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Acquisition of specific antibodies and their influence on cell-mediated immune response in neonatal cord blood after maternal pertussis vaccination during pregnancy

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ABSTRACT

Maternal immunization with pertussis acellular vaccine (Tdap) is an intervention that provides protection to newborns. However, it has been reported that high maternal antibody levels may adversely affect the immune response of infants after active immunization. In this study, we evaluated neonatal passive acquisition of pertussis-specific antibodies and their influence on the neonatal cell-mediated immune response.

Pregnant women were either vaccinated with Tdap vaccine (case group, n = 66) or received no vaccine (control group, n = 101). Whole-cell *Bordetella pertussis* (Bp), pertussis toxin (PT), filamentous hemagglutinin (FHA) and pertactin (PRN)-specific serum IgG were quantified in paired maternal-cord sera, and Bp- and PT-specific IgA were evaluated in colostrum by ELISA. Ex vivo neonatal blood lymphocyte responsiveness after Bp stimulation was assessed in case (n = 17) and control (n = 15) groups using flow cytometry to detect proliferation, cytokine production and activation phenotype of lymphocytes in the context of high specific IgG acquired after maternal vaccination.

Anti-Bp, PT, FHA and PRN IgG concentrations in maternal and cord sera from case group were higher than those in control group with positive correlation indexes in both groups for all pertussis antigens. The control group presented higher placental transfer ratios of specific antibodies and, in the case group, vaccination between 26 and 31 gestation weeks was associated with the best placental transfer ratios. Specific IgA concentrations in colostrum were not affected by vaccine status. Whole blood assays revealed that newborns responded to Bp stimulation with higher expression of CD40L, CD69 and $CD4^+$ T cell proliferation compared to unstimulated cells, and a lower Th1 response, while a preserved Th2 response compared to adults, but there were no differences between the neonatal groups for any of the studied parameters.

Our results indicate that higher pertussis-specific IgG levels in newborn sera after maternal vaccination do not affect the neonatal ex vivo cell-mediated immune response.

1. Introduction

Pertussis cases have increased considerably over the years, and many countries have experienced recent pertussis epidemics [1–3]. Whooping cough was the most frequently reported vaccine-preventable disease in the American continent between 2012 and 2014 [4].

Neonates are the most susceptible to severe forms of the disease, often leading to death, due to a relatively restricted function of cellular immune components regarding the defense from infection, particularly the lack of immunological memory, along with an absent or incomplete vaccination scheme, which is only achieved after the third vaccine dose at 6 months of age [5,6]. In Brazil, from 2010 to 2014, 87.5% of confirmed pertussis cases in children younger than 1 year of age occurred in those younger than 6 months. In addition, 98.7% of all deaths during this period,

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including all age groups, occurred in children younger than 1 year of age, and 97.2% of which were infants less than 2 months old [7].

As in other countries, following the recommendation of ACIP (Advisory Committee on Immunization Practices), in 2014, the Tdap vaccine (diphtheria, tetanus and acellular pertussis) was introduced into the National Vaccination Calendar for pregnant women between the 27th and 36th gestation week in Brazil. Maternal immunization as a strategy to provide protection to the newborn during the period of greatest vulnerability, that is, shortly after birth, is based on the concept of placental transfer of IgG antibodies. IgG present in high concentrations in the maternal circulation is internalized in the apical side of syncytiotrophoblast (which constitutes the fetal portion of the placenta) and then, in the acidic environment, binds to the neonatal Fc receptors (FcRn) expressed on the inner surface of the endosomes. Bound IgG is then transcytosed to the basolateral side, where it is released upon exposure to neutral pH (7.4). Transcytosed IgG may or may not pass through the stroma before reaching the fetal blood vessels [8].

Another way to provide protection to the neonate in the first days of life consists of the practice of breastfeeding. IgA antibodies are transferred to the newborn via human milk and confer protection by binding to pathogenic microorganisms or toxins, inhibiting colonization and invasion of the child's mucous membranes [9,10].

The humoral response is not solely responsible for the protection of *Bordetella pertussis* infection; experiments in animal models have provided convincing evidence that the cellular response is also required for an effective immunity, although the contribution of each T cell subtype after pertussis vaccination is not fully known [11,12]. Immune mechanisms involving T and B cells play relevant roles in protection against pertussis. In particular, the effector mechanisms mediated by IFN-γ are required to prevent the spread of *B. pertussis* in the respiratory tract [11,13]. In summary, cellular immunity along with opsonizing antibodies are the effector mechanisms that eliminate a primary infection and confer protection against secondary exposure to *B. pertussis*.

However, it has been described that high IgG antibody levels transplacentally acquired by the neonate due to maternal immunization may negatively affect the infant's immune response to active immunization. Some proposed hypotheses are epitope masking of vaccine antigens by maternal antibody; elimination of maternal antibody – vaccine antigen complexes by infant antigen-presenting cells by means of phagocytosis; and cross-linking of the inhibitory receptor FcγRIIB to the B cell receptor by maternal antibody – vaccine antigen complexes [14,15]. Nevertheless, several authors have demonstrated results indicating that the impaired antibody response disappears after the completion of primary pertussis vaccination [16].

The present study investigated the presence of serum IgG and colostrum IgA antibodies reactive with whole-cell *B. pertussis,* pertussis toxin (PT), filamentous hemagglutinin (FHA) and pertactin (PRN) in Tdap-vaccinated or unvaccinated mothers and their transmission to newborns via transplacental transfer and breastfeeding. In addition to studying passive immunization, we investigated ex vivo neonatal blood lymphocyte responsiveness to challenge with inactivated whole-cell *B. pertussis*.

2. Materials and methods

This prospective cohort study was approved by the University of Cuiaba Research Ethics Committee and Ethics Committee of the Department of Pediatrics of São Paulo University Medical School (N° 1.005.350 and N° 134/15). Written informed consent was obtained from all participants or their legal guardians. Inclusion criteria were the following: healthy women between 15 and 40 years old with healthy term newborns with adequate weight for the gestational age. Exclusion criteria included the following: placental malformation, maternal chronic or immunological disorder, use of anti-inflammatory, immunosuppressive or immunomodulatory medications, serious infectious diseases during pregnancy or during the delivery period, positive serological reactions for HIV, toxoplasmosis, rubella, cytomegalovirus, herpes, syphilis, hepatitis and malaria and previous pertussis vaccination in adult life. The case group comprised 66 paired maternal serum, cord blood serum and colostrum samples from parturients immunized with a combined Tdap booster vaccine during current gestation. The control group comprised 101 unimmunized mothers who met the same inclusion criteria. Immunized pregnant women were recruited during routine preventive visits, and unimmunized pregnant women were recruited during delivery. At recruitment, demographic data and clinical history were obtained from the women's medical records. Peripheral blood samples from healthy adults were used as controls in whole blood assays. The age range of the healthy adults was 20–40 years-old. They were selected from the laboratory's group and were chosen by the absence of clinical signs of infection at the time of blood withdrawal. In addition, none of these adult volunteers were vaccinated with the Tdap vaccine.

