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Research paper

A novel whole blood assay to quantify the release of T cell associated cytokines in response to *Bordetella pertussis* antigens

Marta Valente Pinto [a,b,1], Alex-Mikael Barkoff [c,*,1], Sagida Bibi [a], Aapo Knuutila [c], Johanna Teräsjärvi [c], Elizabeth Clutterbuck [a], Sophie Gimenez-Fourage [e], Anke Pagnon [e], Jacqueline A.M. van Gaans-van den Brink [f], Veronique Corbiere [g], Aymeric De Montfort [h], Anja Saso [i,j], Haddijatou Jobe [i], Sophie Roetynck [i], Beate Kampmann [j,k], Elles Simonetti [l,m], Dimitri Diavatopoulos [l,m], Eleonora E. Lambert [f], Jussi Mertsola [c,d], Pascal Blanc [e], Cécile A.C.M. van Els [f], Dominic Kelly [a,n], Qiushui He [c], The PERISCOPE Consortium

[a] *Oxford Vaccine Group, Department of Paediatrics, University of Oxford, Oxford, UK*
[b] *Egas Moniz Center for Interdisciplinary Research (CiiEM), Egas Moniz School of Health & Science, Almada, Portugal*
[c] *Institute of Biomedicine, University of Turku, Turku, Finland*
[d] *Department of Paediatrics, Turku University Hospital, Turku, Finland*
[e] *Sanofi Pasteur, Marcy-l'Étoile, France*
[f] *Centre for Infectious Disease Control, National Institute for Public Health and The Environment (RIVM), Bilthoven, The Netherlands*
[g] *Université Libre de Bruxelles, Brussels, Belgium*
[h] *Centre Léon Bérard, Lyon, France*
[i] *Vaccine & Immunity Theme, MRC Unit the Gambia, Banjul, Fajara, Gambia*
[j] *Clinical Research, London School of Hygiene & Tropical Medicine, London, UK*
[k] *Institute of International Health, Charité – Universitätsmedizin Berlin, Berlin, Germany*
[l] *Laboratory of Medical Immunology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, The Netherlands*
[m] *Radboud Center for Infectious Diseases, Radboud University Medical Center, Nijmegen, The Netherlands*
[n] *Oxford NIHR Biomedical Research Centre, Oxford, UK*

A R T I C L E I N F O

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A B S T R A C T

Background: Bordetella pertussis continues to cause whooping cough globally even in countries with high immunisation coverage. Booster vaccinations with acellular pertussis vaccines are thus used in children, adolescents, and adults. T cell immunity is crucial for orchestrating the immune response after vaccination. However, T cell assays can be expensive and difficult to implement in large clinical trials. In this study, a whole blood (WB) stimulation assay was developed to identify secreted T cell associated cytokines in different age groups after acellular pertussis booster vaccination.

Material and methods: Longitudinal WB samples were collected from a small set of subjects ($n = 38$) aged 7–70 years participating in a larger ongoing clinical trial. For assay development, samples were diluted and incubated with purified inactivated pertussis toxin (PT), filamentous haemagglutinin (FHA), inactivated *B. pertussis* lysate, and complete medium (M) as stimulating conditions, with anti-CD28 and anti-CD49d as co-stimulants. Different timepoints around the vaccination (D0, D7, D14, D28), WB dilution factor (1:2, 1:4) and incubation time (24 h, 48 h, 72 h) were compared. Responses to 15 cytokines were tested with Luminex/multiplex immunoassay.

Results: The optimized assay consisted of WB incubation with M, PT, and FHA (including the two co-stimulants). After 48 h incubation, supernatants were collected for measurement of seven selected T cell associated cytokines (IL-2, IL-5, IL-10, IL-13, IL-17 A, IL-17F, and IFN-γ) from samples before and 28 days after vaccination. PT stimulation showed a trend for upregulation of IL-2, IL-13, and IL-17 A/F for adult subjects, whereas the responses of all cytokines were downregulated for the paediatric subjects. Furthermore, PT and FHA-stimulated WB showed diverse cytokine producing profiles.

Conclusions: The developed WB-based cytokine assay was shown to be less costly, easy to perform, and functional in differently aged individuals. Further, it requires only a small amount of fresh blood, which is beneficial

* Corresponding author at: Institute of Biomedicine, University of Turku, Turku, Finland.
E-mail address: ambark@utu.fi (A.-M. Barkoff).

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especially for studies including infants. Our results support the use of this assay for other immunological studies in the future.

