Cellular metabolism constrains innate immune responses in early human ontogeny

Bernard Kan, Christina Michalski, Helen Fu, Hilda H.T. Au, Kelsey Lee, Elizabeth A. Marchant, Maye F. Cheng, Emily Anderson-Baucum, Michal Aharoni-Simon, Peter Tilley, Raghavendra G. Mirmira, Colin J. Ross, Dan S. Luciani, Eric Jan & Pascal M. Lavoie

Pathogen immune responses are profoundly attenuated in fetuses and premature infants, yet the mechanisms underlying this developmental immaturity remain unclear. Here we show transcriptomic, metabolic and polysome profiling and find that monocytes isolated from infants born early in gestation display perturbations in PPAR-γ-regulated metabolic pathways, limited glycolytic capacity and reduced ribosomal activity. These metabolic changes are linked to a lack of translation of most cytokines and of MALT1 signalosome genes essential to respond to the neonatal pathogen Candida. In contrast, they have little impact on housekeeping phagocytosis functions. Transcriptome analyses further indicate a role for mTOR and its putative negative regulator DNA Damage Inducible Transcript 4-Like in regulating these metabolic constraints. Our results provide a molecular basis for the broad susceptibility to multiple pathogens in these infants, and suggest that the fetal immune system is metabolically programmed to avoid energetically costly, dispensable and potentially harmful immune responses during ontogeny.
Infection is a leading cause of neonatal mortality worldwide, responsible for over one million deaths each year in infants under 28 days of age. Infants born prematurely are particularly vulnerable to severe infections. Indeed, one out of six preterm infants born below 37 weeks of gestation will develop a life-threatening infection in their first month or life, owing to immaturity of their immune defenses.

*Candida species* (spp.) are a major neonatal pathogen, and remain an important cause of mortality from sepsis in premature infants despite a decreasing incidence of candidiasis over the last decade. In healthy adults, this micro-organism rarely causes invasive disease. In contrast, newborns are more vulnerable, especially those who are born below 33 weeks of gestation. This warrants research to understand the molecular basis for their increased susceptibility to *Candida*, but also to other neonatal pathogens. Prevention of systemic infection in humans requires immune recognition and phagocytosis via Pattern Recognition Receptors (PRR). PRR-mediated immune responses are profoundly attenuated during gestation, until about 29 to 33 weeks.

Despite the major clinical impact of these functional deficits, we lack a molecular understanding of how these responses are regulated during ontogeny. *Candida* spp. can exist as yeast or hyphae. C-type lectins and Toll-like receptors (TLRs) are the main PRRs involved in the immune recognition of *Candida* spp. Yeast forms are strongly detected by the C-type lectin receptor dectin-1 and predominate in the bloodstream during invasion. In contrast, hyphae can also be recognized via TLR2 and TLR4 in addition to a main role for C-type lectin receptors. Dectin-1 binds the fungal cell wall component β-1,3-glucan, resulting in the production of pro-inflammatory cytokines including interleukin-1β (IL-1β). This cytokine is crucial for rapid innate immune responses, but also for the generation of a long-lasting mucosal immunity against *Candida*. At the cellular level, monocytes (in blood) and macrophages (in tissues) are the main source of IL-1β. Dendritic cells play an important role in presenting fungal antigens to T cells. However, monocytes demonstrate increased reactivity to *Candida albicans* compared to dendritic cells and macrophages, highlighting the importance of the former in preventing disseminated infection through blood.

Production of IL-1β in monocytes can occur via two main cellular pathways: In the canonical pathway, activation of PRRs results in expression of the *Il1b* gene, which is then translated into the pro-IL-1β precursor protein until a second danger-associated signal (e.g., tissue damage) leads to proteolytic cleavage of pro-IL-1β and secretion of its mature IL-1β form via the NLRP3/Caspase-1 inflammasome (reviewed in ref. 6). Alternatively, in the non-canonical pathway, both the production of pro-IL-1β and its cleavage into mature IL-1β by caspase-8 occur via activation of the MALT1/Bcl10/CARD9 signalosome pathway. In humans, the importance of the signalosome is evidenced by data showing increased invasive *Candida* infections in subjects carrying loss-of-function mutations in CARD9.

Despite decades of research into the functional characterization of the neonatal immune system, we lack an understanding of the mechanisms regulating PRR responsiveness during ontogeny. To address this question, we studied responses to *Candida* spp., a major neonatal pathogen that is recognized through multiple PRRs, and for which relatively little is known in human newborns. Combining transcriptomic, metabolic and polysome profiling studies in an unbiased way, we find that neonatal monocytes are metabolically skewed and lack translation of key immune response genes in a gestational age-dependent manner. Our data provide a mechanism whereby the broad attenuation in PRR responses in early gestation can concurrently increase vulnerability of preterm infants to multiple neonatal pathogens. In light of these results, we propose that preterm immune cells are metabolically reprogrammed to avoid innate immune activation signals in early ontogeny, at the same time offering potential therapeutic avenues to restore immune deficits and reduce infections in these high-risk infants.

