INFLUENCE OF TRANSIENT B CELL DEPLETION ON RECIRCULATING B CELLS AND PLASMA CELLS IN RHEUMATOID ARTHRITIS

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Contents

1 Introduction

1.1 B cell developmental stages

B cell development initiates in the bone marrow and the progenitor stem cell undergoes a sequential set of differentiation events till it becomes a naïve/mature B cell. The immunoglobulin genes are assembled through a somatic recombinatorial process to yield the B cell receptor (LeBien 2000; Honczarenko *et al.* 2006; Inlay *et al.* 2006)

The early lineage of B cells is termed to be the pro B cells which derive from the pluripotent hematopoietic stem cells. Heavy chain gene segments are rearranged in the pro B cell stage; the diversity gene – joining gene rearrangement (D-JH) occurs in the early pro B cell stage and during the late pro B cell stage variable segment rearranges with the DJH segment yielding a V-DJH joint. The recombination activation genes products, RAG-1 and RAG-2, play a fundamental role in these events.

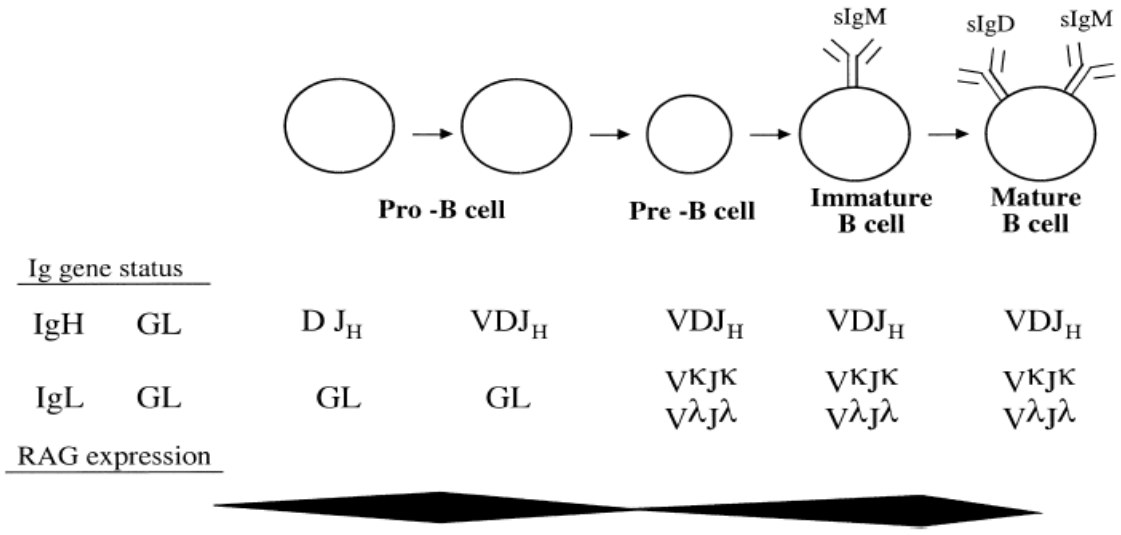


Fig. 1. Rearrangement of heavy and light chain loci- GL= germline; sIgM= surface IgM and sIgD= surface IgD - (Kouskoff *et al.* 2001)

Productive VDJH joining leads to the next main stage of B cell development, the pre B cell stage. An intact μ heavy chain is expressed intracellularly and also in small amounts at the cell surface. The μ heavy chain in combination with the surrogate light chain elements (λ5 and VpreB) forms the Pre B cell receptor (Tsubata *et al.* 1990; Kawano *et al.* 2006). Pre B cell receptor expression stops further rearrangement of the heavy chain segments and provides a signal to proceed with the light chain rearrangement.

Pre B cell divides several times to give rise to pre B cells where the light chain rearrangement (VL-JL) is carried out. Upon rearranging the light chain, a complete IgM molecule is expressed on the cell surface and the cell is defined as an immature B cell. All the developmental stages up to this point take place in the bone marrow and are independent of an antigenic stimulus.

The immature B cell eventually migrates to the periphery and differentiates in to mature/naïve B cell carrying IgM and IgD on the cell surface. In the peripheral lymphoid organs the B cell encounters antigen and further differentiates into antibody secreting plasma cell or memory B cell.

BCR *- a complex comprising a membrane-bound immunoglobulin molecule and two associated signal-transducing α and β chain*

IgM and IgD *- immunoglobulin molecules with mu and delta isotypes respectively*

1.2 Affinity maturation of B cells

In a humoral response to antigen, B cells undergo a series of genetic events to produce a population of antibodies that combat and eradicate the invading pathogens. Three major events in this process are the somatic hypermutation, gene conversion and class-switch recombination (Weill *et al.* 1996; Diaz *et al.* 1998). In this section, these intrinsic aspects in are discussed in detail.

1.2.1 Somatic hypermutation of Ig genes

B cells upon exposure to the invading antigen become activated and attain affinity maturation by the process of somatic hypermutation of immunoglobulin genes. During this event, Ig genes are subjected to a hypermutation mechanism which predominantly introduces random mutations. However deletions and insertions are also occasionally observed (Goossens *et al.* 1998). The frequency of mutations introduced is at the rate of approximately 10^{-3} per base pair per cell division (Rajewsky *et al.* 1987). This rate is 10^{6} fold higher than the spontaneous mutations that occur in somatic cells. In this way, the specificity of the antibody is highly tuned and high affinity variants are propagated which give rise to plasma or memory cell precursors.

Somatic hypermutation is confined to a 1-2 kilobase region in the rearranged Ig gene. At the 5' side, the promoter of variable region is the boundary, with mutations beginning approximately 150 bp downstream of the transcription start site. The 3' boundary is not well defined; however the C region is protected from mutations (Rada *et al.* 2001).

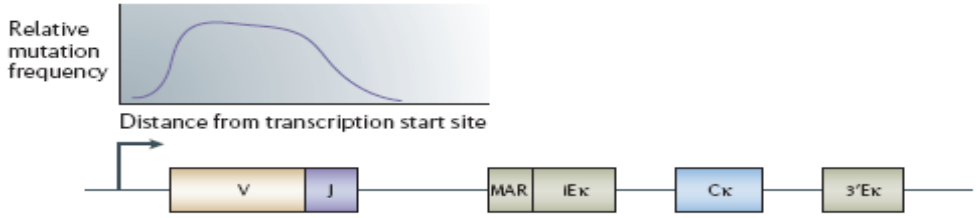


Fig. 2.Somatic hypermutation in the variable region of Ig gene- SHM in the variable (V) and joining (J) gene segments of Ig kappa light chain - (Odegard *et al.* 2006).

1.2.1.1 Mechanisms involved in somatic hypermutation

Findings from several groups summarized together propose a multilayered model of Ig hypermutation that includes the following:

1.2.1.1.1 Ig-hypermutation specific elements and role of transcription

SHM specifically targets the V, D and J segments of the heavy chain and the V and J segments of the light chain that together constitute the antigen binding site. Hypermutation appears to be under the direction of transcription related elements including the promoter and enhancer regions. Deletion of sequences upstream of the promoter region does not influence the hypermutational process (Tumas-Brundage *et al.* 1996) however, eliminating transcription through an Ig locus results in loss of SHM (Fukita *et al.* 1998).

Duplicating the V-gene promoter upstream of the C region and moving the intronic enhancer downstream of the C region in a kappa transgene conferred hypermutation also in the C regions (Peters *et al.* 1996). This emphasizes the role of these elements in the mutational process.

1.2.1.1.2 Introduction of DNA lesions

DNA double strand breaks (DSBs) seem to play an important role in the somatic hypermutation process. Recent reports using ligation-mediated PCR in Ramos B cells and mouse germinal center B cells provide strong evidence on this (Bross *et al.* 2000; Papavasiliou *et al.* 2000). In these studies, the DSBs appeared to occur near the mutational hotspot motif – RGYW (discussed in detail in section 1.4). In another study, the DSBs were found in late-S and G2 phases of cell cycle, indicating that a homologous-recombination mechanism of break repair, with the sister chromatid as template, is possible in Ig hypermutation. However, it does not require the non-homologous DNA end-joining (NHEJ) mechanism of DSB repair.

1.2.1.1.3 Error prone synthesis

The generation of a mismatched pair during synthesis suggests that a putative hypermutation polymerase could insert an incorrect base. Additionally, in sharks, where the mutational pattern is very similar to mammalian hypermutation, many mutations occur in doublets, suggesting two misincorporation and mismatch extension events (Diaz *et al.* 1999).

The polymerases η, ι and ζ – are documented to be error-prone and capable of extending with various efficiencies, from a mismatched terminus (Lawrence *et al.* 2001; Matsuda *et al.* 2001; McDonald *et al.* 2001). All these mechanisms contribute to the mutational machinery in B cells.

NHEJ *– A pathway that rejoins DNA strand breaks without depending on homology. The pathway is generally used in mammalian cells to repair strand breaks that are caused by DNA damaging agents*

Ramos Cells *– A Burkitt's lymphoma human B- cell line that constitutively undergoes high rates of SHM*

1.2.2 Gene conversion

Chicken IgH and IgL loci contain only one functional V region with many upstream pseudogenes. However a diverse repertoire with huge antibody diversity is produced predominantly by gene conversion, a non reciprocal form of intra-chromosomal homologous recombination (Bucchini *et al.* 1987; Reynaud *et al.* 1987). The size of gene conversion may range between 10 and 100 bp. Gene conversion-mediated antibody diversity is also observed in rabbits, pigs and cows, but not in mice and humans.

Although double-stranded DNA breaks induce gene conversion of other genes in yeast and mammalian cells, it is still unclear if DNA breaks occur during Ig gene conversion.

1.2.3 Class-switch recombination (CSR)

CSR detaches a variable (VDJ) region of the Ig gene from the constant region and ligates it to a downstream constant (C) region deleting the DNA between (Manis *et al.* 2002; Li *et al.* 2004). The signal to recombine a particular switch region comes from the cell surface. Cytokines such as IL-4 and co-stimulation (for example with CD40L) induce the production of sterile transcripts from promoters that are upstream of the targeted switch regions and under the regulation of the 3' regulatory region.

C region of the antibody *determines how the antigen is removed from the body. Each class of C region has specific pathways with which it eradicates specific kinds of pathogens*

Ig-3' regulatory region *– Sequences that are located 3' of the last constant region genes of the heavy chain Ig cluster that regulates the transcription of the Ig locus*

1.2.4 Activation induced cytidine deaminase (AID) - a regulator of B cell affinity maturation

Although the precise mechanisms of CSR, gene conversion and SHM are different and are not fully understood, they all require a newly discovered gene that encodes the activation induced cytidine deaminase (AID) and is specifically expressed in germinal centre B cells (Muramatsu *et al.* 2000; Revy *et al.* 2000; Diaz *et al.* 2003). Thus, AID is a pivotal player in generation of secondary antibody diversity which is essential for all the three processes (SHM, gene conversion and CSR).

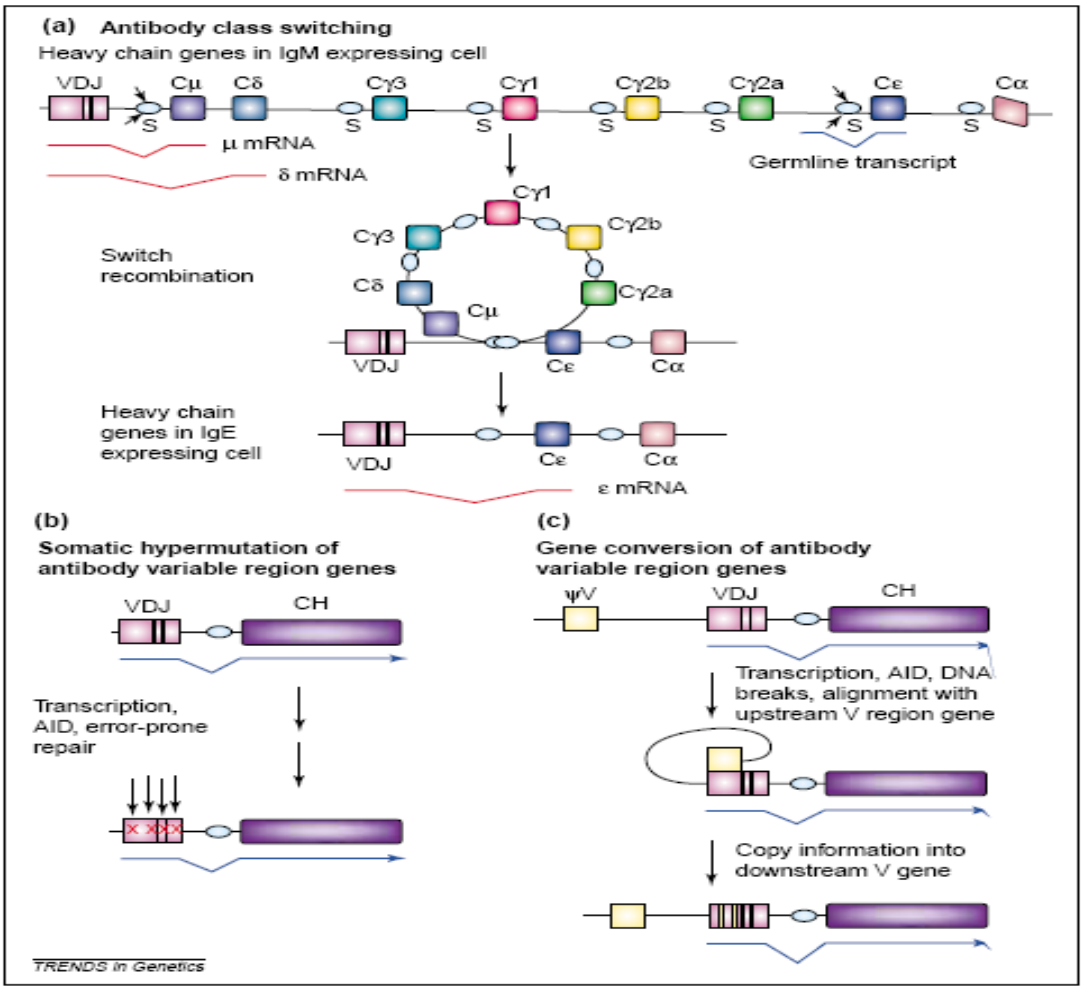


Fig. 3. Events in affinity maturation of a B cell – CSR initiates in the switch S region, leading to deletion of DNA sequence and finally resulting in switching to a different isotype. SHM includes point mutations, and Gene Conversion involves copying of pseudogenes located at 5' end and expressing it n the V region gene. All events require the activity of AID - (Stavnezer *et al.* 2002)

It is speculated that AID might directly deaminate the cytidine residues within the V region genes. This would result in mutations and would also recruit DNA repair processes to create DNA breaks that could initiate SHM and CSR. Furthermore, separate domains of AID interact with specific cofactors to regulate the two distinct genetic events (SHM and CSR). The C- terminal region of AID is responsible for CSR whereas N-terminal of AID regulates SHM (Shinkura *et al.* 2004).

Mutations resulting from SHM are more often transitions (T substitution by C and A substitution by G), which could be due to deamination of cytidine residues in the DNA. Studies on SHM also reveal that transition mutations occur about twice as frequently as transversion mutations (Golding *et al.* 1987).

Affinity maturation *- The mutation of antibody variable region genes followed by selection for higher affinity variants in the germinal centre, leads to an increase in average antibody affinity as an immune response progresses*

Transition mutations *- Base change in the DNA, where a purine is replaced by another purine (adenosine(A) to guanosine(G) or vice versa) or a pyrimidine is replaced by another pyrimidine (cytidine(C) to thymidine (T) or vice versa)*

Transversion mutations *- Base change in the DNA, where a purine base (A or G) is replaced by a pyrimidine base(C or T) or vice versa*

1.3 Sites of Somatic hypermutation

1.3.1 Germinal centre – The site of somatic hypermutation

The B cell follicles of the secondary lymphoid organs develop germinal centres (GCs) during T cell dependent humoral responses. GCs are identified to be a specialized micro-environment where the following events occur in order; i) T cell interaction and clonal expansion of B cells, ii) SHM of immunoglobulin genes, iii) selection of B cells on the basis of antigenic binding ability, and iv) differentiation of B cells in to memory B cells or plasma cells (MacLennan 1994).

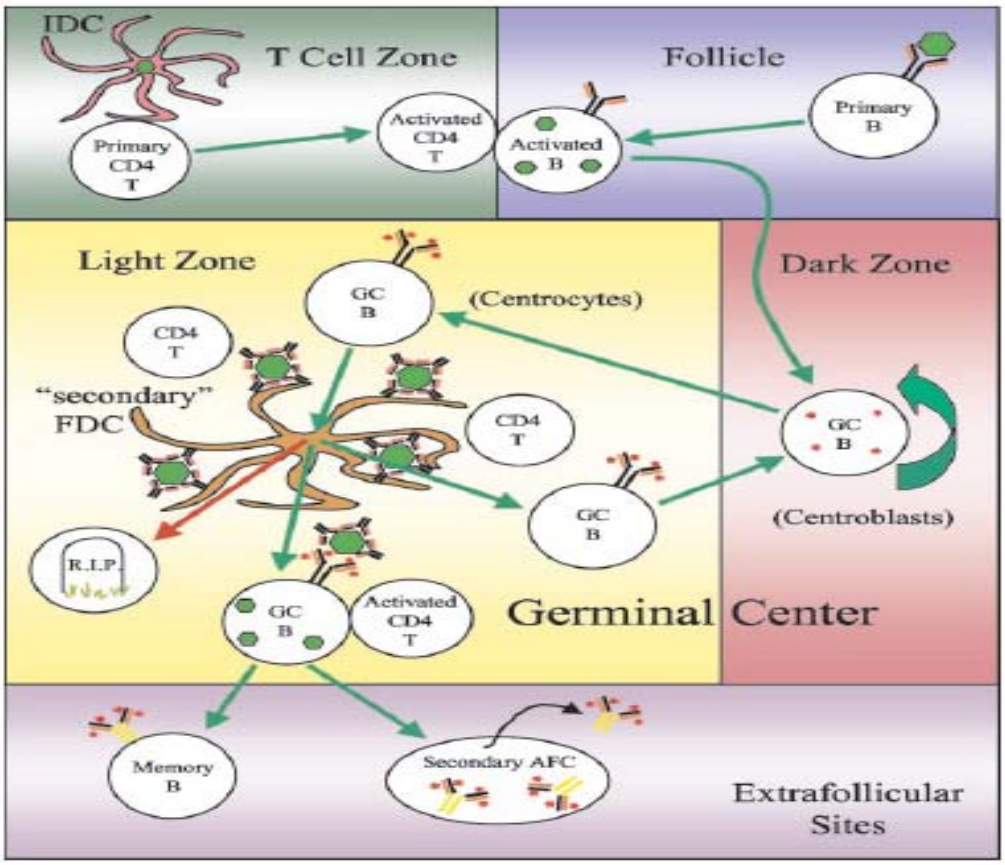


Fig. 4. Germinal centre reaction – various microenvironemental compartments are indicated by different coloured boxes, IDC = Interdigitating dendritic cells, green arrows = foreign antigen driven events, green hexagons = antigens, curved green arrow = rapid cell progression, red asterisk = mutations, red arrow = negative selection leading to apoptosis - (Manser 2004).

GCs initially contain only dividing centroblasts, but later they become mature GCs and are resolved into dark and light zones. A classical model of GC function holds that B cells in the dark zone involve T cell- B cell contact via CD40L- CD40 ligation, undergo rapid rounds of proliferation and SHM of the variable region followed by migration to the light zone that is rich in follicular dendritic cell (FDC) network (Allen *et al.* 2007). In the light zone, the B cells undergo selection on the basis of affinity towards its particular antigen; B cells that adequately bind to the antigen survive, whereas B cells that do not bind die by apoptosis (Kelsoe 1996; Tarlinton 1998). Thus GCs harbour hypermutation of Ig genes mediated by a T cell - B cell interaction.

1.3.2 Somatic hypermutations outside the germinal centre

T cell dependent hypermutation of B cells are thought to occur in the GCs formed by follicular B cells. However, several studies also provide evidence for SHM outside GCs. SHM has been observed in animals such as frogs and sharks that normally do not have GCs (Diaz *et al.* 1998). Also, mice deficient in lymphotoxin-alpha, where no lymph nodes, peyer's patches or GCs are formed, manifested a high-affinity anti-NP IgG1 response upon immunization with high doses of 4-hydroxy-3-nitrophenyl acetyl-ovalbumin (NP-OVA) (Matsumoto *et al.* 1996). Moreover, mice lacking TNF-receptor1 (TNFR1) are unable to form GCs, nevertheless produce high affinity antibodies. The load of mutations observed in the Ig genes of the TNFR1 deficient mice were not extremely different from the wild type mice (Kim *et al.* 2006). Furthermore human beings that do not express CD40L and do not have GCs still have somatic hypermutations (Huang *et al.* 1999). These observations strongly suggest that B cell variable gene mutations can also occur without GCs. Therefore the supposition that GCs are the only sites of Ig mutation and selection of high affinity antibodies remain controversial.

Centroblasts *are activated B cells that proliferate and divide rapidly and start SHM in the dark zones of the GC. They do not display membrane Ig*

Centrocytes *are B cells in the light zone of the GC that are programmed to die unless survival signals are provided by accessory cells*

FDCs *retain antigen on their surfaces in the form of immune complexes*

1.4 Mutational hotspots

1.4.1 RGYW and WRCY motifs

Molecular analysis of the distribution of mutations along the Ig sequence leads to the description of mutational hotspots. Several studies found that mutations in the IgR are predominantly confined to the RGYW consensus motif (R= purine, Y= pyrimidine and W=A or T). Computational analysis of the potential target of hypermutation revealed this evidence and opened new doors in the mutational hotspot determination (Rogozin *et al.* 1992). Further studies unveiled that mutations of G-C base pairs that are in the RGYW and the complementary WRCY motifs, as well as in specific di- and tri- nucleotide motifs also occur at higher frequency (Shapiro *et al.* 1999).

