HIV-1 infection \textit{in vitro}\(^{(4, 5)}\). The uterus contains CD\(^{4+}\) T cells and macrophages that express HIV-1 coreceptors. Uterine cells and uterine explants are also permissive to HIV-1 infection \textit{in vitro}\(^{(6)}\). Thus, as HIV-1 target cells are present throughout the FRT, an effective vaccine should induce protective responses in all FRT compartments.

Recombinant poxviruses such as vaccinia virus and canarypox virus are strongly immunogenic and are often used as vaccine vectors. Partial but significant protection against HIV-1 was observed in the Thai phase III trial (RV144) of a canarypox vector expressing HIV-1 antigens plus a trimeric recombinant gp120 protein, used in a prime/boost strategy\(^{(7)}\). The modified vaccinia virus Ankara (MVA), another recombinant poxvirus, is widely used in vaccines for infectious diseases\(^{(8)}\). Mucosal immunity after MVA vaccination has been studied in the gastrointestinal tract but not in the FRT\(^{(9)}\).
Most studies of vaccine responses in the FRT have been performed in mice (10). However, macaques, which exhibit marked immunological and anatomical similarities to humans in contrast to mice (11), is the reference model for HIV-1 research and vaccine studies. Here, using an MVA vaccine as a model, we examined whether systemic MVA vaccination induced specific local responses in the macaque FRT. After detailed phenotypic characterization of immune cell subpopulations, we examined specific immune responses in the blood, lymph nodes (LNs), vagina, cervix (endo and ecto), uterus and fallopian tubes.
Materials and Methods

Ethics statement

Six sexually mature adult female cynomolgus macaques (*Macaca fascicularis*) imported from Mauritius were housed in the Infectious Disease Models and Innovative Therapies (IDMIT) facilities at Commissariat à l’Énergie Atomique et aux Énergies Alternatives (CEA, Fontenay-aux-Roses, France). Treatment of non-human primates (NHP) at CEA complies with French national regulations (CEA authorization A 92-032-02), with the Standards for Human Care and Use of Laboratory Animals (OLAW Assurance number #A5826-01), and with European Directive 2010/63 (recommendation #9). Experiments were supervised by veterinarians in charge of the animal facility. This study was approved and accredited by the Comité d’Ethique en Expérimentation Animale du CEA (A14-080) and by the French Research Ministry. Animals were housed in pairs under controlled conditions of humidity, temperature and light (12-hour light/dark cycles). Water was available *ad libitum*. The animals were monitored and fed once or twice a day with commercial monkey chow and fruits, by trained personnel, and were provided with environmental enrichment including toys, novel foodstuffs and music, under the supervision of the CEA Animal Welfare Officer.

Experimental design

On day zero (D0) and D58, the macaques received two subcutaneous injections per time point in the right and left side of the upper back, delivering 2 x 1 ml of inoculum containing a total of 4 x 10^8 plaque-forming units (PFU) of recombinant MVA-HIV-1 expressing the Gag, Pol, and Nef proteins from HIV-1 strain LAI (ANRS-MVA HIV-B, MVATG17401, Transgene Ltd, France). The animals were monitored daily for signs of disease, appetite loss and lethargy. A physical examination was performed at each blood sampling and each inoculation. All experimental procedures (handling, immunization, blood sampling) were conducted after
sedation with ketamine hydrochloride (Rhône-Mérieux, Lyon, France, 10 mg/kg). To synchronize their hormonal cycle, an intramuscular injection of a synthetic variant of progesterone (Depoprovera, 30mg, Pfeizer, France) was given 42 days after the first vaccine injection. The animals were sedated 77 days after the first vaccine injection with ketamine hydrochloride (10 mg/kg) then euthanized by intravenous injection of 180 mg/kg sodium pentobarbital.

Sample collection and cell isolation

Blood, serum and vaginal fluid were collected before and after each vaccine inoculation and at the time of euthanasia. Lymph nodes (LNs) and tissues were collected at necropsy. Serum was isolated by centrifugation at 3000 rpm for 10 min and stored at -80°C. Cervicovaginal fluid was collected with a Week-Cel spear (Medtronic, USA) placed in the vaginal vault for 2 minutes. Secretions were recovered from the spears by adding 600 µl of extraction buffer (PBS, NaCl 0.25 M and protease inhibitor cocktail (Merck Millipore, Fontenay-sous-bois, France)) and then centrifuging at 13 000 g for 20 min. Filtered vaginal fluids were stored at -80°C.

