Poster #871

University of Colorado Anschutz Medical Campus

MAVRC Mucosal and Vaccine Research Colorado

MUWRP Makerere University Walter Reed Project

Ontogeny of HIV-Targeted CD4+T Cells During Exclusive and Non-Exclusive Breastfeeding

Elizabeth J. McFarland[1*], Tina Powell[1], Weiming Zhang[1], Carolyne Onyango-Makumbi[2], Kelsey Melander[1], Prossy Naluyima[3], Samuel Okurut[3] , Michael Eller[3], Mary Glenn Fowler[2] , and Edward N. Janoff[1].

[1]University of Colorado Anschutz Medical Campus, Colorado, USA , [2]Makerere University Johns Hopkins University, Kampala, Uganda, [3]Makerere University Walter Reed Project, Kampala, Uganda.

Elizabeth J. McFarland
University of Colorado AMC
Division of Pediatric Infectious Diseases
12700 E. 19th Ave, 8604
Aurora, CO 80045
Tel. 303-724-3447, Fax. 303-724-4485
betsy.mcfarland@ucdenver.edu

ABSTRACT

Background: Mixed breastfeeding (BF) is associated with a higher risk of mother-to-child transmission than exclusive BF (EBF), however the mechanisms of increased risk are unclear. We determined whether feeding practices alter the expression of CD4+ T cell phenotypes with increased susceptibility to HIV.

Methods: We prospectively evaluated 99 HIV-exposed, but uninfected, breastfed Ugandan infants at birth, 2, 6, 12, 18, 24, and 48 wks. T cells in fresh blood were characterized by flow cytometry for naïve, central (CM), effector (EM), and effector RA+ (EMRA) memory phenotypes by differential expression of CD45RA/CCR7, for markers of gut homing (β7), and for activation (HLA-DR+/CD38+) on CD4+ and CD8+ T cells and for HIV susceptibility by expression of surface co-receptor CCR5 on CD4+ cells. FMO tubes were used to define gating. Mothers were counseled to practice EBF but made a study-independent choice of feeding practice. EBF infants (n=49) were reported to have received only breast milk at all visits through 24 wk. Non-exclusively breastfed infants (NX, n=19) received non-breast milk liquids or solids, ± continued BF, at ≥2 visits between 2-24 wk. There was one HIV-infected infant who was excluded from the analysis. Values are expressed as medians; p values determined by paired t test for changes over time and Wilcoxon Rank-Sum test for comparisons between EX and NX.

Results: In the whole cohort at birth, although most CD4+ T cells were naïve (91.7%), memory cells were already detected, particularly CM (6.9%). CD4+ CM T cells expressing CCR5 were present at birth and increased 2.6-fold from birth to 2 wk (3.0% vs. 7.9%, p<0.001). Memory CD4+ cells with gut-homing β7hi expression were rare at birth but increased progressively from birth to 48 weeks among CM (1.3% vs 14.4%, p=<0.001) and EM (1.6% vs 7.0%, p<0.001). We identified specific differences between EBF and non-exclusive BF (NX) by 24 weeks, when feeding practices had diverged. NX had a higher frequency of HIV-targeted β7high/CCR5+ CD4+ T cells than EBF in both CM and EM subsets (p≤0.05). Within the β7hi EM subset, NX had higher frequency of activated cells (p=0.03). In the CD8+ T cell population, β7high CM cells were also higher frequency in NX compared to EBF, as were activated cells in each memory phenotype (p≤0.01).

Conclusions: Non-exclusively breastfed infants had altered frequencies of CD4 and CD8 T cell subsets compared to EBF infants. The higher frequencies of CCR5+, gut-homing, memory CD4+ T cells, thought to be prime targets for HIV, might contribute to the higher transmission rate associated with mixed breastfeeding.

BACKGROUND

- Breastfeeding, despite antiretroviral prophylaxis, remains a significant mode of mother-to-child transmission (MTCT).
- Exclusive breastfeeding (EBF) results in lower rates of MTCT HIV transmission than mixed feeding.
- The mechanisms underlying the increased risk associated with mixed feeding are unclear.

