

Co-ordinate expression of Th1/Th2 phenotypes in maternal and fetal blood: evidence for a transplacental nexus^{*,**}

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Abstract

If maternal atopy and environmental exposure affect prenatal Th cell development, the maternal and fetal immune systems should display common Th1/Th2 phenotypes. To test this hypothesis, we studied maternal and neonatal blood samples from mothers with total serum IgE <300 IU/mL. Basal levels of IFN- γ , IL-4, and eotaxin in paired maternal and fetal sera were tightly correlated. Polyclonal T cell activation *in vitro* by Staphylococcal exotoxin B induced co-ordinate IFN- γ production from paired maternal and fetal mononuclear cells, accompanied by co-ordinate increases in activated CD4+CD69+ cells that display the CCR4+Th2 and CXCR3+Th1 phenotypes. Maternal and fetal CD4+CXCR3+ T cells were subsequently identified as the major producers of IFN- γ . The data established that a transplacental nexus exists during normal pregnancy and that fetal Th cell responses may be biased by the maternal immune system.

Keywords: Chorioamnionitis; perinatal immunity; preterm labor; transplacental nexus.

Introduction

It is known that CD4+ T lymphocytes can be subdivided into Th1 or Th2 cells by their secretory products, with IFN- γ and IL-4 as their signature cytokines [10]. It also has been

determined that immune responses are generally polarized towards either a Th1 or Th2 phenotype. Dysregulation of either subset may cause autoimmune disease, e.g., the Th2 hyperactivity in allergic disorders. Atopy is believed to develop as the result of complex gene-environment interactions. The “Hygiene Hypothesis” claims that reduced exposure to microbial products is related to this process, and predisposes individuals in developed countries to allergic disorders [3, 6, 27, 28]. By the same mechanism, prenatal exposure to a farming environment was significantly associated with a lower frequency of atopic sensitization in early life [26]. Higher levels of TLR2, TLR4 and CD14 gene expression were seen in children whose mothers worked regularly in stables during pregnancy [5], and IFN- γ production was found to be higher in newborns from farm compared to non-farm families [14].

We hypothesize that in order for maternal atopy and/or environmental exposure to impact prenatal development, the maternal immune system must be presenting innate or adaptive signals to the fetal immune system that it receives during gestation. We have previously shown that fetal T cells are capable of responding to activation through syngeneic as well as allogeneic HLA-antigens by the second trimester [25]. If there is maternal programming of the fetus, maternal and fetal lymphocytes should express common Th1/Th2 phenotypes indicative of the maternal influence on fetal Th cell polarization *in utero*. To assess the presence of a transplacental nexus, we collected paired maternal and neonatal peripheral blood specimens and analyzed the basal levels of circulating Th1 vs. Th2 cytokines *in vivo*. We also studied paired samples for the expression of Th1 vs. Th2 cytokines associated with the expression of Th1 vs. Th2 chemokine receptors on CD4+ lymphocytes following polyclonal T cell activation *in vitro*.

Materials and methods

Specimens

Informed consent was obtained from all study subjects recruited at the New York University Langone Medical Center under an Institutional Review Board approved protocol. The ethnic background of our subject population (n=38) was 3% African, 6% Asian, 54% Caucasian, 30% Hispanic, and 7% other, included mixed and unknown ethnicities. The median maternal age was 34 (23–43 years). Three deliveries were preterm (25–31 weeks), one produced twins at full term. All the gravidas had uncomplicated scheduled term cesarean births except for the three preterm patients. Following delivery, 10 mL of fetal blood (FB) was withdrawn from the umbilical cord. At the same time, 10 mL of maternal blood (MB) was obtained by venipuncture. An aliquot of blood was transferred to a vacutainer containing heparin, another aliquot was transferred to an SST vacutainer. Specimens were processed

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and analyzed side-by-side within 12 h. SST vacutainers were centrifuged according to supplier instructions (Becton Dickinson, Franklin Lakes, NJ, USA) to isolate serum. An aliquot of unfractionated whole blood was removed for determination of lymphocyte subsets. The remainder was fractionated over Ficoll-Hypaque (Amersham, Princeton, NJ, USA), and peripheral blood mononuclear cells (PBMC) above the Ficoll interphase were harvested, washed with Dulbecco's PBS (DPBS), and resuspended to the same density ($1\text{--}2 \times 10^6$ PBMC/mL) in culture medium consisting of RPMI 1640 supplemented with antibiotic/antimycotic plus glutamine (Invitrogen, Carlsbad, CA, USA) and 10% autologous serum. Unused serum was stored at -70°C .

