Monitoring the immune responses to vaccination and pertussis: bordetella pertussis and beyond

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Chapter 5

General Discussion

Chapter 5

Immune monitoring is a process in which a diverse set of tools is applied to allow evaluation of the immune system. Monitoring of the immune system, both when an active response is ongoing and when in homeostasis, can yield valuable information about the immune status of an individual or a group. Vaccination is an example of an active response. Here, monitoring can yield information about the efficacy of applied vaccination/immunization method, or it can give information about the functioning of the immune system in one or more individuals (for example when using a neoantigen). The importance of immune monitoring is well-recognized and various efforts are made to harmonize/standardize protocols, yet further improvements are still needed to facilitate direct comparisons between different laboratories and between different clinical studies.

In this thesis, we addressed multiple topics related to immune monitoring. First, we investigated the impact of various pre-analytical and analytical factors on the data quality and robustness of analysis (**Chapter 2**). We formulated a list of guidelines to improve reliability and reproducibility of data regarding immune cell quantity and phenotype. These guidelines find application both in research utilizing human blood, but also in patient diagnostics or industry, e.g., in evaluation of new treatments or vaccination strategies.

Next, we evaluated the longitudinal immune response following a pertussis booster vaccination, or after bacterial challenge with Bo*rdetella pertussis (B*p) (**Chapter 3**). In case of booster vaccination, we investigated the immune response not only in a 'standard' cohort of healthy adults, but also in a cohort of children, adolescents, and older adults. Additionally, we extended our flow cytometry studies with antigen (Ag)-specific assays, such as serology and ELISpot, and showed that they can complement each other.

In **Chapter 3.1**, we investigated kinetics of >250 circulating immune cell subsets after booster vaccination. We observed the most prominent changes in the plasma cell compartment. Consequently, we focused on kinetics in the B-cell compartment in **Chapter 3.2** and found that irrespective of age and primary vaccination background, the expansion and maturation of (primarily) IgG1 plasma cells was the most prominent feature of an immune response initiated by acellular pertussis (aP) booster vaccination. Despite these similarities, specific differences in plasma cell responses appeared to be associated with age and primary vaccination background.

Conclusions drawn from flow cytometry can not only be supplemented by techniques such as ELISpot and serology, but also by more exploratory techniques such as B-cell receptor (BCR) repertoire analysis. In **Chapter 3.3**, we investigated the BCR repertoire of plasma cells by means of single cell sequencing techniques and evaluated to what extent the distribution of plasma cell subsets in this data was reflecting the flow cytometry data. Moreover, we built a query tool to help search for BCRs specific to individual vaccine components.

As diverse cell populations and timepoints can be of importance in different models, we compared the cellular kinetics (within innate immune cells, T cells, and B cells) between Bp booster vaccination or bacterial challenge in **Chapter 3.1** and **3.4**. Here, we found that the expansion and maturation of plasma cells was less prominent and was delayed in time in the bacterial challenge model, but more heterogenous with respect to used plasma cell isotypes. Using the data gathered in **Chapter 3**, we identified informative timepoints and cell populations that could be monitored in future vaccination/challenge studies. This data emphasizes the importance of pilot (time point finding) studies.

Finally, we set out to use immune monitoring to evaluate the immune system in homeostasis to get in-depth information on the leukocyte composition and function in a group of individuals carrying a specific genetic variant, which is associated with decreased risk of developing dementia, and increased chance of longevity (**Chapter 4**). This was supplemented with SARS-CoV-2 vaccination studies to evaluate the induced immune responses.

Several of the conclusions drawn from the here-presented work have implications for future studies and/or experiments. These implications and highlights will be discussed further in this chapter.

The need for (increased) standardization in immune monitoring

Standardization of the pre-analytical, analytical, and post-analytical processes is crucial to improve data quality and reproducibility. The importance of standardization is well-recognized, with many scientific articles, guidelines, protocols and tools being published in the past years, not only for flow cytometry, but also for immune monitoring in general.[1-10] Thus, when designing a clinical trial, there are multiple guidelines available to make informed decisions about the logistics and how to keep the pre-analytical phase as controlled and optimal as required for the type of study.

Pre-analytical factors

To understand the impact of storage and transportation after sample collection, we first determined the optimal storage time and condition. In our case, storage of whole blood at room temperature for <24h, but ideally <6h, was the best for PB-EDTA. Storage from 6h onwards resulted in decreasing absolute cell counts (**Chapter 2.1**). In this entire thesis, we attempted to store samples as short as possible (ideally <6-12h, with exception of the storage experiments in **Chapter 2**) and samples were always kept at room temperature as whole blood until the moment of processing. To avoid introducing any bias in longitudinal data, we aimed to keep storage conditions and used anti-coagulant as similar as possible. In this thesis we only investigated the impact of the pre-analytical factors on the primary immunodeficiency screening and orientation tube (PIDOT). However, the EuroFlow Consortium has further investigated the impact of several pre-analytical factors on other EuroFlow panels (such as tube 1 from the acute myeloid leukemia/myelodysplastic syndrome (AML/MDS) panel, the acute leukemia

orientation tube (ALOT), and the lymphoid screening tube (LST) and published their findings recently.[11]

Analytical and post-analytical factors

In this thesis, the guidelines and protocols as proposed by the EuroFlow Consortium have been applied to all experiments executed on a BD FACS Canto II 3L, a BD FACS LSR Fortessa 4L, or a BD FACS LSR X-20 Fortessa 4L (BD Biosciences, San Jose, CA, USA).[4, 5] In short, this means that standardized protocols were used whenever available, and that CS&T beads (BD Biosciences) as well as SPHERO™ Rainbow calibration particles (Cytognos, Salamanca, Spain) were run daily to check machine performance.