AID is thought to target RGYW/WRCY motifs during transcription and leads to deamination of C to U. Frequent targeting of these motifs by the mutational machinery is observed *in vitro* and *in vivo*. Mutational analysis of the P1-5 hybridoma cell line indicated 62% of all mutations were within the RGYW/WRCY motifs in the variable region of Ig gene (Martin *et al.* 2002). Codon insertion and/or deletion, which represent a mechanism contributing to the affinity maturation of antibodies, also occur in the RGYW/WRCY motifs. More recently, it is reported that insertions/deletions are prevalently located in the complementary determining regions (CDRs) of the heavy and light chain gene segments, VH, Vκ and Vλ. In contrast, framework regions (FRs) were less targeted for insertions/deletions however, a tendency of the mutations to be located in RGYW/WRCY motifs was observed (Reason *et al.* 2006).

1.4.2 Expression of RGYW/WRCY motifs

Beyond targeting of RGYW/WRCY motifs for hypermutation, the occurrence of these motifs is also more frequent in the Variable gene segments (Dorner *et al.* 1998). All individual quartets of the RGYW motif (AGCA, AGCT, AGTA, AGTT, GGCA, GGCT, GGTA and GGTT) occur in the CDR of the germline Vκ light chain segments and all

except AGTA in the framework region (FR) (Foster *et al.* 1999). Moreover, in the germline of VH heavy chain segments, the RGYW motifs, AGCT and GGCT occurred more frequently than expected and especially in the CDR (Dorner *et al.* 1998).

1.5 Replacement and silent mutations

SHM leading to mainly point mutations in the Ig gene could be either of replacement or of silent type. Assessment of the replacement to silent mutation ratio (R/S) is essential to evaluate antigen selection on Ig genes. In the absence of antigen, with no positive or negative selective pressure on Ig genes, a random mutational process resulting in even distribution of R and S mutations would be expected in the variable region of the coding sequence. However, in reality, increased replacement over silent mutations was observed, especially in the CDRs. This notion is supported by a study showing elevated R/S in CDRs in comparison with FRs (4.27 versus 1.50) (Messmer *et al.* 2004). Furthermore, positive selection of replacement mutations was also reported in the CDRs of Ig- Vκ (Foster *et al.* 1999) and Ig-Vλ repertoire (Monson *et al.* 2000) indicating antigen selected antibodies demonstrate a higher frequency of R mutations in CDRs than in FRs.

In order to maintain the structural integrity and preserve the functional Ig molecule, higher frequency of S mutations and paucity of R mutations are expected in the FRs. This mechanism is manifested in the canonical hypermutational process (Lossos *et al.* 2000).

***Framework Region (FR**) is the more conserved region of the Ig variable region. Human Ig consists of FR1, FR2 and FR3 in the variable region*

***Complementary Determining Region (CDR)** is the hypervariable region in the variable region of the Ig gene. Human Ig genet consists of a sequential order of FR1-CDR1- FR2-CDR2-FR3-CDR3 to form the V region*

***Replacement mutation** is the base substitution which on the transcription level eventually leads to change in the amino acid*

***Silent mutation** is the base substitution which on the transcription level does not influence the amino acid*

1.6 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic, autoimmune disease, mainly characterized by destructive inflammation of the lining, or synovium of the joints. It is one of the most common inflammatory diseases with a high incidence, affecting 1% of the world's population. The disease affects women three times more than men. RA can affect people of all ages and races and commonly appears between the ages of 25 and 55. It has serious impacts on a person's life and well-being. If not diagnosed and treated early, RA results in significant morbidity, mortality and disability of the individual (Lee *et al.* 2001).

Progression of RA is accomplished through several stages. The first stage is the swelling of the synovial lining causing pain, warmth, stiffness, redness and swelling around the joint. Second stage is the rapid proliferation of cells, or pannus that causes thickening of the synovium. During the next stage, the inflamed cells secrete enzymes that may digest bone and cartilage leading to the loss of joint shape and alignment. Increased pain and stiffness generally accompany this stage.

The aetiology of RA remains elusive. The combination of genetic susceptibility with yet-unidentified inciting event(s) might lead to the disease manifestation. The association of HLA-DR4 with RA is well-established (Stastny 1978). However more data on the precise role of genetic factors is still pending.

The pathophysiology of RA includes the presence and activity of several pro-inflammatory cytokines and chemokines. Marked infiltration of mononuclear cells, especially T cells and macrophages into the synovial compartment leads to production of Interleukin (IL)-1, -2, -6, -8 and -10, tumor necrosis factor-α (TNF-α) and certain other factors. Another potential player from the lymphocyte arm of immune system, the B cell fraction also infiltrates the synovium and differentiates into plasma cells, producing polyclonal immunoglobulin and rheumatoid facor (RF). The net result of these activities is the pannus formation, cartilage invasion, bone erosion and destruction (Weyand *et al.* 1997; Smeets *et al.* 1998; Lundy *et al.* 2007).

Disease markers such as presence of RF, antibodies to cyclic citrullinated peptide (anti-CCP) and radiographic demonstration of bone erosion are a few diagnostic tools to mention in clinical practice assessing the severity of RA in patients.

The central role of inflammation is illustrated by the clinical observation that the bone compartment in closest proximity to inflamed joints suffers the most severe damage. The skeletal lesions are termed to be local bone erosions and are hall marks depicting the destructive role of RA (Schett 2006).

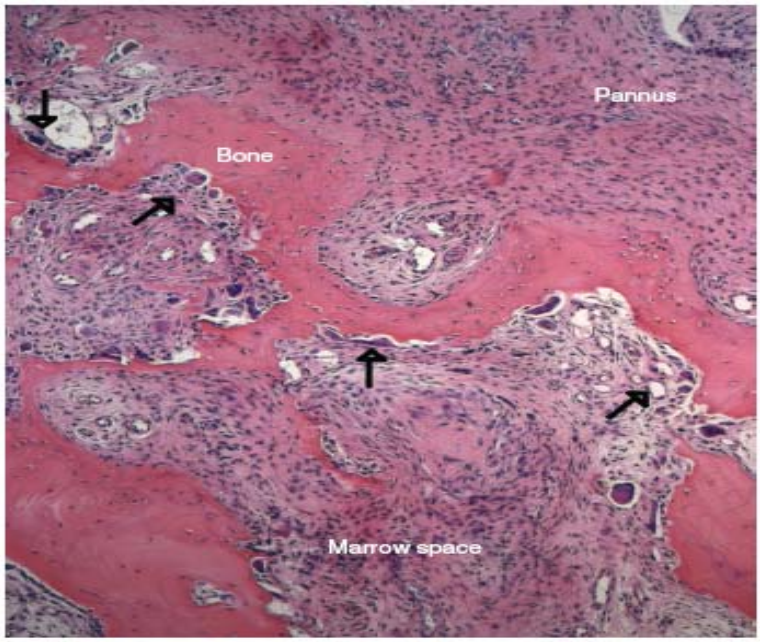


Fig. 5. Focal articular bone erosion in RA – the image depicts pannus invading bone tissue with inflammatory tissues infiltrating the sub-chondral narrow space. Cells with a osteoclast morphology are pointed by arrows - (Walsh *et al.* 2005).

An Osteoclast *is a multi-nucleated cell expressing tatrate-resistant phosphatase (TRAP) and cathepsin K, involved in bone resorption*

Rheumatoid Factor (RF) *is an auto-antibody directed against the Fc portion of autologus IgG. It is generally present in 80% of RA cases*

Anti-CCP *is an antibody against the unusual amino acid citrulline, used in the diagnosis of RA*

1.7 Role of B cell in health and disease

1.7.1 Functions of B cell in healthy subjects

In normal immune responses, B cells have additional roles than being mere precursors of antibody secreting plasma cells. They function as antigen presenting cells to other components of the immune system, for example the T cells. They also regulate T cell activation, produce various cytokines and regulate the differentiation of follicular dendritic cells and lymphoid organization (Gonzalez *et al.* 1998; Harris *et al.* 2000).

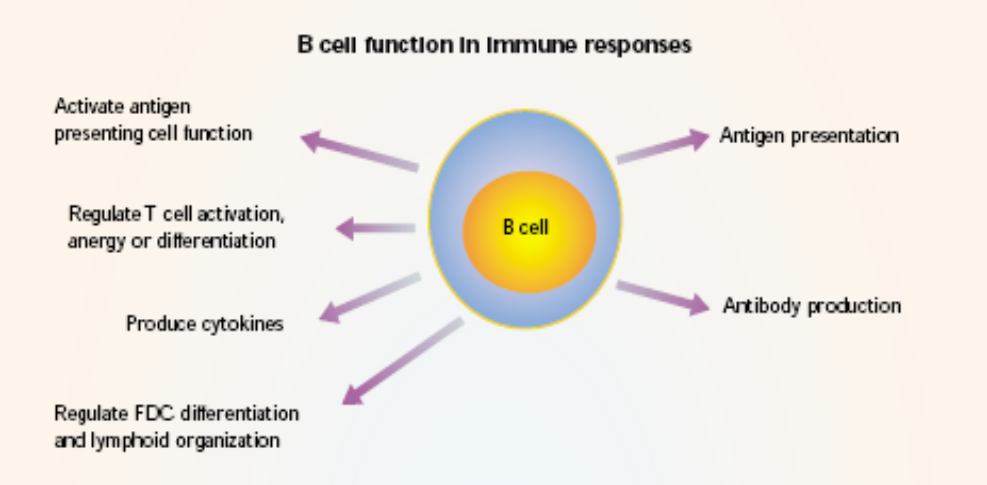


Fig. 6. B cell regulation in normal immune responses - (Lipsky 2001)

1.7.2 Role of B cell in rheumatoid arthritis

B cells play a central role also in the immuno-pathogenesis of various autoimmune diseases. In several ways they regulate the aspects of autoimmune responses (Browning 2006). Recent studies in rheumatoid arthritis, evaluate the functions of B cells which reveal additional roles besides secretion of auto-antibodies. B cells stimulate the rheumatoid factor producing B cells, present auto-antigen to T cells and express co-stimulatory signals for T cell expansion (Wang *et al.* 1999).

Moreover, B cells secrete regulatory cytokines like IL-4 and IL-10 that support the survival of other mononuclear cells, regulate follicular dendritic cells and lymphatic organization in bones (Silverman *et al.* 2003; Martin *et al.* 2004). Recent work demonstrates that B cells secrete the pro-inflammatory cytokines, such as TNF-α, IL-1 and lymphotoxin that inturn enhances the production of IL-6 and IL-10. This further stimulates B cell function via a feedback loop thereby exacerbating chronic inflammation (Pistoia 1997; Duddy *et al.* 2004). Eventually, these events lead to destruction of the bone architecture and finally result in organ damage.

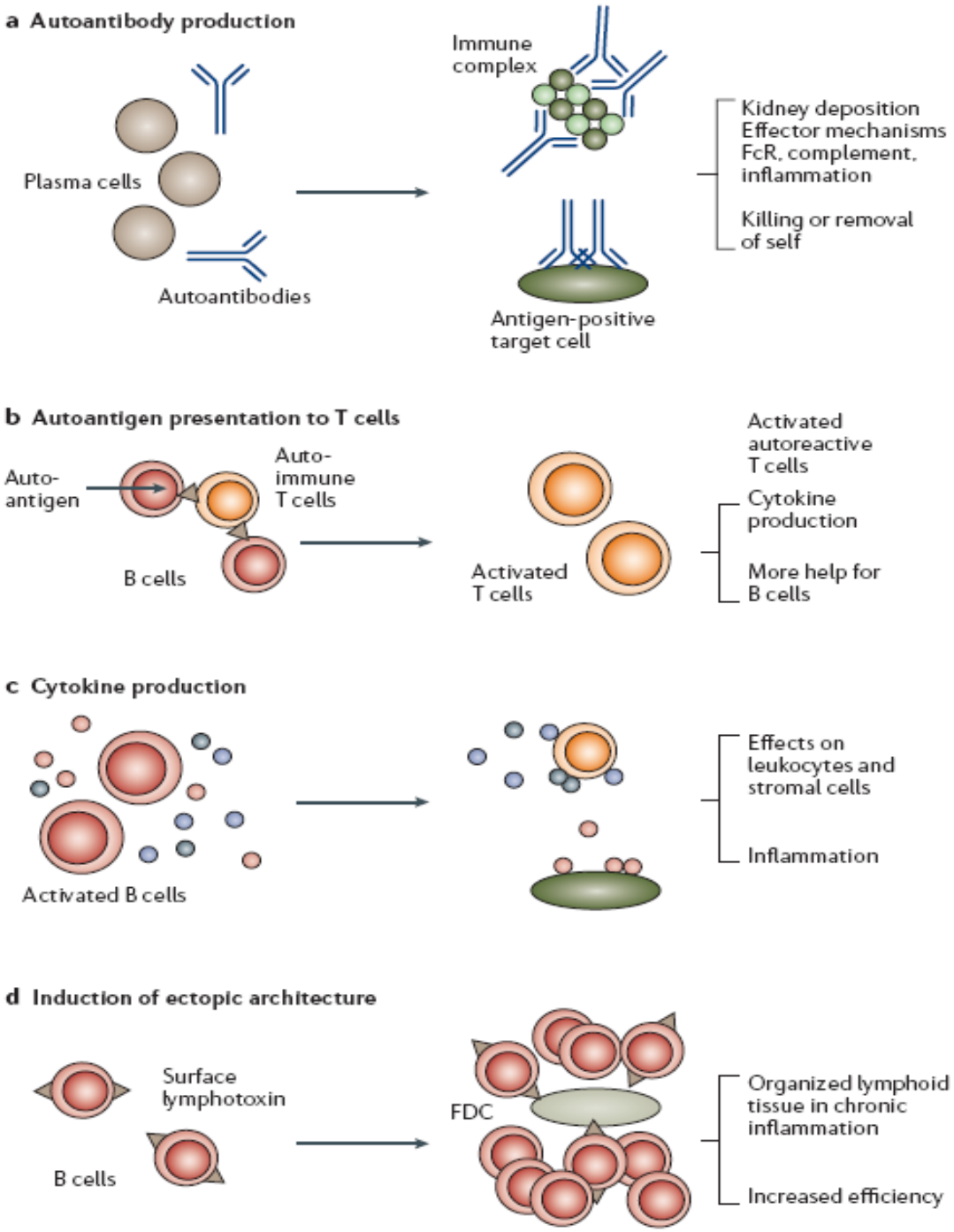


Fig. 7. Role of B cells in autoimmunity- B cells produce self antigen binding auto antibodies, present self antigen to auto reactive T cells, secrete disease mediating cytokines and activates other components of the immune system and also form organized lymphoid structures - (Browning 2006)

1.8 Human Ig- VH locus

The human VH locus is located in the subtelomeric band, q32, on the long arm of chromosome 14. The locus spans 1250 kilobases and consists of 123 VH gene segments classified into seven families (VH1-VH7) based on the sequence homology. Among these, 44 gene segments consist of an open reading frame and 79 are pseudogenes. Ig heavy chain also comprises of 25 to 27 diverse (D) genes grouped into seven families and six functional joining (JH) gene segments (Matsuda *et al.* 1998).

1.8.1 Ig-VH4 gene rearrangements in healthy subject

VH4 is the second biggest VH family (after VH3) consisting of 32 family members among which 9 members are expressed in the antibody repertoire, especially Ig-VH4-34 (or VH4-21) account for 25% of VH4 rearrangements (Suzuki *et al.* 1995). In a previous study, Ig-VH4-34 was found to be the most widely used VH4 family member (Logtenberg *et al.* 1992). B cell repertoire analysis of two normal adults revealed that VH4 family contributed to approximately 28% of VH rearrangements with a prevalent usage of VH4-34 gene (Kraj *et al.* 1995). In contrast, another group reported 20% of the peripheral blood Ig-VH rearrangements belong to VH4 family. Moreover in this study, VH4-59/DP-71/ and 4-4b but not 4-34 was over-expressed in the VH4 repertoire (Brezinschek *et al.* 1995).

1.8.2 Ig-VH4 gene usage in autoimmunity

Abnormal usage of VH genes were observed in autoimmunity. There are indications that VH4-34 gene, which is over-expressed in a healthy repertoire, is also supposed to encode pathogenic auto-antibodies (Hirabayashi *et al.* 1997). Exclusive usage of VH4-34 gene is shown in cold agglutinin disease (Dorner *et al.* 2001; Dorner *et al.* 2002). Another study on autoimmune repertoire analysis also revealed the over representation of VH4-34 gene (Pascual *et al.* 1992). Moreover, VH4-34 is found to be preferentially used in rheumatoid factor producing B cells. In rheumatoid arthritis, the

genes 4-34 and 4-31 are demonstrated to be widely used in the VH4 repertoire (each 26%) followed by VH4-4 gene (8%) (Huang *et al.* 1998).

1.9 B cells as therapeutic targets in autoimmune diseases

The immunologic surveillance of B cell is highly appreciated in a healthy immune response against the foreign organisms. However in case of autoimmunity, given that B cells play a central role in the immuno-pathogenesis, therapeutic targeting of B cell is gaining more importance. Several studies in the recent years prove the efficacy of B cell targeted therapies in treating various autoimmune disorders. B cells are directly or indirectly targeted as explained in the following sections.

1.9.1 B cell depletion

Removal of B cells by an antibody mediated depletive therapy (anti CD20 therapy) has gained more attention in the treatment of several autoimmune disorders. B cell depletive therapy is being investigated intensively in a wide range of autoimmune diseases including rheumatoid arthritis, systemic lupus erythematosus, sjögren's syndrome, ANCA associated vasculitis and others (Cohen 2005; Pitashny *et al.* 2005; Edwards *et al.* 2006). The mechanism of depletion is discussed in detail in section 10.

1.9.2 Inhibition of B cell survival

B cell activating factor (BAFF) also called as BLyS is a key cytokine that sustains B cell survival. BAFF binding to the BAFF-receptor (BAFF-R) is believed to deliver primary survival signal to the B cells. BAFF levels are found to be elevated in the blood of patients suffering rheumatoid arthritis, SLE and sjögren's disease which indicated that excess BAFF might be connected to the expansion of certain B cell subsets related to the pathogenesis. Several approaches are being implemented for BAFF suppression. Data from monkey experiments reveal reduction of B cells by about 50% in both the blood and in the

secondary lymphoid tissues (Halpern *et al.* 2006; Vugmeyster *et al.* 2006). An anti-BAFF antibody, belimumab, reported some efficacy in treating rheumatoid arthritis.

1.9.3 Blockade of CD40 signalling in B cells

Another enticing strategy in B cell targeting is the blockade of T cell- B cell interaction through CD40-CD40L (also known as CD154) binding. This attempts in preventing the germinal-centre specific T cell- B cell interaction which generally might result in acquisition of increased point mutations along the B cell Ig sequence and iso-type switching. These elevated mutations suppose to increase the affinity with which the B cells bind to auto-antigen.

Employment of CD154 monoclonal antibodies has shown safety and efficacy in patients with SLE, idiopathic thrombocytopenic purpura and crohn's disease (Kalunian *et al.* 2002; Kuwana *et al.* 2004). Another study using CD154 antibodies in SLE reported the reduction of circulating plasmablasts, which may be a potential biomarker of the disease (Arce *et al.* 2001).

1.9.4 Suppression of cytokines

Several cytokines are identified to be essential for activation and expansion of B cells. For example, IL-6 plays a pivotal role in the terminal differentiation of B cells into antibody producing plasma cells thereby contribution to the immuno-pathogenesis. IL-6 blockade with an anti-IL-6 receptor antibody has shown efficacy in rheumatoid arthritis and Crohn's disease (Kishimoto 2005; Mihara *et al.* 2005).

In SLE, elevated levels of type I interferon is reported. Psoriatic arthritis and a subset of rheumatoid arthritis revealed the expression of a collection of genes induced by type I IFN- that is, an "IFN signature"(Baechler *et al.* 2006). In such cases, IFN blockade might be a successful tool in treating the disorder.

1.10 Anti-CD20 therapy

1.10.1 Rituximab treatment – A novel B cell depletive therapy

Rituximab is a genetically-engineered chimeric monoclonal antibody with murine heavy and light chain variable regions and human IgG1/κ constant regions and driven against the surface CD20 expressed on B cells. Discovered in 1991, Rituximab is the first FDA- approved antibody for the treatment of low grade non-Hodgkin's lymphoma (Grillo-Lopez 2003). Rituximab has shown to have profound depletion of B cells, selectively and effectively *in vivo* from the peripheral blood (Looney 2002; Eisenberg *et al.* 2005). Depletion in bone marrow, spleen and lymph nodes is not well studied. A pilot study including four patients with lupus and autoimmune thrombocytopenia indicated rituximab-mediated complete B cell depletion in bone marrow and spleen (Kneitz *et al.* 2002) whereas monkey experiments reveal less complete depletion in bone marrow, spleen and lymph nodes.(Vugmeyster *et al.* 2005).

1.10.2 Human CD20 molecule

Human CD20 is a tetraspan molecule B cell surface molecule of 33-37 KDa with an extra cellular loop of 44 amino acids. The binding site of the antiCD20 antibody presumably lies in this loop (Polyak *et al.* 2002). CD20 is phosphorylated and can signal phosphorylation or function as calcium channels (Valentine *et al.* 1989). Mice lacking CD20 do not have abnormal responses. Therefore, the exact physiological role of CD20 in human B cells is yet not understood. On a therapeutic level, CD20 poses to be an effective target due to its high expression and additionally, it does not shed or internalize in response to antibody binding.

