Interleukin 6 receptor alpha expression in PMNs isolated from prematurely born neonates: Decreased expression is associated with differential mTOR signaling.

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Abstract

**Background:** Dysregulated inflammation leads to morbidity and mortality in neonates. Neutrophil-mediated inflammation can cause inflammatory tissue damage. The mammalian Target of Rapamycin (mTOR) pathway governs IL-6Rα protein expression in human neutrophils. Shed IL-6Rα then participates in trans-signaling of IL-6/IL-6Rα to cells not otherwise sensitive to IL-6. Signaling to endothelial cells triggers efferocytosis where macrophages limit persistent inflammation by phagocytizing neutrophils. We hypothesized that preterm neonatal PMNs fail to synthesize IL-6Rα due to alterations in mTOR signaling.

**Methods:** We studied IL-6Rα expression, PAF-receptor expression, and mTOR signaling in plasma and PAF-stimulated PMNs isolated from newborn infants and healthy adults using ELISA, real time RT-PCR, Western blotting, flow cytometry, and immunocytochemistry with phospho-specific antibodies.

**Results:** Compared to healthy adults, plasma from neonates contains significantly less soluble IL-6Rα. IL-6Rα mRNA expression in PAF-stimulated PMNs does not differ between neonates and adults, but IL-6Rα protein expression is decreased in preterm neonatal PMNs. Rapamycin, a mTOR inhibitor, blocks IL-6Rα protein expression. mTOR signaling following PAF-stimulation is decreased in preterm neonatal PMNs.

**Conclusions:** Preterm neonatal PMNs exhibit decreased mTOR pathway signaling leading to decreased IL-6Rα synthesis. Decreased synthesis of IL-6Rα by neonatal PMNs may result in decreased IL-6/IL-6Rα trans-signaling with prolonged inflammatory response and increased morbidity.
Introduction

Premature birth predisposes newborn infants to significant morbidity and mortality(1). The incidences of common complications of prematurity such as sepsis(2, 3), neonatal chronic lung disease (CLD)(4), retinopathy of prematurity (ROP)(5), and necrotizing enterocolitis (NEC)(6, 7) all vary inversely with gestational age at birth. Implicit in the pathogenesis for each of these common sequelae of prematurity is a component of dysregulated inflammation leading to inflammatory tissue damage, morbidity, and potentially death(3–6). Dysregulated inflammation also contributes to the morbidity and mortality associated with sepsis in newborn infants born at a term gestation. Neonates, born either at term or prematurely, account for more cases of sepsis than all other pediatric age groups combined(8).

The polymorphonuclear leukocyte (PMN; neutrophil) serves as the primary effector cell of the acute inflammatory response(9). Following activation, PMNs rapidly migrate to areas of tissue injury or infection and respond to pro-inflammatory signals within the inflammatory milieu by releasing many of the anti-microbial factors and degradative enzymes contained in their granules. PMNs also phagocytize microbes and generate reactive oxygen species in phagolysosomes to kill microbes intracellularly. A subset of PMNs can also generate neutrophil extracellular traps (NETs), lattices of decondensed chromatin decorated with granule contents, to limit the spread of infection and kill microbes extracellularly(10, 11). Deficits in any aspect of PMN function routinely result in overwhelming infection despite optimal use of antimicrobial therapy. However, dysregulation of PMN-mediated acute inflammation leading to a prolonged, over exuberant neutrophil response correlates with inflammatory tissue damage and clinical deterioration(9).

