

# Cytokine Signatures of Innate and Adaptive Immunity in 17DD Yellow Fever Vaccinated Children and Its Association With the Level of Neutralizing Antibody

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**Background.** The live attenuated yellow fever (YF) vaccines have been available for decades and are considered highly effective and one of the safest vaccines worldwide.

**Methods.** The impact of YF-17DD-antigens recall on cytokine profiles of YF-17DD-vaccinated children were characterized using short-term cultures of whole blood samples and single-cell flow cytometry. This study enrolled seroconverters and nonseroconverters after primovaccination (PV-PRNT<sup>+</sup> and PV-PRNT<sup>-</sup>), seroconverters after revaccination (RV-PRNT<sup>+</sup>), and unvaccinated volunteers (UV-PRNT<sup>-</sup>).

**Results.** The analysis demonstrated in the PV-PRNT<sup>+</sup> group a balanced involvement of pro-inflammatory/regulatory adaptive immunity with a prominent participation of innate immunity pro-inflammatory events (IL-12<sup>+</sup> and TNF- $\alpha$ <sup>+</sup> NEU and MON). Using the PV-PRNT<sup>+</sup> cytokine signature as a reference profile, PV-PRNT<sup>-</sup> presented a striking lack of innate immunity proinflammatory response along with an increased adaptive regulatory profile (IL-4<sup>+</sup>CD4<sup>+</sup> T cells and IL-10<sup>+</sup> and IL-5<sup>+</sup>CD8<sup>+</sup> T cells). Conversely, the RV-PRNT<sup>+</sup> shifted the overall cytokine signatures toward an innate immunity pro-inflammatory profile and restored the adaptive regulatory response.

**Conclusions.** The data demonstrated that the overall cytokine signature was associated with the levels of PRNT antibodies with a balanced innate/adaptive immunity with proinflammatory/regulatory profile as the hallmark of PV-PRNT<sup>MEDIUM+</sup>, whereas a polarized regulatory response was observed in PV-PRNT<sup>-</sup> and a prominent proinflammatory signature was the characteristic of PV-PRNT<sup>HIGH+</sup>.

Received 11 January 2011; accepted 2 May 2011.

Potential conflicts of interest: Six of the authors (R. M. M., M. L. S. M., R. H. G. F., M. S. F., R. G., and A. H.) are employed by the 17DD vaccine manufacturer (Bio-Manguinhos, Fundação Oswaldo Cruz) and 3 others worked in other units of Fundação Oswaldo Cruz (L. A. B. C., A. T.-C., and O. A. M.-F.). Bias from competing interest was prevented by (1) participation of members from the Minas Gerais State Health Secretary, with expertise in epidemiological surveillance in vaccine studies (José Geraldo Leite Ribeiro and Jandira Aparecida Campos Lemos) and 2 general clinical physicians with experience in infectious disease, 1 from the São Paulo University (Maria Auxiliadora-Martins) and another from the Federal University of Minas Gerais (Silvana Maria Eloi-Santos), that contributed with critical overview of the study design, volunteer immunization and medical care, blood sample collection, and supervision of data interpretation, working together with the Collaborative Group for the Study of Yellow Fever Vaccines, which conducted the study; and (2) having 6 independent university professionals working as PhD students or postdoctorate

researchers in the field of infectious diseases (Maria Luiza-Silva; Ana Carolina Campi-Azevedo, Maurício Azevedo Batista, Marina Angela Martins; Renato Sathler Avelar, and Denise da Silveira Lemos), responsible for blind sample handling and processing, data collection, and statistical analysis. All other authors: no conflicts.

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**The Journal of Infectious Diseases** 2011;204:873-83

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0022-1899 (print)/1537-6613 (online)/2011/2046-0010\$14.00

DOI: 10.1093/infdis/jir439

Yellow fever (YF) remains an important public health problem and the 17D live, attenuated yellow fever virus vaccine constitutes the single most effective means of limiting impact of the disease [1–3]. The World Health Organization (WHO) recommends that persons aged >9 months who are traveling to or living in areas where yellow fever infection is officially reported should be vaccinated [4–8]. In Brazil, the YF-17DD vaccine is offered for free to every citizen by the National Immunization Program and was introduced into the routine vaccination schedule in endemic and risk areas in 2005, and is currently administered at 9 months of age [9].

The YF vaccine immunogenicity in adults reaches over 95% of vaccinees that may persist for at least 35 years [10]. However, the rates of seroconversion in children ranged from 77.5% to 90%, with distinct seroconversion rates observed according to the age at vaccination [11–17].

The immaturity of the immune system [18–22] and the presence of passively acquired maternal antibody in the serum of the young infant may have an immunosuppressive effect on the development of the infant's own immune response [23–25], and has been presented to account for the decreased humoral immune response of the younger infants.

Despite the extensive literature covering the humoral immune response triggered by the YF vaccines in adults and children, knowledge about the overall cellular immunity following YF immunization still remains to be elucidated, with few studies focusing the multiparametric aspects of the innate and adaptive immunity following YF vaccination [26–33]. Studies performed in primovaccinated adults have demonstrated the coexistence of activation events and modulatory pathways. In addition, a mixed pattern of cytokine profile is also observed as the hallmark of YF-17DD vaccination in adults [31–33]. Regardless of the relevance of the immune response triggered parallel to the establishment of distinct patterns of seroconversion after YF vaccination, there are no studies covering the issue of overall cellular immunity.

The purpose of this study is to evaluate whether the immune response triggered in YF-17DD-vaccinated children resembled the overall pattern previously described in adults and whether the cellular immune response is somehow related to the levels of YF-neutralizing antibodies. To test these hypotheses, we have characterized the impact of YF antigen recall in the intracytoplasmic cytokine pattern of peripheral blood leukocytes and the association between specific cytokine signatures with distinct postvaccination seroconversion status.

## METHODS

### Study Population

This study was coordinated by the Collaborative Group for Studies of Yellow Fever Vaccine [34] and included 60 healthy children (34 males and 26 females) from 9 to 43 months of age,

living in the metropolitan area of Belo Horizonte-Minas Gerais and Brasília-Distrito Federal, Brazil.

The study was approved by the Ethical Committee of Oswaldo Cruz Foundation FIOCRUZ—protocol number 236A/03. The children were formally included in the study if the free and informed consent form was signed by parents or legal guardians.

Prior to and 30 days after YF-17DD vaccination, children were requested to provide a blood sample to detect anti-YF-neutralizing antibodies using the plaque reduction neutralization test (PRNT), performed as described by Stefano et al. [12]. The tests were carried out at Laboratório de Tecnologia Viroológica, Bio-Manguinhos/FIOCRUZ, under the supervision of one of us (M. S. F.). The results are expressed as milli-International Units/mL (mIU/mL). A PRNT result of  $\geq 2.5 \log_{10}$  mIU/mL was used as the cut-off mark to segregate seroconverters from negative and nonseroconverters.