**Results**

**Lack of anti-fungal immune recognition in early gestation.** To examine responses to *Candida species*, we first compared monocytes’ ability to phagocytose clinical strains of this micro-organism between preterm, term neonatal and adult subjects. Notably, preterm cells phagocytosed *Candida albicans* as well as adults (Fig. 1a). On the other hand, mononuclear cells from preterm neonates were unable to produce a cytokine response in presence of *Candida albicans* or *C. parapsilosis*, as demonstrated by a lack of production of IL-1β, IL-6 (Fig. 1b, c) and of other cytokines (Supplementary Figure 1). When assaying specific receptors, preterm neonatal cells also did not respond to dectin-1 (using curdlan), TLR2 (using zymosan) or TLR4 (using LPS) stimulation (Fig. 1d-g) despite a strong response detected in adult and term neonatal cells. At the transcript level, responses to LPS were generally stronger then responses to curdlan across all three age groups (Supplementary Figure 2). However, cytokine gene responses to curdlan were reduced in preterm subjects, suggesting an upstream dectin-1 signaling deficit in the latter age group (Supplementary Figure 2).

The importance of dectin-1 for recognition of *Candida* was confirmed by efficient blocking of cytokine responses using a neutralizing receptor antibody (Fig. 2a). On the other hand, dectin-1 blocking only partially inhibited phagocytosis of this pathogen (Fig. 2b). Moreover, phagocytosis of *Candida* was unaffected by blocking other receptors known to be important for the uptake of this micro-organism (Fig. 2c, d), which is consistent with a functional redundancy of these receptors. Altogether, our data suggest a functional impairment in the PRR mediating the immune recognition of *Candida* in preterm monocytes, thereby abrogating cytokine responses, but not phagocytosis of this pathogen.

**Broad metabolic impairments in preterm monocytes.** The lack of cytokine response to *Candida* in preterm cells was concerning given the major importance of this pathogen mediating invasive disease in these infants. Yet, the molecular basis for this vulnerability is unknown. To investigate this, we used an unbiased, systems approach comparing the transcriptome of adult, term and preterm monocytes, at the genome-wide level. First, a gene ontology analysis comparing the transcriptome of adults, term and preterm monocytes revealed major differences located mainly in pathways involved in glycolysis, oxidative phosphorylation and beta-oxidation metabolism. Notably, differences were seen also with a profound downregulation of ribosomal genes (Fig. 3d) and an over-representation of down-regulated genes involved in translation initiation (adjusted \( p = 8.3 \times 10^{-21} \)), translation (adj. \( p = 2.6 \times 10^{-07} \)), and cell activation pathways (adj. \( p = 2.6 \times 10^{-16} \), Supplementary Data 1).

In light of the reduced responses to curdlan in preterm cells, we opted to compare gene expression responses to LPS instead. Upon stimulation with LPS, a large proportion of genes (~40%) were comparatively upregulated in all 3 age groups (Fig. 4a, b), including strong gene expression of *Il1b*, *Tnfα*, and *Il6* (Fig. 4c), as well as other cytokine/chemokine genes (Supplementary Figure 5). In independent qPCR experiments, expression of the *Il1b* and *Il6* cytokine genes was detectable after 30 min, and followed a
similar kinetics, peaking between 5 and 8 h in all three age groups (Jan R, Sharma A, and Lavoie PM, unpublished data and ref. 24). Strikingly, however, the strong gene expression response in preterm cells contrasted with the lack of corresponding protein expression (Fig. 4d), particularly for IL-1α/β, TNF-α, and IL-10, suggesting a translation defect. Of note, the same changes in metabolic and ribosomal-related genes persisted in LPS-stimulated cells (Supplementary Data 2).

Defective immune response translation in preterm monocytes.

To assess whether preterm monocytes lacked translation of immune response genes, we performed polysome analysis. Monocyte lysates from all three age groups were subjected to sucrose gradient fractionation; the concentration of specific mRNAs in monosome, disome, and light and heavy polysome fractions were measured by RT-qPCR. This experiment was extremely difficult due to the limited number of monocytes obtained from preterm infants. Given the lack of dectin-1 response, we focused on interrogating this pathway (Fig. 5a).

Despite the technical challenge, we were able to perform polysome profiling in all three age groups (Fig. 5b). In general, the overall distribution of mRNAs across the polysome gradient was similar between preterm, term and adult samples, but distinct between genes, suggesting that each mRNA is translated with similar kinetics, peaking between 5 and 8 h in all three age groups (Fig. 5b). Expression of the Malt1, Bcl10, and Card9 genes in total mRNA fractions were comparable between all three age groups (Fig. 5d). In the polysome fractions, Actb, Clec7a, and Card9 mRNAs, which respectively encode β-actin, the dectin-1 receptor, and Caspase recruitment domain-containing protein 9 (CARD9), were present in heavy polysomes in preterm monocytes. This is similar to the distribution in term and adult monocytes, indicating that these mRNAs are translated even at low gestational age. In contrast, the majority of Bcl10 and Malt1 mRNAs was associated with monosomes and disomes in preterm and adult monocytes, and only Malt1 mRNA was detected in heavy polysomes, in term monocytes (Fig. 5c). These results potentially indicate that dectin-1 signaling genes are differentially translated across the gestational age spectrum.

Next, we asked whether these proteins were expressed in monocytes from all three age groups. The dectin-1 protein was comparably expressed between preterm, term, and adult monocytes (Fig. 5e). In contrast, expression of the MALT1 protein, but also the Syk and CARD9 proteins, were severely reduced in preterm monocytes (Fig. 5f, g; Supplementary Figure 6). For Bcl10 protein, expression was undetectable in preterm monocytes by Western blotting (Fig. 5f, h). To confirm whether translation was reduced in preterm cells, pulse-labeling experiments were conducted and showed reduced 35S-methionine/cysteine incorporation in the latter, in response to LPS (Supplementary Figure 7).

