Nucleic acid cytokine responses in obese children and infants of obese mothers☆

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ABSTRACT

Almost a third of Irish children are now overweight and the country ranks 58th out of 200 countries for its proportion of overweight youths. With the rising obesity epidemic, and the impaired immune responses of this population, it is vital to understand the effects that obesity has on the immune system and to design future therapeutics, adjuvants and vaccines with overweight and obese populations in mind. Many current vaccines use adjuvants that have been found to be less effective at stimulating the immune response in children compared with adults and there is now substantial effort to design paediatric-focused adjuvants. Additionally, vaccine responses have been shown to be less effective in obese populations indicating that this is a particularly vulnerable population. We have recently identified cytosolic nucleic acids (CNAs), as novel candidate adjuvants for childhood vaccines. Here we investigated whether immune responses to these candidate adjuvants were adversely affected in infants born to overweight or obese mothers, and in overweight and obese children. Type I Interferon (IFN) and proinflammatory cytokines such as Tumor Necrosis Factor α (TNFα) are vital for driving innate and adaptive immune responses. We found that childhood obesity conferred no significant adverse effect on CNA-induced Type I IFN responses when compared with lean children. Similarly, Type I IFN responses were intact in the cord blood of babies delivered from overweight and obese mothers, when compared with lean mothers. There was also no significant impact of obesity on CNA-induced TNFα responses in children or from cord blood of infants born to overweight/obese mothers. In all cases, there was a tendency towards decreased production of innate cytokine Type I Interferon and TNFα, however there was no significant negative correlation. Interestingly, high maternal BMI showed weak and moderate positive correlation with IL-12p70 and IFNγ, respectively, in response to CNA stimulation. This study demonstrates that future adjuvants can be tailored for these populations through the use of activators of CNA sensors.

1. Introduction

Obesity has become a global epidemic over a period of only two decades. It now represents an urgent medical and a major societal challenge for which the solution certainly requires a multidisciplinary approach. Obese individuals are at higher risk of a wide range of infections including postoperative infections and other nosocomial infections, and also of developing serious complications resulting from common infections [1]. Obese individuals have been shown to be at increased risk of infection from certain vaccine-preventable diseases, such as tetanus, hepatitis B and influenza, due to poorer responses to vaccines in these individuals [2–4]. Although there is limited data concerning the effect of obesity on vaccine efficacy in neonates born to obese mothers, limited data from overweight and obese children suggest that responses to vaccinations are impaired in these groups [2,5]. Data from obese adults would also suggest that obesity increases the chances of a poor vaccine-induced immune response [3,4,6,7]. One study investigating the effect of increased body mass index (BMI) on vaccination responses showed that overweight children had reduced tetanus titers following vaccination when compared to lean children [2]. Another study in adults receiving Hepatitis B vaccination showed that obese adults have limited protection against the disease compared to lean adults [3]. Influenza vaccine responses are also affected by obesity, with increased risk of influenza among vaccinated adults who are obese [4]. Interestingly, initial antibody production is intact in obese individuals, however at later sampling, 12 months post vaccination, these antibody titers decreased below healthy weight individuals [7]. CD4+ and CD8+ T cell responses ex vivo were also decreased in obesity [7,8]. Obesity in adults is defined as a BMI ≥ 30 kg/m [6], while for children it is more appropriate to use a BMI z-score. BMI z-scores are measures of relative weight adjusted for child age and sex [5].

We have recently identified a potential novel adjuvant for childhood vaccines. In adults, Poly(I:C) or Poly(dA:dT), when transfected in to cells, activate cytosolic nucleic acid (CNA) sensors and induce Type I Interferon (IFN) production along with other proinflammatory cytokines such as Tumor Necrosis Factor α (TNFα) [9]. It has previously been shown that while other activators of the innate immune system such as Lipopolysaccharide (LPS) and CpG, TLR4 and 9 ligands respectively, function well in adults, they are less able to activate the immune system of neonates and young children [10–13]. Adding to this defective paediatric immune response to TLRs, maternal obesity has been shown to lead to diminished responses to TLR ligands in cord blood monocytes and dendritic cells, when compared to cord blood of lean mothers [14], suggesting that adjuvants targeting these pathways [15–17] would be less efficient in neonates born to obese mothers. Cytosolic Poly(I:C) and Poly(dA:dT), unlike many Toll Like Receptor (TLR) agonists, can activate the neonatal immune response as efficiently as in adults, thereby opening up the possibility of harnessing these responses for broader use in adjuvant development.

