CHALMERS

Human peripheral B cell development

Characterization of a novel CD45RB+ B cell population

Master of Science Thesis

JOEL HOLMQVIST

Department of Chemical and Biological Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
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This thesis was carried out under the supervision of Dr Mats Bemark at the The mucosal immunology and vaccine center (MIVAC), UNIVERSITY OF GOTHENBURG, Göteborg, Sweden, in 2010.

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I know nothing except the fact of my ignorance.

— Socrates

Dedicated to people like
Robert Bergman and Johan Selander.

If cells never failed the way yours did,
they would not be half as interesting to me.

ABSTRACT

Since the middle of the nineties, our understanding of human peripheral B cell development has gradually increased. Today, the B cell compartment has been divided into three main populations; transitional, naive and memory B cells. In Mats Bemark's laboratorium, a cell surface marker identifying a novel peripheral B cell population has been found. The work performed during this thesis aimed to characterize the novel B cell population by comparing it with the major already described peripheral B cell populations.

Novel B cells were found to be smaller than memory B cells. The antibody genes were found to contain less mutations than those of memory B cells. Furthermore, novel B cells were found to be less prone to enter apoptosis than transitional B cells. A screening experiment using flow cytometry added support to the hypothesis that the novel B cells are different from the already described populations.

The results suggests that the novel B cell population is a distinct subpopulation post- or parallel to the naive population. Novel B cells might be able to differentiate into IgM^+ memory B cells. In order to verify this, new experiments are needed.

SAMMANFATTNING

Sedan mitten på nittiotalet har vår förståelse för human perifer B-cellsutvecklingen ökat gradvis. Idag delas de perifera B-cellerna upp i tre huvudpopulationer; transitionella B-celler, naiva B-celler och minnes-B-celler. I Mats Bemarks laboratorium har en markör som identifierar en ny B-cellspopulation upptäckts. Det arbete som utförts inom ramen för det här examensarbetet har syftat till att karakterisera den nya B-cellspopulationen genom att jämföra den med de tidigare beskrivna B-cellspopulationerna.

De nya B-cellerna visade sig vara mindre än minnes-B-celler. Deras antikroppsgener innehöll färre mutationer än minnes-B-cellernas. De nya B-cellerna överlevde bättre i in vitro-försök än transitionella B-celler. I ett flödescytometriexperiment skilde sig de nya B-cellerna från de övriga, redan kända, B-cellerna.

Resultaten tyder på att den nya B-cellspopulationen är ny och åtskild från de tidigare beskrivna. Utvecklingsmässigt tycks den ligga parallellt med eller strax efter den naiva B-cellspopulationen. Den nya B-cellspopulationen kan eventuellt differentiera till IgM^+ minnesceller. Ytterligare experiment krävs för att bekräfta detta.

Between stimulus and response there is a space.
In that space lies our freedom and power to choose our response.
In those choices lie our growth and our happiness.

— Stephen R. Covey [1]

ACKNOWLEDGMENTS

Note: At times, I might write interesting curiosities in the margin.

It is my belief that we live in fascinating times. On the one hand rumors never cease telling me that the earth suffers in a way it has never suffered before. On the other hand, using technology developed at the earth's expense, new knowledge is created in a speed never before achieved.

Life goes on. While new generations are continuously being born, old ones pass hence. I like to imagine *knowledge* as an *entity*, and *life* as *the process which feeds it*. As individuals, we will all one day evanesce. As individuals, we are not important. However - carefully formulated information has the potential to serve as a springboard for those interested in increasing our common knowledge.

I would like to thank Mats Bemark, not only for giving me the opportunity to join him in his strive to create new knowledge, not only for allowing me to make mistakes, but also for his patience those times I raved in general or whined over less intelligent mistakes I had made.

I would also like to thank Carl Johan Franzén for being supportive and flexible. Some of those mails I wrote could probably have been a bit shorter!

Moreover, thanks goes to my family, people at the lab, my opponents, LaTeX, R and the guy who wrote this thesis template.

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Part I

INTRODUCTION

1

BACKGROUND, AIM & STRATEGY

1.1 BACKGROUND

Mats Bemark is an associate professor focusing on B cells and their development. The work presented in this thesis has been performed in his group at MIVAC, The Mucosal Immunobiology and Vaccine Center at University of Gothenburg, a strategic research center with support from the Swedish Foundation for Strategic Research.

Flow cytometry experiments rely on the knowledge of cell surface marker expression patterns. In Mats Bemark's laboratorium, a successful attempt to identify previously unknown cell surface marker expression patterns in human peripheral B cells has been made. In a cell surface marker screen, an antibody was found to identify an epitope differentially expressed during B cell development. This antibody also identified a novel B cell population which had not been described in the literature at the time.

1.2 AIM

The aim of this thesis has been to identify different ways in which the novel human peripheral B cell population can be characterized and perform an initial characterization.

1.3 STRATEGY

Information about B cells were gathered in published literature. Methods previously used for B cell characterization were identified, developed and applied. Obtained results were evaluated. At the end, a hypothesis regarding the nature of the novel B cell population was formed. The need for future work was discussed.

Part II

THEORY

2 BACKGROUND KNOWLEDGE

2.1 OUR BLOOD

The percentage of the blood that is cells is called the *hematocrit*. The hematocrit normally averages around 40%. These cells are surrounded by plasma [2].

2.1.1 *Hematopoiesis*

The process in which blood cells are formed is called *hematopoiesis*. In adults hematopoiesis occurs mainly in the bone marrow. The process involves several steps of differentiation. In the first step, hematopoietic stem cells (HSC) differentiates into common myeloid progenitors and common lymphoid progenitors [3].

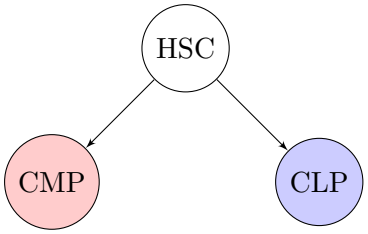


Figure 1: Hematopoietic stem cells (HSC) differentiates into the common lymphoid progenitors (CLP) and the common myeloid progenitors (CMP).

2.1.1.1 *The differentiation of the common myeloid progenitor*

The common myeloid progenitor differentiates into several different types of cells.

THE ERYTHROCYTE These cells make up the majority of the blood cells; women have 3.9-5.2$\times 10^{12}$/L while men have 4.1-5.4$\times 10^{12}$/L [4]. The main purpose of these cells is to serve as oxygene carriers [2].

THE THROMBOCYTE The megakaryocyte fragments into thrombocytes either in the bone marrow or soon after entering the blood. Thrombocytes do not have nuclei and cannot reproduce. They are involved in blood coagulation [2]. The number of platelets in adults are 125-340$\times 10^{9}$/L [4].

THE MAST CELL These cells can be found in connective tissue throughout the body. They have high-affinity Fc-receptors

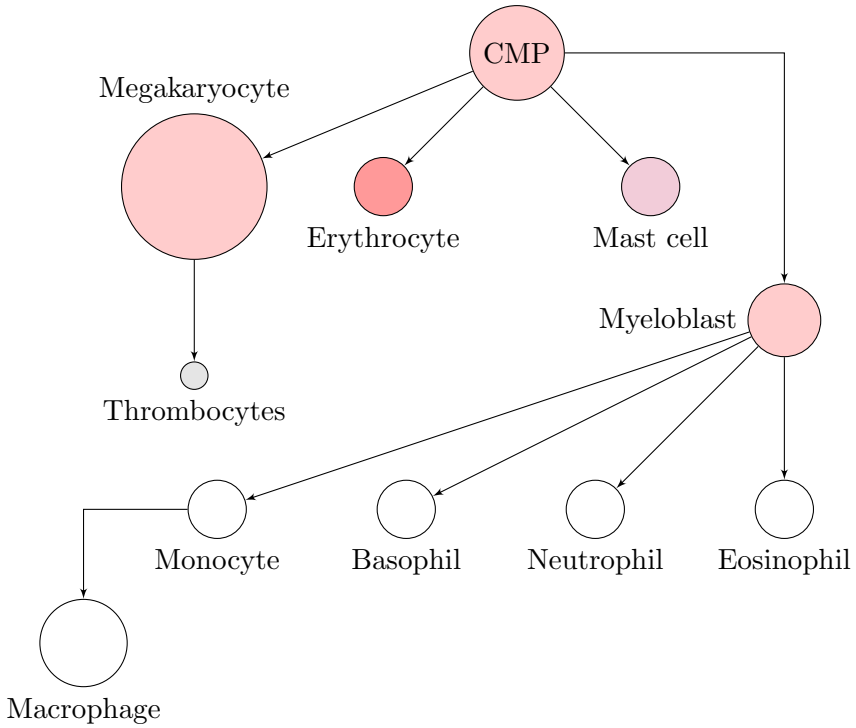


Figure 2: The common myeloid progenitor differentiates into megakaryocytes, erythrocytes, mast cells and myeloblasts.

that bind free IgE. Mast cells have a crucial role in allergic reactions [3].

THE MACROPHAGE Derived from monocytes, macrophages can be found in most tissues. As professional antigen-presenting cells and effector cells in humoral and cell-mediated immunity, they contribute to innate immunity and early nonadaptive phases of host defense [3].

THE BASOPHIL A white blood cell present in small amounts in blood. They contain granules that stain with basic dyes [3].

THE NEUTROPHIL The most abundant white blood cell. They contain granules that stain with neutral dyes and kill extracellular pathogens in infected tissues through phagocytosis [3].

THE EOSINOPHIL These cells contain granules that stain with eosin. They contribute do defense against parasitic infections [3].

2.1.1.2 *The differentiation of the common lymphoid progenitor*

B cells are derived from the common lymphoid progenitor. Also T cells and natural killer cells are formed from this progenitor.

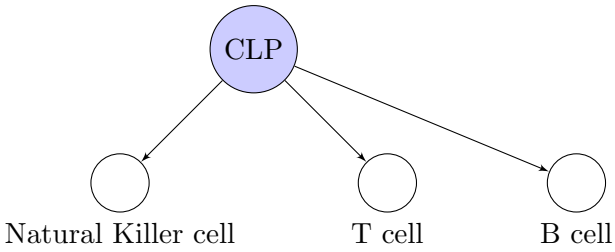


Figure 3: The common lymphoid progenitor differentiates into natural killer cells, T lymphocytes and B lymphocytes.

NATURAL KILLER CELL Large granular cells important in innate immunity to viruses and other intracellular pathogens. They do also kill certain tumor cells and are involved in antibody-dependant cell-mediated cytotoxicity [3].

B CELL All antibodies are produced by B cells. B cell development starts in the bone marrow. B cells just released into peripheral blood express their antibodies as surface molecules. The terminus of B cell differentiation, the plasma cell, secretes antibodies [3]. This thesis focuses entirely on a human peripheral B cell subpopulation.

T CELL There are different types of T cells. In short, T cells are responsible for cell-mediated immunity [3].

2.2 THE CELL

New cells are formed by division of other cells. Therefore, all cells, except for germ cells, have the same set of DNA. Despite this, there are different types of cells.

Our DNA codes for approximately 25000 genes. The cell type depends on the way the individual cell expresses its DNA. When comparing cells from a specific individual, the DNA itself is usually quite uninteresting since all cells once sprung from the very same one and share the same DNA. However, there are exceptions. B cells are, as will be shown later, one of them.

An eukaryotic gene consists of two types of sequences: exons and introns. Exons are the regions that code for actual proteins while introns are noncoding DNA which might have other functions. Genes are transcribed from DNA to RNA in the cell nucleus. Newly transcribed RNA contains both exons and introns. The process in which the introns are removed is called *splicing*. Sometimes *alternative splicing* occurs. This way, the very same gene may give rise to different, but in many aspects similar, proteins [5].

2.2.1 *RNA to protein*

Spliced RNA molecules coding for proteins are called mRNA, short for messenger-RNA. A cell component called ribosome translates mRNA into proteins. Proteins destined to become membrane proteins or to be secreted are placed in the rough endoplasmic reticulum. If a cell needs to produce a lot of a certain protein, large amounts of the corresponding mRNA coding for this protein will be produced. Measurements of the mRNA content of cells give information about the current activities of the cells. Comparisons of mRNA expression of certain genes between different cells have the potential to reveal highly interesting information [5].

2.2.2 *Post-processing of proteins*

Newly formed proteins fold into their functional three-dimensional structures. Certain proteins named chaperones help them out. Incorrectly folded proteins are marked for destruction and removed. Proteins destined to become integral membrane proteins are inserted into the endoplasmic reticulum membrane.

The next halt is the Golgi apparatus. Here, some proteins are modified trough addition of carbohydrates (glycosylation) or phosphates (phosphorylation). As will be seen later in this chapter, sometimes the analysis of protein glycosylation or phosphorylation patterns of a certain protein reveal interesting information about cell differentiation that simply could not have been found in analysis of DNA or RNA. Finally, the finished proteins are to be delivered to different addresses. Some are to be secreted and will leave the Golgi apparatus in secretory vesicles destined to fuse with the outer cell membrane, releasing their contents outside of the cell. This way, membrane molecules integrated in the membrane of the secretory vesicle can be transported to the cell surface [5].

2.2.3 *Functional aspects*

Proteins have certain functions. Some proteins are intended to be secreted in order to communicate with other cells. Other proteins are intended for integration in the outer cell membrane where they have the potential of sending signals to other cells or into its host cell. Others are involved in certain cell signaling paths inside the cell while still others serve completely different purposes.

The fact that cells are autonomous entities responding to signals from the outside makes it possible to study them and their reactions to different stimuli.

2.2.4 *Clusters of differentiation*

Using light microscopy it is possible to distinguish different cells from each other. However, quite diverse cells might have almost the same appearance. To resolve the leukocytes of the blood other methods have to be applied. One approach involves antibodies directed against cell surface proteins. The research performed in this field has identified expression patterns of certain cell surface proteins, making these proteins useful for identification and isolation of different cell populations.

Human Cell Differentiation Molecules (HCDM) is an organization aiming to "characterise the structure, function and distribution of leucocyte surface molecules and other molecules of the immune system" [6]. The work performed in this thesis circulate around the use of a certain cell surface marker whose glycosylation pattern during human peripheral B cell development recently was described in Mats Bemark's laboratorium. The identification of a novel B cell population with the help of an antibody clone directed against a specific glyco-epitope shows that cell surface molecules might hide secrets even after they have been described by HCDM.

2.3 THE ANTIBODY

The purpose of B cells is antibody production. In order to be able to produce antibodies in a controlled way, B cells must undergo changes specific for this type of cells. The idea here is to present some important facts about antibodies, explain why they are important and discuss some aspects of their production before showing what B cells actually do in order to be able to produce antibodies.

2.3.1 *The purpose of antibodies*

Our immune system can be divided into two parts: innate immunity and adaptive immunity. The receptors used by the innate immune system for identification of pathogens are always available and always the same. These receptors recognize structures shared by many different pathogens or alterations to human cells induced by the presence of pathogens. The adaptive part of our immune system uses a different strategy. A vast number of cells (B cells) expressing different receptors (antibodies) are continuously being produced. If the receptor on one of these cells is shown to have an affinity for an invading pathogen, it might become mass produced and optimized in order to take part in the fight against the invading pathogen.

When comparing the immune responses of the innate and adaptive immunity against a pathogen that is new to the immune

system, the response of the adaptive immunity will be slower but more specific and effective (provided that the pathogen isn't killed by the innate immune response alone, in this case the innate immunity might not have time to respond). In addition, the adaptive immunity has a memory which means that it has the potential to react both rapidly and specifically the next time it encounters the same antigen [3].

Thus, the purpose of antibodies could be defined as "recognizing pathogens and assisting in their elimination".

2.3.2 *The structure of antibodies*

Antibodies, also known as immunoglobulins, are globular proteins. Their structure is bilaterally symmetrical. They are composed of two light and two heavy protein chains. Each chain can be divided in two distinct regions where one is constant in sequence (constant region) while the other is very varied (variable region).

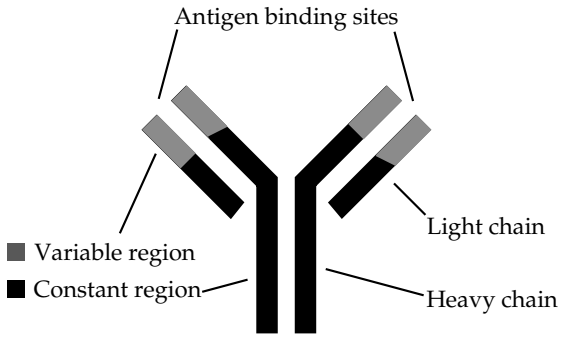


Figure 4: This schematic drawing of an antibody illustrates its bilaterally symmetrical structure.

The variable regions are responsible for binding antigen. The variable regions of one heavy chain and one light chain make one antigen binding site. Each antibody has two antigen binding sites. The variability is not constant within a variable region; each variable region contains three hypervariable regions, also called complementarity-determining regions (CDR). These are responsible for the direct interaction with the antigen. The other parts of the variable regions are called framework regions (FWR). These regions are important for maintenance of appropriate structure.

The constant regions are important for structure maintenance. They are also used for the division of antibodies in different classes. Some of the cells of the immune system have Fc receptors with affinity for different constant regions. This way, constant regions are responsible for some of the immunobiological functions of antibodies [7].

2.3.3 *Antibody classes*

There are five antibody classes in humans; IgA, IgD, IgE, IgG and IgM. The difference between these is their constant region which in some cases determines their immunobiological function. In humans, IgG and IgA are further divided in subclasses with heavy chain amino acid sequences that differ less within than between classes [7].

2.3.3.1 *IgM*

All newly formed B cells express IgM before they encounter antigen. It is secreted as a pentamer. On initiation of an immune response, IgM antibodies are the first to be produced. These early antibodies are often of low affinity. When bound to antigen, IgM antibodies can neutralize pathogens and activate the complement system [3].

2.3.3.2 *IgD*

As IgM, IgD is expressed on early peripheral B cells that are yet to encounter antigen. This antibody class cannot be secreted. It has no known effector functions [3].

2.3.3.3 *IgG*

IgG is the most abundant antibody in internal body fluids. It is secreted as monomers which in general have higher affinities for their antigen than IgM antibodies. The IgG antibodies are more flexible due to an extended hinge region in the heavy class chain. The different subclasses have different effector functions. Examples of effector functions are neutralization, opsonization, sensitization for killing by NK cells and activation of the complement system [3].

2.3.3.4 *IgA*

IgA is made both in monomeric and dimeric form. More of this antibody class is produced than of any other class. It is acting at mucosal surfaces and in secretions. Available effector functions are neutralization, opsonization and activation of the complement system [3].

2.3.3.5 *IgE*

IgE antibodies are produced in small amounts. Fc receptors on mast cells have such a high affinity for these antibodies that they are occupied with IgE antibodies all the time. When an antigen is bound to an IgE molecule bound to a mast cell, histamine and other inflammatory mediators are released from internal granules

of the mast cell. This triggers strong inflammatory responses - a fact most allergic people are sadly aware of [3].

