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

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Chapter 1

General Introduction

NB: parts of this chapter have been published in 'B-cell immunophenotyping to predict vaccination outcome in the immunocompromised - a systematic review' by Diks & Overduin et al.[1]

1.1 The human immune system– general overview

The primary task of our immune system is to protect our body from pathogens. To do so, the immune system utilizes several lines of defense. The first line of defense comprises physical and chemical barriers, such as the epithelium, mucosal surfaces, and secreted antimicrobial products (**Figure 1**). The next line of defense is the innate immune system, which consists of blood proteins (such as the complement system), cytokines, and innate immune cells such as phagocytes (which can eliminate the pathogen by engulfing and digesting it (phagocytosis)), dendritic cells (which can activate the cells of the adaptive immune system via a process called antigen presentation), and natural killer cells (which can kill infected cells). Innate immunity is already present before an infection occurs or can be generated within hours and is therefore able to respond fast upon infection/ pathogen invasion.

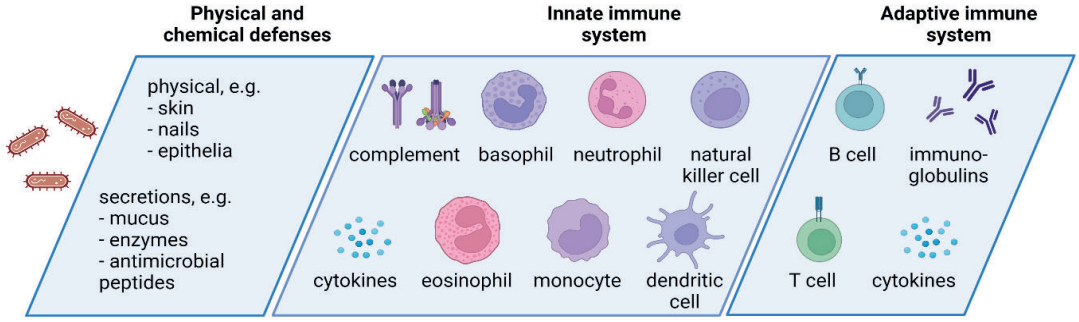


Figure 1. Simplified overview of the three lines of defense that protect the human body from pathogens. The first line comprises physical and chemical defenses, such as skin, nails and epithelial lining, and secretions such as mucus, enzymes, and antimicrobial peptides. The second layer of defense consists out of the cells of the innate immune system. These are phagocytes (neutrophils, monocytes, and dendritic cells), eosinophils, basophils, and natural killer cells. In addition to immune cells, proteins from the complement system and cytokines are part of the innate immune system. The last layer of defense consists of the adaptive immune system, which is characterized by unique receptors and the ability to create memory cells, which leads to a more robust and specific response upon a second encounter with the pathogen. Like in innate immunity, cytokines play an important role in adaptive immunity. The adaptive immune system consists out of B- and T cells, which both exist in various activation and maturation stages. The last part of the adaptive immune system are the immunoglobulins, which are antigen-specific products secreted by the terminally differentiated B cells (plasma cells). Of note, only immune cells that generally can be detected in the periphery are shown. Figure created in Biorender.com.

Although innate responses provide effective initial defenses against infections, many pathogens have evolved mechanisms to escape the innate immune system. To eliminate these pathogens, an adaptive immune response is required. The adaptive immune system is the third line of defense. It is characterized by the presence of unique receptors on the cell surface and the generation of immunological memory. These receptors specifically distinguish parts of microbes and molecules, called antigens. The adaptive immune system launches more vigorous responses upon repeated encounters and comprises B- and T-lymphocytes (B and T cells), and immunoglobulins (also frequently referred to as ‘antibodies’). Like in innate immunity, cytokines play an important role in adaptive immunity.[2]

The components of the innate and adaptive immune system are inter-connected and work together to provide optimal protection against pathogens. For example, the innate and adaptive immune system are connected via antigen-presenting cells. These are innate cells that present an antigen to a naive T cell (or pre-existing memory T cells) in a process called 'antigen presentation'. When the presented antigen is recognized by the unique receptor on the naive T cell, this T cell can become activated.[3,4] Moreover, T cells can instruct innate immune cells by means of cytokine secretions, and the products of B cells (immunoglobulins) can neutralize a pathogen and enhance its degradation by innate immune cells. Thus, there is a continuous interplay between the different components of the immune system.

Immune cells can be found in all tissues in the body, such as the blood, bone marrow, thymus, spleen, and secondary lymphoid organs, but also in the epithelial layers or in mucosa-associated lymphoid tissue (MALT).[2] In addition, released cytokines and chemokines can attract immune cells to specific locations, for example a site of tissue damage or infection. Some types of immune cells are found primarily in the tissues, such as tissue-resident T cells, Langerhans cells, dendritic cells (DCs), mast cells and macrophages.[2] In this thesis, we focus on immune cells that can generally be detected in peripheral blood.

1.2 The innate immune system

The innate immune system consists out of several immune cell types and is responsible for the control or removal of encountered pathogens by starting inflammation and/or the antiviral defense. The main types of innate immune cells found in the periphery are phagocytes (neutrophils, monocytes, and dendritic cells (DCs)), eosinophils, basophils, and natural killer (NK) cells. Upon inflammation or tissue damage, the first cells to arrive are neutrophils and monocytes. When migrating from the blood into the tissue, monocytes differentiate into macrophages. Neutrophils, monocytes and macrophages express receptors on their surface that can sense pathogens via different mechanisms.[3,5] For example, via detection of specific conserved patterns on the outside of the pathogen ('pathogen-associated molecular patterns; PAMP's), complement proteins attached to the pathogen and marking it for degradation, or specific immunoglobulins – often generated in a previous response- bound to the pathogen (a process known as opsonization). As **Chapter 4** of this thesis discusses the phagocytosis of opsonized pathogens, this process will be explained in more detail (**Figure 2**).

Opsonized particles are covered in immunoglobulins that recognized and bound to the particle. Phagocytes express receptors on their surface that can recognize the Fc-part of these immunoglobulins, these receptors are called Fc-Receptor (FcR). Although inhibitory FcRs do exist (FcγRIIB; CD32), the majority of FcRs has activator properties, such as FcγRIII (CD16) and FcγRI (CD64). Binding of multiple immunoglobulins can lead to FcR cross-linking. This results in signal transduction events, which leads to mobilization of the cytoskeleton. One important player in this signaling cascade is phospholipase C gamma 2 (PLCγ2) (we go

more in depth into this in **Chapter 4**).[6] The mobilization of the cytoskeleton enables the phagocyte to engulf the pathogen while keeping it concealed from the rest of the cell in a so-called phagosome. The phagosome then fuses with other vesicles (lysosomes) present in the cell, which contain substances that are harmful for the pathogen. The fusion with the lysosomes leads to a controlled release of enzymes and chemicals, such as reactive oxygen and nitrogen species, which are all meant to destroy and digest the engulfed pathogen (**Figure 2**).[2,5,7]

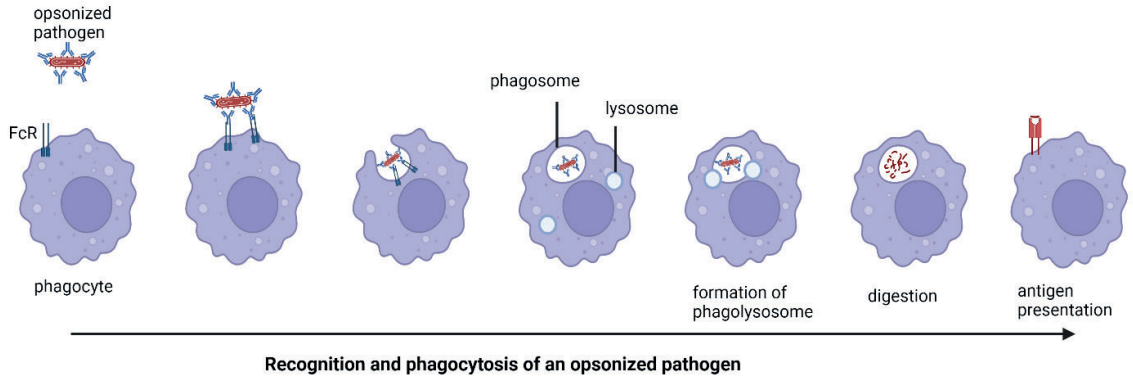


Figure 2. Simplified representation of the phagocytosis of an opsonized pathogen. From left to right: phagocytes express Fc-receptors (FcR), which can recognize the Fc-tail of the immunoglobulins that have opsonized the pathogen. Cross-linking of the FcRs leads to cell activation. The phagocyte adjusts its cytoskeleton to engulf the pathogen. When the pathogen is engulfed, it is contained in a vesicle: the phagosome. Next, the phagosome is fused with lysosomes, resulting in phagolysosomes, which are vesicles filled with substances that are meant to destroy the pathogen. Upon destruction of the pathogen, the phagocyte can present parts of the pathogen on its surface to activate the adaptive immune cells. Figure created in BioRender.com.