Neutrophils respond to inflammatory signals such as lipopolysaccharide (LPS) and platelet-activating factor (PAF) through multiple cell signaling mechanisms. Figure 1 diagrams two major signaling cascades – NFkB and mammalian Target of Rapamycin (mTOR) – which lead to inflammatory gene products and leukocyte recruitment and activation within the inflammatory milieu. The cytokine interleukin 6 (IL-6) and the soluble IL-6/IL-6 receptor trans-signaling system serve as one checkpoint to limit the acute inflammatory response(12–14). Immune and non-immune cells, including endothelial cells, secrete IL-6 as part of the acute inflammatory response. IL-6 expression triggers the release of acute phase reactants such as C-reactive protein, fibrinogen, and serum amyloid A by the liver, and can promote proliferation and differentiation of immune cells such as macrophages, neutrophils, and megakaryocytes. These pro-inflammatory effects result from the binding of IL-6 to its receptor IL-6 receptor alpha (IL-6Rα) which is expressed on the cell surface with the gp130 glycoprotein. Together, these two receptor components transduce IL-6 binding into activation of the JAK/STAT, ERK, and PI3K signaling pathways in cells that express both IL-6Rα and gp130. However, an alternative, trans-signaling pathway exists to mediate IL-6 effects. For IL-6 trans-signaling, secreted IL-6 binds soluble IL-6Rα (sIL-6Rα) found in plasma after being shed from inflammatory cells including PMNs(13). This sIL-6Rα/IL-6 complex can then bind to cells that express gp130 and trigger IL-6 dependent cell signaling. This trans-signaling mechanism allows endothelial cells to respond to IL-6, activate tissue macrophages and monocytes, and promote macrophage efferocytosis of residual “spent” pro-inflammatory neutrophils as a limit on the acute inflammatory response(12–14).
IL-6Rα expression in PMNs is regulated via the mTOR protein translation control pathway(15), with quiescent IL-6Rα mRNA rapidly translated into protein following activation of mTOR in PMNs isolated from healthy adults and stimulated with PAF, a biologically active phospholipid implicated in the pathogenesis of inflammatory syndromes including NEC(16). Here we report decreased sIL-6Rα levels in plasma isolated from prematurely born newborns compared to control plasma isolated from newborns born at term and from healthy adults. Furthermore, we show that differential IL-6Rα protein expression in newborn PMNs compared to PMNs isolated from healthy adults is associated with differential activation of the mTOR pathway following PAF stimulation. These findings may, in part, explain the propensity of neonates to develop syndromes of dysregulated inflammation leading to significant morbidity and mortality.

Methods

Reagents

We purchased the following reagents: Platelet-activating factor (Avanti Polar Lipids, Alabaster, AL); Whole Blood anti-human CD15 microbeads (Miltenyi Biotec, Auburn, CA); Media-199 (Lonza Biologics, Alpharetta, GA); TOPRO-3 (Molecular Probes, Eugene, OR); Rapamycin (Calbiochem, Burlington, MA). We purchased the following antibodies from Cell Signaling Technology (Danvers, MA): anti-mTOR antibody (#2972), anti-phospho mTOR antibody (#2971), anti-4E-BP1 antibody (#9452), anti-phospho 4E-BP1 T37/46 antibody (#9459), anti-phospho 4E-BP1 Ser65 antibody (#9451), anti-phospho 4E-BP1 Thr70 antibody (#9455), anti-β-actin antibody. Anti-human PAF-receptor and anti-human IgG isotype-matched control antibodies were purchased from Abcam (Cambridge, MA). The goat-anti-rabbit Alexa Fluor 488 secondary antibody was purchased from Life Technologies (Carlsbad, CA) and the anti-IL6Rα primary antibody from Santa Cruz Biotechnology (Dallas, TX).

Cord and Peripheral PMN Isolation

As approved by the University of Utah Institutional Review Board and in accordance with the Declaration of Helsinki, we enrolled 27 preterm infants and 30 term infants in our study. We defined preterm infants as being born at < 30 0/7 weeks gestation and term infants as being born at > 36 6/7 weeks gestation. We outline the clinical characteristics for these two donor sources for human umbilical cord PMNs in Supplemental Table S1. The indications for preterm delivery are also listed. We isolated human PMNs from healthy adult voluntary donors who self-reported via questionnaire a lack of chronic diseases and/or medications for chronic diseases such as heart disease, kidney disease, or chronic inflammatory conditions. Their ages ranged from 18 to 45 years. Human PMNs were immediately isolated from umbilical cord blood and healthy adult venous blood after collection in acid-citrate dextrose anticoagulant. For all cord blood samples, preterm or term, we collected umbilical cord blood immediately at the time of birth and proceeded with PMN isolation immediately regardless of time of day. We routinely began experiments using cord blood PMNs within 60–90 minutes of delivery. Human PMNs were isolated using positive immunoselection with anti-human CD15 microbeads and the auto-MACS automated cell sorter (Miltenyi Biotec Inc, Auburn, CA). Human PMNs isolated in this manner were > 98% pure as assessed by
morphology and flow cytometry. We used adult PMNs to compare PMN activation profiles following isolation using CD15 positive immunoselection or Ficoll-Paque differential centrifugation. We found no difference in the expression of IL-6Ra or RARα, two mTOR-dependent rapidly translated mRNA transcripts induced in activated PMNs(15, 17). Positive immunoselection also facilitated our study by dramatically improving our PMN isolation yield even for umbilical cord blood samples < 0.5 mL from the most prematurely born neonates. Isolated PMNs were re-suspended at a concentration of $2 \times 10^6$ cells/mL in 37°C, serum-free M-199 media.