2.3.4 *Immunobiological functions of antibodies*

2.3.4.1 *Neutralization*

Both bacteria and viruses strive to invade our bodies. In most cases, a crucial step of the process of invasion is the binding to the surface of a target cell using cell-surface proteins. The covering of these cell surface proteins with antibodies render them useless, neutralizing the pathogen [3].

2.3.4.2 *Opsonization*

Macrophages and neutrophils have the ability to recognize, ingest and destroy bacteria and viruses. They express Fc-receptors which bind with low affinity to the constant regions of a IgG antibodies. Antibodies specific for a certain pathogen bind to its surface, hence facilitating phagocytosis [3].

2.3.4.3 *Removal of parasites*

Parasites differ from bacteria and virus. As a group, they are good at evading the immune system and they are skilled at establishing long-lasting, persistent infections. Multicellular parasites cannot be eliminated with the mechanisms used for microorganisms. A different mechanism based on IgE has evolved. In summary, the combined actions of IgE, mast cells, basophils and eosinophils serve to physically remove parasites from the body [3].

2.3.4.4 *Sensitization for killing by NK cells*

Natural killer cells express Fc receptors and recognize and kill cells coated with antibodies in experimental situations. Normally, NK cells are regarded as belonging to innate immunity, but, this mechanism suggest that it might take part in adaptive immune responses utilizing antibodies already formed [3].

2.3.4.5 *Complement activation*

The complement system is a part of the innate immune system that complements the antigen-binding function of antibodies. It consists of over 25 proteins and protein fragments. There are three ways in which the complement system can become activated; the classical pathway, the alternate pathway and the lectin pathway. Of these, only the classical pathway involves antibodies. Secreted IgM and IgG antibodies complexed with antigens can activate the complement system this way. Activation of the complement system

leads to a series of enzymatic reactions resulting in covalent bonding of particular complement components to the pathogen surface. Macrophages and neutrophils recognize these components which facilitates phagocytosis of the pathogen. Beyond this, activation of the complement system also leads to the formation of a complex of proteins that attacks the pathogen by poking hole in it [3].

2.4 THE B CELL

2.4.1 *The central part of B cell development*

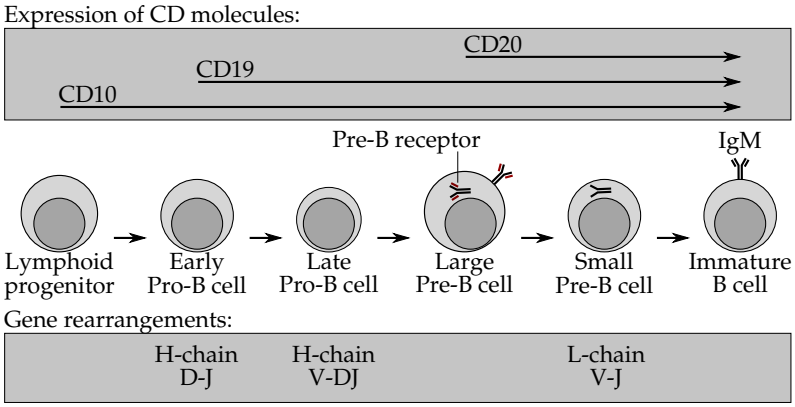


Figure 5: The central part of the B cell development.

Pro-B cells are derived from a common lymphoid progenitor which has the potential to differentiate into several types of cells. At this stage of development the expression of the surface marker CD19 begins. In addition to being a part of the BCR co-receptor, this protein is commonly used for identification of B cells. Another thing occurring at this stage of development is a genomic recombination of the gene coding for the heavy chain. This recombination is called V(D)J-recombination (explained further in 2.4.2). It is responsible for some of the variation between final antibodies.

After the first step of the V(D)J-recombination, which occurs in early pro-B cells, proliferation yields multiple progeny. The second step occurs after the proliferation, leading to a larger increase in varation [8].

When the heavy chain locus has been rearranged, a heavy chain of a type called μ can be produced. The B cell is now known as a pre-B cell. These cells express a surrogate light chain, illustrated in red in Figure 5, Igα and Igβ. The presence of a functional pre-B cell receptor halts both the rearrangement of the heavy chain locus and the expression of surrogate light chains and serves as a quality control: its presence also induces a new proliferation step in which large pre-B cells yield many small pre-B cells. In these cells the light chain loci are rearranged. Once light chains can be made, they

assemble with the μ heavy chain, resulting in an IgM antibody which is transported to the surface of the cell. At this stage, the cell expresses only IgM and is known as an immature B cell [3].

During the central part of B cell development, poly- and auto-reactive antibodies are removed from the repertoire. For example, a process called *receptor editing* is known to occur during the recombination of both heavy- and light-chains. Antigen-binding to a newly formed pre-BCR induces continued recombination which might change the specificity of the previously auto-reactive heavy-chain. Upon BCR-formation, light-chains may undergo further recombination in a similar way [11]. The antibodies expressed by immature B cells have, in spite of this, been shown to be autoreactive to a substantial degree. It has been suggested that the primary cause of autoantibody production is the extra variability induced by junctional diversity, a process which was discussed in 2.4.2.2. The extra variability added here appears to conduce to removal of at least half of the initial antibody repertoire. The development stage of immature B cells serves as a checkpoint for removal of auto-reactive cells since these cells are highly susceptible to deletion by receptor cross-linking [12].

2.4.2 *V(D)J-recombination*

Antibody genes are organized in a way that differs from other genes. In all cells, except in B cells, these genes are in a fragmented form that cannot be expressed. The V(D)J-recombination makes expression of the antibody genes possible in B cells and lays the foundation of the vast variation that is required among antibodies [3]. Estimations of the number of combinations that can be created using the available genomic sequences seem to be of relatively little use since some segments are known to be overrepresentated suggesting that the utilization of each V_H segment may not be completely random [8].

2.4.2.1 *The germline genome*

In humans, the immunoglobulin genes are found at three chromosomal positions: the heavy-chain locus on chromosome 14, the κ light-chain locus on chromosome 2 and the λ light-chain locus on chromosome 22. Every B cell has two copies of the heavy-chain locus and two copies of each light-chain locus. The layouts of these loci are shown in Figure 6. It can be seen that the heavy-chain locus contains D sequences while the light-chain loci do not.

As an immunoglobulin gene is rearranged, its promotor and enhancer are brought into a closer juxtaposition. This enables transcription to proceed. The genomic rearrangement is tightly controlled and rearranges only one heavy-chain locus and one light-

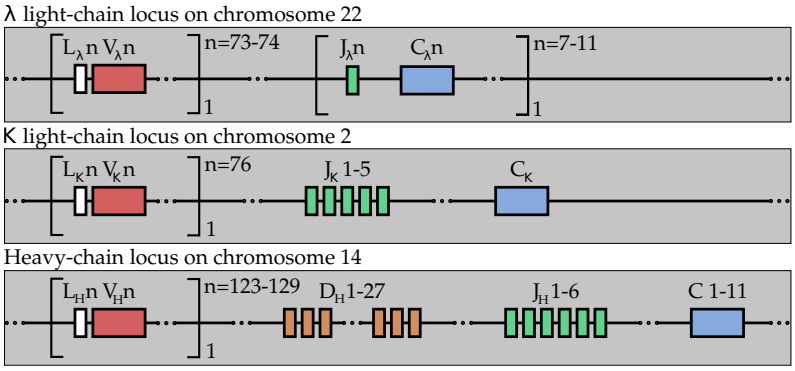


Figure 6: The human heavy and light chain immunoglobulin germline genes. Note that only the heavy chain locus contain D sequences.

chain locus per B cell, a phenomenon known as *allelic exclusion* which ensures that each cell produces only one antibody clone [8, 3].

2.4.2.2 *Heavy-chain V(D)J-recombination exemplified*

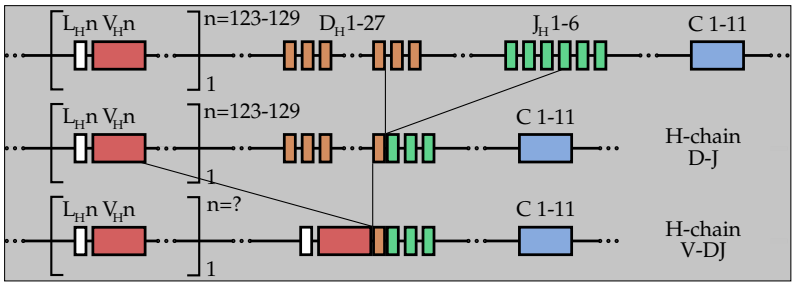


Figure 7: V(D)J-recombination of the heavy locus. First, a J_H sequence is joined with a D sequence. In the next step, the newly formed DJ sequence is joined with a V_H sequence.

The rearrangement of a heavy-chain locus is, as can be seen in Figure 7, a two-step process. The first step in which a D segment is joined with a J_H segment is followed by a second step in which the same D segment is joined with a V_H segment. Since quite a few combinations are possible, it is obvious that this recombination alone will create a variation between the antibodies of the B cells undergoing it. Yet, further varation (not shown in Figure 7) is added during the recombination due to inherent properties of the mechanisms used by the enzymes performing it: Additional sequence diversity not determined by germline DNA is introduced between the D and the J_H segments, into the third hypervariable region (CDR3) of the antibody gene. This contribution to amino acid sequence diversity, called *junctional diversity*, is an important source of antibody variability [3].

2.4.3 *Peripheral part of human B cell development*

As newly formed B cells leave the bone marrow they become part of the peripheral B cell compartment. As can be seen in Figure 8, the major populations are transitional, naive and memory B cells. Since these have been used as control populations in this thesis they have been highlighted in a blue color. Plasma cells are shown in the picture but are normally not found in peripheral blood.

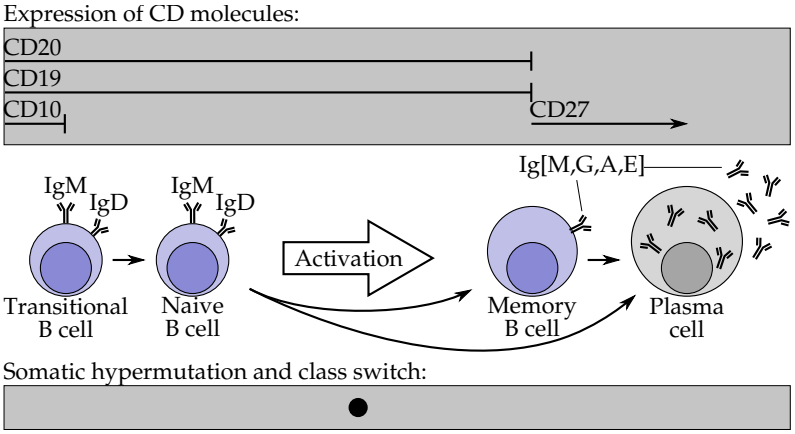


Figure 8: The peripheral development of the B cell development. Populations used as controls have been highlighted in blue.

2.4.3.1 *A dissection of the peripheral B cell compartment*

Cells that have left the bone marrow but not yet matured into naive B cells are called transitional B cells. CD10 combined with the general B cell marker CD19 have been shown to identify these cells. Early characterizations showed that transitional cells die rapidly in culture and that BCR stimulation induces apoptosis rather than cell proliferation [13, 14, 15]. It was seen early on that maturation of transitional B cells seemed to follow a continuum from what was thought to be early transitional B cells to mature naive B cells. This finding has been exploited in recent studies, resolving the transitional population in three development steps; T1, T2 and T3 [16, 17]. Since the proportion of autoreactive B cells is lower in the peripheral mature naive population than in the immature population of the bone marrow, the development stage of transitional cells is thought to harbour a checkpoint in which autoreactive cells are removed [11]. However, the exact position and mechanism of this checkpoint is yet to be determined.

Two other markers used for identification of transitional B cells are CD24 and CD38. Transitional B cells have a high expression of these markers while naive B cells have a lower expression. $CD24^{high}CD38^{high}$ transitional B cells have been shown to arise before $CD24^{low}CD38^{low}$ mature naive B cells after hematopoietic

stem cell transplantation (HSCT). These markers are gradually downregulated as cells become more mature [15].

It has been postulated that expression of the ATP-binding cassette (ABC)B1 transporter is a feature unique for naive B cells. However, while expression of ABCB1 is permanently lost in memory B cells, some overlap in terms of other cell surface markers was seen between transitional and naive B cells [18]. Others have considered gradual acquisition of ABCB1 expression as a natural part of the T3 stage of the phenotypic and functional continuum between immature and mature peripheral B cells and used other cell surface markers to distinguish transitional and naive B cells. It seems to be agreed that full ABCB1-expression is a feature unique for mature naive B cells [16, 17]. Since this small controversy only concerns a very small number of late transitional/early naive B cells, the usage of CD10 to distinguish the relatively small population of transitional cells from the larger population of mature naive B cells can be assessed as being a reasonable method.

Some transitional B cells will eventually mature into naive B cells. These will continue to recirculate between blood, secondary lymphoid tissue and the lymph. Naive B cells that are yet to encounter antigen must pause periodically in secondary lymphoid tissues in order to receive survival signals. Without these signals, they will die within a few days after entering the peripheral circulation. Naive B cells which manage to get access to the structures in which they receive their survival signals seem to have a half-life of 3-8 weeks, unless they encounter their specific antigen [3].

Some naive B cells differentiate directly into plasma cells upon activation. Plasma cells are completely focused on the production and secretion of antibodies. The change of antibody form from membrane-bound to secreted is implemented by a change in the splicing of heavy-chain mRNA. Plasma cells do not divide. Some have a life-span of approximately four weeks while others are long-lived. Long-lived plasma cells migrate to the bone marrow, and B cell development ends where it once started [3].

Activated naive cells form germinal centers where they might undergo class switch and affinity maturation. Both plasma cells and memory B cells form this way. However, it is *not* clear that *all* memory B cells are formed this way. In order to explain this claim, the memory B cell population has to be dissected in subpopulations.

Transitional and mature naive B cells express IgD. In Figure 8, memory B cells have been drawn without expression of IgD. While this is not absolutely true, it reflects the methods once used for identification of memory B cells: Since classic memory B cells are believed to have asserted themselves during the process of affinity maturation, cells of this kind should have mutated V regions. This hypothesis proved to be true in the middle of the 90's when IgD in combination with CD38 was used in order to resolve

the differentiation process as it took place in germinal centers in human tonsils: pre-germinal center IgD^+CD38^- cells were shown to have less mutated V-regions than post-germinal center IgD^-CD38^- cells. (CD38 was used to isolate $CD38^+$ germinal center-specific cells.)

In peripheral blood, studies using IgD in combination with IgM identified three populations. The mature naive population, IgM^+IgD^+, turned out to have almost unmutated V-regions while the V-regions of the classic memory population, IgM^-IgD^-, had a higher mutation frequency. Beyond these populations, a third IgM^+IgD^- population with an intermediate mutation frequency was found. At this time, it was speculated that this third population either consisted of cells that had taken part in germinal centers but left early, before class switch, or cells formed outside of germinal centers.

Next, the cell surface marker CD27 was introduced. Since transitional B cells had not been identified at this time, peripheral B cells were classified as mature naive ($CD27^-IgD^{high}$), IgM^+ memory B cells ($CD27^+IgD^{low}$) or IgM^- memory B cells ($CD27^+IgD^-$) [19]. At this point, it was speculated that CD27 identified all memory B cells. Indeed, using this marker for the identification of memory B cells led to the discovery of new memory B cell populations carrying mutated V regions: a $CD27^+IgM^+IgD^+$ and a very rare $CD27^+IgM^-IgD^+$ [20]. However, the detection of a minor $CD27^-IgM^-$ class switched memory B cell population with mutated V regions made clear that expression of CD27 is not a prerequisite for memory B cells [21].

Thus, memory B cells can be divided in two categories; class switched (40% of memory B cells) and non-class switched (60% of memory B cells). The $CD27^+$ class switched memory B cell constitutes about 85% of the class switched memory B cells and represents the classic memory B cell with an origin which is thought to be well investigated. Less is known about its $CD27^-$ counterpart. The non-class switched memory B cells can be divided in subpopulations expressing various combinations of IgM and IgD, where IgM^+IgD^+-cells constitute about two thirds while IgM^+IgD^--cells constitute about one third. The IgM^-IgD^+-cells is a vanishingly small population [20].

In total, memory B cells normally constitute about 40% of all human peripheral B cells. Mature naive B cells are the most common, ~55%. The transitional B cell population is a small one constituting slightly less than 5% of all peripheral B cells.

2.4.4 *B Cell activation*

When the V(D)J-recombination has been completed, the B cell is able to express its immunoglobulin gene in form of an IgM antibody.

Using alternative splicing, mature naive B cells can express both IgM and IgD antibodies. These are the only antibody classes that can be expressed simultaneously by a single cell.

Antibodies can be expressed in two forms; membrane-bound and secreted. While the end-stage of B cell differentiation—the plasma cell—mass produces its antibodies in its secreted form, unactivated B cells that are yet to encounter antigen only express antibodies in the membrane-bound form.

The membrane-bound form of the antibody is surrounded by proteins which together with the antibody form a complex called the B cell receptor (BCR). Before discussing the events preceding the activation of a B cell, the BCR will be introduced [3].

2.4.4.1 *The B cell receptor and its coreceptors*

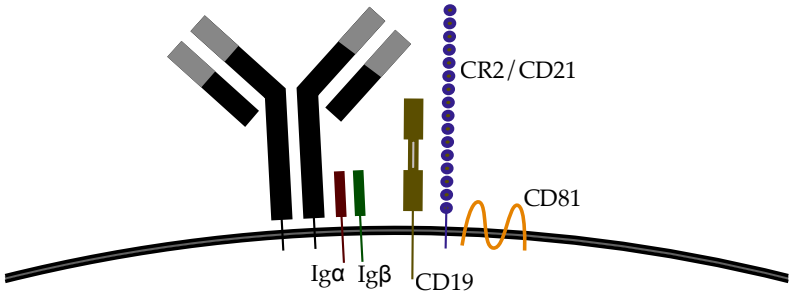


Figure 9: The B cell receptor and some of its coreceptor molecules.

The BCR consists of the membrane-bound antibody together with the proteins Igα and Igβ. These two proteins are responsible for communicating any interactions between antigen and the antibody to the interior of the B cell. When the BCR binds an antigen, the intracellular $[Ca^{2+}]$ is increased. Since the details of the signaling pathways are of little importance for the work performed during this master thesis, these will not be discussed. The BCR also has coreceptors. Some of these enhance signal strength while others, such as CD22, diminishes it [8].

