

ARTICLE

Cytosolic dsRNA improves neonatal innate immune responses to adjuvants in use in pediatric vaccines

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Abstract

Pattern recognition receptors (PRRs) of the innate immune system represent the critical front-line defense against pathogens, and new vaccine formulations target these PRR pathways to boost vaccine responses, through activation of cellular/Th1 immunity. The majority of pediatric vaccines contain aluminum (ALUM) or monophosphoryl lipid A (MPLA) as adjuvants to encourage immune activation. Evidence suggests that elements of the innate immune system, currently being targeted for vaccine adjuvanticity do not fully develop until puberty and it is likely that effective adjuvants for the neonatal and pediatric populations are being overlooked due to modeling of responses in adult systems. We recently reported that the activity of the cytosolic nucleic acid (CNA) sensing family of PRRs is strong in cord blood and peripheral blood of young children. This study investigates the function of CNA sensors in subsets of neonatal innate immune cells and shows that myeloid cells from cord blood can be activated to express T cell costimulatory markers, and also to produce Th1 promoting cytokines. CD80 and CD86 were consistently up-regulated in response to cytosolic Poly(I:C) stimulation in all cell types examined and CNA activation also induced robust Type I IFN and low levels of TNF α in monocytes, monocyte-derived macrophages, and monocyte-derived dendritic cells. We have compared CNA activation to adjuvants currently in use (MPLA or ALUM), either alone or in combination and found that cytosolic Poly(I:C) in combination with MPLA or ALUM can improve expression of activation marker levels above those observed with either adjuvant alone. This may prove particularly promising in the context of improving the efficacy of existing ALUM- or MPLA-containing vaccines, through activation of T cell-mediated immunity.

Abbreviations: ALUM, aluminum hydroxide and magnesium hydroxide.; CBMCs, cord blood mononuclear cells.; CNA, cytosolic nucleic acid.; cPIC, cytosolic Poly(I:C); DC, dendritic cell.; dsDNA, double-stranded DNA.; dsRNA, double stranded RNA.; LDH, lactate dehydrogenase.; Mda5, Melanoma Differentiation-Associated protein 5.; MDM, monocyte-derived macrophage.; moDCs, monocyte-derived dendritic cells.; MPLA, monophosphoryl lipid A.; NMH, National Maternity Hospital.; Poly(I:C), Polyinosinic-polycytidylic acid.; RIG-I, retinoic acid-inducible gene 1.; SEAP, secreted embryonic alkaline phosphatase.

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KEYWORDS

ALUM, MPLA, myeloid cell, Poly(I:C), RIG-I, TLR3

1 | INTRODUCTION

Along with clean water, the development of vaccines has widely been lauded as one of the world's greatest health interventions. According to the WHO, immunization currently prevents 2–3 million deaths every year from diseases like diphtheria, tetanus, pertussis, influenza, and measles. However, it is also estimated that approximately 20 million people, mostly infants, have insufficient access to vaccines.¹ In areas of the developing world, including areas of conflict, a drop-off rate of up to 22% is observed between dose 1 and dose 3 of diphtheria, tetanus, and pertussis vaccine and a rate of 46% between dose 1 and 2 of measles-containing vaccines.² One way of improving access to vaccines, and thereby decreasing deaths associated with some of these infectious diseases, would be to cut down on the need for boosters by making vaccines more efficient, and also tailoring vaccines and adjuvants for deployment in very early life when contact with a healthcare worker is more accessible.

Vaccines in their simplest form are comprised of an antigen alone. Historically, inactivated or live-attenuated whole cell vaccines did not need additional adjuvants to boost the immune response, as bacterial or viral signatures within the whole cell vaccine activated the immune system very effectively. More recently, a move toward highly purified subunit vaccines has improved the safety of vaccines. One downside to these highly purified vaccines is a lack of immunostimulatory properties, leading to a need for addition of adjuvants, which can encourage immune memory and also allow for lower doses of vaccines to be given.³ Empirically, and quite by accident, aluminum (ALUM) was found to improve vaccine responses and encourage immunologic memory⁴ and has been the adjuvant of choice for over 90 years. ALUM is still the most common adjuvant in current vaccine formulations. The mechanism of action of ALUM has been widely investigated and debated. Several mechanisms have been proposed to explain the adjuvant activity of ALUM, including the “depot effect,” enhanced antigen uptake by antigen presenting cells, NLRP3 inflammasome activation, and others.⁵ ALUM adjuvants effectively drive humoral immunity through various mechanisms including IL-33 production,⁶ but induce low levels of cellular immune responses, possibly due to the ability of ALUM to inhibit IL-12 and drive IL-10 in some instances.^{7,8}

In recent years, there has been a move toward developing adjuvants that are better inducers of proinflammatory cytokines and Type I IFN, in the hope of generating a stronger Th1 response.⁹ This increased Th1 response would in turn lead to increased cellular immunity, which is particularly beneficial in combating intracellular infections. TLR ligands and other pathogen-associated molecular patterns became an obvious target due to their ability to induce Type I IFN and proinflammatory cytokine production.¹⁰ Of note, monophosphoryl lipid A (MPLA), a TLR4 agonist, formulated with ALUM triggers an enhanced

Th1 response and is approved for use in Fendrix (Hepatitis B) and Cervarix (Human Papilloma Virus) in Europe. Previous literature has shown that many TLR responses, including TLR3, are attenuated or absent in neonatal and pediatric populations.^{11–16} One notable exception to this are TLR7/8 responses, which tend to remain intact and are being investigated for their adjuvant properties.^{17,18} Interestingly, combinations of TLR agonists with other innate immune activators such as C-type lectin receptor agonists have shown promise in activating neonatal immune cells.¹⁹

At present, most neonatal or pediatric vaccines contain either ALUM or MPLA as an adjuvant, and these may not be optimal for use in neonatal or pediatric populations. Development of pediatric-relevant, effective adjuvants, or combinations of adjuvants could increase efficacy and decrease the need for boosters or delayed vaccination.

Poly(I:C) is a synthetic dsRNA sequence that is associated with viral infections. It can activate TLR3 on the surface of immune cells²⁰ or, when transfected into cells, can activate cytosolic nucleic acid (CNA) sensors such as RIG-I.²¹ In both cases, it induces a robust Type I IFN response in adults. Previous literature has shown that TLR3 responses to Poly(I:C) are attenuated in neonates; however, we have previously shown that intracellular Poly(I:C) responses are intact in mixed populations of neonatal immune cells.²⁵ Slavica et al.²⁶ have previously shown that TLR3 mRNA levels are notably decreased in cord blood mononuclear cells (CBMCs) compared with adult blood mononuclear cells, whereas RIG-I mRNA levels are similar. TLR3 is also absent at the protein level in isolated cord blood NK cells²⁶; however, protein expression levels have not been investigated on cord blood monocytes, monocyte-derived macrophages (MDMs), or monocyte-derived dendritic cells (moDCs).

Targeting different mechanisms that activate the immune response, through combining adjuvants, may result in a more effective or longer lasting immune response.²⁷ Characterization of specific neonatal immune cell responses to intracellular Poly(I:C), or combinations of Poly(I:C) with the most common adjuvants currently used in pediatric vaccines, ALUM and MPLA, and their impact on the adaptive immune system could aid in moving toward targeted adjuvants for the pediatric population. Here, we show that CNA activation through intracellular Poly(I:C) can induce Type I IFN production from cord blood monocytes, MDMs, and moDCs, whereas adjuvants currently in use, such as MPLA or ALUM, cannot. Cytosolic Poly(I:C) alone, or in combination with MPLA or ALUM can enhance the expression of costimulatory molecules on innate immune cells, important for T cell activation. Importantly, T cells transfected with Poly(I:C) or combinations of Poly(I:C) and MPLA or ALUM can activate naïve CD4+ T cells. Furthermore, moDCs treated with Poly(I:C) or combinations of Poly(I:C) with MPLA or ALUM can induce CD4+ T cell proliferation. This implies that Poly(I:C) can activate T cells both directly and through myeloid cells. Although further work is necessary, these results indicate that

Poly(I:C)/dsRNA is a very favorable candidate adjuvant for inclusion in pediatric vaccine formulation.

2 | MATERIALS AND METHODS

2.1 | Participants

Umbilical cord blood samples were obtained from term births following normal pregnancy, labor, and delivery at National Maternity Hospital (NMH), Holles St, Dublin 2. All infants had an uncomplicated postnatal course and Apgar scores of 9 at 5 min. Ethics approval was obtained from the Ethics Committees of NMH and informed consent was obtained from each participant.

