The Journal of Immunology

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J Immunol 2018; 201:1131-1143; Prepublished online 6 July 2018;
doi: 10.4049/jimmunol.1700956
http://www.jimmunol.org/content/201/4/1131

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Type 1 IFN Induction by Cytosolic Nucleic Acid Is Intact in Neonatal Mononuclear Cells, Contrasting Starkly with Neonatal Hyporesponsiveness to TLR Ligation Due to Independence from Endosome-Mediated IRF3 Activation

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Two million infants die each year from infectious diseases before they reach 12 mo; many of these diseases are vaccine preventable in older populations. Pattern recognition receptors represent the critical front-line defense against pathogens. Evidence suggests that the innate immune system does not fully develop until puberty, contributing to impaired response to infection and impaired vaccine responses in neonates, infants, and children. The activity of the pattern recognition receptor family of cytosolic nucleic acid (CNA) sensors in this pediatric population has not been reported. We show that in direct contrast to weak TLR-induced type I IFN in human cord blood mononuclear cells, cord blood mononuclear cells are capable of initiating a potent response to CNA, inducing both antiviral type I IFN and, unexpectedly, proinflammatory TNF-α. A deficiency in Rab11-GTPase endosome formation and consequent lack of IRF3 activation in neonatal monocytes is at least in part responsible for the marked disparity in TLR-induced IFN production between neonatal and adult monocytes. CNA receptors do not rely on endosome formation, and therefore, these responses remain intact in neonates. Heightened neonatal responses to CNA challenge are maintained in children up to 2 y of age and, in marked contrast to TLR4/9 agonists, result in IL-12p70 and IFN-γ generation. CNA sensors induce robust antiviral and proinflammatory pathways in neonates and children and possess great potential for use as immunostimulants or vaccine adjuvants for targeted neonatal and pediatric populations to promote cell-mediated immunity against invasive infectious disease. ***The Journal of Immunology*****, 2018, 201: 1131–1143.**

Neonates and infants are unduly susceptible to a wide variety of infections. Although many viral infections produce disease that is often severe in neonates compared with children or adults, the incidence of admittance to intensive care units (ICU) because of confirmed viral infection is low and has been estimated at 1% of infants admitted to ICU in one Dutch study (1). In contrast, bacterial infections account for the majority of admissions to both neonatal and pediatric ICU (2–4). The observation that viral infections, anecdotally, are less life-threatening in neonates and infants than bacterial infections was of particular interest to us. This implies that the pediatric population may have intact functional responses to viral when compared with bacterial infections, responses that could be harnessed pharmacologically to boost the immune system in neonates and infants.

Differences in the immune responses of adults and neonates have been a subject of interest in recent years (5), with gathering evidence to suggest that TLR-mediated responses elicited by the innate immune system of neonates, infants, and young children are significantly different compared with those in adults (6). TLRs are pattern recognition receptors (PRRs), a receptor family comprising the primary initiators of the immune response bridging innate and adaptive systems to induce rapid and efficient host defense mechanisms. PRRs comprise a number of subfamilies,

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Received for publication July 3, 2017. Accepted for publication June 10, 2018.

This work was supported by The National Children's Research Centre, Health Research Board HRA_PHR/2013/290, and Science Foundation Ireland 15/CDA/3497.

K.B., E.P.B., E.C., E.O., R.M., K.M., and A.M.R. performed experiments. B.D.O., D.M., N.A., D.N.O., A.N.C., A.M., and A.E.H. identified suitable donors and collected data and blood. K.B., E.J.M., S.P., F.M.M., and S.L.D. designed experiments, analyzed data, and wrote the manuscript.

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The online version of this article contains supplemental material.

Abbreviations used in this article: alum, aluminum salt; CBMC, cord blood mononuclear cell; cDC, conventional DC; cGAMP, cyclic guanosine monophosphate–adenosine monophosphate; CNA, cytosolic nucleic acid; DC, dendritic cell; ICU, intensive care unit; IRF, IFN regulatory factor; LDH, lactate dehydrogenase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; NMH, National Maternity Hospital; ODN, oligonucleotide; OLCHC, Our Lady's Children's Hospital, Crumlin; pDC, plasmacytoid DC; Poly(dA:dT), poly(deoxyadenylic-deoxythymidylic) acid; Poly(I:C), polyinosinic–polycytidylic acid; ppp, triphosphate; PRR, pattern recognition receptor; RLR, RIG-I–like receptor; SEAP, secreted embryonic alkaline phosphatase.

including membrane-bound TLRs, and cytosolic Nod-like receptors, RIG-I–like receptors (RLRs), and dsDNA sensors (7–10). Early studies demonstrated impairment of neonatal TLR4-induction of TNF-α (11), the mechanism of which was later attributed to soluble plasma factors, such as adenosine, found in cord blood plasma (12). TLRs activate a number of downstream signaling pathways to orchestrate an immune response. In addition to inducing TNF-α, a number of TLRs engage the IFN regulatory factor (IRF) pathway to induce type I IFNs (9). Like TNF-α, TLR4-induced IFN-β has also been shown to be impaired in cord blood cells compared with adult cells (13). It is likely that these differences contribute to the inability of infants to mount an effective immune response both to certain invasive pathogens and to vaccine formulations.

Our understanding of how the innate immune system recognizes viral nucleic acid in adults has grown exponentially over the past decade (8). Cytosolic RLRs and dsDNA sensors have both been reported to be essential for induction of innate immune responses to viral infection (14, 15). However, to date, there have been no investigations into the presence or activity of the cytosolic intracellular nucleic acid–sensing PRRs and their downstream pathways in neonates and infants. In this study, we show that all cytosolic nucleic acid (CNA) sensor agonists tested in cord blood mononuclear cells (CBMCs) resulted in robust induction of IFN-α production equivalent or greater than those elicited from PBMCs, and unexpectedly, all CNA sensor agonists induced marked, enhanced production of proinflammatory TNF-α production from neonates compared with adults. We show that deficiency in Rab11a-positive endosome formation in neonatal monocytes is, at least in part, responsible for the difference observed in type I IFN production between neonates and adults in response to TLR4 ligand versus CNA. Finally, we show that the heightened neonatal responses to CNA challenge are maintained in healthy children up to 2 y of age and that CNA challenge alone induces IL-12p70 and IFN-γ in neonates, whereas TLR4 and TLR9 agonists did not. Our data indicate that harnessing the proficiencies of CNA sensors may have utility for stimulating the developing immune system in the neonatal and pediatric populations.

Materials and Methods

Subjects

Umbilical cord blood samples were obtained from term births following normal pregnancy, labor, and delivery at National Maternity Hospital (NMH). All infants had an uncomplicated postnatal course and Apgar scores of 9 at 5 min. Venous blood from infants, children, and adolescents of various ages was collected during elective surgical procedures in which no indication of infection was present (such as hydrocele repair, umbilical hernia repair, onychocryptosis, or orchipexy repair) in Our Lady's Children's Hospital, Crumlin (OLCHC). In all cases, children had an extra 1–10 ml of blood drawn postanesthetic, thus avoiding extra venupuncture. Volumes drawn were age and weight dependent according to standard, approved guidelines. Ethics approval was obtained from the Ethics Committees of NMH and OLCHC, and informed consent was obtained from each subject or their parent/guardian. Adult blood samples were from healthy adults, supplied by the Irish Blood Transfusion Service.

Isolation of mononuclear cells and monocytes

Primary PBMCs or CBMCs were isolated from healthy human blood or cord blood. Monocytes were isolated by negative selection using Monocyte Isolation Kit II (Miltenyi Biotec). Monocyte purity was assessed using CD14 staining and was routinely 85–95%. Cells were cultured at 37°C, 5% CO_2, 95% air in RPMI 1640, with stable 2.5 mM L-glutamine and 0.5 mM sodium pyruvate with 10% FBS (all from Sigma-Aldrich).

Stimulation of PBMCs and CBMCs

LPS (100 ng/ml) (Enzo Life Sciences), CL075 (5 μg/ml), and CpG oligonucleotide (ODN) 2395 (1 μM) (both from InvivoGen) were used to activate TLR4, 8, and 9, respectively. Polyinosinic–polycytidylic acid [Poly(I:C)] (5 μg/ml) (InvivoGen), poly(deoxyadenylic-deoxythymidylic) acid [Poly(dA:dT)] (5 μg/ml) (Sigma-Aldrich), 2′3′ cyclic guanosine monophosphate–adenosine monophosphate (cGAMP) (10 μg/ml), 5′ triphosphate (ppp) dsRNA (2.5 μg/ml), and HSV60 (2 μg/ml) (all from InvivoGen) were transfected into PBMCs or CBMCs using TransIT-X2 (Mirus Bio).

Measurement of cytokines

HEK-Blue TNF-α/IFN-α/β assays were performed as per manufacturer's instructions using QUANTI-Blue Detection Reagent. Secreted embryonic alkaline phosphatase (SEAP) levels were determined using a spectrophotometer plate reader at 630 nm. IFN-α (3425-1H-6; Mabtech) and IP-10 (DY266; R&D Systems) were detected by sandwich ELISA. IFN-α ELISA used pan-specific IFN-α Abs, which allows detection of IFN-α subtypes 1/13, 2, 4, 5, 6, 7, 8, 10, 14, 16, and 17. BioLegend LEGENDplex Human Inflammation Panel (13-plex) was carried out as per manufacturer's instructions to determine levels of IFN-γ, IL-10, IL-12p70, and IL-23. A BD LSRFortessa cell analyzer was used to acquire samples, and BioLegend LEGENDplex software was used for analysis.

IRF3/7 activation

Active Motif TransAM IRF3 and IRF7 assays were carried out on nuclear extracts from PBMCs or CBMCs and harvested as per manufacturer's instructions to detect and quantify IRF3 or IRF7 activation.

