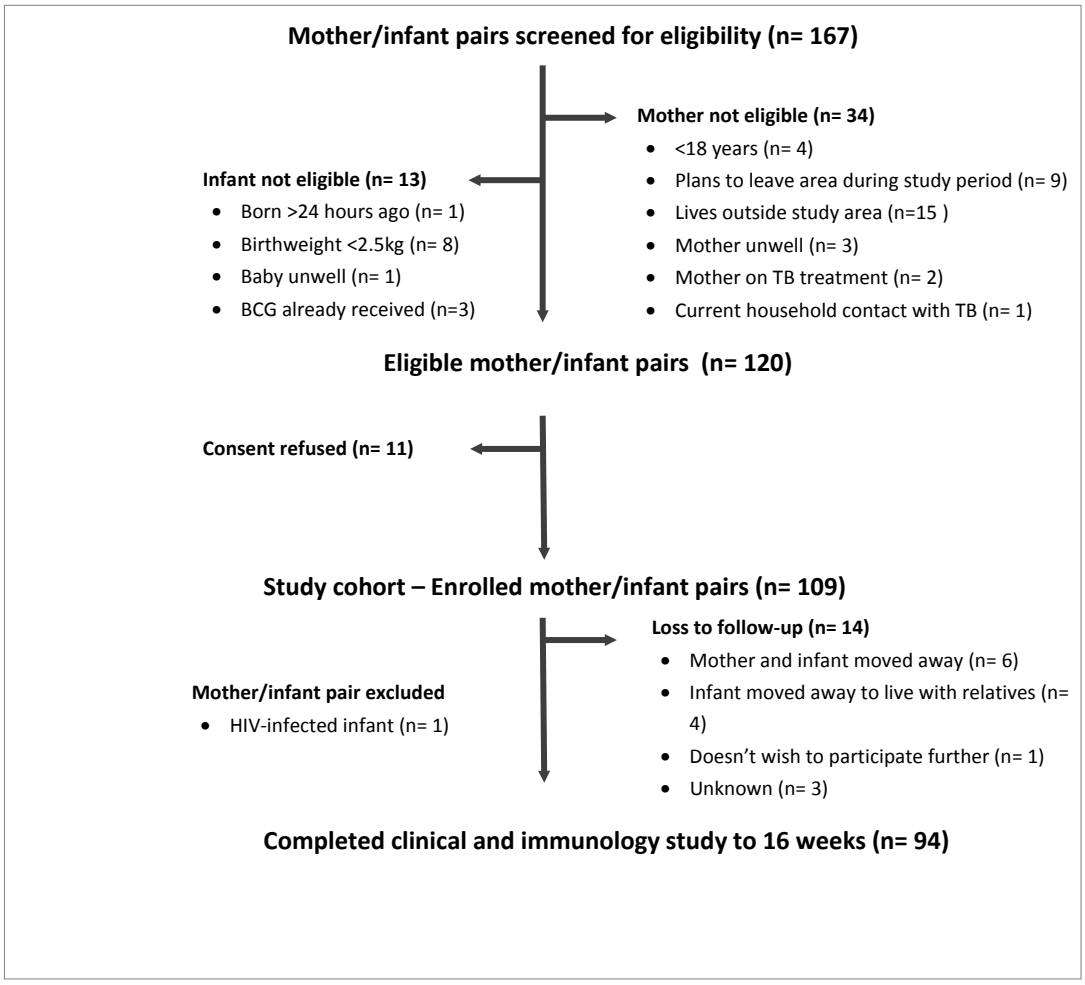
