Published in final edited form as:
J Allergy Clin Immunol. 2012 July ; 130(1): 195–204.e9. doi:10.1016/j.jaci.2012.02.042.

Imidazoquinoline TLR8 agonists activate human newborn monocytes and dendritic cells via adenosine-refractory and caspase-1-dependent pathways

Victoria J. Philbin, D.Phil[a,b,*], **David J. Dowling, PhD**[a,b,*], **Leighanne C. Gallington, BSc**[a], **Guadalupe Cortés, PhD**[a,b], **Zhen Tan, MD**[a,b,f], **Eugénie E. Suter, MA**[a], **Kevin W. Chi, BA**[a], **Ariel Shuckett, MPH**[a], **Liat Stoler-Barak, MSc**[a], **Mark Tomai, PhD**[c], **Richard L. Miller, PhD**[d], **Keith Mansfield, DVM**[e,b], and **Ofer Levy, MD, PhD**[a,b]

[a]Department of Medicine, Divisions of Infectious Diseases, Children's Hospital Boston, Boston, MA, USA

[b]Harvard Medical School Boston, MA, USA

[c]3M Drug Delivery Systems, St Paul, MN, USA

[d]3M Pharmaceuticals, St Paul, MN, USA (retired)

[e]New England Primate Research Centre, Southborough, MA, USA

[f]Shanghai Jiao Tong University, School of Medicine, Shanghai, China

Abstract

Background—Newborns suffer frequent infection and manifest impaired vaccine responses, motivating a search for neonatal vaccine adjuvants. Alum is a neonatal adjuvant, but may confer a Th2 bias. Toll-like receptor (TLR) agonists are candidate adjuvants, but human neonatal cord blood monocytes (Mos) demonstrate impaired Th1-polarizing responses to many TLR agonists due to plasma adenosine acting via cAMP. TLR8 agonists, including imidazoquinolines (IMQs) such as the small synthetic 3M-002, induce adult-level TNF from neonatal Mos, but the scope and mechanisms of IMQ-induced activation of neonatal Mos and Mo-derived dendritic cells (MoDCs) have not been reported.

Objectives—To characterize IMQ-induced activation of neonatal Mos and MoDCs.

Methods—Neonatal cord and adult peripheral blood Mos and MoDCs were cultured in autologous plasma; Alum- and TLR agonist-induced cytokines and co-stimulatory molecules were measured. TLR8 and inflammasome function were assayed using siRNA and western blotting/ caspase-1 inhibitory peptide, respectively. The ontogeny of TLR8 agonist–induced cytokine responses was defined in Rhesus macaque whole blood *ex vivo*.

Corresponding Author: Ofer Levy MD, PhD, Division of Infectious Diseases, Children's Hospital Boston, 300 Longwood Avenue, Boston, MA 02115, Phone : 617-919-2904, Fax : 617-730-0255, ofer.levy@childrens.harvard.edu.
*These authors contributed equally to this study.

Declaration of all sources of funding: This project was supported by grants to OL from the National Institutes of Health RO1 AI067353-01A1 and Administrative Supplement 3R01AI067353-05S1, The Patterson Trust, and by Grand Challenges Explorations Grant 53278, and Global Health Grant OPPGH5284 from The Bill & Melinda Gates Foundation. Reagent support was provided by 3M Pharmaceuticals. VJP was supported by an American Heart Association Postdoctoral Fellowship.

Results—IMQs were more potent and effective than Alum at inducing TNF and IL-1β from Mos. 3M-002 induced robust TLR pathway transcriptome activation and Th1-polarizing cytokine production in neonatal and adult Mos and MoDCs, signaling via TLR8 in an adenosine/cAMP-refractory manner. Newborn MoDCs displayed impaired LPS/ATP-induced caspase-1-mediated IL-1β production, but robust 3M-002-induced caspase-1-mediated inflammasome activation independent of exogenous ATP. TLR8-IMQs induced robust TNF and IL-1β in whole blood of Rhesus macaques at birth and infancy.

Conclusions—IMQ TLR8 agonists engage adenosine-refractory TLR8 and inflammasome pathways to induce robust Mo and MoDC activation and represent promising neonatal adjuvants.

Keywords

TLR8; Innate immunity; Neonate; Newborn; Alum; Adjuvant; Adenosine; IL-1β; Caspase-1; Inflammasome

INTRODUCTION

Each year over 2,000,000 newborns and infants worldwide die of infection (1, 2). Newborns express a distinct T-helper (Th) 2-polarized immune system at birth, potentially limiting the efficacy of crucial immunization efforts. Impairments in Th1-polarizing responses of neonatal antigen-presenting cells (APCs) limit neonatal immune responses (3). In response to most stimuli, neonatal monocytes (Mos) (4–6), monocyte-derived dendritic cells (MoDCs) (7), myeloid DCs (mDCs) (8) and plasmacytoid DCs (pDCs) (9) produce relatively low amounts of Th1-polarizing cytokines (10), including TNF, IFN-γ, interleukin (IL)-12p70, and IFN-α (11). DCs are specialized cells with very high intrinsic antigen-presenting activity important to vaccine responses (12). In this context, there is an unmet need for immunomodulators that can activate robust Th1-polarizing responses from neonatal APCs, to prevent and/or treat infection and allergies (13) or as vaccine adjuvants (14).

