Profound Lack of Interleukin (IL)–12/IL-23p40 in Neonates Born Early in Gestation Is Associated with an Increased Risk of Sepsis

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Background. Infants born prematurely are highly vulnerable to infections and also exhibit a high susceptibility to organ damage due to inflammation.

Methods. To investigate homeostatic immune control early in life, we used advanced multiparameter flow cytometry to compare responses to multiple Toll-like receptor (TLR) ligands in single cells and mononuclear cell populations in term neonates versus preterm neonates born before 29 weeks of gestation.

Results. Preterm neonates had globally attenuated TLR-stimulated interleukin (IL)–6, interferon–alpha, and, to a lesser extent, tumor necrosis factor–alpha responses but demonstrated relative preservation of anti-inflammatory IL-10 responses in monocytes and dendritic cell subtypes. Remarkably, preterm neonates were also profoundly deficient in the common IL-12 and IL-23 cytokines’ p40 subunit, which is critical for immunity against a wide variety of microbial pathogens in mice. Consistent with the increased susceptibility to infections resulting from the lack of IL-12/IL-23 in human newborns, significantly lower serum p40 concentrations were observed at birth in infants who developed early-onset sepsis.

Conclusion. To our knowledge, this study is the first detailed analysis of multiple TLR function in neonates born extremely premature. Although attenuation of proinflammatory pathways may protect against tissue-damaging immunity early in life, this previously unrecognized p40 immune deficiency appears to result in considerably increased susceptibility to infection in human preterm newborns.

Neonates are highly susceptible to infections and heavily rely on innate immune responses to defend against microorganisms [1]. Among newborns, preterm neonates are most likely to have morbidity and mortality due to infections, and nearly one-third of infants who are born extremely premature (ie, before 29 weeks of gestation) develop serious bloodstream infection during their first weeks of life [2, 3]. Understanding the innate immune mechanisms in preterm neonates is critical for improving outcomes in this patient group.

Previous studies examining innate immune functions in preterm neonates largely focused on lipopolysaccharide (LPS)–induced cytokine responses in whole blood or monocytes [4–14]. Toll-like receptor (TLR) 4, which is the receptor for LPS and a member of a family of at least 10 nonopsonic receptors (TLR1–TLR10) in humans, is predominantly involved in sentinel recognition of pathogens by the innate immune system [15]. In preterm neonates, the proinflammatory cytokine response to LPS [4–8, 16], interleukin (IL)–1 [9], or whole microorganisms [10, 11] is significantly reduced.
(mainly reported for IL-6 but, also, for tumor necrosis factor [TNF–α, IL-8, and IL-1β], compared with that in term neonates, whereas data about anti-inflammatory IL-10 or transforming growth factor–β responses are somewhat conflicting [6, 10, 12]. Part of this attenuation in proinflammatory responses has been attributed to a developmental, gestational age-dependent reduction in the surface expression of TLR4 and its coreceptor CD14 [13, 14, 17], as well as a reduced expression of the downstream intracellular signaling components MyD88 and IRF5 [17]. However, it is unclear whether compensatory responses exist in innate immune cell types other than monocytes or through pathogen recognition by other TLRs. Indeed, recognition of intact pathogens by innate immune cells likely involves multiple TLRs and, to our best knowledge, no studies to date have described responses to stimulation via TLRs other than TLR2 and TLR4 [17].

Dendritic cells are the primary source of IL-12 and IL-23, two immune-regulatory cytokines that are critically important for immune defenses against microorganisms [18]. Both cytokines exist as heterodimers with a common p40 subunit. The p40 subunit mainly functions in conjunction with either p35 (to form IL-12) or p19 (to form IL-23) to support differentiation and maintenance of T helper 1 (Th1) or T helper 17 (Th17) cells, respectively [18]. Th1 and Th17 responses are essential for host protection against multiple intracellular and extracellular pathogens, but they can also induce detrimental autoimmunity in the absence of strict regulatory control [18]. In term neonates, p19 and p40 are expressed, but p35 is barely detectable, resulting in a profound lack of IL-12 that is compensated by the production of IL-23 [19, 20]. Data on the expression of IL-12 or IL-23 in preterm neonates are lacking.