PBMC were isolated in heparin CPT tubes (BD biosciences, Le Pont de Claix, France) after centrifugation for 30 min at 3000 rpm. PBMC were collected from the top of the CPT gel surface and washed twice. At euthanasia, LN and FRT tissues were collected. LN cells were obtained by mechanical dissociation. FRT tissues (vagina, cervix, uterus and fallopian tubes) were isolated and cut into small pieces. Each tissue was digested for 1 hour at 37°C with agitation in digestion buffer, consisting of RMPI 1640 (Fisher Scientific, Illkirch, France), collagenase IV (0.3 mg/ml, Sigma Aldrich, St Quantin Fallavier, France), fetal calf serum (5%, Fisher Scientific), Heps (0.025 M, Fisher Scientific), DNase (0.1 mg/ml, Roche, Mannheim, Germany), and antibiotics (Fisher Scientific). Undigested pieces were subjected to up to 3 more digestion steps. Cell suspensions from LNs and FRT tissues were filtered through 70-µm sterile
nylon cell strainers (BD biosciences). The median of cell numbers recovered for each FRT compartment was $14.2 \times 10^6$ cells/g of tissue (ie $64 \times 10^6$ cells) in the vagina, $13.8 \times 10^6$ cells/g of tissue (ie $54 \times 10^6$ cells) in the cervix, $15 \times 10^6$ cells/g of tissue (ie $62 \times 10^6$ cells) in the uterus and $27.7 \times 10^6$ cells/g of tissue (ie $18 \times 10^6$ cells) in the tubes.

**Immune phenotyping**

Whole blood, LN cells and cells from FRT compartments were analyzed by flow cytometry. The cells were incubated with the antibodies listed in Table S1, then washed and fixed with FACS lysing buffer or BD Cell Fix solution. A Fortessa 2-UV 6-Violet 2-Blue 5-Yelgr 3-Red laser configuration was used (BD biosciences), with Diva (BD) and FlowJo 9.8.3 (Tristar, USA) softwares. At least 500 events for rare cell populations (i.e. pDC) were recorded. The gating strategies are illustrated in supplemental figure S1a-d.

**Cellular responses**

Specific cellular immune responses were evaluated with *in vitro* stimulation assays. The cells were incubated for 5 hours at 37°C with medium, with 0.3 PFU/cell of live wild type MVA, or with PMA (5 ng/ml) and ionomycin (500 ng/ml) (Sigma Aldrich) in DMEM medium (Fisher Scientific) supplemented with 10% FCS and antibiotics. Brefeldin A was then added (5 µg/ml, Sigma Aldrich) and the cells were incubated for a further 10 hours at 37°C. For HIV-1 stimulation, cells were incubated with 4 µg/ml overlapping GAG peptide pools in DMEM medium supplemented with 10% FCS, antibiotics and costimulatory antibodies, for 1 hour at 37°C, then for an additional 4 hours with brefeldin A (5 µg/ml). The cells were stained with blue dye (LIVE/DEAD® Fixable Blue Dead Cell Stain, Thermo Fisher) for viability then fixed and permeabilized with BD Fix&Perm reagent (BD Bioscience). The antibodies listed in Table S2 were used for intracellular staining. At least 5,000 events in the CD8+ T cell gate were
recorded. The gating strategy was as described elsewhere (12). Briefly, expression of cytokines and activation markers was evaluated in CD4⁺ and CD8⁺ T cells, and Boolean gate analyses were performed with FlowJo software. The percentages of cells positive for cytokines and activation markers were then compared between unstimulated and MVA- or GAG peptide pool-stimulated cells. Immune response was considering positive against the antigen when the percentage of cells positive for cytokines and activation markers were at least twice superior to the percentage under unstimulated condition.

**Antibody responses**

Specific antibodies were measured by EIA in serum and vaginal fluid collected by Weck-cel spears. 96-well MaxiSorp microplates (Nunc, Thermo Fisher) were coated overnight with 10⁵ PFU/well wtMVA (Transgene, Illkirch, France) in NaHCO₃/Na₂CO₃ buffer, or with 1 µg/ml p24 antigen (kind gift from Bernard Verrier, LBTI UMR5305) in PBS. The plates were then blocked for 1 h with PBS containing 3% (w/v) bovine serum albumin (BSA, Sigma Aldrich) or with PBS containing 10% skimmed milk. The plates were washed 5 times with PBS containing 0.1% Tween 20 and 10 mM EDTA, then incubated with two-fold serial dilutions of macaque fluids diluted in PBS containing 1% BSA for 1 h at RT (to detect anti-MVA IgG/IgA) or in PBS containing 1% skimmed milk and 0.05% Tween 20 for 1 h at 37°C (to detect anti-HIV IgG), starting at 1:50 for serum and 1:20 for vaginal fluid. The plates were then washed 5 times and incubated for 1 h with a 1:20,000 dilution of horseradish peroxidase-conjugated goat anti-monkey H+L chain IgG (Bio-Rad, Marne-la-Coquette, France) or with a 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-monkey IgA (Alpha Diagnostic international, San Antonio, TX). The plates were washed five times, then 100 µL of o-phenylenediamine dihydrochloride (OPD) (Sigma Aldrich) was added and incubated for 30 mins at RT in the dark. The reaction was stopped by adding 2N H₂SO₄. Absorbance was measured at 492 nm with
spectrophotometer (Tecan, Lyon, France), and data were analyzed with Magellan software (Tecan). Antibody titers were calculated by extrapolation from the OD as a function of a serum dilution curve and were defined as the dilution of the test serum reaching 2 OD of the corresponding preimmune serum or vaginal fluid, tested at 1:50 and 1:30, respectively.