In this study, we wished to investigate whether the CNA responses previously observed in neonates and children were intact in neonates born to overweight or obese mothers, and in overweight and obese children.

2. Materials and methods

2.1. Subjects

Umbilical cord blood samples were obtained from term births following normal pregnancy, labour 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. Maternal BMI at booking appointment was recorded. A BMI of 18.5–24.9 was classified as healthy, BMI between 25 and 29.9 was classified as overweight, while a BMI of > 30 was classified as obese. Venous blood from infants,

children and adolescents of various ages was collected during elective surgical procedures, where no indication of infection or underlying genetic or endocrine disease was present, (such as hydrocele repair, umbilical hernia repair, onychocryptosis, orchiopexy repair) in Our Lady's Children's Hospital Crumlin (OLCHC), Dublin 12. In all cases children had an extra 1–10 ml of blood drawn post-anaesthetic, thus avoiding extra venupuncture. Children's BMI, age and sex at blood draw was recorded and BMI z-scores were calculated. 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.

2.2. Isolation of mononuclear cells

Primary PBMCs or CBMCs were isolated from healthy human blood or cord blood. Cells were cultured at 37 °C, 5% CO2, 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).

2.3. Stimulation of PBMCs and CBMCs

Poly(I:C) (5 μg/ml) (Invivogen) and Poly(dA:dT) (5 μg/ml) (Sigma-Aldrich) were transfected into PBMCs or CBMCs using TransIT-X2 (Mirus).

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. BioLegend LEGENDplex™ Human Inflammation Panel (13-plex) was carried out as per manufacturer's instructions to determine levels of IFNγ and IL-12p70. A BD LSR Fortessa cell analyser was used to acquire samples and BioLegend LEGENDplex™ software was used for analysis.

2.5. Statistical analyses

Data was analysed with GraphPad Prism software. Normality testing was carried out using Shapiro-Wilk 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 for comparison of groups. For correlation analysis, Pearson correlation coefficient was used when datasets were found to follow a Gaussian distribution, while datasets with non-normal distribution were tested for correlation using Spearman's rank correlation. Two-tailed tests were used throughout, and the statistical approaches were all deemed to be valid for each individual experiment.

3. Results

It is widely accepted that obesity has immune complications, both co-morbid disease and inflammation, and immune dysregulation, and can often lead to decreased immune response to infection or vaccination [18]. It is also well established that the innate immune systems of children do not always function in the same manner or with the same efficiency as adults [19,20]. Deficiencies in the responses to TLR activation in children is an example of how the immune system of children and adults differs [12,13]. We have recently reported that Poly(I:C) and Poly(dA:dT), activators of the cytosolic nucleic acid sensors of the innate immune system, can activate the immune systems of neonates and infants as efficiently as in adults [9]. Given that many of the adjuvants in current use rely on activating TLR pathways, we hypothesise that

Table 1
Characteristics of maternal lean, overweight & obese study group.

| Characteristic	Lean	Overweight	Obese
BMI, mean (SD), kg/m^2	22.35 (1.82)	27.5 (1.38)***	32.5 (2.10)***
BMI range	17.84–24.96	25.34–29.75	30–36.64
Maternal Age, mean (SD), years	36.5 (2.87)	34.93 (4.68)	33.66 (4.89)
Neonatal Birthweight, mean (SD), kg	3.49 (0.57)	3.87 (0.55)	3.76 (0.57)
Fetal Sex, M:F Ratio (n number)	28:21 (47)	14:8 (22)	6:8 (14)

*** p < 0.005, compared to Lean.

Poly(I:C) or Poly(dA:dT) may be candidates for future childhood vaccines. We were interested in investigating the effect of obesity on the Poly(I:C) and Poly(dA:dT) responses of neonates and children. We therefore re-analysed previously studied cord blood data in the context of BMI, and also added a cohort of overweight or obese children to previously acquired data on childhood responses to CNA. Characteristics of our study groups used to investigate the effect of maternal obesity on neonatal responses are presented in Table 1.