**Soluble IL-6 Receptor ELISA**

Acid-citrate dextrose anti-coagulated whole blood samples were centrifuged at 1000 rpm for 5 minutes to collect plasma, which was then frozen at −80°C prior to analysis. Soluble IL-6Ra expression in preterm, term, and adult plasma samples was measured after one freeze-thaw cycle using a human soluble IL-6 Receptor DuoSet ELISA kit purchased from R&D Systems (Minneapolis, MN).

**RT PCR Analysis**

As previously described(15), real time RT-PCR was performed using a primer pair for IL-6Ra and GAPDH mRNA purchased from Stratagene (La Jolla, CA). We also assessed IL-8 mRNA expression using primer pairs synthesized by our University of Utah oligonucleotide synthesis core. Superscript II reverse transcriptase and oligo(dT) primers were used to perform first-strand synthesis. Real-time quantitative RT-PCR was performed using Sybr Green on a BioRad iCycler PCR machine (Hercules, CA). GAPDH mRNA amplification was used to normalize IL-6Ra and IL-8 mRNA expression.

**Flow Cytometry**

Freshly isolated PMNs were re-suspended in 37°C serum free M-199 media at a concentration of $2 \times 10^6$ cells/mL. To determine the plasma membrane PAF-receptor expression on unstimulated PMNs from neonates and adults, PMNs were incubated with either a rabbit anti-human polyclonal PAF-receptor antibody or rabbit anti-human IgG isotype matched control antibody for 30 minutes in FACS buffer followed by 3 washes with PBS. PMNs were then incubated with goat anti-rabbit secondary antibody, fixed with 4% paraformaldehyde, and resuspended in PBS. PAF-receptor expression was determined using a Beckman-Coulter CytoFlex flow cytometer (Brea, CA). Forward and side scatter plots of positively isolated PMNs allowed for gating on the PMN population. Supplemental Figure S1 demonstrates representative gating on PMNs with PAF-receptor expression on the x-axis. Determination of PAF-receptor positivity, both percentage of PAF-receptor positive cells and Mean Fluorescent Intensity, were accomplished using FlowJo FACS software version 10 (FlowJo, LLC, Ashland, OR).

**Western Blot Analysis**

Expression of IL-6Ra protein in term and preterm PMNs was determined using Western blot analysis. Briefly, PMNs were stimulated for 0 – 240 min with 10 nM PAF at 37°C. To block mTOR signaling in a subset of samples, pretreatment with rapamycin (100 nM) was
performed at 37°C for one hour prior to stimulation with PAF. PMN samples were pelleted, resuspended in Laemmli gel-loading buffer, and boiled for 5 minutes. Protein lysates were loaded on 8% SDS/PAGE gels for electrophoresis and transferred to poly-vinylidene difluoride membranes (Millipore, Burlington, MA). Membranes were blocked and assessed for IL-6Rα protein expression using an anti-human IL-6Rα primary antibody as previously described(15). β-actin protein expression was evaluated on the same membrane as a control for equal protein loading.

**Immunocytochemistry**

Freshly isolated PMNs (2 × 10^6 cells/mL) were incubated with control buffer or PAF (10 nM) at 37°C for varying time points during a one hour incubation. After each time point, 40 μL of cell suspension was added to 125μL of HBS containing 0.2% human serum albumin and spun onto glass coverslips using a Cyto-Spin 4 centrifuge (Thermo Scientific, Waltham, MA). PMNs were fixed with 4% paraformaldehyde and permeabilized with 0.05% Triton-X-100. PMNs were blocked with PBS/10% goat serum for one hour at room temperature. Antibodies against mTOR, phospho-mTOR ser2448, 4EBP1, phospho-4EBP1 Thr2448, phospho-4EBP1 Ser65, and phospho-4EBP1 Thr70 were diluted in PBS/10% goat serum and incubated overnight at 4°C. Samples were washed and incubated with a goat anti-rabbit secondary antibody conjugated to the Alexa488 fluorochrome. TOPRO-3 served as a nuclear DNA counterstain. Samples were examined by confocal microscopy using a Fluoview FV1000 confocal microscope and Fluoview software version 4.02 (Olympus, Center Valley, PA). Image quantitation was accomplished using ImageJ image analysis software (NIH, Bethesda, MD).