According to the PRNT results at 30 days post vaccination, the children were classified as seroconverters (PRNT  $\geq 2.5 \log_{10}$  mIU/mL) or nonseroconverters (PRNT  $< 2.5 \log_{10}$  mIU/mL), and referred as PV-PRNT<sup>+</sup> ( $n = 30$ , 15 males and 15 females, age range 9–12 months, median = 9 months) and PV-PRNT<sup>-</sup> ( $n = 10$ , 6 males and 4 females, age range 12–14 months, median = 12 months), respectively. Children within the PRNT<sup>+</sup> group were subdivided into 2 groups named PV-PRNT<sup>MEDIUM+</sup> ( $2.5 \leq \text{PRNT} \leq 3.5 \log_{10}$  mIU/mL,  $n = 20$ ) and PV-PRNT<sup>HIGH+</sup> (PRNT  $> 3.5 \log_{10}$  mIU/mL,  $n = 10$ ). The PV-PRNT<sup>-</sup> volunteers were submitted to revaccination 1 year after primovaccination and referred to as RV-PRNT<sup>+</sup> ( $n = 9$ , 6 males and 4 females, age range 40–43 months, median = 41 months) because all volunteers seroconvert after revaccination (PRNT  $\geq 2.5 \log_{10}$  mIU/mL).

One year after primo/revaccination, peripheral blood samples (7 mL) were collected into Vacutainer tubes containing sodium heparin (Becton Dickinson, San Jose, CA) for short-term whole blood cultures and intracytoplasmic cytokines analyses by flow cytometry.

Blood samples from 10 unvaccinated seronegative children (PRNT  $< 2.5 \log_{10}$  mIU/mL, 7 males and 3 females, age range 9–12 months, median = 9 months) collected prior to vaccination were included as a control group and referred to as UV-PRNT.

### Short-term Whole Blood Culture and Intracytoplasmic Cytokine Staining

In vitro short-term cultures of whole blood samples were performed as described by Luiza-Silva et al. [33] and modified as follows: aliquots of heparinized peripheral blood were incubated for 6 hours at 37°C, 5% CO<sub>2</sub>, in the presence of RPMI 1640 medium (GIBCO, Grand Island, NY; control culture) or in the presence of live attenuated YF-17DD vaccine (lot 055VFA054P, Bio-Manguinhos, FIOCRUZ) at a final concentration of  $2.5 \times 10^3$  viral particles/mL in RPMI 1640

plus (YF-Ag culture). Following in vitro YF-17DD antigen priming, cells were reincubated in the presence of brefeldin A (BFA; Sigma Chemical Company, St. Louis, MO) at 10 µg/mL for an additional period of 4 hours at 37°C, 5% CO<sub>2</sub>, and then treated with 2 mM final concentration of ethylenediamine tetraacetic acid (EDTA; Sigma Chemical Company) for 10 minutes at room temperature. Cell suspension was washed with fluorescence-activated cell sorting (FACS) buffer (phosphate buffered saline [PBS], pH 7.2, supplemented with 0.5% bovine serum albumin and 0.1% sodium azide, all from Sigma Chemical Company) and aliquots stained with fluorescent antihuman cell surface monoclonal antibodies (all from Caltag, Burlingame, CA): anti-CD4-Tricolor-TC (clone #S3.5), anti-CD8-TC (clone #3B.5), anti-CD14-TC (clone #Tük4), anti-CD16-TC (clone #3G8), and anti-CD19-TC (clone #ST25-C1) for 30 minutes at room temperature. After membrane staining, erythrocyte lysis, and leukocytes fixation, cell suspension was permeabilized with FACS perm-buffer (FACS buffer supplemented with 0.5% saponin) and aliquots incubated for 30 minutes at room temperature, in the dark, with fluorescent anticytokine monoclonal antibodies (all from BD-Pharmingen, San Jose, CA), including anti-IL-12-PE (clone #C11.5), anti-IFN- $\gamma$ -PE (clone #4S.B3), anti-TNF- $\alpha$ -PE (clone #Mab11), anti-IL-4-PE (clone #8D4-8), anti-IL-5-PE (clone #TRFK5), and anti-IL-10-PE (clone #JES3-19F1). After intracytoplasmic cytokine staining, the leucocytes were washed with FACS perm-buffer and FACS buffer and fixed in FACS fixing solution (10 g/L paraformaldehyde, 10.2 g/L sodium cacodylate, and 6.63 g/L sodium chloride, pH 7.2).

### Flow Cytometry Acquisition and Analysis

Immunostained samples were acquired using a FACScalibur flow cytometer, and CELLQuest software was used to perform data acquisition and analysis. A total of 30,000 events/tube were acquired and stored for further analysis. Distinct gating strategies were used to analyze the cytokine-expressing leukocytes subsets, including neutrophils, monocytes, natural killer (NK) cells, and lymphocytes (CD4<sup>+</sup>, CD8<sup>+</sup> T-cell subsets, and B cells) as previously reported by Luiza-Silva et al. [33]. The results were first expressed as percentages of cytokine-positive cells for different gated leukocyte subpopulations analyzed in this study. These values were used to obtain the cytokine index, calculated as the ratio between the percentage of cytokine-positive cells observed in the stimulated culture (YF-Ag) by the percentage of cytokine-positive cells observed in the unstimulated culture (control).

### Cytokine Signature Analysis

The cytokine profile was first assessed to identify low and high cytokine producers, as previously suggested by Luiza-Silva et al. [33]. Briefly, after the establishment of the global median YF-Ag/control index, each leukocyte subsets from all volunteers were tagged as they display low (□), high proinflammatory

(■), or high regulatory (▣) cytokine indexes. The percentage of volunteers showing high cytokine indexes was calculated for each leukocyte subset. The ascendant frequency of high cytokine indexes for PV-PRNT<sup>+</sup> was then used as the reference cytokine curves (—■—■) to identify changes in the overall cytokine patterns from all other groups. Radar charts were further used to summarize the proinflammatory (■) or regulatory (▣) cytokine signatures in a range of leukocyte subsets of innate and adaptive immunity. Each axis represents the frequency (%) of volunteers showing high cytokine indexes.

### Statistical Analysis

Statistical analyses between groups were performed by parametric analysis of variation (ANOVA) followed by Student *t* test or nonparametric Kruskal-Wallis test followed by the Mann-Whitney test. Spearman rank correlation test was performed to assess the association between the levels of PRNT antibodies (log<sub>10</sub> mIU/mL) and the cytokine indexes (YF-Ag/control). In all cases, significance was considered at *P* ≤ .05. All tests were provided by GraphPad Prism version 5.0 (San Diego, CA).