In the present study, we sought to gain new insights into innate immune homeostatic mechanisms in preterm neonates born before 29 weeks of gestation. To achieve this goal, we used multiparameter flow cytometry to directly assess innate responses to well-defined TLR ligands. To account for differences in the proportion of circulating leukocytes throughout gestation, we chose to compare responses in individual innate immune cell types as well as peripheral blood mononuclear cell populations. Our results show that in contrast to term neonates, preterm neonates are profoundly deficient in IL-12/IL-23p40 and provide compelling evidence of a role for this newly recognized preterm p40 deficit in contributing to susceptibility to neonatal sepsis.

MATERIALS AND METHODS

Sample collection. Cord blood samples were collected in sodium heparin–anticoagulated Vacutainer tubes (BD Biosciences), after written, informed consent was obtained from mothers delivering either prematurely (at <29 weeks of gestation, as determined by ultrasound dating or by the first day of the last menstrual period; n = 12) or at full term (n = 18), at the Children’s & Women’s Health Centre of British Columbia (Vancouver, British Columbia, Canada) or the Centre Hospitalier Universitaire Sainte-Justine (Montreal, Quebec, Canada). For all preterm subjects, placenta were rigorously examined by a clinical pathologist. Only samples collected from subjects without clinical or histologic evidence of chorioamnionitis (defined as a fetal or maternal stage ≥1, as determined by use of validated histologic criteria [21]) were included in this study. The research protocol was approved by the institutional ethics review boards of the University of British Columbia, the University of Alberta, and Centre Hospitalier Universitaire Sainte-Justine.

Blood sample processing and in vitro stimulation. Robust standard operating protocols used for the preparation of TLR-stimulation plates and for processing of blood samples have been described elsewhere [22]. In brief, cord blood mononuclear cells (CBMCs) were mixed 1:2 in prewarmed (at 37°C) RPMI 1640 medium (Invitrogen), extracted by ficoll-hypaque gradient centrifugation within 2 h of collection, and stimulated (5 × 10⁵ cells/200 µL) in RPMI 1640 medium supplemented with 10% heat-inactivated human AB serum (Gemini Bio-Products), 100 U penicillin/mL, and 100 µg of streptomycin/mL (Invitrogen). Cytokine responses were compared after stimulation with PAM-CSK, (EMC Microcollections; recognized by TLR1/TLR2), FSL (EMC Microcollections; TLR2/TLR6), 0111: B4 LPS (Invivogen; TLR4), 3M-013 (3M; TLR7), 3M-002 (3M; TLR8), 3M-003 (3M; TLR7 and TLR8), and CpG-A type ODN 2336 (Coley; TLR9). CBMCs were added to premed 96-well round-bottom polystyrene plates (VWR) containing different concentrations of 10-fold dilutions of individual TLR agonists, and they were stimulated at 37°C in an atmosphere of 5% CO₂. For intracellular cytokine detection, cells were initially incubated for 6 h in the presence of brefeldin A (BFA; final concentration, 10 µg/mL), except in the case of CpG-A stimulation, in which BFA was added only for the final 3 h of culture. For cytokine detection in culture supernatants, cells were stimulated for 24 h. After stimulation (for 6 or 24 h), cells were incubated for 15 min at 37°C after the addition of ethylenediaminetetraacetic acid (final concentration, 2 mmol/L); plates were centrifuged; 120 µL of supernatant was harvested; and cell pellets were resuspended in BD FACs lysing solution (BD Biosciences), sealed, and immediately frozen at −80°C until analysis.

Staining, flow cytometry acquisition, and analysis. Stimulated mononuclear cell samples were analyzed for single-cell cytokine responses in monocytes, conventional dendritic cells (cDCs), and plasmacytoid dendritic cells (pDCs), by means of flow cytometry (for supplementary methods, see the Appendix, which does not appear in the print version of the Journal). CompBeads (BD Biosciences) serving as positive and
negative staining controls were stained the same way as cells and were used to standardize voltage settings.