2.2 | Isolation of mononuclear cells, T cells, MDMs, monocytes, and moDCs

Cord blood was collected into 3.2% sodium citrate and processed within 24 h of collection. Primary CBMCs were isolated from healthy cord blood and cultured at 37°C, 5% CO₂, 95% air in complete RPMI-1640 (containing stable 2.5 mM L-glutamine and 0.5 mM sodium pyruvate with 10% FBS). For MDM generation, CBMCs were plated at 2×10^6 cells/ml in complete RPMI-1640 supplemented with 50 ng/ml recombinant human M-CSF (Miltenyi) for 7 or 8 days, with 2 supplements of fresh medium and cytokines at days 3 and 5 of culture (Figure S1(A)). Monocytes were isolated by negative selection using Monocyte Isolation Kit II (Miltenyi). Monocyte purity was assessed using CD14 staining and was routinely >90% (Figure S1(B)). For moDCs, isolated monocytes were cultured in tissue-culture dishes at 1×10^6 cells/ml in RPMI 1640 medium containing 10% FBS supplemented with recombinant human IL-4 (40 ng/ml) and recombinant human GM-CSF (80 ng/ml) with 1 supplement of fresh medium and cytokines at day 3 of culture. After 6–7 days, immature moDCs (CD14^{low}DC-SIGN⁺), routinely >90% purity, were harvested by gently pipetting only the loosely adherent fraction and replated in a 96-well format at the desired cell density (Figure S1(C)). T cells were isolated from CBMCs by negative selection using CD4⁺ T cell isolation kit (Miltenyi) and were routinely >95% CD45⁺CD4⁺ (Figure S1(D)).

2.3 | Stimulation of cells

MPLA (1 µg/ml) or ALUM (5 µg/ml) (Invivogen) was used to activate monocytes, MDMs, moDCs, or T cells directly. Poly(I:C) (5 µg/ml) (Invivogen) was either added to cells directly to activate TLR3 or transfected into cells using TransIT-X2 (Mirus), either alone or in combination with other stimuli, in order to activate CNA sensors. Dose-response curves and cytotoxicity assays were used to determine optimal concentrations of each agonist (Figure S2). For TCR activation, round-bottomed plates were coated with anti-CD3 (OKT3) and anti-CD28 (15E8), both at 5 µg/ml in PBS for 3 h at 37°C. Prior to addition of

T cells, at 1×10^6 /ml, antibodies were removed and wells were washed 1× with PBS.

2.4 | Measurement of cytokines

HEK Blue TNFα/IFNα/β Assays were performed as per manufacturer's instructions using Quanti-Blue Detection Reagent. SEAP levels were determined using a spectrophotometer plate reader at 630 nm. For IFNα/β SEAP assays, results were normalized to 50 U/ml IFNα to give a relative arbitrary unit (AU). IFNγ (88-7316-88; ThermoFisher) and IL-17A (900-K84; Peprotech) were detected by sandwich ELISA. BioLegend LEGENDplex™ Human Inflammation Panel 1 (13-plex) was carried out as per manufacturer's instructions to determine levels of IL-6, IL-10, and IL-1β from monocyte, MDM, or moDC cell culture supernatant. A BD LSR Fortessa cell analyzer was used to acquire samples and BioLegend LEGENDplex™ software was used for analysis.

2.5 | 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 on a 96 well plate spectrophotometer and background (absorbance at 680 nm) was subtracted.

2.6 | DC-CD4⁺ T cell cocultures

CD4⁺ T cells were stained with Vybrant™ CFDA SE (Life Technologies) according to the manufacturer's instructions. CD4⁺ T cells were cocultured with allogeneic moDC that had been pretreated with MPLA, ALUM, or transfected Poly(I:C) for 24 h, as described above. Cells were cocultured at a ratio of 1:10 moDC to CD4⁺ T cells with no stimulations present. Cells were collected after 5 days for analysis of CD4⁺ T cell proliferation by flow cytometry.

2.7 | Flow cytometry

Monocytes, MDMs, moDCs, or T cells were assessed for purity or were labeled for the investigation of surface marker expression with the following fluorochrome-labeled antibodies: CD4 (REA623), CD45 (2D1; BioLegend), CD14 (REA599), DC-SIGN (REA617), CD80 (REA661), CD86 (REA968), CD83 (REA714), CD40 (REA733), and HLA-DR (AC122) (All from Miltenyi). Cells were stained with LIVE/DEAD™ Aqua (Molecular Probes), followed by staining for 20 m at 4°C, washed, and analyzed by flow cytometry immediately.

For intracellular staining, cells were stained with LIVE/DEAD™ Aqua, fixed, and stained with fluorochrome-labeled antibodies to TLR3 (TLR-104; from BioLegend) or RIG-I (D-12; from Santa Cruz) using the eBioscience™ Foxp3/Transcription Factor Fixation/Permeabilization

kit as per manufacturer's instructions. Flow cytometry was carried out on a BD LSR Fortessa or BD FACSCanto cell analyzer and analyzed using FlowJo software (TreeStar).

2.8 | 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 non-normal distribution, a Kruskal–Wallis with Dunn's multiple comparison test or Mann–Whitney *U* test was carried out. Statistical analysis on normally distributed datasets was using one-way ANOVA with Dunnett's post-test or Bonferroni's post-test. The statistical approaches were all deemed to be valid for each individual experiment.

3 | RESULTS

3.1 | RIG-I is abundantly expressed and functional on cord blood monocytes, MDMs, and moDCs, whereas TLR3 is absent

Our previous work showed that CBMCs had intact responses to CNA sensor activation through Poly(I:C).²⁵ TLR3 and RIG-I are the main receptors for Poly(I:C), and as such we were interested in characterizing the expression of TLR3 and RIG-I on neonatal myeloid cells, along with comparing direct activation of cells with untransfected Poly(I:C) to transfected, cytosolic Poly(I:C). RIG-I expression was high in neonatal monocytes, MDMs, and moDCs (Figures 1(A)–1(C)), whereas we found no expression of TLR3 on any of the neonatal myeloid cells tested (Figures 1(D)–1(F)). In support of this expression data, there was a significant difference in the production of Type I IFN when cells were stimulated with transfected, cytosolic Poly(I:C) versus untransfected Poly(I:C), suggesting that any responses observed to cytosolic Poly(I:C) are predominantly due to CNA sensor activation rather than TLR3 activation.

3.2 | Cytosolic Poly(I:C) is more efficient at inducing Type I IFN production in human neonatal innate immune cells than adjuvants currently used in pediatric vaccines

Cytosolic Poly(I:C) activation of CNA receptors is a potent activator of Type I IFN responses in neonatal cord blood cells.²⁵ We were interested in comparing the activation of a variety of neonatal innate immune cells by adjuvants currently in use in pediatric vaccines, namely MPLA and ALUM, with cytosolic Poly(I:C) activation.

As expected, monocytes activated with cytosolic Poly(I:C) produced a significant amount of Type I IFN. Neither MPLA nor ALUM induced any Type I IFN production (Figure 2(A)). Similar results were seen in

both MDMs and moDCs (Figures 2(B) and 2(C), respectively), with Poly(I:C) driving Type I IFN production, whereas MPLA or ALUM did not. Importantly, none of the stimulations caused any increase in LDH production from neonatal cells (Figures 2(D)–2(F)).

3.3 | MPLA is the most efficient adjuvant at inducing proinflammatory cytokines in human neonatal cells

The ability of cytosolic Poly(I:C) to elicit production of other proinflammatory cytokines was also examined. Poly(I:C) transfection or ALUM stimulation did not induce significant levels of TNF α production from monocytes, MDMs, or moDCs. MPLA induced TNF α from all cell types (Figure 3(A)). Similar to TNF α production, IL-6 production was not observed in response to Poly(I:C) or ALUM in monocytes, MDMs, or moDCs, whereas MPLA induced significant levels of IL-6 in all cell types (Figure 3(B)). In the case of IL-1 β production, high levels of IL-1 β were produced in response to MPLA in monocytes but not to Poly(I:C) or ALUM (Figure 3(C), left hand panel), whereas there was very little cytokine production observed in MDMs (Figure 3(C), center panel). When examining moDCs, MPLA was again the only adjuvant that induced low, but significant levels of IL-1 β production (Figure 3(C), right hand panel). We also examined IL-10 production in response to Poly(I:C), due to the anti-inflammatory effect of IL-10. Poly(I:C) did not induce any IL-10 production from neonatal monocytes, MDMs, or moDCs, whereas significant levels of IL-10 were produced from all cell types in response to MPLA. Surprisingly, ALUM did not induce any IL-10 production from any of the cell types examined (Figure 3(D)).

