

Expression of X-linked Toll-like receptor 4 signaling genes in female vs. male neonates

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BACKGROUND: Male neonates display poorer disease prognosis and outcomes compared with females. Immune genes which exhibit higher expression in umbilical cord blood (UCB) of females may contribute to the female immune advantage during infection and inflammation. The aim of this study was to quantify expression of Toll-like receptor (TLR) 4 signaling genes encoded on the X-chromosome in UCB from term female vs. male neonates.

METHODS: UCB samples were collected from term neonates ($n = 26$) born by elective Caesarean section and whole blood was collected from adults ($n = 20$). Leukocyte RNA was isolated and used in quantitative PCR reactions for I κ B kinase γ (IKK γ), Bruton's tyrosine kinase (BTK), and IL-1 receptor associated kinase (IRAK)1. IRAK1 protein was analyzed by Western blot and confocal microscopy.

RESULTS: In neonates there was no significant difference in the relative expression of IKK γ or BTK mRNA between genders. IRAK1 gene and protein expression was significantly higher in female vs. male UCB, with increased cytosolic IRAK1 expression also evident in female UCB mononuclear cells. Adults had higher expression of all three genes compared with neonates.

CONCLUSION: Increased expression of IRAK1 could be responsible, in part, for sex-specific responses to infection and subsequent immune advantage in female neonates.

Due to ontogenic deficiencies in adaptive immunity, neonates depend largely on innate immunity to protect against infection. It is well known that infants display a gender disparity in morbidity and overall mortality, particularly at the borders of viability. For example male neonates have higher rates of infections and sepsis compared with females of matched gestational age, irrespective of higher birth weight distributions (1). Neonatal sepsis is a clinical manifestation of a dysregulated innate immune response to invasive pathogens.

Toll-like receptors (TLRs) are signaling pattern recognition receptors that represent an integral part of innate immunity.

Once activated by recognition of their cognate ligands, TLRs initiate intracellular signaling cascades leading to transcription factor activation e.g., nuclear factor κ B (NF κ B), and induction of cytokine expression. The prototypical TLR, TLR4, is critically important in signaling the inflammatory response to Gram-negative bacteria through recognition of lipopolysaccharide (LPS/endotoxin). TLR4 signals via various intermediate adaptor proteins and kinases (2). One of the most common TLR4 pathways involves signaling via myeloid differentiation factor 88 (MyD88), interleukin-1 receptor-associated kinases (IRAKs) 1 and 4, TNF-receptor-associated factor (TRAF) 6, and inhibitors of NF κ B Kinases (IKKs) leading to activation of NF κ B; this can be summarized as TLR4/MyD88/IRAKs/TRAF6/IKKs/NF κ B. Bruton's tyrosine kinase (BTK) can participate in this pathway at various levels via individual interactions with TLR4, MyD88, and IRAK1 (3). BTK, and both IRAK1 and IKBKG (the gene that encodes IKK γ , also known as NEMO) are encoded on the X chromosome at Xq21 and Xq28, respectively (4).

Sex is determined during embryogenesis by the sex chromosome complement; XX in females and XY in males. The Y chromosome contains ~100 protein-coding genes, whereas the X chromosome encodes at least 1,100 including many that are involved in immunity (4). A combination of random silencing of one X chromosome in females and increased expression levels of dosage-sensitive X chromosome genes in both sexes achieves dosage compensation. Escape from X chromosome inactivation (XCI) leads to a sex bias in gene expression, and some of these genes are likely to be implicated in the gender differences in certain diseases. Although ~15% of X-linked genes consistently escape XCI in humans, a further 10% show variable escape, at least in cell lines (5,6). In females this may confer an advantage in responding to and resolving acute infections.