Currently, aluminum salt (Alum) is the major U.S. Food and Drug Administration-approved human vaccine adjuvant (15). Administered as part of vaccines to millions of people, it enhances antibody (Ab) production, effectively reducing morbidity and mortality. However, Alum-adjuvanted vaccines typically require multiple booster doses, fail to enhance cell-mediated immunity and may bias subsequent immune responses towards Th2 (16). Alum may exert its adjuvant effects in part via activation of the *n*ucleotide-binding domain *l*eucine-rich repeat-containing *r*eceptor containing a *p*yrin domain-3 (NLRP3) cytosolic inflammasome protein complex that mediates IL-1β and IL-18 production (17–19). Alum is given at birth as part of the hepatitis B vaccine, yet its activity towards primary neonatal leukocytes has not yet been reported with little known regarding expression/function of the inflammasome at birth (20).

Toll-like receptors (TLRs), sentinel components of innate immune defense, are activated by microbial products, synthetic compounds or endogenous danger signals (21). Engagement of TLRs activates APCs. Thus TLRs agonists can serve as stand-alone immune enhancing agents or as vaccine adjuvants (22, 23). The synthetic compound imiquimod (TLR7) is approved as a stand-alone topical agent for the treatment of human papilloma virus and certain skin cancers (24). A number of vaccines contain TLR agonists, including those against *Haemophilus influenzae* type b containing Neisseria-derived outer membrane proteins (TLR2) and Bacille Calmette-Guérin (*Mycobacterium bovis*; TLR2/4/8) (25), as well as certain hepatitis B vaccines that contain lipid A (TLR4) (12). Efforts are on going to identify TLR agonists that enhance adaptive immune responses of newborns and infants (26).

Impaired Th1 responses of human neonatal cord blood Mos and mDCs to agonists of TLRs 1-7 suggest that agonists of these TLRs may have limited immune adjuvant efficacy early in life (5, 6). Impairment of TLR2-mediated TNF production by human neonatal blood Mos is mediated in part by high extracellular concentrations of inhibitory adenosine in human neonatal cord blood plasma (27). Adenosine is an endogenous purine metabolite that acts via cognate adenosine receptors to induce intracellular production of cAMP, a secondary messenger that inhibits production of TNF and other Th1-polarizing cytokines, while maintaining/enhancing Th2 and anti-inflammatory cytokines (28, 29). Our prior studies of neonatal whole cord blood (CB), cord blood mononuclear cells (CBMCs) and Mos demonstrate that, in contrast to the impaired neonatal TNF response to agonists of TLRs 1-7, TLR8 agonists, including the IMQ resiquimod (R848; TLR7/8), induce adult-level TNF and IL-12p40/70 (30).

The TLR7, -8 and -9 subfamily is endosomal and activated by nucleic acids (31). Single-stranded viral RNAs (ssRNAs), such as those of influenza and human immunodeficiency viruses, are natural TLR7 and TLR8 agonists (32). Imidazoquinolines (IMQs) are small (<400 Da) synthetic antiviral compounds, which bear structural homology to the purine adenosine and activate mammalian leukocytes via TLR7 and/or TLR8 leading to MyD88-dependent NF-κB activation (24, 33). Whereas TLR7-agonist imiquimod activates Th1-polarizing responses from pDCs, including IFN-α, TLR8 agonists such as the small synthetic (Mr 243.33) IMQ 3M-002 activate Mos and mDCs to induce robust TNF production (34). Of note, IMQs engage the TLR pathway in a species-specific fashion: human and non-human primate TLR8 is activated by the same agonists (35), but murine TLR8 is divergent from human TLR in the expression of LRR repeats and therefore is not activated by agonists of human TLR8 (36). In addition to their TLR-mediated effects, IMQs also induce NLRP3 inflammasome-dependent IL-1β production in mice (37) as well as caspase-1 and IL-1β expression in human Mos and pDCs (38).

Our prior study demonstrated that TLR8 agonists might have unique stimulatory effects towards human neonatal mononuclear cells (39), but much remained unknown. In this study, we characterized the pattern of IMQ TLR8 agonist-induced activation of human neonatal Mos and MoDCs and the mechanisms underlying this activation. We show that IMQs induce, via TLR8- and caspase-1-mediated pathways refractory to inhibitory effects of adenosine/cAMP, Th1-type responses from neonatal APCs that exceed those induced by Alum or other TLR agonists. We also characterized the post-natal age range during which TLR8 agonists retain superior activity relative to other TLRs agonists Rhesus in macaque whole blood tested *ex vivo*. These results shed new light on the mechanisms governing neonatal Mo and MoDC responses, establish functional expression of the neonatal caspase-1/inflammasome as an adenosine-refractory pathway, and suggest that IMQ TLR8 agonists are promising candidate neonatal and infant immune adjuvants for the prevention and/or treatment of infection in the very young.

METHODS

See the Methods section in this article's Online Repository (OR) for additional method details.

TLR agonists, assay reagents and Human Blood

TLR agonists included ultra-pure LPS from *Salmonella minnesota*, (TLR4; List Biological Laboratories, Campbell, CA) and IMQs 3M-013, R848 and 3M-002 (TLR7, TLR7/8 and TLR8 respectively; 3M Pharmaceuticals, St. Paul, MN). Human blood was collected under human experimentation guidelines of the U.S. Department of Health and Human Services.

Rhesus macaque blood

Rhesus macaque peripheral blood was collected in accordance with local institutional review board-approved animal study protocols.

Isolation of mononuclear cells and monocytes and Flow cytometry

Heparinized human blood was layered onto Ficoll-Hypaque gradients the cord or peripheral blood mononuclear cell (CBMC or PBMC, respectively) layer collected as previously described (40). Mos were isolated by negative selection. Flow cytometry analysis employing a MoFlo Legacy cytometer (DakoCytomation, Fort Collins, CO).