Engagement of the BCR results results in activation of protein tyrosine kinases and rapid phosphorylation of their substrate protein. Three proteins—complement receptor 2 (CR2 or CD21), CD19 and CD81—form a complex that can enhance the signal strength of the BCR. In 2.3.4.5 it was stated that activation of the complement system could lead to the deposition of particular complement components to the pathogen surface. One of these components, named C3d, is the ligand for CD21. As the BCR binds to an antigen present on a pathogen surface, CD21 has the potential to bind any present C3d. This binding serves to bring the BCR and its coreceptor into juxtaposition which enables in-

tracellular signal components of CD19 to generate signals that synergize with those generated by the BCR complex. This has the potential of increasing signal strength by 1000-10000-fold. However, usually even the combined efforts of the BCR and this coreceptor are insufficient to activate the naive B cell [3].

2.4.4.2 *T Cell dependent activation*

Most pathogen-specific antibodies are produced by B cells that have been activated by the help of helper CD4 T cells. This activation occurs in secondary lymphoid tissue where specific antigen, B cells and T cells are all brought together. Here, a second role of the BCR becomes apparent: After binding to the antibody, antigen is internalized by receptor-mediated endocytosis, processed and presented to helper T cells as antigenic peptides bound to MHC II molecules on the B cell surface. As this interaction occurs, CD40L on helper T cells is allowed to interact with CD40 on B cells, activating the transcription factor NFκB in the B cell which increases the surface expression of ICAM-1, strengthening the bond between the cells. A signal produced in the T cell causes it to secrete cytokines which induce proliferation and differentiation in the now activated B cell [3].

2.4.4.3 *T Cell independent activation*

A B cell can become activated also without help from T cells. There are certain antigens that binds to both the BCR and other receptors on the B cell. An example is the lipopolysacharide (LPS) of Gram-negative bacteria which bind to LPS-binding protein and CD14 which in turn associates with another receptor, called a Toll-like receptor, to produce activating signals. An antigen that triggers other receptors on the B cell this way may also facilitate the activation of B cells expressing antibodies with affinities for other structures on the pathogen.

Other antigens, typically repetitive carbohydrate or protein epitopes, might be present in high density on the surface of a pathogen. These antigens only stimulate B cells specific for it and activation is probably induced due to extensive cross-linking of the BCR and its coreceptors [3].

2.4.4.4 *Post activation*

What happens after activation of a B cell depends on how it was activated. If it was activated without T cell help, it will proliferate and differentiate into plasma cells expressing IgM. These cells will not undergo somatic hypermutation and switch antibody class only seldom. They do not contribute to the immunological memory. Still, they might serve as a useful early low affinity-response against an invading pathogen.

Cells that have been activated by helper CD4 T cells in secondary lymphoid tissue form germinal centers in which they proliferate and differentiate. These centers of dividing lymphocytes can be felt as swollen lymph nodes about a week after the start of an infection. This is where class switch and somatic hypermutation leading to affinity maturation are taking place [3].

2.4.5 *Class switch & somatic hypermutation*

2.4.5.1 *Class Switch*

The effector function of an antibody is determined by its constant region. The constant region of antibodies is also used to divide them into different classes. During their development, some B cells will change the class of their antibodies. In order to change class, a second genomic recombination is performed. This recombination brings a different C gene into juxtaposition with the assembled V-region sequence, resulting in the expression of the same variable region combined with another constant region [3].

2.4.5.2 *Somatic hypermutation and affinity maturation*

Somatic hypermutation occurs in germinal centers. One of the enzymes required for somatic hypermutation, Activation-Induced (Cytidine) Deaminase (AID), is required also for class switch recombination [9].

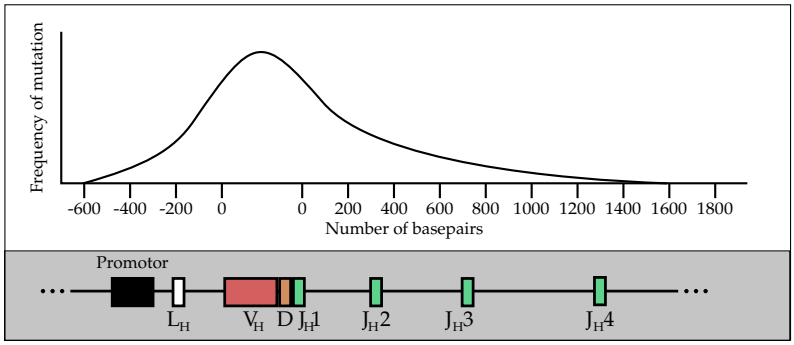


Figure 10: Somatic hypermutation has been shown to induce mutations downstream of the promotor. Mutation frequency is highest in the variable region. Although not shown in the picture, CDR regions are more mutated than FWR regions [10].

The process of somatic hypermutation induces mutations in V(D)J-recombined antibody genes. The distribution of these mutations has been investigated. A graphical interpretation is shown in Figure 10. The mutation intensity is most intense over the variable region but the region affected by mutations extended over 1 kb from the V(D)J-gene in the 3' direction [10].

Cells undergoing somatic hypermutation in germinal centers are programmed to enter apoptosis unless they receive certain survival signals. A B cell that is activated by a helper T cell will proliferate. In order to evade apoptosis, newly formed B cells have to find a follicular dendritic cell carrying its antigen. Since the incorporation of a mutation in the antibody gene might affect the affinity of the antibody for its antigen, the germinal center B cell population will contain cells expressing antibodies with different affinities for the same antigen. This leads to a competition where B cells compete for antigen, resulting in affinity maturation, a process where the B cells with the highest antigen affinities will be selected for further proliferation. This is the last variation-inducing step of the B cell development [3].

2.5 A NOVEL B CELL POPULATION

The ultimate aim of this thesis is a further characterization of a novel, not previously described, B cell population. When this work began, a few things about this population were already known.

2.5.1 *The detection of a novel B cell population*

As has already been discussed, the definition and identification of B cell populations rely on utilization of the fact that different cells exhibit different expression patterns of cell surface molecules. As new cell surface markers are identified, new populations are found and old ones are broken into subpopulations. When a new population or subpopulation is identified, it can be compared to others. As more knowledge is gradually acquired using this and other approaches, the development of human peripheral B lymphocytes becomes better defined. A better understanding of B cell development might for example lead to increased understanding of autoimmune diseases.

A screening experiment was made in Mats Bemark's laboratorium. The experiment was an attempt to find cell surface markers whose expression changed during B cell development. B cells were identified using the marker CD19 and separated using CD27 and IgM. Using flow cytometry, the expression of over 75 different cell surface markers were investigated in these populations. While most of these cell surface markers did not provide any additional information, one of them did: The monoclonal antibody CD45RB$^{\text{MEM-55}}$.

The content of this paragraph is quite amazing!

When investigated further, this cell surface marker was shown to be highly expressed on CD27^{+} memory B cells and CD27^{-} class switched memory B cells. Neither naive nor transitional B cells expressed it. However, a novel CD27^{-}CD10^{-} B cell population consistently constituting ~5% of the peripheral B cell compartment in healthy individuals expressed CD45RB$^{\text{MEM-55}}$.

2.5.2 *Introduction of the cell surface marker CD45RB*

In order to understand the work performed during this thesis, it is enough to be aware of the fact that CD45RB$^{\text{MEM-55}}$ identifies a novel B cell population. However, since other members of the group recently completed a study regarding CD45RB and the way it is differentially glycosylated during human peripheral B cell development, a few words regarding this subject will be written here.

CD45 is specifically expressed in hematopoietic cells. This transmembrane protein has been shown to be a regulator of BCR signaling. Absence of functional CD45 leads to immunodeficiencies in both human and mouse. Alternative splicing results in expression of different combinations of three exons, resulting in different proteins. In addition, glycosylation post-processing creates even more diversity, resulting in expression of large numbers of isoforms. The work performed shows that while CD45RB$^{\text{MEM-55}}$ does identify a novel B cell population, other CD45RB-reactive antibodies do not. The reason for this is the affinity of the antibody clone, it binds to a glyco-epitope on the CD45RB molecule whose expression is strictly regulated during B cell development.

2.5.3 *HSCT patients in a clinical study*

Certain diseases can be cured with hematopoietic stem cell transplantation (HSCT). To immunologists, newly transplanted patients are interesting. In these patients they get the opportunity to study the immune system as it re-develops.

In another research project performed in Mats Bemark's laboratorium, a cohort of pediatric patients treated with HSCT were followed for two years. Here, quite a few interesting findings where made. While the concentration of class switched memory B cells reached normal levels at the end of the study, their IgM$^+$ counterpart stayed low in most of the patients. Normally, IgM$^+$ memory B cells constitute 60% of the memory B cell compartment. In these patients, an average of only 40% of the memory B cells expressed IgM.

Patients that have undergone HSCT are known to suffer from recurrent infections by polysaccharide encapsulated bacteria. IgM$^+$ memory B cells have been speculated to take part in immunological responses against these bacteria. In addition, low numbers of blood IgM+ memory B cells have been linked to poor responses to encapsulated bacteria. While further studies are needed to confirm this, the low number of IgM$^+$ memory B cells in patients recovering from HSCT might explain at least a part of their sensitivity to infections by encapsulated bacteria.

Another interesting finding was that the novel B cell population identified by the cell surface molecule identified in the earlier screening experiments, CD45RB$^{\text{MEM-55}}$, was highly enriched in these patients: During the first year after HSCT treatment, the majority of the B cells expressed this isoform of CD45RB.

2.5.4 *The novel B cell*

When the work of this thesis begun, some things were already known about the novel B cell population. This information led to the hypothesis that these cells were late transitional B cells.

2.5.4.1 *The novel B cell is not an early transitional B cell*

Human transitional T1 and T2 B cells express CD10 and CD5. The novel B cell population has been shown to be CD10$^-$CD5$^-$. However, Late T3 transitional/pre-naive B cells have been shown to down-regulate their expression of these surface molecules. Therefore, it could not be excluded that novel B cells might be late transitional B cells [16].

2.5.4.2 *The novel B cell is not a naive B cell*

As was discussed in 2.4.3, naive B cells have been shown to express ABCB-1. This distinguishes them from novel B cells which do not express this surface molecule. Beyond this, novel B cells have been shown to have a higher expression of IgM than naive B cells.

2.5.4.3 *The novel B cell does not resemble memory B cells*

Memory B cells have been shown to have a CD27-expression, to be class switched or both. The novel B cell is CD27$^-$ and expresses IgM. While a discovery of a new memory B cell would be possible, the fact that these cells have been shown to be enriched in children and in patients recovering from hematopoietic stem cell transplantation suggests that this is an immature cell type rather than a mature one.

3

MATERIALS & METHODS

3.1 ACQUISITION OF STARTING MATERIAL

3.1.1 *Obtaining blood*

Cell samples have been purified from peripheral human blood. Small amounts of up to 50 mL were drawn from people at the laboratory using evacuated equipment. Buffy coats purified from 450 mL blood were used when larger amounts were needed.

The fact that buffy coats often cannot be fetched before 1:30PM has a potential of slightly skewing the daily rythm of researchers.

3.1.2 *Purification of B cells*

In most cases, the purification of B cells can be divided in two steps. First, erythrocytes and platelets are removed. Next, B cells can be purified.

3.1.2.1 *Removal of erythrocytes and platelets*

Centrifugation of diluted blood on Ficoll Paque™ PLUS (GE Healthcare) as described in the supplied manual removes erythrocytes, platelets and granulocytes, leaving only peripheral blood mononuclear cells (PBMC). In contrast, removal of erythrocytes with HetaSep™ (Stem Cell Technologies) and platelets by centrifuging do not remove granulocytes.

3.1.2.2 *Positive selection*

Using EasySep® immunomagnetic technology (Stem Cell Technologies), B cells were labeled with CD19-antibodies which in turn were linked to magnetic particles. Using a magnet, labeled cells where then positively selected. This method was assessed as being suitable for smaller samples and resulted in acceptable purity for the intended downstream applications.

For larger samples, an autoMACS® Pro Separator (Miltenyi Biotec) was used for positive selection of $CD19^+$ cells. While this method was perceived as being extremely convenient, giving samples of high purity in short time, it is also a quite expensive purification method.

Dynabeads® CD19 Pan B followed by DETACHaBEAD® CD19 (Invitrogen) was also used for positive selection. Although samples of acceptably high purity was often achieved, the results were less consistent than when the autoMACS® Pro Separator was used.

3.1.2.3 *Negative selection*

The autoMACS® Pro Separator was used also for negative selection. In this case, yield and purity was remarkably high.

Using RosetteSep® (Stem Cell Technologies), an antibody cocktail was applied before centrifugation on Ficoll Paque™ PLUS. The antibodies bind to surface antigen specifically expressed on unwanted cells and crosslinks these to erythrocytes. While this method is extremely convenient when working with cell samples of reasonable size, it is also quite expensive.

3.1.3 *Identification and isolation of B cell populations*

Purified B cells were either analyzed using a BD™ LSR II Flow Cytometer or sorted in subpopulations using a BD FACSAria™ Flow Cytometer. Antibodies directed against the cell surface markers CD19, CD20, CD27, CD10, $CD45RB^{MEM\text{-}55}$ and IgM were used in different combinations depending on the needs of the current application. For some of these cell surface markers, multiple antibodies with different fluorochromes were ordered.

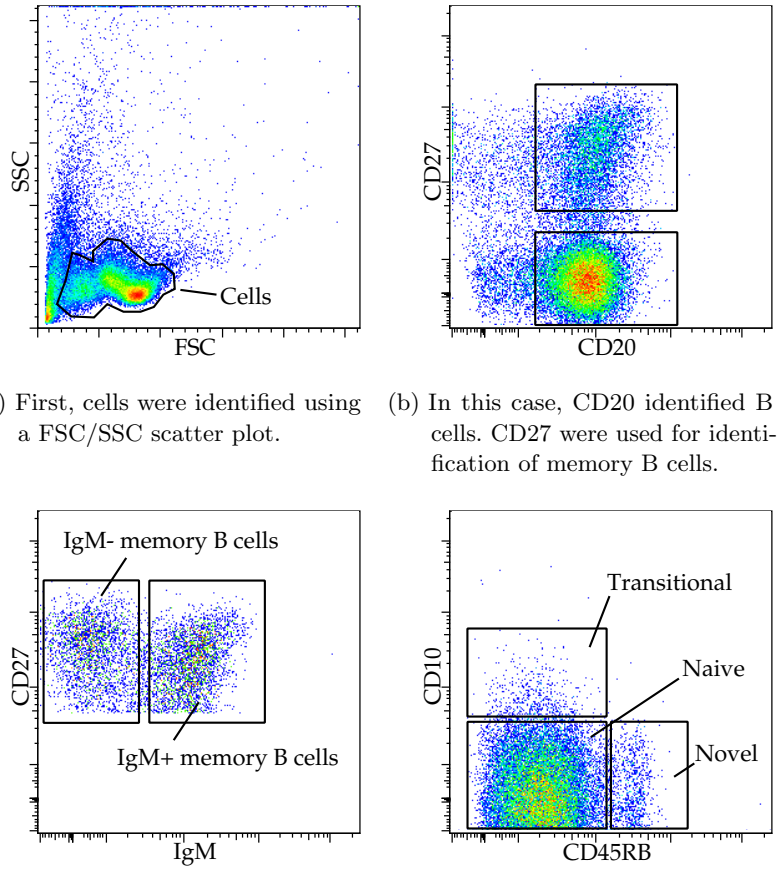
3.1.3.1 *FACS analysis*

The FlowJo Flow Cytometry Analysis Software was used for all FACS analysis. Events representing cells were identified in a forward scatter/side scatter-plot. Forward scatter depends on cell volume while side scatter depends on the inner complexity of the cell [7]. An example of a FSC/SSC scatter plot is provided in Figure 11a.

The events representing cells were separated into B cells and non-B cells using their expression of either CD19 or CD20. B cells were divided into memory B cells and non-memory B cells using the expression of CD27. In Figure 11b, these two steps are performed simultaneously. Figure 11c illustrates the separation of memory B cells into IgM^- and IgM^+ subpopulations. $CD27^-$ B cells were divided into transitional, naive and novel cells using their expression of CD10 and $CD45RB^{MEM\text{-}55}$as shown in Figure 11d.

3.1.3.2 *Cell sorting*

Since the BD FACSAria™ Flow Cytometer is able to sort four cell populations at a time, four populations were sorted for most experiments; general memory ($CD19^+CD27^+$), naive ($CD19^+CD27^-CD10^-CD45RB^-$), novel ($CD19^+CD27^-CD10^-CD45RB^+$) and transitional B cells ($CD19^+CD27^-CD10^+CD45RB^-$). In cases where B cells have been sorted in more than four subpopulations, two consecutive sortings were performed. Consecutive sortings of different cell types demand more starting material.



(a) First, cells were identified using a FSC/SSC scatter plot.

(b) In this case, CD20 identified B cells. CD27 were used for identification of memory B cells.

(c) Expression of IgM were sometimes used for identification of memory B cell subpopulations.

(d) CD10 and CD45RB were used to separate transitional and novel B cells from CD27$^-$ naive B cells.

Figure 11: An example of a typical FACS analysis. In this case, peripheral blood mononuclear cells were purified from a buffy coat using Ficoll Paque™ PLUS. From these, B cells were purified using an autoMACS® Pro Separator for CD19 positive selection.

3.2 CHARACTERIZATION OF CELL SAMPLES

3.2.1 *Light microscopy*

The cell samples used in these experiments were all obtained using the FACSAria cell sorter. Using the procedure described above four types of B cells were sorted from PBMC; general memory, naive, novel and transitional B cells.

3.2.1.1 *Method 1 - May Grünwald/Giemsa staining*

From the sorted cell samples suspensions of 100 µL each containing 25 000 cells were prepared. Using a cytospin centrifuge set at 500 g for seven minutes the cell suspensions were spun onto slides. These slides were allowed to air dry during the night. The following day

the slides were stained with May Grünwald and Giemsa (Sigma-Aldrich) using the supplied protocol.

Pictures of these cells were obtained using a light microscope equipped with a high quality 63x oil immersion objective. Since there was no use for the slides once the photos had been obtained cover slips were not used. Thus, immersion oil was applied directly on the slides.

3.2.1.2 *Method 2 - size measurement*

Cell samples were transferred to a 96-well plate and PBS was added until each separate well contained about 25 000 cells in 200 µL PBS. The plate was put on ice until the cells had sedimented to the bottom of the wells, a process which took about 45 minutes. This procedure made it possible to obtain photos of the cells using an inverted light microscope equipped with a camera.

The photos were imported into Adobe Illustrator. This software has a tool for drawing ellipses which was used to obtain the vertical and horizontal diameter of 20 random cells from each sample. Since the aim of these measurements was to obtain data that could be used for size comparisons only the data was normalized against the smallest available data point. The normalized data was used to calculate a fictive cross-sectional area.

3.2.2 *Gene cloning*

The DNA samples used in these experiments were all prepared using the AllPrep DNA/RNA Micro Kit from Qiagen as described in the manual. Cell samples were obtained from three buffy coats from different donors which were collected at different times. Using the FACSAria cell sorter, five different B cell populations were sorted from these buffy coats; $IgM^{+}CD27^{+}$ memory B cells, $IgM^{-}CD27^{+}$ memory B cells, naive B cells, novel B cells and transitional B cells.

