LONGTERM IMPACT OF ANTI-CD20 MEDIATED TRANSIENT B CELL DEPLETION ON MEMORY B CELLS IN PATIENTS WITH RHEUMATOID ARTHRITIS

LANGZEITVERÄNDERUNG DER GEDÄCHTNIS B-ZELLEN NACH ANTI-CD20 VERMITTELTER B-ZELLDEPLETION IN PATIENTEN MIT RHEUMATOIDER ARTHRITIS

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To my parents, the dearest in my life!

To my wife Asifa and our lovely son Rayyan, with love!

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1. Introduction

Despite their microscopic appearance that belies their functional heterogeneity and development, lymphocytes known as T (thymus derived) and B cells (bursa or bone marrow derived) have boosted the curiosity and challenged the experimental skills in many disciplines. A simplified definition of B cells is a population of cells expressing clonally diverse cell surface immunoglobulin (Ig) receptors which recognize specific antigenic epitopes having their functional end point in antibody production by terminally differentiated plasma cells (LeBien *et al*, 2008). The discovery and characterization of B cells occurred in mid 1960s and early 1970s using animal models by cellular surface molecule characterization (Good *et al,* 1956, Cooper *et al*, 1965, 1966).

1.1 B-cell development in Human

B cell development begins in the fetal liver and continues in the bone marrow throughout our lives. Lymphoid progenitors are descendant of hematopoietic stem cells (HSCs) with ability to develop into all lymphohematopoietic lineages. These lymphoid progenitor cells receive signals from bone marrow stromal cells through cell-cell contacts and secreted signals. This bone marrow microenvironment is responsible for B cell development where B cell precursors start development in bone marrow which later on migrates to peripheral lymphoid organs for becoming mature naïve B cell which continuously circulate in lymphoid tissue and blood until encounter an antigen. Once binding antigen to BCR (B cell receptor), this complex (antigen: BCR) is internalized, processed and presented as an antigen-MHC class II complex to T cells. This antigen specific T cell causes co-stimulation of the B cell which further develops into a memory B cell or plasma cells to be a continuous source of antibody production (LeBien, 2000; LeBien *et al*, 2008; Janeway *et al,* 2005).

1.1.1 B cell ontogeny

The earliest lineage of B cells is termed pro B cells in which heavy chain gene segments rearrangement takes place. During further differentiation of pro B cell, rearrangement of V_H segment to D_HJ_H-rearranged segments of IgH chain occurs. The

recombinase-activating genes, RAG-1 and RAG-2, play a fundamental role in these events (kawano *et al*, 2006). RAG-1 and RAG-2 enzymes are very crucial in development of the BCR and without these enzymes no lymphocytes can develop. A productive VDJ_H joining leads to the next B cell development stage called Pre B cell stage.

During Pre B cell stage, μH chains produced in early phase combines with invariant VpreB/y5 surrogate light to form the pre-B cell receptor. Pre BCR provides a signal to proceed with light chain rearrangement. If the B cell fails to express the receptor ultimately causes cell apoptosis. Thus the pre BCR formation serves as checkpoint for positive selection of Pro B cells (Karasuyama *et al*, 1996; Kawano *et al*, 2006).Upon light chain rearrangement complete IgM molecule is expressed on cell surface which becomes Immature B cell (Fig. 1.1).

An Immature B cell expressing surface IgM if binds strongly to self molecules is directed to die by apoptosis. Alternatively, the cell gets survival chance through receptor editing of IgM molecule and if this is successful the cell survives.

Finally the B cell that does not bind self molecule continue its journey as transitional B cell by expressing both IgM and IgD on its surface and develop into a naïve mature B cell and migrates through blood to the peripheral lymphoid organs where final maturation occur where B cell is again tested for its self-reactivity.

1) If it strongly binds self-antigen that ultimately cause cell death by apoptosis due to receptor cross linking.
2) Soluble self antigen which does not cross link the receptor cause cell anergy and ultimately the cell dies. If the cell survives this final test, it is ready to patrol for antigens (Janeway *et al,* 2005).

