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Title: Tuberculosis IFN-γ Responses in Breastmilk of HIV-infected Mothers

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Tuberculosis (TB) cellular immune responses were examined in breastmilk of HIV-infected mothers using the T-SPOT.TB interferon gamma release assay (IGRA). Positive TB IFN-γ responses were detected in 6 of 8 (75%) valid breast milk assays. Among 7 mothers with paired breastmilk and blood assays, TB IFN-γ responses were higher in breastmilk compared to blood (p=.02). The magnitude of TB IFN-γ responses in maternal breastmilk and blood were correlated. Elucidating the influence of breastmilk TB immune responses on infant TB susceptibility and immunity may inform future maternal TB vaccine strategies.

INTRODUCTION

Cell-mediated immunity in breastmilk has been associated with protection from infectious pathogens and modulation of infant immune development.[1,2] Tuberculosis (TB)-specific cellular immunity in breastmilk has not been well-characterized and its role in infant TB immunity is undefined. Small historical studies suggest that tuberculin sensitivity can be transferred by breastmilk to infants.[3,4] Breastfed infants born to tuberculin positive mothers had evidence of tuberculin sensitivity, while infants of non-breastfed tuberculin positive or tuberculin negative mothers did not.[4] We adapted a standardized TB interferon-gamma (IFN-γ) release assay to measure breastmilk IFN-γ responses in HIV-infected mothers and compared these to TB IFN-γ responses in peripheral blood of mothers and their infants.

STUDY POPULATION AND METHODS

In a historical perinatal HIV cohort, TB IFN-γ responses were measured in cryopreserved maternal breastmilk cells (BMCs) and peripheral blood mononuclear cells (PBMCs) using the T-SPOT.TB ELISPOT assay (Oxford Immunotec, Oxfordshire, UK). This study was approved by the University of Washington Institutional Review Board and the Kenyatta National Hospital Ethics and Research Committee. HIV-infected women in Nairobi, Kenya were enrolled during pregnancy in 2002 and mother-infant pairs followed monthly for one year postpartum.[5] Breastmilk and peripheral blood were collected at 1 month postpartum, centrifuged, and cryopreserved in 10% dimethyl sulfoxide-90% fetal calf serum (FCS) (Sigma, St. Louis, Missouri, USA). In 2011, cryopreserved PBMCs and BMCs were thawed and incubated for 4 hours in RPMI 1640 supplemented with 10% FCS (Sigma). Lymphocytes were counted manually in the presence of Trypan Blue and viable cells were plated at mean concentration 1.5 x 10^5 BMCs and 2.5 x 10^5 PBMCs per well to perform the T-SPOT.TB assay as previously described.[1,6] Infant T-SPOT.TB results from 6 months of age were available from a prior study.[6] Total BMC or PBMC spot-forming cells (SFCs) were normalized to the number of spots per 2.5 x 10^5 cells, and an assay was considered valid if there were $\geq$20 SFCs in the positive control well. A test was positive if $\geq$6 spots above the nil control for either antigen ESAT-6 or CFP-10 when the nil control was <10 SFCs. If the

nil control was >10 SFCs, a test was considered positive if the SFCs for either ESAT-6 or CFP-10 were at least twice the SFCs in the nil control.

Statistical analyses were performed using STATA software, version 11.2 (StataCorp, College Station, Texas, USA). After exclusion of invalid assays, TB IFN-γ responses were described as dichotomous (positive/negative) and continuous (SFCs above nil) measures. Median IFN-γ SFCs in response to ESAT-6 and CFP-10 above nil in BMCs and PBMCs were compared using Wilcoxon signed-rank tests. Data were log-transformed and Spearman's correlation of BMC and PBMC IFN-γ responses to ESAT-6 and CFP-10 were assessed.

RESULTS

T-SPOT.TB assays were conducted in paired BMC and PBMC specimens from 11 HIV-infected mothers at one month postpartum. Prior T-SPOT.TB results were available for 10 of their infants at 6 months of life.[6] Median maternal CD4 count was 676 cells/mm^3. In the first 6 months postpartum, mothers exclusively breastfed for a median of 6 months (IQR 3-6); one mother delayed breastfeeding initiation until 1 month postpartum. No mothers or infants were diagnosed with active TB disease during follow-up. All infants were HIV negative by HIV DNA PCR at 6 months of age.