One strategy that could help reporting the data in a more harmonized and standardized manner is the use of database-driven analysis (Automated Gating & Identification; AGI), possibly combined with comparison with reference cell counts and/or automated reporting of findings. The AGI tool makes use of algorithms that cluster flow cytometry data based on an established reference database.[8] Although events that cannot be automatically assigned still require manual checking, we and others have shown that the use of AGI strongly reduced the variability as compared to manual analysis, where each operator introduces subjectivity (**Chapter 2.2**).[12-14]

The calibration and daily quality control (QC) of instruments, and the use of standardized protocols for sample preparation and acquisition are a prerequisite for a successful automated analysis. When using the database-driven approach, it is of importance that evaluated samples have undergone similar preparation/ treatment as the samples in the reference database. When sample treatment or used anti-coagulant deviates from the samples used to construct the reference database, this may influence the scatter properties or fluorescence of the acquired sample. As a result, automated analysis may be suboptimal, with many events requiring a manual check.

In case no database-driven approach is available, which is often the case for (highly) exploratory research, the use of highly standardized analysis strategies can be a solution. In this thesis, we started with manual analysis of flow cytometry data acquired in strictly controlled settings. In the meantime, a database-driven approach, using a highly similar gating strategy, became available. In two consecutive clinical trials (one using manual gating and one using AGI, **Chapter 3.1** and **3.2**, respectively), we found that (major) findings were reproducible between the two approaches, confirming that the degree of standardization was sufficiently high.

Additionally, several commercially available databases contain not only fully annotated flow cytometry datafiles of healthy individuals, but also fully annotated patient material or reference values, which aid clinical laboratories to establish a diagnosis. For example, when staining a (suspected) patient sample with the

ALOT panel and using the automated analysis in the Infinicyt Software, the Compass Tool performs immunophenotypic diagnosis and classification in patients with acute leukemia (AL). Based on the outcome, consecutive steps can be taken to finalize the diagnosis.[15] Another example is the inclusion of well-annotated files of patients with PIDs in the reference database for the PIDOT.[16]

Cohort selection and definition

Cohort selection is inherently related to clinical trials. While selecting a cohort, the aim is to make it representative of the total target group. In the introduction of this thesis, we referred to a systematic review that we conducted.[17] Here, we found that the extent of cohort description varied between evaluated studies, even though the reported metadata (such as comorbidities, age range, number of individuals, sex distribution, ethnicity, current health status, etc.) is an important factor showing how well the cohort represents the total target population.

One example of the importance of the cohort definition became apparent in **Chapter 4** of this thesis, where we determined the calcium release in memory B cells (MBCs) upon BCR stimulation. This assay was performed for a set of the healthiest individuals ('Cohort II'), but also in the total cohort ('Cohort I'), which had far less strict inclusion criteria, and more reported comorbidities. Results differed between the two cohorts. Non class-switched MBCs from Cohort I showed no difference in calcium flux upon stimulation between *PLCG2* p.P522R carriers and non-carriers. However, when only selecting Cohort II (the healthiest individuals, whose cells were used for several functional experiments), we observed a clear trend towards a more robust calcium flux in stimulated non class-switched MBCs in carriers. This marks the importance of clearly stating the inclusion criteria. Thus, standardization of cohort description should be an ongoing effort.

To gain more confidence in the representativeness of a cohort, it is a good practice to compare baseline parameters of the cohort to available reference values. With the introduction of high dimensional flow cytometers, the number of reported cell populations (percentages and cell counts) is growing. It would be highly beneficial to establish and keep updating such 'reference values' for healthy controls of different age categories as distribution of multiple leukocyte subsets changes during lifetime. Several of such reference values in healthy controls of several age ranges have already been published, both as tables and as database-implemented reference values, e.g. for diagnostic purposes.[12, 16, 18, 19] [van der Pan et al, manuscript accepted] Importantly, reference values may also be impacted by other factors such as sex and ethnicity.[20, 21] Therefore, these factors should ideally be included as metadata as well.

It is not unimaginable that in the (near) future, reference ranges of various studies reporting on the same cell populations and subsets will be merged into large databases. In fact, such efforts are already ongoing, and tools are being developed. For example, in 2018 Hu and colleagues introduced a bioinformatic tool for automated meta-analysis of mass and flow cytometry files.[20] Of note, the

heterogeneity between studies was marked as a challenge. To facilitate such meta-analysis, it is important that cohort description is clearly stated. Moreover, the used antibody cocktail, phenotypic identification of cell populations and mode of reporting (e.g., IU/mL, ratio compared to baseline, % of total cells, or cells/μL blood) should be clearly stated to enable merging of (sub)data, although the latter may be less of an issue when raw data files are used. This clarity is as important when comparing or merging data in-between different studies to draw valid conclusions. In an era where bioinformatics play an important role in handling these high-dimensional datafiles, consistency and transparency is becoming increasingly important.