2.1. Vaccine

All pregnant women in the case group were vaccinated with the licensed Tdap vaccine (Boostrix® – GSK Biologicals, Rixensart, Belgium) provided by the Ministry of Health, containing tetanus toxoid (≥20 IU), diphtheria toxoid (≥2 IU), inactivated PT (8 μg), formaldehyde-treated FHA (8 μg) and formaldehyde-treated PRN (2.5 μg) adjuvanted with aluminum hydroxide.

2.2. Laboratory specimens

A maternal peripartum blood sample and a venous umbilical cord blood sample (both routinely discarded) were collected. Whole umbilical cord blood used in cellular assays were used within 24 h, and serum samples for ELISA were centrifuged and stored at −80 °C until the moment of use. Colostrum samples were collected from all women up to 96 h postpartum, defatted by centrifugation and the liquid phase was stored in aliquots at −80 °C. Two control pools were used: a human serum pool prepared with sera from healthy 18- to 40-year-old blood donors with negative results for conventional serological tests and a human colostrum pool prepared with colostrum from healthy mothers; both were already available in our laboratory.

2.3. Bacterial antigens

An inactivated whole *B. pertussis* (Bp) suspension (strain 137; NIH, Bethesda, MD, USA) was used for in vitro tests. This inactivated suspension is currently included in the DTPw vaccine, which is still in use for child immunization in Brazil. The suspension was kindly provided by the Instituto Butantan, Brazil.

2.4. Total antibody determination

Total IgG concentrations were measured in maternal and umbilical cord serum according to standard automatized nephelometry protocols (Roche Cobas, Mannheim, Germany) [17]. The results were expressed in mg/dL. Total IgA antibodies present in the maternal colostrum were measured by ELISA as previously described [18], and the results were expressed in g/L.

2.5. Anti-pertussis antibody determination

Maternal and umbilical cord serum IgG antibodies and colostrum IgA antibodies reactive with Bp (2.6×10^8 bacteria/mL) or reactive to purified antigens—PT at 0.05 μg, FHA at 0.1 μg and PRN at 0.1 μg (PT, cat. 180; FHA cat. 170; and PRN cat. 187; List Biological Laboratories)—were determined by ELISA, as described by Quinello et al. [19], with some modifications. IgG and IgA results directed against Bp were expressed in arbitrary units (AU/mL), determined by comparison to OD values of the serum or colostrum pool, both defined to contain anti-Bp IgG or IgA at 1000 AU/mL, respectively. IgG and IgA antibody concentrations directed against purified antigens were determined from the standard curve values obtained using the first International WHO standard (WHO International Standard Pertussis Antiserum, human, 1st IS NIBSC Code 06/140) and expressed in International Units (IU/mL). The lower limit of detection of the assays was 0.026 IU/mL for PT, 0.02 IU/mL for FHA, 0.01 IU/mL for PRN and 0.63 AU/mL for Bp.

2.6. Whole umbilical cord blood assay

The ex vivo assay of neonatal T and B lymphocytes was performed with umbilical cord blood to maintain a microenvironment that more accurately reflects the normal physiology and possible effects of high levels of antibodies present in cord serum. The number of total blood leucocytes was determined with an automatic counter (Sysmex XP-300, Sysmex Corporation, Kobe, Japan), and 1×10^6 leukocytes/well were stimulated or not (negative control) with the inactivated Bp suspension at 0.5 mg/mL or with 5 μL of PHA (positive control) diluted in RPMI-1640 medium (Gibco®, Life Technologies, Foster City, CA, USA) supplemented with 1% L-glutamine, gentamicin (50 mg/L) and 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO, USA) (R-10 medium) and incubated for 48 h at 37 °C. BD GolgiPlug™ (BD Biosciences, San Jose, CA, USA) was added during the last 4 h of culture. Then, cells were subjected to a 30-min erythrocyte lysis step using BD Lysing Solution™ (BD Biosciences). After two wash steps with staining buffer (phosphate-buffered saline (PBS) with 1% fetal calf serum and 0.1% sodium azide), the supernatant was discarded, and the cells were resuspended in 100 μL of the same solution. Cells were labeled for 30 min with monoclonal antibodies directed against T and B surface antigens and their activation markers: CD3 V500 (clone UCHT1), CD4 FITC (SK3), CD8 V500 (SK1), CD69 APC (FN50), CD40 PE (5C3), CD40L PECy5 (TRAP1), and CD19 PECy7 (SJ25C1). After 2 wash steps and a 30-min incubation with Cytofix/Cytoperm (BD Biosciences), monoclonal antibodies for intracellular labeling were then added:IFN-γ V450 (B27), Ki67 V450 (B56) and isotype control (MOPC-21) for 30 min. After two washes, the cells were resuspended and analyzed by flow cytometry. Acquisition was performed on a FACS LRS II Fortessa (BD Biosciences), and 20,000 events were acquired in the lymphocyte gate using forward- (FSC) and side-scatter (SSC) parameters. The analyses were done in FlowJo software (Tree Star, Ashland, OR, USA). T cell subsets were identified using combinations of markers gating on $CD3^+/CD4^+$ or $CD3^+/CD8^+$ cells within the total lymphocyte population. Within the $CD3^+/CD4^+$ or $CD3^+/CD8^+$ T lymphocyte gate, the activation markers CD69 and CD40L, the proliferation marker Ki67 and intracellular IFN-γ production were analyzed. B cells were identified as $CD19^+$ cells within the total lymphocyte gate, and the expression of CD40 was evaluated within the B cell population. The results obtained from the analyses were expressed as frequency (%) and Mean Fluorescence Intensity (MFI), corresponding to the number of molecules of a given marker displayed by each cell.

2.7. Cytokine concentrations in culture supernatant

During the whole blood ex vivo assay, as described in the previous Section 2.6, BD GolgiPlug™ was not added to some wells in the last 4 h to obtain the secreted cytokines in the extracellular medium. After 48 h, the supernatants were collected and stored at −80 °C until use. IFN-γ and IL-6 were evaluated by ELISA (BD OptEIA™ Human ELISA Set, BD Biosciences) according to the manufacturer's instructions, and the results were expressed in pg/mL. IL-2, IL-4, IL-10, TNF-α and IL-17A were measured by flow cytometry using a BD™ Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Cytokine Kit (BD Biosciences) according to the manufacturer's instructions, and the concentrations were expressed in pg/mL.

2.8. Statistical analysis

Total and specific IgG and IgA antibodies were expressed as geometric mean concentrations (GMCs) with 95% confidence intervals (CIs). Statistical analysis was performed using GraphPad Prism version 7.0 software for Windows (GraphPad Software Inc., San Diego, CA, USA). In addition to descriptive analysis, the normality of the data was tested by the D'Agostino-Pearson normality test. Non-parametric tests, such as the Wilcoxon and Mann-Whitney tests, were used to examine differences between paired and unpaired samples, respectively, and Student's *t*-test was performed for parametric data. Spearman's or Pearson's correlation analysis was performed according to the normality of data. All statistical tests were performed assuming a confidence limit of 95% and a significance level of $p < 0.05$. Placental transfer ratios of IgG antibodies were defined as the ratio of cord serum concentrations/maternal serum concentrations, multiplied by 100.