**Results**

**Plasma collected from preterm infant umbilical cord blood contains lower levels of soluble Interleukin-6 receptor alpha as compared to cord blood and peripheral blood isolated from healthy term infants and adults, respectively.**

Prematurely born newborn infants develop inflammatory syndromes such as sepsis, neonatal CLD, and NEC(2, 4, 6), which result from persistent pathogenic inflammation. The sIL-6Rα/IL-6 trans-signaling system participates in the recruitment of mononuclear cells to areas of acute inflammation, an essential first step towards resolution of potentially injurious persistent inflammation (Fig. 2a)(14). We, therefore, hypothesized that plasma sIL-6Rα expression would be lower in preterm infants compared to healthy term infants and healthy adults. We used ELISA to determine the plasma sIL-6Rα protein expression levels in plasma collected from umbilical cord blood samples of prematurely born infants at risk for sepsis, neonatal CLD, and NEC (gestational age < 30 weeks), from umbilical cord blood plasma of healthy term infants, and from peripheral blood plasma from healthy adults. We found significantly decreased sIL-6Rα levels in preterm infant plasma as compared to plasma obtained from healthy term infants or adults (Fig. 2b). Given the association of PAF with persistent inflammation in newborn infants(6, 10, 16), we next explored the effect of PAF-stimulation on IL-6Rα expression by PMNs isolated from preterm infants compared to healthy term infants and adults.
PAF induces IL-6Rα protein expression in PMNs isolated from healthy term newborn infants, but fails to augment expression of IL-6Rα protein in PMNs isolated from prematurely born infants.

We next determined whether PAF induces expression of IL-6Rα in PMNs isolated from newborn infants, whether term and preterm, as it does in PMNs isolated from healthy adults(15). We found that PAF-stimulation fails to induce increased IL-6Ra mRNA expression in PMNs isolated from newborn infants, whether born at term or prematurely, as assayed quantitatively via real time RT-PCR (Fig. 3a). These PMNs do express IL-6Ra mRNA at baseline (Fig. 3a). PAF-stimulation does, however, induce a robust transcriptional response with increased IL-8 mRNA expression in PMNs isolated from both term and preterm infants, suggesting that the lack of increased IL-6Ra mRNA expression does not result from a global failure of the transcriptional response in preterm infants (Fig. 3b). We also used flow cytometry to determine the expression of the PAF-receptor on PMNs isolated from both term and preterm infants as well as healthy adults. We found that while the PAF-receptor expression on PMNs isolated from the newborn infants was significantly decreased compared to PMNs isolated from healthy adults, PMNs isolated from preterm infants express the receptor for PAF with greater frequency than those isolated from term infants (Fig. 3c). Furthermore, PMNs isolated from preterm infants demonstrated a higher mean fluorescent intensity of PAF-receptor expression than did PMNs isolated from term neonates or from adults (Fig. 3d). Decreased PAF-receptor expression does not, therefore, completely explain these findings.

We next found that PAF-stimulation induces de novo IL-6Rα protein expression within 120 minutes of stimulation in PMNs isolated from healthy term infants, and this IL-6Rα protein expression persists and increases throughout a 240 minute time course (Fig. 3e). PAF-stimulated PMNs isolated from preterm infants, however, show decreased IL-6Rα protein expression over a 240 minute time course (Fig. 3E). Furthermore, given the rapidity of PAF-induced IL-6Rα protein expression in term PMNs and the unchanged but present expression of IL-6Ra mRNA, we hypothesized that IL-6Rα protein expression is regulated at the translational level via the mTOR pathway as it is in PMNs isolated from healthy adults(15). Western blotting demonstrated that rapamycin (100 nM; 120 minutes), a potent and specific inhibitor of mTOR signaling, blocks IL-6Rα protein expression in experiments performed in parallel (Fig. 3e). Taken together, these results suggest altered mTOR signaling in PAF-stimulated PMNs isolated from preterm infants.