OBJECTIVE

To compare CD4 and CD8 T lymphocyte phenotypes (maturation, gut-homing, and activation) in peripheral blood between infants receiving only breast milk versus infants receiving non-breast milk feeds.

METHODS

Study population

The study enrolled 100 HIV-exposed, breastfed Ugandan infants recruited at Mulago Hospital in Kampala. One HIV-infected infant was excluded.

Feeding group categorization:

Mothers were counseled to practice exclusive breast feeding (EBF) but made a study-independent choice of feeding practice. Infant Feeding Exposure was assessed at each study visit for the preceding weeks. Feeding Exposure Code id below:

1: **EBF** (Only breast milk (≤3day water, non-milk liquids, drops of syrups, vitamins, minerals and drugs allowed).

Non-Breast Milk Exposures:
2: Breastfeeding plus water/juice/tea/sugar
3: Breastfeeding plus non-human milk
4: Breastfeeding plus solids with or without non-human milk
5: Non-human milk/NO breastfeeding
6: Solids with or without non-human milk/ NO breastfeeding

Feeding Cohorts:
EX: Exclusive breast feeding (EBF), n=49, category 1 at all visits through week 24
NX: Non-Exclusive breast feeding, n=19, category 2-6 on ≥2 visits between weeks 2-24
U: Unclassified, n=32, category 2-6 at only 1 visit or missing data at ≥ 1 visit; **U** group excluded from EX vs NX comparison but included in kinetic analysis of maturation, homing and activation.

	Feeding exposure at each study visit				
Study ID	2 Week	6 Week	12 Week	18 Week	24 Week
BMF056C	1	1	3	1	3
BMF100C	1	1	1	2	3
BMF149C	1	2	1	3	1
BMF020C	1	1	1	2	2
BMF039C	1	1	1	5	5
BMF040C	1	1	1	5	6
BMF046C	1	1	1	5	6
BMF006C	1	5	5	5	5
BMF007C	2	1	1	1	6
BMF031C	1	1	1	5	6
BMF067C	1	1		6	5
BMF096C	1	1	1	6	6
BMF097C	1	1	1	6	6
BMF098C	1	1	1	5	5
BMF104C	1	1	1	5	5
BMF119C		1	1	6	6
BMF145C	1	5	5	5	6
BMF151C	1	1	5	5	6
BMF154C	1	1	1	5	6

* EX cohort not displayed as they are all category 1 from week2-24

Statistics:

Baseline infant and maternal characteristics were compared using means and tested by Fisher's Exact Test. The frequencies of T lymphocyte subsets are expressed as medians; p values determined by paired t test for changes over time and Wilcoxon Rank-Sum test for comparisons between EX and NX.

Flow Cytometric Analysis

CD4+ and CD8+ T cell subsets in fresh whole blood obtained at birth, 2, 6, 12, 18, 24, and 48 weeks were characterized as:

Maturation
- Naïve (CD45RA+/CCR7+),
- Central memory (CM; CD45RA-/CCR7+)
- Effector memory (EM; CD45RA-/CCR7-)
- Effector Memory RA (EMRA; CD45RA+/CCR7-)

Homing
- β7
- CCR5

Activation:
- CD38
- HLA-DR

Flow Gating Strategy

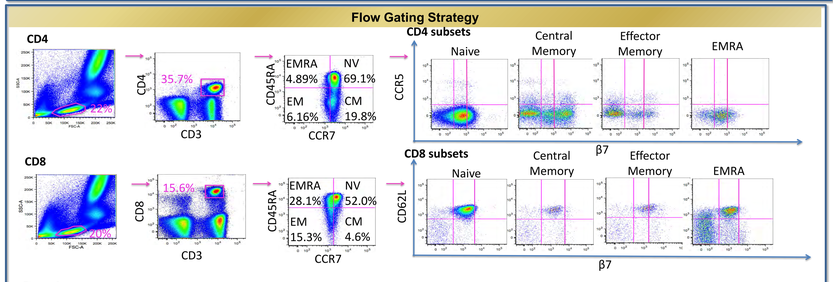


Figure 1.
Forward and Side scatter are used to gate lymphocytes. Next, CD3+CD4+ or CD3+CD8+ are isolated and evaluated for memory using a CD45RA/CCR7 schema. Naïve and memory phenotypes are evaluated for β7 and CCR5 expression for CD4 cells or β7 and CD62L for CD8 cells.

