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Characterization of biological and functional features of a new type of human CD27$^-$ memory B lymphocytes

Charakterizace biologických a funkčních vlastností nového typu lidských CD27$^-$ paměťových B lymfocytů

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Tuto diplomovou práci jsem vypracovala samostatně, pouze s použitím citované literatury a pod vedením vedoucího diplomové práce.

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Abstrakt

V periferní krvi pacientů s běžným variabilním imunodeficitem (CVID) v porovnání se zdravými dárci byly pozorovány zvýšené frekvence dvou nových B buněčných populací definovaných jako $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{+}$ a $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{-}$. Cílem studie bylo nalezení takových B buněk u pacientů s revmatoidní artritidou (RA), jejich následná charakterizace a vzájemné porovnání.

Produkce imunoglobulinové mRNA u jednotlivých B lymfocytů byla analyzována pomocí průtokové cytometrie s následným tříděním jednotlivých buněk, RT-PCR na úrovni jedné buňky, IgV_H-specifické PCR amplifikace, cyklického sekvenování a statistické analýzy. Zaměřili jsme se na analýzu variabilních oblastí těžkého řetězce imunoglobulinů a zjistili jsme významné rozdíly v zastoupení V_H, D_H a J_H genových segmentů, mutační frekvenci, distribuci tichých a záměnných mutací, délce a složení CDR3 oblastí, klonální příbuznosti a expresi RAG genů mezi výše zmíněnými B buněčnými populacemi.

Analyzované populace byly považovány za naivní a to zejména z důvodu absence povrchové CD27 molekuly považované za marker B lymfocytů, které prošly antigenně řízenou germinální reakcí. Nicméně rozložení a typ mutací naznačují, že tyto buňky představují nový typ paměťových/antigenně zkušených B lymfocytů (u CVID méně diferencovaných) hrající roli při ochraně organismu proti infekcím nebo s dosud neznámou regulační funkcí.

Nejzajímavějším nálezem bylo omezení repertoáru V_H genů pouze na 10 V_H genových segmentů s převahou V_H3-48 a V_H4-34 genů v důsledku extrémně vysokého stupně klonální příbuznosti B buněk u pacienta s RA. Protože V_H4-34 gen kóduje anti-dsDNA autoprotilátky, naše data by mohla naznačovat, že tyto B buňky mohou za určitých podmínek uniknout negativní selekci a stát se autoreaktivními.

Tyto nálezy však u zdravých kontrol a pacientů s CVID nebyly potvrzeny a data svědčí pro to, že $CD24^-$ B lymfocyty představují více diferencované a antigenně zkušené buňky oproti $CD24^+$ populaci.

U zdravých jedinců jsou pravděpodobně námi identifikované $CD27^-$ B lymfocyty zapojeny do udržování homeostázy imunitního systému (u CVID porušené), zatímco u autoimunitních chorob vykazují spíše autoreaktivní rysy.

K potvrzení obecnější role těchto B lymfocytů v rozvoji autoimunitních nemocí bude třeba další výzkum např. u pacientů se systémovým lupusem erytematosus nebo Sjögrenovým syndromem.

Klíčová slova

B lymfocyty, CD antigeny, běžný variabilní imunodeficit, revmatoidní artritida, VDJ přestavba, průtoková cytometrie, single-cell RT-PCR

Abstract

The increased frequencies of two novel B cell populations defined as $IgM^+CD19^+CD27^-CD21^{low}CD38^{low}CD24^+$ and $IgM^+CD19^+CD27^-CD21^{low}CD38^{low}CD24^-$ in peripheral blood of patients with common variable immunodeficiency (CVID) compared to healthy donors were found. The aim was to search for such B cells in patients with rheumatoid arthritis (RA) and their further characterization.

The production of immunoglobulin (Ig) mRNA in single B cells was analyzed using flow cytometry, single cell sorting and RT-PCR, IgV_H-specific PCR, cycle sequencing and statistical analysis. The study was focused on analysis of variable regions of the heavy chains of Igs and significant differences in the usage of V_H, D_H and J_H gene segments, mutational frequencies, distribution of silent and replacement mutations, length and composition of CDR3 regions, clonal relation and RAG gene expression in above mentioned B cell populations were found.

Because of lack of the surface CD27 molecule being regarded as marker of B cells that have undergone antigen-driven germinal reactions, analyzed populations were considered as naive. However, the pattern and type of mutations suggested that these cells could represent a new type of differentiated memory/antigen-experienced B lymphocytes (in CVID less maturated) with the likely role in protecting of organism against infections or with so far unknown regulatory function.

The most interesting finding was remarkable restriction of V_H gene repertoire to only 10 V_H genes with predominance of V_H3-48 and V_H4-34 genes as the result of extremely high degree of clonal relation in B cells of patient with RA. Since V_H4-34 gene is coding for anti-dsDNA autoantibodies the data might suggest for susceptibility of these cells to escape negative selection and to become autoreactive.

However, this was not confirmed in controls and in patients with CVID, and our data indicate that $CD24^-$ B cells might represent more

differentiated and antigen-experienced cells as compared to their $CD24^+$ counterparts.

Discovered $CD27^-$ B cells could be included in healthy subjects into maintenance of homeostasis of immune system (in CVID disturbed) whereas in autoimmune diseases they display rather autoreactive features.

Detailed analysis of these particular B cells in patients with other autoimmune diseases could confirm the role of these cells in physiological or pathological process.

Key words

B lymphocytes, CD antigens, common variable immunodeficiency, rheumatoid arthritis, VDJ rearrangement, flow cytometry, single-cell RT-PCR

Contents

1. Introduction

The immune system of humans is complex of interconnected mechanisms that provide resistance to infections from the external environment and regulate internal homeostasis. Defects in individual components of the immune system are caused by a number of more or less serious injuries, and they are manifestated in the form of either autoimmune process or immunodeficient status.

One of the most frequent primary immunodeficiency is common variable immunodeficiency (CVID). Although this primary immune defect is at least in some cases genetically determined however, the underlying causes are different and it remains still rather enigmatic. The result of these defects is that the patient does not produce sufficiently effective amount of antibodies in response to exposure to pathogens. Therefore, the patient's immune system fails to protect organism against common bacterial and viral (and occasionally parasitic and protozoan) infections. Another characteristics of CVID are changes in the frequencies of different populations of B cells.

The increased frequencies of two novel B cell population in peripheral blood of CVID patients compared to healthy donors were observed using flow cytometry. In our previous study (Vlková et al., 2010), these cells with the immunophenotype $IgM^{+}CD19^{+}CD27^{-}CD21^{low}$ $CD38^{low}CD24^{+}$ and $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{-}$ were found to be in their properties controversial. The most interesting finding was CD27 negativity which suggested for naive nature of these cells.

Therefore, we have planned to describe these populations at a single cell level using a "single-cell" Ig-specific RT-PCR. Employing the IgM/IgG mRNA expression we addressed the question whether these cells have undergone antigen-driven selection, affinity maturation and isotype switching. As a disease control, B cells of the same immunophenotype from patient with prototype autoimmune disease (rheumatoid arthritits, RA) were analyzed by the same way.

The aim of this work was not only contribute to clarifing of the nature of discovered B lymphocyte populations in terms of their differentiation in general, but also to understanding of the significance of B cells in the pathogenesis of CVID and RA.

2. Literary Review

2.1. The immune system of humans

The immune system is a collection of highly versatile, variable and adaptive mechanisms that ensures the integrity of the organism. Basicly it has three elementary functions:

1) identification and destruction of microorganisms and their toxic products in the body, blood circulation or on the mucosal surface
2) continuous identification and removal of old, damaged or mutated cells
3) identification of the body's own tissues and maintenance of tolerance of them.

The immune system consists of the specific and non-specific component that can work together to protect the human body against infection and a large variety of pollutants (Beck et al., 1996).

2.1.1. Innate immunity

Non-specific or innate immunity is evolutionaly older and plays an important role in the early stages of infection. These immune mechanisms are responsible for the ability to recognize and destroy a large variety of contaminants and infectious organisms due to their common structural and functional features. Moreover, the mechanisms of innate immunity are in the body prepared in advance, therefore their activation is very fast. On the other hand, this type of response is not affected by previous exposition to the antigen, which means that there is no immunological memory. Thus, the rate and the strength of the immune response during re-infection is identical as during the first pathogen challenge.

The innate immunity is represented as protective component in anatomical and physiological barriers and consists of cellular and humoral components. Cellular immunity is mediated mainly by phagocytes, macrophages, monocytes, granulocytes, dendritic cells, mast cells

and other cells. The humoral components include complement, interferons, lectins and other serum proteins (Beck et al., 1996).

2.1.2. Adaptive immunity

Adaptive (specific) immunity is phylogenetically younger and develops throughout entire life of the individual. Its response to the antigen takes place in the hours to days. It displays antigenic specificity and immunological memory. In contrast to non-specific immunity, adaptive type of responses is highly variable and and leads to the production of great amount of highly specific molecules that have crucial role in destroying of invading pathogens and their toxic products. Adaptive immune responses have the key ability to distinguish which of these molecules are foreign to the host and which not. The failure of immune system at this point can lead to destruction of host's own (self) molecules and thereby to development of autoimmune diseases.

As in above mentioned case, also adaptive response is composed of cellular and humoral components. Cell-mediated immunity is represented mainly by T lymphocytes, which have variety of functions depending on their characteristic features. Humoral immunity is mediated by immunoglobulins called antibodies that are produced by B lymphocytes (Beck et al., 1996).

2.2. B lymphocytes

B lymphocytes (cells) belong to lymphoid cell line (Fig. 1.) and their main activity is focused to production of antibodies against antigens during humoral immune response (Litman et al., 1993). In this context so-called mature $CD19^+$ B cells after recognition of antigen differentiate from naive (antigen unexprerienced B cells) into either memory B cells or terminally differentiated plasma cells, which is associated with changes of expression of surface molecules CD27 and CD20 (see later in Chapter 2.2.4.1.; Klein et al., 1998).

In addition, B cells are known as antigen presenting cells (APC) and represent more effective antigen presenters in comparison to professional

APCs such as dendritic cells (Kotzin et al., 2005). Besides this it was shown that B cells under specific conditions display the same features as natural killers or they can mediate osteoclastogenesis (Hagn et al., 2009; Li et al., 2007). With respect to the topic of current thesis, these B cell activities will not be further discussed.

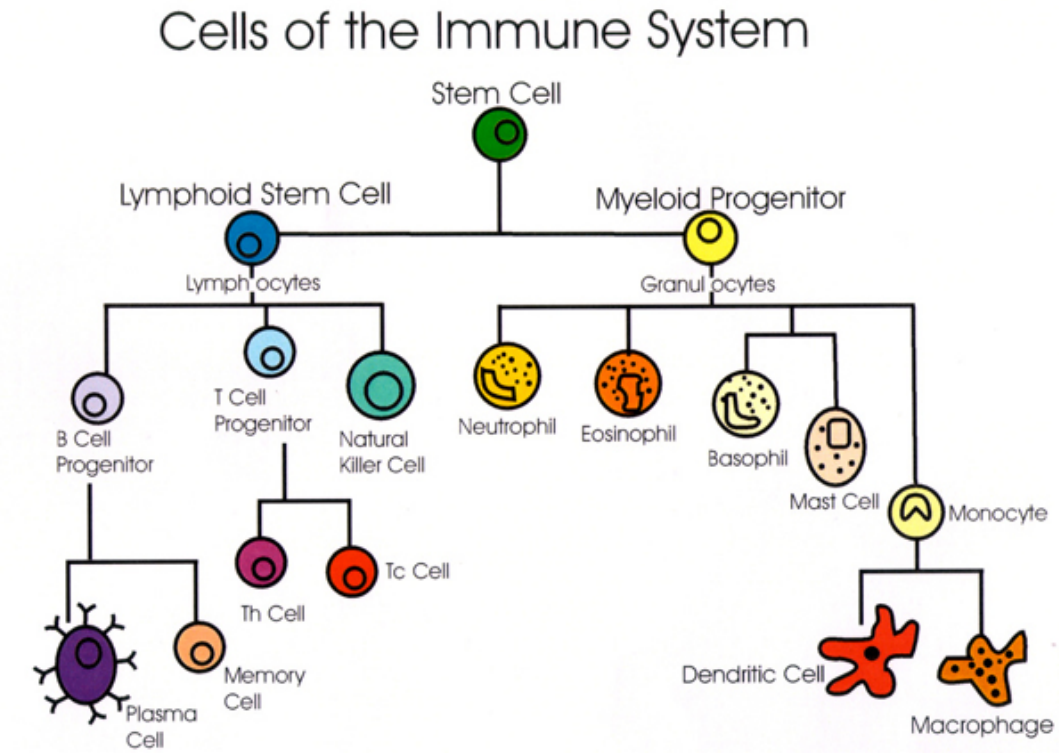


Figure 1. Cells of the immune system
http://textbookofbacteriology.net/cellsindefenses75.jpg

2.2.1. Immunoglobulins

Immunoglobulins (Ig; also known as antibodies, Ab) represent the most important part of specific immunity. They occur in two forms – soluble (secreted) and membrane-bound and are produced by plasma cells and memory B cells in response of the immune system to antigen challenge.

Soluble immunoglobulins arise after cleavage of the leader sequence, see below. Soluble Ig are secreted from the cell into

the bloodstream and tissue fluids in large amounts. The main functions of antibodies is to prevent toxin effect by binding to its epitopes or to opsonize microorganisms, which are then absorbed by phagocytes, and finally antibodies activate the classical complement pathway (with exception of antibodies of IgG4 and IgA isotype; Russell et al., 1989).

Membrane bound immunoglobulin molecules are located on the outer surface of B cells as part of a specific receptor for antigen – B cell receptor (BCR) complex. The BCR is a multimeric complex containing two Ig heavy chains, two Ig light chains and two heterodimeric transmembrane molecules Igα, Igβ (CD79α, CD79β; Fig. 2.).

Entire BCR is then able to bind antigens in native form and thus acts as the key receptor that regulates the fate of B lymphocyte. Fully functional BCR signaling is necessary for the proper conduct of the development of B lymphocytes.

2.2.1.1. Immunoglobulin structure

Typical Ig molecule consists of two large heavy chains (H) and two small light chains (L) which are held together by disulphide bridges to form final Y structure.

Heavy chains contain four or five domains, one of which is variable domain (V_H, 110 amino acids) and 3 to 4 constant domains (C_H, 330 – 440 amino acids) depending on the Ig isotype. C_H domains are termed μ, δ, γ, α and ε and they determine a particular isotype of antibody – IgM, IgD, IgG, IgA or IgE, see below (Janeway, 2001). A molecular weight of the heavy chains is approximately 50 – 75 kDa.

Light chains contain only two domains – the variable (V_L, 110 amino acids) and constant (C_L, 110 amino acids), which can be isotype kappa (κ) or lambda (λ). Molecular weight of the light chain is about 25 kDa.

Every variable and constant domain of H and L chains of imunoglobulin is composed of a sequence of 110 – 120 amino acids. A N-terminus (NH_2) is situated at the start of variable region and a C-terminus (COO) at the end of the constant region. The C-terminus

of the heavy chain includes a hydrophobic stretch of 20 amino acids, which is called the leader (signalling) sequence and is anchored in the membrane of B lymphocyte.

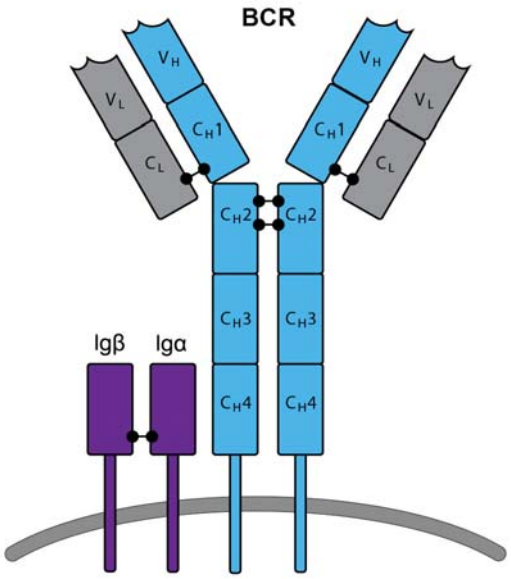


Figure 2. BCR structure

In the primary sequence of immunoglobulin genes for variable regions can be distinguished three hypervariable regions (complementarity determing region, CDR), which are separated by four relatively conservative regions called framework regions (FR). While the FR are involved in the maintaining of the stability of immunoglobulin molecules, CDR regions together form the antigen binding site (mainly CDR1 and 2) and are responsible for recognition and binding of (auto)antigen (especially CDR3). CDR3 region is made up of the 3' end of V_H, entire D_H and 5' end of J_H segments (Zemlin et al., 2005; Fig. 3.). CDR3 is a very variable structure determining the final specificity of the Ig molecule and represent a "hot spot" for the changes (mutations, duplications, inversions, deletions or nucleotide aditions) leading to higher affinity for antigen. B lymphocytes that have been selected by same (auto)antigen are characterized by the same length and amino acid sequence of Ig molecule, ie. they are clonally related (Klein et al., 1997).

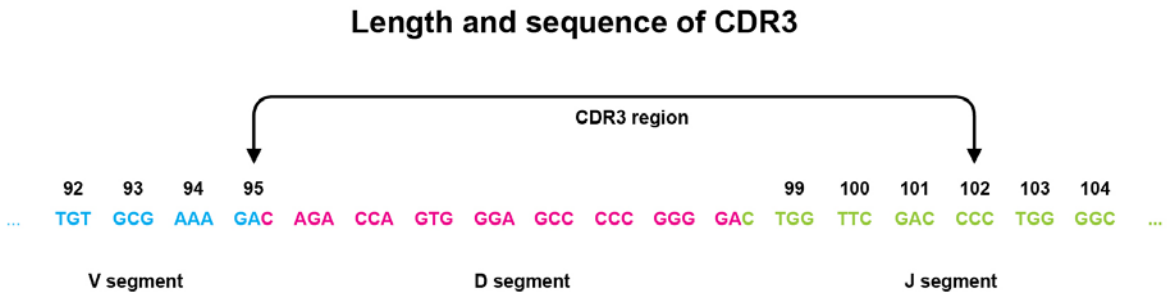


Figure 3. CDR3 structure

2.2.1.2. Immunoglobulin isotypes

As it was mentioned above, depending on the type of heavy chain constant region there are five different isotypes of antibodies - IgM, IgD, IgG, IgA or IgE.

IgM immunoglobulins are formed as the first antibody in response to an antigenic stimulus. IgM antibodies are present in the membrane-bound form and in the secreted form. They are produced mainly by B cells, that have not yet met antigen and therefore have not undergone the somatic hypermutation (see later in Chapter 2.2.2.2.). IgM forms monomers or polymers – mostly pentamers that display increased avidity and the greatest ability to stimulate the complement system (Parkhouse and Della Corte, 1973). Due to a formation of polymeric structures, soluble IgM has a larger size than other isotypes and therefore it occurs most frequently in the peripheral blood rather than in the tissues. IgM antibodies represent very efficient tool in the elimination of microorganisms from the body.

IgD together with IgM are expressed on the majority of mature B cells leaving the bone marrow. Once B lymphocyte produces IgD (post-switched cells), the expression of IgD is permanently suppressed. IgD does not bind complement and its specific function is still unknown (Janeway, 2001).

IgG exists in four subtypes (IgG1 - IgG4) that differ in the ability to bind complement fragment C1 (IgG1 and IgG3 are involved in the activation of the complement system; Parkhouse and Della Corte, 1973).

IgG antibodies opsonize pathogens and bind very well to Fc receptors present on certain immune cells (macrophages, monocytes; Quan et al., 2009). IgG antibodies can pass across the human placenta and thus they ensure the immune protection of the foetus during pregnancy.

IgA occurs in two isotypes, IgA1 and IgA2, their ratio varies in the different lymphoid tissues of the human body (Simell et al., 2006). We can distinguish also serum and secretory form. Mucosal (secretory) IgA forms dimers containig so-called J chain and is present on the surface membrane of mucosa, which is responsible for binding to microbial surface receptors and thus for blocking the adhesion of bacteria to the host tissue (Parkhouse and Della Corte, 1973). The serum form of IgA is monomeric, dimeric or trimeric (monomers are linked by joining chain). Generally, the main function of IgA antibodies is neutralization of bacterial and viral Ag.

IgE antibodies are present in the blood only at very low concentrations, they have great ability to bind to Fc receptors expressed on mast cells and basophils (Johansson, 1975). Binding of antigen to IgE bound to Fc receptor of mast cell then triggers the release of chemical mediators of these cells to induce defensive responses in particular against multicellular parasites. On the other hand, IgE is responsible for the early phase of atopic (allergic) reactions (Johansson, 1975).

2.2.2. B cell development

B cell development includes several differentiating stages. The first part of the development of B cells is independent of the presence of antigen. The pluripotent hematopoietic stem cells in the bone marrow starts to differentiate into the immature B cells, which is associated with a gradual process of rearrangement of genes coding for variable parts of immunoglobulin chains (Warnatz and Schleiser, 2008). This is followed by the development in the periphery in presence of Ag. Development of these mature B cells is based mainly on somatic hypermutation and isotype switching.

2.2.2.1. Rearrangement of immunoglobulin genes

Rearrangement of immunoglobulin genes is a fundamental process necessary for the creation of a broad repertoire of antigen-specific B cell receptors.

Genes encoding light and heavy chain antibodies occur in the human genome as so-called gene segments (Togenawa, 1983). Gene segments for heavy chains are located on the chromosome 14, gene segments for the κ light chain on the chromosome 2 and for the λ light chain on the chromosome 22.

Ig molecule consists of variable domains that are encoded by segments V_H (variation), D_H (diversity) and J_H (joining) and constant domains. Individual segments are present in so-called germinal DNA sequence in greater numbers. In detail, the DNA sequence of pluripotent stem cell contains 51 V_H genes (in 7 families), 27 D_H segments (in 7 families), 6 J_H segments (in 6 families) and 9 C_H segments (1 μ, 1 δ , 4 γ, 1 ε and 2 α). Variable domains of both light chain genes never contain segment D_L and have different number of V_L and J_L segments (40 Vκ , 31 Vλ , 5 Jκ , 4 Jλ; Link and Schroeder, 2002).

Recombination signal sequences (RSS) are located at the 3' end of each V exon, at both ends of each D exon and at 5' end of each J exon (Fig. 5.). RSS nucleotide sequences are conservative palindromic heptamers and AT-rich conserved nonamers that are separated by sequences of length 12 or 23 base pairs (so-called spacer; Zhang et al., 2003). RSS is a signal sequence complex for recombination proteins, which are collectively called recombinase. Recombinase has two main component – highly specific enzymes RAG1 and RAG2, which are expressed mostly in bone marrow but moreover in germinal center lymphocytes (see later in Chapter 6.2.4.; Girschick et al., 2001; Dörner et al., 1998a).

The recombination is highly regulated process, which is initiated at the stage of B lymphocyte progenitor cells (pro-B) on both chromosomes simultaneously by rearrangement of IgH locus (Ghia et al., 1996). The initial steps of $V_HD_HJ_H$ recombination are carried out by above mentioned

recombinase. The function of this enzyme complex is to split, shuffle and rejoin the V(D)J genes (Zhang et al., 2003). In early pro-B cell occur the first recombination event between random D_H and J_H gene. After the recognition of the RSS sequences, associated recombinase induces cleavage on one DNA strand. This action of recombinase leads to a creation of a hairpin loop that resulted in the formation of D_HJ_H complex. This is followed by the joining of one random V_H gene and transition of early pro-B cell to late pro-B cell (Fig. 4.). All other genes not included in the newly formed $V_HD_HJ_H$ region are deleted (Bassing et al., 2002; Fig. 5).