Differentiation of monocyte-derived dendritic cells (MoDCs)

Mos were cultured at 10^6/mL in RPMI (Gibco, Carlsbad, CA) with 10% fresh autologous plasma, supplemented with IL-4 and GM-CSF (each at 20 ng/ml) for 7 days. To assess caspase-1 activation, MoDCs were stimulated for 2 or 24 hours with a TLR agonist initially and 5 mM ATP (Sigma-Aldrich) added for 15 minutes prior to collection of supernatants (41, 42).

Measurement of pDC activation

Human newborn cord and adult peripheral blood was stimulated with TLR agonists at 37°C, with end-over-end rotation for 19 hours.

Quantitative RT-PCR array

Total RNA was isolated from lysates of TLR-stimulated Mos or DCs (QIAshredder spin column and RNeasy kit; Qiagen, Valencia, CA).

Cytokine, chemokine and cAMP measurement

Supernatants derived from human leukocyte stimulations were assayed by multiplex bead assay or where stated, by ELISA. cAMP was measured in lysates of neonatal Mos by competitive immunoassay as per manufacturer's instructions (Thermo Scientific, Waltham, MA).

Small interfering (si) RNA

Employed siRNAs were targeted against TLR7 or TLR8 and a non-targeting siRNA (ON-TARGETplus SMARTpool, Dharmacon) to MoDCs on day 6/7 of cell culture using electroporation (human DC-electroporation program, Amaxa Nucleofection System, Basel, Switzerland).

Caspase-1 western blotting

Supernatants of TLR agonist-stimulated MoDCs (10^6/mL) cell lysates were collected for protein determination (BCA protein assay, Pierce, Waltham, MA). Clarified lysate was subjected to acetone precipitation at −20°C for 90 minutes, SDS-PAGE and western blot (43).

Statistical analysis and graphics

Data were analyzed using Prism for MacIntosh v. 5.0b (GraphPad Software Inc.; San Diego, CA). Data represent means ± SEM. For normal sample sets, two-tailed t-test or One-way ANOVA with Bonferroni post-test were applied as appropriate. Non-normal sample sets were analyzed by Wilcoxon signed-rank test or by Mann-Whitney test as appropriate. p values < 0.05 were considered significant.

RESULTS

Imidazoquinolines are more potent and efficacious than Alum in inducing IL-1β and TNF from human neonatal and adult monocytes

We compared the *in vitro* bioactivity of IMQs to that of Alum. Human newborn cord and adult peripheral blood-derived Mos were cultured in autologous plasma and stimulated with Alum, 3M-013 (TLR7), R848 (TLR7/8) or 3M-002 (TLR8). Compared to Alum, IMQs were ~10–100 fold more potent and effective at inducing both TNF and IL-1β from both newborn and adult Mos, inducing ~1,000 to 10,000 pg/mL of these cytokines at 5–50 μM. In marked contrast, Alum failed to induce comparable cytokine concentrations even at 500–5,000 μM (Fig 1).

Imidazoquinoline TLR8 agonist induces robust expression of a TLR transcriptome and Th1-polarizing cytokine/chemokines by human cord blood monocytes

We compared human newborn Mos responses to 3M-013 with those to 3M-002 (Fig 2). We selected the agonist concentrations required to achieve TNF production per our prior studies (30), and a stimulation time-point (4h) based on kinetic data (and unpublished observations) (5). In comparison to buffer control, 3M-002 greatly increased levels of mRNAs encoding IL-6 (8603-fold change, $p < 0.001$), pro-IL-1β (82-fold change, $p < 0.001$), IFN-β1 (21-fold change $p < 0.01$), IFN-γ(21- fold change, $p < 0.05$) and TNF (32-fold change, $p < 0.0001$) (data not shown). 3M-002 induced significantly greater overall increases in TLR pathway mRNAs in newborn Mos than did 3M-013, with a mean log-fold difference in mRNA levels of +0.1862 ($p < 0.0001$; Fig 2, *A*). Compared to 3M-013, 3M-002 induced greater expression of mRNAs encoding Th1-polarizing cytokines/chemokines, including CXCL10 (IP-10), IFN-γ, TNF, IFN-β1, IL-12α (p35) as well as TICAM-1 (Fig 2, *A*). 3M-002 also induced greater Th1-polarizing responses than MALP (TLR2/6), with a mean log-fold difference in mRNA levels of +0.2156 ($p < 0.0001$), including greater induction of TNF, IFN-β1, and lymphotoxin α (Fig E1). Effects of 3M-002 and 3M-013 on neonatal Mo cytokine expression were further compared at the protein level. Consistent with mRNA results, 3M-002 induced significantly greater protein concentrations of Th1-polarizing cytokines/chemokines including, IL-2, TNF, IFN-γ CXCL10 and CCL3 (MIP-1α) (Fig 2, *B*). In contrast, cytokines/chemokines with Th2 (±Th1)-polarizing potential (including IL-4, IL-7, IL-12p40 and IL-13), Th17-polarizing potential (IL-1β) or anti-inflammatory-polarizing potential (IL-6, IL-10) were induced to similar levels by both agonists (Fig 2, *C-D*). Similarly, when compared to MALP (TLR2/6), 3M-002 induced significantly greater concentrations of Th1-polarizing cytokines such as IL-12p70, TNF, IFN-γ and CXCL8 (IL-8) (Fig E1, *B*). With respect to Th2 (±Th1), Th17-polarizing, or anti-inflammatory cytokines/chemokines, 3M-002 induced significantly greater concentrations of IL-1α, IL-1β, IL-4 and CCL11 (eotaxin) and also trended towards higher concentrations of the other cytokines tested (Fig E1, *C-D*).