Role of MALT1 in the monocyte response against Candida spp.

The role of the MALT1 signalosome in dectin-1 responses in myeloid cells has been mainly studied in mice macrophages and dendritic cells, but it has not been formally established in human monocytes.20,25 Moreover, due to the major role of MALT1 in T and B cells activation, it is also unclear whether signaling through
this molecule is also essential for myeloid responses to *Candida* in humans. To confirm this, we examined the effect of blocking MALT1 on both cytokine responses and phagocytosis of *Candida species*, in human monocytes. Complete loss of cytokine response to curdulan was observed with MALT1 inhibition (Fig. 6a). When testing responses to clinical strains of *Candida*, MALT1 blocking also completely abrogated cytokine responses to *C. albicans* or *C. parapsilosis* (Fig. 6b–d). Blocking of Syk also partially abrogated responses to these two micro-organisms, indicating that the response to *Candida* primarily involves signaling through dectin-1 (Fig. 6b–d). On the other hand, these responses were neither affected by blocking of MyD88 (downstream of TLRs) nor Raf-1 (mediating MALT1-independent dectin-1 signaling; Fig. 6b–d).

Given that preterm monocytes are fully able to phagocytose *Candida*, we also examined the role of MALT1 in this function. MALT1 inhibition did not block uptake of *Candida spp.*, indicating a non-essential role in phagocytosis (Fig. 6e). Altogether, these results confirm an essential role of the MALT1 signalosome in the recognition, but not the phagocytosis of whole *Candida* in human monocytes.

**Altered energy metabolism in preterm monocytes.** Recent data has demonstrated that a shift in basic cellular energy metabolism from oxidative phosphorylation to glycolysis is required during immune cell activation, in order to provide the rapid energy and metabolic intermediates necessary for translation of immune response proteins. While these studies have been mainly conducted in macrophages and dendritic cells, we confirmed the importance of this mechanism also in human monocytes by showing inhibition of cytokine production with 2-DG, a non-functional glucose analog that inhibits glycolysis (Fig. 7a).

In light of the reduced translation in preterm monocytes, we asked whether glycolysis could be impaired. To this end, we compared the glycolytic capacity of preterm, term, and adult monocytes. Notably, glycolysis was severely diminished in preterm monocytes (Fig. 7b, c). The reduced glycolytic activity in preterm monocytes was also reflected in reduced lactate production (Fig. 7d) and reduced glucose uptake (Supplementary Figure 8), at lower gestation, upon LPS but also at rest (in unstimulated cells). In term monocytes, glycolysis was variably affected, suggesting a transitional functional state.

Given that phagocytosis is preserved in preterm cells, these data raise an important question: do these metabolic constraints differ between cytokine responses and phagocytosis? Indeed, the requirement for glycolysis and/or translation has been mainly studied for PRR-mediated cytokine responses, but only sparsely for phagocytosis. Consequently, we showed that phagocytosis of *Candida* was unaffected by blocking glycolysis, or even by blocking of de novo protein synthesis (Fig. 7e). These results indicate a specific requirement of glycolysis for PRR-mediated responses, but not for phagocytosis.

Human adult monocytes are capable of rewiring their metabolism to adapt their energy requirements in a function-specific manner. The nuclear receptor PPAR-γ plays an important role in this function, regulating lipid uptake. PPAR-γ has also been shown to regulate phagocytosis in addition to promoting anti-inflammatory responses in human monocytes. In preterm monocytes, we observed a net down-regulation of mitochondrial electron transport (Supplementary Figure 9) and oxidative phosphorylation transcripts (Supplementary Figure 10A). Corresponding measures of oxygen consumption rates in preterm monocytes suggested decreased ATP-linked respiration (Supplementary Figure 10B), consistent with a metabolically quiescent state. Interestingly, expression of PPAR-

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**Fig. 2** Importance of dectin-1 in response to *Candida* in neonatal monocytes. a Antibody blocking of dectin-1 reduces cytokine production to *C. albicans* (*Ca*) in adult mononuclear cells (one-sided t-test with Welch’s correction for unequal variance; 6 to 12 subjects per condition; boxes and whiskers); b Phagocytosis of *C. albicans* upon blocking with anti-dectin-1 receptor antibody, same-isotype control or cytochalasin-D (Cyto-D); (11 to 18 subjects per age group; boxes and whiskers); Blocking of α *C. albicans* (*Ca*) or d *C. parapsilosis* (*CP*) phagocytosis using laminarin (blocking dectin-1), mannan (blocking dectin-2 and mannose receptor), anti-DC-SIGN, and anti-CD206 antibodies, or a combination of all three antibodies (bar graph with mean ± standard deviation (SD); 2 to 3 subjects per age group).
γ was increased in preterm monocytes (Supplementary Figure 10A). Furthermore, increased PPAR-γ activity in preterm monocytes is supported also by upregulation of its target genes mapping to metabolic pathways (Supplementary Figure 11). Altogether, these data indicated a rewiring of metabolic pathways favoring phagocytosis instead of pathogen-mediated pro-inflammatory cytokine responses, in preterm monocytes.