1.10.3 B cell subsets depleted by rituximab therapy

B cells arise from the stem cells in the bone marrow and undergo a series of maturation and activation steps as they migrate to the periphery, germinal centre, memory

compartment and finally as plasma cells, to the bone marrow. The early progenitor cell, namely the pro-B cell lineage does not express CD20 on the surface. Expression of CD20 surface molecules on the B cells start at the pre-B cell stage and continues to express during different stages of maturation. However, during the later stages of differentiation, for example in the pre-plasma- cell stage CD20 expression is down-regulated. Thus, the pre-B cells, plasmablasts and plasma cells are not directly targeted by the anti-CD20 rituximab therapy (Browning 2006). Effective Rituximab therapy targets the CD20 expressed in pre-B, immature, naïve, activated, germinal centre and memory B cells and depletes these subsets selectively.

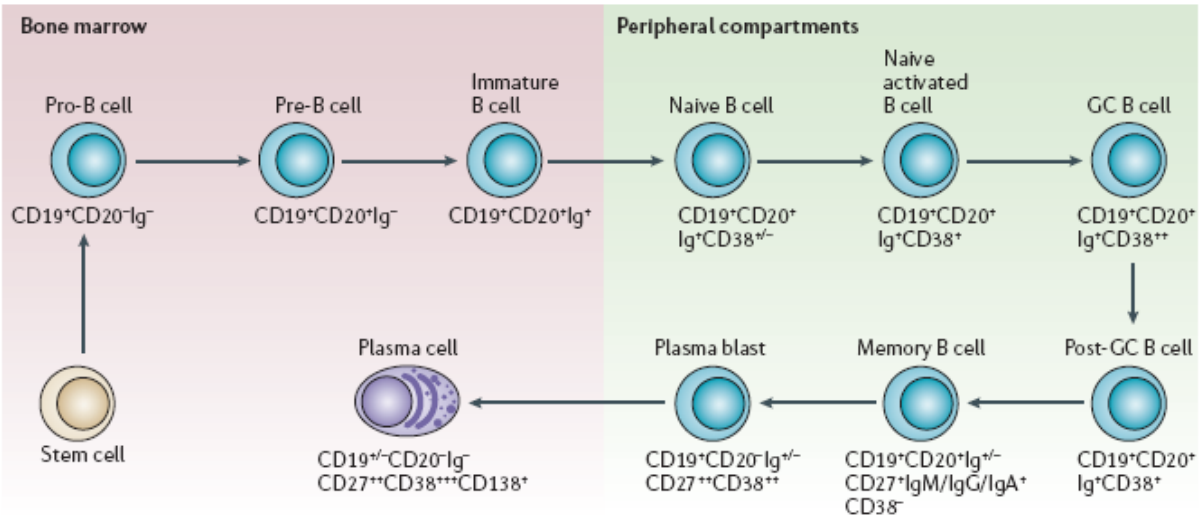


Fig. 8. B cell subsets, their anatomical site and expression of surface markers - (Edwards *et al.* 2006)

1.10.4 Mechanism of B cell depletion by rituximab therapy

Rituximab mediated depletion of B cells seems to include a combination of antibody-dependent cellular toxicity (ADCC) and complement-dependent cytotoxicity (CDC) mechanisms. Direct induction of apoptosis is also proposed to play a role in the depletion mechanism. Although ADCC reflects to be the dominant mode, mouse models, show a range of other depleting mechanisms (Cartron *et al.* 2004; Hamaguchi *et al.* 2006). Successful depletion lowers the number of B cells to below detectable range in the peripheral blood.

1.11 Rituximab therapy in treating rheumatoid arthritis

Though rituximab mediated B cell depletive therapy was first approved for treating lymphoma, later it also extended its application in the treatment of various diseases especially in autoimmune disorders. In the treatment of autoimmunity, particularly in rheumatoid arthritis, B cell depletive therapy has been appreciated for its profound efficacy and promising results in the recent years. Rituximab has a good safety record in almost all the studies except for mild infusion reactions. No significant treatment associated side effects were documented and in general the drug is well tolerated. This also holds well with repeated treatments over a five year period (Popa *et al.* 2006). However as with other biological immunomodulatory reagents, the incidence of serious infections seems to be doubled.

An open label study showed reduction in the Disease Activity Score (DAS)-28 and decreased RF level in RA patients treated with rituximab (Kneitz *et al.* 2004). Serologic changes assessment in rituximab-treated RA patients, showed a marked reduction in IgA-RF, IgG-RF and anti-CCP antibodies (Cambridge *et al.* 2003). Another study comprising 15 patients indicated elevated levels of BAFF following rituximab therapy and reduced serum levels of RF, though those of anti-tetanus toxoid or anti-pneumococcal polysaccharide do not fall significantly (Cambridge *et al.* 2006). Other components of the immune system, for example, the proportion of T cells remained unaltered by rituximab-mediated B cell depletion. More recently, a multi-center, randomized, placebo-controlled phase III trial documented a significantly meaningful clinical response by a single course of rituximab in treating active, longstanding RA which had an inadequate response to anti-TNF therapies (Cohen *et al.* 2006).

During the regeneration phase following the therapy, a characteristic pattern of B cell re-emerging was observed. Elevated levels of circulating immature B cells were observed (Leandro *et al.* 2006). In parallel, increased circulation of CD38hi plasmablasts were also reported. The immature B cells during the later time points of regeneration differentiate into mature/naïve B cells and dominate the peripheral blood (Roll *et al.* 2006).

2 Objectives of the project

B lymphocytes play a central role in the pathogenesis of various autoimmune diseases. Therefore, therapeutic targeting of B cells in treating autoimmune disorders is significantly rising over the last years. Recently, B cell depletive therapy using anti CD20 antibody has opened new doors in the treatment of autoimmunity. Especially, in RA, a more prevalent autoimmune disorder, B cell depletion seems to be a promising tool for treatment. Phase III clinical trials document the safety and efficacy of the drug. Owing to the increased interest in depleting B cells in autoimmunity, it is essential to understand the modulation in the immune system following such a therapy.

However, to date, the regenerative mechanism of B cell depletive therapy for the complex autoimmune response in RA is not fully understood. In one such attempt in our lab, we carried out a phenotypic analysis of the B cell subsets during the regeneration process. Regeneration process post treatment follows a characteristic pattern with circulation of immature B cells during the early recovery phase in addition to abundance in plasma cell phenotype. During the late recovery phase, the periphery is dominated by naïve B cell phenotype. Nevertheless, data on the molecular level of B cell regeneration is still meagre. There is a hint that during the early recovery phase, the peripheral blood B cell compartment comprises of highly mutated B cell receptor (BCR) sequences.

In order to gain insights into the mode of action of rituximab, we studied the regeneration of peripheral B cells. We were particularly interested to know how the B cell system is modulated by rituximab. To delineate this, we framed a detailed molecular analysis of the BCR before therapy and during the early and late recovery phases. Furthermore, we investigated the pattern of mutations in the B cell- Ig receptors at these time points.

The main questions we wanted to answer are:

1*) Does the therapy induce alterations in the variable gene usage in the Ig-VH4 repertoire?*

2) How does the B cell subsets regenerate and is there any dynamic change in the replenishing B cells?

3) In regard to the mutations in the BCR: To what extent does the targeting of mutations to previously defined hotspot motifs occur and what is the nature of mutations?

4) Is there any therapy-induced qualitative modulation of the BCR which could be used as a potential bio marker in B cell depletive studies?

3 Material and methods

3.1 Patients

Five patients, (A-E) were included in the study of treating RA with monoclonal anti-CD20 antibodies. Peripheral blood of patients A-C, was studied at multiple time points (one pre treatment and two post treatment) using a total genomic DNA amplification protocol. In patients, D and E, only one time point (post treatment) was analyzed using a single cell RT-PCR method.

All the patients were female and met the American College of Rheumatology (ACR) criteria for RA. The ages of patients ranged between 30 and 66 years (Patient A, B, C, D and E were 49, 66, 45, 30 and 44 years old respectively during the start of the study).The patients were refractory to previous treatments with Disease Modifying Anti-Rheumatic Drugs (DMARD) including methotrexate and /or TNF-α- antagonists.

Informed consent was obtained from all patients before entering the study in accordance with the protocol approved by the ethics committee of the University of Wuerzburg, Germany. Four patients stayed on concomitant Methotrexate, one patient received only concomitant steroids. All patients received two infusions of rituximab (Mabthera ®, Hoffmann La – Roche, Grenzach – Whylen, Germany) at a dose of 1000 mg, 2 weeks apart.

Disease activity was regularly determined by monitoring DAS28 activity, CRP levels and RF values. Molecular analysis of immunoglobulin receptor (IgR) from peripheral blood of the patients was performed in 3 different time points, namely before therapy, early regeneration phase (ERP - the time point after B cell depletion when for the first time more than 1% B cells in lymphocyte population was detected) and late regeneration phase (LRP – 2 to 3 months later to ERP).

3.2 Ig analysis from total genomic DNA

3.2.1 Isolation of Peripheral blood mononuclear cells (PBMCs)

Peripheral blood (40ml) of RA patients was collected in heparinized syringes. PBMCs were isolated by following a FICOL gradient protocol as described below:

Blood was diluted with equal volume of isotonic solution (NaCl). Equal volume of FICOL was laid over the blood-NaCl mix followed by centrifugation at 900g for 25 min without brake. At the end of centrifugation the various fractions of blood were separated into different layers based on the density. The ring-shaped, white-layer, rich in PBMCs, was separated and washed twice with HBSS medium with 10% FCS and penicillin-streptomycin and L-glutamine. The corresponding centrifugation speed was 1300g for 10 min. The PBMCs count was determined microscopically by counting the viable PBMCs stained with trypan blue. The PBMCs were finally suspended in PBS buffer and stored at -20^0C till further use.

3.2.2 Extraction of total genomic DNA

Total genomic DNA was extracted from approximately 1.10^7 PBMCs using QIAamp DNA Blood Mini Kit (Qiagen) according to manufacturer's instructions. The kit employs a fast and easy way to purify total DNA from PBMCs for reliable PCR. The pure and concentrated DNA at the final stage of extraction is eluted in buffer AE provided in the kit and is free of protein, nucleases and other contaminants or inhibitors and stored at 4^0C until further analysis.

3.2.2.1 Determination of nucleic acid concentration

The concentration of genomic DNA was measured photometrically using a Ultraspec 1000 apparatus (Pharmacia Biotech). The DNA was diluted in dH_2O and the absorbance was measured at a wavelength of 260 nm. The purity of the sample was

assessed by absorbance measurement at 280 nm. Absorbance ratio (A260/A280) in the range of 1.6 to 2.0 assures sample purity.

Nucleic acid concentration was calculated as follows:

C = A260 x (dilution factor) x (50) µg/ml

250 ng DNA (usually in 5 µl) was utilized as template in the PCR amplification of Ig genes. The Ig-VH4 specific primers and PCR conditions are explained in the following sections.

3.3 Ig-VH4 gene amplification

A nested PCR was employed for the amplification of VDJ rearrangements of Ig genes of VH4 family with the total DNA as template. The primers employed were described in previous publications (Brezinschek *et al.* 1995; Foster *et al.* 1997; Farner *et al.* 1999). The primers were synthesized by MWG Biotech AG.

3.3.1 Oligonucleotide sequences

3.3.1.1 External primers

Variable region primer

VH4 E (20): ATG AAA CAC CTG TGG TTC TT

Constant region primers

JH1,2,4,5 E (21): TGA GGA GAC GGT GAC CAG GGT

JH3 E (20): TAC CTG AAG AGA CGG TGA CC

JH6 E (19): ACC TGA GGA GAC GGT GAC C

3.3.1.2 Nested primers

Variable region primer

VH4 N (27): GGT GCA GCT GCA GGA GTS GGG GSC AGG

Constant region primers

JH1,3,4,5 N (25): CGA CGG TGA CCA GGG TBC CYT GGC C

JH2 N (25): CGA CAG TGA CCA GGG TGC CAC GGC C

JH6 N (24): CGA CGG TGA CCG TGG TCC CTT GCC

IUB codes mentioned in the primer sequences correspond to the following mixed base sites: S = G,C; B = G,T,C; Y = C,T

All primers as above are provided 5' to 3'. VH4 E and VH4 N primers hybridize to the leader peptide and frame work region 1 of Ig VH4 genes respectively.

3.3.2 External amplification round

The PCR mixture was prepared as follows:

5 µl genomic DNA template (50 ng/µl)

8 µl $MgCl_2$

1.6 µl deoxynucleoside triphosphate (dNTPs 10 mM each)

5 µl VH4 E primer (10 µM)

5 µl JH E mixed primer (10 µM)

8 µl 10X buffer II, without $MgCl_2$

0.5 µl AmpliTaq DNA polymerase (5U/µl)

46.9 µl dH_2O

The AmpliTaq polymerase, 10X buffer II and $MgCl_2$ were purchased from Applied Biosystems where as dNTPs was purchased from Peqlab.

PCR amplification was performed on Gene Amp PCR System 2400 (Perkin Elmer).

The amplification temperature and cycles are as following:

denaturation at 95^0C for 5 min
annealing at 50^0C for 1 min — 1 cycle
extension at 72^0C for 1 min

followed by,

denaturation at 94^0C for 1 min
annealing at 50^0C for 30 s — 35 cycles
extension at 72^0C for 1 min

followed by a final extension at 72^0C for 5 min and stored at 4^0C

3.3.3 Internal amplification round

The nested round was carried out in an identical manner to the external round except for the following changes:

a) Nested primers as described previously (see section 3.1.2) were used.
b) The template for the internal round is 5 µl of external PCR product
c) Annealing temperature was raised to 65^0C to yield more specific Ig-VH product.
d) Number of cycles in the PCR program was reduced to 30

The volume of $MgCl_2$, dNTPs, 10x Buffer and dH_2O remained the same as in the case of external amplification round.

Amplified DNA of Ig-VH4 genes was stored at 4^0C until further use.

3.4 PCR product isolation

Amplified Ig-VH4 gene products were isolated as explained below:

The PCR product was separated by electrophoresis through 1% agarose gel. The product was allowed to migrate at 100V for about an hour. The amplified Ig-VH4 gene product corresponding to 350 bp was stained with ethidium bromide and visualized under UV light at a wavelength of 254 nm. The product was excised with a clean scalpel, extracted and purified by using MinElute Gel Extraction kit (Qiagen) as per manufacturer's instructions. The MinElute system gel extraction system combines the convenience of spin-column technology with the selective binding properties of a uniquely designed silica gel membrane. MinElute columns are designed to give high-end concentration of purified DNA fragments. The procedure involves dissolving of agarose, binding of DNA to the silica membrane, washing steps with different buffers and a final elution of pure DNA in 10µl elution buffer.

3.5 Sub cloning of Ig-VH4 gene rearrangements

3.5.1 Polishing

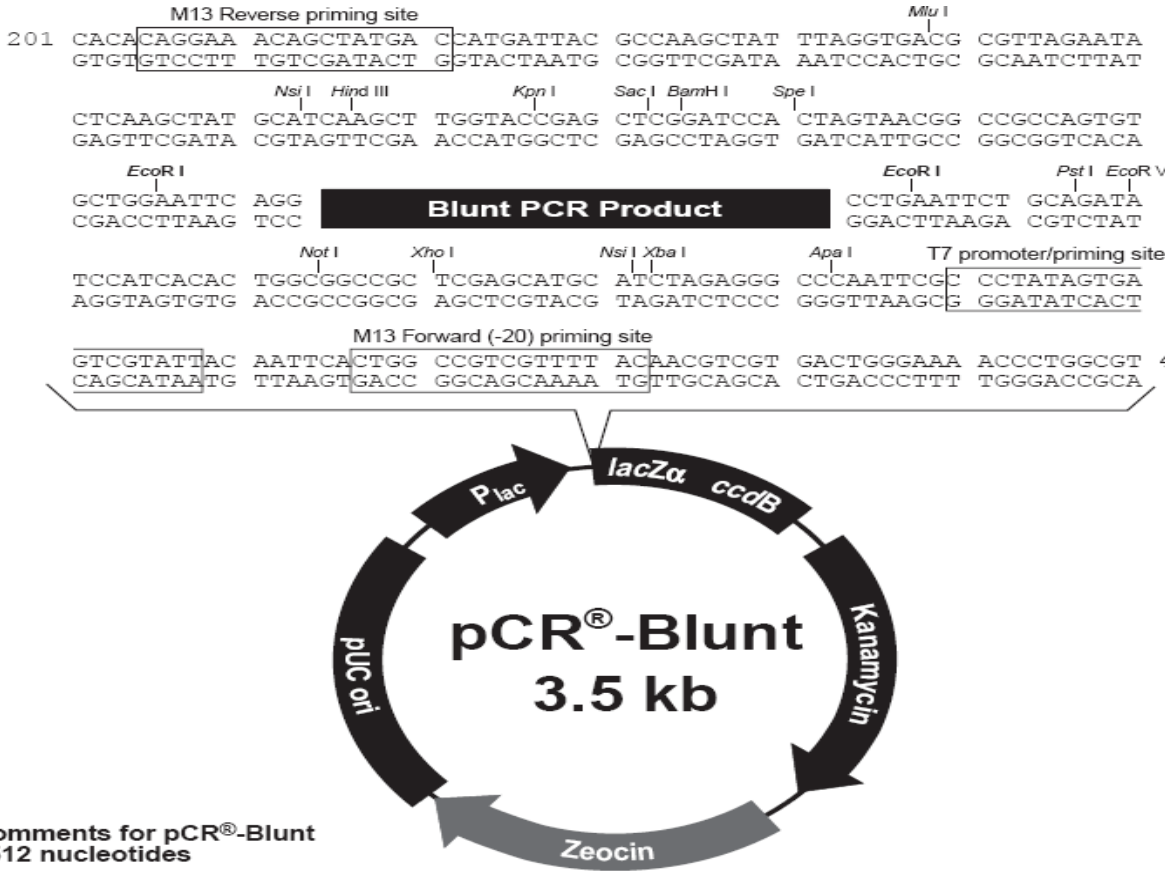
TAQ polymerase employed in the PCR amplification of Ig-VH4 genes generates 3'-overhang extensions of DNA fragments that have to be polished before the product is ligated. Stratagene polishing kit is designed to polish the ends of the 3'- overhang extensions and to generate blunt ends which could be further ligated and cloned.

Component	Volume µl
DNA fragment	9
Nucleotide mix (10 mM, 2.5 mM each)	1
Polishing buffer (X10)	1.3
Cloned *pfu* polymerase	1

The above-mentioned mixture was incubated at 72^0C for 30 min. Polishing reaction yields blunt-ended DNA fragments which could be further ligated.

3.5.2 Subcloning and transformation

A subcloning step was performed using Zero Blunt-PCR cloning kit (Invitrogen, Life Technologies). In this process, the blunt PCR fragments with a low background of non-recombinant were cloned in to a pCR-Blunt vector. The features of the pCR-Blunt vector are represented in the figure below. The vector allows direct selection of positive recombinants via disruption of the lethal gene, *ccd*B that belongs to *E.coli*. The product of the *ccd*B gene is expressed as a fusion protein to the LacZα peptide fragment. Ligation of the blunt PCR product disrupts the expression of LacZα-ccdB gene fusion permitting the growth of only positive recombinants upon transformation. The vector also contains both kanamycin and zeocin resistant genes for selection in E.coli.



Comments for pCR®-Blunt
3512 nucleotides

Lac promoter/operator region: bases 95-216
M13 Reverse priming site: bases 205-221
*Lac*Z-alpha ORF: bases 217-570
T7 promoter priming site: bases 400-419
M13 Forward (-20) priming site: bases 427-442
Fusion joint: bases 571-579
*ccd*B lethal gene ORF: bases 580-882
Kanamycin resistance ORF: bases 1231-2025
Zeocin resistance ORF: bases 2231-2605
pUC origin: bases 2673-3386

Fig. 9. pCR blunt vector

Subcloning and transformation involved the following steps:

(i) Ligation of the DNA fragment into the vector

(ii) Transformation into competent cells

3.5.2.1 Ligation into pCR-Blunt vector

After polising, the concentration of the insert is generally, ≤10 ng/µl. For a higher cloning efficiency, blunt end ligations require high insert to vector ratios. In our case, 5:1 insert to vector ratio was used. The corresponding ligation protocol was as follows:

Component	Volume µl
Linearized pCR-Blunt vector (25 ng)	1
Blunt DNA fragment insert	1-5
Ligation buffer 10X	1
T4 DNA ligase (4U/µl)	1
Water to make upto 10 µl	

The ligation reaction was carried out by incubation the mixture at 16^0Cfor 1 hour

3.5.2.2 Transformation into One Shot TOP 10 Competent cells

Transformation was carried out by following the instructions in the subcloning kit provided by Invitrogen. Briefly, 5 µl of the ligation product was pipetted into a vial containing 50 µl of One Shot Top 10 competent cells. Competent cells are highly sensitive to changes in temperature and mechanical lysis by pipetting. So, these cells were thawed on ice and the ligation product was pipetted gently into the cells. The vial was incubated on ice for 30 min and a thermal shock was applied for exactly 45 seconds at 42^0C. To this, 250 µl of SOC medium was added and the vial was shaked at 37^0C for 1 hour at 225 rpm. In the mean time the agar plates were set ready.

As the transformation reaction ends, 80 µl of the mixture was streaked over the LB agar plate with kanamycin and the plate was incubated at 37^0C overnight.

3.6 Liquid culture

Following overnight incubation at 37^0C, antibiotic resistant transformants were observed as individual colonies on the agar plates. Individual colonies were randomly picked- up from all over the agar plate and set up into a liquid culture in culture tubes containing 2 ml of LB brothbase medium. These tubes were incubated at 37^0C for 12-15 hours.