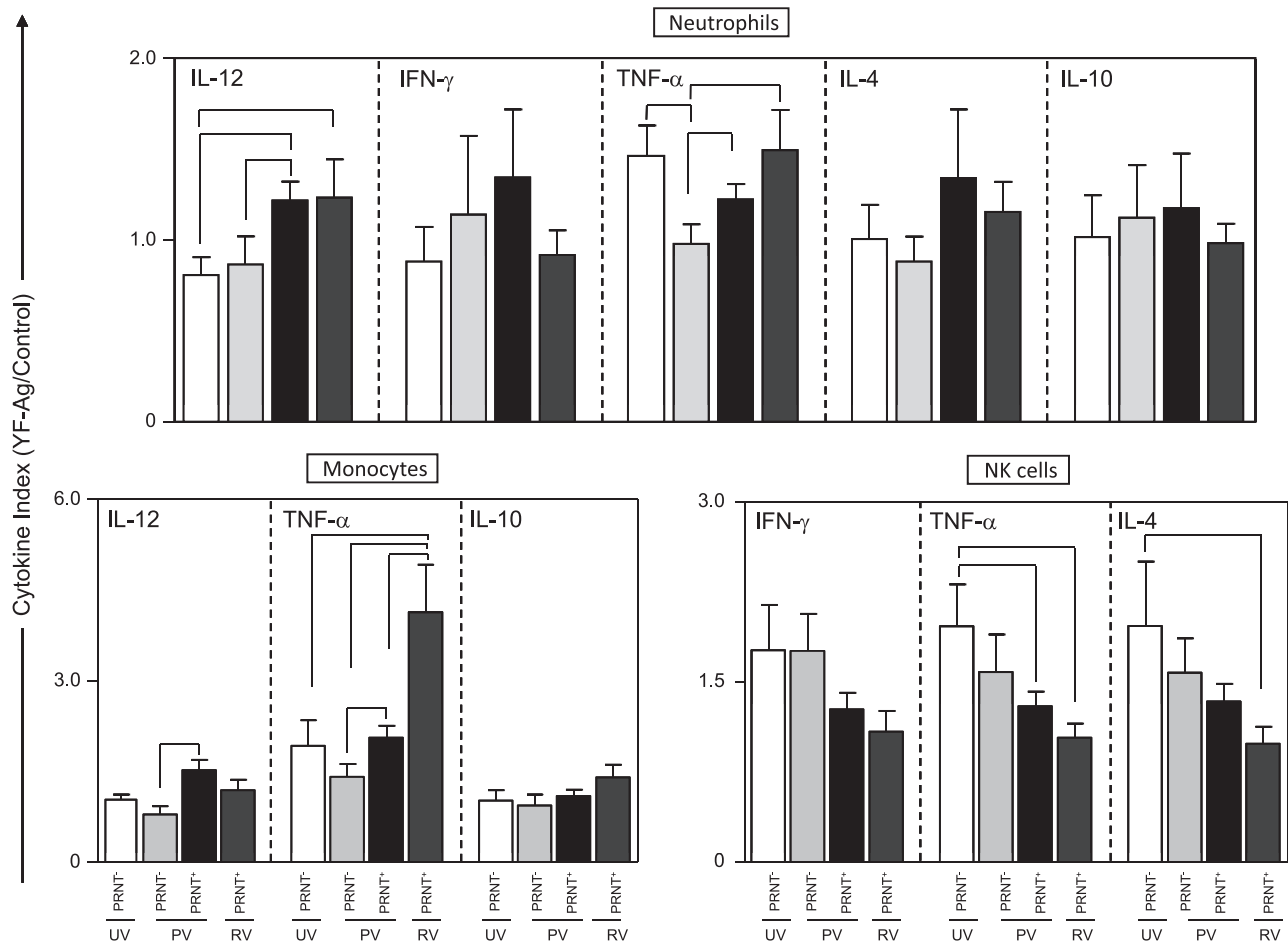
The analysis of cytokine signatures was performed using the PV-PRNT<sup>+</sup> cytokine signature as the reference curve, and significant differences were considered when the values emerged outside the quartile of the reference signature.

## RESULTS

### Impact of In Vitro YF-Vaccinal Antigen Stimuli on the Cytokine Profile of Innate and Adaptive Immunity in YF-17DD Primovaccinated Children

The frequency of cytokine<sup>+</sup> cells (IL-12<sup>+</sup>, IFN- $\gamma$ <sup>+</sup>, TNF- $\alpha$ <sup>+</sup>, IL-4<sup>+</sup>, IL-5<sup>+</sup>, and IL-10<sup>+</sup>), focusing on circulating neutrophils, monocytes, NK cells, CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, and B cells, was determined after short-term in vitro incubation of whole blood samples. Our data demonstrated that the YF-Ag recall was able to increase the synthesis of IL-12 by neutrophils in PV-PRNT<sup>+</sup> compared with PV-PRNT<sup>-</sup> and UV-PRNT<sup>-</sup>, as well as IL-12 by monocytes compared with PV-PRNT<sup>-</sup>. In addition, whereas the YF-Ag recall decreased the synthesis of TNF- $\alpha$  by neutrophils and monocytes in PV-PRNT<sup>-</sup>, the levels of TNF- $\alpha$  in PV-PRNT<sup>+</sup> was higher and comparable to that observed in UV-PRNT<sup>-</sup> (Figure 1). Furthermore, the YF-Ag recall decreased the synthesis of IL-4 by CD4<sup>+</sup> T cells in PV-PRNT<sup>+</sup> compared with UV-PRNT<sup>-</sup> (Figure 2). Together, these findings demonstrated that the seroconversion after YF-17DD primovaccination in children is able to guarantee, upon YF-Ag recall, the establishment of a proinflammatory microenvironment via enhanced synthesis of IL-12 and TNF- $\alpha$  by neutrophils and monocytes and down-regulated the production of IL-4 by CD4<sup>+</sup> T cells.

Conversely, the lack of seroconversion after YF-17DD primovaccination promote a regulatory status upon YF-Ag recall, with lower production of IL-12 by neutrophils and monocytes



**Figure 1.** Impact of YF-Ag in the cytokine profile of innate immune cells from UV-PRNT<sup>-</sup> (in white), PV-PRNT<sup>-</sup> (in light gray), PV-PRNT<sup>+</sup> (in black), and RV-PRNT<sup>+</sup> (in dark gray) children groups 1 year after YF-17DD vaccination or revaccination. Intracytoplasmic cytokine studies were performed after *in vitro* short-term incubation. To identify neutrophils (top panels) and NK cells (bottom panels), anti-CD16 TC mAbs were used, and to identify monocytes (middle panels) CD14 TC mAbs were used. Anti-cytokines-PE mAbs were used to determine inflammatory and regulatory cytokines<sup>+</sup> of innate immunity leukocytes in peripheral blood samples. The index ratio was calculated as the ratio of percentage of cells that express the cytokine of interest in the stimulated culture (YF-Ag) to the percentage of cytokine<sup>+</sup> cells in the unstimulated (control) culture. Results are expressed in bars representing mean values  $\pm$  SEM. Significant differences (connecting lines) at  $P < .05$  are shown on the figure.

