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# Isolation of Leukocytes from Human Breast Milk for Use in an Antibody-Dependent Cellular Phagocytosis Assay of HIV Targets

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**TITLE:**

# Isolation of Leukocytes from Human Breast Milk for Use in an Antibody-Dependent Cellular Phagocytosis Assay of HIV Targets

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**KEYWORDS:**

leukocytes, breast milk, cell isolation, phagocytosis, lactation, neutrophils

**SUMMARY:**

Breast milk transmits human immunodeficiency virus (HIV), though only ~15% of infants breastfed by HIV-infected mothers become infected. Breastfed infants ingest ~$10^5$–$10^8$ maternal leukocytes daily, though these cells are understudied. Here we describe the isolation of breast milk leukocytes and an analysis of their phagocytic capacity.

**ABSTRACT:**

Even in the absence of antiretroviral drugs, only ~15% of infants breastfed by HIV-infected mothers become infected, suggesting a strong protective effect of breast milk (BM). Unless access to clean water and appropriate infant formula is reliable, the WHO does not recommend cessation of breastfeeding for HIV-infected mothers. Numerous factors likely work in tandem to reduce BM transmission. Breastfed infants ingest ~$10^5$–$10^8$ maternal leukocytes daily, though what remains largely unclear is the contribution of these cells to the antiviral qualities of BM. Presently we aimed to isolate cells from human BM in order to measure antibody-dependent cellular phagocytosis (ADCP), one of the most essential and pervasive innate immune responses, by BM phagocytes against HIV targets. Cells were isolated from 5 human BM samples obtained at various stages of lactation. Isolation was carried out via gentle centrifugation followed by careful removal of milk fat and repeated washing of the cell pellet. Fluorescent beads coated with HIV envelope (Env) epitope were used as targets for analysis of ADCP. Cells were stained with the CD45 surface marker to identify leukocytes. It was found that ADCP activity was significant above control experiments and reproducibly measurable using an HIV-specific antibody 830A.

**INTRODUCTION:**

Human breast milk (BM) is comprised of maternal cells that are >90% viable[1]. Cell composition is impacted strongly by stage of lactation, health status of mother and infant, and individual

variation, which remains poorly understood[1-4]. Given that BM contains ~$10^3$–$10^5$ leukocytes/mL, it can be estimated that breastfed infants ingest ~$10^5$–$10^8$ maternal leukocytes daily[5]. Various in vivo studies have demonstrated that maternal leukocytes provide critical immunity to the infant and are functional well beyond these sites of initial ingestion[5-11]. All maternally-derived cells ingested by the infant have the potential to perform immune functions alongside or to compensate for the infant's own leukocytes[12].

Mother-to-child transmission (MTCT) of human immunodeficiency virus (HIV) remains a crisis in resource-limited countries. As diarrheal and respiratory diseases are responsible for substantial rates of mortality among infants in resource-limited countries, and these illnesses are significantly reduced by exclusive breastfeeding, the benefits to HIV-infected mothers of breastfeeding far outweigh the risks[13-15]. Unless access to clean water and appropriate infant formula is reliable, the WHO does not recommend cessation of breastfeeding for HIV-infected mothers[16]. Approximately 100,000 MTCTs via BM occur annually; yet, only ~15% of infants breastfed by their HIV-infected mothers become infected, suggesting a strong protective effect of BM[17-21]. Numerous factors likely work in tandem to prevent transmission. Importantly, HIV-specific antibodies (Abs) in BM have been correlated with reduced MTCT and/or reduced infant death from HIV infection[22,23]. What remains largely unclear is the contribution of the cellular fraction of BM to its antiviral qualities.

Many Abs facilitate a variety of anti-viral activities mediated by the 'constant' region of the immunoglobulin molecule, the crystallizable fragment (Fc), via interaction with Fc receptors (FcRs) found on virtually all innate immune cells, virtually all of which are found in human BM[24]. Antibody-dependent cellular phagocytosis (ADCP) has been demonstrated as necessary for the clearance of viral infections and has been understudied in the case of prevention of MTCT of HIV[25-29]. Given the paucity of knowledge about the potential contribution of ADCP activity by BM phagocytes to prevention of MTCT of HIV, we aimed to develop a rigorous method to isolate cells from human BM in order to undertake a study of ADCP mediated by cells from BM obtained at various stages of lactation.

**PROTOCOL:**

Each participant in this study was recruited and interviewed in accord with the ethical and institutional review board (IRB) approval with the guidance and authorization of Mount Sinai's Program for the Protection of Human Subjects (PPHS) using an IRB-approved protocol for obtaining breast milk samples.