Developmental regulation through mTOR. The data above raise an additional important question: how are glycolysis and
transcription translation negatively regulated in preterm monocytes. Mechanistic target of Rapamycin (mTOR) is a key regulator of the metabolic switch towards glycolysis during immune activation (depicted in Fig. 7f)\(^3\). Therefore, we examined the mTOR regulator node developmentally. Interestingly, preterm monocytes showed reduced phosphorylation of mTOR following LPS stimulation (Fig. 7g). These cells also displayed reduced expression of its main downstream target 4EBP1, which is important in mediating the effect of mTOR on translation downstream of PRR activation (Fig. 7g). Given the difficulty in getting sufficient amounts of monocytes for efficient detection of mTOR phosphorylation by Western blot in preterm cells, we employed a flow cytometry assay, on a larger number of subjects. Using this approach, we confirmed the reduced mTOR phosphorylation, but also the gestational age-dependent functional dissociation between mTOR phosphorylation and cytokine responses in preterm monocytes (Supplementary Figure 12).

Next, we more closely examined regulatory gene expression within the mTOR pathway. Again, due to the absence of dectin-1/MALT1 signaling, we focused our analyses on LPS-stimulated cells. Major differences were observed in neonatal monocytes in the mTOR pathway (Supplementary Data 3), including a decreased expression of the upstream mTOR activator RAC-alpha serine/threonine-protein kinase (encoded by the Akt1 gene). Expression of the insulin receptor substrate 2 (Irs2) gene, which is an upstream regulator of mTOR, the genes encoding the adenosine monophosphate-activated protein kinase (prkaa2) and the hypoxia-inducible factor 1-alpha (Hif1α) were increased in
preterm monocytes compared to term or adults especially after LPS stimulation, which may represent compensatory mechanisms.

Most notably, expression of the negative mTOR regulators NAD-dependent deacetylase sirtuin-1 (Sirt1) and DNA damage inducible transcript-4-like (Ddit4l) were profoundly upregulated in preterm monocytes. Ddit4l is a paralog of the DNA damage inducible transcript-4 (Ddit4) that has also been shown to inhibit mTOR35,36 (Fig. 7h). Upregulation of Ddit4l was further confirmed at the protein level in neonatal preterm monocytes by Western blot (Supplementary Figure 13), suggesting that this molecule may represent an important developmental-specific negative mTOR regulator.

Glycolysis is required for MALT1 protein expression. The experiments described above reveal two main deficits in preterm monocytes: (i) a lack of dectin-1 signaling resulting in a lack of cytokine responses to *Candida*, and (ii) a reduced metabolic state leading to reduced innate immune responsiveness to LPS activation. In order to investigate whether these two functional deficits are linked, we tested whether blocking of glycolysis impaired translation of MALT1. In both Western blot and flow cytometry analyses, MALT1 protein levels were significantly reduced after 2-DG treatment (Fig. 8a–c). In contrast, protein expression of β-actin, a highly abundant gene transcript (see above) was unaffected (Fig. 8a). Conversely, inhibition of MALT1 did not affect the glycolytic capacity of these cells, as shown by sustained lactate levels both at rest and after LPS (Fig. 8e). In the latter experiment, LPS-induced cytokine responses correlated with lactate levels (Fig. 8f). Together these data are consistent with reduced glycolysis limiting cytokine responses and MALT1 protein expression in preterm cells rather than the other way around.

Immune response and risk of invasive *Candida* infection. Finally, we sought to determine how reduced cytokine responses in preterm cells may impact infants’ risk of invasive infections. In order to assess this, we reviewed data from 39,336 infants born below 33 weeks of gestation in Canada over 10 years. We found that rates of invasive infections exponentially increased with decreasing gestational age (Supplementary Table 1). When specifically looking at *Candida* infections, rates of candidemia also increased exponentially at lower gestation (Supplementary Table 1). Rates of invasive *Candida* infections also inversely correlated with in vitro responses to this pathogen (Supplementary Figure 14). Altogether, our data provide further evidence that the increased risk of invasive infections in these infants may be related to a lack of immune responses to *Candida* species.

Discussion
Infection and prematurity are thought to kill more newborns than any other cause globally (about one million infants die from infections each year)35. Despite this, we lack insights into the very basic mechanisms responsible for the high immunological vulnerability of preterm neonates, which restrain innovation towards new therapeutic interventions. In this study, we provide the first evidence of a role for cellular energy metabolism regulating neonatal innate immune responsiveness during ontogeny, in a gestational age-dependent manner. We also provide insights into how this may occur, through a lack of expression of key immune response proteins in the context of reduced glycolytic metabolic capacity. Our data suggest that multiple PRR responses could be broadly affected through these mechanisms in preterm myeloid cells. In light of our data, we posit that constraining innate immune reactivity in utero during development is physiologically important, possibly to limit adaptive immune co-activation signals during the early establishment of self-tolerance, but also to...
avoid energetically costly immune responses early in gestation. Indeed, translation consumes up to ~45% of cellular ATP. Immune activation triggers an upregulation of glycolytic pathways to rapidly supply the high energy needs and metabolic intermediates required for protein synthesis. Preventing an invasive *Candida* infection is also relatively costly energetically, and may not be specifically advantageous prior to a viable fetal stage. On the other hand, the preservation of other immune intermediates required for protein synthesis may be less than the rate of translation of immune response proteins may be less than the rate of their turn-over due to cis-acting elements in their 5’UTRs. Consistent with this model, pharmacological inhibition of mTOR using PP242, reduced translation of MALT1. The precise mechanisms that limit expression of immune proteins in preterm cells require future studies.