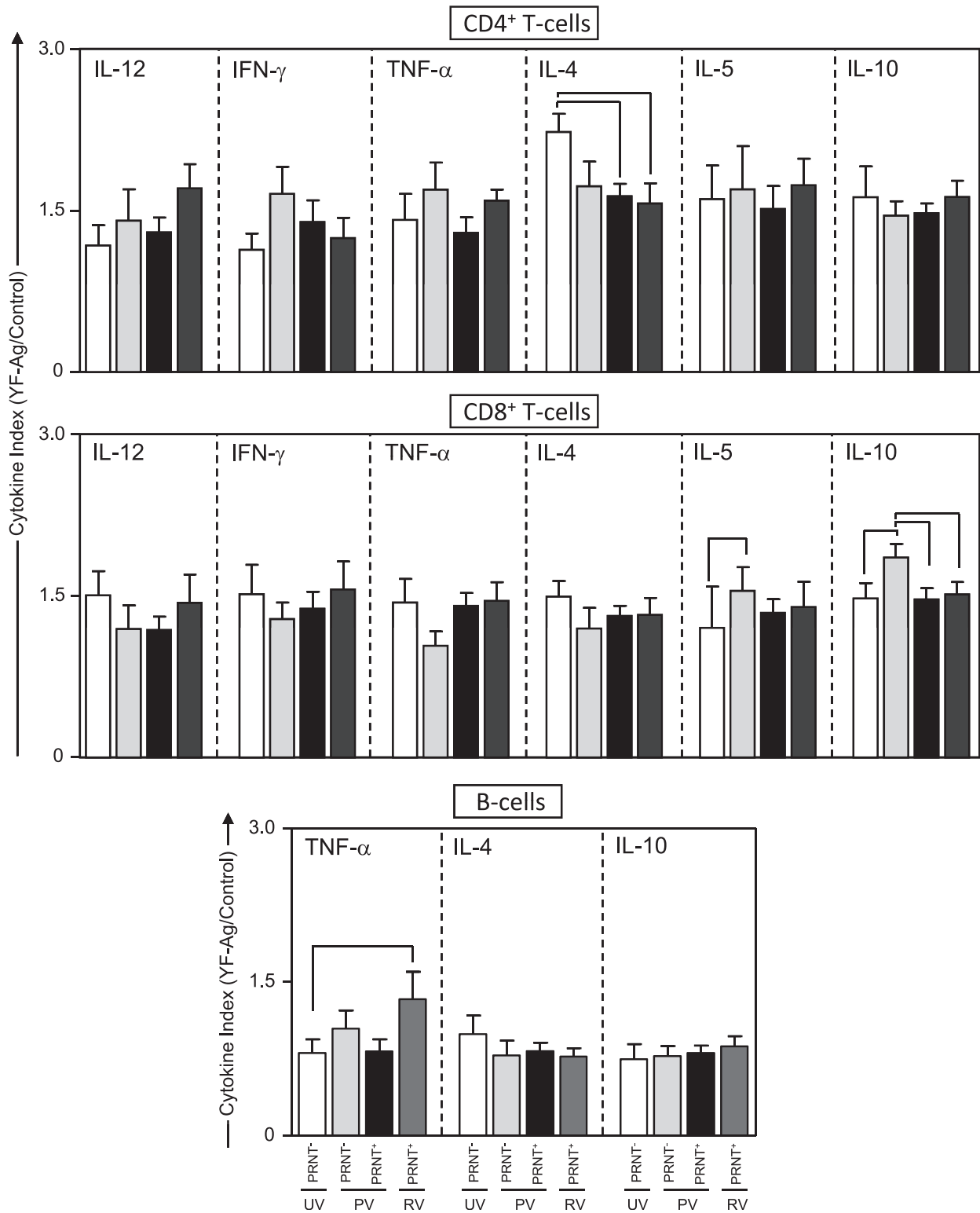
compared with PV-PRNT<sup>+</sup>, and lower synthesis of TNF- $\alpha$  by neutrophils compared with all other groups and TNF- $\alpha$  by monocytes compared with PRNT<sup>+</sup> groups (Figure 1). Moreover, the PV-PRNT<sup>-</sup> also displayed enhanced synthesis of IL-5 by CD8<sup>+</sup> T cells compared with UV-PRNT<sup>-</sup>, and IL-10 by CD8<sup>+</sup> T cells compared with all other groups (Figure 2).

It was interesting to notice that the revaccination accompanied by protective levels of neutralizing antibodies modify the cytokine response of innate immunity toward increased synthesis of IL-12 and TNF- $\alpha$  by neutrophils compared with UV-PRNT<sup>-</sup> and PV-PRNT<sup>-</sup>, respectively (Figure 1). Moreover, a marked increase in the synthesis of TNF- $\alpha$  by monocytes was also observed in RV-PRNT<sup>+</sup> compared with all other groups. A general inhibition of cytokine synthesis by NK cells (IFN- $\gamma$ , TNF- $\alpha$ , and IL-4) was observed in RV-PRNT<sup>+</sup> (Figure 1). Additionally, in response to YF-Ag recall, the RV-PRNT<sup>+</sup> also displayed a putative

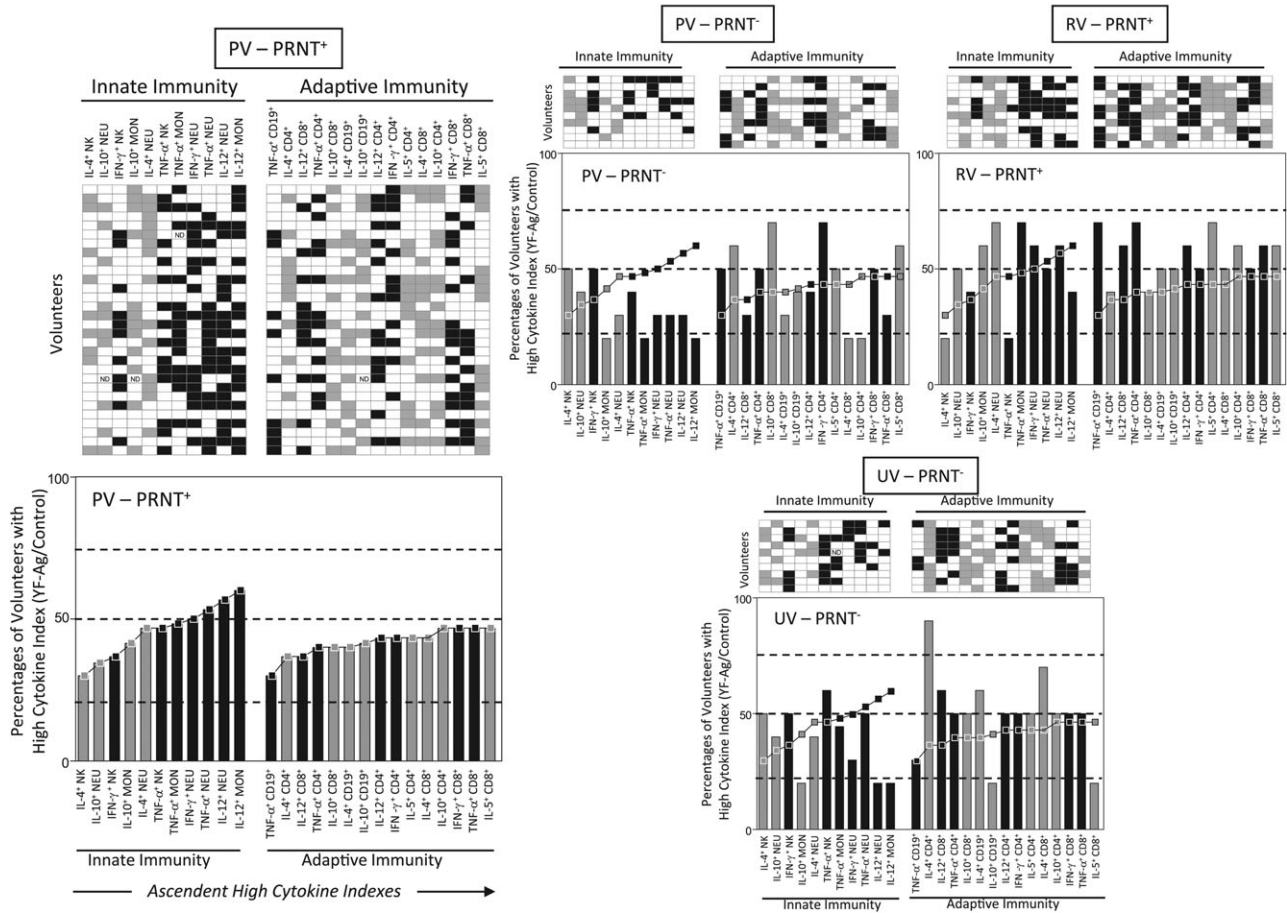
proinflammatory pattern in the adaptive immunity, with increased production of TNF- $\alpha$  by B cells and lower production of IL-4 by CD4<sup>+</sup> T cells compared with UV-PRNT<sup>-</sup>, along with basal synthesis of IL-10 by CD8<sup>+</sup> T cells (Figure 2). Together, it seems that the revaccination of YF-17DD nonseroconverter shifted the microenvironment triggered by the YF-Ag recall toward a proinflammatory profile, with higher synthesis of TNF- $\alpha$  by neutrophils, monocytes, and B cells along with lower production of IL-4 by CD4<sup>+</sup> T cells and IL-10 by CD8<sup>+</sup> T cells, despite the overall inhibition of cytokine synthesis by NK cells.