RNA extraction and hybridization
Tissue biopsies collected at euthanasia were immediately immersed in RLT-beta-mercaptoethanol 1/100 lysis buffer (Qiagen, Courtaboeuf, France), then disrupted and homogenized with a TissueLyser LT (Qiagen). RNA was purified with Qiagen RNeasy microkits. Contaminating DNA was removed by using the RNA Cleanup step of the RNeasy microkit. Purified RNA was quantified with a ND-8000 spectrophotometer (NanoDrop Technologies, Fisher Scientific, Illkirch, France) before being checked for integrity on a 2100 BioAnalyzer (Agilent Technologies, Massy, France). cDNA was synthesized and biotin-labelled using the Ambion Illumina TotaPrep RNA amplification kit (Applied Biosystem/Ambion, Saint-Aubin, France). Labelled cRNA was hybridized on Illumina Human HT-12V4 BeadChips, that target 47 323 probes corresponding to 34 694 genes. The manufacturers’ protocols were followed.

Transcriptome analysis
Microarray data were analyzed with R/Bioconductor software. Gene expression values were quantile normalized. Differentially expressed genes were identified with a paired non-parametric t-test (q-value<0.05), based on a fold-change cutoff of 1.2. Functional enrichment analysis used QIAGEN’s Ingenuity Pathway Analysis (IPA, QIAGEN, Redwood City, ww.qiagen.com/ingenuity). Hierarchical clustering presented in the heatmaps were generated with the Euclidian metric and complete linkage methods. Microarray raw data are available.
from the EBI-ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-5663. The transcriptomic and cellular co-expression network was generated by using the Spearman correlation coefficients, based on the abundance of cell populations and normalized gene expression values across the whole dataset. Significant correlations (R>0.70 and p-value<0.01) were restricted to correlations between cell populations, and between cell populations and gene expression levels.
Results

Characterisation of FRT leukocytes

To characterize the vaccine responses at the mucosal level, an extensive identification of the cell subpopulations present in the different compartments was first performed. The phenotypes of immune cells collected from FRT compartments and lymph nodes were analyzed by flow cytometry. The proportions of immune cell subsets were characterized in four sites of the FRT (vagina, cervix, uterus and tubes) by comparison with whole blood and both proximal and distal lymph nodes (iliac, axillary and inguinal). Leukocytes were present in all the FRT compartments (Figure 1a),

- Innate immune cells

Two subpopulations of innate lymphoid cells (ILC) were identified on the basis of NKG2A (NK cells) and NKp44 (ILC-3) expression (Supplementary Figure S1b). NK cells composed less than 4.0±2.0% (median±SD) of leukocytes in blood and LN. This percentage was higher in the FRT compartments, especially in the uterus (13.9±5.2%) (Figure 1b). The NK phenotype differed between the mucosa and blood, as mucosal NK cells did not express CD16 Fc-γ receptor but expressed CD69. In the LN and FRT, NKp44 was expressed by ILC-3 cells but not by NK cells (Figure 1c and Supplementary Figure S1b). Although few in number, ILC-3 cells were found in all LN and FRT compartments, and preferentially in the tubes (0.5±0.4%) (Figure 1d).

The distribution of three main APC populations (CD14+ APC, CD123+ plasmacytoid dendritic cells (pDC) and CD11c+ myeloid dendritic cells (mDC)) is shown in Supplementary Figure S1c accordingly to the gating strategy.

CD14+ APC were the main APC subtype in all FRT compartments (Figure 1e) (from 1.5±1.0% to 5.3±5.6% of total leukocytes) and were principally located in the uterus. The proportions of
these cells were similar in the lower FRT and blood, but only mucosal CD14+ cells expressed
the activation marker CD69 and the Fc-γ receptor CD16, particularly in the vagina (Figure 1f).
The distribution of mDC (CD11c-1) was similar to that of CD14+ cells, and mDC were mainly
found in the uterus (3.4±2.2% of total leukocytes) (Figure 1g). The proportion of pDC
(CD123+) was similar in all the compartments, where they represented less than 1% of total
leukocytes (Figure 1h).

Neutrophils were the main leukocyte subtype in blood (43.8±14.1% of CD45+ cells). In the
FRT, neutrophils were mainly found in the cervix and upper compartments (8.9±6.8% in cervix,
3.4±1.8% in uterus and 2.3%±4.0 in the fallopian tubes) (Figure 1i).

Antigen-specific immune cells

T lymphocytes were the main leukocyte subpopulation in the LN and FRT compartments. The percentage of CD8+ T cells in the FRT mucosae was higher than the percentage of CD4+ T cells, contrasting with blood and LN (Figure 2a and 2d). CD4+ T cells represented about 50% of total leukocytes in LN, nearly 20% in the lower FRT and 10% in the upper FRT (Figure 2a). The T cell memory phenotype was defined by CD28, CD95 and CD45RA expression (Supplementary Figure S1d). In contrast to blood and LN, the majority of CD4+ T cells in the FRT exhibited the central memory phenotype (CD28+/CD95+), while naïve cells (CD28+/CD95neg) were rare (Figure 2b). In the FRT, up to 40% of leukocytes were CD8+ T cells (Figure 2d). These cells expressed markers of central memory (CD28+/CD95+) and effector memory (CD28neg/CD95+), contrary to blood and LN, where most CD8 T cells were naïve (Figure 2e). Mucosal CD4+ and CD8+ T cells frequently expressed CD69 (Figures 2c and 2f).