**1. Breast milk cell isolation**

1.1. Obtain human breast milk from healthy lactating women, expressed using double electronic or manual pumps. Isolate cells within ~4 h of expression, keeping milk at room temperature.

1.2. Using 50 mL tubes, centrifuge 50 mL milk at 800 x *g* for 15 min. Carefully pour off the skim

milk and fat while leaving the cell pellet undisturbed. Wipe the inside of the tube with a lint-free wipe to remove all fat from the tube wall.

1.3. Add 10 mL of 2% human serum albumen in Hank's balanced salt solution (2% HSA HBSS [without $Ca^{2+}$ or $Mg^{+}$]). Resuspend the pellet by gentle pipetting to avoid cell activation and apoptosis. Transfer to a 15 mL tube and centrifuge at 450 x *g* for 10 min.

1.4. Pour off supernatant and repeat step 1.3. Then, gently resuspend cells in 1−2 mL of 2% HSA HBSS depending on expected cell number, and count cells by a hemocytometer or an automated cell counter, noting also the cell viability.

**2. ADCP assay**

NOTE: Methods described here are adapted from Ackerman et al.[30].

2.1. Select a relevant target antigen.

NOTE: In this example, the recombinant protein V1V2-2F5K was used, which was designed to mimic the trimeric apex of native HIV envelope[31].

2.2. Perform biotinylation using a commercial kit (**Table of Materials**) according to the manufacturer's protocol.

2.2.1. Calculate mmol of the biotin reagent to add to the reaction for a 5-fold molar excess using the formula: mmol biotin = mL protein x (mg protein/mL protein) x (mmol biotin/mg protein) x (5 mmol biotin/mmol protein)[31,32]. Then calculate µL of the biotin reagent to add using the formula: µL biotin = mmol biotin x (1,000,000 µL/L) x (L/10 mmol).

2.2.2. Equilibrate biotin to room temperature before opening. Dissolve protein in 0.5−2.0 mL of phosphate-buffered saline (PBS) according to the calculation made above.

2.2.3. Prepare a 10 mM solution of biotin reagent in dimethylsulfoxide (DMSO) and add the appropriate volume of 10 mM biotin reagent to the protein solution, and incubate reaction on ice for 2 h or at room temperature for 30 min.

2.3. Remove excess biotin using protein concentrators (polyethersulfone [PES] membranes, 3 kDa molecular weight cut-off [MWCO], 0.5 mL; **Table of Materials**) according to the manufacturer's instructions.

2.3.1. Deposit sample into the upper chamber of spin column and add PBS up to 400 µL. Cap, then insert this sample chamber into a collection tube. Centrifuge at 12,000 x *g* at room temperature for 30 min.

2.3.2. Discard flow-through and add PBS to 400 µL. Repeat centrifugation. Discard flow-through

and add PBS to 100 µL. Measure protein concentration by a spectrophotometer.

NOTE: Protein can be aliquoted and frozen at -80 °C until used.

2.4. Conjugate biotinylated protein to 1 µm microspheres ('beads'; **Table of Materials**) according to the manufacturer's instructions.

2.4.1. Per plate of conjugated beads, incubate 6 µg of protein with 12 µL of stock beads in 200 µL of 0.1% bovine serum albumin (BSA)-PBS at room temperature for 1 h, vortexing gently every 20 min.

2.4.2. Centrifuge at 13,000 x *g* for 5 min. Discard supernatant, vortex gently and resuspend in 1200 µL of 0.1% BSA-PBS. Repeat spin and wash step 2x. Resuspend in 1200 µL of 0.1% BSA-PBS.

2.5. Aliquot 10 µL of bead solution per well in a round bottom 96-well plate. Prepare dilutions of antibody or immune sera of interest in 12 µL of 2% HSA HBSS, typically starting at 50 µg/mL of antibody or a 1/100 serum dilution.

NOTE: In the sample data, monoclonal antibody (mAb) 830A was used.

2.6. Add 10 µL of titrated antibody/sera to the bead plate and incubate for 2 h at 37 °C. Add 200 µL of 2% HSA HBSS to each well and centrifuge plate at 2,000 x *g* for 10 min.

2.7. Carefully remove supernatant by a rapid overturning and decanting of liquid from the plate wells into a sink to avoid disturbing the invisible bead pellet. Add 50,000 freshly isolated BM cells to each well in 200 µL of 2% HSA-HBSS. Incubate for 2 h at 37 °C.

2.7.1. For control experiments, pre-incubate cells at 37 °C with 10 µg/mL actin inhibitor (cytochalasin-D), 50 µg/mL FcR blocking agent (FcBlock), or a combination of both prior to their addition to the plates.