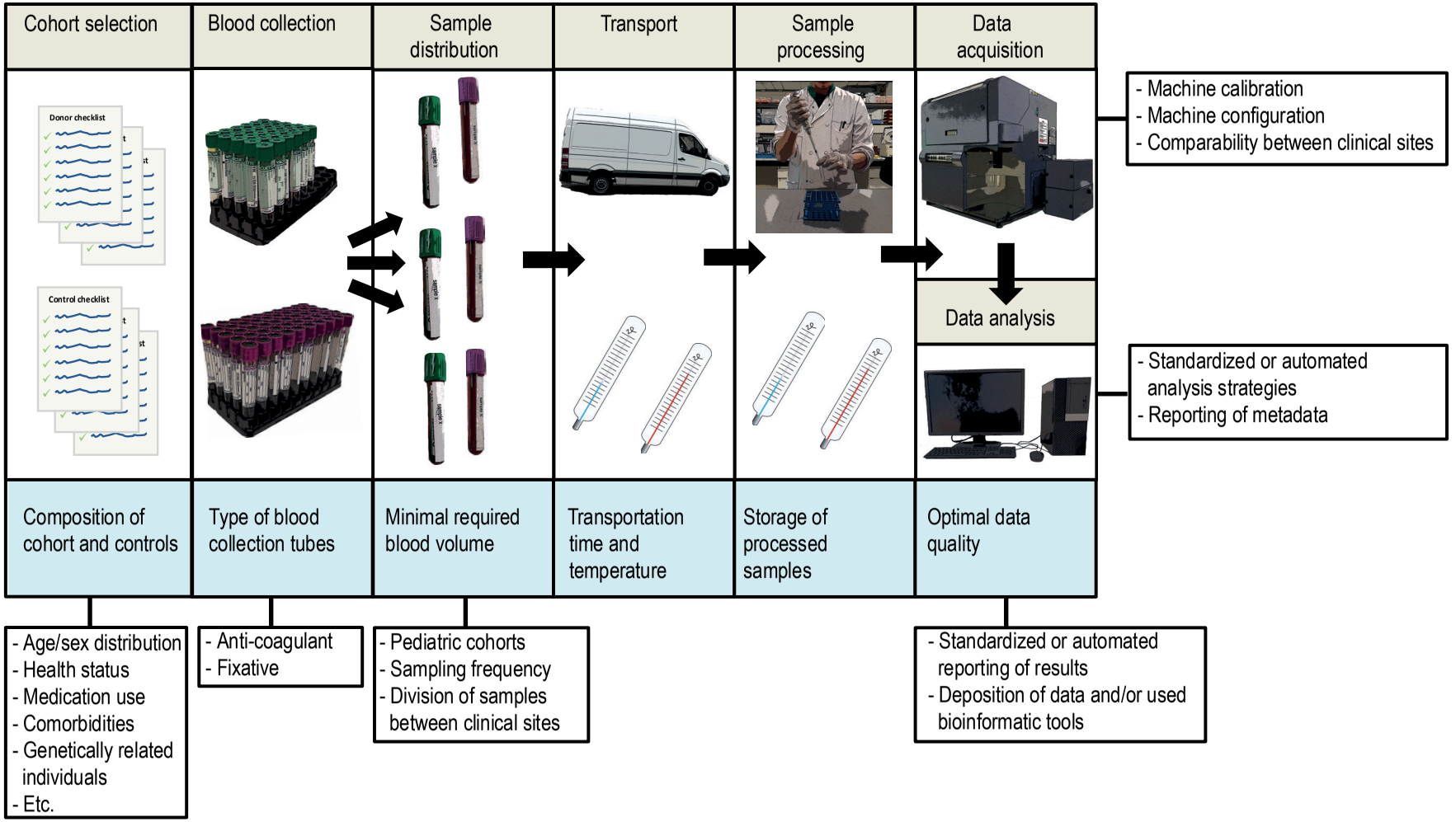
In addition to clearly stated methods, it is becoming more standard to deposit raw data (or to provide this upon request) in repositories such as ImmPort (Immport.org).[22] This results in increased data transparency, and enables comparison or alignment of data analysis strategies between studies where initially different approaches were used. Additionally, guidelines outlining the minimal required information are available.[1] Although we mainly focus on flow cytometry data here, this statement also applies for other fields, such as mass cytometry or single-cell sequencing data.

Based on the findings in this thesis, I highly encourage other researchers to be as complete and consistent as possible when describing their methods, data, and readouts. In **Figure 1**, I indicated which pre-analytical, analytical, and post-analytical factors can influence the results of (meta-)analysis and should thus be carefully decided (please beware that this figure does not aim to give a complete overview of all factors known to influence results of the (meta-)analysis). To provide a better overview of the experimental procedure used in individual studies throughout this thesis, I have included a comprehensive list of used gating strategies, used flow cytometry panels and list of population definitions as appendices in this thesis.

Role of bioinformatic tools in analysis

With the ever-increasing size and dimensionality of experimental data, bioinformatics is becoming an important tool in the objective interpretation of data. Especially the spectral flow cytometers and mass cytometers yield high-dimensional data that may require specialized software for objective analysis.[23-27] Various software and analysis packages are available to attune to everyone's needs. In this thesis we primarily used (direct) prototypes of flow cytometry panels of which standardized gating strategies and databases for automated analysis were developed in the Infinicyt Software. Some of these were developed specifically

Figure 1 (next page). Overview of factors in the pre-analytical, analytical, and post-analytical process that can influence the quality of results and should ideally be decided before start of large experiments, such as a clinical trial. To increase transparency and help re-use of data (e.g., meta-analysis or to construct a database), a clear cohort definition and data availability statement are of importance. Figure modified from Diks et al (this thesis).


Cohort selection
Blood collection
Sample distribution
Transport
Sample processing
Data acquisition
Donor checklist
Control checklist
- Machine calibration
- Machine configuration
- Comparability between clinical sites
Data analysis
- Standardized or automated analysis strategies
- Reporting of metadata
Composition of cohort and controls
Type of blood collection tubes
Minimal required blood volume
Transportation time and temperature
Storage of processed samples
Optimal data quality
- Age/sex distribution
- Health status
- Medication use
- Comorbidities
- Genetically related individuals
- Etc.
- Anti-coagulant
- Fixative
- Pediatric cohorts
- Sampling frequency
- Division of samples between clinical sites
- Standardized or automated reporting of results
- Deposition of data and/or used bioinformatic tools

for the PERISCOPE program in a collaboration with Cytognos, the company that developed the Infinicyt Software. Therefore, we used the Infinicyt software for all flow cytometry analysis in this thesis. Yet we recognize that there are many different analysis software packages/analysis methods (such as instrument-packages software FACS Diva, stand-alone software FlowJo or FSC Express, or methods such as t-SNE, vi-SNE, FlowSOM, SPADE or PhenoGraph), all with their own strengths.[27] Although several of these approaches are relatively accessible, not all are equally user-friendly, therefore collaboration and crosstalk with bioinformaticians is highly encouraged. In this thesis, we aimed for objective analysis by means of applying (bio)informatic strategies, such as correlation networks and the use of database-driven analysis.