Within the monocyte compartment, several subsets can be defined based on phenotypic and functional differences. Monocytes mature from classical monocytes (cMos, CD14++CD16-), via intermediate (iMos, CD14++CD16+) to nonclassical monocytes (ncMos, CD14+CD16++) and can be further subdivided into different functional subsets or activation stages (**Figure 3**).[8,9] cMos play an important role in the initiation and progression of the inflammatory response, whereas ncMos are involved in the resolution of inflammation and have the ability to clear dead cells.9 The function of iMos seems to be intermediate, combining both pro- and anti-inflammatory properties.[9] Within the cMos, two subpopulations can be defined based on expression of surface marker CD62L+ (also known as L-selectin) and FcεR1.[10,11] The CD62L+ cMos are thought to be recent bone marrow emigrants, whereas CD62L- cMos most likely have been activated, and have shed the CD62L upon activation. Within the ncMos, several subpopulations can be defined based on markers such as CD9, CD36 and SLAN.[9-11] Within these ncMo subsets, it was shown that SLAN+ ncMos had higher efferocytosis (removal of apoptotic cells by phagocytes) capacities compared to SLAN- ncMos, suggesting they play a role in clearance of apoptotic cells.[9]

Another important set of innate cells that can be found in the circulation are DCs. Although a large part of the DCs resides within the tissues, they can be detec-

ted in low quantities in the circulation. DCs can be subdivided into myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) with different functional properties.[8] In short, mDCs are considered 'the classical DCs': immature DCs that circulate through the body in search of antigens. Upon encounter of antigens, these antigens are taken up via several mechanisms such as phagocytosis, macropinocytosis or receptor-mediated endocytosis.[4] Next, the DCs migrate to the lymph node. During this process, the DC matures, and its main function becomes antigen presentation via MHC molecules (major histocompatibility complex) on the cell surface to engage the adaptive immune system.[4] Although pDCs can also present antigen to T cells, they are considered to specialize in the production of type 1 Interferon and promote antiviral immune responses.[12]

Eosinophils are innate immune cells known to have a role in defense against parasite infections, but their numbers are also known to increase in allergic diseases, drug reactions and in hypereosinophilic syndrome.[13] Basophils play a role in allergic responses and in the host defense against parasites.[13] Lastly, NK cells respond to viral infections in a non-antigen specific way and play a role in the elimination of malignant cells from the body.[5]

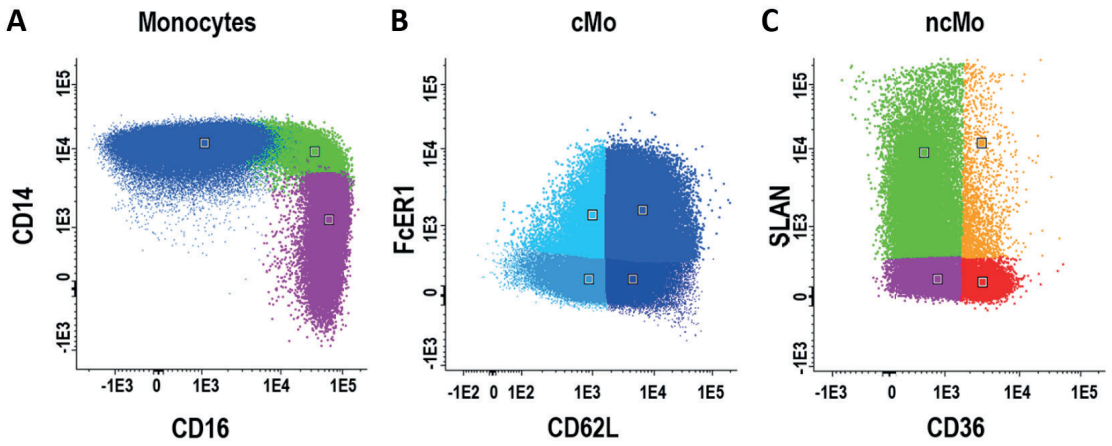


Figure 3. Different monocyte subsets discriminated based on differential expression of surface markers. (A) Based on expression of CD14 and CD16, classical monocytes (cMo), intermediate monocytes (iMo), and non-classical monocytes (ncMo) can be defined. (B) Within cMo, four subpopulations can be defined based on expression of CD62L (L-selectin) and the FcER1. (C) Within ncMo, four subpopulations can be defined based on expression of SLAN and CD36.

1.3 The adaptive immune system

1.3.1 Generation of unique receptors

The adaptive immune system consists out of two main players: T cells and B cells. Both are generated in the bone marrow from common lymphoid progenitor cells. However, T cells mature in the thymus, and B cells mature in the bone marrow. Both B and T cells express a unique receptor on their surface (the B- and T-cell receptor, BCR and TCR, respectively).[14] The genes encoding these unique receptors are generated by the rearrangement of the different variable (V) genes with the diversity (D) and joining (J) genes. An example of this recombination is shown for the BCR heavy chain- encoding genes (IGH) in **Figure 4** (adapted from

[15]). IGHV, IGHD and IGHJ genes are present in the DNA of every cell in the body (germline genes) but only when a single random V gene, D gene and J gene have rearranged to form a functional V(D)J exon, the DNA can be transcribed into a functional receptor.[16,17] Of note, the D gene is not included in every recombination; the BCR Ig heavy chain (IgH), TCR β and TCR δ chains are encoded by a V, D and J gene, while the BCR Ig light chain (IgL), TCR α and TCR γ chains are encoded by a V and J gene only.[17,18] This rearrangement of genes– called V(D)J recombination- results in an exon encoding a unique receptor. In addition to the use of different V(D)J combinations, junctional diversity (caused by non-homologous end joining; NHEJ) is an important contributor to the generation of unique receptors.[17] In this process, there is a random removal and addition of nucleotides at the joints where double stranded DNA breaks were introduced and where the DNA pieces were ligated together to form the functional V(D)J exon. Moreover, the heavy and light chains are paired randomly. The combination of

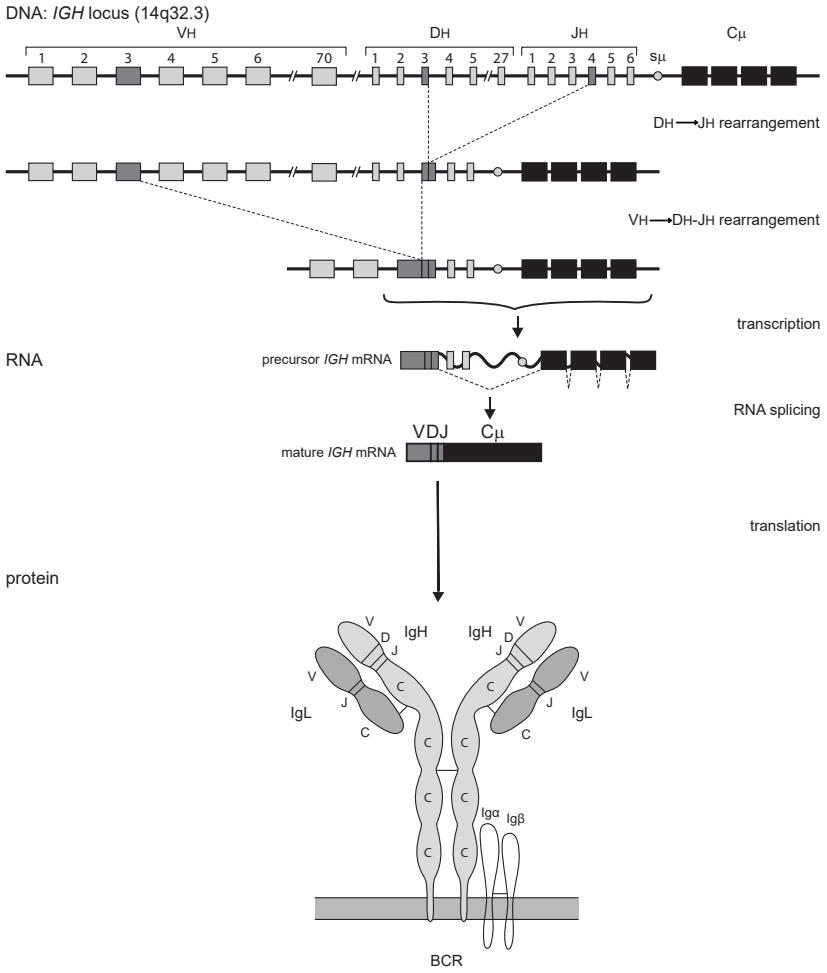


Figure 4. Schematic representation of IGH gene rearrangement. In the first step of V(D)J recombination in the IGH a D gene is coupled to a J gene. Subsequently, a V gene is coupled to the DJ joint. The VDJ exon is transcribed and spliced to the IGHM exons. A functional IgM protein is transported to the plasma membrane with anchoring molecules Igα and Igβ (also known as CD79Aa and CD79b, respectively) and a functional Ig light chain protein in immature and mature B cells. Adapted from van Dongen et al, 1991.