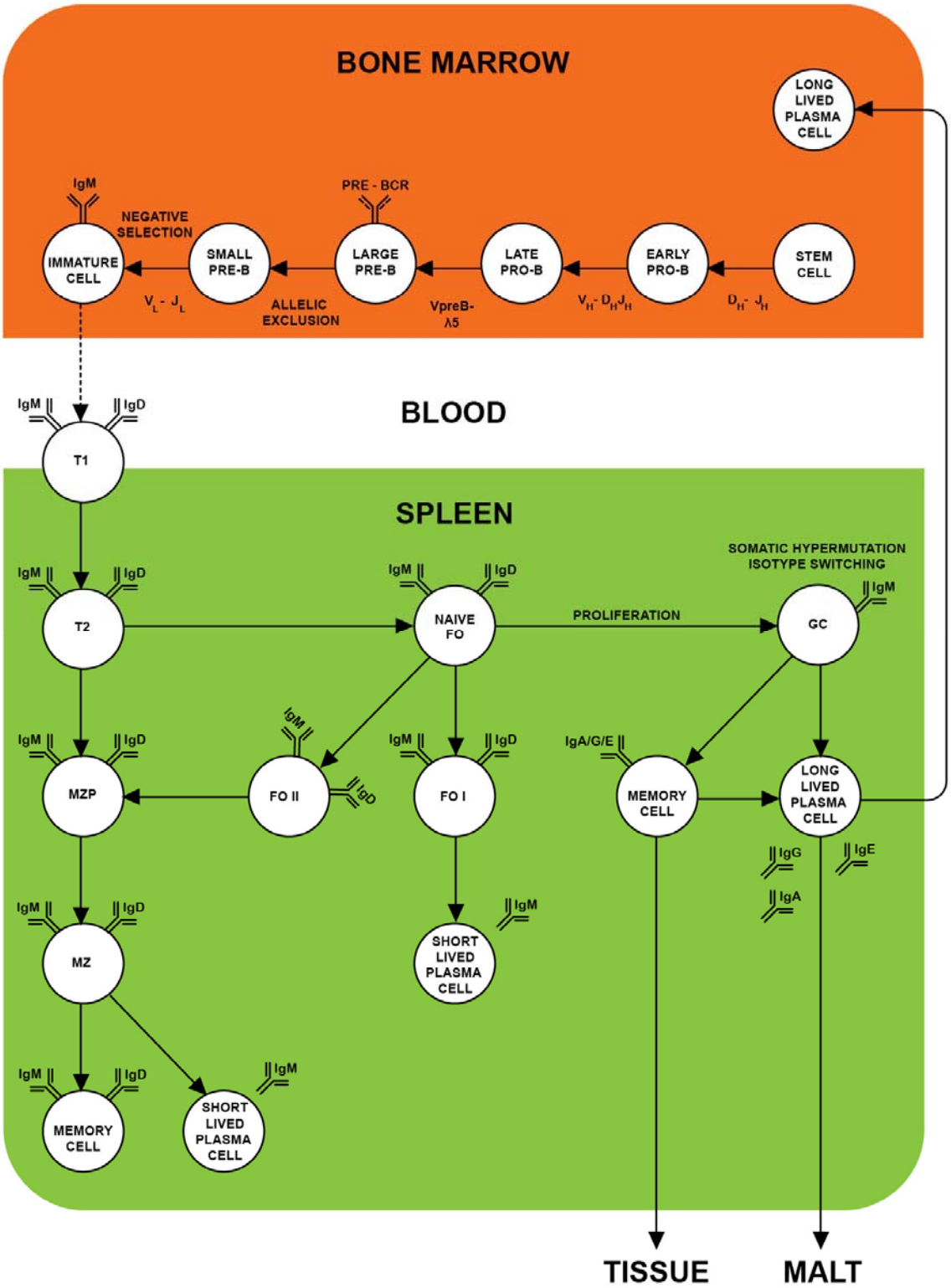


Figure 4. B cell development

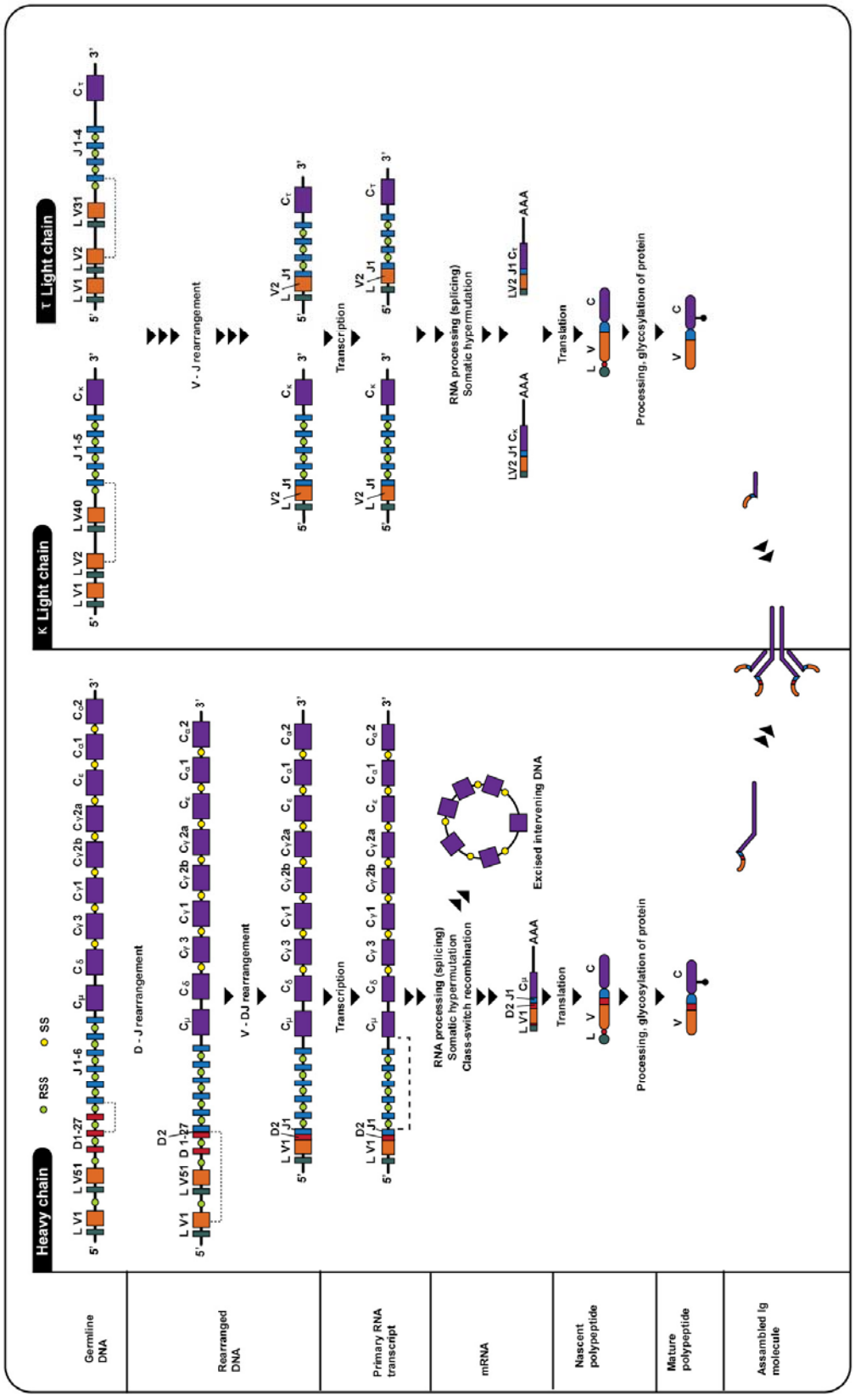


Figure 5. Rearrangement of immunoglobulin genes

The successful rearrangement of genes on one chromosome and mRNA coding functional protein is followed by allelic exclusion that prevents expression of the allele on the second chromosome. This suppression of H chain gene rearrangement ensures only one specifity of Ab expressed per cell.

At this time, late pro-B cell requires surrogate (pseudo) light chain for surface expression istead of the light chain gene that has not been yet rearranged (Fig. 4.). Surrogate (ψL) light chain is created from VpreB and λ5 genes and unlike conventional light chains do not undergo through the gene rearrangement (Wang et al., 2002). Large pre-B lymphocyte expresses on its surface so-called pre-B receptor (pre-BCR) consisting of IgM, VpreB and λ5, which associates with the signaling protein dimer Igα - Igβ (CD79α, CD79β). Signals from this signaling complex promote limited clonal expansion and after that initiate the rearrangement of genes for light chain.

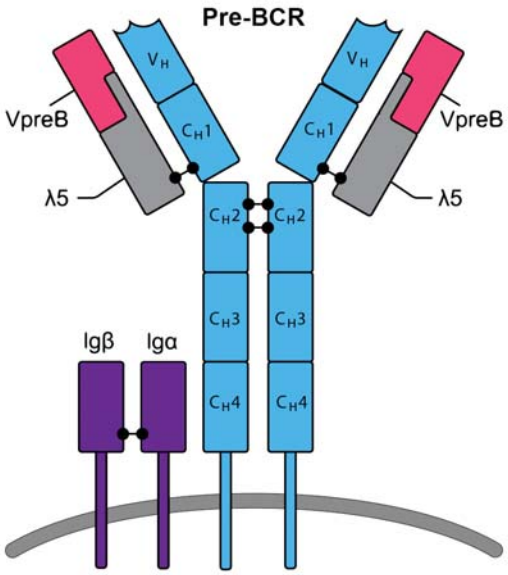


Figure 6. Pre-BCR

The rearrangement of genes for light chain is based on similar principal and also takes place on both chromosomes. It is initiated by rearrangement of κ light chain (Fig. 5.). If the rearrangement of none of κ gene segments is not successful it is followed by the rearrangement of genes for the λ gene segments. If this process is nonproductive, pre-B lymphocyte dies by apoptosis (Hořejší and Bartůňková, 2009). If the rearrangemet is productive, the light chain substitutes ψL and confers the right BCR and cells continue to the stage of immature B cells (Wang et al., 2002).

At this point, all immature B cells carrying the BCR, that recognizes the body's own (self) antigens are eliminated from the immune repertoire by negative selection. Negative selection of potentially autoreactive cells leads to the apoptosis (clonal deletion) or anergy (hyporesponsiveness to BCR signals, no proliferation, no secretion of antibodies). Autoreactive cells can evade this mechanism by using receptor editing to rearrange immunoglobulin genes on the second chromosome.

Individual variability of Ig molecules, that ensures the capacity of human organism to protect itself against a wide spectrum of different pathogens and bind to a variety of antigen binding sites, is based on the combinatorial assembly of V, D, J gene segments, somatic hypermutation (see later in Chapter 2.2.2.2.) and also on random addition of nucleotides (non-templated aditions, N-additions; Rösner et al., 2001; Ivanov et al., 2005).

2.2.2.2. Somatic hypermutation

Further increasing of different Ig variants is mediated by somatic hypermutation (SHM) in periphery (germinal centers; Fig.6.). B cell that is activated by contact with antigen presented by follicular dendritic cell of germinal centers initiates the process of SHM that generates nucleotide substitutions in variable regions of Ig genes. Mutations occur in all of 3 loci on chromosomes 2, 14 and 22 in areas with a length of about 2 kb (Wabl

et al., 1985). Somatic hypermutations include transitions (purine → pyrimidine, pyrimidine → purine) as well as transversitions (purine → purine, pyrimidine → pyrimidine).

The whole process is initiated by activation-induced cytidine deaminase (AID) that is expressed only in B lymphocytes and establishes a mutation rate of about 10^{-3} nt per cell division (Muramatsu et al., 1999). In the process of somatic hypermutation participate also different subtypes of DNA polymerases, uracil DNA glycosylase and other enzymes.

Type of SHM and their pattern were shown to be crucial for differentiation of mature B cells and they are characteristic for memory/Ag-experienced B cells (Klein et al., 1998).

2.2.2.3. Isotype switching

Final process that improves the efficiency of antibody response is isotype switching (class switching). Isotype switching allows a generation of other Ig isotypes than primary created IgM and typically occurs subsequent by to a first exposure to antigen. During this process only the constant region of immunoglobulin molecule is changed and thereby the antigen specifity remains unchanged.

Introns separating the genes for the heavy chain constant region (excluding the Cδ intron) contain so-called switch sequences (SS; Kataoka et al., 1980). Isotype switching occurs by recombination between S sequences and the region between two random S sequences is eliminated from IgH gene and finally the nearest C segment is included in the final transcript (Fig. 5.).

In appropriately activated cells is isotype switching regulated by T_H (T helper lymphocytes) cells. This regulation is mediated by switching factors, realized by physical contact between T_H and B cells (CD40L + CD40, CD28 + CD80, 86.) and promoted by cytokines (IL-1, IL-2, IL-4, etc.). Particularly, cytokines regulate the process and decide on a specific Ig isotype. For example in humans, the presence of IL-4 enhances IgG and IgE production, TGF-β and IL-10 promote the formation of IgA (McHeyzer-Williams et al., 2006).

2.2.3. B cell types

B cell development starts in the fetal liver and after birth continues in the bone marrow that provides microenviroment for B cells maturation and differentiation. Bone marrow stromal cells nurture developing B cells by specific cell-to-cell contact and secretion of cytokines, and thus they are the key regulators of developing B cells. Each stage of developing B cells is defined by rearrangements of immunoglobulin heavy and light chain genes, expression of surface Ig, adhesion molecules and cytokine receptors (as was discussed in Chapter 2.2.2.; Ghia et al., 1996).
In the stage of immature B cells leave the bone marrow and migrate as a so-called transient cells through the bloodstream to secondary lymphoid organs, particularly to the spleen and lymph nodes, where they maturate into folicular or marginal B cells (Smith et al., 1997; Fig. 6.). Further differentiation of these B cells can take several pathways, the major mechanism that differentiate developing B cells is the resulting affinity to antigen. High-affinity cells develop into plasma cells or into the memory cells, and low-affinity cells die by apoptosis (Smith et al., 2000).

2.2.3.1. Transient B cells

They are called Transient - T (transitional = transition) or NF (newly formed) B lymphocytes.

Transient B cells are short-lived, functionally immature and characterized by a high density of expression of surface molecules IgM, CD24 and CD38. In addition, transient cells express on their surface a molecule CD21, CD23 and CD62L (L-selectin; Loder et al., 1999). According to their phenotype and functional characteristics, transient cells can be further divided into two or three B cell subpopulations - T1, T2 and T3, see below. T1 cells are $IgM^{high}IgD^{-}CD19^{+}CD27^{-}CD21^{low}CD38^{high}$ $CD24^{high}CD23^{-}$. T2 cells phenotype is $IgM^{high}IgD^{high}CD19^{+}CD27^{-}$ $CD21^{high}CD38^{high}CD24^{high}CD23^{high}$ (Loder et al., 1999; Srivastava et al., 2005) and T3 differs from T2 by lower expression of surface IgM (IgM^{low}; Allman et al., 2001).

T1 cells migrate from the bone marrow into the blood, spleen and lymph nodes, while the presence of T2 cells is limited to the spleen (Fig. 5). Futhermore, during BCR-mediated signaling, T1 cells die by apoptosis, whereas T2 cells proliferate, differentiate and increase their size (Chung et al., 2002). Some authors distinguish based on IgM expression a third transient stage – T3. This population is phenotypically very similar to B cells at the stage of anergy and is not yet clear whether T3 cells represent really a transient or an anergized autoreactive B cells (Merrell et al., 2006).

2.2.3.2. Follicular B cells

Follicular (FO) B cells are recirculating small lymphocytes, that form B cell follicles in the white pulp in the spleen. They represent a subpopulation of B cells that produces specific antibodies.

T2 cells migrate into the spleen, where they differentiate into mature follicular B cells. These B cells have not yet encountered antigen and therefore are referred as naive. The population of naive follicular cells ($IgM^+IgD^+CD19^+CD27^-CD21^+CD38^+CD24^+CD23^+$) actively circulates between the secondary lymphoid organs in the body.

When follicular dendritic cells (FDC) display Ag on their surface, activate Ag-specific $CD4^+$ T cells which proliferate and mature into effector cell capable to activate Ag specific FO B cells. Once activated by $CD4^+$ T cell, the FO B cells proliferate to form primary follicule of Ag specific B cells. These B cells migrate to nearly follicules and proliferate. Other B cells from primary focus (FO I) persist in a T cell area, differentiate to short-lived plasma cells secreting Abs.

The B cells that enter the follicul begin to proliferate rapidly to form highly specialized structure – germinal center (GC; Schwickert et al., 2009). Here in, during so called GC reaction B cells, also undergo somatic mutation to introduce new variations into the BCR followed by a process

of selection when BCR are tested for their ability to bind Ag. For further developments are then selected only those B cells that recognize antigen with high affinity, while other B cells die by apoptosis. Subsequently, the GC B cells differentiate into isotype switched long-lived memory B cells and plasma cells (Smith et al., 1997).

2.2.3.3. Marginal zone B cells

T2 cells remain in the marginal zone (MZ) – a specialized region on the border of white and red pulp of the spleen. It is a unique part of the spleen and is not present in any other primary or secondary lymphoid organs. The marginal zone B cells, myeloid, dendritic and stromal cells are a major component of the marginal zone. At first, T2 cells mature to precursor MZ B cells (MZP B cells), with $IgM^{high}IgD^{high}CD19^{+}CD27^{-}$ $CD21^{high}CD38^{high}CD24^{high}$ phenotype that differs from mature MZ B cells ($IgM^{low}IgD^{high}$ $CD19^{+}$ $CD27^{+}$ $CD21^{high}$ $CD38^{high}$ $CD24^{high}$) in the expression of IgD.

Although they provide important signals during T lymphocyte dependent response, their main function is to provide the first line of defense against T independent (TI) pathogens such as polysaccharide coated bacteria (Streptococcus pneumoniae, Haemophilus influenzae; Srivastava et al., 2005). High expression of CD21 receptor (complement receptor for C3d component of complement) on the surface of MZ B cells is a prerequisite for binding of polysaccharide antigens, which are mostly opsonized by C3d component of complement. Although MZ B cells in mice were present only in the marginal zone, in humans are generally detected even in the peripheral blood circulation (Weller et al., 2004).

2.2.3.4. Memory B cells

Memory B cells are susceptible to rapid stimulation and production of specific immunoglobulin in secondary antigen challenge. Memory B cells are recirculating between peripheral lymphoid organs and bone marrow (Paramithiotis and Cooper, 1997). The hallmark of these cells is the expression of surface molecule CD27 and the presence of somatic

mutations in variable regions of immunoglobulin genes. Phenotype of these cells that are differentiated in the germinal centers is $IgM^{-}IgD^{-}CD19^{+}$ $CD27^{+}CD21^{high}CD38^{+}CD24^{high}$ (Klein et al., 1998). When isotype switched memory B cells are exposed to the antigen that stimulated their development after the first challenge, they rapidly activate and differentiate into plasma cells. Thus, immune response is faster and more efficient (Klein et al., 1997).

Other B cell memory population found in peripheral blood is derived from marginal zone B cells. They are $IgM^{high}IgD^{+}CD19^{+}CD27^{+}CD21^{high}$ $CD38^{+}CD24^{high}$ B lymphocytes, and although they undergo germinal center reaction they somatically have mutated genes for the variable immunoglobulin chains (Schereen et al., 2008).

Unlike conventional memory cells, whose activity is directed mainly against protein antigens, IgM^{+} memory B cells rather recognize polysaccharide antigens and trigger T-independent immune responses (see above; Klein et al., 1998).

2.2.3.5. Plasma B cells

Plasma B cells (plasmocytes) with phenotype $IgM^{-}IgD^{-}CD19^{+}$ $CD27^{high}CD21^{-}CD38^{high}CD24^{-}$ act as the key source of antibodies. Plasma cell can differentiate from follicular FO I and MZ B cells and also from long-lived memory cells (Fig. 6.). The main function of plasma cells is the formation and secretion of immunoglobulins, which circulate in the peripheral blood and specifically bind to the antigen that originally initiated and stimulated their differentiation. The activation of plasma cells requires additional signals, especially those mediated by T lymphocytes and cytokines (Agematsu et al., 1999).

Plasma cells normally live for a short period of time, but also the existence of long-lived plasma cells was described. These special cells colonize the bone marrow, where they are supported by stromal cells. Physiological significance of long-lived plasma cell has not yet been fully

elucidated, but they can be responsible for long-lasting production autoantibodies (Arce et al., 2004). In this context, these cells due to absence of CD20 molecule on their surface represent the most important complication during anti-B cell depletion therapy of autoimmune diseases such as RA and SLE (Hoyer et al., 2004; Edwards et al., 2004).

2.2.4. B lymphocytes in peripheral blood

In human peripheral blood circulate various developmental stages of B cells, that can be identified by specific expression of surface molecules, known as CD (cluster of differentiation) antigens. Up to 60% of peripheral B cells are naive B cells with $CD27^-$ phenotype and about 40% are memory B cells expressing CD27 ($CD27^+$; Klein et al., 1997).

About half of the memory cells are isotype switched memory B cells expressing surface immunoglobulins other than IgM and IgD ($IgM^-IgD^-CD19^+CD27^+CD21^{high}CD38^+CD24^{high}$). The second half of the memory cells are cell with phenotype $IgM^{high}IgD^+CD19^+CD27^+CD21^{high}CD38^+CD24^{high.}$ Very rarely can be detected subpopulations of memory cells that express only IgM or only IgD (Klein et al., 1998).

Plasma cells do not circulate in the bloodstream, their precursors may be detected in small amounts in peripheral blood via a high density of surface molecules CD27 and CD38 (Avery et al., 2003).

Other population present in peripheral blood are transient B cells regarded as main and divided into two, according to some authors into three subpopulation (see above in Chapter 2.2.3.1.; Loder et al., 1999). The main fraction of $CD27^-$ cell population is formed by naive follicular cells that express surface CD23, CD21, IgM and IgD (Odendahl et al., 2000; Warnatz and Schleiser, 2008).

Monitoring of differentiation of B cells in peripheral blood is important not only for diagnostics of immunodeficiency, but also for autoimmune diseases. Currently, there are several schemes for monitoring the differentiation of B cells in the periphery, using different surface markers (CD21, CD23, CD38; Jacobi et al., 2003).

2.2.4.1. Markers of B cell populations

As was mentioned above, the various developmental stages of B lymphocytes express on their surface a variety of membrane-bound molecules and CD antigens. These are signaling molecules and complexes, receptors, ligands and other molecules.

Molecules on B cell surface are used as markers by which individual cell subpopulation can be identified and separated from the others. Furthermore, there is a list of some CD antigens that are relevant to the focus of this thesis.

Molecule CD19 is a marker of the entire B cell line in humans (pan-B cell marker). CD19 has a conserved structure, it is a 95 kDa transmembrane glycoprotein with two Ig-like domains and 240 amino acid cytoplasmic tail. An early stage of B cells in bone marrow are already expressing CD19 and this expression continues until the stages of memory B cells and plasmablasts. CD19 molecules with CD21, CD81 and CD225 generates a signaling complex that positively or negatively regulate signals transmitted by the BCR. The function of CD19 molecule is the activation of signaling cascades by binding to CD21 molecule in the membrane (Matsumoto et al., 1991). Signals mediated by CD19 and the BCR are essential for the development, activation and differentiation of B lymphocytes (Matsumoto et al., 1991). Mutations in CD19 are associated with some immunodeificiency or lead to development of autoimmune diseases such as SLE (systemic lupus erythematosus) and ANCA (Anti-neutrophil cytoplasmic antibodies; Culton et al., 2007).

CD21 is also known as C3d receptor (C3dR) or complement receptor type 2 (CR2). Its expression first appears in the stage of T1 cells, which express only small amounts of CD21 and the expression increases during subsequent maturation of B cells. MZ B cells have especially high density of CD21 molecules on their surface. During development of plasma cell CD21 expression is down-regulated. CD21 is as a part of the BCR signaling complex acts as ligand binding C3d (g) fragment of complement and thus is involved for activation of B cells (Matsumoto et al., 1991).

The membrane glycoprotein CD24 is expressed on most developmental stages of B lymphocyte and as in the case of other CD molecules its expression is down-regulated during B cell differentiation. CD24 molecule transmits signals that modulate the response of B cells to activation signals (Kay et al., 1991) and can induce the apoptosis (Suzuki et al., 2001). CD24 polymorphism is associated with susceptibility to multiple sclerosis (MS, Zhou et al., 2003).

CD27 molecule belongs to the family of TNF (tumor necrosis factor) receptors. B cells begin to express the CD27 receptor during their development in germinal centers and continue its expression to the final stages of differentiation. Ligand of CD27 is CD70 molecule expressed on T lymphocytes. Signaling through CD27 on memory B cells stimulates their differentiation to plasma cells (Agematsu et al., 1999). As it was mentioned this molecule represents a hallmark of classical memory B cells (Klein et al., 1998).

CD38 is expressed on B lymphocytes especially during their early differentiation and activation. The high density of surface CD38 is characteristic mainly for plasma cells (Medina et al., 2002). This molecule functions as surface ligand for CD21 and also as an enzyme catalyzing a number of cellular responses negatively and possitively regulating (according to the environment) the activation and proliferation of cells (Jackson and Bell, 1990). Signaling through CD38 may prevent apoptosis of B cells in germinal center. In contrast, CD38 signaling in the bone marrow leads to inhibition of proliferation and subsequent apoptosis precursor B cells (Shubinsky and Schlesinger, 1997). Because the molecule CD38 can act as an enzyme and a surface receptor, and it was detected also in the nuclear membrane, not all aspects of functions of this glycoprotein are still fully understood (Jackson and Bell, 1990).

2.2.5. Autoreactive B cells

Tolerance to self-antigens is critical in preventing autoimmunity in the organism. A significant part of the B cell repertoire is autoreactive and elimination of autoreactive B cells is mediated by negative selection based on clonal deletion, receptor editing and anergy in BM and by competitive elimination in periphery (Wardemann et al., 2003). B cells expressing self-reactive BCRs that occure in peripheral blood belong to either to short-lived plasma cells, isotype switched memory cells or long-lived plasma cells (Hoyer et al., 2004).

Autoreactive B cells are typical and major source of autoantibodies and they play other important pathogenetic roles like presenting of autoantigens (autoAg) to T cells and secreting proinflammatory cytokines. These B cells are crucial cells for initiation and promotion of autoimmune diseases such as SLE, RA (Sfikakis et al., 2005; Edwards et al., 2004). Research on autoreactive B cells is recently becoming more important since these cells may offer the possibility of highly specific and effective treatment of autoimmune disorders (Sfikakis et al., 2005).

The prototype autoAb produced by autoreactive B cells is represented by rheumatoid factor (RF) characteristic for RA.
RF is an antibody against the Fc portion of self IgG. RF is not only specific for RA it can be found in the patients with SjS, SLE, chronic infectious diseases (tuberculosis, syphilis, infectious mononucleosis, hepatitis, AIDS, etc.) or after vaccination (Symmons et al., 1993). It healthy individuals, RFs have probably only physiological role in the removal of IgG bound in immune complexes (Jarvis et al., 1993). In healthy controls RF represent low affinity IgM while in patients with RA predominates high affinity form of IgG isotype (Jarvis et al., 1993).

In sera of RA patients The RA have been described by a number of other autoantibodies: anti-keratin (AKA), anti-perinuclear factors (APF), anti-cyclic citrullinated peptide, etc. (anti-CCP; Youinou et al., 1985; Janssens et al., 1988; Shellekens et al., 2000).

Other typical autoantibodies are represented by anti-dsDNA autoAbs that recongnize self double-stranded DNA and are characteristic for patients with SLE. In these peripheral blood B cells are found high titres of anti-dsDNA autoAbs. Decreased frequencies of such B cells possitively correlate with disease activity and V_H segments used in their variable regions of heavy Ig chains are predominantly encoded by V_H4-34 gene segment (Odendahl et al., 2000).