In addition to the flow cytometry data, other techniques, such as single-cell sequencing, require a certain level of expertise in bioinformatic approaches. In **Chapter 3.3**, our bioinformatician developed an analysis pipeline to evaluate and interpret the whole transcriptome and BCR sequencing data. Moreover, the PLCG2 variant investigated in **Chapter 4** was a result of a large genome-wide association study (GWAS) by Sims et al., where researchers from different fields and institutes worked together to identify rare coding variants associated with Alzheimer's Disease, which again emphasizes the need for inter-field collaboration.[28] Although I acknowledge that it may be challenging for both parties to meet at 'the border of their comfort zone', I believe this is how we can advance objective analysis of big data and -in fact- science in general. Therefore, I highly recommend researchers to collaborate with one another, and learn from and with each other.

In-depth immune monitoring to evaluate the functioning of the immune system
Although majority of this thesis focused on the immune system during an antigen challenge, there are many cases where monitoring of the immune system in homeostasis is performed. For example, to confirm or exclude an immunological disorder (e.g., with the PIDOT panel as discussed in **Chapter 2**) or to diagnose hematological malignancies. Aside from diagnostics, the immune system can also be monitored in more exploratory settings, as was done in **Chapter 4**. There, we investigated the immune system both in presence and absence of stimulation in carriers of a genetic variant of the PLCG2 gene (variant p.P522R/ rs72824905), which was associated with decreased risk of several forms of age-related neurodegenerative diseases, including Alzheimer's Disease.[29-31] Moreover, this variant was associated with increased chance of longevity.[29] As the immune system is (in)directly involved in several of these diseases, we hypothesized that evaluation of the immune system may give information about the (protective) mechanism of action.

Age is an important risk factor in many neurodegenerative diseases, and individuals of the same metric age can present with differently pronounced signs of biological aging. Based on the findings presented in **Chapter 4**, we speculate that p.P522R carriers are somehow more resilient to the effect of biological aging.

There are many markers that can be used as marker of aging, and the function and composition of the immune system is one of them. Even though most disease-related pathologies in dementia occur in the brain, immune cells -isolated from the blood- can provide valuable information about aging processes. There are several similarities between blood phagocytes and microglia, such as the expression of *PLCG2* and the mechanisms used to remove damaged cells, pathogens, or (cellular) debris. Therefore, some findings in the blood phagocytes might give insight in the microglia of the same individual as well. Moreover, a recent conference abstract from the 63rd ASH Annual Meeting described the tracing of somatic mutations (occurring in blood or bone marrow), and found these mutations in cells that resided in the brain and could not be distinguished from microglia.[32] This may suggest that during lifetime, (progenitor) cells originating from the blood and bone marrow can find their way into the brain and contribute to the microglial pool. Thus, insights into the functional capacities of circulating immune cells (mainly phagocytes) may translate in part to neurodegenerative diseases and possibly the functioning of microglia in these individuals. The clear advantage of blood is that it can be obtained in a minimally invasive way, whereas human microglia can generally only be obtained post-mortem or from brain biopsies. Of note, iPSC-derived human microglia are an alternative to primary human-derived microglia and have been shown to have a highly similar transcriptomic profile, protein expression, and functional ability to their brain-derived counterpart.[33] Lastly, immunoglobulins (Igs) are also able to enter the brain and exert their functions there, making them potential candidates for intervention strategies for several types of dementia.[34-36]

Certain *PLCG2* variants can be associated with the pathological conditions. For example, there is a condition called *PLCG2* associated antibody deficiency and immune dysregulation (PLAID),[37] which is generally associated with loss- or gain-of-function deletions in *PLCG2*. Most patients are thought to have a deletion in the autoregulatory domain, which leaves the active site of the enzyme continuously exposed.[38] To the best of our knowledge p.P522R, which is a gain of function variant, has not been associated with any disease/disorders, but in fact reduces the chance to develop several neurodegenerative diseases.[29-31] It should however be noted that this is a rare variant that only recently gained attention, and data on human subjects are still limited. Thus, it may be wise to further characterize p.P522R carriers (e.g., the prevalence of malignancies, immune disorders and/or other morbidities). Here, immune monitoring can play an important role by combining immunophenotyping with an *ex vivo* functional analysis using blood as a minimally invasive source of material.

Immune monitoring to evaluate ongoing immune responses - complementarity of different approaches

The efficacy of vaccines has been demonstrated by the reduction of infectious-disease related deaths since their introduction.[39] In addition to neutralizing Igs, cellular responses play an important role in the protective immunity generated by vaccines.[40, 41] However, not all underlying cellular mechanisms are fully

understood. Generally, serology is considered the golden standard to assess vaccine efficacy. Serological tests may evaluate the bactericidal activity of produced Igs, their neutralization or inhibition activity, or the level of Ag-specific Igs in the serum.[40]

In many (exploratory) vaccination studies, serology is complemented with other readouts, such as ELISpot and flow cytometry.[42-45] Occasionally, approaches such as DNA/RNA sequencing or mass cytometry are used.[46, 47] Although these various techniques can complement each other, one can wonder to what extent these assays are redundant or unique in their findings, and which assays should be included in a certain setup. Aside from costs associated with each technique, the required time and expertise for analysis of raw data, as well as the extent of standardization may play a role, especially when application in clinical trials or diagnostic purposes is the end goal.

