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Abstract

Introduction: Maternal antibody interference of the infant's humoral immune responses raises some concern to the strategy of maternal Tdap (tetanus, diphtheria, acellular pertussis [aP]) vaccination. This study assessed the impact of maternal Tdap antibodies on the infant's pertussis-specific T lymphocyte responses following infant vaccination with an aP containing vaccine, in a term and preterm born cohort.

Methods: Heparin samples (±0.5mL) were conveniently drawn from infants of a Belgian prospective cohort study (N=79, NCT02511327), including Tdap vaccinated (Boostrix®) and non-vaccinated women (no Tdap vaccine in the last 5 years) that delivered at term or prematurely. Sampling was performed before and one month after primary (8-12-16 weeks) and booster vaccination (13 or 15 months) with DTaP-IPV-HB-PRP~T vaccine (Hexyon®). Pertussis toxin (PT)-specific $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphoblasts and their cytokine secretions were measured using a flow cytometric assay on whole blood (FASCIA) and multiplex technology (Meso Scale Discovery), respectively.

Results: 57% of all infants were considered PT-specific $CD3^+CD4^+$ lymphoblasts responders after primary and booster vaccination, whereas 17% were $CD3^+CD8^+$ lymphoblast responders. IFN-γ, IL-13, IL-17A and IL-5 cytokine secretions after primary and booster vaccination were indicative of a mixed T helper (Th) 1/Th2/Th17 cell profile. Lymphoblast and cytokine levels were comparable between term and preterm infants. Non-responders for IL-13 after booster vaccination had higher maternal PT IgG levels at birth when compared to responders.

Conclusions: Term and preterm born infants are capable of inducing Th1, Th2 and Th17 responses after aP vaccination, yet maternal vaccination modulate these responses. Evaluation of this effect in larger trials is needed.

Key points of the manuscript:

- Acellular pertussis vaccination induces comparable cellular immune responses in term and preterm born infants
- Establishment of Th1, Th17 cytokines, next to Th2 cytokines, suggest bacterial clearance functions are established
- Maternal antibodies could modulate the infant's Th1/Th2 balance after booster vaccination

Keywords: cell-mediated immune response, maternal antibodies, maternal immunization, preterm born infants, Tdap

Introduction

Pertussis vaccination during pregnancy ensures efficient and timely transplacental transfer of protective pertussis antibodies from the mother to the fetus, conferring additional protection against *Bordetella pertussis* until the start of the infants' primary vaccinations or until the most vulnerable period passes [1, 2]. Next to the protective properties of these actively acquired Tetanus, Diphtheria, acellular Pertussis (Tdap) maternal antibodies (Mabs), they are known to interfere with the infants' humoral immune response after their own primary [3, 4] and booster vaccinations [5-7]. Here, significantly lower vaccine-antibody levels are observed in infants born to vaccinated when compared to unvaccinated women. Although, one surveillance study from England has demonstrated no evidence of an increased burden of disease [8], it remains challenging to determine the clinical significance of Mabs interference because there is no serological correlate of protection for pertussis.

T cells also play a prominent role in the protection against *B. pertussis* infection, as they confer important functions like bacterial clearance and neutrophil recruitment [9]. Unfortunately, the influences of Mabs on the T cell repertoire have been studied less extensively compared to the humoral responses. Current research indicates that the T cell compartment remains largely unaffected by Mabs [10]. Although, inhibition of the Tfh cell expansion in mice [11] and alterations of the cytokines secretions in human [12, 13] in the presence of Mabs (against influenza, tetanus and pertussis, respectively), have been observed. These observations demonstrate that determining the impact of Mabs on all aspects of the infant's humoral and cellular immune system are essential. This Belgian clinical study investigated the effect of pertussis-specific Mabs on the humoral and cellular immune response after primary and booster DTaP-IPV-HB-PRP~T (Hexyon®) vaccination in

term and preterm infants. In a first report, comparable humoral responses in term and preterm infants of vaccinated women after primary vaccination were demonstrated. Yet, when comparing term and preterm infants from vaccinated and non-vaccinated women, Mabs interference was observed for some of the Tdap antigens [14]. This paper covers the parallel evaluation of the infants' pertussis-specific T responses before and after primary and booster DTaP-IPV-HB-PRP~T vaccination in the presence of vaccine-induced Mabs.

Materials and Methods

Study design

A prospective controlled cohort study (N=234, NCT02511327) conducted in Flanders (Belgium) recruited mother-infant pairs, assigning them to 4 different cohorts based on the women's vaccination status (did or did not receive Tdap vaccine [Boostrix®, GSK Biologicals] during pregnancy) and their gestational age (GA) at delivery (Supplementary Figure 1.A):

- VT Cohort: Vaccinated women and their Term (GA ≥ 37 weeks) infant
- VP Cohort: Vaccinated women and their Preterm (GA < 37 weeks) infant
- UnVT Cohort: UnVaccinated women (no pertussis containing vaccine in the last 5 years) and their Term (GA ≥ 37 weeks GA) infant
- UnVP Cohort: UnVaccinated women (no pertussis containing vaccine in the last 5 years) and their Preterm (GA < 37 weeks) infant

From this cohort, infants were conveniently selected to participate in a sub-study where the cell-mediated immune (CMI) responses were evaluated.