3.2.2.1 *Method 1 - cloning the intron*

This PCR reaction was designed to amplify an intron sequence. The upstream primer was placed above the CDR3 region and the downstream primer was placed in the intron.

The Phusion® Hot Start II High-Fidelity DNA Polymerase (Finnzymes) was chosen because of its high accuracy. All optimizations were performed following the manual. During the first development phase of this method several different primer pairs, composed of primers already available at the laboratorium, intended to amplify about the same region were tried. When one of these pairs was shown to amplify the wanted region it was chosen

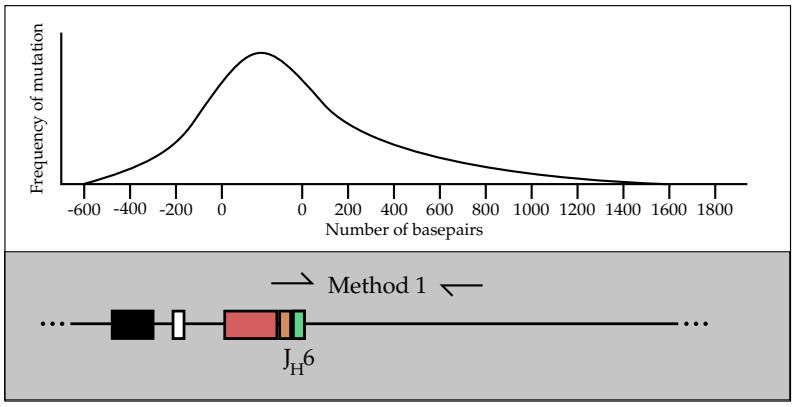


Figure 12: Primer placement for cloning method #1. Please note that primer size and placement are approximations intended to provide an overview rather than disclose details.

for further optimization. This resulted in the PCR protocol shown in Table 1.

Table 1: PCR protocol #1

PCR protocol cloning #1			
Upstream primer: 5'-GMG GAC ACG GCY GTG TAT TAC-3' Downstream primer: 5'-GTC CTC GAG GTC AGG ACG GCA GCC ACC GTT T-3'			
Initial denaturation	98°C	0:30	
Denaturation	98°C	0:10	
Annealing	65°C	0:30	
Extension	72°C	0:30	35 cycles
Final extension	72°C	10:00	

The PCR product was cleaved with the restriction enzyme Stu1 and loaded into a 1.5% agarose gel which was run for approximately 45 minutes at 100V whereupon the wanted band was cut out and purified using the QIAquick Gel Extraction Kit (Qiagen). In this step, only sequences with a DJ_H6 sequence were selected for further experimentation, since sequences with other DJ_H-combinations were at least 300bp longer.

The obtained product was cloned following the manual for Zero Blunt® TOPO® Cloning kit for Sequencing (Invitrogen). Relevant colonies were identified with probe hybridization [29] using a primer specific for the interesting sequence; 5'-ACC CAA CCT GAG TCC CAT TTT CC-3'. 15 relevant colonies of each cell type were chosen

for plasmid preparation using GenElute™ Plasmid Miniprep Kit (Sigma Aldrich). Obtained plasmids were sent to Eurofins MWG Operon for sequencing.

The upstream primer of this PCR reaction is constructed to bind to as many different FWR3 regions as possible. This means that amplified sequences contain the CDR3 region of the antibody gene and a part of the intron. Through junctional diversity, discussed in 2.4.2.2, sequence diversity not determined by germline DNA is added in the CDR3 region during V(D)J-recombination. Using comparisons of CDR3 regions, duplicates were removed from the obtained sequence databank, leaving only unique sequences for analysis.

For each cell type, cloned sequences were analysed using the Staden Package. The number of mutations were counted for the same 500bp long region in each sequence and the results were compiled in pie charts.

3.2.2.2 *Method 2 - cloning the variable region*

This PCR reaction was designed to amplify the antibody locus. The upstream primer was placed in the FWR1 region and the downstream primer was a J_H consensus primer. The amplified sequences were 310-360 bp long.

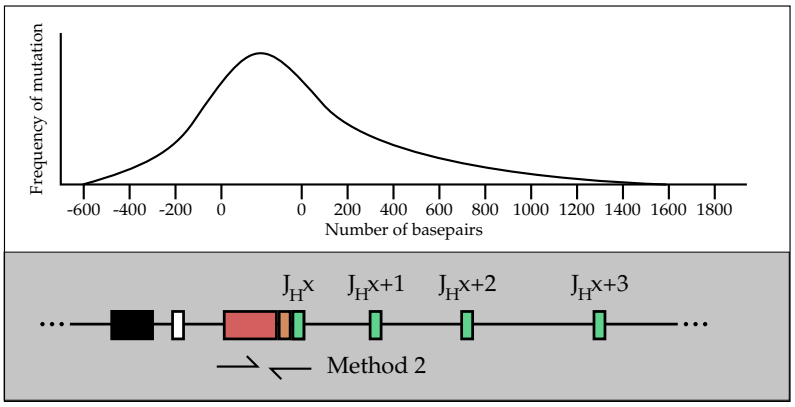


Figure 13: Primer placement for cloning method #2. Please note that primer size and placement are approximations intended to provide an overview rather than disclose details.

Primers amplifying the variable region of the antibody locus were obtained from the literature [30]. This primer set contained upstream primers specific for the different V_H-families and a consensus downstream primer. Primers supposed to amplify the three largest V_H-families were ordered. Despite the fact that these primers were designed to be run together in order to amplify as many different sequences as possible, V_H1-FR1 combined with the consensus J_H-primer seemed to give the best results during early optimization

experiments. Because of this, all reactions were run using this primer pair.

Table 2: PCR protocol #2

PCR protocol cloning #2			
Upstream primer: 5'-GGC CTC AGT GAA GGT CTC CTG CAA G-3' Downstream primer: 5'-CCA GTG GCA GAG GAG TCC ATT C-3'			
Initial denaturation	98°C	0:30	
Denaturation	98°C	0:10	
Annealing	68-70°C	0:20	
Extension	72°C	0:14	40-45 cycles
Final extension	72°C	10:00	

Amplified sequences were analyzed using IgBlast. Only sequences with a unique combination of V-, D- and J-regions were selected for further analysis.

3.2.3 *qRT-PCR*

Eight genes specifically expressed in mice transitional B cells with a high fold change were chosen and qRT-PCR primers for their human counterparts were ordered. A couple of other genes were added to the set due to their differential expression in mice B cell subpopulations [23, 24].

A batch of RNA for calibration purposes was prepared. Standard curves for the primer pairs were developed. $IgM^{-}CD27^{+}$ memory B cells, $IgM^{+}CD27^{+}$ memory B cells, naive B cells, novel B cells and transitional B cells were sorted using the BD FACSAria™. However, preparation of RNA from peripheral B cells failed.

Several different strategies and commercial kits were tried and evaluated. There are, for example, amplification kits intended to give high cDNA yields. The QuantiTect Whole Transcriptome Kit (Qiagen) is one which was tried without consistent reproducible results. Since the RNA preparation step was thought to be a problem, this step was eliminated using a one-step qRT-PCR procedure. The CellsDirect™ One-Step qRT-PCR kit (Invitrogen) was tried, giving no results.

3.2.4 *FACS cell surface marker screen*

B cells were prepared from a buffy coat using Ficoll Paque™ PLUS followed by CD19 positive selection using an autoMACS® Pro Separator. Using antibodies against cell surface markers CD10, CD20, CD27, CD45RB$^{MEM\text{-}55}$ and IgM, the expression of a cell surface molecule could be analyzed individually on IgM^{+} and IgM^{-} memory B cells, naive B cells, novel B cells and transitional B cells through the addition of another antibody. Figure 11 is based on a sample from this experiment and illustrates the gating strategy.

The stained cells were divided into FACS tubes. The content of each tube was stained with one of the antibodies used in the original screening experiment. A summary of the antibodies used are presented in Table 3. Control antibodies have been excluded.

Table 3: This is a summary of the antibodies used for the cell surface marker screen.

Flow cytometry screen			
CD1a	CD13	CD34 #1	CD57
CD2	CD14 #1	CD34 #2	CD59
CD3 #1	CD14 #2	CD35	CD61
CD3 #2	CD15	CD36	CD63
CD4 #1	CD16 #1	CD37	CD65
CD4 #2	CD16 #2	CD41a	CD66acde
CD5	CD18 #1	CD42b	CD66b
CD6	CD18 #2	CD43	CD71
CD7 #1	CD21	CD44	CD72
CD7 #2	CD22	CD45 #1	CD80
CD8 #1	CD23	CD45 #2	CD103
CD8 #2	CD25 #1	CD45RA #1	CD95
CD9 #1	CD25 #2	CD45RA #2	CD122
CD9 #2	CD28	CD45RO	CD138
CD11a #1	CD29	CD49B	CD235a
CD11a #2	CD31	CD54 #1	
CD11b	CD33	CD54 #2	

3.2.5 *Functional aspects*

3.2.5.1 *BCR Stimulation and Intracellular [Ca^{2+}]*

B cells were purified from whole blood using Ficoll-Paque™ PLUS followed by CD19 positive selection using Dynabeads® CD19 Pan

B and DETACHaBEAD® CD19. After purification, the purified B cells were incubated for one hour at room temperature with 4µM Fluo-4 AM and <0.1% Pluronic F-127. Finally, the cells were stained with antibodies directed against CD19, CD27, CD10 and $CD45RB^{MEM\text{-}55}$. For stimulation of the BCR, an $F(ab')_2$ goat anti-human antibody was used. Before the stimulations were performed, cells were suspended in HBSS and prewarmed to 37°C. The ionophore ionomycin was used as a positive control.

3.2.5.2 *Cell stimulation*

APOPTOSIS EXPERIMENT #1 PBMCs were prepared from a buffy coat using Ficoll Paque™ PLUS (GE Healthcare). Memory, naive, novel and transitional B cells were sorted using the BD FACSAria™ Flow Cytometer. Each cell sample was diluted in medium supplemented with 10% fetal calf serum and transferred to a 96-well plate. Eight wells each containing 11 000 cells were prepared for each cell type. Two wells of each cell type were harvested at 0h, 24h, 48h and 72h. Apoptosis and necrosis was evaluated using Annexin/7-AAD-staining. Proliferation was evaluated using CountBright™ absolute counting beads (Invitrogen). Before each flow cytometer experiment, cells were restained with the antibodies used for sorting (against CD19, CD27, CD10 and CD45RB) for evaluation of differentiation.

APOPTOSIS EXPERIMENT #2 PBMCs were prepared from a buffy coat using Ficoll Paque™ PLUS (GE Healthcare). B cells were prepared using the autoMACS® Pro Separator (Miltenyi Biotec) for positive selection of $CD19^+$ cells. Memory, naive, novel and transitional B cells were sorted using the BD FACSAria™ Flow Cytometer. Eight wells each containing 11 000 cells were prepared for each cell type. Two wells of each cell type were harvested at 0h, 24h, 48h and 72h. Apoptosis and necrosis was evaluated using the Vybrant® DyeCycle™ Violet/SYTOX® AADvanced™ Apoptosis Kit (Invitrogen).

APOPTOSIS EXPERIMENT #3 PBMCs were prepared from a buffy coat using Ficoll Paque™ PLUS (GE Healthcare). B cells were prepared using the autoMACS® Pro Separator (Miltenyi Biotec) for negative selection of $CD19^+$ cells. Memory, naive, novel and transitional B cells were sorted using the BD FACSAria™ Flow Cytometer. Eight wells each containing 11 000 cells were prepared for each cell type. Two wells of each cell type were harvested at 0h, 24h, 48h and 72h. Apoptosis and necrosis was evaluated using the Violet Ratiometric Membrane Asymmetry Probe/Dead Cell Apoptosis Kit (Invitrogen).

PROLIFERATION AND DIFFERENTIATION PBMCs were prepared from a buffy coat using Ficoll Paque™ PLUS (GE Healthcare). Memory, naive, novel and transitional B cells were sorted using the BD FACSAria™ Flow Cytometer. Each cell sample was diluted in medium supplemented with 10% fetal calf serum and transferred to a 96-well plate. Sixteen wells each containing 13 500 cells were prepared for each cell type. Two wells of each cell type were supplemented with CpG, CpG + IL-2, R848, R848 + IL-2, BAFF, CD40L or IL-4 leaving two wells of each cell type without stimulant [13, 14, 16, 17, 18, 32, 33, 34]. Cells were harvested at day five. Proliferation was evaluated using CountBright™ absolute counting beads (Invitrogen). Cells were restained with the antibodies used for sorting (against CD19, CD27, CD10 and CD45RB) for evaluation of differentiation.

Part III

RESULTS & DISCUSSION

4

RESULTS

4.1 LIGHT MICROSCOPY

4.1.1 *Method 1 - May Grünwald/Giemsa*

PURPOSE CD27^{+} and CD27^{-} memory B cells have been shown to be larger and have more cytoplasm than naive and transitional B cells [21]. The purpose of this experiment was to find out if these results could be reproduced and, if so, find out whether the phenotype of novel B cells were more similar to the one of naive and transitional B cells or the one of memory B cells.

COMMENTS ON THE METHOD The May Grünwald / Giemsa-staining is commonly used for the staining of peripheral blood cells. Nuclei are stained strongly violet while cytoplasm is lighter stained, enabling visualization of cells and differentiation of cell nucleus and cytoplasm using light microscopy.

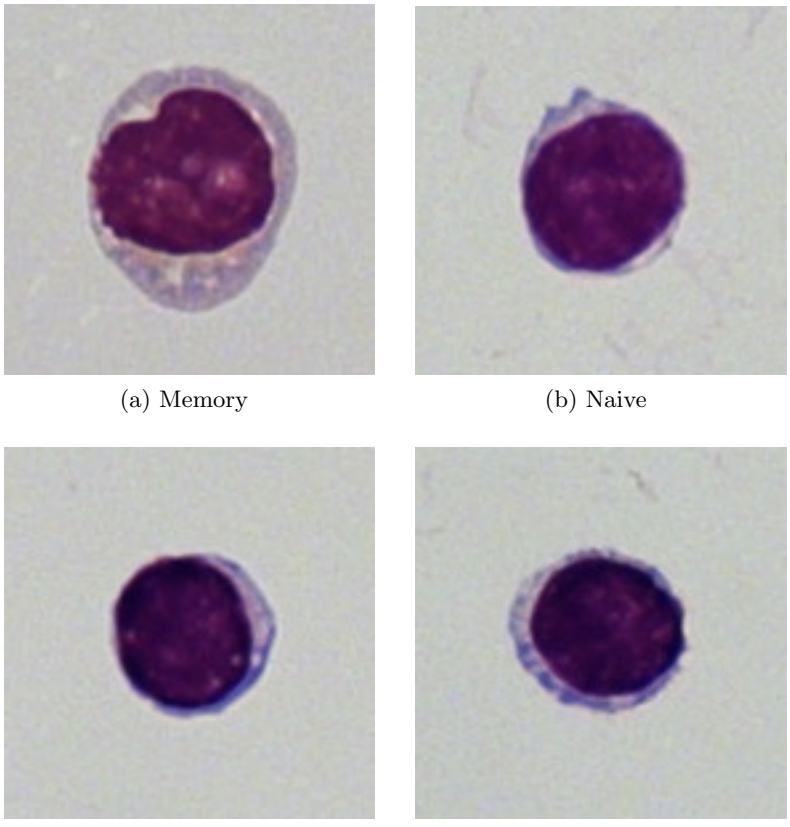
4.1.1.1 *Results*

This experiment was repeated twice using blood drawn from different donors. Both times, the centrifugation step deformed cells positioned in the periphery of the slides. Centrally placed cells had a circular shape. These cells were assessed as accurate representatives of their kind.

In both sample sets, memory B cells were found to be generally larger with larger nuclei and more cytoplasm than naive, novel and transitional B cells. In addition the color of memory B cell nuclei seem to be a bit brighter than the color of the nuclei of the other cell types. Since each cell type appeared to be homogenous in size, a typical example of each cell type is shown in Figure 14.

Among the cells sorted as novel and naive B cells a few larger cells similar to the cells sorted as memory B cells could be found. An example of such a cell is shown in Figure 15a. Note that the incidental findings shown in Figure 15 really were incidental – only a small percentage of cells with a strikingly different appearance were found.

Some of the cells sorted as transitional cells had an appearance whose counterpart couldn't be found in the other cell samples. This can be seen in Figure 15b. Both the cells and their nuclei appear to be swollen and blebby. A possible explanation of this cell morphology is that the cells might have been apoptotic.



(a) Memory (b) Naive

(c) Novel (d) Transitional

Figure 14: Since each one of the cell samples had a homogenous appearance, only one typical example of each cell type is shown. Memory B cells tend to look a bit larger with brighter nuclei and more cytoplasm than the other cell types.

4.1.2 *Method 2 - size measurements*

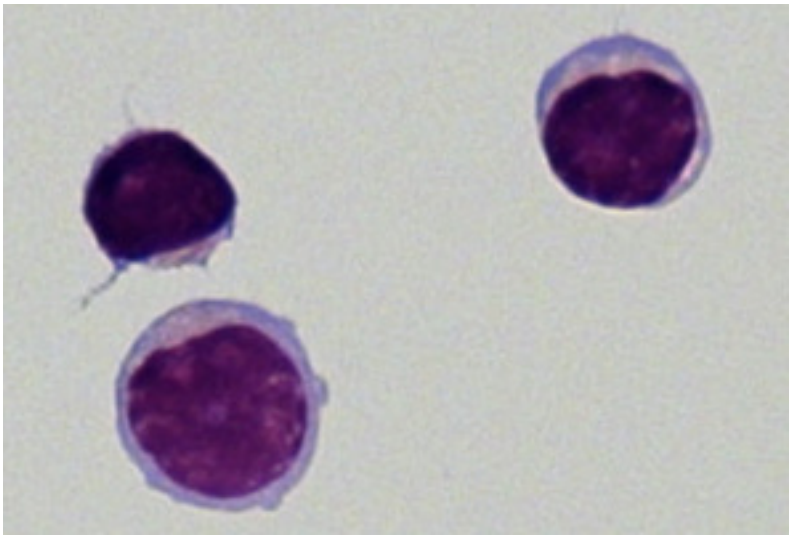
PURPOSE The method in which the cells were stained with May Grünwald/Giemsa contained a centrifugation step which placed the cells on microscope slides before the staining step. This centrifugation step made size measurements unreliable. The purpose of this experiment was to obtain as reliable size measurements as possible.

COMMENTS ON THE METHOD Since this method did not involve any staining of the cells, the centrifugation step could be replaced with a sedimentation step.