2.8. Centrifuge plate at 930 x *g* for 10 min. Add 200 µL of 2% HSA HBSS and repeat centrifugation. Carefully remove supernatant as in step 2.7 and repeat wash.

2.9. Carefully remove supernatant and stain cells for viability using 0.5 µg/mL (final concentration) fixable viability stain 450 per well in 50 µL of 2% HSA HBSS. Incubate 20 min at room temperature in the dark. Centrifuge plate at 930 x *g* for 10 min and remove supernatant as in step 2.7. Add 200 µL of 2% HSA HBSS and centrifuge plate again followed by removal of supernatant as in step 2.7.

2.10. After viability staining, stain cells for leukocyte markers of interest, minimally including a CD45-specific stain such as PE-mouse anti-human CD45 (clone HI30) at an optimized concentration (1 µg/µL in 50 µL of 1% BSA HBSS in the example data).

NOTE: Any lineage-specific markers of interest can be included.

2.11. Incubate 20 min at room temperature in the dark. Centrifuge plate at 930 x *g* for 10 min and remove supernatant as in step 2.7. Add 200 µL of 1% BSA HBSS and repeat centrifugation. Remove supernatant. Fix cells in 200 µL of 0.5% formaldehyde in the dark at room temperature for 30 min or overnight at 4 °C. Refrigerate in the dark until analysis.

**3. Analysis by flow cytometry**

3.1. Perform initial gating to eliminate doublets on a forward scatter (FSC) vs. side scatter (SSC) plot and debris (material smaller than FSC = 5000) (see **Figure 1**). Use an SSC vs. viability stain (V450 in this case) plot to eliminate dead cells (those that are positive for viability stain).

3.2. Use an SSC vs. CD45 plot to differentiate the major leukocyte classes (granulocytes, monocytes, lymphocytes) as extensively described[33,34].

NOTE: This classification is only suggestive and lineage-specific markers are needed to confirm cell type.

3.3. For all CD45+ cells, or for each leukocyte subset of interest, measure ADCP activity by gating with a marker on the bead-positive cells in a histogram of the fluorescein isothiocyanate (FITC) channel, where the fluorescent beads are detected.

NOTE: The negative control wells in which beads were not added will indicate where the bead-positive cells are apparent in the histogram and therefore where to place the gating marker.

3.4. Calculate ADCP scores as (median fluorescence intensity [MFI] of bead-positive cells) x (% of total CD45 + cells in the positive population). Use graphics software to plot scores at each Ab/serum concentration and to perform an area-under-the-curve (AUC) analysis.

NOTE: ADCP is considered positive if the AUC is greater than 3x standard deviation of the ADCP score AUC of a non-specific negative control mAb (in this case, 3865).

**REPRESENTATIVE RESULTS:**

Milk can be kept at room temperature or cooler (though not frozen); however, given that we have observed reduced viability when milk has been kept very cold (data not shown), and that it is simpler to collect, store briefly, and transport at ambient temperatures, it is recommended that samples are not refrigerated in order to reduce sample-to-sample variability. In milk obtained 7−183 days post-partum, cell concentration determined by automated cell counter ranged from 16,083−222,857 cells/mL. **Figure 1** illustrates the gating strategy eliminating doublets, debris, and dead cells. Viability was ~90−99%. Approximately 1.6−12.3% of total live cells were CD45+ leukocytes (**Table 1**). Most purported monocytes appeared to be precursors/immature cells as previously described, based on the suggestive SSC vs. CD45

gating[34]. The purported monocytes were defined as $SSC^{low\text{-}intermediate}/CD45^{low}$, though few exhibited the higher CD45 staining levels distinct from the purported lymphocyte population ($SSC^{low}/CD45^{low}$) more typically associated with blood monocytes (**Figure 1**), similar to previous studies[33,34]. The purported granulocytes were defined as $SSC^{high}/CD45^{intermediate}$[33,34] (**Table 1**). Note that this classification is only suggestive and that lineage-specific markers would be needed to confirm cell type.