3. Results

A total of 66 women immunized with Tdap (case group) and 101 unimmunized women (control group) were included in study. All women were healthy at the time of sample collection and had no history of chronic diseases. The deliveries occurred without complications, leading to healthy term infants with adequate weight for gestational age. The demographic and clinical characteristics of the study population are displayed in Table 1. There were

Table 1
Demographic and clinical characteristics by Tdap vaccination during pregnancy.

	Case group (n = 66)	Control group (n = 101)	*p-value*
Maternal age at delivery (y)[a]	28 (15–39)	24 (15–38)	*<0.05*
Gestational age at delivery (w)[b]	39 $^2/_7$ ± 1 (38 $^2/_7$–40 $^2/_7$)	39 $^2/_7$ ± 1 $^5/_7$ (37 $^4/_7$–41)	*0.851*
Gestational age at vaccination (w)[b]	33 ± $^3/_7$ (30–36)	–	–
Interval between vaccination and delivery (d)[a]	46 (6–115)	–	–
Birth weight (g)[b]	3344 ± 457 (2887–3801)	3335 ± 442 (2983–3777)	*0.903*
Delivery (NSVD/C-section) [c]	26 (39.4%)/40 (60.6%)	94 (93.1%)/7 (6.9%)	*<0.0001*
Apgar at 5 min[a]	9 (5–10)	9 (6–10)	*0.589*
Parity[a]	2 (1–8)	2 (1–7)	*0.414*

Case Group, Tdap vaccination during pregnancy; Control group, no Tdap vaccination during pregnancy; Tdap, tetanus toxoid, diphtheria toxoid, and acellular pertussis vaccine; y, years; w, weeks; d, days; g, grams; NSVD, normal spontaneous vaginal delivery; min, minutes; -, not applicable.
[a] Median (Minimum and maximum value).
[b] Mean ± SD (Variance).
[c] Number (%).

Table 2
Geometric mean concentration (GMC) with 95% confidence interval (CI) of IgG antibodies reactive with *B. pertussis*, PT, FHA and PRN and placental transfer ratios (%) in maternal and cord serum samples from the case and control groups.

IgG	Case group(n = 66)		Control group(n = 101)		Case vs Control group Mann-Whitney test		Transfer ratios (%) [a] Mann-Whitney test	
	Maternal	Cord	Maternal	Cord	Maternal	Cord	Case Group	Control Group
Total (mg/dL)	923.0(876.5–972.9)	1045.0(987.7–1106.0)	1075.0(1034.0–1118.0)	1188.0(1150.0–1228.0)	*p < 0.001*	*p < 0.001*	114(31.0–196.0)	112(63.0–211.0)
Wilcoxon test	*p < 0.0001*		*p < 0.0001*				*0.938*	
Anti-*Bp* (AU/mL)	944.5(718.1–1242.0)	838.2(630.8–1114.0)	239.4(204.9–279.7)	257.8(220.2–301.7)	*p < 0.0001*	*p < 0.0001*	94(87.2–105.2)	105(105.9–124.7)
Wilcoxon test	*0.054*		*p < 0.05*				*p < 0.01*	
Anti-PT (IU/mL)	59.6(42.5–83.5)	55.0(39.3–77.1)	4.7(3.4–6.6)	6.2(4.5–8.7)	*p < 0.0001*	*p < 0.0001*	98.7(94.9–119.8)	128.4(131.3–174.7)
Wilcoxon test	*0.878*		*p < 0.0001*				*p < 0.01*	
Anti-FHA (IU/mL)	339.2(250.8–458.6)	329.6(251.1–432.8)	23.7(18.7–30.1)	32.1(25.3–40.6)	*p < 0.0001*	*p < 0.0001*	107(96.8–151.5)	132.1(124.3–209.6)
Wilcoxon test	*0.697*		*p < 0.0001*				*p < 0.01*	
Anti-PRN (IU/mL)	257.5(153.8–431.2)	225.9(137.3–371.8)	7.5(5.4–10.5)	8.9(6.3–12.7)	*p < 0.0001*	*p < 0.0001*	99.2(88.0–136.2)	114.7(92.9–222.5)
Wilcoxon test	*0.230*		*p < 0.0001*				*p < 0.01*	

Case Group, Tdap vaccination during pregnancy; Control group, no Tdap vaccination during pregnancy; *Bp, Bordetella pertussis*; PT, pertussis toxin; FHA, filamentous hemaglutinin; PRN, pertactin; mg/dL, milligram/ deciliter; AU/mL, Arbitrary Unit/Milliliter; IU/mL, International Unit/Milliliter.
[a] Median (95% CI).

Table 3
Geometric mean concentration (GMC) with 95% confidence interval (CI) of total IgA and IgA antibodies reactive with *B. pertussis* and PT in maternal colostrum from the case and control groups.

IgA	Case Group (n = 65)	Control Group (n = 100)	*p-value*
Total (mg/mL)	50.9(41.2–62.8)	62.8(56.3–70.0)	*0.059*
Anti-*B. pertussis* (AU/mL)	4299.0(3338.0–5537.0)	6238.0(5185.0–7505.0)	*<0.05*
Anti-PT (IU/mL)	118.1(88.7–157.2)	143.2(120.3–170.3)	*0.415*

Case Group, Tdap vaccination during pregnancy; Control group, no Tdap vaccination during pregnancy; PT, pertussis toxin; mg/mL, milligram/milliliter; AU/mL, Arbitrary Unit/milliliter; IU/mL, International Unit/milliliter.

no significant differences between the Tdap-immunized and unimmunized women and their newborns in terms of gestational age at delivery and birth weight. Significant differences were observed in age at delivery and type of delivery. However, these two parameters did not cause statistical differences between groups regarding total and specific antibody levels, nor in the evaluation of cellular immune response of newborns.

3.1. Total antibody determination

As expected, maternal serum showed significantly lower total IgG concentrations than cord serum samples in both groups (*p < 0.0001*), demonstrating positive correlation indexes in the case and control groups (Pearson test: r = 0.51, *p < 0.001* and r = 0.31, *p < 0.01,* respectively). Comparison of total maternal IgG GMCs revealed lower concentrations in the case group than in the control group, and the same trend was observed in the newborns. No significant differences were observed between the groups regarding the transplacental transfer ratios of total IgG antibodies (Table 2).

GMC and 95% CI of total IgA concentrations in maternal colostrum did not differ between the groups (Table 3). Among the samples collected, two parturients had selective IgA deficiency (IgAD), one belonging to the Tdap-immunized group and the other to the control group; therefore, these parturients were not included in the colostrum analyses.

3.2. Anti-pertussis antibody determination

Table 2 provides an overview of the anti-pertussis IgG results and placental transfer ratios of these antibodies. Mann-Whitney analysis revealed significantly higher titers of anti-Bp IgG and anti-PT, FHA and PRN IgG concentrations in maternal and umbilical cord serum samples from the case group than the control group. The Wilcoxon test revealed significantly lower anti-Bp, anti-PT, anti-FHA and anti-PRN IgG concentrations in unimmunized pregnant women than in their respective newborns.

