BASIC SCIENCE ARTICLE

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

Robert A. Campbell¹, Mark J. Cody², Bhanu K. Manne¹, Guy A. Zimmerman¹ and Christian C. Yost^{1,2}

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, an 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.

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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 antimicrobial 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, overexuberant 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—nuclear factor-κB (NFκB) 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

¹Program in Molecular Medicine, University of Utah, Salt Lake City, UT, USA and ²Department of Pediatrics/Neonatology, University of Utah, Salt Lake City, UT, USA
Correspondence: Christian C. Yost (christian.yost@u2m2.utah.edu)

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Fig. 1 Platelet-activating factor (PAF) and lipopolysaccharide (LPS) signal to nuclear factor-κB (NFκB) and mammalian target of rapamycin (mTOR) in neutrophils. We depict two major inflammatory signaling pathways described in neutrophils—NFκB acting as a transcription factor to induce the 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

two receptor components transduce IL-6 binding into activation of the Janus-activated kinase/signal transducer and activator of transcription factor, extracellular signal-regulated kinase, and phosphoinositide-3 kinase 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: PAF (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), and 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 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 min 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-6Rα 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 resuspended at a concentration of 2×10^6 cells/mL in 37 °C, serum-free M-199 media.

Soluble IL-6 receptor enzyme-linked immunosorbent assay (ELISA) Acid-citrate dextrose anti-coagulated whole-blood samples were centrifuged at 1000 rpm for 5 min to collect plasma, which was then frozen at –80 °C prior to analysis. Soluble IL-6Rα 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).

Reverse transcriptase-PCR (RT-PCR) analysis

As previously described,¹⁵ real-time RT-PCR was performed using a primer pair for *IL-6Rα* 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 resuspended in 37 °C, serum-free M-199 media at a concentration of 2×10^6 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 min in FACS buffer followed by 3 washes with phosphate-buffered saline (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 Fig. 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 the 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 1 h prior to stimulation with PAF. PMN samples were pelleted, resuspended in Laemmli gel-loading buffer, and boiled for 5 min. Protein lysates were loaded on 8% sodium dodecyl sulfate/polyacrylamide gel electrophoresis gels for electrophoresis and transferred to poly-vinylidene difluoride membranes (Millipore, Burlington, MA). Membranes were blocked and assessed for IL-6Ra protein expression using an anti-human IL-6Ra 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 1-h incubation. After each time point, 40 μ L of cell suspension was added to 125 μ L 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 1 h at room temperature. Antibodies against mTOR, phospho-mTOR ser²⁴⁴⁸, 4E-BP1, phospho-4E-BP1 Thr^{37/46}, phospho-4E-BP1 Ser⁶⁵, and phospho-4E-BP1 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 the ImageJ image analysis software (NIH, Bethesda, MD).

RESULTS

Plasma collected from preterm infant umbilical cord blood contains lower levels of sIL-6Ra 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-6Ra/IL-6 trans-signaling system participates in the recruitment of mononuclear cells to areas of acute inflammation, an essential first step toward resolution of potentially injurious persistent inflammation (Fig. 2a).¹⁴ We, therefore, hypothesized that plasma sIL-6Ra expression would be lower in preterm infants compared to healthy term infants and healthy adults. We used ELISA to determine the plasma sIL-6Ra 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-6Ra 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-6Ra expression by PMNs isolated from preterm infants compared to healthy term infants and adults.

PAF induces IL-6Ra protein expression in PMNs isolated from healthy term newborn infants but fails to augment expression of IL-6Ra protein in PMNs isolated from prematurely born infants. We next determined whether PAF induces the expression of IL-6Ra 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-6Ra protein expression within 120 min of stimulation in PMNs isolated from healthy term infants, and this IL-6Ra protein expression persists and increases throughout a 240-min time course (Fig. 3e). PAF-stimulated PMNs isolated from preterm infants, however, show decreased IL-6Ra protein expression over a 240-min time course (Fig. 3e). Furthermore, given the rapidity of PAF-induced IL-6Ra protein expression in term PMNs and the unchanged but present expression of *IL-6Ra* mRNA, we hypothesized that IL-6Ra 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; 60 min), a potent and specific inhibitor of mTOR signaling, blocks IL-6Ra 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.