ADCP activity of the freshly isolated BM cells was measured using the HIV-specific human mAb 830A, which is specific for the V2 region of the HIV envelope and binds to the V1V2-2F5K antigen tested here. ADCP activity was measured for the example here using milk obtained at 1 month post-partum (**Figure 2A**). Example data shows the expected FITC (bead+) histograms seen when gating on CD45+ cells (data generated using 1 µg/mL mAb is shown). The black markers indicate the populations used to calculate ADCP scores. In the sample 830A data (first panel of **Figure 2A**), percentage of CD45+ cells and mean fluorescence of that population are shown, which were used to calculate the ADCP score using the equation in step 3.4. Cells pre-incubated with actin inhibitor cytochalasin-D (cytoD) and/or FcR-blocking Abs (FcBlock) prior to their incubation with the Ab-bound/antigen-coupled beads exhibited ADCP activity at the level of the control mAb 3865 or below, indicating ADCP was FcR and actin-dependent (**Figure 2**). The ADCP score determined for total CD45+ cells was ~25−35-fold above background levels defined using the negative control anti-anthrax mAb 3865. Each major subset was analyzed separately as well. The purported granulocytes exhibited ADCP activity ~12−29-fold higher than background. The purported monocyte ADCP was ~2−3-fold above background (**Figure 2**). The purported lymphocytes as expected did not exhibit any measurable ADCP activity (less than 3x standard deviation of the ADCP score AUC of the non-specific negative control mAb 3865; data not shown).

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Sample flow cytometry data of cells isolated from breast milk.** Cells were processed and stained as described in the protocol. (**A**) Single cells were gated on to eliminate doublets in an FSC-H vs. FSC-A plot as shown, also gating out the small debris <5000 in FSC-A. (**B**) This population was used to gate on live cells (which do not stain with the viability dye) in an SSC vs. V450 (viability stain) plot. (**C**) These live cells were used in an FSC vs. SSC plot. The expected position of non-leukocytes, likely to be predominantly mammary epithelial cells, is highlighted ("E"). (**D**) The same FSC vs. SSC plot is shown only with CD45+ cells. The major leukocyte subsets noted are only purported identities based on well-established and expected SSC parameters (G: granulocytes; M: monocytes; L: lymphocytes). (**E**) Viable cells were used for an SSC vs. CD45 plot with the major leukocyte subsets noted. Back-gating from this plot yielded the data shown in panel D. Note that this classification is only suggestive and that lineage-specific markers are needed to confirm cell type.

**Figure 2: Sample ADCP data using cells isolated from breast milk.** The ADCP assay performed is based on the assay adapted from Ackerman et al.[30]. The assay was performed as outlined in the protocol above. (**A**) Sample FITC histograms at 1 µg/mL mAb, with markers indicating the

bead/FITC+ populations used to determine the ADCP score. Scores were calculated as (MFI of bead-positive cells) x (% of total CD45+ cells in the bead/FITC+ positive population). The first panel using mAb 830A alone also indicates the percentage of total CD45+ cells and the mean fluorescence intensity value used to calculate the ADCP score in that example. (**B**) ADCP scores at each mAb dilution assayed were used to calculate area-under-the-curve (AUC) values in graphics software. For control experiments, actin inhibitor cytochalasin-D (cytoD), FcR blocking agent (FcBlock), or a combination of both were pre-incubated with cells prior to their addition to the immune complexes (see legends). Note that this cell classification is only suggestive and that lineage-specific markers are needed to confirm cell type.

**Table 1: Examples of typical breast milk cell counts and characteristics.**

**DISCUSSION:**

The flow cytometry-based technique for measuring ADCP activity described herein was first described in 2011[30] and has since been proven robust and cited in more than 80 studies. The protocol described here adapts this technique for use with primary BM cells for the first time. Previous studies of Fc-mediated functionality by BM cells have been largely limited to measurement of oxidative bursts or histology-based phagocytosis assays using cells isolated from colostrum (0–4 days after birth). Virtually no studies have examined cells in human BM past the colostrum phase. Studies using colostral cells have generally concluded that the granulocytes in colostrum are less active than those isolated from blood, behaving as an 'exudate cell' that has moved into the extravascular space[35], though conflicting studies have reported similar phagocytic and bactericidal capacities[36].

For decades, traditional microscopy was used to identify BM leukocytes, and this type of visual identification may have led to cell misidentification[1]. Few studies have compared BM leukocyte composition beyond the first month of lactation, and most have focused on colostrum. The use of flow cytometry to identify cells is likely to accurately identify cells, though only a small number of BM studies have been done using this method, often with a very small sample number. Current studies have indicated that the leukocyte content of BM at all stages of lactation varies widely, ranging from ~$10^4$–7 x $10^5$ leukocytes/mL in early colostrum, decreasing to $10^3$–5 x $10^4$ leukocytes/mL in mature milk, though all studies confirm that cell concentration and composition is impacted strongly by the stage of lactation[1-5]. As milk transitions to its mature composition, neutrophil concentration appears to increase, though such studies have not typically extended beyond the first month postpartum[34].

