Article

Evaluation of B-cell kinetics after acellular pertussis vaccination in four cohorts of different age and priming background

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Abstract: Pertussis is a vaccine-preventable disease caused by the bacterium Bordetella pertussis. Over the past years, the incidence and mortality of pertussis increased significantly. A possible cause is the switch from whole cell to acellular pertussis vaccines, although other factors may also contribute. To develop future vaccines and improve current vaccination strategies, it is critical to understand factors influencing the generation of immunological memory. We applied high-dimensional flow cytometry to investigate changes in B cells in individuals of different ages and distinct priming backgrounds upon administration of an acellular pertussis booster vaccine. These findings were correlated to vaccine-specific plasma cells and serum Ig levels. Expansion and maturation of plasma cells 7 days post-vaccination was the most prominent cellular change in all age groups, and was most pronounced for more mature IgG1+ plasma cells. Cellular responses were stronger in individuals primed with whole cell vaccine than in individuals primed with acellular vaccine. Moreover, IgG1+ plasma cell expansion weakly correlated with Prn- and PT- specific serum IgG levels. Our study points at plasma cells as a potential early cellular marker of an immune response and contributes to understanding differences in immune responses between age groups and priming backgrounds.

Keywords: Tdap, flow cytometry, acellular pertussis vaccine, whole cell pertussis vaccine, plasma cells

1. Introduction

Pertussis is a vaccine-preventable respiratory disease caused by the bacterium Bordetella pertussis (Bp). Since the introduction of the first pertussis vaccines in 1940/50 containing whole inactivated bacteria (whole cell pertussis; wP), the incidence and mortality due to pertussis have dramatically decreased [1]. However, the wP vaccine itself has a relatively high reactogenicity profile [2, 3]. Therefore, from early 1980s onwards, many (developed) countries started to replace the wP vaccine by an acellular pertussis (aP) vaccine which had a more favorable reactogenicity profile [3, 4]. In the Netherlands, this

change took place on January 1st, 2005. Acellular variants contain purified Bp components, such as pertussis toxoid (PT), filamentous hemagglutinin (FHA), pertactin (Prn), Fimbriae 2 and 3 (Fim2/3). In the Netherlands, the combined DTaP-IPV-Hib-HepB vaccine (providing protection against diphtheria, tetanus, pertussis, polio, Haemophilus influenzae type b (Hib) and hepatitis B) is used for primary vaccination, where boosters at later age are often Tdap vaccines [5].

Immune surveillance data showed that despite high vaccination coverage in many countries, there has been an increase of pertussis in the past decennia [6, 7]. This increase is not only seen in aP-using countries, but it has also been reported in countries where primarily wP is used [6, 8]. Several possible explanations for this increase have been proposed. First of all, improved awareness, surveillance and diagnostics may increase the detection rate [9]. Furthermore, several new Bp strains have been identified, i.e. that lack antigens present in the aP vaccine (such as FHA or Prn deficient strains), or PtxP3 strains that have adapted to suppress host immunity by producing higher levels of PT [10-13]. Lastly, there may be increased carriership within the population as well as faster waning of protective immunity in aP-primed individuals. Initial studies comparing efficacy of aP vs wP vaccines showed a similar short-term protection [14, 15]. However, later long-term studies showed that protection lasted shorter when using aP vaccines [16-19]. Also baboon models have shown that aP-induced immunity does not prevent transmission, immunity induced by wP vaccines leads to a faster clearance of bacteria,   and immunity generated by infection prevents colonization [20]. These combined data point at the need for mucosal immunity to prevent or reduce colonization and carriership.

An improved vaccine, immunization program, and/or route of administration seem necessary to combat pertussis. This implies a need to first understand the mechanism underlying protection induced by aP and wP vaccines (their differences and similarities). So far, no true correlate of protection (neither serological nor cellular) has been established for pertussis, and this would greatly aid evaluation of newly developed vaccines. This is one of the pillars of the Innovative Medicines Initiative (IMI)2 PERISCOPE consortium (PERtussIS COrrelates of Protection Europe), which aims to increase the scientific understanding of pertussis-related immunity in humans, identify new biomarkers of protection and to generate technology and infrastructure for the future development of improved pertussis vaccines [21].

Several (recent) studies both within and outside the IMI2 PERISCOPE consortium have shown that initial priming against pertussis (aP or wP vaccine) influences protection against disease as well as the immune response to (future) booster vaccinations [16, 18, 22-25]. For example, Hendrikx et al found that in aP-primed children, Ag-specific IgG4 serum levels were higher compared to wP-primed children [23]. Furthermore, Da Silva et al showed that -even after receiving aP booster vaccinations- initial priming (wP or aP vaccine) determined the antigen-specific CD4 T-cell response [24]. Similarly, Lambert et al showed that CD4 T cells isolated from recently aP-boostered individuals could be separated in a PCA view based on priming background, where an aP priming background resulted in a more Th2-related response as compared to a wP priming background [22].

As neither vaccine-induced nor infection-induced immunity leads to life-long protection against pertussis, the use of booster vaccinations later in life is a topic relevant for public health, as people with waned immunity can become carriers and thereby a source of transmission. Moreover, older adults can be more vulnerable to severe disease outcomes [26]. Several studies have shown that aP boosters are effective and well-tolerated in (older) adults [27, 28]. Recently, Versteegen et al investigated the specific serological response to an aP booster vaccination in four cohorts of different ages and priming backgrounds in the Netherlands, Finland and the UK (IMI2 PERISCOPE study acronym: BERT; Booster pertussis vaccination study) [29]. Here, they found all age cohorts showed a good response upon booster vaccination, with only limited differences between the different age cohorts for the Bp-specific IgG levels. However, Ag-specific serum IgA (both pre- and post-vaccination) increased with age, likely caused by over-time (mild) exposures to Bp.

Previous studies on influenza have shown that up to 80% of the circulating IgG plasma cells 7 days after vaccination can be vaccine-specific [30, 31]. This, combined with the low numbers of circulating plasma cells at baseline (median counts <5cells/ul [32]), implies that the plasma cell system is a relatively 'clean' system to monitor. Thus, flow cytometry may serve as a faster and less laborious approach to study vaccination-elicited plasma cells than typically Ag-specific approaches such as ELISpot. Recently, our team used high-dimensional flow cytometry to investigate over-time cellular kinetics in 10 healthy (wP-primed) adults upon aP vaccination. There, we were able to demonstrate a clear expansion and maturation of plasma cells (especially IgG1+) and a strong correlation between IgG1+ memory B-cell expansion and the magnitude of the Ag-specific IgG serum response [33]. Here, we extended our exploratory studies by the analysis of participants of different ages and different priming background after receiving an aP booster (Boostrix-IPV, GlaxoSmithKline (GSK), Wavre, Belgium). We included 48 individuals enrolled in the Dutch cohort used in the IMI2 PERISCOPE- BERT study (periscope-project.eu/patients/study-2-bert/) at pre-defined time points, with the primary objective to describe the kinetics of circulating B-cell populations in four cohorts of different ages and with different priming backgrounds. This may lead to new insights on the impact of priming at infancy with aP or wP vaccines on future immune responses.

2. Materials and Methods

2.1 Study design and sample collection

This study comprises one of the exploratory objectives of the Dutch 'BERT study', which was initiated by the IMI2 PERISCOPE consortium. It was approved by Medical Research Ethics Committees United (MEC-U, NL60807.100.17-R17.039) and registered at the EU Clinical trial registry (EudraCT number 2016-003678-42). To be eligible for this study, participants had to (1) be generally healthy, (2) have no recent evidence of serious disease, i.e. requiring the use of immunosuppressive or immunomodulating medication, within the 3 months prior to inclusion, (3) received all regular vaccines according to Dutch National Immunization Program (www.rivm.nl/en/national-immunisation-programme) as appropriate for their age. An extensive description of the cohort and exclusion criteria have recently been published by Versteegen and colleagues [29]. A fraction of the participants of this aP vaccination study was subjected to additional exploratory analysis, such as mass cytometry, evaluation of mucosal antibodies, NGS or in-depth flow cytometry. The exploratory sub-study monitoring the fluctuations in circulating B-cell subsets after vaccination is discussed in this manuscript. Here, 48 individuals were selected from 4 cohorts of different age and distinct priming background at infancy (children, 7-10 y/o, aP-primed; adolescents, 11-15 y/o, aP or wP-primed; young adults, 20-34 y/o, wP-primed; older adults 60-70 y/o, in whom vaccination history was unknown (presumably: wP-primed or not vaccinated)).

The study was conducted by the Spaarne Academy (Spaarne Hospital, Hoofddorp, the Netherlands). Written informed consent was obtained at the start of the study. Participants were vaccinated intramuscularly with the Boostrix-IPV vaccine after their first blood donation (baseline). Boostrix-IPV is a reduced-antigen Tdap-IPV booster vaccine, which contains diphtheria toxoid (Diph) (≥2.5 Limit of flocculation (Lf)), tetanus toxoid (Tet) (≥5Lf), three Bordetella pertussis proteins, being PT (8μg), FHA (8μg), Prn (2.5μg) and inactivated poliovirus (Mahoney strain; 40 D-Antigen units (DU), MEF-1 strain; 8 DU, Saukett strain; 32 DU) and aluminum hydroxide as adjuvant [34]. Peripheral blood samples were collected in blood collection tubes using heparin as anticoagulant and in serum collection tubes at baseline, day 7 and 28 after vaccination. An additional peripheral blood sample was taken at day 14 in participants aged 20-34 and aged 60-70. Individuals were excluded and replaced by a new participant if a blood sample at day 0 or 28 could not be obtained.

2.2 Evaluation of antigen-specific immunoglobulin levels in serum

Serological analysis was performed in all collected samples. Levels of IgG directed against Tet, PT, FHA, Prn, and Fim2/3, and levels of IgA directed against PT, FHA, Prn and Fim2/3 were determined by multiplex immunoassay (MIA) at the Dutch National Institute for Public Health and the Environment (RIVM, The Netherlands) [35]. The serum antibody responses raised against Bp-antigens during the PERISCOPE-BERT study have been extensively discussed by Versteegen and colleagues [29].

Detection of vaccine-specific antibody-producing plasma cells and memory B cells

Analysis of numbers of IgG and IgA producing plasma cells and memory B cells was performed in majority of the samples included in this study. B cells producing IgG directed against PT, FHA, Prn and Tet, and B cells producing IgA direct against PT, FHA and Prn were measured using an Enzyme-Linked Immunospot (ELISpot) assay at the RIVM. This procedure has been described previously [36]. In short, peripheral blood mononuclear cells (PBMCs) were isolated using a density gradient. For antibody producing plasma cells, PBMCs at day 7 post-vaccination were directly transferred to Ag-coated ELISpot filter plates (duplicates). For the detection of vaccine-specific memory B cells, PBMCs collected at day 0 and day 28 were collected and stored at -135°C. Thawed PBMCs were stimulated for 5 days using a culture medium containing CpG, IL-2 and IL-10. Next, cells were transferred to Ag-coated plates (duplicates). Numbers of Ag-specific antibody-producing cells – appearing as spots- were measured using an ImmunoSpot S6 Ultra-V analyzer (Cellular Technology Limited, Cleveland, OH). Uncoated wells filled with PBS served as negative control and were used to subtract background signal. Wells with a signal below the limit of quantification were set at 0.1 cell/105 PBMCs. Cumulative IgG and IgA spot counts for all antigens measured were used for analyses.