Cell viability

A lactate dehydrogenase (LDH) cytotoxicity assay kit (Pierce) was used to measure cell death in response to stimulation as per manufacturer's instructions. The absorbance was read at 490 nm, and background (absorbance at 680 nm) was subtracted. A CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay] (Promega) was also used to measure cell viability through assaying mitochondrial function, according to manufacturer's instructions. Absorbance was read at 490 nm on a 96-well plate spectrophotometer.

Quantitative RT-PCR

Total RNA from PBMCs or CBMCs was extracted using TRIzol extraction as previously described (16) and was reverse transcribed using M-MLV Reverse Transcriptase (Promega) according to the manufacturer's protocol. This cDNA served as template for amplification of target genes, along with the housekeeping gene *βActin,* by real-time PCR with SensiFAST SYBR Green (Bioline) to determine the relative amounts of CXCL10, IFN-α, and TNF-α mRNA. The ABI 7900HT system (Applied Biosystems) was used for real-time PCR, and the cycling threshold method ($2^{-(\Delta\Delta Ct)}$) was used for relative quantification by comparative method after normalization to *βActin* expression. The primers used were: CXCL10 forward 5′-AGCAGAGGAACCTCCAGTCT-3′, reverse 5′-ATGCAGGTACAGCGTACAGT-3′; IFN-α forward 5′-TGAAGGACAGACATGACTTTGG-3′, reverse 5′-TCCTTTGTGCTGAAGAGATTGA-3′; Rab5 forward 5′-ACGGGCCAAATACGGGAAAT-3′, reverse 5′-AGAAAAGCAGCCCCAATGGT-3′; Rab7 forward 5′-CAGACAAGTGGCCACAAAGC-3′, reverse 5′-AAGTGCATTCCGTGCAATCG-3′ Rab10 forward 5′-CCTCAGAAAGCCCGAGTGAG-3′, reverse 5′-GTCGTACGTCTTCTTCGCCA-3′; Rab11 forward 5′-CTTCGGCCCTAGACTCTACA-3′, reverse 5′-CACTGCACCTTTGGCTTGTT-3′; TNF-α forward 5′-CTGGGCAGGTCTACTTTGGG-3′, reverse 5′-CTGGAGGCCCCAGTTTGAAT-3′. IFN-α primers were designed to detect IFN-α subtypes 2, 5, 6, 8, 14, 16, 17, and 21.

Flow cytometry

PBMCs or CBMCs were labeled for the investigation of dendritic cell (DC) or monocyte subsets with the following fluorochrome-labeled Abs: CD45 (2D1), CD16 (3G8), CD14 (M5E2), CD66b (G10F5), CD11c (3.9), Lineage (CD3, CD14, CD16, CD19, CD20, CD56), HLA-DR (L243), and CD123 (6H6) (All from BioLegend). Each staining well contained 4×10^5 cells; cells were stained with LIVE/DEAD Aqua (Molecular Probes) followed by staining for 20 m on ice, washed, and analyzed by flow cytometry immediately. Gating during analysis was based on fluorescence minus one controls. For transfection efficiency investigation, PBMCs or CBMCs were transfected with Poly(I:C) fluorescein (5 μg/ml) (InvivoGen) for 24 h and analyzed for percentage fluorescein expression as a measure of transfection efficiency. Flow cytometry was carried out on a BD LSRFortessa cell analyzer and analyzed using FlowJo software (Tree Star).

Confocal imaging

$CD14^+$ monocytes, negatively selected from PBMCs or CBMCs, were seeded on Nunc Lab-Tek II Chamber Slide system. LPS was sonicated for 30 s and preincubated in serum-containing medium at 37°C for 5 m before being added to cells. Cells were stimulated with LPS (1 μg/ml) for 1 h. Cells were fixed in 2% paraformaldehyde, permeabilized in 0.05% Triton X-100, and stained with goat anti-human Rab11 (K-15) (Santa Cruz Biotechnology) at 10 μg/ml and anti-goat Alexa Fluor 647 at 4 μg/ml or, for costaining, rabbit anti-human Rab11 (ab3612) and mouse anti-human TLR4 (ab22048), and anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 647. Cells were mounted using ProLong Gold Antifade Mountant with DAPI. Images were captured using an Axio Observer Z1 inverted microscope equipped with a Zeiss LSM 700 T-PMT scanning unit and a 40× plan apochromat objective. Image analysis was carried out using LSM Image Browser.

Statistical analyses

Data were analyzed with GraphPad Prism software. Normality testing was carried out using Shapiro–Wilk, Kolmogorov–Smirnov, and D'Agostino–Pearson omnibus normality testing. When datasets were found to follow a nonnormal distribution, a Kruskal–Wallis with Dunn multiple comparison test or Mann–Whitney *U* test was carried out. Statistical analysis on normal datasets was performed with Student *t* test when two individual experimental groups were analyzed. For multiple comparisons, ANOVA was used with a Bonferroni posttest. Two-tailed tests were used throughout, and the statistical approaches were all deemed to be valid for each individual experiment.

Results

Previous studies have reported that LPS- and CpG-induced type I IFN production is decreased in neonatal cells in comparison with adult cells (13, 17). Using HEK-Blue–IFN-α/β reporter cells, we confirmed this observation in PBMCs and CBMCs ($n \geq 30$) (Fig. 1A, 1B). Data in Fig. 1A–E are represented as both bar charts and dot plots. CL075, a TLR7/8 agonist, also induced significantly lower levels of IFN-α/β production from CBMCs compared with PBMCs ($n = 35$), albeit in a less marked manner (Fig. 1C). In contrast, mimicking viral infection through activation of cytosolic RLR/dsDNA sensors with transfected Poly(I:C) and Poly(dA:dT) showed no inhibition of type I IFN production in CBMCs when compared with PBMCs ($n \geq 39$) (Fig. 1D, 1E). Transfection-only controls were also carried out (Supplemental Fig. 1A). Untransfected Poly(I:C), as a TLR3 agonist, was also used to stimulate PBMCs and CBMCs or isolated monocytes from adult and cord blood, and, as expected from the literature (17), type I IFN responses were decreased in neonatal cells (Supplemental Fig. 1B, 1C). MTS and LDH assays were performed on stimulated PBMCs and CBMCs to assess whether cell death occurred in either cell type in response to TLR, RLR, or dsDNA sensor ligation that might skew the results; however, no differences were observed between cell types (Fig. 1F, 1G). Dot plots for MTS and LDH assays are shown in Supplemental Fig. 1D and 1E.

To ensure we were comparing like with like, we assessed the subpopulations of mononuclear cells in our neonatal CBMC preparations and our adult mononuclear cell preparations. Leukocyte percentages in PBMCs and CBMCs were measured via flow cytometry, and, based on $CD45^+$ staining, no significant difference in leukocyte percentage frequency was found between adult and neonatal mononuclear cells (Fig. 2A). DC populations were also measured, as plasmacytoid DCs (pDCs) are excellent producers of Type I IFN (18). Lineage $HLA\text{-}DR^+$ DC cells were analyzed for expression of CD123 and CD11c, markers of pDCs and conventional DCs (cDCs), respectively. No significant difference in the populations of either pDCs or cDCs that might account for differences in cytokine production was observed when comparing adult and neonatal cells (Fig. 2B). Potential differences in monocyte populations between adult and cord blood were also investigated. Total monocytes were characterized as $CD66b^-CD14^+CD11c^+$ and, based on their expression of CD16 and level of expression of CD14, were further characterized as classical ($CD14^+CD16^-$), patrolling ($CD14^{intermediate}CD16^+$), or inflammatory ($CD14^+CD16^+$) monocytes. No significant differences in any of these monocytes populations were observed when comparing mononuclear cells isolated from adult and cord blood (Fig. 2C). We next investigated whether there was any differential transfection efficiency between neonatal and adult mononuclear cells. Fluorescein-labeled Poly(I:C) was transfected into cells, and fluorescein uptake was measured via flow cytometry. No significant difference was noticed in transfection efficiency of adult versus neonatal cells (Fig. 2D). Monocytes were isolated through negative selection from PBMCs or CBMCS to a purity of >85%. As observed in Fig. 2E, monocytes showed similar trends of IFN production as those observed from total adult and neonatal mixed mononuclear cell populations. Dot plots are shown in Supplemental Fig. 2. We were unable to isolate sufficient numbers of pDCs from our preparations to check pDC responses by ELISA, and flow cytometric analysis of type I IFN was unfeasible. Our data indicated that differences observed between neonatal and adult responses to ligands of TLRs and similarities observed between neonatal and adult responses to ligands of CNA sensors were a result of differences in intracellular signaling pathways and not due to alterations in cell population, viability, or transfection efficiency.

Next, we performed IFN-α ELISAs on cells supernatants from adult or neonatal mononuclear cells treated with LPS, CpG ODN, transfected Poly(I:C), or transfected Poly(dA:dT) ($n \geq 23$). Similar trends and significant differences were seen for the IFN-α ELISA as had been observed in the IFN-α/β SEAP assays, with significantly decreased TLR4/9-induced IFN-α responses but equivalent or enhanced CNA sensor responses in neonates compared with adults (Fig. 3A–D). Alternative preparations of nucleic acid (e.g., 5′ ppp dsRNA and HSV60) are suggested to specifically activate RIG-I and IFI16, respectively; similarly, 2′3′ cGAMP is the direct ligand for STING (19–21). These agonists were tested for their ability to induce IFN-α from adult and neonatal cells ($n \geq 18$). In all cases, the levels of IFN-α produced in response to these ligands was lower than that in response to either Poly(I:C) or Poly(dA:dT); nevertheless, CBMCs responded equally well or with enhanced IFN-α production when compared with PBMCs (Fig. 3E–G). To ensure that this increase in neonatal IFN-α production was not linked to an increase in cell death, cell viability was assayed via MTS assay in cells treated with CNA agonists and compared with unstimulated controls. No difference in cell viability was observed between PBMCs and CBMCs following nucleic acid stimulation (Fig. 3H).