1. Introduction

Bordetella pertussis continues to cause whooping cough (or pertussis) globally, even in countries with high immunisation coverage (European Centre for Disease Prevention and Control, 2016; Bordet and Gengou, 1906; Ratner et al., 2010). The first vaccines, composed of killed bacteria [whole cell (wP) pertussis vaccine], were developed in the 1920–1930s and led to a significant decrease in reported cases and deaths. (Cherry, 2013). The high reactogenicity of the wP vaccines led to a change to acellular pertussis (aP) vaccines around the 1990s, particularly in high income countries. However, a resurgence of cases in the last decade has prompted questions around the relative effectiveness of aP versus wP in the long-term control of pertussis within a routinely immunised population (Warfel et al., 2014). The differences in the type of immune response induced by aP and wP are still actively investigated.

The formation of antigen-specific T helper cell (CD4+) populations after different vaccinations has been extensively studied, showing e.g., that circulating T follicular helper cells (type 1) are related to formation of neutralizing antibodies after yellow fever vaccination, and that aging changes the T cell population balance after immunisation with different vaccines (Gustafson et al., 2020; Huber et al., 2020). In humans, further understanding of T cell associated cytokine responses induced after aP booster vaccinations is crucial for a better understanding of vaccine immunology. Preclinical models suggested that wP vaccines induced a T cell helper profile orientation closer to natural infection responses (Th1/Th17-skewed) than induced by aP vaccines (Th2/Th17-skewed) (Warfel and Merkel, 2014; Warfel et al., 2014).

Specific T cell assays can be expensive and difficult to implement in large clinical trials due to their technical complexity. Measurement of T cell associated cytokines in response to antigenic restimulation allows an indirect measure to estimate the type of possible T helper cell response, Th1/Th2/Th17, after immunisation. Th1 cells produce primarily IL-2, IL-12, and IFN-γ whereas Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13. Th17 cells, which are of high relevance for mucosal immunity, can be measured by the production of IL-17 A and IL-17F (da Silva Antunes et al., 2018). In previous studies, *B. pertussis* antigen-specific T cell responses have been measured by various cytokine readouts from stimulated peripheral blood mononuclear cells (PBMC) (Bancroft et al., 2016; Lambert et al., 2021; Hougardy et al., 2007; van der Lee et al., 2018). These PBMC-based approaches include white blood cell isolation steps and need a relatively large volume of blood for testing, and therefore are not feasible for infants or other young participants. Recently, a new set of assays was set up to detect and measure *B. pertussis* antigen specific T cells using whole blood (WB). These WB assays are mainly intended to detect intracellular cytokine production in low-frequency T cell populations by flow cytometry after short term restimulation of blood cells (Corbiere et al., 2023; Lambert et al., 2020).

Whole blood is a good choice for identification of immunological processes after vaccination as it captures circulating immune cells, which are migrating to and from organs and tissues. As collection of WB is easy, it also allows longitudinal sampling in clinical settings. Further, compared to PBMCs, it contains all host cells and serum factors, and allows cell-to-cell communication during the stimulation phase (Mûller et al., 2024). However, although flow cytometry-based assays are expensive and time consuming, they can be linked to T cell populations, whereas in sole cytokine assays performed with WB, the origin of the cytokines cannot be directly linked to T cells. Therefore, addition of co-stimulants such as anti-CD28, which mimics the antigen-specific T cell activation through CD28 and B7 molecule interaction, and anti-CD49d, which has been shown to regulate e.g. proliferation and effector functions of T cells, intends to make the results of this cytokine assay closer to T cell specific responses (Lee et al., 2020; Thakur et al., 2013).

In this paper we describe the development of a new whole blood (WB) stimulation assay, optimized to detect secreted *B. pertussis*-antigen-specific T cell associated cytokine responses, and designed to have a semi-high throughput readout while using a small volume of blood. The assay could be applied for clinical trials involving differently aged individuals, including infants.

2. Material and methods

2.1. Study design and participants

A modified WB T cell assay was developed as part of the PERISCOPE (PERtussIS COrrelates of Protection Europe) project, and was applied to a sub-set of samples from the BERT (Booster against pertussis) multicentre study (in Finland, the Netherlands, and the United Kingdom) (Lambert et al., 2020; Versteegen et al., 2021). The modified assay is based on the original WB assay, in which two readouts, multiplex cytokine measurement and flow cytometry for determination of T cell populations, were performed on supernatants and cells harvested after 19 h of WB stimulation, and performed within 12 h from the sampling, excluding cytokines which were frozen and measured later (Lambert et al., 2020). We anticipated that this novel assay has advantages to the previous as it consumes less blood, is quicker, and does not need expensive equipment. However, the original assay allowed the detection of *B. pertussis*-specific CD4+ T cell populations from functional Th lineages at low frequencies for different aged subjects (Lambert et al., 2020).