2.3. Common variable immunodeficiency

Immunodefincies are disorders of the immune system characterized by inadequate immune response after antigenic stimulus and typical clinical manifestation is primarily increased susceptibility to the infectious agents. Immunodeficiency can be divided into primary and secondary, the most common primary immunodeficiency is common variable immunodeficiency (CVID). The onset of primary immunodeficiences occurrs mainly in the second year of life, however CVID is usually diagnosed after the 65th year of the life (Conley et al., 1999; Warnatz and Schleiser, 2008).

The prevalence of CVID is about 1: 10000 – 50000 and its incidence is the same for men and women (Cunningham-Rundles et al., 1999). Surprisingly this disease is rare among Asian populations where the prevalence is 1: 2000000 (Hayakawa et al., 1981).

The clinical manifestations in almost all patients include an increased susceptibility to bacterial infections of the upper and lower respiratory tract caused by different types of microbes (Streptococcus pneumoniae, Haemophilus influenzae, Staphylococcus aureus, etc.), recurrent bronchitis or frequent infections of gastrointestinal tract (Di Renzo et al., 2004).

CVID is further characterized by low concentrations of serum immunoglobulins (IgG, IgA, the concentration of IgM is variable, Conley et al., 1999). Various abnormalities in the function or in the representation of immunocompetent cells, the primary defect in the function of dendritic cells, changes in representation and function of T and B lymphocytes have

been indetified in patients with CVID (Warnatz et al., 2002; Vlková et al., 2010).

This disease in about 22% of patients is accompanied by several autoimmune diseases (RA, SLE, etc.) which also exacerbate the severity of the disease. The most commonly are attacked by autoantibodies against platelets, red cells and sometimes neutrophils. Treatment of CVID patient with autoimmunity is complicated since the commonly used immunosuppressive agents further reduces function of already deficient immune system, and thus actually increase the risk of infection (Di Renzo et al., 2004).

The ethiopathogenesis of CVID is not clear, only in up to 5% patients certain genetic defects were identified and heritability of the diseases reaches only 20%. Mutations in genes coding for TACI (transmembrane activator and CAML interactor, calcium-modulating ligand; Salzer et al., 2005), CD19 (differentiation marker of B cell lines; van Zelm et al., 2006; Grimbacher et al., 2003), ICOS (inducible co-stimulator molecule) and BAFF receptor (B cell activating factor; Losi et al., 2005) were observed in some patients. In adition, association between particular alelles for human leukocyte antigens (HLA) such as HLA-DQ/DR (especially HLA-DQB1/DRA region) and HLA-A and predisposition to CVID was found (Kralovicova et al., 2003).

2.4. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune disease affecting up to 1% of world population being 2.5 times more frequent in women than in men, and accompanied by symmetrical inflammation and destruction of joints and synovial membrane proliferation (Lawrence et al., 1998).

The first evidence of genetic predisposition to autoimmune diseases has been made just in this disease. Epidemiological analysis of twins showed 15-30% concordance for monozygotic twins and only 5-7% in dizygotic twins (Wordsworth and Bell, 1992). Predisposition to RA is thus polygenic and the number of genes are unlikely to be large. The first

confirmed was the association between RA and HLA II genes in patients of the Caucasian population (people of European origin), namely with HLA-DR1 and HLA-DR4 genes, in particular with DRB1*0101, DRB1*0404 and DRB1*0401 alleles (Stastny 1978, Weyand et al., 1992).

Since up to 70% of RA patients is HLA DR4 positive and the frequency of this gene is 20-25% in the healthy population, this association is not absolute (Deighton et al., 1989). Therefore other non-HLA genes such as genes coding for immunoglobulins (Ig) or cytokines will be included into pathogenesis of RA. Thus for instance, G/A substitutions have been described in promoter of TNF-α gene at positions -308 and -238, and the correlation between the degree of joint destruction and the genotype -238GG was found (Kaijzel et al., 1998). Another example is the allele -1082A in the gene coding for IL-10, which was shown to be associated with lower production of IL-10 in vitro, whereas the -1082G allele was associated with higher production of this cytokine. In this regard, the contribution of IL-10 to the development of RA will be dependent on the level of its production, which is given by the specific genotype (Turner et al., 1997).

The prototypic autoantibody for RA is rheumatoid factor (RF) recognizing the Fc part of self IgGs (Waaler 1940). In healthy individuals the RF levels are low and predominantly of IgM isotype with low affinity whereas in patients with RA are of high affinity and IgG isotope, and they occur in periphery at high titers (Soltys et al. 1997).

Although the nature of the contribution of B cells to the pathogenesis of RA is unknown, their participation in the development of the disease was demonstrated by therapeutic effect of anti-CD20 therapy, eliminating $CD20^+$ B cells (Edwards et al., 2001). In addition, it was shown that B cells directly infiltrate the synovial membrane of rheumatic joints in RA, they form so-called ectopic (pseudo-) germinal centers, mature into plasma cells and activate T cells (Kim et al., 1999, Takemura et al., 2001).

3. Aims of the thesis

The aim of this thesis, which is also part of the current project of grant IGA MZ CR NT/11414-3 was to characterize $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{+/-}$ B cell populations at the molecular level by analyzing of genes for the variable regions of immunoglobulin heavy chain. The individual B cells of interest from patients with CVID, RA and controls were analyzed and following parametres were investigated:

1) Analysis of genes for the variable regions of immunoglobulin heavy chain focused on:
   a) the mutation frequency (stimulation of antigen)
   b) the usage of specific V_H, D_H, J_H gene segments
   c) the length of CDR3 regions
   d) nucleotide and amino acid composition of the CDR3 region (clonal relationship of B lymphocytes)
2) Analysis of isotype rearrangement
3) Comparison of molecular characteristics of these populations in CVID patients, healthy donors and RA patients

4. Materials and methods

Peripheral blood processing and preparation of cell suspension for flow cytometry were performed in the laboratories of Children's Hematology and Oncology Clinic, FN Motol (Prague). Cell sorting of studied B cell populations in peripheral blood using flow cytometer with sorting module FACS Aria (Becton, Dickinson and Company, USA) were also carried out in the laboratories of Children's Hematology and Oncology Clinic, FN Motol.

4.1. Peripheral blood processing

Peripheral blood samples were collected from 3 CVID patients, 2 healthy individuals and 1 RA patient. They were fully informed on the procedure and signed an informed consent. Peripheral blood mononuclear cells (PBMCs) were separated using centrifugation in density gradient of Ficoll-Paque (Pharmacia, Uppsala, Sweden) and used for flow cytometry as well as for sorting of individual B cells of interest.

In detail, peripheral blood was taken into blood collecting tube containing K_2EDTA and diluted with1x PBS (pH 7.2 – 7.4) in ratio 1:1 (9ml of blood with 9ml 1x PBS).The diluted blood sample was carefully layered on Ficoll-Paque in 50ml test tube (BD Biosciences, Le Pont De Claix, France) and centrifuged at 400 x g for 30 minutes at 10°C. The ring consisted of lymphocytes, monocytes and immature granulocytes was transferred to a clean 50 ml centrifuge tube (BD Biosciences) using a Pasteur pipette. Further, 5ml of 1% albumine (Calbiochem, San Diego, USA) in 1x PBS was added to the lymphocytes in the test tube. The sample was centrifuged at 190 x g at 10°C for 10 minutes. The supernatat was removed, the sediment was resuspended in 4ml of 1x PBS with 1% albumine (Calbiochem) repeatedly spinned at 190 x g at 10°C for 10 minutes. Supernatant was further resuspended in volumes of 100µl and stained for flow cytometry or cell sorting.

4.2. Cell staining for flow cytometry and single-cell sorting

Staining of cell suspension for flow cytometry and single-cell sorting was performed in the laboratories of Children's Hematology and Oncology Clinic, FN Motol. PBMC suspension of 100µl was incubated in dark at 4°C with selected mouse monoclonal antibodies conjugated with fluorochromes against surface antigens on human B lymphocytes (Tab. 1.). It concerned antibodies anti-CD27 Pacific Blue, LT27 clone (Exbio Praha a.s., Czech Republic); anti-CD38 Alexa Fluor 700, HIT2 clone (Exbio); anti-human IgM FITC, G20-127 clone (BD Pharmingen, San Jose, USA); anti-CD21 APC, B-ly4 clone (BD Pharmingen), anti-CD24 PE, ALP9 clone (Immunotech, Marseille, France); anti-CD19 PC7, clone J3-119 (Immunotech).

One sample was always stained with a mixture of mentioned monoclonal antibodies. The content of particular antigens is shown in Tab. 1.

antibodies	volume of antibodies
IgMFITC, CD21APC, CD24PE, CD19PC7, CD27Pacific Blue, CD38Alexa Fluor 700	20/20/20/10/4/2

Table 1. The volume of monoclonal antibodies used to staining of selected populations of B cells

Compensation controls and controls for particular monoclonal antigens used for setting of flow cytometer before the actual measuring and/or sorting were included in staining protocols.

After staining the cell suspension was washed two times in 1x PBS (pH 7.2 – 7.4) and centrifuged at 190 x g at 10°C for 10 minutes. The suspension was then transferred into 100µl of WSB (wash and staining buffer) solution.

Measurement and frequency of particular B cell population in peripheral blood perfomed flow cytometer with sorting module FACS Aria (Becton, Dickinson and Company, NJ, USA) in the laboratories of Children's Hematology and Oncology Clinic, FN Motol. Sorted B cells were subsequenly used for Ig-specific RT-PCR.

The gating method of B cell populations of interest is shown in Fig. 7.

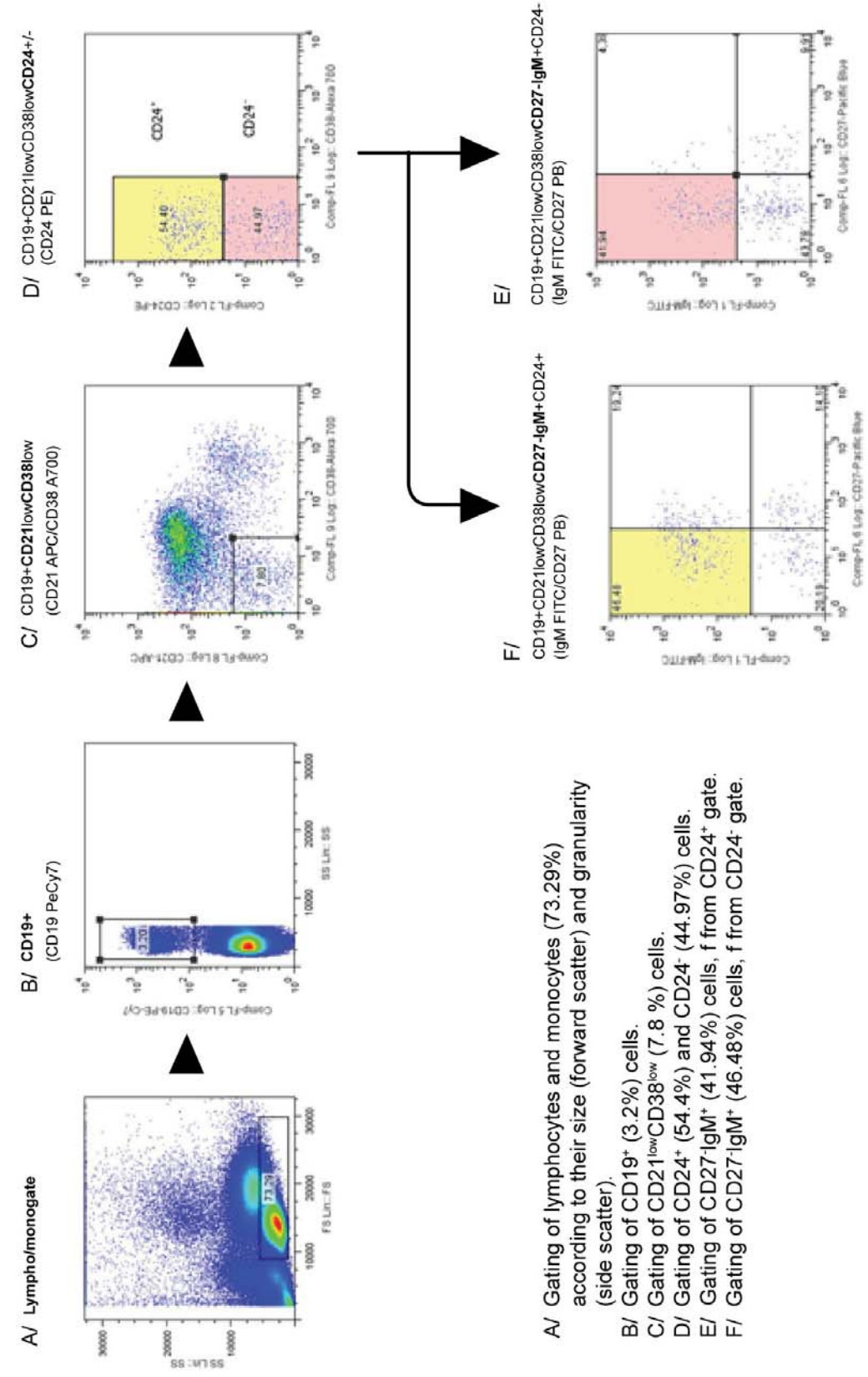


Figure. 7: Example of B cell sorting by flow cytometry

4.3. Single cell RT-PCR

B lymphocytes of both analyzed populations were sorted using flow cytometer with sorting module (FACS Aria) into the 96-well plates (BD Biosciences, Le Pont de Claix, France) so that each well contained exactly one single cell. Each well contained 50µl RT-PCR Lysis Buffer (Tab. 2.).

Synthesis of the first-strand cDNA synthesis was initiated immediately after cell sorting in PCR cycler (Bio-Rad, Hercules, USA) under the conditions shown in Fig. 8.

	volume (µl / well)	final concentration
H_2O	29,5	-
0.1 M DTT	2.5	5mM
RNasin (40U/µl)	0.5	20U
50nM Spermidin	0.5	0.5nM
10µg µl BSA	0.05	0.5µg
800ng/µl Oligo dT	0.45	400ng
10% Triton X	5.0	1%
5x RT PCR buffer	10.0	1x
10mM dNTPs	1.0	0.2mM
reverse transcriptase	0.5	

Table 2. Composition of lysing buffer for RT-PCR

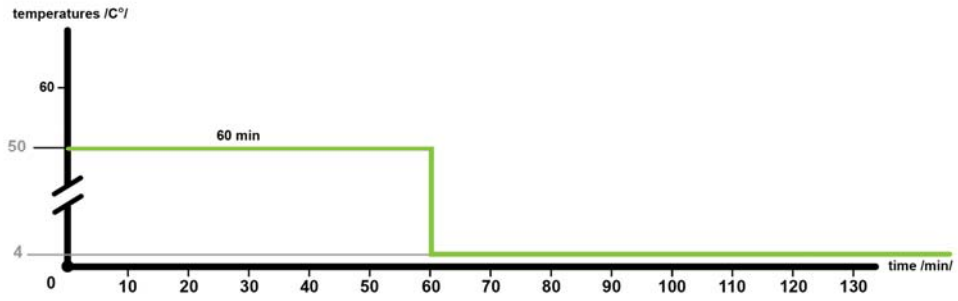


Figure 8. Profile of the first-strand c DNA sythesis

4.4. Amplification of genes for variable regions of immunoglobulin heavy chain

Rearranged $V_HD_HJ_H$ segments were amplified by nested PCR as decribed (Ruzickova et al., 2002).

4.4.1. Amplification by external primers

For the external PCR was used a mixture of primers (Invitrogen Corporation, Carlsbad, USA) for the so-called signal (leader) sequence of variable gene segments (SVH1-SVH6) together with one of the primers for the constant region of Ig genes (mju or GA, specific either IgM or IgG, respectively; Küppers et al., 1993).

SVH1 5´- CCA TGG ACT GGA CCT GGA - 3´
SVH2 5´- ATG GAC ACA CTT TGC TMC AC - 3´
SVH3 5´- CCA TGG AGT TTG GGC TGA GC - 3´
SVH4 5´- ATG AAA CAC CTG TGG TTC TT - 3´
SVH5 5´- ATG GGG TCA ACC GCC ATC CT - 3´
SVH6 5´- ATG TCT GTC TCC TTC CTC AT - 3´
Mju 5´- TCA GGA CTG ATG GGA AGC CC – 3´
Ga 5´- CAG GCC GCT GGT CAG AGC G - 3´

A total of 75µl of the prepared reaction mixture, 5µl cDNA and a drop of mineral oil (Top-Bio, Prague, Czech Republic) was added to each well of the 96-well plates (BD Biosciences). The final volume of each well for the external PCR was 80µl (Tab. 3.).

Amplification reaction was performed in PCR cycler (Bio-Rad) in the steps shown in Fig. 9.

	volume (µl/well)	final concentration
H_2O	52.98	-
25mM $MgCl_2$	8	2.5mM
100µM SVH1	0.56	0.7µM
100µM SVH2	0.56	0.7µM
100µM SVH3	0.56	0.7µM
100µM SVH4	0.56	0.7µM
100µM SVH5	0.56	0.7µM
100µM SVH6	0.56	0.7µM
100µM Mju/(Ga)	0.56	0.7µM
10mM dNTPs	1.6	0.2mM
10x PCR buffer	8	1x
Taq polymerase (5U/µl)	0.5	2,5 U/well

Table 3. The reaction mixture for the external PCR

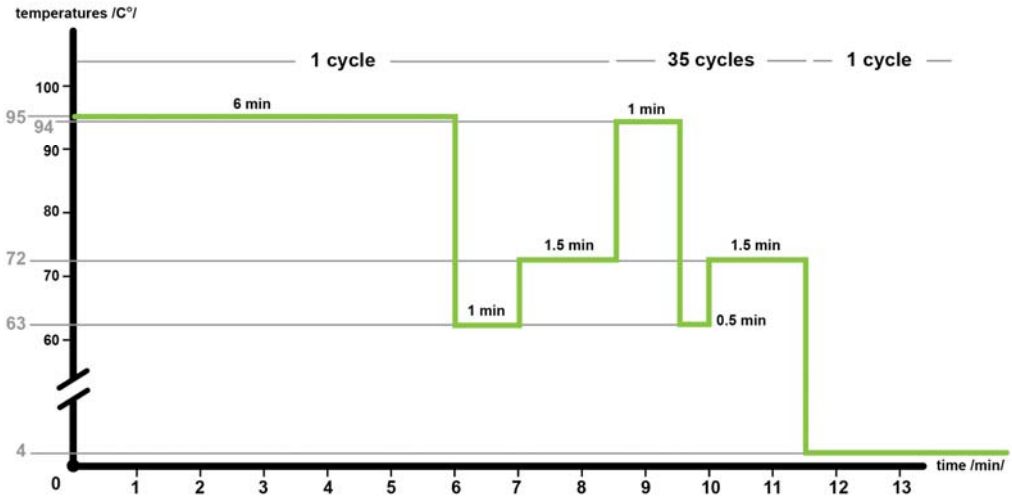


Figure 9. PCR profile for amplification by external primers

4.4.2. Amplification by internal primers

Internal PCR reaction was performed separately for each of 6 families using one primer (Invitrogen Corporation) specific to a particular area of V_H FR1 segment of one V_H family (i.e. for each family separately) in combination with constitution of primers for all possible J_H segment (Küppers et al., 1993).

iVH1 FRI	5´- CCT CAG TGA AGG TYT CCT GCA AGG C - 3´
iVH2 FRI	5´- GTC CTG CGC TGG TGA AAC CCA CAS A - 3´
iVH3 FRI	5´- GGG GTC CCT GAG ACT CTC CTG TGC AG - 3´
iVH4 FRI	5´- GAC CCT GTC CCT CAC CTG CRC TGT C - 3´
iVH5 FRI	5´- AAA AAG CCC GGG GAG TCT CTG ARG A - 3´
iVH6 FRI	5´- CTC ACT CAC CTG TGC CAT CTC CGG - 3´
iJH 1/3/4/5	5´- CGA CGG TGA CCA GGG TBC CYT GGC C - 3´
iJH 2,5	5´- CGA CAG TGA CCA GGG TGC AC GGC C - 3´
iJH6	5´- CGA CGG TGA CCG TGG TCC CTT GCC - 3´

A total of 75µl of the prepared reaction mixture, 5µl cDNA and a drop of mineral oil (Top-Bio, Prague, Czech Republic) was added to each well of the 96-well plates (BD Biosciences). The final volume of each reaction for the internal PCR was 80 µl (Tab. 4.).

Amplification reaction performed in PCR cycler (Bio-Rad) in the following steps shown in Fig. 10.

	volume (µl/well)	final concentration
H_2O	54.98	-
25mM $MgCl_2$	8	2.5mM
10mM dNTPs	1.6	0.2mM
100µM iVH primer	0.48	0.6µM
100µM iJH 6 primer	0.48	0.6µM
100µM iJH2/5 primer	0.48	0.6µM
100µM iJH1/3/4/5 primer	0.48	0.6µM
10x PCR buffer	8	1x
Taq polymerase (5U/µl)	0.5	2.5 U/well

Table 4. The reaction mixture for the internal PCR

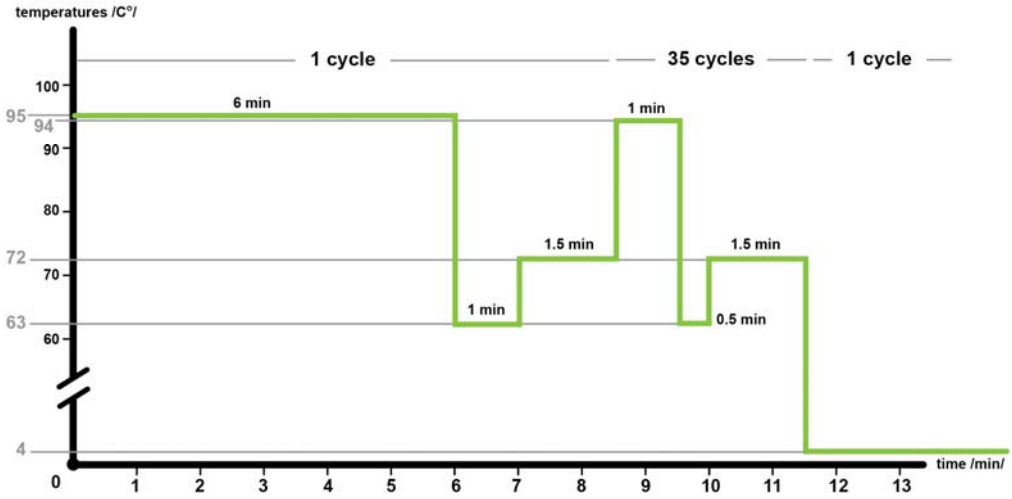


Figure 10. PCR profile for amplification by internal primers

4.5. Monitoring of presence of specific PCR product

The presence of PCR producted was screened using electrophoresis in 1.5% agarose gel. Appropriate amount of agarose powder was dissolved in amount of 1x TBE and than boiled in a microwave. Ethidium bromide (final concentration 0.01µg/ml gel, Sigma, St. Louis, USA) was added to cooled gel and poured onto electrophroretic gel tray (Bio-Rad). Total of 20µl of PCR product with 5µl of SLB (Sigma Loading Bufer, Sigma) was loaded in ge where the PCR products were separated. As a marker of molecular weight of 4µl standard DNA Molecular Weight Marker XIV ladder (Roche Diagnostics GmBH, Manhein, Germany) with 1µl SLB (Sigma) was loaded into gel. The PCR products were separated at 80V

for 75 minutes. DNA bands were visualized with UV lightbox using transluminator (Ultra-Lum, Claremont, Canada) and photographed with a camera (Uvitec, Cambridge, UK). In the case of possiting the remaining 60µl of PCR products was used for purification (see later).

4.6. Purification of PCR products

Purification of PCR products from agarose gel was performed using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) using the same electrophoretic conditions.

PCR products were loaded into every second well to avoid cross-contamination of PCR products.

Each band of about 280bp were cut out from the gel using a scalpel and placed in a 1.5ml tube. We added 600µl of QG Buffer into tubes and incubated then for 10 minutes at 50°C in a water bath until the gel still was completely dissolved.

After that, 200µl of isopropanol (Penta, Chrudim, Czech Republic) was added into each tube and the tubes were vortexed (Janke & Kunkel, Staufen, Germany). Part of the tube content was poured onto a spin column (special collection tube) and spinned on microcentrifuge (Beckman Coulter, Fullerton, USA) at 11.000 x g for 1 minute. Tube was taken out of spin cuvette and the solution was removed. The second part ot the tube volume was processed in the same way. 500µl of QG buffer was loaded onto tibes with columm spinned at 11.000 x g for 1 minute and the tube content was removed. Further, 750µl of PE-WASH buffer with ethanol (Penta, Chrudim, Czech Republic) was added and tubes with columm were incubated for 5 minutes at room temperature (RT). After that, these tubes were two times spinned and the tube contents were removed. Tubes with columms were transferred into special collection tubes with screw caps and poured with 30µl of EB buffer incubated for 1 minute at RT and then PCR product from columm was eluted by centrifugation at 18.000 x g for 1 minute. The purified PCR products were further used for sequencing.

4.7. Sequencing of PCR products

For cycle sequencing of PCR products (10µl), we used the Big Dye Terminator Cycle Sequencing Kit version 1.1 (Applied Biosystems, Foster City, USA). Prepared reaction mixture and 5µl of purified PCR product was added into 96-well plate (BD Biosciences; Tab. 5.).