Table 1: Maternal Enrollment Characteristics

	All	All "n"	Exclusive (EX) Feeders	n	Non-Exclusive (NX) Feeders	n	P
Maternal age mean (range)	24.6 (18-40)	100	24.7(18-36)	49	26.3 (18-40)	19	0.226
WHO stage % Stage 1 %Stage 2	 89% 11%	100	 86% 14%	49	 89% 11%	19	1.000
Maternal CD4 cells/mm3 at screen; Mean (range)	657 (354-2095)	100	710 (362-2095)	49	532 (367-798)	19	0.769
% with >500 CD4+ % with ≥350-500 CD4+	68% 32%		73% 27%		58% 42%		
Maternal VL at screen Mean (range)	12968 (20-408,901)	97	7364 (20-73,931)	49	12,496 (20-60,990)	17	0.344
% VL < 400 %VL ≥400-<10,000 %VL ≥10,000	35/97 (36%) 37/97 (38.1%) 24/97 (24.7%)		18/49(36.7%) 20/49 (40.8%) 11/49 (22.4%)		5/17(29.4%) 6/17(35.3%) 6/17(35.3)		
Maternal NVP	93/100 (93%)	100	47/49 (96%)	49	17/19 (89%)	19	0.310
Additional ARV prophylaxis**	99/100 (99%)	100	49/49 (100%)	49	19/19 (100%)	19	N/A
Maternal ARV for treatment ever during study	4/100 (4%)	100	1/49 (2%)	49	3/19 (16%)	19	0.063
Maternal co-trimoxazole treatment at week 2	95/99 (96%)	99	47/47(100%)	47	18/18 (100%)	18	N/A
Maternal mastitis ever	0%	99	0%	49	0%	19	N/A

** additional maternal ARV prophylaxis was zidovudine or zidovudine plus lamivudine

RESULTS

Table 2: Infant Enrollment Characteristics

	All	All "n"	EX Group	EX "n"	NX Group	NX "n"	P
Infant gender % male	48%	100	45%	49	47%	19	1.000
Infant birth wt (kg) mean (range)	3094(2500-4000)	100	3108(2500-4000)	49	3100(2600-3600)	19	0.815
Infant birth BMI mean (range)	13.4(10.5-16.9)	98	13.6(10.5-16.9)	49	13.4(11.7-14.9)	19	.652
Infant week 24 wt (kg) mean (range)	7.4 (4.9-10.1)	95	7.54 (5.6-10.1)	49	6.9 (4.9-8.4)	19	**0.009**
Infant week 24 BMI Mean (range)	18.2 (13.6-24)	95	18.7 (13.6-24)	49	17.2 (13.8-20)	19	**0.007**
Infant post-natal ARV prophlyaxis Zidovudine Nevirapine unknown	 53/91 (58%) 35/91 (39%) 3/91 (4%)	91	 26/49 (53%) 20/49 (41%) 3/49 (6%)	49	 7/19 (37%) 9/19 (47%) 3/19 (16%)	19	0.294
Infant co-trimoxazole prophylaxis at week 12	92/96 (96%)	96	49/49 (100%)	49	16/18 (89%)	19	0.069
Infant received BCG vaccine at birth	86/89 (97%)	89	41/43 (95%)	43	17/18 (94%)	18	1.000
Infant ever ill between wk 2-24	13/97 (13.4%)	97	5/49 (10.2%)	49	4/19 (21.1%)	19	0.253
Infant ever with diarrhea between wk 2-24	43% (43/100)	100	33% (16/49)	49	53% (10/19)	19	0.167