Various studies have examined expression of X-linked genes involved in TLR signaling in infants and umbilical cord blood (UCB) (7–11). However, to our knowledge, the levels

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of expression of specific X-linked TLR4 signaling intermediate genes in UCB have not been compared between male and female neonates, or adults. In this study, we investigated quantitative differences in BTK, IKK γ , and IRAK1 mRNA levels in male and female leukocytes from UCB and compared these to levels in adult whole blood. We also explored IRAK1 protein expression in more detail in mononuclear cells isolated from UCB. The data indicate that IRAK1 is expressed more highly in female vs. male UCB and this may underlie, in part, the differences in inflammatory outcomes in infants.

METHODS

Patient Population and Sample Collection

Ethical approval for the study was received from the institutional ethics committee at the National Maternity Hospital, Holles Street, Dublin. Informed written consent for blood sampling was obtained in all cases and completed by a single investigator. The study population included the following groups:

1. *Adult controls*: 10 nonpregnant healthy women and 10 men (laboratory and medical colleagues) aged 20–30 y. Peripheral venous blood (9 ml) was obtained by venepuncture and collected in EDTA-coated vacutainers.
2. *Elective lower segment caesarean sections (LSCS)*: 9 ml UCB samples ($n = 13$ males, $n = 13$ females, $n = 26$) collected in EDTA-coated vacutainers immediately following elective (no labor) LSCS delivery from uncomplicated term ($\geq 37 \pm 0$ wk) pregnancies. All elective LSCS were performed under spinal anesthesia. All neonates had an uncomplicated postnatal course (Table 1).

RNA Isolation

Whole blood samples were centrifuged at 1,800g for 20 min at room temperature to extract plasma and total mononuclear cell fraction (monocytes and lymphocytes). RNA was extracted from 200 μ l of buffy coat using 750 μ l/sample Trizol BD reagent (Sigma-Aldrich, Wicklow, Ireland) as per the manufacturer's protocol. This was followed by phase separation using chloroform (200 μ l/sample, Sigma-Aldrich), RNA isolation using isopropanol (500 μ l/sample, Sigma-Aldrich) and finally, washing of the RNA pellet in 70% ethanol, centrifugation to obtain the RNA pellet, and dissolution in 0.1%

diethyl pyrocarbonate (DEPC)-treated (Sigma-Aldrich) water as per the manufacturer's protocol. Quantification of RNA was performed on an 8-channel Nanodrop-8000 spectrophotometer (Thermo Scientific, Dublin, Ireland). RNA was stored at -80°C until required.

Quantitative PCR

Equal quantities of RNA were reverse transcribed into cDNA using the Quantitect Reverse Transcription Kit (Qiagen, Manchester, UK) and used for quantitative PCR (qPCR). Primers were designed using BLAST on-line resources. Primer efficiencies were determined from cDNA pooling and serial dilutions (1, 1 : 5, 1 : 25, 1 : 125, and 1 : 625) (Table 2). qPCR reactions contained 5 μ l cDNA, 10 μ l SyBr Green mastermix, 3 μ l PCR H₂O and 1 μ l of each a forward and reverse primer (IRAK1, BTK, IKK γ , or RPLP0) in a 20 μ l volume. Amplification was performed using the LightCycler-480 PCR system (Roche, Dublin, Ireland) at an annealing temperature of 56°C for 45 cycles. Quantitative expression of the target genes was determined using the $2^{(-\Delta\Delta\text{Ct})}$ method.

Western Blot

Total protein was isolated from leukocyte samples ($n = 5$ male, $n = 5$ female). Protein concentrations were determined using the Bicinchoninic acid (BCA) Protein Assay kit (Thermo Scientific) as per the manufacturer's instructions. Samples were denatured by the addition of sample buffer (62.5 mmol/l Tris (pH 6.8), 2% sodium dodecyl sulfate (SDS), 0.002% bromophenol blue, 1.54% dithiothreitol, 1% sucrose, 1 mmol/l EDTA in dH₂O), and were boiled at 95°C for 5 min prior to separation by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were transferred to a polyvinylidene-fluoride (PVDF) membrane (Thermo Scientific) and incubated with mouse monoclonal IRAK1 antibody (Santa Cruz sc-55530, 1:2000, Heidelberg, Germany) in 1% bovine serum albumin (BSA)-tris-buffered saline (TBS) and 0.1% Tween-20 overnight at 4°C . Following a series of washes, membranes were incubated with rabbit polyclonal glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz FI-335, 1:2000, Heidelberg, Germany) and washed as before. Membranes were incubated with horseradish peroxidase-conjugated IgG secondary antibodies (antimouse Cell Signaling 7076, or antirabbit Cell Signaling 7074, both 1:2000, Dublin, Ireland). Following washing, signal detection was determined using HRP substrate on the Syngene G:Box chemi-XL gel documentation system. Densitometry was analyzed using GeneTools software.