Study procedures

Maternal vaccination was planned within the regular healthcare system following Belgian recommendation, i.e. between 24-32 GA. Women were excluded when suffering from mental illness, immunological disorders or when receiving experimental medication during pregnancy. Infants with serious medical condition or immune disorders were excluded from the study (full list of inclusion/exclusion criteria available in Maertens et al. [14]). Informed consent was obtained. Within the regular planned well-baby clinic visits, newborns received the hexavalent DTaP-IPV-HB-PRP~T vaccine (Hexyon®, Sanofi Pasteur) at the age of 8, 12, and 16 weeks (primary vaccination). A fourth dose (booster vaccination) was administered at 13 months for preterm born infants or at 15 months for term born infants. Hexyon® contains Diphtheria Toxoid (≥ 20 international units [IU]), Tetanus toxoid (≥ 40 IU), Pertussis Toxoid (PT; 25µg) and Filamentous Haemagglutinin (FHA; 25µg), Inactivated Poliovirus type 1, 2, and 3 (40, 8, and 32 D-antigen units, respectively), Hepatitis B surface antigen (10µg) and Hemophilus influenzae type b polysaccharide (12µg) conjugated to tetanus protein (22-36µg). Cord and maternal blood samples (8mL) were collected 72 hours after delivery. Infants were sampled (serum tube, ±5mL) before and 28-35 days after their primary and booster vaccination. This report includes infants of whom an additional heparin tube (±0.5mL) at each timepoint was collected (convenience sample; Supplementary Figure 1.B). This study was approved by the Ethical Committee at the University Hospital Antwerp (B300201422982).

Serological assessment

Serum samples were evaluated for PT-specific immunoglobulins G (IgG) at the Global Clinical Immunology laboratory of Sanofi Pasteur in Swiftwater, Pennsylvania, using an in-house electrochemiluminescent assay (Meso Scale Discovery technology). Samples below the lower limit of quantification (LLOQ) for PT (2EU/mL) were assigned LLOQ/2 [15]. A full report on maternal and infant antibody titers is available in Maertens et al. [14].

Flowcytometric acquisition of PT-specific lymphocytes

PT-specific lymphocytes ($CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ blasts) were measured on heparin blood samples using a specialized Flowcytometric Assay that detects the Specific Cell-mediated Immune response in Activated whole blood (FASCIA). Based on previous protocols [16-18] whole blood was diluted 1/10 in RPMI 1640 (Life technologies) supplemented with 50 µg/mL gentamycine, 2 mM L-glutamine, MEM non-essential AA, Na pyruvate, 2B-mercapthoethanol and Fetal Bovine Serum. Diluted blood was either left unstimulated (negative control) or stimulated with 5 µg/mL PT antigen (heat-inactivated PT, *Bordetella pertussis* strain 165, List Biological) or 1 µg/mL Staphylococcal enterotoxin B (SEB, positive control). Per condition, 2x200µl diluted blood was incubated for 6–7 days (37 °C, 5% CO^2) on a 96-well plate in duplicate. After incubation, cell culture supernatant was removed and stored at −80°C for the cytokine analysis (see below). Cell pellets from duplicates were pooled and stained with APC-CD3 (BD Biosciences), PE-Cy7-CD4 (BD Biosciences) and Pacific Blue CD8 (Life technologies) at room temperature for 15 minutes. Red blood cells and debris were removed after a 10-minute incubation with IOTest lysis solution (Beckman Coulter), followed by centrifugation at 350G for 5 minutes. Supernatant was removed and the pellet

was washed with 2mL FACS buffer (BD FACS sheat supplemented with 0.1% Bovine Serum Albumin and 0.05% azide). After centrifugation, samples were acquired on CyFlowML (Sysmex) in 1mL FACS buffer. Proliferation was identified using FlowJo software, measuring blast cells based on forward and side scatter (Figure 1). Similar to Palazzo et al. [19], data was expressed as percentage of PT-stimulated blasts subtracted by the percentage of blasts in the unstimulated culture. FASCIA protocol and PT concentrations were validated and optimized on blood samples from healthy adults before and after TdaP (Boostrix®) vaccination.

Cytokine detection

Cytokine secretions of Interferon-γ (IFN-γ), interleukin (IL)-10, IL-13, IL-2, IL-4, TNF-α, IL-17A and IL-5 were measured on stored culture medium using the V-PLEX Cytokine and Proinflammatory Panel 1 Human Kit (Meso Scale Discovery), following the manufacturers recommendations. Lower limits of detection (LLOD) for IFN-γ, IL-10, IL-13, IL-2, IL-4, TNF-α, IL-17A and IL-5 were 0.37 pg/mL, 0.04 pg/mL, 0.24 pg/mL, 0.09 pg/mL, 0.02 pg/mL, 0.04 pg/mL, 0.31 pg/mL and 0.14 pg/mL, respectively. Similar to the flowcytometric data, cytokine secretions were controlled with the negative controls. Values below the LLOD were assigned the adapted LLOD/2 [15].