V(D)J recombination, introduction of junctional diversity, and random pairing of the heavy and light chains occurs in individual lymphocytes during their maturation process, and results in a large variety of BCRs and TCRs expressed.[17] Importantly, not every successful receptor rearrangement will lead to a naive B or T cell being released into the circulation. There are extensive selection processes ongoing to prevent release of potentially harmful B- and T cells into the circulation. Continuous production of new B- and T cells (naive cells) leads to a constant renewal of the available repertoire. Only cells that encountered a matching antigen become activated and can further develop into memory or effector cells. Cells that did not find a matching antigen will not receive activation or survival signals and will generally go into programmed cell death, thereby creating space for newly generated B- and T cells to enter the circulation. It is estimated that at any given moment, the available repertoire (i.e., the number of unique receptors) present in an individual is approximately 10^7, although it should be noted that this depends on the age of the given individual (higher in younger individuals, lower in the elderly).[2,19-21]

1.3.2 T-cell subsets and functions

When a T cell has successfully generated a unique TCR and has passed the selection processes, it is released in the circulation as a naive T cell. Of note, most T cells generate a TCR consisting of α and β chain (TCRαβ), and a small part of T cells generates a TCR consisting of a γ and δ chain (TCRγδ). The main goal of the naive T cell is to encounter antigen, which occurs via antigen presentation on MHCI or MHCII molecules on other cells. Upon antigen recognition the naive T cell will get activated and start proliferating and differentiating into effector T cells and memory T cells. The effector T cells are short-lived, whereas the memory T cells stay in the body for a longer time, providing immunological memory in case of antigen re-encounter.[22] Aside from naive, effector and memory cells, there are many subsets defined within the T-cell compartment, each with specific characteristics.[22] Although many T-cell subsets can be monitored in the blood, most T cells are present in the bodily tissues, and only part of T cells is thought to be (re)circulating.[23]

In healthy adults, majority of T cells found in the blood are of the CD4 'helper' T (Th) cell or CD8 'cytotoxic' T cell phenotype. CD4 T cells recognize MHCII-antigen complexes, and CD8 T cells recognize MHCI-antigen complexes. Cytotoxic T cells scan the surface of cells in the tissues. When a cell is damaged or infected, the CD8 T cell gets activated and induces apoptosis of the damaged/infected cell.[2] The CD4 Th cells play a role in the activation of innate immune cells and -in the case of follicular Th (Tfh) cells- in the activation and shaping of the B-cell and immunoglobulin response.[24] There are different subtypes of Th cells, each of which is directed to the defense against specific types of pathogens (**Table 1**, based on ref [2,23,25,26]). Importantly, although we can distinguish many different Th cell subsets, there seems to be a high plasticity between them. Likewise, each Th subset has signature cytokines, yet there is overlap between the cytokine production in different Th subsets.[23]

Table 1. Overview of main helper T-cell subsets, their signature cytokines, and functions. Table based on ref [2,23,25,26].

Type Th cell	Signature cytokine expression	Associated with
Th1	IFN-γ	Defense against intracellular microbes
Th2	IL-4, IL-5, IL-13	Defense against helminthic parasites
Th17	IL-17, IL-22, (IL-10)	Defense against extracellular bacteria; fungi; Tissue repair
Th22	IL-22	Tissue repair

The most well-known Th subsets are Th1, Th2, Th17 and Th22 cells, which are generated in different responses and can each be identified by the secretion of signature cytokines, or by the expression of specific surface markers (**Table 1**). A recent study by Botafogo et al. (2020) described a flow cytometry panel in which many of these T-cell subsets can be identified based on the surface expression of various markers.[27] This flow cytometry approach identified an even larger heterogeneity within the Th subsets, and has defined several new possible Th subsets, primarily described by their marker expression pattern (based on expression of CXCR3, CCR4, CCCR6 and CCR10).

Aside from cytotoxic T cells and Th cells, other T-cell subsets can be found in the blood too. Examples hereof are the Tfh cells, regulatory T cells (Tregs) and TCRγδ T cells.[27-31]

Tfh cells are primarily present in the secondary lymphoid organs (especially in germinal centers), but can also be detected in the blood, albeit in lower quantities. Like in Th cells, there is a heterogeneity within the Tfh cells.[30] Based on marker expression patterns, subpopulations similar to Th cells can be defined, e.g. Th1-like cells, Th2-like cells, etc.[27] Next are the Tregs, which are thought to play an important role in the development and maintenance of immune tolerance. In a healthy situation, they regulate ongoing immune responses and prevent autoimmunity. Like Th and Tfh cells, Tregs have been shown to be a heterogenous population.[29] Lastly, TCRγδ T cells can be identified in the circulation. TCRγδ T cells are a small distinct T-cell subset/lineage and although their exact function in immunity is not fully understood, it is known that TCRγδ T cells have features of innate and adaptive immune cells and seem to have a multifaceted role in immunity.[28] And– in contrast to TCR αβ T cells- these cells are not completely restricted by MHC presentation for antigen recognition.[31]

In this thesis, we used the flow cytometry panel as published by Botafogo et

al.[27] to monitor the CD4 T-cell compartment and describe the kinetics of CD4 T-cell populations that can be defined with that marker combination.

1.3.3 B-cell functions and subsets- general overview

As explained in **§1.3.1**, the BCR is formed during a process called V(D)J recombination. First the genes encoding the immunoglobulin heavy chain (IgH), and then the genes encoding the immunoglobulin light chain (Igκ or Igλ) rearrange to form a unique BCR.[15,32,33] From the immunoglobulin light chain genes, each developing B cell will first initiate recombination of the Igκ light chain. When this does not result in a functional light chain, the cell will initiate recombination of the Igλ light chain. If both Igκ and Igλ rearrangements fail, the cell will go into programmed cell death.

After generation of a functional BCR the B cell leaves the bone marrow and enters the blood as a naive B cell in search for antigen. As each generated B cell expresses a unique BCR, there is a large variety in the available repertoire. Upon encounter of an antigen that matches the BCR, the naive B cell gets activated and develops into an effector cell (known as plasma cell) or a memory cell. Dependent on the type of antigen, this process can be T-cell dependent (in case of protein antigens) or T-cell independent (in case of nucleic acid, lipid, and polysaccharide antigens).[34]

1.3.4 B-cell functions - Activation of the BCR

The BCR forms a complex with the Igα and Igβ molecules. The Igα and Igβ molecules contain an immunoreceptor tyrosine-based activation motif (ITAM) in the cytoplasmic tail. Upon binding of antigen, the involved BCRs start cross-linking (**Figure 5**). This leads to phosphorylation of the ITAMs in the Igα and Igβ molecules, which triggers subsequent signaling events downstream of the BCR.[35] For example, the PLCγ2 protein gets activated, which leads to the production of second messengers such as diacylglycerol (DAG) and inositol-1, 4, 5-trisphosphate (IP3).[35,36] IP3 generation leads to the increase in intracellular calcium, whereas DAG is an activator of protein kinase C (PKC).[37,38] The production of second messenger molecules and the increased calcium levels result in enzyme activation. These enzymes activate several transcription factors, such as NFAT and NF-κB, which results in activation of genes whose products are required for functional B-cell responses, such as proliferation and differentiation.[35]

Upon activation, the B cell starts proliferating and actively modifying its BCR to generate a BCR with a better fit to the antigen. This modification occurs via the introduction of mutations in the regions of the BCR that come into direct contact with the antigen. This process is called 'Somatic Hypermutation' (SHM) (**Figure 6A**).[24] Moreover, the B cell can change the isotype of their heavy chain in order to alter the effector functions of the BCR and secreted immunoglobulins in a process called 'Class-Switch Recombination' (CSR) (**Table 2**, based on [39-42]).[24] As this process involves the removal of genomic fragments, this is an irreversible process that goes in one direction (**Figure 6B**, based on [2,15,43]).

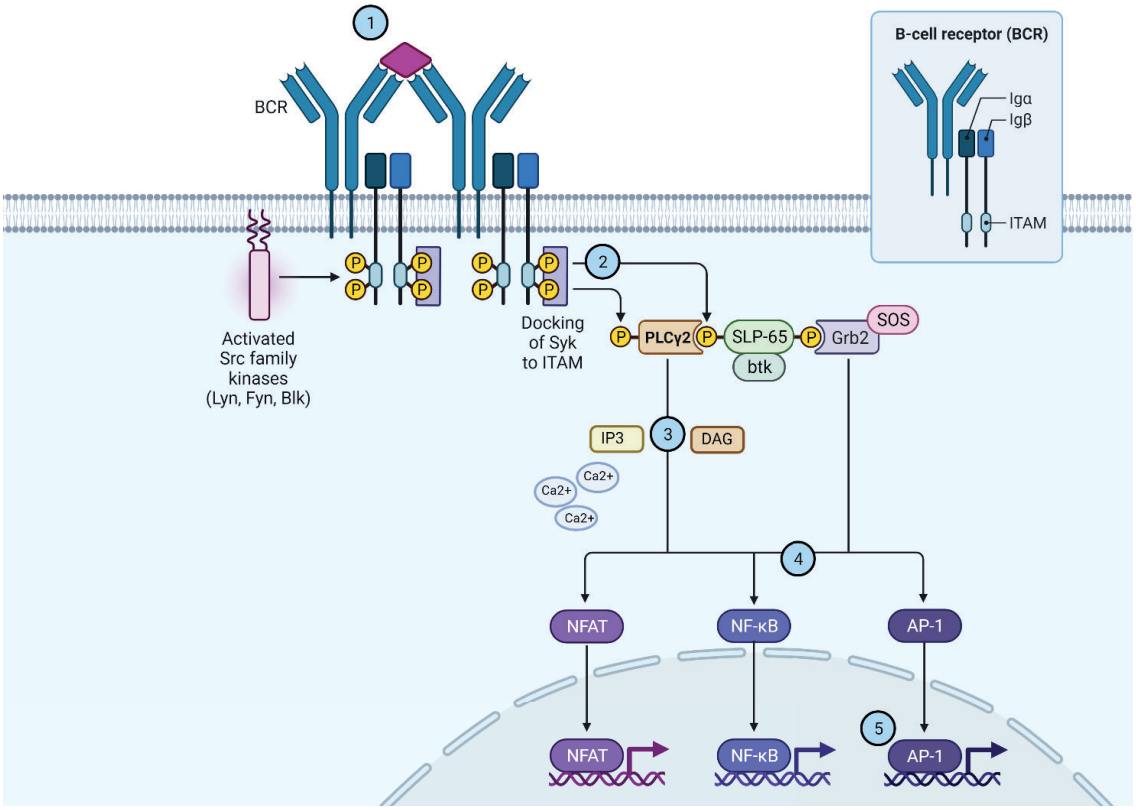


Figure 5. Simplified representation of the major steps in BCR activation. (1) Cross-linking of the BCRs by means of antigen binding (or via use of Fab-binding fragments). (2) Phosphorylation of the ITAM parts of the Igα and Igβ chain by the activation Src family kinases. (3) Release of secondary messenger molecules/biochemical intermediates, such as DAG or the release of calcium. These lead to the activation of (Ca-dependent) enzymes. (4) Activation of transcription factors. (5) Activation/transcription of genes whose products are required for functional B-cell responses. Adapted from 'BCR downstream signaling' by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates.

