Cervicovaginal specimen collection for evaluation of mucosal immune responses: standardization and comparative assessment of sampling techniques

Florian Hladik, Seattle

NIH / NIAID
Specific Aims

• To standardize the procurement and processing of non-invasive cervicovaginal lavage and cervical cytobrush specimens for yields of viable leukocytes and their subtypes (15 women each at four sites)

• To compare the yield of viable leukocytes and their subtypes between non-invasive cervicovaginal specimens and ectocervical biopsies (20 women each at three sites)
Participating study sites and investigators

Optimizing Viable Leukocyte Sampling from the Female Genital Tract for Clinical Trials: An International Multi-Site Study

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Seattle  Chicago  Nairobi  Cape Town
Sampling

- Women 18 – 55 of age
- Negative for STDs
- 16-24 days after start of last menstrual cycle
- 10 mL cervicovaginal lavage
- Two 360° cytobrushes (Digene)
- One Baby Tischler cervical biopsy
- Cell isolation protocols are appended to PLOS One paper
Endpoints

- Total viable CD4$^+$ T cells
- Total viable CD8$^+$ T cells
- Total viable CD19$^+$ B cells
- Total viable CD14$^+$ macrophages
- Total viable CD14$^-$ CD19$^-$ HLA-DQ$^+$ DCs

Cell counts are accurately calculated by adding TruCount counting beads to every staining tube.
<table>
<thead>
<tr>
<th>Marker</th>
<th>Color</th>
<th>Clone</th>
<th>Catalogue #</th>
<th>Manufacturer</th>
</tr>
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<tbody>
<tr>
<td>Viability</td>
<td>Aqua</td>
<td>n. a.</td>
<td>L34957</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>CD45</td>
<td>APC</td>
<td>HI30</td>
<td>555485</td>
<td>BD</td>
</tr>
<tr>
<td>CD3</td>
<td>V450</td>
<td>UCHT1</td>
<td>560366</td>
<td>BD</td>
</tr>
<tr>
<td>CD4</td>
<td>ECD</td>
<td>SFC112T4D11</td>
<td>6604727</td>
<td>Coulter</td>
</tr>
<tr>
<td>CD8</td>
<td>PE</td>
<td>SK1</td>
<td>340046</td>
<td>BD</td>
</tr>
<tr>
<td>CD19</td>
<td>APC-AF750</td>
<td>J3-119</td>
<td>A78838</td>
<td>Coulter</td>
</tr>
<tr>
<td>CD14</td>
<td>PE-Cy7</td>
<td>M5E2</td>
<td>557742</td>
<td>BD</td>
</tr>
<tr>
<td>HLA-DQ</td>
<td>FITC</td>
<td>SK10</td>
<td>347453</td>
<td>BD</td>
</tr>
</tbody>
</table>
Gating scheme

- CD3 (V450)
- CD14 (PE-Cy7)
- CD8 (PE)
- CD4 (ECD)
- CD19 (APC-AF750)
- HLA-DQ (FITC)
- CD45 (APC)
- Live/Dead (Aqua)
- FSC-A
- SSC-A
- TruCount beads
AIM 1: CVL vs CYTOBRUSH

CD45 (APC) vs SSC-A
Absolute yields of leukocyte populations

Chicago
Nairobi
Seattle

Cell Number (log10)

CD45  |  CD4  |  CD8

CB      |  CVL  |  CB      |  CVL  |  CB      |  CVL

*
Absolute yields of leukocyte populations

[Graph showing cell numbers in log10 for CD19, CD14, and DC from CB and CVL in Chicago, Nairobi, and Seattle.]
Cell phenotype as a percentage of CD45+

- Unknown
- DC
- CD19
- CD14
- CD4
- CD8

Comparison between CB and CVL.
AIM 2:
CYTOBRUSH vs BIOPSY

CD45 (APC)

CB

Biopsy

SSC-A
Absolute yields of leukocyte populations

Cell Number (log10)