Our data shed additional light on the roles of MALT1 in the response of myeloid cells to *Candida spp.* in humans. Previous studies have been conducted in murine dendritic cells where blocking of MALT1 using siRNA or using a knock-out in mice abrogates cytokine responses to multiple strains of *Candida spp.* as well as to *curdlan*. In humans, the role for MALT1 in protecting against invasive *Candida* infections has been obscured by the major importance of MALT1 in T cells and B cells. Our
Untreated treatment may be essential to protect against organ damage in utero,\textsuperscript{44} that limits innate immune responsiveness during fetal development that activates responses via MALT1.

Metabolic constraints may also be reversed with pre-treatment of endotoxin challenge or sepsis also display a similar transcriptome in preterm cells likely precludes this approach.\textsuperscript{47} Alternatively, whole bacterial micro-organisms (\textit{C. epidermidis}) were partially abrogated by blocking Syk may suggest the existence of a Syk-independent, yet unidentified, non-dectin receptor that activates responses via MALT1.

Our data have potential clinical relevance. While a mechanism for inhibition glycolysis results in loss of MALT1 protein expression. Effect of blocking glycolysis (using 2-DG) or of blocking translation (using cycloheximide, as control) on MALT1 protein expression (monocytes). a MALT1 protein was detected by Western blot (left panel; representative of two experiments; cropped images from same blot probed with each antibody) at 8 h and 19 h. Lymphoblastoid cell line (LCL) lysate used as positive control for MALT1 protein expression; MALT1 protein detection (b) at 16 h and (c) over time (intracellular staining by flow cytometry, gated on CD14-expressing cells; MFI mean fluorescence intensity; dotted line: signal for fluorescence-minus-one staining control MFI level; boxes and whiskers with a paired 2-sided \textit{t}-test in b, mean ± SD in c and d, d corresponding cell viability over time (mean ± SD); 6 subjects. e Effect of MALT1 inhibition on IL-1\textbeta, IL-6, and lactate production at rest and following LPS (mononuclear cells; boxes and whiskers with 2-sided paired \textit{t}-tests); f correlation between LPS-induced IL-1\textbeta and IL-6, and lactate production (Spearman’s \textit{r}; \textit{p} < 0.05; with dotted regression line); 8 subjects. All experiments were conducted in adult cells.

work confirms a central role for MALT1 in the immune recognition of \textit{Candida} also in human myeloid cells. Moreover, our data suggest that the loss of MALT1 function in preterm cells impairs the ability to respond to \textit{Candida spp.}, although the extent to which impaired translation of additional immune response genes limit other immune pathways remains to be determined. Of note, our observation that \textit{Candida} responses were partially abrogated by blocking Syk may suggest the existence of a Syk-independent, yet unidentified, non-dectin receptor that activates responses via MALT1.

Our data have potential clinical relevance. While a mechanism that limits innate immune responsiveness during fetal development may be essential to protect against organ damage in utero,\textsuperscript{44}, it becomes a serious problem when a preterm birth occurs, due to increased infections in the premature infant. One key question is: can these constraints be reversed therapeutically, and if so, how? The comparably strong transcriptomic response in preterm monocytes is not unique to LPS, and has been reported with whole bacterial micro-organisms (\textit{Escherichia coli} and \textit{Staphylococcus epidermidis}).\textsuperscript{45} Monocytes from adult subjects after an endotoxin challenge or sepsis also display a similar transcriptome profile.\textsuperscript{46} In this latter case, immune training using low dose stimulation through the dectin-1 receptor could reverse the metabolic constraints,\textsuperscript{46} though the absence of dectin-1 signaling in preterm cells likely precludes this approach.\textsuperscript{47} Alternatively, metabolic constraints may also be reversed with pre-treatment of cells with IFN-\gamma.\textsuperscript{46,48,49} Interestingly, clinical trials suggest that IFN-\gamma treatment may ameliorate clinical outcomes in adults post sepsis.\textsuperscript{51,52} Newborn immune cells produce reduced IFN-\gamma, especially in absence of exogenous polarization.\textsuperscript{53} One study showed that preterm whole blood pre-treated with IFN-\gamma show augmented immune responsiveness to LPS in vitro, thus representing a potential therapeutic avenue that could be explored to restore immune defenses after birth.\textsuperscript{54} Alternatively, SIRT1 inhibitors have also been shown in pre-clinical trials to augment glycolysis and immune responses post sepsis, through a stabilization of HIF-1\alpha.\textsuperscript{55} Despite the multiple challenges that lie between our studies and a potential therapeutic application in preterm infants, our studies represent an important discovery and reveal potential translational research avenues to tackle the high vulnerability of these infants to infections. However contrary to adults, our data suggest that the lack of glycolytic and mTOR activity in preterm cells may be actively suppressed, possibly through the putative mTOR regulator DDIT4L. In this context, further studies are required to determine how a metabolic reprogramming of preterm myeloid cells can be sustainably achieved at the cellular level.