IFN-α is an antiviral cytokine particularly relevant for host defense against intracellular pathogens as it promotes a Th1 type immune response (22). TNF-α also promotes a Th1-type response. TNF-α has previously been studied in the neonatal setting in response to TLR agonists but not in response to CNA sensors. Following our novel observation that CNA induced an increase in production of IFN-α in neonatal compared with adult mononuclear cells, TNF-α production in response to CNA was investigated in more detail. TNF-α was measured in supernatant of PBMCs and CBMCs treated with Poly(I:C)-, Poly(dA:dT)-, dsRNA-, cGAMP-, or HSV60-transfection or LPS, CL075, or CPG ODN for 4, 8, or 24 h ($n \geq 29$). Interestingly, all CNA tested induced significantly more TNF-α production from neonatal cells than from adult cells (Fig. 4A–E). In contrast, no significant difference in TNF-α production was observed from CBMCs and PBMCs treated with TLR agonists LPS or CL075 (Fig. 4F, 4G). CpG ODN did not induce any production of TNF-α from either PBMCs or CBMCs (Fig. 4H).

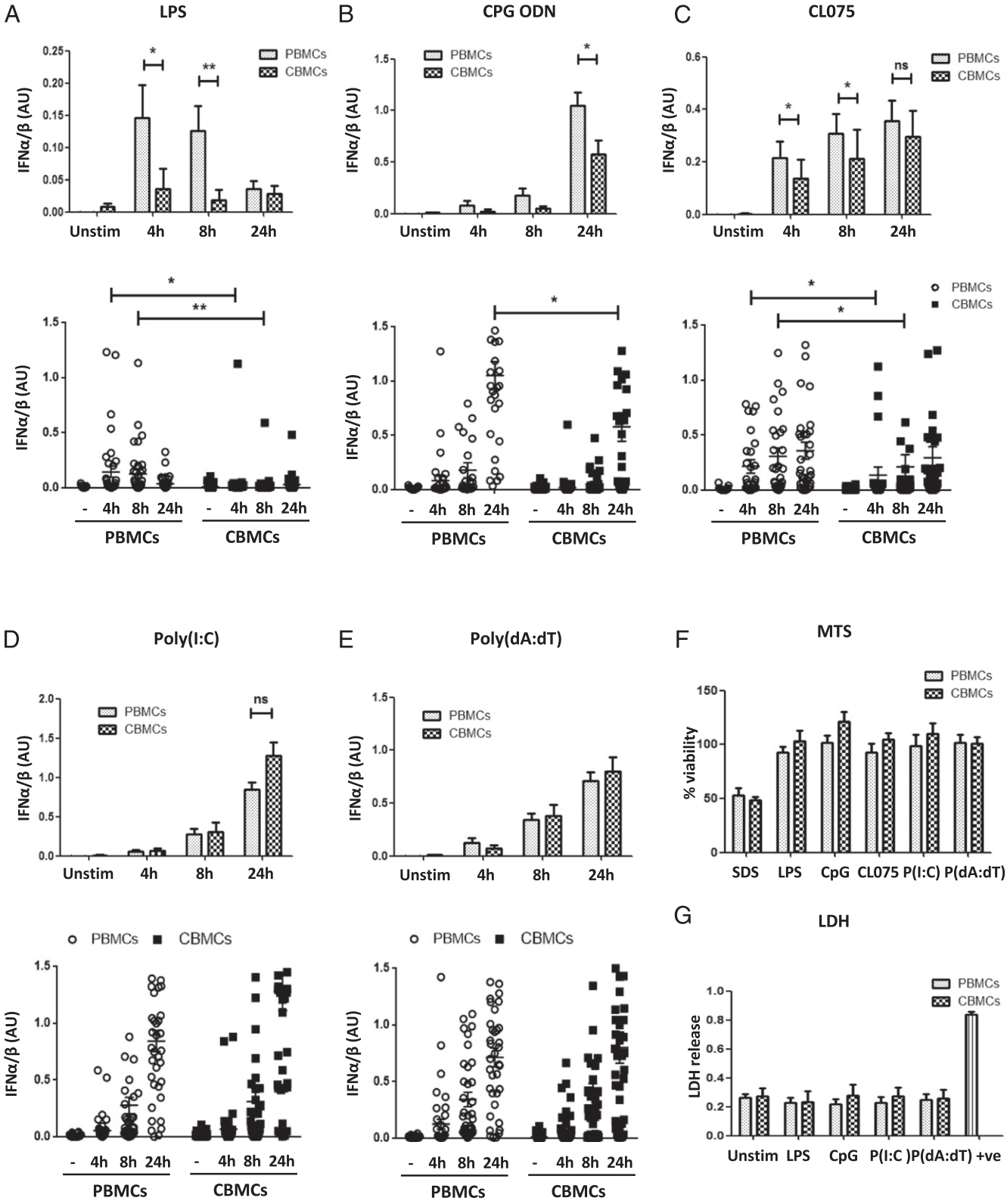

FIGURE 1. Differential regulation of neonatal IFN-α/β secretion between membrane-bound TLRs and CNA sensors. (**A**–**G**) Adult PBMCs or neonatal CBMCs were stimulated with (A) LPS, (B) CpG ODN, (C) CL075, (D) Poly(I:C) transfection, or (E) Poly(dA:dT) transfection for 4, 8, or 24 h. Levels of IFN-α/β were assayed via HEK-Blue IFN-α/β SEAP assay. Cell viability, following 24 h stimulation with ligands, was assayed using (F) MTS assay or (G) LDH assay. Data in the upper panels of (A)–(E) are mean ± SEM ($n \geq 30$ donors in each group), with individual responses shown in lower panels. Data in (F) and (G) are mean ± SEM ($n = 10$ donors in each group). (A–E) Kruskal–Wallis nonparametric test with Dunn posttest was used to compare groups. (F and G) The p value was determined by ANOVA and Bonferroni posttest. *$p < 0.05$, **$p < 0.01$.

Thus far, we had observed differential regulation of IFN-α that was broadly delineated between responses to cytosolic receptors versus responses to membrane-bound receptors, with type I IFN responses intact or enhanced in neonates in response to CNA but attenuated in response to TLR4/7/8/9 activation. The ability of neonatal cells to induce equal levels of TNF-α as adult PBMCs in