Mimicking viral infection through activation of cytosolic RLRs/dsDNA sensors with transfected Poly(I:C) and Poly(dA:dT) resulted in equivalent Type I IFN production in CBMCs regardless of whether maternal BMI was in the healthy, overweight or obese range (Fig. 1A and C). Correlation analysis confirmed our grouping analysis, showing

no positive or negative correlation between BMI and Poly(I:C)- or Poly(dA:dT)-induced Type I IFN responses (Fig. 1B and D). We had previously published that cytosolic Poly(I:C)-induced Type I IFN levels in CBMCs were higher than levels induced in adult PBMCs [9]. Here, stratification of responses by maternal BMI grouping demonstrates that this boost in immune stimulation is lost with increasing BMI, however levels of IFN production never drop below adult levels previously shown (0.842 AU ± 0.092) [9], as illustrated by the dashed lines on Fig. 1A.

When TNFα was measured in response to Poly(I:C) or Poly(dA:dT) transfection, in CBMCs from babies delivered from mothers with different BMIs, we again observed no significant difference in cytokine production between CBMCs from healthy, overweight or obese mothers when grouped according to BMI (Fig. 2A and C). Similar to Type I IFN production, levels of TNFα production tend to decrease with increasing BMI groups, though not significantly and no negative correlation was found between maternal BMI and levels of CNA-induced TNF following correlation analysis. Levels of TNFα production in response to CNA also remain greater than or equal to previously observed adult levels (Poly(I:C): 33.65 pg/ml ± 0.8.58, Poly(dA:dT): 48.51 pg/ml ± 14.18 (dashed lines Fig. 2A and C)) [9], regardless of maternal BMI. Taken together these results suggest that maternal BMI, while not having a negative correlation with innate cytokine output, does have the effect of blunting the cord blood responses to transfected Poly(I:C) or Poly(dA:dT) thereby eliminating the previously observed enhancement in