Peripheral Blood Mononuclear Cell Isolation

Freshly collected UCB samples ($n = 3$ male, $n = 3$ female) were diluted in dPBS at room temperature. UCB samples were overlaid onto Lymphoprep (Stemcell Technologies, Cambridge, UK) and centrifuged at 800g for 10 min at room temperature (RT). The mononuclear cell interphase band containing monocytes and lymphocytes was aspirated and washed in Hank's Balanced Salt Solution (HBSS) medium and resuspended in Roswell Park Memorial Institute (RPMI) 1640. 2X Freezing Media (10% dimethylsulfoxide (DMSO), 40% fetal calf serum (FCS), 50% RPMI 1640) was added to cell suspensions. Samples were transferred into cryovials and placed at -80°C . After 24 h, cryovials were transferred into liquid nitrogen where they were stored until further use.

Confocal Microscopy

Isolated peripheral blood mononuclear cells (PBMCs) were incubated for 30 min in 4% paraformaldehyde (Sigma-Aldrich) in 1XPBS, washed in 1XPBS, and permeabilised with 0.5% Triton X-100 in 1XPBS for 10 min. Cells were washed twice in 1XPBS and stained with 50 μ g/ml fluorescent rhodamine phalloidin conjugate solution (Thermo Scientific) to stain F-actin in 1XPBS for 40 min at room temperature and washed three times with 1XPBS to remove unbound phalloidin conjugate. Nunc 8 well chamber slides (Thermo Scientific) were coated in poly-L-Lysine (Sigma-Aldrich) at 37°C for 30 min and left to dry at room temperature. Isolated PBMCs were resuspended and allowed to adhere to the slides overnight. Chamber slides were incubated with blocking buffer (4% BSA in 1XPBS) for 30 min at 37°C to minimize nonspecific absorption of the antibodies. The slide was incubated overnight at 37°C in primary IRAK1 antibody (Santa Cruz, sc-55530, 1:250, Heidelberg, Germany) in 1XPBS, the antibody was removed and the slide was washed twice

Table 1. Characteristics of neonates enrolled in the study

Demographics	$n = 26$
Male	13
Female	13
Delivered by elective Caesarean section	26
Vaginal delivery	0
Mean gestational age, weeks (SD ^a)	39 \pm 0 (0.63)
Evidence of labour?	0
Mean Apgar score at:	
1 min	9.0
5 min	9.0
10 min	10.0
Mean age of mother, years (SD)	35.9 (3.49)
Birthweight (average)	3.48 \pm 0.27 kg
Postnatal complications?	0
Admission to NICU ^b ?	0

^aSD, standard deviation; ^bNICU, Neonatal Intensive Care Unit.

Table 2. Primer sequences and efficiency values

Gene		Sequence (5'–3')	Primer efficiency
<i>IκBKγ</i>	F ^a	GAGAAGCTGGCCGAGAAGAA	15 μ mol/l; Efficiency = 94.5%
	R ^a	CTCATGTCCTCGATCCTGGC	–
<i>IRAK1</i>	F	CGTGTCCAGGTTTCGTACAC	5 μ mol/l; Efficiency = 100%
	R	CCGTACACCAGGCAGTAGAA	–
<i>BTK</i>	F	GCTCAAAAACGTAATCCGGTCA	15 μ mol/l; Efficiency = 99.6%
	R	GTCTCCGGTGAGAACTCCC	–
<i>RPLP0</i> ^b	F	AATCTCCAGGGGCACCATT	10 μ mol/l; Efficiency = 110%
	R	CGCTGGCTCCCACTTTGT	–

^aF, forward; R, Reverse. ^bRPLP0, ribosomal protein lateral stalk subunit P0.