CB  Biopsy  CB  Biopsy  CB  Biopsy

CD45  CD4  CD8

Chicago
Nairobi
Seattle
Absolute yields of leukocyte populations

Cell Number (log10)

CD19  CD14  DC
CB    Biopsy    CB    Biopsy    CB    Biopsy

Chicago
Nairobi
Seattle
Cell phenotype as a percentage of CD45+

- Unknown
- DC
- CD19
- CD14
- CD4
- CD8

CB
Biopsy

0 25 50 75 100%
Cytobrushes from repeat donors

CD45+ Leukocytes (log_{10})

Spearman r = 0.49
p = 0.1063
Duplicate simultaneous biopsies

Biopsy I CD45+ (log_{10})

Biopsy II CD45+ (log_{10})

Nairobi

Spearman r=0.89
p=0.0123
α4β7 expression

CD4  CD8  DC  B cells

Chicago  Seattle
Three different tissue digestion procedures

CD45^+ (x1000)

Percent of CD45

CD3
CD14

Collagenase  Cocktail  Emigration

Collagenase  Cocktail  Emigration
Impact of blood on cytobrushes

CD45+ Leukocytes (log_{10})

Blood  No Blood

Cape Town
Chicago
Nairobi
Seattle

Unknown
DC
CD19
CD14
CD4
CD8

Blood  No blood

0  25  50  75  100%
Impact of DMPA on cytobrush cell yields

- CD45⁺ Cell Number (log_{10})
  - DMPA
  - None

- Unknown
- DC
- CD14
- CD19
- CD4
- CD8

Comparison between DMPA and None treatment groups.
Preservation and recovery of cytobrush cells

Claire Levy

### Neutrophils

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Preservation method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>No preservative</td>
</tr>
<tr>
<td>Frozen</td>
<td>DMSO + Ethylene Glycol + Trehalose</td>
</tr>
<tr>
<td>Stabilized</td>
<td>SmartTube Proteomic Stabilizer</td>
</tr>
</tbody>
</table>

### Macrophages

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<td>Fresh</td>
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### T cells

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**All cytobrush samples**

- Stabilized samples stained after thawing
- Stabilized samples stained before freezing
## Preservation and recovery of cytobrush cells

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<th></th>
<th>Neutrophils</th>
<th>Macrophages</th>
<th>T lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>median (range)</td>
<td>median (range)</td>
<td>median (range)</td>
</tr>
<tr>
<td>Fresh</td>
<td>127,589 (5,841 - 596,748)</td>
<td>1,602 (184 - 13,473)</td>
<td>1,401 (373 - 2,962)</td>
</tr>
<tr>
<td>Frozen</td>
<td>56,595 (294 - 240,276)</td>
<td>997 (8 - 10,695)</td>
<td>851 (78 - 1,923)</td>
</tr>
<tr>
<td>Stabilized</td>
<td>7,779 (62 - 185,772)</td>
<td>50 (0 - 2,899)</td>
<td>231 (12 - 2,009)</td>
</tr>
</tbody>
</table>

Claire Levy
SUMMARY I

- Cervical cytobrushes yield more cells than CVLs
- One cervical biopsy yields approximately equal numbers of viable leukocytes (~10,000) than two sequential cytobrushes
- Cytobrushes contain more macrophages and biopsies more T cells
- Sample yields were overall consistent between sites
• Visible red blood cells increased leukocyte yields more than three-fold, but did not change their subpopulation profile

• More than half of genital T cells, B cells and DCs express α4β7 in cervical cytobrushes, and ~80% in biopsies

• Cytobrush cell yields as a group are consistent over time, but yields from individual women vary

• Leukocyte yields from same-day replicate biopsy biopsies were consistent

• DMPA contraception did not increase leukocyte yields in cytobrushes (but numbers were too small for to be sure)

• Cytobrushes also contain a large number of neutrophils

• Isolated cytobrush leukocytes can be cryopreserved with approximately 30-40% cell loss
Function of vaginal T cells after cryopreservation

% TNFα, IFN and/or IL2+

Stimulation:
- DMSO
- PMA/ionomycin