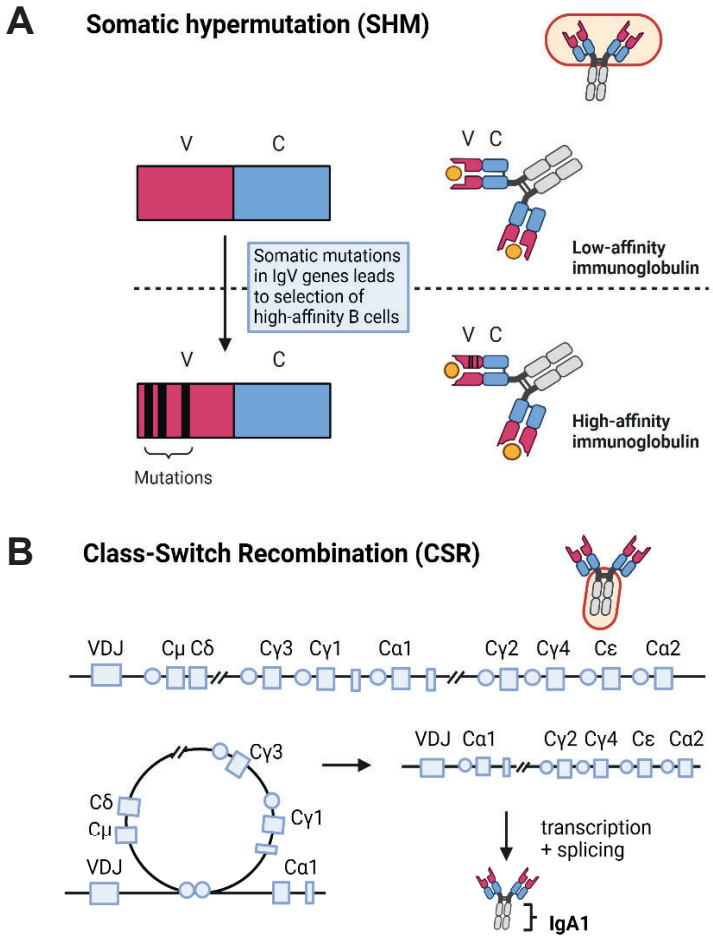
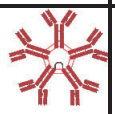


Figure 6. Simplified representation of the processes of Somatic Hypermutations (SHM) and Class-Switch Recombination (CSR). (A). Introduction of somatic hypermutations can lead to structural changes in the V-region of the immunoglobulin, which can lead to increased or decreased affinity, or no change in affinity. Immunoglobulins with higher affinity are preferentially selected, and thus SHM leads to the generation of higher affinity immunoglobulins. V; Variable region, C; Constant region (B) The human IGH locus contains nine constant regions (IGHC). When an antigen-activated B cell gets T-cell help, the B cell will undergo switching to another isotype, depending on the T-cell help. In this figure, the proximal genes Cμ, Cδ, and Cγ3 are deleted in a circle of DNA, which leads to recombination of the VDJ with the Cα1 gene. Transcription of the newly formed exon results in generation of immunoglobulin with the IgA1 constant region. As part of the DNA is deleted, next class switching events can only go in one direction. Adapted from 'Somatic Hypermutation allows for generation of higher affinity BCRs' by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates.

Table 2. General overview of different immunoglobulin (sub)classes. In the left column, simplified representations of the immunoglobulin format(s) present are indicated. The primary known binding receptors and effector functions are indicated per immunoglobulin subclass.[39-42] Immunoglobulin representations generated in BioRender.com.

Immuno-globulin	format	binding receptor	function
IgM 	Pentamer	FcμR	Complement fixation; opsonization; primary adaptive responses
IgG    	Monomer	FcγR	**IgG1-4:** secondary adaptive responses **IgG1,IgG3:** complement fixation, strong opsonization, strong FcγR binding **IgG2:** no complement binding or opsonization capacity, reduced binding to most FcγRs **IgG4:** no complement binding, reduced binding to most FcγRs, *in vivo* Fab exchange resulting in bispecific immunoglobulins
IgA  	Monomer, dimer	FcαR	**IgA1:** mainly mucosal immunity, immune protection of newborns, larger distances between Fab tips allows binding that are further apart **IgA2:** mainly mucosal immunity, immune protection of newborns
IgD 	Monomer	FcδR	Function not defined, possibly involved in regulation of B-cell fate and shaping of B-cell repertoire
IgE 	Monomer	FcεR	Involved in allergies, hypersensitivity and worm infections

1.3.5 Generation of kappa-deleting recombination circles (KRECs)

Each developing B cell will first initiate recombination of the Igκ light chain and will only initiate recombination of the Igλ light chain if Igκ is not successfully recombined. This knowledge can be used to our advantage in immune monitoring. We know that when the Jκ-Cκ intron recombination signal sequence (intron RSS) rearranges to the kappa-deleting element (Kde), the Igκ locus is not rearranged any further (thus rendering this locus 'non-functional').[44,45] In humans, this rearrangement is quite common in mature B cells with an Igκ light chain, and present in almost all B cells with an Igλ light chain.[46-48] During this specific rearrangement, part of the DNA is excised and remains as an 'excision circle' in the cell (**Figure 7**, duplicated with permission from [46]). These excision circles – also known as kappa-deleting recombination circles; KRECs- are stable elements which do not replicate during cell divisions. This means that on the

cell population level they dilute upon each cell division. Thus, by calculating the ratio between the 'coding joint', which is left in the genome and the 'signal joint' on the KREC, the average number of cell divisions that a B-cell population has undergone since generation of the BCR (≈ since they left the bone marrow) can be determined.[46] This concept and the first results are described in the patent filed by van Dongen and Szczepanski in 2004 (PCT/NL2005/00761, priority date 25.10.2004).[49] For T cells, a similar approach can be used when detecting the T cell receptor excision circles (TRECs).[50,51] In terms of immune monitoring the number of detected KRECs or TRECs could give insight in the dynamics of

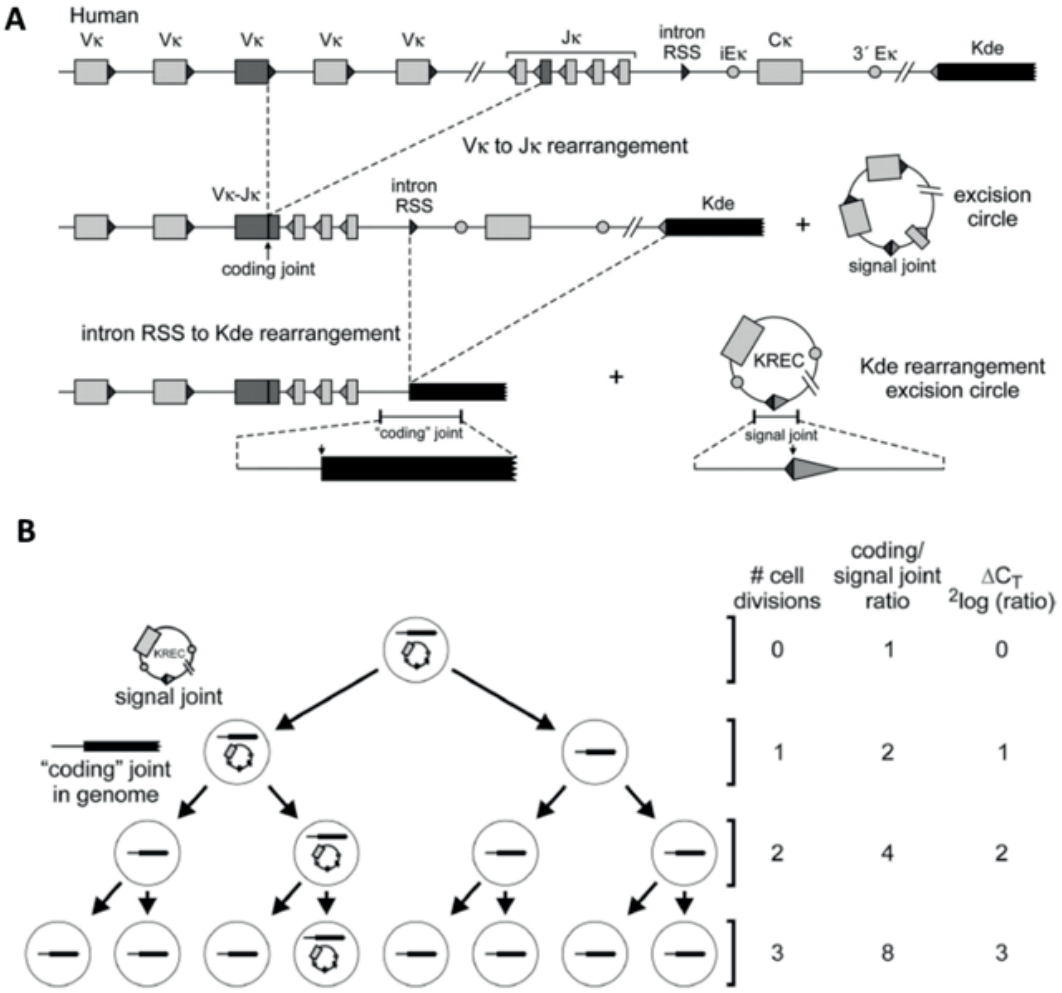


Figure 7. Formation of kappa-deleting recombination circles during the generation of the human B-cell receptor. (A) V(D)J recombination of the IGK locus results in a Vk-Jk coding joint. Subsequent rearrangement between the intron RSS and the Kde elements can make the IGK allele nonfunctional by deleting the ck exons and the enhancer. Consequently, the coding joint precludes any further rearrangements in the IGK locus and therefore remains present in the genome. The KREC with the corresponding signal joint is a stable double-stranded, circular DNA structure, which will not be degraded, but neither be duplicated during the cell cycle. (B) Therefore, upon each cell division, the number of KRECs will be diluted. The coding joint in the genome and the signal joint on the KREC can be quantified via RQ-PCR and their ratio can be used to determine the number of cell divisions a B-cell subset has undergone since they left the bone marrow. Figure and legend duplicated with permission from Van Dongen (2004).