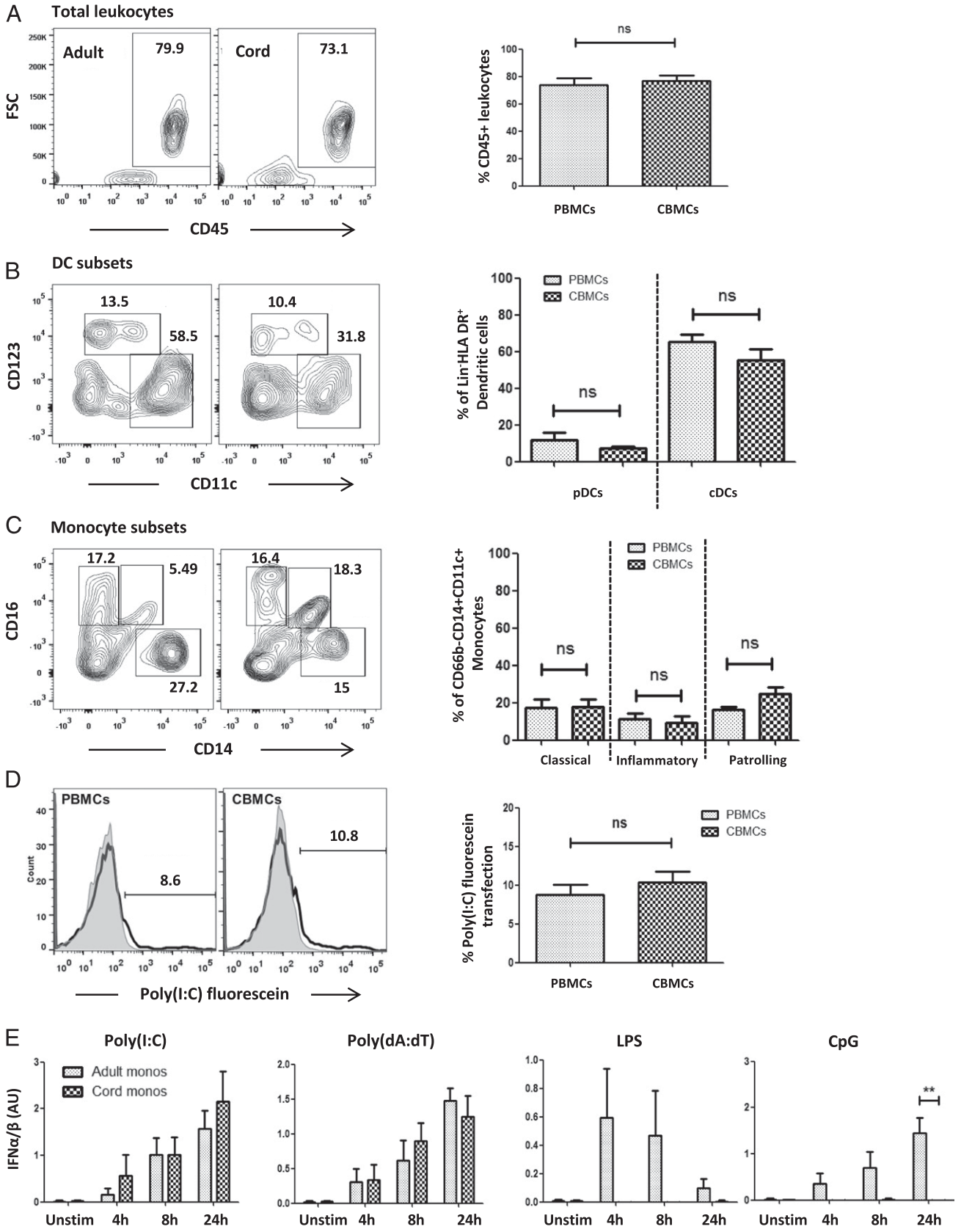

FIGURE 2. Frequencies of IFN-producing leukocyte subsets are similar in adult and cord blood. Adult PBMCs or neonatal CBMCs were isolated and stained with Abs to distinguish between cell subsets. (**A**) Leukocytes were gated on based on expression of CD45, and percentage leukocyte population was compared between PBMCs and CBMCs. (**B**) DCs were gated on as follows: $CD45^+Lin\text{-}HLA\text{-}DR^+$ and analyzed for percentage frequency of $CD11c^+CD123^-$ (cDCs) or $CD11c^-CD123^+$ (pDCs). (**C**) Monocytes were gated on as follows: $CD45^+CD66b^-CD11c^+$ and analyzed for percentage frequency of $CD14^+CD16^-$ (classical monocytes), CD14 intermediate $CD16^+$ (patrolling monocytes), or $CD14^+CD16^+$ (inflammatory monocytes) (**D**) PBMCs or CBMCs were transfected with Poly(I:C) fluorescein. Following 24 h incubation, the percentage of $fluorescein^+$ cells was analyzed. (**E**) Monocytes, isolated from adult or cord blood, were transfected with Poly(I:C) or Poly(dA:dT) or treated with LPS or CpG as indicated. Levels of *(Figure legend continues)*

response to TLR activation (Fig. 4F, 4G) indicated that cell signaling from these receptors to NF-κB is fully intact. Furthermore, the ability of neonatal cells to secrete IFN-α in response to CNA indicates that the process of type I IFN secretion is not affected. The implication is that factors specific for the signaling pathway to IFN-α/β induction downstream of TLRs (but not CNA sensors) are inhibited in some manner. To verify this, real-time quantitative PCR was used to measure levels of mRNA expression of TNF-α, IFN-α, and CXCL10 in response to Poly(I:C), Poly(dA:dT), LPS, or CpG ODN ($n \geq 4$). There was no significant difference in TNF-α levels in response to either CNA transfection or LPS stimulation between PBMCs or CBMCs (Fig. 5A, dot plots shown in Supplemental Fig. 3A). In contrast, gene expression levels of IFN-α and CXCL10, an IFN–inducible gene, were inhibited in CBMCs in response to CpG and LPS, respectively, when compared with the PBMC response. In contrast, there was no inhibition of transcription of either IFN-α or CXCL10 in CBMCs in response to CNA stimulation (Fig. 5B, 5C, Supplemental Fig. 3B, 3C). Together, these data indicate the TLR-induced signaling pathway to IFN gene expression is inhibited in a manner that does not affect the signal transduction pathway used by CNA sensors to induce gene expression of IFN.

IRF3 and IRF7 are implicated as transcription factors activated downstream of PRR ligation to induce type I IFN. IRF7, in particular, is thought to be important for the induction of IFN-α (23, 24). We hypothesized that IRF7 activation would be enhanced in CBMCs in response to Poly(I:C) transfection when compared with IRF7 activated by TLR4 or TLR9 ligation. Intriguingly, we found that IRF7 was not significantly activated by LPS, CpG, or Poly(I:C) transfection in the CBMCs ($n = 4$); by striking contrast, LPS, CpG, and Poly(I:C) all activated IRF7 in adult PBMCs ($n = 5$), with percentage increases in activation over basal IRF7 between 150 and 250% (Fig. 5D, Supplemental Fig. 3D). Aksoy et al. (13) have previously shown that impaired IRF3 DNA binding and CBP interaction in neonatal DCs exposed to LPS are associated with impaired expression of IFN-β and IFN-inducible genes. As mentioned, both IRF3 and IRF7 are implicated as transcription factors for both IFN-α and IFN-β, so we next investigated whether, in contrast to LPS-induced impaired IRF3 activation in neonates, Poly (I:C) transfection might promote activation of IRF3 DNA binding. We observed an ∼20% increase in IRF3 activation above basal levels in response to either LPS or CpG stimulation in CBMCs, indicating significant but low levels of IRF3 activation ($n = 4$). On the other hand, Poly(I:C) transfection resulted in an ∼80% increase in IRF3 activation above basal levels in CBMCs ($n = 4$) (Fig. 5E, Supplemental Fig. 3E).

Together, these results indicate that IRF7, although expressed, may not be activated in neonates and that the signaling pathway to activate IRF3 to induce IFN-α transcription is muted in CBMCs in response to LPS and CpG when compared with the efficient response to CNA. In support of this observation, we assessed the production of CXCL10 from PBMCs and CBMCs in response to CNA transfection or LPS stimulation ($n \geq 29$). There was no significant difference in the CXCL10 response between PBMCs or CBMCs to either Poly(I:C) or Poly(dA:dT) transfection (Fig. 5F, 5G, Supplemental Fig. 3F, 3G); however, LPS-induced CXCL10 secretion was significantly decreased in CBMCs compared with PBMCs (Fig. 5H, Supplemental Fig. 3H). This observation highlights that induction of IFN-α transcription and the subsequent IFN response appear fully functional downstream of CNA transfection in CBMCs, in direct contrast to TLR stimulation of CBMCs, in which transcription of IFN-α appears to be significantly inhibited with consequent subduing of the IFN response.

A key difference between TLR induction of IFN-α/β and CNA induction of IFN-α/β is TLR dependence on endosomal localization to engage with TRAF3 and the IRFs. Several Rab GTPases have been reported to be involved in trafficking TLRs to endosomes (25). Of particular interest, in the absence of Rab11a, IFN-α/β production in response to *Escherichia coli* is significantly decreased, whereas TNF-α induction is unchanged (26). Therefore, we investigated the expression and function of Rab11a in PBMCs versus CBMCs. Real-time quantitative PCR was used to investigate basal levels of Rab GTPase mRNA in PBMCs and CBMCs. Rab11a levels were found to be significantly lower in CBMCs compared with PBMCs (Fig. 6A) Strikingly, Rab11a was the only Rab GTPase with an expression profile that was significantly different between CBMCs and PBMCs, and Rab5, Rab7, and Rab10 all showed comparable mRNA expression between CBMCs and PBMCs ($n \geq 9$) (Fig. 6B–D, Supplemental Fig. 4B–D). We next investigated if $Rab11^{+ve}$ endosome formation was impaired in neonatal monocytes when compared with adult monocytes. $CD14^{+ve}$ monocytes were isolated from CBMCs and PBMCs ($n = 3$). Adult monocytes showed abundant accumulation of $Rab11^{+ve}$ endosomes in response to LPS stimulation (Fig. 6E, upper panels). $Rab11^{+ve}$ endosomes were not observed to the same extent in neonatal monocytes in response to LPS (Fig. 6E, lower panels). Fig. 6F (Supplemental Fig. 4E) depicts the significant difference between the percentage of adult and neonatal cells with detectable Rab11 following LPS treatment. There is a clear inhibition of $Rab11^{+ve}$ endosome formation in neonatal monocytes in response to LPS stimulation when compared with adult monocytes ($p = 0.00011$). To further support our findings, we investigated whether LPS-induced TLR4 colocalization with Rab11a would be less evident in neonatal monocytes. Accumulation of $Rab11^{+ve}$ endosomes in response to LPS stimulation was observed in adult monocytes, with colocalization with TLR4 indicated by white arrowheads (Fig. 6G, left-hand side panels). Again, $Rab11^{+ve}$ endosomes were difficult to observe in neonatal monocytes in response to LPS and are indicated by red arrow heads and did not appear to colocalize with TLR4 (Fig. 6G, right hand side panels). Given the literature on the reliance of LPS-induced type I IFN on Rab11a, it seems likely that this is one mechanism by which neonatal cells constrain the immune response to Gram-negative bacteria.

To assess if CNA-induced IFN-α and TNF-α were a peculiarity of neonatal cord blood or whether these responses were maintained into infancy and early childhood, blood samples from healthy children in various age groups (4–24 mo; 2–5 y; 6–11 y) undergoing elective surgery were collected and assayed for their ability to produce both type I IFN ($n \geq 17$) and TNF-α ($n \geq 16$). As can be seen in Fig. 7A and 7B, the production of type I IFN in response to Poly(I:C) and Poly(dA:dT) was maintained throughout childhood. This maintenance of cord blood responsiveness to CNA was also observed for both Poly(I:C)- and Poly(dA:dT)-induced TNF-α production to the age of 24 mo (Fig. 7C, 7D).

IFN-α/β were assayed via HEK-Blue IFN-α/β SEAP assay. Dot plots and histograms in (A)–(D) are representative of ≥5 individual donors. Graphs in (A)–(E) are mean ± SEM ($n \geq 5$ donors in each group). (A–D) Student t test was used to test for significant difference between groups. (E)The p value was determined by ANOVA and Bonferroni's posttest. $^{**}p < 0.01$.