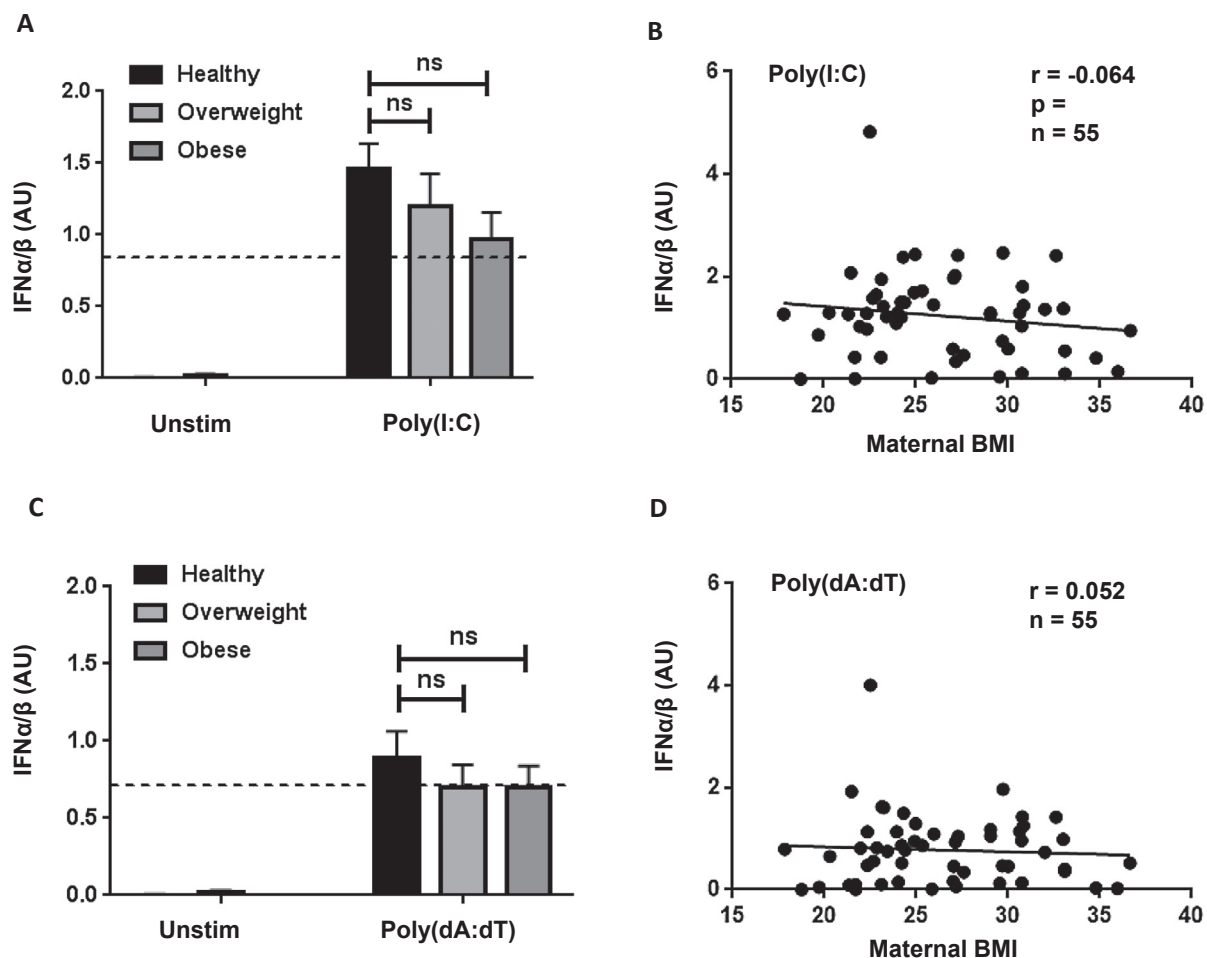
Fig. 1. Relationship of Type I IFN production in CBMCs to increasing maternal BMI. Neonatal cord blood mononuclear cells (CBMCs) from babies born to healthy, overweight or obese mothers were stimulated with (A and B) Poly(I:C) or (C and D) Poly(dA:dT) transfection for 24 h. Levels of IFNα/β were assayed via HEK-Blue™ IFNα/β SEAP assay. (A and C) Data are mean ± SEM (n ≥ 14 donors in each group). Kruskal-Wallis non-parametric test with Dunn's post-test was used to compare groups. (B and D) Correlation analysis of Type I IFN production and maternal BMI was carried out using Spearman's rank correlation testing. Dashed line represents adult Type I IFN production following relevant stimulation.
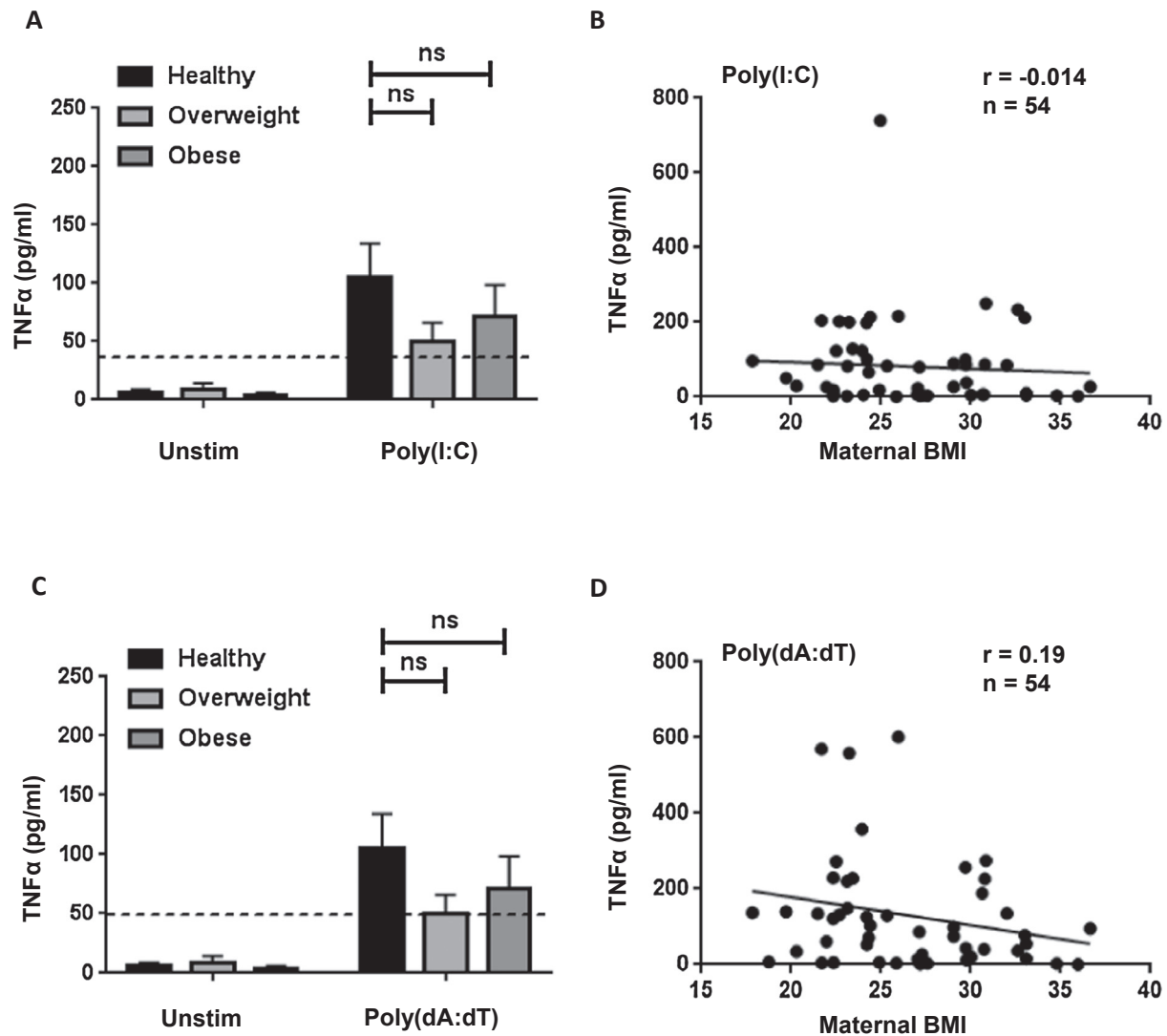
Fig. 2. Relationship of TNFα production to maternal BMI in CBMCs activated with cytosolic nucleic acid. Neonatal cord blood mononuclear cells (CBMCs) from babies born to healthy, overweight or obese mothers were stimulated with (A and B) Poly(I:C) or (C and D) Poly(dA:dT) transfection for 24 h. Levels of TNFα were assayed via HEK-Blue™ TNFα SEAP assay. (A and B) Data are mean ± SEM (n ≥ 14 donors in each group). Kruskal-Wallis non-parametric test with Dunn's post-test was used to compare groups. (B and D) Correlation analysis of TNFα production and maternal BMI was carried out using Spearman's rank correlation testing. Dashed line represents adult TNFα production following relevant stimulation.