2.3 Longitudinal flow cytometric analysis of circulating B-cell subsets

All peripheral blood samples were subjected to high-throughput flow cytometric immunophenotyping of the B-cell compartment. Here, we used the recently developed BIGH-tube: the B-cell and plasma cell tube (BIGH) allows identification of >100 populations of B- and plasma cells distinguished based on their maturation stage and expressed Ig subclasses [32, 37] (Antibody panel and phenotypic description of the identified B-cell subsets: Supplemental Table 1 and 2).

Samples were processed according to the bulk lysis protocol using 10 x 106 cells followed by intracellular staining as described before [33] (protocols available on www.EuroFlow.org), with the addition of membrane staining with CD45-AlexaFluor700.

In short, based on the white blood cell count (as determined by an automated hematological analyzer (Sysmex XP-300, Sysmex Europe GmbH, Norderstedt, Germany)), one or multiple tubes were filled with up to 2mL of blood, after which ammonium chloride was added up to a total volume of 50mL. After a 15 min incubation at room temperature on a roller bank to lyse non-nucleated red blood cells, cells were washed, counted on a Sysmex XP-300 and pooled to a total of 10x106 cells. Next, cells were stained with an antibody cocktail directed against surface markers for 30 min in the dark with the BIGH panel (Supplemental Table 1). This was followed by a cytoplasmic staining for intracellular Igs using the Fix & Perm reagent kit (Nordic MUbio, Susteren, The Netherlands) according to manufacturers' protocol. Finally, samples were washed and resuspended in PBS for immediate acquisition (or stored for max ~ 3h at 4°C).

For precise enumeration of cell numbers, we used Perfect-Count Microspheres™ (Cytognos) according to the EuroFlow SOP (protocol available on www.EuroFlow.org). In short, exactly 50ul of well-mixed Perfect-Count Microspheres™ were added to exactly 50ul of peripheral blood. Then, antibodies directed against CD19, CD3 and CD45 were added and the sample was incubated for 30 min in the dark. Next, 500uL of NH4Cl was added and after 10 minutes incubation samples were ready for immediate acquisition. Using this tube, we could identify and quantify total leukocytes and lymphocytes, B-, T- and NK cells in each sample. All samples were acquired at the Flow cytometry Core Facility of LUMC, using a BD FACS LSR Fortessa 4L (BD Biosciences, San Jose, CA, USA)

or a BD FACS LSR Fortessa X-20 flow cytometer (BD Biosciences), which were calibrated daily according to EuroFlow guidelines, as previously described [38, 39].

2.4 Data analysis and statistics

To ensure objective data analysis and minimize operator-induced variability, all data were analyzed using the automated gating and identification (AGI) module of the Infinicyt software (Infinicyt™ Software v2.0, Cytognos). This AGI module makes use of clustering algorithms and comparison with fully annotated reference flow cytometry (FCS) data files of healthy individuals to assign clusters of events to a population [40] . Importantly, when there was no perfect fit for a cluster of events, this was marked as a 'check' population and the software indicated to which populations this cluster may correspond. These check events were assigned manually according to the proposed gating strategies for the BIGH panel (Supplemental Table 2) [32, 37].

For visualization and statistical analysis, the GraphPad Prism 8.1.1 software (GraphPad, San Diego, CA, USA) was used. To test longitudinal data within a cohort, the Wilcoxon signed-rank test for paired samples was used. Correlations were determined using Spearman's Ranking Correlation (correlation coefficients of <0.6 or – in case of negative correlation >-0.6 were considered weak correlations). Finally, to compare differences between the four cohorts, the Kruskal-Wallis approach was used, followed by Dunn's test, and, as only BU and BV samples were measured at day 14, the Mann-Whitney test was used instead of Kruskal-Wallis at day 14. To correct for multiple testing, tests were followed by Bonferroni correction when appropriate.

3. Results

3.1. Study cohorts

All subjects enrolled in the study between October 2017 and March 2018. In total, 12 children (age: 7-10, aP-primed, m/f ratio: 6/6), 12 adolescents (age: 11-15, 7 individuals wP-primed, m/f ratio: 2/5, 5 individuals aP-primed, m/f ratio: 4/1), 12 young adults (age 20-34, wP-primed, m/f ratio: 7/5) and 12 older adults (age 60-70, presumably wP-primed or not vaccinated, m/f ratio: 4/7) completed this study (as part of the PERISCOPE BERT study). Three additional children, who were initially enrolled, did not participate beyond the first visit and were replaced by three new participants. From all acquired samples, two baseline B-cell samples were lost due to technical problems (one child and one young adult). Finally, one older adult was excluded due to (potentially) clonal expansion of B cells and replaced by a new participant.

For most participants the leukocyte, lymphocyte, T-cell, B-cell and NK-cell counts at baseline were within the normal age-matched range (Supplemental Table 3, Table 1), or, in case of minor deviations, fell into the normal range at later time points [41, 42]. Leukocytes, lymphocytes and T cells remained mostly stable over the time of analysis. Although NK-cell numbers showed a minor decrease at day 28, this was most likely not related to the vaccination response (day 0 vs 28, p≤0.01 and day 7 vs 28, p≤0.05, Supplemental Figure 1). There were no statistically significant differences in absolute leukocyte, lymphocyte, T-cell and NK-cell counts at baseline in-between age cohorts. Thus, regarding the numbers of leukocytes, lymphocytes, B cells, T cells and NK cells, our donors were healthy representatives of the general population.

Table 1. Baseline distribution of normal B-cell and plasma cell subsets (cells/µl) in each age cohort. Ages of the cohorts of which reference values were used: children 5-9 y/o, adolescents 10-17 y/o, young adults 18-39 y/o, older adults 60-79 y/o. The reference values as indicated in this table were selected from the study performed by Blanco et al. [32] In the before-mentioned study, reference values for B-cell subsets are provided for all age ranges (from cord blood until older adults >80 y/o). When referring to the reference values indicated here, please refer to the original source data.

Cohort	Children (ch)			Adolescents (ad)			Young adults (yo)			Older adults (ol)			Statistical differences between cohorts				
Age	7-10 y/o			11-15 y/o			20-34 y/o			60-70 y/o			No significant differences found within the other combination (yo vs ol)				
Priming background	aP			Mixed aP and wP			wP			No vaccination history available (presumably wP-primed or not vaccinated)							
N=	11†			12			11†			12							
	median	min.	max.	median	min.	max.	median	min.	max.	median	min.	max.	ch vs yo	ch vs ol	ch vs ad	ad vs yo	ad vs ol
total B cell																	
reference cell counts	451	157	725	360	174	630	220	41	470	173	36	384					
cell counts	496.0	346.0	966.0	430.0	133.0	667.0	279.0	141.0	481.0	244.0	53.5	396.0	**	***	ns	ns	*
pre-GC B cell																	
reference cell counts		NI			NI			NI			NI						
cell counts	391.0	173.0	731.0	326.5	96.3	529.0	194.0	53.2	342.0	195.0	31.5	273.0	**	**	ns	*	**
immature B cell																	
reference cell counts	41	12	84	37	11	111	5.6	0.25	24	6.1	0.69	36					
cell counts	26.6	9.5	70.4	20.8	1.6	43.7	5.9	1.1	19.8	4.7	0.6	9.2	**	****	ns	*	**
CD5+ naive B cell																	
reference cell counts		NI			NI			NI			NI						
cell counts	89.7	25.0	248.0	49.8	5.2	166.0	30.9	7.8	152.0	15.3	2.4	47.0	*	***	ns	ns	*

naive B cells																	
reference cell counts	265	68	505	189	75	401	111	13	288	109	20	280					
cell counts	260.0	124.0	483.0	238.5	89.4	335.0	160.0	44.0	186.0	150.0	24.9	250.0	**	*	ns	ns	*
memory B cell																	
reference cell counts	123	64	282	68	31	160	91	23	221	56	13	128					
cell counts	160.0	76.1	252.0	96.2	33.6	193.0	85.7	31.3	282.0	63.8	21.1	171.0	ns	**	ns	ns	ns
IgMD+ memory cells																	
reference cell counts	54	23	147	29	17	78	38	7.9	122	27	7.4	72					
cell counts	65.7	30.7	129.0	48.8	16.0	141.0	37.4	10.0	121.0	20.8	8.6	93.2	*	****	ns	ns	ns
IgG1+ memory cells																	
reference cell counts	30	12	86	18	7	42	18	3.2	40	9.1	1.3	22					
cell counts	47.8	15.2	81.7	17.3	6.8	44.7	13.2	5.4	72.5	16.5	2.8	27.8	*	****	ns	ns	**
IgG2+ memory cells																	
reference cell counts	4.4	0.7	15	3.0	0.7	10	5.9	1.6	30	3.6	1.0	11					
cell counts	3.8	1.6	10.3	2.8	1.4	4.4	3.1	1.1	12.3	2.8	0.6	13.4	*	***	ns	ns	ns
IgG3+ memory cells																	
reference cell counts	7.4	2.4	16	3.0	1.1	8.3	3.0	0.5	8.4	2.3	0.4	8.1					
cell counts	7.9	3.2	13.0	3.1	0.6	7.1	1.8	0.8	6.4	2.1	0.5	7.4	*	**	ns	ns	ns
IgG4+ memory cells																	
reference cell counts	0.4	<0.01	2.0	0.2	<0.01	2.9	0.4	<0.01	2.4	0.4	<0.01	2.1					
cell counts	0.2	<0.05	2.2	0.3	<0.05	1.0	0.5	0.1	6.1	0.3	0.1	1.2	ns	***	ns	ns	ns
IgA1+ memory cells																	
reference cell counts	12	4.5	24	9.0	2.9	21	11	2.1	43	6.2	2.2	22					
cell counts	16.3	6.4	34.7	11.4	4.4	14.7	8.0	3.8	42.4	11.2	4.2	30.4	ns	ns	ns	ns	ns
IgA2+ memory cells																	
reference cell counts	3.2	1.0	13	2.7	0.8	5.9	4.1	1.2	18	3.4	0.7	9.0					
cell counts	4.8	1.4	10.3	2.7	0.5	6.2	3.1	0.7	17.5	2.7	0.8	9.6	ns	**	ns	ns	ns
IgD+ memory cells																	
reference cell counts	1.1	<0.01	2.9	0.3	<0.01	1.7	0.2	<0.01	2.4	0.01	<0.01	1.2					
cell counts	1.0	0.3	2.1	0.4	<0.05	1.4	0.2	<0.05	2.7	0.2	<0.05	0.8	*	**	ns	ns	ns