A limitation of our study is the strict use of cord blood which may not adequately represent peripheral blood immune responses during the neonatal period. However, obtaining sufficient volumes of peripheral blood from premature neonates is ethical questionable and could not be achieved for safety reasons. Indeed, only a handful of studies have measured immune parameters in peripheral blood from premature neonates, and these studies confirm the marked attenuation in PRR responses compared to term neonates, with correlation of the expression of phagocytosis and monocyte activation marker between cord and peripheral blood.\textsuperscript{35,56–60} Therefore, it appears that cord blood is a reasonable alternative to using peripheral blood, though important differences exist and the former does not reflect the dynamic changes occurring over time when infants are most susceptible to sepsis.\textsuperscript{56,60} A second limitation is the focus on monocytes, which incompletely recapitulates the complex cell-microbes interactions during sepsis in vivo. For example, IL-1\beta can be cleaved by serine proteases (e.g., PR3, cathepsin G) expressed by neutrophils, in addition to caspases. Because of the crucial role of neutrophils in preventing \textit{Candida} invasion in humans,\textsuperscript{58} specific studies are required to determine the contributions of other immune cells.
In summary, our data reveal important metabolic constraints that limit immune activation against major neonatal pathogens in monocytes before the term of gestation. This mechanism provides a unifying pathway to explain the broadly suppressed innate immune responsiveness during human fetal development. Future studies are required to understand how reduced mTOR function impairs translation in preterm monocytes, and how the putative negative regulator of mTOR, DDIT4L, may regulate this process. Finally, the availability of metabolic reprogramming drugs in pre-clinical studies may offer therapeutic avenues to augment immune reactivity and reduce the major burden from infections in these high-risk preterm newborns.

Methods

Human Subjects. This study was conducted according to the Canadian Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans at http://www.pre.ethics.gc.ca/eng/policy-politique/initiatives/tcp2-epct2/Default/. Cord blood was obtained from preterm infants (<33 weeks) and term infants (>38 weeks) born by elective cesarean section in absence of labor, and peripheral blood was obtained from healthy adult volunteers recruited at the BC Children’s Hospital Research Institute. All blood samples were collected in sodium heparin vacutainers. Cord blood from more than 104 preterm deliveries were used in the experiments included here. To assess whether the lack of dectin-1 response in preterm subjects may have been due to an enrichment of loss-of-function genetic variants in the dectin-1 receptor, genotyping for the common Y238X mutation (SNP rs16910526) was performed on a previous cohort of infants born before 31 weeks of gestation. Only 2 out of the 177 infants were homozygous for the rare variant, a rate similar to the adult population.

The clinical characteristics of the preterm subjects included in each of the figures are provided in aggregated form, in the accompanying Supplementary Tables 2 to 11. All cord blood samples were collected following delayed cord clamping clinical standards, and after the placenta was detached from the infant. Written consent was obtained from all participants, except for eight preterm cases where parents were unavailable/non-reachable in person or by phone. In these cases, no clinical data was obtained on completely de-identified cord blood samples, in accordance with the University of British Columbia Children’s & Women’s Research Ethics Board (C&W REB). The study complied with all relevant ethical regulations and was approved by the C&W REB (#H07-01698).

Cells. Mononuclear cells (MNCs) were isolated from between 0.5 and 20 mL of whole blood (depending on each ensuing experiment) by density centrifugation using Lymphoprep (STEMCELL Technologies). Monocytes were purified from MNCs generally from at least 1 mL of whole blood, using an EasySep positive CD14 selection kit (STEMCELL Technologies, #18058) and 1 mM EDTA to prevent cell clumping. Monocyte purity was regularly determined by flow cytometry (FSC/SSC plus staining for CD14 surface expression) throughout the study, and strictly confirmed to be > 95% for example, for microarray studies.

Reagents. Pediatric clinical isolates of Candida albicans and Candida parapsilosis identified by mass spectrometry (Bukker Daltonics, Billerica, MA) were obtained from the BC Children’s Hospital Microbiology Lab (Vancouver, Canada). We used fixed Candida spp yeast particles which are closer antigenically to yeast forms. Fungi were grown on 4 days at 30 °C in BHI (Brain-Heart Infusion) broth (Oxoid, Nepean, ON). Yeast in exponential growth phase were harvested after 4 days, centrifuged and fixed in 10% paraformaldehyde. To ensure reproducibility, batches of Candida particles were prepared and used throughout the study. Human dectin-1 and dectin-2 neutralizing antibodies were purchased from R&D Systems (#AB18859) and Invivogen (#Mabg-hdect2), respectively, and used at 5 µg mL⁻¹ for cytokine studies, and 10 µg mL⁻¹ for phagocytosis assays. Human CD-206 antibody (eBioscience, #48-0149-42, 1:100 dilution) and a dectin-1-specific agonist, was obtained from Wako (#002-09902) and used at 10 µg mL⁻¹ in cytokine studies. Lipopolysaccharide (LPS), a TLR-4 agonist purified from Escherichia coli (Oxoid), was used as positive control at 10 µg mL⁻¹. Mannan was purchased from Sigma-Aldrich (#M3640-1G), and used at 3 mg mL⁻¹ in phagocytosis assays. Laminarin was purchased from Sigma-Aldrich (#L6364-100G) and used at 5 µg mL⁻¹ in phagocytosis assays. Assays were performed in triplicate.