Cytokine and serum IgE measurements

Serum IFN- γ , IL-4 and eotaxin were assayed using multiplexed Bio-Plex kits (BioRad Laboratories, Hercules, CA, USA) and analyzed on a Luminex 200. Culture supernatants were assayed for secreted IFN- γ , TNF- α , IL-10, IL-5, IL-4 and IL-2 using a Th2 cytometric bead array kit (BD Biosciences, San Jose, CA, USA) and analyzed on a FACSCalibur. Supernatants from flow-sorted PBMC cultures were assayed using a single IFN- γ Bio-Plex kit and analyzed on a Luminex 200 (Luminex Corporation, Austin, TX, USA). Total IgE in maternal sera was quantitated using an ELISA kit (ALerCHECK, Luminex Corporation, Austin, TX, USA), with a detection range of 0–400 IU/mL.

Leukocyte and lymphocyte subsets

Lymphocyte subset distribution was determined on a FACSCalibur using 50 μL whole blood with MultiTest CD3/CD56/CD16/CD45/CD19 and CD3/CD8/CD45/CD4, according to supplier instructions (BD Biosciences, San Jose, CA, USA). Data acquisition and analysis was performed using MultiSet software (BD Biosciences, San Jose, CA, USA).

Polyclonal T cell activation

Fetal and maternal PBMC were cultured at 37°C with 5% CO_2 in the absence or presence of 10 $\mu\text{g/mL}$ Staphylococcal enterotoxin B (SEB), purchased from Sigma-Aldrich (St. Louis, MO, USA). After 20–24 h, cells were pelleted by centrifugation. Culture supernatants were removed and stored at -70°C .

Th1 vs. Th2 surface phenotype

Pelleted PBMC were reacted with a premixed cocktail containing FITC-anti-CD69, PE-Cy7-anti-CD4, PE-anti-CXCR3 (clone 1C6/CXCR3) and Alexa Fluor 647-anti-CCR4 (clone 1G1) for 30 min

at 4°C , washed with DPBS, then fixed with 1% formaldehyde. Fluorophore-conjugated mAbs were obtained from BD Biosciences. To identify Th1 and Th2 cells. Data were acquired on a FACSCalibur and processed using CellQuest software (BD Biosciences, San Jose, CA, USA). PBL were discriminated by forward (FS) vs. 90° angle (SS) scattered laser light intensities. Fluorescence spillover detected by inappropriate channels was corrected by electronic compensation determined using PBL labeled in a single fluorophore.

Fluorescence-activated cell sorting

To identify IFN- γ producing cells, paired FBMC and MBMC were reacted with premixed FITC-anti-CD3, PE-anti-CXCR3, PerCP-Cy5.5-anti-CD45, and allophycocyanin-anti-CD4 for 30 min at 4°C , washed and resuspended in RPMI supplemented with 10% FCS and sorted on a FACS Vantage (BD Biosciences, San Jose, CA, USA). Nucleated erythroblasts were excluded by triggering on PerCP-Cy5.5, and FS vs. SS were used to discriminate PBMC from cellular debris. Fluorescence spillover was corrected as described above. PBMC were selectively depleted of either CD3+, CD3+CD4+, or CD3+CD4+CXCR3+ PBL, washed, resuspended in culture medium and activated with 10 $\mu\text{g/mL}$ SEB. After 20–24 h, cells were pelleted by centrifugation. Culture supernatants were removed and stored at -70°C .

Statistical analysis

GraphPad Prism software (LaJolla, CA, USA) was used for all statistical analyses. Paired *t*-tests, Spearman's correlations, and linear regressions between quantitative variables were performed using two-tail *P* values at 95% confidence interval. Significance was determined by $P < 0.05$.

Results

IFN- γ and atopic markers

To assess systemic production, we measured IFN- γ , IL-4, and eotaxin in paired fetal and maternal sera ($n=38$) by multiplex analyte detection. Fetal IFN- γ levels (2.5 ± 1.8 pg/mL) were not significantly different from maternal IFN- γ levels (2.9 ± 2.1 pg/mL), but co-ordinate expression ($r=0.61$, $P < 0.001$) between fetal and maternal pairs was found (Figure 1A). While maternal IL-4 levels (2.9 ± 5.9 pg/mL) were 2.4-fold higher than fetal IL-4 levels (1.2 ± 1.2 pg/mL), this difference was not significant ($P=0.07$). However, similar to IFN- γ , co-ordinate expression ($r=0.97$, $P < 0.0001$)