Determination of cytokine secretion in culture supernatants was performed on 10 randomly chosen plates from term neonates and nine 24-h plates from preterm neonates, and it was sufficiently powered to allow sound statistical comparison between term and preterm groups (see below). Supernatants were diluted 1:2 and 1:20 in RPMI, to obtain signals in the linear detection range. Measurements were performed using a “Flex Kit” multiplex bead array cytokine detection assay (Millipore), which allowed the detection of IL-6, IL-10, TNF-α, IL-12p40 monomers, the IL-12p35/p40 heterodimer (also referred to herein as “IL-12p70”), and IFN-α-2B with overnight incubation at 4°C. For detection of human IL-23, a human IL-23 enzyme-linked immunosorbent assay specific for the p19/p40 heterodimer (eBioscience) was used. Cytokine detection was performed on a Luminex analyzer (PerkinElmer), except for IL-23, which was analyzed by spectrophotometry at 450 nm with a 570-nm subtraction. IL-23 production with stimulation of TLR9 by CpG is not reported because of background interference on IL-23p19/p40 detection in the assay used (unpublished data, which were also confirmed by the manufacturer). For all cytokines, sigmoid logistic curves were used to generate standard curves. Except for IL-6 levels, cytokine levels in unstimulated culture supernatants were below the limit of detection and therefore are not presented.

Detection of p40 in the serum of neonates at risk of early-onset neonatal sepsis. After parental informed consent was obtained, serum samples were collected at 12–21 h of age from neonates who were born at 24–41 weeks of gestation \( n = 425 \) at the Royal Alexandra Hospital, the Misericordia Hospital, or the Grey Nuns Hospital (Edmonton, Canada) and whose mother had risk factors for early-onset (ie, at <72 h) neonatal sepsis (EONS; risk factors were defined as either a positive maternal vaginal culture for group B streptococcus, preterm labor, prolonged rupture of membranes for >18 h, or clinical chorioamnionitis, which was defined as foul-smelling amniotic fluid, a maternal body temperature >38.0°C, or fetal tachycardia >180 beats/min). Cases with culture-proven infection were compared with a group of randomly selected controls matched by frequency for gestational age and birth weight and in whom sepsis was excluded (ie, those with a negative blood culture result, a C-reactive protein level <5 mg/L, no clinical signs of infection, and a favorable outcome without antibiotic treatment after 48 h). Concentrations of IL-6, IL-10, and IL-12/IL-23p40 in serum samples were corroborated using 2 multiplex Luminex assays (Panomics and Medicorp/Invitrogen) on a Luminex analyzer (PerkinElmer), and only data from the former multiplex assay are presented.

Statistical analyses. Proportions of monocytes, cDCs, and pDCs relative to CBMCs were compared using Student’s \( t \) test as they were normally distributed. Cytokine levels in culture supernatants were compared using the Mann-Whitney \( U \) test. The Mann-Whitney \( U \) test was also used to compare flow cytometry results in preterm and term neonates. Bonferroni-corrected \( P \) values were used to correct for multiple comparisons in the analysis of TLR-stimulated cytokine responses, with \( P < .01 \) considered to denote statistical significance. Statistics were calculated using SPSS software for Windows (version 11; Lead Technologies).

RESULTS

Proportions of innate immune circulating cell types in cord blood from term and preterm neonates. Because differential blood counts of leukocytes vary greatly across gestational age and may directly influence the magnitude of cytokine responses, the proportion of monocytes, cDCs, and pDCs in CBMCs was compared between preterm and term neonates. Consistent with previous reports [7, 23], a 2-fold lower proportion of monocytes was detected in CBMCs from preterm neonates, compared with term neonates (mean ± standard deviation [SD], 15% ± 4.3% vs. 8% ± 4.8%) \( P = .002 \). Similarly, the proportions of pDCs were marginally lower (mean ± SD, 0.19% ± 0.16% vs. 0.25% ± 0.11%) \( P = .03 \) in preterm neonates versus term neonates, whereas no difference in the proportions of cDCs was detected (mean ± SD, 0.63% ± 0.39% vs. 0.55% ± 0.24%) \( P > .05 \).