In this study, the original assay was adapted by omitting the secretion blocking step, and by cryopreserving the harvested supernatant at −80°C after WB stimulation. Development was carried out in three phases: 1) determination of assay conditions with BERT samples collected at the University of Turku (UTU), 2) verification of the conditions with BERT samples collected at the University of Oxford (UOXF), and 3) final protocol determination, and evaluation with BERT samples from different age cohorts from UTU and UOXF. The study population consisted of 38 samples, collected from four different age cohorts as follows: paediatric cohort A (children aged 7–10 years, $n = 8$), paediatric cohort B (adolescents aged 11 to 15 years, $n = 12$), adult cohort C (younger adults aged 20 to 34 years n = 8), and adult cohort D (older adults aged 60 to 70 years, $n = 10$). All study subjects were randomly selected from the original cohorts, and received an aP booster immunisation with combined diphtheria, tetanus, pertussis toxoid, filamentous hemagglutinin (FHA), pertactin, and poliomyelitis (IPV) vaccine (Boostrix-IPV®) at D0 with follow-ups on D7, D14, and D28. The study was run in accordance with the Declaration of Helsinki (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/doh-oct1996/) and the International Conference on Harmonisation Guidelines for Good Clinical Practice, registered at the EU Clinical Trial database (EudraCT number 2016–003678-42) and approved by the Medical Research Ethics Committee of three countries (MEC-U, NL60807.100.17-R17.039; MERC OX, South Central Hampshire B Research Ethics Committee (REC) 19/SC/0368; MREC UTU 129/1800/2017), and informed consents signed (Lambert et al., 2020; Versteegen et al., 2021).

2.2. Protocol development steps

The WB samples were collected in sodium heparin tubes (Vacuette, Greiner Bio-one, Kremsmünster, Austria), processed within four hours from blood draw, and prediluted at different ratios in complete medium

(M), consisting of RPMI 1640 with GlutaMax with HEPES (Gibco, Life Technology Corporation, Grand Island, NE, USA), 10 % fetal bovine serum (heat inactivated, Sigma Aldrich, St. Louis, MO, USA), and 100 U/mL penicillin/ 100 μg/mL streptomycin (Sigma Aldrich, St. Louis, MO, USA). The prediluted WB was distributed on 48 well tissue culture plates and stimulated with purified genetically detoxified pertussis toxin (PT, 5 μg/mL, heat inactivated at 80 °C for 10 min, LIST Biological Laboratories Inc., Campbell, CA, USA), FHA (5 μg/mL, Sanofi Pasteur, Lyon, France) and *B. pertussis* lysate (Bp lysate, 10 μg/mL, heat inactivated at 80 °C for 10 min, QB, St Louis, MO, USA). Complete medium was used as a negative control. It has been previously shown that WB stimulation with PT and FHA induced clear readouts for different cytokines (Lambert et al., 2020). Purified mouse anti-human CD28 (clone L293, BD Biosciences, San Jose, CA, USA) and CD49d antibodies (clone L25, BD Biosciences, San Jose, CA, USA) at 1 μg/mL were used as co-stimulants in each phase of the protocol development (Lambert et al., 2020).

The diluted and stimulated WB samples (800 μL in total volume, 200 μL per antigen) were cultured in plates for a pre-defined period of 24, 48, or 72 h at 37 °C/5 % CO_2. At each timepoint, the supernatant of each sample was harvested (from above the cell layer, no centrifugation), divided in three aliquots (V = 100 μL), and stored at −80 °C. Levels of different cytokines (N = 15) were determined using a customized 'human Th-cell lineage indicator cytokine' kit from Millipore/Luminex (Luminex® Millipore Corporation, Hayward, CA, USA). This kit included the following cytokines: IL-1β, IL-2, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-13, IL-17 A, IL-17F, IL-21, IL-22, CCL20, IFN-γ, and TNF-α. The concentration of cytokines per supernatant was determined based on interpolation of the standard curve of the kit. The lower limits of quantification (LLOQ), ranged from 0.5 to 1.3 pg/mL per cytokine, which were based on kit recommendations. Values below the LLOQ limit were converted to 0.1 pg/mL for the statistical analysis.

2.3. Statistical analysis

A descriptive analysis of all samples (incubation periods, WB dilutions, and time after immunisation) was performed. Regarding development phase data, we performed a comparison between different settings and conditions using the non-parametric Kruskal-Wallis *t*-test. For all sets of samples, we performed a comparison between each timepoint at similar conditions using the paired non-parametric Wilcoxon statistical test (no post-hoc correction). A p-value <0.05 was considered statistically significant. For the combined results, a similar approach was used to determine statistical significance of each cytokine readout within the adult and paediatric cohorts. No cross-cohort analysis was performed. Statistical analysis was performed using Microsoft Excel (MS office, USA), GraphPad Prism (version 10.0, GraphPad Software, Boston, Massachusetts USA), and SAS statistics (SAS/STAT®, North Carolina, USA).