cytokine production in healthy CBMCs when compared with lean adult PBMCs [9]. Notably, maternal BMI does not affect the cord blood responses to transfected Poly(I:C) or Poly(dA:dT) to such a degree that it inhibits cytokine responses to below that of an adult.

We were also interested in whether obesity in childhood had an effect on Type I IFN and TNFα responses to transfected Poly(I:C) and Poly(dA:dT). Characteristics of our study groups used to investigate the effect of paediatric obesity on cytokine responses are presented in Table 2.

Interestingly, we found that while there was a slight tendency towards decreased Type I IFN production in overweight/obese children, there was no significant difference in Type I IFN production between

Table 2
Characteristics of lean & overweight/obese paediatric groups.

| Characteristic	Lean	Overweight/Obese	P value
BMI z-score, mean (SD)	0.06 (0.67)	2.29 (0.52)	< 0.001
Age, mean (SD), years	12.21 (2.99)	13.13 (2.66)	0.35
Fetal Sex, M:F ratio	12:3	10:10	N/A

our healthy and overweight/obese groups in response to either Poly (I:C) or Poly(dA:dT) transfection (Fig. 3A and B). We also tested the TNFα responses of healthy or overweight/obese children to Poly(I:C) or Poly(dA:dT) transfection and found that there was no significant difference in the levels of TNFα produced regardless of BMI z-scores (Fig. 3C and D). In all cases, there was no correlation between BMI and CNA-induced cytokine production.