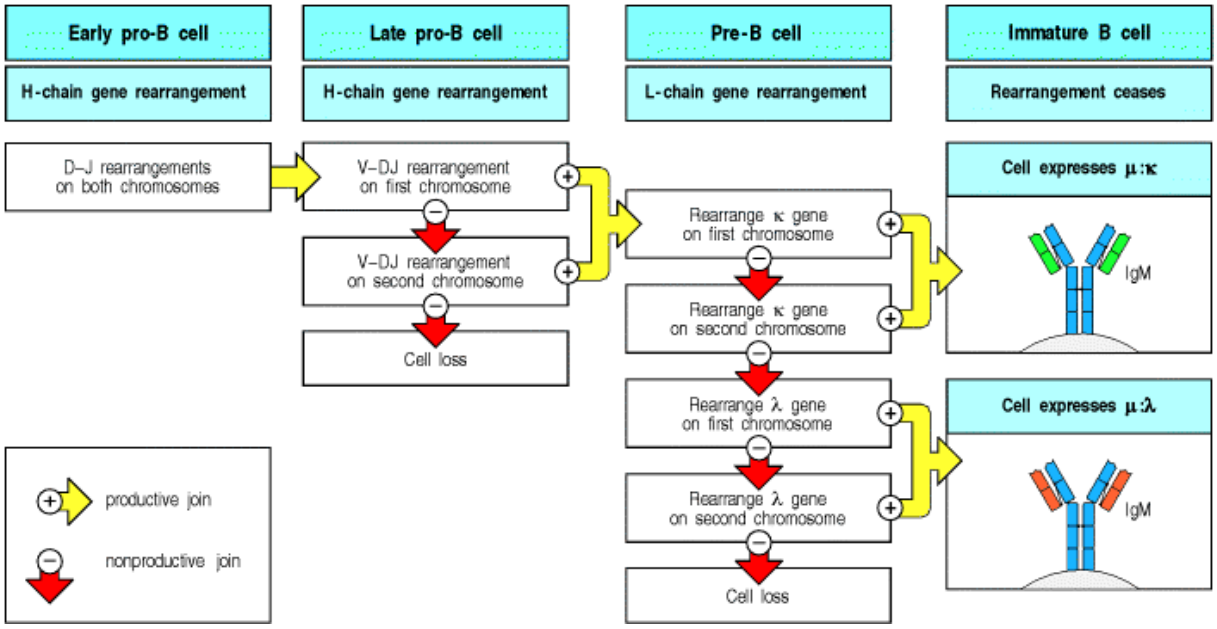


Figure: 1.1 The steps of B cell development: involving checkpoints for generation of productive immunoglobulin gene rearrangement (Adopted from Immunobiology; Janeway *et al*, 2005)

1.2 B cell activation

Mature B cells and their products, mainly antibodies, constitute the backbone of humoral immunity in protecting extracellular spaces from pathogenic invasions. As mature naïve B cells patrol in the blood and lymphoid follicles of spleen and lymph nodes until encounter of invading antigens or the antigenic binding to B cell receptor (BCR). The BCR is composed of an immunoglobulin antigen binding unit which is associated with Igα/Igβ heterodimers which function as signal transduction unit and provide a connection of BCR to a well organized complex of cytoplasmic signalling molecules (Fig.1.2). This binding provides first B cell activation signal which also require a secondary signal for fully activation, this signal is provided either by T cells dependent or independent ways (Carsetti *et al*, 2001).