|																	
IgH- memory cells																	
reference cell counts		NI			NI			NI			NI						
cell counts	2.2	0.6	4.4	1.5	0.4	2.7	1.2	0.5	1.8	0.9	0.3	3.0	ns	ns	ns	ns	ns
total plasma cells																	
reference cell counts	13	3.5	45	8.5	1.3	27	4.4	1.1	25	1.2	0.3	7.1					
cell counts	8.5	3.8	12.9	3.4	1.6	13.1	2.6	1.2	8.5	1.6	0.2	6.1	ns	*	ns	ns	ns
IgM+ plasma cells																	
reference cell counts	1.4	0.6	14	0.8	0.2	5.7	0.4	0.05	4.7	0.1	0.01	0.8					
cell counts	1.3	0.3	2.9	0.6	0.4	2.0	0.4	0.1	2.8	0.1	<0.05	0.8	ns	ns	ns	ns	ns
IgG1+ plasma cells																	
reference cell counts	1.9	0.1	7.7	1.1	0.1	4.8	0.4	0.05	4.4	0.1	0.01	0.6					
cell counts	1.5	0.2	2.7	0.4	0.1	5.6	0.4	0.1	1.3	0.2	0.1	0.6	**	**	ns	ns	ns
IgG2+ plasma cells																	
reference cell counts	0.7	0.07	2.3	0.5	0.08	0.8	0.2	<0.01	2.6	0.09	<0.01	1.6					
cell counts	0.8	0.2	1.1	0.3	0.1	1.5	0.2	<0.05	0.6	0.1	<0.05	1.2	ns	ns	ns	ns	ns
IgG3+ plasma cells																	
reference cell counts	0.2	<0.01	1.3	0.08	<0.01	0.4	0.03	<0.01	0.3	<0.01	<0.01	0.2					
cell counts	0.2	<0.05	0.5	<0.05	<0.05	0.9	<0.05	<0.05	0.1	<0.05	<0.05	0.1	*	**	ns	ns	ns
IgG4+ plasma cells																	
reference cell counts	0.02	<0.01	0.2	<0.01	<0.01	0.2	<0.01	<0.01	0.4	<0.01	<0.01	0.1					
cell counts	<0.05	<0.05	0.2	<0.05	<0.05	0.2	<0.05	<0.05	0.7	<0.05	<0.05	0.1	ns	ns	ns	ns	ns
IgA1+ plasma cells																	
reference cell counts	4.4	0.6	16	3.1	0.5	14	1.7	0.3	6.9	0.4	0.04	3.3					
cell counts	3.4	0.6	5.6	1.3	0.4	4.1	1.1	0.4	3.3	0.7	0.1	3.0	ns	ns	ns	ns	ns
IgA2+ plasma cells																	
reference cell counts	1.5	0.3	3.5	1.0	0.3	3.6	0.7	0.2	4.2	0.3	0.06	1.2					
cell counts	1.0	0.3	2.7	2.7	0.5	6.2	0.3	0.1	1.1	0.2	0.1	1.2	ns	ns	ns	ns	ns
IgD+ plasma cells																	
reference cell counts	0.04	<0.01	0.8	<0.01	<0.01	2.0	<0.01	<0.01	1.1	<0.01	<0.01	0.2					
cell counts	0.1	<0.05	0.6	<0.05	<0.05	0.1	<0.05	<0.05	0.1	<0.05	<0.05	<0.05	**	**	*	ns	ns

3.2 Higher counts of naive B cells and plasma cells in children

B-cell numbers are known to decrease over time from on average 1400 cells/µl in children <2 years to on average 200 cells/µl in adults [32, 41]. This trend was also visible in our data set where children had more B cells than the adult groups (Table 1). This difference was mainly due to high numbers of pre-germinal center (naive) B cells in children. Although memory B-cell numbers were also higher in children than in adults (160 cells/µl in children vs 85.7 cells/µl in young adults, n.s., and 160 cells/µl in children vs 63.8 cells/µl in older adults, p≤0.01), these differences were less prominent, and mainly restricted to IgG1+ and IgG3+ memory B cells. Finally, several plasma cell subsets were significantly more abundant in children than in adults (IgG1+, IgG3+ and IgD+ plasma cells), but, due to their overall low frequencies, this did not have a major impact on total B-cell numbers. Limited differences in B-cell subset numbers were observed between adolescents and adults (Table 1). These differences were predominantly found in pre-germinal center (naive) B cells and individual plasma cell subsets. Thus, baseline cell numbers of B-cell subsets differed between the cohorts, which was in line with previously published data of age-matched individuals [32].

3.3 Expansion of plasma cells as the most prominent cellular B-cell change after vaccination

We have recently shown that administration of an aP booster vaccination in (wP-primed) adults triggers several cellular changes, of which the expansion of (predominantly) IgG1+ plasma cells at day 7 is most prominent [33]. Now, we set out to determine whether the same type of changes occurs in vaccinated individuals, irrespective of age and primary vaccination background.

While total B cells, pre-germinal center B cells and memory B cells remained relatively stable and did not show any consistent fluctuations over time following vaccination, plasma cells in all participants underwent significant expansion between baseline and day 7 (p≤0.01 for children, and p≤0.001 for adolescents and the adult cohorts, Figure 1), which returned to baseline at day 14 or day 28. The magnitude of this expansion was highly similar in children, adolescents and young adults (ratio to baseline: 2.6-3.4) and was significantly higher in older adults as compared to children (ratio: 2.6 in children vs 5.7 in older adults, p≤0.01). Total plasma cell numbers returned to baseline at day 14 or -if day 14 was not measured- day 28 (p≤0.001 for adolescents, young and older adults, in the children cohort, plasma cell counts were still slightly elevated at day 28). Thus, the expansion of plasma cells 7 days post-vaccination was the most prominent change in all age groups.
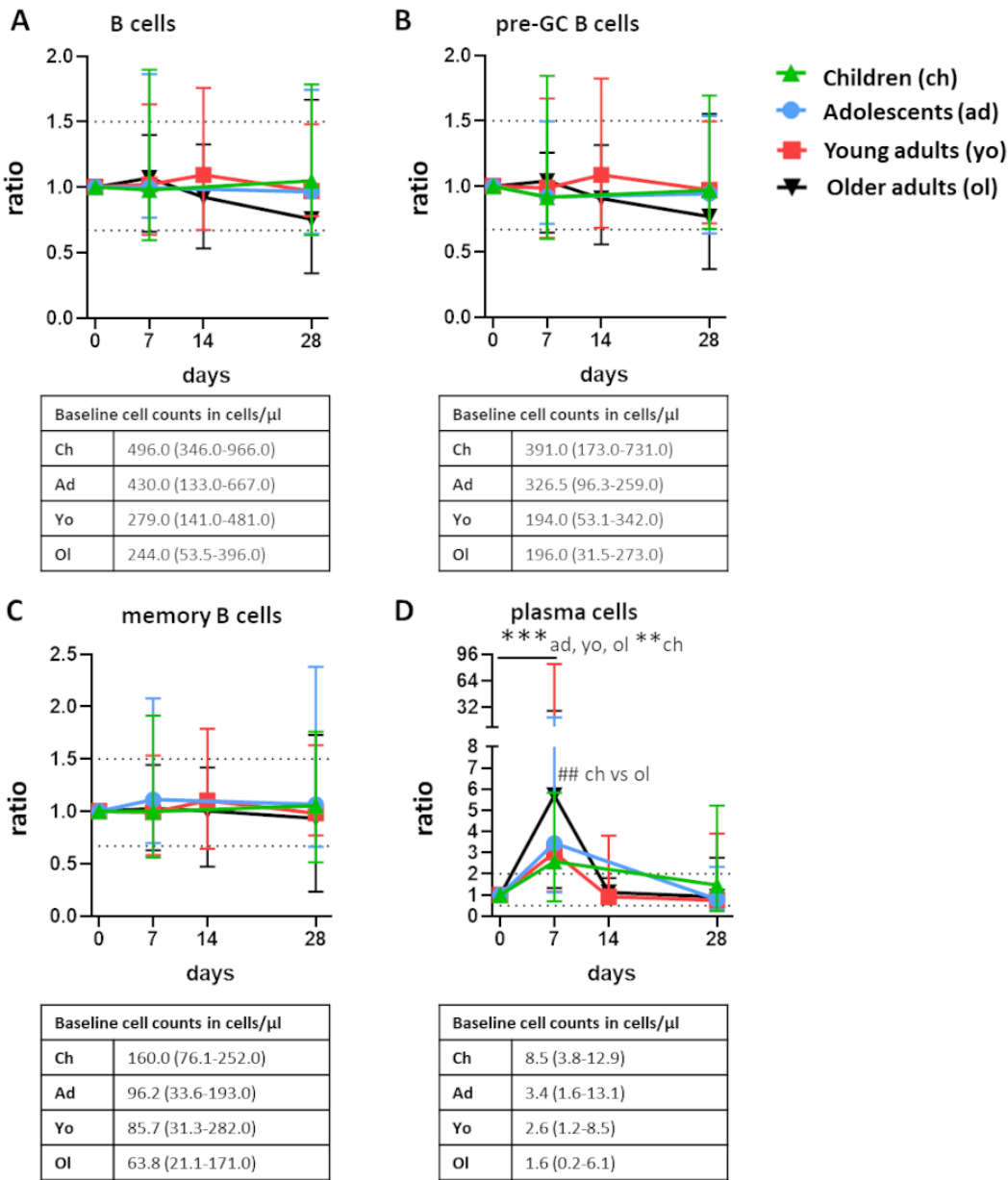
Figure 1. Post-vaccination fluctuations of major B-cell subsets. Fluctuations of (**a**) total B-cells (**b**) pre-germinal center (pre-GC) B cells, (**c**) memory B cells and (**d**) plasma cells post-vaccination, presented as ratio over baseline (median, min-max). For total B cells, pre-GC B cells and memory B cells, the dashed lines indicate a ratio over baseline of 0.67 and 1.5. For plasma cells, the dashed lines indicate a ratio over baseline of 0.5 and 2.0. Underneath each graph, the baseline cell counts per cohort are presented in cells/μL (median, min-max). To assess longitudinal changes within each cohort, Wilcoxon matched pair signed-ranked test followed by Bonferroni correction was used. To test differences between cohorts at one timepoint, Kruskal-Wallis followed by Dunn's test was used, with exception of the comparison at day 14. At day 14, only blood samples from the adult cohorts were collected. Here, the Mann-Whitney test followed by Bonferroni correction was used. For longitudinal changes, only significant differences compared to baseline are shown. Significant longitudinal differences within a cohort as indicated with *, $p<0.05$; **, $p\leq0.01$; ***, $p\leq0.001$. Significant differences between cohorts at the same time point are indicated with #, $p<0.05$; ##, $p\leq0.01$; ###, $p\leq0.001$.