Diminished proinflammatory TLR responses in monocytes and cDCs from preterm neonates. The overall proinflammatory (IL-6) (Figure 1A) and TNF-α (Figure 1C) cytokine response generated as a result of TLR stimulation was compared in CBMC culture supernatants. Preterm neonates produced significantly less IL-6 upon stimulation via TLR1, TLR2, TLR4, and TLR6, whereas the response to stimulation via TLR7 and TLR8 was comparable to that in term neonates. For TLR9 stimulation, there was a trend toward higher IL-6 responses in term neonates.

Stimulation with TLR1, TLR2, TLR4, and TLR9 ligands also generally yielded lower TNF-α responses in preterm supernatants; however, these differences did not reach statistical significance. TNF-α responses to stimulation of TLR7/TLR8 using 3M-003 were comparable between preterm and term neonates. The reduction in IL-6 production in response to stimulation via TLR1, TLR2, TLR4, and TLR6 in preterm neonates was also confirmed at the single-cell level in both monocytes and cDCs (Figure 1B), when comparisons with term neonates were made. Intracellular TNF-α responses were similar between preterm and term neonates on a per-cell basis, and when either expressed as a percentage of cytokine-expressing cells (data not shown) or as mean fluorescence intensity (MFI; data not...
Figure 1. Interleukin (IL)-6 and tumor necrosis factor (TNF)-α proinflammatory cytokine responses. IL-6 (A) and TNF-α (C) responses in cord blood mononuclear cell culture supernatants (mean ± standard error of the mean). Background levels of IL-6 in unstimulated culture supernatants were low (range, 34–6364 pg/mL) and did not differ significantly between term and preterm neonates; therefore, for simplicity, graphs denote levels after subtracting background unstimulated cytokine levels. B, Peak IL-6 responses (expressed as the mean percentage of cytokine-producing cells) in monocytes and conventional dendritic cells (cDCs). Agonists used for stimulation are indicated above each graph, with their respective stimulated TLR(s) in brackets. For monocytes, CD14 staining for one preterm infant was not available for technical reasons, and data from only 11 preterm infants therefore are presented. LPS, lipopolysaccharide; NS, unstimulated (bar graphs). *P < .01.

Similar IL-10, but reduced type I IFN, responses in preterm versus term neonates. To assess whether preterm neonates exhibit a deficit in anti-inflammatory cytokine responses, we examined the TLR-induced production of IL-10, a prototypic

shown). IL-6 and TNF-α were detected in both monocytes and cDCs with similar MFI, indicative of an equal contribution of the 2 cell types to the overall culture supernatant response, in both preterm and term newborns (data not shown).
anti-inflammatory cytokine. In response to all TLR ligands tested, preterm and term neonates demonstrated generally similar IL-10 production (Figure 2), except perhaps for a marginally reduced preterm response to stimulation of TLR2/TLR6 by use of the agonist R-FSL.

For type I interferon, significant responses were detected only after stimulation with either TLR7 or TLR9 agonists and in pDCs. A trend toward reduced IFN-α production was apparent in preterm neonates when analyzing culture supernatants, but biological variability in this assay precluded differences from reaching a Bonferroni-adjusted threshold for statistical significance within our sample size (Figure 3A). However, when IFN-α production was compared between preterm and term neonates in a single-cell, flow cytometry–based analysis, the former clearly produced significantly lower responses on a percentage cytokine-producing (pDC) basis (Figure 3B).

**Profound deficiency of IL-12/IL-23p40 in preterm infants.** Next, we examined TLR-triggered IL-12/IL-23 cytokine production in monocytes and cDCs. Overall, IL-12/IL-23p40 was mainly detected in cDCs, with 5- to 8-fold greater production, compared with monocytes (as determined based on MFI; not shown), and consistent with previous data [20]. In addition, term neonates were able to produce detectable amounts of IL-12/IL-23p40 but negligible amounts of IL-12p70, except after TLR8 stimulation (Figure 4A), as reported elsewhere [19, 20].