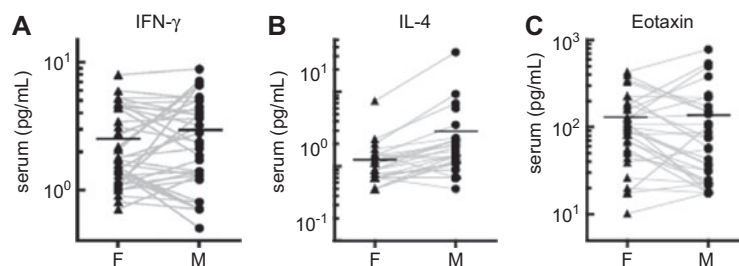


Figure 1 IFN- γ , IL-4 and eotaxin in fetal and maternal blood. Serum levels ($n=38$) were determined using Bio-Plex kits, with minor modifications. (A) IFN- γ ; (B) IL-4; (C) eotaxin. Sensitivity of detection was >0.8 pg/mL for IFN- γ , >0.7 pg/mL for IL-4 and >29 pg/mL for eotaxin. F=fetal, M=maternal.

between fetal and maternal pairs was found (Figure 1B). There was also no significant difference in eotaxin levels between fetal (128 ± 109 pg/mL) and maternal (136 ± 175 pg/mL) sera, but similar to IFN- γ and IL-4, co-ordinate expression ($r=0.59$, $P<0.001$) between fetal and maternal pairs was found (Figure 1C). IgE was detected by ELISA in only 3 (3, 3, 19 IU/mL) of 38 fetal sera. All maternal serum IgE levels were <300 IU/mL.

Fetal eotaxin levels were inversely correlated with IFN- γ levels ($r=-0.57$, $P<0.001$) but no correlation with IL-4 levels was found. Maternal eotaxin levels were inversely correlated with IFN- γ levels ($r=-0.45$, $P=0.01$) and directly correlated with IL-4 levels ($r=0.40$, $P=0.02$). No correlation was seen between IFN- γ and IL-4 levels in either the fetal or maternal cohort, and no correlation was found between maternal IgE levels and any of the circulating cytokines analyzed.

Cytokine production and co-ordinate IFN- γ expression following polyclonal activation *in vitro*

The capacity of FBMC and MBMC to produce Th2/Th1 polarizing and effector cytokines is shown in Figure 2A. With the exception of IL-2, SEB-induced cytokine production was significantly higher (3.7 $\pm$ 1.0-fold, $P<0.05$) for the maternal compared to fetal cohort. IFN- γ was the predominant cytokine. Upregulation ($P<0.05$) of IFN- γ , IL-10, IL-5 and IL-2 was seen with SEB incubation in the fetal as well as maternal cohort.

Spearman's correlation coefficients (Table 1) showed significant covariation in SEB-induced IFN- γ production between paired fetal and maternal specimens. The relationship between fetal and maternal IFN- γ expression was determined by regression analysis of the log-transformed maternal and fetal IFN- γ levels, and shown in Figure 2B. Correlation between other cytokines was similar for maternal and fetal samples as well (Table 2).

Table 1 Covariation in fetal and maternal cytokine expression.

IFN- γ TNF- α	IL-10		IL-5	IL-4		IL-2
r	0.59	-0.08	0.41	0.38	0.19	0.38
P	<0.01	0.75	0.08	0.11	0.43	0.10

Cytokines were produced by paired FBMC and MBMC following SEB activation. r =Spearman's correlation coefficient.

Th2 vs. Th1 surface phenotype following polyclonal activation

Fetal PBL ($n=21$) contained the same %CD3+, %CD3+CD4+ and %NK (CD3-D56+CD16+) cells as their maternal counterparts ($n=20$), but 1.3-fold more B cells (CD19+), and slightly fewer cytotoxic/suppressor T cells (CD3+CD8+). There was no significant difference in PBL subsets when preterm and full-term PBL were compared.

Th cells responding to SEB stimulation *in vitro* were detected as CD4+ PBL expressing the early activation marker CD69 by FACS. Th2 cells were identified as CCR4+CXCR3- and Th1 cells were identified as CXCR3+CCR4-. CD69 expression was significantly higher for the maternal compared to fetal cohort ($28 \pm 12\%$ vs. $20 \pm 8\%$, $P<0.01$). There were significantly more activated fetal compared to maternal Th2 cells ($28 \pm 31\%$ vs. $16 \pm 25\%$, $P=0.002$) and significantly more activated maternal compared to fetal Th1 cells ($20 \pm 23\%$ vs. $14 \pm 16\%$, $P=0.005$). The distribution of *in vitro* SEB-activated Th2 and Th1 cells in both the maternal and fetal cohort was inversely correlated (Figure 3).