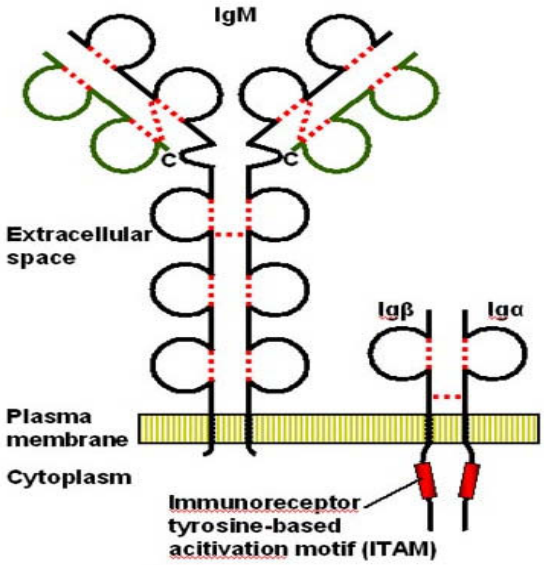


Figure 1.2 Cell membrane IgM on the surface of mature B cell is associated with Igα and Igβ molecules. The resulting complex constitutes the B cell antigen receptor complex. Igα and Igβ contain ITAMs in their cytoplasmic tails that mediate signal transduction (Adapted from Vasile *et al*, 1994).

T cell independent responses (TI) are induced when antigen itself binds to Toll like receptor (TLR) on B cell surface or BCR cross linking. Long polysaccharide chain containing antigens usually results TI responses. In contrary to TI responses, T cell help is required to induce T cell dependent responses. When this signal is induced by T cells, antigen: BCR complex is internalized and endosomally processed to present antigen-derived peptides in MHC II on the B cell surface where T cell recognize MHC:antigen complex and are activated and upregulate CD40L which co-stimulates the activated B cells (Weller *et al*, 2001).

The pathway of B cell functioning is a complex road with multiple factors involvement for successful responses. B cells undergo a complex series of genetic events including somatic hypermutation, gene conversion and class-switch recombination which are now discussed in details.

1.3 Somatic hypermutation

B cells have a unique ability to fine tune their receptors to creat antigenic specificity in order to improve their ability to bind a diverse variety of antigens. This ability of generating repertoire diversity is result of somatic hypermutation process which modifies variable segment of rearranged immunoglobulin (Ig) genes during an immune response. A distinctive difference between naïve and memory B cells is attributed to this process. In primary immune responses, Ig genes have germline configuration but numerous point mutations are notable in Ig genes of antibodies during secondary immune responses (Longo *et al,* 2006). The process of somatic hypermutation is associated with DNA strand breaks and induction of mostly single nucleotide exchange. During this process small deletions and duplications are produced in rearranged IgV region at the rate of 10^{-3} per base pair per generation (Klein *et al,* 2008).

1.3.1 Sites of somatic hypermutation

Circulating B cells after antigenic stimulation migrate through the helper T cell rich areas of secondary lymphoid organs e.g. Spleen and lymph nodes to form germinal centres. B cells proliferation occurs in dark zone of GC, while switching of effector functions i-e re-expression of surface Ig (sIg) and antigenic driven selection occurs in light zone (Liu *et al*, 1997). Somatic hypermutation of Ig V genes occurs in dark zone (Figure 1.3) which indicates that DNA replication and /or repair mechanisms are involved in the mutational mechanism. The enzyme Activation induced cytidine Deaminase (AID) is crucial for the process of somatic hypermutation which deaminates cytosine at hotspots within the variable (V) region of the immunoglobulin DNA. A mechanistic approach to understand the process of somatic hypermutation in addition to involved mechanisms is briefly described below.