B- and T-cell subsets. One example is the case of severe combined immunodeficiency (SCID) or primary immunodeficiencies (PID), where the level of TREC and KREC numbers can aid the diagnosis. In fact, this approach has implemented in newborn screening programs in several countries.[52,53]

As indicated earlier in this introduction, naive B cells differentiate into memory B cells (that can persist in the body for many years) and plasma cells, which produce large quantities of immunoglobulins to neutralize antigen. In the blood, several maturation stages of plasma cells can be observed based on expression of several cellular markers. The surface marker CD20 (its exact function is not known, but CD20 interacts with the BCR and may be involved in B-cell activation [54]) and CD138 (a proteoglycan that is considered a hallmark of (mature) plasma cells [55]) play an important role in defining the plasma cell maturation stage. Immature plasma cells express CD20 but not CD138. During maturation, CD20 is downregulated and CD138 is upregulated. Most mature plasma cells are CD20 negative, and CD138 positive (**Figure 8A**).[56] Plasma cells of all these maturation stages can be detected in circulation, and thus a heterogenous expression of CD138 is observed. As CD138 expression is considered a hallmark of plasma cells, this mixed circulating plasma cell population is often referred to as plasmablasts. In this thesis, we will refer to the circulating plasmablast/plasma cell populations simply as plasma cells.

Aside from the changes in CD20/CD138 expression, maturing plasma cells gradually lose the expression of surface immunoglobulins (the BCR) and various other surface markers (**Figure 8B**) and initiate massive immunoglobulin production (production and secretion of BCRs).[55] After their generation in follicles or germinal centers, plasma cells migrate via the blood stream to find a niche that supports their survival (bone marrow, spleen, MALT, lymph node).[55] Although most generated plasma cells are short-lived, the plasma cells that find a niche become long-lived plasma cells and can stay in the body for many years. It is thought that, upon antigen-challenge, a fraction of the newly generated plasma cells will compete with the long-lived plasma cells for a place in the niches, pro-

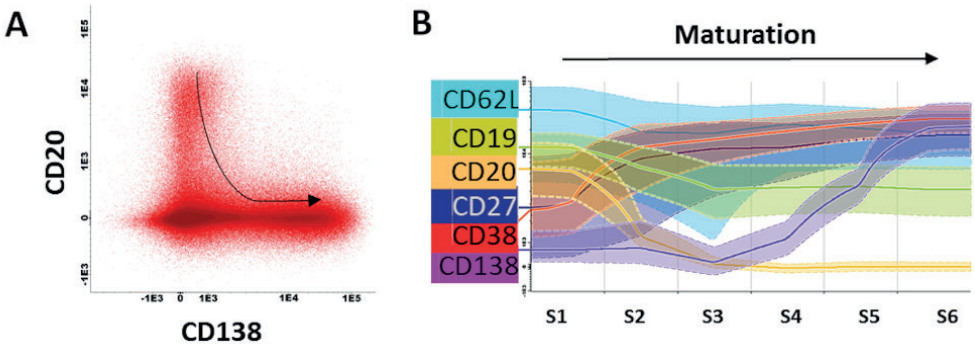


Figure 8. (A) Heterogenous expression of CD20 and CD138 in plasma cells, depending on their maturation status. (B) Gradual loss or gain of expression of various surface markers in plasma cells upon their maturation.

bably expelling some of these plasma cells from their location. As a result hereof, the pool of long-lived plasma cells is a dynamic entity.[57] The immunoglobulins secreted by plasma cells play an important role in the neutralization of the pathogen/antigen. Depending on the type of antigen, the type of encounter and the location in the body (e.g., mucosal vs. systemic), different BCRs are generated, and thus different types of immunoglobulins are generated.

1.3.6 B-cell functions and subsets- distribution in the general population

In healthy adults, majority of B cells are of the naive mature or memory B-cell phenotype (**Figure 9**, modified from [1,46,48]). Additionally, low numbers of transitional/immature B cells (recent bone marrow migrants) and plasma cells can be detected in peripheral blood.[58] While the breadth of the naive B-cell repertoire (number of naive B cells carrying a unique BCR) is crucial in the response to neoantigens, the diversity within the memory B-cell compartment, shaped by previous antigen encounters, plays an important role in recall responses, such as to a booster vaccination. Within memory B cells, two major subpopulations can be defined: the non class-switched memory B cells and class-switched memory B cells. Most non class-switched memory B cells are believed to be (partly) derived from T-cell independent immune responses. In contrast, formation of class-switched memory B cells is mostly T-cell dependent and takes place in germinal centers upon recognition of protein antigens.[34] However, it should be noted that several exceptions to these general observations have been described, such as T-cell independent IgA responses in the MALT or T-cell dependent origin of a part of IgM+ memory B cells.[59]

1.4 Immune monitoring

All the immune cells mentioned so far can be detected in the peripheral blood, which is considered the 'highway' for immune cells to make their way through our body.[60,61] The blood can be sampled in a minimally invasive manner, making it suitable to use for immune monitoring. Immune monitoring is a process where the immune system is monitored at one or several timepoints to gain more information about its status or composition. Detailed monitoring of the immune system and ongoing processes can be a valuable source of information, both when the system is in homeostasis and when it is not, e.g. in case of infection, disease, or when an individual is receiving treatment or medication.[62-64] Immune monitoring can be performed in vivo and in vitro, with a functional or phenotypic assessment or a combination of both, and at different levels; the cellular level, the molecular level, or by means of e.g. serum analysis or repertoire studies.[65-70] The preferred method of immune monitoring may be influenced by the available material, equipment, timeframe, funding and knowledge.

In this thesis, we primarily used flow cytometry-based immune monitoring tools to assess baseline and ongoing cellular immune kinetics, with peripheral blood as source of material. Therefore, we will further explain this method in the following paragraph (**§1.5**). To a lesser extent, serology, ELISpot, qPCR, activation/