Between 6.7% and 15.9% of blood and LN leukocytes were B cells (CD20+) (Supplementary Figure S1d), whereas B cells were infrequent in all the FRT compartments (from 0.1±0.1% to 1.0±1.4% of leukocytes) (Figure 2g).
Thus, cells involved in initiating immune responses, and effector cells, were present throughout the macaque FRT, with specific distribution according to the compartment.

Vaccine-specific CD4$^+$ T cells found mainly in lymph nodes draining the inoculation site

The anti-MVA T cell response was monitored in blood by using an *in vitro* re-stimulation assay. Antigen-specific CD4$^+$ T cells were identified as CD154$^+$ cells. Their percentage increased in blood two weeks after the first and second vaccine injections (respectively 0.84% and 0.85% among total CD4$^+$ T cells; mean) (Figure 3a). Three weeks after the second vaccine injection, the anti-MVA response was analyzed in all compartments. Antigen-specific CD4$^+$ T cells were significantly detected in PBMC and LNs (Figure 3b). The largest percentage of MVA-specific CD4$^+$ T cells was found in the axillary LNs (from 0.23% to 3.24% of total CD4$^+$ T cells). No MVA-specific CD4$^+$ T cell response was detected in FRT tissue, as the percentage of CD154$^+$ CD4$^+$ T cells did not significantly change after MVA re-stimulation (Figure 3b). In all compartments, CD154$^+$ cells represented a large percentage of CD4$^+$ T cells after stimulation with PMA and ionomycin (data not shown). The anti-HIV-1 response was also analyzed after *in vitro* stimulation with gag peptide pools and co-stimulatory antibodies (anti-CD28 and anti-CD49d mAbs). The addition of co-stimulatory antibodies induced non-specific activation of T cells and thus increased background CD154 expression, even in non-stimulated conditions. As this could have masked weak responses, we measured the HIV-1-specific CD4$^+$ T cell response by analyzing the percentage of CD4$^+$ T cells that expressed CD154 and produced IFN-γ (i.e. only specific T cells). The Gag-specific CD4$^+$ T cell response was very weak and only detected in PBMC (Figure 3c).

Thus, MVA-specific CD4$^+$ T cells were mainly found in blood and in lymph nodes draining the vaccine inoculation site.
Systemic and mucosal polyfunctional vaccine-specific CD8\(^+\) T cell responses

Like the CD4\(^+\) T cell response, the vaccine response mediated by CD8\(^+\) T cells was monitored by \textit{in vitro} re-stimulation assays in blood over time, and in all compartments at euthanasia. The percentage of MVA-specific CD8\(^+\) T cells that produced IFN-\(\gamma\) increased in blood after the first vaccine inoculation and rose strongly after the second injection (respectively 1.79% and 5.38% of total CD8\(^+\) T cells; mean) (Figure 4a). A similar profile was observed for MIP-1\(\beta\) and TNF-\(\alpha\) production by antigen-specific CD8\(^+\) T cells over time (data not shown). MVA-specific CD8\(^+\) T cells were detected in PBMC and LNs of all animals three weeks after the second vaccine injection. They represented from 0.15% to 7.16% of total CD8\(^+\) T cells, depending on the animal (Figure 4b-c). Interestingly, MVA-specific CD8\(^+\) T cell responses were also detected in all FRT compartments (Figure 4b and 4d) and especially in the vagina (from 0.04% to 1.08% of total CD8\(^+\) T cells for the IFN-\(\gamma\) response). The anti-MVA response mediated by CD8\(^+\) T cells was polyfunctional. Importantly, most of the CD8\(^+\) T cells which had only one function (produced one cytokine/chemokine) secreted MIP-1\(\beta\), while those with two functions produced MIP-1\(\beta\) and IFN-\(\gamma\), and those with three functions produced MIP-1\(\beta\), IFN-\(\gamma\) and TNF-\(\alpha\) (Figure 4e). MVA-specific CD8\(^+\) T cells from the FRT produced less TNF-\(\alpha\) than their blood and LN counterparts (Figure 4c-e).

HIV-1 antigen-specific CD8\(^+\) T cells were also measured in the different compartments. The background signal was high in all conditions, as noted for CD4\(^+\) T cell responses. To detect HIV-1 antigen-specific CD8\(^+\) T cells, the analyses focused on CD8\(^+\) T cells which produced both MIP-1\(\beta\) and IFN-\(\gamma\). Anti-gag CD8\(^+\) T cells were detected above background in one animal's PBMC (triangle), LNs and uterus (Fig. 4f).

Thus, MVA-specific CD8\(^+\) T cell responses were polyfunctional and found in all FRT compartments, in addition to blood and LNs.
Vaccine-specific IgG and IgA detected in vaginal fluid after the second vaccine inoculation.