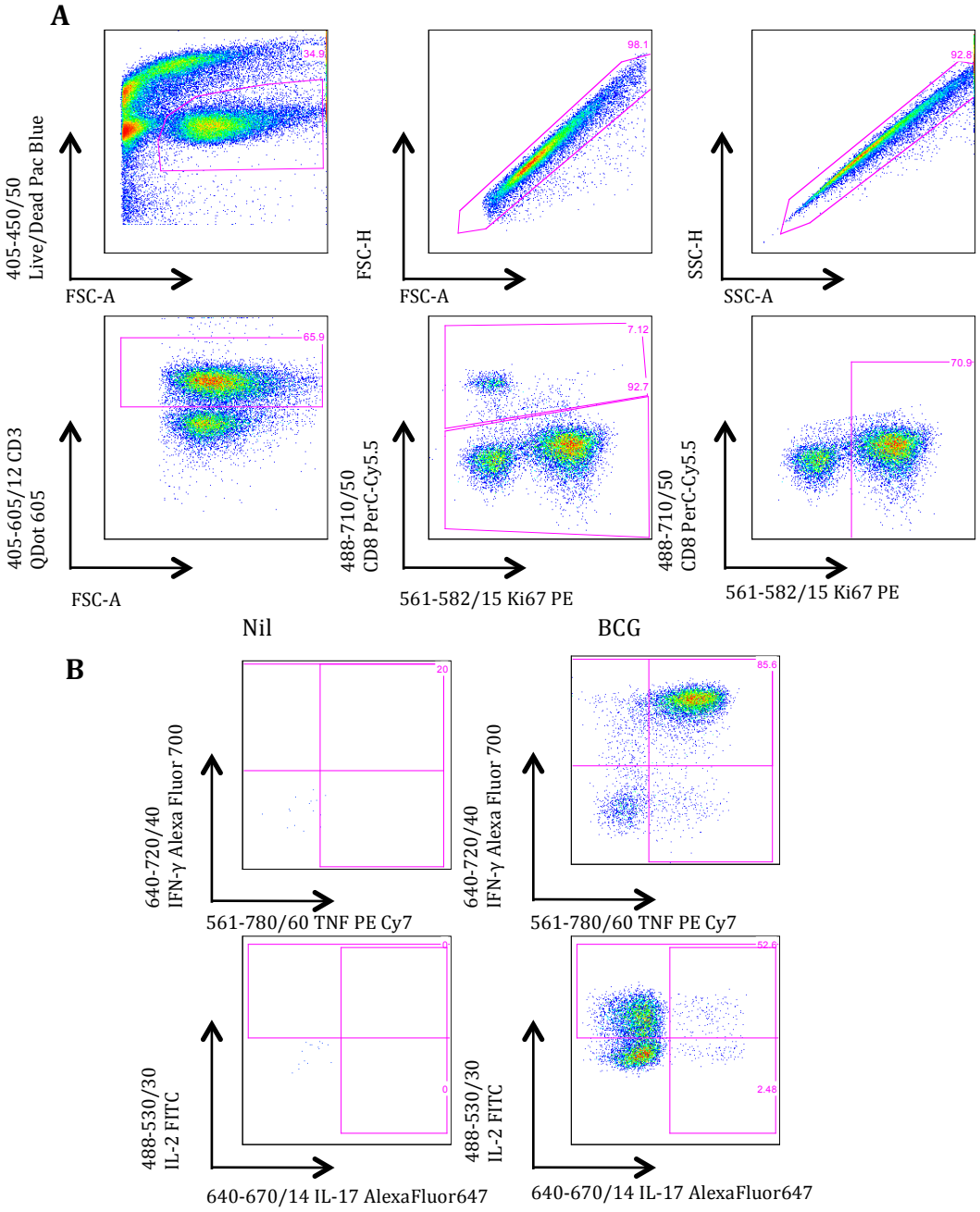
Supplemental Digital Content