In this thesis, we applied various techniques to measure vaccine-induced responses. In all Bp studies, we measured the increase in Ag-specific serum Ig levels and the longitudinal changes in circulating immune cells, with a special focus on the B-cell compartment. Although in **Chapter 3.1** we found that the maximum expansion of total IgG1 MBCs (between d7 and 28) positively correlated with the increase in Ag-specific serum Ig, we could not confirm this in **Chapter 3.2**. A possible explanation for this can be that in **Chapter 3.1**, we were able to choose maximum expansion from several close timepoints, whereas in **Chapter 3.2** only 1 or 2 timepoints were available. Here, we may have missed the peak of IgG1 MBC expansion. Additionally, in **Chapter 3.2**, we correlated the increase in Ag-specific IgG or IgA MBCs (ELISpot) with the increase in total IgG or IgA MBCs (flow cytometry). However, no correlation was observed between these two techniques.

Monitoring of the vaccine-induced kinetics in the MBC compartment seems to require an Ag-specific approach, such as ELISpot or flow cytometry with (fluorescently) labeled antigens. These antigens could comprise of fluorescently labeled pathogens or pathogen-derived molecules, tetramers composed of pathogen-derived molecules, of fluorescently labeled Klickmers (multiple identical antigens coupled to a dextran molecule). Both ELISpot and Ag-specific flow cytometry approaches have been reported in studies before, have high potential to be standardized, and can be optimized relatively fast, provided that (labeled) antigens are readily available or can easily be produced.[48-50] When such Ag-specific flow cytometry panels are established, these allow for longitudinal monitoring and immunophenotyping of Ag-specific cells, but also for more exploratory strategies such as BCR repertoire studies of Ag-specific cells. With regards to the work done in **Chapter 3** of this thesis, it would be of interest to evaluate the Ag-specific B-cell response in individuals of different age groups (**Chapter 3.2**). As individuals are expected to encounter Bp multiple times during life, the specific response may change over the course of our lifetime. From a primarily vaccine-based systemic response in children to a mixed vaccine-based/natural encounter-based res-

ponse in adults, where local mucosal immunity may play an important role too. Moreover, based on the studies discussed in **Chapter 3.1** and **Chapter 3.4**, we could get new insights in the longitudinal Ag-specific B-cell response generated after a booster vaccine or bacterial challenge, respectively.

Expansion of IgG1 MBCs observed in **Chapter 3.1**, which correlated with an increase in Ag-specific serum IgG level, was minor when expressed as ratio over baseline (at most 1.9-fold increase). Similar levels of expansion were observed in the innate compartment and T-cell compartment but varied between donors. It can be debated if such minor expansions are biologically relevant, especially when variable between individuals. Although we kept factors known to influence the numbers of circulating cells, such as circadian rhythm, recent plasma or blood donation, or a vaccination unrelated to this study, tightly controlled, there could still be other factors influencing the fluctuation that may not be controlled that easily, such as the season, subclinical infections, undiagnosed co-morbidities, dehydration, the impact of repetitive blood donation within the time of the study, etc. Understanding natural fluctuations of the immune cells can be achieved by inclusion of a cohort of healthy individuals who donate blood at the exact same timepoints without receiving a Tdap booster vaccine at d0, ideally over a timespan that covers all seasons when vaccinated donors were included. By monitoring the natural fluctuations of cell populations, we can be more certain whether observed fluctuations are vaccine-induced or may simply be natural fluctuations. Immune monitoring to evaluate ongoing immune responses - plasma cell response to vaccination

In contrast to MBC monitoring, which requires an Ag-specific approach, we found that the expansion and maturation of plasma cell subsets does correlate with Ag-specific Ig serum levels. In **Chapter 3.1**, the correlation network analysis showed clear correlations between the kinetics of (especially more mature) IgG1, IgG4, and IgA1 plasma cells and vaccine component-specific serum IgGs and IgAs. However, when correlating the increase in total vaccine-specific Ig levels with the increase in plasma cells, this correlation was not considered significant as it did not pass the FDR (false discovery rate) correction. However, in a next study with fewer variables (**Chapter 3.2**), we observed a correlation between the expansion of IgG1 and IgA1 plasma cells (expressed as ratio over baseline) and the vaccine-component specific serum IgGs and IgAs. Thus, the expansion and maturation of plasma cells may be a good indication of ongoing immune responses and a correlate of serological data. Given the function of plasma cells (massive Ig production), their expansion is not surprising and has been reported before, especially the plasma cell expansion at d7 post booster vaccination is a frequent finding.[49, 51-53] However, their monitoring/description is usually limited to the total plasma cell population, or to major classes (IgG, IgA, and IgM plasma cells). The in-depth approach used in this thesis, allowing us to determine the expressed Ig subclass and maturation stage, yields additional information in the induced immune response and difference between individuals. More in-depth knowledge of the induced plasma cell response may be of added

value during the early/exploratory stages of vaccine research. An example hereof is the generation of IgG1 vs IgG4 plasma cells, although both will be identified as 'IgG' plasma cells, the Igs they secrete have different functional capacities regarding complement fixation and binding of FcRs.[54] This may have consequences for the generated immune responses, e.g. when Ag-specific IgG1 and IgG4 would both be present, this may lead to competition for the antigen, and thus may reduce opsonization- and complement-mediated removal of the pathogen. Likewise, generation of IgA1 or IgA2 plasma cells would both be detected as IgA, although they have different chemical and biological properties.