BTK, Bruton's tyrosine kinase; IKK γ , I κ B kinase γ ; IRAK1, interleukin-1 receptor associated kinase 1; qPCR, quantitative PCR; UCB, umbilical cord blood.

in 1XPBS. Cells were incubated for 2h at room temperature in goat antimouse DyLight-488 secondary antibody (abcam, ab96879, 1:500, Cambridge, UK) in 1XPBS, washed, fluorescent mounting media containing 4,6-diamidino-2-2-phenylindole (DAPI) was added and a coverslip was placed onto the slide and sealed for imaging. Images were captured using a Zeiss 510 meta confocal microscope at X40 magnification and X4 zoom. Scans were performed at 1 μ m interval depths through the fixed cells, and single or merged images are presented as XY single planes through the mid-section of the images unless otherwise specified.

Statistical Analyses

All statistical analyses were performed using GraphPad Prism 4.0 software package (San Diego, CA). Significance was assumed for values of $P < 0.05$ (Student's t -test, ANOVA).

RESULTS

Patient Demographics

The mean duration of pregnancy for this cohort ($n = 26$) was 39 ± 0 wk (0.63 SD). The indications for elective LSCS were breech/transverse lie, previous LSCS, and previous obstetric perineal (third degree) tear and this maternal cohort did not labor (no rupture of membranes/premature rupture of membranes, and no cervical dilatation). Recruited mothers were of Caucasian ethnicity and did not have a history of acute or chronic disease. Delivery of all recruited neonates by LSCS was uncomplicated. All neonates had Apgar scores of 9 at 1 and 5 min, and 10 at 10 min. Average birth weight was 3.48 ± 0.27 (SD) kg (3.56 kg \pm 0.33 (SD) in male neonates and 3.37 kg \pm 0.29 (SD) in female neonates; $P = 0.127$). All neonates had an uncomplicated postnatal course and were discharged with their mothers.

Neonates have Lower BTK, IKK γ , and IRAK1 Gene Expression Compared with Adults

The X chromosome encodes a large number of genes involved in immunity (4). Here the levels of expression of three X-linked genes involved in TLR4 signaling were quantified in leukocytes from neonatal UCB and adult whole blood. **Figure 1** shows the relative expression levels of BTK, IKK γ , and IRAK1 mRNA in cord blood vs. adult whole blood quantified by qPCR. For all three genes neonates had significantly lower levels compared with adults (**Figure 1**, $P < 0.0001$).

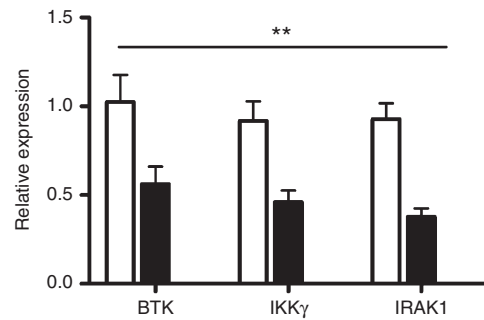


Figure 1. Relative expression of BTK, IKK γ , and IRAK1 in adult blood vs. UCB. RNA was isolated from the buffy coat of UCB or adult whole blood and used in BTK, IKK γ , and IRAK1 qPCRs in triplicate ($n = 20$ per group). Relative gene expression (mean \pm S.E.) was calculated for these target genes by comparison with the reference gene *RPLP0* using the $2^{-\Delta\Delta Ct}$ method. ** $P < 0.0001$ ANOVA with Bonferroni's multiple comparison tests. White = adults, black = neonates. BTK, Bruton's tyrosine kinase; IKK γ , I κ B kinase γ ; IRAK1, interleukin-1 receptor associated kinase 1; qPCR, quantitative PCR; UCB, umbilical cord blood.