3.4 Despite individual differences, skewing towards IgG1 plasma cells responses in all cohort

Cell expansion at day 7 was not equally pronounced in all plasma cell subsets. It was the   60
most prominent in IgG1+ plasma cells (ratio to baseline: 5.7-17.6, depending on cohort,   61
$p \leq 0.001$ in all cohorts, with a higher increase in older adults compared to children, $p < 0.05$).   62
This was followed by IgG3+ plasma cells (ratio: 3.6-6.5, $p \leq 0.01$ in children and adolescents,   63
and $p \leq 0.001$ in young and older adults, Figure 2A). IgG4+ plasma cells were significantly   64
increased in children and adolescents (ratio: 5.1 and 7.2 respectively, $p \leq 0.01$). IgG2+   65
plasma cells were significantly increased in adolescents only (ratio:1.8, $p \leq 0.001$). IgA1+   66
plasma cells were significantly increased in both adult cohorts (ratio: 2.1 and 3.9 in   67
younger and older adults, $p \leq 0.01$ and $p \leq 0.001$, respectively). Although IgM+ plasma cells   68
showed a tendency to expand at day 7 in older adults, there was a large variation in-be-   69
tween individuals and the difference was not statistically significant.   70

Despite individual changes in response patterns between the cohorts, IgG1+ plasma cells   71
were the single most expanded subset. They constituted between 43% (young adults) and   72
61% (adolescents) of all plasma cells at the peak of expansion, while only at most 18% at   73
baseline (Figure 2B). Moreover, although the ratio (to baseline) of plasma cells was often   74
higher in adults than in children, children had higher baseline IgG1+ plasma cell numbers,   75
and also higher IgG1+ plasma cell numbers at day 7 (median cell count at day 7: 7.64   76
cells/µl in children vs 3.81 cells/µl in older adults, ns). Thus, the expansion of IgG1+   77
plasma cells was the most prominent in all cohorts. Both an increase over baseline (ratio)   78
and in absolute cell numbers were observed.   79
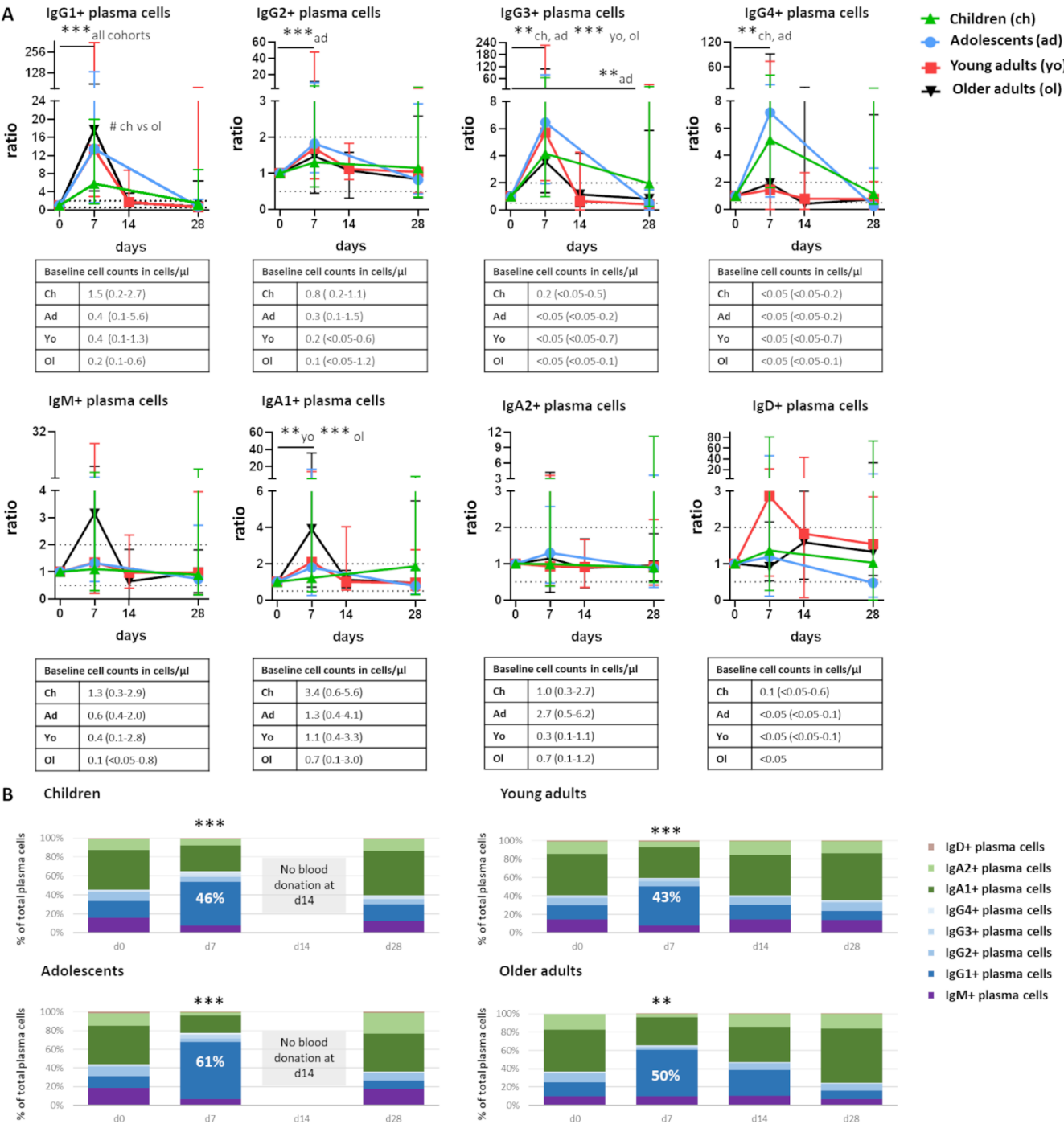
Figure 2. Individuals of all age groups undergo expansion of IgG1+ plasma cells at day 7 post-vaccination. (**a**) Quantitative changes in plasma cells expressing different Ig subclasses, presented as ratio over baseline (median, min-max). Each dot represents a median value with range. Dashed lines indicate a ratio over baseline of 0.5 and 2. Underneath each graph, the baseline cell counts per cohort are presented in cells/µL (median, min-max). Wilcoxon matched pair signed-ranked test followed by Bonferroni correction was used to assess longitudinal differences within each cohort. Differences in ratio at day 7 between cohorts were assessed using Kruskal-Wallis followed by Dunn's test. (**b**) Over-time distribution of plasma cells expressing different Ig subclasses. Median values for each population were used to construct the plots. Wilcoxon matched pair signed-ranked test followed by Bonferroni correction was used to assess longitudinal differences in the percentage of IgG1+ cells in the total plasma cell compartment within each cohort. Differences in the percentage of IgG1+ cells in total plasma cell compartment between cohorts were assessed using Kruskal-Wallis followed by Dunn's test, but did not yield significant differences. For pediatric cohorts, no blood samples were collected at day 14. There, the Mann-Whitney test followed by Bonferroni correction was used. For longitudinal changes, only significant differences compared to baseline are shown. Significant longitudinal differences within a cohort as indicated with *, p<0.05; **, p≤0.01; ***, p≤0.001. Significant differences between cohorts at the same time point are indicated with #, p<0.05; ##, p≤0.01; ###, p≤0.001. d= days after vaccination.

3.5 Maturation of plasma cells over time following vaccination   94

Newly generated plasma cells migrate from germinal centers via the blood stream to be-   95
come long-lived antibody-secreting plasma cells in the bone marrow and other peripheral   96
tissues. Over time, they gradually lose expression of CD20 and gain expression of CD138   97
[43] (Figure 3A). We used this information to divide plasma cells into consecutive matu-   98
ration stages and to trace their maturation over time after booster vaccination.   99

Plasma cells representing all maturation stages were expanded at day 7 (Supplemental   100
Figure 2 for total plasma cells, Figure 3 for IgG1+ plasma cells). This expansion was limited   101
in the least mature CD20+CD138- plasma cells (ratio to baseline: up to 3.1 in IgG1+ plasma   102
cells in children, Figure 3B), more clear in intermediate CD20-CD138- plasma cells (ratio   103
to baseline: up to 22.7 in IgG1+ plasma cells in young adults, in all cohorts p≤0.01 or   104
p≤0.001) and most prominent in the most mature CD20-CD138+ plasma cells (ratio to base-   105
line: up to of 115.6 in young adults, in all cohorts p≤0.01 or p≤0.001). Similar to what was   106
observed for total plasma cells, expansion of IgG1+ plasma cells belonging to different   107
maturation stages was least prominent in children. In all cohorts a significant increase in   108
the percentage of the most mature plasma cells was observed, except for the adolescent   109
cohort, showing just a trend. At day 7 post-vaccination most mature plasma cells consti-   110
tuted between 42% and 53% of IgG1+ plasma cells (Figure 3C). Thus, the expansion of   111
plasma cells at day 7 post-vaccination was accompanied by a shift towards a more mature   112
plasma cell phenotype in all cohorts.   113