Sequencing reactions were performed in PCR cycler (Bio-Rad) under the following conditions shown in Fig.11.

	volume (µl/well)
H_2O	2µl
Big Dye buffer	2µl
5´ primer (1 : 20)	1µl

Table 5. The reaction mixture for cycle sequencing

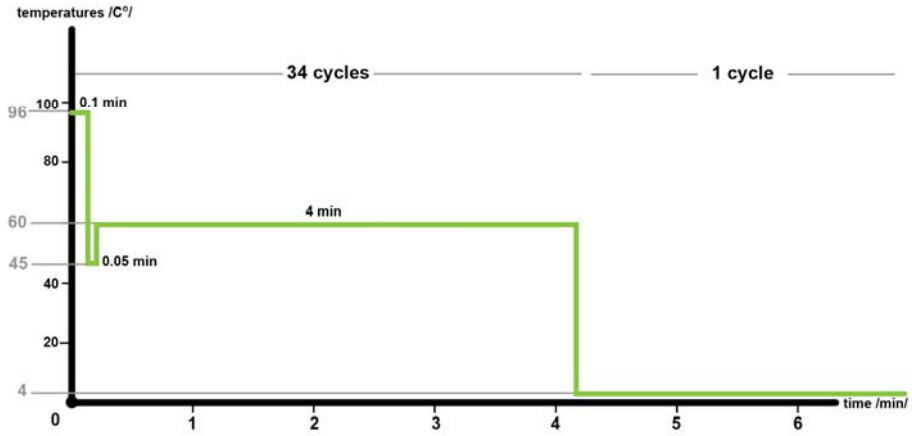


Figure 11. Profile of sequencing of PCR product

4.8. Ethanol precipitation of sequencing product

Prepared precipitation mixture of the total volume of 350µl and 10µl of sequencing product were added 1.5ml tubes (Tab. 6.).

	volume (µl/sample)
H_2O	90µl
3 M NaAc (pH 5.2)	10µl
100 % EtOH	250µl

Table 6. Precipitation mixture

The samples were vortexed and spinned at 18.000 x g for 18 minutes. The supernatant was carefully removed and 350µl of 70% ethanol (Penta) was added to the sediment. Sample was spinned at 18.000 x g for 8 minutes and the sediment was left to dry in the laminar box (Flow Laboratories). After complete evaporation of EtOH, all samples were centrifuged at 18.000 x g for 10 minutes.

4.9. Sequencing analysis

Sequencing was carried out at the Centre for DNA Sequencing at the Institute of Microbiology ASCR (www.biomed.cas.cz/mbu/lab119/index.htm) using the ABI PRISM 3130xl sequencer (AME Bioscience, Toroed, Norway).

The overview of all laboratory steps is summarized in scheme in Fig. 12.

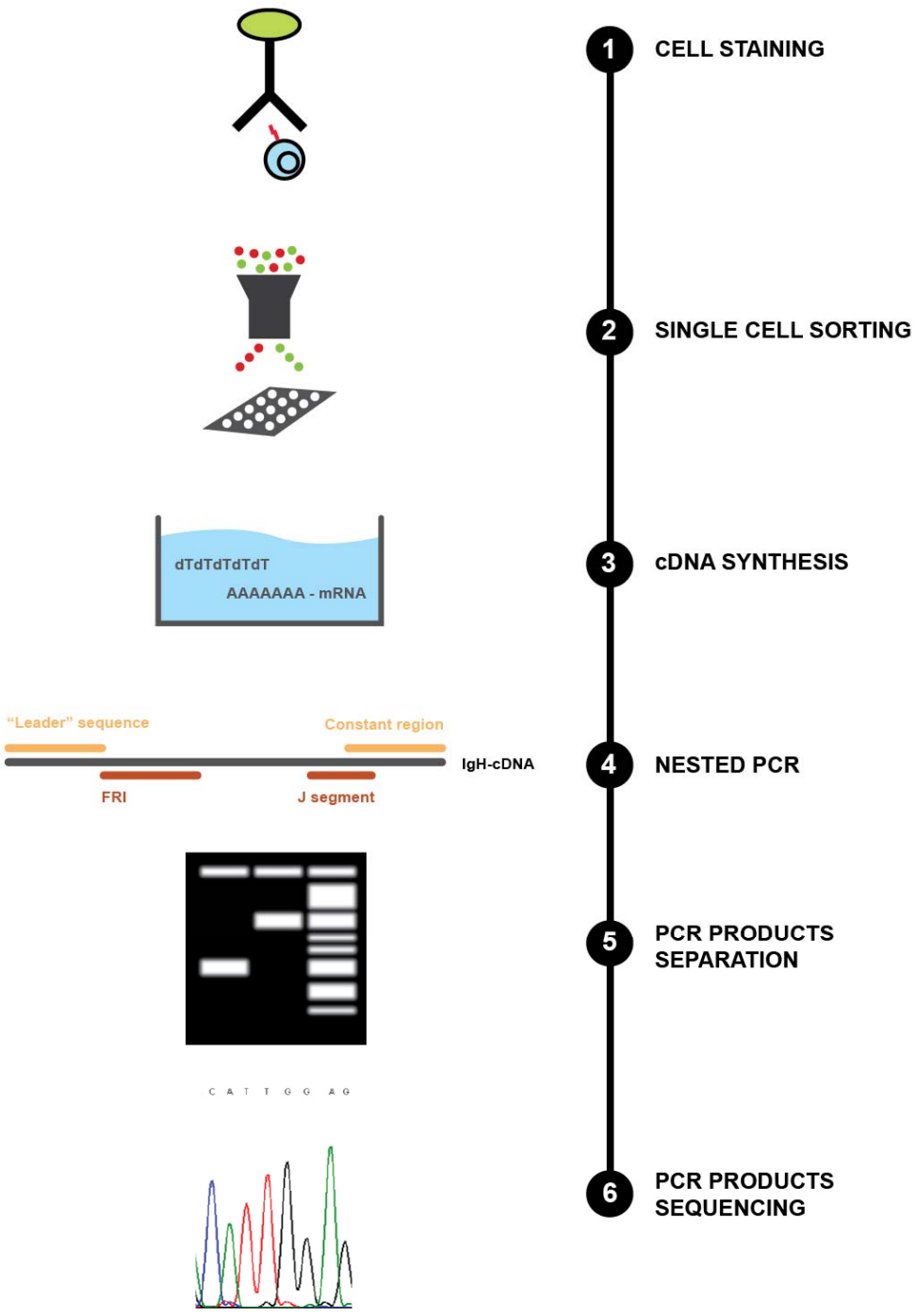


Figure 12. Scheme of the approach

4.10. Evaluation and analysis of rearrangement of V_H, D_H and J_H genes

The sequences of $V_HD_HJ_H$ rearrangement were compared to germline (non-mutanted) sequences, which are accessible in the database V-BASE (vbase.mrc-cpe.cam.ac.uk). Based on this comparison the mutations were identified and the mutation frequency (mf) of $V_HD_HJ_H$ rearrangements was calculated.

mf (%) = n/ N x 100

(n = number of substituted nucleotides in the V_H region of the transcript, N = total length of the V_H region of the detected transcript)

By submitting of our sequences to the V-BASE, particular used V_H, D_H and J_H gene segments, sequence of CDR1 - CDR3 regions and FR2 and FR3 regions were identified.

Nucleotide sequence of CDR3 regions was translated into amino acid sequence using translate tool of ExPASy Proteomics Server (Swiss Institute of Bioinformatics, available at http://expasy.org/tools/dna.html).

4.11. Statistical analysis

For statistical analysis of results Fisher's exact test and nonparametric Mann-Whitney test and GraphPad software, version 3.1 were used (GraphPad, San Diego, USA).

4.12. List of chemicals and equipment

4.12.1. Chemicals and kits

Albumine	Calbiochem, San Diego, USA
Agarose	Top-Bio, Praha, Czech republic
Big Dye Terminator Cycle Sequencing Kit, version 1.1	Applied Biosystems, Foster City, USA
BSA	Serva, Heidelberg, Germany
DNA Molecular Weight Marker XIV	Roche Diagnostics GmBH, Manhein, Germany
dNTPs 10 mM	Sigma, St. Louis, USA
DTT 0.1 M	Roche Diagnostics GmBH, Manhein, Germany
EB buffer	QIAGEN GmBH, Hilden, Germany
Ethanol	Penta, Chrudim, Czech Republic
Ethidium bromide	Sigma, St. Louis, USA
Ficoll-Paque	Pharmacia, Uppsala, Švédsko
Gelatine	Sigma, St. Louis, USA
Isopropylalcohol	Penta, Chrudim, Czech Republic
Mineral Oil	Top-Bio, Praha, Czech Republic
$MgCl_2$ 25 mM	Roche Diagnostics GmBH, Manhein, Germany
NaAc 3M	Sigma, St. Louis, USA
Oligo dT primer	Promega Corporation, Madison, USA
PCR buffer 10x	Roche Diagnostics GmBH, Manhein, Germany
PCR H_20	Top-Bio, Praha, Czech Republic
PE – WASH buffer	QIAGEN GmBH, Hilden, Germany
Primers for nested PCR 100μM	Invitrogen Corporation, Carlsbad,

	USA
PrimeScript buffer 5x	TaKaRa, Shiga, Japan
QG buffer	QIAGEN GmBH, Hilden, Germany
QIAquick Gel Extraction Kit	QIAGEN GmBH, Hilden, Germany
Reverse transcriptase	TaKaRa, Shiga, Japan
RNasin plus RNase inhibitor	Promega Corporation, Madison, USA
Sodium acetate (NaAC, 3M, pH 5,2)	Sigma, St. Louis, USA
Sodium azide	Sigma, St. Louis, USA
SLB	Sigma, St. Louis, USA
Spermidine 50 nM	Roche Diagnostics GmBH, Manhein, Germany
Taq polymerase	Roche Diagnostics GmBH, Manhein, Germany
Tris base ($C_4H_{11}NO_3$)	Serva, Heidelberg, Germany
Triton X-100	Serva, Heidelberg, Germany

4.12.2. Antibodies

BD Pharmingen, San Jose, USA

- Monoclonal Mouse Anti-Human IgMFITC, clone G20-127
- Monoclonal Mouse Anti-Human CD21APC, clone B-ly4

Immunotech, Marseille, France

- Monoclonal Mouse Anti-Human CD24PE, clone ALP9
- Monoclonal Mouse Anti-Human CD19PC7, clone J3-119

Exbio Praha a.s., Vestec, Czech republic

- Monoclonal Mouse Anti-Human CD27Pacific Blue, clone LT27
- Monoclonal Mouse Anti-Human CD38Alexa Fluor 700, clone HIT2

4.12.3. Solutions

1.5% agarose gel with ethidium bromide:

chemicals	amount	final concetration
agarose	6g	1.50%
1x TBE	400ml	-
ethidium bromide (1µg/ml)	0.04ml	0.01µg/ml

5x TBE:

chemicals	amount	final concetration
Tris base	54g	450mM
borid acid	27.5g	450mM
0.5M EDTA (pH 8)	20ml	10mM
H_2O	ad 1000 ml	-

1x TBE:

200ml 5x TBE + 800 ml H_2O

10x PBS:

chemicals	amount	final concetration
NaCl	80g	1.37M
KCl	2g	0.027M
Na_2HPO_4 . 12 H_2O	29g	0.081M
KH_2PO_4	2g	0.015M
H_2O	ad 1000 ml	-

1x PBS:

10 ml 10x PBS + 90 ml H_2O

WSB:

chemicals	amount	final concetration
gelatine	4ml	0.18%
sodium azide	1g	0.10%
1x PBS	ad 1000 ml	-

4.12.4. Instruments and equipment

Automatic Pipettes	Labnet, NJ, USA Gilson, Middleton, USA
Autosampler	Eppendorf, Hamburg, Germany
Centrifuge Microfuge 18	Beckman Coulter, Fullerton, USA
Centrifuge Alegra X-22R	Beckman Coulter, Fullerton, USA
96-well plates	BD Biosciences, Le Pont De Claix, France
Electrophoretic tank	Bio-Rad, Hercules, USA
Sartorius electronic weigh	Sartorius AG, Gottingem, Germany
CD camera with a therm printer	Uvitec, Cambridge, UK
Laminar flow box	Flow Laboratories Ltd, Surrey UK
Microcentrifuge	Hermle Labortechnik, GmbH, Wehingen,Germany
Microwave Goddess MOM 717	Goddess, Zlín, Czech Republic
Microtubes	Eppendorf, Hamburg, Germany
Multichannel pipette	Finnpipette, Vantan, Finland
PCR cycler	Bio-Rad, Hercules, USA
Flow cytometer with sorting module	Becton, Dickinson and Company, FACS Aria, Franklin Lakes, USA
Latex gloves	Semperit, Vienna, Austria
Sequencer ABI PRISM 3130xl	AME Bioscience, Toroed, Norway
PCR strips with caps	Eppendorf, Hamburg, Germany
Tips for automatic pipettes Eppendorf	Hamburg, Germany
UV Transilluminator	Ultra-Lum, Claremont, Canada
Water thermobath with thermostat	Dittmann, Bytom, Poland
Vortex	Janke& Kunkel, Staufen, Germany
Voltage source	Bio-Rad, Hercules, USA
Tubes 15 ml, 50 ml	BD Biosciences, Le Pont De Claix, France

5. Results

5.1. Analysis of the frequency of $IgM^+CD19^+CD27^-$ $CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, controls and RA patient using a flow cytometer

The frequency of B cell populations of $IgM^+CD19^+CD27^-$ $CD21^{low}CD38^{low}CD24^+$ (referred to CD24+) and $IgM^+CD19^+CD27^-$ $CD21^{low}CD38^{low}CD24^-$ (referred to CD24-) was measured using flow cytometry in 48 CVID patients, 51 healthy donors and 8 RA patients (Fig 13.). A total of 10 CVID patients were measured twice, in the range of at least 6 months, and measured values were used for statistical analysis alone. Measurements were performed in the Department of Pediatric Hematology and Oncology, FN Motol.

The frequency values that are then used for statistical analysis represent the percentage of the particular population from the gating scheme (Fig. 8.).

The highest frequency of these B cells was obtained in CVID patients (median 3.08% ± SE 1.27%). In COs and RA patient were the frequencies similar (0.98% ±0.17% and 0.68% ±0.33%; Fig. 13., Tab. 7.).

Statistically significant differences between CVID patients and healthy donors B cells (3.08% ± 1.27% vs. 0.98% ±0.17%, $P<0.0001^a$) and also between CVID patients and RA patient B cells (3.08% ± 1.27% vs. 0.68% ±0.33%, $P=0.0005^b$; Fig.13.) were found using the nonparametric Mann-Whitney test.

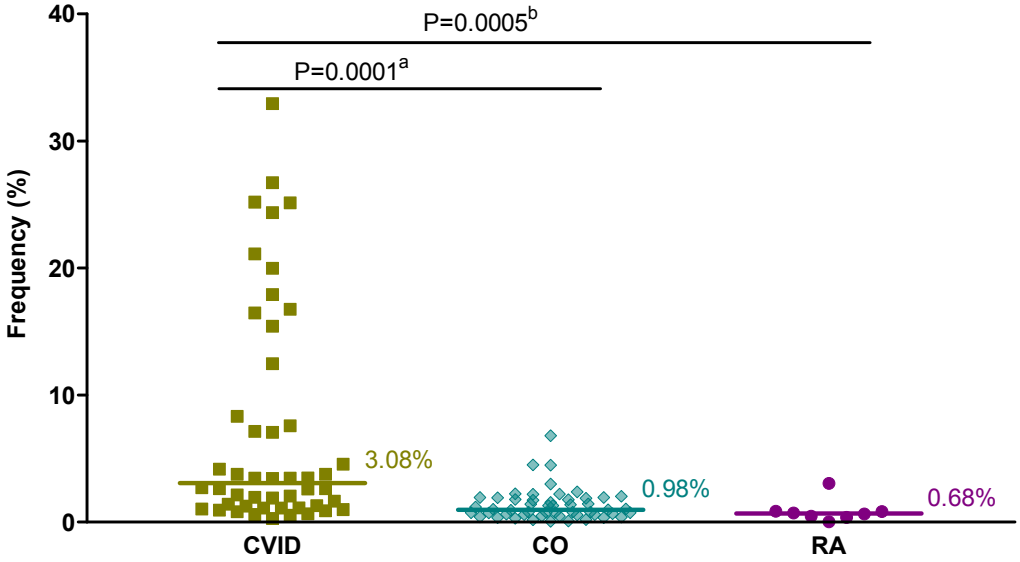


Figure 13. The frequency of $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{+/-}$ B cell populations in peripheral blood of CVID patients, controls and RA patients, medians and P values are indicated

patient	CVID	CO	RA
median ± SE (%) 95% CI	**3.08 ± 1.27[a]**	**0.98 ± 0.17 [ab]**	**0.68 ± 0.33 [b]**
minimum – maximum (%) 95% CI	4.72– 9.83	1.00 – 1.70	0.10 – 1.64

Table 7. The frequency of $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, controls and RA patients (SE = standard error, CI = confidence interval)

5.2. Analysis of genes for variable regions of immunoglobulin heavy chain of cell populations of the $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, controls and RA patient

A total of 96 B lymphocytes were sorted using flow cytometer with sorting module FACS Aria (Becton, Dickinson and Company) from all patients and controls. One half of this amount constituted $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{+}$ and the second $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{-}$ B cell populations.

5.2.1. Distribution of IgM and IgG mRNA transcripts

Both IgM and IgG transcripts were discovered in all of analyzed $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{+/-}$ B cells obtained from in CVID patients, COs and RA patient (Fig. 14., Tab. 8.).

The total of 39 transcripts was detected in the $CD24^{+}$ B lymphocytes of CVID patients, of which were found 37 (94.87%) was ofIgM and 2 (5.13%) of IgG isotype In a population of $CD24^{-}$ B cells from CVID patients, we detected the presence of a total of 34 transcripts, of which 30 (88.24%) was of IgM isotype and 4 (11.76%) of IgG isotype.

A total of 29 productive $V_HD_HJ_H$ rearrangements was obtained in $CD24^{+}$ B cells of healthy donors separated from B lymphocytes, of which 28 (96.55%) transcripts was of IgM isotype and 1 (3.45%) IgG isotype. In a population of $CD24^{-}$ B cells of healthy donors, we found 21 transcripts – 15 (71.43%) IgM-specific and 6 (28.57%) IgG-specific.

A total of 30 transcripts from the $CD24^{+}$ B cells from RA patients was obtained, including 29 (96.67%) of IgM isotype and 1 (3.33%) of IgG isotype. In $CD24^{-}$ B cells a total of 50 transcripts was detected; 49 (98.00%) and 1 (2.00%) of IgG isotype.

The significantly higher proportion of IgG transcripts present in the $CD24^{-}$ population of a healthy donor compared with their $CD24^{+}$ counterparts (3.45% vs. 28.57%, $P=0.0446^{a}$) was found using Fisher's exact test. Comparison of the frequency of IgG^{+} $CD24^{-}$ B cells of healthy individuals (28.57%) and patients with RA (2.00%) also reached statistical significance ($P=0.0061^{b}$).

In CVID patients all IgG+ B cells co-expressed IgM mRNA which was not detected in CO and in RA patient one of two IgG was expressed in the same cell as IgM.

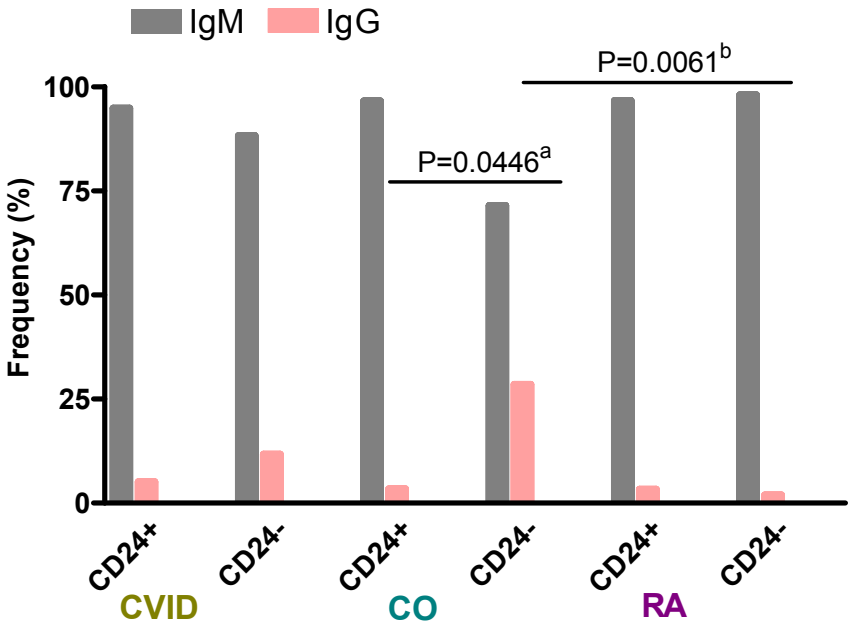


Figure 14. The frequency of IgM and IgG transcripts of the $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, controls and RA patients (nd = not determined segments), P values are indicated

patient	CVID		CO		RA	
population	$CD24^{+}$	$CD24^{-}$	$CD24^{+}$	$CD24^{-}$	$CD24^{+}$	$CD24^{-}$
IgM (%)	37 (94.87)	30 (88.24)	28 (96.55)	15 (71.43)	29 (96.67)	49 (98.00)
IgG (%)	2 (5.13)	4 (11.76)	**1 (3.45)[a]**	**6 (28.57)[ab]**	1 (3.33)	**1 (2.00)[b]**
total (%)	39 (100.00)	34 (100.00)	29 (100.00)	21 (100.00)	30 (100.00)	50 (100.00)

Table 8. The number and proportional representation of IgM and IgG transcripts of the $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, controls and RA patients (nd = not determined segments)

5.2.2. Analysis of $V_HD_HJ_H$ rearrangement in transcripts of IgM isotype

5.2.2.1. Distribution of V_H families

The distribution of V_H family members in Ig heavy chain mRNA transcripts derived from individual-sorted B cells from the patients with CVID, the healthy controls (COs) and the patient with RA reflected the germline complexity of the V_H families. Most of V_H families except the V_H6 and V_H7 were found to be productively rearranged.

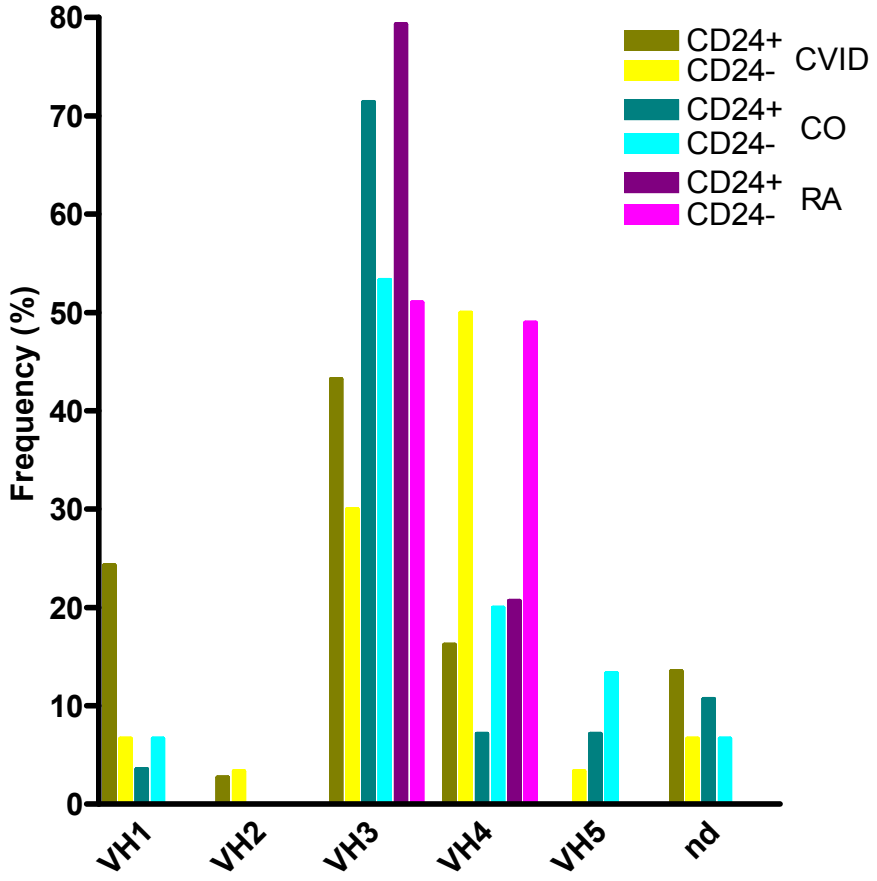
V_H1 – V_H4 families were found in the transcripts derived from the $CD24^+$ B lymphocytes of CVID patients. In the $CD24^-$ B lymphocytes were reported V_H members of V_H1 - V_H5 families. In both B cells population from COs were found members from V_H1, V_H3, V_H4 and V_H5 families. We found only two V_H gene families – V_H3 and V_H4 in RA patients. In healthy controls and also in RA patients were not identified any IgM transcripts containing segments of V_H2 family (Fig. 15., Tab. 9.).

In $CD24^+$ B cells of CVID patients the most frequent gene segments belonged to V_H3 (16 of 37 transcripts, 43.24%) and V_H1 families (9 of 37 transcripts, 24.23%). In $CD24^-$ B cells the most frequent gene segments were derived from V_H4 (15 of 30 transcripts, 50.00%) and V_H3 families (9 of 30 transcripts, 30.00%).

In the both of observed populations of COs was discovered also a predominance of gene segments of the V_H3 family (20 of 28 transcripts, 71.43% in the $CD24^+$ B cells and 8 of 15 transcipts, 53.33% in the $CD24^-$ B cells). Gene segments of V_H1, V_H4 and V_H5 families were detected much less frequent by than those ones of V_H3 family.

The similar situation was described in RA patient. The V_H3 family was the most frequent family with frequencies 23 of 29 transcripts (79.31%) in $CD24^+$ B cells and 25 of 49 transcripts (51.02%) in $CD24^-$ B cells.