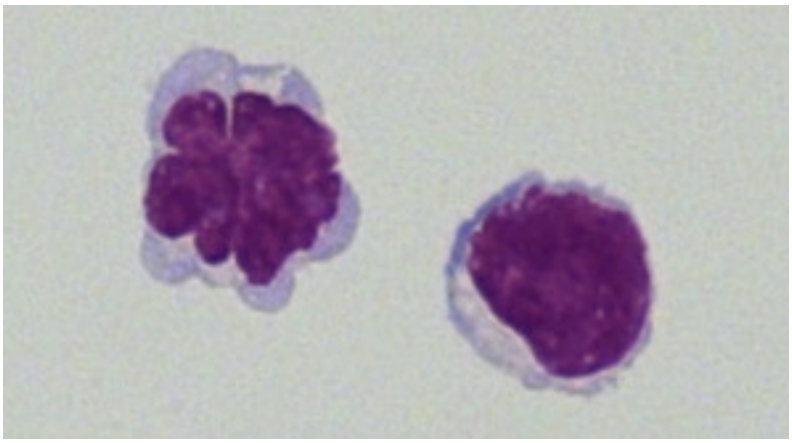
4.1.2.1 *Results*

Due to a technical failure of the camera of the microscope used for this experiment it was only performed once.

As a first analysis, obtained data sets were normalized and plotted in scatter plots. The result is shown in Figure 16. Memory



(a) A small minority of the cells sorted as novel B cells resemble memory B cells. Similar cells were also found among naive B cells. (Picture not shown.)

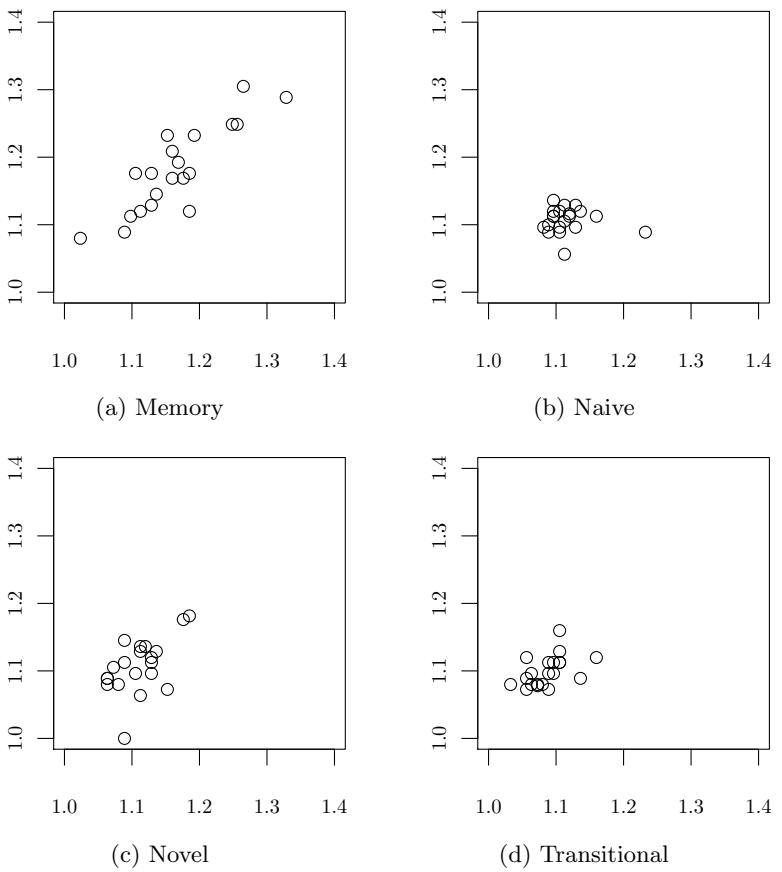


(b) Some of the cells sorted as transitional B cells had an appearance that could not be found among the other cell types. It has been speculated that these cells might be apoptotic.

Figure 15: Incidental findings among cells sorted as memory, naive, novel and transitional B cells.

B cells are shown to have larger cell size variance than the other B cell types. The obtained data sets were also used to calculate fictive cross–sectional cell areas. These dimensionless data sets are illustrated in a boxplot in Figure 17. The means and standard deviations of these data sets are shown in Table 4.

The cell sorting procedure is known to sort $CD27^-$ memory B cells with naive and novel B cells. Some plasmablasts may also be sorted with memory B cells. In general, these contaminating cells are thought to be larger than the desired cells; plasmablasts are thought to be larger than memory B cells, $CD27^-$ memory B cells are thought to be larger than naive and novel B cells. In Figure 17 the three samples in which contaminating cells might be present is shown



(a) Memory (b) Naive

(c) Novel (d) Transitional

Figure 16: Height (y) and width (x) of each data point is plotted against each other. It can be seen that memory B-cells in average are larger and vary more in size than the other B cell subpopulations.

to contain one outlier each. None of these outliers are extreme and when the scatter plots in Figure 16 are taken into account questions regarding the relevance of the outlier detection can be raised. Nevertheless, calculations aiming to determine whether cell size differs significantly between the samples or not have been performed both with and without these outliers.

The comparison of the memory and novel B cell data sets with the Welch–Shutterworth t–test shown in Table 5 results in p values indicating a significant difference in size between these cell types regardless of whether outliers are included or not. Thus, novel B cells can be considered being significantly smaller than memory B cells.

Using a one way ANOVA for comparison of several size distributions reduces the risk of Type 1 errors. This analysis was applied to the naive, novel and transitional B cell size measurement data sets to test whether they were of different sizes. The calculations resulted in non significant p values stating that no significant dif-

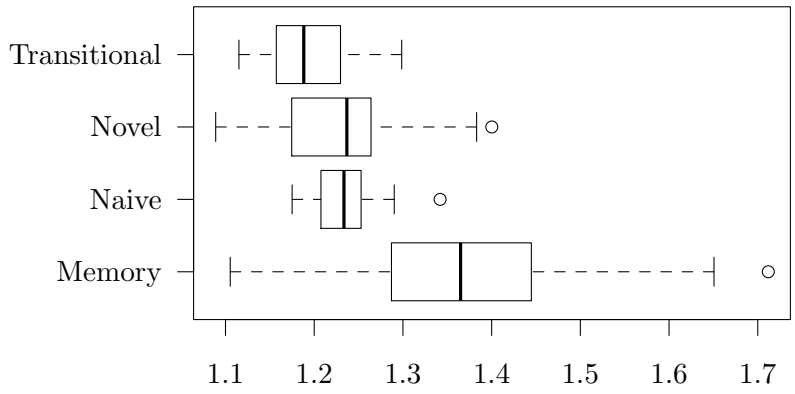


Figure 17: The measurements of height and width have been used to calculate a fictive "cell area". The presence of outliers in the memory B cell subpopulation might indicate the presence of plasmablasts in the sample. The outliers in the naive and novel B cell subpopulation might reflect the presence of $CD27^-$ memory cells.

Table 4: Sample means and standard deviations are shown for the different populations with and without outliers.

Calculated "fictive cell area"				
	With outliers		Without outliers	
Cell type	Mean	SD	Mean	SD
Memory	1.379	0.1535	1.362	0.1356
Naive	1.235	0.03989	1.230	0.03186
Novel	1.230	0.07490	1.221	0.06515
Transitional	1.195	0.04918	1.195	0.04918

Table 5: P values were calculated using the Welch–Shutterworth t–test.

Memory and novel B cell size comparison	
Outliers included	P=0.0005647
Outliers excluded	P=0.0003991

ferences in size could be found between these cell types in this experiment. The result is presented in Table 6.

These size measurement results have been confirmed in flow cytometry experiments. Using the FSC channel, memory B cells have been shown to be consistently larger than naive, novel and transitional B cells. (Data not shown.)

Table 6: A one-way ANOVA was used to compare naive, novel and transitional B cells.

Naive, novel and transitional B cell size comparison.		
	F value	P value
Outliers included	3.0603	0.05464
Outliers excluded	2.5667	0.08596

4.2 GENE CLONING

Roughly 1000 toothpicks were consumed during these experiments. Not a joke.

PURPOSE Memory B cells have been shown to have more mutations in their antibody genes than transitional and naive B cells. The purpose of these experiments was to find out whether novel B cells were more similar to naive and transitional or memory B cells.

COMMENTS ON THE METHOD Two PCR methods amplifying different regions were developed.

4.2.1 *Method 1 - cloning of the intron of the heavy chain gene*

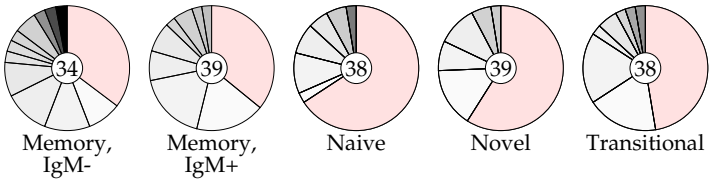


Figure 18: The results for each cell type have been pooled. The slices of the pie charts representing totally unmutated sequences have been highlighted. The number in the middle circle of each pie chart represents the number of sequences used to construct it. The level of blackness in each slice represents its relative mutation frequency.

Two thirds of the sequences cloned from the mature naive population were unmutated. In the memory B cell populations, only one third of the sequences were unmutated, suggesting that these cell populations had undergone somatic hypermutation. This suggestion is also supported by the fact that the memory B cell data sets contain the most mutated sequences. Novel B cells carry antibody genes which are about as unmutated as those of naive B cells. About half of the sequences cloned from transitional B cells were unmutated.

In Figure 19 the results for each donor is shown separately. It can be seen that the method gave reproducible results despite the fact that the total mutation frequency varied between individuals.

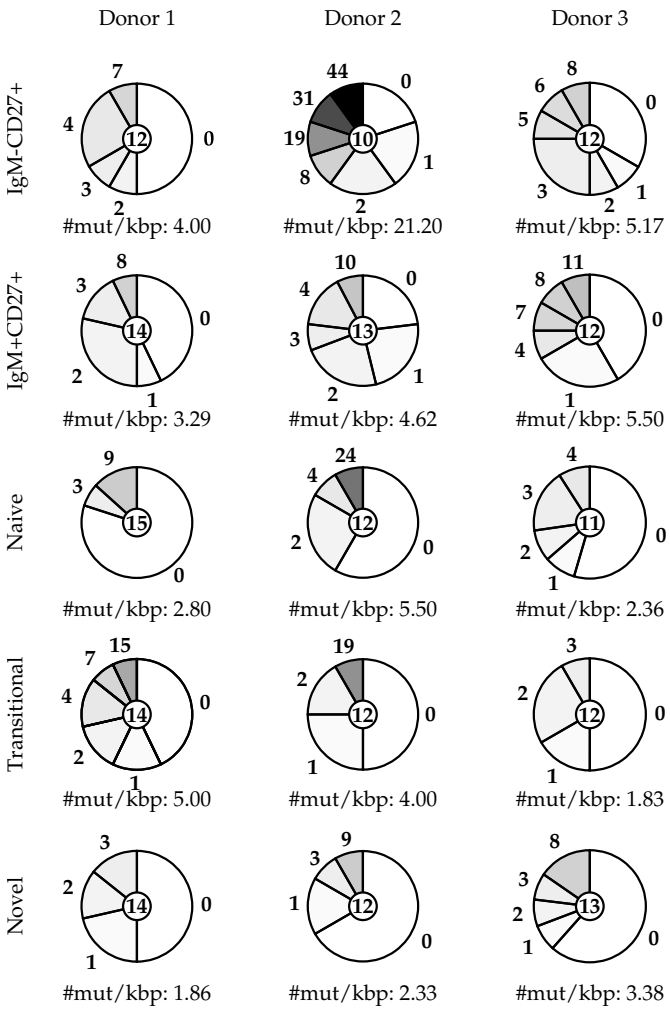


Figure 19: Results obtained using cloning method #1 shown for each donor and cell type.

Class switched memory B cells were in general more mutated than IgM^+ memory B cells which in turn were more mutated than the other cell types. Novel B cells tended to follow naive B cells.

Somatic hypermutation is known to create mutations following a certain pattern. Introduced mutations are predominantly substitutions rather than inserts or deletions. A $\leftrightarrow$ G or C $\leftrightarrow$ T transitions are more common than transversion mutations. In addition, G bases are mutated more often than C bases and A bases more often than T bases. Mutations also occur in clusters, defined by local "hotspot" genetic sequences. The tendency of mutations to occur in clusters was evident during analysis (data not shown). In Table 7 the mutation pattern is reported. The pattern is in accordance with the literature, ensuring the quality of the method [35].

As a quality control, the error rate of the DNA polymerase and its effect on the experiment was investigated. According to Finnzymes, the Phusion® Hot Start II High-Fidelity DNA Polymerase has an error rate of 4.4×10^{-7} in the provided buffer. Using their PCR

Table 7: An analysis of mutation pattern.

Cloning method #1 - analysis of mutation type

IgM$^-$ memory

From \ To	A	C	G	T	Σ
A	—	11	15	11	37
C	2	—	6	26	34
G	18	16	—	17	51
T	9	19	11	—	39
Σ	29	46	32	54	161

IgM$^+$ memory

From \ To	A	C	G	T	Σ
A	—	2	9	5	16
C	4	—	5	20	29
G	12	12	—	9	33
T	2	4	2	—	8
Σ	18	18	16	34	86

Naive

From \ To	A	C	G	T	Σ
A	—	3	6	7	16
C	1	—	6	11	18
G	12	12	—	4	28
T	1	1	3	—	5
Σ	14	16	15	22	67

Novel

From \ To	A	C	G	T	Σ
A	—	5	4	2	11
C	2	—	4	9	15
G	7	5	—	6	18
T	3	0	0	—	3
Σ	12	10	8	17	47

Transitional

From \ To	A	C	G	T	Σ
A	—	6	6	2	14
C	3	—	5	5	13
G	15	3	—	5	23
T	5	15	1	—	21
Σ	23	24	12	12	71

Summary of all

From \ To	A	C	G	T	Σ
A	—	27	40	27	94
C	12	—	26	71	109
G	64	48	—	41	153
T	20	39	17	—	76
Σ	96	114	83	139	432

fidelity calculator, the estimated percentage of PCR products having an error (i.e. DNA molecules with 1 error) was found to be 1.155% in this case. A value of this magnitude can most likely be ignored.

4.2.2 *Method 2 - cloning of the variable region*

This method has been applied on two of the three donors.

The results are visualized in Figure 20. The mutation frequency is generally higher in these sequences than in the sequences amplified using cloning method #1. Conclusions regarding the mutation frequency of IgM$^+$ memory B cells in donor 1 should probably

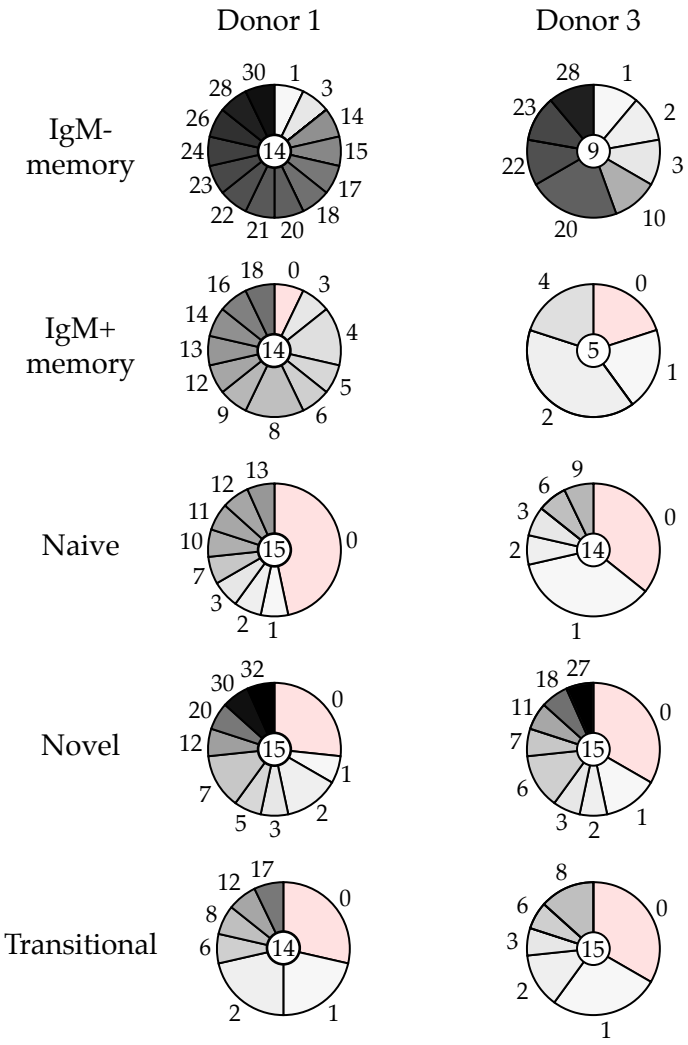


Figure 20: Summary of cloning method #2. The slices of the pie charts representing totally unmutated sequences have been highlighted. The number in the middle circle of each pie chart represents the number of sequences used to construct it. The level of blackness in each slice represents its relative mutation frequency.

not be drawn due to the low sequence count. While all sequences amplified from class switched memory B cell samples have mutations, about one third of naive, novel and transitional sequences do not. The mutation frequency of transitional B cells is similar to the level of naive and novel B cells. These results supports both the hypothesis that the novel B cell populations is an immature population and the results obtained using cloning method #1.

The mutations were summarized in a mutation type frequency table. This summary is shown in Table 8. The mutation frequencies are adequate for somatic hypermutation which indicates that the results are reliable.

Table 8: An analysis of mutation pattern.

Cloning method #2 - analysis of mutation type

	IgM$^-$CD27$^+$					IgM$^+$CD27$^+$				
From	To					To				
	A	C	G	T	Σ	A	C	G	T	Σ
A	—	32	59	27	118	—	7	12	6	25
C	16	—	27	61	104	8	—	3	16	27
G	60	37	—	19	116	28	22	—	5	55
T	8	41	9	—	58	6	13	3	—	22
Σ	84	110	95	107	396	42	42	18	27	129

	Naive					Novel				
From	To					To				
	A	C	G	T	Σ	A	C	G	T	Σ
A	—	4	11	3	18	—	13	21	20	54
C	3	—	7	5	15	9	—	13	25	47
G	18	9	—	6	33	32	31	—	10	73
T	2	14	2	—	18	7	22	7	—	36
Σ	23	27	20	14	84	48	66	41	55	210

	Transitional					Summary of all				
From	To					To				
	A	C	G	T	Σ	A	C	G	T	Σ
A	—	5	11	0	16	—	61	114	46	221
C	3	—	12	3	18	39	—	62	110	211
G	20	9	—	5	34	158	108	—	45	311
T	3	10	4	—	17	26	100	25	—	151
Σ	26	24	27	8	85	223	269	201	201	894

The genomic sequences are divided in framework regions (FWR) and complementarity determining regions (CDR). Using IgBlast, sequences can be compared against germline V_H-regions. While junctional diversity adds diversity not coded by the germline genome to CDR3 regions, the mutation frequence of FWR1, FWR2, FWR3, CDR1 and CDR2 were analyzed separately.