Co-ordinate activation of fetal and maternal Th cells

The CCR4+CXCR3- Th2 surface phenotype was found to be co-ordinately expressed ($r=0.68$, $P<0.001$) on CD4+CD69+ PBL from paired fetal and maternal specimens (Figure 4A). However, no correlation was found between fetal and maternal

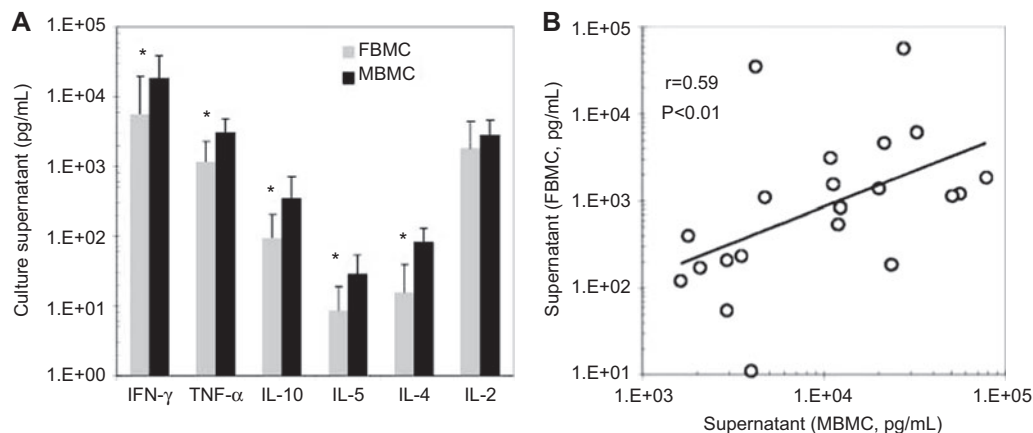


Figure 2 Cytokine production by fetal and maternal mononuclear cells *in vitro*. Culture supernatants were harvested from paired PBMCs incubated for 20–24 h in the presence of 10 μ g/mL SEB ($n=21$). Samples were assayed simultaneously for multiple cytokines using a CBA kit. Sensitivity of detection was >10 pg/mL for IFN- γ , and >3 pg/mL for the other cytokines. (A) Bars represent means $\pm$ SD. *Significant difference ($P<0.05$) between fetal and maternal specimens. P values were determined using paired t -tests. (B) Co-ordinate expression of IFN- γ by paired MBMC and FBMC following SEB activation.

Table 2 Covariation between cytokines in FBMC and MBMC following polyclonal T cell activation.

	IFN- γ		TNF- α		IL-10		IL-5		IL-4	
	r	P	r	P	r	P	r	P	r	P
Fetal										
TNF- α	0.34	0.15								
IL-10	0.74	<0.001	0.52	0.02						
IL-5	0.52	0.02	0.65	<0.01	0.77	<0.0001				
IL-4	-0.15	0.53	-0.16	0.53	-0.06	0.79	-0.07	0.78		
IL-2	0.50	0.03	0.36	0.13	0.44	0.06	0.34	0.15	0.03	0.90
Maternal										
TNF- α	-0.30	0.21								
IL-10	0.54	0.02	0.19	0.43						
IL-5	0.53	0.02	-0.29	0.23	0.38	0.11				
IL-4	0.10	0.67	-0.23	0.34	0.16	0.52	0.36	0.13		
IL-2	0.70	<0.001	0.38	0.11	0.56	0.01	0.60	<0.01	0.22	0.36

Cytokines produced by FBMC and MBMC following 20–24 h activation with 10 flg/mL SEB were measured using a Th2 CBA kit. r =Spearman's correlation coefficient. Values indicating significant covariation between two cytokines are in bold.

PBL expressing the CXCR3+CCR4⁻ Th1 phenotype (Figure 4B), whereas the CXCR3+CCR4⁺ “intermediate” Th surface phenotype was found to be co-ordinately expressed ($r=0.64$, $P=0.02$) on CD4+CD69⁺ PBL from paired fetal and maternal specimens (Figure 4C).