#### Overall Signature of High Cytokine Producers in the Innate and Adaptive Immune Compartments Triggered by YF-Ag Recall in 17DD Vaccinees

Aiming to further characterize the overall cytokine signature of innate and adaptive immunity compartments, we have calculated



**Figure 2.** Impact of YF-Ag in the cytokine profile of adaptive immune cells from UV-PRNT<sup>-</sup> (in white), PV-PRNT<sup>-</sup> (in light gray), PV-PRNT<sup>+</sup> (in black), and RV-PRNT<sup>+</sup> (in dark gray) children groups 1 year after YF-17DD vaccination or revaccination. Intracytoplasmic cytokine studies were performed after in vitro short-term incubation. To identify CD4<sup>+</sup> T cells (top panels), CD8<sup>+</sup> T cells (middle panels), and B cells (bottom panels), anti-CD4 TC, anti-CD8 TC, and anti-CD19 FITC mAbs were used, respectively. Anticytokines PE mAbs were used to determine proinflammatory and regulatory cytokines<sup>+</sup> of adaptive immunity leukocytes in peripheral blood samples. The index ratio was calculated as the ratio of the percentage of cells that express the cytokine of interest in the stimulated culture (YF-Ag) to the percentage of cytokine<sup>+</sup> cells in the unstimulated (control) culture. The results are expressed in bars representing mean values  $\pm$  SEM. Significant differences (connecting lines) at  $P < .05$  are shown.



**Figure 3.** Overall signature of high cytokine producers in the innate and adaptive immune compartments triggered by in vitro YF-Ag stimulation. The diagrams were plotted using the global median cytokine index (YF-Ag/control) as the cut-off mark to identify each volunteer as a low (□) or high (■) cytokine producer in the PV-PRNT<sup>+</sup>, PV-PRNT<sup>-</sup>, RV-PRNT<sup>+</sup>, and UV-PRNT<sup>-</sup> groups. The ascendant frequency of high cytokine producers at the innate and adaptive immunity of each group was demonstrated by bar graphics. The ascendant frequency of high cytokine producers of the PV-PRNT<sup>+</sup> group was used to generate the reference cytokine signature curves (—■—■—) that were applied to identify changes in the overall cytokine signature from all other groups.

the global median cytokine index (YF-Ag/control) for each cell population (IL-12<sup>+</sup>NEU = 0.9; IFN- $\gamma$ <sup>+</sup>NEU = 0.8; TNF- $\alpha$ <sup>+</sup>NEU = 1.1; IL-4<sup>+</sup>NEU = 0.9, IL-10<sup>+</sup>NEU = 0.9; IL-12<sup>+</sup>MON = 1.1; TNF- $\alpha$ <sup>+</sup>MON = 1.8; IL-10<sup>+</sup>MON = 1.0; IFN- $\gamma$ <sup>+</sup>NK = 1.2; TNF- $\alpha$ <sup>+</sup>NK = 1.3; IL-4<sup>+</sup>NK = 1.3; IL-12<sup>+</sup>CD4<sup>+</sup> = 1.2; IFN- $\gamma$ <sup>+</sup>CD4<sup>+</sup> = 1.1; TNF- $\alpha$ <sup>+</sup>CD4<sup>+</sup> = 1.4; IL-4<sup>+</sup>CD4<sup>+</sup> = 1.6; IL-5<sup>+</sup>CD4<sup>+</sup> = 1.3; IL-10<sup>+</sup>CD4<sup>+</sup> = 1.4; IL-12<sup>+</sup>CD8<sup>+</sup> = 1.1; IFN- $\gamma$ <sup>+</sup>CD8<sup>+</sup> = 1.2; TNF- $\alpha$ <sup>+</sup>CD8<sup>+</sup> = 1.2; IL-4<sup>+</sup>CD8<sup>+</sup> = 1.3; IL-5<sup>+</sup>CD8<sup>+</sup> = 1.2; IL-10<sup>+</sup>CD8<sup>+</sup> = 1.5; TNF- $\alpha$ <sup>+</sup>CD19<sup>+</sup> = 0.8; IL-4<sup>+</sup>CD19<sup>+</sup> = 0.8; IL-10<sup>+</sup>CD19<sup>+</sup> = 0.8; where NEU = neutrophil, MON = monocyte, and NK = natural killer) and used these values as the cut-off mark to tag the cell population from each volunteer as being a low (□) or high (■) cytokine producer. The assembling of the ascendant frequency of high cytokine producers at the innate and adaptive immunity of PV-PRNT<sup>+</sup> vaccinees generated the reference cytokine signature curves (—■—■—) applied to identify changes in the overall

cytokine signature from all other groups (PV-PRNT<sup>-</sup>, RV-PRNT<sup>+</sup>, and UV-PRNT<sup>-</sup>). Our findings demonstrated that the cytokine signature curves triggered by the YF-Ag recall in PV-PRNT<sup>+</sup> was characterized by a prominent involvement of innate immunity (mainly represented by NEU and MON) along with an uniform participation of the adaptive immunity cells (T-cell subsets and B lymphocytes) (Figure 3).

The comparative analysis of the cytokine signature, taking the PV-PRNT<sup>+</sup> as reference curves, demonstrated that the PV-PRNT<sup>-</sup>, likewise the UV-PRNT<sup>-</sup>, presented an impaired involvement of innate immunity cells upon YF-Ag recall (specially represented by NEU and MON) with punctual enhancement in the adaptive immunity cells (especially CD4<sup>+</sup> and CD8<sup>+</sup> T cells) (Figure 3). Interestingly, the RV-PRNT<sup>+</sup> group are able to almost overturn the deficient involvement of the innate immunity, restoring almost all the cytokine profile of NEU and MON, leading to even over-frequency of high cytokine producers in

the innate and adaptive immunity, despite the overall impairment of NK cells (Figure 3).