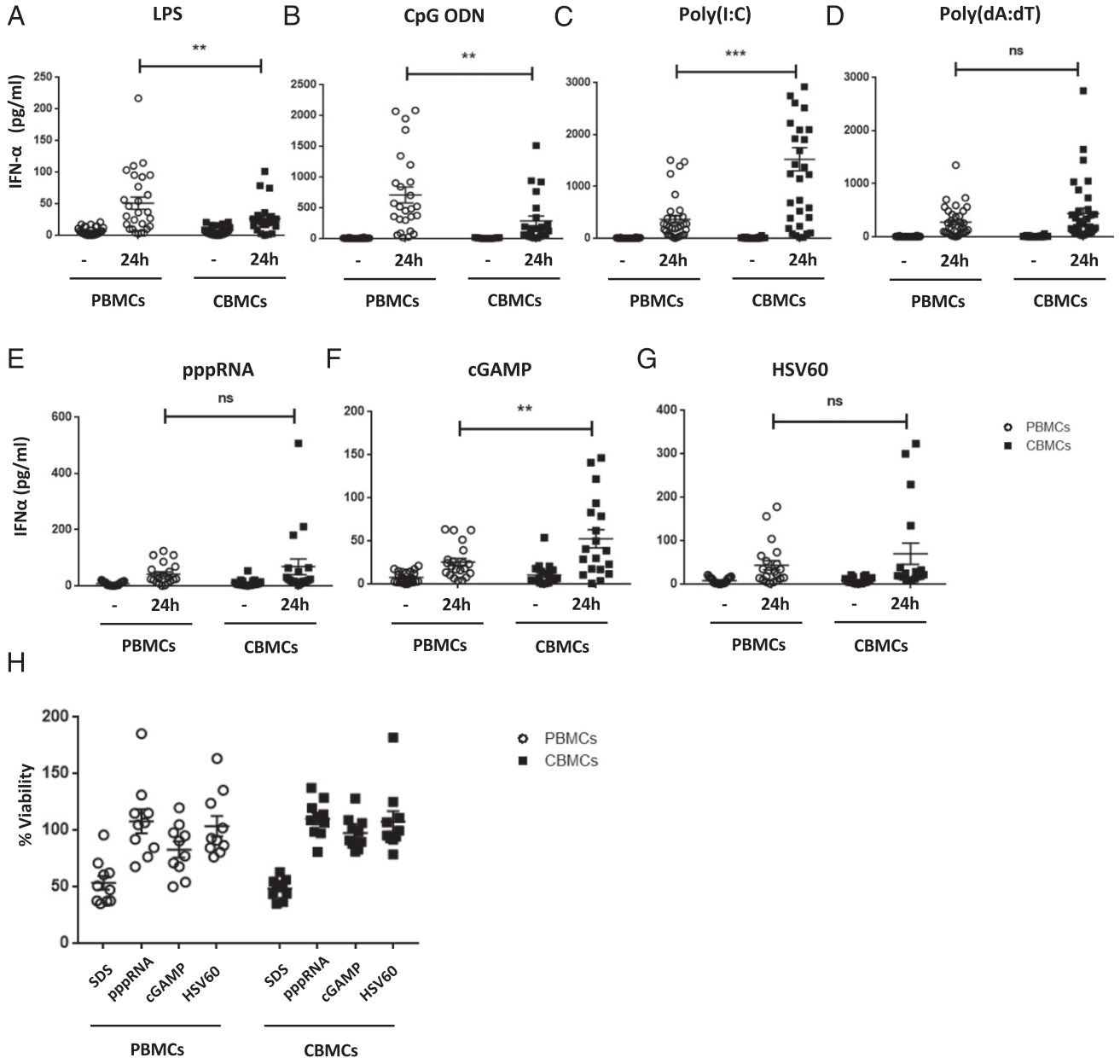

FIGURE 3. Neonatal IFN-α secretion is attenuated in response to TLR ligation but enhanced in response to CNA. PBMCs or CBMCs were treated with (**A**) LPS or (**B**) CpG ODN or transfected with (**C**) Poly(I:C), (**D**) Poly(dA:dT), (**E**) 5′ ppp dsRNA, (**F**) 2′3′ cGAMP, or (**G**) HSV60 for 8 or 24 h, as indicated, and IFN-α was measured by ELISA. (**H**) Cell viability following 24 h treatment was assayed using MTS assay. (A–G) Graphs show mean ± SEM ($n \geq 18$ donors in each group). (H) Graph compares mean ± SEM of $n = 10$ donors in each group $^{**}p < 0.01$, $^{***}p < 0.005$ as determined by ANOVA and Bonferroni posttest.

Given the potential of Poly(I:C) transfection to induce substantial IFN-α and TNF-α production in neonates and infants and consequently give rise to a Th1-type immune response desirable in vaccine-induced adaptive immunity, we sought to explore the polarizing ability of Poly(I:C) transfection on IL-12p70, an important mediator of such responses ($n = 21$) (27–31). IL-12p70 levels were low, as expected in a mixed population of cells; however, despite this, a similar significantly enhanced response to Poly(I:C) transfection was found in CBMCs compared with PBMCs (Fig. 8A). IL-12p70 was measured in a multiplex assay which included the analyte IFN-γ. IFN-γ is the primary cytokine that defines Th1 cells and is produced predominantly by $CD4^+$ Th1 and $CD8^+$ cytotoxic T cells, creating a positive feedback loop causing naive $CD4^+$ cells to differentiate into Th1 cells (32). To our great surprise, Poly(I:C) transfection induced striking levels of IFN-γ, which was also significantly increased in CBMCs compared with PBMCs (Fig. 8B), despite the lack of a T cell activator (e.g., anti-CD3). IFN-γ is secreted at low concentrations by macrophages, NK cells, and NKT cells as part of the innate immune response, is an important activator of both macrophages and NK cells, and induces expression of MHC class I and II (33). Levels of Th17-supporting cytokine IL-23 and anti-inflammatory IL-10 production were low but detectable, with no significant difference between adult and cord blood cells noted (Fig. 8C, 8D). The source of the observed IFN-γ secreted by CBMCs in response to Poly(I:C) transfection remains to be identified in future experiments; however, the high levels of IFN-γ and enhanced IL-12p70 secretion implies that the environment created through activation of CNA sensors, in a mixed blood cell population, would be tailored toward promoting a Th1-mediated cellular

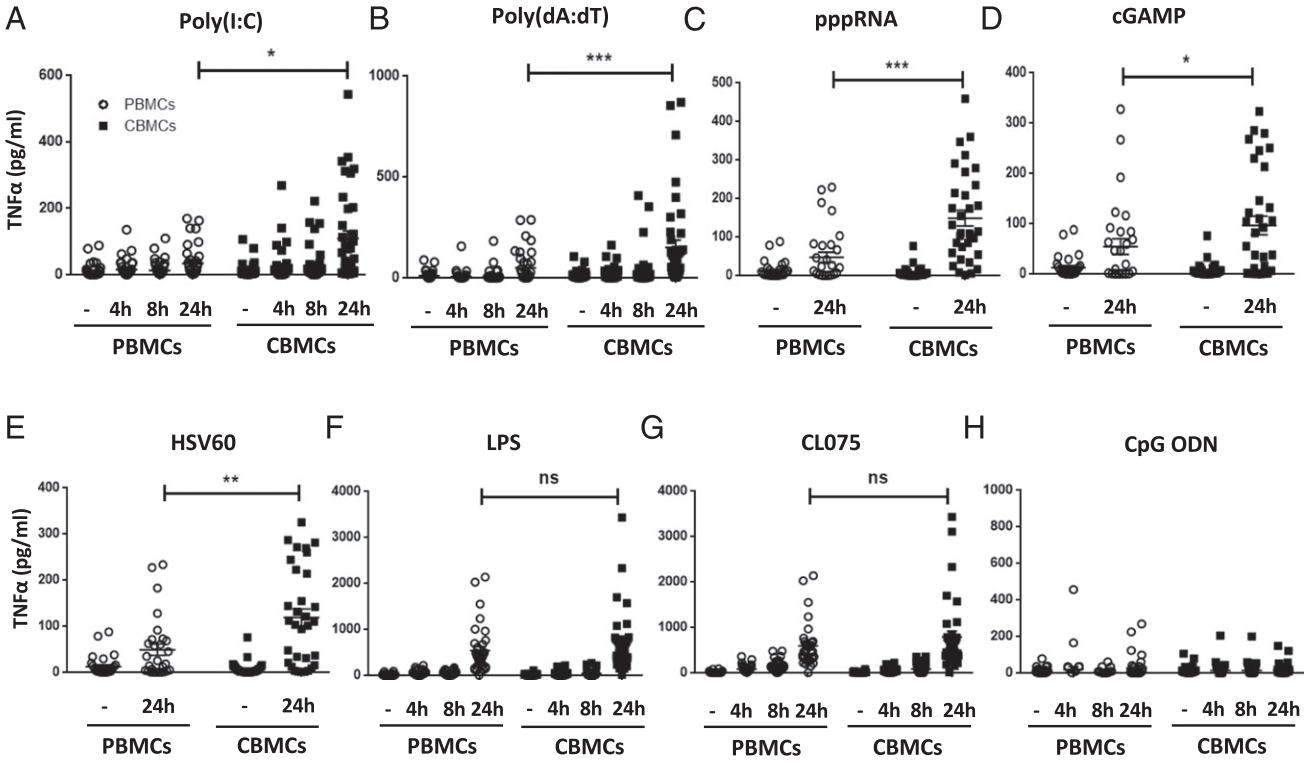

FIGURE 4. TNF-α secretion from CBMCs is enhanced compared with adult PBMCs in response to a range of CNA receptor agonists. PBMCs or CBMCs were transfected with (**A**) Poly(I:C), (**B**) Poly(dA:dT), (**C**) 5′ ppp dsRNA, (**D**) 2′3′ cGAMP, or (**E**) HSV60 or treated with (**F**) LPS, (**G**) CL075, or (**H**) CpG ODN for the indicated timepoints. Following stimulation, supernatant was harvested from the cells, and TNF-α was measured using HEK-Blue TNF-α assay and made relative to 125 pg/ml TNF-α. Graphs show means ± SEM ($n \geq 29$ donors in each group). Kruskal–Wallis nonparametric test with Dunn posttest was used to test for significant difference between groups. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$.

immune response. To determine whether Poly(I:C) transfection might present a better adjuvant option for pediatric vaccine design than LPS and CpG analogs currently in use or in development (34–36), IL-12p70, IFN-γ, IL-10, and IL-23 were also measured in CBMCs in response to LPS ($n = 20$) or CpG ($n \geq 16$). In direct contrast to the cytokine profile observed in CBMCs transfected with Poly(I:C), neither LPS nor CpG activation of PBMCs or CBMCs resulted in detectable IL-12p70 (Fig. 8E, 8I) or secretion of IFN-γ (Fig. 8F, 8J). Again, IL-23 and IL-10 levels were low, with no significant difference between adult and neonatal mononuclear cells noted in response to LPS or CpG (Fig. 8G, 8K, 8L). Dot plots are shown in Supplemental Fig. 4F–Q.