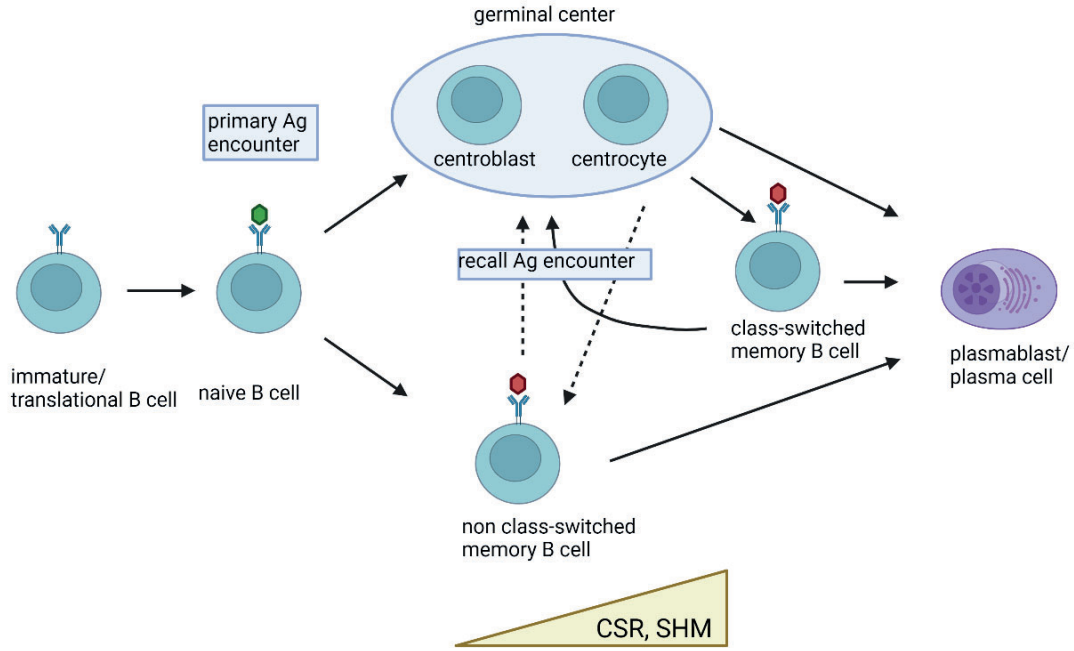


Figure 9. Simplified representation of major B-cell subsets detectable in blood. From left to right: Immature/Transitional B cells are recent bone marrow migrants and present in low numbers in the peripheral blood. They mature into naive B cells, which constitute a major part of the circulating B-cell population. The number of naive B cells with unique B-cell receptors forms the naive B-cell repertoire, which is crucial for recognition of neoantigens (primary antigen encounters). Naive B cells which encountered T-cell dependent antigens (e.g., a protein), will enter germinal centers to receive T-cell help. Consequently, they will upregulate AID (Activation Induced Cytidine Deaminase) and subsequently improve affinity for antigen by initiating Somatic Hypermutation (SHM) and change effector functions in the process of Class-Switch Recombination (CSR). Only B cells that express receptors with increased affinity to the encountered antigen survive and can leave the germinal center as class-switched memory B cells or as plasma cells. When a B cell is activated by a T-cell independent antigen (e.g., a polysaccharide, nucleic acid or lipid), it does not enter the germinal center, but differentiates into a non class-switched memory B cell instead. Class-switched and non class-switched memory B cells make up a large part of the circulating B-cell compartment, and are present in other parts of the peripheral lymphoid system, such as the spleen or lymph nodes. These memory B cells are important during recall responses (recall antigen encounter) when they can re-enter germinal centers and undergo further processes of affinity maturation and class-switching. Lastly, plasma cells are the terminal effector B cells and responsible for massive immunoglobulin production after antigen encounter. Upon infection or vaccination, a transient peak of plasma cell numbers is observed, but in steady state, plasma cell numbers are low. Figure created in BioRender.com. Modified from Diks et al., 2021, van Zelm et al., 2005, van Zelm et al., 2007. 1,46,48

stimulation assays and single cell-sequencing technologies were used for immune monitoring, again using peripheral blood as source of material.

Serology, or the detection of antigen-specific serum immunoglobulins is considered a golden standard when it comes to assessing vaccine effectiveness; the (increased) levels of vaccine-specific immunoglobulins in the serum are known to correlate with protection against disease in numerous cases.[71] To detect and quantify the number of antigen-specific B cells, the ELISpot method was applied in this thesis. Significant correlations between the number of Ag-specific B cells and the Ag-specific Ig serum levels have been reported for multiple vaccines.[72]

The qPCR-based methods that we use in this thesis are related to the detection of KRECs. The number of KRECs detected in a B-cell population can give information about the number of cell divisions that a B-cell population has undergone since it left the bone marrow.[46] Activation and stimulation assays were used in this thesis to determine the 'fitness' of cells. We evaluated the activation of cells upon stimulation at various locations during the activation pathway, such as phosphorylation events, calcium release, and phagocytosis/ROS production (upon stimulation with opsonized particles).

Lastly, we made use of single cell sequencing techniques to investigate the B-cell repertoire during an immune response. By investigating the B-cell repertoire, we can get insight in the selection processes that occur upon antigen encounter. This way we may identify structures that are most immunogenic during natural pathogen encounter and evaluate if these are also present in current vaccines or vaccine candidates.

1.5 Flow cytometry as a tool for immune monitoring

Flow cytometry is a frequently used immune monitoring method which allows identification of cells/particles in (single cell) suspensions. Cell identification is based on the detection of cellular markers by fluorescently labelled (monoclonal) antibodies or detection of nucleic acids by fluorescent dyes (**Figure 10**).[73] Frequently used cell suspensions for flow cytometry include, but are not limited to, mechanically or enzymatically digested tissues, cell lines and/or co-cultures, bone marrow, cerebrospinal fluid, saliva, urine and (peripheral) blood.

In the early days, the number of cellular markers that could be detected was rather limited. However, over time the number of fluorescent labels and the ability of flow cytometers to pick up multiple signals has increased significantly, leading to the development and publication of several high-dimensional/multicolor flow cytometry panels (van der Pan et al, manuscript accepted).[27,74,75] Such panels allow identification of many different cell entities, such as subsets, maturation stages or different activation stages. Aside from the detection of surface/intracellular markers, the availability of bioparticles coupled to a sensor dye or sensor dyes in general, such as pHrodo, DHR123 or calcium-sensor dyes, allow for detection of processes such as phagocytosis, generation of reactive oxygen species (ROS) or calcium release. Thus, the use of (intra)cellular markers, bioparticles and sensor dyes can be a valuable source of information about the function of evaluated cells. Additionally, the introduction of high-throughput flow cytometers allows analysis of high cell numbers, and when counting beads are used, one can retain information about absolute numbers. The large availability of fluorochrome-linked detection antibodies, number of fluorescent dyes and the opportunity to adjust the optical configuration of the machine result in great flexibility. Lastly, the introduction of spectral flow cytometers has resulted in an even higher number of marker combinations that can be used simultaneously. Spectral flow cytometers differ from conventional flow cytometers regarding their optics and detectors, resulting in detection of the complete emission spectrum from a

fluorochrome/dye instead of only the light passing a set of filters, as is the case in conventional flow cytometers.[76] The combination of high-throughput, flexibility and the relatively fast acquisition of cells make flow cytometry a very suitable tool for exploratory research.

Although much information can be retrieved from such complex flow cytometry panels and large cell numbers, this increased complexity of antibody panels has resulted in an increased subjectivity when it comes to data analysis and interpretation.[74] To ensure data quality and reproducibility, it is important that protocols and phenotypic descriptions are standardized between laboratories/clinical sites. To this end, several groups and consortia proposed the use of harmonized or standardized protocols for sample collection, acquisition, and (automated) analysis.[77-80] In this thesis, the guidelines and protocols as proposed by the EuroFlow Consortium have been applied to all experiments executed on

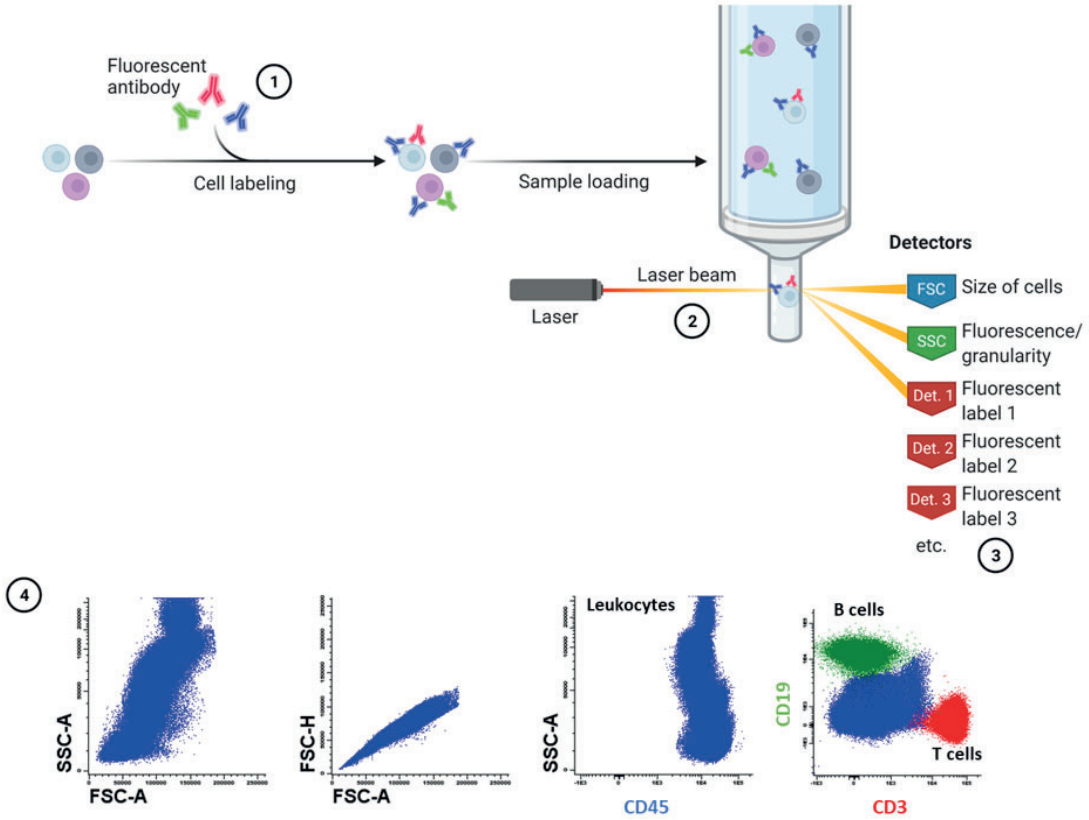


Figure 10. Simplified overview of flow cytometry principles. The main steps are indicated and labeled sequentially. (1) A mixture of cells is fluorescently labeled with antibodies specific for each cell type. (2) In the flow cytometer, the labeled cell mixture is passing a laser beam in a stream of fluid. The laser beam strikes the stream and the cells that are passing by at that moment. Based on the type of cell that is hit at that moment, different light signals are hitting the detectors. The FSC detector identifies the cell size. The SSC detector identifies the (auto)fluorescence and the granularity of the cell. The other detectors identify signals originating from the fluorescently labeled antibodies that bound to the cells. (3) For each interrogated cell (also called "event"), the outcome of each detector is saved as a separate parameter. (4) Using flow cytometry analysis software, each cell type can be identified based on its unique combination of parameters. Adapted from 'Fluorescence-activated Cell Sorting (FACS)' by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates.

a BD FACS Canto II 3L or a BD FACS LSR (X-20) Fortessa 4L (BD Biosciences, San Jose, CA, USA).[77,81] In short, this means that standardized protocols were used whenever available, and that machine performance was monitored daily with CS&T beads (BD Biosciences) as well as SPHERO™ Rainbow calibration particles (Cytognos, Salamanca, Spain).