Statistics

Sample size calculation was performed for the for the assessment of the humoral responses (primary aim) in the study [14], secondary aims were based on convenience samples. JMPpro version 14, R studio (version 1.3.1093) and GraphPad Prism version 7 statistical

software was used. Demographic and descriptive analyses were carried out using chi-square, (paired) students t-test, Mann Whitney-U or Wilcoxon tests, where appropriate. Statistical significance was defined by P-value < 0.05. Group comparisons were analyzed with non-parametric Wilcoxon test. Here, cutoff for statistical significance was set at 1% which accounted for multiple testing. Infants were considered positive responders when the *in vitro* CMI response (lymphoblast or cytokine response) after vaccination was higher than the 95% Confidence Interval (CI) of the responses before vaccination. Principal component analysis (PCA) was performed on lymphoblast calculations ($CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$), normalized serum PT IgG and cytokine data (IFN-γ, IL-10, IL-13, IL-2, IL-4, TNF-α, IL-17A and IL-5; natural logarithm) to examine the variance within the data. Correlations between symmetrized serum PT IgG and each immune component separately were examined using a simple linear regression (LR). Next, linear mixed effect model (LMM) was made to illustrate the different immune components over the course of the infant's vaccination schedule and accounted for more complexity. Model selection was performed based on Akaike Information Criterion (AIC). Age, cohort, subject and interaction between serum PT IgG levels and the sampling timepoints were included in the model (Model specifications in supplement) [20]. Model analysis was followed by Tukey pairwise comparisons to analyze possible differences between the cohorts.

Results

Demographics of the CMI subpopulation

Heparin blood samples were conveniently drawn from 79 infants (8 twins; N=28 VT; N=38 VP; N=6 UnVT; N=7 UnVP cohort). Baseline characteristics of infants and their mothers (N=71) did not significantly differ from the total study population [14]. Most differences

between cohorts were related to preterm delivery (GA at delivery, interval between maternal vaccination and delivery, stay at neonatal care unit, birth weight and weights at the sampling timepoints, age of vaccination; Table 1).

PT-specific lymphoblast proliferation and cytokine secretion

T cell proliferation after *in vitro* stimulation with PT showed that primary vaccination significantly increased the infants' PT-specific $CD3^+$ and $CD3^+CD4^+$ lymphoblasts, while PT-specific $CD3^+CD8^+$ lymphoblasts did not increase at any timepoint. Over time slight waning of $CD3^+$ and $CD3^+CD4^+$ lymphoblasts populations was observed, which rose again after booster vaccination (Figure 2). Overall, this corresponded to 57% of infants being $CD3^+CD4^+$ lymphoblasts responders, while only 17% $CD3^+CD8^+$ lymphoblasts responders were reported (Table 2). Analysis of the infant's cytokine secretions revealed that lymphoblasts non-responders were still capable of producing cytokines. Primary vaccination resulted in significantly increased IFN-γ, IL-2, IL-13, IL-5 and IL-17A cytokine secretions after PT stimulation in infants (Figure 2). IFN-γ, IL-13, IL-5 and IL-17A secretions remained stable at the different points in time, with 60-70% of the infants being responders (Table 2). A gradual increase of IL-2 secretions was observed (from 56% to 72%, and 80% responders after booster vaccination). Primary vaccination did not induce IL-10, TNF-α and IL-4 cytokine secretions. Yet, booster vaccination significantly increased IL-4 secretions in all cohorts (92% responders). Lymphoblast populations and cytokines secretions were similar between all cohorts.

PT IgG levels in cord blood of responders versus non-responders

Infants born to vaccinated or unvaccinated women, who were non-responders for IL-13 one month after booster vaccination (±36% of the infants), had significantly higher PT IgG levels in their cord blood when compared to the levels of responders (P-value = 0.002; Figure 3). A more detailed analysis showed that mainly preterm not term infants who were non-responders for $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, IFN-γ, IL-13 and/or IL-5 after booster vaccination had significantly higher PT IgG levels at birth (Supplementary Figure 2).

Principal component analysis of the immune components

Most of the variability between the different immune factors was explained by PCA1 and PCA2 (41.8% and 14.1%, respectively). Primary and booster vaccination induced a cluster shift (Supplementary Figure 3), which can be explained by a change in immune components correlated to PCA1 ($CD3^+$, $CD3^+CD4^+$, IFN-γ, IL-13, IL-17A and IL-5, 60% positively correlated), and to a lesser extent to PCA2 (IL-10 and TNF-α, 70% positively correlated). A separate PCA for the vaccinated cohorts (VT and VP) demonstrated similar findings. No PCA was performed for the unvaccinated cohorts due to sample size limitations.

Correlation between the infant's immune components and serum PT IgG

Positive correlation slopes were detected between the infants' PT IgG levels and several immune components after primary ($CD3^+$, $CD3^+CD4^+$, IL-13, IL-4, IL-17A and IL-5) and booster vaccination ($CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, IL-13, IL-17A, IL-5 and IFN-γ; Figure 4). At all

other time points no correlations were observed. Although, goodness of fit was achieved by testing normality of the residuals, a lot of the variance remained unexplained with the LR model.

Linear mixed models of the different immune components

The LMM was used to study the overall differences of the immune components over time between and within the cohorts, estimating approximately 18-73% of the variability (depending on the immune component) within the data (Figure 5). The best model fit was observed when accounting for the individual subject's variability with a random intercept, and when the respective age of the infants and serum PT IgG levels at each sampling point were added as model predictors. The model predicted that when serum PT IgG levels were high after primary or booster vaccination, also high $CD3^{+}CD4^{+}$, IFN-γ, IL-13, IL-17A and IL-5 levels were observed. Tukey pairwise comparison showed no differences between the cohorts. Parameter estimates for each modeled immune component can be found in Supplementary Table 1.