3.7 Plasmid DNA isolation

Plasmid DNA from the overnight liquid culture was isolated using the Wizard Plus SV Minipreps DNA Purification System kit (Promega). The process involved the following steps: Pelletizing the cells, production of a clear lysate with an alkaline protease solution, neutralization, binding of the plasmid DNA to a spin column, washing thoroughly with column wash solution and elution of the DNA in elution buffer.

3.8 Analysis of the transformants

The presence of inserts were checked by different procedures: restriction digest using the enzyme EcoR1 or PCRs on plasmid DNA using M13 forward and M13 reverse universal primers.

3.8.1 PCR on plasmid DNA

Plasmid DNA was amplified using M13 forward and M13 reverse primers. The PCR mix consisted of:

1 µl (100 ng) plasmid DNAgenomic DNA template (50 ng/µl)
0.5 µl dNTPs
0.5 µl M13F primer (10 µM)
0.5 µl M13R primer (10 µM)
2 µl $MgCl_2$ (25 mM, Applied Biosystems)
2.5 µl 10X buffer II, without MgCl2 (Applied Biosystems)
0.25 µl AmpliTaq DNA polymerase (5 U/µl)
17.75 µl dH_2O

And the thermocycler program comprised of:

denaturation at 94^0C for 5 min
annealing at 56^0C for 1 min
extension at 72^0C for 1 min

} 1 cycle

followed by,

denaturation at 94^0C for 1 min
annealing at 56^0C for 30 s
extension at 72^0C for 1 min
} 25 cycles

followed by a final extension at 72^0C for 10 min and stored at 4^0C

The PCR products were analysed by gel electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining under UV light at 254 nm.

3.9 Sequencing

The transformants obtained in the form of plasmid DNA were sequenced using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). This kit consists of a ready to use terminator mix with dye terminators, deoxynucleoside triphosphates, AmpliTaq DNA polymerase, FS, rTth pyrophosphatase (a component in AmpliTaq DNA Polymerase, FS), magnesium chloride and buffer in a single tube. These reagents are suitable for performing a fluorescence based cycle sequencing reaction.

Sequencing reaction mix

The following components were mixed to set up a sequencing reaction:

4µl ABI Prism BigDye Terminator
0.5µl M13F or M13R primer (10 pmol/µl)
2-7 µl template DNA (corresponding to 500 ng)
9.5-13.5 µl dH_20 (to make upto 20 µl)

Thermoocycler program comprised of:

denaturation at 95^0C for 5 min
annealing at 50^0C for 1 min
extension at 60^0C for 1 min
} 1 cycle

followed by,

denaturation at 95^0C for 30 s
annealing at 50^0C for 1 min
extension at 60^0C for 3 min
} 25 cycles

followed by a final extension at 72^0C for 5 min and stored at 4^0C

3.9.1 Purification of sequencing reaction products

3.9.1.1 Preliminary purification

The PCR products were first purified using AutoSeq G-50 columns (GE Health care). This was done to remove the excess dye-labelled dideoxynucleotides from the sequencing reaction. The resin in the column was first resuspended by vortexing gently. The resin preservative was removed by pre spinning the column for 1 min at 2000 x g. The PCR product was then loaded to the centre of the angled surface of the compact resin bed and the column was spun for 1 min at 2000 x g. The eluate consists of purified DNA.

3.9.1.2 Isopropanol precipitation

The product was further purified by 75% isopropanol. 80 µl of 75% isopropanol was added to 20 µl of the PCR product, shaked and incubated for 15 min at room temperature. After 15 min, the samples were centrifuged at a speed of 14000 rpm for 20 min. The supernatants were discarded and the pellets were dissolved again in 250 µl of 75% isopropanol and centrifuged at 14000 rpm for 6 min. The supernatants were discarded and the pellets, rich in pure DNA, were air-dried. These pellets were then suspended in 20 µl Template Suppression Reagent (Applied Biosystems), denatured for 3 min at 95^0C and placed on ice for 2 min. Later, the products are loaded on an automated ABI PRISM 310 Genetic Analyser (Applied Biosystems).

3.10 Analysis of Ig sequences

3.10.1 Sequence analysis software

Heavy chain Ig genes were analyzed by JOINSOLVER®, a web-based software program developed for human immunoglobulin V(D)J recombination and mutational analysis. This program was created by the National Institutes of Health, National Institute of Arthritis and Musculosketelal and Skin Diseases (NIAMS) and the Center for Information Technology (CIT). JOINSOLVER® is used for performing immunoglobulin nucleotide and amino acid alignment as well as extensive mutation and CDR3 analysis. (http://joinsolver.niams.nih.gov/index.htm)

3.10.1.1 Parameters analyzed

The following parameters were analyzed:

1. **VDJ segments usage**

In each Ig rearrangement, the heavy chain variable region analysis was undertaken. The variable gene used in every rearrangement was delineated.

2. **Mutational frequency**

The number of mutations in the variable region was determined by comparing the germline sequence and the mutational frequency is assessed by dividing the number of mutations by total nucleotides in the rearrangement, multiplied by 100.

3. **Mutations in RGYW and WRCY motifs**

Previous studies by several groups (Rogozin *et al.* 1992; Martin *et al.* 2002) demonstrated the accumulation of mutations in RGYW and WRCY hotspot motifs. (R = purine, Y= pyrimidine and W= A/T). The number of mutations lying in the hotspot motifs was assessed and the percentage of RGYW/WRCY mutations was calculated.

4. Replacement to Silent mutation (R/S)

The replacement and silent mutations in the complementary determining regions (CDR) were delineated and the replacement to silent mutational ratio in CDR was calculated.

A model Ig gene sequence with the analyzed parameters is represented as follows:

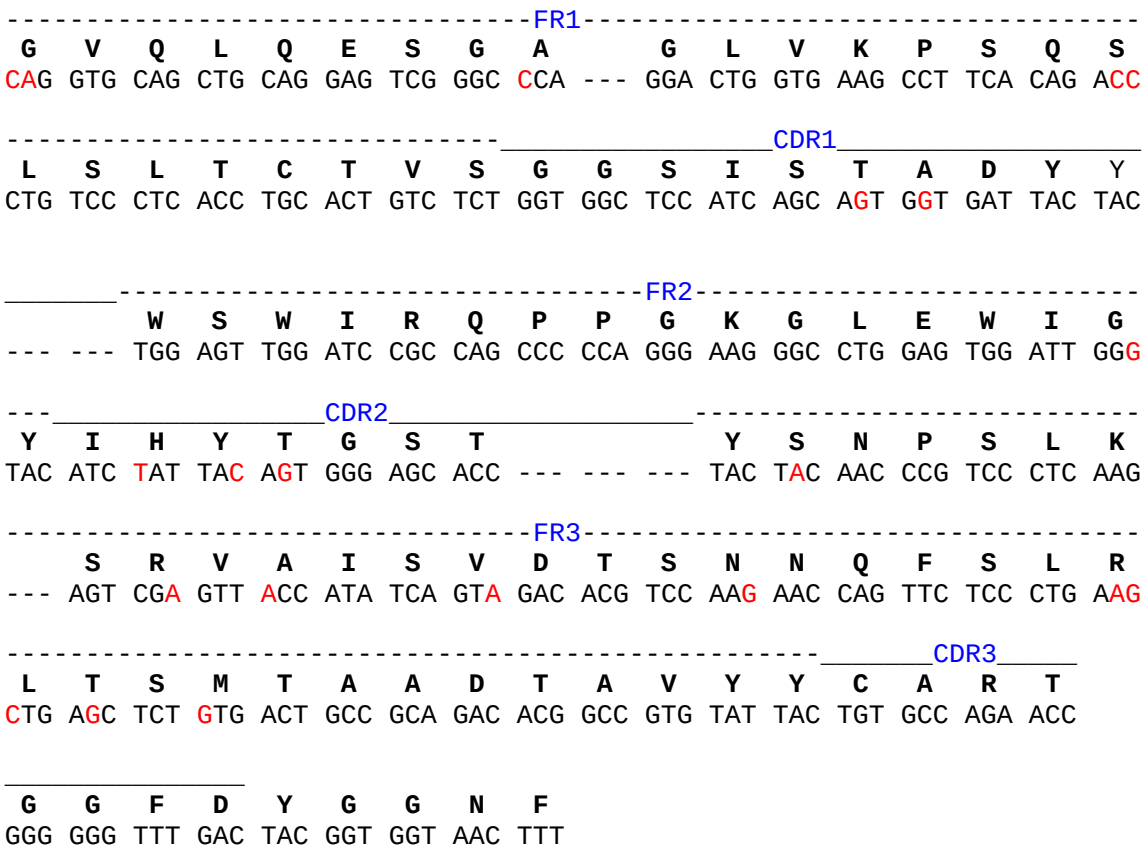


Fig. 10. Representative of an immunoglobulin VH4 gene sequence

The alphabets above the codons in figure 10 represent the corresponding amino acids and the VDJ segments and mutations are delineated by comparing the sequence to the germline sequences. The mutated bases are indicated in red.

Results

V gene usage: VH4-30; D gene usage: D-01; J gene usage: JH4-03

Number of mutations in the variable region: 22

VH mutational frequency: 7.56%

Number of RGYW motifs: 11

Number of mutations in RGYW motifs: 7

Number of WRCY motifs: 13

Number of mutations in WRCY motifs: 4

Number of RGYW/WRCY mutations: 11

RGYW/WRCY mutational frequency: 50%

R/S ratio in FR (1-3): 10/7

R/S ratio in CDR (1-2): 4/1

3.11 Single cell RT-PCR

3.11.1 Cell preparation

PBMCs from peripheral blood were isolated as described in section 2.1. After determination of the PBMCs count, staining of the B cell surface markers with fluorescence labeled antibodies was carried out. In this study, we sorted individual B cells of the plasma cell, naïve B cell, immature B cell and memory B cell subsets. The different surface markers expressed on these B cell subsets and the fluorescence labeled antibodies used against these surface markers were provided in the following table.

Table 1. B cell subsets, their surface expression and corresponding fluorescence labeled antibodies

B cell subset	Surface expression	Respective antibody employed
Plasma cell	CD 19+, CD 38hi, IgD-	APC+, PE+, FITC-
Memory B cell	CD 19+, CD 38med/low	APC+, PE med/low
Immature B cell	CD 19+, IgD+,CD 10+ CD38+	APC+, FITC+, PerCp+, PE+
Naïve B cell	CD 19+, IgD-, CD38-	APC+, FITC-, PE-

To approximately 1.10^7 PBMCs, 50 µl of each antibodies were added and incubated for 15 min on ice followed by washing with 10 ml RPMI medium(with 10% FCS and 1% penicillin- streptomycin and 1% glutamine) at 1300 rpm for 10 min. The supernatant was discarded and the stained cells were suspended in 1.5ml of RPMI medium and filtered through Miltenyi column (pore diameter 30 µm). These cells were stored on ice until further sorting.

3.11.2 Preparation of lysis buffer

The stained cells were sorted into each well of the 96 well plate which contains lysis buffer. The lysis buffer was prepared as following:

Component	**Stock concentration**	**Volume µl (for 100 wells)**	**Final concentration (in each well)**
DTT	100 mM	150	5 mM
RNAsin	40 U/µl	50	20 U
BSA	10 µg/µl	5	0.5µg
Oligo dT(15)	800 ng/µl	100	400 ng
Triton	10%	300	1%
RNAse free H_2O	-	2445	make up volume of 30µl

DTT was provided in Titan One Tube RT-PCR kit and BSA and Triton were purchased from Sigma. RNAsin and Oligos dT15 were purchased from Promega and Roche respectively.

To each well of the 96 well PCR plate, 30µl of the lysis buffer was added the PCR plate was stored on ice until the sorting procedure.

3.11.3 Cell sorting

Sorting of individual B cell of different subsets was carried out in a FACSDiVa single cell sorter which was outfitted with an automated cell deposition unit. Various B cell subsets were identified on the basis of their surface expression (recognized by the fluorescence- labeled antibody binding) and individual cells were sorted into the wells of the 96 well plate. After the sorting procedure the plate was shortly spun down and the cells are lysed in the buffer which leads to the disruption of the cell membrane and release of mRNA.

3.11.4 Upper reaction buffer

To the plate with the sorted B cells, an upper reaction buffer containing nucleotides, reverse transcriptase and buffer were added. The composition of the upper reaction buffer was as following:

Component	**Stock concentration**	**Volume µl (for 100 wells)**	**Final concentration (in each well)**
RT-PCR buffer	5 X (with $MgCl_2$)	400	1X
dNTPs	10 mM each	40	0.2 mM
Reverse transcriptase	20 U/µl	100	20 U
RNAse free H_2O	-	1460	make up volume of upto 20 µl

RT-PCR buffer and reverse transcriptase were provided in Titan One Tube RT-PCR kit and dNTPs was purchased from peqLab. To each well, 20 µl of the upper reaction mix was added and cDNA synthesis was started.

3.11.5 Synthesis of cDNA

The plate was incubated at 50^0C for 1h and the first strand of cDNA was synthesized from mRNA by a reverse transcription reaction. The plate is stored at 4^0C till further use.

3.11.6 Amplification of the VH4 genes

VH4 genes from cDNA were amplified and determined using the same procedure explained in section 3.

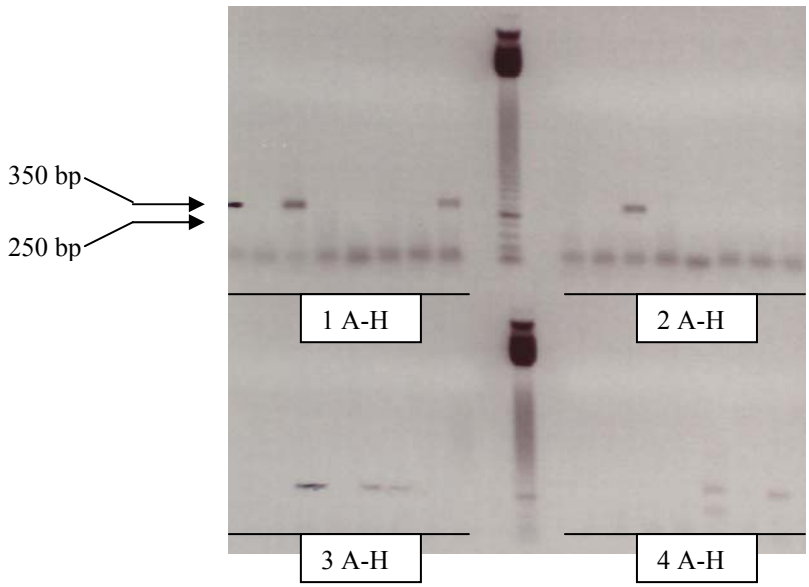


Fig. 11. PCR amplification of Ig-VH4 genes

Depicted above is a representative of Ig amplification following single cell RT-PCR. Shown were the positive signals for Ig-VH4 gene amplification products in a 96 well plate PCR amplification method. The desired product corresponds to a size of approximately 350 bp. The gel shows the products belonging to lanes 1-4 (8 wells A-H in each lane, totally 32 wells) of a 96 well PCR plate. All the wells do not show positive signal for Ig-VH4 products indicating that the cells without a positive signal for VH4 might use other Ig-VH family rearrangements. Lanes 1 & 2 and 3 & 4 are separated by a 50 bp ladder marker.

3.11.7 Direct sequencing of Ig-VH4 genes

The products from the wells showing positive signal for VH4 gene products were extracted by MinEluteGel extraction protocol as explained in section 3.4 and directly sequenced and analyzed as explained in sections 3.9 and 3.10. The sequential steps involved in single cell sorting of individual B cells, synthesis of cDNA, amplification of Ig-VH4 genes and subsequent sequencing steps are depicted as below:

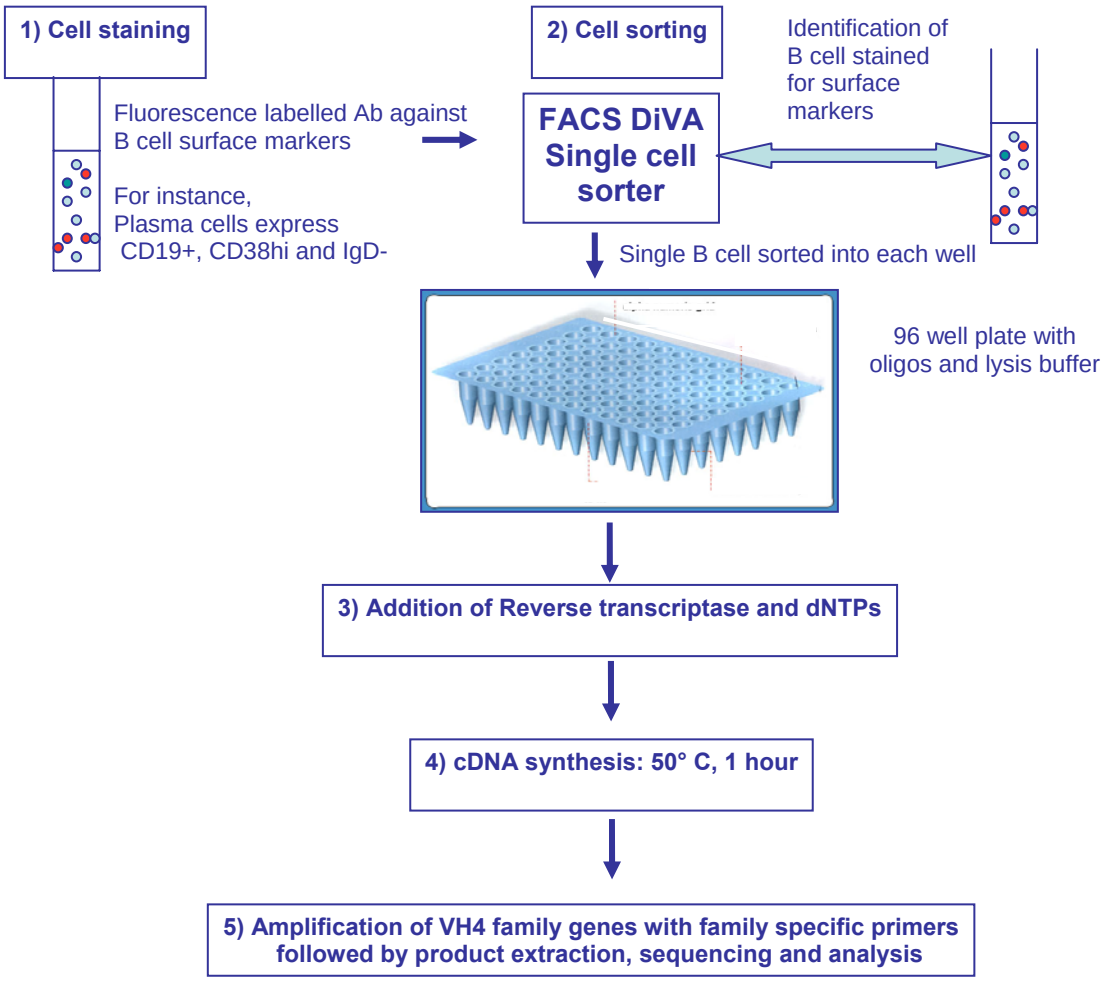


Fig. 12. Schematic representation of single cell sorting of B cells and subsequent analysis

3.12 Statistical analyses

Graphpad software which is available online (http://www.graphpad.com/) was used for detemining the statistical significance of various parameters analyzed in the study. Individual VH gene segments usage before and after therapy was compared by Fischer's exact test. The overall mutational frequency of Ig-VH4 genes, RGYW/WRCY mutations and R/S mutational ratios pre and post-treatments were compared by chi square test.

3.13 Materials

Buffers

TAE electrophoresis buffer (50X)	242 g Tris base 57.1 ml glacial acetic acid 37.2 g $NA_2EDTA.2H_2O$ made upto 1l with H_2O
Loading dye (6X)	0.25% bromophenol blue 0.25% xylem cyanol 30% glycerol in water
TE (10 mM Tris, 1 mM EDTA)	100 µl EDTA 0.5 M pH 8 500 µl Tris 1M made upto 50 ml with water
LoTE (3 mM Tris, 0.2 mM EDTA)	20 µl EDTA 0.5 M pH 8 150 µl Tris 1 M made upto 50 ml with water

Agarose gel

1.5% Agarose UltraPure (Gibco BRL)
1X TAE
0.5 µg/ml ethidium bromide

Sequencer

Genteic analyzer buffer (Applied Biosystems)
Pop-6 gel (Applied Biosystems)

Media and reagents

Ficoll- Pague (Amersham Biosciences)
Trypan Blue (Merck)
HBSS (Gibco BRL)
RPMI (Sigma)
Penicillin-streptomycin (Biochrom AG)
L-glutamine (Gibco BRL)
LB Broth Base (Gibco BRL)
LB Agar (Gibco BRL)
Kanamycin (USB Corporation)

4 Results

4.1 B cell regeneration

4.1.1 Identification of B cells in the periphery following rituximab therapy

In the entire patient group, effective rituximab-mediated peripheral B cell depletion was observed. Before the therapy was initiated, all patients showed a consistent proportion of B cells in the peripheral lymphocyte population. Flow cytometric analysis with B cell specific surface markers revealed that pre-treatment, 6%, 10.9%, 14.5%, 7.5% and 10.4% of the lymphocyte population was comprised of B cells in patients A, B, C, D and E respectively. This was in accordance with the range expected in normal healthy subjects (5-15%). Rituximab therapy led to complete depletion of B cells in the periphery. No appreciable number of B cells was noticed in the peripheral blood following the therapy. This became evident with the observation that the corresponding B cell proportion in the peripheral lymphocyte population declined to almost-zero value.

B cell depletion lasted for several months in the patients. 8 months post-treatment was the earliest time point in patient A, where appreciable number of B cells was noticed in the peripheral blood.