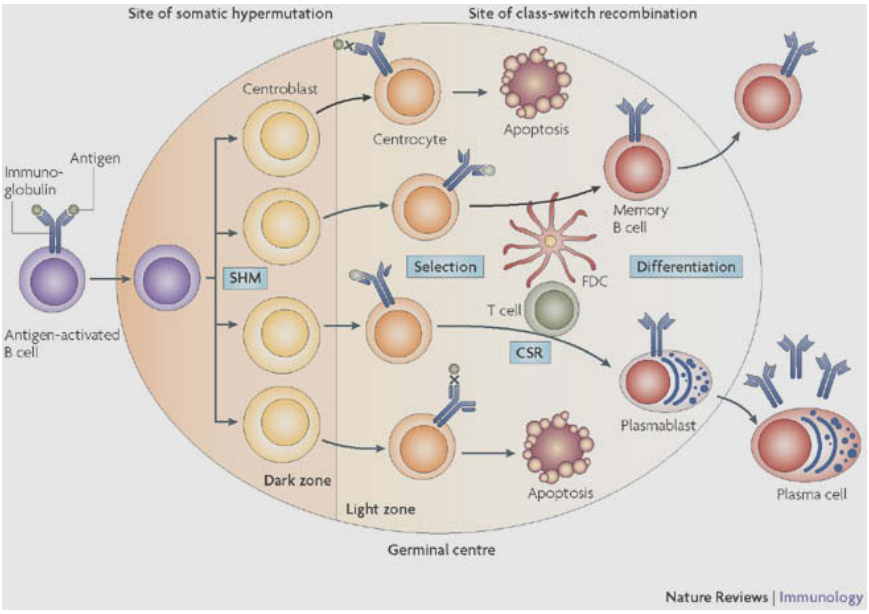


Figure 1.3 Germinal centre microenvironment: Antigen-activated B cells differentiation and clonal expansion in the dark zone of the germinal centre. During proliferation, the process of somatic hypermutation (SHM) introduces base-pair changes into the V (D) J region of the rearranged genes encoding the immunoglobulin variable region (IgV) of the heavy chain and light chain occurs (adopted from Klein *et al,* 2008).

1.3.2 Role of Transcription in somatic hypermutation

The process of Somatic hypermutation is confined to 1-2 kilobase region in the rearranged Ig gene and mutations are introduced in regions which encompasses ~2 kb downstream of transcriptional region. These mutations are non-random (Klein *et al,* 2008). Several studies have implicated the role of transcription in somatic hypermutation.

The enhancer/matrix attachment region in JC intron in heavy chain locus is believed to be indispensible for this process while 3 prime enhancers which are downstream from C exons seem to facilitate somatic hypermutation reaction (Azuma *et al*, 1994). Replacement of Ig promotor with heterogeneous promotor and movement of promotor either to downstream or upstream of V region displaces mutations to the regions 3 prime of the promotor (Diaz *et al,* 1998). However deletion of sequences which are upstream of the promotor region does not influence the process of hypermutation (Tumas-brundage *et al*, 1996). As concluding remarks, the frequency of mutations increases rapidly in the leader intron peaks at rearrangement V (D) J exon and decreases into J-C intron of Ig genes.

1.3.3 DNA double strand breaks and error-prone DNA repair

DNA double strand breaks (DSBs) also play important role in inducing somatic hypermutation process based on deletion and duplication analysis. This DNA double strand breaks occurs in the V gene of Ig genes of GC B cells (Goossens *et al,* 1998).DSBs could be introduced as direct single nucleotide substitutions via base modification mechanism without interference with the sugar-phosphate backbone of the DNA double helix (Wu *et al,* 2003).

These DSBs enhance a high transcriptional activity and their distribution along V_HDJ_H parallels the mutations in hypermutation domain. Mostly DSBs (around 50%) occur at RGYW consensus motifs(R=purine, Y= pyrimidine, and W=A or T) which are intrinsic hot spots of somatic hypermutations (Bross *et al,* 2000) indicating that DSBs appears to depend on transcriptional activity.