PMNs isolated from preterm infants demonstrate dysregulated mTOR pathway signaling following PAF stimulation.

We next used semi-quantitative immunocytochemistry with a phospho-specific antibody raised against the activating serine2448 residue of the mTOR complex to determine mTOR activation status in PMNs isolated from preterm infants as well as healthy term infants and adults. We first demonstrated low level baseline phosphorylation of mTOR serine2448 and no increase in phosphorylation through 60 minutes following PAF stimulation in PMNs isolated from preterm infants (Fig. 4a, b). In PMNs isolated from healthy term infants and adults assayed in parallel, however, we demonstrated minimal baseline phosphorylation of mTOR ser2448 with significantly increased mTOR ser2448 phosphorylation at 30 and 60 minutes.
following PAF stimulation for term infant PMNs, and at 30 minutes for adult PMNs (Fig. 4a, b). This differential activation of mTOR by PAF in preterm PMNs as compared to PMNs isolated from healthy term infants and adults led us to assay the activation of one downstream effector molecule in the mTOR pathway, elongation factor 4E – binding protein 1(4EBP1) in these primary human neutrophils.

**PAF fails to induce the hierarchical phosphorylation pattern leading to activation of 4EBP1 in PMNs isolated from preterm infants.**

We next assayed phosphorylation of 4EBP1 using semi-quantitative immunocytochemistry with phospho-specific antibodies against four separate phosphorylation sites. Following activation of the mTOR pathway, Gingrich, et al. demonstrated a hierarchical phosphorylation pattern for 4EBP1 with the threonine$^{46}$ and threonine$^{37}$ sites phosphorylated first, then the threonine$^{70}$ site, and finally the serine$^{65}$ site(18). Only after all sites are phosphorylated in order does 4EBP1 dissociate from eIF4E and allow incorporation of eIF4E into the initiation complex thus enabling translation of select mRNA molecules such as IL-6Ra(15). We consistently found decreased 4EBP1 phosphorylation at three of the four prospective phosphorylation sites in PAF-stimulated preterm PMNs over 60 minutes as compared to PAF-stimulated PMNs isolated from healthy adults (Fig. 5). 4EBP1 phosphorylation at the thr$^{37}$/thr$^{46}$ sites in adult PAF-stimulated PMNs increases over the 5, 15 and 30 minute time points before decreasing, while PAF-stimulated PMNs isolated from term and preterm infants fail to increase dramatically over a 60 minute time course (Fig. 5a). 4EBP1 phosphorylation at the thr$^{70}$ site increases for PAF-stimulated PMNs from all three groups over the 5 and 15 minute time points with decreases back to baseline by the 30 and 60 minute time points in PAF-stimulated adult PMNs. PAF-stimulated PMNs isolated from newborn infants, both preterm and term, demonstrated increases in 4EBP1 thr$^{70}$ phosphorylation throughout the 60 minute time course (Fig. 5b). Phosphorylation of 4EBP1 at the ser$^{65}$ site, the final signaling event in the hierarchical phosphorylation pattern of 4EBP1 before its release of eIF4E as a binding partner, remains low throughout the 60 minute time course in PAF-stimulated PMNs from preterm infants, while increases of greater than 5 fold are seen following PAF stimulation in PMNs isolated from healthy term infants and healthy adults (Fig. 5c).

We also demonstrated that 4EBP1 phosphorylation at the ser$^{37}$/thr$^{46}$ sites requires mTOR kinase activity by completely inhibiting PAF-stimulated thr$^{46}$/thr$^{37}$ phosphorylation following pretreatment with rapamycin (Fig. 6). As expected, rapamycin does not inhibit the PAF-induced phosphorylation of mTOR at its ser$^{2448}$ residue (Fig. 6).