Supplemental Digital Content 1



Study cohort flow chart

Supplementary Digital Content 2



Hierarchical gating strategy for selection of CD4$^+$ proliferating T ells expressing intracellular cytokines in response to BCG antigens.

Representative dot plots from a single 16 week old infant are shown. (A) Gating strategy used to identify CD4$^+$ proliferating cells T cells. From left to right, live cells were identified from dead cells using a viability dye and cell doublets excluded with forward scatter area

versus forward scatter height parameters, followed by side scatter area versus side scatter height. T cells were identified by positive staining for the CD3 cell surface marker and subsequently differentiated into $CD4^+$ and $CD8^+$ T cells. Proliferating $CD4^+$ T cells were then identified by selecting $CD4^+Ki67^+$ T cells. (B) Representative dot plots of cytokine expression in $CD4^+Ki67^+$ T cells from unstimulated and BCG stimulated conditions in the same 16 week old infant. Gates for proliferating $CD4^+$ T cells expressing cytokines were set using the unstimulated control. The filters used are specified in association with the laser, eg 488-530/30 indicates the blue laser and the 530/30 filter.

Supplementary Digital Content 3

Customised plates (MILLIPLEX MAP) were used to quantify 20 cytokines and chemokines in supernatants collected after 24 hours of culture: Epidermal growth factor (EGF), fractalkine, granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin(IL)-1 receptor antagonist (IL-1RA), IL-12p40, IL-17, interferon-γ (IFN-γ), inducible protein 10 (IP-10 or CXCL10), monocyte chemoattractant protein-1 (MCP-1 or CCL2), monocyte derived chemokine (MDC), macrophage inhibitory protein-1 β (MIP-1β, CCL4), soluble CD40 ligand (sCD40L), tumournecrosis factor-α (TNF-α), IL-1β, IL-2, IL-6, IL-7, IL-8 (CXCL8), IL-10 and IL-13.

Supplemental Digital Content 4

	HIV-infected mothers and their uninfected infants (n=46)	HIV-uninfected mothers and their infants (n=62)	p value
Median age in years (IQR)	27.5 (24 – 31.25)	24 (20 - 27.25)	.001
Normal vaginal delivery	46 (100%)	62 (100%)	1.00
Primigravidity	12 (26%)	30 (48%)	.02
Informal housing structure	36 (79%)	34 (55%)	.01
Infant gestation, weeks (IQR)	38^{+3} (37^{+1} - 39^{+6})	38^{+4} (35^{+1} - 40^{+1})	.36
Female infant	26 (57%)	34 (55%)	1.00
Infant birth weight, kg (IQR)	3.13 (2.90 - 3.45)	3.12 (2.98 - 3.47)	.59
Infant breast fed at birth	0 (0%)*	62 (100%)	<. 001

Characteristics of study cohort.