Notably, the frequency of the V_H1 family members of the $CD24^+$ B cell differed significantly between patients with CVID and RA (24.32% vs. 0.00%, $P=0.0104^a$) as it was demonstrated using Fisher's exact test.



VH1.....CVID CD24+/RA CD24+.....P=0.0104[a]

Figure 15. The frequency of V_H families of the $IgM^+CD19^+CD27^-CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, controls and RA patients (nd = not determined segments), P values are indicated

	CVID		CO		RA	
V_H family	$CD24^+$	$CD24^-$	$CD24^+$	$CD24^-$	$CD24^+$	$CD24^-$
V_H1 (%)	**9 (24.32)[a]**	2 (6.67)	1 (3.57)	1 (6.67)	**0 (0.00)[a]**	0 (0.00)
V_H2 (%)	1 (2.70)	1 (3.33)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
V_H3 (%)	16 (43.24)	9 (30.00)	20 (71.43)	8 (53.33)	23 (79.31)	25 (51.02)
V_H4 (%)	6 (16.22)	15 (50.00)	2 (7.14)	3 (20.00)	6 (20.69)	24 (48.98)
V_H5 (%)	0 (0.00)	1 (3.33)	2 (7.14)	2 (13.33)	0 (0.00)	0 (0.00)
nd (%)	5 (13.51)	2 (6.67)	3 (10.71)	1 (6.67)	0 (0.00)	0 (0.00)
total (%)	37 (100.00)	30 (100.00)	28 (100.00)	15 (100.00)	29 (100.00)	49 (100.00)

Table 9. The number and proportional representation of V_H family members of the $IgM^+CD19^+CD27^-CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, controls and RA patients (nd = not determined segments)

5.2.2.2. Distribution of V_H gene segments

A spectrum of different V_H genes as detected in the Ig V_H transcripts obtained from $IgM^+CD19^+CD27^-CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, COs and RA patients (Fig. 16. – 18., Tab. 10.).

The most abundant V_H gene segments of the $CD24^+$ B lymphocytes of CVID patients were V_H3-23 (5 of 37 transcripts, 13.51%), V_H1-18, V_H1-2, V_H 3-30.5 and V_H4-34 (all – 3 of 37 transcripts, 8.11%). Noticable overpresentation of rearranged V_H4-34 was detected in the $CD24^-$ B cells (9 of 30 transcripts, 30.00%; Fig. 16.).

The most commonly used V_H gene segments in the $CD24^+$ B cells of COs were V_H3–23 (8 of 28 transcripts, 29.00%) and V_H3-7 (3 of 28 transcripts, 10.71%). V_H5-51 gene segment (2 of 15 transcripts, 13.33%) was found to be dominated by $CD24^-$ B cells. In none of CO's B cell populations was found V_H4-34 segment (Fig. 17.).

In contrast, in patient with RA this segment represented the most abundant V_H segment of $CD24^-$ subset (21 of 49 transcipts, 42.86%). In $CD24^+$ B lymphocytes was the most frequent V_H3-48 gene segment (14 of 29 transcripts, 48.28%; Fig. 18.).

Comparison of frequencies for V_H gene segments was performed using the Fisher's exact test. Statistically significant higher numbers of V_H3-11 and V_H 4-30.4 containig B cells were found in $CD24^+$ B cells of RA patient with this $CD24^-$ B cells (24.14% vs. 0.00%, P=0.0017 and 17.24% vs. 2.04%, P=0.0375). In contrast, significantly lower numbers of V_H 4-34 containing B cells were described in $CD24^+$ compared with $CD24^-$ B cells (3.45% vs. 42.86%, P=0.0030).

We found significant absence of V_H4-34 gene segment in $CD24^-$ in COs compared with CVID (0.00% vs. 30.00%, P=0.0463[k]) and RA (0% vs. 42.86%, P=0.0092[l], Tab. 10.).

The frequence of V_H3-48 (48.28%) gene segment from the $CD24^+$ B cells of RA patient was found to be significantly greater than in CVID patients (2.70%, P=0.0004[g]), as well as in COs (3.57%, P=0.0027[h]). Comparison of $CD24^-$ B cells of this segment was statistical significance

only between CVID and RA patinets (3.33% vs. 32.65%, P=0.0017[i]). The frequency of V_H3-11 gene was statistically higher in the population of $CD24^+$ B cells of RA patient (24.12%) compared with the same population of CVID patients (2.70%, P=0.0263[d]) and COs (0.00%, P=0.0153[e]). The last two significant differences were found fot the V_H3-23 segment between $CD24^+$ B cells of COs and RA patient (28.57% vs. 0.00%, P=0.0068[f]) and in the V_H4-30.4 segment between $CD24^+$ B cells of CVID and RA patiets (0.00% vs. 17.24%, P=0.0214[j]).

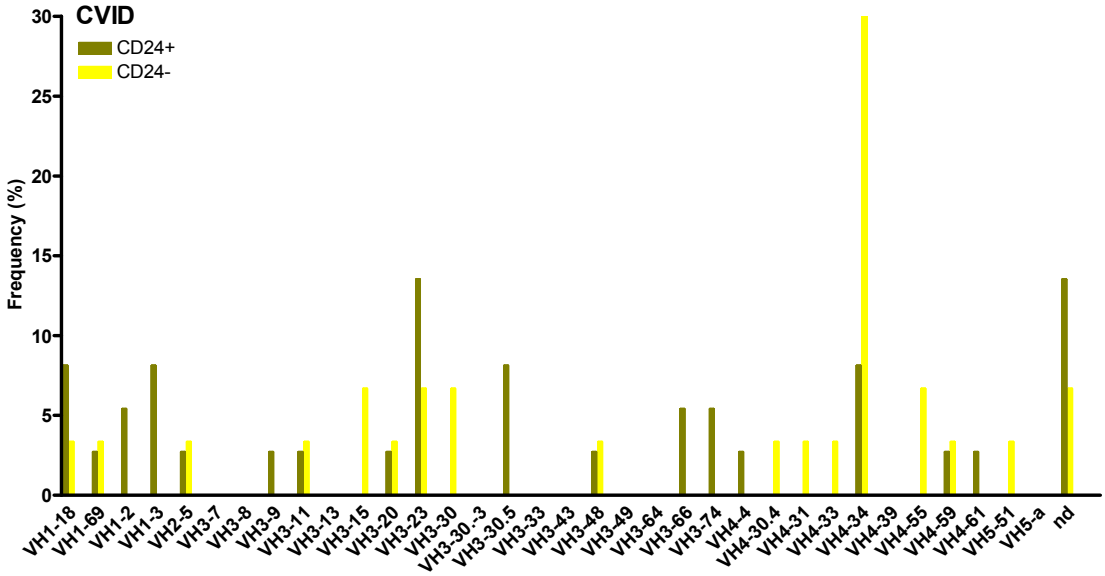


Figure 16. The frequency of V_H genes of $IgM^+CD19^+CD27^-CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients (nd = not determined segments)

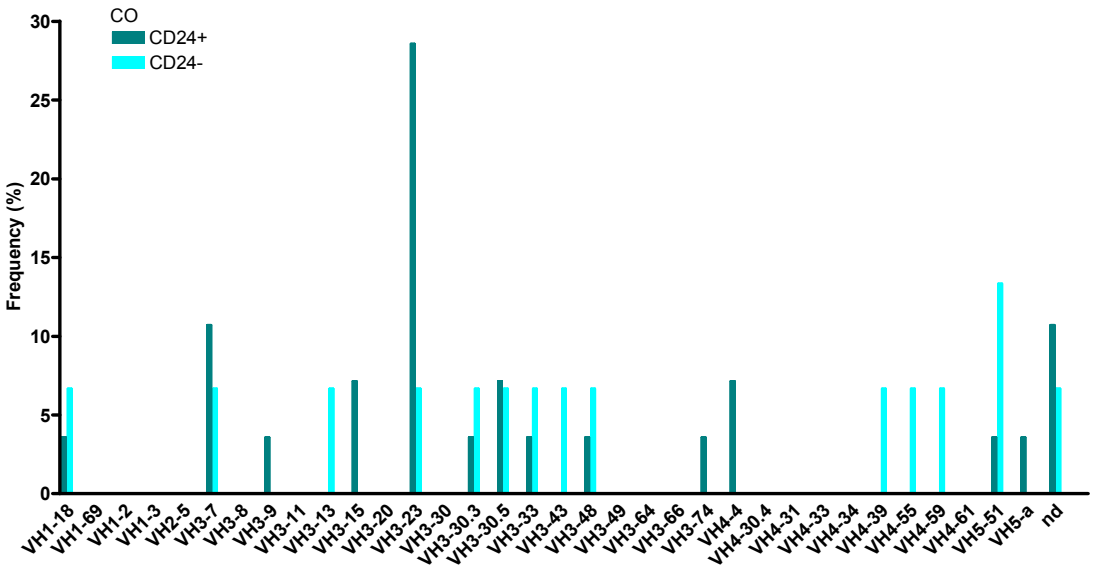
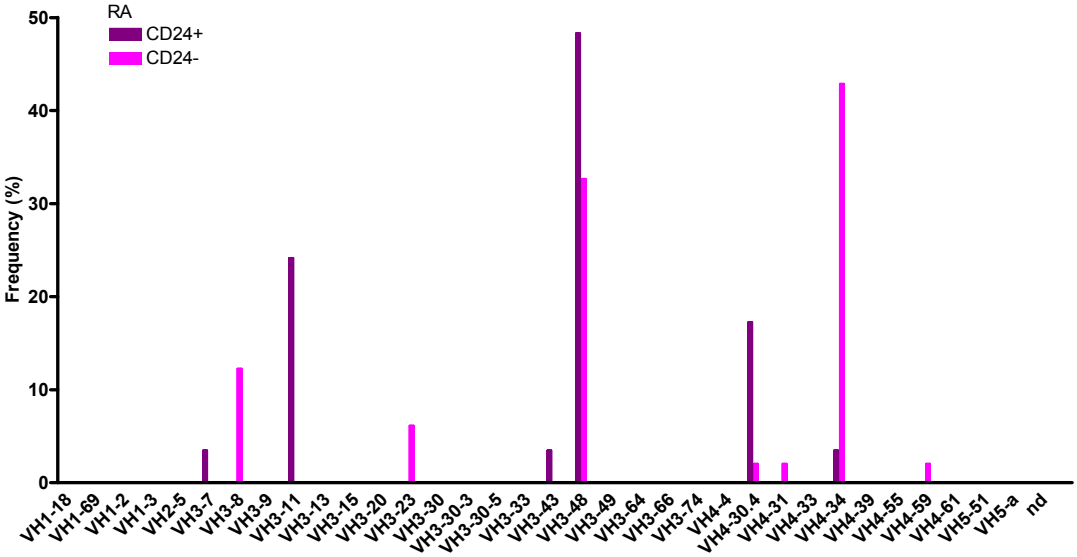


Figure 17. The frequency of V_H genes of $IgM^+CD19^+CD27^-CD21^{low}CD38^{low}CD24^{+/-}$ B cells in Controls, (nd = not determined segments), P values are indicated



VH3-11.....RA CD24+/CD24-.....P=0.0017[a]
VH4-30.4.....RA CD24+/CD24-.....P=0.0375[b]
VH4-34.....RA CD24+/CD24-.....P=0.0030[c]

Figure 18. The frequency of V_H genes of $IgM^+CD19^+CD27^-CD21^{low}CD38^{low}CD24^{+/-}$ B cells in RA patients, (nd = not determined segments), P values are indicated

	CVID		CO		RA	
V_H segments	$CD24^+$	$CD24^-$	$CD24^+$	$CD24^-$	$CD24^+$	$CD24^-$
V_H1-18 (%)	3 (8.11)	1 (3.33)	1 (3.57)	1 (6.67)	0 (0.00)	0 (0.00)
V_H1-69 (%)	1 (2.70)	1 (3.33)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
V_H1-2 (%)	2 (5.41)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
V_H1-3 (%)	3 (8.11)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
V_H2-5 (%)	1 (2.70)	1 (3.33)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
V_H3-7 (%)	0 (0.00)	0 (0.00)	3 (10.71)	1 (6.67)	1 (3.45)	0 (0.00)
V_H3-8 (%)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	6 (12.24)
V_H3-9 (%)	1 (2.70)	0 (0.00)	1 (3.57)	0 (0.00)	0 (0.00)	0 (0.00)
V_H3-11 (%)	**1 (2.70)**[d]	1 (3.33)	**0 (0.00)**[e]	0 (0.00)	**7 (24.14)**[ade]	0 (0.00)[a]
V_H3-13 (%)	0 (0.00)	0 (0.00)	0 (0.00)	1 (6.67)	0 (0.00)	0 (0.00)
V_H3-15 (%)	0 (0.00)	2 (6.67)	2 (6.67)	0 (0.00)	0 (0.00)	0 (0.00)
V_H3-20 (%)	1 (2.70)	1 (3.33)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
V_H3-23 (%)	5 (13.51)	2 (6.67)	**8 (28.57)**[f]	1 (6.67)	**0 (0.00)**[f]	3 (6.12)
V_H3-30 (%)	0 (0.00)	2 (6.67)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
V_H3-30-3 (%)	0 (0.00)	0 (0.00)	1 (3.57)	1 (6.67)	0 (0.00)	0 (0.00)
V_H3-30-5 (%)	3 (8.11)	0 (0.00)	2 (6.67)	1 (6.67)	0 (0.00)	0 (0.00)
V_H3-33 (%)	0 (0.00)	0 (0.00)	1 (3.57)	1 (6.67)	0 (0.00)	0 (0.00)
V_H3-43 (%)	0 (0.00)	0 (0.00)	0 (0.00)	1 (6.67)	1 (3.45)	0 (0.00)
V_H3-48 (%)	**1 (2.70)**[g]	**1 (3.33)**[i]	**1 (3.57)**[h]	1 (6.67)	**14 (48.28)**[gh]	16 (32.65)[i]
V_H3-49 (%)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
V_H3-64 (%)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
V_H3-66 (%)	2 (5.41)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
V_H3-74 (%)	2 (5.41)	0 (0.00)	1 (3.57)	0 (0.00)	0 (0.00)	0 (0.00)
V_H4-4 (%)	1 (2.70)	0 (0.00)	2 (6.67)	0 (0.00)	0 (0.00)	0 (0.00)
V_H4-30.4 (%)	0 (0.00)[j]	1 (3.33)	0 (0.00)	0 (0.00)	**5 (17.24)**[bj]	1 (2.04)[b]
V_H4-31 (%)	0 (0.00)	1 (3.33)	0 (0.00)	0 (0.00)	0 (0.00)	1 (2.04)
V_H4-33 (%)	0 (0.00)	1 (3.33)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
V_H4-34 (%)	3 (8.11)	9 (30.00)	0 (0.00)	**0 (0.00)**[k]	**1 (3.45)**[c]	**21 (42.86)**[ck]
V_H4-39 (%)	0 (0.00)	0 (0.00)	0 (0.00)	1 (6.67)	0 (0.00)	0 (0.00)
V_H4-55 (%)	0 (0.00)	2 (6.67)	0 (0.00)	1 (6.67)	0 (0.00)	0 (0.00)
V_H4-59 (%)	1 (2.70)	1 (3.33)	0 (0.00)	1 (6.67)	0 (0.00)	1 (2.04)
V_H4-61 (%)	1 (2.70)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
V_H5-51 (%)	0 (0.00)	1 (3.33)	1 (3.57)	2 (13.33)	0 (0.00)	0 (0.00)
V_H5-a (%)	0 (0.00)	0 (0.00)	1 (3.57)	0 (0.00)	0 (0.00)	0 (0.00)
nd (%)	5 (13.51)	2 (6.67)	3 (10.71)	1 (6.67)	0 (0.00)	0 (0.00)
total (%)	37 (100.00)	30 (100.00)	28 (100.00)	15 (100.00)	29 (100.00)	49 (100.00)

Table 10. The number and proportional representation of V_H gene segments in populations of the $IgM^+CD19^+CD27^-CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, controls and RA patients (nd = not determined segments)

V_H segment		P values
V_H3-11	CVID $CD24^+$/RA $CD24^+$	P=0.0263[d]
	CO $CD24^+$/RA $CD24^+$	P=0-0153[e]
V_H3-23	CO $CD24^+$/RA $CD24^+$	P=0-0068[f]
V_H3-48	CVID $CD24^+$/RA $CD24^+$	P=0.0004[g]
	CO $CD24^+$/RA $CD24^+$	P=0.0027[h]
	CVID $CD24^-$/RA $CD24^-$	P=0.0017[i]
V_H4-30.4	CVID $CD24^+$/RA $CD24^+$	P=0.0214[j]
V_H4-34	CO $CD24^-$/CVID $CD24^-$	P=0.0463[k]
	CO $CD24^-$/RA $CD24^-$	P=0.0092[l]

Table 11. Comparison of frequency of V_H gene segments in B cells derived from CVID patients, controls and RA patient

5.2.2.3. Distribution of D_H families

In the transcripts derived from the both of analyzed populations of B lymphocytes of CVID patients were detected similar proportional representation of the D_H family members (Fig. 19., Tab.12.). We found D_H1 – D_H3 and D_H6 family members in the $CD24^+$ B cells and the D_H1 – D_H4 and D_H6 family members in the $CD24^-$ B cells. In both populations of B cells of COs, we observed gene families D_H1 – D_H6; D_H7 gene family was present only in $CD24^-$ B cells. The relative usage of the D_H gene families in RA pacient was also similar between $CD24^+$ and $CD24^-$ B cells. The gene families D_H3,D_H4 and D_H6 were present in both of B cell populations. Moreover, in $CD24^-$ B cells was described D_H1 gene family.

Generally, the two most frequent D_H gene families of B cells of CVID patients were D_H3 (13 of 37 transcripts, 35.14%) and D_H6 (7 of 37 transcripts, 18.92%). The same situation occured in repertoire of $CD24^-$ B cells with 10 of 30 transcripts, i.e. 33.33% (D_H3) and 7 of 30 transcripts, i.e. 23.33% (D_H6).

In RA pacient, D_H2 family members were recorded in 44.83% of cases (13 of 29 transcripts) of $CD24^+$ B cells and 34.69% of cases (17 of 49 transcripts) of $CD24^-$ B cells.

D_H2 family members of $CD24^+$ B cells of RA were significantly higher than those of CVID patients (44.83% vs 5.41%, P=0.0035[a]; Fischer's exact

test) and COs (44.83% vs 10.71%, P=0.0442[b]). Moreover, similar significant difference was found in RA patient compared with CVID patients in $CD24^-$ B cells (34.69% vs. 3.33%, P=0.0099[c]).

There were significant differencies in the frequency of D_H3 family members from both (P=0.0131[d] in $CD24^+$, P=0.0020[e] in $CD24^-$) B cell populations of CVID patients (35.14% in $CD24^+$, 33.33% in $CD24^-$) compared with those from RA (3.45% in $CD24^+$, 2.04% in $CD24^-$). The absence of D_H5 gene segments observed in CVID patients was statistically significant in the case of $CD24^+$ population as compared to COs (0.0% vs. 14.29%, P=0.0416[f]). Significantly higher expression of D_H6 family was found in $CD24^-$ B cells from CVID patients compared to RA patient (23.33% vs 2.04%, P=0.0095[g]).

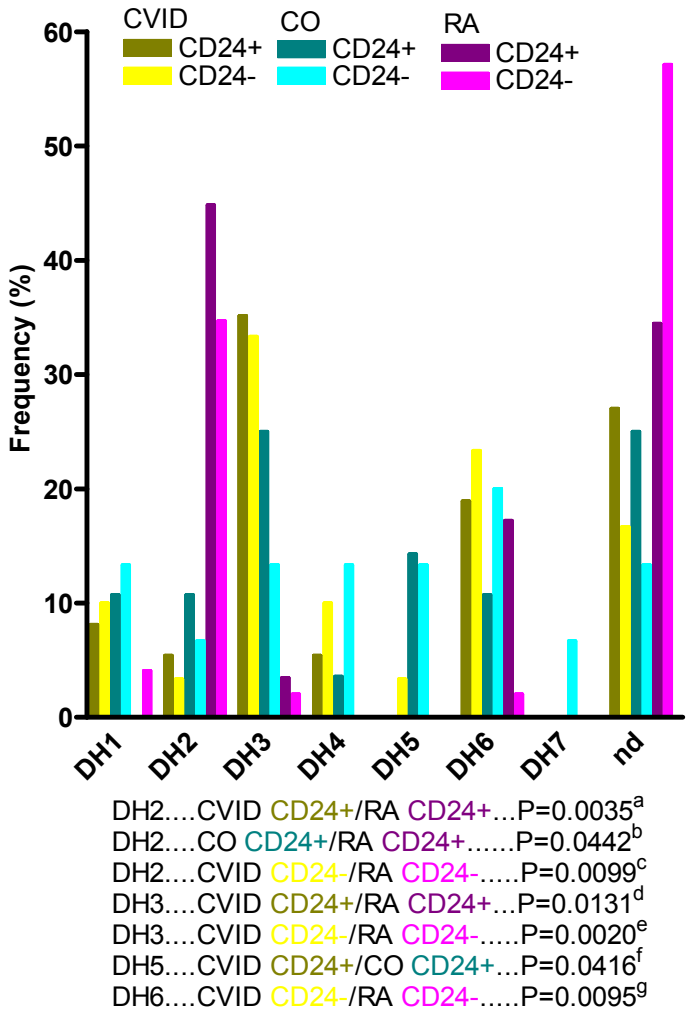


Figure 19. The frequency of D_H segments of the $IgM^+CD19^+CD27^-CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, controls and RA patients (nd = not defined segments), P values are indicated

	CVID		CO		RA	
D_H family	$CD24^+$	$CD24^-$	$CD24^+$	$CD24^-$	$CD24^+$	$CD24^-$
D_H1 (%)	3 (8.11)	3 (10.00)	3 (10.71)	2 (13.33)	0 (0.00)	2 (4.08)
D_H2 (%)	**2 (5.41)[a]**	**1 (3.33)[c]**	**3 (10.71)[b]**	1 (6.67)	**13 (44.83)[ab]**	**17 (34.69)[c]**
D_H3 (%)	**13 (35.14)[d]**	**10 (33.33)[e]**	7 (25.00)	2 (13.33)	**1 (3.45)[d]**	**1 (2.04)[e]**
D_H4 (%)	2 (5.41)	3 (10.00)	1 (3.57)	2 (13.33)	0 (0.00)	0 (0.00)
D_H5 (%)	**0 (0.00)[f]**	1 (3.33)	**4 (14.29)[f]**	2 (13.33)	0 (0.00)	0 (0.00)
D_H6 (%)	7 (18.92)	**7 (23.33)[g]**	3 (10.71)	3 (20.00)	5 (17.24)	**1 (2.04)[g]**
nd (%)	10 (27.03)	5 (16.67)	7 (25.00)	2 (13.33)	11 (37.93)	28 (57.14)
total (%)	37 (100.00)	30 (100.00)	28 (100.00)	15 (100.00)	29 (100.00)	49 (100.00)

Table 12. The number and proportional representation of D_H family members of the $IgM^+CD19^+CD27^-CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, controls and RA patients (nd = not defined)

5.2.2.4. Distibution of J_H families

The distribution of J_H family members in IgM mRNA transcripts derived from CVID and RA groups of patients and COs was similar in both of analyzed B cell populations (Fig. 20., Tab. 13.).

The members of J_H4 family were more frequently detected in the B cells of CVID patients (14 of 37 transcripts, 37.84% in $CD24^+$; 16 of 30, 53.33% in $CD24^-$).

J_H4 and J_H6 gene segments were frequently detected (both 10 of 28 transcripts, 35.71%) in $CD24^+$ B cells of COs. J_H4 gene segments also significantly prevailed in $CD24^-$ cells (9 of 15 transcripts, 60.00%). Both of B cells subsets of RA contained the highest number of J_H4 gene segments (21 of 29 transcripts, 72.41% in $CD24^+$ and 40 of 49, 81.63% in $CD24^-$) as compared to those in CVID and COs.
The absence of J_H6 gene segment in RA patient (0.00%) was statistically significant in the case of the $CD24^+$ population as compared to CVID (21.62%) and also to COs (35.71%) ($P=0.0192^a$ for CVID and $P=0.0035^b$ for COs; Fischer's exact test).