Table 1. Early time point of detectable B cells in the peripheral blood following therapy

Patient population (%)	Early B cell detection in peripheral blood following therapy	B cell fraction in lymphocyte
A	8 months	2.6
B	6 months	2.4
C	6 months	2.3
D	11 months	1.6
E	15 months	1.7

The early time point of peripheral B cell re-circulation post treatment varied between patients and the corresponding B cell proportion varied between 1.6% and 2.6% in the lymphocyte population.

Phenotypic analysis of the lymphocyte population using fluorescence-labelled antibodies against B cell and T cell specific surface markers revealed that before the therapy was initiated, the lymphocyte fraction had consistent number of both B (CD19 expressing) and T (CD3 expressing)cells. Following therapy, for several months, no appreciable number of B cells was observed and the lymphocyte fraction was almost comprised only of T cells. Earlier circulation of B cells was found around 8 months after therapy in patient A. Depicted below is an example of Patient A, wherein almost no CD19+ B cells were detectable between months 1 and 7 following rituximab therapy. Appreciable number of B cells was observed during month 8 following therapy and in the subsequent months, the B cell proportion increased gradually (data not shown).

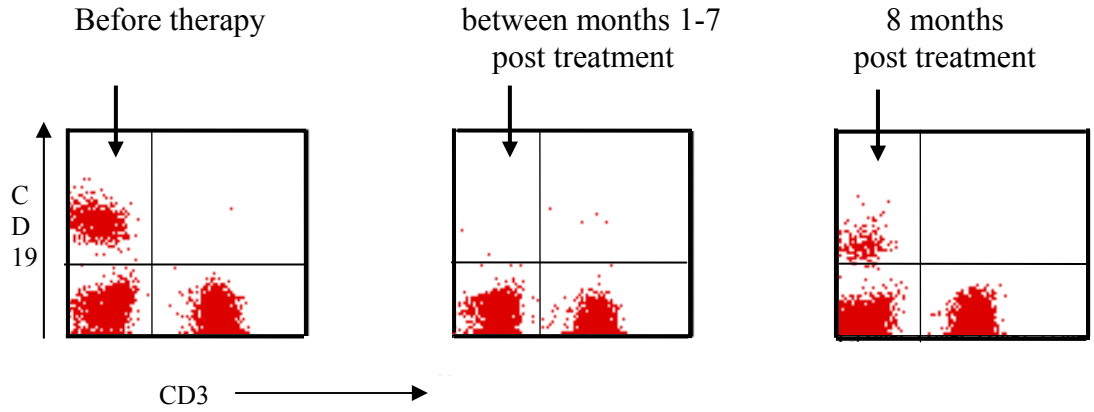


Fig. 13. FACS profile of lymphocyte population pre and post treatment – CD19 and CD3 were the B cell and T cell marker used respectively

4.1.2 Detection of B cells by PCR amplification of Ig genes

In parallel to flow cytometric analysis, peripheral absence of B cells following therapy and slow regeneration of B cells, several months after therapy were also determined by PCR amplification of B cell Ig-VH genes using total genomic DNA from PBMCs as template. Ig-VH family specific primers were used.

B cells, if present, were detected by corresponding Ig amplification which yields a PCR product in the size of approximately 350 bp.

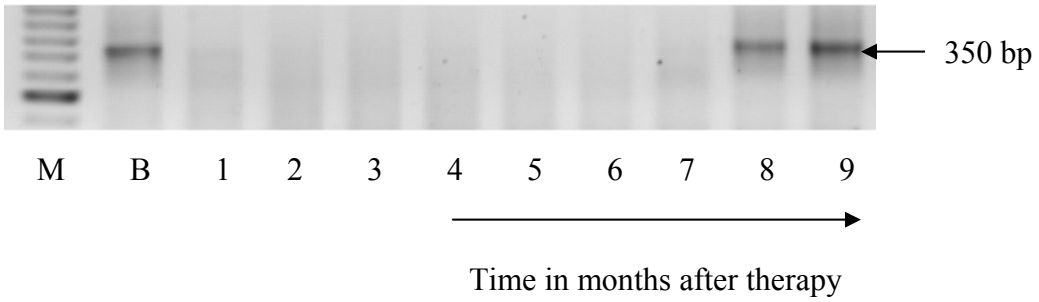


Fig. 14. Figure Amplification of Ig-VH4 gene at different time points in patient A – M = 50 bp ladder, B = before therapy.

As shown in the figure above, PCR amplification of B cell Ig-gene revealed undetectable level of B cells in the peripheral blood up to 7 months after therapy in patient A. Starting from month 8, B cells were detectable.

4.2 Clinical data

The clinical response of the patients was studied at all time points of molecular analysis. Disease activity score, C reactive protein level and rheumatoid factor value were measured and shown as tabulated below.

Table 2. Patient characteristics at different time points

Patient	Timepoint	DAS28	CRP (mg/dl)	RF (U/ml)
Patient A	before therapy	4.95	0.66	42.3
	ERP	6.42	3.59	39.6
	LRP	4.96	0.29	33.4
Patient B	before therapy	5.98	1.83	195
	ERP	4.65	0.65	n.d
	LRP	4.37	0.66	130
Patient C	before therapy	6.00	0.29	81.7
	ERP	3.30	0.26	67.9
	LRP	5.69	0.52	45.2
Patient D	before therapy	5.17	1.88	115.
	ERP	2.70	0.64	91.5
Patient E	before therapy	6.66	7.38	125
	ERP	4.59	2.07	59

ERP= early regeneration period; LRP= late regeneration period, n.d= not determined

The disease activity score DAS28 showed an improvement in the ERP in patient B, C, D and E compared with the baseline. Patient A did not respond well in the ERP however following a subsequent therapy with azulfidine baseline disease activity was noticed in this patient during LRP. RF activity declined in all five patients after therapy. Similarly the inflammatory parameter CRP declined in four of five patients in the ERP.

4.3 Kinetics of B cell subsets regeneration

In order to investigate the kinetics of B cell regeneration, B cell subsets were examined by immunophenotypic studies before therapy and during the regeneration phase following therapy.

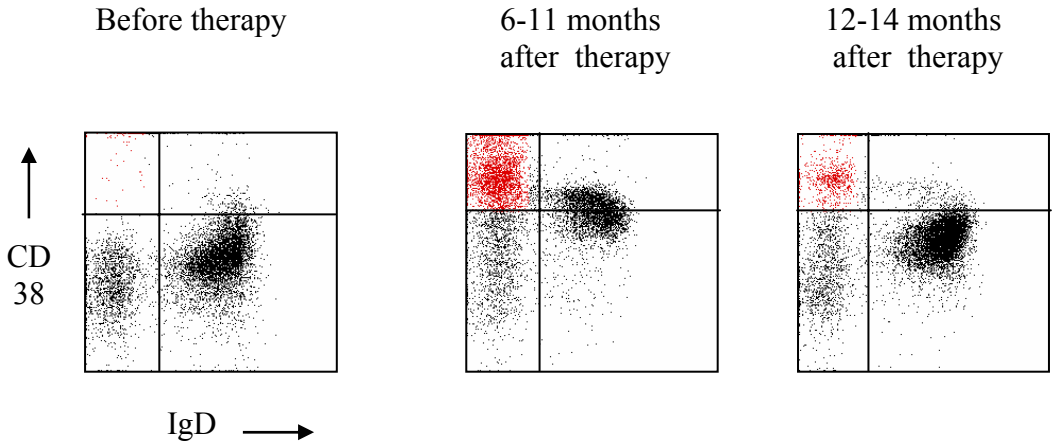


Fig. 15 Kinetics of B cell subset regeneration – depicted above is dot plot of various B cell subsets distinguished by IgD and CD38 expression. The quadrants are: lower left: class-switched memory B cells, upper left (red dots): plasma cells, lower right: naïve B cells, upper right: immature B cells

CD 19 positive B cells were grouped into two categories on the basis of IgD surface expression and of particular interest the four subsets as following were studied: 1. IgD+, CD19+, CD38hi, CD10+ Immature B cells, 2. IgD+, CD19+, CD38med/low, CD10- naïve B cells, 3. IgD-, CD19+, CD38hi plasma cells and 4. IgD-, CD19+, CD38low class-switched memory B cells. It was observed that before therapy was initiated, the IgD+ and IgD- compartments comprised of B cell subsets proportion which were closely comparable to healthy individuals. In the IgD+ compartment, the majority of cells carried a naïve phenotype and in the class-switched, IgD- compartment, low levels of plasma cells were observed. But during the early recovery phase (6-11 months after rituximab therapy), the IgD+ compartment was predominated by significantly increased levels of CD38hi immature B cells. They also co-expressed CD10 which is consistent with an immature B

cell phenotype. In the late recovery phase, (12-14 months after therapy) the IgD+ compartment comprised of majority of naïve B cells which were more likely to be differentiated from immature B cells. In parallel, the early recovery phase IgD- class-switched compartment was marked by increased levels of CD38hi plasma cells. The circulation of plasma cells in the periphery was transient which was marked by low levels of these cells during late recovery phase.

This characteristic pattern of B cell regeneration was similar in the entire group of patients included in the study.

4.4 Immunoglobulin analysis by total genomic DNA amplification

4.4.1 Analysis of Ig-VH4 gene rearrangement pre and post treatments

Ig-VH4 genes amplified from total genomic DNA was analysed in the three patients group at three different time points (before therapy, ERP and LRP). In total, a sum of 376 sequences was analyzed wherein at least 112 sequences were analyzed from each time point. In all patients included in the study, a diverse set of VH4 family genes (VH4-04, VH4-30, VH4-31, VH4-34, VH4-39, VH4-4, VH4-59 and VH4-61) were employed by the repertoire and these genes were overall very similar before and after B cell depletion (Fig.16). Particularly, VH4-34 and VH4-39 gene segments known to encode for auto-antibodies (Dorner *et al.* 2001) were predominantly used in these patients before therapy. These two genes accounted for approximately 60% of the total VH4 rearrangements prior to B cell depletion.

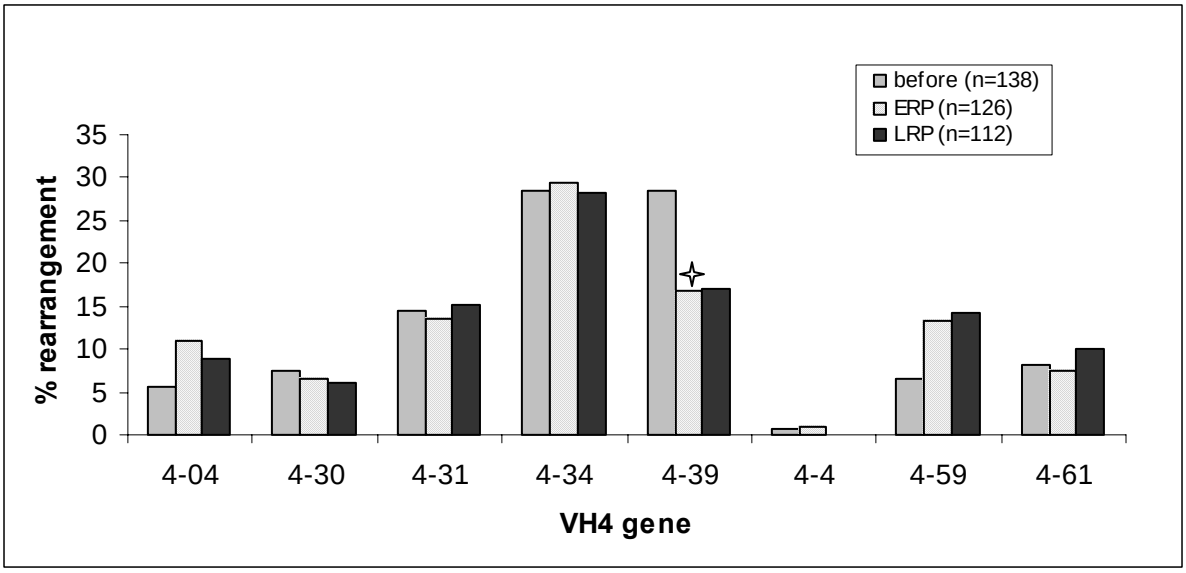


Fig. 16. Ig-VH4 gene usage in patients A-C before and after rituximab therapy
✧ $P<0.05$ versus before therapy by Fischer's exact test

In the re-emerging peripheral B cells following depletion, frequency of rearranged Ig-VH4-34 remained unaltered (28.4% versus 29.4%), whereas the frequency of rearranged Ig-VH4-39 declined significantly after therapy (28.5% before rituximab versus 16.8% in ERP and 17.1% in LRP, $P<0.05$).

4.4.2 Comparison of mutational frequency of immunoglobulin genes

The mutational frequency of Ig-VH4 genes were analyzed and compared pre and post treatments. It was observed that before therapy was initiated, the mutational frequency in the patients were relatively low (1.59%, 1.29% and 1.19% in patients A, B and C respectively). During the ERP, all the three patients showed an increased mutational frequency in comparison to before therapy. In patient A, a drastic increment (four-fold) was observed and in patients B and C, a three-fold increment was observed. These changes observed were also statistically significant.

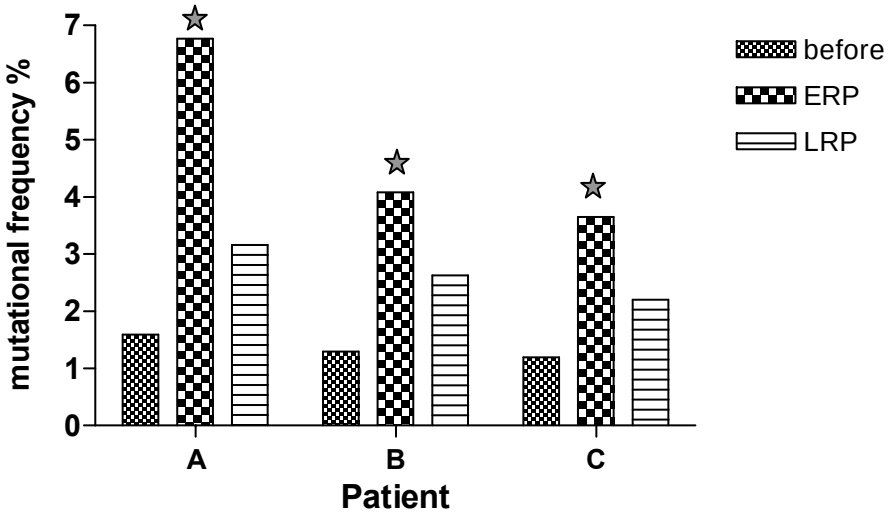


Fig. 17. Mutational frequency of Ig-VH4 genes before and after therapy
☆ $P<0.001$ versus before therapy by chi square test

However, three months later to this time point, during LRP, the overall mutational frequency of the Ig genes declined. Nevertheless, the mutational frequencies pertaining to this time point were higher in comparison to before therapy status. (3.16 versus 1.59, 2.63 versus 1.29 and 2.2 versus 1.19 for patients A, B and C respectively).

Thus, on the basis of mutational frequency, profound changes were noticed during the early regeneration phase wherein a significantly higher number of mutations of the re-emerging Ig genes, was found in the peripheral blood.

4.4.3 Grouping of Ig sequences on the basis of number of mutations

Significantly increased mutational frequency during the ERP, allowed for grouping of the Ig rearrangements based on the number of mutations in individual sequences belonging to various time points in all three patients.

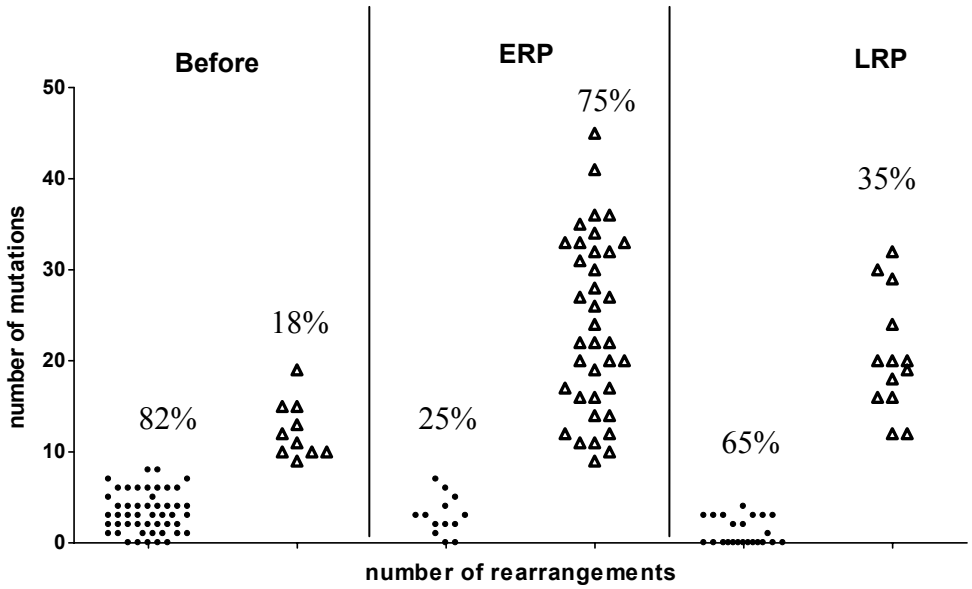


Fig. 18. Ig rearrangements grouping based on number of mutations in patient A

In patient A, 50 out of 60 Ig rearrangements had 0-8 mutations before therapy. Only 9 sequences were observed with mutations in the range of 10 and 15. In contrast to this observation, ERP exhibited 37 out of 50 rearrangements with ≥9 mutations (9-45 m). The frequency of rearrangements with <9 mutations declined (14 out of 50). However during LRP, the number of rearrangements ≥9 mutations started to decline.13 out of 36 Ig sequences possessed ≥9 mutations whereas 23 Ig sequences showed <9 mutations.

Patient B also showed increased numbers of rearrangements with ≥9 mutations during ERP in comparison to before therapy. The mutations were in the range of 11 and 40. During LRP, the rearrangements with ≥9 mutations declined.

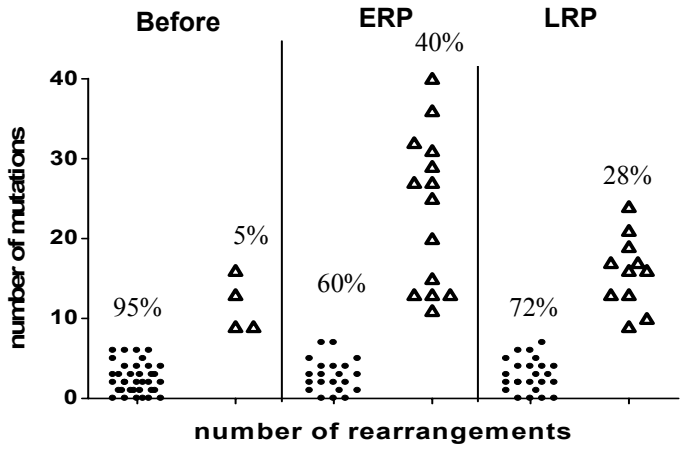


Fig. 19. Ig rearrangements grouping based on number of mutations in patient B

Similar effect was noticed also in patient C with elevated levels of rearrangements with ≥9 mutations during ERP. During LRP a higher proportion of sequences had 0-8 m.

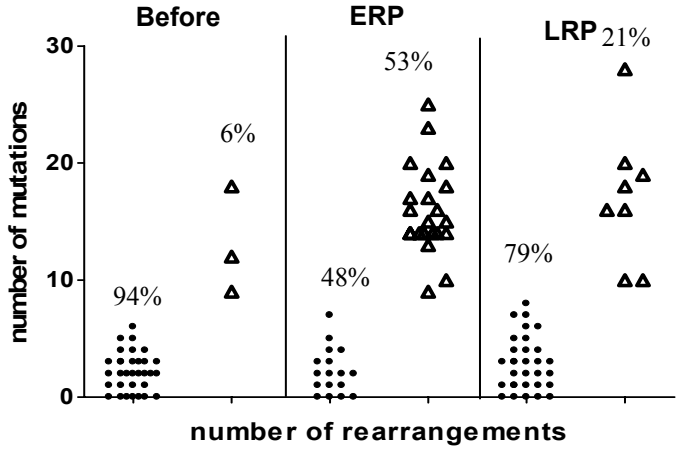


Fig. 20. Grouping of Ig rearrangements on the basis of number of mutations in patient C

It was revealed from these depictions that there existed two distinct populations of B cells based on the number of mutations in Ig sequences, which allowed further grouping the sequences in to two categories; one highly mutated and the other low mutated. All

sequences which harboured less 9 mutations were grouped to be low-mutated whereas the sequences with ≥9 mutations were categorized under the highly mutated group.

Moreover, analysis from these three patients demonstrated that the ERP had an elevated number of highly mutated Ig sequences, which eventually contributed to the increased mutational frequency at this time point.

4.4.4 Phenotypic analysis of plasma cells pre and post treatment

In parallel to total genomic DNA amplification and analysis of Ig genes, phenotypic analysis of B cell subsets was also performed wherein PBMCs belonging to time points before therapy, during ERP and LRP were stained for CD19, CD38 and IgD surface expression. The percentage of CD19+, CD38hi and IgD- expressing plasma cell were determined by flow cytometry.