Additionally, using the Spearman test, positive correlation indexes between maternal and cord concentrations were observed in the case and control groups for anti-Bp IgG (case: r = 0.92; *p < 0.0001* and control: r = 0.88; *p < 0.0001*), anti-PT IgG (case: r = 0.84; *p < 0.0001* and control: r = 0.96; *p < 0.0001*), anti-FHA IgG (case: r = 0.79; *p < 0.0001* and control: r = 0.89; *p < 0.0001*) and anti-PRN IgG (case: r = 0.91; *p < 0.0001* and control: r = 0.90; *p < 0.0001,* respectively).

The placental transfer ratios of IgG reactive with Bp, PT, FHA and PRN were significantly higher in the control group (*p < 0.01*) and the median ratios were above 100% for all antibodies, which means higher antibody levels in the neonates when compared to their mothers.

Regarding the colostrum samples, a large range of variation in anti-Bp IgA levels was observed in both groups. Statistical analysis revealed lower colostrum anti-Bp IgA concentrations in the case

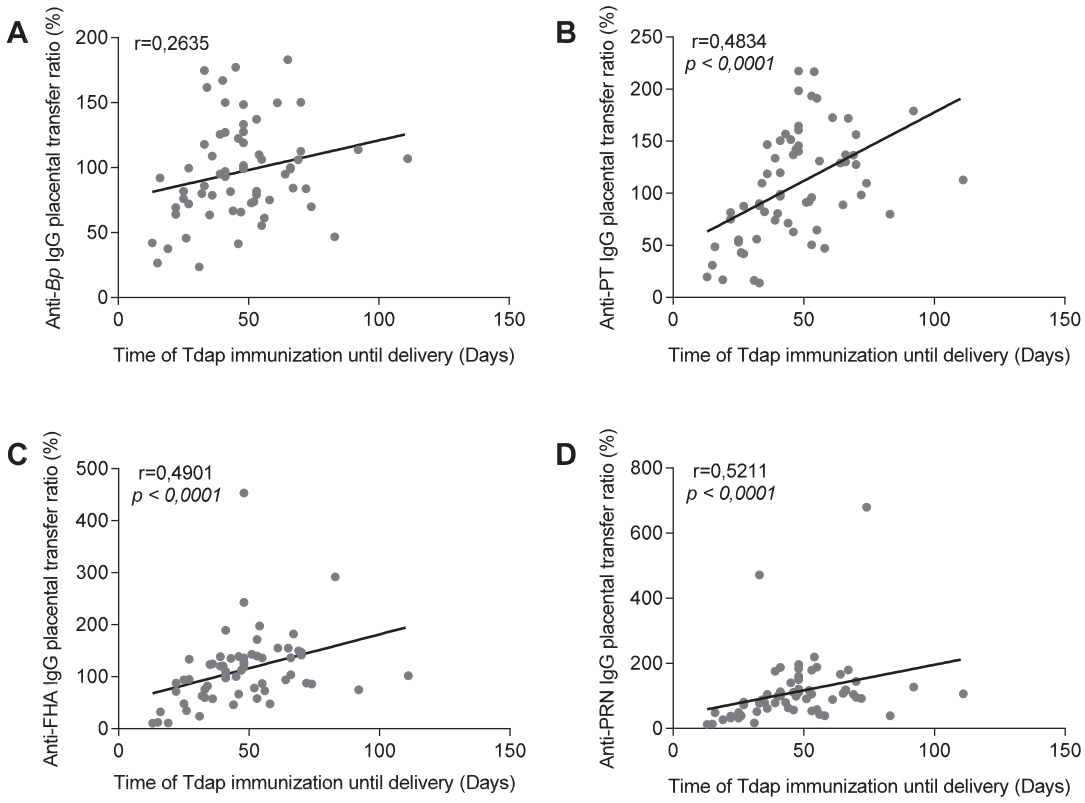

Fig. 1. Spearman correlation indexes between placental transfer ratios of (A) anti-*B. pertussis* (Bp), (B) anti-pertussis toxin (PT), (C) anti-filamentous hemagglutinin (FHA) and (D) anti-pertactin (PRN) IgG antibodies and the time of Tdap immunization until delivery (days) in the case group (Tdap vaccination during pregnancy).

group than in the control group. No significant differences in anti-PT IgA concentrations were detected in the colostrum samples between the groups (Table 3). There was a positive correlation between anti-Bp and anti-PT IgA antibody concentrations in colostrum samples (Spearman: r = 0.67, $p < 0.0001$ in case and r = 0.62, $p < 0.0001$ in control group).

3.3. Timing of gestational Tdap immunization

As expected, pregnant women vaccinated at 26–31 and at 32–36 gestation weeks presented significantly higher IgG placental transfer ratios than women vaccinated after 37 gestation weeks (Bp: $p < 0.01$ and $p < 0.05$; PT: $p < 0.0001$ and $p < 0.001$; FHA: $p < 0.01$ and $p < 0.05$; PRN: $p < 0.01$ and $p < 0.01$, respectively). Significant moderate correlation indexes were observed between the time interval from vaccination to delivery and IgG placental transfer ratios for all antigens, with the exception of anti-Bp IgG (Fig. 1).

3.4. Umbilical cord blood T and B lymphocyte activation

As parameters of pertussis-induced T cell activation, we assessed T and B lymphocyte activation markers, T cell proliferation and cytokine production for a complete characterization of T cell function after *B. pertussis* stimulation. Whole blood assays were performed using 17 umbilical cord blood samples from the case group and 15 cord blood samples from the control group followed by 11 peripheral blood samples from healthy adults as controls.

To study antigen-induced T cell activation, the T lymphocyte activation markers CD40L (CD154) and CD69 were used. The results in Fig. 2 show a statistically significant increase in the percentages of $CD40L^+CD4^+$ and $CD40L^+CD8^+$ T cell subsets after Bp stimulation in both newborn groups. For the adult group, an increase was observed only in the percentage of $CD40L^+CD4^+$ T lymphocytes. An increase was also observed in $CD69^+CD4^+$ T lymphocytes after Bp stimulation in both groups of newborns. B lymphocyte analysis results were consistent with those of the T cell analysis, with a significant and equivalent increase in the percentage of $CD40^+$ B cells in all groups after Bp stimulation.

Assessment of T cell proliferation capacity was performed using anti-Ki67 antibodies, which bind a nuclear protein that is expressed only during the phases of cell division and is absent in quiescent cells or during DNA repair [20]. Notably, *B. pertussis* was able to generate significant $CD4^+$ T cell proliferation only in the case group and in adults (both $p < 0.01$), and proliferation of $CD8^+$ T cells was noted only in adults ($p < 0.01$). Regarding PHA stimulation, $CD4^+$ and $CD8^+$ T lymphocyte proliferation was observed in both neonatal groups and adults (Fig. 3).

Given the importance of IFN-γ production in the immune response against *B. pertussis*, quantification of this cytokine was carried out intra- and extracellularly (Fig. 4). Intracellular IFN-γ staining demonstrated that newborns from the two groups presented higher IFN-γ-producing $CD4^+$ T cell percentages after Bp stimulation than unstimulated cells ($p < 0.05$), but this result was not observed for $CD8^+$ T cells. Regarding the total intracellular production of IFN-γ, represented by MFI, the newborn samples from the case and control groups showed significant increases after Bp stimulation in $CD4^+$ T cells ($p < 0.05$ and $p < 0.01$, respectively) and in $CD8^+$ T cells ($p < 0.05$ in both groups). However, no differences were detected in the percentage and MFI of IFN-γ-producing cells between the groups. Notably, no significant increase was observed in the percentage and MFI of IFN-γ^+ T cells after Bp stimulation in the adult group, contrary to what was observed after PHA stimulation.