Phagocytosis assay. MNCs were incubated in 96-well plates in the presence of Candida strains pre-stained for 15 min with DAPI (Biotium, #422801) at room temperature. After a 1-h incubation with Candida species, cells were quenched in Trypan Blue/PBS of any residual fluorescence of non-phagocytosed Candida spp, and then washed once in PBS before staining with a CD14 PE-Cy7-conjugated antibody (BD Biosciences, #565575) and CD80 Alexa Fluor 700 (BD Biosciences, #742541), respectively, and used at 10 µg mL⁻¹ for phagocytosis assays. Cells were washed 3 times in PBS before acquisition on a BD LSRII flow cytometer (Becton Dickinson). Live cells were gated on singlets. Data were analyzed using FlowJo v10 (FlowJo, LLC, Ashing OR). CD14-expressing monocytes were identified from live cells based on FMO. Cells were analyzed on a BD Fortessa or a 4-laser customized LSRII flow cytometer (Becton Dickinson). Data were analyzed using FlowJo v10 (FlowJo, LLC, Ashing OR).

Flow cytometry. MNCs were washed in PBS (3×) and stained for surface CD14 (Bioscience #25-0149; 1:100 dilution), and dectin-1 (AbD Serotec, #MA6661A1488; 1:100 dilution) as indicated. Intracellular cytokine staining for pro-IL-1β was performed by fixing/permeabilizing cells with Foxp3 Staining Buffer (Bioscience, #500522-00), and staining with an anti-IL-1β antibody (BioLegend, #508208, 1:50 dilution). After 30 min of incubation, cells were washed 3 times in PBS before analysis. Nontarget expression and phosphorylation was performed using 10 µg mL⁻¹ for phagocytosis, 5 µg mL⁻¹ and LPS for cytokine studies, and 10 µg mL⁻¹ for phagocytosis assays. Human CD-206 antibody (eBioscience, #48-0149-42, 1:100 dilution) was added during the last 15 min of incubation. Incubation was stopped and cells were stained intracellularly using Transcription Factor Phospho Buffer Set (BD Biosciences, #565575) for native (Cell Signaling Technology, #5043 S, 1:50 dilution) and phospho-mTOR (Cell Signaling Technology, #5534 S, 1:50 dilution). Cells were washed 3 times in PBS before acquisition on a BD LSRII flow cytometer (Becton Dickinson). Data were analyzed using FlowJo v10 (FlowJo, LLC, Ashing OR).

Phagocytosis assay. MNCs were incubated in 96-well plates in the presence of Candida strains pre-stained for 15 min with DAPI (Biotium, #422801) at room temperature. After a 1-h incubation with Candida species, cells were quenched in Trypan Blue/PBS of any residual fluorescence of non-phagocytosed Candida spp, and then washed once in PBS before staining with a CD14 PE-Cy7-conjugated antibody (BD Biosciences, #565575) and CD80 Alexa Fluor 700 (BD Biosciences, #742541), respectively, and used at 10 µg mL⁻¹ for phagocytosis assays. Cells were washed 3 times in PBS before acquisition on a BD LSRII flow cytometer (Becton Dickinson). Live cells were gated on singlets. Data were analyzed using FlowJo v10 (FlowJo, LLC, Ashing OR).

Western blots. After stimulation (LPS or curcumin), monocytes were washed, and then lysed in RIPA buffer in presence of phosphatase/protease inhibitors (Santa Cruz Biotechnology, #sc-294984). Protein quantification was performed using a Pierce 660 nm protein assay (Thermo Fisher Scientific). Lysates boiled in 4X Laemmi buffer (Bio-Rad) supplemented with 2-mercaptoethanol were run on 4–20% mini PROTEAN TGX gradient gels (Bio-Rad), transferred to PVDF membranes, and incubated with primary and secondary antibodies. Blots were imaged on LiCOR Odyssey Infrared Imaging system. Image Studio Ver. 5.2 and adjusted for brightness. Uncut Western blot images are provided in Supplementary Figures 16 to 19.

Polyosomometry. For polyosome profiling experiments, a minimum of 3 × 10⁶ monocytes (corresponding to at least 5 mL of cord blood) were pre-cultured with cycloheximide (100 µg mL⁻¹) for 10 min at 37 °C, washed once in PBS/cycloheximide and lysed in polyosome lysis buffer (Mammalian ARTseq Ribosome Profiling Kit, Illumina, #15123612260, 0.3 M NaCl, 10 mM HEPES, pH 7.5), followed by 2× to 50× sucrose gradients in buffer (0.3 M NaCl, 15 mM Tris-Cl pH 7.5, 15 mM MgCl2, 0.1 mg mL⁻¹ cycloheximide) in the presence of heparin (1 mg mL⁻¹) and spun in an ultra-centrifuge (SW41, Beckman Coulter) for 3.5 h at 4 °C (35,000 rpm) in order to separate RNA strands based upon ribosomal occupancy. After fractionation, the purified mRNAs were transcribed into cDNA, was pooled into pools of monosomic, disomic, or heavy or light polysome fractions as internal control. The cyclization of separate RNA strands based upon ribosomal occupancy. After fractionation, the purified mRNAs were transcribed into cDNA, was pooled into pools of monosomic, disomic, or heavy or light polysome fractions as internal control. The monosome, disome, or heavy or light polysome fractions were subsequently used for microarray studies.