Type I IFN and TNFα can promote a Th1-type immune response which is highly desirable in shaping immunity in response to intracellular bacteria. Due to the potential of Poly(I:C) transfection to induce substantial Type I IFN and TNFα in neonates regardless of maternal BMI, we sought to explore whether Poly(I:C) could induce IL-12p70, another important Th1-polarising cytokine, in the cord blood of babies delivered from overweight/obese mothers. Fig. 4A shows IL-12p70 production in CBMCs from overweight/obese mothers in response to Poly(I:C) compared to CBMCs from healthy weight mothers. There was no significant difference between the groups but when analysed for correlation, IL-12p70 production was found to positively correlate with BMI (Fig. 4B). IFNγ, considered both an innate and adaptive cytokine, is the primary cytokine that defines Th1 cells and is
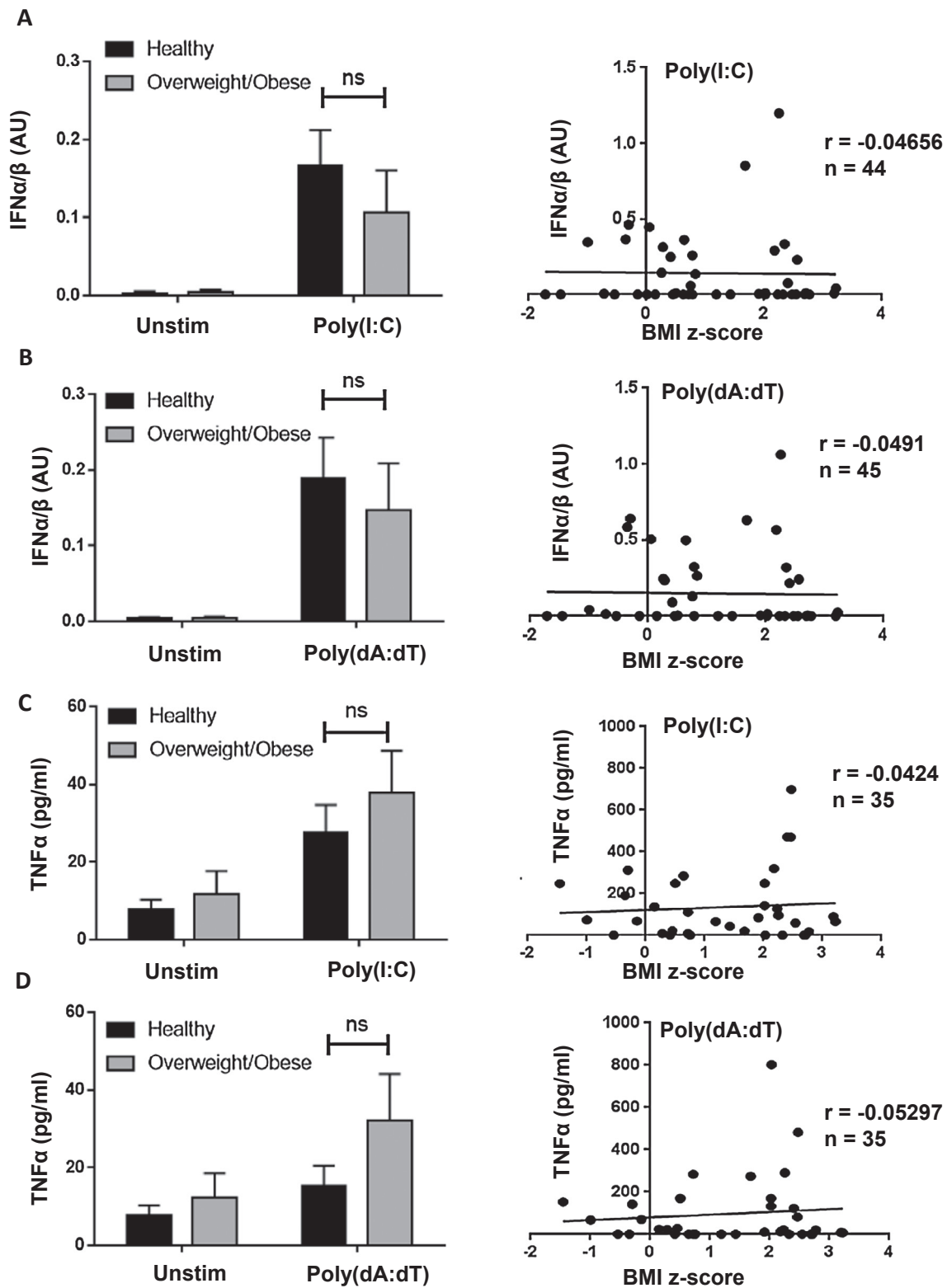
Fig. 3. Cytosolic nucleic acid-induced Type I IFN and TNFα production remain intact in overweight and obese children. PBMCs from healthy, overweight or obese children were stimulated with (A and C) Poly(I:C) or (B and D) Poly(dA:dT) transfection 24 h. Levels of IFNα/β or TNFα were assayed via HEKBlue™ IFNα/β SEAP