Fetal and maternal IFN- γ producing cells

Labeled PBL subsets were removed by flow-sorting. Levels of IFN- γ induced from the selectively depleted PBMC isolates are shown in Figure 5, which identified CD4+CXCR3⁺ cells as the majority of IFN- γ producing PBL in both FBMC and MBMC following SEB activation *in vitro*.

Discussion

There is mounting evidence for the contribution to fetal immune development of maternal atopy or maternal exposure to environmental modifiers that may affect Th2 polarization

of fetal CD4⁺ lymphocytes [17]. Thus, cord blood from atopic pregnancies contained higher levels of eotaxin [11], while PBMC of neonates born to mothers with atopy produced lower levels of IFN- γ when stimulated with allergens [8, 16], T cell mitogens [13, 23], or incompatible HLA antigens [18]. In contrast, whole blood leukocytes or PBMC of neonates born in a farm environment, with elevated levels of endotoxin in home dust [2, 7], produced higher levels of IFN- γ when stimulated with T cell mitogens [14, 21, 24] than neonates from a non-farm environment. Our study shows mutual fetal and maternal Th1/Th2 polarization of immunological phenotypes in paired fetal and maternal blood samples.

Cord blood IgE was detected in 8% of our subject population which is consistent with that reported for the northeastern US [15]. Our data shows that in normal pregnancy cord blood eotaxin levels correlated with maternal levels, as reported for a cohort of pregnant women with physician diagnosed asthma [19]. We also show that before SEB stimulation cord blood IFN- γ and IL-4 levels correlated with maternal levels.

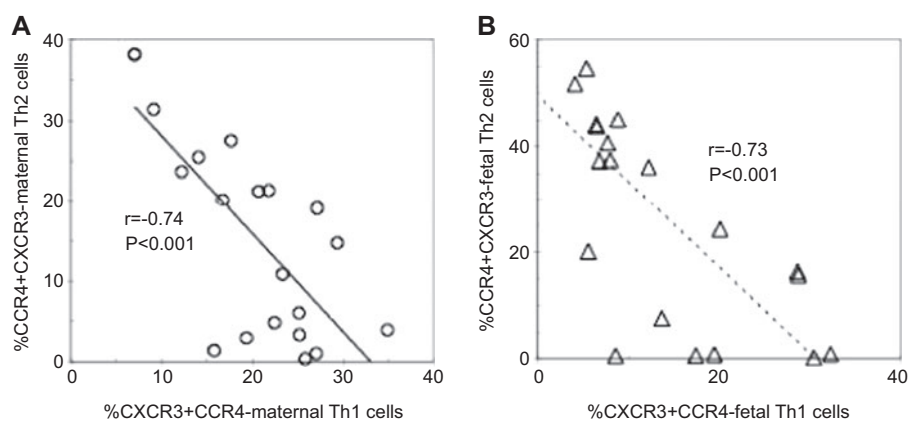


Figure 3 Inverse relationship between SEB-activated Th1 and Th2 cells in MBMC and FBMC. Activated Th cells were identified as CD4+CD69⁺. Th1 cells were identified as CXCR3+CCR4⁻ and Th2 cells were identified as CCR4+CXCR3⁻ within the CD4+CD69⁺ subset.

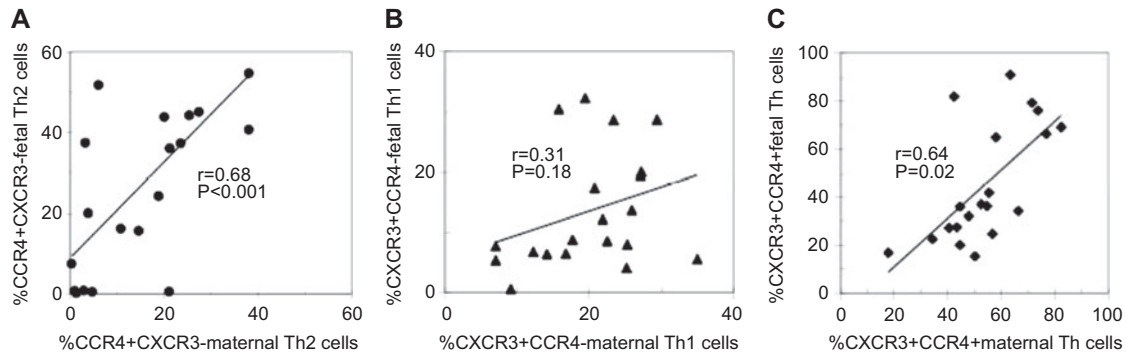


Figure 4 Co-ordinate activation of fetal and maternal Th cells following SEB activation. (A) Frequency of CCR4+CXCR3⁺ Th2 cells in the fetal and maternal cohort following SEB activation. (B) Frequency of CXCR3+CCR4⁺ Th1 cells in the fetal and maternal cohort following SEB activation. (C) Frequency of CXCR3+CCR4⁺ “intermediate” Th cells in the fetal and maternal cohort following SEB activation.