Discussion

Noting the potential disparity in neonatal and pediatric morbidity between bacterial and viral infections in ICU admissions, we speculated that infants were perhaps better equipped to fight viral infection than bacterial infection. Viruses and bacteria are both recognized by a variety of PRRs; however, as viruses produce dsRNA in the cytosol to replicate, CNA sensors are highly adapted to generate a robust antiviral response, including the induction of type I IFNs and cellular immunity. We were interested in assessing the ability of CNA to induce a type I IFN response in neonates and children. In this article, we report for the first time, to our knowledge, that stimulating CBMCs with a variety of dsRNA or dsDNA mimics results in strong induction of type I IFN comparable with responses in adults. A previous report by Renneson et al. (37) showed that CMV-infected moDCs produce high levels of IFN-α but lower levels of IL12 and IFN-β. Interestingly, we found no evidence of IFN-β production in our system in response to CNA activation (data not shown), and, similar to CMV infection, the type I IFN produced in response to CNA activation was predominantly IFN-α. We also confirm published reports that neonatal TLR4 and TLR9 are less able to induce a proinflammatory response when compared with adult PBMCs (5, 11, 17, 38). It is important to note that the relative contributions of TLRs and CNA sensors in the detection of many viruses are incompletely understood.

Several studies have reported conflicting data on the subpopulations of various cell types and total cell numbers in adult versus cord blood (39–42). Of particular interest, Drohan et al. (40) have reported increased frequency of pDCs in cord blood, although several recent studies observed similar frequencies of pDCs between adult and cord blood (43, 44). Owing to the ability of pDCs to produce high amounts of Type I IFN (18), it was important to characterize the DC subsets in our cohort. Following isolation of mononuclear cells, we found no differences in leukocyte percentages and no differences in DC subsets between adult and cord blood. We also found that classical and inflammatory monocyte subsets are the same between cord and adult mononuclear cells, an observation previously reported in the literature (42, 45, 46) but in conflict with another report that suggested that decreased populations of monocytes could be responsible for observed hyporesponsiveness to LPS seen in neonates (41). Importantly, within our cohort, we found that monocytes isolated from either adult or neonate stimulated with CNA or TLR ligands mirrored the IFN response observed in the PBMC/CBMC system. Unfortunately, technical limitations precluded the defining of the cell subsets responsible for the production of intracellular Type I IFN, and we were unable to isolate sufficient numbers of DCs from our samples to check their responses by ELISA; therefore, at this time, we cannot rule in or out the role of pDCs. Data from

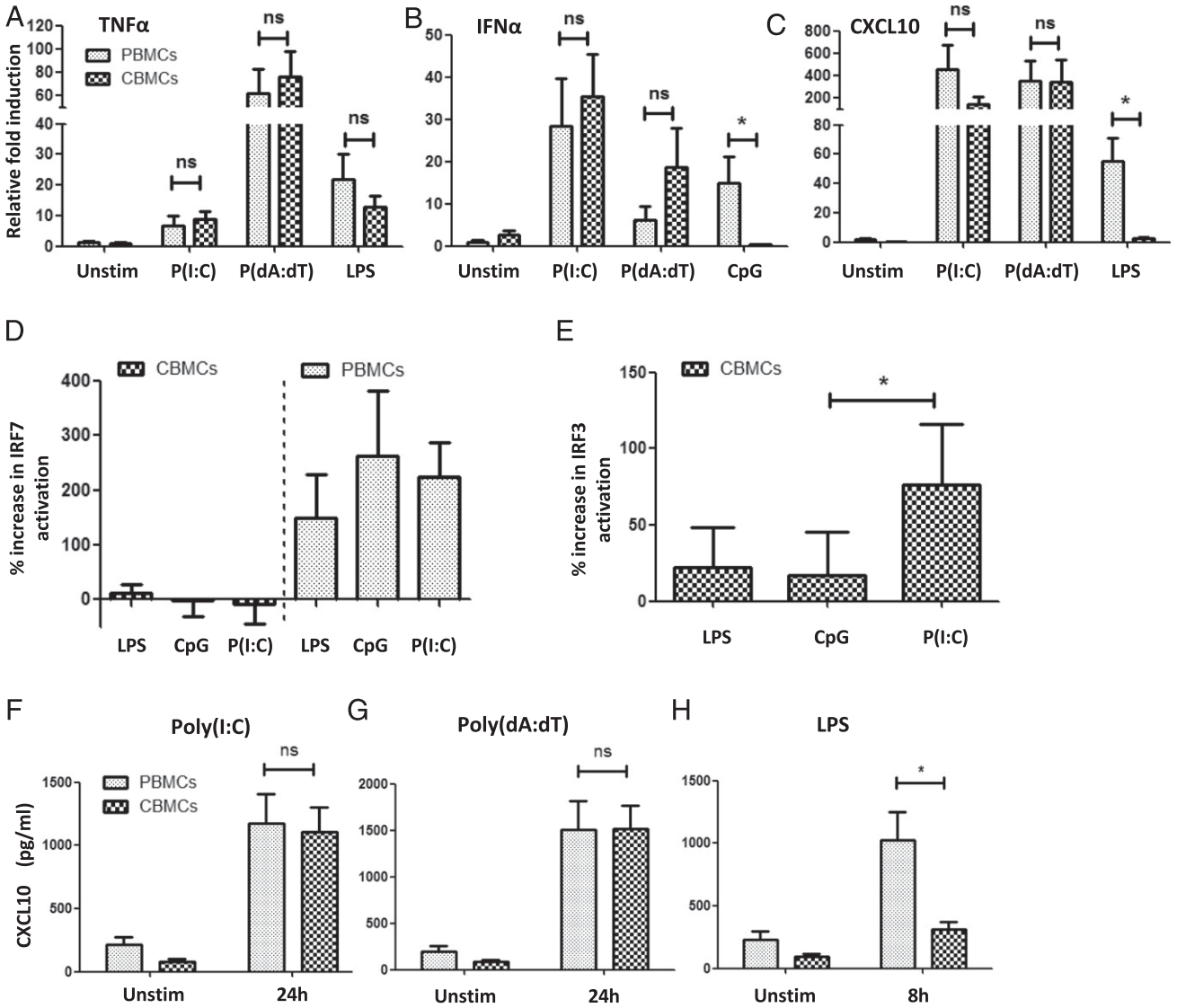

FIGURE 5. Impaired TLR-induced IRF3 activation in CBMCs leads to attenuated IFN and ISG responses not observed in response to CNA. PBMCs or CBMCs were transfected with Poly(I:C) or Poly(dA:dT) for 4 h (**A** and **C**) or 24 h (**B**), treated with LPS for 2 h (A) or 4 h (C), or treated with CpG ODN for 24 h (B), as indicated. Following stimulation, TRIzol extraction of RNA was carried out, and induction of (A) TNF-α, (B) IFN-α, and (C) CXCL10 mRNA expression was assayed by quantitative RT-PCR, normalized to β-actin, and presented relative to untreated, unstimulated adult PBMCs. (**D** and **E**) PBMCs or CBMCs were treated with LPS for 15 min, CpG for 1 h, or transfected with Poly(I:C) for 1 h, and nuclear extracts were harvested and assayed for IRF7 or IRF3 activation via TransAM assays. (**F**–**H**) Cells were stimulated with Poly(I:C) or Poly(dA:dT) transfection or LPS as indicated, and protein levels of CXCL10 were measured by ELISA. (A–E) Graphs show mean ± SEM ($n \geq 4$ donors in each group). (F–H) Graphs show mean ± SEM ($n \geq 29$ donors in each group). *$p < 0.05$ as determined by Student t test or Kruskal–Wallis with Dunn posttest.

Figs. 1 and 2E, however, demonstrate that monocytes are unquestionably involved in the production of Type I IFN in our experimental system.

It appears that in our system, altered cell subsets are unlikely to play a role in the divergent regulation of IFN in response to CNA or TLR ligands. In fact, our data demonstrating parity in cell signaling downstream of TLR4/7/8/9 to TNF-α induction in both neonates and adults highlighted the striking disparity in cell signaling to IFN induction downstream of TLR4/7/8/9 and the CNA sensors. The signaling pathways leading to the production of IFN-α are largely shared between the various receptors beyond adaptor level. A key difference between TLR-induced type I IFN and CNA induction is TLR dependence on endosomal localization to engage with TRAF3 and the IRFs. Several Rab GTPases have been reported to be involved in trafficking TLRs (25). Of particular interest, in response to LPS stimulation or *E. coli* infection, type I IFN production relies on the regulation of TLR4 transport to sorting phagosomes by Rab11a. In the absence of Rab11a, IFN production is significantly attenuated, whereas TNF-α induction is unchanged (26). Conversely, type I IFN responses downstream of cytosolic receptors have no dependency on endosome formation because of receptor location in the cytoplasm. We therefore investigated the expression and function of Rab11a in neonatal CBMCs compared with adult PBMCs. Of four Rab GTPases tested, basal expression of Rab11 was significantly lower in CBMCs than in adult mononuclear cells. As mentioned previously, monocytes are unquestionably involved in Type I IFN production in our system. This, along with previous reports on the role of Rab11 in IFN production focusing on monocytes and macrophages, led us to believe that monocytes were the appropriate cell to investigate our hypothesis that Rab11 was reduced in cord blood cells.