3.4 | Combination of Poly(I:C) and MPLA increases the diversity of cytokine responses from human neonatal myeloid cells

Given the importance of both Type I IFN and proinflammatory cytokines in directing the adaptive immune response, we hypothesized that combinations of Poly(I:C) with adjuvants currently in use in pediatric vaccines may be a useful strategy for eliciting the optimal combinations of Th1-directing cytokines. When we compared the activation of neonatal myeloid cells with MPLA or ALUM in combination with transfected Poly(I:C), to assess whether a more diverse cytokine response could be encouraged, we found that combinations of MPLA or ALUM with Poly(I:C) produced a significant amount of Type I IFN from neonatal monocytes, compared with control (Figure 4(A), left panel). Combinations of MPLA or ALUM with Poly(I:C) were similarly capable of inducing production of significant amounts of Type I IFN from either MDMs (Figure 4, center panel) or moDCs (Figure 4, right panel). However, in all cases, the amount of IFN induced was not significantly higher than that induced by Poly(I:C) transfection alone (dotted line).

We had previously observed that MPLA could induce proinflammatory cytokines TNF α , IL-6, and IL-1 β in all myeloid cells examined

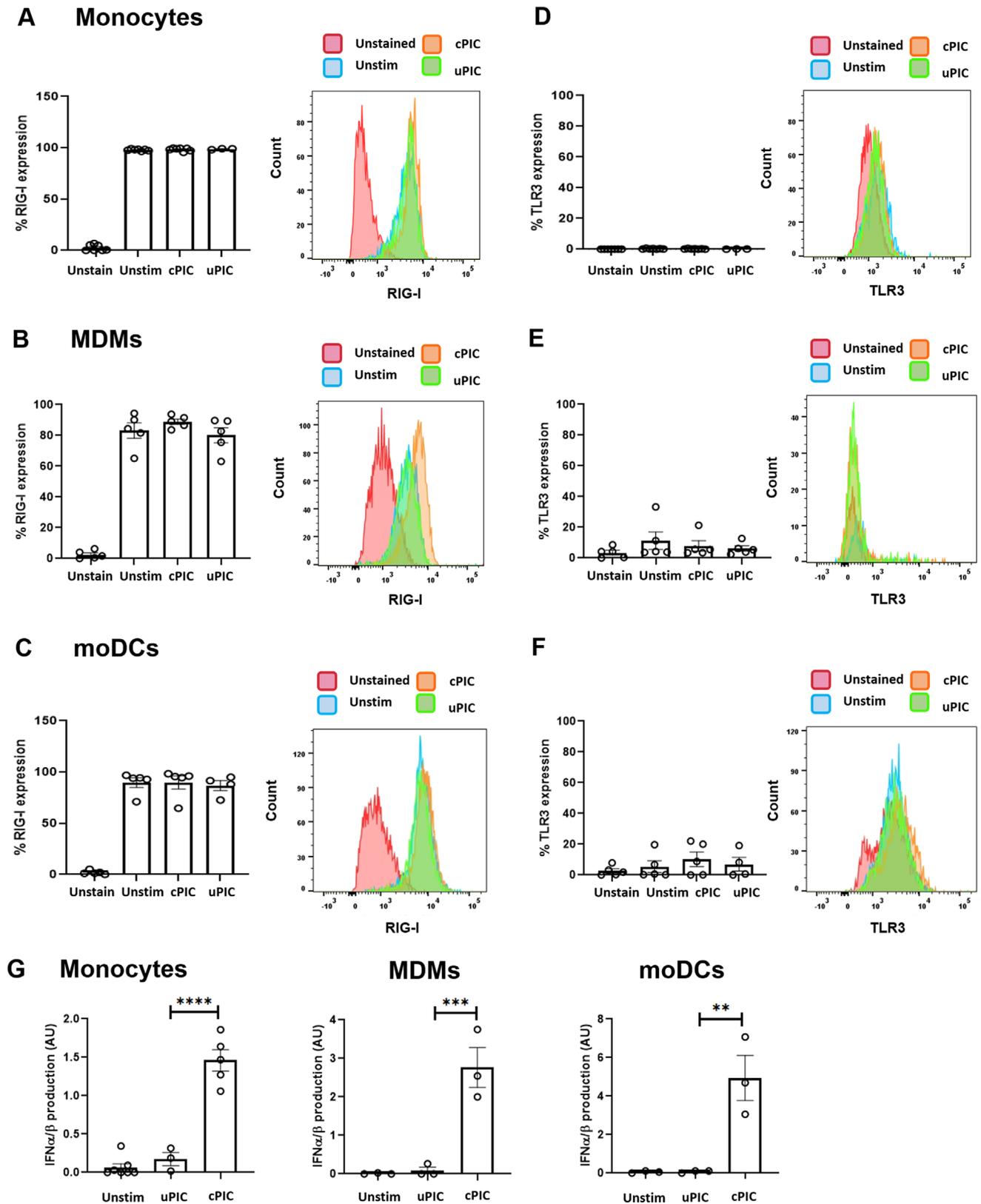


FIGURE 1 RIG-I is abundantly expressed and functional on cord blood monocytes, MDMs and moDCs, whereas TLR3 is absent. Cord blood monocytes (A and D), monocyte-derived macrophages (MDMs) (B and E), or monocyte-derived dendritic cells (moDCs) (C and F) were left unstimulated or stimulated with untransfected Poly(I:C) (uPIC) or cytosolic Poly(I:C) (cPIC) for 18 h. Levels of RIG-I expression (A–C) or TLR3 expression (D–F) were assayed via intracellular flow cytometry. Histograms depict expression of receptors on cells from 1 representative donor. Pooled data ($n \geq 4$) shown are mean \pm SEM of the percentage of cells positive for each marker. Levels of Type I IFN were assayed from cord blood monocytes, MDMs, or moDCs in response either uPIC or cPIC stimulation (G). Data are presented as mean \pm SEM. $n \geq 3$ donors in each group. ** $p < 0.01$, *** $p < 0.05$, **** $p < 0.001$. One-way ANOVA with Bonferroni's post-test was used to compare groups