Sex-specific BTK, IKK γ , and IRAK1 Gene Expression Levels in Adults and Neonates

Next, we stratified the samples by sex in order to determine if there are any sex-specific differences in expression of BTK, IKK γ , or IRAK1 mRNA in leukocytes from adult blood or UCB. **Figure 2a** shows that although levels of each of the three transcripts tended to be lower in adult females vs. males, there was no significant difference overall. In UCB samples there was no difference in BTK expression between the sexes (**Figure 2b**). Levels of IKK γ mRNA were slightly higher in female neonates (ns, $P = 0.119$) however IRAK1 relative gene expression was significantly higher in female neonates compared with male neonates ($P = 0.0198$).

IRAK1 Protein Levels in Female vs. Male Neonates

In order to investigate whether IRAK1 protein, like IRAK1 mRNA expression, was also increased in female UCB we performed western immunoblotting for IRAK1 on cells isolated from male and female UCB that were previously analyzed by qPCR. IRAK1 protein levels were variable but detectable

in whole cell extracts isolated from leukocytes isolated from female UCB (Figure 3). IRAK1 protein was barely, if at all, detectable in the male samples.

IRAK1 Levels and Localization in Female vs. Male PBMCS

IRAK1 is a cytosolic protein that participates in TLR4 and other TLR signaling pathways. To further confirm our observations regarding increased IRAK1 mRNA and protein expression in female vs. male UCB leukocytes, we next isolated mononuclear cells from additional cord blood samples and used confocal microscopy, which is more sensitive than western blotting, to visualize IRAK1 localization within these cells. Figure 4 shows that compared with male mononuclear cells, IRAK1 protein is more highly expressed in female cord blood mononuclear cells where it colocalizes with F-actin in the cytosol.

DISCUSSION

Females have a well-recognized survival advantage compared with males throughout the entire lifecycle. The female immune advantage is particularly evident during the newborn period, as evidenced by Ulizzi et al. who described a generalized male overmortality in the early period of extrauterine life (12).

Here we assessed the expression levels of three X-linked TLR4 signaling genes in female vs. male UCB in order to observe whether differences exist in transcript levels between the sexes. Having detected higher IRAK1 gene and protein expression in female leukocytes, we further demonstrated increased cytosolic IRAK1 protein expression in female vs. male mononuclear cells isolated from UCB. The data presented here support our hypothesis that expression of TLR4 signaling intermediates encoded on the X chromosome might be altered in monocytes from female UCB. Based on these observations we speculate that increased basal expression of IRAK1 could contribute to sex-specific responses to infection and an immune advantage in female neonates.

Both monocytes and neutrophils are major cells of the innate immune response; in this study we were interested in the behavior of monocytes. These cells originate from precursors in the bone marrow, and circulate in the bloodstream, until they are attracted to infection or inflammatory signals where they differentiate into macrophage or dendritic cell populations. The monocyte/macrophage lineage of myeloid cells has three primary roles to this effect: phagocytosis, antigen presentation, and immunomodulation. Our initial observations