**Discussion**

Dysregulated inflammation represents a significant cause of morbidity and mortality in humans of all ages, not just neonates. Studies performed in our laboratory defined the role of the mTOR protein translation regulatory pathway in neutrophils isolated from healthy adults, demonstrating that quiescent mRNAs such as IL-6Ra or the transcription factor retinoic acid receptor alpha are translated into functional protein within minutes of stimulation leading to alterations in the PMN response within the inflammatory milieu(15, 17). Additional reports
now confirm the importance mTOR as a regulator of the IL-6/IL-6Rα trans-signaling system. Garbers et al. showed that mTOR is a central molecular switch with governs both the classic and trans-signaling of IL-6R with implications for cellular senescence and tumor development(19). Ruwanpura et al. recently targeted sIL-6Rα trans-signaling in a mouse model of emphysema and demonstrated mTOR hyperactivation in emphysema when sIL-6Rα levels were elevated(20). With this report, we implicate for the first time the mTOR pathway in the complex neutrophil dysfunction exhibited by PMNs isolated from human neonates. We found significantly decreased mTOR phosphorylation at the ser2448 site in PMNs isolated from preterm neonates compared to PMNs isolated from term newborns and healthy adults. Furthermore, downstream signaling from mTOR to 4EBP1 was also decreased at three out of the four phosphorylation sites studied in preterm PMNs. While these semi-quantitative techniques do not prove causation for failed IL-6Rα protein expression in PMNs isolated from preterm infants, these studies represent the first association of differential mTOR signaling with aberrant protein expression in neutrophils isolated from prematurely born infants. Indeed, a strength of this report is the use of primary human PMNs isolated from the umbilical cord blood of healthy term neonates and from neonates born with significant prematurity (gestational age < 30 weeks). This is the patient population most at risk for the inflammatory syndromes associated with prematurity including neonatal CLD, early and late onset sepsis, NEC, and ROP(3–7). These findings are, therefore, translational in nature. Primary human PMNs isolated from healthy adults serve as a further control for these studies of umbilical cord blood isolated neutrophils.

One weakness of our study, however, is our inability to correlate predisposing prenatal factors for infection and inflammation with these studies of mTOR activity. As described in Supplemental Table S1, we did track the indication for preterm delivery as well as the mode of delivery. While we did recruit 27 different preterm infant umbilical cord blood donors, we performed separate experiments with a sample size of 4–5 for most of the experiments. Consequently, our sample size for the individual experiments remains too low to assess the role of prenatal infection or placental insufficiency in altering mTOR signaling in response to PAF. In addition, in our cohort, we did not track the type of anesthesia used at delivery(21) or the maternal group B Streptococcus colonization status(22), all of which can potentially affect neutrophil inflammatory responses. As such, we cannot determine whether the reason for preterm birth explains our findings. This deficiency of our study will require further exploration in a larger cohort of preterm infants.

While the role of the IL-6/IL-6Rα trans-signaling system remains essentially unexplored in newborn infant PMNs, alterations in its trans-signaling components represent a potential mechanism to mediate both the pro- and anti-inflammatory effects of IL-6. Indeed, the importance of the IL-6/IL-6Rα trans-signaling system is well established for cells of the hematopoietic lineage; hematopoietic cells depend on sIL-6R for self-renewal(23), and ablation of IL-6R leads to a reduction in sIL-6R levels of > 60% with decreased wound healing in mice(24). In addition, intra-amniotic inflammation increases levels of IL-6 trans-signaling components such as IL-6 and sIL-6Rα in amniotic fluid(25). Here we suggest that decreased synthesis and shedding of IL-6Rα by stimulated neonatal PMNs isolated from preterm infants may decrease IL-6/sIL-6R trans-signaling to endothelial cells. This may in turn lead to decreased macrophage efferocytosis of “spent” neutrophils resulting in
neutrophil enrichment in the inflammatory milieu with subsequent and persistent inflammatory tissue damage. Rabe et al. demonstrated that the IL-6/IL-6Rα trans-signaling system participates in the recruitment of macrophages to areas of inflammation where they abrogate persistent inflammatory responses(26). Macrophage participation in modulating the inflammatory milieu may also extend to the clearance of NETs, which are implicated in inflammatory tissue damage caused by infection and chronic inflammation as well as with development of autoimmune disorders. Several recent reports demonstrate the importance of macrophage-mediated clearance of NETs in vitro and in models of influenza pneumonitis and acute respiratory distress syndrome(27–29). While this hypothesis is not tested in these studies, our findings lay the foundation for future studies to examine the role of differential IL-6/sIL-6Rα trans-signaling in inflammatory conditions pertinent to prematurely born infants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Platelet-activating factor and lipopolysaccharide signal to NFκB and mTOR in neutrophils.
We depict two major inflammatory signaling pathways described in neutrophils – NFκB acting as a transcription factor of induce expression of inflammatory gene products – and mTOR – regulating translation and expression of additional regulatory molecules key to the acute inflammatory response by neutrophils. Together, signaling through Toll-like Receptor 4 or the PAF-receptor regulate multiple aspects of neutrophil responses to inflammation through activating the NFκB and mTOR signaling cascades.
Figure 2. Umbilical cord blood plasma from preterm infants has decreased circulating levels of soluble IL-6 receptor.