Discussion

Previously we showed that Tdap vaccination during pregnancy increased the Tdap antibody titers in cord blood of term and preterm born infants, which persisted before primary vaccination [14]. The presence of these persisting Mabs were shown to interfere with the infant's humoral immune responses at primary vaccination, as significantly lower antibody titers for FHA and DT in infants of in-pregnancy vaccinated women were observed when compared with infants of unvaccinated women (in preterm infants only interference for DT

was observed). Here, we provide evidence that pertussis-specific Mabs do not significantly interfere with the infant's CMI responses after primary vaccination, as no major differences were detected between the cohorts for any of the cellular immune components. However, after booster vaccination infants who were considered IL-13 non-responders had significantly higher concentrations of Mabs at birth (PT IgG). Specifically, preterm infants with high PT IgG Mabs in their cord blood were observed to be non-responders for $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, IFN-γ, IL-13 and IL-5 after booster vaccination. It is possible that this effect is caused by earlier booster vaccination in preterm infants. However, Rowe et al. described similar kinetics, as tetanus Mabs at two months of age were positively correlated with IL-4, IL-13 and IL-5 cytokine responses in the infant at 18 months, but not at 6 months of age [12]. This implies that Mabs do allow priming of the infant's CMI responses after primary vaccination, yet modulate the infant's T helper (Th) 1/Th2 cytokine balance later in life. The communication between the maternal-antibody: antigen complexes and the Fc receptors on different immune cells might promote this mechanism, as was suggested by Rice et al. [13]. They reported the impact of maternal Tdap-IPV on the infants' cytokine and innate immune responses, demonstrating lower IL-10 and IL-4 responses after infant primary vaccination when born to Tdap-IPV vaccinated women. In accordance, our results show that the infants' serum PT IgG positively correlated with $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphoblast proliferation, and IFN-γ, IL-13, IL-4, IL-17A and IL-5 cytokine secretions after primary and/or booster vaccination (LR model and LMM model). These findings again strengthened the evidence that interference by Mabs could modulate the infant's CMI responses, especially after booster vaccination.

Next to these possible influences of the womens vaccination status, we demonstrate that prematurity did not influence the CMI responses, as preterm born infants had comparable responses to those of term born infants. This complements our previous serological findings, showing that primary and booster vaccination with Hexyon® vaccine is immunogenic in both preterm and term born infants [14]. These results confirm previous literature describing the immune competence of term and preterm infants after acellular pertussis (aP) vaccination [21, 22].

aP vaccines are known to induce a mixed Th1/Th2 profile after vaccination [23, 24]. A similar profile was observed in this study, as more than 57% of all infants responded to primary and/or booster vaccination with $CD3^+CD4^+$ lymphoblast proliferation, IFN-γ, IL-2, IL-13 and IL-5 cytokine secretions. Booster, not primary vaccination elevated the levels of IL-4 in nearly all infants, confirming more Th2 responses after aP booster vaccination shown by previous studies [22, 23, 25]. IL-2 cytokine secretions significantly increased over time, indicating the induction and persistence of Th1 responses. The lack of anti-inflammatory IL-10 cytokines after vaccination, suggest that no suppressive regulatory T cells are induced, which in turn allows for more protective Th1 responses to take place [24]. However, these lower levels might be caused by our experimental set-up, as IL-10 is mainly induced by FHA not PT antigens [26]. On the other hand, the low TNF-α levels (primarily induced by PT antigens) imply poorer pro-inflammatory responses [27]. Next to these Th1 and Th2 responses, also the importance of Th17 cells in the protection against *B. pertussis* at the lung and nasal mucosae, has been recognized over the last years [24, 25]. More than 60% of all infants produced IL-17A, suggesting that Th17 cells are systemically induced after

vaccination. However, whether Hexyon® also provides protective mucosal Th17 cells, remains to be investigated. Furthermore, it should be noted that cytokine secretions dependent on the administered vaccine type [28, 29] and the duration of *in vitro* cell-culture. Moreover, they could also be the secretion products of other immune cells within the culture. Other weaknesses of the study, like the small sample size of the unvaccinated cohorts and the different immunization schedule for the preterm infants, were recognized. However, these limitations were inherent to real-life situations of the Belgian population, as in Flanders vaccinating pregnant women with Tdap reached a coverage of ±70% in 2016 and earlier booster vaccination for preterm infants is nationally recommended. Limitations within our experimental set-up, like the limited amount of T cell markers, the mitogenic characteristics of the PT-stimulating antigen, the absence of other stimulating vaccine-antigens, and the strict responder definitions, might have resulted in a deviation from the *in vivo* situation. Regardless, this study provides a unique insight into the CMI responses of term and preterm born infants, which was made possible through assessing small volume samples with FASCIA.

Conclusion

This study identified that primary and booster vaccination with aP vaccine induced a mixed Th1, Th2 and Th17 CMI response, which was comparable in term and preterm born infants. The induction and persistence of IFN-γ, IL-2 and IL-17A cytokines after vaccination provides indirect evidence that bacterial clearance functions can be established by Th1 and Th17 cells. Moreover, we demonstrate that Mabs might modulate the infants' booster CMI responses. Even though, the proven benefit of vaccination during pregnancy should not be

discarded, these observations raise questions on Mab interference and its possible long-term effects. Proliferation assays like FASCIA combined with cytokine assessment can be an advantage to future large-scale and long-term surveillance studies involving the evaluation of both humoral and CMI responses.