Of 11 paired maternal BMC and PBMC assays, 8 BMC and 10 maternal PBMC assays were valid. Of valid assays, TB IFN-γ responses to either ESAT-6 or CFP-10 were present in 6 (75%) BMCs and 4 (40%) maternal PBMCs. For BMC assays, median response to ESAT-6 was 27 SFCs (IQR 2-221) and CFP-10 was 18 SFCs (IQR 4-240). Among maternal PBMC assays, median IFN-γ response to ESAT-6 was 3 SFCs (IQR 1-38) and CFP-10 was 3 SFCs (IQR 0-21) (Table 1). Median IFN-γ response to ESAT-6 and CFP-10 were higher in BMCs than PBMCs (p=0.02 for both antigens) (Figure 1a). Maternal BMC and PBMC TB IFN-γ SFCs were highly correlated (ESAT-6 r=.91, p=.005 and CFP-10 r =.86, p=.01) (Figure 1b).

We previously conducted T-SPOT.TB assays on the PBMCs of 10 infants of these mothers.[6] Three of 8 infants (37%) with a valid assay had a positive T-SPOT.TB result. Median infant IFN-γ response to ESAT-6 was 0 SFCs (IQR 0-4) and CFP-10 was 0 SFCs (IQR 0-2). Magnitude of infant PBMC TB IFN-γ response to ESAT-6 and CFP-10 did not correlate with maternal BMCs (r=.53, p=.36 and r=0, p>.9, respectively). Of 5 mother-infant pairs with valid assays, 3 were positive in both maternal BMC and infant PBMC, 1 was negative in both maternal BMC and infant PBMC, and 1 pair was discordant (maternal BMC positive, infant negative in pair with delayed breastfeeding).

DISCUSSION

In this proof of concept study, we measured TB-specific cellular immunity in breastmilk using the T-SPOT.TB assay. We found TB IFN-γ responses in breastmilk cells of most HIV-infected mothers examined. Breastmilk IFN-γ responses to ESAT-6 and CFP-10 were of higher magnitude and strongly correlated with maternal PBMC IFN-γ responses.

Measurement of TB IFN-γ in breastmilk may be useful in future prospective studies to determine if breastmilk cell-mediated immunity may alter infant TB immunity or TB susceptibility. TB-specific immune cells in breastmilk could provide passive infant TB protection in the oral or respiratory mucosa or enhance development of infant TB immune responses. Maternal immunity may shape antigen sensitization, as observed in children aged 12 to 36 months who were tuberculin positive after breastfeeding from tuberculin positive mothers.[3] We did not find a significant association between maternal breastmilk and longitudinal infant TB IFN-γ production. However our finding that 4 of 5 mother-infant pairs had concordant TB IFN-γ responses in maternal breastmilk and infant blood at 6 months of age is intriguing.

Use of cryopreserved samples may have decreased T-SPOT.TB assay sensitivity[7], and small sample size limits the generalizability of our findings. Future studies to determine whether and how breastmilk TB cellular immunity influences infant immunity and susceptibility to TB will be important, and may inform complementary maternal vaccine strategies that potentially enhance protection of infants via breastmilk.

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Tables

Table 1: IFN-γ SFCs per 2.5 x 10^5 cells in response to *M. tuberculosis* in BMCs and maternal and infant PBMCs

ID	Cells	PHA	ESAT-6	CFP-10	Nil	Result	Breastfed* (months)
189	Maternal BMC	2432	7.5	15	2.5	Positive	
	Maternal PBMC	888	0	0	0	Negative	
	Infant PBMC	211	169	2	0	Positive	6
181	Maternal BMC	48	5	9	0	Positive	
	Maternal PBMC	665	3	3	0	Negative	
	Infant PBMC	200	20	44	16	Positive	6
143	Maternal BMC	1661	0	0	0	Negative	
	Maternal PBMC	348	0	0	0	Negative	
	Infant PBMC	0	4	4	4	Invalid	6
134	Maternal BMC	1207	373	297	17	Positive	
	Maternal PBMC	474	83	204	0	Positive	
	Infant PBMC	nd	nd	nd	nd	nd	5
194	Maternal BMC	168	0	0	0	Negative	
	Maternal PBMC	1	0	0	0	Invalid	
	Infant PBMC	288	0	1	0	Negative	2
173	Maternal BMC	0	0	0	0	Invalid	
	Maternal PBMC	921	1	1	0	Negative	
	Infant PBMC	708	24	52	77	Negative	6
141	Maternal BMC	1	0	0	0	Invalid	
	Maternal PBMC	961	59	31	0	Positive	
	Infant PBMC	0	0	0	0	Invalid	6
118	Maternal BMC	1087	744	488	115	Positive	
	Maternal PBMC	625	38	21	0	Positive	
	Infant PBMC	93	235	28	4	Positive	1
113	Maternal BMC	458	86	24	0	Positive	
	Maternal PBMC	576	39	6	1	Positive	
	Infant PBMC	69	0	0	0	Negative	3 ¶
140	Maternal BMC	800	50	200	0	Positive	
	Maternal PBMC	973	4	4	0	Negative	
	Infant PBMC	6	0	0	0	Invalid	3
107	Maternal BMC	0	0	0	0	Invalid	
	Maternal PBMC	861	1	0	0	Negative	
	Infant PBMC	82	0	0	0	Negative	6