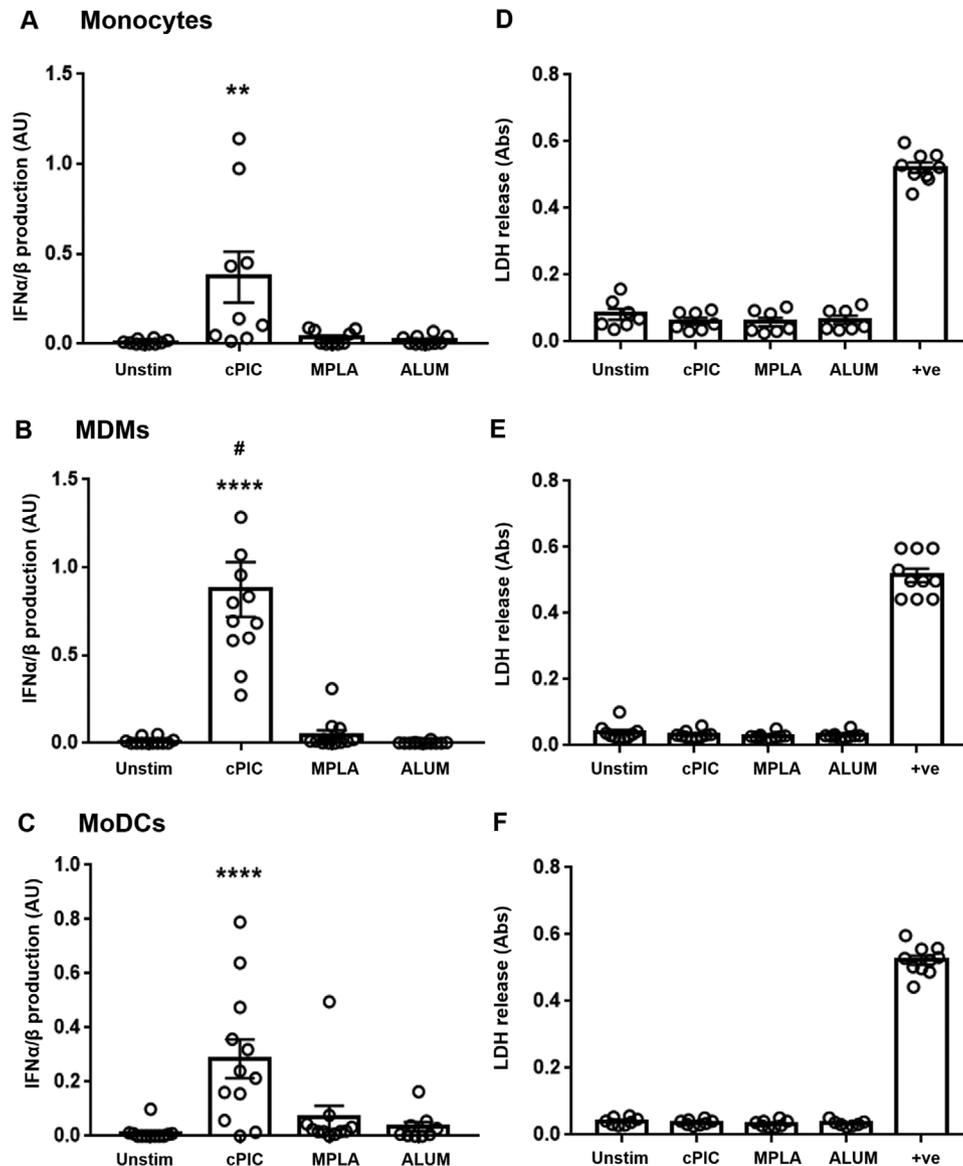


FIGURE 2 Cytosolic Poly(I:C) is more efficient at inducing Type I IFN production in human neonatal innate immune cells than adjuvants currently used in pediatric vaccines cord blood monocytes (A and D), MDMs (B and E), or moDCs (C and F) were left unstimulated or stimulated with Poly(I:C) transfection, MPLA, or ALUM for 24 h. Levels of IFN α/β were assayed via HEK-Blue™ IFN α/β SEAP assay (A–C). Cell viability, following 24 h stimulation with ligands, was assayed using LDH assay (D–F). Data are presented as mean \pm SEM. $n \geq 7$ donors in each group. ** $p < 0.01$, **** $p < 0.001$. Kruskal–Wallis nonparametric test with Dunn’s post-test was used to compare groups. # indicates 1 data point outside limit of graph

(dotted line, Figures 4(B)–4(E)), whereas Poly(I:C) could not. MPLA in combination with Poly(I:C) induced significantly more TNF α , IL-6, and IL-1 β production than control cells. However, in all cases, the amount of TNF α , IL-6, or IL-1 β induced was not significantly higher than that induced by MPLA alone (dotted line) (Figures 4(B)–4(D)). IL-10 levels were also unchanged by combination of MPLA with Poly(I:C) compared with MPLA alone in all myeloid cells tested (Figure 4(E)). These data indicate that combinations of adjuvants do not act together to increase individual cytokine levels but instead the combinations can encourage myeloid cells to expand their cytokine repertoire enabling secretion of both Type I IFN as well as proinflammatory cytokines.

Given the low responsiveness of the neonatal cells to ALUM, we assayed IL-33, which is known to be induced in the peritoneal lavage of mice injected with ALUM.⁶ Though not significant, there was a clear induction of IL-33 in neonatal monocytes in response to ALUM, 5-fold over unstimulated, leading us to the conclusion that ALUM was functioning in our neonatal cell systems but simply not to anticipated levels (Figures S3(A)–S3(C)). To further demonstrate that ALUM was active in our experimental setting, we carried out inflammasome activation assays in adult PBMCs and observed significant IL-1 β production in response to ALUM, in LPS-primed PBMCs (Figure S3(D)).

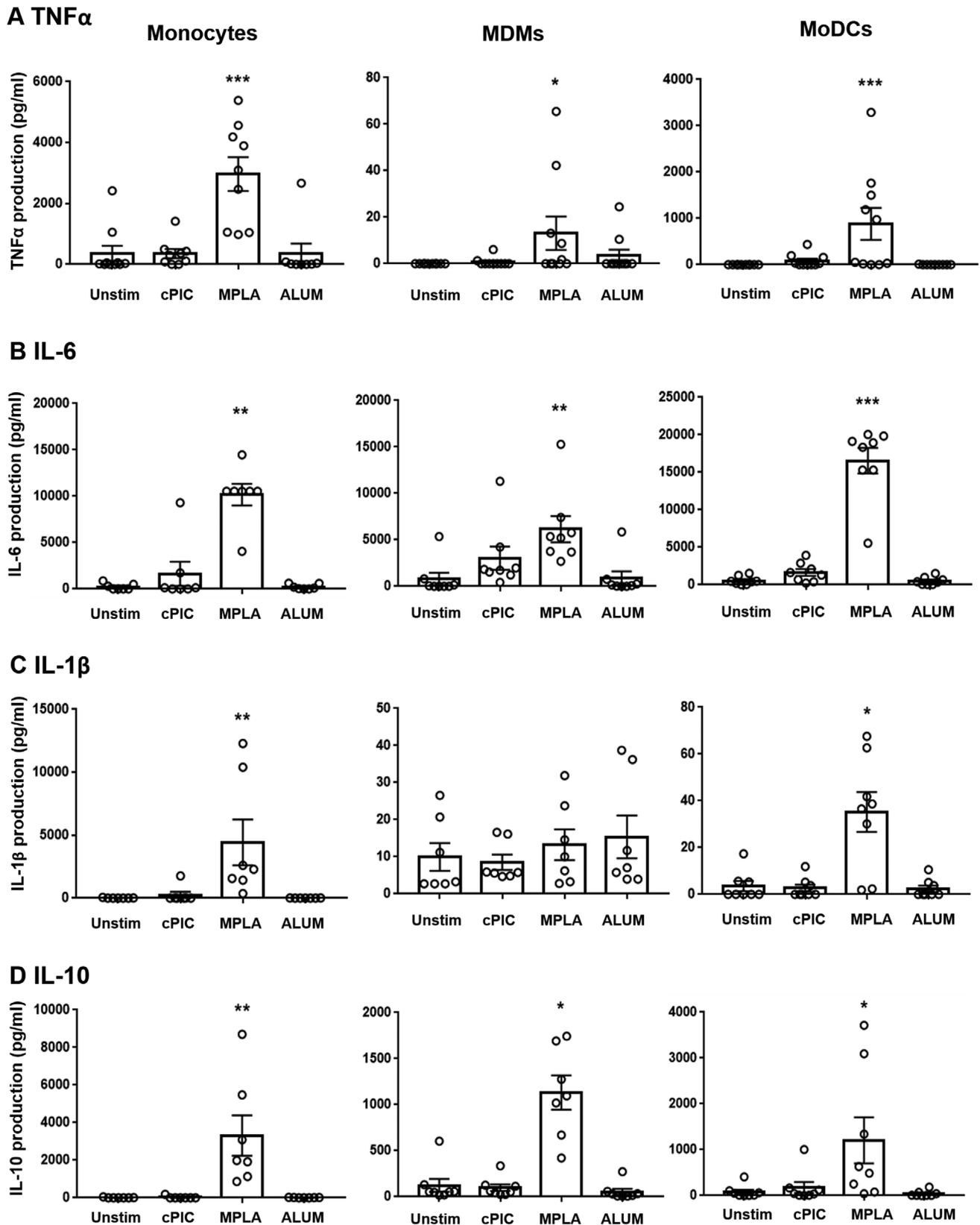


FIGURE 3 MPLA is the most efficient adjuvant at inducing proinflammatory cytokines and IL-10 in human neonatal cells. Neonatal monocytes (left hand panels), MDMs (center panels) or moDCs (right hand panels) were unstimulated or treated with Poly(I:C) transfection, MPLA, or ALUM for 24 h. (A) Levels of TNF α were assayed via HEK-Blue™ TNF α SEAP assay. BioLegend LEGENDplex™ Human Inflammation Panel I was used to measure the levels of (B) IL-6, (C) IL-1 β , or (D) IL-10. Data are presented as mean \pm SEM. $n \geq 8$ donors in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. Kruskal-Wallis nonparametric test with Dunn's post-test was used to compare groups