Notes

Financial support. This study was funded by the Research Foundation – Flanders (FWO G064015N). An unrestricted grant from Sanofi Pasteur was received for the laboratory analysis of the serum samples. Kirsten Maertens is the beneficiary of a postdoctoral mandated fellowship from the Research Foundation – Flanders (FWO 12R5719N). Benson Ogunjimi acknowledges funding by the Research Foundation – Flanders (FWO 1861219N).

Acknowledgements. The authors gratefully acknowledge all participating women and the study nurse, Mrs. Aline Bontenakel.

Conflicts of interest.

The Universities of Antwerp and Hasselt obtain grants from several vaccine manufacturers (GSK Biologicals, Pfizer, Merck and J&J) for specific studies aimed at modelling the spread of infectious diseases for which Niel Hens is the PI. Niel Hens obtains no personal renumeration. The University of Antwerp obtains grants from several SME and vaccine manufacturers (GSK Biologicals, Pfizer, SANOFI, Merck, Takeda, Baxter, CanSino China, Themis, Osivax, J&J and Abbott) for the conduct of vaccine trials for which P Van Damme is the investigator and for the support of the Viral Hepatitis Prevention Board. P Van Damme obtains no personal remuneration. The University of Antwerp obtains grants from foundations, EU and Government (The Bill & Melinda Gates Foundation, PATH, Flemish Government, and European Union) for the conduct of trials and vaccine research for which P Van Damme is the (principal) investigator.

No potential conflicts of interest were declared by the authors.

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Figures and legends

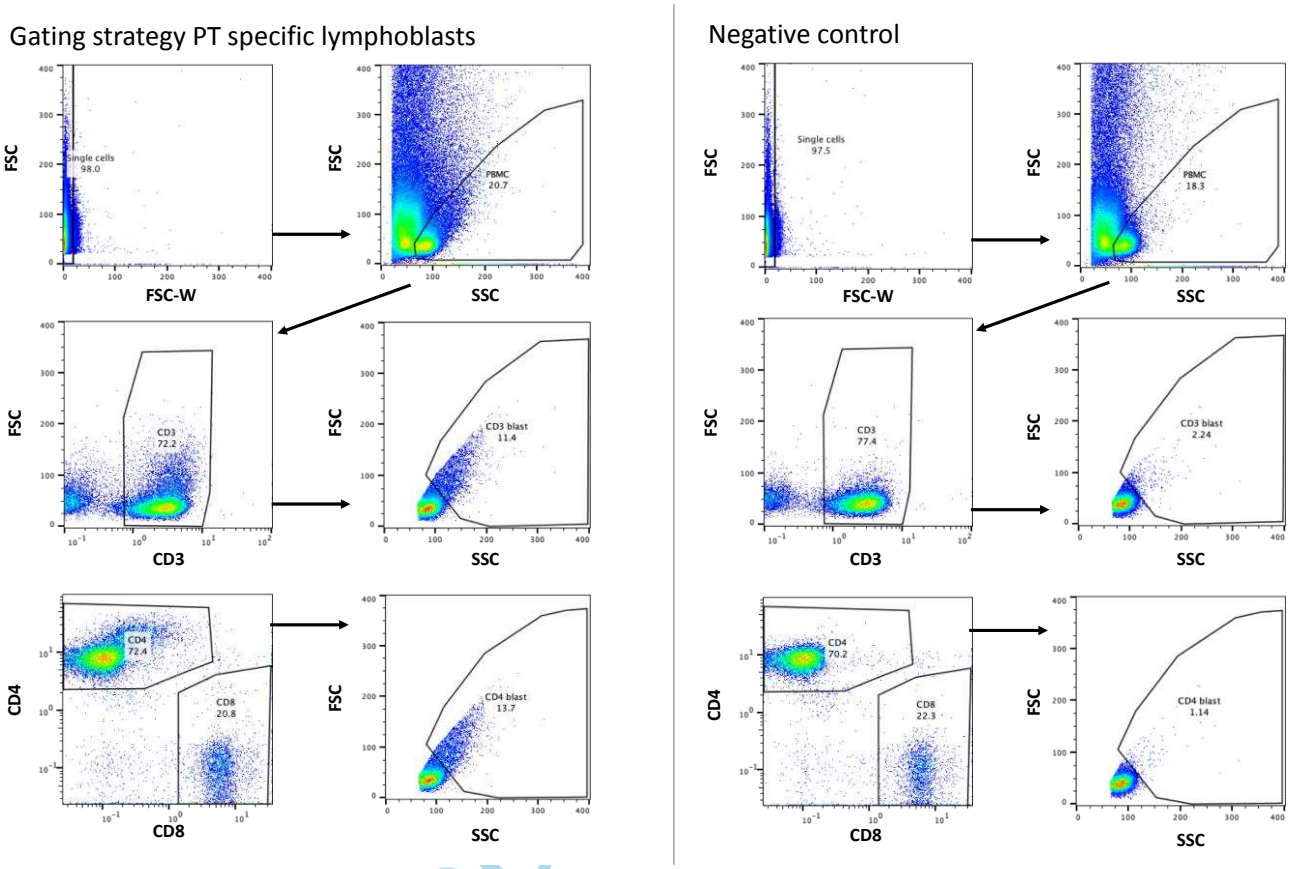

Figure 1: Flow cytometry gating strategy of the pertussis toxin (PT)-specific lymphoblast populations (left), which were compared against the negative control (right). Single cells were selected using a narrow gate on the forward scatter (FSC)/FSC-width (W) plot, after which the total lymphocytes were gated on a FSC/side scatter (SSC) plot. Next, $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ populations were selected and the percentage of lymphoblasts were identified based on their morphologic characteristics using the FSC/SSC plot (FSC/SSC lymphoblast plot not shown for $CD8^+$).