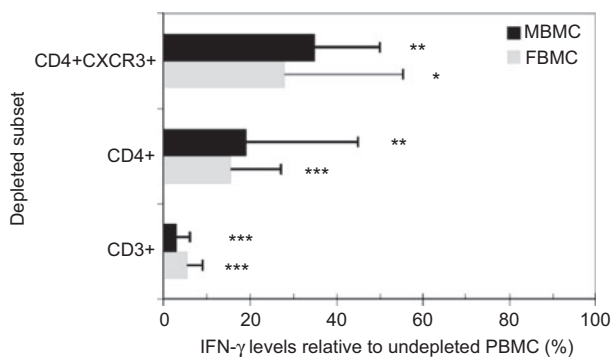


Figure 5 Identification of IFN- γ producing fetal and maternal Th cells. Paired FBMC and MBMC ($n=5$) were labeled with FITC-anti-CD3, PE-anti-CXCR3, PerCP-Cy5.5-anti-CD45, and allophycocyanin-anti-CD4. Indicated PBL subsets were identified and removed by flow-sorting. Selectively depleted PBMC isolates were cultured in the presence of 10–11g/mL SEB for 20–24 h and compared to unsorted controls. Secreted IFN- γ was measured using a Bio-Plex kit. Sensitivity of detection was >6 pg/mL. Bars represent means $\pm$ SD. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

Furthermore, inverse correlation between IFN- γ and eotaxin levels implies that IFN- γ was down-regulating Th2 effector responses in both fetal and maternal cohorts.

Following *in vitro* SEB polyclonal T cell stimulation, we observed co-ordinate IFN- γ up-regulation between fetal and maternal PBMC accompanied by co-ordinate increases in activated CD4⁺ cells that display the CXCR3⁺ Th1 surface phenotype, identified as the major producers of IFN- γ . Since we ascertained that differences in the frequency of cord blood Th cells in our cohort did not contribute to lower cytokine production, our results confirm earlier findings that naïve fetal CD4⁺ lymphocytes are less effective in mounting an IFN- γ response [29]. The increased percentage of B cells we previously reported as characteristic of fetal blood [25] was confirmed by the present study. Our findings established that transplacental communication exists during normal pregnancy, which implies maternal regulation of the developing fetal immune system.

Our previous report that fetal CD4⁺ lymphocytes are capable of responding to TCR-mediated triggering by the second trimester [25] can account for the positive relationship between fetal Th responses to allergens and allergen-specific IgG which reflects their transplacental passage during gestation [15, 16]. Also, the data presented in this report can account for the negative relationship between neonatal Th1 responses and maternal atopy [8, 13, 16, 18, 23]. Since $>85\%$ of cord blood Th cells are CD45RA⁺ [4], our observations imply that upon antigen encounter before birth, fetal responses are likely to be polarized along the same pathway as the maternal response. However, as the neonates respond autonomously to exogenous immune stimuli following parturition, the maternal imprint can be lost or maintained [5, 12, 16, 20, 22]. In addition, genomic variation would be expected in immune responses, despite maternal programming.

While the contributions of T regulatory and Th17 cells continue to be defined, the role for IFN- γ in suppressing Th2 polarization and effector function has been elucidated in human studies and corroborated using murine models [9, 10, 30–32]. It was proposed that in the absence of Th1 inducing signals, such as IL-12 from endotoxin exposed antigen-presenting cells, Th2 is the default pathway with constitutive maturation sustained by endogenous IL-4 from the activated Th cells [1]. Upon induction towards the Th1 pathway, CpG sites in the IFN- γ promoter are progressively demethylated, a process that is considerably more marked in neonatal compared to adult Th cells [31]. Since cytokines and chemokines do not cross the placenta [1], the exact mechanisms by which a Th1 or Th2 promoting milieu in the mother is transmitted to the developing fetus and the fetal feedback occurs, await further investigation. It is clear that there is maternal-fetal crosstalk *via* a transplacental nexus, with major implications for tolerance of the fetal homograft, fetal programming, and immune responsiveness.

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