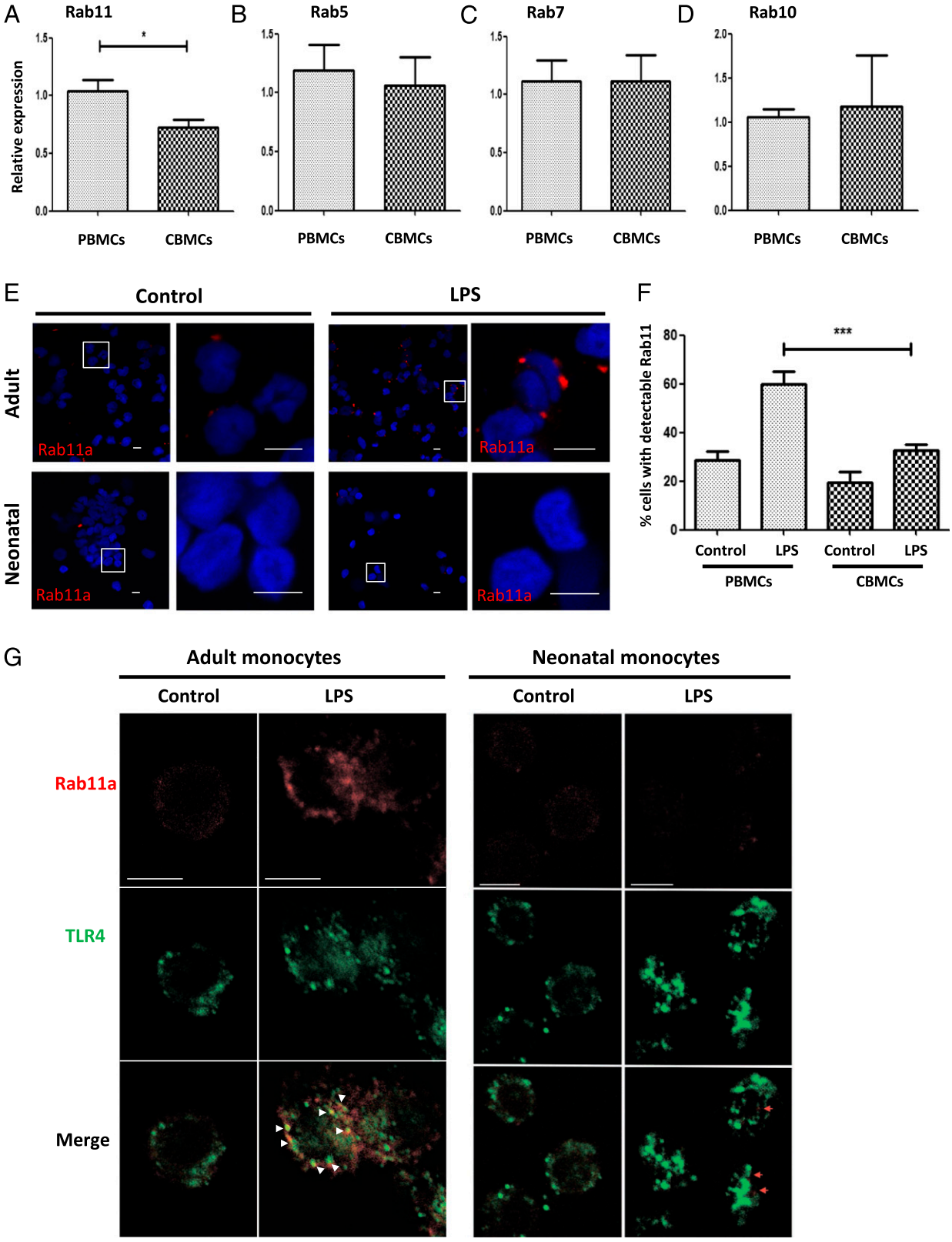

FIGURE 6. Attenuation of Rab11-positive endosome formation in LPS-stimulated cord blood monocytes. (**A**–**D**) TRIzol extraction of RNA from PBMCs or CBMCs was carried out, and expression of (A) Rab11, (B) Rab5, (C) Rab7, or (D) Rab10 mRNA was assayed by quantitative RT-PCR, normalized to β-actin, and presented relative to adult PBMCs. (**E** and **G**) CD14-positive cells were isolated from adult PBMCs or neonatal CBMCs and left unstimulated or treated with 1 μg/ml LPS for 1 h, as indicated. Cells were stained for Rab11 expression (red), DAPI (blue), or TLR4 (green) as indicated. (**F**) Cells expressing detectable levels of Rab11 were counted and presented as a percentage of total cells (using >200 cells). (A–D) Graphs show mean ± SEM ($n \geq 9$ donors in each group). (E and G) Images are representative of three separate experiments; white arrows denote colocalization and red arrows denote Rab11 alone. Scale bars, 10 μm (E) and 5 μm (G). (F) Graph shows mean percentage of cells expressing Rab11 of ≥200 cells from three individual donors (± SEM). $*p < 0.05$, $***p < 0.005$ as determined by unpaired Student t test (A)–(D) or ANOVA and Bonferroni posttest (F).

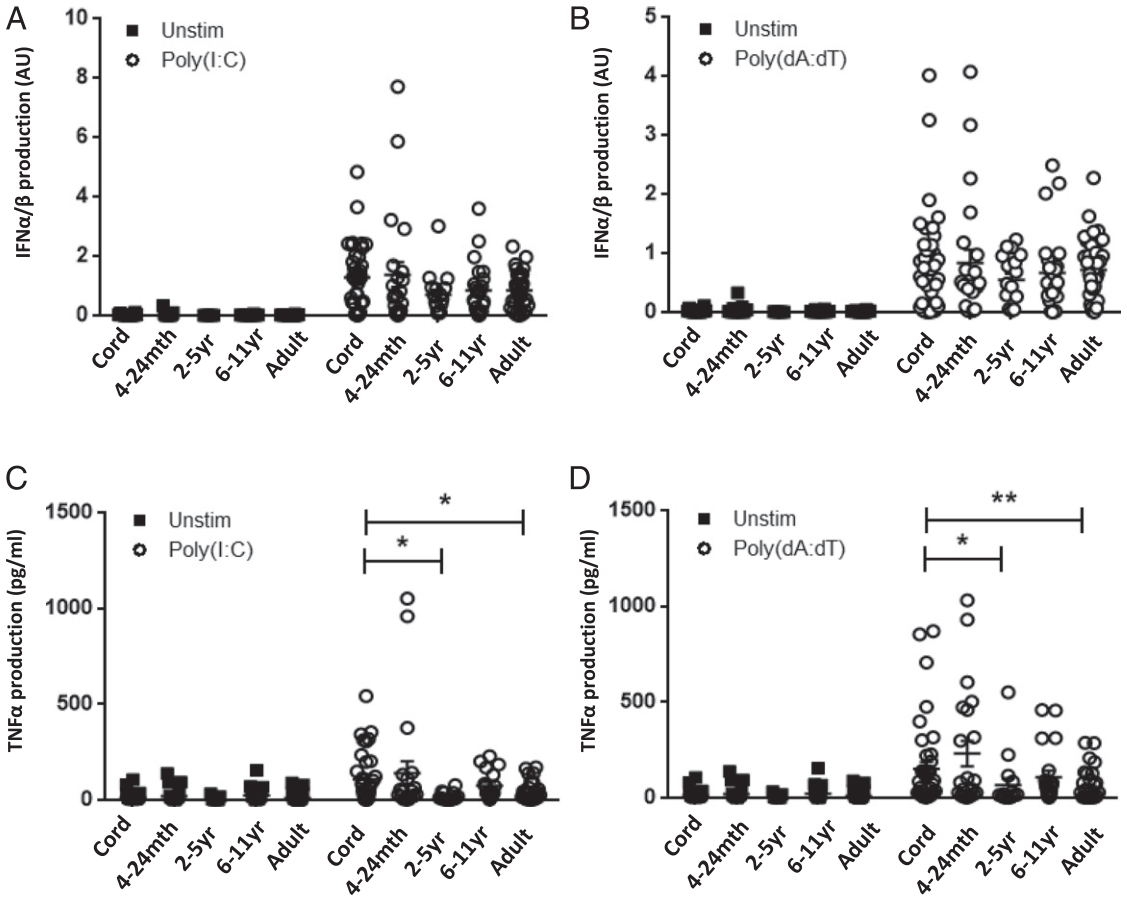

FIGURE 7. CNA maintain strong type I IFN and TNF-α responses in healthy infants and children up to and beyond 24 mo. Mononuclear cells were isolated from cord blood, adult blood, or from children's blood collected from otherwise healthy children undergoing surgery for hydrocele repair, umbilical hernia repair, onychocryptosis, or orchipexy repair in OLCHC. Cells were transfected with Poly(I:C) or Poly(dA:dT) for 24 h. Following stimulation, supernatant was harvested from the cells, and (**A** and **B**) IFN-α/β and (**C** and **D**) TNF-α were measured using HEK-Blue IFN-α/β or TNF-α assay and made relative to 25 U/ml IFN-α or 500 pg/ml TNF-α, respectively. (A)–(D) show mean ± SEM (cord, $n \geq 35$; 4–24 mo, $n = 22$; 2–5 y, $n \geq 16$; 6–11 y $n \geq 18$; adult, $n \geq 35$). Stimulated populations were compared with stimulated cord mononuclear cells by Mann–Whitney U test. $^{*}p < 0.05$, $^{**}p < 0.01$.