In summary, our data reveal important metabolic constraints that limit immune activation against major neonatal pathogens in monocytes before the term of gestation. This mechanism provides a unifying pathway to explain the broadly suppressed innate immune responsiveness during human fetal development. Future studies are required to understand how reduced mTOR function impairs translation in preterm monocytes, and how the putative negative regulator of mTOR, DDIT4L, may regulate this process. Finally, the availability of metabolic reprogramming drugs in pre-clinical studies may offer therapeutic avenues to augment immune reactivity and reduce the major burden from infections in these high-risk preterm newborns.
Real-time qPCR. Total RNA was isolated using TRIzol LS (Thermo Fisher Scientific) followed by chloroform extraction and cleaned using RNeasy Mini spin columns (Qiagen) followed by ethanol precipitation. The RNA sample was reverse transcribed using SuperScript® III First-Strand Synthesis SuperMix (Thermo Fisher Scientific, #18080400), and qPCR experiments were carried out in triplicates on a Viia 7 system (Applied Biosystems) using both Power SYBR® Green (Thermo Fisher Scientific, #4368706) or TaqMan. Data were normalized on dsRED (ActB method) as detailed in 63. Briefly, ActB was reverse transcribed on each sample against dsRED. 2^−ΔΔCt was then calculated, and the sum of all fractions determined. Gene expression (mRNA) was expressed as (2^−ΔΔACT/sum of each fraction).

Pulse-labeling experiments. Monocytes were stimulated for 2.5 h and pulsed with [35S]-methionine/cysteine for the last 30 min of the experiment. Cells were washed with PBS and lysed in RIPA (Santa Cruz Biotechnology); equivalent amounts of protein were loaded on polyacrylamide gel. Blots were imaged on a fluoimager.

Metabolic assays. Glucose uptake in monocytes was measured by flow cytometry. Cryopreserved MNCs were rested for 1 h at 37°C in ERPMI after gentle thawing, and then counted before stimulation for 2 h and 40 min in presence of LPS (10 ng ml⁻¹), with 2-(N-[7-Nitrobenz-2-oxa-1,3-diazol-4-yl]Amino)-2-Deoxyglucose (2-NBDG, 15 μM) added for the last 40 min. Cells were stained with CD14 (eBioscience #25-0149: 1:100 dilution) and CD16 (eBioscience #48-0168: 1:100 dilution) at the beginning of LPS stimulation. Cells were washed twice before acquisition on a LSFortessa™ flow cytometer (Becton Dickinson). Data was analyzed using Flowjo® (Flowjo, LLC). For extracellular flux analyses, monocytes were plated on Cell-Tak™ (Corning, #452400)-coated XF96 Cell Culture (IXP6 Analyser). Extracellular acidification rate (ECAR) was normalized to protein content. L-lactate was measured by colorimetry (Abcam, #ab65331).

Microarray analysis. Total RNA from purified CD14+ monocytes was extracted using the MaglEt RNA purification kit (Thermo Fisher Scientific). RNA was quantified using NanoDrop™ 1000 spectrophotometer, and its integrity was determined using the Agilent 2100 Bioanalyzer. Samples were hybridized onto an iScan System (Illumina, San Diego, CA, USA). Data were analyzed using R. Data was preprocessed by quantile normalization and log2 transformation. Probes that were non-expressed (based on their detection p-values) were adjusted for multiple comparisons using the Bonferroni step-down method. Gene Ontology analysis and Network images were generated using the ClueGO plugin for Cytoscape 3.5.19

Statistical analysis. For each experiment, sample size was estimated based on the variance from 3 to 5 replicates in any age group (assuming comparable variance between age groups). To avoid a selection bias, we balanced distribution at adult, term, and preterm samples in batch experiments over time. GraphPad Prism v6.07 was used for graphs and statistical analyses. To simplify the figures, we present p values only when indicated by the data. Differences between groups were analyzed using 2-tailed t-tests, ANOVA (for groups) or as otherwise specified. Normality of data was assumed in most statistical testing, unless grossly skewed. Statistical significance was determined at p values of <0.05. When data is shown in boxes and whiskers, boxes are spanning from the first quartile to the third quartile, with the center line depicting the median. Whiskers are extending to the highest and lowest points.

Data availability

Microarray data used in Figs. 3, 4a-c, 7h, Supplementary Data 1, 2, and 3, and Supplementary Figures 3.4, 5, 9, 10A and 11 have been deposited in the NCBI Gene Expression Omnibus under accession code GSE104510. All other data, including raw data used in each figure will be provided upon reasonable request to the corresponding author, and provided that the nature of the request complies with our institutional ethics board policy.

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Author contributions

B.K. and C.M. led the majority of the experiments as part of their PhD theses. B.K. drafted the first version of the manuscript with help from C.M. H.F. helped with the Candida experiments. H.A. helped with polysome profiling and pulse-labeling experiments. K.L. enrolled research participants and helped with the generation of transcriptome data, together with B.K. E.A.M. and M.F.C. helped with sample processing and data acquisition. E.A.B. helped with experimental design and polysome profiling. M.A.S. provided technical and experimental support for metabolic experiments. P.T. provided clinical strains of Candida as well as design input and clinical relevance. R.G.M. helped design polysome experiments and supervised E.A.B. C.J.R. supervised the generation of microarray data. D.S.L. helped design and supervise metabolic studies. E.J. helped design and supervise the translation experiments, including final polysome profiling and pulse-labeling experiment, and data analysis. P.M.L. developed the original concept of the study together with B.K. and C.M., provided general oversight including data analysis and manuscript writing. All authors have reviewed, edited, and approved the final version of the manuscript.

Additional information

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