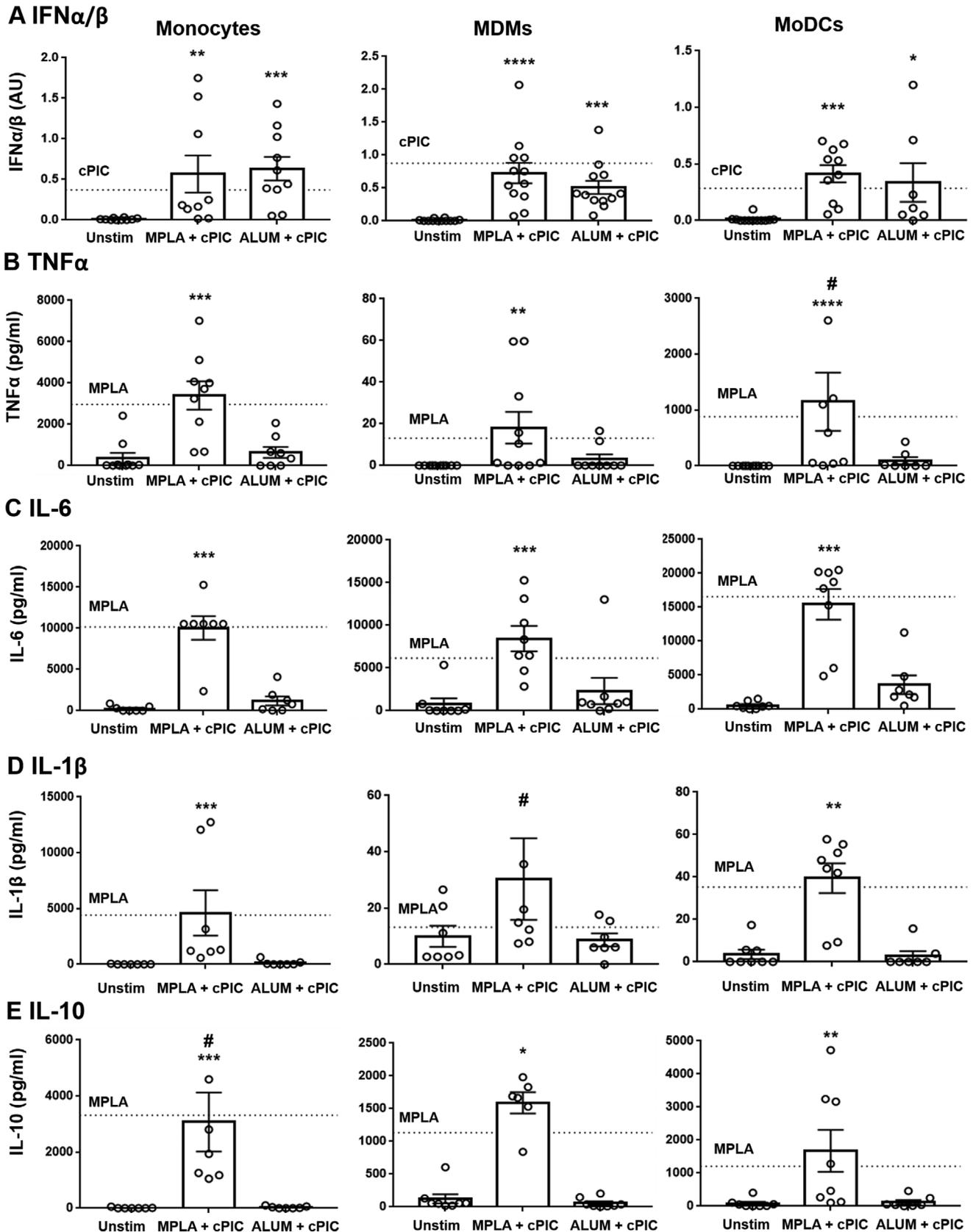


FIGURE 4 Combination of cytosolic Poly(I:C) and MPLA increases the diversity of cytokine responses in human neonatal myeloid cells. Cord blood monocytes were left untreated or stimulated with combinations of Poly(I:C) transfection with MPLA or Poly(I:C) transfection with ALUM for 24 h. Following 24 h stimulation, supernatants were harvested and analyzed for production of (A) IFN α/β or (B) TNF α were assayed via HEK-Blue™ IFN α/β or HEK-Blue™ TNF α SEAP assay. BioLegend LEGENDplex™ Human Inflammation Panel I was used to measure the levels of (C) IL-6, (D) IL-1 β , or (E) IL-10. Dotted lines show previously observed levels of cytokines produced in the presence of Poly(I:C) transfection alone (A) or MPLA treatment alone (B-E). Data are presented as mean \pm SEM. $n \geq 8$ donors in each group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.0001$. Kruskal-Wallis nonparametric test with Dunn's post-test was used to compare groups. # indicates 1 data point outside limit of graph

3.5 | Cytosolic Poly(I:C) increases costimulatory marker expression on human neonatal monocytes and MDMs in combination with MPLA or ALUM

One downstream consequence of Type I IFN production is the up-regulation of costimulatory molecules, such as CD80 and CD86, on myeloid cells.²⁸ CD80 and CD86 are found on the surface of many immune cells and work together to bind to CD28 on T cells to provide a costimulatory signal to these T cells.²⁹ Having examined cytokine production from neonatal myeloid cells in response to Poly(I:C), MPLA, and ALUM, we were interested in looking at the expression of these important costimulatory markers on neonatal cells in response to activation with these adjuvants.

In response to Poly(I:C) transfection, CD86 expression was slightly increased on monocytes, when MFI was made relative to unstimulated cells; however, this increase was not significant. Neither MPLA nor ALUM induced any increase in CD86 expression whatsoever. MPLA in combination with Poly(I:C) again showed no increase in expression, whereas a combination of ALUM and Poly(I:C) induced a significant increase in expression of CD86 on the surface of monocytes compared with unstimulated cells (Figure 5(A)). Similarly, CD80 expression appeared to be somewhat increased on monocytes in response to Poly(I:C) transfection, though not significantly, whereas MPLA stimulation of monocytes did induce a significant increase in CD80 expression. Interestingly, combinations of MPLA or ALUM with Poly(I:C) induced significant increases of CD80 expression on monocytes compared to unstimulated cells or single adjuvants alone (Figure 5(B)).

When examining MDMs, only Poly(I:C) or Poly(I:C) in combination with ALUM induced significantly higher expression of CD86 over unstimulated cells (Figure 5(C)), and none of the adjuvants tested increased expression of CD80 significantly (Figure 5(D)). CD40 is a costimulatory marker on the surface of APCs and is required for their activation by Th cells. While no adjuvant, or combination of adjuvants, increased expression of CD40 significantly, the combinations of MPLA or ALUM with Poly(I:C) did appear to increase expression slightly more than any adjuvant individually (Figure 5(A)).

Cytosolic Poly(I:C) induced CD86 significantly on moDCs in comparison with unstimulated cells. This increase in CD86 expression was also observed in MPLA treated cells but not in ALUM treated moDCs. Combinations of Poly(I:C) with MPLA also induced a significant increase in CD86 expression (Figure 6(A)).

CD80 expression on moDCs was increased following Poly(I:C) transfection compared with unstimulated cells, though not significantly ($p = 0.07$). When moDCs were stimulated with combinations of MPLA or ALUM with Poly(I:C), a modest but not significant increase in CD80 expression was seen (Figure 6(B)).

CD83 is a marker of DC maturation, expressed on functionally mature DCs, and is involved in promoting prolonged activation of CD8+ T cells. moDCs treated with MPLA showed an increase in expression of CD83 and this increase was enhanced when MPLA was combined with Poly(I:C), though Poly(I:C) induced very little CD83

expression alone. ALUM did not appear to have any effect on CD83 levels alone, but a significant increase was observed when ALUM was combined with Poly(I:C) (Figure 6(C)).

In the case of HLA-DR, a molecule essential for antigen presentation of peptide to the TCR, Poly(I:C) and MPLA, but not ALUM, induced a significant increase in expression on moDCs. The combination of Poly(I:C) with MPLA also induced a significant increase in HLA-DR expression (Figure 6(D)). CD40 expression on moDCs was also analyzed and whereas some increase in expression was seen with Poly(I:C), MPLA, or combinations of Poly(I:C) with MPLA or ALUM, there was no significant increase with any stimulation (Figure 54(B)).