To analyze humoral responses, vaccine-specific Ig titers were serially determined in serum and vaginal fluid by ELISA. MVA-specific IgG was detected in all the animals' sera two weeks after the first and second vaccine inoculations (respective titers of 1,216±430 and 50,079±11780; mean of 6 animals) (Figure 5a), and in vaginal fluid only after the second inoculation (titer of 634±386, mean of 6 animals) (Figure 5b). Similarly, MVA-specific IgA was detected in serum after the two vaccine inoculations (respective titers of 282±250 and 1,960±1532, mean of 6 animals) and in vaginal fluid only after the second inoculation (titer of 231±132, mean of 6 animals) (Figure 5c-d). The anti-HIV-1 humoral response was estimated by measuring Gag-specific IgG (Figure 5e-f). These antibodies were detected in the serum of 4 of 6 animals after the first vaccine inoculation, and in all 6 animals' serum after the second inoculation. In contrast, Gag-specific IgG was not detected in vaginal fluid (Figure 5f).

Thus, systemic MVA vaccination induced detectable vector-specific IgG and IgA in vaginal fluid after the second vaccine injection.

Transcriptomic analyses highlight FRT compartmentalization of immune cells

To better characterize the events at the molecular level, we compared the transcriptomic profiles from vaginal, cervical and uterine tissue samples. The numbers of differentially expressed genes (DEG) in each comparison are represented in Figure 6a. We found that respectively 3,810 and 4,800 genes were differentially expressed in the cervix and uterus compared to vagina. We identified 3,804 DEG in the uterus versus the cervix. The Venn diagram in Figure 6b represents the common DEG between the comparisons, showing that 624 genes were shared between the three comparisons (i.e. cervix vs vagina, uterus vs vagina and uterus vs cervix). The relative expression of genes found as DEG in at least one comparison is represented by a heatmap in Figure 6c. Four main branches (gene clusters) were identified by
hierarchical clustering. The clustering branch #2 was mainly driven by DEGs over-expressed in the uterus. Similarly, branch #1 was driven by DEGs over-expressed in the vagina, while branches #3 and #4 were driven by DEGs over-expressed in the cervix. Each gene set was analyzed using Ingenuity Pathway Analysis (IPA). Branches #3 and #4 were merged for the enrichment analysis. Functional enrichment analysis of canonical pathways and upstream regulators are represented on the side of each branch. Upstream regulator analyses highlighted that beta-estradiol (branch #1 p=9.69e^{-30}; branch #2 p=2.7e^{-21}; branches #3-4 p=8.39e^{-8}), progesterone (branches #3-4 p=1.16e^{-10}) and estrogen receptor (ESR1) (branch #2 p=8.77e^{-17}; branches #3-4 p=1.45e^{-10}) constituted top upstream regulators in at least one of the four branches, confirming that hormones are involved in central regulation pathways in the FRT.

Enrichment analyses were then filtered to reveal immune-related pathways. Significant immune-related canonical pathways and associated p-values are represented in Figure 6d. The results showed that numerous pathways are associated with the uterus (branch #2) and are linked to NK cells and antigen-presenting cells (natural killer cell signaling p=8.51e^{-4}; IL-15 signaling p=2.04e^{-3}; Crosstalk between dendritic cells and natural killer cells p=2.04e^{-3}; production of nitric oxide and reactive oxygen species by macrophages p=9.77e^{-4}). Similarly, in the vagina (branch #1), immune canonical pathways are associated with macrophages/monocytes and T cells (CTLA4 signaling in cytotoxic T cell p=2e^{-4}; TCR signaling p=4.47e^{-3}; Fcγ-R mediated phagocytosis in macrophages and monocytes p=9.12e^{-4}; CCR5 signaling in macrophages p=4.57e^{-3}). No significant immune-related pathway was identified for the cervix (clustering branches #3 and #4).

To integrate flow cytometry and transcriptomic data, we generated a co-expression network. We restricted the correlations to DEG associated to the pathways in bold on Figure 6D. Co-expression network revealed that frequent immune populations in the vagina (T and B cells) correlated positively with these branch #1 immune pathways and negatively with the
branch #2 immune pathways (Figure 6e). Conversely, frequent immune populations in the uterus (APC and ILC) correlated positively with the branch #2 immune pathways and negatively with the branch #1 immune pathways.

Thus, transcriptome and correlation analyses highlighted the specificity of each FRT compartment and the compartmentalization of FRT immune cells.
Discussion

As male-to-female transmission via the FRT mucosae is the main route of HIV-1 transmission, it is essential to study vaccine responses in the FRT. We conducted a detailed characterization of the immune cells involved in MVA-HIV-1 vaccine responses in the cynomolgus macaque, and the vaccine responses themselves in all female reproductive tract compartments during the luteal phase, by comparison with blood and draining lymph nodes. Previous studies of mucosal responses to MVA vaccination have been limited to the gastrointestinal tract (9), whereas the FRT is the main portal of entry for sexually transmitted pathogens. To our knowledge, we show for the first time that subcutaneous MVA injections induce specific immunoglobulin (IgG and IgA) and polyfunctional CD8\(^+\) T cells in the FRT of female macaques. This study reveals that each FRT compartment has its own characteristics, as shown by immune cell phenotyping and transcriptomic analyses.