The protocol described herein uses fluorescent beads as the phagocytic target, though it likely can be applied to study BM ADCP of a variety of more biologically relevant targets such as immune complexes and infected cells, triggered by various Ab isotypes and subclasses. A larger cell staining panel can be employed to further differentiate the leukocytes. Large studies will be essential to develop a comprehensive understanding of ADCP by these relevant primary cells. This protocol allows for the establishment of ADCP by BM leukocytes as a potential mechanism for reduction of MTCT of HIV, as well as other pathogens.

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**DISCLOSURES:**

The authors have nothing to disclose.

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Figure 1



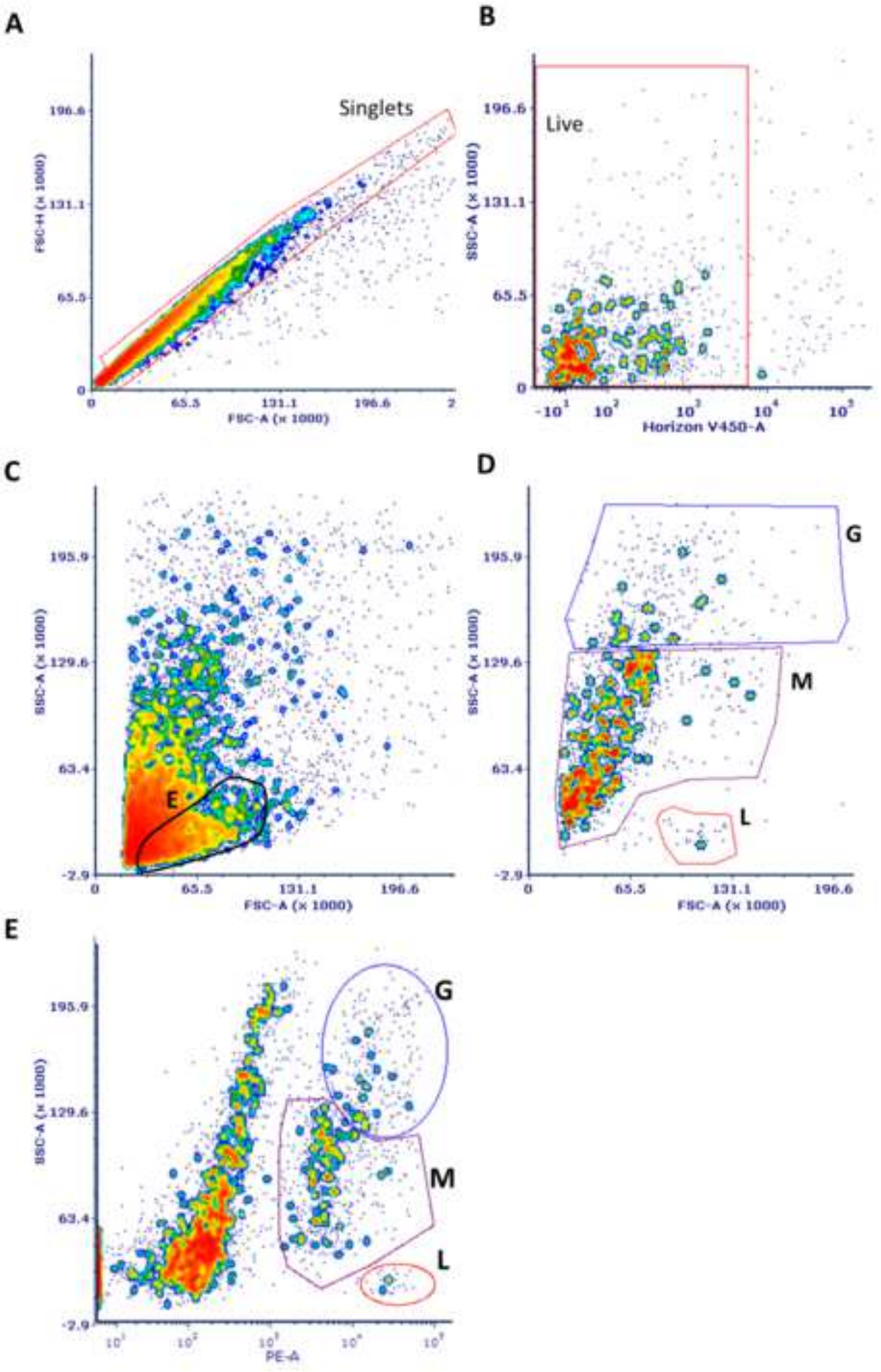
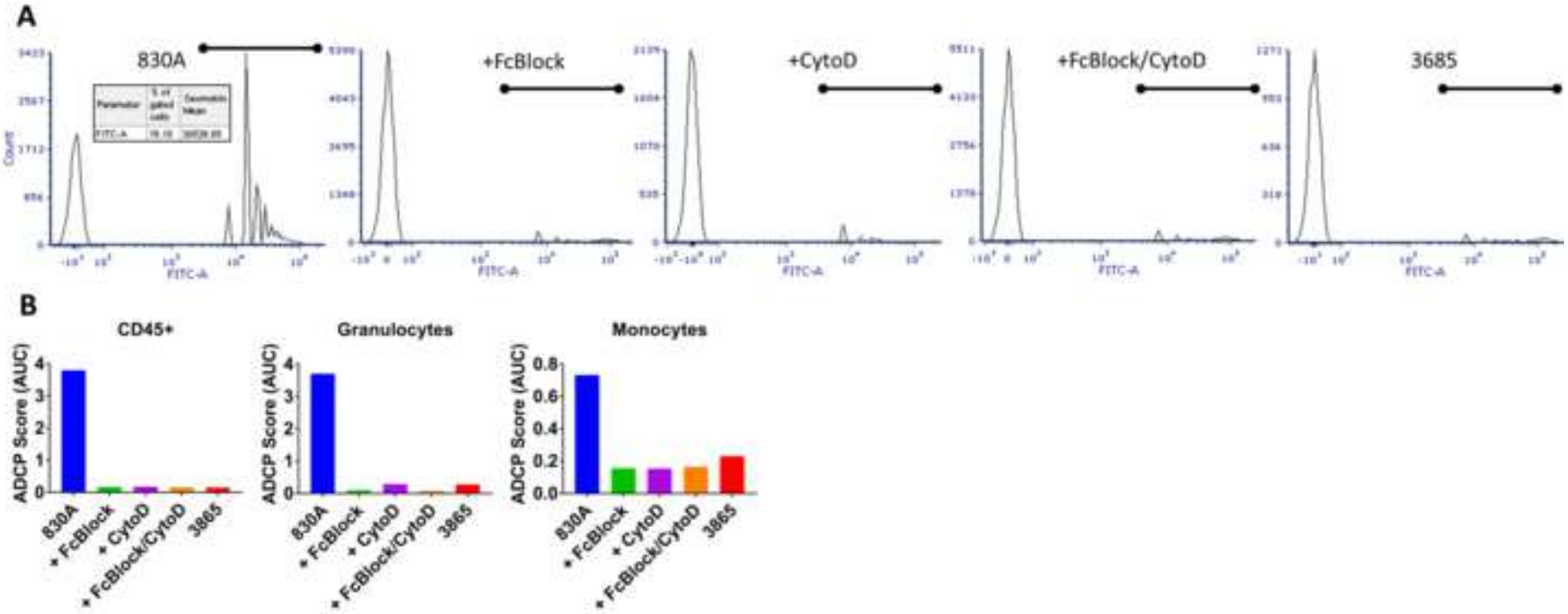