The CDR regions of antibody genes are coding for the protein regions responsible for direct interaction with antigens (2.3.2). Mutations in these regions have the potential to change the affinity of the antibody for its antigen dramatically. Memory B cells have been shown to accumulate amino acid replacement mutations in their CDR regions and silent mutations in their FWR regions. This

gives memory B cells a higher CDR/FWR replacement mutation ratio than naive B cells [36].

Table 9: To the left, the number of mutations per base pair is given for each cell type and donor. To the right, the CDR/FWR replacement mutation ratio is given for each cell type and donor.

FWR & CDR mutation frequency analysis				
	Mutations/kbp		CDR/FWR-ratio	
	Donor 1	Donor 3	Donor 1	Donor 3
IgM^- memory	182	139	2.2	2.1
IgM^+ memory	90	15	3.0	2.8
Naive	36	11	2.0	0.2
Novel	76	51	2.2	1.9
Transitional	36	15	2.1	0.8

Two interesting findings are visualized in Table 9. First, the total number of mutations per base pair was higher in novel B cells than in naive and transitional B cells, almost at the level of IgM^+ memory B cells. This was reproduced twice. The second finding is that the replacement CDR/FWR-ratios of naive and transitional B cells differed between donor 1 and donor 3.

Replacement mutation CDR/FWR-ratios of individual sequences were calculated in an attempt to investigate the latter finding further. These results are visualized in Figure 21. Memory B cells had a greater proportion of CDR-weighted sequences than the other cell types. Novel B cells tended to have a slightly higher proportion of CDR-weighted sequences than naive and transitional B cells. The fact that novel B cell samples are expected to contain some class switched $CD27^-$ memory B cells might explain both this finding and the finding that novel B cells had a higher total mutation frequency than naive and transitional B cells.

Finally, an effort to find amino acid replacement patterns was made. It was theorized that mutations in CDR regions might be more radical than mutations in FWR regions due to the fact that conservation of FWR regions is thought to enhance the stability of the antibody. Using different scoring systems taking changes in type (basic/acidic etc.) and size into account no obvious differences were found. This does not mean that there are no differences, but, it means that no such differences were found.

A calculation of PCR fidelity similar to the one performed for cloning method #1 estimated the percentage of PCR products having an error (i.e. DNA molecules with 1 error) to 0.7128%. This decrease in error rate in spite of the increase of number of cycles is due to the shorter length of this sequence compared to the sequence amplified in method #1.

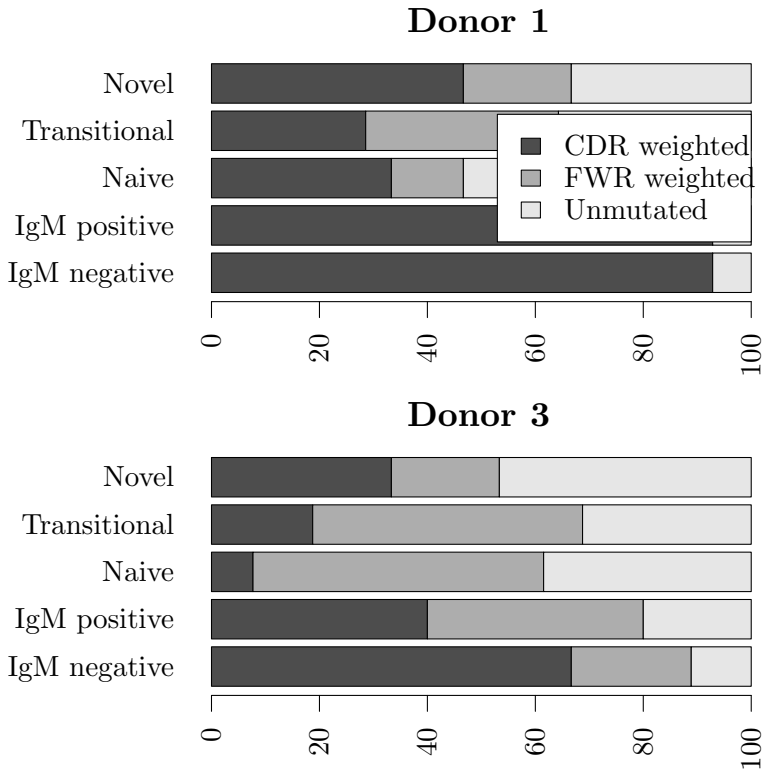


Figure 21: Replacement CDR/FWR-ratios have been calculated for individual sequences. The sequences have been classified as either unmutated (having no mutations), FWR-weighted (having more FWR than CDR replacement mutations per base pair) or CDR-weighted (having more CDR than FWR replacement mutations per base pair).

4.3 FACS CELL SURFACE MARKER SCREEN

PURPOSE The novel B cell population was once found in a screening experiment where B cells were separated in $CD27^+$ and $CD27^-$ populations. The two populations were stained with 75 different antibodies. In this experiment, the same antibodies were used to stain B cells divided in transitional, naive, novel and memory populations. The intention was to investigate whether there were any antibodies with interesting staining patterns.

COMMENTS ON THE METHOD This screening experiment was only performed once.

4.3.1 *Uniform results*

This category consists of two subcategories; antibodies staining no B cells and antibodies staining all B cells uniformly. Some findings will be closer discussed.

4.3.1.1 *Antibodies staining no B cells*

Figure 22 serves as an example of a typical negative result. In general, memory B cells have an upper tail on their graphs. It has been speculated that this finding is due to the larger size of memory B cells: a larger surface area could lead to a proportionally larger unspecific binding of antibodies.

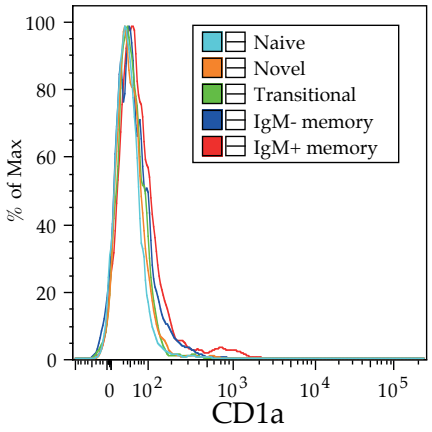


Figure 22: Expression of CD1a on IgM^- memory, IgM^+ memory, naive, novel and transitional B cells. These histograms display a frequency distribution of the data, as a function of fluorescence intensity. This is an example of a uniformly negative result. Since all negative results are very similar in appearance, no others are shown.

Antibodies that gave consistently negative results are summarized in Table 10. Most of these antibodies are known to be expressed on T cells rather than B cells. However, one antibody worth a closer look is the one against CD138. This cell surface marker is used for the identification of plasma cells. The fact that these cells did not express any CD138 shows that none of the populations contained plasma cells.

Table 10: Antibodies giving negative results.

Negative results			
CD1a	CD13	CD36	CD65
CD2	CD15	CD41a	CD66b
CD3 #1	CD16 #1	CD42b	CD71
CD3 #2	CD16 #2	CD45RO	CD95
CD4 #1	CD28	CD49b	CD103
CD4 #2	CD33	CD57	CD122
CD7 #1	CD34 #1	CD61	CD138
CD7 #2	CD34 #2	CD63	CD235

4.3.1.2 *Antibodies staining all B cells uniformly*

Antibodies with affinity for CD22, CD37 and CD59 were found to stain all cell types uniformly (Figure 23).

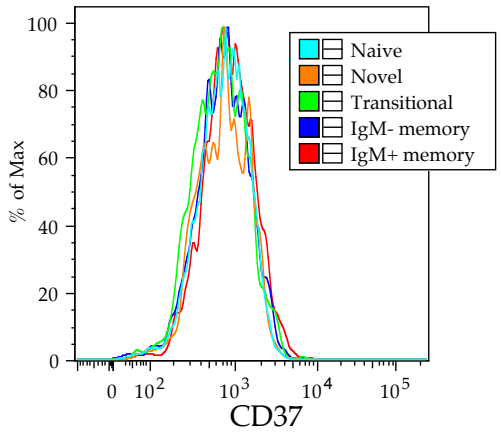


Figure 23: Expression of CD37 on IgM^- memory, IgM^+ memory, naive, novel and transitional B cells. Since the three uniformly positive results of CD22, CD37 and CD59 were very similar in appearance, the others are not shown.

4.3.2 *Non-uniform results*

These results have been assessed as interesting enough for further investigation. However, since they have not been shown to be reproducible, they should only be considered as indicative results.

4.3.2.1 *Transitional B cells stand out*

Transitional B cells are known to express CD5 [14]. As expected, only transitional B cells were shown to express CD5 in this experiment (Figure 24). However, late transitional B cells (T3) have been shown to downregulate their expression of CD5 [16]. Thus, this finding excludes that the novel B cell population is an early transitional B cell.

4.3.2.2 *IgM^+ memory B cells stand out*

IgM^+ memory B cells had the highest expression of CD6 and CD9 (Figure 25 and 26). Both markers separated them from class switched memory B cells.

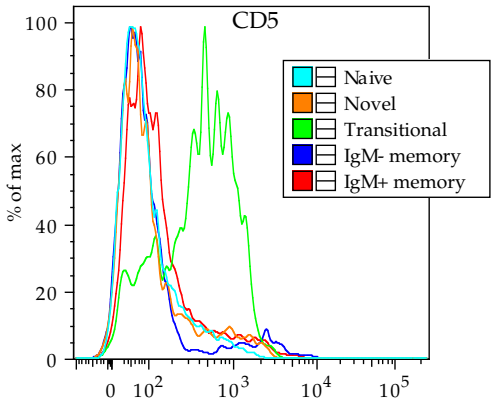


Figure 24: Expression of CD5 on IgM$^-$ memory, IgM$^+$ memory, naive, novel and transitional B cells.

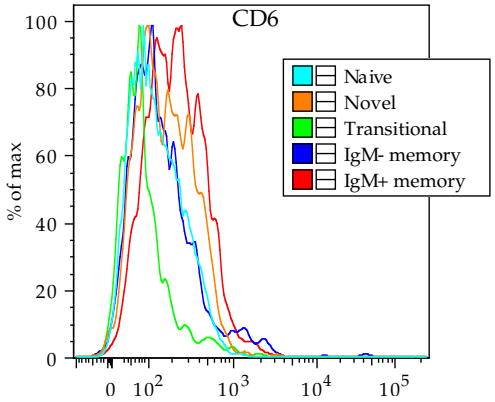


Figure 25: Expression of CD6 on IgM$^-$ memory, IgM$^+$ memory, naive, novel and transitional B cells. While transitional B cells hardly expressed any CD6 at all, IgM$^+$ memory B cells and novel B cells seemed to have a higher expression of this cell surface marker than IgM$^-$ memory B cells and naive B cells.

4.3.2.3 *IgM$^+$ & IgM$^-$ memory B cells stand out*

The expression of CD8, CD11b, CD14 and CD80 was higher in both IgM$^+$ and IgM$^-$ memory B cells than in the other peripheral B cell types (Figure 27-30).

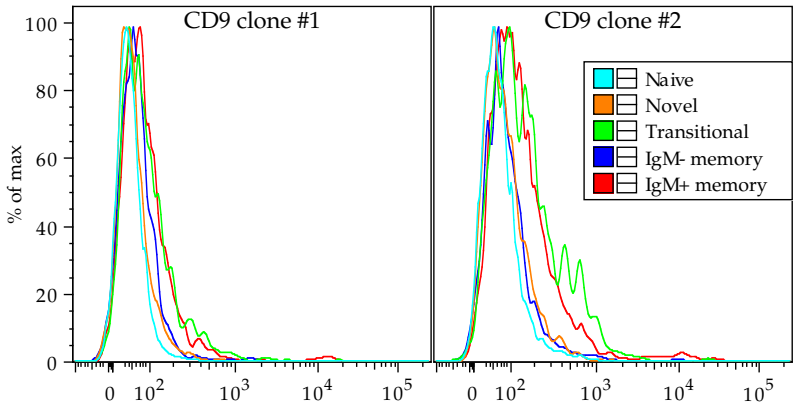


Figure 26: Expression of CD9 measured with two antibody clones on IgM$^-$ memory, IgM$^+$ memory, naive, novel and transitional B cells. The expression of CD9 seem to be slightly higher in IgM$^+$ memory B cells and transitional B cells than in the other peripheral B cell types. Two different antibody clones were used, showing the same staining pattern.

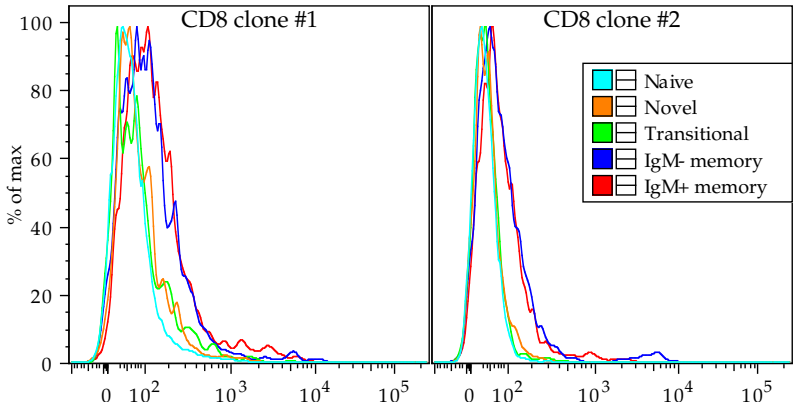


Figure 27: Expression of CD8 measured with two antibody clones on IgM$^-$ memory, IgM$^+$ memory, naive, novel and transitional B cells.

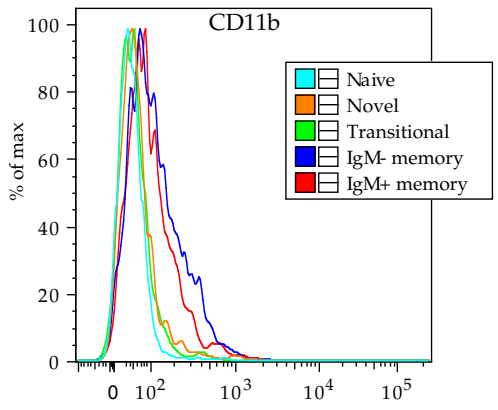


Figure 28: Expression of CD11b on IgM$^-$ memory, IgM$^+$ memory, naive, novel and transitional B cells.

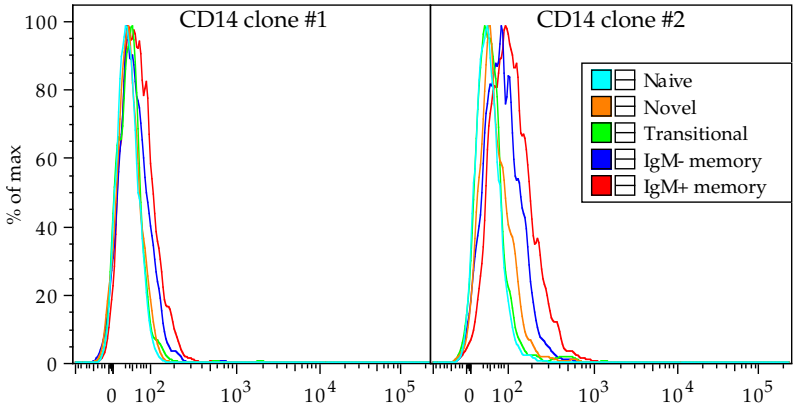


Figure 29: Expression of CD14 on IgM$^-$ memory, IgM$^+$ memory, naive, novel and transitional B cells.

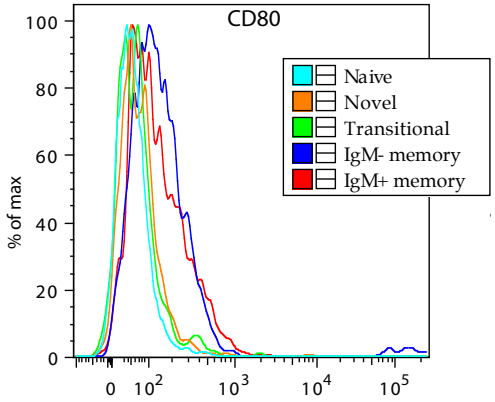


Figure 30: Expression of CD80 on IgM$^-$ memory, IgM$^+$ memory, naive, novel and transitional B cells. The higher expression of CD80 on memory B cells than on the other peripheral B cell types is somewhat expected, since CD80 is used as a memory B cell marker in mice. This cell surface marker has also been shown to be expressed on some human B cell cancers.

4.3.2.4 *The novel B cell population is found "in between"*

The expression of CD11a, CD18, CD21, CD25, CD29, CD35, CD44, CD45RA, CD54 and CD66acde turned out to be higher in novel B cells than in transitional and naive B cells (31-40). However, in most cases both IgM^+ and IgM^- memory B cells had a higher expression.

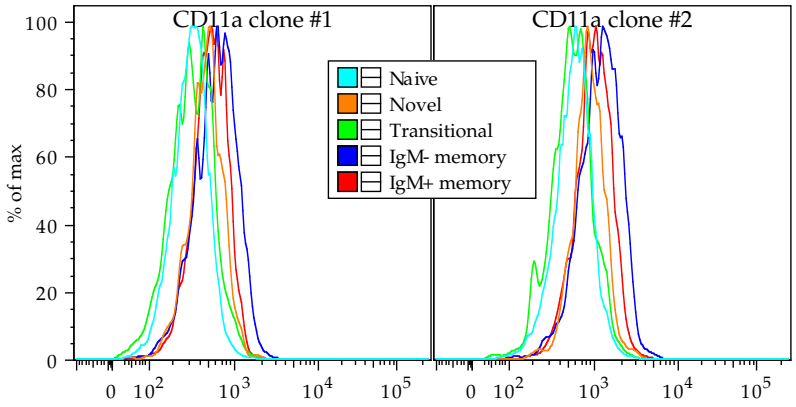


Figure 31: Expression of CD11a clone #1 and clone #2 on IgM^- memory, IgM^+ memory, naive, novel and transitional B cells.

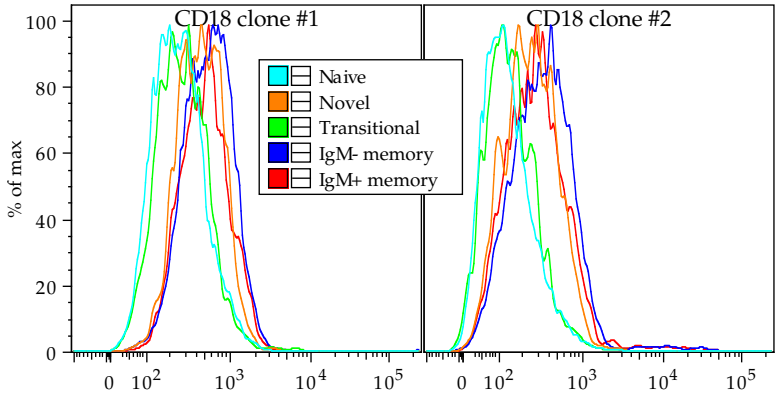


Figure 32: Expression of CD18 clone #1 and clone #2 on IgM^- memory, IgM^+ memory, naive, novel and transitional B cells.