Additionally we characterized siRNA-mediated inhibition of TLR8 gene expression in human neonatal Mos (see results section and Fig E2 in this article's Online Repository), which to our knowledge is the first report of the use of siRNA technology in primary neonatal APCs. 3M-002 also induced greater TNF, IFN-γ and IL-12p70 than Alum in human newborn MoDCs (see results section and Fig E3 in this article's Online Repository). 3M-002 and R848 demonstrated greater activation of neonatal MoDCs than 3M-013 with respect to up-regulation of CD40, 80, 86, and 83 as well as IL-12p70 production. As TLR7-mediated pDC activation is important for enhancing Th1 responses, including cross-presentation to CD8-bearing T cells (44), it is notable that R848 also induced robust neonatal pDC activation in whole blood as measured by IFN-α production and up-regulation

of CD40, to adult-like levels (see OR results section and Fig E4 in this article's Online Repository).

TLR8 agonist 3M-002 is refractory to the inhibitory adenosine-cAMP axis

To further characterize the mechanisms by which IMQs activate neonatal Mos, whose activity can be limited by adenosine acting via cAMP production (27), we assessed the relative effects of adenosine on TLR7- vs. TLR8-mediated TNF and IL-12p70 production by newborn Mos cultured in autologous plasma. Pre-incubation of newborn Mos with adenosine inhibited 3M-013-induced TNF and IL-12p70 production by up to ~70% (Fig 3, *A*). In contrast, 3M-002 was refractory to the inhibitory effect of adenosine. Adenosine did not reduce IL-1β production induced by either compound (Fig 3, *A*), suggesting that the inflammasome pathway may be refractory to adenosine inhibition. Analyzed as normalized percentage, 3M-002 was significantly more refractory to adenosine inhibition than to 3M-013 (Fig 3, *B*). As adenosine can mediate inhibitory effects via induction of cAMP (45), we measured intracellular cAMP concentrations in newborn Mos exposed to TLR7 or TLR8 agonists. Exposure of neonatal Mos to 3M-002 resulted in significantly less early (5 min, $p < 0.05$) cAMP accumulation than did exposure to 3M-013 (Fig 3, *C*). We determined whether pharmacologic enhancement of intracellular cAMP concentrations using the cell-permeable molecule dibutyryl-cAMP (db-cAMP) affected TLR-mediated TNF production. Whereas addition of db-cAMP inhibited TLR7 agonist-induced TNF production ($p < 0.05$), TLR8 agonist-induced TNF was largely refractory (Fig 3, *D*). Overall, the standard errors of the mean for these conditions were relatively large, likely reflecting study of primary cells in autologous plasma, rather than cell lines in defined culture medium, but the effects described were statistically significant. Thus stimulation 3M-002 not only resulted in less cAMP accumulation in neonatal Mos, but also engaged a pathway for TNF production that was relatively less sensitive to intracellular cAMP concentrations.

Newborn MoDCs demonstrate impaired caspase-1-dependent IL-1β production in response to LPS/ATP

Little is known about expression or function of the inflammasome in neonatal leukocytes. Newborn and adult MoDCs were stimulated with LPS (TLR4) followed by ATP, a combination known to activate inflammasome-dependent IL-1β production in adult MoDCs (19, 41). Of note, neither buffer alone nor ATP alone induced IL-1β production. When exposed to LPS (alone) for 2 hours, both newborn and adult MoDCs produced low levels of IL-1β (Fig 4, *A* and active) caspase-1 (Fig 4, *B* and *C*). Addition of ATP to LPS-stimulated MoDCs significantly increased IL-1β production for both newborn and adult MoDCs (Fig 4, *A*, $p < 0.01$), but to a significantly lesser extent in newborn as compared to adult MoDCs (Fig 4, *A*, $p < 0.05$). Accordingly, western blotting of lysates derived from LPS/ATP treated MoDCs demonstrated diminished production of the active 10 kDa caspase-1 fragment in neonatal as compared to adult MoDCs (Fig 4, *B* and *C*). These results suggest a limitation in neonatal inflammasome activation with respect to certain stimuli.

3M-002 activates robust caspase 1-dependent IL-1β production in neonatal MoDCs independent of exogenous ATP

We next evaluated the ability of 3M-002 to activate the neonatal inflammasome. In contrast to LPS, which required addition of exogenous ATP to boost IL-1β production, 3M-002, when added alone, induced robust IL-1β production in both adult MoDCs and neonatal MoDCs. Exogenously added ATP did not further increase 3M-002-induced production of IL-1β (Fig 4, *D*). Analysis of inflammasome pathway mRNA expression indicated that there was no significant difference of in amounts of 3M-002 induced compared to LPS induced pro-IL-1β mRNA (Fig E5), suggesting that differences in IL-1β production were not due to differences at the mRNA level. Accordingly, in contrast to LPS (Fig 4, *B, C*), 3M-002

induced caspase-1 activation in both newborn and adult MoDCs independently of exogenous ATP as assessed by western blotting (Fig 4, *E, F*). In contrast to LPS/ATP induction of IL-1β, which peaked at 2 hours, 3M-002-induced IL-1β demonstrated distinct kinetics with a marked increase in IL-1β production at 24 hrs of stimulation (Fig. E6, *A*). Independence of 3M-002-induced IL-1β production from exogenous ATP across this timeframe was also evident when the data were expressed as %IL-1β production in the ATP-treated condition relative to that induced without ATP (Fig. E6, *B*). To further characterize the action of 3M-002 on neonatal MoDCs, we determined whether 3M-002-induced IL-1β production was caspase-1-dependent. To this end, neonatal MoDCs were pre-incubated with or without the caspase-1-selective antagonist Z-WEHD-FMK prior to stimulation (Fig 4, *G*). Z-WEHD-FMK inhibited both LPS/ATP- and 3M-002 (alone)-induced IL-1β, an effect that was also evident when analyzed as % IL-1β production (Fig. 4, *H*, $p < 0.001$). Effects of Z-WEHD-FMK were specific to IL-1β production as TNF production was unaffected (data not shown).