3. Results

3.1. Determination and verification of assay conditions

The first set of samples included five subjects from cohort C (20–34 years). For initial verification of a wide set of test and readout conditions, whole blood samples were longitudinally collected before and at three different timepoints after aP booster immunisation. Samples were first diluted under two conditions (1:2 and 1:4) in complete medium. After this, 700 μL of each dilution was pipetted into four wells, after which the two co-stimulants, anti-CD28 and anti-CD49d, were added into each well. Lastly, for each dilution, either PT, FHA, BP lysate or complete medium (negative control) was added to each well. To identify the optimal incubation time for stimulation, three sets of stimulation were performed in parallel and supernatants were harvested after 24 h, 48 h, and 72 h of incubation at 37 °C/5 % CO_2.

Based on the acquired cytokine readouts, the cytokines CCL20, IL-1β, IL-6, IL-9, IL-12p70, IL-21, IL-22, and TNF-α were excluded from further analysis. The concentrations obtained for these cytokines/chemokines provided no significant differences between timepoints, most of them being high throughout the time points. The negative control showed almost no background at all four timepoints after aP booster with the final panel of selected cytokines (IFN- y, IL-2, IL-5, IL-10, IL-13, IL-17 A and IL-17F). The negative control values were subtracted from responses to other antigens. Bp lysate stimulation did not provide significant differences between timepoints, and was not selected for further testing.

Regarding incubation time, for example with PT, the 48 h and 72 h periods showed a more consistent increase in the cytokine levels from D0 compared to other timepoints (D7, D28), and were therefore selected for further assessment (Fig. 1A). Although both 1:2 and 1:4 dilutions showed good performance with cytokine readouts (data not shown), we assume the viability of cells is better preserved with more diluted samples (no shortage of nutrients). Therefore, the 1:4 dilution was selected. Of note, we did not test direct stimulation of undiluted blood, as previous study indicates that diluted blood facilitates use of smaller blood volumes and longer incubation periods (van Crevel et al., 1999).

Cytokine kinetics (IFN-γ, IL-5, IL-10, IL-13, IL-17 A, IL-17F) produced against PT showed an increase from D0 to D7 after aP booster with a decrease at D14, approaching similar levels to D0 (Fig. 1A). A second increase at D28 after immunisation was observed, achieving levels slightly higher than the ones detected at D7, except for IFN-γ, which achieved similar levels to D7. However, IL-2 showed a progressive increase from D0 to D28 post-immunisation (Fig. 1B).

The conditions for measuring T cell associated cytokines to pertussis antigens (PT, FHA) after aP booster were evaluated at D0 and D28, using a 1:4 dilution, with an incubation period of 48 h or 72 h. To verify these conditions, an additional eight adult samples from cohort C (20–34 years, N = 3) and cohort D (60–70 years, N = 5) were included and analysed. Results from this additional experiment showed individual differences, but also showed that with the previous conditions and with 48 h incubation (data not shown), differences between the two study timepoints, different stimulants, and cytokines can be observed. Final assay conditions are described in the supplementary file 1.

3.2. Cytokine results of adult and paediatric cohorts

After establishing the final assay conditions, 25 additional samples were tested from adults (Cohort D, 60–70 years, N = 5) and paediatric participants (children's Cohort A, 7–10 years, N = 8; adolescent Cohort B, 11–15 years, N = 12). The data from these samples were analysed together with the previous data from the development phase, using the optimal culture conditions. The cytokine results to identify different Th sub-populations for PT and FHA are presented for the adult (20–70 years) (Table 1) and paediatric (7–15 years) (Table 2) cohorts. The median cytokine concentrations are presented in the two tables per stimulant and per timepoint.

We noted individual variations in cytokine concentrations, D0 responses, as well as variation between adult and paediatric cohorts. For the adult cohort, when WB was stimulated with PT, there were no significant differences for any cytokine at D28 versus D0, although the median concentrations were higher for IL-2, IL-13, IL-17 A, and IL-17F at D28, and lower for IFN-γ, IL-5, and IL-10. For FHA stimulation, the results showed a significant increase for IL-2 and IL-13, whereas no increased concentration was detected for other cytokines at either timepoint (Table 1). For the paediatric cohort, the outcome for PT stimulated cytokine readouts was distinct to the adults, as a significant decrease was noticed for all cytokines, excluding IL-17F, between D0 and D28. However, FHA stimulation showed similar results to adult cohorts, with increased concentrations for IL-2 and IL-13. In addition, the FHA results showed responses to IFN-γ, IL-17 A, and IL-17F, with a decreasing trend (although low concentration of cytokines detected) from D0 to D28. Further, IL-10 did not show any response among the