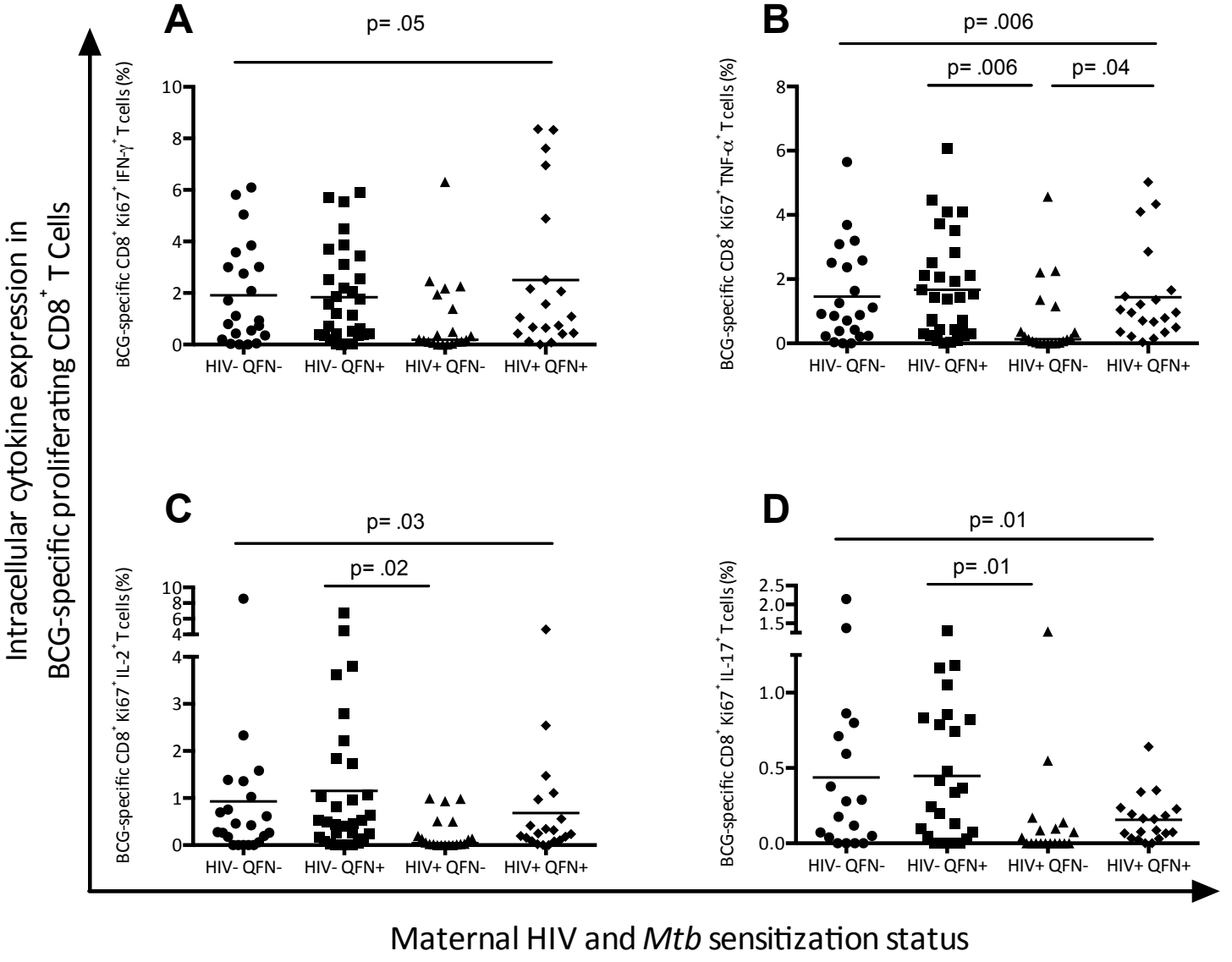
* All HIV-infected mothers chose exclusive replacement feeding and continued this practice through the study period; this was routine practice at this time in this setting

Supplemental Digital Content 5

Group (maternal infection status)	n	$CD8^-Ki67^+$ $IFN\text{-}\gamma^+$	$CD8^-Ki67^+$ $TNF\text{-}\alpha^+$	$CD8^-Ki67^+$ $IL\text{-}2^+$	$CD8^-Ki67^+$ $IL\text{-}17^+$
HIV- QFN -	15	0.07% (0.00 – 0.37)	0.23% (0.09 – 0.87)	0.22% (0.08 – 1.08)	0.07% (0.00 – 0.22)
HIV- QFN $^+$	20	0.11% (0.00 – 0.97)	0.30% (0.02 – 1.31)	0.18% (0.00 – 1.15)	0.00% (0.00 – 0.08)
HIV^+ QFN -	9	0.12% (0.00 – 0.65)	0.38% (0.01 – 1.59)	0.14% (0.02 – 1.00)	0.00% (0.00 – 0.21)
HIV^+ QFN $^+$	8	0.44% (0.13 – 1.84)	1.96% (0.30 – 5.29)	0.50% (0.21 – 1.49)	0.07% (0.00 – 0.36)