*Months mother reported exclusive breastfeeding during the first 6 months postpartum;
¶ Mother delayed initiation of breastfeeding until 1 month postpartum; nd = not done

R1 Figure

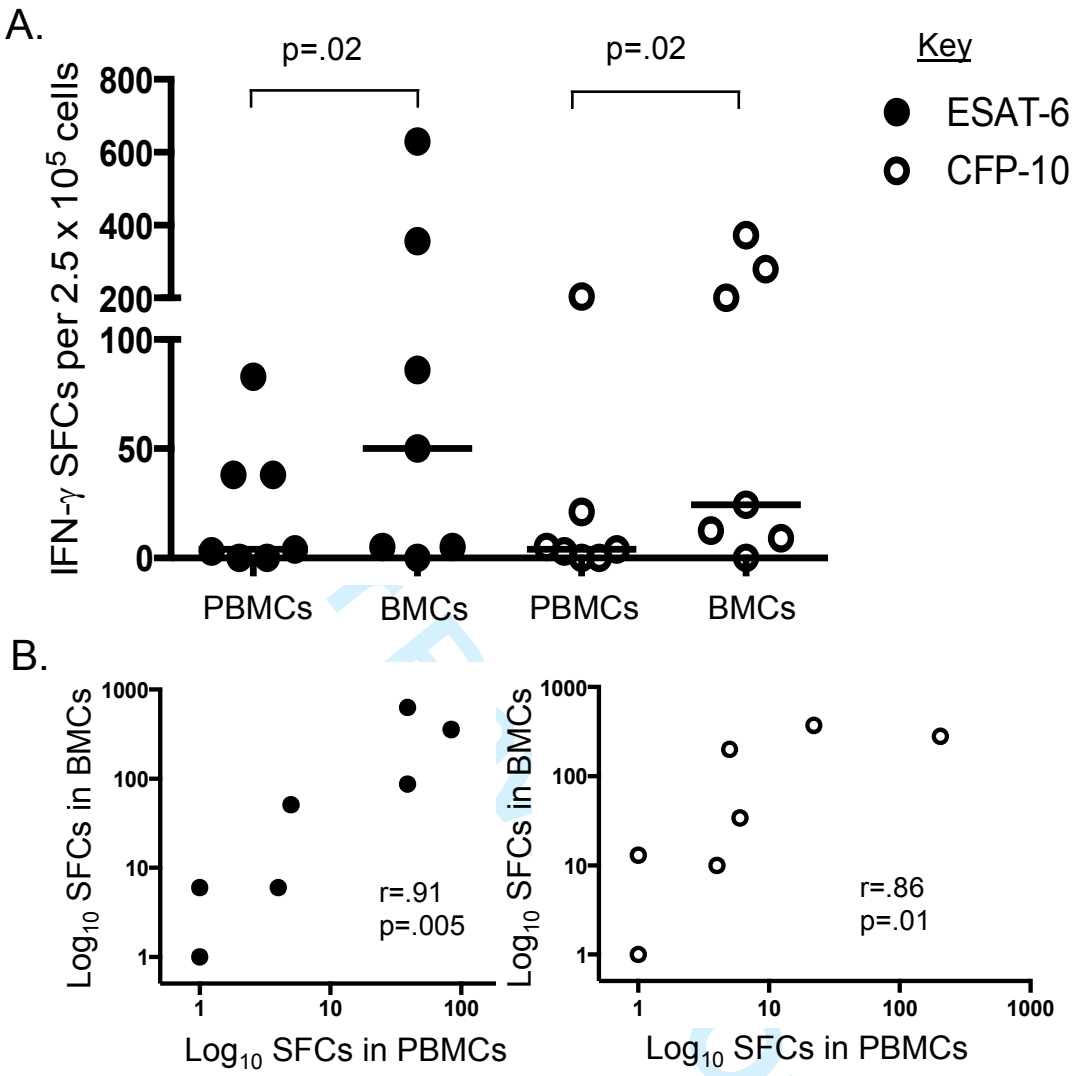

Figure 1: Magnitude and correlation of IFN-γ response to ESAT-6 and CFP-10 in Maternal BMCs and PBMCs. A. The T-SPOT.TB assay was performed on maternal PBMCs and BMCs. SFCs per 2.5 x 10^5 cells in response to antigens ESAT-6 (closed circles) and CFP-10 (open circles) are shown after subtraction of background in the nil control. Horizontal lines represent the median. Differences were determined using the Wilcoxon signed-rank test. B. Data were log-transformed and Spearman's correlation of BMC and PBMC IFN-γ responses to ESAT-6 (closed circles) and CFP-10 (open circles) were assessed.