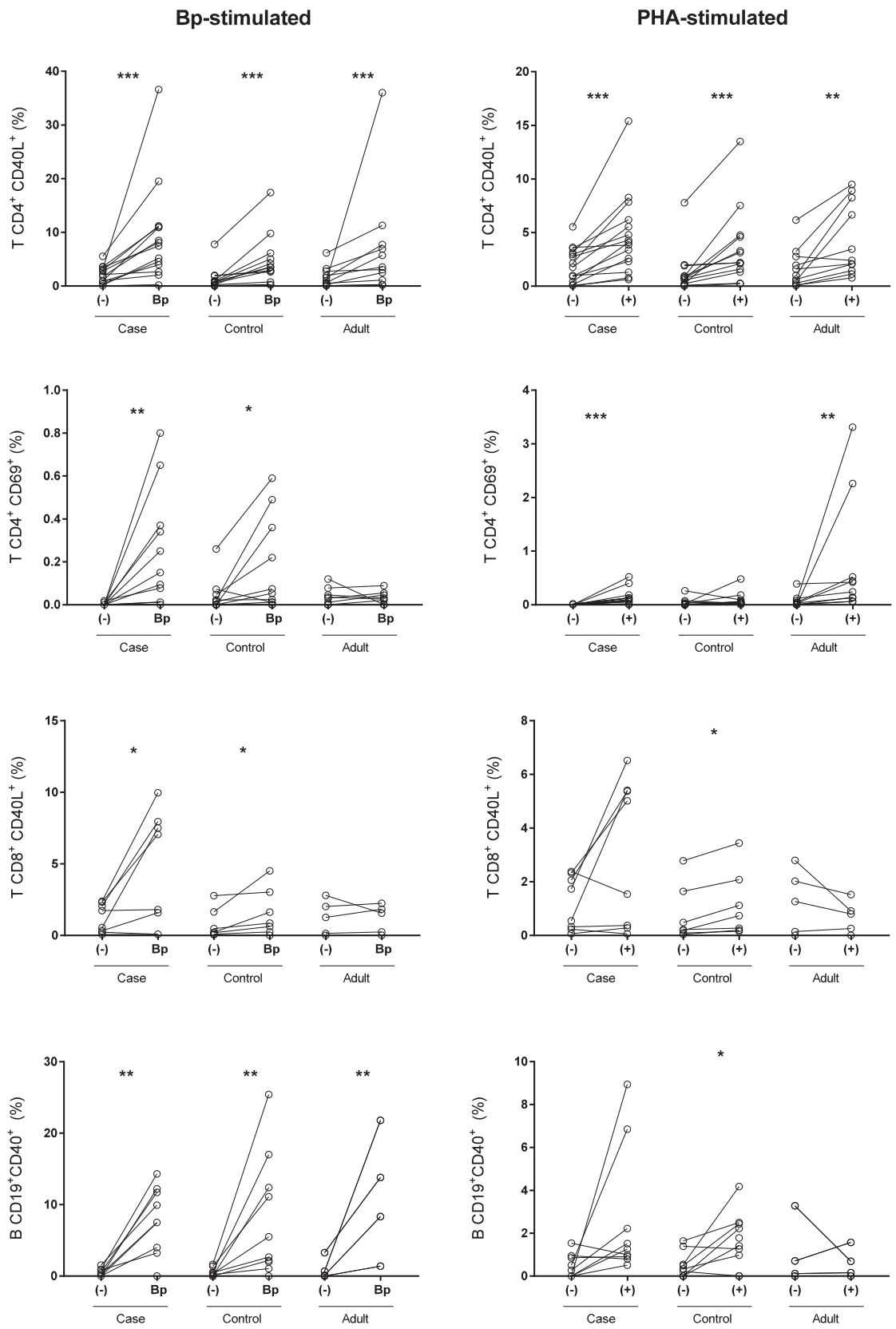

Fig. 2. Percentages of $CD40L^+CD4^+$ T cells, $CD69^+CD4^+$ T cells, $CD40L^+CD8^+$ T cells and $CD40^+CD19^+$ B cells within the lymphocyte population in cord blood samples from the case (Tdap vaccination during pregnancy, n = 17) and control groups (no Tdap vaccination during pregnancy, n = 15) and in peripheral blood of adults (n = 11) before and after *B. pertussis* (Bp) (left column) or PHA (right column) stimulation. Blood leukocytes were stimulated in vitro for 48 h with 5 μL of PHA or 0.5 mg/mL Bp, and $CD4^+$ and $CD8^+$ T or $CD19^+$ B lymphocytes were detected by flow cytometry within the lymphocyte population. The lines join the results obtained for the same neonate or adult before (−) and after stimulation with PHA (+) or Bp. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (Wilcoxon matched-pairs signed rank *U* test).