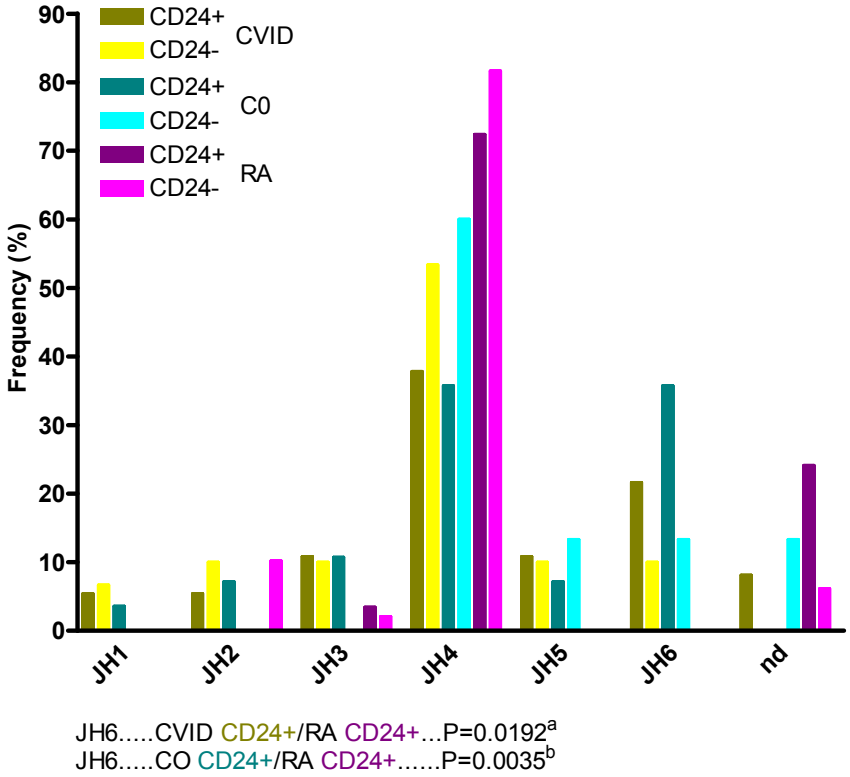


Figure 20. The frequency of J_H segments of the $IgM^+CD19^+CD27^-CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, controls and RA patients (nd = not defined segments), P values are indicated

	CVID		CO		RA	
J_H family	$CD24^+$	$CD24^-$	$CD24^+$	$CD24^-$	$CD24^+$	$CD24^-$
J_H1 (%)	2 (5.41)	2 (6.67)	1 (3.57)	0 (0.00)	0 (0.00)	0 (0.00)
J_H2 (%)	2 (5.41)	3 (10.00)	2 (7.14)	0 (0.00)	0 (0.00)	5 (10.20)
J_H3 (%)	4 (10.81)	3 (10.00)	3 (10.71)	0 (0.00)	1 (3.45)	1 (2.04)
J_H4 (%)	14 (37.84)	16 (53.33)	10 (35.71)	9 (60.00)	21 (72.41)	40 (81.63)
J_H5 (%)	4 (10.81)	3 (10.00)	2 (7.14)	2 (13.33)	0 (0.00)	0 (0.00)
J_H6 (%)	**8 (21.62)**[a]	3 (10.00)	**10 (35.71)**[b]	2 (13.33)	**0 (0.00)** [ab]	0 (0.00)
nd (%)	3 (8.11)	0 (0.00)	0 (0.00)	2 (13.33)	7 (24.14)	3 (6.12)
total (%)	37 (100.00)	30 (100.00)	28 (100.00)	15 (100.00)	29 (100.00)	49 (100.00)

Table 13. The number and proportional representation of JH segments of the $IgM^+CD19^+CD27^-CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, controls and RA patients (nd = not defined segments)

5.2.2.5. Mutational frequencies in Ig V_H mRNA transcripts

$CD24^+$ B cells from CVID patient were found to express the most mutated IgM transcripts (mean 2.76% ± SD 2.44%). In contrast, $CD24^-$ cells from the same patients were identified as those with the lowest mutational frequencies (mf, 1.19% ± 1.85%) of V_H gene segments detected in all analyzed groups of patient (Fig. 21., Tab. 14.). In the transcripts derived from $CD24^+$ and $CD24^-$ B cells of healthy controls V_H gene segments with means for mutation rates of 2.13% ± 2.35% and 2.16% ± 1.96 were recorded, respectively (Fig. 21., Tab. 14.). The values of mf were evaluated also for $CD24^+$ (1.74% ± 1.25%) and $CD24^-$ (1.94% ± 1.47%) subsets from RA (Fig. 21., Tab14.).

Using the nonparametric Mann-Whitney test we found statistically significant differences in mutation frequencies of CVID patients between populations $CD24^+$ and $CD24^-$ B cells (2.76% vs. 1.19%, $P=0.0025^a$). $CD24^-$ cells from CVID displayed lower numbers of mutations than those from RA (1.19% vs. 2.05%, $P=0.0026^b$).

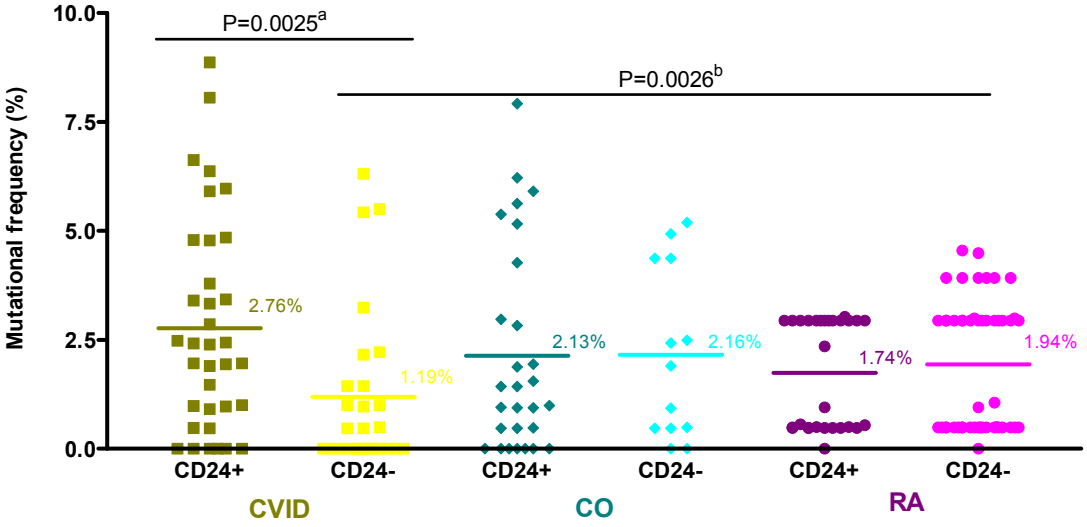


Figure 21. Mutation frequency of $IgM^+CD19^+CD27^-CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, controls and RA patients (nd = not defined segments), means and P values are indicated

patient	CVID		CO		RA	
population	$CD24^+$	$CD24^-$	$CD24^+$	$CD24^-$	$CD24^+$	$CD24^-$
mean ± SD (%) 95% CI	**2.76 ±2.44[a]**	**1.19 ±1.85[ab]**	2.13 ±2.35	2.16 ±1.96	1.74 ±1.25	**1.94 ±1.47[b]**
minimum - maximum (%) 95% CI	0 – 8.87	0 – 6.31	0 – 7.92	0 – 5.19	0 – 3.03	0 – 4.55

Table 14. Mutation frequency of mutated V_H segments of $IgM^+CD19^+CD27^-CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, controls and RA patients (SD = standard deviation, CI = confidence interval)

5.2.2.6. Distribution of R and S mutations in Ig V_H mRNA transcripts

In CVID patients, CDR2 (for $CD24^+$ B cells) and FR3 (for $CD24^-$ B cells) were the most frequently targeted by R (replacement) mutations whereas CDR1 ($CD24^+$) and FR3 ($CD24^-$) region contained S (silent) mutations (Fig. 22., Tab. 15.). The highest load of both types of mutations in $CD24^+$ B cells of COs was found in CDR1 region but in $CD24^-$ the highest load of R and S was found in CDR1, CDR2 respectively (Fig. 23., Tab. 15.). Interestingly, in RA patient FR3 region showed the highest load of R mutations and in CDR1 region S prevailed mutations and this was apparent in for both of B cell populations (Fig. 24., Tab. 15.).

We performed statistical analysis using Fischer's exact test in all patients. In CVID, R and S mutations were compared between $CD24^+$ and $CD24^-$ B cells. A significantly higher incidence of R mutations in CDR1 (2.63% vs. 1.24%, P=0.0330[a]) and also in CDR2 was found (3.31% vs. 1.33%, P=0.0010[b]) where also higher incidence of S mutations was detected (0.89% vs. 0.07%, P=0.0049[c]; Fig. 22.).

In healthy controls significant difference in numbers of S mutations between $CD24^+$ and $CD24^-$ B cells was observed (0.09% vs. 1.19%, P=0.0039[d]). Other significant differences were observed comparison of R and S in CDR2 region of both of B cell subsets with the high P value (1.88% vs. 0.21%, P<0.0001[e] in $CD24^+$ and 2.30% vs. 0.00%, P<0.0001[e] in $CD24^-$) and moreover in FR3 region of $CD24^+$ (1.60% vs. 0.59%, P=0.0008[g] ;Fig. 23.).

The discrepancy in numbers of R and S mutations was found also in CDR1 region of $CD24^+$ and $CD24^-$ RA patient's B cells (0.55% vs. 3.85%, P=0.00039[h] and 0.00% vs. 3.44%, P<0.0001[i]). Generally, values of R mutations were lower in $CD24^+$ than in $CD24^-$ B cells in FR2 (0.00% vs. 0.45%, P=0.0423[j]), CDR2 (0.34% vs. 1.22%, P=0.0080[k]) and FR3 regions (0.89% vs. 1.24%, P=0.0106[l]; Fig. 24.).

The frequency of R mutations in both population from CVID was significantly higher than in RA in CDR1 (2.63% vs. 0.55%, P=0.0284[m] in $CD24^+$ and 1.24% vs. 0.00%, P=0.0088[r] in $CD24^-$), FR2 (0.54% vs. 0.00%, P=0.0297[o] in $CD24^+$ and 0.18% vs. 0.45%, P=0.0049[t] in $CD24^-$), CDR2 (3.31% vs. 0.34%, P<0.0001[p]) and FR3 regions (1.44% vs. 0.89%, P=0.0144[q] in $CD24^+$ and 1.54% vs. 1.24%, P=0.0016[v] in $CD24^-$), in spite of lower incidence of S mutations in CDR1 region (1.32% vs. 3.85%, P=0.0381[n] in $CD24^+$ and 0.00% vs. 3.44%, P<0.0001[s] in $CD24^-$) and CDR2 (0.07% vs. 1.22%, P=0.0004[u] in $CD24^-$; Tab.16., 17.).

The numbers of R mutations were also higher in COs as compared to RA patient. Noticeable differences were described in CDR1 (2.47% vs. 0.55%, P=0.0412[w] in $CD24^+$ and 3.89% vs. 0.00%, P<0.0001[4] in $CD24^-$), FR2 (0.78% vs. 0.00%, P=0.0066[y] in $CD24^+$), CDR2 (1.88% vs. 0.34%, P=0.0002[z] in $CD24^+$) and FR3 (1.60% vs. 0.89%, P=0.0274[2] in $CD24^+$) regions. The incidence of S mutations of these compared patients and controls reached statistical significance in CDR1 (1.23% vs. 3.85%, P=0.0342[x] in $CD24^+$ and 0.56% vs. 3.44%, P=0.0401[5] in $CD24^-$), FR2 (1.19% vs. 0.10%, P=0.0067[6] in $CD24^-$), CDR2 (0.21% vs. 0.89%, P=0.0271[1] in $CD24^+$ and 0.00% vs. 1.22%, P=0.0129[7] in $CD24^-$) and FR3 regions (0.59% vs. 1.72%, P=0.0002[3] in $CD24^+$; Tab. 18., 19.).

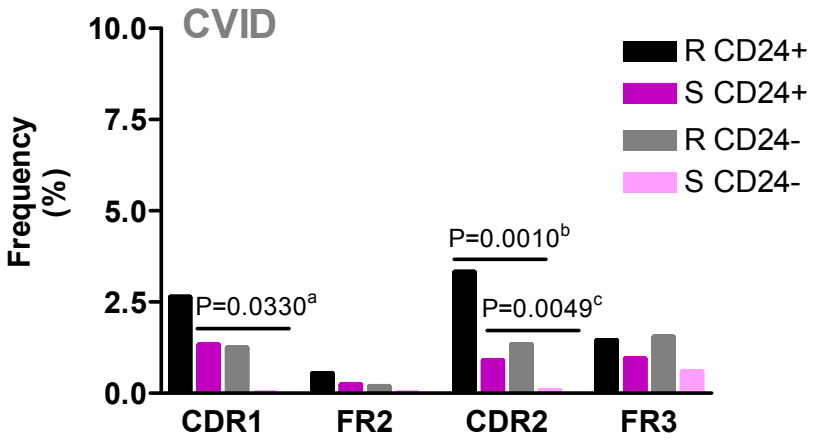


Figure 22. The frequency of R and S mutations in the $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, P values are indicated

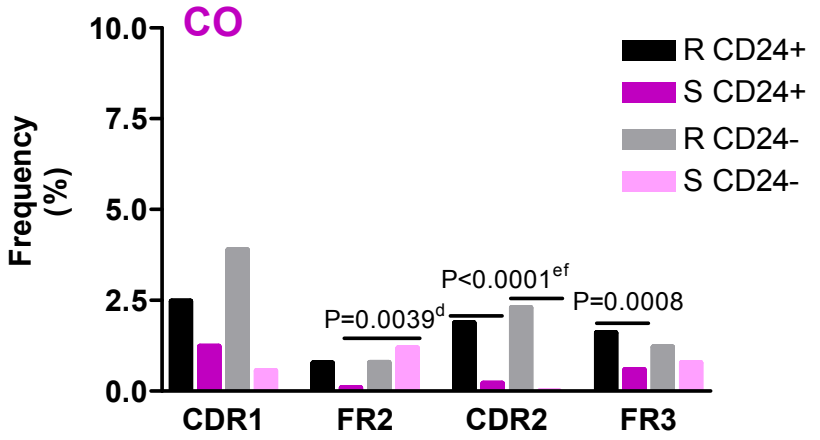


Figure 23. The frequency of R and S mutations in the $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{+/-}$ B cells in controls, P values are indicated

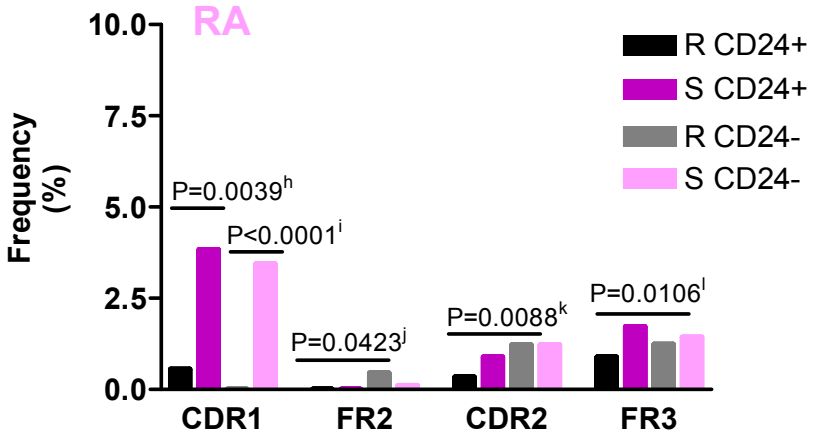


Figure 24. The frequency of R and S mutations in the $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{+/-}$ B cells in RA pacient, P values are indicated

patient	CVID		CO		RA	
population	$CD24^+$	$CD24^-$	$CD24^+$	$CD24^-$	$CD24^+$	$CD24^-$
lenght of CDR1 (nt)	456	402	405	180	366	639
R mutations (%)	**12 (2.63)^{am}**	**5 (1.24)^{ar}**	**10 (2.47)^{w}**	**7 (3.89)^{4}**	**2 (0.55)^{hmw}**	**0 (0.00)^{ir4}**
S mutations (%)	**6 (1.32)^{n}**	**0 (0.00)^{s}**	**5 (1.23)^{x}**	**1 (0.56)^{5}**	**14 (3.85)^{hx}**	**22 (3.44)^{is5}**
lenght of FR2 (nt)	1302	1134	1172	504	1215	1983
R mutations (%)	**7 (0.54)^{o}**	**2 (0.18)^{t}**	**9 (0.77)^{y}**	4 (0.79)	**0 (0.00)^{joy}**	**9 (0.45)^{jt}**
S mutations (%)	3 (0.23)	0 (0.00)	**1 (0.09)^{d}**	**6 (1.19)^{d6}**	0 (0.00)	**2 (0.10)^{6}**
lenght of CDR2 (nt)	1569	1356	1440	609	1464	2382
R mutations (%)	**52 (3.31)^{bp}**	**18 (1.33)^{b}**	**27 (1.88)^{ez}**	**14 (2.30)^{f}**	**5 (0.34)^{koz}**	**29 (1.22)^{k}**
S mutations (%)	**14 (0.89)^{c}**	**1 (0.07)^{cu}**	**3 (0.21)^{e1}**	**0 (0.00)^{f7}**	**13 (0.89)^{1}**	**29 (1.22)^{u7}**
lenght of FR3 (nt)	2986	2605	2691	1161	2797	4611
R mutations (%)	**43 (1.44)^{q}**	**40 (1.54)^{v}**	**43 (1.60)^{g2}**	14 (1.21)	**25 (0.89)^{lq2}**	**57 (1.24)^{lv}**
S mutations (%)	28 (0.94)	15 (0.58)	**16 (0.59)^{g3}**	9 (0.78)	**48 (1.72)^{3}**	66 (1.43)

Table 15. Frequency of R and S mutations in the $IgM^+CD19^+CD27^-CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, controls and RA patients

CVID $CD24^+$/RA $CD24^+$					
type of mutations	R	S	R	R	R
compared regions	CDR1	FR2	CDR2	FR3	
P values	P=0.0284^{m}	P=0.0381^{n}	P=0.0297^{o}	P<0.0001^{p}	P=0.0144^{q}

Table 16. Comparison of frequency of R and S mutations in V_H regions between $CD24^+$ B cells from CVID patients and controls

CVID $CD24^-$/RA $CD24^-$					
type of mutations	R	S	R	S	R
compared regions	CDR1	FR2	CDR2	FR3	
P values	P=0.0088^{r}	P<0.0001^{s}	P=0.0049^{t}	P=0.0004^{u}	P=0.0016^{v}

Table 17. Comparison of frequency of R and S mutations in V_H regions between $CD24^-$ B cells from CVID patients and RA patient

CO $CD24^+$/RA $CD24^+$							
type of mutations	R	S	R	R	S	R	S
compared regions	CDR1		FR2	CDR2		FR3	
P values	P= 0.0412^{w}	P= 0.0342^{x}	P= 0.0066^{y}	P= 0.0002^{z}	P= 0.0271^{1}	P= 0.0274^{2}	P= 0.0002^{3}

Table 18. Comparison of frequency of R and S mutations in V_H regions between $CD24^+$ B cells from controls and RA patient

CO $CD24^-$/RA $CD24^-$				
type of mutations	R	S	S	S
compared regions	CDR1		FR2	CDR2
P values	P<0.0001^{4}	P=0.0401^{5}	P=0.0067^{6}	P=0.0129^{7}

Table 19. Comparison of frequency of R and S mutations in V_H regions between $CD24^-$ B cells from controls and RA patient

5.2.2.7. Distribution of lengths of CDR3 regions

The lenght of CDR3, in a total of 188 productive IgM transcripts was analyzed (Fig. 25., Tab. 20.).

In the transcripts of patients with CVID this length ranged from 8 to 22 codons (median ± SE, 5% ± 0.60%) in $CD24^+$ B cells and 7 to 21 codons (13% ± 0.61%) in $CD24^-$ cells. Healthy controls contained CDR3s considered of 5 – 20 codons (15% ± 0.77%) in $CD24^+$ cells and 8 – 16 (13% ± 0.60%) in $CD24^-$ B cells. In these cell subsets in RA the length varied from 8 to 14 codons (8% ± 0.53%) and 8 to 16 (10% ± 0.28%) respectively.

Using the nonparametric Mann-Whitney test, significant differences were observed in the CDR3 lengths of $CD24^+$ and $CD24^-$ B cells between CVID and RA patient (14.50% ± 0.60% vs. 8.00% ± 0.53%, $P<0.0001^b$ and 13% ± 0.61% vs. 10% ± 0.28%, $P<0.0001^c$) and COs and RA (15.00% ± 0.77% vs. 8.00% ± 0.53%, $P<0.0001^d$ and 13.00% ± 0.64% vs. 10.00% ± 0.28%, $P=0.0012^e$). In COs, significantly shorter CDR3 regions were observed in $CD24^-$ B cells as compared to $CD24^+$ (15.00% ± 0.77 vs. 13.00% ±0.64, $P=0.0285^a$).

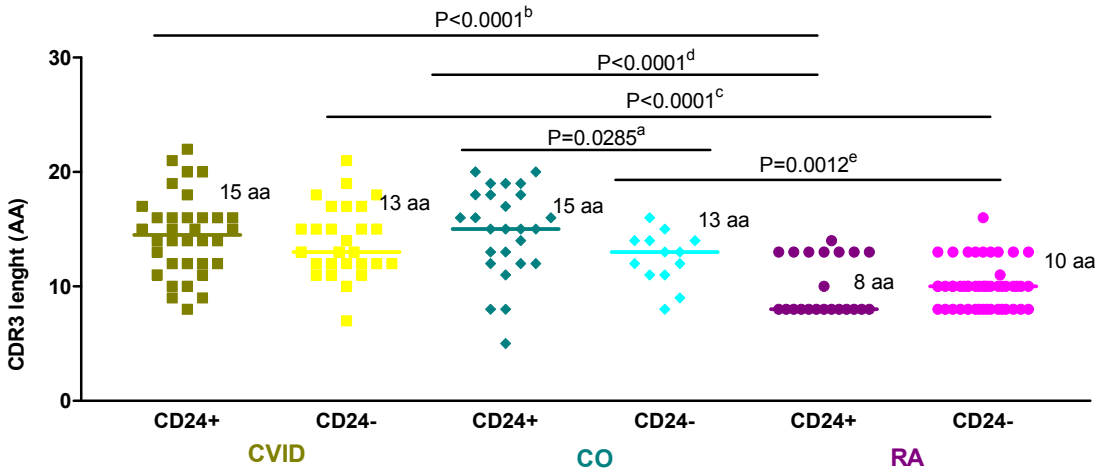


Figure 25. The distribution of lengths of CDR3 regions of the $IgM^+CD19^+CD27^-CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, controls and RA patients, medians and P values are indicated

patient	CVID		CO		RA	
population	$CD24^+$	$CD24^-$	$CD24^+$	$CD24^-$	$CD24^+$	$CD24^-$
median ± SE (%) 95% CI	**14.50 ±0.60[b]**	**13.00 ±0.61[c]**	**15.00 ±0.77[ad]**	**13.00 ±0.64[ae]**	**8.00 ±0.53[bd]**	**10.00 ±0.28[ce]**
minimum – maximum (%) 95% CI	8.00 – 22.00	7.00 – 21.00	5.00– 20.00	8.00– 16.00	8.00 – 14.00	8.00 – 16.00

Table 20. Lenghts of CDR3 regions of $IgM^+CD19^+CD27^-CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, controls and RA patients (SE = standard error, CI = confidence interval)

5.2.2.8. Clonal relation

Finally, the sequences of CDR3s suggested for polyclonality of tested B cell populations in CVID. In detail, among detected IgM transcripts of both studied B cell populations in CVID patients or healthy donors we did not found any transcripts with identical nucleotide or amino acid sequence in their clonally related CDR3 regions. Only two of such transcripts were found in IgG^+CD24^- B cells from COs.

By contrast, in RA a majority of IgM transcripts were found to be clonally related (68.97% in $CD24^+$ and 93.88% in $CD24^-$; Fig. 26., Tab. 21). Moreover, we identified 4 RA patients´ B cell clones with same CDR3 region that were derived from the same V_H gene segments (V_H 3-8, V_H 3-11,V_H 3-48 and V_H 4-34) with the same shared mutations (Fig. 27.).