The first part of this study clearly shows that immune cells are compartmentalized. Two subpopulations of ILC were identified according to their phenotypes. Thus, NK cells, defined by NKG2A expression, were mainly found in the upper FRT (uterus and tubes) and expressed low levels of CD16 Fc-\(\gamma\) receptor, as previously described (13), and no NKp44, in contrast to gut mucosae (14) (personal communication by Mariangela Cavarelli). Localization of NK cell activity within the uterus was confirmed by transcriptomic analyses, as NK cell-related pathways were associated with the uterus (branch #2) and correlated with uterine immune cell populations (co-expression network, Figure 6e). A second subtype of ILC, called ILC-3, which expressed NKp44 but not NKG2A (14), were also mainly found in the upper FRT.

We detected three main populations of professional APC, according to their phenotypic markers: i) CD14\(^+\) APCs, ii) mDC that expressed CD11c\(^+\), and iii) plasmacytoid DC (pDC) that
expressed CD123+. These APCs were distributed throughout the FRT, but differences in their
distribution and phenotype were noted. The largest percentage of mDC CD11c+ cells and
CD14+ cells was found in the uterus. Moreover, phenotypic analyses of APC subtypes
confirmed that CD14+ APCs from the vagina expressed CD16 Fc-γ receptor, contrary to
intestinal CD14+ APCs(15). This characteristic of vaginal CD14 APCs was supported by
transcriptomic analysis, as the “Fc-γ receptor-mediated phagocytosis in macrophages” pathway
was enriched in the vagina.

B lymphocytes were the main leukocyte subtype in LNs, while they were detected at a very low
percentage throughout the FRT, mainly in the vagina. This low frequency of B cells in the FRT
was not due to the enzymatic digestion procedure, as very few B cells were detected by
immunostaining of tissue biopsies (data not shown). Here, we detected B cells by their CD20
e expression rather than the CD19 marker usually used in humans, as the available antibodies do
not cross-react in cynomolgus macaques. As CD20 is not expressed by all B cell subsets(16),
this could explain the low percentage of B cells found in our samples. The distribution of
CD20neg B cells such as plasma cells and antibody-secreting cells in FRT compartments will
require further studies with specific markers.

T lymphocytes were the main immune cell populations in all the FRT compartments. CD8+ T
cells were more abundant than CD4+ T cells within the mucosae, in contrast to blood and LNs.
In particular, the vagina exhibited the largest percentage of CD8+ T cells, as well as a specific
transcriptomic signature related to T cell pathways. Co-expression networking showed that T
cell abundance correlated positively with these T cell pathways (branch #1) (Figure 6e). We
confirm that both CD4+ and CD8+ T cells express memory markers in the FRT(17), whereas
naïve cells were mainly found in blood and LNs. Among the memory T cells, resident memory
lymphocytes have been described in the tissues such as the vagina(18). Our analyses performed
in one vaccinated animal showed that CD8+ resident memory T cells were mainly present in
the vagina (39.8% among the effector memory T cells) and in the cervix (22.2% among the effector memory T cells) and were less frequent in the uterus (2.5% among the effector memory T cells) (data not shown). Together, these data show that immune cells exhibit tissue specificity in the macaque FRT.

Our results match some published data on the human FRT, including the observations that i) mucosal NK cells exhibit a unique phenotype and are mainly found in the uterus (19); ii) ILC-3 are detected in the upper FRT (20); iii) APCs are distributed throughout the FRT, particularly in the upper tract (21) and ectocervix (22); iv) CD20+ B cells are rare throughout the FRT (23); v) memory CD8+ T cells represent a large proportion of immune cells in all compartments of the human FRT. Thus, the localization and phenotype of immune cell subtypes are similar in the macaque and human FRT, validating the cynomolgus macaque as a model for human reproductive biology and genital immunity, including FRT mucosal immune responses to vaccination.

We therefore vaccinated female cynomolgus macaques subcutaneously with MVA-HIV-1, selected as a vaccine model, and analyzed specific responses in the FRT, LNs and blood. Analyze of humoral responses confirmed that MVA vaccination induces strong anti-MVA IgG responses in serum, whereas anti-HIV IgG was not detected in all animals. In contrast to other mucosal fluids, cervicovaginal fluid contained more IgG than IgA. MVA-specific IgG and IgA were detected in vaginal fluid after the second vaccine injection. Local anti-MVA IgG titers were lower than in serum, as the vaccine was administered subcutaneously. Cervicovaginal IgG has been shown to come mainly from the systemic compartment (24). Anti-HIV IgG titers in serum were much lower than anti-MVA IgG titers, which could explain why anti-HIV IgG was not detected in vaginal fluid, given their systemic origin.

Anti-MVA responses mediated by CD4+ T cells were weaker than those mediated by CD8+ T cells in blood and LNs, and were not detectable in the FRT mucosae. Since the peak of CD4+
T cell responses precede the one of CD8\(^+\) T cell responses(25), immune responses analysed 77 days after the prime may not be optimal to detect a strong CD4\(^+\) T cell response. The largest anti-MVA CD4\(^+\) T cell responses were measured in axillary LNs, i.e. those draining the vaccine injection site (upper back). However, MVA-HIV vaccination may induce non-specific CD4\(^+\) T cell activation, as CD4\(^+\) T cells exhibited a high CD154 expression in some animals in unstimulated conditions.