As in the adult cohorts an expansion of IgA1+ plasma cells was observed, we evaluated   114
maturation of IgA1+ plasma cells at the peak of expansion as well. In children, no increase   115
in more mature (CD20-CD138- and CD20-CD138+) IgA1+ plasma cells was observed at   116
the peak of expansion. In adolescents, a small increase of most mature (CD20-CD138+)   117
IgA1+ plasma cells was observed, and in both adult cohorts, an increase in both interme-   118
diate and most mature (CD20-CD138- and CD20-CD138+) IgA1+ plasma cells was ob-   119
served at the peak of plasma cell expansion (adolescent cohort: p<0.05, adult cohorts: 120
p≤0.01). When comparing all cohorts at 7 days post-vaccination, a higher expansion of   121
most mature IgA1+ plasma cells was observed in older adults as compared to children   122
(p≤0.01). Thus, the expansion and maturation of IgA1+ plasma cells seemed to increase   123
with age of the cohort. However, within the oldest cohort, no correlation was found be-   124
tween total IgA plasma cells, IgA1+ plasma cells, or vaccine-specific IgA cells and age.   125
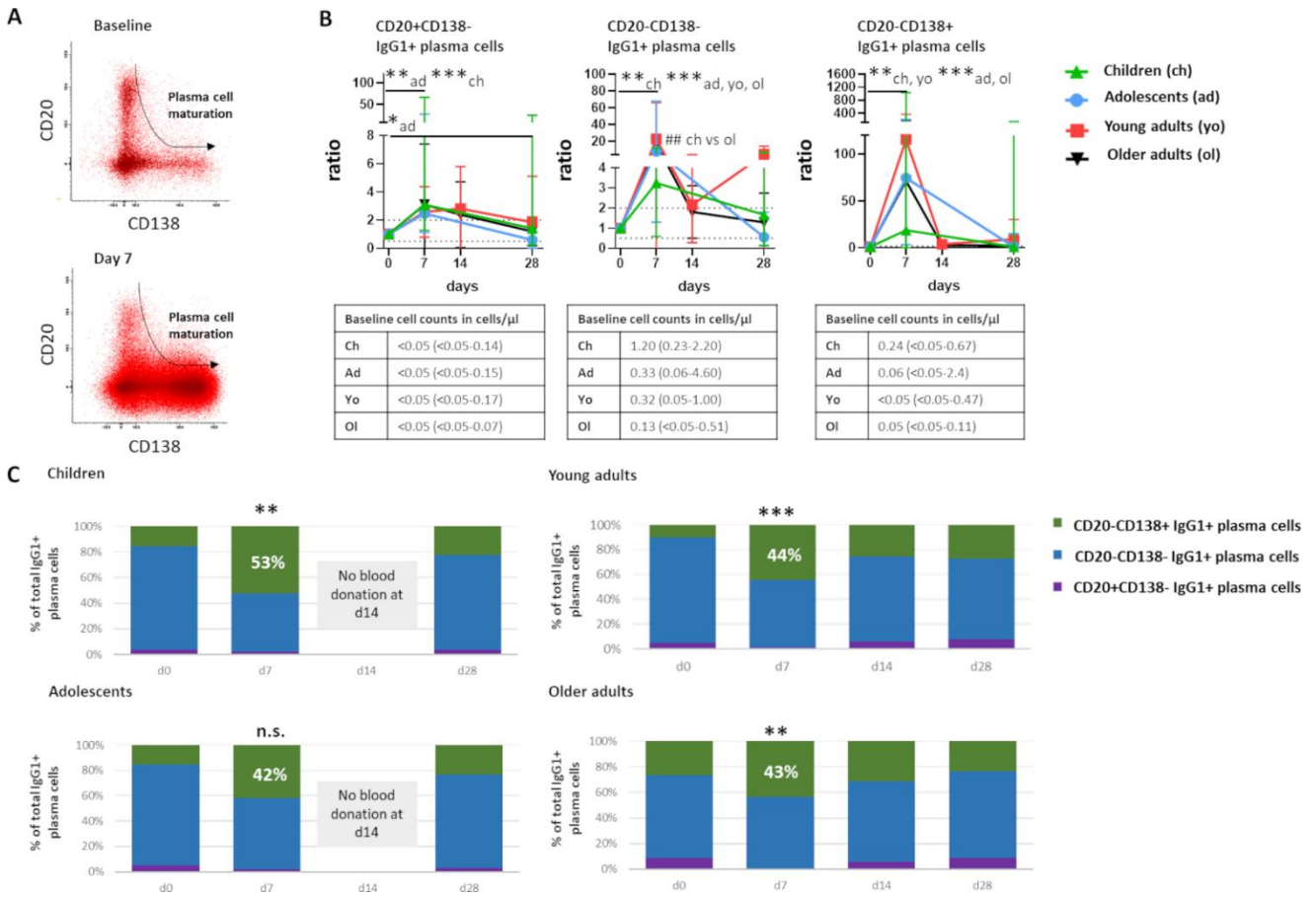
Figure 3. Over time maturation of IgG1+ plasma cells. (**a**) Representative dot plots showing the phenotypical changes during plasma cell maturation. The top plot shows a baseline situation, and bottom plot shows a situation at day 7 post-vaccination. Each dot represents an individual cell. The arrow indicates the direction of changes during maturation. (**b**) Over-time quantitative changes in IgG1+ plasma cells belonging to different maturation stages, presented as ratio over baseline (median, min-max). Dashed lines indicate a ratio over baseline of 0.5 and 2. Underneath each graph, a table shows the baseline cell counts of that population in cells/μL (median, min-max). (**c**) Over-time distribution of IgG1+ plasma cells representing different maturation stages with total IgG1+ plasma cells based on expression of CD20 and CD138. Median values for each population were used to construct the plots. Wilcoxon matched pair signed-ranked test followed by Bonferroni correction was used to assess longitudinal differences in percentage of CD20-CD138+ cells in total IgG1+ plasma cells within each cohort. Differences in the percentage CD20-CD138+ cells in total IgG1+ plasma cells between cohorts were assessed using Kruskal-Wallis followed by Dunn's test, but did not yield significant differences. For pediatric cohorts, no blood samples were collected at day 14. There, the Mann-Whitney test followed by Bonferroni correction was used. For longitudinal changes, only significant differences compared to baseline are shown. Significant longitudinal differences within a cohort as indicated with *, p<0.05; **, p≤0.01; ***, p≤0.001. Significant differences between cohorts at the same time point are indicated with #, p<0.05; ##, p≤0.01; ###, p≤0.001. d= days after vaccination.

3.6 No clear changes in the memory B-cell compartment over time following vaccination

While mature antibody-secreting plasma cells predominantly reside in bone marrow and only transiently appear in blood, memory B cells form the circulating component of immunological memory. In the steady state, memory B cells (directed against various antigens) are known to be abundant in blood of both children and adults, and undergo limited quantitative changes following antigen exposure [44, 45]. Still, memory B cells with specific reactivities can be detected in the blood stream [45-47]. In our previous study we have shown that even minor expansions in circulating memory B cells can strongly correlate with a post-vaccination increase in Bp-specific serum Ig levels [33]. Therefore, we set out to determine whether any quantitative changes can be observed at here-selected time points and whether the same pattern is shared by different cohorts.

Neither total memory B cells nor any of the major memory B-cell subsets underwent con- 152
sistent quantitative changes over time following vaccination (Figure 4A), with exception 153
of IgG4+ memory B cells in adolescents, which showed a minor but significant increase at 154
day 28 compared to baseline levels. Moreover, at 14 days post-vaccination, there was a 155
minor but significant difference between the number of IgG3+ memory B cells between 156
young and older adults, although there were no significant longitudinal changes within 157
any of the groups. Within each cohort the distribution of memory B-cell subsets was stable 158
over the time of analysis (Figure 4B). However, upon further subdivision of memory B 159
cells based on the expression of CD20, CD21, CD24 and CD27, two memory B-cell subsets 160
underwent significant fluctuations over time (Supplemental Figure 3). In adolescents, 161
there was a significant increase in IgG1+ CD20++CD21-CD24+ memory B cells 28 days 162
after vaccination. Moreover, in adolescents and older adults, there was a significant in- 163
crease of IgG1+ CD20++CD21-CD24-CD27+ memory B cells 14 or 28 days after vaccination 164
as compared to baseline. Therefore, it is possible that memory B cells specific to Boostrix- 165
IPV vaccine reside within these CD20++CD21- memory B cells. Here, the use of an antigen- 166
specific approach should lead to additional insights. No significant differences were ob- 167
served between the cohorts. Although most of subsets defined within IgG1+ memory B 168
cells were significantly more numerous in children than in both adult cohorts (data not 169
shown), this was mainly due to higher numbers of IgG1+ memory B cells in children at 170
baseline. Thus, except for a few minor fluctuations, no differences in the number of 171
memory B cells were observed after Tdap booster vaccination. 172
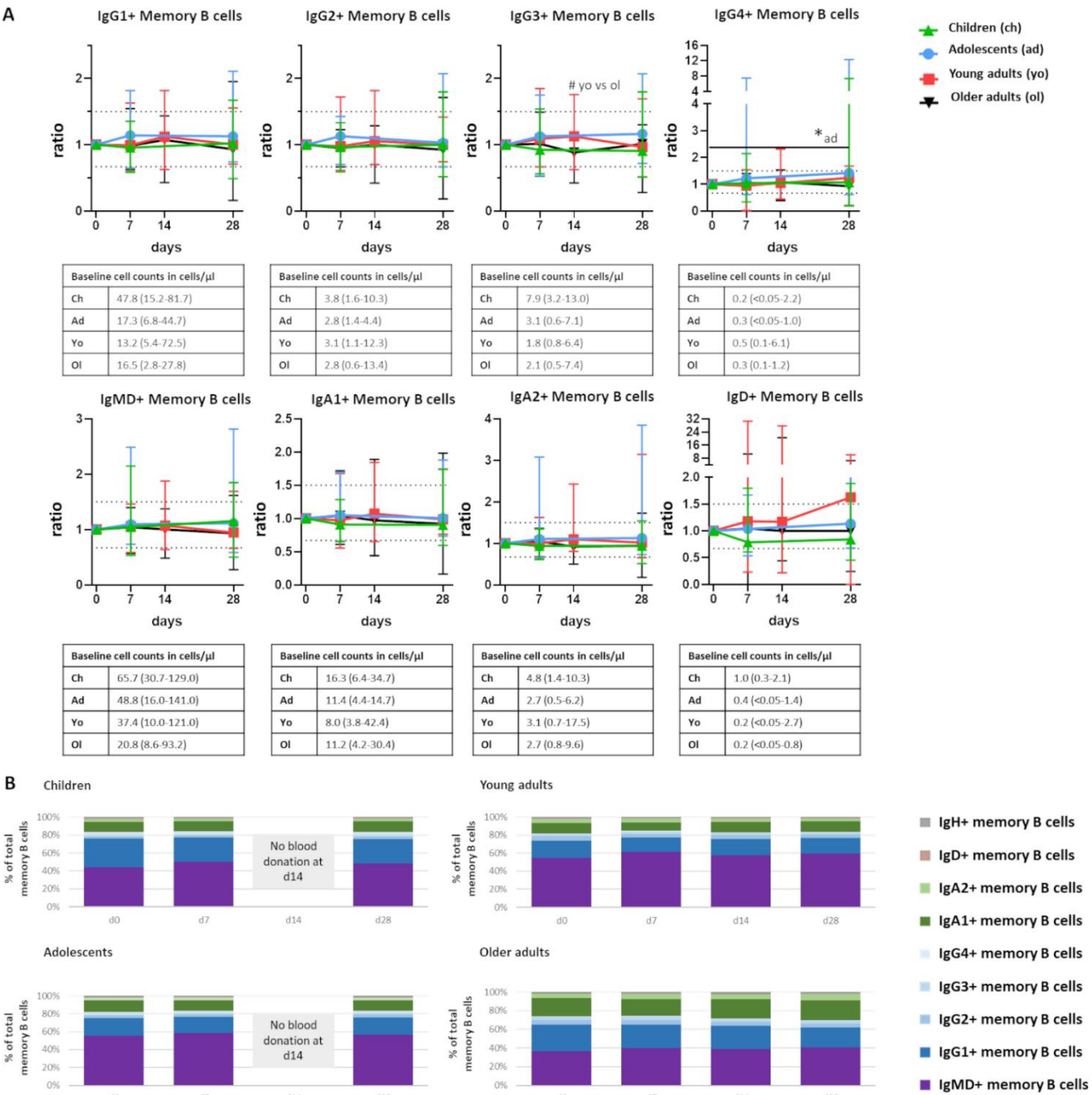
Figure 4. Stable distribution of memory B-cell subsets over time after vaccination. (**a**) Quantitative changes in memory B cells expressing different Ig subclasses, presented as ratio over baseline (median, min-max). Dashed lines indicate a ratio over baseline of 0.67 and 1.5. Underneath each graph, a table shows the baseline cell counts of that population in cells/µL (median, min-max). (**b**) Over-time distribution of memory B cells expressing different Ig subclasses within the total memory B-cell compartment. Median values for each population were used to construct the plots Wilcoxon matched pair signed-ranked test followed by Bonferroni correction was used to assess longitudinal differences within each cohort. Differences between cohorts were assessed using Kruskal-Wallis followed by Dunn's test, but did not yield significant differences. For pediatric cohorts, no blood samples were collected at day 14. There, the Mann-Whitney test followed by Bonferroni correction was used. For longitudinal changes, only significant differences compared to baseline are shown. Significant differences between cohorts at the same time point are indicated with #, $p<0.05$; ##, $p≤0.01$; ###, $p≤0.001$. d= days after vaccination.