DSBs are repaired through two competitive pathways which include opposing and rejoining broken DNA ends with no or little regard for sequence homology or by template directed homologous recombination process. Research has also shown that DSBs in hypermutating B cells are mainly done during S/G2 cell populations and mutations are also occur during DSB repair by homologous recombination after recruitment of error-prone DNA polymerase(Papavasiliou *et al,* 2000). Error-prone DNA polymerase inserts incorrect bases and capable of extension of newly generated mismatched terminus. These error-prone

translation or lesion bypass polymerases affect continuous strand synthesis opposite to DNA lesions that install a replication fork. Several studies have shown that translation DNA polymerases like β, μ, δ, κ and λ are not involved while polymerase ι and ζ play important role in somatic hypermutation. Polymerase ι is involved to insert an incorrect base while ubiquitous polymerase ζ extends from mismatch repair for stabilizing the mutation (Wu *et al,* 2003; Holbeck *et al,* 1997)

1.3.4 Class-switch recombination

All mature B cells starting out as $IgM^{+}IgD^{+}$ and after encountering antigen and CD40-CD40L interaction they can exchange the IgM and IgD with a receptor of different isotype i-e IgA, IgG or IgE which is facilitated by a process of class switch recombination. Class-switch recombination is recombination between two different switch regions which are present upstream from each C region of IgH with deletion of the intervening DNA. The DNA between switch region of choice get looped out and forms excised intervening DNA. Recombination signal for a particular switch region comes from the cell surface. Class switching is irreversible recombination of DNA and during this process of exchange constant regions of the heavy chains but leaves the variable regions intact (Klein *et al,* 2008; Longo *et al,* 2006).

1.4 Regulation of somatic hypermutation process

Activation induced cytidine Deaminase (AID) enzyme is believed to be a master regulator of secondary antibody diversification processes (Figure 1.4) by exerting its important role in immunoglobulin somatic hypermutation process and class-switch recombination. AID is a 24kDa enzyme which removes the amino group from the cytidine base in DNA (Bransteitter *et al,* 2003). AID is normally expressed in germinal centre B cells but its forced expression in B cells at wrong differentiation stage results enhanced mutagenesis and mutational spectra mimicking somatic hypermutation. AID expression in non-B cells suggest that its operation does not require either B cell specific V gene targeting elements and other normal B cell proteins for its natural functioning (Martin *et al,* 2002).

AID is believed to initiate process of somatic hypermutation by different multi-step processes including catalyzing dC ⟶ dU deamination activity on double stranded DNA containing small 'transcription like' single stranded DNA substrates during somatic hypermutation and mutational hotspots RGYW are preferentially deaminated resulting mutations and mismatchs which are recognized by mismatch repair machinery followed by error-prone DNA polymerases (Bransteitter *et al,* 2003; Poltoratsky *et al,* 2000)