Kinetics of T cell Maturation: Memory Cells Present at Birth

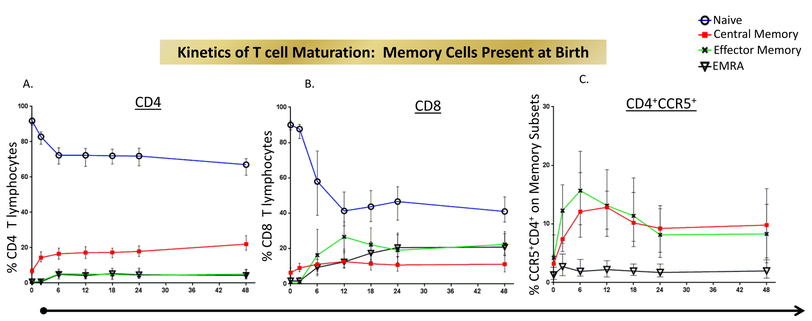


Figure 2.
Plot A and B: At birth, infants have low frequencies of CD4+ and CD8+ memory cells that rapidly increase by 2-6 wks. At 6wks, steep decline in CD8+ Naïve cells compared to CD4+ cells C. Low frequencies of CD4+CCR5+ present at birth predominately in EM (green) and CM (red) memory cells with peak levels at approximately 6 wks. Median percents shown with IQR.

Kinetics of T cell Activation: Rapid Increase by 2-6 Weeks

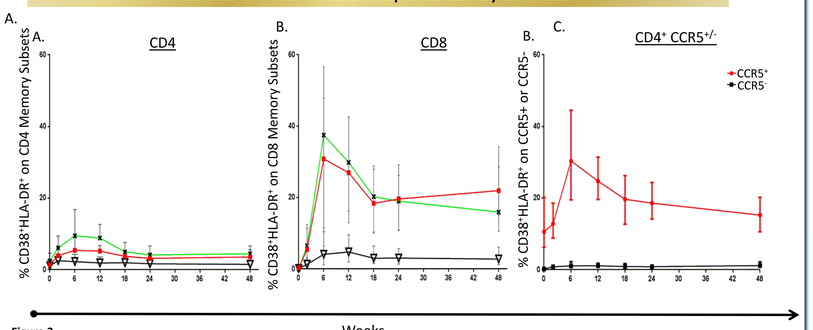


Figure 3.
Plot A and B: Rapid increase in activated CD4+ and CD8+ T cells by age 2-6wks with increase variability in the CD8+ cells. Plot C. Activated CD4+CCR5+ cells (red) present at birth with rapid increase from 2-6wks. In contrast, CD4+CCR5− cells (black) show negligible activation. Median percent shown with IQR in error bars

Kinetics of β7 high expression: Progressive Increase in Central and Effector Memory Cells

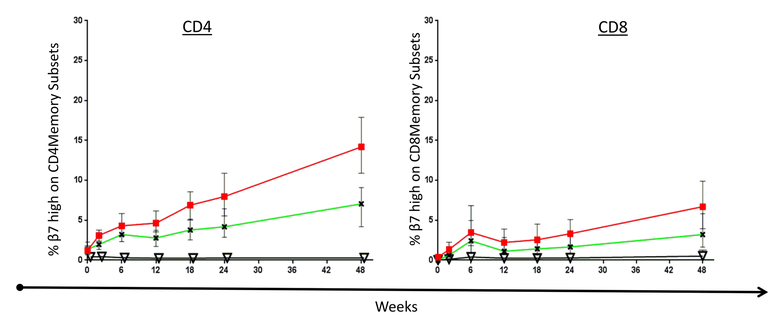


Figure 4.
Plots A and B: β7 high+ CD4+ and β7 high+CD8+ cells are rare at birth but progressively increase during first year, especially, in the Central memory (red) and Effector memory (green) and to a lesser extent in the EMRA (black) compartments. β7 high cells were rare or negligible in the Naïve CD4+ and CD8+ cells (Data not shown). Percentages are represented as medians with IQR error bars.