Fig. 2 Umbilical cord blood plasma from preterm infants has decreased circulating levels of soluble interleukin (IL)-6 receptor (IL-6R). **a** A model of neutrophil participation in the IL-6R α /IL-6 *trans*-signaling system. The synthesis of IL-6R α by polymorphonuclear leukocytes is regulated at the translational level via the mammalian target of rapamycin pathway. Membrane-bound IL-6R α is cleaved by ADAM17 and released into the circulation. Soluble IL-6R α 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-6R α *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-6R levels (ng/mL, mean $\pm$ SEM) were determined using enzyme-linked immunosorbent assay. We applied the one-way analysis of variance statistical test with Tukey's Multiple Comparison Test post hoc analysis. * $p < 0.05$, *** $p < 0.001$

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 serine 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 min following PAF stimulation in PMNs isolated from preterm infants (Fig. 4a, b). In PMNs isolated from healthy term infants and adults, however, we demonstrated minimal baseline phosphorylation of mTOR ser²⁴⁴⁸ with significantly increased mTOR ser²⁴⁴⁸ phosphorylation at 30 and 60 min following PAF stimulation for term infant PMNs and at 30 min 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 (4E-BP1), 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 4E-BP1 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 4E-BP1 with the threonine⁴⁶ and threonine³⁷ sites phosphorylated first, then the threonine⁷⁰ site, and finally the serine⁶⁵ site.¹⁸ Only after all sites are phosphorylated in order does 4E-BP1 disassociate from eIF4E and allow incorporation of eIF4E into the initiation complex thus enabling translation of select mRNA molecules,

such as IL-6R α .¹⁵ We consistently found decreased 4E-BP1 phosphorylation at three of the four prospective phosphorylation sites in PAF-stimulated preterm PMNs over 60 min as compared to PAF-stimulated PMNs isolated from healthy adults (Fig. 5). 4E-BP1 phosphorylation at the thr³⁷/thr⁴⁶ sites in adult PAF-stimulated PMNs increases over the 5-, 15-, and 30-min time points before decreasing, while PAF-stimulated PMNs isolated from term and preterm infants fail to increase dramatically over a 60-min time course (Fig. 5a). 4E-BP1 phosphorylation at the thr⁷⁰ site increases for PAF-stimulated PMNs from all three groups over the 5- and 15-min time points with decreases back to baseline by the 30- and 60-min time points in PAF-stimulated adult PMNs. PAF-stimulated PMNs isolated from newborn infants, both preterm and term, demonstrated increases in 4E-BP1 thr⁷⁰ phosphorylation throughout the 60-min time course (Fig. 5b). Phosphorylation of 4E-BP1 at the ser⁶⁵ site, the final signaling event in the hierarchical phosphorylation pattern of 4E-BP1 before its release of eIF4E as a binding partner, remains low throughout the 60-min time course in PAF-stimulated PMNs from preterm infants, while increases of greater than five-fold are seen following PAF stimulation in PMNs isolated from healthy term infants and healthy adults (Fig. 5c).

We also demonstrated that 4E-BP1 phosphorylation at the thr³⁷/thr⁴⁶ sites requires mTOR kinase activity by completely inhibiting PAF-stimulated thr³⁷/thr⁴⁶ phosphorylation following pretreatment with rapamycin (Fig. 6). As expected, rapamycin does not inhibit the PAF-induced phosphorylation of mTOR at its ser²⁴⁴⁸ 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-6Ra *trans*-signaling system. Garbers et al. showed that mTOR is a central molecular switch that 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-6Ra *trans*-signaling in a mouse model of emphysema and demonstrated mTOR hyperactivation in emphysema when sIL-6Ra 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 4E-BP-1 was also decreased at three out of the four phosphorylation sites

Fig. 3 Interleukin-6 receptor alpha (*IL-6Ra*) mRNA and protein expression in newborn polymorphonuclear leukocytes (PMNs) are consistent with translational regulation of gene expression via the mammalian target of rapamycin (mTOR) pathway. **a** We determined *IL-6Ra* mRNA expression in platelet-activating factor (PAF)-stimulated PMNs (10 nM; 120 min) isolated from term ($n = 6$) and preterm ($n = 4$) newborn infants using quantitative reverse transcriptase-PCR (RT-PCR). mRNA expression levels are shown as fold change over baseline after normalization to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (mean $\pm$ SEM) with cycle-to-threshold values also shown (mean $\pm$ 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 min) 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 $\pm$ SEM) with cycle-to-threshold values also shown (mean $\pm$ 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. y axis shows the percentage of PMNs positive for PAF receptor fluorescence (mean $\pm$ SEM). We employed the one-way analysis of variance statistical tool with Dunnett's Multiple Comparisons post hoc testing. $*p < 0.05$ and $***p < 0.001$. **d** We also determined the mean fluorescent intensity (MFI) of PAF receptor expression for the same PMNs studied in **c** using flow cytometry. y axis shows MFI for PAF receptor expression (mean $\pm$ SEM). No statistically significant differences were detected. **e** *IL-6Ra* protein expression was determined using western blotting for PAF-stimulated PMNs (10 nM; 0–240 min) isolated from term and preterm newborns. β -Actin protein expression is shown as a control for equal protein loading. We used rapamycin pretreatment for 60 min (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