Our study clearly shows that MVA vaccination induces strong specific CD8\(^+\) T cell responses in blood, LNs and all FRT compartments. In the FRT, they were mainly localized in the vagina, but specific responses were also detected in the cervix, uterus and tubes. Responses mediated by CD8\(^+\) T cells were polyfunctional, as specific CD8\(^+\) T cells positive for two or more cytokines were detected. Boolean gating analyses were used to sort specific CD8\(^+\) T cells according to the number of functions they displayed (single, double, triple or quadruple cytokine producers). The majority of single producer cells were MIP-1\(\beta\)^, double producers were MIP-1\(\beta\)\(^+\) and IFN-\(\gamma\)^, triple producers were MIP-1\(\beta\)\(^+\), IFN-\(\gamma\)\(^+\) and TNF-\(\alpha\)^. These findings correspond to reports of blood CD8\(^+\) T cell responses(26). TNF-\(\alpha\) and IL-2 are thus produced only by highly polyfunctional cells. We noted that percentage of triple producer cells was lower in the FRT than in blood and LNs. Together, these results demonstrate that TNF-\(\alpha\)^ specific CD8\(^+\) T cells are less abundant in the FRT than in blood and LNs.

Previous study has demonstrated that the MVA-HIV-1 vaccine induced T cell responses mainly against Gag and Pol genes(27). Due to limited amount of mucosal cells recovered to perform the different experiments and antigen stimulations, cellular and humoral immune responses induced against the HIV-1 insert in our present study were focused on anti-Gag responses. HIV-1 (Gag)-specific responses were mediated by CD4\(^+\) T cells and were detected mainly in blood. These responses were weaker than MVA-specific CD8\(^+\) T cell responses and were not detectable in the FRT, apart from the uterus of one animal. The MVA-HIV-1 vaccine was used
in this study as a model, and animals were vaccinated subcutaneously with two injections of
the same vaccine construct. Therefore, specific responses mainly targeted the immunogenic
vector. To enhance insert-specific responses, it will be essential for the boost or the prime to
use another type of vaccine construct, such as a DNA vaccine, in addition to MVA(27).

The local environment of the FRT mucosae is under the influence of several factors, including
hormones during the menstrual cycle, semen during intercourse, and sexually transmitted
pathogens(28–30). As these factors impact mucosal immune cells and their environment, it will
be crucial to study their possible influence on mucosal vaccine responses.
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References


Footnotes

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**Figure legends**

**Figure 1. Innate cell distribution in blood, LNs and FRT of vaccinated animals**
(a) Distribution of leukocytes among living cells. Percentage of NK cells (b), ILC-3 (d), CD14+ cells (e), CD11c+ mDC (g), CD123+ pDC (h) and neutrophils (i) among leukocytes in the different compartments. Each symbol represents one animal and the bar represents the median.

Phenotypic marker expression by NK cells (c) and CD14+ cells (f) in the different compartments are represented as a heat map. Each horizontal coloured line indicates one animal (n=6).

**Figure 2. Adaptive immune cell distribution and phenotype**
Percentage of CD4+ T cells (a), CD8+ T cells (d) and CD20+ B cells (g) among CD45+ cells in the different compartments. Each symbol represents one animal and the bar represents the median. Distribution of naïve (CD28+CD95-) and memory subsets (CM CD28+CD95+, EM CD28+CD95-, EMRA CD28-CD95+CD45RA+) among CD4+ (b) and CD8+ (e) T cells (mean of n=6). Histograms represent CD69 expression by CD4+ (c) and CD8+ (f) T cells in the different tissues. (Mean +/- SEM of n=6)

**Figure 3. Specific immune responses mediated by CD4+ T cells**
(a) Percentage of CD154+ CD4+ T cells over time in PBMC after *in vitro* stimulation with medium (dotted lines) or wt MVA (full lines). Purple bold line indicates the mean of n=6 and purple arrows indicate vaccine injections. (b) Percentage of CD154+ CD4+ T cells after *in vitro* stimulation with medium (grey) or wt MVA (red) in the different compartments. (c) Percentage of CD154+/IFN-γ+ CD4+ T cells after *in vitro* stimulation with medium (grey) or gag peptide pools (red) (GAG1 and GAG2) in blood and LNs (top panel c) and FRT mucosae (bottom panel c). Each symbol represents one animal.
Figure 4. Vaccine-specific CD8+ T cell responses in the blood, LNs and FRT
(a) Percentage of IFN-γ+ CD8+ T cells over time in PBMC after in vitro stimulation with medium (dotted lines) or wt MVA (full lines). Purple bold line indicates the mean of n=6 and purple arrows indicate vaccine injections. (b) Dot plots of one representative animal (▽) for IFN-γ staining after wt MVA stimulation in the different compartments. Percentages of IFN-γ+ cells are indicated. (c-d) Percentage of IFN-γ+, MIP-1β+, TNF-α+ and IL-2+ cells among CD8+ T cells after in vitro stimulation with medium (grey) or wt MVA (red) in blood and LNs (c) and FRT tissues (d). (e) CD8+ T cell polyfunction analysed by Boolean gating is represented as a heat map. Each horizontal coloured line indicates one animal (n=6). (f) Percentage of MIP-1β+/IFN-γ+ CD8+ T cells after in vitro stimulation with medium (grey) or gag peptide pools (red) (GAG1 and GAG2) in the different compartments. Each symbol represents one animal.