Figure 2

Click here to access/download;Figure;Fig. 2 revised.tif



Table



| Sample | Cells/mL | % CD45+ | Granulocytes* | % Monocytes* |
|---|---|---|---|---|
| 1 | 222,857 | 12.3 ± 1.9 | 13.6 ± 3.8 | 65.9 ± 5.6 |
| 2 | 27,361 | 1.6 ± 0.01 | 25.2 ± 4.0 | 9.1 ± 5.6 |
| 3 | 161,486 | 3.6 ± 1.1 | 47.8 ± 6.8 | 24.3 ± 4.3 |
| 4 | 16,083 | 2.7 ± 0.1 | 17.9 ± 3.5 | 34.4 ± 1.0 |
| 5 | 25,155 | 4.0 ± 0.7 | 29.7 ± 2.6 | 20.5 ± 1.4 |

***of CD45+ cells**

Table of Materials



| Name of Material/ Equipment | Company | Catalog Number |
|---|---|---|
| 1 µm FluoSpheres NeutrAvidin-Labeled Microspheres | Thermo Fisher | F8776 |
| BD Pharmingen PE Mouse Anti-Human CD45 | BD | 560975 |
| Bovine serum Albumin | MP Biomedicals | 8810025 |
| Corning V-bottom polystyrene 96-well plate | Corning | 3894 |
| Cytochalasin D | Sigma | 22144-77-0 |
| EZ-Link NHS-LC-LC-Biotin kit | Thermo Fisher | 21338 |
| Falcon 15mL Conical Centrifuge Tubes | Corning | 352196 |
| Falcon 50mL Conical Centrifuge Tubes | Corning | 352070 |
| Fixable Viability Stain 450 | BD | 562247 |
| Formaldehyde solution | Sigma | 252549 |
| HBSS without Calcium, Magnesium or Phenol Red | Life Technologies | 14175-095 |
| Human BD Fc Block | BD | 564219 |
| Human Serum Albumin | MP Biomedicals | 2191349 |
| Kimtech Science Kimwipes Delicate Task Wipers | Kimberly-Clark Professional | 34120 |
| PBS 1x pH 7.4 | Thermo Fisher | 10010023 |
| Polystyrene 10mL Serological Pipettes | Corning | 4488 |
| Protein Concentrators PES, 3K MWCO, 0.5 mL | Pierce | 88512 |