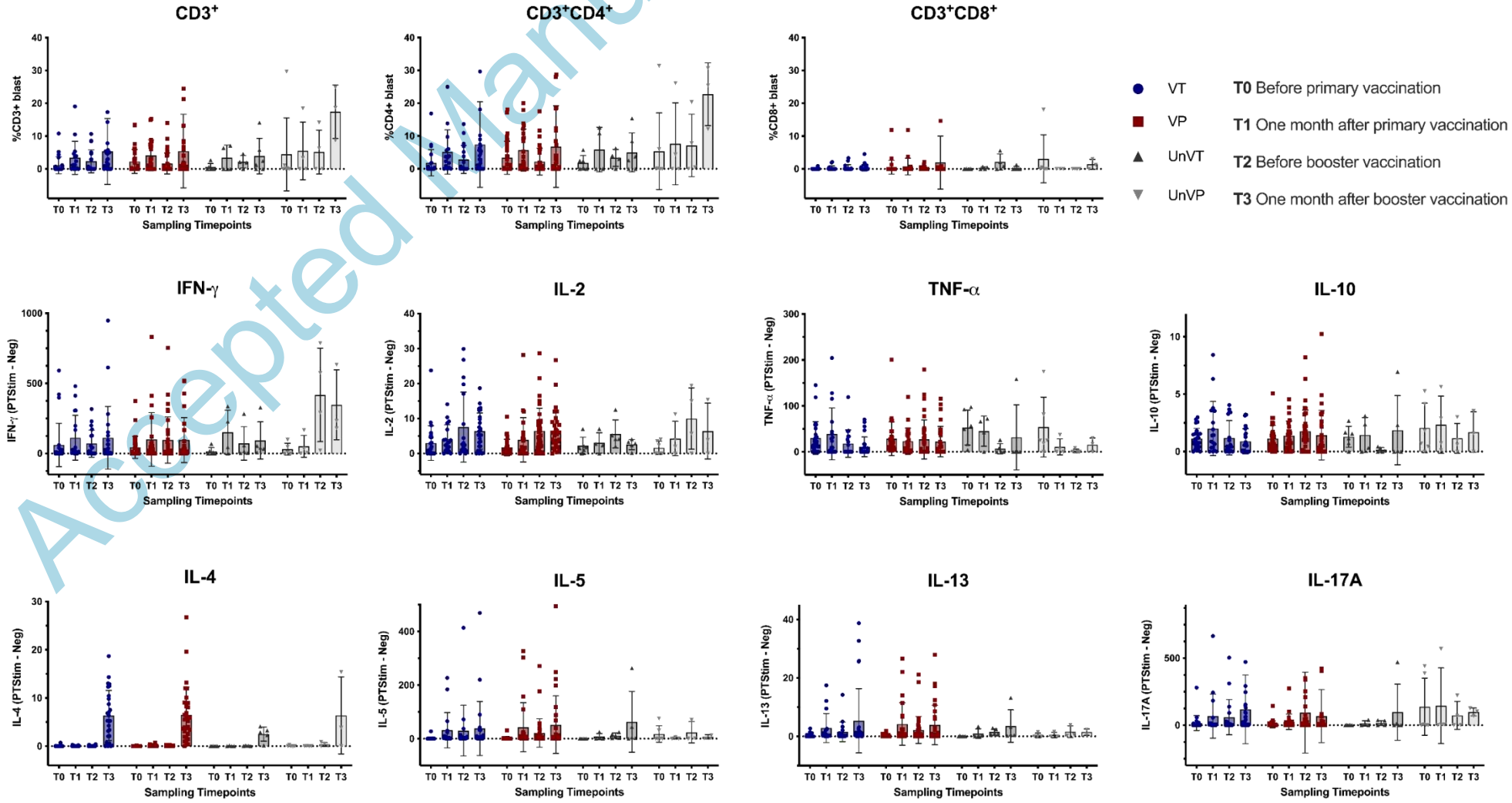

Figure 2: General overview of the $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphoblasts populations (in percentages) and the IFN-γ, IL-10, IL-13, IL-2, IL-4, TNF-α, IL-17A and IL-5 cytokine secretions (pg/mL) over time. Outliers were excluded from the graphs. No significant differences were observed between the different cohorts.