3.6 | Cytosolic Poly(I:C) increases moDC-induced T cell proliferation and increases IFN γ and IL-17A production in neonatal T cells

The interaction between moDCs and T cells plays an instructive role in generating the adaptive immune response. Having observed significant increase in cytokine production and costimulatory molecules on moDCs in response to Poly(I:C), and combinations of Poly(I:C) with MPLA or ALUM, we next assessed the effect of adjuvant-treated APCs on naïve T cells. In order to assess the impact of adjuvant treatment on the ability of moDC to direct T cell activation, immature neonatal moDCs were stimulated with our range of agonists or combinations of adjuvants as previously described and subsequently cocultured with purified allogeneic CD4+ T cells in a mixed leukocyte reaction. After 5 days, CD4+ T cell proliferation was measured by CFDA SE fluorescence dilution. Analysis of CD4+ T cell proliferation revealed that T cells cultured with allogeneic moDC, previously matured with Poly(I:C), demonstrated a higher level of proliferation compared with T cells cultured with immature DC (Figures 7(A), second panel and 7(B)). In contrast moDCs activated with either MPLA or ALUM alone induced only basal levels of allogeneic CD4+ T cell proliferation (Figures 7(A), third and fourth panels respectively and 7(B)). Significantly, CD4+ T cells cultured with moDCs, previously treated with combinations of MPLA and Poly(I:C), or ALUM and Poly(I:C), showed significantly higher levels of proliferation than either MPLA or ALUM alone (Figures 7(A) and 7(B)). These data confirm that Poly(I:C), in combination with established adjuvants, can act on innate immune cells to enhance their capacity to initiate CD4+ T cell responses. Interestingly, no differences in cytokine levels produced by CD4+ T cells were detected in this assay (data not shown) therefore to determine whether tested adjuvants could also influence qualitative responses we used direct stimulation assays.

When treating T cells directly, we unexpectedly found that Poly(I:C), or MPLA alone, was capable of significantly increasing the amount of IFN γ produced from TCR-activated isolated CD4+ T cells (Figure 7(C)). Surprisingly, no such effects were observed when both stimuli were used in combination. Over recent years, Th17 responses have been implicated in many infectious diseases, along with many autoimmune disorders and recognition of the significance of this proinflammatory cytokine in vaccine immunology has steadily grown.³⁰ Interestingly,

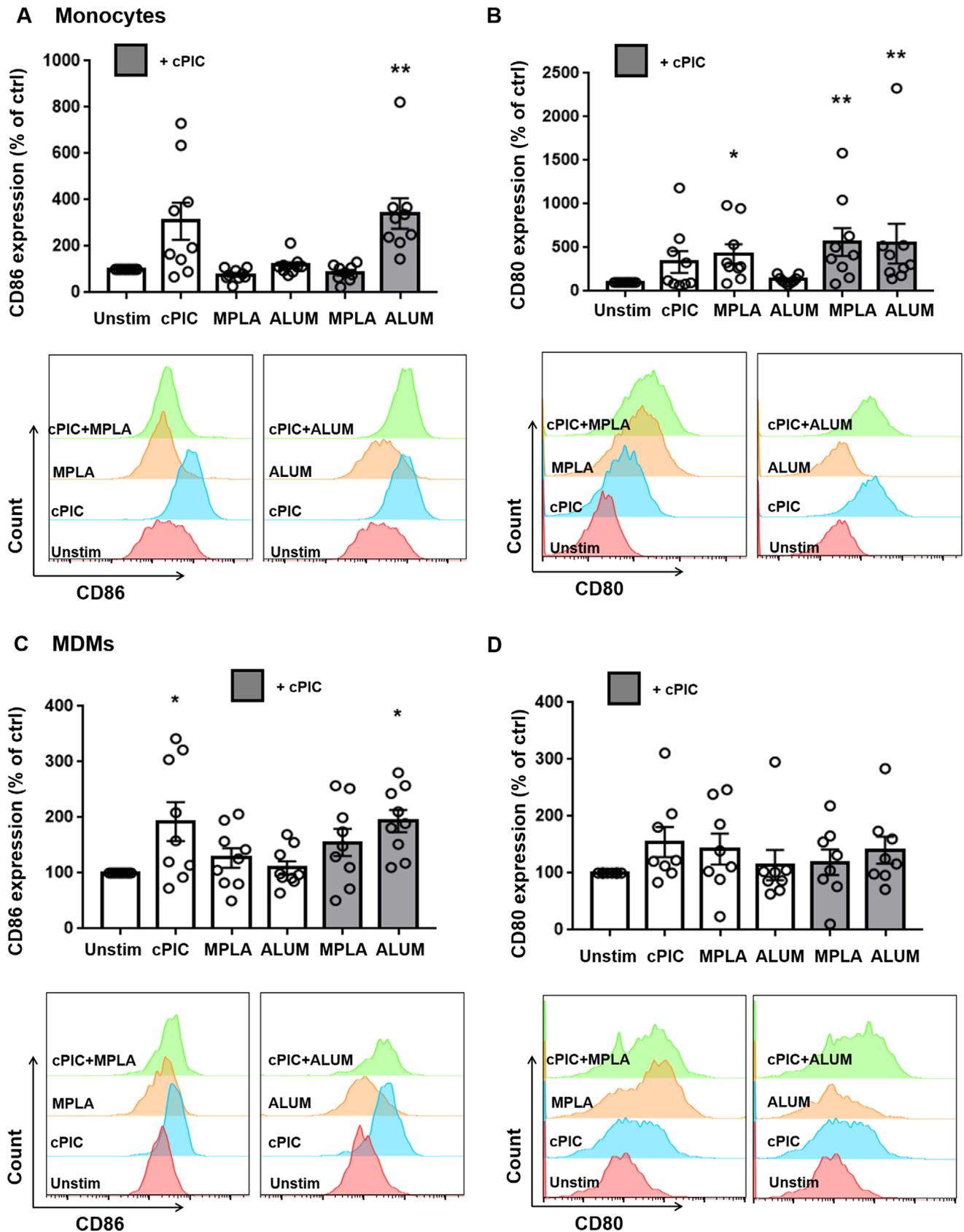


FIGURE 5 Cytosolic Poly(I:C) increases costimulatory marker expression on human neonatal monocytes and MDMs. Cord blood monocytes or MDMs were stimulated with Poly(I:C) transfection, MPLA or ALUM, or combinations of MPLA and transfected Poly(I:C), or ALUM and transfected Poly(I:C) for 24 h. Following 24 h stimulation, the expression of maturation markers (A and C) CD86 and (B and D) CD80 was measured by flow cytometry. Histograms depict expression of CD86 and CD80 in MPLA- and ALUM-treated monocytes, with or without transfected Poly(I:C), compared with control monocytes from 1 representative experiment. Pooled data ($n \geq 8$) shown are mean \pm SEM of the measured MFIs, expressed as percentages of the vehicle control. * $p < 0.05$, ** $p < 0.01$. Kruskal–Wallis nonparametric test with Dunn’s post-test was used to compare groups