Figure 11 shows an overview of different aspects in the preanalytical, analytical and postanalytical process that can influence the results and should ideally be decided before start of large experiments, such as a clinical trial. In short, the type of anticoagulant can influence cell distribution over time.[82] The presence of a fixation agent can influence cell identification markers (e.g. altered scatter properties), but allows for extended sample stability.[83,84] The amount of blood used for sample processing and acquisition (and thus cell numbers evaluated) determines depth of analysis, moreover, protocol adjustment may be needed when sample volume changes. With increased sample age (time since donation), quality reduces, but this differs per evaluated cell type and is influenced by type of anti-coagulant, storage temperature, storage time and mode of storage (e.g. full blood, cell suspension, or fixed cells).[84-86] During sample acquisition, the type of flow cytometer used (including configuration and calibration), the used protocols, and the antibody cocktail can influence the generated flow cytometry files.[81] Lastly, the analysis of the flow cytometry files is dependent on the (experience of the) operator and population definition. Thus, standardization of these aspects leads to increased data quality and reproducibility.

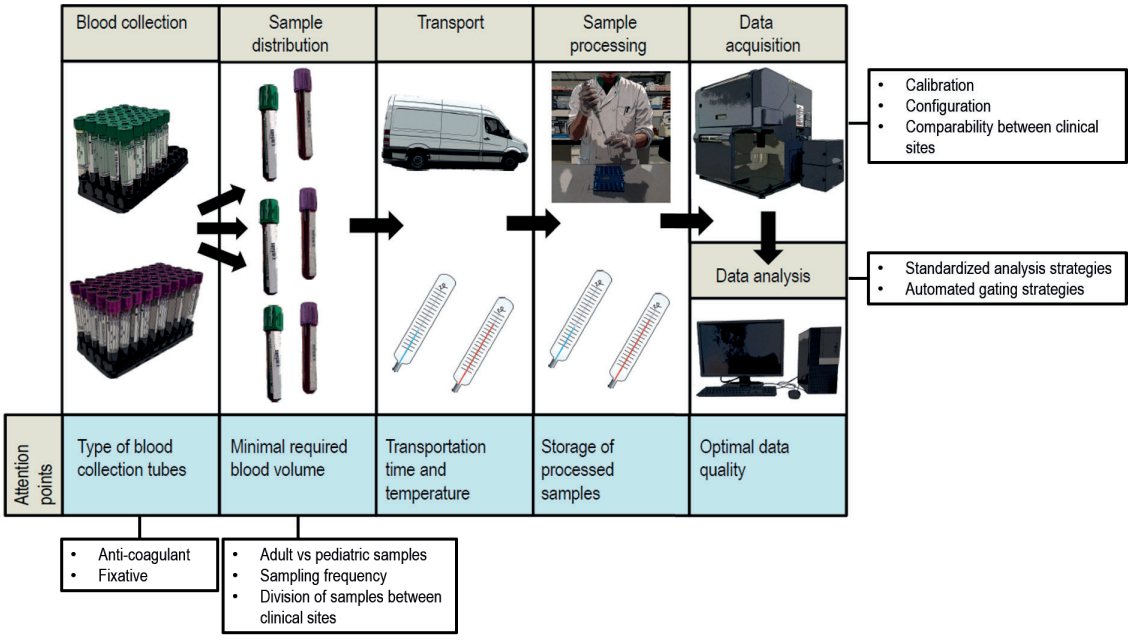


Figure 11. Different aspects in the preanalytical, analytical and postanalytical process that can influence the quality of results and should ideally be decided before start of large experiments, such as a clinical trial. Figure modified from Diks et al (this thesis).

1.6 Immune response to antigen – vaccines

Exposure to a pathogen or vaccine component initiates an immune response which effectiveness depends on the cooperation of multiple cell types. During the immune response, innate immune cells are rapidly recruited to the place of damage or infection.[62,87,88] They initiate the immune response by means of local inflammation, but also act as antigen (Ag)-presenting cells.[87,88] The innate response is followed by activation of adaptive immune cells (T and B cells), which results in the formation of effector and memory cells.[24] The generation of immunological memory is the basis for vaccine-induced protection. By actively exposing the immune system to a part of a pathogen, or a weakened/dead pathogen, the immune system is triggered and generates an immune response against the pathogen, while induction of disease is avoided. In this process, immunological memory is built, and when the pathogen is encountered in real-life, the tools required to neutralize/destroy the pathogen are already present. This leads to a more efficient immune response and can prevent (major) disease. The efficacy of vaccines has been demonstrated by the reduced number of infectious-disease related deaths since their introduction.[89]

However, the use of vaccines is often based on empirical data, and not all underlying (protective) mechanisms are fully understood. Moreover, the vaccine composition such as type of adjuvant, variety and concentration of antigen, and type of antigen (e.g., peptide, polysaccharide-conjugates, or whole pathogen) can lead to differences between generated immunity versus optimal protective immunity.

1.7 The difference between vaccine-induced and natural infection-induced immunity- *Bordetella pertussis*

The main immune response monitored in this thesis is the response launched against *Bordetella pertussis* (Bp), a gram-negative bacterium responsible for the respiratory tract disease pertussis, also known as whooping cough. Infection with Bp occurs via airways and transmission of the bacterium occurs via respiratory droplets. Although the introduction of vaccines against pertussis (1940s/1950s) have resulted in a great reduction in reported cases, there is still a high disease burden (**Figure 12**). In 2018, there were 35.627 reported pertussis cases in Europe, of which 10 had a fatal outcome.[90] However, it is generally assumed that there is an underreporting of pertussis cases, and thus that actual number of cases is higher. Worldwide, an estimated 300.000-400.000 pertussis related deaths occur annually, mostly affecting infants and people in developing countries.[91,92]

The initial pertussis vaccines were whole cell pertussis vaccines (wP vaccines), which contained inactivated Bp. However, due to the unfavorable reactogenicity profile of the wP vaccine, many countries switched to acellular pertussis vaccines (aP vaccines).[93,94] The aP vaccines contain one or more Bp antigens, such as pertussis toxin, pertactin, filamentous hemagglutinin and fimbriae2/3, and are adjuvanted with aluminum hydroxide. Vaccines against pertussis are generally combined vaccines that also provide protection against diphtheria, tetanus and

in some cases also *Haemophilus influenzae* type B, polio or hepatitis B.[95] In the US, the first aP vaccine was licensed in 1996, and in Europe, transition towards aP vaccines started in 1995 (Netherlands: 2005).[96]

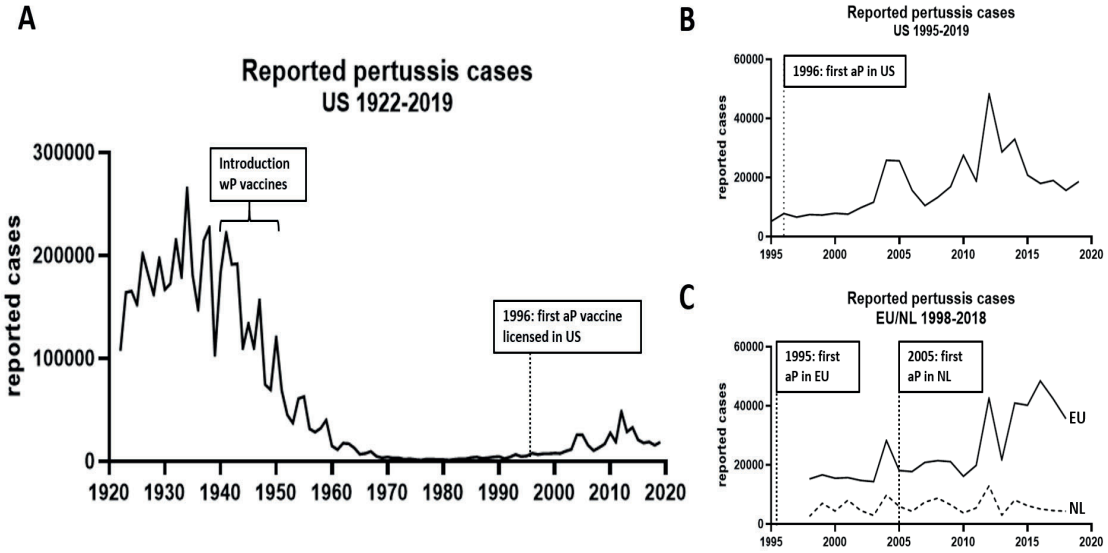


Figure 12. Number of reported pertussis cases in (A) the US (1922-2019), (B) the US (1995-2019), and (C) Europe (EU) and the Netherlands (1998-2018, timeline starts at 1995). Reported pertussis cases were retrieved from the Center for Disease Control.[99] Reported pertussis cases for Europe/Netherlands were retrieved from the European surveillance atlas for infectious diseases.[100]

Nowadays, most high income countries use aP vaccines, whereas several low- and middle income countries still use the wP vaccine.[96,97] Although the introduction of pertussis vaccines in 1940s/1950s resulted in a strong reduction of the number of cases, the incidence of pertussis has increased in the past decennia with a shift in reported cases from young children to adolescents and adults (**Figure 12**).[92,98-100] This may be explained by the fact that protection against Bp is not life-long. Moreover, several studies suggested that aP-induced immunity wanes faster than wP vaccine-induced immunity.[92,93] Dependent on the type of vaccine (wP or aP vaccine), different cellular and serological responses were detected, such as skewing of the Th-response and differences in induced vaccine-specific serum immunoglobulin subclasses.[101-103] In addition, baboon studies have shown that wP and aP vaccines do not prevent transmission of Bp (with wP vaccines being more efficient in reducing colonization and transmission than aP vaccines).[104] Thus, although vaccinated individuals were protected from disease, they could still transmit the pathogen upon infection. Indeed, most pertussis cases with severe outcome are found in unprotected individuals, especially unvaccinated or incompletely vaccinated infants.[92,98] Thus, it is of importance to develop new pertussis vaccines that protect against colonization and transmission.