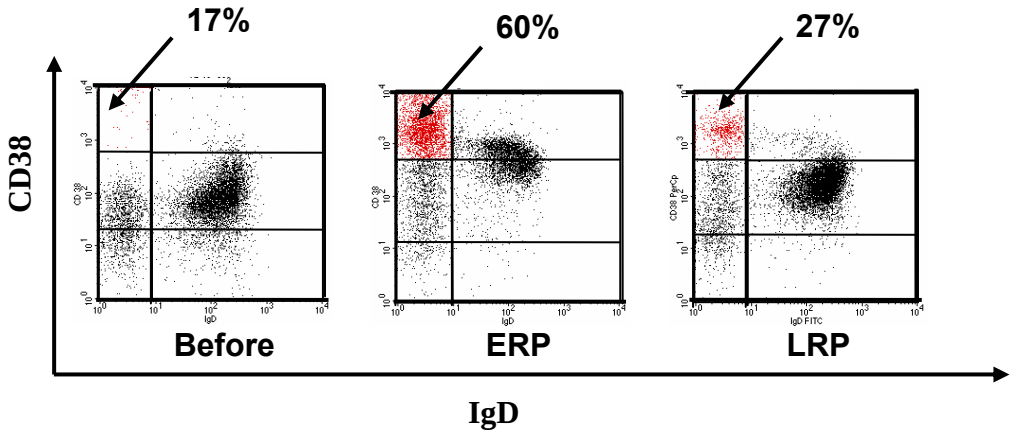


Fig. 21. Fraction of plasma cells before therapy and during regeneration process

It was revealed that before therapy in patient A, 17% of the B cells carried a CD19+, CD38hi and IgD- plasma cell phenotype. Following therapy during the regeneration profile, especially during ERP, approximately 60% of the re-circulating B cells carried a plasma cell phenotype. However, 3 months later, during LRP, this proportion declined and approximately 27% of the re-circulating B cells pertaining to this time point expressed plasma cell phenotype.

Further analysis in patients B and C also showed an increased proportion of plasma cells during ERP in comparison to before therapy. Later time points of regeneration were marked by relative decline of the plasma cell fraction and gradual increase of the relative naïve B cells.

4.4.5 Correlation of highly mutated sequences to the plasma cell fraction

In order to determine if the highly mutated sequences emerge from a particular B cell subset, B cells were stained with specific surface markers which differentiate between different B cell subsets and the percentage of each subset was analyzed.

Upon correlating the highly mutated Ig rearrangements with different B cell subset fractions, it was noticed that the percentage of highly mutated Ig rearrangements during every time point correlated well with the corresponding plasma cell fraction which was recognized by surface expression of CD19+, CD38hi and IgD-.

Table 3. Correlation of highly mutated Ig rearrangements to plasma cell fraction in individual patients

	Before	ERP	LRP
Patient A			
High mutations (≥9) (%)	18	75	35
Plasma cell fraction (%)	17	75	27
Patient B			
High mutations (≥9) (%)	5	40	28
Plasma cell fraction (%)	11	39	33
Patient C			
High mutations (≥9) (%)	6	53	21
Plasma cell fraction (%)	14	49	16

From the table above, it could be seen that before therapy the fraction of plasma cells in the patients ranged between 11 to 17%. In contrast, ERP was marked with a significant increased circulation of plasma cells (39-75%). Nevertheless, the plasma cell fraction had a close correlation with the percentage of highly mutated Ig rearrangements of corresponding time points.

Figure 22 correlates the highly mutated Ig rearrangements to the fraction of plasma cells during different time points in the patient group. It is evident that the quantitative proportion of the highly mutated rearrangements equals that of the plasma cell identified by CD38hi expression.

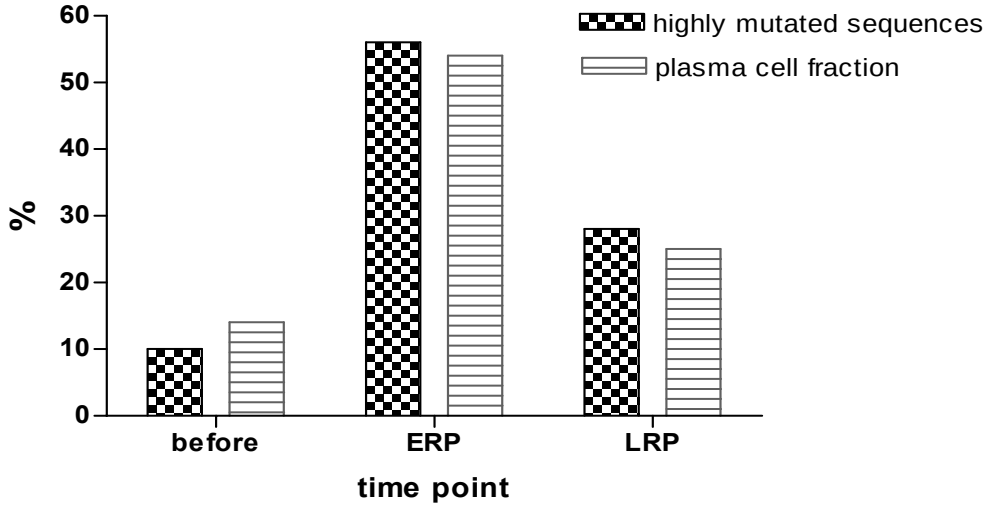


Fig. 22. Correlation of highly mutated Ig rearrangements to plasma cell fraction in patient group

This finding indicated for a more likelihood of the highly mutated Ig sequences arising from the plasma cell fraction pertaining to the corresponding time points.

4.5 Single cell analysis of B cell subsets during ERP

Under steady state condition the IgD-, CD38hi phenotype is described to be plasma cells that are usually highly mutated. But it is not known if this surface phenotype marker also define plasma cells under conditions of the dynamic process during B cell regeneration. Therefore we sorted individual B cells circulating during the early regeneration phase and determined the mutational imprints in patients D and E. using a RT-PCR method. cDNA was synthesized from individual B cells and the Ig-VH4 genes were amplified and the mutations were defined.. That approach allowed us to determine the amount of mutations in their Ig receptor which would further strengthen the notion that the highly mutated sequences observed in total DNA amplification method emerged from the plasma cell subset of B cells.

Individual B cells belonging to four different B cell subsets as represented in figure 23 were sorted, amplified and analyzed. Notably, the analyses were performed during ERP where the B cell compartment is not fully repopulated.

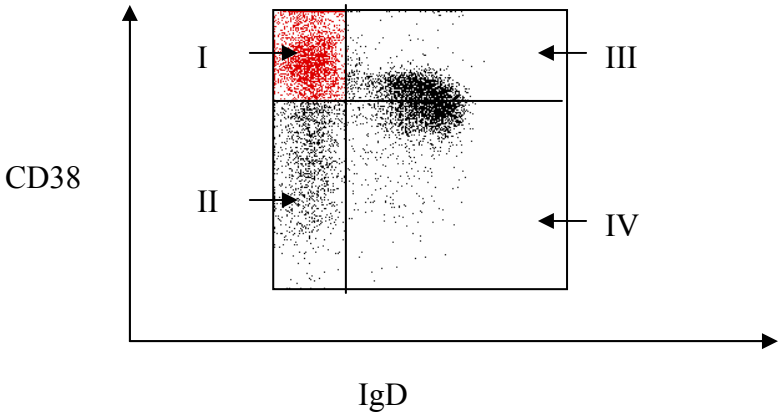


Fig. 23. Single cell sorting of various B cell subsets - identified by expression of CD19, CD38, CD10 and IgD surface markers were: (I) CD19+/IgD-/CD38hi class-switched plasma cell phenotype, (II) CD19+/ IgD-/ CD38med/low class-switched memory subtype, (III) CD19+/IgD+/CD10+/CD38+ immature B cells and (IV) CD19+/IgD+/CD38med/low-naïve B cells .

4.5.1 Patient D

4.5.1.1 Mutations in class-switched compartment

4.5.1.1.1 Mutations in individual IgD-, CD38hi B cells (Quadrant I in Fig.23)

Analysis of CD38hi, IgD- B cells in patient D revealed a predominant utilization of VH4-39 and VH4-59 genes in the rearrangement process (each approximately 35%). Besides these, other genes like VH4-b, VH4-04 and VH4-31 were also employed (7%, 7% and 14% respectively)

In terms of mutation, 79% Ig-VH4 rearrangements from this compartment exhibited higher mutations (≥9 m). The corresponding mutational frequency is 7.0%

Table 4. Mutations in heavy chain of individual CD38hi, IgD- B cells in patient D

Sequence No	VH gene	Mutations	Mutational frequency (%)
1	4-b	13	5.60
2	4-04	15	6.38
3	4-31	14	5.56
4	4-31	30	13.27
5	4-39	0	0.00
6	4-39	0	0.00
7	4-39	0	0.00
8	4-39	26	11.71
9	4-39	16	6.67
10	4-59	12	1.72
11	4-59	20	8.44
12	4-59	17	7.23
13	4-59	9	3.80
14	4-59	7	3.27

4.5.1.1.2 Mutations in IgD- CD38 med/low B cells (Quadrant II in Fig.23)

Class-switched B cells as defined by IgD- and CD38med/low surface expression, showed usage of VH4-04, VH4-31, VH4-34, VH4-39 and VH4-59 Ig genes. Moreover, 50% of the sequences harboured more than 9 mutations. The overall mutational frequency of this subset was 4.9% and from the highly mutated sequences is 9.4%. This is consistent with class-switched memory B cell mutations.

Table 5. Mutations in individual class-switched CD38med/low B cells in patient D

Sequence No	VH gene	Mutations	Mutational frequency (%)
1	4-04	0	0.00
2	4-31	4	1.59
3	4-31	0	0.00
4	4-31	18	7.06
5	4-34	0	0.00
6	4-39	15	6.17
7	4-59	33	13.92
8	4-59	25	10.55

4.5.1.2 Mutations in the naïve B cell compartment

4.5.1.2.1 Mutations in IgD+, CD38+, CD10+ B cells (Quadrant III in Fig.23)

In the immature B cells compartment that express CD38, IgD and CD10 on the surface, similar genes (VH4-04, VH4-31, VH4-34, VH4-39 and VH4-59) which were used in the IgD- class-switched compartment were observed. However, in terms of mutations, the immature B cells harboured no mutations in all the rearranged Ig genes studied. Totally seven Ig sequences with an immature B cell phenotype were amplified and absolutely no mutations were observed. Thus, the overall mutational frequency of the individual immature B cells remained zero.

Table 6. Mutations in individual immature B cells of patient D

Sequence No	VH4	Mutations	Mutational frequency (%)
1	4-04	0	0.00
2	4-31	0	0.00
3	4-31	0	0.00
4	4-34	0	0.00
5	4-34	0	0.00
6	4-39	0	0.00
7	4-59	0	0.00

4.5.1.2.2 Mutations in IgD+, CD38med/low naïve B cells (Quadrant IV in Fig.23)

Naïve B cell subset of patient D identified with IgD+, CD38med/low expression, was marked by a prevalent usage of VH4-04 genes (41%).

Table 7. Mutations in individual mature/naïve B cells of patient D

Sequence No	VH4	Mutations	Mutational frequency (%)
1	4-04	0	0.00
2	4-04	0	0.00
3	4-04	0	0.00
4	4-04	0	0.00
5	4-04	0	0.00
6	4-04	0	0.00
7	4-04	0	0.00
8	4-31	0	0.00
9	4-34	0	0.00
10	4-34	0	0.00
11	4-34	0	0.00
12	4-39	0	0.00
13	4-39	0	0.00
14	4-39	1	0.41
15	4-39	1	0.41
16	4-59	2	0.84
17	4-59	2	0.85

No Ig sequence belonging to the naïve B cell compartment was highly mutated. 76% Ig sequences had totally no mutations where as only 24% sequences had very little mutations (1-2 m).

4.5.1.3 Proportion of highly mutated Ig sequences in various B cell subsets during ERP

The extent of mutational imprint on the Ig genes varied between different B cell subpopulations during the early recovery period. B cells belonging to the IgD+ naïve compartment (Quadrants III and IV) acquired few or no mutations, whereas the IgD- class-switched compartment comprised of highly mutated IgR sequences. The proportion of the highly mutated (≥9 m) B cell Ig sequences were also relatively higher in this compartment, especially, the CD38hi subset (Quadrant I) consisted a major proportion of highly mutated cells. Therefore the CD38hi, IgD- B cells can be assigned to be plasma cells as described in steady state conditions.

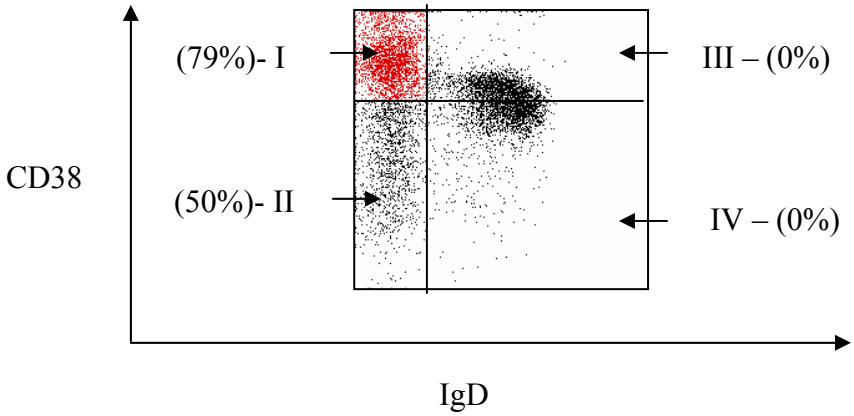


Fig. 24. Percentage of highly mutated IgR sequences within various B cell subsets during ERP – (values shown in brackets)

4.5.2 Patient E

To confirm the highly mutated nature of CD38hi, IgD- B cells and few or no mutations in the IgD+ B cell compartment, individual B cells from these subsets were sorted and amplified in another patient E.

4.5.2.1 Gene distribution in individual IgD-, CD38hi plasma cells

Similar to patient D, individual IgD-, CD38hi B cells of patient E again predominantly utilized VH4-39 and VH4-59 genes. (29% and 36% respectively)

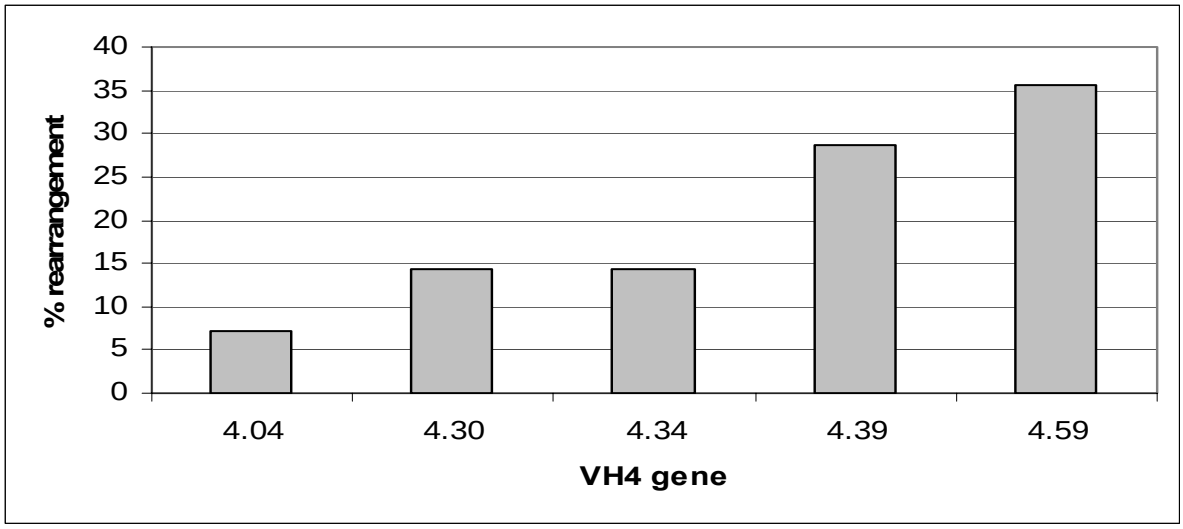


Fig. 25. Ig-VH4 gene distribution in IgD-, CD38hi B cells during ERP in patient E

Of note, the gene segment VH4-04 which was less frequently used in the CD38hi B cell compartment of patient D was found to be low expressed also in patient E.

4.5.2.2 Mutations in individual IgD-, CD38hi plasma cells

Similar to patient D, the IgD-, CD38hi Ig rearrangements of patient E were also highly mutated. 13/14 sequences (93%) had ≥9 mutations. The mutational frequency of these cells varied between 4.96 and 14.75.

Table 8. Mutations in individual plasma cells of patient E

Sequence No	VH4	Mutations	Mutational frequency (%)
1	4-04	26	11.02
2	4-30	3	1.23
3	4-30	21	8.64
4	4-34	32	13.50
5	4-34	12	5.06
6	4-39	12	4.96
7	4-39	34	13.99
8	4-39	36	14.75
9	4-39	19	7.82
10	4-59	23	9.70
11	4-59	26	11.06
12	4-59	19	8.05
13	4-59	13	5.51
14	4-59	29	12.34

4.5.2.3 Mutations in IgD+ naïve compartment

As expected, the Ig gene rearrangements belonging to the IgD+ naïve compartment (n=3) harboured little or no mutations. This substantiates the hypothesis that the mutational mechanisms are less or not active in IgD+ naïve B cells.

Table 9. Mutations in individual naïve B cells of patient E

Sequence No	VH4	Mutations	Mutational frequency (%)
1	4-34	0	0,00
2	4-39	1	0,41
3	4-04	0	0,00

Single cell analysis of B cell subsets of patients D and E during the early regeneration phase is summarized as follows:

Table 10. Single cell analysis of various B cell subsets during ERP- Summary

Surface marker	B cell subset	% Ig sequences with ≥9 m	Overall mutational Frequency (%)
Patient D (ERP)			
CD19+/IgD-/ CD38hi	plasma cell (n=18)	79	5.54
CD19+/IgD-/ CD38med/low	memory B cell (n=8)	50	4.91
CD19+/IgD+/ CD38+/CD10+	immature B cell (n=9)	0	0.00
CD19+/IgD+/ CD38med/low-	naive B cell (n=16)	0	0.15
Patient D (ERP)			
CD19+/IgD-/ CD38hi	plasma cell (n=14)	97	9.12
CD19+/IgD+	naive B cell (n=4)	0	0.00

Monitoring various B cell subsets using specific surface markers and molecular analysis of each subsets circulating during the early recovery period made evident that the surface phenotype defined to B cell subsets under steady state B cell homeostasis assigns also to that of B cells which recirculate in the dynamic early recovery phase where the B homeostasis is disturbed. Similar mutational frequencies exhibited by the B cell subsets in the steady state and regenerating phase strengthen this postulate. Moreover mutational analysis also provided critical information regarding the origin of the highly mutated B cells from the class-switched compartment especially from the plasma cell subset identified by a IgD-, CD38hi surface phenotype.

4.6 Detailed analysis of the highly mutated (plasma cell) rearrangements

It became evident from the single cell analysis that the highly mutated B cells belong to the IgD- compartment, especially the plasma cell compartment. Plasma cells are CD 20 negative and are not directly targeted by rituximab therapy. Nonetheless these cells disappear in the periphery during the depletion period. However during the start of B cell regeneration they shortly dominate the repopulating B cell pool. Therefore we wanted to ascertain if the reemerging plasma cell compartment is modulated by rituximab treatment.

4.6.1 Plasma cells from total genomic DNA amplification method

4.6.1.1 Identification of mutations in the RGYW/WRCY hotspot motif

To evaluate modulation of the plasma cell compartment, firstly the confinement of mutations into the pre-defined hotspot motifs RGYW and WRCY (R= purine, Y= pyrimidine, W=A/T) along Ig rearrangements was assessed in the plasma cells circulating before therapy and during ERP.

Table 11. Comparison of RGYW/WRCY targeting before and after therapy

	Before		**ERP**		**LRP**	
	Total mutations	RGYW/WRCY mutations	Total mutations	RGYW/WRCY mutations	Total mutations	RGYW/WRCY mutations
Patient A	134	39%	950	**57**%	248	61%
Patient B	87	55%	346	**64**%	178	47%
Patient C	42	37%	335	**55**%	130	58%

As shown in the table above, all the three patients showed a reduced mutational targeting of RGYW/WRCY motifs before therapy in comparison to after therapy status. Especially, in patients A and C, a significantly higher number of mutations were confined to the RGYW/WRCY motifs, during ERP. It is further increased during LRP in these patients. On the other hand, patient B also showed an increased RGYW/WRCY targeting

during ERP, however it was not statistically significant. During LRP the targeting effect declined in this patient and reached a lower level in comparison to before therapy status. Of note, patient B had an increased targeting of RGYW/WRCY motifs even pre treatment (55%), in contrast to patients A and C who has 39% and 37% of mutations lying in this motif (pre treatment).

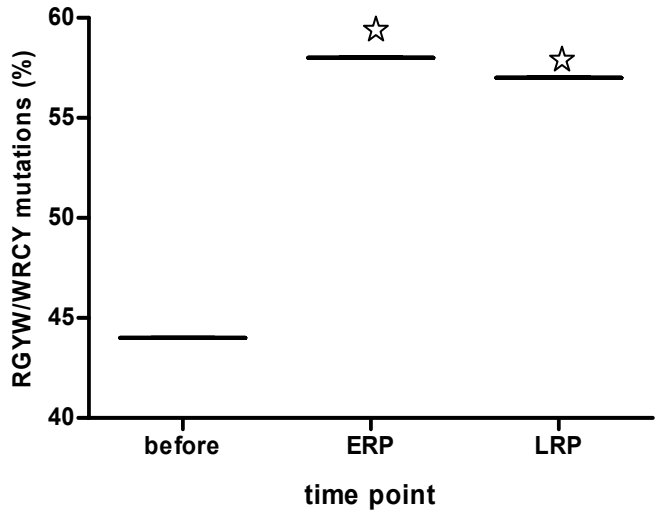


Fig. 26. Average RGYW/WRCY targeting in patients A-C before and after therapy
☆ $P<0.05$ versus before therapy by chi square test

Taken together in patients A-C, prevalent confinement of mutations into the RGYW/WRCY motifs during the ERP was noticed. This effect was also consistent with the LRP. The increment in the targeting effect was statistically significant in comparison to before therapy status.