or HEK-Blue™ TNFα SEAP assay. (Right panels) Data are mean ± SEM (n ≥ 15 donors in each group). Kruskal-Wallis non-parametric test with Dunn's post-test was used to compare groups. (Left panels) Correlation analysis of Type I IFN or TNFα production and maternal BMI was carried out using Spearman's rank correlation testing.
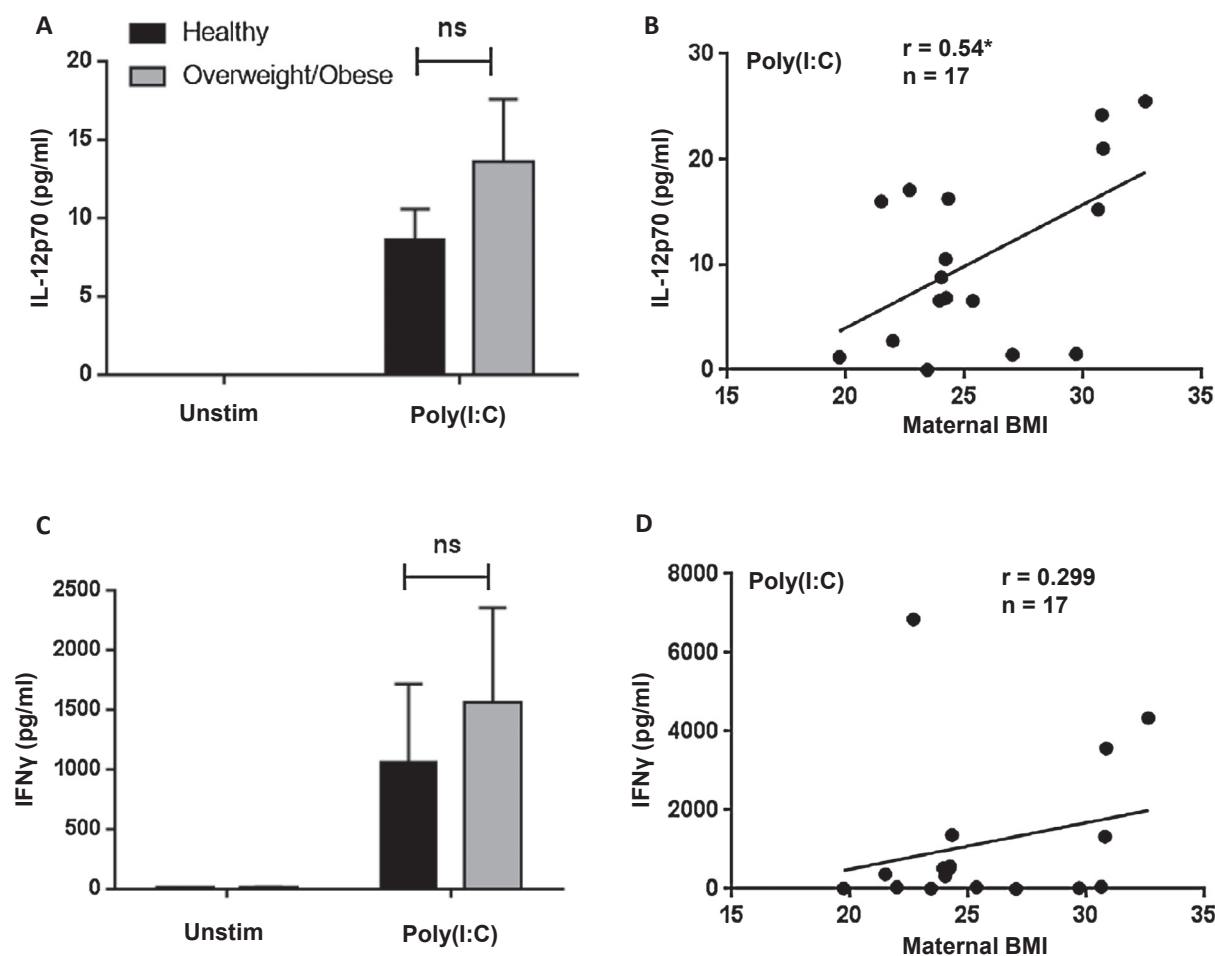
Fig. 4. High Maternal BMI has a positive correlation with Poly(I:C)-induced Th1-polarising cytokines. CBMCs from babies born to healthy, overweight or obese mothers were transfected with Poly(I:C) for 24 h and BioLegend LEGENDplex™ Human Inflammation Panels were used to measure the levels of (A and B) IL-12p70 or (C and D) IFN-γ. (A and C) Graphs show mean ± SEM (n ≥ 16). Two-way ANOVA with Bonferroni's post-test (A) or Kruskal-Wallis non-parametric test with Dunn's post-test (C) was used to test for significant differences between treated cell groups. (B and D) Correlation analysis of Poly(I:C)-induced IL-12p70 production and maternal BMI was carried out using Pearson's correlation coefficient. Correlation analysis of Poly(I:C)-induced IFNγ production and maternal BMI was carried out using Spearman's rank correlation testing. $^*p < 0.05$.