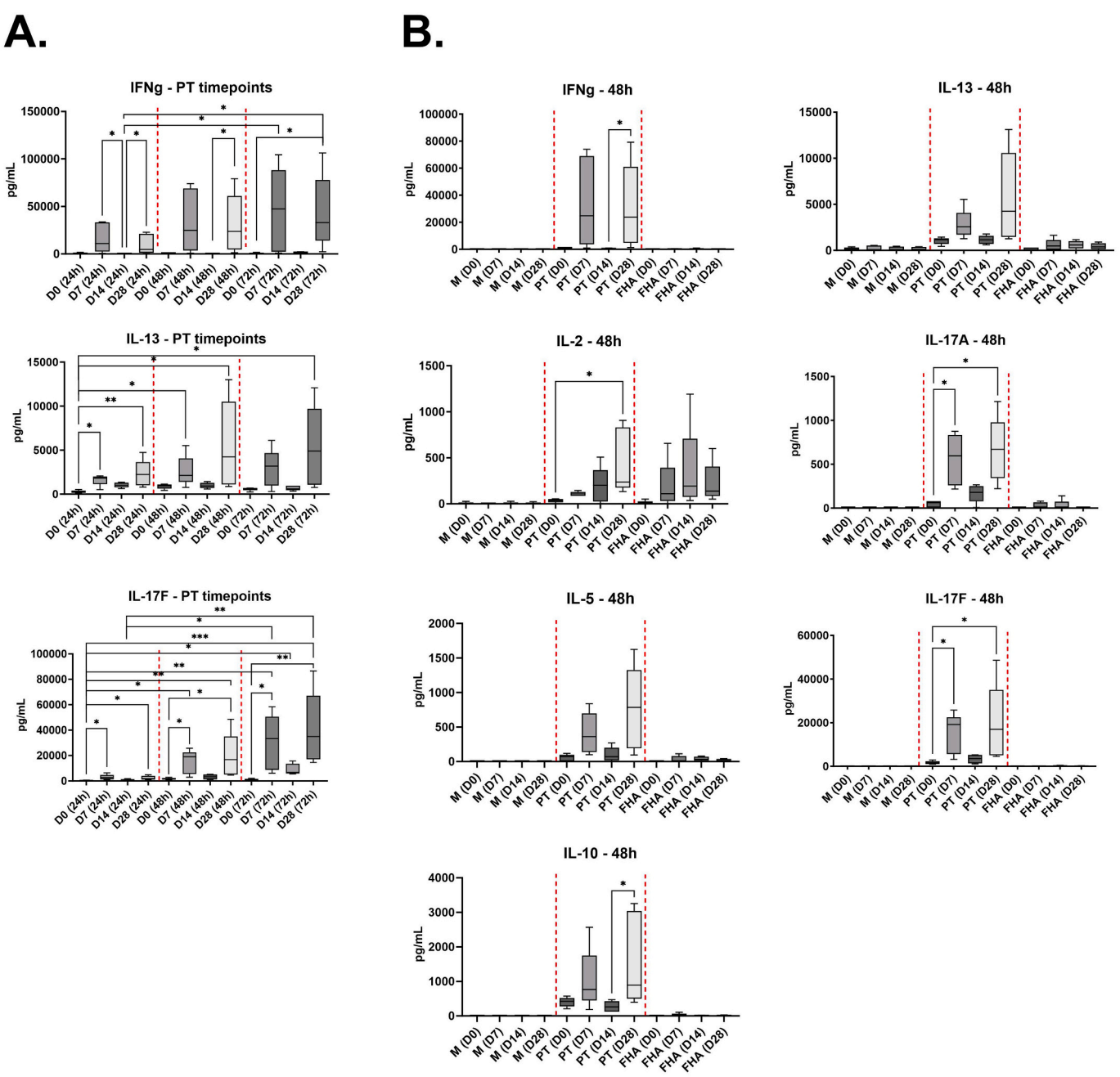

Fig. 1. Adult cohort participants (N = 5) recruited as part of the BERT study at the University of Turku research site received a booster dose of Boostrix®-IPV and were studied for A) cytokine levels of IFN-y, IL-13, and IL-17F measured at baseline and 7 days, 14 days and 28 days after booster dose. Cytokines measured against PT antigen are presented after 24, 48 and 72 h incubation period of WB diluted 1:4. Red-dotted lines separate different stimuli with different incubation and timepoints; B) cytokine levels measured at baseline and 7 days, 14 days and 28 days after booster dose. Cytokines measured against PT and FHA antigens, a negative control (M) after 48 h incubation period of WB diluted 1:4. NB! background not subtracted for Fig. 1B. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
*two-sided *P* value <0.05, ** two-sided P value <0.01, ***two-sided P value <0.001 with the non-parametric Kruskal-Wallis *t*-test.

study subjects post aP booster, which may be related to high D0 cytokine levels (Tables 1 & 2).