4.6.1.2 Replacement to silent mutational ratio

Assessment of the nature of mutation (replacement or silent) reveals if a nucleotide change results in the change of the amino acid that is encoded by the nucleotide. The ratio of replacement to silent mutation in the CDRs was determined in the highly mutated Ig rearrangemens (plasma cell sequences). R/S mutation ratio in plasma cells pre and post treatment was compared.

Table 12. Comparison of R/S mutations in the CDRs before and after therapy

	Before			ERP			LRP		
	R	S	**R/S**	R	S	**R/S**	R	S	**R/S**
Patient A	19	14	**1.36**	170	64	**2.66**	61	16	**3.81**
Patient B	18	6	**3.00**	66	29	**2.28**	18	5	**3.60**
Patient C	12	6	**2.00**	69	21	**3.29**	18	6	**3.00**

CDRs are the hypervariable regions in the Ig sequence which are responsible for the antigenic binding. Mutations occur in the CDRs in order to derive affinity maturation towards the particular invading antigen. Determination of the replacement to silent mutational ratio in the CDRs revealed that post treatment during ERP patients A and C showed an increased R/S mutation ratio. This ratio increased further during LRP in these patients. Only patient B did not show similar effects as this patient had an increased R/S ratio even before the treatment was initiated. R/S ratio pre treatment in patient B was relatively higher in comparison to patients A and C (3.00 Vs 1.36 and 2.00 respectively)

Figure 27 depicts the total R/S mutational ratio in the CDRs pre and post treatment in the patient group. The ERP was marked with an increased R/S ratio in the CDRs as compared to pretreatment, which is also consistent with the LRP arguing for a long term modulation of the recirculating plasma cells.

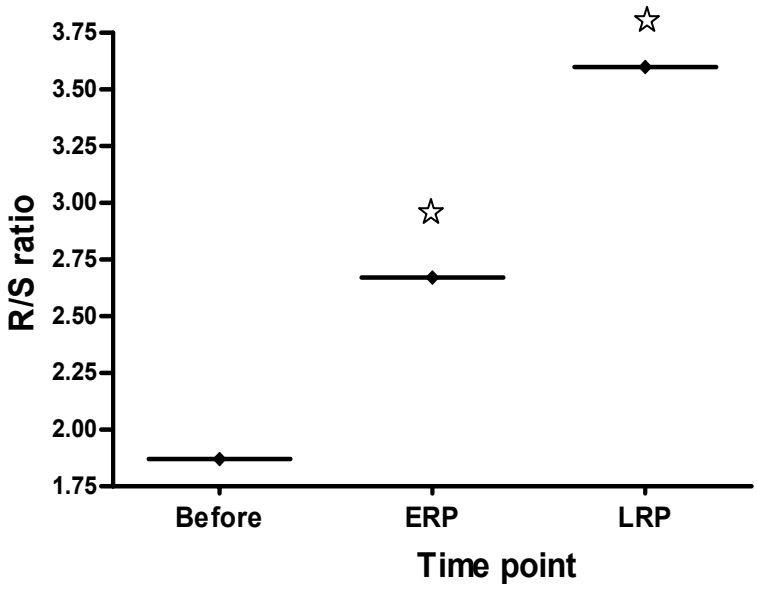


Fig. 27. Average R/S mutations in CDRs in patients A-C before and after therapy
☆ $P<0.005$versus before therapy by chi-square test

4.6.2 Plasma cell IgR rearrangements from single cell analysis

Highly-mutated Ig sequences from the total genomic DNA amplification method showed increased RGYW/WRCY targeting and elevated R/S mutations in the CDR post therapy. Therefore individual plasma cells during ERP which correlate to the highly mutated overall B cells (total genomic DNA) were also investigated for a similar elevated mutational targeting of RGYW/WRCY motifs.

4.6.2.1 RGYW/WRCY targeting in individual plasma cell Ig rearrangements

Individual plasma cell Ig sequences during ERP of patients D and E showed RGYW/WRCY mutational targeting in the range of 68.5% and 61.2 respectively. This correlated well with the RGYW/WRCY mutational targeting of the highly mutated sequences of total genomic DNA method wherein the corresponding RGYW/WRCY targeting was 58%. (patients A-C)

Table 13. RGYW/WRCY targeting in individual plasma cells

	Early regeneration phase	
	Patient D	Patient E
Number of mutations	172	304
Mutational frequency (%)	7.05	9.12
RGYW mutations (%)	43.29	37.95
WRCY mutations (%)	25.22	23.29
RGYW/WRCY mutations (%)	**68.5**	**61.2**

4.6.2.2 R/S mutations of individual plasma cell Ig rearrangements during ERP

Ratio of replacement to silent mutations in the hyper variable regions of the plasma cells that recirculate during the early recovery period was assessed.

Table 14. R/S mutations in individual plasma cells during ERP

	Early regeneration phase	
	Patient D	Patient E
Number of mutations in CDR	61	120
Replacement mutations in CDR	51	96
Silent mutations in CDR	10	24
R/S mutation in CDR	**5.1**	**4.0**

The average R/S mutations in plasma cell CDRs of patients D and E during ERP was found to be 4.55. This was relatively higher in comparison with the average R/S mutations in CDRs of patients A-C (1.68) pre treatment (total genomic DNA amplification method) thereby indirectly suggesting that during ERP, the re-emerging plasma cell Ig genes have an increased R/S mutations in CDRs which more likely leads to selection of these cells.

5 Discussion

B cells play a central role in the progression of self-destruction in autoimmune diseases. Since the pathogenic role of B cells in autoimmunity is being revealed, numerous approaches targeting the B lymphocyte directly or indirectly are in clinical practice (Anolik *et al.* 2004; Mariette 2004). In this connection, temporal depletion of B cells by rituximab seems to be a novel and promising tool and is gaining more attention in recent years (Leandro *et al.* 2006; Silverman 2006). The efficacy of rituximab that targets CD20 expressing B cells has been appreciated in the treatment of various autoimmune disorders in multi-center trials.

During B cell ontogeny, CD20 expression prevails in all B cell subsets between pro B cell and terminally differentiated plasma cells. Depleting B cells by rituximab in autoimmune diseases aims at two main purposes: Firstly, rituximab therapy results in B cell depletion which eventually might introduce a temporal pause in the progression of B cell related autoimmune pathogenesis. Secondly, it might also lead to restoration of a new B cell repertoire which is not yet deviated to autoimmunity.

B cell regeneration starts from pro B cells which are spared by rituximab-mediated B cell depletion since they do not yet express the CD20 surface antigen. At the Pro B cell stage of B cell development, the IgR genes start rearranging the heavy chain gene segments by a random recombination mechanism. Thus, it is expected that rituximab-mediated B cell depletion might allow, the newly forming B cell repertoire to shape their Ig gene arrangements and mutational mechanisms in such a way that they do not contribute to the process of self-destruction. In this measure, to date, a detailed analysis of B cell regeneration profile, in particular the molecular nature of the repopulating B cells remains yet to be explored. Therefore, the prime aim of the study was to scrutinize the pattern of B cell repopulation following B cell depletive therapy with emphasis on the mutational development of the immunoglobulin B cell receptors. Comparison of the fashion of mutations in the Ig genes before and after B cell depletion would comprehend if the therapy can modulate the mutational pattern of B cells.

Five patients suffering from active RA were selected and treated with rituximab. All the patients included in the study were refractory to several other means of treating RA, including anti TNF alpha therapy. Usage of anti CD20 antibodies was associated with various advantages in the cohort. Firstly, the drug was well tolerated in all patients. No relevant infusion related adverse effects were reported. Additionally, effective B cell depletion was observed in the peripheral blood which was revealed by the decline of B cell count in the peripheral blood to <0.0002%. B cell depletion in the peripheral blood lasted up to several months. The earliest time point, following therapy, when appreciable number of peripheral B cells could be detected varied between patients (6-15 months). In response to rituximab treatment the group of patients reported descending rheumatoid factor levels and improved their DAS28 clinical disease activity index. All these measures account for a good clinical response under rituximab therapy.

1. Pattern of B cell regeneration following temporal B cell depletion

Firstly, we analyzed the pattern of B cell regeneration following B cell depletion using rituximab in RA patients. Three different time points were chosen for this evaluation. The first time point was shortly before the patients underwent rituximab therapy. The second time point was set during B cell reconstitution, in specific, the early time point during B cell regeneration following B cell depletion, when an appreciable number of B cells could be detected in the periphery. This time point referred to as “early regeneration period” (ERP), consists of peripheral B cell contribution of just above 1 % of peripheral lymphocytes and was about 6-10 months after rituximab treatment. A third time point namely “late regeneration period” (LRP), commenced at three months after ERP. During these three time points, the proportion of B cell subsets was examined by immunophenotyping with specific markers. Of particular interest, were the composition of four different B cell subsets namely the plasma cells (CD19+, IgD- and CD38hi), class-switched memory cells (CD19+, IgD- and CD38 med/low), immature B cells (CD19+, IgD+, CD10+ and CD38hi) and differentiated naïve B cells (CD19+, IgD+, CD10- and CD38med). It was observed that before the therapy was initiated, the patients B cell subsets manifested the phenotypic characteristics very similar to healthy individuals. However after treatment, a very dynamic change in B cell subset composition was

observed with a characteristic pattern during the early recovery period (ERP) which follows the steps of B cell development from undepleted pro B cells. We observed that during B cell repopulation, the IgD+ compartment was marked with a significant increased number of CD38hi, CD10+ immature phenotype B cells. The levels of CD38med, CD10- naïve B cells remained low in comparison to before therapy status. Increased expression of immature B cells during ERP following rituximab treatment in RA was also recently demonstrated by another group (Leandro *et al.* 2006). In order to substantiate, if these cells were newly regenerated, we determined the mutational load on these B cell subsets. For this assessment, we employed a single cell reverse transcriptase PCR technique to amplify the Ig rearrangements. Single cell analysis of these subsets revealed that the Ig receptors of all individual B cells belonging to the immature B cell subset were absolutely not mutated. Cells from the naïve B cell compartment harboured only few or no mutations (0-2 m). The non-mutated status of these reemerging cells in the periphery argues for the fact that they are newly rearranged B cells which did not yet enter the germinal centers wherein they usually acquire mutations. During the later phases of B cell regeneration, the IgD+ compartment showed reduced levels of CD10 expressing immature B cells whereas CD10 negative naïve B cells were expanded. This supports the notion that during the B cell repopulation period, as time proceeds, the immature B cells differentiate into a naïve phenotype and recapitulate stages of normal B cell development (Roll *et al.* 2006).

In parallel to the evolution of the IgD positive cells also the IgD negative, class-switched compartment was simultaneously replenished during ERP, characterized by a relative expanded population of cells with a plasma cell phenotype, as compared to before therapy. Within the IgD- compartment, individual B cells expressing the plasma cell and class-switched memory phenotype were also amplified and analyzed using single cell technology. Mutational analyses disclosed that cells with the plasma cell phenotype were very intensely mutated that was similar to mutations in plasma cells belonging to healthy subjects. Almost every plasma cell Ig sequence harboured ≥ 9 mutations. In another patient included in the study, we assessed the mutations in IgD-, CD38med memory type B cells where approximately 50% of these B cells had acquired ≥ 9 mutations, which is consistent with their memory phenotype. In summary this set of experiments shows that early in B cell recovery also highly mutated memory B cells and plasma cells recirculate

simultaneously with the newly regenerated immature B cells. The surface marker constellation used under steady state conditions identifies the B cell subsets also under the dynamic phase of B cell repopulation as revealed by their mutational IgR status.

Following B cell subset development over time the absolute number of memory B cells displayed a significant reduction in the periphery following therapy. Even two years post therapy, memory B cells did not achieve base line values and remained numerically reduced although overall CD19 positive B cell levels were already normalized again. Therefore even a single cycle of B cell depletion by rituximab has a long term impact on the homeostasis of B cell subset composition. This has been also observed in Lupus where a similar expansion of transitional immature B cells and paucity of memory B cells in the peripheral blood during B cell reconstitution were reported (Anolik *et al.* 2004). Thus this unique pattern of regeneration following B cell depletion in various autoimmune diseases is supposed to have important implications on humoral immunity in the patients.

2. Therapy-induced modulation of the B cell repertoire

In addition to the characteristic pattern of B cell regeneration, we investigated if B cell depletion by rituximab therapy induced also a modulation in the reemerging B cell repertoire. To address this, a longitudinal VH4 repertoire analysis was performed in three patients under rituximab therapy. Peripheral blood was collected during the time points before therapy, ERP and LRP. Ig genes belonging to one of the more abundant heavy chain families, namely the VH4 family, were amplified from total genomic DNA, cloned, sequenced and analyzed. First, we delineated the gene segments usage in the B cell receptor before and after therapy. Assessment of gene segments used in the recombination of BCR might provide an idea of the genes linked with autoimmune and healthy state of the individuals. Observations from three patients were evident for the usage of a diverse set of VH4 genes before and after rituximab treatment. We examined the presence of the mini gene segments VH4-34 and VH4-39, which were previously reported to be connected with autoimmunity (Dorner *et al.* 2001). It was shown that in three patients studied, the mini gene segments VH4-34 and VH4-39 were prevalently used in the VH4 repertoire before

therapy. These two genes comprised 60% of peripheral VH4 rearrangements. Abundant usage of VH4-39 gene in RA was also previously reported (Kim *et al.* 2000). Following therapy, we noticed, significant reduction in the frequency of VH4-39 utilization in the VH4 repertoire of the recovering B cells (28.5% before rituximab versus 16.8% in ERP). This argues for a therapy-induced modulation of this gene segment. As far as the mini gene VH4-34 is concerned, no significant change in the usage frequency was detected in the patients included in this study. However, heavy chain repertoire analysis of another RA patient in our lab during another study provided evidence of continued lower frequency of VH4-34 in the regeneration phase (Rouziere *et al.* 2005). This indicates that B cell depletion induces changes in the Ig gene repertoire which are heterogeneous between single patients. There is so far no individual gene directly related to disease manifestation. Nevertheless, our observations show that relevant genes like VH4-39 can be modulated, even though it is yet to be shown if this gene is directly involved in the pathogenesis of RA.

Potential modulation of B cell receptor by rituximab-mediated B cell depletion has been documented also in the treatment of other autoimmune disorders. In lupus a large fraction of anti-DNA antibodies express the 9G4 idiotype. 9G4 idiotype-expressing autoreactive B cells, which mostly use the VH4-34 gene product in their heavy chains, are found to be expanded in the in the post GC and plasma cell compartments in SLE (Cappione *et al.* 2005). Successive B cell depletion by rituximab led to relative down-expression of 9G4 expressing anti dsDNA antibodies in the regenerating Ig repertoire (Cambridge *et al.* 2006).

Therefore temporal B cell depletion by rituximab seems to be capable of modulating the expressed Ig repertoire in different autoimmune diseases. This may be an important feature for the underlying pathomechanism and eventually lead to the clinical benefit seen in the patients.

3. Mutational analysis of B cell IgR pre and post treatments

The prime aim of the study reported here was to evaluate prospectively the evolution of mutations in the IgR genes of the patients during temporal B cell depletion particularly in the repopulating B cell pool. For that we analysed one of the larger VH families, namely the VH4 family and examined the mutational pattern of Ig genes belonging to this family in detail. We assessed the mutational frequency of VH4 B cells from total genomic DNA during all three time points. Evaluation of the rate of mutations in the Ig genes revealed that the overall IgR mutational frequency increased in all the three patients (A-C) during the early recovery period. This increment was initially unexpected, since we expected preferentially newly formed cells with not yet mutated Ig receptors to be the first cells recirculating through the periphery. Nevertheless the mutation rate of IgR in the early regeneration period was significantly higher in comparison to before therapy values (3.16 versus 1.59, 2.63 versus 1.29 and 2.2 versus 1.19 in patients A, B and C respectively). Owing to the change in overall mutational frequency during the early recovery period, we thoroughly examined the mutational distribution in individual Ig sequences. This investigation made evident that the B cell pool is made up of two distinct populations namely a low mutated and a highly mutated B cell population, which were segregable on the basis of number of mutations in the Ig rearrangement. Rearrangements with <9 mutations were grouped to be low-mutated sequences whereas ≥9 mutations were categorized into highly mutated sequences. Before the therapy was initiated, all the three patients (A-C) had low numbers of Ig rearrangements possessing high mutations (≥9 m). Specifically patients A, B and C had 18%, 5% and 6% of Ig rearrangements with ≥9 mutations. During the early recovery period, the proportion of highly mutated Ig rearrangements were elevated in all three patients (75%, 40% and 53% in A, B and C respectively) which contributed to the raised levels of overall mutational frequency. So, it is apparent that the elevated overall B cell mutational frequency observed during ERP is due to the presence of relatively increased highly mutated B cells at this time point.

Since there was a dynamical change in the BCR mutational aspects during B cell replenishment period following rituximab therapy, we tried to assign the highly mutated IgR to specific B cell subset(s). Using flow cytometry, recognition of different B cell

subpopulations by surface markers was performed and it became evident that the percentages of highly mutated Ig rearrangements were in close comparison to the proportions of B cells with the phenotype CD19+, IgD-, CD27+ and CD38hi pertaining to the respective time points. Under steady state conditions, these cells are assigned to the plasma cell subset. To confirm that the highly mutated B cell sequences found during the ERP arise from these plasma cell phenotypic cells, individual plasma cells belonging to two patients (D and E) during ERP were sorted using a single cell reverse transcriptase PCR method, amplified and sequenced. Individual plasma cells from these two patients, which were recognized by CD38hi, IgD- expression, were highly mutated and mutations exhibited by these plasma cell Ig genes (patients D and E) were comparable to the highly mutated B cell Ig sequences during ERP in patients A-C. These findings are in line with the assumption that the highly mutated B cells observed during ERP emerge from recirculating plasma cells pertaining to the corresponding time point.

The increased frequency of highly mutated plasma cells during early recovery period garners further attention towards this B cell subset. Actually, plasma cells do not express CD20 on their surface, therefore are not directly targeted by anti CD20 rituximab therapy (Eisenberg *et al.* 2005). Nevertheless they also disappeared in the peripheral blood during the B cell depletion period following rituximab treatment. Currently, it is not clear why these plasma cells leave the peripheral circulation and where they home in the interim period. One speculation is that they might home in other survival niches such as bone marrow and secondary lymphoid organs like lymph nodes and spleen. There is a recent study to support this hypothesis which reports of long-lived plasma cells residing in the additional survival niches like kidneys or tissues of inflammation in autoimmune NZB/W mice(Hoyer *et al.* 2004). Thus, it seems likely that the plasma cells home in such survival niches during the depletion period and recirculate in the peripheral blood during the early recovery phase. However it is unclear what makes the plasma cells to recirculate in parallel to the first wave of reemerging immature and naïve B cells following therapy. In this aspect, it is intriguing to learn about the expression of chemokine receptors on the plasma cells during the period of depletion and availability of the corresponding cognate ligand (chemokine), which plays a key role in the homing and recirculation of B cells. The precise events underlying the navigation process are yet to be understood. Even though this

process of plasma cells residing in additional survival niches is assumed to be the more likely scenario, there is still the possibility that these early circulating plasma cells are also newly generated. It is possible that these newly regenerating B cells acquire intense somatic hypermutations and subsequently differentiate into plasma cells and enter the peripheral circulation. Currently, it is not clear if these plasma cells observed during the ERP are actually the undepleted, long-lived cells which are recirculating or newly regenerated cells which are released in the periphery.

4. Therapy-induced qualitative modulation of plasma cells

Since peripheral plasma cells are CD20 negative and therefore not directly targeted by rituximab therapy we wanted to determine if the dynamic change in different B cell subsets induced by rituximab also influences the peripheral plasma cell compartment. We hypothesized that it may be possible also to modulate the highly mutated peripheral plasma cells. To address this, we evaluated if a qualitative change in the mutational aspects of the peripheral plasma cells (≥9 m) was introduced during B cell reconstitution.

For that we investigated particular motifs along the plasma cell Ig sequence wherein the mutations are shown to be preferably localized. This was performed by assessment of the pre-defined hot spot motifs RGYW and its inverse WRCY, (R=purine, Y=pyrimidine, W=A/T) which are usually the prevalent targets of somatic hypermutation that is induced in T cell dependent B cell activation. It is known that during a classical immune response in a healthy individual specific B cell-T cell interaction in the germinal centres is predominant. T cell-B cell collaboration in the GCs subsequently leads to somatic hypermutations of B cell Ig genes. This process of SHM is marked by prevalent targeting of RGYW/WRCY motifs along the Ig gene (Dorner *et al.* 1998; Farner *et al.* 1999). This notion is further substantiated by observations in hyper IgM children, where the classical CD40-CD40L pathway is impaired, leading to a break up in the T cell-B cell collaboration. Heavy chain IgR analysis of hyper IgM children showed a reduction in the frequency of hot spot targeted mutations, indicating that RGYW/WRCY mutations are critically dependent on T-B interactions (Brezinschek *et al.* 2000). In addition to T cell

dependent hypermutation, there are also T cell independent mechanisms for SHM reported that do not include T cell activation of B cells. This T-independent type of B cell differentiation garners more attention in recent years and seems to operate more predominantly in autoimmunity. Several groups have previously reported that T-independent type of SHM is particularly observed in the Ig repertoire under autoimmune conditions (Dorner *et al.* 1998; Brezinschek *et al.* 2000). One micro environment where T cell independent mutations can be acquired seems to be provided by the marginal zone areas (William *et al.* 2002). The process of T-independent differentiation results in mutations which cluster to a lesser extent in RGYW/WRCY motifs.