Imidazoquinoline TLR8 agonists induce robust cytokine and co-stimulatory responses in blood from newborn and infant Rhesus macaques

We next characterized the ontogeny of TLR8 agonist activity towards peripheral blood leukocytes during the first weeks of life by employing whole blood derived from newborn and infant Rhesus macaques, a species that expresses TLR8 that is functionally similar to that of humans (35, 46, 47). Rhesus macaque blood was drawn at birth (newborn cord), weekly basis for the first three weeks of life (peripheral blood from same animals) as well as from dam and non-dam adults. We compared the ability of MALP, 3M-013, R848 and 3M-002 to induce TNF and IL-1β production *ex vivo*. In marked contrast to the TLR2 and TLR7 (only) agonists, R848 and 3M-002 demonstrated greater TNF induction (Fig E7, *A-D*), inducing cytokine levels comparable to those in adult blood. Similarly, although demonstrating an apparent age-dependent maturation in production from birth (cord) to 1 week of life, R848 and 3M-002 induced robust IL-1β production, at least as great as adult levels, at weeks 1-3 of life (Fig E7. *EH*). R848 also demonstrated efficacy in inducing up-regulation of CD40 in both infant (1–4 months) and adult Rhesus macaque mDCs in whole blood (Fig E4, *D*).

DISCUSSION

In this study, we characterized the scope and mechanisms of neonatal Mo and MoDC activation by IMQ TLR7 and/or -8 agonists. In neonatal Mos, 3M-002 was more effective than 3M-013 at inducing mRNA and protein expression of Th1-polarizing cytokines, suggesting that IMQ TLR7/8 agonists may offer greater adjuvant potency and efficacy than Alum. Since 3M-002-induced TNF production from neonatal primary MoDCs was at least in part TLR8-dependent, we further characterized the mechanism of TLR8-induced APC activation and cytokine production in neonatal Mos by assessing the relative sensitivity of TLR7 and TLR8 agonists to the inhibitory adenosine-cAMP axis. Adenosine modulates TLR-mediated cytokine production (28) and DC activity (48) and can differentially regulate DC-primed Th cell responses, increasing Th2 responses in atopic study subjects while reducing Th1 and increasing Treg activity in non-atopic donors (49). High neonatal plasma concentrations of adenosine, coupled with sensitivity of neonatal mononuclear cells to adenosine-induced intracellular cAMP accumulation, suppresses the ability of neonatal Mos to produce pro-inflammatory/Th1-polarizing cytokines (11). Remarkably, neonatal Mos stimulated with TLR8 agonists were refractory to the inhibitory effects of adenosine (Fig 3) and accumulated less cAMP than those exposed to a TLR7 agonist (Fig 3, *C*). TLR8 agonists were also refractory to pharmacologic enhancement of cAMP using cell-permeable db-cAMP (Fig 3, *D*). As cAMP is downstream of adenosine in an inhibitory pathway, this

suggests that TLR8 activation engages a cAMP refractory pathway, potentially as an adenosine receptor antagonist (50). Collectively, these data suggest that an IMQ TLR8 agonist can act refractory to the adenosine/cAMP axis by: 1) reducing intracellular cAMP accumulation and 2) engaging pathways insensitive to cAMP (Fig 3).

In addition to the TLR-NF-κB pathway, increasing attention has focused on the inflammasome's role in immune responses (51). Inflammasome-mediated IL-1β production may contribute to vaccine responses by enhancing Ab production (17). Little is known regarding the effects of IMQs on inflammasome activation in primary human leukocytes, with published studies largely confined to adult cells cultured in serum-free media or in fetal calf serum (38, 52, 53) that have largely focused on IL-1β production, with the exception of one study of caspase-1 activation in human adult Mos and pDCs (38). We have compared for the first time the action of Alum and IMQs in primary human newborn and adult MoDCs cultured in fresh autologous human plasma. Of note, in contrast to the production of TNF and IL-12p70, both TLR7- and TLR8-mediated Mo IL-1β were largely refractory to adenosine, to our knowledge the first time the effects of adenosine on the inflammasome pathway have been evaluated.

Little is known about the functional expression of the inflammasome at birth. Neonatal CBMCs demonstrate lesser LPS-induced IL-1β than adult PBMC (6), but there are no published reports of caspase activity in neonatal leukocytes. Here, we demonstrated for the first time that IMQs are effective inducers of IL-1β in human cord blood-derived Mos and MoDCs. 3M-002 and R848 were ~10-100-fold more potent and effective inducers of IL-β in human newborn and adult Mos than Alum, whose adjuvant properties may in part be inflammasome-dependent (17), though additional mechanisms are under study (54). We demonstrate for the first time the functional expression of a caspase-1 inflammasome in human newborn MoDCs. Primary human neonatal MoDCs demonstrated TLR agonist-induced expression of pro-IL-1β mRNA, as well as caspase-1 activation and IL-1β protein production. Within our limited cohort of study subjects, we discovered diminished LPS/ATP-induced caspase-1 activation and IL-1β production in neonatal MoDCs compared to adult MoDCs, indicating a stimulus-specific impairment in caspase-1/inflammasome pathway at birth.