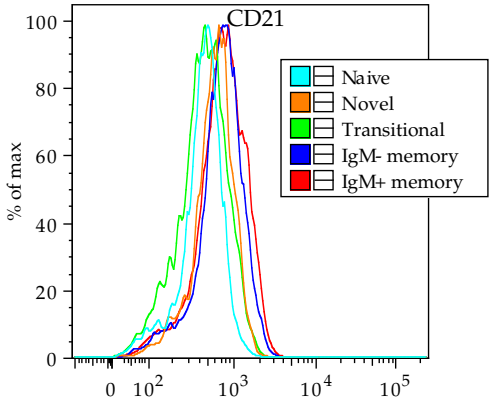


Figure 33: Expression of CD21 on IgM$^-$ memory, IgM$^+$ memory, naive, novel and transitional B cells.

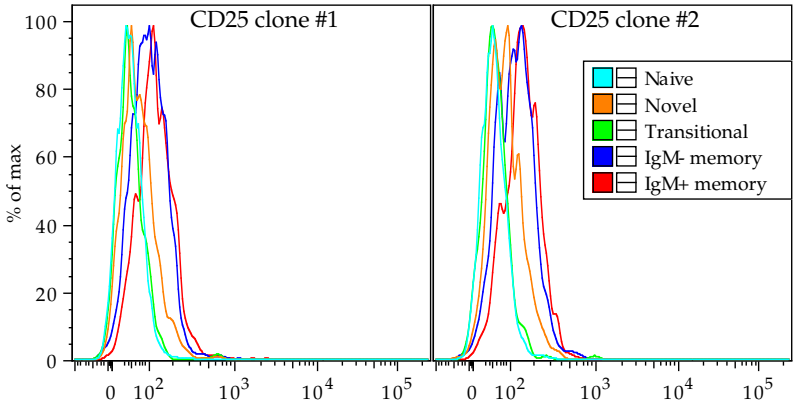


Figure 34: Expression of CD25 clone #1 and clone #2 on IgM$^-$ memory, IgM$^+$ memory, naive, novel and transitional B cells.

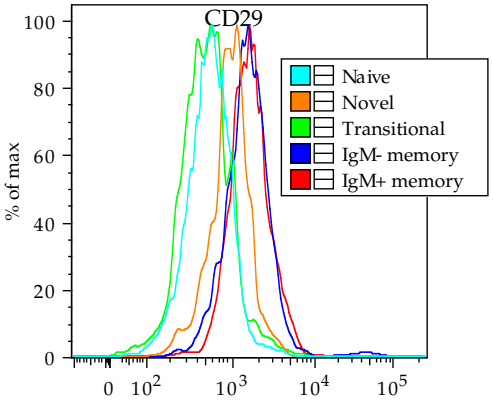


Figure 35: Expression of CD29 on IgM$^-$ memory, IgM$^+$ memory, naive, novel and transitional B cells.

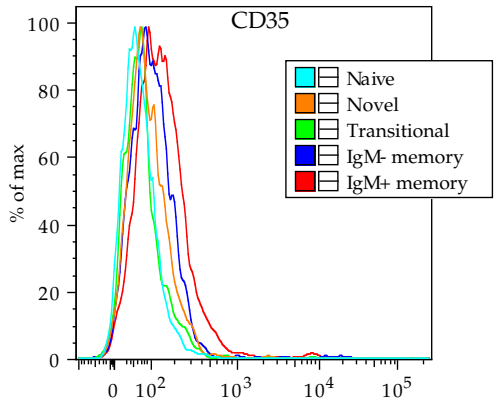


Figure 36: Expression of CD35 on IgM$^-$ memory, IgM$^+$ memory, naive, novel and transitional B cells.

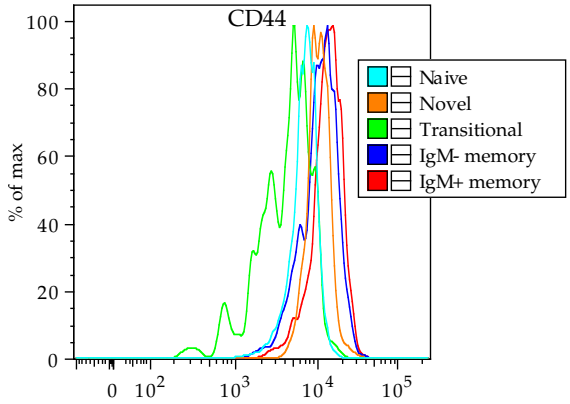


Figure 37: Expression of CD44 on IgM$^-$ memory, IgM$^+$ memory, naive, novel and transitional B cells.

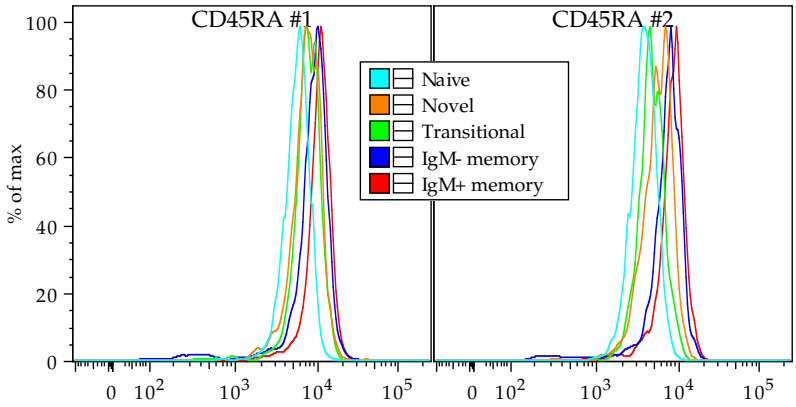


Figure 38: Expression of CD25 clone #1 and clone #2 on IgM$^-$ memory, IgM$^+$ memory, naive, novel and transitional B cells.

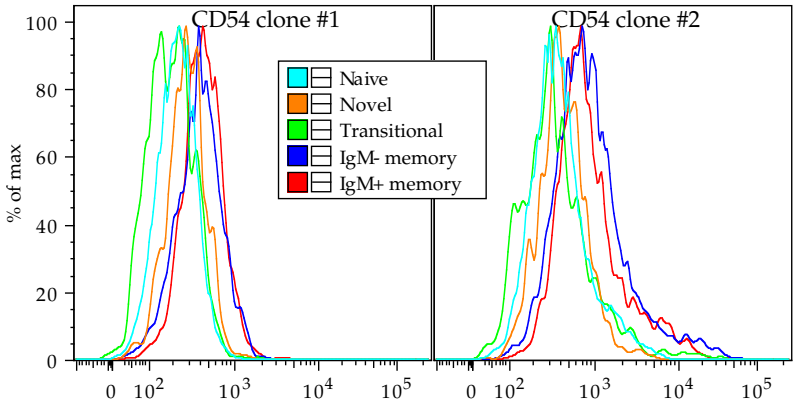


Figure 39: Expression of CD54 clone #1 and clone #2 on IgM^- memory, IgM^+ memory, naive, novel and transitional B cells.

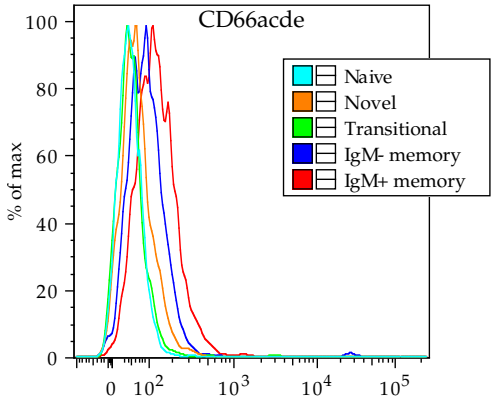


Figure 40: Expression of CD66acde on IgM^- memory, IgM^+ memory, naive, novel and transitional B cells.

4.3.2.5 *Special Cases*

Cases that were impossible to place in any of the categories above have been placed in this subsection. They are be individually discussed in the legends of Figures 41-45.

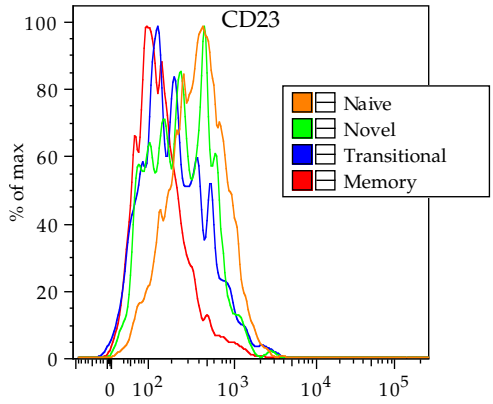


Figure 41: Expression of CD23 on memory, naive, novel and transitional B cells. No marker for differentiation of IgM^- and IgM^+ memory B cells were used. Memory B cells and transitional B cells had a lower expression of CD23 than naive B cells. Novel B cells seemed to have a broader expression interval than the other B cell types.

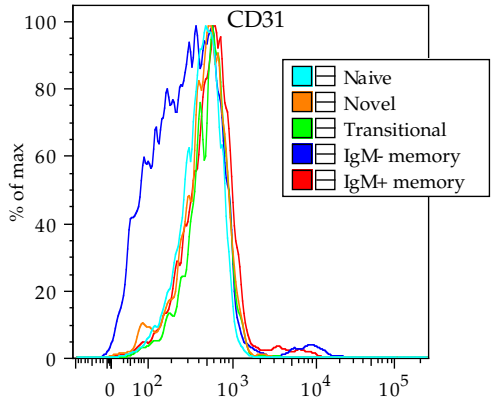


Figure 42: Expression of CD31 on IgM^- memory, IgM^+ memory, naive, novel and transitional B cells. IgM^- memory B cells seemed to have a lower expression of CD31 than the other cell types.

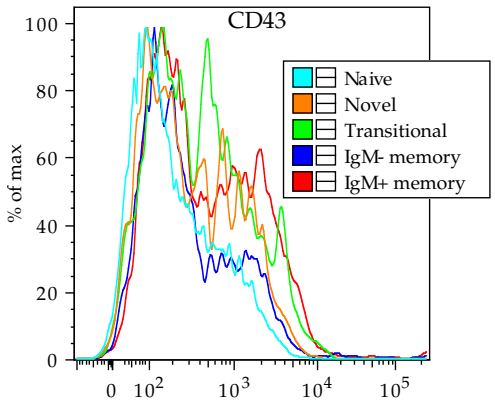


Figure 43: Expression of CD43 IgM^- memory, IgM^+ memory, naive, novel and transitional B cells. All peripheral B cell types could be divided in $CD43^{low}$ and $CD43^{high}$ populations. Novel and IgM^+ memory B cells had a similar expression, as did naive and transitional B cells. IgM^- memory B cells had the lowest number of $CD43^{high}$ cells.

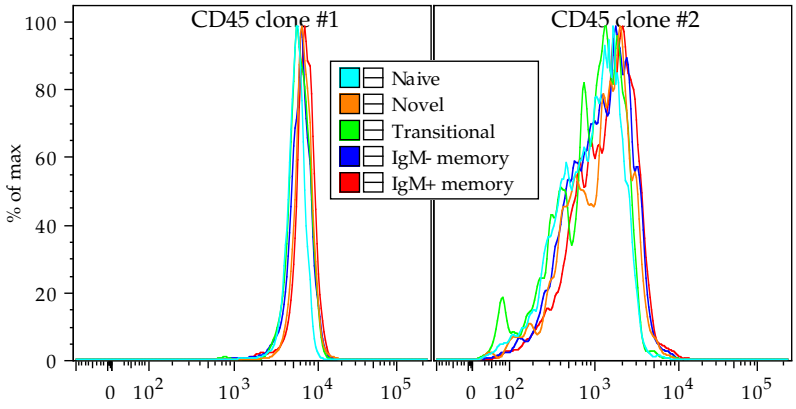


Figure 44: Expression of CD45 clone #1 and clone #2 on IgM^- memory, IgM^+ memory, naive, novel and transitional B cells. Two antibodies directed against CD45 were used. Each one stained B cells uniformly but in different ways.

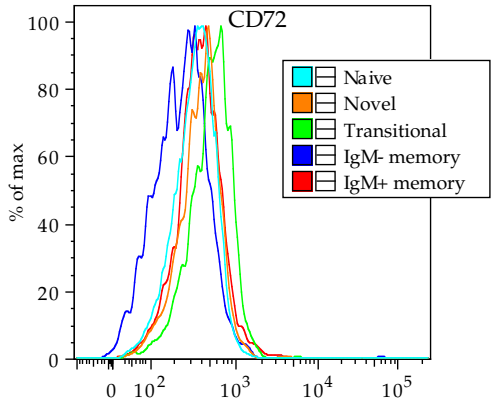


Figure 45: Expression of CD72 IgM^- memory, IgM^+ memory, naive, novel and transitional B cells. Transitional B cells had the highest expression of CD72 while IgM^- memory B cells had the lowest. The other cell types were all somewhere in between.

4.4 FUNCTIONAL ASPECTS

4.4.1 *B cell receptor stimulation and intracellular [Ca^{2+}]*

PURPOSE Increases in [Ca^{2+}] upon BCR stimulation have been shown to be smaller in early transitional B cells than in T3 late transitional and naive B cells [16, 17]. The intention of this experiment was to compare the novel B cell population to the other peripheral B cell types using an approach based on these articles.

COMMENTS ON THE METHOD No results were obtained.

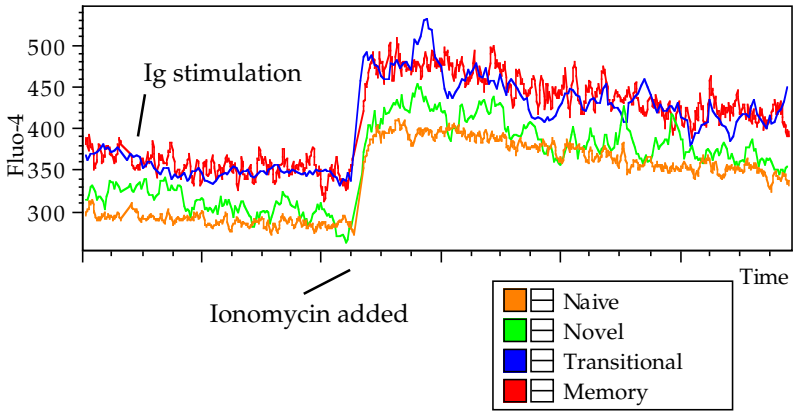


Figure 46: Ig stimulation was added after 30 seconds. Ionomycin was added after about two minutes.

While the addition of ionomycin gave the expected rise in fluorescence intensity and could be considered as a functioning positive control, Ig stimulation did not. Thus, no results.

4.4.2 *Cell stimulation*

PURPOSE Transitional B cells are known to die faster in vitro than the other peripheral B cell types. A survival of novel B cells matching the one of naive B cells would rule out the possibility of novel B cells being late transitional B cells.

COMMENTS ON THE METHOD Three different methods were used to stain apoptotic cells. They all gave similar results.

4.4.2.1 *Apoptosis experiment #1*

Transitional B cells were shown to become apoptotic and necrotic faster than the other cell types. However, finding out whether cells were alive, apoptotic or necrotic using the Annexin/7-AAD-staining turned out to be hard. Since these results were unclear they are not shown here.

When only those cells which were assessed as being healthy by the Annexin/7-AAD-staining were observed, novel B cells seemed to start expressing CD27. This is illustrated in Figure 47. This has been seen in other experiments too, however, since none of these experiments aimed to investigate this particular subject, no reliable and reproduced results have been obtained.

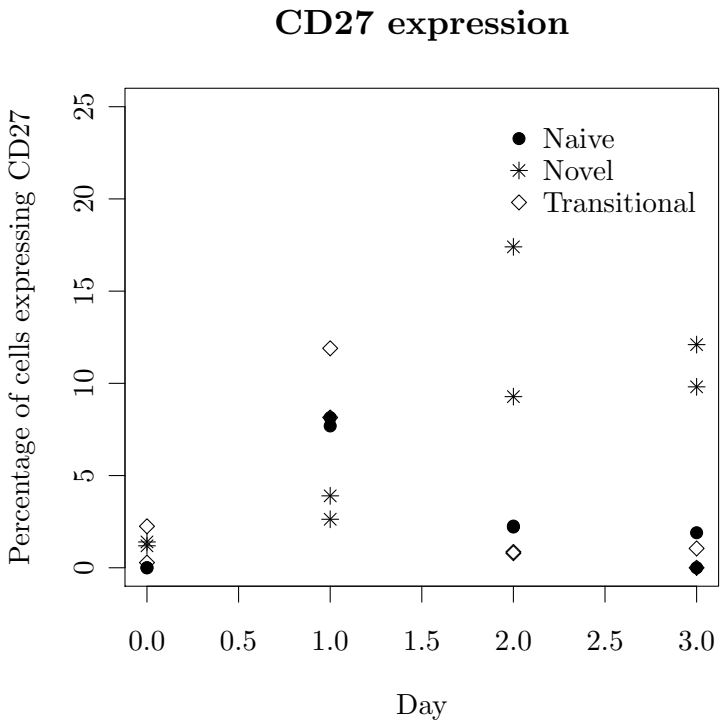


Figure 47: Percentage of non-apoptotic and non-necrotic cells expressing CD27 at each evaluation point as determined by Annexin/7-AAD-staining. Memory B cells have been excluded due to their inherent CD27 expression.

4.4.2.2 *Apoptosis experiment #2*

In Figure 48, the fraction of each cell sample being stained as alive by the Vybrant® DyeCycle™ Violet/SYTOX® AADvanced™ Apoptosis Kit (Invitrogen) is shown for all days and cell types. Transitional B cells were shown to die the fastest.

4.4.2.3 *Apoptosis experiment #3*

In Figure 49, the acquired samples are plotted against the day of harvesting. Transitional B cells died most rapidly. Memory B cells tended to have a slightly better survival. Naive and novel B cells showed similar survival.