4. Discussion

In this study, we have developed a simple and cost-effective WB-based cytokine assay, built on a previous approach by Lambert et al. (Lambert et al., 2020). This novel assay requires only a small amount of blood per stimulation condition (200 μL), can be performed without non-centralized laboratories, and the harvested supernatants can be cryopreserved before cytokine measurements. Furthermore, the functionality of the WB-based cytokine assay was tested among subjects with a wide age range, 7–70 years. The assay is performed without PBMC isolation, which has previously been the main approach to study T cell responses for pertussis (Grondahl-Yli-Hannuksela et al., 2016; Guiso et al., 2007; Smits et al., 2013; Warfel and Merkel, 2013). Furthermore, in previous studies, a longer incubation time (3–5.5 days) was used to stimulate PBMCs. For pertussis, WB-based assays were recently developed for real-time flow cytometry approaches to detect T cell populations of interest (Corbiere et al., 2023; Diks et al., 2021; Lambert

Table 1
Cytokine results from the adult cohorts*.

Adults (Cohort C and D) Median (interquartile range -IQR)							
		PT			FHA		
	Antigen	D0	D28	*p values*	D0	D28	*p values*
		N = 18	*N* = 18		N = 18	N = 18	
Th1	IL-2	39.9	245.8	*0.5226*	0.1	477.9	*< 0.0001*
	(pg/mL)	(0.1–580.74)	(140.8–414.3)		(0.1–48.8)	(135.0–765.0)	
	IFN-γ	2495.0	887.5	*0.5509*	0.1	0.1	*0.8203*
	(pg/mL)	(666.2–40,403.0)	(59.3–8218.7)		(0.1–0.1)	(0.1–0.1)	
Th2	IL-5	70.8	31.8	*0.8817*	0.1	0.1	*0.1875*
	(pg/mL)	(0.1–215.9)	(0.1–287.1)		(0.1–0.1)	(0.1–4.4)	
	IL-10	1101.4	877.8	*0.4683*	0.1	0.1	*0.5000*
	(pg/mL)	(464.2–3734.2)	(279.8–2566.6)		(0.1–0.1)	(0.1–0.1)	
	IL-13	428.7	595.5	*0.6705*	0.1	39.2	*0.0410*
	(pg/mL)	(31.1–2283.6)	(314.6–1233.8)		(0.1–0.1)	(0.1–274.6)	
Th17	IL-17A	72.1	115.5	*0.8482*	0.1	0.1	*0.3750*
	(pg/mL)	(0.1–679.0)	(60.0–456.2)		(0.1–0.1)	(0.1–0.1)	
	IL-17F	1700.7	3899.0	*1.000*	0.1	0.1	*1.000*
	(pg/mL)	(1081.2–24,820.6)	(3049.1–13,735.0)		(0.1–0.1)	(0.1–0.1)	

* Background data has been subtracted from the results.

Table 2
Cytokine results from the paediatric cohorts*.

Paediatrics (Cohort A and B) Median (interquartile range -IQR)							
		PT			FHA		
	Antigen	D0	D28	*p values*	D0	D28	*p values*
		N = 20	N = 18		N = 5	N = 8	
Th1	IL-2	278.4	164.2	*0.0312*	1028.0	1622.0	*0.25*
	(pg/mL)	(150.5–715.5)	(89.0–356.1)		(657.9–2431.1)	(935.9–4988.8)	
	IFN-γ	2150.6	454.3	*0.0182*	28.0	23.0	*0.875*
	(pg/mL)	(628.3–12,866.9)	(156.0–1364.1)		(21.3–94.8)	(7.2–106.8)	
Th2	IL-5	304.6	36.0	*0.0003*	36.7	55.5	*0.125*
	(pg/mL)	(105.7–1434.0)	(0.1–85.4)		(5.0–95.4)	(37.0–506.8)	
	IL-10	2210.0	1153.4	*0.0120*	0.1	0.1	*1.000*
	(pg/mL)	(1248.4–3221.4)	(258.0–2342.9)		(0.1–0.1)	(0.1–0.1)	
	IL-13	2126.2	266.5	*0.0001*	375.1	942.7	*0.125*
	(pg/mL)	(1258.8–4740.6)	(0.1–916.0)		(323.3–1284.8)	(400.3–3731.0)	
Th17	IL-17A	256.0	79.5	*0.0012*	26.1	11.8	*0.875*
	(pg/mL)	(65.6–638.7)	(0.1–203.0)		(0.1–66.7)	(2.7–38.1)	
	IL-17F	185.7	6.5	*0.2160*	114.6	0.1	*0.25*
	(pg/mL)	(8.5–2762.8)	(2.0–2317.1)		(0.1–239.4)	(0.1–0.1)	