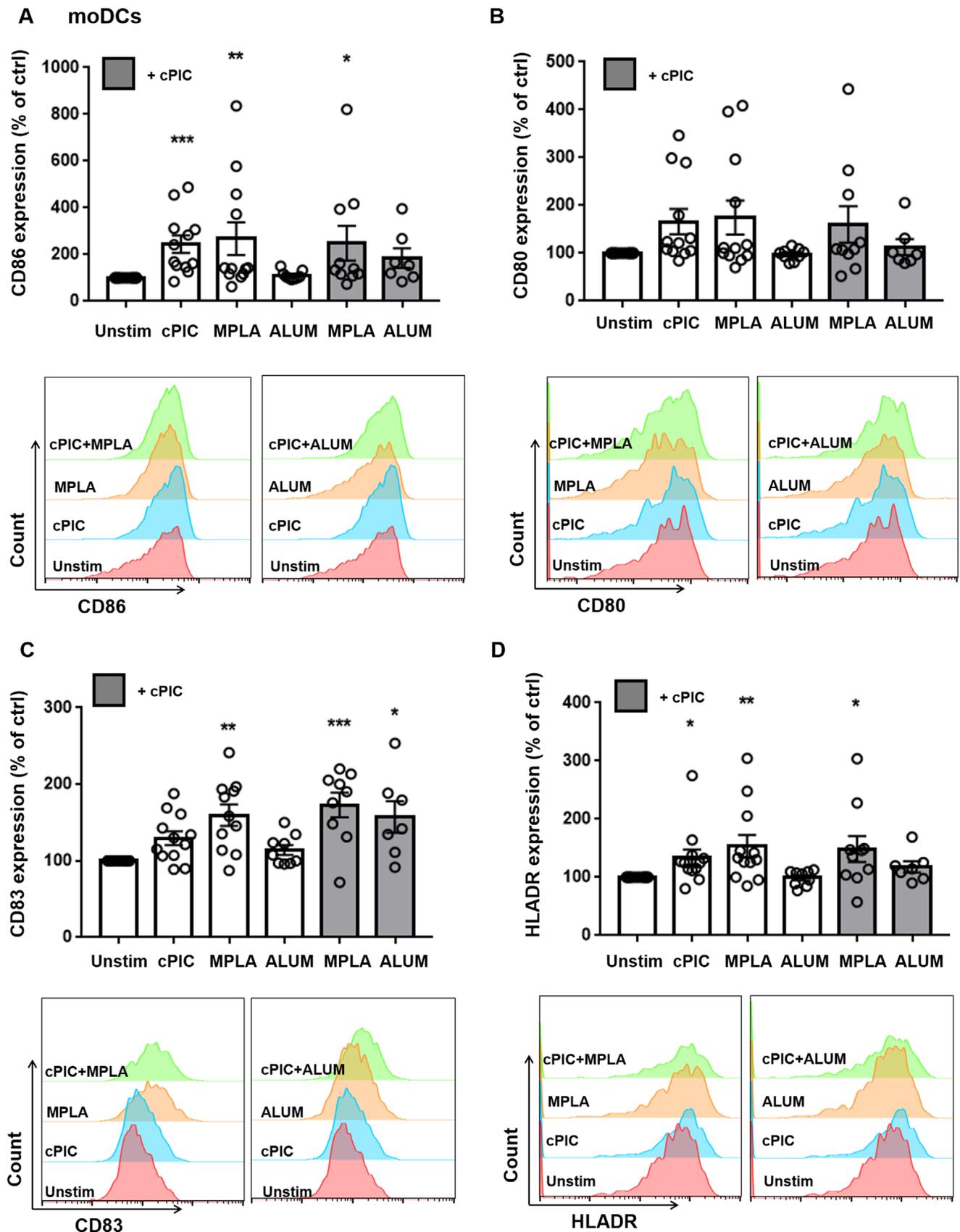


FIGURE 6 Cytosolic Poly(I:C) increases costimulatory molecule expression on human neonatal moDCs. Human neonatal moDCs were stimulated with either Poly(I:C) transfection, MPLA or ALUM, or combinations of MPLA and transfected Poly(I:C), or ALUM and transfected Poly(I:C) for 24 h. Following 24 h stimulation, the expression of surface markers (A) CD86, (B) CD80, (C) CD83, and (D) HLA-DR was measured by flow cytometry. Histograms depict expression of surface markers on MPLA- and ALUM-treated moDCs, with or without transfected Poly(I:C), compared with control moDCs from 1 representative experiment. Pooled data ($n \geq 7$) shown are mean \pm SEM of the measured MFIs, expressed as percentages of the vehicle control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$. Kruskal–Wallis nonparametric test with Dunn’s post-test was used to compare groups

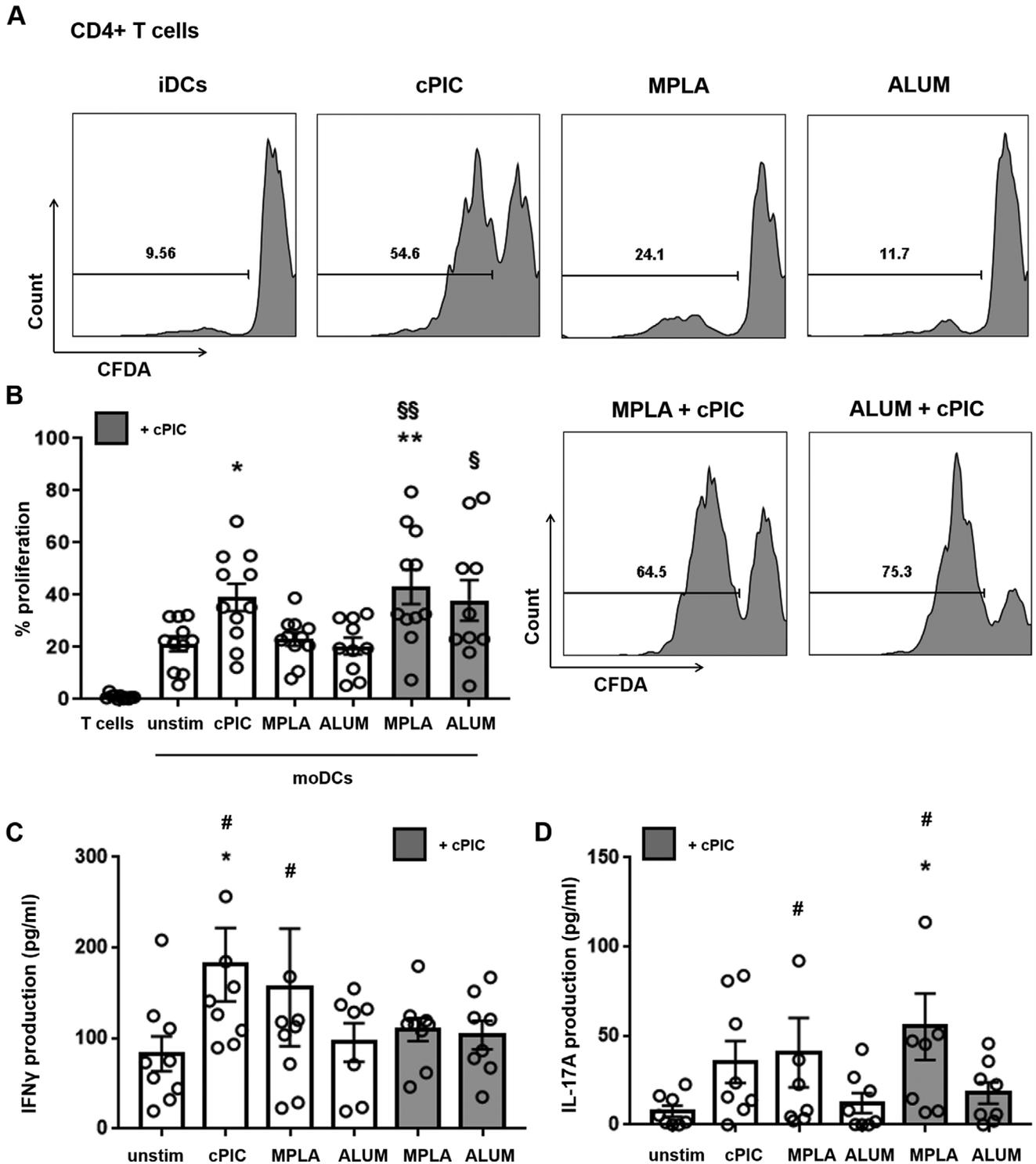


FIGURE 7 Cytosolic Poly(I:C) increases moDC-induced T cell proliferation and increases IFN γ and IL-17A production in neonatal T cells. (A and B) moDCs from healthy cord blood ($n = 8$) were left untreated or treated with MPLA, ALUM, or transfected Poly(I:C), or combinations of treatments, for 24 h. After 24 h, moDCs were washed and cultured at a 1:10 ratio with allogeneic purified neonatal CD4+ T cells. After 5 days CD4+ T cells were analyzed for proliferation, using Vybrant™ CFDA SE Cell Tracer Kit, by flow cytometry. (A) Histograms depicting proliferation of CD4+ T cells cocultured with either control, Poly(I:C), MPLA, ALUM, or combination treated allogeneic DC, as measured by CFDA fluorescence dilution. Data shown are from 1 healthy donor. (B) Pooled data depicting mean \pm SEM percentage proliferation of CD4+ T cells from 11 allogeneic combinations of T cells and moDCs. * indicates comparison with unstimulated control, § indicates comparison with MPLA or ALUM alone. (C and D) Isolated CD4+ T cells from healthy cord blood were treated with Poly(I:C), MPLA, ALUM, or combinations of Poly(I:C) with MPLA or ALUM, along with immobilized CD3/CD28 stimulation. Following 5 days of culture, supernatants were analyzed for production of (A) IFN γ or (B) IL-17A by ELISA. Data are presented as mean \pm SEM ($n \geq 7$) donors in each group. */§ $p < 0.05$, **/§§ $p < 0.01$. Statistical significance was determined by one-way ANOVA, with Dunnett's multiple comparisons post hoc test or by Kruskal-Wallis nonparametric test with Dunn's post-test. # indicates 1 data point outside limit of graph

while direct treatment of CD4+ T cells with MPLA alone significantly enhanced IL-17A secretion, these levels were enhanced even further when a combination of MPLA with Poly(I:C) were present (Figure 7(D)). These data demonstrate that potential vaccine adjuvants under investigation have the capacity to directly stimulate activated neonatal CD4+ T cell responses.