### Overall Pattern of Proinflammatory and Regulatory High Cytokine Producers Triggered by YF-Ag Recall in 17DD Vaccinees

In order to further characterize the overall cytokine pattern of YF-17DD-vaccinated children, we have constructed radar charts to characterize the frequency of high proinflammatory and regulatory cytokine producers in the PV-PRNT<sup>+</sup> group (Figure 4, top chart) to be used as a standard to identify changes in the overall cytokine pattern in PV-PRNT<sup>-</sup>, RV-PRNT<sup>+</sup>, and UV-PRNT<sup>-</sup> (Figure 4, middle and bottom charts).

Our data demonstrate that the overall cytokine pattern of the PV-PRNT<sup>+</sup> group was characterized by a prominent participation of the proinflammatory compartment of the innate immune response (especially driven by IL-12<sup>+</sup> and TNF- $\alpha$ <sup>+</sup> NEU and MON along IFN- $\gamma$ <sup>+</sup> NEU). This profile was counterbalanced by a uniform microenvironment of regulatory cytokine (driven by uniform synthesis of IL-4, IL-5, and IL-10 from CD4<sup>+</sup> and CD8<sup>+</sup> T cells) (Figure 4, top chart).

The comparative analysis of the overall cytokine pattern observed in PV-PRNT<sup>-</sup> demonstrated a relevant deficiency in the synthesis of proinflammatory cytokines at innate immunity (mainly IL-12<sup>+</sup> and TNF- $\alpha$ <sup>+</sup> NEU and MON) along with punctual enhancement of regulatory cytokines (especially IL-4 from NK and CD4<sup>+</sup> T cells along with IL-10 and IL-5 from CD8<sup>+</sup> T cells) (Figure 4, middle left chart).

Interestingly, the RV-PRNT<sup>+</sup> was able to overturn the impaired involvement of proinflammatory cytokines and restore regulatory profile, leading to an over-frequency of high cytokine producers (Figure 4, middle right chart).

The cytokine pattern of UV-PRNT<sup>-</sup> somehow resembles the profile observed for the PV-PRNT group (Figure 4, bottom chart).

### Association Between the Serum Levels of Anti-YF-Neutralizing Antibodies After 17DD Primovaccination and the Overall Signature of High Cytokine Producers Triggered by YF-Ag Recall

Based on the serum levels of anti-YF-neutralizing antibodies, the 17DD primovaccinees were categorized into 3 distinct subgroups, referred to as PRNT<sup>-</sup>, PRNT<sup>MEDIUM+</sup>, and PRNT<sup>HIGH+</sup>. Using this criterion, our data demonstrated that all UV-PRNT<sup>-</sup> and PV-PRNT<sup>-</sup> were confined to a level below the cut-off mark ( $<2.5 \log_{10}$  mUI/mL). The PV-PRNT<sup>+</sup> and RV-PRNT<sup>+</sup> groups presented levels of neutralizing antibodies above the cut-off mark ( $\geq 2.5 \log_{10}$  mUI/mL). The PV-PRNT<sup>+</sup> group presented distinct patterns of neutralizing antibodies, including MEDIUM (2.5–3.5  $\log_{10}$  mUI/mL) or HIGH ( $>3.5 \log_{10}$  mUI/mL) (Figure 5, top panel). Our findings demonstrated that PV-PRNT<sup>MEDIUM+</sup> presented an overall balanced cytokine signature confined to the second quartile. It was interesting to notice that the polarization of humoral immune response toward PRNT<sup>-</sup> or PRNT<sup>HIGH+</sup>

was associated with dichotomic cytokine signatures. While the PV-PRNT<sup>-</sup> presented a cytokine profile with a slight increase in the regulatory pattern on the adaptive immunity (IL-4<sup>+</sup>CD4<sup>+</sup> T cells along with IL-5<sup>+</sup> and IL-10<sup>+</sup>CD8<sup>+</sup> T cells), the PV-PRNT<sup>HIGH+</sup> displayed a cytokine signature associated with an enhanced proinflammatory profile, mainly in the innate immunity compartment (IL-12<sup>+</sup> NEU and MON along with IFN- $\gamma$ <sup>+</sup> NEU) (Figure 5, middle panel). Additional correlation analyses validate these findings, demonstrating a positive correlation between the inflammatory response of innate immunity (IL-12<sup>+</sup> NEU and MON along with IFN- $\gamma$ <sup>+</sup> NEU) and the levels of PRNT antibodies as well as TNF- $\alpha$ <sup>+</sup>CD8<sup>+</sup> T cells. On the other hand, negative correlation indexes were observed between IL-4<sup>+</sup>CD4<sup>+</sup> and IL-10<sup>+</sup>CD8<sup>+</sup> T cells (Figure 5, bottom panels).

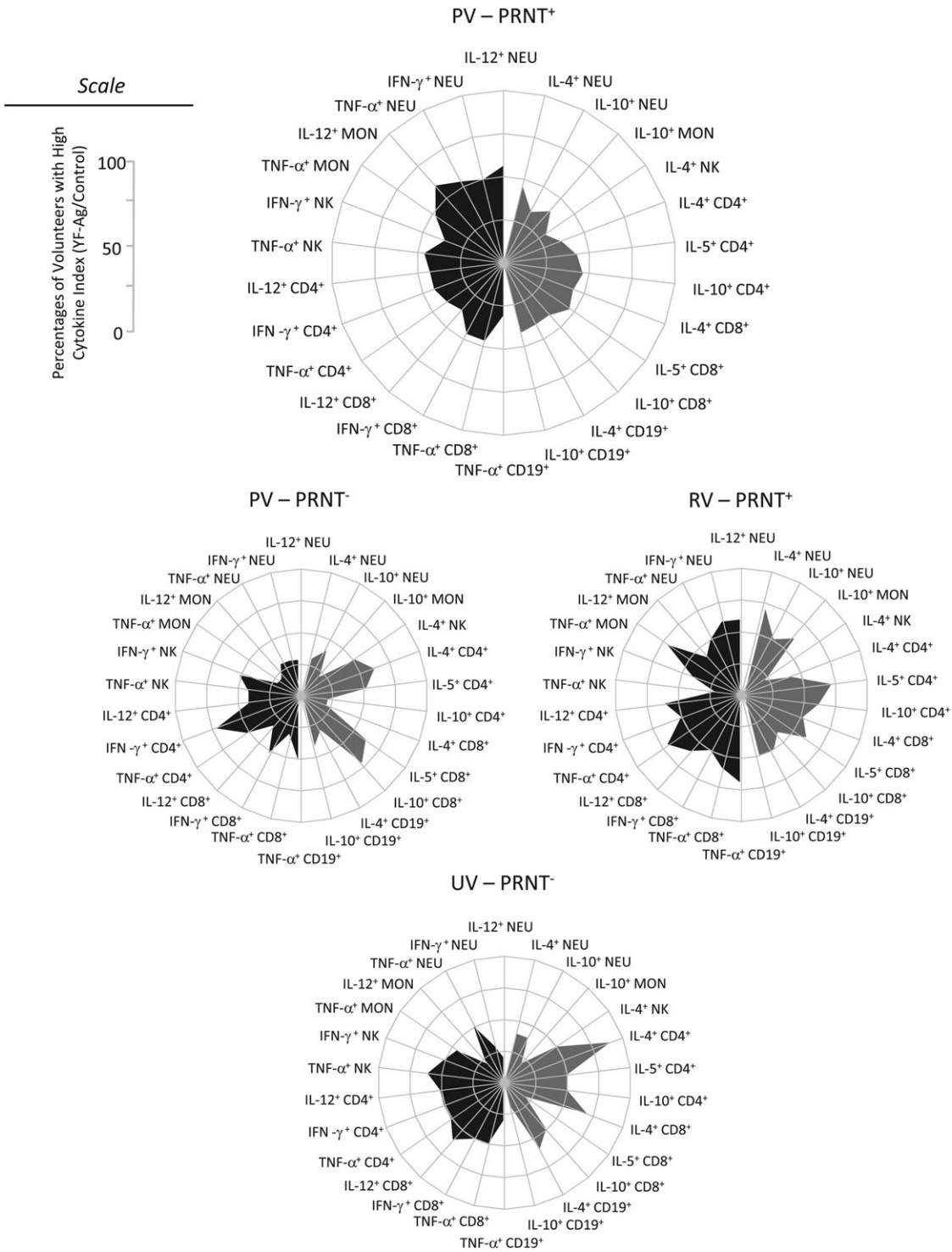
Taken together, our results demonstrated in YF-17DD primovaccinees that the overall signature of high cytokine producers triggered by YF-Ag recall is associated with the levels of anti-YF-neutralizing antibodies, with a balanced proinflammatory and regulatory profile of innate and adaptive immunity being the hallmark of PV-PRNT<sup>MEDIUM+</sup>, whereas a polarized regulatory signature is observed in PV-PRNT<sup>-</sup> and a prominent proinflammatory signature is characteristic of PV-PRNT<sup>HIGH+</sup>.