3.7 Good correlation between the increase in plasma cell numbers with the vaccine-specific antibody producing cells

Plasma cells are the main producers of antibodies, and, in a recall response, are mainly generated from Ag-specific memory B cells originating from a previous encounter. To support our flow cytometry-based monitoring of memory B and plasma cell fluctuations, we determined the increase in vaccine-specific plasma and memory B cells via ELISpot.

Most plasma cells induced by vaccination are believed to be Ag-specific; we tested this hypothesis by correlating the flow cytometry data with ELISpot analysis. Indeed, the absolute increase in IgG and IgA plasma cells (as measured by flow cytometry) from baseline to day 7 and the number of vaccine-specific IgG and IgA producing plasma cells (as measured by ELISpot) at day 7 showed a positive correlation (IgG: r= 0.59, p<0.0001; IgA: r=0.60, p<0.0001) (Figure 5).
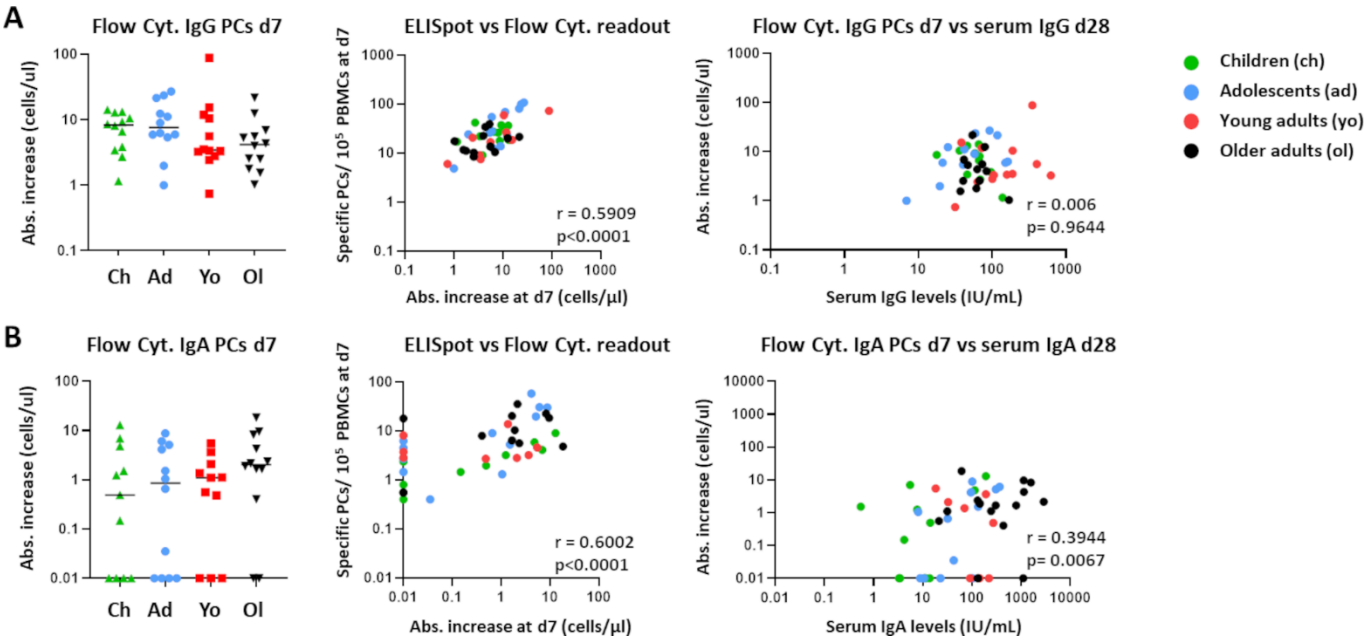
Figure 5. Correlation between cellular changes as measured by flow cytometry and ELISpot, and the correlation between plasma cell expansion and vaccine-specific serum Igs 28 days post-vaccination. (**a**) Left panel: expansion of IgG+ plasma cells (day 7) per individual, expressed as absolute increase in cells/ul. Middle panel: correlation between the ELISpot and flow cytometry readout for IgG+ plasma cells. Right panel: correlation between the increase in plasma cells (as measured by flow cytometry) and the vaccine-specific serum IgG levels (day 28) (**b**) Left panel: expansion of IgA1+ plasma cells (day 7) per individual, expressed as absolute increase in cells/ul. Middle panel: correlation between the ELISpot and flow cytometry readout for IgA+ plasma cells. Right panel: correlation between the increase in plasma cells (as measured by flow cytometry) and the vaccine-specific serum IgA levels (day 28). Each dot represents a single donor. Of note; for visualization purposes, all absolute increases lower than 0.01 were set to 0.01. The original values were used to calculate the Spearman Correlations. Flow Cyt. = flow cytometry; PCs = plasma cells; d= days after vaccination; Abs. = absolute.

Next, we evaluated whether the expansion of circulating IgG+ and IgA+ plasma cells at day 7 could predict the increase in serum IgG and IgA at day 28 and year 1 post-vaccination. We correlated the increase in IgG+ and IgA+ plasma cells (measured by flow cytometry) with the levels of vaccine-specific serum IgG and IgA at day 28 and year 1. Although plasma cells had no predictive value for serum IgG levels, a weak but positive correlation was found for serum IgA levels (r=0.3944, p=0.0067). This correlation was lost at 1-year post-vaccination (r=0.3403, p=0.0207).

For memory B cells, no correlation was observed between ELISpot readout and flow cytometry readout (Supplemental Figure 4). Neither flow cytometry IgG nor IgA memory readout correlated with vaccine-specific serum Igs at day 28 or year 1 (data not shown). These findings indicate that for analysis of memory B cells by flow cytometry an Ag-

specific approach is required. As differences in cellular changes could be found between   218
the different age groups, we also tested for correlations between cell expansion (ratio over   219
baseline) and vaccine specific serum Ig (IU/mL) within each age group (Supplemental   220
Figure 5). No correlations between cell expansion and serum Ig levels were found. Thus,   221
the expansion of plasma cells as measured by flow cytometry seems reflective of the num-   222
bers of Ag-specific plasma cells, and in this regard flow cytometry and ELISpot can pro-   223
vide complementary data. This is not observed for memory B cells.   224

3.8 Weak positive correlation between plasma cell expansion and vaccine component-specific IgG   225
levels   226

As the response to individual vaccine components may differ, we next correlated the   227
IgG1+ and IgA1+ plasma cell expansions at day 7 post-vaccination with the levels of serum   228
IgG and IgA directed against individual pertussis vaccine components. Additionally, we   229
tested whether the degree of maturation correlated with vaccine component-specific IgG   230
or IgA levels. The strongest correlations (with a correlation coefficient between 0.3-0.5)   231
were found between PT- or Prn-specific IgG levels and total IgG1+ plasma cells or CD20-   232
CD138- IgG1+ plasma cells (Supplemental Table 4 (IgG) and 5 (IgA)). No correlations were   233
observed between IgG1+ memory B cell expansion and vaccine component-specific serum   234
IgG, nor between the expansion of IgA memory B cells and vaccine component-specific   235
serum IgA.   236

3.9 More prominent cellular responses in participants primed with wP vaccine   237

Due to the change in the national immunization program at January 1st 2005, all children   238
and 5 out of 12 adolescents were primed with an aP vaccine, while 7 adolescents and all   239
young adults received the Dutch wP vaccine in childhood (presumably, older adults were   240
vaccinated with wP or received no pertussis vaccination during childhood). It has been   241
previously shown that the type of priming can have an impact on both T-cell and antibody   242
responses [16, 18, 22-24]. To study whether cellular B-cell responses can also be affected   243
by the type of priming, while avoiding the effect of age, we first compared both subgroups   244
of adolescents.   245

Over time changes in total, naive and memory B cells were minor and comparable be-   246
tween wP and aP-primed groups (Figure 6A). In contrast, changes in plasma cells were   247
much more prominent in the group which was primed with the Dutch wP vaccine (ratio   248
to baseline: 4.8 vs 1.5 at day 7 in total plasma cells). From all plasma cells, the differences   249
were the clearest for IgG1+ (ratio: 23.9 vs 4.5 at day 7), IgG3+ (ratio: 19.7 vs 3.7 at day 7)   250
and IgG4+ (ratio: 9.2 vs 3.2 at day 7) plasma cells. Only the difference in IgG1+ plasma cell   251
expansion reached statistical significance (p≤0.01), possibly due to the low number of par-   252
ticipants in both groups. Moreover, plasma cell maturation at the peak of expansion was   253
more prominent in wP-primed adolescents in whom most mature CD20-CD138+ plasma   254
cells constituted 46% of IgG1+ plasma cells in contrast to 39% in aP-primed adolescents   255
(Figure 6A+B). Thus, the type of priming background seems to influence the plasma cell   256
response to later booster vaccinations. In our study, the plasma cell response was stronger   257
and more diverse in wP-primed adolescents.   258

To exclude that differences observed in the adolescent cohort were caused by the different   259
sex distribution between aP- and wP-primed individuals, we assessed the impact of sex   260
on the cell expansion in the young adult cohort and extrapolated this to the adolescent   261
cohort. The young adult cohort was well sex-balanced and had a homogenous priming   262
background. We evaluated the expansion of total B cells, plasma cells, IgG1-3+ plasma   263
cells, and IgA1+ plasma cells in males and females. No significant differences were ob-   264
served between male and female responses (Supplemental Figure 6). Therefore, we con-   265
cluded that the sex imbalance in the adolescent cohort did not influence the plasma cell   266
expansion as found in this study.   267

The adolescent cohort only consisted of 12 individuals, which is rather small for statistical analysis. Therefore, although we found some age-dependent differences in cellular responses, we next grouped all aP-primed (children and adolescents) and all wP-primed individuals (adolescents, younger and the presumably wP-primed older adults) to increase the size of the study cohorts (Supplemental Figure 7A). Also in this comparison, expansion of total and IgG1+ plasma cells was more pronounced in wP-primed participants. Differences in other plasma cell subsets did not reach statistical significance. Finally, based on ratio to baseline the (total) plasma cell maturation was more prominent in wP-primed individuals as compared to aP-primed individuals, nevertheless the percentage of most mature cells in the IgG1+ plasma cell population did not significantly differ between the two groups (Supplemental Figure 7).