Fig. 4 Platelet-activating factor (PAF) does not induce mammalian target of rapamycin (mTOR) phosphorylation in polymorphonuclear leukocytes (PMNs) isolated from preterm infant cord blood. **a** We assessed mTOR phosphorylation at the serine²⁴⁴⁸ residue in PAF-stimulated PMNs (10 nM; 0–60 min) 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 μ m. The baseline (0 min) and 30-min images are representative of 5 separate experiments conducted using PMNs from 5 separate donors in each group. **b** Semiquantitative analysis of the immunocytochemistry images was performed using the ImageJ software. y axis shows mTOR phosphorylation in relative fluorescence units as a fold change over baseline, which was arbitrarily set at 1. x axis shows the time points assessed over the 60-min experiment. We used the two-way analysis of variance statistical tool with Bonferroni post hoc testing. $**p < 0.01$ and $*p < 0.001$

studied in preterm PMNs. While these semiquantitative techniques do not prove causation for failed *IL-6Ra* 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 delivery²¹ 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-6Ra 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-6Ra trans*-signaling system is well established for cells of the hematopoietic lineage; hematopoietic cells depend on *sIL-6R* for self-renewal²³ 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-6Ra* in amniotic fluid.²⁵ Here we suggest that decreased synthesis and shedding of *IL-6Ra* 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

Fig. 5 Platelet-activating factor (PAF) fails to induce the hierarchical phosphorylation of elongation factor 4E-binding protein 1 (4E-BP-1) in polymorphonuclear leukocytes (PMNs) isolated from preterm infant cord blood. **a** We assessed 4E-BP-1 phosphorylation at the threonine³⁷ and threonine⁴⁶ residues in PAF-stimulated PMNs (10 nM; 0–60 min) isolated from preterm infants, healthy term infants, and healthy adults using immunocytochemistry. Blue fluorescence shows nuclear DNA and yellow fluorescence shows 4E-BP-1 phosphorylated at the threonine³⁷ and threonine⁴⁶ sites. The baseline (0 min) and 15-min images are representative of at least 3 separate experiments conducted using PMNs from 3–5 separate donors in each group. Semiquantitative analysis of the immunocytochemical images was performed using the ImageJ software. y axis shows phosphorylation of 4E-BP-1 at the threonine³⁷ and threonine⁴⁶ sites in relative fluorescence units as a fold change over baseline, which was arbitrarily set at 1. x axis shows the time points assessed over the 60-min experiment. **b** We assessed 4E-BP-1 phosphorylation at the threonine⁷⁰ residue in PAF-stimulated PMNs (10 nM; 0–60 min) isolated from preterm infants, healthy term infants, and healthy adults with immunocytochemistry using identical techniques, semiquantitation, and staining as in **a**. The baseline (0 min) and 15-min images are representative of at least 3 separate experiments conducted using PMNs from 3–5 separate donors in each group. **c** We assessed 4E-BP-1 phosphorylation at the serine⁶⁵ residue in PAF-stimulated PMNs (10 nM; 0–60 min) isolated from preterm infants, healthy term infants, and healthy adults with immunocytochemistry using identical techniques, semiquantitation, and staining as in **a**. The baseline (0 min) and 30-min images are representative of at least 3 separate experiments conducted using PMNs from 3–5 separate donors in each group

enrichment in the inflammatory milieu with subsequent and persistent inflammatory tissue damage. Rabe et al. demonstrated that the IL-6/IL-6Ra *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-6Ra *trans*-signaling in inflammatory conditions pertinent to prematurely born infants.

Fig. 6 Mammalian target of rapamycin (mTOR) activation is required for the phosphorylation of elongation factor 4E-binding protein 1 (4E-BP1) in polymorphonuclear leukocytes (PMNs) isolated from healthy adults. We assessed 4E-BP1 phosphorylation at the threonine³⁷ and threonine⁴⁶ residues (top row) and mTOR phosphorylation at the serine²⁴⁴⁸ residue (bottom row) in platelet-activating factor (PAF)-stimulated PMNs (10 nM; 0–30 min) isolated from healthy adults using immunocytochemistry. PMNs were pre-incubated with the mTOR inhibitor rapamycin (100 nM, 1 h) or vehicle control prior to stimulation with PAF. Blue fluorescence shows nuclear DNA and yellow fluorescence shows 4E-BP1 phosphorylated at the threonine³⁷ and threonine⁴⁶ sites (top row) or mTOR phosphorylated at the serine²⁴⁴⁸ site (bottom row). The control and 30-min images are representative of 4 separate experiments conducted using PMNs from 4 separate donors in each group

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AUTHOR CONTRIBUTIONS

C.C.Y. and G.A.Z. conceived of the project and designed the experiments. C.C.Y. performed the patient phenotyping and recruited study participants. R.A.C., M.J.C., B.K.M., and C.C.Y. conducted experiments. R.A.C. and C.C.Y. wrote the manuscript.

ADDITIONAL INFORMATION

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