Our observation of hotspot mutations in the plasma cell Ig genes reveal that before rituximab therapy the confinement of mutations to the RGYW/WRCY motifs in our patient group was relatively low in comparison to after therapy status. During recovery phase, the mutations were significantly more restricted to the RGYW/WRCY motifs (59% versus 44%, ERP versus before therapy). These results support the hypothesis that during active disease the patients plasma cells are marked by mutations induced preferentially in a T cell independent fashion which is revealed by reduced RGYW/WRCY targeting. However, temporal B cell depletion by rituximab modulates also the recirculating peripheral plasma cells which then have an increased mutational targeting in the RGYW/WRCY motifs. Therefore the plasma cell repertoire seems to be more formed by a T cell dependent process after rituximab therapy, which seems to re-establish a state that is seen in healthy persons.

In order to find additional arguments to support this hypothesis we assessed also the affinity of the plasma cell IgR to antigenic binding and selection of these cells in the active repertoire. These properties of the plasma cells can be indirectly studied by delineating the replacement and silent mutations in the CDRs of Ig sequence. Point mutations in form of base substitutions leading to a change in the amino acid transcribed are termed replacement mutations and if the base substitution does not result in change in the amino acid, it is said to be a silent mutation. In general, antigen-selected antibodies within a T cell dependent germinal center reaction demonstrate a higher frequency of R mutations in the CDRs as compared to mutations induced in a T-cell independent manner. The repertoire selection

for a ubiquitous autoantigen seems less marked by positive antigen selection but rather by negative selection, which then would result in lower R/S ratio. This has been reported previously by other investigators that demonstrate decreased R/S mutations in the CDRs and negative selection of R mutations in autoimmunity as a feature manifested in self-destruction (Randen *et al.* 1995; Stott *et al.* 1998). Furthermore, existing data from heavy chain (VH) and light chain variable (Vκ and Vλ) region analyses in healthy subjects also provide strong evidences for positive selection of R mutations and elimination of S mutations in the CDRs (Dorner *et al.* 1998; Foster *et al.* 1999; Monson *et al.* 2000). Therefore we analyzed the R/S ratio prospectively in highly mutated plasma cells under rituximab therapy. The mean R/S ratio in the CDRs of three patients was relatively low (1.87) before rituximab treatment. Interestingly this ratio significantly increased in the plasma cells that recirculate during B cell repopulation to values of 2.67 and 3.60 in ERP and LRP status respectively. Our results argue again for a significant modulation of the plasma cell repertoire after rituximab therapy. The increase in R/S ratios can be interpreted as a shaping of the Ig-repertoire by positive antigen selection as seen in healthy individuals.

In conclusion, the current study demonstrates that B cell depletive therapy has provided desirable effects in patients suffering from RA. Apart from the modulations in the Ig-VH4 gene repertoire, it also became evident that during the period of early B cell repopulation (ERP) following anti CD20 therapy a pool of recirculating plasma cells is observed which contributed to the increased overall mutational frequency of B cell Ig receptors found at that time point. Interestingly, rituximab induced also a qualitative modulation in the mutational aspects of these plasma cells. The plasma cells observed in the periphery during ERP reflected characteristics of a more T cell-mediated mutational pattern as indicated by increased targeting of the RGYW/WRCY hot spot motifs. Similarly, an elevated R/S mutational ratio in the CDRs of the plasma cell Ig genes was displayed during peripheral repopulation in comparison to before therapy status. This argues for an increased positive selection influence on the B cell repertoire. Therefore the mutational pattern in the plasma cell Ig genes found after rituximab-induced temporal B cell depletion, manifests a repertoire which resembles patterns found in healthy individuals.

Taken together, transient B cell depletion by rituximab therapy seems to modulate also the plasma cell compartment which is not directly targeted by the therapy. This may indicate a therapy-induced alteration of the class-switched memory B cell pool and particularly relate to the long term clinical benefit observed in patients including reduction of disease specific autoantibodies. Modulation of plasma cells in RA could be also used as a potential bio-marker in studying the effective response in RA treatment. This needs to be further explored to gain deeper insights into the underlying processes which may be influenced by future therapies.

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Summary

B cells play diverse roles in the immunopathogensis of autoimmune diseases by production of autoantibodies, presentation of autoantigens, regulation of T cell effector responses and secretion of chemokines and cytokines involved in the differentiation of follicular dendritic cells. Since the central role of B cell has been highlighted, several approaches targeting B cell directly or indirectly are in clinical practice in the treatment of autoimmunity. In this regard, B cell depletion by rituximab (anti CD20 antibody) is being appreciated and gaining more importance in recent years. It has been hypothesized that B cell depletion might reset the abnormalities rendered by the autoimmune B cell repertoire.

Rituximab mediated B cell depletion poses to be a promising tool in the treatment of rheumatoid arthritis (RA), a prevalent autoimmune disorder. To date, little is known about the regeneration profile of B cells following temporal B cell depletion. We wanted to investigate the early replenishing B cells and examine the dynamic changes in the repertoire. Of particular interest, we wanted to determine the modulation in the mutational pattern of the B cell receptor (BCR).

To address this issue, we studied the immunoglobulin receptor (IgR) modulation of Ig-VH4 genes as representative of the heavy chain family. In total, five patients were included in the study and therapy induced alterations were assessed. Three time points namely before therapy, early regeneration phase (ERP- the early time point during regeneration where just above 1% B cells were found in the peripheral lymphocyte pool) and later regeneration phase (LRP- which commenced 2-3 months following ERP) were chosen. In three patients (A-C) Ig-VH4 genes were amplified from total genomic DNA during the above-mentioned three time points and in another two patients (D and E), Ig genes before therapy and during ERP were studied by single cell amplification technique.

Firstly, B cell regeneration followed the characteristic regeneration pattern as reported by several groups, with a predominant circulation of CD38hi expressing plasma

cells and immature B cells in the early recovery period. During LRP, the proportion of these cells reduced relatively and the levels of naïve B cells rose gradually.

On a molecular level, Ig-VH4 variable gene usage prior and post B cell depletion was determined and it was noticed that a diverse set of Ig-VH4 genes were employed in the repertoire before and after therapy. Mini gene segments such as VH4-34 and VH-4-39 which were reported to be connected with autoimmunity were over expressed in the B cell repertoire before therapy. Rituximab induced some changes in the regenerating repertoire which was revealed by reduction in the frequency of VH4-39 usage in the reemerging B cells. However to date no particular gene is known which is involved in the pathomechanism in RA.

Profound changes were noticed in the early reemerging repertoire with a relatively increased population of intensely mutated B cells. These B cells acquired ≥9 mutations in the Ig genes. Immunophenotyping with specific surface markers revealed that these highly mutated B cells evolve from the isotype-switched memory compartment especially the plasma cells. In order to substantiate the hypothesis that the highly mutated B cells observed during ERP were plasma cells we carried out single cell amplification of individual plasma cells in another two patients during ERP and compared the mutational load which remained similar.

Actually plasma cells do not express CD20 on their surface and therefore are not eliminated by rituximab therapy. However they were not observed in the peripheral blood following B cell depletion. The earliest time point when plasma cells are found again in peripheral circulation is the early recovery period (ERP). During this time point, the plasma cells were found to recirculate in parallel with the newly formed B cells. Therefore, it was intriguing to ascertain if the plasma cells were also modulated by rituximab therapy although they were not directly targeted by the therapy.

In this connection, we performed a detailed analysis of the pattern of mutations in the plasma cells that circulate before therapy and during the early recovery phase. We investigated if there is a therapy mediated mutational modulation of the plasma cells

though these are not directly targeted by the therapy. We thoroughly examined the confinement of mutations to the pre-defined RGYW/WRCY hotspot motifs (R=purine, Y=pyrimidine, W=A/T) in the plasma cells which provides information on the involvement of T cells in B cell somatic hypermutation (SHM). Plasma cells before rituximab manifested the characteristics of active disease which was revealed by restricted mutational targeting to the RGYW/WRCY motifs. The reemerging plasma cells during ERP had an increased targeting of the RGYW/WRCY motifs which indicated for a more pronounced T cell mediated B cell mutations which is the scenario observed in the healthy subjects. To secure additional arguments to support the hypothesis of rituximab-mediated plasma cell modulation, we delineated the replacement to silent mutations ratio (R/S) in the hypervariable regions (CDRs) of the plasma cell Ig sequences. Affinity of the plasma cell IgR to antigenic binding and selection of these cells in the active repertoire could be indirectly studied by R/S analysis in the CDR. Elimination of R mutations and increased S mutations in CDRs were previously reported in autoimmunity. Within our study, the mean R/S ratio in the plasma cell CDRs of the patient group was relatively low (1.87) before rituximab treatment and interestingly this ratio increased significantly in the recirculating plasma cells to values of 2.67 and 3.60 in ERP and LRP status respectively. The increase in R/S ratios in reemerging plasma cells can be interpreted as a shaping of the Ig-repertoire by positive antigen selection as seen in healthy individuals.

To conclude, our study demonstrates temporal B cell depletion by rituximab therapy seems to modulate also the plasma cell compartment which is not directly targeted by the therapy. This may indicate a therapy-induced alteration of the class-switched memory B cell pool and particularly relate to the long term clinical benefit observed in patients including reduction of disease specific autoantibodies. Modulation of plasma cells in RA could be also used as a potential bio-marker in studying the effective response in RA treatment. This needs to be further explored to gain deeper insights into the underlying processes which may be influenced by future therapies.

Zusammenfassung

B-Zellen spielen eine wichtige Rolle in der Pathogenese von Autoimmunerkrankungen. Neben der Produktion von Autoantikörpern, präsentieren sie Autoantigene, regulieren T-Zell Effektorfunktionen und produzieren Chemokine und Zytokine. Daneben sind sie in die Differenzierung von follikulär dendritischen Zellen beteiligt. Die zentrale Rolle der B-Zellen in der Pathogenese von Autoimmunerkrankungen hat in den letzten Jahren zu unterschiedlichen therapeutischen Ansätzen geführt, B-Zellen direkt oder indirekt zu targetieren. Ein Beispiel hierfür stellt der monoklonale anti-CD20 Antikörper Rituximab dar.

Die B-Zelldepletion mit Rituximab stellt ein vielversprechendes Therapieverfahren in der Behandlung der rheumatoiden Arthritis dar (RA). Nach Gabe von Rituximab kommt es zu einer passageren, in der Regel 6-9 Monate anhaltenden peripheren B-Zelldepletion. Derzeit ist wenig über das Regenerationsverhalten von B-Zellen nach Therapie mit Rituximab bekannt. Daher untersuchten wir die frühe Regnerationsphase und die Veränderungen des B-Zellrepertoirs. Insbesondere wollten wir die Modulation des Mutationsmusterns des B-Zellrezeptors untersuchen (BCR).

Am Beispiel der VH4 Familie der Immunglobulin schweren Ketten analysierten wir die Modulation des Immunglobulinrezeptor Repertoires durch die passagere B-Zelldepletion.. Insgesamt wurden im Rahmen der Studie bei 5 Patienten 3 Zeitpunkte analysiert: vor Therapie, in der frühen Regenerationsphase (ERP- early regeneration period, mit einem B-Zellanteil > 1% im peripheren Blut) und in der späten Regenerationsphase (LRP- late regeneration period, 2-3 Monate nach der frühen Regenerationsphase). Bei 3 Patienten (A-C) wurden die Ig-VH4 Gene aus genomischer DNA amplifiziert und zu o.g. Zeitpunkten analysiert. Bei weiteren 2 Patienten (D und E) erfolgte die Analyse der Ig Gene in einzelnen B-Zellen mittels Einzelzellsortierung und Einzelzell RT-PCR.

Die B-Zellregeneration nach Therapie mit Rituximab zeigte ein charakteristisches Regenerationsmuster mit einer Dominanz von unreifen CD10+ B-Zellen und CD38hi

Plasmazellen während der frühen Phase der B-Zellrekonstitution. Im weiteren Verlauf kam es zu einer Abnahme dieser Zellen und einem Anstieg von naiven B-Zellen.

Auf der molekularen Ebene zeigte sich vor und nach B-Zelldepletion eine unterschiedliche Nutzung der Ig-VH4 Gene. Mini Gene wie VH4-34 und VH4-39, die in Verbindung mit Autoimmunität stehen, waren vor Einleitung der Therapie überexprimiert. Durch die Behandlung mit Rituximab kam es zu einer Veränderung des Repertoires der regenerierenden B-Zellen mit einer reduzierten Benutzung der VH4-39 Gene im B-Zellpool. Ein bestimmtes Gen, welches in die Pathogenese der RA involviert ist, ist bis heute allerdings nicht bekannt.

Tief greifende Veränderungen fanden sich im regenerierenden Repertoire, mit einem relativen Anstieg von stark mutierten B-Zellen. Diese B-Zellen wiesen über 9 Mutationen in ihren Ig-Genen auf. Die Immunphänotypisierung zeigte, dass diese hochmutierten B-Zellen den Ig-klassengeswitchten Gedächtnis B-Zellkompartiment, insbesondere den Plasmazellen zughörig sind. Um diese Hypothese zu untermauern, erfolgte bei 2 Patienten eine Einzelzellsortierung dieser Plasmazellen während der frühen Regenerationsphase, welche einen vergleichbaren Mutationsstatus zeigte.

Da Plasmazellen kein CD20 Molekül exprimieren, werden sie durch eine Therapie mit Rituximab nicht direkt eliminiert. Allerdings zirkulieren sie nicht im peripheren Blut während der Phase der B-Zelldepletion. Während der frühen Regenerationsphase (ERP) lassen sie sich in der Peripherie erneut nachweisen. Zu diesem Zeitpunkt finden sich neben Plasmazellen auch regenerierende unreife B-Zellen. Es wurde deshalb untersucht ob auch Plasmazellen durch die Therapie moduliert werden, obwohl sie nicht direkt durch Rituximab targetiert werden.

In diesem Zusammenhang erfolgte eine detaillierte Analyse des Mutationsmusters der Plasmazellen vor Therapie und während der frühen Regenerationsphase. Die Analyse der Mutationshäufigkeit in RGYW/WRCY Hotspot Motive (R=purine, Y=pyrimidine, W=A/T) erlaubt Abschätzung in wieweit die somatische Hypermutation der B-Zellen durch T-Zell abhängige Differenzierung erfolgte. Die Plasmazellen vor Therapie zeigten

Charakteristika einer aktiven Erkrankung an mit einem verminderten Targeting der RGYW/WRCY Motive. Im Gegensatz hierzu zeigte sich in den rezirkulierenden Plasmazellen während der frühen Regenerationsphase ein zunehmendes Targeting der RGYW/WRCY Motive. Dies spricht für einen Repertoire Shift zu mehr T-Zellabhängigen B-Zell Mutation. Ein Zusatand, wie er bei Gesunden beobachtet wird. Um die Hypothese der Rituximab-induzierten Plasmazell Modulation zu stützen wurde die R/S- Ratio (replacement to silent mutations ratio) der hypervariablen Regionen (CDRs) der Plasmazell Ig Sequenzen bestimmt. Die Affinität des Plasmazell IgR bzgl. Antigenbindung und Selektion kann indirekt über die Analyse der R/S Ratio der CDR analysiert werden. Die Elimination von R-Mutationen und verminderte S Mutationen der CDRs finden sich im Rahmen von Autoimmunerkrankungen. In unserer Studie war die mittlere R/S Ratio der CDRs der Plasmazellen vor Therapie entsprechend relativ niedrig (1.87). Interessanterweise kam es in der frühen und späten Regenerationsphase zu einer signifikant erhöhten R/S Ration in den rezirkulierenden Plasmazellen mit Werten von 2.67 bzw. 3.60. Die verminderte R/S Ratio in den CDRs der Plasmazellen kann als Entwicklung des Ig-Repertoires durch positive Antigenselektion interpretiert werden und weist damit eine Therapie induzierte Veränderung auf, die dem entspricht wie man sie bei Gesunden findet.

Zusammenfassend zeigt unsere Studie, dass die passagere B-Zelldepletion mit Rituximab zu einer Modulation des Plasmalzellkompartimentes führt, welches nicht direkt durch die Therapie targetiert wird. Dies zeigt eine Therapie induzierte Veränderung des Ig-klassengeswitchten Gedächtnis B-Zellpools an und ist möglicherweise auch mit der Reduktion krankheitsspezifischer Autoantikörper verknüpft. Die Modulation der Plasmazellen bei der RA kann eventuell auch als möglicher Biomarker entwickelt werden, um ein Ansprechen auf die Therapie vorherzusagen. Dies muss im Weiteren untersucht werden, um tiefer greifende Einblicke in Prozesse zu erlangen, die durch zukünftige Therapien beeinflussbar werden.

Abbreviations

ACR	american college of rheumatology
ADCC	antibody dependent cellular cytotoxicity
AE	adverse effects
AID	activation induced cytidine deaminase
ANCA	antineutrophil cytoplasmic antibody
APC	antigen presenting cell
BAFF	B cell activating factor
BCR	B cell receptor
BLyS	B lymphocyte stimulator
bp	base pair
C	constant
CCP	cyclic citrullinated peptide
cDNA	complementary DNA
CDC	complement dependent cytotoxicity
CDR	complementary determining region
CRP	C reactive protein
CSR	class switch recombination
D	diversity gene
DAS	disease activity score
DMARD	disease modifying anti rheumatic drug
DNA	deoxyribonucleic acid
DSB	double strand break
FCS	fetal calf serum
FDC	follicular dendritic cell
FR	framework region
FACS	fluorescence activated cell sorting
GL	germline
GC	germinal centre
HLA	human leukocyte antigen

IDC	interdigitating dendritic cell
IFN-γ	interferon gamma
Ig	immunoglobulin
IgH	immunoglobulin heavy chain
IgL	immunoglobulin light chain
IgR	immunoglobulin receptor
IL	interleukin
J	joining gene
Jk	kappa light chain joining gene
Jλ	lambda light chain joining gene
KDa	kilodalton
L	ligand
m	mutation
mRNA	messenger RNA
NHEJ	non-homologous end joining
NP-OVA	nitro-phenyl-ovalbumin
PB	peripheral blood
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
R	purine
RA	rheumatoid arthritis
RAG	recombination activating gene
RF	rheumatoid factor
R/S	replacement to silent mutation
RTPCR	reverse transcriptase polymerase chain reaction
SHM	somatic hypermutation
SLE	systemic lupus erythematosus
SS	sjögren's syndrome
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
V	variable
VH	heavy chain variable region

VL	light chain variable region
Y	pyrimidine
W	adenosine or thymidine

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Curriculum Vitae

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Publications

1. Original articles

- **Influence of transient B cell depletion on recirculating plasma cells in rheumatoid arthritis**
  (Manuscript under submission)
- **Regeneration of B cell subsets after transient B cell depletion using anti-CD20 antibodies in rheumatoid arthritis.** Arthritis Rheum. 2006; 54(8): pp 2377-2386.
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  Anne-Sophie Rouzière, Christian Kneitz, **Arumugam Palanichamy**, Thomas Dörner, and Hans-Peter Tony

2. Published abstracts

- Roll P., **Palanichamy A.,** Rouziere A.S., Dörner Th.1, Kneitz C., Tony H.-P. B cell regeneration after B cell depletion with rituximab in patients with rheumatoid arthritis: transient recirculation of plasmablasts and increased levels of pre germinal centre cells in the early regeneration phase (Abstract)
  Annual meeting American College of Rheumatology November 2005 San Diego, USA
- **Palanichamy A.,** Theiss R., Roll P., Longo N., Kneitz C., Dorner T., Tony H.-P. Change in the mutational pattern of the B cell immunoglobulin receptor during the regeneration phase after transient B cell depletion with anti-CD20 antibodies in rheumatoid arthritis
  Annual meeting American College of Rheumatology November 2006, USA

3. Posters and oral presentations at congresses and symposia

- **Palanichamy A**., Rouziere A.-S., Kneitz C., Tony HP. Ig-VH analysis of patients suffering autoimmune diseases by fluorescent multiplex PCR (Poster)- 3rd Joint retreat "Immunomodulation meets lymphocyte activation" July 2003 Markttaschendorf
- **Palanichamy A.,** Rouziere AS., Kneitz C., Tony HP. "Regeneration of B cells after transient B cell depletion using anti- CD20 antibodies" (Oral presentation)- 4th Joint retreat "Immunomodulation meets lymphocyte activation" July 2004 Markttaschendorf
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- Roll P., **Palanichamy A.,** Rouziere A.S., Dörner Th.1, Kneitz C., Tony H.-P. B-Zellregeneration nach passagerer B-Zelldepletion mit Rituximab bei Patienten mit Rheumatoider Arthritis: transiente Rezirkulation von Plasmablasten während der Regenerationsphase (Poster)- Jahrestagung Deutsche Gesellschaft für Rheumatologie in September 2005 Dresden
- **Palanichamy A.,** Theiss R., Roll P., Longo N., Kneitz C., Dorner T., Tony H.-P. Change in the mutational pattern of the B cell immunoglobulin receptor during the regeneration phase after transient B cell depletion with anti-CD20 antibodies in rheumatoid arthritis (Oral presentation)- Annual meeting American College of Rheumatology November 2006 , Washington DC, USA

Eidesstattliche Erklärungen

Hiermit erkläre ich ehrenwörtlich, dass die vorliegende Dissertation ***" Influence of transient B cell depletion on recirculating B cells and plasma cells in rheumatoid arthritis "*** Selbständig an der Medizinische Klinik und Poliklinik II der Universität Würzburg angefertigt wurde und dass ich keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Weiterhin versichere Ich, dass die vorliegende Dissertation weder in gleicher oder ähnlicher Form noch nicht in einem andern Prüfungsverfahren vorgelegen hat und ich bisher noch keine akademische Grade erworben oder zu erwerben versucht habe.

Hiermit bewerbe ich mich erstmals um den Doktorgrad der Naturwissenschaften der Bayerischen Julius-Maximilians-Universität Würzburg

Würzburg, 30. July 2007

Arumugam Palanichamy