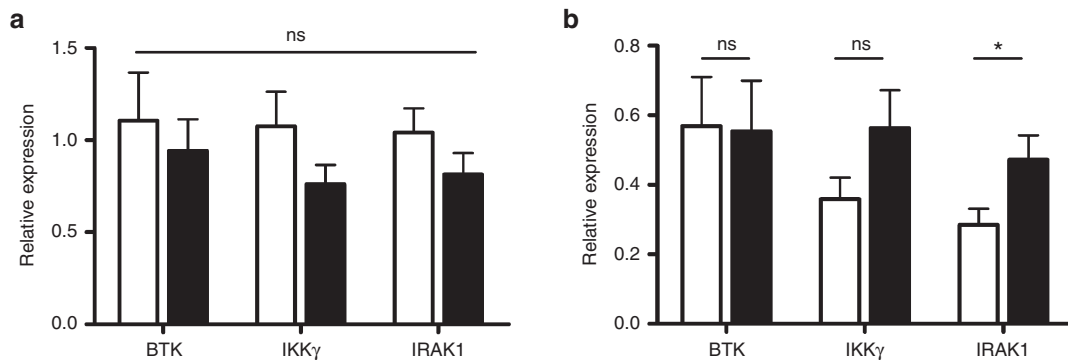


Figure 2. Sex-specific BTK, IKKγ, and IRAK1 gene expression levels in adults and neonates. RNA was isolated from the buffy coat of male and female (a) adult whole blood or (b) UCB (n = 10 per group) and used in triplicate BTK, IKKγ, and IRAK1 qPCRs. Relative gene expression (mean ± S.E.) was calculated for these target genes by comparison with the reference gene *RPLP0* using the 2^{-ΔΔCt} method. *P = 0.0198 unpaired t-test, ANOVA. White = males, black = females. BTK, Bruton's tyrosine kinase; IKKγ, IκB kinase γ; IRAK1, interleukin-1 receptor associated kinase 1; qPCR, quantitative PCR; UCB, umbilical cord blood.

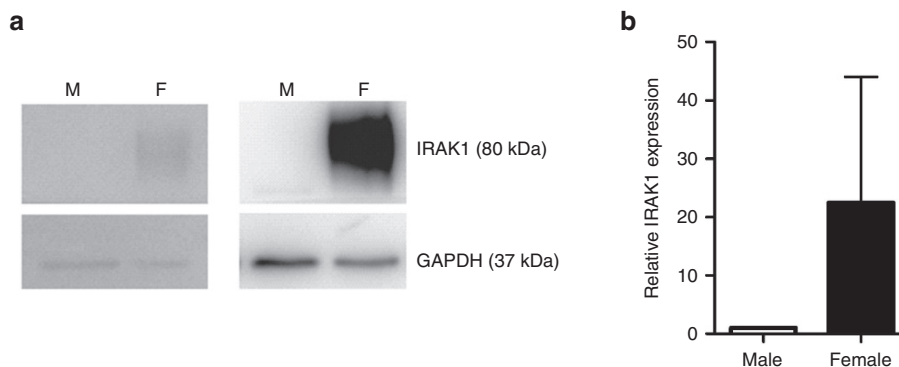


Figure 3. IRAK1 protein levels in female vs. male neonates. Protein extracts (20 μg) from the buffy coat of male and female UCB (n = 5 per group) were electrophoresed on 10% SDS-PAGE, transferred onto nitrocellulose and probed with anti-IRAK1 and anti-GAPDH antibodies by western blotting. (a) Representative blots (n = 2) and (b) densitometric analysis of IRAK1 protein expression (n = 5 per group, mean ± S.E.) normalized to GAPDH for all samples. M = males, F = females. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IRAK1, interleukin-1 receptor associated kinase 1; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; qPCR, quantitative PCR; UCB, umbilical cord blood.