In contrast to Alum's limited ability to activate primary MoDCs *in vitro,* IMQs activated caspase-1 and induced IL-1β production. Similarly to LPS, 3M-002-induced IL-1β protein production was caspase-1-dependent as it was sensitive to the specific inhibitor Z-WEHD-FMK. However, in contrast to LPS, 3M-002 demonstrated: a) distinct kinetics with a marked increase in IL-1β production at 24 hrs of stimulation and b) independence from ATP. 3M-002 may require time to diffuse into the endosomal compartment to activate endosomal TLR8 (needed for pro-IL-1β mRNA). In contrast, the combination of LPS/ATP results in rapid surface activation of TLR4-NF-κB pathway and exogenous ATP inflammasome activation respectively. The ability of IMQs to induce IL-1β without exogenous ATP, directly through ligand-receptor interaction or through yet to be determined secondary effects, may contribute to relatively high IMQ-induced IL-1β. In addition, the ability of TLR8-signaling IMQs to trigger both NF-κB and inflammasome pathways in an adenosine- and exogenous ATP-independent manner (see model in Fig 5), respectively, may contribute to the ability of these agents to consistently induce TNF and IL-1β responses not only at birth but throughout infancy as demonstrated in Rhesus macaque blood tested *ex vivo* (see OR results section and Fig E7 in this article's Online Repository). Further investigation of crosstalk in the NF-kB and inflammasome signaling pathways in early life is warranted, including the study of combinatorial effects of Alum and TLR agonists.

Relatively high TLR8 agonist-induced TNF and IFN-γ induction was also evident in a recent study of whole blood derived from human infants in The Gambia (55). IMQs carry substantial potential as vaccine adjuvants that can be formulated either as covalently conjugated to antigen (56), or as topical adjuvants with subsequent injection of antigen (57) to enhance antigen-specific responses (58). Our findings may thus have translational implications. 3M-002-induced IFN-γ production is of particular interest given the impaired ability of newborn Mos/APCs to produce this key Th1-poalirzing cytokine in response to many stimuli (59). TLR8 IMQ-induced IL-1β production is also notable, given that inflammasome-dependent production of this cytokine may contribute to the *ex vivo* effects of adjuvants (17, 37). Future studies will characterize how biochemically distinct TLR8 agonists, such as ssRNAs, induced IL-1β production, and in combination with agonists of other TLRs, may induce synergistic maturation of neonatal and adult DCs (52, 53) that may increase antigen-specific responses *in vivo* (60).

As neonatal APC responses to many stimuli are impaired and/or skewed (6, 11), our work on agents that potentially induce Th1-polarizing responses from neonatal APCs is novel and of potential translational importance, but further work remains to be done. We demonstrate that IMQs activate neonatal APCs *in vitro* to produce cytokines with Th1-polarizing properties (e.g, IFN-γ , IL-12p70). Future work should assess whether this activity extends to actual DC-T cell interactions. *In vitro* modeling of the effects of insoluble Alum crystals may incompletely reflect the activity of Alum *in vivo,* and additional approaches such as animal models may provide further insight in comparing the adjuvant activities of Alum and IMQs.

Identifying novel neonatal vaccine adjuvants is key to the development of more effective vaccines against common infections of neonates and infants (61). We demonstrated that imidazoquinoline TLR8 agonists are effective inducers of TNF and IL-1β in both cord blood and infant peripheral blood of Rhesus macaques. These data demonstrate that TLR8 agonists maintain greater immunostimulatory properties not only at birth (cord blood) but also in peripheral blood obtained during infancy, an important phase of susceptibility and immunization schedules. Future translational studies should evaluate the safety and efficacy of vaccine formulations containing TLR7/8 agonists in newborns animals, focusing on species such as Rhesus macaques that express TLR8 with structural and functional similarities to human (56). Given the high global burden of infections in newborns and infants (20, 62) and the unmet medical need for more effective neonatal vaccines that target individuals at birth, the most frequent point of healthcare contact (61, 63), this approach now merits timely evaluation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Drs. Christine Palmer, Matthew Pettengill, Adi Reske, Isaac Kohane, for expert technical advice and helpful discussions. Drs. Michael R. Wessels, Raif Geha, Richard Malley, and Christopher Wilson are acknowledged for helpful conversations and mentorship. Amity M. Paye, Amy Li, Sibel Guzel, Erika Levis, Emily Babendreier, Kamila Naxerova, Christy Mancuso, and Elizabeth Boush provided expert technical assistance. The assistance of the labor and delivery staff at The Brigham & Women's Hospital is gratefully acknowledged.

Abbreviations used

Ab antibody

Alum	Aluminum hydroxide and magnesium hydroxide (Imject Alum)
APC	Antigen-presenting cells
ATP	Adenosine-5'-triphosphate
BCA	bicinchoninic acid
cAMP	Cyclic adenosine monophosphate
DCs	dendritic cells
db-cAMP	dibutyryl-cAMP
IFN	Interferon
IL	Interleukin
IMQ	Imidazoquinoline
LPS	Lipopolysaccharide
MALP	Macrophage-activating lipopeptide-2
mDCs	Myeloid dendritic cells
Mos	Monocytes
MoDCs	Monocyte-derived DCs
NALP3	Nacht domain leucine-rich repeat and PYD-containing protein 3 (also known as Nlrp3)
pDCs	Plasmacytoid DCs
Sal	Saline
siRNA	Small interfering (si) Ribonucleic acid (RNA)
Th	T-helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor

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Clinical Implications

Imidazoquinoline TLR8 agonists are more effective than the conventional vaccine adjuvant Alum at activating human neonatal antigen-presenting cells (APCs) and are promising candidate adjuvants to enhance neonatal vaccine responses.