A clear advantage of using plasma cells as biomarker of ongoing immune responses is that the baseline levels of plasma cells are very low. This makes the plasma cell system a relatively 'clean' system, in which an Ag-specific approach is not strictly required to get a fair impression of the ongoing responses. Indeed, when correlating the absolute increase in Ag-specific plasma cells (as determined by ELISpot) with the absolute increase in total plasma cells (as determined by flow cytometry), we found a clear positive correlation between the two methods (Spearman's Ranking Correlation test, $r \approx 0.6$, $p < 0.0001$), indicating complementarity of the two assays (**Chapter 3.2**). Moreover, ELISpot findings have previously been shown to correlate with the levels of Ag-specific serum Igs, albeit with various degrees of correlation.[43, 49] Based on the findings in this thesis, we believe that flow cytometric evaluation of the plasma cell compartment may serve as a worthy, fast, in-depth alternative of plasma cell ELISpot evaluation. Another important conclusion is that the d7 expansion of plasma cells has been reported in various age groups (e.g., children, adolescents, young and older adults), and after various (booster) vaccinations (e.g., rabies, tetanus, pertussis, influenza, and hepatitis B), indicating that this may be a widely applicable biomarker.[17, 49, 51-53] It should be noted however that in several occasions, such as a primary vaccination, the expansion of plasma cells may be delayed. This was shown for rabies vaccination, where plasma cells after primary vaccination were observed at d10, and for vaccination with life-attenuated yellow-fever virus, where plasma cells were observed at d14 after primary vaccination.[52, 55] In contrast, upon primary vaccination with a life-attenuated *Vibrio cholerae* vaccine, plasmablasts were observed at d7 after vaccination.[56] These findings highlight the importance of an initial time-finding study as pilot before starting a large-scale immune monitoring study.

Vaccination vs bacterial challenge – *Bordetella pertussis*

In this thesis, we had the opportunity to evaluate the longitudinal kinetics of immune cells after a booster vaccination against Bp and after bacterial challenge with live bacterium (Bp strain B1917). Evaluation of immune cell kinetics after controlled bacterial infection can mimic natural infection but has several advantages over the (retrospective) monitoring of confirmed pertussis cases. The main advantages are (a) known baseline immune status of the individual, (b) defined inoculum dose and time (c) homogenous infection in all individuals regarding pathogen strain, reducing microbiological variation. Moreover, *in vivo* monitoring

may result in additional insights compared to in vitro stimulation experiments, as in this setting, not only the isolated immune cells, but the entire organism is responding to the pathogen. It is true that a human model is more restricted compared to an animal model, for example regarding the type of sampling, and the degree of disease-related symptoms that are tolerated by ethical protocols (in the human model monitored in **Chapter 3.4**, inducing bacterial carriage without Bp disease was the objective). However, no extrapolation to another species is required, and the cohort will much better represent the human population (broader age-range, genetic diversity, and human). Thus, although the human challenge model may have its own limitations, we do believe that it is the best model for monitoring of the immune response to controlled Bp infection. Once fully established, this human challenge model can not only be used to study the infection, colonization, translocation, transmission and/or shedding of Bp in challenged individuals, but also the induced immune responses (protective vs non-protective). More importantly, controlled bacterial challenge could be an important step to evaluate the efficacy of novel Bp vaccine candidates. Before such studies can be performed, the safety and efficacy of the original challenge model should be established. For this model, it was done by de Graaf and colleagues who have published their findings in 2019.[57] In **Chapter 3.4** of this thesis, we add to these findings and describe the induced immune responses in challenged individuals.

We thoroughly compared the immunological responses between individuals receiving a booster vaccine and those who were challenged. In short, differences observed in the generated immune response concerned three major aspects: *timing, magnitude*, and *diversity*.

In short, we found that the fluctuations of immune cell subsets following vaccination and bacterial challenge occurred at different time points. Within the innate immune cells in both cases most prominent changes were found in the monocyte compartment, but timing and type of cells differed (for a full description of kinetics, please refer to **Chapter 3.1** and **Chapter 3.4**). After bacterial challenge, we observed a decrease of CD62L+ cMos and an increase of CD62L- cMos, which may indicate activation and maturation of cMos from d1 onwards.[58] After booster vaccination, the earliest sampling point was only at d3. However, another part of the BERT study (B-cell kinetics were discussed in **Chapter 3.2**) focused on the innate immune response and included d1 in the study. Although not available yet, comparison of the kinetics at d1 after booster vaccination and bacterial challenge could lead to new insights in the early cellular immune response.

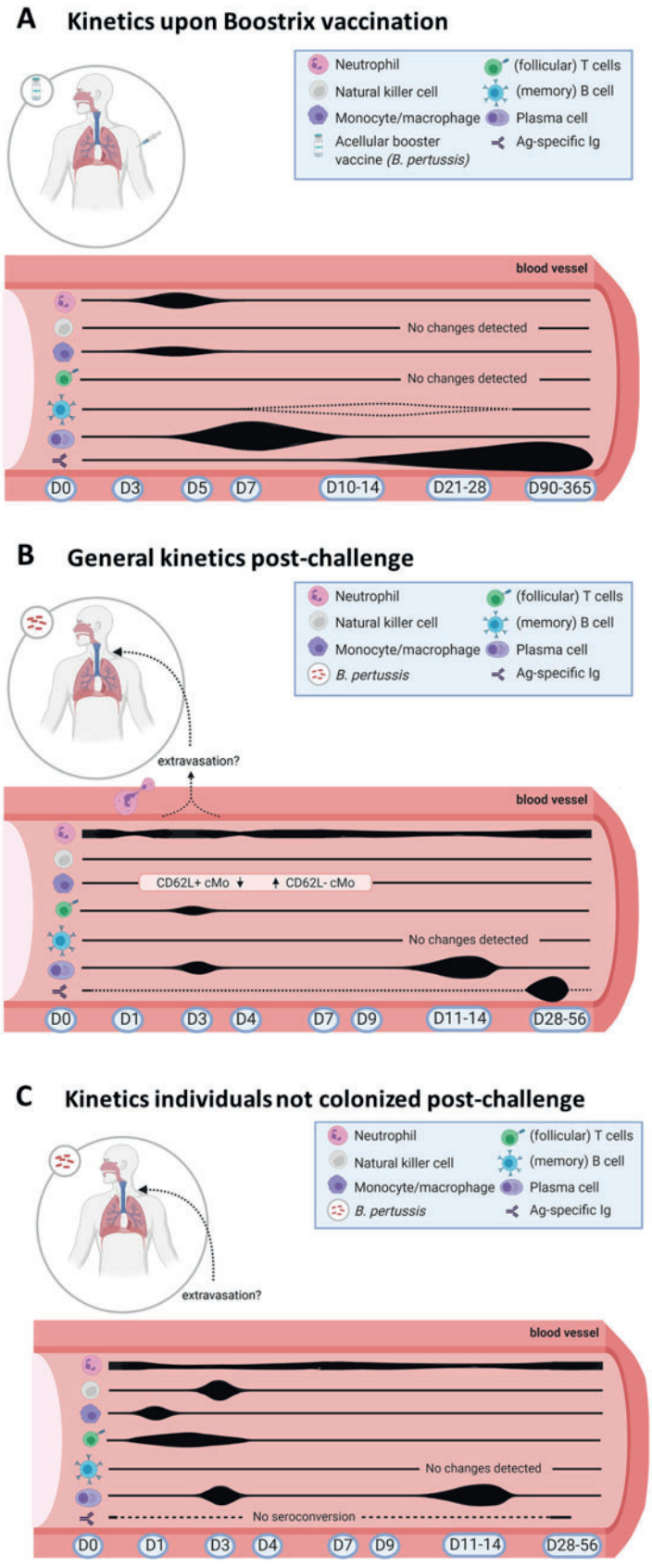
Moreover, within the B-cell compartment, differences between bacterial challenge and vaccination were the most visible regarding time, magnitude, and diversity. Plasma cell expansion after bacterial challenge was less pronounced (1.6-fold compared to baseline (**Chapter 3.4**)) compared to the vaccination studies, where we observed a 5.4-fold compared to baseline (**Chapter 3.1**) and a 2.6-, 3.0-, 3.4-, and 5.7-fold compared to baseline (**Chapter 3.2**, children, adolescents, young adults, and older adults, respectively)). Moreover, the plasma cell response