In summary, here we showed that irrespective of the age of vaccinated individuals, the most prominent cellular changes occurred numbers of in circulating plasma cells. Despite some age-related differences, expansion and maturation of IgG1+ plasma cells at day 7 post vaccination is a shared phenomenon. This expansion of plasma cells measured by flow cytometry was complementary to the increase of vaccine-specific plasma cell numbers determined by ELISpot. No correlations between cell expansion and post-vaccination Ag-specific serum Ig levels were observed. Finally, plasma cell responses were stronger in individuals who were wP-primed.
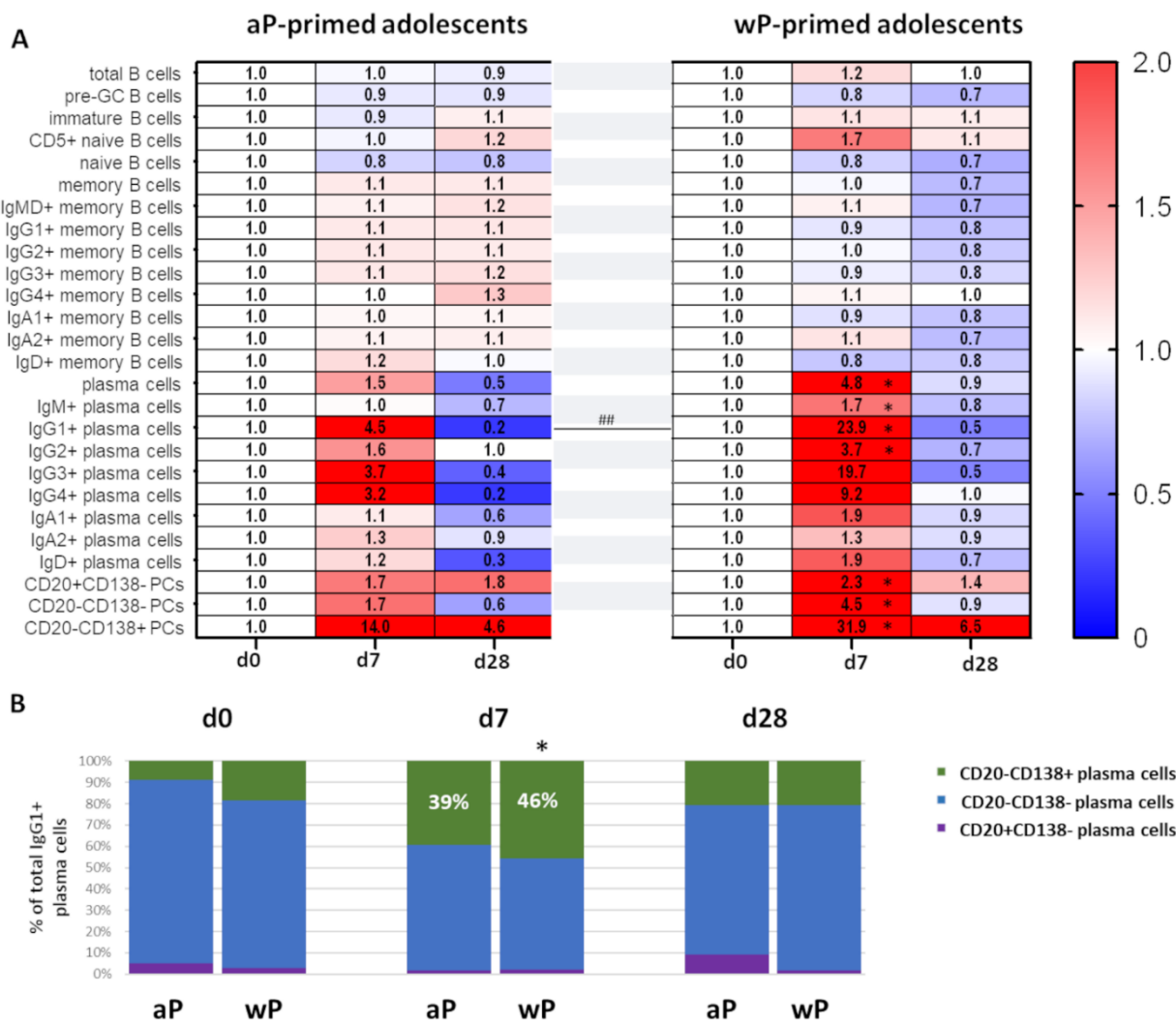
Figure 6. IgG1+ plasma cell expansion and maturation are more prominent in age-matched participants after wP priming. (**a**) Heatmap showing over-time changes in memory B-cell and plasma cell subsets in aP-primed (n=5) or wP-primed (n=7) adolescents (median values). (**b**) Over-time distribution of IgG1+ plasma cells representing different maturation stages within total IgG1+ plasma cells. Median values for each population were used to construct the plots. Wilcoxon matched pair signed-ranked test followed by Bonferroni correction was used to assess longitudinal differences in percentage of CD20-CD138+ cells in total IgG1+ plasma cells within each cohort. Differences in the percentage CD20-CD138+ cells in total IgG1+ plasma cells between cohorts were assessed using Kruskal-Wallis followed by Dunn's test, but did not yield significant differences. For longitudinal changes, only significant differences compared to baseline are shown. Significant longitudinal differences within a cohort as indicated with *, p<0.05; **, p≤0.01; ***, p≤0.001. Significant differences between cohorts at the same time point are indicated with #, p<0.05; ##, p≤0.01; ###, p≤0.001. d= days after vaccination; aP = acellular pertussis vaccine; wP = whole cell pertussis vaccine; pre-GC = pre- Germinal Center.

4. Discussion

In this study, we applied high-dimensional flow cytometry to investigate changes in B cells in individuals of different ages and priming backgrounds upon administration of an aP booster vaccine and correlated these findings with vaccine-specific Ig levels in serum. In all age groups expansion and maturation of plasma cells 7 days post-vaccination was the most prominent cellular change. Although in children the expansion of plasma cells was less prominent than in adults, when expressed as ratio to baseline, they had more plasma cells at peak levels due to their initially high plasma cell numbers. Furthermore, cellular responses were stronger in individuals primed with the Dutch wP vaccine than in individuals who were initially primed with aP vaccines. No consistent over-time memory B-cell fluctuations were observed. No direct correlation between plasma cell expansion or memory B-cell expansion with vaccine-specific serum Ig levels was observed. Nevertheless, weak positive correlations were observed between the expansion of IgG1+ plasma cells and Prn- and PT-specific serum IgG levels post-vaccination. Although serology provides insight in Ag-specific Ig levels and function, analysis of circulating immune cells may result in a deeper understanding of the processes induced by the vaccine and the cellular changes preceding Ig production. Our study points at plasma cells as a potential cellular marker of an immune response and contributes to a better understanding of differences in immune responses between age groups and primary vaccination backgrounds.

To ensure objective data analysis, we used the automated gating and identification (AGI) tool in the Infinicyt software, which has been demonstrated to reduce intra- and inter-operator variability and increase reproducibility of the analysis [40, 48-50]. This is especially important for studies with big data from multiple samples, which cannot be analyzed by a single operator within a reasonable timeframe. Irrespective of the new analysis strategy, these data corroborated major findings from our previous study, where data were subjected to manual analysis [33]. This automated analysis approach, in combination with the standardized EuroFlow sample processing and acquisition procedures, allows for identification of fluctuations in small populations of cells e.g. different plasma cell maturation stages.

Levels of Ag-specific serum Igs are routinely used as readout for vaccine efficacy. In many cases a rise in Ag-specific IgG levels is associated with response to vaccination, and for several vaccines, e.g. against rotavirus, an increase in IgA levels has been indicated as correlate of protection [23, 45, 51-53]. As Igs are the product of terminally differentiated B cells (plasma cells), the B-cell compartment may harbor new correlates or biomarkers of ongoing immune responses. Indeed, we found that expansion and maturation of circulating plasma cells 7 days after booster vaccination was the most prominent cellular change. The generation of mainly IgG1+ plasma cells is in line with previous serology-based studies where within Bp-specific serum IgGs mostly IgG1 antibodies were found, with minor contribution of IgG2,-3 and -4 [51, 54]. The positive correlation between the numbers of total plasma cells with the vaccine-specific plasma cell numbers supports the assumption that most of the plasma cells at the peak of expansion are vaccine specific.

The observed IgA response, mostly in the adult cohorts, is most likely a result of immunological memory generated by previous (subclinical) infection of the respiratory tract, where a mucosal response against Bp was launched. As Bp circulates within the population, causing outbreaks every 2-5 years, the adult cohorts likely encountered Bp multiple times during life, which explains the more prominent IgA1+ plasma cells response in these groups [55-58]. In contrast, the expansion of IgG4+ plasma cells was mostly seen in the pediatric cohorts, which may be explained by the predominantly aP priming in these cohorts; this has been shown to induce a more Th2-related response as well as increased vaccine-specific serum IgG4 [23, 24].

In addition to the expansion of IgG1+ and IgA1+ plasma cells in adults, and the IgG1+ and IgG4+ response in the pediatric cohorts, which are in line with previous (cellular and serology-based) studies, we also observed a prominent increase in IgG3+ plasma cells in all cohorts [23, 33, 51, 54]. A potential explanation for this phenomenon might be that – in

addition to the memory B cells-   there are naive B cells recognizing the antigens and undergo first-step IgG3 class switching and affinity maturation [59].

The difference in plasma cell –and thus antibody- production can have consequences for the type and efficacy of the launched immune response. IgG1 and IgG3 antibodies have stronger opsonizing capacities as compared to IgG4 antibodies [60]. The mixed IgG1-IgG3-IgG4 response observed in the mostly aP-primed pediatric cohorts may lead to competition for Bp antigens in future encounters, possibly leading to less efficient bacterial clearance as compared to the IgG1-IgG3 (and IgA1) response observed in the adult cohorts [59, 61]. Lastly, the prominent contribution of IgA1+ plasma cells to responses observed in the adult cohorts, which is likely an indicator of previous pertussis encounters, may imply existence of effective mucosal defense mechanisms, and more efficient protection against bacterial translocation in IgA-producing individuals. Comparison of repertoires and reactivities of IgA in mucosa and in circulation could provide better insights into this phenomenon and value of IgA as a biomarker of protection.

Maturation of plasma cells (total and IgG1+) was observed irrespective of age and priming background. This clear expansion and maturation of total and IgG1+ plasma cells is in line with our previous findings and may be explained by the prolonged retention of newly generated plasma cells in the periphery as well as the competition for bone marrow niches with pre-existing long lived plasma cells [33, 62].

In this study, several differences between the aP and wP-primed cohorts were observed. Although the size of the age-matched cohort was too limited to reach statistically significant conclusions, major observations were confirmed by analysis of the total cohort, divided based on the priming background. Remarkably, this difference based on priming background was not observed in the overarching part of the BERT study, where the Bp-specific Ig responses of 85 Dutch and Finnish adolescents pre- and post-vaccination were evaluated [29].