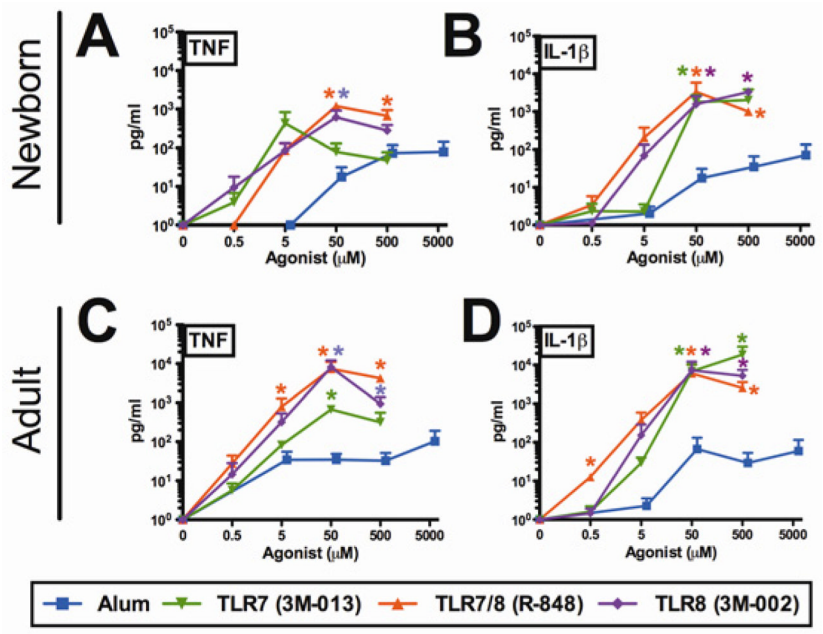

FIG 1. TLR7/8 and TLR8 agonists are more potent and effective than Alum in inducing TNF and IL-1β from human newborn and adult monocytes

A, and **B**, newborn or **C** and **D**, adult stimulated monocytes for 4 hrs (TNF) or 18 hrs (IL-1β) and supernatants were assayed by ELISA. $n = 3–4$; * $P \leq 0.05$ (Mann-Whitney test comparing TLR agonist to Alum).

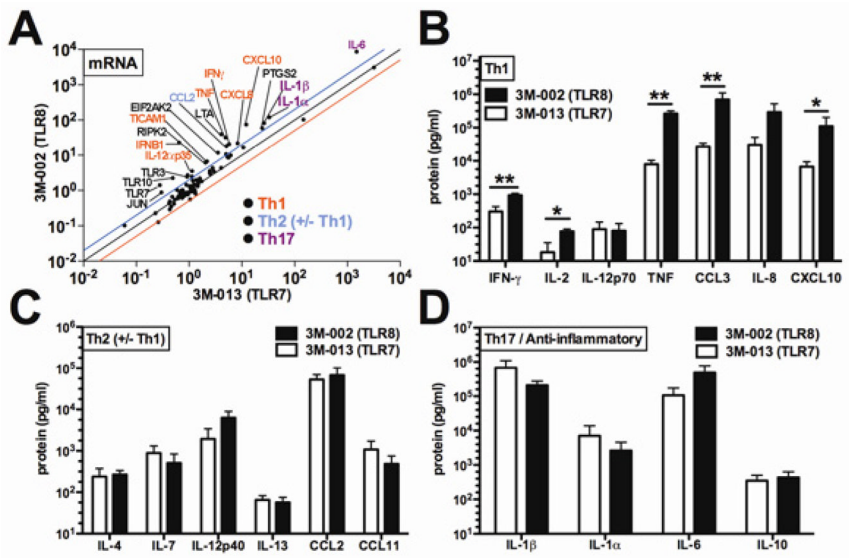

FIG 2. The TLR8 agonist 3M-002 induces greater TLR pathway mRNA up-regulation and Th1-polarizing cytokines/chemokines from neonatal Mos than a TLR7 agonist

Monocytes (5×10^7 cells/mL) stimulated with indicated agonist (50 μM), 4 h. **A**, Agonist-induced mRNA fold change relative to control (colored lines indicate 2-fold change). **B-D**; Th1, Th2 (±Th1), Th17 and anti-inflammatory proteins. $n = 3–6$; * $P < 0.05$, ** $P < 0.01$.

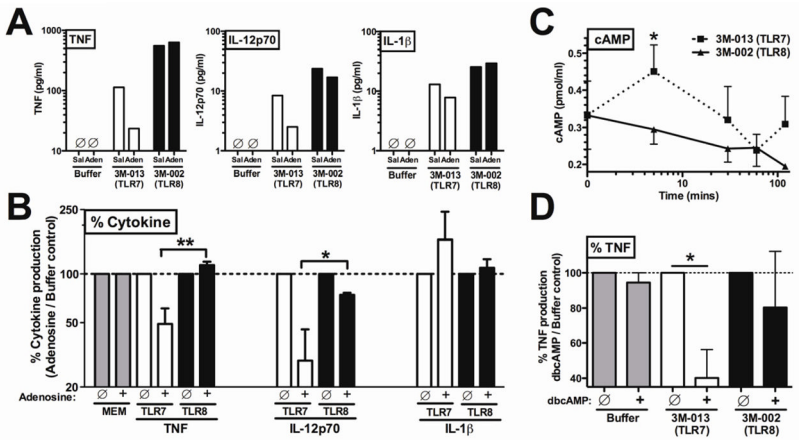

FIG 3. TLR8 agonists are refractory to inhibitory adenosine/cAMP and associated with lesser cAMP accumulation in newborn Mos

A, Monocytes, to which adenosine (10 μM) or saline (Sal) added prior to stimulation with agonists (50 μM) for 2 hrs (representative of 3-6 experiments). **B**, % cytokine. **C**, cAMP concentrations in agonist treated monocytes. **D**, 30 min db-cAMP (10 μM) treated monocytes, 4 hr stimulation. n =3–6; * $P < 0.05$, ** $P < 0.01$.