Total	52	0.14% (0.00 – 0.56)	0.39% (0.05 – 1.62)	0.21% (0.08 - 1.06)	0.02% (0.00-0.20%)
Group (maternal infection status)	**n**	$CD8^+Ki67^+$ $IFN\text{-}\gamma^+$	$CD8^+Ki67^+$ $TNF\text{-}\alpha^+$	$CD8^+Ki67^+$ $IL\text{-}2^+$	$CD8^+Ki67^+$ $IL\text{-}17^+$
HIV- QFN -	15	0.11% (0.00 – 0.44)	0.16% (0.00 – 1.09)	0.13% (0.00 – 0.80)	0.06% (0.00 – 0.58)
HIV- QFN +	20	0.13% (0.00 – 0.81)	0.24% (0.00 – 0.62)	0.24% (0.03 – 1.41)	0.00% (0.00 – 0.10)
HIV+ QFN -	9	0.15% (0.06 – 0.69)	0.34% (0.00 – 0.53)	0.11% (0.00 – 0.34)	0.07% (0.00 – 0.29)
HIV+ QFN +	8	0.01% (0.00 – 3.07)	0.49% (0.05 - 4.01)	0.02% (0.00 - 0.20)	0.37% (0.00 – 0.48)
Total	52	0.14% (0.00 – 0.66)	0.24% (0.00 – 0.65)	0.10% (0.00 – 0.79)	0.06% (0.00 – 0.37)