## DISCUSSION

The purpose of our study is to evaluate immune responses in infants vaccinated at 9 months of age with YF-17DD vaccine through detailed investigation of the in vitro YF antigens impact in the intracytoplasmic cytokine pattern of peripheral innate and adaptive leukocytes, aiming to characterize the cytokines profile involved with status vaccination.

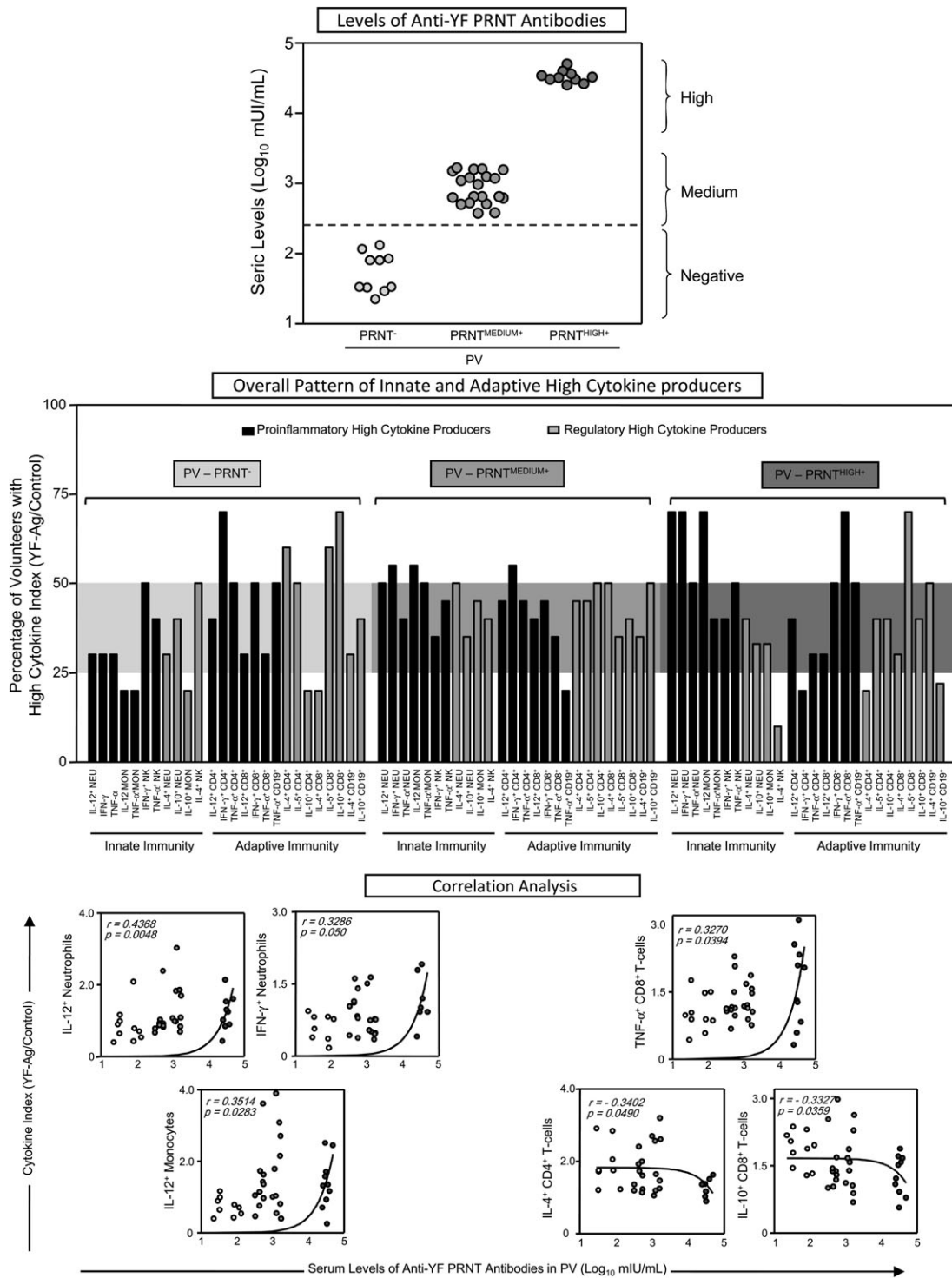
Little is known about the immune response in children following YF vaccination. Previous reports have postulated that infants have deficiencies in primary antigen presentation by dendritic cells, limited T-cell proliferation, impaired B-cell function, and reduced production of cytokines by helper T cells of the type 1 subset (TH1), including interleukin 2 (IL-2) and interferon gamma (IFN- $\gamma$ ) [35–41]. In the present investigation, our data demonstrated that the impact of in vitro YF-vaccinal antigen stimuli on the cytokine profile of innate and adaptive immunity in YF-17DD primovaccinated children promoted distinct immunological profiles characterized by a balanced involvement of proinflammatory/regulatory adaptive immunity with a prominent participation of the innate immunity proinflammatory events in the PV-PRNT<sup>+</sup> group mediated by IL-12<sup>+</sup> and TNF- $\alpha$ <sup>+</sup>NEU and MON, along with down-regulation of IL-4<sup>+</sup>CD4<sup>+</sup> T cells. Conversely, the absence of seroconversion in the PV-PRNT<sup>-</sup> children was accompanied by a regulatory status with lower index of IL-12<sup>+</sup> and TNF- $\alpha$ <sup>+</sup>NEU and MON, along with up-regulation of IL-5<sup>+</sup> and IL-10<sup>+</sup>CD8<sup>+</sup> T cells. Moreover, the analysis of the cytokine signature confirmed that these subjects presented a striking lack of proinflammatory response