was delayed after bacterial challenge (d11/14) compared to post-vaccination (d7). Lastly, the plasma cell response seemed more diverse (i.e., no strong polarization towards a specific Ig subclass) when compared to the post-vaccination response.

The different kinetics of immune cells between vaccination and bacterial challenge (**Figure 2**) are not surprising, as both models differ in the type of antigen as well as route of administration. In the case of vaccination, a high dose of selected antigens is directly injected into the muscle, which will lead instantly to the recruitment of innate cells and initiate an immune response. In contrast, bacterial challenge may not lead to such instant cell recruitment and activation. In fact, bacterial colonization itself might not be sufficient to induce a systemic (anamnestic) immune response, for which translocation of the bacterium through the mucus layer is required. This may happen at a different pace, or not happen in part of the donors. In **Chapter 3.4** we showed that not all colonized donors showed signs of seroconversion, which may indicate that a systemic response was not initiated (or at least not to the same extent) in every donor with confirmed colonization. Moreover, we found that although the d3 plasma cell peak was most prominent in non-colonized donors, it could also be found in several colonized donors. And the expansion of plasma cells at d11/14 was not unique for colonized donors, although a trend towards higher plasma cell expansion was found in high-density colonized donors. Thus, the link between colonization and systemic immunity may not be as straightforward as we might hope.

In this thesis, we did not evaluate the (local) mucosal responses. As Bp is transmitted via air droplets and infects the airways, mucosal immunity is expected to play a major role in prevention of infection and transmission. Despite high vaccination coverage the carriage and transmission of Bp persist, which may explain the reported resurgence of pertussis in the last decades.[59-61] I believe that increased mucosal immunity should be an important aim for future Bp vaccine candidates, as this may reduce transmission. The benefit of reduced carriage has already been suggested for other nasopharyngeal-microbe related infectious diseases, such as meningococcal disease and *Haemophilus influenzae* type b.[62, 63] There, intramuscular vaccination resulted in reduced carriage, which was suggested to lead to increased herd immunity/protection because of interrupted transmission. This interrupted transmission was most likely caused by increased mucosal immunity. Indeed, vaccination against meningococcal disease did not

Figure 2 (next page). Summarizing figure showing the main differences and similarities detected in our studies in a cohort of individuals vaccinated against pertussis or challenged with *Bordetella pertussis*. (A) Schematic overview of main observed immune cell kinetics in donors that received aP booster vaccination. Of note, in Chapter 3.1 of this PhD thesis, we observed an increase in the number of memory B cells after vaccination, but this was not confirmed in later studies (Chapter 3.2), therefore, this is indicated with dashed lines. Levels of Ag-specific Ig were measured at all time points. (B) Schematic overview of main observed kinetics in donors that were challenged with Bordetella pertussis, irrespective of inoculum dose (10^4 or 10^5). Levels of Ag-specific Ig were only measured at d0 and d28 post-challenge. (C). Schematic overview of main observed kinetics in donors that were not colonized after challenge with Bordetella pertussis. Levels of Ag-specific Ig were only measured at d0 and d28 post-challenge.


A Kinetics upon Boostrix vaccination
Neutrophil
Natural killer cell
Monocyte/macrophage
Acellular booster vaccine (B. pertussis)
(follicular) T cells
(memory) B cell
Plasma cell
Ag-specific Ig
blood vessel
No changes detected
No changes detected
D0
D3
D5
D7
D10-14
D21-28
D90-365
B General kinetics post-challenge
Neutrophil
Natural killer cell
Monocyte/macrophage
B. pertussis
(follicular) T cells
(memory) B cell
Plasma cell
Ag-specific Ig
extravasation?
blood vessel
CD62L+ cMo
CD62L- cMo
No changes detected
D0
D1
D3
D4
D7
D9
D11-14
D28-56
C Kinetics individuals not colonized post-challenge
Neutrophil
Natural killer cell
Monocyte/macrophage
B. pertussis
(follicular) T cells
(memory) B cell
Plasma cell
Ag-specific Ig
extravasation?
blood vessel
No changes detected
No seroconversion
D0
D1
D3
D4
D7
D9
D11-14
D28-56