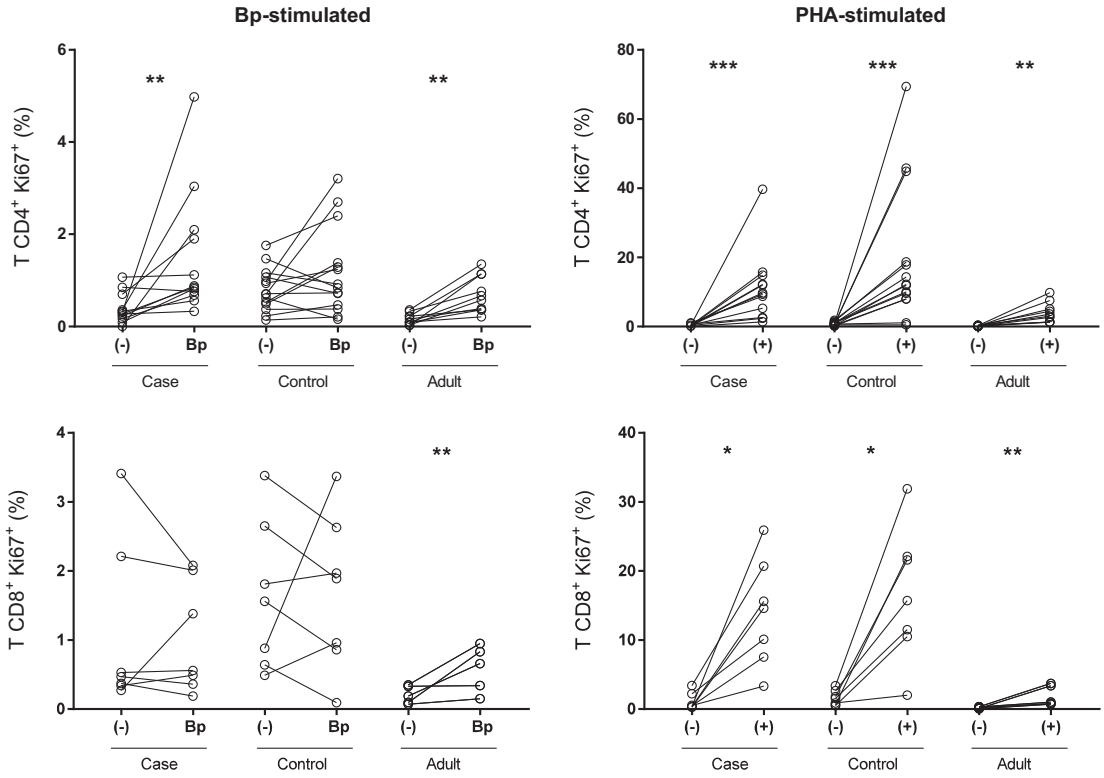

Fig. 3. Proliferation percentages were measured by the incorporation of Ki67 in $CD4^+$ and $CD8^+$ T cells from cord blood samples from the case (Tdap vaccination during pregnancy, n = 17) and control groups (no Tdap vaccination during pregnancy, n = 15) and from the peripheral blood of adults (n = 11) before and after *B. pertussis* (Bp) (left column) or PHA (right column) stimulation. Blood leukocytes were stimulated in vitro for 48 h with 5 μL of PHA or 0.5 mg/mL Bp, and $CD4^+$ and $CD8^+$ T or $CD19^+$ B lymphocytes were detected by flow cytometry within the lymphocyte population. The lines join the results obtained for the same neonate or adult before (-) and after stimulation with PHA (+) or Bp.$^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ (Wilcoxon matched-pairs signed rank *U* test).

Evaluation of IFN-γ concentrations in the whole blood assay supernatants showed that some newborn samples from both groups did not respond to PHA stimulation (12% in case and 20% in control group), and to Bp (6% in case and 13% in control group), contrary to the trend seen in adult blood samples. However, the medians presented significant results, indicating the production of IFN-γ by all groups after *B. pertussis* stimulation (case group: $p < 0.01$; control group: $p < 0.001$; adult group: $p < 0.001$). There were no significant differences in IFN-γ levels in the supernatants between the two groups of neonates. The adult group revealed higher IFN-γ secretion after Bp stimulation than both newborn groups ($p < 0.0001$) (Table 4).

Table 4 also shows the other cytokine concentrations in the whole blood assay supernatants, which were analyzed to compare the response profiles of newborns from immunized or unimmunized pregnant women. The cytokines analyzed were TNF-α and IL-2, which belong to a Th1 profile, in association with IFN-γ; IL-4, IL-6 and IL-10, which contribute to the Th2 response profile; and IL-17 secreted by Th17 cells [21]. Comparison of unstimulated cells with those stimulated in the same group showed that in the presence of Bp, neonatal cells responded with good production of IL-6, IL-10, TNF-α and IFN-γ, while IL-2 and IL-4 production was very low. There was no production of IL-17A after bacterial stimulation. The same results were observed for the adult group. PHA stimulation was much more effective for the adult group, leading to the production of higher levels of almost all cytokines, with the exception of IL-6. There were no differences between the case and control groups regarding the production of all cytokines in response to Bp or PHA. The adult group produced higher amounts of TNF-α than both groups of neonates after stimulation with Bp ($p < 0.01$) or PHA ($p < 0.05$).

4. Discussion

To the best of our knowledge, this report is the first to describe maternal Tdap vaccine responsiveness in Brazil since its introduction in 2014. It is worth mentioning that it was possible to collect a large sample number of unvaccinated parturients because our work began shortly after the implementation of the Tdap vaccine for pregnant women. However, over time, we observed a greater difficulty in increasing the number of the control group, due to greater adhesion to the vaccine, which is extremely beneficial for the population.

Pertussis-specific IgG antibody concentrations in the case group highlighted the ability of the Tdap vaccine to generate a specific humoral immune response. Higher specific antibody concentrations in mothers and newborns after Tdap immunization is an incontestable observation described by other groups [16,22,23]. The PT protein presented lower immunogenicity than the other antigens present in the vaccine, revealed by lower specific antibody concentrations in the case group. This may be a consequence of the specificity of this protein, produced only by *B. pertussis* [24], whereas FHA and PRN antigens can be found in other *Bordetella* species, which would generate cross-reactive antibodies [25]. In particular, anti-FHA antibodies can also be specific to epitopes present in other bacteria such as *Haemophilus influenzae, Mycoplasma pneumoniae* and *Chlamydophila pneumonia* [26]. The PRN protein had the highest immunogenicity, as anti-PRN IgG median concentrations were 56-fold higher in the mothers and almost 34-fold higher in the newborns from the case group than in the control group. The presence of these antibodies may have great beneficial potential, since anti-PRN antibodies appear to help in bacterial phagocytosis by the host immune system [27].