Remarkably, production of IL-12/IL-23p40 was substantially lower in preterm neonates, in response to most TLR agonists tested (Figure 4B). Although the percentage of IL-12/IL-23p40–producing cDCs was comparable between preterm and term neonates (data not shown), the amount of cytokine produced on a per-cell basis (ie, as measured by MFI) was highly consistent with our results obtained from culture supernatants: levels were markedly reduced in cDCs of preterm neonates, compared with cDCs of term neonates (Figure 4C). This marked reduction in p40 observed in preterm neonates suggested a profoundly impaired production of the IL-23 cytokine in addition to the lack of IL-12 (Figure 4A). This was confirmed by direct measurement of the IL-23p19/p40 heterodimer by use of the enzyme-linked immunosorbent assay (Figure 4D), where no IL-23 was detectable in culture supernatants of preterm neonates, except for a weak response to stimulation of TLR7/TLR8.

**IL-12/IL-23p40 is significantly reduced in neonates with early-onset neonatal sepsis.** To determine the clinical relevance of IL-12/IL-23p40 for protection against infections in neonates, we conducted a nested case-control analysis in a large prospective cohort of infants (n = 425) at risk for EONS. In this at-risk cohort, the overall incidence of EONS was 18 cases per 1000 neonates. Clinical characteristics were similar between the 8 EONS cases (Table 1) and 42 matched controls (mean gestational age ± SD, 32.9 ± 3.8 weeks versus 32.2 ± 3.8 weeks; mean birth weight ± SD, 2038 ± 636 g versus 2010 ± 788 g; frequency of maternal-infant pair exposure to antenatal corticosteroids, 62% versus 48% [95% confidence interval {CI}, −52% to 22%]) (P = .70). In EONS cases, serum concentrations of IL-12/IL-23p40 were significantly lower at birth, compared with gestational age- and birth weight–matched control neonates who did not show any clinical or laboratory signs of infection (P = .018) (Figure 5). The difference in serum p40 levels between cases and controls persisted when considering only neonates without prolonged rupture of membranes over 18 h or clinical signs of chorioamnionitis (28 ± 16 pg/mL versus 74 ± 34 pg/mL) (P = .02). On the other hand, levels of IL-6 (P = .002), IL-10 (P = .001) (Figure 5), and diagnostic C-reactive protein (mean, 66.5 mg/L versus <5 mg/L) that were simultaneously sampled were higher in cases than in controls, indicating that the reduction in serum levels of IL-12/IL-23p40 was not the result of a generally blunted inflammatory response to infection.

**DISCUSSION**

In the present study, we systematically interrogated individual TLR-stimulated innate immune cytokine responses in preterm neonates born most early in gestation. Overall, our results revealed attenuated IL-6 production in monocytes and cDCs from preterm neonates and, to a much lesser extent, a reduction in TNF-α production, after stimulation via TLR1, TLR2, TLR4,
TLR6, or TLR9. In contrast, comparison of preterm and term neonates revealed equivalent levels of IL-10 production and similarly weak IL-12p70 and IFN-α responses. Entirely novel was the finding of a profound reduction in IL-12/IL-23p40 production in preterm neonates. The lack of IL-12/IL-23p40 in preterm neonates appears to play a critical role in susceptibility to infections, as demonstrated by the reduction in serum IL-12/IL-23p40 levels in preterm infants with EONS.

Most of the previously published data on TLR-stimulated responses in infants are derived from cord blood samples obtained from term neonates. Studies of term neonates have consistently reported deficits in IFN-α [20, 24, 25] and IL-12p70 responses [20, 26, 27]. In contrast, IL-6 and IL-10 responses were generally comparable or even higher when compared with those in adults [5, 20, 28–32]. However, data are very scarce for preterm neonates, and relatively large discrepancies are reported among studies. This high level of variation could be explained in part by the varying conditions that lead to premature birth. In animal models, chronic antenatal inflammation through repeated LPS exposure induces a state of innate immune tolerance manifested by a blunted response to subsequent TLR stimulation [33], which may affect measurements of TLR responses in cord blood. To avoid this potential pitfall and specifically examine the potential intrinsic developmental difference, we conducted our analysis exclusively in samples from subjects in whom no clinical or histological signs of chorioamnionitis (as defined using rigorous histological criteria) were detected.