predominantly produced by CD4$^+$ Th1 and CD8$^+$ cytotoxic T cells, driving further differentiation of T cells into Th1 cells. Interestingly, we found that IFNγ production in the cord blood of babies, in response to Poly(I:C) transfection, was slightly increased when samples were grouped according to BMI (Fig. 4C). Further analysis showed that IFNγ also correlates positively with BMI (Fig. 4D)

4. Discussion

Most literature evaluating the effect of obesity on vaccine efficacy has been carried out in adults. Obesity impacts negatively on vaccine efficacy and has been shown to give rise to an inflamed phenotype, including increased levels of circulating proinflammatory cytokines [18]. Most vaccines are given to children in the first months & years of life, when obesity of the child has not become a factor in vaccine efficacy. However, studies have shown dysregulated immunity in obese children of school going age, an age when important booster vaccinations are given [21]. While trends in obesity continue to rise, women tend to be more prone to obesity than men [22,23]. As the frequency of obesity in young women increases, so too does the importance of our understanding the effects of obesity on the offspring during and post-pregnancy.

Maternal obesity as a factor in vaccine efficacy has not been reported on to date. Given the fact that obesity is a risk factor for

decreased vaccine efficacy in adults, it is possible that maternal obesity could affect early vaccine responses in their infants, as levels of circulating cytokines are similar in mothers and their offspring, early in life [24–28]. Importantly, maternal obesity has been linked to decreased cord blood monocyte and dendritic cell responses to TLR activation, including decreased TNFα production to LPS [14]. The importance of being able to efficiently produce proinflammatory cytokines, such as TNFα and Type I IFN, in response to infection or vaccination is well recognised. Type I IFNs activate cellular immunity by activating DCs and driving their maturation. This DC maturation allows antigen presentation to CD4$^+$ T cells, cross-priming CD8$^+$ T cells, and induction of IFNγ and opsonizing antibodies [29,30]. Initiating Th1 responses and cytotoxic T cells is a major target in vaccine development. Having previously observed robust Th1 responses to cytosolic Poly(I:C) and Poly(dA:dT) in cord blood samples, we were interested in evaluating any impact of increased maternal BMI on these responses. Our initial measurements of Type I IFN and TNFα found no correlation of increasing maternal BMI on cytokine responses, or indeed increasing childhood BMI on PBMC responses. We tested the potential of cytosolic Poly(I:C) to promote this innate Th1 response and to encourage an adaptive response through measuring the production of Th1-polarizing IL-12p70 and induction of IFN-γ in CBMCs from healthy and overweight/obese women. Interestingly, we found a moderate positive correlation of IL-12p70 production with maternal BMI suggesting that

neonates from overweight/obese mothers might be better able to induce a cellular immune response to Poly(I:C). Similarly, IFNγ production correlated positively, albeit weakly, with maternal BMI. While the effect of maternal obesity on vaccine responses in neonates has not yet been studied, a previous report has observed decreased CD4 T-cells in neonates born to obese mothers along with reduced monocyte and dendritic cell responses to TLR ligands [14]. The limited studies examining the effect of obesity on vaccination in children have focused on Hepatitis B and influenza vaccines and have shown deficiencies in the response to these vaccines in obese children. A recent paper from Wu, Y et al. has shown that LPS-induced cytokine responses are decreased in PBMCs from obese children compared to lean children [31]. This blunted cytokine response to LPS is paralleled by the previously noted study reporting that LPS-induced TNFα production is decreased in cord blood monocytes and myeloid DCs of babies born to obese mothers [14]. Given the use of LPS-derivatives such as MPLA in current adjuvant development, this has implications for the obese population. With the rise in obesity in general, and particularly childhood obesity, choice of adjuvant for paediatric vaccines may gain importance to ensure the best possible efficacy is attained in the breadth of stakeholder populations. Collectively, our data highlights that neonates from obese mothers, and importantly also obese children display functional cytokine responses to the cytosolic nucleic acid Poly(I:C) demonstrating that activation of CNA sensors may have potential in future vaccine development for this at-risk population.

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Author contributions

KB performed experiments. BDOL, DMcL, EM & DK identified suitable donors, collected data and bloods. KB, DC, SP, FMM, AEH & SD designed experiments, analysed data and wrote the manuscript.

Conflict of interest

No conflict of interest.

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