Median (IQR) frequencies of BCG-specific $CD4^+$ and $CD8^+$ $Ki67^+$ T cells expressing intracellular cytokines in infants at birth.

Groups of infants based on maternal HIV infection and *Mtb* sensitization, defined by the QFN test. There were a reduced number of samples analysed due to the reduced viability of T cells in the infant birth samples after 6 days of culture. Number of samples analysed are given in table, except for $IL\text{-}17^+$ $CD4^+$T cells were the number of samples analysed were: HIV- QFN - 11; HIV- QFN+ 17; HIV+ QFN – 7; HIV+ QFN + 7. Differences between groups were not statistically significant as calculated by the Kruskal-Wallis test.


A
p= .05
BCG-specific CD8+ Ki67+ IFN-γ+ T cells (%)
HIV- QFN-
HIV- QFN+
HIV+ QFN-
HIV+ QFN+
B
p= .006
p= .006
p= .04
BCG-specific CD8+ Ki67+ TNF-α+ T cells (%)
C
p= .03
p= .02
BCG-specific CD8+ Ki67+ IL-2+ T cells (%)
D
p= .01
p= .01
BCG-specific CD8+ Ki67+ IL-17+ T cells (%)
Intracellular cytokine expression in BCG-specific proliferating CD8+ T Cells
Maternal HIV and Mtb sensitization status

Supplemental Digital Content 7

Cytokine / chemokine		Mother and newborn infant correlations				Mother and 16 week old infant correlations			
		HIV- QFN- (n=27)	**HIV- QFN+ (n=34)**	**HIV+ QFN- (n=24)**	**HIV+ QFN+ (n-21)**	**HIV- QFN- (n=22)**	**HIV- QFN+ (n=32)**	**HIV+ QFN- (n=17)**	**HIV+ QFN+ (n=17)**
IL-1β	r_s	**0.53**	**0.59**	**0.63**	**0.57**	0.22	-0.08	-0.13	-0.13
	p	**<0.01**	**<0.01**	**<0.01**	**<0.01**	0.32	0.66	0.61	0.62
IL-6	r_s	**0.46**	**0.51**	0.3	**0.61**	0.10	0.24	-0.13	-0.13
	p	**0.02**	**<0.01**	0.15	**<0.01**	0.65	0.18	0.61	0.62
IL-10	r_s	**0.47**	**0.52**	0.32	0.11	0.22	-0.01	-0.15	0.37
	p	**0.01**	**<0.01**	0.12	0.64	0.33	0.96	0.56	0.14
IL-12p40	r_s	0.32	0.24	0.44	0.36	0.14	0.49	0.25	0.48
	p	0.11	0.18	0.03	0.11	0.55	<0.01	0.32	0.05
IL-1ra	r_s	**0.51**	0.31	0.35	0.21	0.30	0.14	0.07	0.16
	p	**<0.01**	0.07	0.09	0.36	0.17	0.43	0.79	0.54
GM-CSF	r_s	**0.5**	0.33	0.06	0.4	0.31	-0.02	-0.04	0.2
	p	**<0.01**	0.06	0.79	0.07	0.17	0.23	0.87	0.44
IFN-γ	r_s	0.13	0.35	-0.06	0.07	0.43	-0.1	0.0	0.40
	p	0.50	0.04	0.77	0.78	0.05	0.58	1.0	0.11
MCP-1	r_s	-0.04	**0.37**	0.34	0.08	**0.51**	0.25	-0.20	-0.04
	p	0.86	**0.03**	0.10	0.72	**0.01**	0.17	0.44	0.89
TNF-α	r_s	0.37	**0.59**	**0.46**	**0.45**	0.06	**0.43**	**0.01**	0.21
	p	0.06	**<0.01**	**0.02**	**0.04**	0.79	**0.01**	**0.97**	0.42