In mice, IL-12/IL-23p40 deficiency induces susceptibility to a wide variety of microbial pathogens [18]. In contrast, older p40-deficient humans demonstrate no major disease susceptibility apart from infections involving salmonella and mycobacterium [34]. In term neonates, it was postulated that elevated production of p40 provided sufficient protective immunity via IL-23 [19, 20], but this seemingly is not the case in IL-12− and IL-23−deficient preterm neonates. The relatively mild clinical phenotype in human adults, compared with preterm neonates, is likely a result of the effect of compensatory immune mechanisms that may mask the contribution of TLRs to the protection conferred by the immune system. Preterm neonates lack these compensatory adaptive immune mechanisms, including maternally transferred antibodies, and therefore uniquely rely on TLR pathways to defend against microorganisms. The significantly lower serum level of p40 in neo-
Figure 4. Interleukin (IL–12 and IL–23 responses. Data are the mean ± standard error of the mean for IL–12p70 (A) or IL–12/IL–23p40 (B) responses in cord blood mononuclear cell (CBMC) culture supernatants. C, IL–12/IL–23p40 responses in conventional dendritic cells (cDCs). D, IL–23p40/p19 responses in CBMC culture supernatants. Responses to graded concentrations of individual Toll-like receptor agonists in term (black bars) and preterm (white bars) neonates were measured by gating on cDCs and are reported as mean fluorescence index (MFI). Agonists used for stimulation are shown above each graph, with their respective stimulated Toll-like receptor(s) shown in brackets. *P < .01. LPS, lipopolysaccharide.

Apart from its role in immune protection, IL–23 clearly plays a pathogenic role in autoimmune disorders through the promotion of proinflammatory, potentially tissue-damaging, IL–17-secreting T cells [18]. Attenuation of the IL–23 pathway may be particularly critical to the fetus to prevent Th17 inflammatory–mediated self-organ damage or a potential life-threatening immune response against maternal tissues. Indeed, evidence support an exquisite susceptibility of preterm infants to inflammation-mediated injury to the brain [35–37] or lung.
Table 1. Clinical Characteristics of Infants with Early-Onset Neonatal Sepsis

<table>
<thead>
<tr>
<th>Case</th>
<th>Time of onset of symptoms</th>
<th>Gestational age, weeks</th>
<th>Birth weight, g</th>
<th>Infection</th>
<th>PROM</th>
<th>ANC</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>&lt;6 h</td>
<td>34</td>
<td>2485</td>
<td>E. coli</td>
<td>Yes</td>
<td>&gt;72 h</td>
</tr>
<tr>
<td>2</td>
<td>&lt;6 h</td>
<td>28</td>
<td>1100</td>
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<td>Yes</td>
<td>&lt;72 h</td>
</tr>
<tr>
<td>3</td>
<td>&lt;6 h</td>
<td>40</td>
<td>2760</td>
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</tr>
<tr>
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<td>&lt;6 h</td>
<td>32</td>
<td>1940</td>
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<td>Yes</td>
<td>&gt;72 h</td>
</tr>
<tr>
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<td>&lt;6 h</td>
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</tr>
<tr>
<td>6</td>
<td>&lt;6 h</td>
<td>34</td>
<td>2060</td>
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<td>Yes</td>
<td>&lt;72 h</td>
</tr>
<tr>
<td>7</td>
<td>&lt;6 h</td>
<td>34</td>
<td>2870</td>
<td>GBS</td>
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</tr>
<tr>
<td>8</td>
<td>&lt;6 h</td>
<td>30</td>
<td>1520</td>
<td>H. influenzae</td>
<td>No</td>
<td>&gt;72 h</td>
</tr>
</tbody>
</table>

**NOTE.** ANC, timing of maternal exposure to antenatal corticosteroids relative to delivery; E. coli, *Escherichia coli*; GBS, group B streptococcus; H. influenzae, *Haemophilus influenzae*; PROM, prolonged rupture of membranes for >18 h.