Overall Pattern of Inflammatory/Regulatory High Cytokine Indexes



**Figure 4.** Overall pattern of proinflammatory and regulatory high cytokine producers triggered by YF-Ag recall in 17DD vaccines. Radar chart summarizes the cytokine signatures the proinflammatory (■) or regulatory (▒) cytokine balance in a range of cell subsets of innate and adaptive immunity from the PV-PRNT<sup>+</sup>, PV-PRNT<sup>-</sup>, RV-PRNT<sup>+</sup>, and UV-PRNT<sup>-</sup> groups. Each axis displays the proportion of each cytokine balance category within a given leukocyte subset.





**Figure 5.** Association between the serum levels of anti-YF-neutralizing antibodies after 17DD vaccination and the overall signature of high cytokine producers triggered by YF-Ag recall. Top panel represents the categorization of study group according to the serum levels of anti-YF-neutralizing antibodies of 17DD after primo/revaccination in PV-PRNT<sup>-</sup>, PV-PRNT<sup>MEDIUM+</sup>, and PV-PRNT<sup>HIGH+</sup>. Middle panel displays the association between the overall signature of high cytokine producers and the serum levels of anti-YF-neutralizing antibodies in PV-PRNT<sup>-</sup>, PV-PRNT<sup>MEDIUM+</sup>, and PV-PRNT<sup>HIGH+</sup> groups; bottom panels demonstrates the positive correlation between IL-12<sup>+</sup> neutrophils and monocytes, and IFN- $\gamma$ <sup>+</sup> neutrophils and the levels of PRNT antibodies (bottom left panels). Bottom right panels display negative correlation of IL-4<sup>+</sup>CD4<sup>+</sup> T cells, as well as the positive correlation of TNF- $\alpha$ <sup>+</sup> and negative correlation of IL-10<sup>+</sup> in CD8<sup>+</sup> T cells and the levels of PRNT antibodies. The data are expressed as scattering of all individual values (UV; PRNT<sup>-</sup>; and low, moderate, and high PRNT<sup>+</sup> groups) and include the frequency of cytokines<sup>+</sup> cells and the levels of PRNT antibodies (mIU/mL). Correlation indexes ( $r$  and  $P$  values) are provided in the figure.

in the innate immunity compartment besides an overall increased adaptive regulatory profile. This resultant regulatory profile observed in PV-PRNT<sup>-</sup> children, similar to that observed in unvaccinated children (UV-PRNT<sup>-</sup>), suggests a putative vulnerability of these subjects to develop a risky immune response upon contact with the wild-type YF virus, favoring viral replication, especially due to the lack of proinflammatory pathways in the innate immunity.

Interestingly, when the PV-PRNT<sup>-</sup> children were revaccinated, all volunteers seroconverted and the overall regulatory cytokine signature were shifted toward a proinflammatory status, similar to that observed in the seroconverter group (PV-PRNT<sup>+</sup>), especially mediated by TNF- $\alpha$ <sup>+</sup>NEU, MON, and B cells, along with lower levels of IL-4<sup>+</sup>CD4<sup>+</sup> and IL-10<sup>+</sup>CD8<sup>+</sup> T cells. These data demonstrated that along with the seroconversion event, the 17DD-YF vaccine booster was able to restore almost all immunological events observed in the ideal reference cytokine signature characterized by a balanced involvement of proinflammatory/regulatory immunity. The presence of neutralizing antibodies along with the pro-inflammatory/regulatory cytokine signature observed in the re-vaccinated children may represent a relevant biomarker of protective immunity upon the contact with the YF wild virus. This shift in the immunological status suggests that revaccination could be helpful to guarantee the seroconversion besides a protective cellular immune response, especially in endemic areas where vaccination is the primary way to prevent infection and children are constantly exposed to wild YF virus.

Our data demonstrated that the overall cytokine signature observed in the PV-PRNT<sup>+</sup> group was somehow associated with the levels of PRNT antibodies, with a predominant proinflammatory pattern observed in those individuals that displayed a higher PRNT profile. These finding may suggest that higher levels of PRNT antibodies may be a good biomarker, and may be associated with a prominent antiviral immune response.

It is important to note that this study focused mainly on the interface of the humoral and cellular immune response with regard to major consequences that distinct patterns of seroconversion may have in the overall cytokine signatures—biomarkers of the cellular immune response. However, our investigation did not accomplish an important issue regarding the YF-17DD vaccination in children—the mechanisms underlying the establishment of distinct status of seroconversion observed in YF vaccinees [11–17]. Several proposals have been presented to explain this phenomenon, including the vaccine substrain (17D-204, 17D-213/77, or 17DD), the presence of residual maternal antibodies, the baseline cytokine signature at the individual level, and the interference of simultaneous viral vaccination [18–25]. The Collaborative Group for the Study of Yellow Fever Vaccines has already reported in an observational multicenter study [17] that the age at primovaccination is a relevant variable that would contribute to the seroconversion rate.

In that investigation, the 17DD-YF vaccine reached higher seroconversion rates among children older than 2 years: 97% among children >10 years and 94% among children 2–9 years old; data showed lower seroconversion rates in children <2 years: 88% for 12–23-month-olds, 72% for 9–11-month-olds, and 82% for 6–8-month-olds. In the present study, the age was not related to the lack of seroconversion after YF-17DD, since the mean age in the PV-PRNT<sup>-</sup> was even higher in comparison with the PV-PRNT<sup>+</sup>. Complementary kinetic studies early after YF-vaccination are still required to elucidate the mechanism responsible for the distinct profile of seroconversion.

## Funding

This research was supported by Bio-Manguinhos/Oswaldo Cruz Foundation (FIOCRUZ; grant 05/05, 05/08, and CC#009/2010), Council for Scientific and Technological Development (CNPq; grant #479663/2004-1, grant #308651/2006-5, grant #472782/2009-6, and PQ# 303316/2009-8), and the Minas Gerais State Research Foundation (FAPEMIG; grant# APQ 01183-08, APQ 01877-10, and PDJ 00265-09).

## Acknowledgments

We thank Dr Ivone Perez de Castro from the Secretaria de Saúde do Distrito Federal for coordinating the fieldwork in Brasília, DF, Brazil, and Rosângela Aparecida de Azevedo for the logistical support during fieldwork in Minas Gerais, Brazil; the Program for Technological Development of Health Products (PDTIS) for technological development in tools for health; and the Oswaldo Cruz Foundation (FIOCRUZ) for use of its facilities. E. G. K., A. T. C., and O. A. M. F. thank the National Council for Scientific and Technological Development (CNPq) for fellowships PQ. A. C. C. A. acknowledges the Minas Gerais State Research Foundation (FAPEMIG) for the fellowship PDJ 00265/09.

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