Associations between concentrations of cytokines and chemokines in mothers and infants at infants at birth and at 16 weeks of post-natal age.

Only cytokines or chemokines will any significant association found in any group or time points are shown.

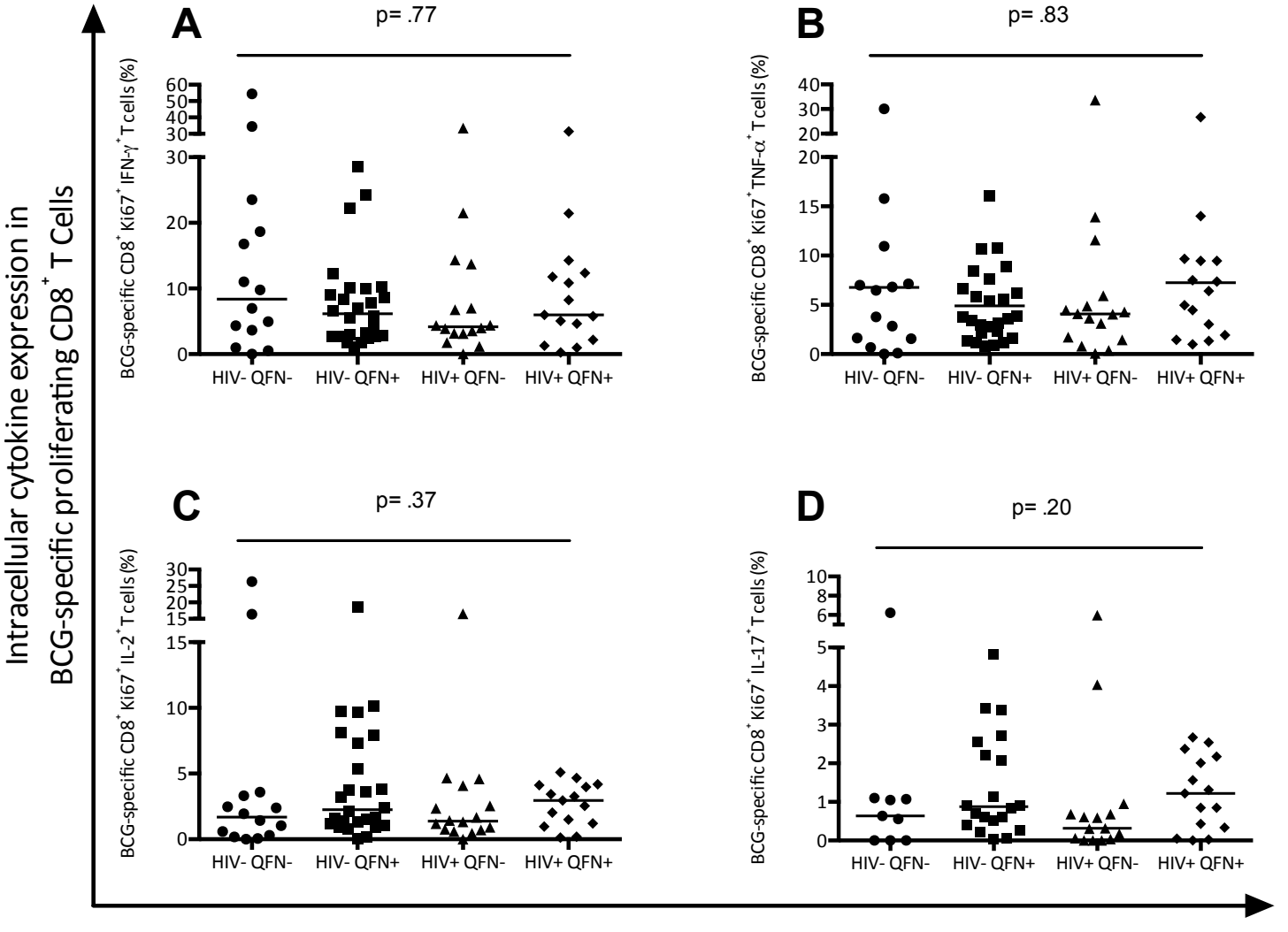
Supplemental Digital Content 8

Group	Time point	n	CD8⁻Ki67⁺ IFN-γ⁺	CD8⁻Ki67⁺ TNF-α⁺	CD8⁻Ki67⁺ IL-2⁺	CD8⁻Ki67⁺ IL-17⁺
HIV- QFN -	Birth	7 (4)	0.08 (0.06)	0.18 (0.21)	0.49 (0.86)	0.82 (2.68)
HIV- QFN -	16 weeks		24.1 (3.93)	22.75 (2.94)	14.78 (1.65)	8.02 (6.67)
HIV- QFN +	Birth	16(13)	0.29 (0.27)	0.66 (0.57)	0.48 (0.48)	0.09 (0.84)
HIV- QFN +	16 weeks		21.12 (4.76)	20.93 (4.75)	13.27 (2.56)	3.47 (1.31)
HIV+ QFN -	Birth	4 (3)	0.16 (0.16)	0.50 (0.36)	0.39 (0.5)	0.05 (0.15)
HIV+ QFN -	16 weeks		30.54 (0.24)	33.71 (0.35)	13.7 (1.1)	8.46 (4.03)
HIV+ QFN +	Birth	5 (5)	1.36 (0.48)	3.03 (0.88)	1.24 (0.88)	0.03 (0.05)
HIV+ QFN +	16 weeks		22.88 (9.72)	22.75 (7.5)	14.69 (6.59)	2.73 (2.59)
Cohort (Birth)		32(25)	0.32 (0.3)	0.74 (0.64)	0.57 (0.58)	0.13 (0.84)
Cohort (16 weeks)			23.14 (4.43)	23.06 (4.03)	13.87 (2.52)	4.42 (2.5)
Effect of time		**p**	**< .0005**	**< .0005**	**< .0005**	**< .0005**
Effect of time and maternal infection status		**p**	**0.76**	**0.55**	**0.98**	**0.76**