Upon LPS stimulation, the percentage of monocytes with detectable Rab11^{+} endosomes was strikingly higher in adult monocytes when compared with cord blood monocytes. Given our data showing that the transcription of IFN-α was inhibited in CBMCs in response to LPS, it seems plausible that a deficiency in Rab11-positive endosome formation may account for the attenuated IFN-α production in response to LPS in neonates. A requirement for Rab11-positive endosome formation has not yet been attributed to TLR7/8/9, and as such, the mechanism underlying the attenuation of IFN-α/β induction in response to CL075 or CpG in neonates remains to be investigated.

Previously, Aksoy et al. (13) reported that LPS cannot induce IFN-β in neonatal moDCs because of a lack of binding of IRF3 to CBP. Efficient interaction of IRF3 with its coactivator CBP is essential for transcriptional activity of IFN genes. Our data demonstrating that type 1 IFN induction is equivalent between adult and neonatal mononuclear cells/monocytes downstream of CNA stimulation would strongly imply that there is no integral or inborn defect in the neonatal IRF3–CBP interaction in these cells when compared with adults. This discrepancy could potentially be due to cell type differences (i.e., moDCs versus monocytes) or it could be that downstream of LPS, posttranslational modifications of IRF3 required for CBP binding are defective in neonates because of the attenuation of the Rab11^{+} endosome trafficking of IRF3; or it could be simply a limitation of the inherent variability observed between cells isolated from human blood.

To assess whether the response to CNA observed in cord blood was unique to cord blood or persisted through infancy and childhood, we isolated PBMCs from children undergoing elective surgery. The enhanced production of type I IFN in response to cytosolic Poly(I:C) was maintained up to the age of two, at which point it appears to level off to match adult levels. Poly(dA:dT)-induced production of type I IFN was maintained across all age groups. Interestingly, the induction of TNF-α in response to CNA also remained steady up until the age of two, at which point it drops to adult levels. Type I IFNs have critical roles in the induction of adaptive immunity; they promote the generation of cytotoxic T cell responses as well as a Th1-biased CD4^{+} T cell phenotype (22). Type I IFNs drive cellular immunity by promoting the activation and functional maturation of DCs, facilitating Ag presentation to CD4^{+} T cells, cross-priming CD8^{+} T cells, and inducing IFN-γ and opsonizing Abs (22, 47). Initiating Th1 responses and cytotoxic T cells is vital in the fight against intracellular pathogens, infections to which neonates are most susceptible, and are the holy grail for vaccine development (48). Currently, a major obstacle for improving the impaired vaccine responses observed in infants appears to be the lack of age-specific adjuvants that can safely drive potent cellular immunity against intracellular pathogens, resulting in the need for multiple booster injections and in some cases delaying the administration of a vaccine until a certain age has been reached. Aluminum salts (alum) find wide clinical application as adjuvants and promote

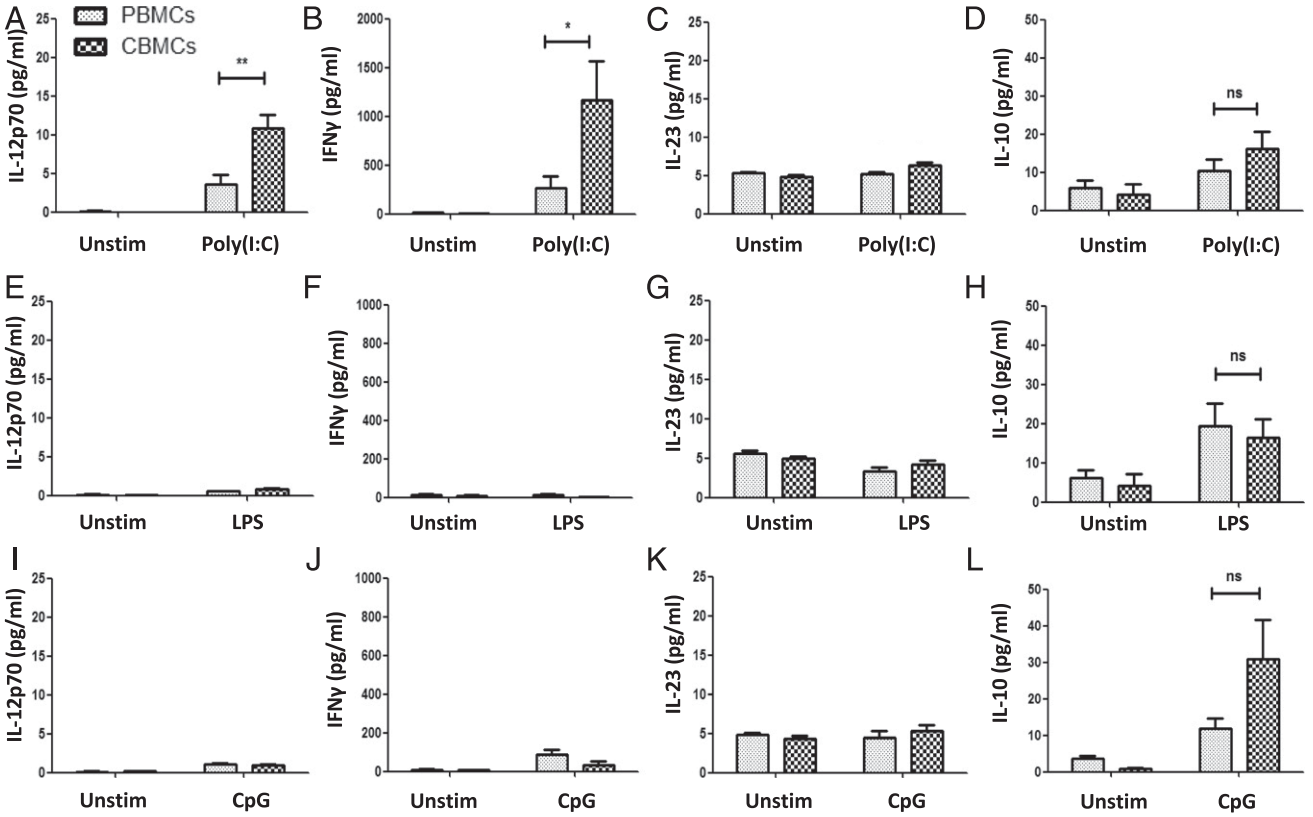

FIGURE 8. Cytosolic Poly(I:C) induces Th1-polarizing IL-12p70 and IFN-γ in CBMCs, whereas TLR4/9 activation does not. PBMCs or CBMCs were transfected with (**A**–**D**) Poly(I:C) for 24 h, treated with (**E**–**H**) LPS for 4 h or (**I**–**L**) CpG ODN for 24 h, and BioLegend LEGENDplex Human Inflammation Panels were used to measure the levels of (A, E, and I) IL-12p70, (B, F, and J) IFN-γ, (C, G, and K) IL-23, and (D, H, and L) IL-10. Graphs show mean ± SEM ($n \geq 16$). Kruskal–Wallis nonparametric test with Dunn posttest was used to test for significant differences between groups. *$p < 0.05$, **$p < 0.01$.

humoral immunity and Th2 cell responses. However, a major disadvantage of alum is its limited ability to efficiently drive Th1 responses (49), motivating the search for targeted neonatal and pediatric vaccine adjuvants, an unmet need recently highlighted in a European Union–commissioned report on vaccines (50). We tested the potential of cytosolic Poly(I:C) to promote a Th1 response through measuring the production of Th1-polarizing IL-12p70 and induction of IFN-γ in adult and CBMCs. We found that neonates produced significantly higher levels of both IL-12p70 and IFN-γ in response to CNA than adult PBMCs. Conversely, there was no difference in levels of anti-inflammatory IL-10 or Th17-polarizing IL-23 between neonates and adults basally or in response to cytosolic Poly(I:C). By comparison, neither LPS nor CpG activation of CBMCs induced either IL-12p70 or IFN-γ. A recent study by Borriello et al. (51) has investigated the adjuvancy potential of combining alum with activation of STING in mice. This study, along with our work in human neonatal cells, highlights the potential of these intracellular receptors in future vaccine development. From a physiological perspective, it is interesting to speculate that infants have evolved constrained responses to bacterial infections to allow for appropriate commensal colonization of the skin and gut without major inflammatory response, while at the same time broadly conserving effective defense against inherent viral pathogenicity by enabling CNA sensors to mount an efficient immune response against invasive infection. Sharma et al. (52) have previously proposed a hierarchical maturation of innate immune defenses in very preterm neonates, resulting in predominance of specific microbial infections in this age group. It is possible that a hierarchical development of early cytosolic/endosomal PRR function, followed later by extracellular/surface PRR function, may be essential to balance the need to limit inflammatory signals to the adaptive immune system during the critical period of the establishment of self-tolerance in early life while preserving some responses to viruses. Together, our data indicate that CNA and activators of CNA sensors possess major potential to guide the neonatal immune response in the direction of cell-mediated immunity and that this response is active and robust in neonates and persists throughout childhood.

Acknowledgments

We thank the children in OLCHC, their parents, and the women in the NMH who agreed to donate peripheral and cord blood for this research project.

Disclosures

The authors have no financial conflicts of interest.

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