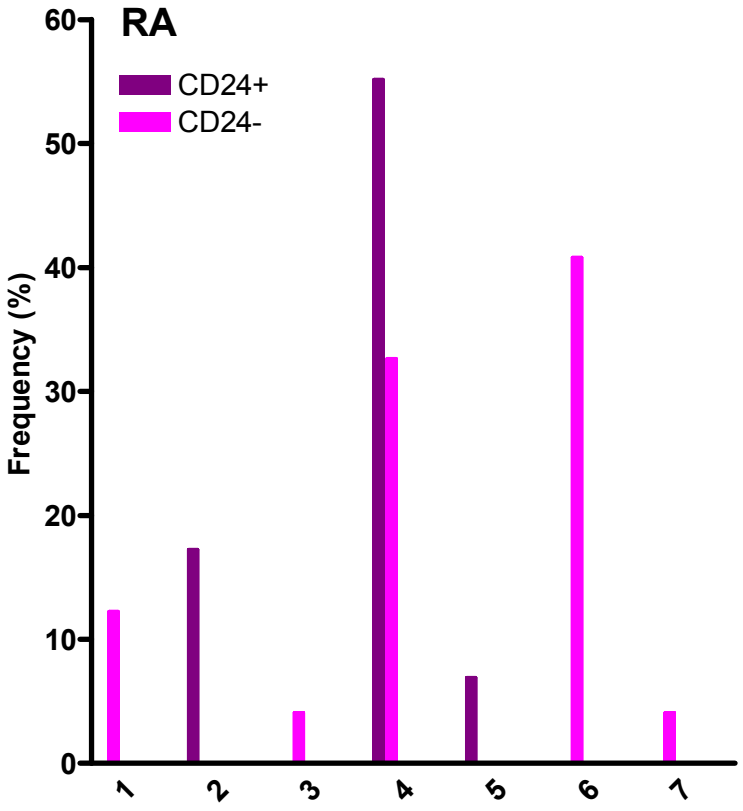


Figure 26. The frequency of clonally related IgM transcripts of $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{+/-}$ B cells in RA patients

	V_H gene segment	$CD24^{+}$	$CD24^{-}$	CDR3 nt sequence
1	V_H3-8 (%)	0 (0.00)	6 (12.24)	GCGGTGGCTACGATGGGGGCCTACTGGTACTTCGATCTC
2	V_H3-11(%)	5 (17.24)	0 (0.00)	GCATTAGGGGGAGCAGTGGCTGGTGGTCTCTTTGACTAC
3	V_H3-23 (%)	0 (0.00)	2 (4.08)	GTTTATGGTGGGAACTACAACTTTGACTAC
4	V_H3-48 (%)	13 (55.17)	16 (32.65)	TTGTGTGGTGCTGACTGCGACTAC
5	V_H4-30.4	2 (6.90)	0 (0.00)	AAGTGGGCCTATGGTTCGGGGAGTTACGGTATGGACTTG
6	V_H4-34 (%)	0 (0.00)	20	AGGGGGGGTGACTCGTACTACTTTGACTAC
7	V_H4-30-1(%) V_H4-30-4(%)	0 (0.00)	2 (4.08)	AAGTGGGCCTATGGTTCGGGGAGTTACGGTATGGACGTT
	clonally related transcripts (%)	20 (68.97)	46 (93.88)	
	total (%)	29 (100.00)	49 (100.00)	

Table 21. V_H gene segment, frequency of clonally related CDR3 region and nucleotide sequence of IgM transcripts detected in $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{+/-}$ B cells in RA patient

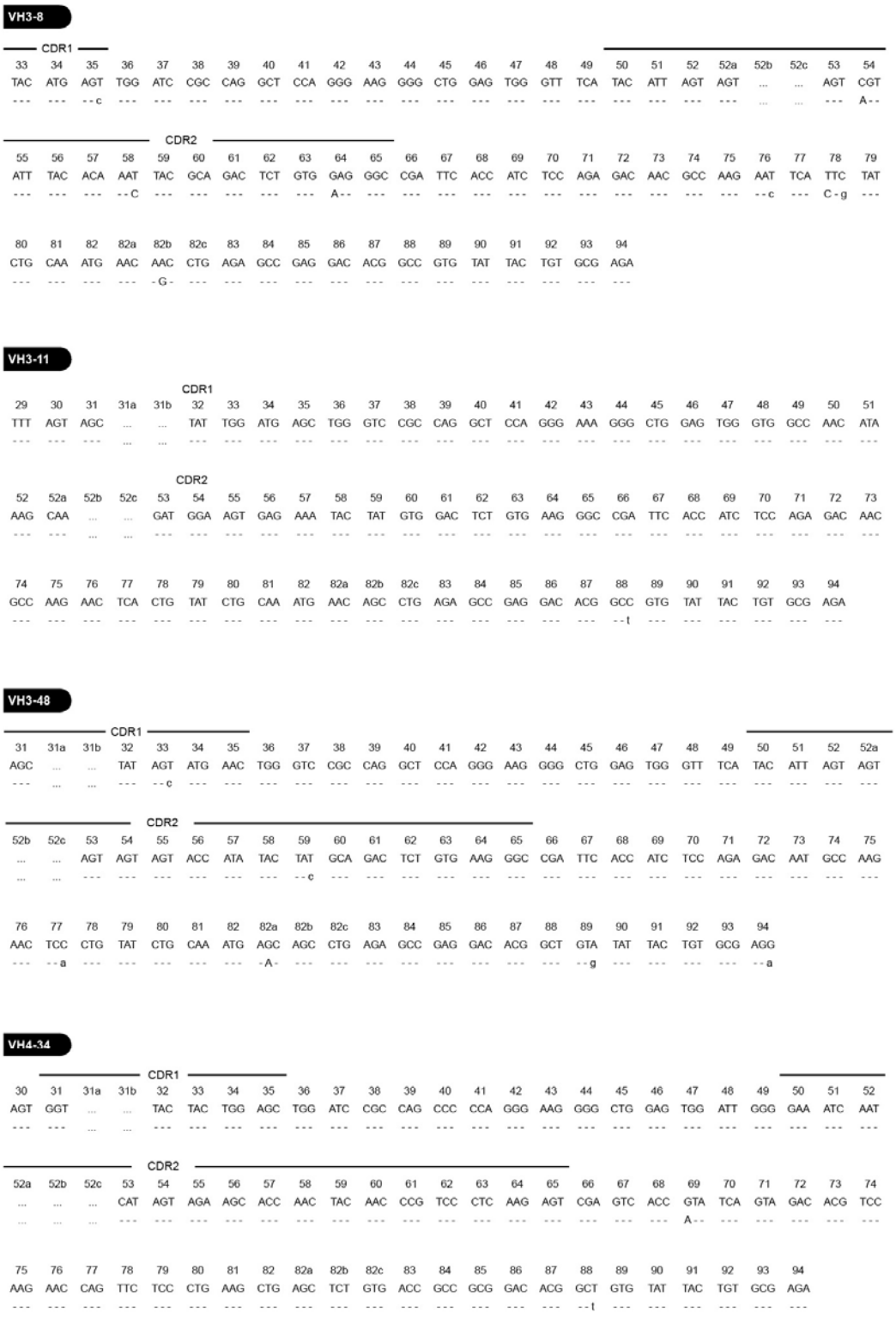


Figure 27. The sequence of the V_H region in IgM transcripts of $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{+/-}$ B cells in RA patient (somatic mutations are shown either as small letters for S or as capital letters for R mutations)

5.2.2.9. Amino acid composition in the CDR3 region

Representation of positively and negatively charged and neutral amino acids (AA) in the CDR3 regions of detected Ig transcripts derived from $CD24^+$ and $CD24^-$ B cells of CVID patients, healthy controls and RA patients was analyzed. Neutral AA constituted approximately 71 – 81% AA in of CDR3 regions, negatively charged AA 5 – 13% and positively charged AA remaining 12 – 18% amino acids in CDR3 regions (Fig. 28. – 30., Tab. 22.).

The significant difference in the frequency of positively charged AA was observed between $CD24^+$ and $CD24^-$ B cell derived from COs using Fisher's exact test was observed ($P=0.0251^a$; Fig. 29.).

Statistically significant higher proportion of positively charged AA in CDR3 regions of transcripts from $CD24^-$ B cells of RA patient compared with CVID patients was found (18.13% vs. 12.10%, $P=0.0463^b$). In contrast, the significant reduction of negatively charged AA in RA patient compared with CVID patients (6.77% vs 1.83%, $P=0.0086^c$ in $CD24^+$ and 9.51% vs. 5.00%, $P=0.0261^e$ in $CD24^-$) and COs was observed (6.04% vs. 1.83%, $P=0.0229^d$ in $CD24^+$ and 12.58% vs. 5.00%, $P=0.0043^f$ in $CD24^-$; Tab. 23.).

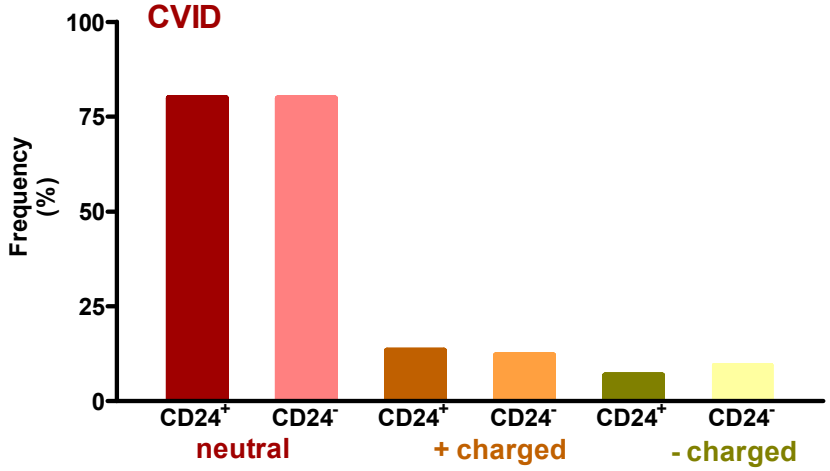


Figure 28. Relative distribution of positively and negatively charged and neutral amino acid in CDR3 regions of immunoglobulin transcripts of $IgM^+CD19^+CD27^-CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients

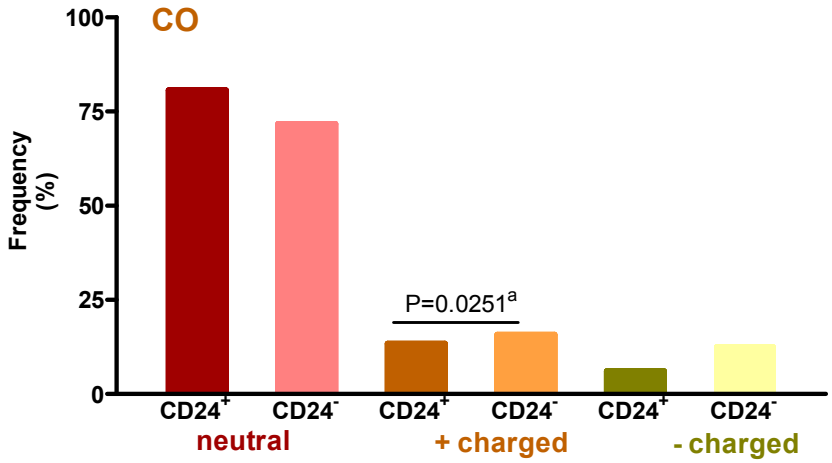


Figure 29. Relative distribution of positively and negatively charged and neutral amino acid in CDR3 regions of immunoglobulin transcripts of $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{+/-}$ B cells in controls, P values are indicated

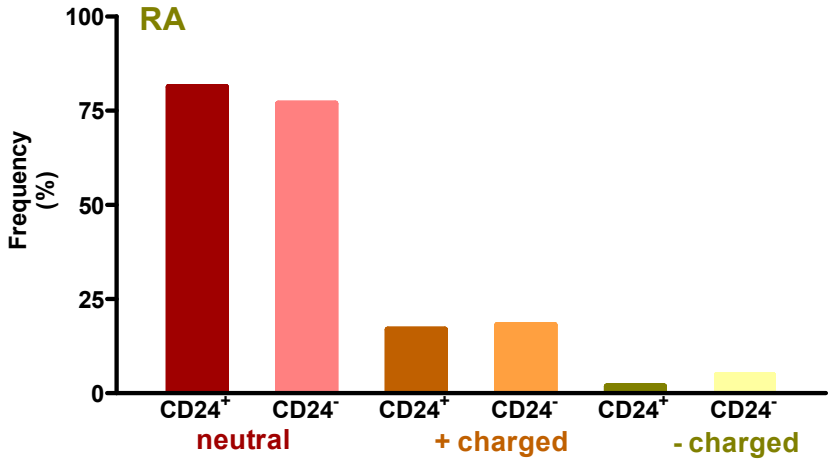


Figure 30. Relative distribution of positively and negatively charged and neutral amino acid in CDR3 regions of immunoglobulin transcripts of $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{+/-}$ B cells in RA patient

Patient	CVID	CVID	CO	CO	RA	RA
population	$CD24^{+}$	$CD24^{-}$	$CD24^{+}$	$CD24^{-}$	$CD24^{+}$	$CD24^{-}$
neutral (%)	378 (79.92)	277 (79.83)	307 (80.58)	144 (71.70)	178(81.28)	369 (76.86)
+ charged (%)	63 (13.32)	**42 (12.10)^{b}**	**51 (13.39)^{a}**	**25 (15.72)^{a}**	**37 (16.89)^{b}**	87 (18.13)
- charged (%)	32 (6.77)	33 (9.51)	23 (6.04)	20 ˇ12.58)	4 (1.83)	24 (5.00)
total (%)	473 (100.00)	347 (100.00)	381(100.00)	159 (100.00)	219 (100.00)	480 (100.00)

Table 22. The number and proportional representation of AA in the $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, controls and RA patient

AA		P values
+ charged	CVID $CD24^-$/RA $CD24^-$	P=0.0436[b]
- charged	CVID $CD24^+$/RA $CD24^+$	P=0-0086[c]
	CO $CD24^+$/RA $CD24^+$	P=0-0229[d]
	CVID $CD24^-$/RA $CD24^-$	P=0.0261[e]
	CO $CD24^-$/RA $CD24^-$	P=0.0043[f]

Table 23. Comparison of frequency of V_H gene segment among B cells derived from CVID patients, controls and RA patient

5.2.3. Analysis of $V_HD_HJ_H$ rearrangement in IgG transcipts

In CVID patients 2 IgG transcripts out of total 39 were obtained in $CD24^+$ B cells and 4 out of 34 in $CD24^-$ B cells (Tab. 24.). In both investigated populations of B cells in CVID patients, all IgG transcripts were detected in cells in which also transcripts of IgM isotype transcripts were present. Mutational frequency of V_H genes of IgM and IgG transcripts found to be completely identical.

sample	population	V_H family	V_H gene segment	V_H gene	D_H gene segment	D_H gene	J_H gene segment	mf (%)	Lenght of CDR3 (AA)
1A	$CD24^+$	V_H3	3-23	DP-47/V3-23	5-12	D5-12/DK1	J_H4b	4,9	8
3H	$CD24^+$	V_H4	4-34	DP-63/VH4.21	nd	nd	nd	1.00	nd
8C	$CD24^-$	V_H1	1-18	DP-14/V1-18+	3-09	D3-9/DXP1	J_H5b	0,5	21
9G	$CD24^-$	V_H4	4-34	DP-63/VH4.21	5-05	D5-5/DK4	J_H4b	0,0	13
12E	$CD24^-$	V_H5	5-51	DP-73/V5-51	2-08	D2-8/DLR1	J_H3b	3,2	11
9G	$CD24^-$	V_H4	nd	H3	4-23	D4-23	J_H6b	1.49	15

Table 24. Usage of V_H, D_H and J_H segments, mf (mutation frequency) and the length of CDR3 in IgG transcripts of $IgG^+CD19^+CD27^-CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patient (nd = not defined)

In healthy donors one IgG transcript out of total 29 in $CD24^+$ B cells and 6 of 21 in $CD24^-$ B cells were found (Tab. 25.).

Unlike CVID patients, IgG transcripts in B cells were detected in healthy donor only in the absence of transcripts of IgM isotype.

Notably, two clonally related $CD24^-$ B cells (7H and 7C) were found.

sample	population	V_H family	V_H gene segment	V_H gene	D_H gene segment	D_H gene	J_H gene segment	mf (%)	Lenght of CDR3 (AA)
6A	$CD24^+$	V_H1	1-69	DP-10/hv1051	2-15	D2-15/D2	J_H4a	2,4	19
7C	$CD24^-$	V_H5	5-a	$V_H32Sanz+$	5-12	D5-12/DK1	J_H5a	2,9	7
7H	$CD24^-$	V_H5	5-a	$V_H32Sanz+$	5-12	D5-12/DK1	J_H5a	2,9	7
10A	$CD24^-$	V_H3	3-30-5	DP-49/1.9III	1-26	D1-26	J_H4b	2,8	12
10B	$CD24^-$	V_H3	3-15	DP-38/9-1	6-06	D6-6/DN4	J_H4b	0,0	11
10H	$CD24^-$	V_H2	2-70	S12-10	nd	nd	nd	5,2	13
12H	$CD24^-$	V_H1	1-03	DP-25/VI-3b+	2-02	D4	J_H5b	3,3	18

Table 25. Usage of V_H, D_H and J_H segments, mf (mutation frequency) and the length of CDR3 in IgG transcripts of $IgG^+CD19^+CD27^-CD21^{low}CD38^{low}CD24^{+/-}$ B cells of healthy controls (nd = not defined)

In patient with RA 1 IgG transcript out of total 30 in $CD24^+$ B cells and 1 out of 50 in $CD24^-$ B cells were found (Tab. 26.)

In this case, IgG transcripts were detected in B cells of RA both lacking of IgM isotype ($CD24^+$) and also in the presence of IgM ($CD24^-$).

sample	population	V_H family	V_H gene segment	V_H gene	D_H gene segment	D_H gene	J_H gene segment	mf (%)	Lenght of CDR3 (AA)
1H	$CD24^+$	V_H3	3_15	DP-38/9-1	nd	nd	J_H6_b	0.5	13
12G	$CD24^-$	V_H3	3_48	V3-48/hv3d1	2_21	D2-21	J_H4_b	3.43	8

Table 26 .Usage of V_H, D_H and J_H segments, mf (mutation frequency) and the length of CDR3 in IgG transcripts of $IgG^+CD19^+CD27^-CD21^{low}CD38^{low}CD24^{+/-}$ B cells of RA patients (nd = not defined)

5.2.4. RAG mRNA expression

RAG mRNA analysis was performed in all analyzed B cells of both subset RAG mRNAs were detected in all B cell subsets from all analyzed patients. Representation of RAG1 mRNA transcripts was very similar in $CD24^+$ and $CD24^-$ B cells of CVID patients (144 of 189 (76.20%) and 144 of 199 (72.36%) and RA patients (96 of 134 (71.64%) and 93 of 123 (75.61%)), whereas the frequency was decreased in COs (56 of 130 (43.07%) and 53 of 134 (39.55%); Fig. 31.– 33.,Tab. 27.).

The differences in RAG1 mRNA expression in the $CD24^+$ and $CD24^-$ subsets were statistically significant in COs in comparison with CVID patients (43.07% vs. 76.20%, $P=0.0035^a$ in $CD24^+$ and 39.55% vs.72.36%, $P=0.0019^b$ in $CD24^-$) and also in comparison with RA patient (43.07% vs.

71.64%, P=0.0107[c] in $CD24^+$ and 39.55% vs.75.61%, P=0.0026[d] in $CD24^-$; Fisher exact test; Fig. 31.).

Both type of RAG2 mRNA were expressed in B cell populations from all donors (Fig. 32. – 33., Tab. 27.). RAG2a mRNA was found in 30 of 189 (15.87%) $CD24^+$ B cells and in 39 of 199 (19.60%) $CD24^-$ B cells from CVID patients. RAG2a mRNA expression was detectable also in $CD24^+$ (41 of 130 (31.54%) and $CD24^-$ (50 of 134 (37.31%) B cells from normal subjects, whereas RAG2a mRNA was found in only 21 of 134 (15.67%) $CD24^+$ B cells and 17 of 123 (13.82%) $CD24^-$ B cells from RA patient.

The differences in RAG2a expression reached significant levels upon comparison of both B cells subsets between COs and RA (31.54% vs. 15.67%, P=0.0233[e] in $CD24^+$ and 37.31% vs. 13.82%, P=0.0009[f] in $CD24^-$).

Analysis of individual $CD24^+$ and $CD24^-$ B cells from CVID patients revealed that 15 of 189 (7.94%) and 16 of 199 (8.04%) of individual cells expressed RAG2b mRNA. The increased numbers of $RAG2b^+$ B cells were found in COs (33 of 130 (25.38%) in $CD24^+$ and 31 of 134 (23.13%) in $CD24^-$). Finally, in RA patient this expression was found in 17 of 134 (12.69%) $CD24^+$ and 13 of 123 (10.57%) $CD24^-$ B cells.

A significant by higher number of RAG2b positive cells was identified in $CD24^+$ and $CD24^-$ B cells from COs in comparison with those from CVID patients (25.38% vs. 23.13, P=0.0003[g] and 23.13% vs. 8.04%, P=0.0014[h]) and RA patient (25.38% vs. 12.69%, P=0.0316[i] and 23.13% vs. 10.57%, P=0.0323[j]).

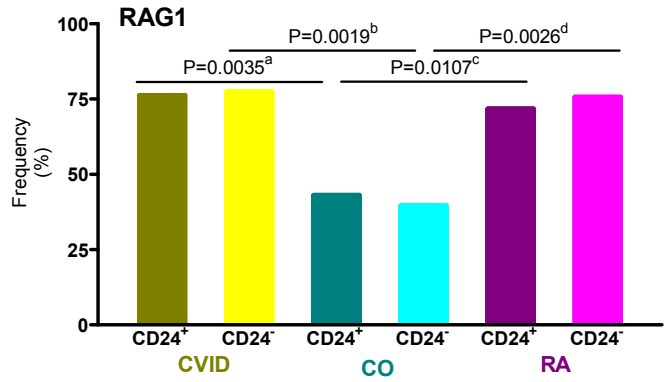


Figure 31. RAG1 mRNA expression in the $IgM^+CD19^+CD27^-CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, controls and RA patient, P values are indicated

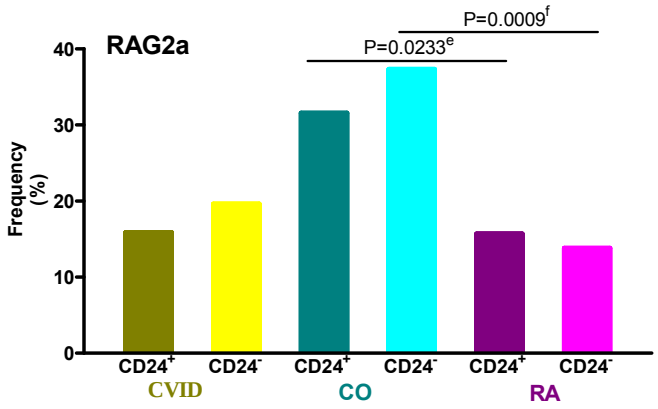


Figure 32. RAG2a mRNA expression in the $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, controls and RA patient, P values are indicated

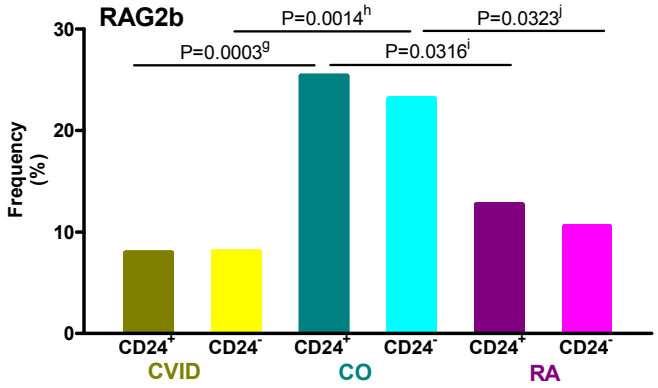


Figure 33. RAG2b mRNA expression in the $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, controls and RA patient, P values are indicated

patient	CVID	CVID	CO	CO	RA	RA
B cells	$CD24^{+}$	$CD24^{-}$	$CD24^{+}$	$CD24^{-}$	$CD24^{+}$	$CD24^{-}$
RAG1 (%)	**144 (76.20)[a]**	**144 (72.36)[b]**	**56 (43.07)[ac]**	**53 (39.55)[bd]**	**96 (71.64)[b]**	**93 (75.61)[d]**
RAG2a (%)	30 (15.60)	39 (19.60)	**41 (31.54)[e]**	**50 (37.31)[f]**	**21 (15.67)[e]**	**17 (13.82)[f]**
RAG2b (%)	**15 (7.94)[g]**	**16 (8.04)[h]**	**33 (25.38)[gi]**	**31 (23.13)[hj]**	**17 (12.69)[i]**	**13 (10.57)[j]**
Total (%)	189 (100.00)	199 (100.00)	130 (100.00)	134 (100.00)	134 (100.00)	123 (100.00)

Table 27. The number and proportional representation of RAG 1, RAG2a and b mRNA in the $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, controls and RA patient

6. Discussion

6.1. Analysis of the frequency of $IgM^+CD19^+CD27^-$ $CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, controls and RA patient using a flow cytometer

$IgM^+CD19^+CD27^-$ $CD21^{low}CD38^{low}CD24^{+/-}$ B cells were observed in all analyzed groups of donors. The frequency of these B cells in peripheral blood of CVID patients was significantly higher than in healthy donors and RA patients. In the case of CVID disease these cells could be likely affected by a particular defect that prevents their further development and causes their accumulation in the peripheral blood or other body sites (Vlkova et al., 2010). The decreased frequency of these cells in RA might be caused by their accumulation in synovial tissue, the main site of inflammation (Tak et al., 1996).

6.2. Analysis of genes for variable regions of immunoglobulin heavy chain of cell populations of the $IgM^+CD19^+CD27^-$ $CD21^{low}CD38^{low}CD24^{+/-}$ B cells in CVID patients, controls and RA patients

6.2.1. Distribution of IgM and IgG mRNA transcripts

Immunoglobulin isotype class switch is a mechanism that occurs after activation of a mature B cell and changes production of antibodies for example, from IgM isotype to IgG.

Both IgM and IgG transcripts were discovered in all analyzed B cells but those expressing IgM mRNA predominated. $CD24^-$ B cells from COs expressed the largest number of IgG transcripts in comparison to $CD24^+$ counterparts and such a tendency was found also in CVID patients. In this context $CD24^-$ B cells could represent more mature B cell population. Such findings were not observed in patient with RA and both B cell subsets here from unknown reason displayed similar frequency of IgG expressing B cells. One of the explanations could be disturbed process in isotype switching

n this particular population of increased or by migration of IgG B cells into the site of inflammation (Tak et al., 1996). On the other hand, IgG production in CVID patients and COs could be a result of exposition of immune system to (auto)antigen mediated by similar mechanism as in patients with RA (Kokkonen et al., 2011)

6.2.2. Analysis of $V_HD_HJ_H$ rearrangement in transcripts of IgM isotype

Using statistical methods, we assessed the usage of V_H, D_H and J_H gene families, mf and characteristics of the CDR3 regions in transcripts of IgM isotype that were detected in analyzed populations of B cells of CVID patients, healthy donors and RA patient.

6.2.2.1. Distribution of V_H families

A total number of 51 V_H segments in human IgM locus located on chromosome 14 can be divided into seven distinct families (V_H1 – V_H7). Interpersonal differences found between people are due to the possible presence of non-functional alleles of certain segments (Link and Schroeder, 2002).

Proportional distribution of V_H families in $V_HD_HJ_H$ rearrangements in mature B cells in the normal physiological state of the immune system is equivalent to germinal repertoire of V_H families, although slight changes during further development of B cell populations may occur (Brezinschek et al., 1997; Tian et al., 2007). Significant variations in the distribution of individual families are associated with diseases in which the immune system is disturbed such as acute lymphoblastic leukemia or systemic lupus erythematosus and Sjögren's syndrome (Morutza et al., 2001; Hansen et al., 2002; Dörner et al., 1998b).