a. A model of neutrophil participation in the IL-6Rα/IL-6 trans-signaling system. The synthesis of IL-6Rα by PMNs is regulated at the translational level via the mTOR pathway. Membrane bound IL-6Rα is cleaved by ADAM17 and released into the circulation. Soluble IL-6Rα then binds IL-6 in the circulation and can confer IL-6 sensitivity to any cell expressing the gp130 glycoprotein, an endothelial cell in this schematic. IL-6/IL-6Rα trans-signaling modulates the acute inflammatory response.

b. We isolated plasma from the umbilical cord blood of newborn infants, both term (n=26) and preterm (n=30), and from peripheral blood samples from healthy adults (n=18). Plasma soluble IL-6 receptor levels (ng/mL, mean ± SEM) were determined using ELISA. We applied the one-way ANOVA statistical test with Tukey’s Multiple Comparison Test post hoc analysis. * denotes p<0.05, *** denotes p<0.001.
IL-6Rα mRNA and protein expression in newborn PMNs are consistent with translational regulation of gene expression via the mTOR pathway.

**a.** We determined IL-6Rα mRNA expression in PAF-stimulated PMNs (10 nM; 120 minutes) isolated from term (n=6) and preterm (n=4) newborn infants using quantitative RT-PCR. mRNA expression levels are shown as fold change over baseline after normalization to GAPDH (mean ± SEM) with cycle-to-threshold values also shown (mean ± SEM). The baseline expression level was arbitrarily set at 1.

**b.** We also determined IL-8 mRNA expression in PAF-stimulated PMNs (10 nM; 120 minutes) isolated from term (n=6) and preterm (n=4) newborn infants using quantitative RT-PCR. mRNA expression levels are shown as fold change over baseline after normalization to GAPDH (mean ± SEM) with cycle-to-threshold values also shown (mean ± SEM). The baseline expression level was arbitrarily set at 1.

**c.** Plasma membrane PAF-receptor expression on unstimulated PMNs isolated from healthy adults, term infants, and preterm infants was determined using flow cytometry. The y-axis shows the percentage of PMNs positive for PAF-receptor fluorescence (mean ± SEM). We employed the one-way ANOVA statistical tool with Dunnett’s Multiple

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**Figure 3.** IL-6Rα mRNA and protein expression in newborn PMNs are consistent with translational regulation of gene expression via the mTOR pathway.

- **a.** We determined IL-6Rα mRNA expression in PAF-stimulated PMNs (10 nM; 120 minutes) isolated from term (n=6) and preterm (n=4) newborn infants using quantitative RT-PCR. mRNA expression levels are shown as fold change over baseline after normalization to GAPDH (mean ± SEM) with cycle-to-threshold values also shown (mean ± SEM). The baseline expression level was arbitrarily set at 1.
- **b.** We also determined IL-8 mRNA expression in PAF-stimulated PMNs (10 nM; 120 minutes) isolated from term (n=6) and preterm (n=4) newborn infants using quantitative RT-PCR. mRNA expression levels are shown as fold change over baseline after normalization to GAPDH (mean ± SEM) with cycle-to-threshold values also shown (mean ± SEM). The baseline expression level was arbitrarily set at 1.
- **c.** Plasma membrane PAF-receptor expression on unstimulated PMNs isolated from healthy adults, term infants, and preterm infants was determined using flow cytometry. The y-axis shows the percentage of PMNs positive for PAF-receptor fluorescence (mean ± SEM). We employed the one-way ANOVA statistical tool with Dunnett’s Multiple.
Comparisons post hoc testing. * denotes $p<0.05$ and *** denotes $p<0.001$. d. We also determined the mean fluorescent intensity (MFI) of PAF-receptor expression for the same PMNs studied in c using flow cytometry. The y-axis shows MFI for PAF-receptor expression (mean ± SEM). No statistically significant differences were detected. e. IL-6Rα protein expression was determined using Western blotting for PAF-stimulated PMNs (10 nM; 0–240 minutes) isolated from term and preterm newborns. β-actin protein expression is shown as a control for equal protein loading. We used rapamycin pretreatment for 120 minutes (100 nM) to block mTOR signaling. These Western blots are representative of > 4 separate experiments performed in PMNs isolated from 4 different term and preterm newborn umbilical cord blood samples.
Figure 4. PAF does not induce mTOR phosphorylation in PMNs isolated from preterm infant cord blood.