* Background data has been subtracted from the results.

et al., 2020). However, WB assays to study secreted cytokines from specifically-restimulated T cell populations have been developed previously for other diseases to measure e.g. IL-2, IFN-y, and IL-10 responses among coeliac disease subjects, IL-12 and IFN-γ ex-vivo production stimulated with BCG in those osteitis survivors after BCG vaccination, and IFN-y and IL-17 responses to *Aspergillus fumigatus* antigens (Hanekom et al., 2004; Hardy et al., 2021; Kagina et al., 2015; Lauruschkat et al., 2021; Poyhonen et al., 2016). The latter study also included the same co-stimulants (anti-CD28 and anti-CD49d) used in this study, and showed enhanced production of IFN-y and IL-17 by T cell activation. However, when co-stimulants are used, the risk of bystander effect in cell activation cannot be excluded. Previously, it has been shown that TCR-independent activation of CD4+ T cells happens especially to memory T cells, which probably consists most of the T cells among the WB used. Still, this effect may also be more regulated by Toll-like receptors than the co-stimulants (Lee et al., 2020). In comparison to the approach by Lambert et al. (Lambert et al., 2020), the newly developed method has advantages: Golgi Stop is not needed to detect internal cytokines, half the volume of WB is needed, no expensive instruments are required (such as flow cytometry), and a longer incubation (48 h vs 19 h) increases the ability to detect a broader array of cytokine responses. Using this approach, similar trends in responses among T cell associated cytokines between the studies were noted, e.g. with IL-10 responses only detected with PT stimulation.

PT-based stimulation resulted in a mixed trend of T cell associated cytokine profiles at D0 and D28 within the adult cohort, whereas this was not seen for the paediatric cohort, as all cytokines significantly decreased from D0 to D28. The difference between PT and FHA stimulation was remarkable, as for the adult cohort there were low levels of cytokines at D0, but a significant increase for IL-2 and IL-13 at D28. FHA stimulation from samples of the paediatric cohort indicated detectable levels for six cytokines at D0, and an increase for IL-2, IL-5, and IL-13 at D28, although this did not reach statistical significance. The reasons for these differences are unclear. In the paediatric cohort it seems that PT is a strong inducer of innate immunity and pre-existing memory. For FHA, one possibility for low responses among adults could be other factors included in the WB, such as high anti-FHA antibodies neutralizing the stimulants (partly the same for paediatric cohort), as the amount of these

is significantly higher when compared to anti-PT antibodies (Carlsson et al., 1999; Versteegen et al., 2021). Furthermore, anti-FHA antibodies are not fully specific to *B. pertussis*, as cross-reactivity to FHA produced by *B. parapertussis* and to surface proteins of *Haemophilus influenzae* has been shown (Carlsson et al., 1999). This cross-reactivity may also affect cytokine responses. Another reason could be related to antigen preparation, but in such case, we should not detect differences between timepoints, cytokines, subjects, and stimulants. However, it seems that PT and FHA behave differently in cell activation, which could be related to their different function in the host microbe interactions, as suggested by Higgs et al. (Higgs et al., 2012). Additionally, the possible effect of heterogenic and limited study subjects cannot be excluded. A recent study suggested that individuals primed with wP vaccine, like the adult cohort in this study, have higher amounts of IFN-y and IL-17 producing T resident memory cells in the tonsils than in the blood (McCarthy et al., 2024). This could partly explain why a poor response was noted to these cytokines with the WB assay. Other white blood cells such as regulatory B cells, neutrophils, natural killer cells, and monocytes also produce cytokines (e.g., IL-10, IFN-y) measured in this study (Arango Duque and Descoteaux, 2014; de Gruijter et al., 2022; Tecchio et al., 2014), which may influence the activation and proliferation of different subsets of cells, and affect the final cytokine readouts. Indeed, we observed kinetic differences in several cytokine productions induced at D7, D14 and D28 after booster vaccination (Fig. 1A). Another issue could be repeated boosting, as it has been speculated to dampen the cytokine responses to re-vaccination (Grondahl-Yli-Hannuksela et al., 2016). Further, when compared to the previous pertussis WB assays, our assay lacks the T cell specificity, as no flow cytometry-based assays were used for identification of different T cell populations (Corbiere et al., 2023; Lambert et al., 2020). Among our results, the IL-2 outcome was one of the most promising findings, as there were differences in both cohorts and to both antigens of PT and FHA. When compared to the other cytokine responses, IL-2 has an important regulatory role in balancing and maintenance of differentiated CD4+ Th cell subpopulations (Spolski et al., 2018). One puzzling question in the pre-phase was the lack of cytokine expression on D14, excluding IL-2. As the subjects in this study presumably already have generated (partial) immunological memory to the pertussis antigens through prior vaccination, the memory recall response is probably quite rapid, which may lead to expansion of CD4+ T cell type already at D4-D7, followed by contraction by day 14. It is also known that cytokine levels vary in healthy individuals. The population in this study consisted of individuals from two countries, Finland and the UK, with different vaccination schedules and distinct national pertussis epidemiology throughout the years (Versteegen et al., 2021). These conditions and other unknown individual factors are expected to affect the outcome of our measurements, which can be seen as high variation within the study population (Tables 1 and 2). Considering the individual differences, age seems to have a clear role in immune responses to pertussis vaccines, that can partially justify some of our findings. As described by *van Twillert* et al, T cell responsiveness seems to be in a steady-state at paediatric ages, with a tendency to decay in older ages due to immunosenescence, among other reasons, (van Twillert et al., 2015).