RESULTS

Higher Expression of β7 on CD4+ and CD8+ Central Memory T Cells in Non-Exclusive Breastfeeding Infants

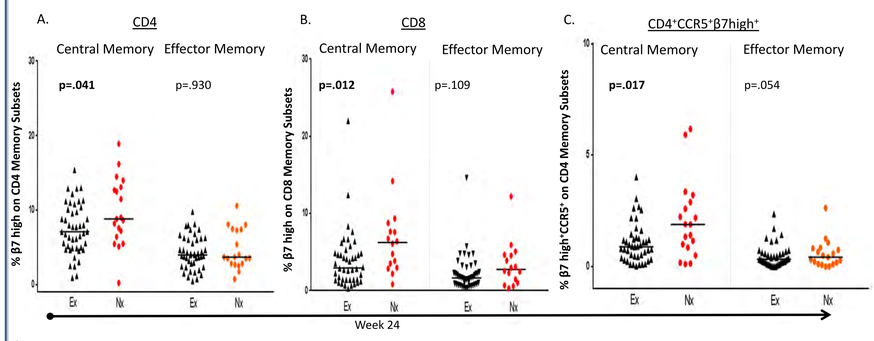


Figure 5.
Plot A and B: Central Memory cells for CD4+ and CD8+ T cells have significantly higher co-expression of β7 high cells for the NX (Non-Exclusively breastfeeding) group versus the EX (Exclusively breastfeeding) group. depict the expression of β7 high on CD4 and CD8 cells respectively. Exclusively breastfeeding infants (EX) are represented as triangles for the central and effector memory subsets whereas non-exclusively breastfeeding infants (NX) are shown in red circles (central memory) or orange circles (effector memory). Plot C also shows higher expression of β7+CCR5+ for CD4+ Central Memory cells in the NX versus the EX cohort.

Higher Activation of CD8 Memory cells in Non-Exclusive Breastfeeding Infants

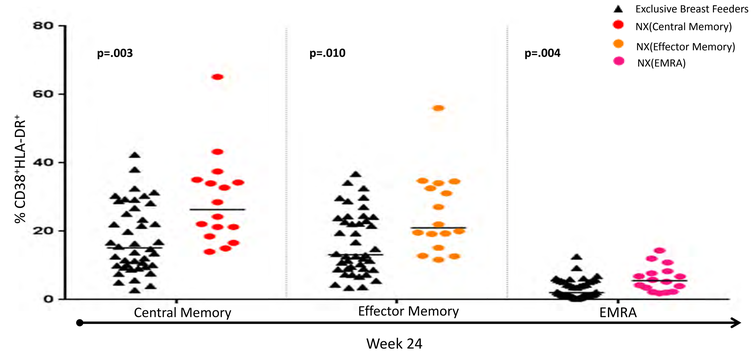


Figure 6.

At 24weeks of life, there is a significantly higher frequency of activated memory cells for the NX group compared to the EX group in all three memory subsets: Central Memory (red), p=.003; Effector Memory (orange), p=.010, and EMRA (pink), p=.004

CONCLUSIONS

- Newborns show low but detectable frequencies of CD4+ memory T cells as well as CCR5 expression, indicating the presence of circulating CD4 T lymphocytes susceptible to HIV.
- Circulating memory and activated T cells rapidly increase in frequency by age 2-6 wks and plateau after 12 weeks of life through the first year indicating an early shift from naïve to memory phenotypes in these HIV-exposed Ugandan infants.
- Gut homing (β7 high) CD4 and CD8 T lymphocytes are rare at birth but progressively increase throughout the first year of life.
- Infants receiving non-breast milk feeds (NX) had higher frequencies of β7 high, CCR5+ CD4 T lymphocytes as well as higher frequencies of activated CD8 T memory lymphocytes.
- These observations suggest that exposure to non-breast milk feeds is associated with an increase in gut homing T cells and higher activation.
- Higher frequencies of T cells that are targets for HIV infection may contribute to higher rates of MTCT among infants exposed to mixed feeding in the first six months of life.

ACKNOWLEDGEMENTS

We wish to express our gratitude to all the study participants. We also would like to acknowledge the physicians and staff at the Makerere University – Johns Hopkins University/MU-JHU for participant recruitment, data collection, and the investigators and staff at the Makerere University Walter Reed Project Laboratory for flow cytometry specimen analysis. This study was supported by National Institutes of Health Grants 1RO1 HD059527 and RO1 AI097265 and Mucosal and Vaccine Research Colorado Program (MAVRC)