In general, V_H3 was found as the most present family in all $CD24^+$ B cells and also in $CD24^-$ B cells of COs and RA patient whereas in $CD24^-$ B cells of CVID patients it was V_H4 family. When we compared the distribution of V_H4 family between $CD24^+$ and $CD24^-$ B cells of CVID patients, COs and RA patient, the overpresentation of V_H4 family was found in $CD24^-$ subset of all analyzed subjects. Such as predominance of V_H4

family was demonstrated also in patients with SLE or SjS and it was proposed that B cells expressing this particular family could represent autoreactive population (Hansen et al., 2002; Dörner et al., 1998b). The higher frequency of V_H4 family was detected in $CD24^-$ B cell subset of all individuals, which could suggest that these cells are more differentiated or they are susceptible to escape negative selection and become autoreactive (Hansen et al., 2002; Dörner et al., 1998b).

Only significantly higher representation of V_H1 family segments was found in transcripts obtained from the cell population $CD24^+$ B cells of CVID patient compared with $CD24^+$ B cells of RA patient.

V_H3 family is the largest and the most frequently used V_H family in B cell repertoire and moreover some studies have shown that the members of this family are also included in humoral defense against parasitic infections (Tachibana et al., 2003).

V_H4 family was repeatedly described to encode autoantibodies (against self dsDNA or erythrocytes) and therefore V_H4 containing B cells were suggested to be more susceptible to become autoreactive (Stevenson et al., 1993). V_H4 segments were found to be more prevalent in naive cells than in mature memory B cells (Stevenson et al., 1993; Hansen et al., 2002; Dörner et al., 1998b).

Our findings according to the distribution of V_H families suggest that all B cell populations exhibit features as conventional B lymphocytes and the population of $CD24^-$ B lymphocytes of all groups of pacients is more differentiated than the population of $CD24^+$ B lymphocytes.

6.2.2.2. The distribution of V_H gene segments

The specificity of the antibodies is determined by nucleotide sequence at DNA and mRNA level, susequently by amino acid sequence and finally by three-dimensional structure of antigen binding sites at the protein level. Differential usage of V_H gene segments directly affects the specifity of Ig molecules. It was shown that some antibodies against certain

antigens used in their $V_HD_HJ_H$ rearrangements preferentially particular V_H gene segments (Lucas et al., 2003; Pugh-Bernard et al., 2001; Baxendale and Goldblatt, 2005).

We described utilization of different V_H gene segments in detected Ig transcripts. In general, majority of B cells of CVID patients were V_H3-23 or V_H4-34 positive whereas in B cells from healthy donors the most frequent gene segment was V_H3-23. This was true particularly for $CD24^+$ B cells of CVID patients and COs. On the other hand, in RA patient $CD24^-$ B cells expressed V_H3-23. In RA patient, were found mainly V_H3-48 and V_H4-34 possitive cells. However, in COs was observed complete absence of V_H4-34 gene segment. The second most interesting finding was remarkable restriction of V_H gene repertoire in RA B cells with 10 V_H genes being present in mRNAs, whereas distribution of V_H genes of CVID and COs was similar and included wider spectrum of particular genes.

The gene segment V_H3-23 was detected in $V_HD_HJ_H$ rearrangements that encode antibodies against Haemophilus influenzae (Lucas et al., 2003). It can be possible that some B cells of healthy donors and CVID patients were stimulated by antigens of these bacteria and therefore positively selected and present in the circulation.

V_H3-48 is used rather rarely in Ig repertoire of the circulating B cells however it was shown as dominant gene segment in the memory B cells responsing to Pneumococcal polysaccharide (Baxendale and Goldblatt, 2005). It can be possible that RA patient favorized this positively selected segment after natural exposure to pneumococcus.

V_H4-34 gene segment encodes the variable part of Ig chains that are directed against red blood cells or self dsDNA and in peripheral blood B cells from patients with SLE represents predominant gene in VDJ rearrangements correlating with disease activity (Stevenson et al., 1993; Odendahl et al., 2000; Hansen et al., 2002).

V_H4-34 is more frequently found in non-mutanted $V_HD_HJ_H$ rearangements, whereas in B cell populations that have undergone somatic hypermutation representation of this gene segment was lower than

expected, likely due to deletion of B cells expressing the receptor encoded by this segment (Brezinschek et al., 1997; Hansen et al., 2002). This assumption is supported by observed higher detection of the V_H4-34 sequence in the $CD24^+$ B cells obtained from CVID and RA and its absence in COs.

6.2.2.3. Distribution of D_H families

In analyzed B cells we could distinguish 27 D_H gene segments grouped into seven D_H families however D_H assignment is very problematic. This is probably caused due to activity of TdT (terminal deoxynucleotidyl transferase), DNA exonucleases and DNA polymerases during the processes of recombination or somatic hypermutation which are responsible for remarkable changes in the sequence of D_H segments that are subsequently non-detectable. Notably, it has been shown that difficulty in assignment of D_H gene segments can be also closely correlated with CDR3 regions with short lengths (Clausen et al., 1998).

D_H3 and D_H6 families were the most frequently described in CVID patients and also in COs and their distribution corresponded to normal distribution (see later; Link and Schroeder, 2002; Tian et al., 2007). Interestingly, D_H2 family was overpresented in $V_HD_HJ_H$ rearrangements in RA patient.

Significantly lower number of D_H2 segments was found in transcripts obtained from both of B cell populations of CVID patients and COs as compared with RA. Higher numbers of obtained D_H3 segments from $CD24^+$ and $CD24^-$ B cells in CVID patients in comparison with RA patient reached the statistical significance.

The gene segments from D_H3 and D_H6 families are the most frequent segments in $V_HD_HJ_H$ rearrangements found in peripheral B lymphocytes (Link and Schroeder, 2002; Tian et al., 2007). Notably, it was postulated that D_H3 and D_H2 families are overpresented also under pathological conditions in patients with multiple myeloma (MM; Gonzalez et al., 2005).

In summary CVID patients´ and controls´ B cells exhibited features similar rather for normal B lymphocytes whereas RA patient´s B cells

resembled more B lymphocytes that potentially cause some diseases such as MM.

6.2.2.4. Distribution of J_H families

Six functional J_H gene segments of human Igs differ in length and nucleotide sequence and strongly affect the final length of entire CDR3 region. J_H6 contributes to the total length of the CDR3 segment with nine AA, J_H1 and J_H2 with six AA, J_H5 with five AA, J_H3 and J_H4 with four AA. The J_H6 segments are more frequently presented in the $V_HD_HJ_H$ rearrangements of naive B lymphocytes than the J_H4 segments in comparison to B cells that have undergone germinal reaction (Brezinschek et al., 1997; Tian et al., 2007).

A predominance of J_H4 segments was found in the transcripts derived from both of B cell populations observed in CVID and RA patients and in healthy donors which is in accordance with published data (Brezinschek et al., 1997; Tian et al., 2007). Similar representation of J_H4 and JH6 segments was found in $CD24^+$ B cells of COs. We found significantly increased number of J_H6 segments in Ig transcripts obtained from $CD24^+$ B cells of CVID patients and COs as compared with the patient with RA which displayed the highest frequency of J_H4 segment usage.

6.2.2.5. Mutational frequency and distribution of replacement and silent mutations

As mentioned in the introduction, the antigenic stimulation of B cells leads to intense mutational processes in genes coding for variable regions of heavy and light chains of antibodies. The mutations occur in DNA throughout the entire length of the variable regions however, during the antigen-driven selection of B cells mutations preferentially accumulate in CDR regions rather than in FR regions.

Nucleotide substitutions (replacement – R or silent mutation – S) may result in amino acid substitutions at the protein level. B cells that undergo antigenic stimulation and selection possess more S than R mutations in their FR regions to minimize dramatic alterations of the final Ig structure.

In addition, they care more frequently R mutations in their CDR regions than in FR regions because of increasing of the specifity of BCR and secreted antibodies (van Es et al., 1992).

Analysis of distribution of mutations in the V_H segments of Ig transcripts derived from CVID patients and healthy donors demonstrated that more R mutations was presented in CDR1 and 2 regions than in FR, which was not found in RA patient´s B cells.

Mutational frequencies in CVID patients were observed to be significantly increased in $CD24^+$ B cells as compared to $CD24^-$ counterparts whereas in COs and RA patient mutational rates were similar between both analyzed subsets. The significant difference in mutational frequency was found between $CD24^-$ B cells of CVID patients and RA patient. Since $CD24^-$ B cells in CVID patients showed significantly less mutated patterns as compared to $CD24^+$ population and in COs and RA patient $CD24^-$ population displayed slight increase of this value, we speculate that at least in healthy donors and patient with RA this cell subset might represent more diffentiated B cell population. This is supported by evidences that higher mutational rates correspond rather to (auto)antigen-experienced B cells (Hansen et al., 2002; Dörner et al., 1998b).

6.2.2.6. Distribution of lengths of CDR3 regions

Differences in VDJ utilization directly determine variability of sequences and lenght of CDR3 regions which also finally influences the affinity of particular antibodies to given antigen. The typical length of the CDR3 regions of immunoglobulin in healthy adults generally ranges from 6 to 28 amino acids, shorter CDR3 regions are presented in B lymphocytes that have undergone (auto)antigen-stimulated somatic hypermutation (Link and Schroeder, 2002; Lee et al., 1994). CDR3 regions play a critical role in the final antigen recognition and binding, and in general it is assumed that shorter CDR3 regions more efficiently bind to antigen than longer ones (Rösner et al., 2001).

CDR3 lengths of $V_HD_HJ_H$ transcripts observed in our analyzed populations ranged from 5 to 21 amino acids. Notably, $CD24^+$ B cells had longer CDR3 regions than $CD24^-$ B cells in CVID patients and COs and the medians of $CD24^+$ and $CD24^-$ B cells reached the same values for both groups of patients. An opposite situation was described in RA patient where both $CD24^+$ and $CD24^-$ B cells showed shorter CDR3 regions and this shortening was significant.

In general, the shortest CDR3 regions have been described in B cells from RA patients (especially in $CD24^+$ subset) which suggests that these cells are more maturated than those from CVID and COs, likely due to stimulation by autoantigen as the result of autoimmune condition.

6.2.2.7. Clonal relation

As mentioned above, B lymphocytes derived from a common "progenitor" B cell, stimulated by one particular antigen show the same specificity of produced antibodies, which is manifested at the DNA/RNA level by using identical V_H, D_H, J_H segments and therefore the same sequence of nucleotides and subsequently AA at protein level of CDR3 regions. Thus, CDR3 region defines clonal relatedness based on nonradom usage of variable domains and high levels of R mutations. These facts together may predicate about an ongoing antigen-driven B cell response (Clausen et al., 1998). The higher frequency of clonal relation was demonstrated to be typical for autoimmune diseases such as RA, SjS or SLE (Stott et al., 1998; van Esch et al., 2003; Hansen et al., 2002; Dörner et al., 1998b).

In the current study, the extremely high degree of clonal relation (69% in $CD24^+$ and 94% $CD24^-$ in B cells) was described in RA patients´ B cells whereas in COs and CVID such IgM transripts were not found. Especially, we identified four RA patients´ B cell clones which were derived from the same V_H gene segments, shared identical mutations and used the same of D_H and J_H segments.

Taken together, these data provide evidence for an (auto)antigen-driven immune response ongoing in RA and suggest for oligoclonality of B cells in RA patient and on the other hand for polyclonality of tested B cell populations in both CVID and COs (Clausen et al., 1998; Hansen et al., 2002; Dörner et al., 1998b).

6.2.2.8. Amino acid composition in the CDR3 region

Neutral hydrophilic amino acids such a tyrosine, glycine, serine are used the most commonly. By constrast hydrophobic and positively charged amino amino acids such as arginine, lysine and histidine are rare in CDR3 regions. It has been demonstrated that the presence of charged amino acid could disrupt the stability of immunoglobulin heavy chain and its association with light chains (Ippolito et al., 2006). The frequency of charged AA is diminished during the development and selection of B lymphocytes. As they become atigen-experienced charged AA are often present in CDR3 regions od autoreactive Ig (Ippolito et al., 2006; Schwartz et al., 1999).

Observed relative amino acid composition in the CDR3 domains of analyzed Ig transcripts was in accordance with the above mentioned facts. However, significantly higher frequency of positively charged amino acids in the CDR3 regions of $CD24^-$ B cells as compared with $CD24^+$ counterparts was found in healthy donors. Similar, although non-significant tendency was found in patient eith RA. This might suggest that the population of $CD24^+$ B cells is in the more advanced stage of development and reflect certain degree of Ag-stimulation of these B cells. In CVID patients an inverse trend was observed however the difference was not statistically significant. This finding can result from disturbed immune system and B cell biology.

6.2.3. Analysis of $V_HD_HJ_H$ rearrangement in IgG transcipts

In CVID patients, all IgG transcripts were detected in B cells, simultanously producting IgG mRNAs. This suggest that the B cells have undergone class switching from IgM to IgG and still maintained IgM

transcripts in detectable amounts. Like in CVID patients, B cells from healthy donors also have undergone isotype switching however with no detectable IgM mRNA. In RA patient one $IgM^{-}IgG^{+}$ cell and one $IgM^{+}IgG^{+}$ cell was found.

The result suggests that a majority of analyzed B cells have not yet undergone isotype switching and the predominant by utilized Ig isotype was IgM.

6.2.4. RAG mRNA expression

As was discussed in Chapter 2.2.2.1., RAG enzyme complex is directly responsible for the V(D)J rearrangement of BCR in the developing B cells. Generally, it is assumed that the level of RAG expression is diminished in immature B lymphocytes and is rare in mature naive B cell repertoire (Girshick et al., 2001; Zhang et al., 2003). However, RAG enzymes can be re-expressed in germinal center B cells after their activation by Ag and it has been also proposed that mature B cells from patients with SLE regain the RAG expression (Dörner et al., 1998a). This phenomenon could represent a rescue mechanism for B cells with decreased receptor avidity after somatic hypermutation (Han et al., 1997).

The V(D)J recombination can be also accompanied by presence of N-addition introduced by TdT. No TdT mRNA expression could be detected in all analyzed B cell populations although N-additions in $V_HD_HJ_H$ rearrangements were commonly presented. This finding can be caused by down-regulated expression of TdT or by low amount of TdT-specific mRNA under detectable levels.

All types of RAG transcripts were routinely detected in B cells of all individuals. Comparable differences were noted when individual B cells were analyzed. B cells of CVID and RA patients expressed RAG1 mRNA as compared to normal peripheral blood B cells. Different results was revealed in RAG2b mRNA expression which was in all patients lowered in comparison to controls. The expression of RAG2a mRNA was different in all tested subjects showing no biased pattern.

The discordance in expression of RAG1 and RAG2 in individual B cells has been described however, the explanation remains still unknown (Girschick et al., 2001). All our analyzed B cell populations exhibited above mentioned features and therefore these results are consistent with the conclusion that not only immature B lymphocytes express RAG enzymes.

Our results are in accordance with data published by other groups and support the postulation that expression of CD27 molecule is not an exclusive feature of memory B cells. It has been shown that $CD27^-$ B cell subpopulation might also represent $CD38^{low}$ IgG secreting precursors of various $CD38^{+/high}$ plasma cell populations (Arce et al., 2004). In this context, observed $CD27^-$ B cells subpopulation might represent a new type of differentiated memory-like B cells, likely acting in protection of organism against bacterial and viral infection (Rakhmanov et al., 2009; Weston-Bell et al., 2009).

7. Conclusions

Two novel so far undescribed B cell populations were detected in the peripheral blood of controls (CO) and patients with CVID and RA, and characterized by immunophenotyping as $IgM^{+}CD19^{+}CD27^{-}$ $CD21^{low}CD38^{low}CD24^{+}$ and $IgM^{+}CD19^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{-}$. Both of B cell subsets were found to be overrepresented in the peripheral blood of patients with CVID in comparison to COs and patients with RA.

In order to find whether these cells share some common molecular characteristics, parameters such as produced immunoglobulin isotype, mutational rates, distribution of VH, DH and JH segments, and sequence and length of CDR3 were analyzed in variable regions of immunoglobulin heavy chain.

Surprisingly, mutations preferentially accumulated in CDR regions were observed in all analyzed Ig transcripts in spite of absence of CD27 expression which might indicate that B cells of our interest represent antigen-experienced or memory-like B cells. The main difference with respect to conventional memory B cells defined according to Klein´s criteria is that they are present in the periphery as a rare population reaching very low final frequences (below 0.1%). The hypothesis that these cells have undergone antigen-driven selection and possibly affinity maturation is also supported by detected expression of IgG mRNAs.

According to our molecular data, $CD27^{-}$ B lymphocytes of patients with CVID and COs are more close to each other sharing the similar characteristics of their $V_HD_HJ_H$ rearrangements. Moreover, $CD24^{-}$ subset of these B cells displayed a higher degree of differentiation status as compared to their $CD24^{+}$ counterparts in the same individuals. In addition, both subsets appeared to be very heterogenous intrinsically and polyclonal.

This was in contrast to our observations in the same B cell subsets of patient with RA. The most prominent difference was remarkably biased V_H gene usage restricted only to 10 genes and the predominance of occurence of V_H4-34 gene segment in Ig mRNAs. Most interestingly, these cells intrisically displayed high degree of homogeneity and clonal relation. Finally,

the length of CDR3 regions of these B cells was significantly shorter suggesting the ongoing process of (auto)antigen-driven selection.

Thus in conclusion, our observed particular B cells may play an important role in regulating of homeostasis of the immune system of healthy individuals or they might be responsible for the pathogenesis of the CVID. On the other hand, they also might under certain condition escape negative selection and produce autoreactive antibodies initiating development of autoimmune diseases such as RA.

Detailed understanding to the biology of these particular B lymphocyte subsets in patients with other autoimmune diseases such as systemic lupus erthematosus and Sjögren´s syndrome will be necessary to confirm the role of these B cells in physiological and/or pathological process.

8. Appendix – abstract presented

8.1. Abstract No. 1

Human B cell population escaping negative selection – from immunodeficiency to autoimmunity.

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In our previous study new $CD21^{low}CD38^{low}$ and $CD24^{pozitive/negative}$ B cell subpopulations were observed in CVID patients and controls (CO). The presence of B cells expressing VH4-34 gene known as coding for anti-dsDNA autoantibodies has been confirmed only in patients with CVID. The aim of the study was to search for such B cells in patients with rheumatoid arthritis (RA) using single-cell Ig specific RT-PCR.

All analyzed RA B cells overexpressed IgM transcripts The mutational frequency in both $CD24^{+}$ and $CD24^{-}$ RA B cells was decreased as compared to COs (1.74% and 1.47% vs. 2.35% and 1.96%). The most interesting finding was remarkable restriction of VH gene repertoire in RA B cells to only 10 VH genes in detected transcripts. Notably, the signifiant predominance of VH4-34 gene was found in analyzed B cells of both subsets in RA patiens whereas in COs this gene was completely absent (0% vs. 43%; p=0.0092). The most surprising observation was extremely high degree of clonal relation in RA patients´ B cells while in COs such transripts were not found (76% vs. 0%; p<0.0001). Over 60% of clonally related RA B cell used VH 4-34 gene segment and their CDR3 regions were shortened (5-11 amino acids).

Observed clonal relation of analyzed B cell populations in RA patients and their used VH genes such as VH4-34 that coding for autoantibodies suggest the susceptibility of these cells to escape negative selection and become autoreactive under autoimmune condition.

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10th ESID Prague Spring Meeting, May 9 and 10, 2011

Monday 9.5.2011

Lymphoproliferation and autoimmunity

chairs Andrew Cant, Václava Gutová

Aleš Janda (Germany): B-cell activating factor (BAFF) in autoimmunity and lymphoproliferation. 10 min

Václava Gutová, T.Votava, E. Mejstrikova, M.Sukova , A. Janda (Czech Rep.): CVID and lymphoproliferative syndrome in a child. Case report. 10 min

Andrea Poloučková, P. Pohunek, E. Kabíčková, R. Zachová (Czech Rep): EBV associated lymphoproliferation in CVID? – case report. 10 min

Šárka Růžičková, M. Bajzíková, A. Brundu, T. Kalina, J. Litzman, M. Vlková (Czech Rep.): Human B cell population escaping negative selection – from immunodeficiency to autoimmunity. 20 min

8.2. Abstract No. 2

The expanded $CD19^{+}IgM^{+}CD27^{-}CD21^{low}CD38^{low}CD24^{+/-}$ B cell populations in CVID patients and controls show the signs of antigen-experienced memory B cells

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Objectives:

Common variable immunodeficiency (CVID) is the most common primary immunodeficiency syndrome in adults and is characterized by the severe depletion of immunoglobulins however, its etiology remains unknown. Using polychromatic flow cytometry, a new subpopulations of naive $IgM^{+}CD27^{-}$ B-lymphocytes were observed as overrepresented in the peripheral blood (PB) of patients with CVID in comparison to controls (CO) and characterized as $CD21^{low}CD38^{low}$ $CD24^{pozitive/negative}$. However, their molecular characteristics such as produced immunoglobulin isotype, mutational rates, distribution of VH, DH and JH segments, and sequence and length of CDR3 have not been analyzed yet.

Aim:

Therefore the aim of the study was to search for signs of antigen-driven selection, affinity maturation and isotype switching at the IgM/IgG mRNA level.

Material and methods:

Single-cell Ig and Rag 1 and 2 specific RT-PCR was employed and individual

$IgM^{+}CD27^{-}CD21^{low}CD38^{low}$ $CD24^{+/-}$ B cells of two CVID patients and COs were examined. Obtained Ig mRNA sequences are available in GenBank under accession numbers GU552683-GU552781. The CVID patients were stratified according to Freiburg classification.

Results:

Both IgM and IgG transcripts were discovered in all analyzed B-cells, in COs the frequency of B-cells producing IgG mRNA was significantly higher in **$CD24^{-}$** subset as compared to **$CD24^{+}$** (4% vs. 31.6%; p= 0.0316). Mutational rates in CVID patients were significantly increased in **$CD24^{+}$** B-cells as compared to **$CD24^{-}$** (2.5% vs. 0.5%; p=0.0003) whereas in COs mutational rates were similar (1.8% for **$CD24^{+}$** and 2.2% for **$CD24^{-}$**). Significant absence of VH4-34 gene segment was found in COs in comparison to CVID (0% vs. 35%; p=0.0266).

Significantly higher proportion of positively charged amino acids in CDR3 regions was detected in **CD24$^-$** B-cells in comparison to **CD24$^+$** in COs (13% vs. 5.7; p=0.0095).

CDR3 regions were significantly shorter in **CD24$^-$** B-cells as compared to **CD24$^+$** in COs (16±0.69 aa vs. 13±0.64; p=0.0086) the sequences suggested for polyclonality of B cell populations in both CVID and COs.

Conclusions:

All molecular parameters suggest that both of populations might represent a new type of differentiated memory-like or antigen-experienced B cells, with less differentiated phenotype n CVID patients. This together with observed expansion of these cells in patients implies the hypothesis on their role for the pathogenesis of the disease. Since these cells share similar features between CVID and COs they might contribute to immune homeostasis in general.

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Abstract has been selected as an poster presentation in **ESF-EMBO Symposium:**

B Cells and Protection: Back to Basics, 12-17 June 2011, Hotel Eden Roc, Sant Feliu de Guixols, Spain

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9. Abbreviations

A	adenine
AID	activation-induced cytidin deaminase
AA	amino acid
APC	allophycocyanine
APRIL	a proliferation-inducing ligand
AV ČR	Akademie věd České Republiky
BAFF	B cell activating factor
BCR	B cell receptor
BSA	bovine serum albumin
C	constant region of gene complex of immunoglobulin; cytosine
CAML	calcium-modulating ligand
CD	cluster of diferentiation
CD40L	CD40 ligand
cDNA	complementary deoxyribonucleic acid
CDR	complementarity determing region
CDR-H	complementarity determing region of heavy chain
CDR-L	complementarity determing region of ligt chain
CO	control
CVID	common variable immunodeficiency
D	diversity region of gene complex of immunoglobulin
Da	dalton
DNA	deoxyribonucleic acid
dNTP	deoxynucleotidetriphosphate
dT	deoxythimidine
DTT	1,4-dithiothreitol
EDTA	ethylenediammine tetraacetic acid
FACS	fluorescence-activated cell sorter
FDC	folicular dendritic cell
FITC	fluoresceinisothiocyanate
FR	framework region
FSC	forward scatter

G	guanine
HLA	human leukocyte antigen
ICOS	inducible costimulator molecule
ID	immunodeficiency
Ig	immunoglobulin
IGA MZ ČR	Interní grantová agentura Ministerstva zdravotnictví České republiky
IgAD	IgA deficiency
IL	interleukin
IS	immune system
IVIG	intravenous immunoglobulins
J	joining region of gene complex of immunoglobulin
MB	memory B cell
mf	mutational frequency
MM	Multiple myeloma
mRNA	messenger ribonucleic acid
MZ	marginal zone
MZP	precursor of marginal zone B cell
nd	not defined
MSH2-MSH6	MutS homolg proteins
P	palindromic
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PC	phycoerythrine-cyanine
PCR	polymerase chain reaction
PE	phycoerythrine
pre-B	precursor B lymphocyte
pro-B	progenitor B lymphocyte
RA	rheumatoid arthritis
RAG	recombination activating gene
RSS	recombination signal sequence
RT	room temperature

RT-PCR	reverse transcriptase PCR
S	switch sequence
SD	standard deviation
SE	standard error
SHM	somatic hypermutation
SLB	Sigma loading buffer
SLE	systemic lupus erythematosus
smB	switched memory B cell
SSc	Sjögren syndrome
SSC	side scatter
ssDNA	single-strand DNA
T	thymine
T1, T2, T3	transient cells 1,2,3
TACI	transmembrane activator and CAML interactor
TBE	tris-borate buffer with EDTA
TdT	terminal deoxynucleotidyl transferase
T_H	helper T lymphocytes
TI	T-independent
TNF	tumor necrosis factor
Tr	transient
U	uracil
V	variability region of gene complex of immunoglobulin
WSB	wash and staining buffer

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