The cytokines used in this study are described to have a high variability in the baseline levels between individuals, including IFN-y and IL-2 (Wu et al., 2017). A study from Karanfilov et al. showed that baseline cytokine levels associated with both Th1 and Th2 responses tend to be significantly higher in young individuals compared with older individuals (Karanfilov et al., 1999). This was partly seen with our study population, as the paediatric cohort had high baseline cytokines with a decreasing trend to D28. For the adult cohort, immunosenescence can partly explain the decline in different Th responses, which might be the case especially among the group D subjects, aged 60–70 years, who are shown to have different responses in antibodies compared to group C (20–34 years) (Versteegen et al., 2021). However, the behaviour of cytokines produced as part of Th1 and Th2 stimulation is not consistent between studies (Gardner and Murasko, 2002).

There are several limitations of the study. The number of clinical samples was low, with a different number of subjects within the age groups and within the PT and FHA categories. We did not include a separate positive control, as the functionality of PT and FHA as stimulants to produce immune responses has been proven in the previous study (Lambert et al., 2020). In the future staphylococcal enterotoxin B may be used for this purpose. We did not measure the original cell counts in the WB, so the results are based on the antigen responses from a small fraction of WB. Although we have measured indicator cytokines for T cell associated cytokines, we cannot demonstrate that these are purely T cell derived. The effect of pre-existing (baseline) antibodies (as neutralizing agents for stimulants), and the effect of circulating baseline Tregs and other ongoing minor infections may increase white blood cells at baseline.

To conclude, the method is easy to perform with fresh blood samples, needing only a fraction of WB, and is adaptable for all age-groups, and would therefore be feasible for different types of studies. Further improvements to the protocol in the future require bigger-scale studies with mixed age-groups to present better cut-offs for the conditions and results obtained.

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CRediT authorship contribution statement

Marta Valente Pinto: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Validation, Writing – original draft, Writing – review & editing, Visualization. **Alex-Mikael Barkoff:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Validation, Writing – original draft, Writing – review & editing, Visualization. **Sagida Bibi:** Investigation, Validation, Writing – review & editing. **Aapo Knuutila:** Investigation, Validation, Writing – review & editing. **Johanna Teräsjärvi:** Investigation, Validation, Writing – review & editing. **Elizabeth Clutterbuck:** Investigation, Validation, Writing – review & editing. **Sophie Gimenez-Fourage:** Validation, Writing – review & editing. **Anke Pagnon:** Validation, Writing – review & editing. **Jacqueline A.M. van Gaans-van den Brink:** Validation, Writing – review & editing. **Veronique Corbiere:** Methodology, Validation, Writing – review & editing. **Aymeric De Montfort:** Validation, Writing – review & editing. **Anja Saso:** Methodology, Validation, Writing – review & editing. **Haddijatou Jobe:** Validation, Writing – review & editing. **Sophie Roetynck:** Validation, Writing – review & editing. **Beate Kampmann:** Conceptualization, Methodology, Validation, Writing – review & editing. **Elles Simonetti:** Conceptualization, Methodology, Validation, Writing – review & editing. **Dimitri Diavatopoulos:** Conceptualization, Methodology, Validation, Writing – review & editing. **Eleonora E. Lambert:** Validation, Writing – review & editing. **Jussi Mertsola:** Conceptualization, Methodology, Resources, Validation, Writing – review & editing. **Pascal Blanc:** Methodology, Formal analysis, Resources, Validation, Writing – review & editing. **Cécile A.C.M. van Els:** Conceptualization, Methodology, Validation, Writing – review & editing. **Dominic Kelly:** Conceptualization, Methodology, Resources, Validation, Writing – review & editing. **Qiushui He:** Conceptualization, Methodology, Resources, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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