*No symptoms.

...[38]. Termlike anti-inflammatory IL-10 responses and reduced IL-6, TNF-α, IL-12p70, and IFN-α responses, together with the almost-complete deficit in IL-12/IL-23p40 reported herein, may thus purposefully serve to protect the fetus (or preterm neonate) against potentially harmful inflammation.

Noticeably, our observations confirm and expand upon the findings of Levy et al [39], who had first reported high levels of activation of the TLR7/TLR8 pathway in term neonates compared with adults. Indeed, the TLR7/8 agonist 3M-003 stimulated a strong TNF-α response and IL-12 production in term neonates but, also, to a lesser extent in preterm neonates. Results suggest a potential therapeutic benefit of using TLR7/8 agonists to promote the development of protective vaccine-induced immunity against pathogens in these age groups, as has also been suggested by others [40]. However, given the damage that poorly regulated inflammation can cause in the preterm infant, one would first need to understand the effect that augmentation of innate immunity may have on susceptibility to inflammation-mediated organ injury in early life. The similarity between responses observed after intracellular (ie, TLR7 and TLR8), but not extracellular, TLR stimulation (ie, TLR1, TLR2, TLR4, and TLR6) on a per-cell basis suggests the existence of differential signaling requirements located proximally along the TLR signaling cascade, an aspect that we are currently investigating in purified cell populations.

Our study has potential limitations. A high proportion of mothers who deliver prematurely received antenatal systemic corticosteroids, which may affect the measurement of immune responses in cord blood. However, although antenatal corticosteroids may partially explain reduced levels of IL-6 and TNF-α in preterm neonates, reductions in IL-10 levels could have been expected as well but were not observed [41]. For production of IL-12/IL-23p40, equivalent concentrations of dexamethasone higher than that persistently observed in the serum of pregnant women after administration of exogenous corticosteroids [42] blunted the response in cord blood cell cultures [41]. However, in our study, the median time from the administration of antenatal corticosteroids to cord blood collection for preterm subjects was 16 days (interquartile range, 6.8–22 days). All but one mother of preterm infants had received antenatal corticosteroids more than 72 h before delivery, which is longer than the anticipated pharmacological effect of short-course corticosteroid therapy. In addition, we found no correlation between the timing of antenatal corticosteroid admin-

Figure 5. Serum cytokine concentrations in neonates at risk of early-onset neonatal sepsis. Levels of interleukin (IL)-6, IL-10, and IL-12/IL-23p40 were measured in serum samples simultaneously obtained from infants with culture-proven sepsis (cases; n = 8) and infants who did not present with any clinical or laboratory signs of infection (controls; n = 42) but were selected from a prospective cohort of neonates at-risk for early-onset neonatal sepsis. Bars denote medians and interquartile ranges.

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istration and the level of the IL-12/IL-23p40 TLR response (data not shown), suggesting that the deficit in p40 is intrinsic to the immune system of the preterm neonate. The exact mechanism by which the IL-12/IL-23p40 response is suppressed in preterm neonates deserves further investigation. Although it is plausible that remote antenatal systemic corticosteroid administration may affect measured preterm neonatal TLR responses, persistence of such an effect is likely of high clinical relevance in determining the susceptibility of preterm neonates to sepsis.

In conclusion, our results identify a novel, profound deficit in IL-12/IL-23p40 production, which may at least in part explain the greater risk of infections clinically observed in preterm infants. On the other hand, the overall attenuation of inflammatory response and relative preservation of anti-inflammatory responses may be part of a remarkably efficient mechanism aimed at protecting the fetus against harmful effects of untoward immunity before the full term of gestation is completed.

Acknowledgments

We thank Jennifer Claydon for administrative support with the coordination of this research; Chandra Pham and Sophie Perreault for recruitment of subjects and help with cord blood collection; Deborah McFadden for histological examination of placenta for preterm subjects; Darren Blimkie, Martine Caty, and members of the Wilson Laboratory for experimental assistance; Philippe Chessex for support and mentorship; and parents and nursing staff, who have been invaluable in the success of this study.

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