In general, larger proportions of the cell samples were stained as apoptotic in this experiment than in experiment #2. (Data not

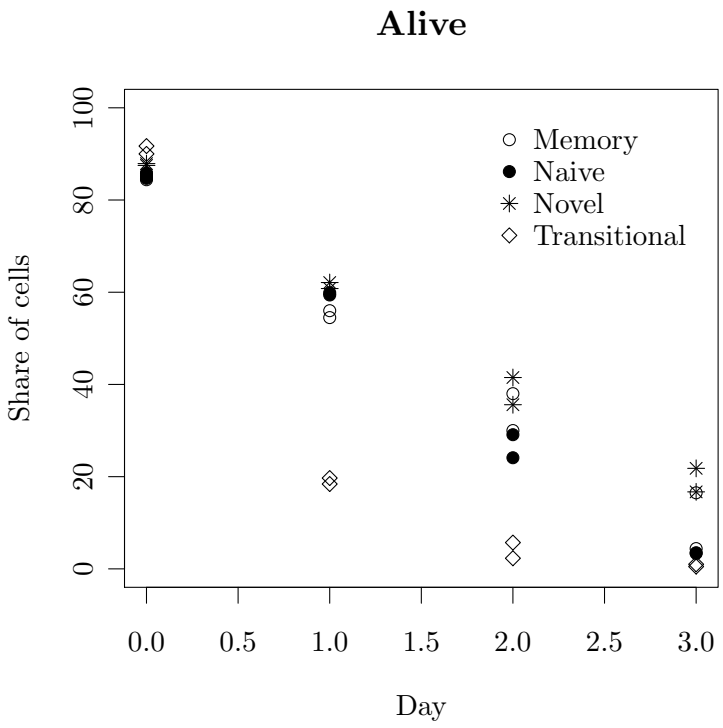


Figure 48: The proportion of the cells being alive at certain times. Alive, apoptotic and necrotic cells were identified using the Vybrant® DyeCycle™ Violet/SYTOX® AADvanced™ Apoptosis Kit.

shown.) This effect might be caused by the fact that another kit, the Violet Ratiometric Membrane Asymmetry Probe/Dead Cell Apoptosis Kit (Invitrogen), was used for identification of apoptotic and necrotic cells in this experiment. Another difference between these experiments was the use of negative selection of B cells instead of positive selection as in experiment #2.

4.4.2.4 *Proliferation and differentiation*

PURPOSE Certain substances are known to induce proliferation and differentiation in B cells. CpG is, as an example, known to induce proliferation in memory B cells. The purpose of this experiment was to investigate the behavior of novel B cells in comparison with transitional, naive and memory B cells.

COMMENTS ON THE METHOD Only a few screening experiments were made. Protocols were find in different articles [13, 16, 17, 18, 32, 33, 34].

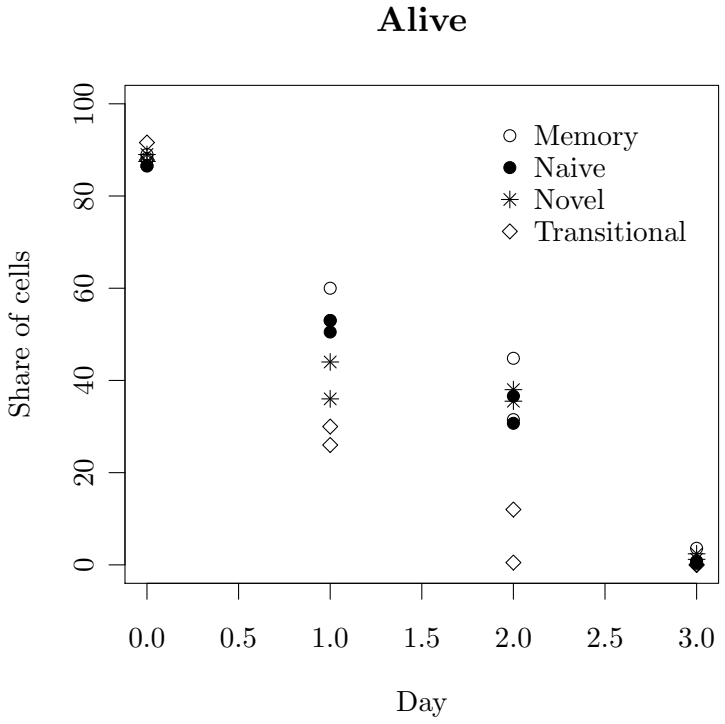


Figure 49: The proportion of the cells being either alive or apoptic at certain times. Alive, apoptotic and necrotic cells were identified using the Violet Ratiometric Membrane Asymmetry Probe/Dead Cell Apoptosis Kit.

No proliferation was induced by R848, R848 + IL-2, BAFF, CD40L or IL-4. CpG and CpG + IL-2 induced proliferation in memory B cells only.

The expression of CD27 was investigated in non-apoptotic and non-necrotic naive, novel and transitional B cell samples. The only samples in which a CD27 expression could be found were novel B cell samples supplemented with CpG or CpG+IL-2. In novel B cell samples supplemented with CpG, 20% och 34.5% of the cells stained positively for CD27. In novel B cell samples supplemented with CpG+IL-2, 38.7% and 60.8% stained positively for CD27.

5 DISCUSSION

5.1 LIGHT MICROSCOPY

5.1.1 *Method 1 - May Grünwald/Giemsa*

Among the naive and novel B cells a few cells more similar to memory B cells were found. These cells were not found in transitional B cell samples. As was mentioned in 2.4.3, an IgM^-CD27^- memory B cell exists. The staining protocol used before cell sorting is known to sort IgM^-CD27^- memory B cells in naive and novel B cell samples. This makes evaluation of the quality of these samples an important task. Since IgM^-CD27^- memory B cells have been shown to be morphologically similar to class switched memory B cells, the few larger cells found in the naive and novel B cell samples are likely to be IgM^-CD27^- memory B cells [21]. The fact that only few cells of this kind were seen in the samples makes it tempting to suggest that they were unlikely to interfere.

In vitro, transitional B cells are known to enter apoptosis much faster than the other peripheral B cell types [13]. The swollen and blebby cells found among transitional B cells have been speculated to be apoptotic cells. The fact that no similar cells were found in novel B cell samples suggest that novel B cells might survive better in vitro than transitional B cells. However, this speculation does not prove this.

5.1.2 *Method 2 - size measurement*

The aim of this method was to obtain more reliable size data than the one obtained using the May Grünwald/Giemsa-staining approach. The replacement of the centrifugation step with a sedimentation step affected the cells less but made staining impossible. Since photos of unstained cells could be obtained, this was not a problem. For each cell type, size calculations were based on size measurements of a set of 20 randomly chosen cells. This is a less arbitrary approach than the one which was used for picking a cell ”typical” for its kind.

Both the naive and the novel B cell sample contained outliers in the memory B cell size range. These might be IgM^-CD27^- memory B cells. Since these cells were shown to be few, these results strengthen the claim that IgM^-CD27^- memory B cells were unlikely to be able to interfere in other experiments.

5.2 GENE CLONING

The results presented here raise a few questions. On the one hand, the novel B cell population has been shown to carry more unmutated sequences and fewer total mutations than memory B cells. In this way, this population is more similar to the immature transitional and the mature naive populations. On the other hand, the mutations in novel B cell sequences have been shown to exhibit a pattern slightly more similar to that of memory B cells than transitional or naive B cells.

5.2.1 *Method 1 - cloning of the intron of the heavy chain gene*

The only real question mark among the results obtained using this method was the tendency of transitional B cells to have a mutation frequency higher than both naive and novel B cells. This tendency was seen in all three experiments. The share of unmutated sequences were smaller in transitional samples than in naive and novel samples. In addition, two of the experiments contained one highly mutated sequence each. Since transitional B cells are known to be prone to enter apoptosis in vitro, it was speculated that some of these mutations might have occured in apoptotic cells. However, Table 7 does not suggest that these mutations follow any other pattern than that of somatic hypermutations. Another possible explanation is that there might be an early expression of AID (one of the enzymes responsible for somatic hypermutation, as discussed in 2.4.5.2) in transitional B cells. Still, this does not explain the fact that naive B cells seem to carry less mutated genes than transitional B cells in these experiments. Yet another explanation would be that some of these transitional B cells later evolve into a B cell subpopulation corresponding to the B1 cells in mice. However, that discussion is beyond the scope of this thesis.

In the experiment performed on Donor 1, transitional B cell sequences seemed to carry antibody genes even more mutated than the ones found in class switched memory B cells. It cannot be excluded that these samples might have been mixed up.

The occasional highly mutated sequences among naive and novel B cells might originate from class switched $CD27^-$ memory B cells.

5.2.2 *Method 2 - cloning the variable region*

The results obtained using this method indicate that novel B cells have a higher mutation frequency than naive and transitional B cells. It has also been shown that novel B cells tend to accumulate mutations in their CDR regions. This can be explained in two different ways:

1. A contamination of novel B cell samples with IgM$^-$CD27$^-$ memory B cells could result both in a higher total mutation frequency and in a higher share of CDR-weighted sequencies.

2. Novel B cells might be undergoing somatic hypermutation.

Before final conclusions can be drawn, the mutation pattern of novel B cells should be investigated also in Donor 2. In order to investigate whether IgM$^-$CD27$^-$ memory B cells are contaminating the samples or not, a sample from a fourth donor should be obtained. It is possible to remove these memory B cells during the cell sorting provided that the cells are stained with antibodies against IgM.

Sequences from memory B cells, especially IgM$^+$ from Donor 3, were consistently hard to amplify. The V_H repertoire is known to be affected during B cell development. A diversity is created by V(D)J-recombination, but, not all B cells are selected to become memory B cells. It has been suggested that the class switched memory B cell V_H repertoire differs from the V_H repertoire of IgM$^+$ memory B cells [37]. With this in mind the failure to amplify sequences from the IgM$^+$ memory B cell sample does not have to mean that the method is dysfunctional. It might rather indicate that the PCR reaction is amplifying only a fraction of the existing V_H sequences, and that B cells carrying these sequences are not selected to become IgM$^+$ memory B cells.

5.3 qRT-PCR

Results obtained using this approach would complement the work of this thesis in an excellent way. Also, a working method could easily be extended to investigate the expression of other genes. Therefore, I feel sad that this did not work out.

Despite numerous attempts using different methods, purification of RNA from B cells failed. In general, novel and transitional B cell samples gave the worst results. The reason for this was probably that these cell samples were small, 100 000 cells at best but often <40 000 cells. The amount of naive B cells that were sorted at the same time were often 10-15 times higher.

Nevertheless, it was hard to amplify RNA also from naive cells. The fact that these cells are known to be inactive and have low RNA expression might explain this. Another reason might be that most of the selected genes code for transcription factors which probably are expressed in minute amounts.

RNA preparation did work once. However, the RNA seemed to degrade a little more during each freeze-thaw cycle before new repetitions of the same qRT-PCR experiment, rendering the final results unreproducible and useless.

5.4 FACS cell surface marker screen

Since it has been argued that the novel B cell population might contain IgM$^-$CD27$^-$ memory B cells, the expression of IgM in the cells gated as novel B cells was investigated. A portion of

the population did indeed have a lower IgM expression, however, no obvious division of the novel B cell population in IgM^- and IgM^+ cells could be performed. For some samples, the novel B cell population was gated as containing more or less of the cells with a lower IgM expression. When the expression of the cell surface marker currently screened for was determined in these truncated novel B cell samples and compared with the expression in the original not truncated population, no differences were seen. This finding was interpreted as indicating that the population gated as novel B cells was a homogeneous population.

For some surface markers, the novel B cell population is shown to have an expression similar to the one of naive and transitional B cells. For ten of the surface markers, the expression is higher in novel B cells than in naive and transitional B cells but lower than in memory B cells. For two surface markers, CD18 and CD43, novel B cells have an expression similar to that of IgM^+ memory B cells. However, novel B cells do not seem to share any similarities with IgM^- memory B cells.

Since this experiment has been performed only once, the results have not been shown to be reproducible. Nevertheless, the high CD5 expression of transitional B cells and the fact that antibody clones give similar results strengthen the results. It is interesting that novel B cells seem to exhibit a cell surface marker expression pattern intermediate to that of the already known cell types. Further experiments where other antibodies with stronger fluorochromes are used will tell whether these initial findings are for real or not.

5.5 FUNCTIONAL ASPECTS

5.5.1 *BCR stimulation and intracellular $[Ca^{2+}]$*

Binding of monoclonal antibodies to CD19 or CD20 is known to affect calcium signaling. In spite of this, an antibody against CD20 has been used for identification of B cells in the literature. It is not known whether binding of the monoclonal antibody $CD45RB^{MEM\text{-}55}$ affects calcium signaling or not. However, since this experiment was aimed at finding differences between the tested cell types, results might be useful in spite of the possible influence the experiment conditions might have on the outcome. At the time of writing, it is not known why this experiment did not work. As soon as reasonable ideas regarding method changes arise, new attempts can be made.

5.5.2 *Cell stimulation*

5.5.2.1 *Apoptosis*

Three experiments with different kits for identification of alive, apoptotic and necrotic cells were performed. The obtained results were consistent. This suggests that the results are reliable.

The hypothesis during this thesis has been that the novel B cell population is an immature peripheral B cell population. It was speculated that it might equal the T3 transitional B cell population [16]. After the three apoptosis experiments performed during this thesis, there is no doubt that transitional B cells die faster in vitro than the other cell types. While memory B cells seem to have slightly better survival than naive and novel B cells, it was not possible to sort out whether novel B cells have better survival than naive B cells or not. However, nothing is indicating that novel B cells have worse in vitro survival than naive B cells. Since the T3 transitional population is known to have worse in vitro survival than naive B cells, in this aspect novel B cells differ from T3 transitional B cells.

5.5.2.2 *Proliferation and differentiation*

The only stimulant that has been shown to affect proliferation of B cells is CpG. The only cell types in which proliferation was induced were IgM^+ and IgM^- memory B cells. But, in the only experiment in which the expression of CD27 was evaluated after stimulation with CpG, novel B cells started to express CD27. This was not seen in naive or transitional B cells. Novel B cells were also found to express CD27 in one of the apoptosis experiments. Since contamination of novel B cell samples with class switched $CD27^-$ memory B cells could induce similar results, further experiments are needed in order to find out whether the induction of CD27 expression is an inherent and reproducible feature of novel B cells or not.

Part IV

CLOSING THE LOOP

6 CONCLUSION

6.1 REJECTION OF THE INITIAL HYPOTHESIS

Initially, it was not possible to exclude that novel B cells were late transitional B cells. The small size of novel B cells suggests that they are indeed relatively young. However, since novel B cells show in vitro survival matching the one of naive B cells, it can be concluded that they have already passed the transitional stage of development [16].

6.2 FORMATION OF A NEW HYPOTHESIS

It is not possible to form a reliable and exact hypothesis regarding the functions of the novel B cell population from the results presented in this thesis report. It is however possible to suggest a direction for further experimentation.

6.2.1 *Hypothesis #1 - IgM^+CD27^+ memory B cell precursor*

The origin of IgM^+CD27^+ memory B cells is still unknown. Different origins have been suggested. As of now, it cannot be excluded that it is a heterogenous population with several origins. The results presented in this thesis suggest that the novel B cell population might be a precursor to IgM^+CD27^+ memory B cells.

IN VITRO SURVIVAL The in vitro survival of the novel B cell population matches the one of naive B cells. This indicates that the novel B cell population is past the transitional stage.

SIZE The size of novel B cells is similar to transitional and naive B cells. The fact that it is an early peripheral population suggests that it might have potential for further differentiation.

IG-GENE MUTATIONS The immunoglobulin genes of novel B cells are less mutated than those of class switched memory B cells but more mutated than those of naive and transitional B cells, almost matching the mutation frequency of IgM^+CD27^+ memory B cells. Furthermore, one of the cloning experiments indicate that novel B cells accumulate mutations in their CDR regions, an expected feature of memory B cells.

CELL SURFACE MARKERS The expression of some cell surface markers seem to place novel B cells between on the one

hand transitional and naive B cells and on the other hand memory B cells. This might suggest that the population is differentiating.

EXPRESSION OF CD27 In several independent experiments, novel B cells have been found to express CD27 after a couple of days of in vitro-stimulation, giving them a phenotype similar to the one of IgM^+CD27^+ memory B cells.

EXPRESSION OF CD45RB^{MEM-55} All human memory B cells express CD45RB^{MEM-55}. The fact that novel B cells are the only human peripheral non-memory B-cells expressing this cell surface marker is what once led to the discovery of them. In this aspect, they are more similar to memory B cells than naive and transitional B-cells.

6.2.2 *Hypothesis #2 - IgM^-CD27^- memory B cell contamination*

The total number of mutations per base pair is higher in novel B cells than in naive and transitional B cells. However, when the results from the experiments in which the immunoglobulin gene is cloned is analyzed more closely, a few heavily mutated sequences is seen to increase the average mutation frequency of novel B cells. These sequences might come from contaminating IgM^-CD27^- memory B cells. This weakens the hypothesis of novel B cells being a precursor of IgM^+CD27^+ memory B cells.

Memory B cells are known to proliferate when stimulated with CpG. Very little is known about IgM^-CD27^- memory B cells. Novel B cells are thought to express CD27 in in vitro-stimulations. It cannot be excluded that this CD27-expression originate from a minute amount of proliferating and differentiating IgM^-CD27^- memory B cells.

6.3 THE FINAL CONCLUSION

The novel B cell population seems to be a unique human peripheral B cell population different from the already described populations which have served as control populations. However, in order to fully understand the role of these cells, further experiments are needed.

7 FUTURE WORK

7.1 SHORT TERM

7.1.1 *Light microscopy*

No further experiments are needed.

7.1.2 *Gene cloning*

1. Cloning method #2, cloning of the variable region, has to be applied on samples from Donor 2.
2. A new DNA sample in which all IgM^- memory B cells are removed in the cell sorting process should be prepared. Cloning method #2 should be applied on this sample.

7.1.3 *qRT-PCR*

This method has potential to reveal high impact information. If, for example, novel B cells could be shown to express AID, this would strengthen the possible claim that novel B cells accumulate mutations in their CDR regions. However, since it is easier to make other experiments work, making this particular experiment work is not first priority.

7.1.4 *FACS cell surface marker screen*

The interesting findings have to be shown to be reproducible, preferably with new antibodies with stronger fluorochromes. Examination of the expression of cell surface markers not included in this screen might give interesting results.

7.1.5 *Functional aspects*

7.1.5.1 *BCR Stimulation and Intracellular $[Ca^{2+}]$*

New attempts to make this experiment work should be made since it has potential of giving results supporting the claim that the novel B cell differs from memory, naive and transitional B cells.

7.1.5.2 *Cell stimulation*

So far, only one experiment investigating differentiation and proliferation has been performed beyond the unstimulated survival experiments. During these experiments some novel B cells were found to differentiate into CD27-expressing cells. This finding has to be further investigated. Additional experiments investigating stimulated survival, proliferation, differentiation and antibody production should also be made.

7.2 LONG TERM

Flow cytometry provides a way to investigate cell populations. As new cell surface markers are identified, populations can be broken into subpopulations. Comparisons of these cell populations reveal differences which increases our understanding of for example immunology.

There are labs in which single cell RNA purification is being performed in microfluidic systems. A microfluidic high-throughput lab-on-a-chip-approach for single cell cDNA-production has the potential to revolutionize cell differentiation research. However, these methods do not exist yet.

If a device for single B cell cDNA-production could be constructed, human peripheral B cell development could be investigated more efficiently. If cDNA from individual novel B cells could be produced, these cDNA samples could be divided in groups according to their gene expression profiles. For example, in which aspects do CD27-expressing novel B cells differ from novel B cells that lack CD27 expression? In what ways do the different development stages of transitional B cells differ from one another?

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