only reduce carriage, but also lead to an increase in secretory IgG and IgA after booster vaccination, leading to authors to conclude that mucosal immunity was boosted.[64] Although IMI2-PERISCOPE-colleagues have investigated the levels of Igs in the mucosal lining fluid (MLF) of the nose at various time points post bacterial challenge (unpublished data), there was limited overlap between participants, and comparisons between the flow cytometry data and the MLF are yet to be done. Future studies should consider combining both outcomes in the same set of individuals. Aside from data generated in each individual assay, the combination may give insight whether the generated IgA plasma cell response can function as a (surrogate) marker for the generation of local mucosal responses. In addition to this comparison, it would be interesting to add further markers to the flow cytometry panel that increase insight in the origin and/or destination of these generated plasma cells. For example, detection of the joining (J)-chain, which regulates the multimerization of IgM and IgA, could help identify plasma cells that are probably destined to migrate to mucosal surfaces (when associated with J-chain, IgA is secreted as a dimer, which is the typical form in mucosa).[65] Moreover, simultaneous expression of chemokine receptor CCR10 (ligand: CCL28) and integrin receptor α4β1 (ligand: VCAM1) could indicate homing to the mucosal surfaces.[66] Another way to gain insight in the relationship between the peripheral plasma cells and the mucosal lining Igs, is by comparing the BCR sequences between isolated mucosal Igs and isolated peripheral plasma cells, ideally at several time points following bacterial challenge.

One remaining question is whether the early response observed in donors protected from colonization is indeed protective, and to what extent this information can be used in future studies. To confirm whether a protective response is observed in these individuals, in fact a rechallenge would be required (e.g., an infection-reinfection cohort). As we know that natural infection gives the best protection (compared to wP and aP vaccination)[67, 68], it may be that this controlled infection leads to a similar level of protection. Based on our data, we assume that the early expansion of plasma cell/T cells in the non-colonized donors is associated with protection. When re-challenging the same group of individuals, we would expect a higher number of participants to be protected against colonization, and thus to show this same early expansion of plasma cells/T cells. Moreover, we could compare the BCRs of d11/14 after primary challenge with the BCRs at d3 after re-challenge. If these plasma cells are indeed protective, we would expect (highly) similar BCRs at d3 after re-challenge.

Moreover, although environmental sampling did not reveal any shedding of Bp from the colonized individuals [57], it would be extremely insightful to know if challenged individuals transmit Bp to others. Such efforts are ongoing within the PERISCOPE. These studies may be performed in the clinic or as outpatient study, where challenged individuals are ‘co-housed’ with non-challenged individuals (e.g., housemate or partner, but without contacts with vulnerable individuals, such as the elderly or (unvaccinated) infants). Naturally, the non-challenged individuals should be screened beforehand and give informed consent. By moni-

toring both the challenged and non-challenged individuals, we could investigate whether transmission takes place.

When this challenge model has been fully defined, the next step would be to evaluate (novel) pertussis vaccines in a vaccination-infection model. In such a model, healthy donors would be vaccinated with a new pertussis vaccine candidate, and 1-2 months later they would be challenged with Bp to evaluate the level of protection induced by the vaccine. When combined with immune monitoring, this would lead to better understanding of the mode of action and protection of the vaccine candidate. Aside from the human challenge model that has been established by de Graaf et al., similar efforts are made by Halperin et al. at the Dalhousie University (Nova Scotia, Canada), where a study was initiated in Jan 2022, and is expected to be completed in Nov 2023 (ClinicalTrials.gov identifier (NCT number): NCT05136599). When writing this thesis, no findings have been published on this latter model yet. Both challenge models use a different well-defined clinical isolate (Bp B1917 by de Graaf et al., and Bp D420 by Halperin et al.). These strains are genetically close and a small comparative study in baboons did not observe major differences after challenge with B1917 or D420.[69] To summarize: establishment of a safe and effective human (re)challenge model can have great potential in the evaluation of future pertussis candidate vaccines, as this can be the ultimate way to evaluate the vaccine efficacy and blocking of transmission in a safe, controlled, and ethical manner.

Future of vaccines against respiratory diseases – towards induction of mucosal immunity

In the past years the induction of mucosal immunity to reduce transmission and carriage has been mentioned repeatedly to improve the pertussis vaccines and to reduce the circulation of Bp in the population.

The induction of mucosal immunity may lead to rapid clearance of the pathogen, and thus reduce the replication and transmission of the bacterium. Although this increased clearance may not be a direct benefit for the individual itself (the current vaccines are already effectively preventing disease and mucosal immunity is generated during natural encounters), the transmission to vulnerable groups, such as the elderly, the immunocompromised, and unvaccinated children, may be strongly reduced from the moment of vaccination. Thus, by inducing mucosal immunity already at priming, one is not dependent on natural encounter to generate mucosal immunity and reduce transmission. This may lead to a much faster reduction in circulation of the pathogen.

Multiple studies investigated the induction of mucosal immunity against pertussis in mouse and/or humans (reviewed in [70]). Of these, several candidate vaccines are already being evaluated in humans in phase I/II clinical trials. With the data gathered in this thesis, we hope to provide researchers currently working on novel and improved (pertussis) vaccines with informative timepoints and populations to monitor. I would like to emphasize:

- The importance of time-finding pilot studies, as our data suggested that the induced immune response and its kinetics depend on the route of vaccine administration.
- The potential of longitudinal flow cytometry-based immunophenotyping for in-depth investigation of the ongoing cellular immune responses to supplement current approaches
- That in the case of vaccination studies, especially monitoring of the innate immune cells and the plasma cell compartment may be a quick and informative tool, whereas monitoring of MBCs and T cells would require an Ag-specific approach.

Lastly, I strongly recommend researchers to make efforts to increase the (sample, and thus final) quality and reproducibility of their work, for example by introducing standardized (multi-center validated) SOPs and to be transparent in their cohort selection and data analysis strategies.

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