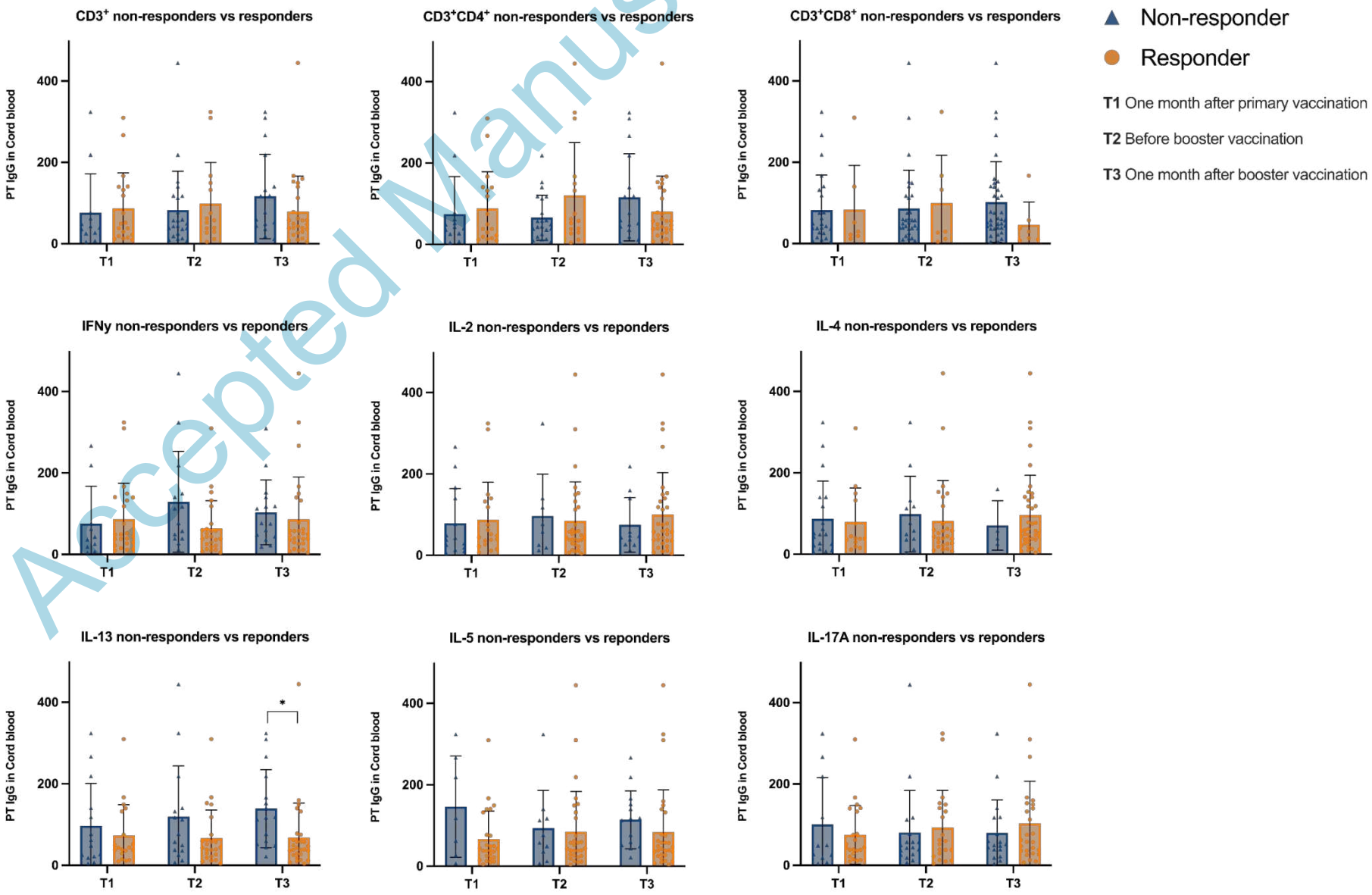

Figure 3: Comparison of the PT IgG levels (EU/mL) in cord blood between responders and non-responders for the different immune components ($CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphoblasts and the IFN-γ, IL-13, IL-2, IL-4, IL-17A and IL-5 cytokines; IL-10, TNF-α not shown) one month after primary vaccination (T1), before booster vaccination (T2) and one month after booster vaccination (T3). * indicating significant differences after correction for multiple testing (P-value<0.01)