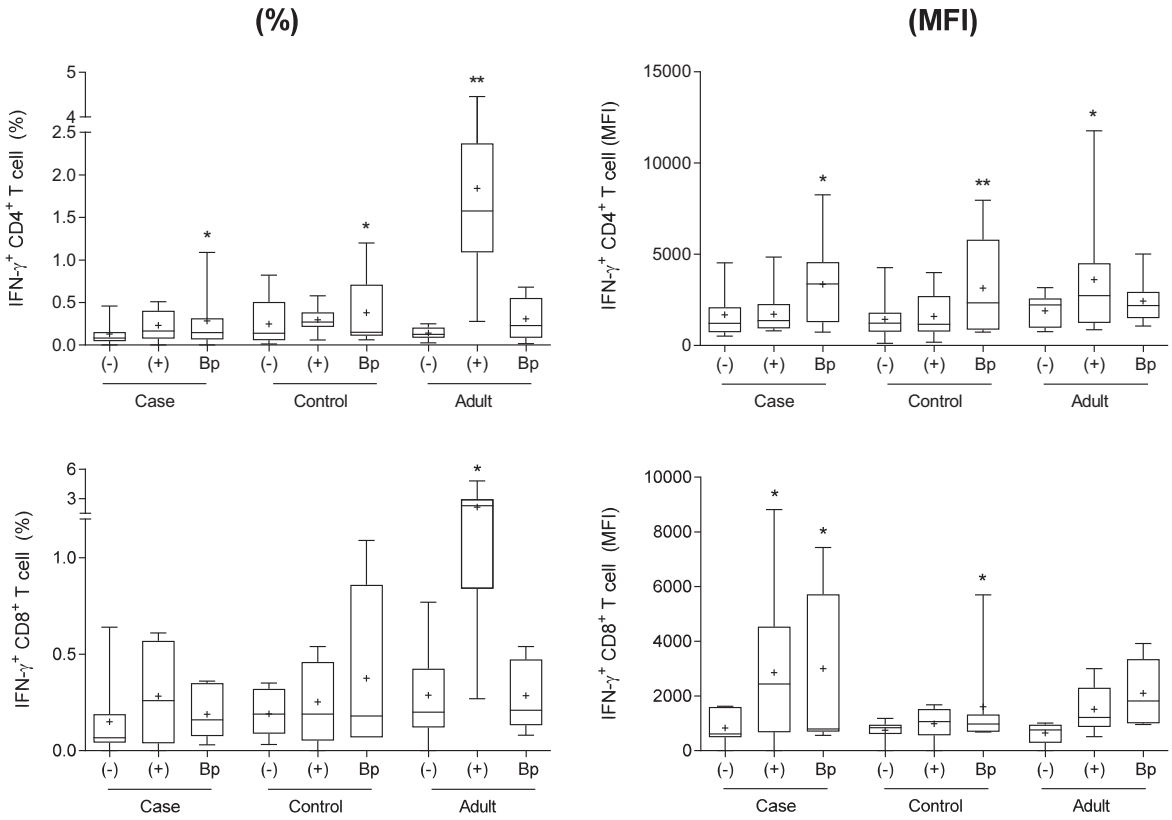

Fig. 4. Production of IFN-γ measured by intracellular staining in CD4 or CD8 T cells from cord blood samples from the case (Tdap vaccination during pregnancy, n = 17) and control groups (no Tdap vaccination during pregnancy, n = 15) and from peripheral blood of adults (n = 11) before (−) and after PHA (+) or *B. pertussis* (Bp) stimulation. The results were expressed as frequency (%) of IFN-γ$^+$CD4$^+$ or IFN-γ$^+$CD8$^+$ T cell as well as Median Fluorescence Intensity (MFI) of IFN-γ$^+$ expression on these populations. Comparison of the data is presented as box and whisker plots. The box represents the 25th–75th percentiles, and the median is represented by the line within the box. The whiskers represent the 5th–95th percentiles. $^*p < 0.05$; $^{**}p < 0.01$ (Wilcoxon matched-pairs signed rank *U* test).

Maternal vaccination is an important factor that can affect placental antibody transfer rates. High IgG concentrations in response to a vaccine are related to reduced transmission rate to the fetus, probably due to the limited number of FcRn receptors, which leads to saturation of these receptors and lower antibody transmission to the newborn [28]. This phenomenon was observed in our study, since the placental transfer ratios of pertussis-specific IgG were lower in the case group. Reinforcing this idea, inverse correlations were observed between maternal pertussis-specific IgG concentrations and placental transfer ratios to the newborns.

In agreement with Naidu et al. [29], we found that pregnant women with the highest IgG antibody transfer ratios were those vaccinated between 26 and 31 gestation weeks, demonstrating that longer exposure to vaccine antigens results in higher maternal specific antibody concentrations and a more efficient transplacental transfer to the infant. Pregnant women vaccinated at more than 37 gestation weeks were those with the lowest rates of placental transmission; therefore, the protection given to the newborn in these cases would alternatively be given by the cocoon strategy, which also would prevent the parturient from contracting the disease and transmitting it to her child in the early days of life [27,30]. Following this strategy, some countries recommend vaccination not only for the pregnant woman but for all people who have close contact with the newborn, such as siblings and grandparents [27]. However, for developing countries, such as Brazil, this measure is not practiced in public services, as it involves a considerably greater expense for public health.

There are few data regarding IgA antibodies specific to *B. pertussis* in breast milk [31,32]. In the present study, the unexpected lower anti-*B. pertussis* IgA concentrations in the case group probably occurred because colostrum samples from this group were collected significantly later than those from the control group (median hours: 26.0 h and 22.0 h, respectively, $p < 0.001$). Although total IgA concentrations did not differ in both groups, the case group showed slightly lower total IgA concentrations than the control group ($p = 0.0725$). The composition of breast milk undergoes changes during 3 lactation periods, defined as colostrum, transition and mature milk, and the concentrations of bioactive factors gradually decay over this time [33].

Regarding anti-PT IgA antibodies in the colostrum, there were no significant differences between the groups, which differs from the data of Raya et al. [31], who observed higher anti-PT and anti-FHA IgA levels in the colostrum of an immunized group, and these antibodies were detected for up to 8 weeks following delivery; however, there was an expressive decline during the first two weeks postpartum. Even in the face of conflicting results, our group did not expect that the Tdap vaccine would raise specific SIgA concentrations. As seen in natural symptomatic or subclinical infection, or previous microorganism colonization [34,35], vaccines capable of raising the production of SIgA and, consequently, promoting its presence in breast milk are those administered by the oral or nasal route that induce the immune response in mucosal sites via plasma cells that preferentially migrate from the intestine and respiratory tract to the lactating breast [32,36].

So far there is no correlate of protection after vaccination with pertussis, probably due to the presence of several antigens in the vaccine formulation, and due to the involvement of the cellular immune response. Although not a consensus, concentrations of anti-PT antibodies greater than 5–10 IU/mL have been reported to function as a relevant indication of protection [37]. Seropositiv-

Table 4
IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ and IL-17A concentrations in culture supernatant of cord blood leukocytes from the case and control groups and from peripheral blood of adults.

IL (pg/mL)	Case Group (n = 17)			Control Group (n = 15)			Adult Group (n = 11)		
	(−)	(+)	Bp	(−)	(+)	Bp	(−)	(+)	Bp
IL-2	0.1 (0–0.7)	6.0*** (28.6–645.8)	0.7 (0.4–2.1)	0.3 (0.2–1.3)	19.2*** (0–1628.0)	0.8 (0.5–2.5)	0.7 (0.4–1.7)	287.2*** (33.7–1787.0)	2.0 (1.0–6.9)
IL-4	0.3 (0.2–0.5)	0.8* (0.6–6.1)	1.2** (1.0–3.2)	0.6 (0.3–0.8)	1.2** (0–21.9)	1.5** (1.0–3.2)	0.6 (0.4–0.9)	75.4*** (54.9–202.8)	2.4*** (1.5–5.6)
IL-6	974.6 (227.4–4176.0)	4818.0*** (1488.0–15603.0)	10589.0*** (6058.0–18510.0)	491.1 (81.9–2946.0)	6380.0* (2345.0–17359.0)	13234.0** (6583.0–26606.0)	1754.0 (505.4–6088.0)	3925.0 (2732.0–5637.0)	6781.0 (4271.0–10766.0)
IL-10	22.6 (36.1–263.8)	316.5** (105.0–934.5)	488.0** (325.2–726.6)	5.3 (25.3–272.1)	427.2** (0–1279.0)	439.1*** (238.8–826.4)	322.8 (132.8–680.0)	1422.0*** (974.5–2345.0)	557.1 (322.9–902.2)
TNF-α	2.7 (0–156.1)	10.3* (68.4–543.4)	325.0** (133.2–1599.0)	4.3 (0–68.5)	34.7** (0–1099.0)	382.9*** (200.8–1359.0)	28.1 (3.4–96.3)	599.3*** (162.6–2072.0)	1742.0*** (1179.0–3268.0)
IFN-γ	15.2 (15.2–15.2)	34.4* (24.9–354.4)	46.6** (13.5–161.4)	15.2 (15.2–15.2)	476.1*** (120.2–806.6)	35.8*** (16.9–73.0)	22.1 (4.8–190.2)	805.1*** (735.3–987.7)	465.7*** (307.6–742.9)
IL-17A	11.4 (7.0–25.3)	8.9 (5.6–16.8)	10.0 (8.8–28.4)	24.8 (17.8–38.1)	12.9** (9.9–26.8)	7.7** (5.7–19.3)	12.0 (7.3–21.3)	157.5** (100.1–440.9)	6.6 (3.8–10.9)

Case Group, Tdap vaccination during pregnancy; Control group, no Tdap vaccination during pregnancy; pg/mL, picogram/milliliter.
Blood leukocytes were stimulated in vitro with 5 μL PHA (+) or 0.5 mg/mL *B. pertussis* (Bp), or left unstimulated (-) for 48 h and the supernatants collected to measure cytokine concentrations by flow cytometry (IL-2, IL-4, IL-10, TNF-α and IL-17A) or ELISA (IFN-γ and IL-6).
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

ity against PT can be defined as any increase for individuals who initially had anti-PT antibody concentrations below 5 IU/mL and for individuals who already have pre-vaccine concentrations above this value that demonstrate a two-fold or higher increase [37,38]. In our study, we did not have pre-vaccination values to permit this type of analysis, which is a limitation. Therefore, using seronegative results as a threshold value (<5 IU/mL), 52.5% of parturients and 41.6% of newborns in the control group were prone to getting pertussis, while only 7.6% of parturients and newborns in the case group were prone to pertussis.