The formulation of aP and wP vaccines differs with regards to number of antigens and the total antigenic load, with wP vaccines containing the broad variety of pertussis antigens and aP vaccines containing high concentrations of a restricted number of antigens. In consequence, wP priming is likely to trigger a more diverse antibody response. Since consecutive boosters lead to a more specific, but also more restricted response, this initial broad priming can be beneficial in case of encountering future (mutated) bacterial strains [10, 12, 13]. Interestingly, in this study we showed that, compared to aP-primed individuals, individuals primed with the Dutch wP vaccine have a stronger response upon aP booster vaccination. It would be of interest to visualize potential differences in breadth of an immune response against Bp antigens. Moreover, since the initial type of priming seems to imprint future responses to given antigens, it should be carefully considered in the design of future vaccines and vaccination strategies. This may also hold true for diseases other than pertussis, such as COVID-19.

To identify unique and shared patterns in-between groups, we primarily focused on normalized data (represented as the ratio to baseline). However, we also showed that, in line with published studies, children had overall higher leukocyte and B-cell counts compared to (older) adults [32, 41]. Specifically, the cell count of naive B and T cells -and thus the available naive repertoire- is substantially higher in youth [32, 48]. Therefore, despite a lower increase in cells expressed as ratio to baseline, children and adolescents may still produce a stronger and more diverse immune responses than adults.

In this study, memory B-cell fluctuations were limited. As the frequencies of Ag-specific memory B cells are low, as demonstrated by previous studies using ELISpot assays, an increase in only these Ag-specific memory B cells may not have an impact on the total memory B-cell population [63, 64]. Indeed, in this study no correlation was found between the memory B-cell fluctuations measured by flow cytometry and the vaccine-specific memory B cells by ELISpot. However, we observed an increase in CD20++CD21- IgG1+ memory B cells in older adults and adolescents at day 14 and 28 after vaccination, respectively. Interestingly, several studies reported an increased percentage of Ag-specific CD27+CD21-/dim B cells 14 days after influenza vaccination [65, 66]. There is no

consensus about the exact function of these CD21-/dim B cells, but, it has been suggested that CD21-/dim B cells are exhausted cells or – as described in autoimmunity and chronic infection- are anergic [67, 68]. In this context it is not unlikely, that cells which responded to an antigen multiple times would acquire this phenotype. However, Lau and colleagues suggest that CD21-/dim B cells are primed for plasma cell differentiation [65]. Ag-specific flow cytometry studies should give insight into the exact function of this B-cell subset.

No correlation was found between expansion of memory B cells and Ag-specific serum IgG levels at day 28. Previously, we observed a clear correlation between the expansion of IgG1+ memory B cells and the vaccine-specific IgG levels at day 21 in a cohort of 10 healthy adults [33]. Moreover, we found that although in the majority of participants memory B cells showed maximum expansion at 14 days after vaccination, the expansion of memory B cells was not as synchronized in time as the plasma cell expansion, implying that -in some participants- we may not have sampled at the most optimal timepoint [33], especially in the children and adolescent cohorts, where the sampling times were limited to day 0, 7 and 28. This difference in timing of memory B-cell responses might be related to the immune status of each individual at baseline and it makes the use of memory B cells as correlates of protection more difficult.

Neither aP nor wP vaccination yields a response that fully mimics natural infection, especially the IgA response seems to be limited upon vaccination and mostly relies upon previous encounters with Bp. To overcome this limitation, multiple novel pertussis vaccines and alternative delivery routes are being developed, such as nasal delivery of a vaccine or the use of life attenuated Bp strains (BPZE1) [69-71]. It would be of interest to evaluate how (cellular) immune responses induced by these vaccine candidates compare to cellular kinetics induced by intramuscular aP and wP vaccines, as well as (controlled) human infection. Such comparison between aP booster vaccination and (controlled) human infection is currently ongoing within the IMI2 PERISCOPE program [72, 73]. These studies will create a solid basis for evaluation of novel vaccination approaches.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/

Supplemental Table 1. Composition of the EuroFlow B-cell panel and technical information on the reagents for the IMI2 PERISCOPE BERT study.

Supplemental Table 2. Phenotypic descriptions used to define B-cell subsets stained with the EuroFlow B-cell panel by manual analysis.

Supplemental Table 3. Baseline distribution of leukocytes, lymphocytes, T cells and NK cells in donor groups.

Supplemental Table 4. Spearman Ranking Correlation between IgG1+ plasma cell and memory B-cell kinetics and vaccine component-specific serum IgG.

Supplemental Table 5. Spearman Ranking Correlation between IgA1+ plasma cell and IgA memory B-cell kinetics and vaccine component-specific serum IgA.

Supplemental Figure 1. No clear over-time post-vaccination changes in major populations in any of the donor groups.   The post-vaccination fluctuations of (A) leukocytes, (B) lymphocytes, (C) T cells, and (D) NK cells, presented as ratio over baseline (median, min-max). Dashed lines indicate a ratio of 0.67 and 1.5 compared to baseline. Underneath each graph, the baseline cell counts per cohort are presented in cells/μL (median, min-max). To assess longitudinal changes within each cohort, Wilcoxon matched pair signed-rank test followed by Bonferroni correction was used. To test differences between cohorts at one timepoint, Kruskal-Wallis followed by Dunn's test was used, with exception of the comparison at day 14. At day 14, only blood samples from the adult cohorts were collected. Here, the Mann-Whitney test followed by Bonferroni correction was used. For longitudinal changes, only significant differences compared to baseline are shown. *, $p<0.05$; **, $p≤0.01$.

Supplemental Figure 2. Over time maturation of total plasma cells. (A) Representative plots show-ing the phenotypical changes during plasma cell maturation. Each dot represents an individual cell. The arrow indicates the direction of changes during maturation. (B) Over-time quantitative changes in plasma cells belonging to different maturation stages, presented as ratio over baseline (median, min-max). Dashed lines indicate a ratio of 0.5 and 2.0 compared to baseline. Underneath each graph, the baseline cell counts per cohort are indicated in cells/μL (median, min-max). (C) Over-time dis-tribution of plasma cells representing different maturation stages within total plasma cells. Median values for each population were used to construct the plots. Wilcoxon matched pair signed-ranked test followed by Bonferroni correction was used to assess longitudinal differences in percentage of CD20-CD138+ cells in total plasma cells within each cohort. Differences in the percentage CD20-CD138+ cells in total plasma cells between cohorts were assessed using Kruskal-Wallis followed by Dunn's test, but did not yield significant differences. At day 14, only blood samples from the adult cohorts were collected. Here, the Mann-Whitney test followed by Bonferroni correction was used. For longitudinal changes, only significant differences compared to baseline are shown. Significant longitudinal differences within a cohort as indicated with *, p<0.05; **, p≤0.01; ***, p≤0.001. Signifi-cant differences between cohorts at the same time point are indicated with #, p<0.05; ##, p≤0.01; ###, p≤0.001.

Supplemental Figure 3. No significant changes in IgG1+ memory B-cell subsets upon vaccination. Over-time quantitative changes in IgG1+ memory B-cell subsets, presented as ratio over baseline (median, min-max). Dashed lines indicate a ratio over baseline of 0.67 and 1.5. Underneath each graph, the baseline cell counts per cohort are presented in cells/μL (median, min-max). Wilcoxon matched pair signed-ranked test followed by Bonferroni correction was used to assess differences in ratio compared to baseline over time. For longitudinal changes, only significant differences com-pared to baseline are shown. *, p<0.05; **, p≤0.01; ***, p≤0.001.

Supplemental Figure 4. Correlation between cellular changes as measured by flow cytometry and ELISpot.   (A) Left panel: expansion of IgG+ Memory B cells (day 28) per individual, expressed as absolute increase in cells/ul. Right panel: correlation between the ELISpot readings and the flow cytometry readout for IgG+ Memory B cells. (B) Left panel: expansion of IgA+ Memory B cells (day 28) per individual, expressed as absolute increase in cells/ul. Right panel: correlation between the ELISpot readings and the flow cytometry readout for IgA+ Memory B cells. Of note; for visualization purposes, all absolute increases lower than 0.01 were set to 0.01. The original values were used to calculate the Spearman Correlations. Flow Cyt. = flow cytometry; spec.= specific (in this case, spe-cific for the tested vaccine antigens); MBC = Memory B cells; PBMC= peripheral blood mononuclear cells; d= days after vaccination; r= Spearman's Correlation coefficient; abs.= absolute.

Supplemental Figure 5. Correlation between cellular changes and the vaccine-specific serum IgG level post-vaccination as determined by Spearman's Ranking Correlation per age cohort. Per cohort the left plot shows the correlation between the maximum expansion of IgG1 plasma cells (day 7) and vaccine-specific serum IgG (directed against FHA, Prn, PT and Tet) (day 28). The right plots show the correlation between the maximum expansion of IgG1 memory B cells (day 14 or day 28) and vaccine-specific serum IgG (directed against FHA, Prn, PT and Tet) (day 28). MBC = memory B cell; r= Spearman's correlation coefficient; d= days after vaccination.

Supplemental Figure 6. Impact of sex on cellular responses after vaccination in the young adult cohort (all wP-primed). Flow cytometry-derived cell numbers (absolute count in cells/ul (A) and ratio over baseline (B)) and their changes over time in an age-matched, wP-primed male (n= 7) and female (n= 5) cohort. Of note, for one male participant, no baseline B-cell data was available. There-fore, in the graphs showing the ratio over baseline, data of 6 males are shown, whereas absolute counts include the data of 7 males. Graphs indicate median + range. Dashed line indicates ratio of 1.0 (baseline value).

Supplemental Figure 7. IgG1+ and total plasma cell expansion is more prominent in non-age-matched donors after wP priming. (A) Heatmap showing over-time changes in memory B-cell and plasma cell subsets in aP-primed (12 children + 5 adolescents) and wP-primed (7 adolescents, 12 young adults and 12 older adults) donors. (B) Over-time distribution of IgG1+ plasma cells repre-senting different maturation stages with total IgG1+ plasma cells. Median values for each popula-tion were used to construct the plots. Wilcoxon matched pair signed-ranked test followed by Bon-ferroni correction was used to assess longitudinal differences in percentage of CD20-CD138+ cells in total IgG1+ plasma cells within each cohort. Differences in the percentage CD20-CD138+ cells in

total IgG1+ plasma cells between cohorts were assessed using Kruskal-Wallis followed by Dunn's   519
test, but did not yield significant differences. For longitudinal changes, only significant differences   520
compared to baseline are shown. Significant longitudinal differences within a cohort as indicated   521
with *, $p<0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$. Significant differences between cohorts at the same time point   522
are indicated with #, $p<0.05$; ##, $p \leq 0.01$; ###, $p \leq 0.001$. D= days after vaccination; aP = acellular per-   523
tussis vaccine; wP = whole cell pertussis vaccine.   524

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