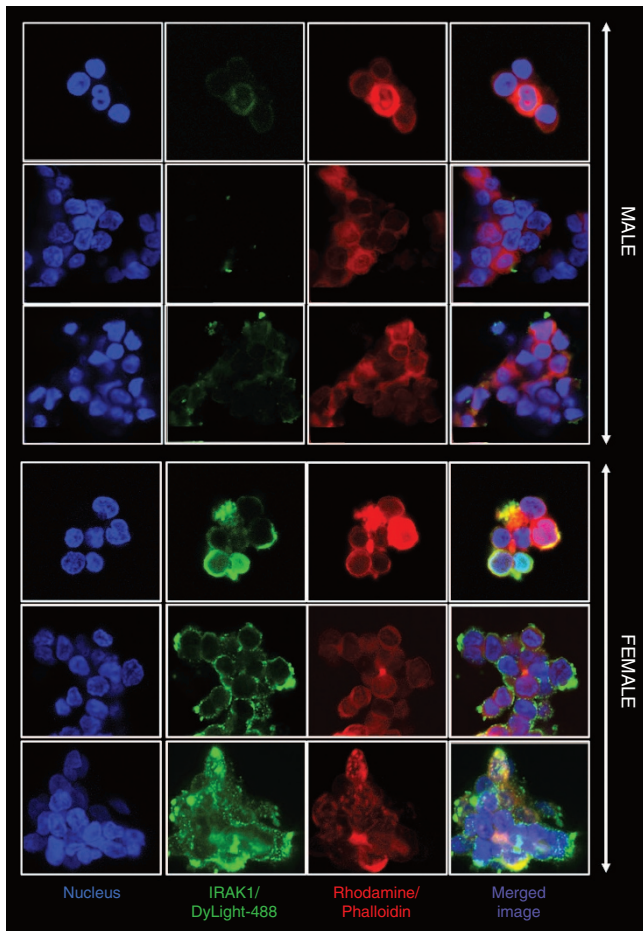


Figure 4. Confocal microscopy images of IRAK1 in PBMCs. PBMCs were isolated from male and female UCB ($n = 3$ per group), fixed, permeabilized, and labelled with DAPI (blue, nucleus), rhodamine phalloidin conjugate solution (red, cytosol) and anti-IRAK1 antibody primary and secondary antibodies (green). Images were captured using a Zeiss 510 meta confocal microscope at X40 magnification and X4 zoom. Scans were performed at 1 μ m interval depths through the fixed cells and single or merged images are presented as XY frontal focal planes. DAPI, 4,6-diamidino-2-2-phenylindole; IRAK1, interleukin-1 receptor associated kinase 1; PBMCs, peripheral blood mononuclear cells; UCB, umbilical cord blood.

pointed to a difference in IRAK1 expression in female vs. male UCB leukocytes. In order to focus more closely on monocytes, we performed subsequent analyses on total mononuclear rather than granulocytic cells. Within the mononuclear fraction lymphocytes as well as monocytes are also present. Thus our observations also potentially relate to adaptive immune responses, however these are not evident in very early life.

The X chromosome encodes many genes that are involved in immune functions such as as TLR7, CD40 ligand, the IL-1 receptor accessory protein, IL-2 receptor γ , and the IL-9 receptor, amongst many others (4). In females protein-coding genes on the X chromosome are susceptible to XCI however despite chromosome-wide transcriptional silencing of one X chromosome, some genes remain expressed from both the active and inactive X alleles, resulting in a double gene dosage. It has been estimated that in women, ~15% of X-linked genes escape XCI and are biallelically expressed (13). Interestingly this selection

of genes includes genes encoding for several components of the TLR4 pathway that are essential for NF κ B signaling. Amongst these is a subset of genes with specific roles in innate immunity, such as *BTK* located at Xq22.1, and a cluster of genes that maps to Xq28, where *IKBKG* and *IRAK1* are located (5). Thus, higher expression of these genes in females compared with males may confer an advantage in responding to and resolving acute infections and could be implicated in gender differences in certain diseases.

Neonates depend largely on the innate immune system as the first line of defense against microbial invasion. The TLR4 pathway recognizes and responds to LPS, and abnormal LPS responses are associated with many diseases in infants such as sepsis, bronchopulmonary dysplasia, necrotizing enterocolitis and cerebral white matter damage with resultant cerebral palsy—all of which are noted to preferentially affect male neonates (14–18). Immunologically immature neonates suffer the highest incidence of sepsis (19–22). Maturation differences in the immune response can affect a neonate’s ability to mount an appropriate inflammatory response to infection and clear pathogens (19,21). Clinical studies suggest that a hypo-inflammatory state is associated with an increased risk of secondary infections and mortality in neonates (19,23). Evaluation of downstream signaling components in the TLR4/MyD88 pathway has revealed differential expression of signal transducing proteins based on immune maturation status (19). Yan *et al.* demonstrated that MyD88 expression is significantly reduced in neonatal monocytes and is associated with decreased tumor necrosis factor- α production following low-dose LPS stimulation (24). IRAK1 is also a key signal transducer in the TLR4 pathway and increased expression of IRAK1, as observed here in female UCB, may enhance TLR4 functional responses, thereby facilitating a more effective innate immune response in the face of infection. It would be interesting to determine the effect of LPS on *IRAK1*, *IKBKG*, and *BTK* gene expression; future studies will attempt to investigate this. What also remains to be determined is the mechanism(s) by which IRAK1 expression is increased. Future studies should investigate the potential roles of epigenetic influences (e.g., promoter methylation) and differential transcription factor activation. It is also possible that neonatal TLR4 pathway ontogeny may be responsible for lower IRAK1 expression in male neonates. IRAK1 is a validated target of miR-146a (25) and decreased miR-146a levels may be accountable for the observed effect of IRAK1 overexpression in females, however this remains speculative. Importantly neonates are subjected to very high levels of sex steroids resulting from pregnancy, in particular 17 β -estradiol, which might influence their immune system. Therefore 17 β -estradiol and progesterone may also have a role here, however addressing these questions are beyond the scope of this study.