To develop new vaccination strategies, it is important to gain a thorough understanding of differences and similarities in the immune response induced by per-

tussis vaccines and natural infection. Previous studies showed that the cellular and serological responses post-vaccination differed from cellular and serological responses measured in patients with a natural infection.[101] However, patient settings are not optimal due to heterogeneity in the cohort and the number of unknown factors (for example, time since infection, time since symptoms, baseline parameters). In contrast, human challenge studies allow for safe and controlled infection, with the option to monitor/predefine many parameters, and with standardized clinical readout. Therefore, such studies can be an immense source of new information regarding immune responses to "natural infection" and the induction of protective immunity.

In the case of pertussis, there are several differences between vaccination and natural infection that can influence the induced immune response. The major differences between current Bp vaccines and natural infection are the site of antigen encounter (deltoid muscle vs respiratory tract) and the variety/concentration of antigens (as indicated above; pertussis vaccines are combination vaccines). Upon vaccination, a high load of Bp (and other) antigens is injected in the muscle (skipping the first line of defense), whereas upon natural infection, the antigenic load of Bp may be a lot lower and more gradually encountered, as the Bp initially encounters the first line of defense (physical en mechanical defenses, mucosal tract). The encounter of bacteria in mucosal areas may initiate immune processes (mucosal responses) that are not induced upon primary vaccination but may be of importance for the induction of protective immunity against infection and transmission. Prevention of transmission is not only of importance to pertussis vaccines but may play a role in other infectious respiratory diseases, such as SARS-CoV-2.

1.8 Immune monitoring outside the healthy adult population

Aside from vaccine evaluation (generally occurring in healthy adults), immune monitoring can be applied in many other situations. In the field of diagnostics, immune monitoring is a valuable tool that can help with the identification or exclusion of hematological malignancies or immune deficiencies. Moreover, the reconstitution of immune system after hematopoietic stem cell transplantation or immunosuppressive treatments and targeted immunotherapies can be subjected to immune monitoring. Thus, immune monitoring has many applications.

One specific group in which monitoring is becoming more important is the aging population. Increased welfare, because of economical and medical improvements, has resulted in people all around the globe living longer. The WHO has estimated that in the coming 25-30 years, the number of individuals aged 60 or older will double, from 1 billion in 2020 to about 2.1 billion in 2050.[105] In majority of people, aging goes hand in hand with a decline in cognitive and/or physical health. There are many age-related impairments, such as lowered resistance to infection, dementia, osteoporosis, atherosclerosis and diabetes, which are directly or indirectly related to the aging immune system, especially to the low grade inflammation that is often observed in older individuals (inflammaging).[20,106]

This overall increase in age creates new challenges for our medical system and the way we monitor the immune system of these individuals (for example; when a population is reaching older age, should we adjust the parameters defining 'healthy reference values' in such individuals?).

Upon aging, the numbers and distribution of circulating immune cells and the functioning of immune cells change. The output of naive B and T cells into the periphery decreases, which leads to a decrease in naive cells in the circulation. This, in combination with the reported increase in oligoclonality in older adults results in a decreased B and T-cell repertoire.[19-21] Moreover, impaired germinal center formation, reduced affinity maturation, memory B-cell formation and lowered levels of plasma cells in the bone marrow have been reported in both human and animal models.[19] Additionally, the functioning of innate immune cells declines. Several studies reported a lowered capacity to phagocytose opsonized particles and produce ROS in neutrophils derived from aged individuals.[21,107] Moreover, reduced phagocytic capacity, lowered ROS production and lower anti-tumor properties were reported for monocytes derived from older adults.[20,108,109] Lastly, immune cells with unusual phenotypes can get a more prominent place during the aging process, as was shown for the so-called 'age-associated B cells'.[1,110,111] Altogether, these processes indicate that upon aging, individuals become less equipped to deal with infection and inflammation. This does not only influence 'natural encounters' but may also negatively impact the induction of protective immunity by means of vaccination.

Indeed, several studies evaluating vaccine responses in the elderly have shown reduced vaccination responses, such as reduced Ag-specific serum immunoglobulins, immunoglobulin functioning (neutralizing or inhibiting capacity), and plasma cell counts.[1] Of note, although reduced immune responses were observed in several of these studies, there was still an expected benefit of the vaccination. However, the impact of aging was not consistent between these studies. A possible explanation is the stringency of the inclusion criteria. As indicated above, majority of individuals experience a cognitive and physical decline upon aging. However, we know from studies that this decline varies between individuals and can be influenced by several factors, such as lifestyle, stress factors and genetics. Depending on these factors, a person can age well or poorly. Therefore, not only the age in years, but also the 'biological age' influences the ability of an individual to response to antigen-challenge. Thus, when a study has stringent health-related inclusion criteria, it may automatically select for individuals that are aging well, and thus reduce the impact of the age in years. In order to harmonize the study cohorts in immunogerontological studies, protocols have been composed to better select and describe the elderly cohort, for example the SENIEUR protocol.[112]

Although majority of individuals show physical and cognitive decline upon aging, there is a selected group of individuals that ages well. Although their successful aging is most likely a combination of several factors, multiple studies have sear-

ched for factors that are associated with good or poor aging.[113,114] The ApoE4 allele is a well-known genetic factor negatively associated with longevity, but there are many more gene variants associated with increased or decreased likelihood of longevity. Examples of gene variants positively associated with longevity are the 1082GG polymorphism in the gene encoding IL-10, the Asp299Gly polymorphism in the gene encoding TLR4, and the P522R polymorphism in the gene encoding PLCγ2.[114,115] As indicated before, aging is in part an immunological process. Therefore, it is relevant to understand the impact of these factors associated with successful aging/increased resilience against aging. Better insight in these processes may help guide and design future therapies for age-related complications.

1.9 Outline of this thesis

Standardization in clinical trials is crucial to obtain data that is reliable and comparable in-between study locations. Therefore, we first evaluated the influence of pre-and post-analytical procedures on the quality of flow-cytometric immunophenotyping in blood. In **Chapter 2**, we assessed the impact of delayed sample processing and acquisition, storage temperature and fixating agents on quantity and distribution of leukocyte subsets (**2.1**). We showed that blood samples stored for <24h at room temperature before processing and staining seem most suitable for reliable immunophenotyping, despite observed losses in absolute cell numbers. Next, we measured the impact of delayed sample processing on the performance of a database-driven analysis (**2.2**). Here, we showed that automated analysis is superior to manual analysis regarding reproducibility and robustness, although expert revision of these automated files remains necessary in samples with numerical alterations and aberrant B- and T-cell maturation and/or marker expression profiles.

Knowledge gathered in studies presented in **Chapter 2** was utilized in several clinical trials where we aimed to dissect the cellular immune responses following controlled pertussis infection or vaccination. These clinical trials were initiated locally at the LUMC or within the IMI-2 PERISCOPE Consortium and their major outcomes are summarized in **Chapter 3**. Initially, within LUMC, we performed an extensive trial, in which we investigated kinetics of multiple leukocyte subsets over time following vaccination (**3.1**). This allowed us to determine the most relevant timepoints and populations for the follow up analysis. Additionally, this set of data could be used as a 'framework', on which we projected data of subsequent vaccination study, where we collected samples from fewer timepoints, but from more diverse cohorts (different ages and priming backgrounds) (**3.2**). There, we showed that the plasma cell response initiated after Tdap booster vaccination differed with age and priming background. Cellular analysis of the immune response to pertussis vaccination was further extended by molecular studies using single cell sequencing technologies on highly enriched plasma cell samples from one vaccinated individual (**3.3**). In this study, we developed a pipeline for analysis of such samples, including hallmarks of antigen exposure, such as somatic hypermutation and clonal expansion. Additionally, we established a query tool to

identify B-cell receptors related to anti-tetanus and anti-pertussis toxoid responses. Finally, in **3.4**, we compared the findings from the vaccination studies with the kinetics found in individuals that were or were not protected against colonization in a controlled bacterial challenge with the same pathogen (*B. pertussis*). Here, we showed that donors that were protected from colonization showed distinct early cellular kinetics. Moreover, we showed that the plasma cell response generated after booster vaccination or bacterial challenge differed with regards to timing, magnitude, maturation, and polarization to specific Ig subsets. With this information we can better understand immunity against pertussis and use this knowledge for future pertussis vaccine evaluations.

In **Chapter 4** we evaluated the SARS-CoV-2 vaccination responses in a cohort of older adults/elderly with or without a specific variant of the PLCG2 gene. This variant is associated with cognitively healthy aging and longevity, but its impact on the immune system remains unknown. Therefore, we extended this study by an in-depth analysis of the quantity and functionality of the circulating immune cells. Although no differences were observed in the responses against SARS-CoV-2 vaccination, carriers of this variant tended to show less signs of immunosenescence compared to age-matched non-carriers.

Lastly, in **Chapter 5**, we discuss the general findings of this thesis and outline future directions that, we believe, should be pursued to reliably gain more insights in the human immune system.

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