As revealed by our whole blood assays, no differences between neonatal groups were observed for any of the studied parameters, but newborns responded very well to *B. pertussis* stimulation, exhibiting higher expression of activation markers and better proliferation of CD4 T cells than their respective unstimulated cells. Exceptions to this similarity between groups were CD40L expression on CD4 and CD8 and the proliferation of CD4 T cells, which showed higher but not significant percentages in the case group. These results showed that newborns from pregnant women who were vaccinated responded equally well, or even better than, those from the control group. FcRn receptors, which are responsible for IgG transplacental transport, are susceptible to the binding of immune complexes from the maternal circulation, which resist the acidic pH of the endosome and thus reach the fetal circulation [39]. Based on this mechanism, it is possible that maternal pertussis vaccination generates the first contact between neonates and bacterial antigens, causing sensitization of the neonatal immune cells that, during secondary contact with whole bacteria through the vaccine or during ex vivo stimulation, which was evaluated in our study, could lead to a greater and presumably more efficient immune response.

Knowing that we observed increased expression of CD40L and CD69 on CD4 T cells and CD40 on B cells, it is thought that the lower proliferation of all groups after pertussis stimulation than after with PHA stimulation occurs due to low IL-2 production in the culture supernatants of all groups, including adults. In fact, IL-2 results revealed values comparable with those obtained from proliferation assays. In contrast, it was shown that in vivo PT effects on T cell differentiation and expansion in mice were promoted in an obligatory manner via costimulatory molecules affecting multiple pathways and that cytokines are redundant for the adjuvant activity of PT, although there may be some additive effects [40].

Cytokines are also extremely important for targeting the immune response profile [40]. In summary, in agreement with other studies, we observed that the neonatal Th1 profile was lower than that of adults, with lower TNF-α and IFN-γ concentrations after *B. pertussis* stimulation, while the Th2 response was preserved, with similar or higher IL-4, IL-10 and IL-6 concentrations [41]. However, although the CD4+ T cell response in infants classically shows a shift to a Th2 profile, Vermeulen et al. [42] demonstrated that newborn vaccination with acellular or whole-cell pertussis vaccine induces an antigen-specific IFN-γ response, which is of great importance for mounting an effective response. Comparing intra- and extracellular IFN-γ production, we noticed that neonates have the capacity to produce this cytokine at similar levels to those of adults; however, neonates secrete IFN-γ at much lower concentrations. These lower levels of IFN-γ in the culture supernatants of neonates after stimulation with *B. pertussis* are in agreement with results described in the literature, since the lower production capacity of IFN-γ by this age group than by older children and adults is well-documented [6].

B. pertussis infection leads to the generation of regulatory T lymphocytes (Treg), which recognize FHA molecules and secrete IL-10, leading to the suppression of Th1 cells [43]. Good IL-10 production by neonates and adults may also explain the reduced IFN-γ pro-

duction by *B. pertussis*-stimulated lymphocytes in both neonatal groups. The adult group had surprisingly high basal levels of IL-10 secretion, as well as IL-6, probably due to the cell manipulation required to perform ex vivo assay.

Our data showed that TNF-α was also produced at relevant concentrations after Bp stimulation for all groups. Experiments with pertussis-infected mice indicate that TNF-α limits neutrophil accumulation and enhances airway resistance in these animals, causing mild infections, and TNF-α knockout mice die of these infections [44].

The observation that Th17 cells play an important role in mucosal immunity, especially for respiratory pathogens, has brought great attention to the participation of these cells in protection against pertussis [45]. However, in our study, no IL-17 production was observed after pertussis stimulation, except for PHA in the adult group.

Comparison of our data with studies of the neonatal cellular response to *B. pertussis* stimulation found in the literature has some limitations. Most of the published data have sought to evaluate the cellular immune response using peripheral blood mononuclear cells stimulated with purified PT [46,47]. As one of our objectives was to study possible influence caused by the presence of maternal antibodies in the fetal circulation, we chose to perform ex vivo assay using whole umbilical cord blood samples. In addition, the choice to use intact and inactive whole *B. pertussis* as a stimulus is justified by the fact that this bacterial suspension is identical to part of the composition of the DTP vaccine, which is still used for infant vaccination at 2, 4, 6 and 15 months of age in Brazil. Thus, it would be possible to mimic the microenvironment that would presumably be found when these newborns were vaccinated for the first time. Nonetheless, another limitation of our ex vivo experiment that should be considered is that from birth until the moment that the first vaccination dose is received at 2 months, an infant's immune system undergoes maturation processes that cannot be reproduced [6]. So, the correct approach would be to study follow up samples from the children at 2, 4 and 6 months of age, which was not possible in this work. Notwithstanding, a relevant point is that if maternal antibodies do not influence the cellular immune response of the newborn soon after delivery, we can hypothesize that at 2 months, when the newborn is first vaccinated, any possible influence would be even lower, since maternal antibody levels significantly decrease over the course of the days after birth, with 36 days being the half-life of maternal anti-PT IgG immunoglobulin [5]. However, we must consider that studies showing maternal antibody interference in the infant antibody response at the time of active vaccination report that this phenomenon is only reversed after the last dose of the first series of vaccines or after the first booster [16,48,49]. In Brazil, a pregnant woman is vaccinated with Tdap and her child with whole-cell pertussis vaccine. Therefore, the whole-cell pertussis stimulus used in our assays may induce a different antigenic repertoire to be recognized by newborn lymphocytes. This situation may render the interference of maternal antibodies transferred via placental transfer irrelevant or, at least, less significant, since these antibodies have specificity only for the three antigens present in the Tdap vaccine.

5. Conclusions

Our findings show that vaccination against pertussis during pregnancy induces a significant increase in antibody concentrations targeting all of pertussis antigens in mothers and consequently in newborns. Our results also indicate that maternal vaccination does not affect the cellular immune response of newborns at birth; that is, the presence of high levels of maternal antibodies in the neonatal circulation in the case group did not alter the cellular immune response of newborns to ex vivo challenge with *B. pertussis*.

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Disclosures

The authors declare that there is no conflict of interests regarding the publication of this paper, which has been approved by all authors.

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