Here we saw no significant difference in BTK and IKK γ transcript levels in female vs. male UCB, although IKK γ mRNA levels showed a trend toward being higher in females ($P = 0.119$), suggesting that this may be true in a larger powered study. Indeed both *IRAK1* and *IKBKG* are closely located on Xq28,

a region known to display XCI escape (5). BTK is involved in TLR4 signaling and has been shown to interact with TLR4, MyD88, and IRAK1 to facilitate signal transduction in certain instances (3). The absence of any observable difference in BTK mRNA levels in this pilot study indicates that its expression in UCB may not be gender-related. We also saw no significant difference in BTK mRNA levels in male vs. female adult whole blood. Surprisingly, none of the X-linked genes studied here were expressed at a higher level in adult females compared with males, however the sample size was small.

It is important to consider that IRAK1 variant haplotypes exist with different functional activities *in vivo*. Generally the IRAK1 haplotype with increased activity (single nucleotide polymorphism in which T is replaced with C at nucleotide 1,595 within exon 12 of the *IRAK1* gene) is associated with poorer outcomes in sepsis in humans (26,27). Yet females heterozygous for the “high-activity” or wild-type IRAK1 haplotypes show no adverse clinical consequences. In our study cohorts, it is not known which IRAK1 variants are present. Preclinical data describes both protective and detrimental effects of IRAK1 deficiency in models of sepsis (28,29). In an adult mouse model of caecal ligation and puncture-induced sepsis, IRAK1 deficiency can protect against sepsis (28). However those authors also reported that IRAK1 levels have been shown to have both protective and deleterious effects in other animal injury models. Overall, it appears that the effect is model-dependent.

In this study adults expressed higher levels of all three genes compared with neonates. Various studies have reported overall reduced immune-related gene expression in neonates compared with adults. For example, expression of Transforming Growth Factor- β 1, macrophage inflammatory protein-1 α , and IL-8 protein were all shown to be lower following agonist-stimulation in human neonatal vs. adult mononuclear cells; however that effect was thought to be post-transcriptional (30). De Wit *et al.* reported that CpG-induced Interferon- γ mRNA and protein in plasmacytoid dendritic cells was dramatically impaired in cord vs. adult blood, and described how these cells are intrinsically deficient in CpG-induced IFN- γ production at birth (31). The gene encoding TLR3 has also been shown to display developmental regulation in dendritic cells derived from cord blood (32). Novel data presented here indicate that *BTK*, *IKBKG*, and *IRAK1* are also expressed at lower levels in neonates compared with adults.

One limitation of this observational study is the small number of patients in all groups. Future work should aim to cover a broader clinical cohort, including preterm infants. We are also aware that in this study the entire patient cohort consisted of neonates born by Caesarean section without labor. Since labor promotes neonatal neutrophil survival and LPS-responsiveness, for example (33), it would be interesting to determine whether it also affects TLR4 functional responses in PBMCs. In conclusion the novel observations from this study regarding increased *IRAK1* gene and protein expression in female vs. male cord blood mononuclear cells provide a sound basis for future mechanistic and disease-related studies in larger cohorts of term and preterm infants.

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