| Comments/Description |
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Title of Article: Isolation of leukocytes from human breast milk for use in HIV antibody-dependent cellular phagocytosis assay of HIV targets

Author(s): Rebecca LR Powell, Alisa Fox

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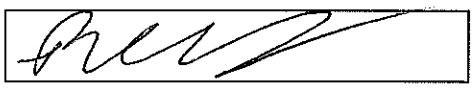
| Signature: |  |
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- Manuscript has been proofread

2. Authors and affiliations: Please provide an email address for each author.

- Now provided on cover page

3. Keywords: Please provide at least 6 keywords or phrases.

- 6th keyword added

4. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Please use the micro symbol µ instead of u and abbreviate liters to L (L, mL, µL) to avoid confusion.

- SI abbreviations edited

5. Please include a space between all numbers and the corresponding unit: 15 mL, 5 g, 7 cm, 37 °C, 60 s, 24 h, etc.

- units edited

6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. Step 1 followed by 1.1, followed by 1.1.1, etc. Each step should include 1–2 actions and contain 2–3 sentences. Use subheadings and substeps for clarity if there are discrete stages in the protocol. Please refrain from using bullets, dashes, or indentations.

- numbering edited and detail added

7. All methods that involve the use of human or vertebrate subjects and/or tissue sampling must include an ethics statement. Please provide an ethics statement at the beginning of the protocol section indicating that the protocol follows the guidelines of your institution.

- ethics statement added

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- commercial language removed

9. 1.2: What volume of milk is used?

- volume added to protocol

10. 2.3: Please describe how.

- more description added to protocol for this section

11. 3.1: Please describe how initial gating is done.

- gating is now described in more detail and figure 1 is expanded

12. Section 3: Please write the text in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "NOTE".

- tense edited

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- please note that the figures have now been changed and are new figures, not reproductions or alterations of those included in a previous publication

14. Figure 2: Please use the micro symbol µ instead of u and include a space between all numbers and the corresponding unit (1 µg/mL).

- figure has been altered.

15. Supplemental Table 1: Please upload it to your Editorial Manager account as an .xlsx file.

- tables are now in xlx form

16. Please remove the embedded Table of Materials.

- removed

17. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol. Please remove trademark (™) and registered (®) symbols. Please sort the items in alphabetical order according to the name of material/equipment.

- table has been expanded and alphabetized

18. References: Please do not abbreviate journal titles; use full journal name.

- the Jove endnote file online does not appear to allow this? I can't seem to get the full titles to display.

**Reviewer #1:**

Major Concerns:

1. Show the entire gating strategy in a stepwise manner showing the gated population that is used in the preceding analysis by using arrows. Include doublets, debris and dead cells. From the gates shown in figure 1 it is not clear what populations are being analyzed and how the gating was done.

- figure 1 has been expanded to show detailed gating strategy

2. Please show staining with epithelial cell antibodies or citation demonstrating the gate marked E in Figure 1A does correspond to epithelial cells. In the graph shown, this gate looks like a classic lymphocyte gate.

- in the altered figure, it is now made more clear where the lymphocytes fall in the SSC v FSC plot, based off of back-gating from the SSC v CD45+ plot. We did not repeat this with an epithelial stain but it is clear the population that is not CD45+ are not lymphocytes.

3. Relying solely on FSC and SSC for the determination of cell populations is not rigorous to claim that a certain population was responsible for the effect. From the gating strategy all that can be said is that CD45lo, medium and high populations had differences in ADCP. It can be suggested that these correspond to granulocytes, monocytes and lymphocytes, but not stated as such, unless lineage specific antibodies are used e.g. CD14, CD3.

- throughout the text and figure legends we have now emphasized that this cell identification is only purported or suggestive and that lineage-specific markers would need to be used to confirm identity. We also cite other studies that have shown these SSC v CD45 populations to correspond with particular cell types.

4. Show individual histograms (as in Fig 2a) for ADCP activity, comparing the activity of the 830A antibody, FcBlock, CytoD, 3865 Ab, etc. so that readers can see the shifts in the peaks and understand what is being measured.