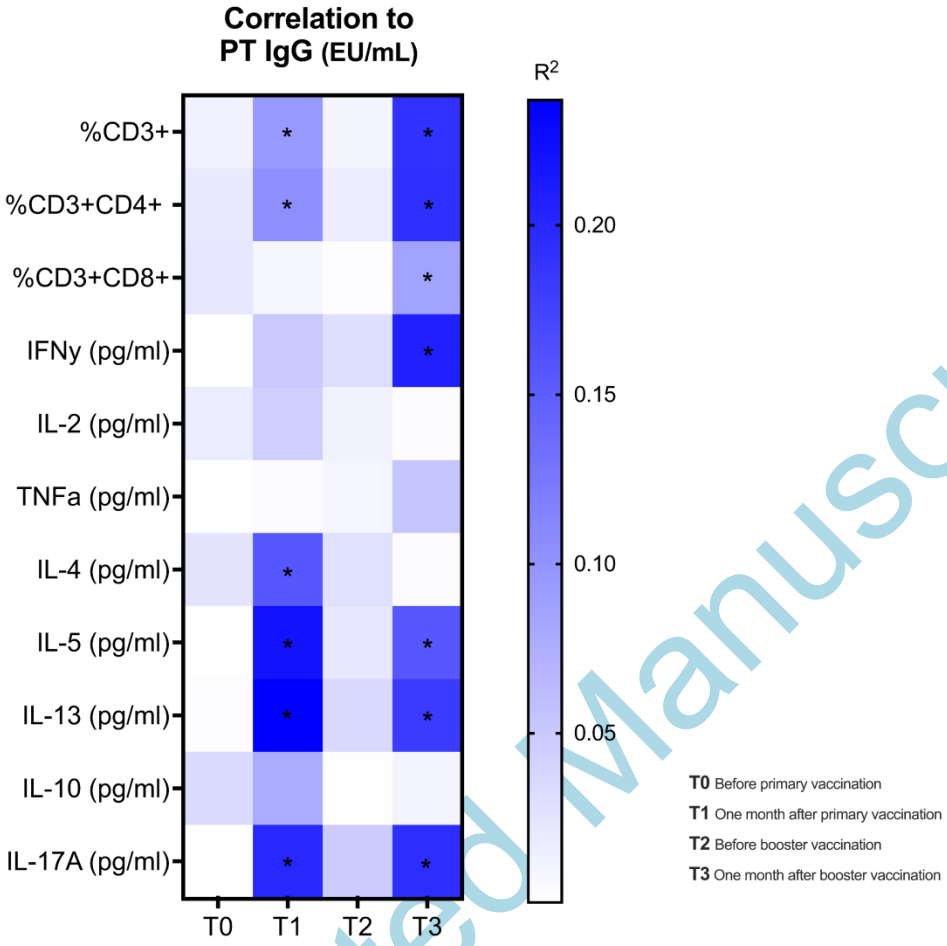

Figure 4: Pearson correlation of the different immune components ($CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ lymphoblasts and the IFN-γ, IL-10, IL-13, IL-2, IL-4, TNF-α, IL-17A and IL-5 cytokines) with PT IgG in the infant's serum over time. Serum PT IgG and cytokine was normalized (natural logarithm). *indicating a significant positive Pearson correlation ($p<0.05$), R^2 represents the variability explained by the PT IgG predictor.

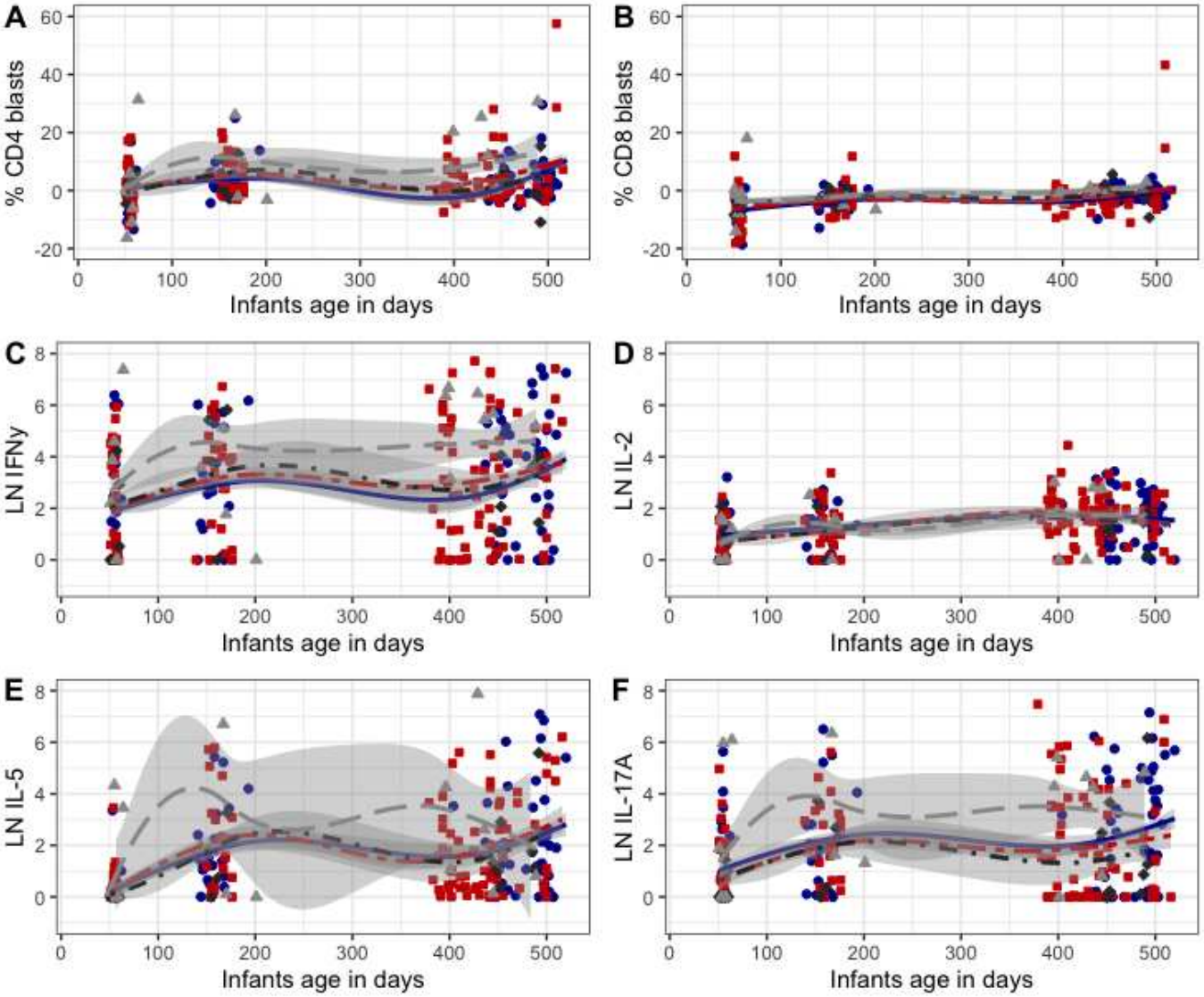

Figure 5: Representation of the linear mixed effect model for the different immune components: $CD3^+CD4^+$ (A) and $CD3^+CD8^+$ lymphoblasts (B) in percentages and the IFN-γ (C), IL-2 (D), IL-5 (E), and IL-17A (F) cytokines (pg/mL) expressed in natural logarithm.