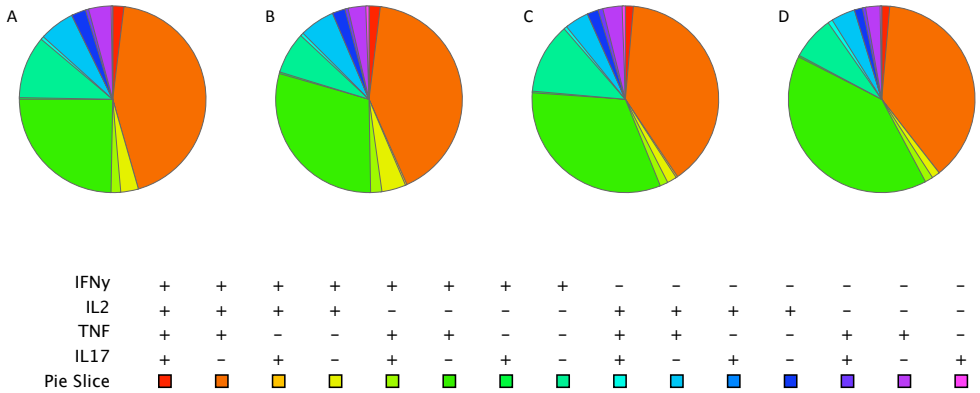
Group	Time point	n	CD8⁺Ki67⁺ IFN-γ⁺	CD8⁺Ki67⁺ TNF-α⁺	CD8⁺Ki67⁺ IL-2⁺	CD8⁺Ki67⁺ IL-17⁺
HIV- QFN -	Birth	7 (4)	0.09 (0.06)	0.1 (0.17)	0.22 (0.11)	0.1 (0.28)
HIV- QFN -	16 weeks		5.24 (3.32)	2.42 (1.62)	0.79 (0.57)	0.32 (0.19)
HIV- QFN +	Birth	16(13)	0.32 (0.47)	0.39 (0.45)	0.43 (0.4)	0.12 (0.81)
HIV- QFN +	16 weeks		4.66 (0.86)	3.74 (0.82)	2.34 (0.67)	2.00 (2.28)
HIV+ QFN -	Birth	4 (3)	0.29 (0.04)	0.22 (0.1)	0.14 (0.08)	0.02 (0.02)
HIV+ QFN -	16 weeks		10.17 (1.56)	8.14 (0.7)	2.69 (0.37)	4.94 (10.71)
HIV+ QFN +	Birth	5 (5)	1.31 (1.33)	1.72 (1.01)	0.17 (0.41)	0.10 (0.07)
HIV+ QFN +	16 weeks		5.30 (1.14)	4.88 (0.93)	2.17 (0.64)	2.09 (1.44)
Cohort (birth)		32(25)	0.35 (0.49)	0.41 (0.49)	0.29 (0.29)	0.10 (0.46)
Cohort (16 weeks)			5.47 (1.46)	4.04 (1.05)	1.94 (0.63)	1.91 (2.5)
Effect of time		**p**	**< .0005**	**< .0005**	**< .0005**	**0.003**
Effect of time and maternal infection status		**p**	**0.45**	**0.33**	**0.29**	**0.47**

The frequency of BCG-specific CD4⁺ and CD8⁺ T cells expressing IFN-γ, TNF-γ, IL-2 or IL-17 in paired infants at birth and 16 weeks of age.

A reduced number of paired samples were available for analysis of IL-17 CD4⁺ T cells and are indicated in parentheses. A mixed ANOVA model was used to assess differences between groups.


Intracellular cytokine expression in BCG-specific proliferating CD8+ T Cells
A
p= .77
BCG-specific CD8+ Ki67+ IFN-γ+ T cells (%)
HIV- QFN-
HIV- QFN+
HIV+ QFN-
HIV+ QFN+
B
p= .83
BCG-specific CD8+ Ki67+ TNF-α+ T cells (%)
C
p= .37
BCG-specific CD8+ Ki67+ IL-2+ T cells (%)
D
p= .20
BCG-specific CD8+ Ki67+ IL-17+ T cells (%)
Infant group determined by maternal HIV and Mtb sensitization status

Supplemental Digital Content 10



Relative frequencies of BCG-specific $CD4^+$ T cell subsets in 16 week old infants.

Groups of infants defined by maternal infection status: (A) HIV- QFN - (B) HIV- QFN + (C) HIV+ QFN- and (D) HIV+ QFN+