- figure 2 has been changed to now include histograms of each experiment

5. Clarify how ADCP score is calculated, it is not clear what is meant by % CD45 cells in the positive population. In the Ackerman et al. manuscript the calculation is as follows: % bead+ cells x MFI of Bead+ cells. Showing and labelling the populations used for this calculation in the histogram (Figure 2a) would be helpful to readers.

- figure 2a now includes the numbers for the percent of total CD45+ cells and the MFI given in the FACS analysis so that the reader might better understand how to calculate ADCP score. We are using the same calculation as Ackerman et. al, but for leukocyte subsets we use percent of CD45+ cell rather than

percent of total cells to account for the relative activities of the different cell types in the milk.
6. What is the criteria for a "positive" ADCP value relative to the negative controls? Is there background ADCP that is too high that would suggest assay is not reliable? Showing the data for lymphocytes would also be helpful since these do not show ADCP activity and ex vivo unstimulated lymphocytes should not express FcR. Using a cell line that does not express FcR should be used as a negative control as well.
- we have now included in the protocol and sample results that ADCP is considered positive if the AUC is greater than 3x standard deviation of the ADCP score AUC of a non-specific negative control mAb (in this case, 3865). We have not included the lymphocyte data but hope that the amended figure 2 will help illustrate what a negative histogram might look like.
7. In Figure 2B it is not clear from the scale what the concentration of mAb was used. If started at 50ug/ml and then did 5 fold dilutions, it would correspond to 10 ug/ml and 2 ug/ml is that correct? The scale does not appear in the right place for 10 and figure 2a shows data for 1ug/ml, please clarify.
- figures have been amended
8. In Figure 2B addition of the the mAb does not show a dose response and the ADCP for 830A never approaches that of the negative controls suggesting a non-specific effect. Can you show a positive control, with a dose response, demonstrating the assay is working? Can you titrate antibody further do demonstrate activity is reduced with diminishing Ab concentration?
- as the figure has been amended to now show AUC, this issue isn't apparent anymore, however we do agree that the mAbs would need to be further diluted to show that ADCP scores do indeed drop down to background. We do not typically have enough cells to do this, although it could be planned for. We have data with other conditions and mAbs that show a decrease in activity down to zero but it doesn't match up with the described protocol so we chose to leave it out of the current manuscript at this time.
9. How does the ADCP activity from breast milk isolated cells compare to that seen in PBMC using your samples? How does this compare to published studies?
- we do not know the answer to this, as the number of cells used and the targets vary in the literature tremendously; as well, there is a paucity of data with primary cells.

Minor Concerns:
1. Methods need to be a bit more specific so that others can reproduce:
a. How much breast milk volume is usually collected? You should at least give a range so someone who has never worked with breast milk knows what are reasonable volumes from which cells can be obtained. If process between 5-10 mls of breast milk should centrifuge in 15 ml conical tube.
- this has now been included
b. Other manuscripts including Trend et. al. leave milk at 4°C . What is these authors experience? This is important in the case where samples may take longer to process.
- we have now noted temperature concerns more clearly at the beginning of the protocol and results
c. Do you determine cell viability before performing assay? Usually if viability is not at least 80% results from functional assays are not reliable.
- yes we get a viability reading in the cell counter but since much time passes after cells are counted and some cell death can occur we rely on the viability staining. We have added that to step 1.2
d. Need concentration used for CD45 antibody, not just volume.
- this has been edited
e. Should mention fat may sometimes not pour off and will need to scoop out with sterile spatula.
- we find that pouring off followed by wiping the tube works well, as described
f. Kimwipes are usually not sterile, please clarify what you mean by "sterile".
- the word sterile has been removed
g. Provide manufacturer for 3K spin columns.

- added

2. Give percentages of the gated populations on the histograms in Figure 1 and 2A, this helps orient reader.

- the figures have now been amended and we hope that the gating is more clear with the added detail. We have refrained from including percentages because every sample is so variable that we do not want to give a sense that what the person following the protocol might see would be wrong even if it is not similar to what is shown in the example. Percentages of leukocyte subsets for the 5 samples tested in the pilot study are shown in Table 1.

3. For Figure 2B, I would make the symbols larger and possibly use open symbols as well so that data is easier to read.

- Figure has been amended

**Reviewer #2:**

The authors do not address how long they are able to keep their biotinylated antigen in culture before use.

-it is now noted in the protocol that protein should be frozen in aliquots

It might be helpful to show the calculations with the representative data to make it clearer as to how ADCP scores are generated.

-numbers for calculation are now included in Figure 2a, first panel as an example

*note that other comments were addressed in the response to the Editor or reviewer 1.