Figure 5. Specific humoral responses in serum and vaginal fluid of vaccinated animals
Titers of MVA (a-d) and HIV-1 gag (e,f)-specific IgG and IgA over time in serum (a, c, e) and vaginal fluid (b, d, f). Each symbol represents one animal, the bold line indicates the mean titer of the six animals, and the dotted line indicates the detection limit. The purple arrows indicate vaccine injections.

Figure 6. Transcriptomic profiling of vaginal, cervical and uterine tissues of vaccinated animals
(a) Bar charts showing the numbers of down-regulated (green) and up-regulated (red) genes in comparison between vaginal, cervical and uterine tissues. (b) Venn diagram showing overlaps between the set of differentially expressed genes found in the three comparisons. (c) Heatmap showing the expression of the genes found to be differentially expressed in at least one condition. Hierarchical clustering was performed at the gene level to identify 4 main sets
(clustering branches) of genes having similar expression profiles. Canonical pathways and upstream regulators found to be statistically over-represented in each clustering branch are indicated. (d) Immune-related canonical pathways and p-values associated with clustering branches #1 and #2. (e) Transcriptomic and cellular co-expression network. Each node of the graph corresponds to a biological variable and links between the nodes correspond to significant correlations (Spearman correlation coefficient). Genes are represented by circles and cell populations by squares. Gene circles are coloured based on their clustering branch associations (represented in (c) and (d)). Positive correlations are presented by red links and negative correlations by green links.
Figure 3
anti-MVA IgG titer (log₁₀) days post vacc.

MVA IgG in serum

-10 10 30 50 70

1 2 3 4 5

anti-MVA IgG titer (log₁₀) days post vacc.

MVA IgG in vaginal fluids

-10 10 30 50 70

1 2 3 4 5

anti-MVA IgA titer (log₁₀) days post vacc.

MVA IgA in serum

-10 10 30 50 70

1 2 3 4 5

anti-MVA IgA titer (log₁₀) days post vacc.

MVA IgA in vaginal fluids

-10 10 30 50 70

1 2 3 4 5

anti-gag IgG titer (log₁₀) days post vacc.

HIV-1 gag IgG in serum

-10 10 30 50 70

1 2 3 4 5

anti-gag IgG titer (log₁₀) days post vacc.

HIV-1 gag IgG in vaginal fluids

-10 10 30 50 70

1 2 3 4 5

Figure 5
Figure 6

(a) Number of differentially expressed genes

Cerv. vs Vag.

Ut. vs Vag.

Ut. vs Cerv.

(b) Relative gene expression

Cerv. vs Vag.

Ut. vs Vag.

(c) Ingenuity Canonical Pathways

(d) Ingenuity Canonical Pathways -log(p-value)

CXCR4 Signaling 8.35
Leukocyte Extravasation Signaling 5.19
Clathrin-mediated Endocytosis Signaling 4.44
Oncostatin M Signaling 3.96
IL-8 Signaling 3.77
 Caveolar-mediated Endocytosis Signaling 3.50
Virus Entry via Endocytic Pathways 3.49
GM-CSF Signaling 3.34
Role of JAK2 in Hormone-like Cytokine Signaling 3.30
Agranulocyte Adhesion and Diapedesis 3.18
Granzyme B Signaling 3.16
Natural Killer Cell Signaling 3.07
IL-3 Signaling 3.07
Chemokine Signaling 3.03
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages 3.01
fMLP Signaling in Neutrophils 2.77
PI3K Signaling in B Lymphocytes 2.76
B Cell Receptor Signaling 2.75
IL-15 Signaling 2.69
Crosstalk between Dendritic Cells and Natural Killer Cells 2.69
CCR3 Signaling in Eosinophils 2.67
IL-6 Signaling 2.48

CTL4 Signaling in Cytotoxic T Lymphocytes 3.70
p38 MAPK Signaling 3.28
Virus Entry via Endocytic Pathways 3.07
Fc-g Receptor-mediated Phagocytosis in Macrophages and Monocytes 3.04
T Cell Receptor Signaling 2.35
CCR5 Signaling in Macrophages 2.34
Supplementary Figure 1. Gating strategy used to analyze immune cells.
(a) Gating strategy used to analyze CD45+ leukocytes. The same strategy was applied to all the tissues and a representative illustration for the uterus is indicated. (b) ILC subsets were characterized after exclusion of T cells, B cells and APC (HLA-DR+ cells). NK cells are defined as NKG2A+ cells and ILC-3 as NKp44+ / NKG2Aneg cells. (c) APC subsets were defined as lineage negative and HLA-DR+ after exclusion of neutrophils, T cells, B cells and NK cells. (d) T cells are defined as CD3+ lymphocytes and B cells as CD20+ / HLA-DR+ lymphocytes. Memory subsets of CD4+ and CD8+ T cells were characterized by expression of CD28, CD95 and CD45RA markers.
Table SI. Antibodies used in immune phenotyping experiments.

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Table SII. Antibodies used in ICS experiments.

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