**a.** We assessed mTOR phosphorylation at the serine$^{2448}$ residue in PAF-stimulated PMNs (10 nM; 0–60 minutes) isolated from preterm infants, healthy term infants, and healthy adults using immunocytochemistry. Blue fluorescence shows nuclear DNA and yellow fluorescence shows phosphorylated mTOR. Scale bar denotes 20 μm. The baseline (0 minute) and 30 minute images are representative of 5 separate experiments conducted using PMNs from 5 separate donors in each group.

**b.** Semi-quantitative analysis of the immunocytochemistry images was performed using ImageJ software. The y-axis shows mTOR phosphorylation in relative fluorescence units as a fold change over baseline, which was arbitrarily set at 1. The x-axis shows the time points assessed over the 60 minute experiment. We used the two way ANOVA statistical tool with Bonferroni post-hoc testing. ** denotes $p<0.01$ and * denotes $p<0.001$. 

**Campbell et al.**

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Figure 5. PAF fails to induce the hierarchal phosphorylation of 4EBP-1 in PMNs isolated from preterm infant cord blood.

a. We assessed 4EBP-1 phosphorylation at the threonine\(^{37}\) and threonine\(^{46}\) residues in PAF-stimulated PMNs (10 nM; 0–60 minutes) isolated from preterm infants, healthy term infants, and healthy adults using immunocytochemistry. Blue fluorescence shows nuclear DNA and yellow fluorescence shows 4EPB-1 phosphorylated at the threonine\(^{37}\) and threonine\(^{46}\) sites. The baseline (0 minute) and 15 minute images are representative of at least 3 separate experiments conducted using PMNs from 3–5 separate donors in each group. Semi-quantitative analysis of the immunocytochemistry images was performed using ImageJ software. The y-axis shows mTOR phosphorylation in relative fluorescence units as a fold change over baseline, which was arbitrarily set at 1. The x-axis shows the time points assessed over the 60 minute experiment. 

b. We assessed 4EBP-1 phosphorylation at the
threonine\textsuperscript{70} residue in PAF-stimulated PMNs (10 nM; 0–60 minutes) isolated from preterm infants, healthy term infants, and healthy adults with immunocytochemistry using identical techniques, semi-quantitation, and staining as in \textbf{a}. The baseline (0 minute) and 15 minute images are representative of at least 3 separate experiments conducted using PMNs from 3–5 separate donors in each group. \textbf{c}. We assessed 4EBP-1 phosphorylation at the serine\textsuperscript{65} residue in PAF-stimulated PMNs (10 nM; 0–60 minutes) isolated from preterm infants, healthy term infants, and healthy adults with immunocytochemistry using identical techniques, semi-quantitation, and staining as in \textbf{a}. The baseline (0 minute) and 30 minute images are representative of at least 3 separate experiments conducted using PMNs from 3–5 separate donors in each group.
Figure 6. mTOR activation is required for the phosphorylation of 4EPB-1 in PMNs isolated from healthy adults.

We assessed 4EBP-1 phosphorylation at the threonine$^{37}$ and threonine$^{46}$ residues (top row) and mTOR phosphorylation at the serine$^{2448}$ residue (bottom row) in PAF-stimulated PMNs (10 nM; 0–30 minutes) isolated from healthy adults using immunocytochemistry. PMNs were pre-incubated for 60 minutes with the mTOR inhibitor rapamycin (100 nM, 1 hour) or vehicle control prior to stimulation with PAF. Blue fluorescence shows nuclear DNA and yellow fluorescence shows 4EBP-1 phosphorylated at the threonine$^{37}$ and threonine$^{46}$ sites (top row) or mTOR phosphorylated at the serine$^{2448}$ site (bottom row). The control and 30 minute images are representative of 4 separate experiments conducted using PMNs from 4 separate donors in each group.