4 | DISCUSSION

PRR stimulation is now an accepted mechanism for immune activation by potential vaccine adjuvants.⁹ An example of the successful targeting of PRR pathways for adjuvanticity is activation of TLR4 through MPLA, which is now a component of several licensed vaccines including Cervarix.³¹ The issue arising when targeting many PRR pathways as potential adjuvants is their compromised efficacy at activating the immune system in newborns and young children.^{14,15,32} TLR7/8 agonists are one exception to this decreased activation in neonates and are being developed as pediatric-specific adjuvants.^{17–19} Our previous study has shown that activation of another family of PRRs, the CNA receptors, induces strong Type I IFN responses in cord blood cells and young children.²⁵ Type I IFNs have important effects on both the innate and adaptive cellular immune response. Type I IFNs have roles in promoting the maturation of DCs.³³ DCs themselves can produce IFN α in response to TLR stimulation, which in turn leads to activation of T cells, which can be independent of IFN γ .³⁴ Downstream of viral infection, Type I IFNs promote CD4+ and CD8+ T cell responses, along with supporting the function and survival of NK cells, reviewed in Ref 35.³⁵ Type I IFNs have also been shown to induce human adult and neonatal CD4+ T cells to produce IFN γ .^{36,37} Interestingly, Type I IFNs have also been shown to have a protective role for T cells which may be targeted by NK cell cytotoxicity. As a result of this viral-induced Th1-driving capacity, it would be of great advantage for a vaccine adjuvant to induce Type I IFNs. We were interested in confirming and further characterizing the viral-sensing, innate CNA responses of myeloid cells in cord blood to transfected Poly(I:C) and comparing these responses to those elicited by MPLA or ALUM, two of the more popular adjuvants currently in use in pediatric vaccines.

As expected, cytosolic Poly(I:C) activation of RIG-I was an excellent driver of Type I IFN in neonatal myeloid cells and induced stronger expression of the costimulatory molecule CD86 on MDMs and moDCs than either MPLA or ALUM. We observed consistent proinflammatory cytokine responses from isolated populations of neonatal cells in response to activation of TLR4 with MPLA, consistent with previous reports of TLR4 activation by MPLA in neonatal moDCs. Monocytes can produce IL-1 β in the absence of a priming signal,³⁸ as evidenced by the robust IL-1 β production in response to MPLA in monocytes. Interestingly, MPLA alone was capable of inducing very low levels of IL-1 β in moDCs, although this low level of activation (~30 pg/ml) has previously been shown in neonatal and adult moDCs.³⁹ In contrast, MPLA failed to induce any Type I IFN response in neonatal myeloid cells. Combining adjuvants is a very appealing strategy to encourage cellular responses,^{40–42} with one particularly interesting study involv-

ing neonatal cells showing that dual activation of TLR7/8 and Mincle, a receptor for C-type Lectin, can induce a strong Th1 response.¹⁹ It was interesting that combining the adjuvants did not enhance levels of individual cytokines secreted, instead parallel activation of CNA sensors and TLR4 through the combination of cytosolic Poly(I:C) and MPLA enhanced the ability of neonatal monocytes, MDMs and moDCs to increase their repertoire of secreted cytokines to encompass both Type I IFN and proinflammatory cytokines.

Cytokine activation impacts expression of costimulatory molecules on the surface of myeloid APCs. In fact, prevaccine assessment of TLR-induced CD80 and CD86 expression on monocytes can predict vaccine response, highlighting the importance of these costimulatory molecules at the interface of the innate and adaptive immune response.⁴³ Interestingly, TLR-induced CD80 and CD86 expression decreases with age, with older adults having less responsiveness to TLR stimulation than younger adults.⁴³ Cord blood monocytes and DCs have been reported to have decreased CD80 expression, in response to LPS and CpG compared with adult samples.⁴⁴ CD86 expression on neonatal and adult blood cells is comparable,^{45,46} although decreased CD86 expression in polarized macrophages from neonates has been shown.⁴⁷ The ability of cytosolic Poly(I:C) alone, or in combination with MPLA or ALUM, to induce expression of costimulatory markers on the surface of neonatal myeloid cells is desirable in the context of combining adjuvants for pediatric vaccines. Expression of these markers in response to Poly(I:C) with MPLA or ALUM is increased on moDCs, and consequently moDCs treated with these combinations induced robust proliferation of CD4+ T cells over treatment with MPLA or ALUM alone.

Our previous work has shown that mixed populations of cord blood cells produced IFN γ in response to Poly(I:C). In this study, we examined myeloid cells for Poly(I:C)-induced IFN γ production and found that IFN γ production was below the level of detection (~2 pg/ml, data not shown). Interestingly however, Poly(I:C) can induce IFN γ from naïve CD4+ T cells directly. Poly(I:C) alone was also able to induce some IL-17A production from naïve CD4+ T cells. When Poly(I:C) and MPLA were combined to treat CD4+ T cells, IFN γ was no longer induced. The decreased IFN γ response in the presence of Poly(I:C) and MPLA could be due to the inhibitory effect of IL-10 produced in response to MPLA. While IFN γ was no longer induced, IL-17A production was significantly increased with the combination of adjuvants. Historically, IL-17A has been deemed very important in the mucosa but IL-17A also plays a critical role in vaccine-induced immunity against bacterial infections.³⁰ The role of IL-17A in the context of vaccines against bacterial infections seems to be in the induction of chemokines that recruit Th1 cells, neutrophils, macrophages, and results in enhanced phagocytosis.^{48,49} Although it has previously been shown that IL-10 can suppress Th17 cytokines secreted by T cells,⁵⁰ IL-17A is significantly induced in the presence of Poly(I:C) and MPLA so is perhaps unaffected by IL-10 in this neonatal context. These data in particular highlight the delicate balance between stimulatory and inhibitory signals.

Our results so far suggest that Poly(I:C), either alone or in combination with MPLA or ALUM, may have potential as a vaccine adjuvant that can enhance neonatal monocyte, macrophage, moDC, and

T cell function. In general, Poly(I:C) activation of CNA sensors can enhance responses of MPLA and ALUM, adjuvants currently used in pediatric vaccines, thereby supporting the idea that combinations of adjuvants may be a useful way of promoting a more robust cellular immune response within a vaccine formulation for the neonatal population.

The data presented here support the adjuvant potential of activation of CNAs in neonatal cells *in vitro*. An activator of RIG-I is currently in phase 1 clinical trials as an adjuvant immunotherapy in advanced/metastatic solid tumors in adults⁵¹ so information on safety of specific activation of RIG-I in humans will soon be readily available. Poly(I:C) has been tested in many capacities as a vaccine adjuvant,⁵² but primarily as an activator of TLR3. Specifically, several studies examining Poly(I:C)-loaded liposomes have shown promise in vaccination studies in adult mice.^{53,54} Although these studies were carried out on the premise that Poly(I:C) was stimulating the immune response through TLR3, we suspect that RIG-I detection of Poly(I:C) was occurring due to the uptake of liposomes into APCs. Recent advances in mRNA vaccine technology have allowed intramuscular delivery of RNA vaccines and so have opened up the possibilities of utilizing intracellular stimulation of RNA receptors as a viable route for adjuvants. Investigating a role for Poly(I:C) alone or in combination with other adjuvants in an *in vivo* neonatal preclinical setting will be an important next step in analyzing Poly(I:C) combinations as an adjuvant to improve pediatric vaccines.

AUTHORSHIP

K. B., E. N., and J. O. C. performed the experiments. S. C., M. C., and D. K. identified suitable donors and collected data and bloods. K. B., E. J. M., P. T. W., F. M. M., and S. D. designed the experiments, analyzed the data, and wrote the manuscript.

DISCLOSURE

S. L. and K. B. are inventors on a patent application for the use of CNA in neonatal vaccines.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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