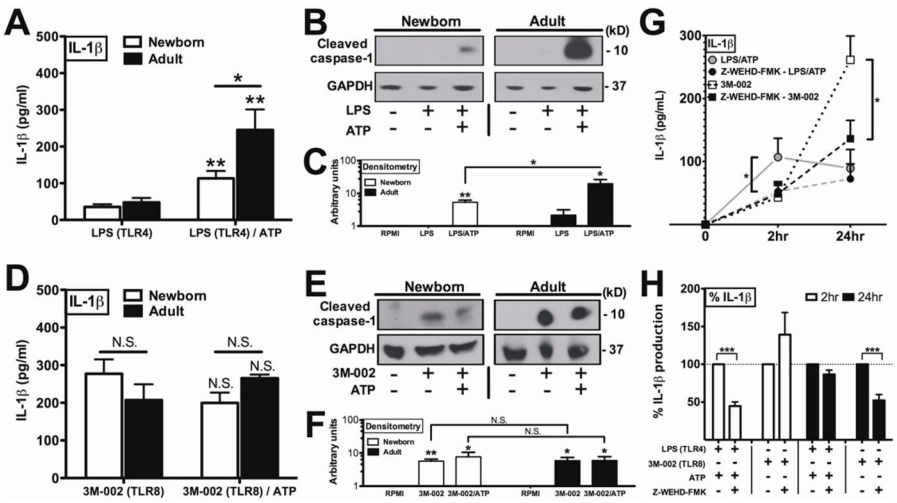

FIG 4. Newborn MoDCs display diminished LPS/ATP-induced but robust IMQ-induced caspase-1 activation and IL-1β production

Newborn and adult MoDCs stimulated with LPS (2 hrs) (**A, B, C**) or 3M-002 (24 hrs) (**D, E, F**), plus 5 mM ATP as indicated. Supernatant assayed IL-1β (ELISA) (**A, D**), cell lysates caspase-1 by western blotting/densitometry (**B, C, E, F**). **C, F** representative of $n = 3$; **A, D** $n = 4–6$, Asterisk denotes comparison between treatment conditions. Line-bar indicates comparison between groups (newborn and adult). **G,** Caspase-1 inhibitor Z-WEHD-FMK pretreatment of neonatal MoDCs treated as above. **H,** IL-1β data from G shown as %. $n = 4–6$; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

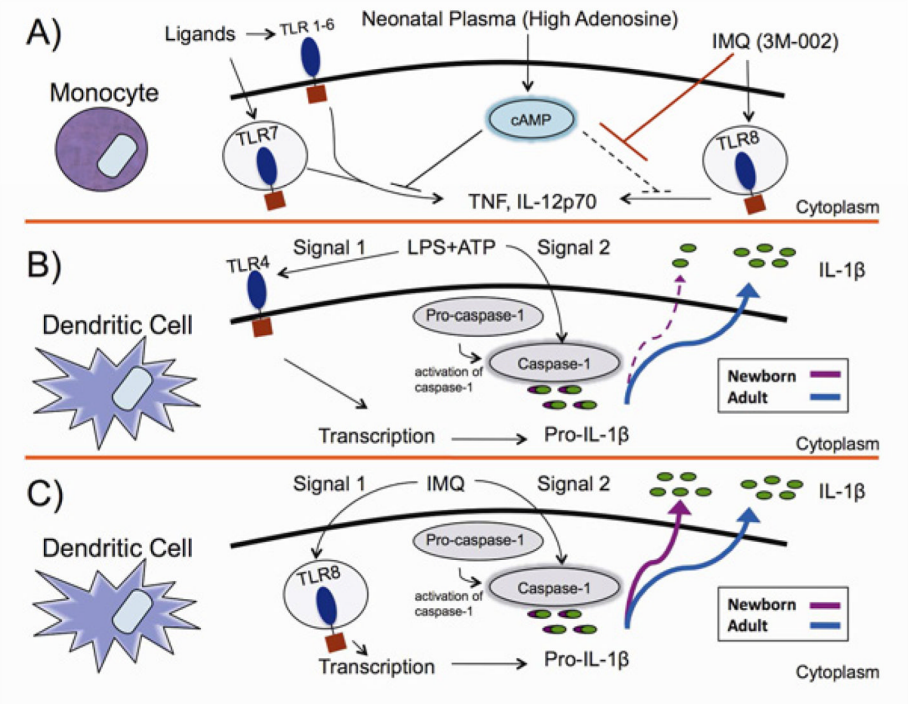

FIG 5. Proposed mechanisms of Imidazoquinoline TLR8 agonist activation of human newborn monocytes and DCs via adenosine-refractory and caspase-1-dependent pathways

3M-002 agonists are refractory to inhibitory effects of the adenosine/cAMP pathway, which skews neonatal immunity against Th1-polarizition (**A**), can overcome impaired IL-1β responses of neonatal DCs to LPS/ATP (**B**) independently of exogenous ATP (**C**).