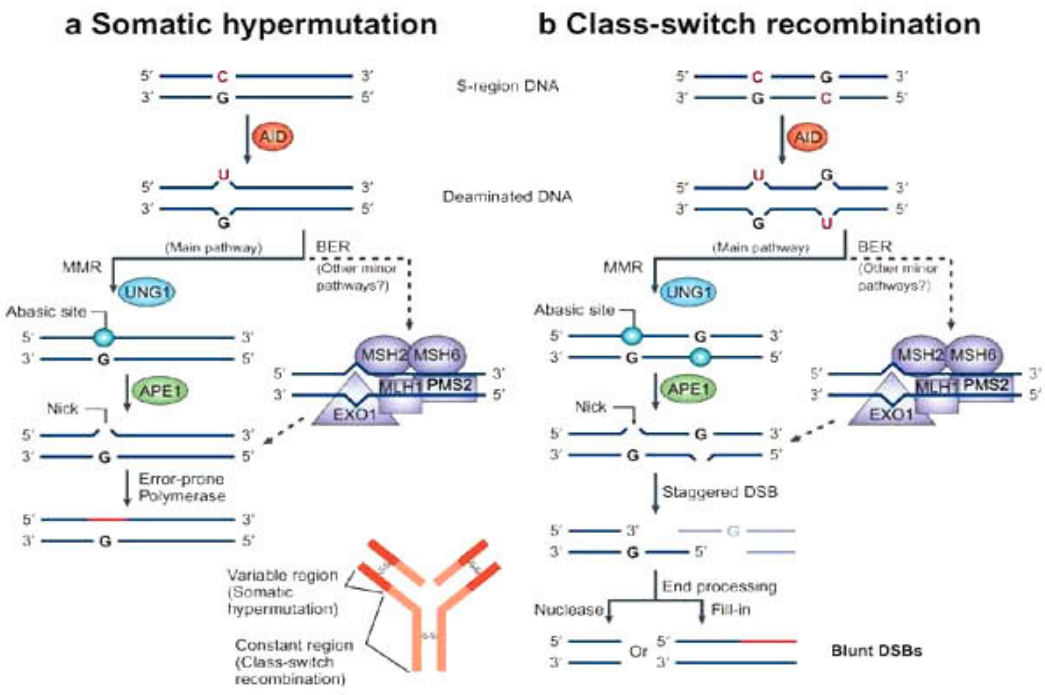


Figure 1.4 Activation-induced cytidine deaminase (AID): deaminates cytidine residues in DNA to be processed either by base excision repair (BER) or mismatch repair (MMR). In somatic hypermutation, the mismatch can be replicated to produce a C-to-T mutation, nick processing by UNG can produce an abasic site, or a gap can be filled in by error prone polymerases. (Adapted from Nussenzweig *et al,* 2004)

1.5 Peripheral B cell pool

After passing highly strict phases during development, successful B cells ultimately reach the peripheral Blood. Different B cell subpopulations are identified in blood based on surface molecular expression to differentiate them mainly into naïve and memory B cells. Fig. 1.5 represents all B cell subpopulations in peripheral blood with an average percent presence of these populations. Below is phenotypic description of B cells subpopulations and their products:

1) Naïve B cells: $CD19^{+}CD20^{+}CD27^{-}CD5^{+/-}IgD^{+}IgM^{+}$

Naïve mature B cells have not yet encountered antigen. These B cells have unmutated V genes and comprise 60% of peripheral B cell pool. Majority of naïve B cells are CD5 negative but small fraction is $CD5^{+}$ (~15%).$CD5^{-}$ B cells are precursors of GC-B cells in T cell dependent immune responses while CD5+ B cells are believed to not involve in T cell dependent responses (Klein *et al*, 1998).

2) Memory B cells: $CD19^{+}CD20^{+}CD27^{+}IgD^{+/-}$ or IgG^{+}, IgA^{+}, IgM^{+} or IgE^{+}

Memory B cells are mature B cells who have encountered antigen and have somatically mutated Ig receptors.CD27 is representative key surface marker to differentiate memory B cells from naïve population (Agematsu *et al*, 2000). More recently in patients suffering from autoimmune disease SLE (systemic lupus erythematosus) have shown $CD27^{-}$ /IgD^{-} cells have been identified displaying features of memory B cells (Wei *et al*,2007; Jacobi *et al*, 2008). Human memory B cells are mainly distinguished into post-switched memory B cells ($IgD^{-}CD27^{+}$ cells) and pre-switched ($IgD^{+}CD27^{+}$ cells) memory B cells. Post-switched memory B cells under maturation process in classical germinal centers in secondary lymphoid organs(Klein *et al*,1998) but very little is known about the origin of $IgD^{+}CD27^{+}$ memory B cells.

3) Plasmablasts ($CD19^{+}CD20^{+}CD27^{+}CD38^{+++}CD138^{-}$ B cells) and Plasma cells: $CD19^{-}CD20^{-}$ $CD27^{+}CD38^{++}CD138^{+}$ B cells

Plasma cells are factories of soluble antibodies production. These include both short and long lived plasma cells. Short lived plasma cells survive for few days while long lived plasma cells can survive for months. Plasma cells are believed to survive and reside in so called survival niches in bone marrow, inflammatory sites and secondary lymphoid organs (Radbruch *et al*, 2006)

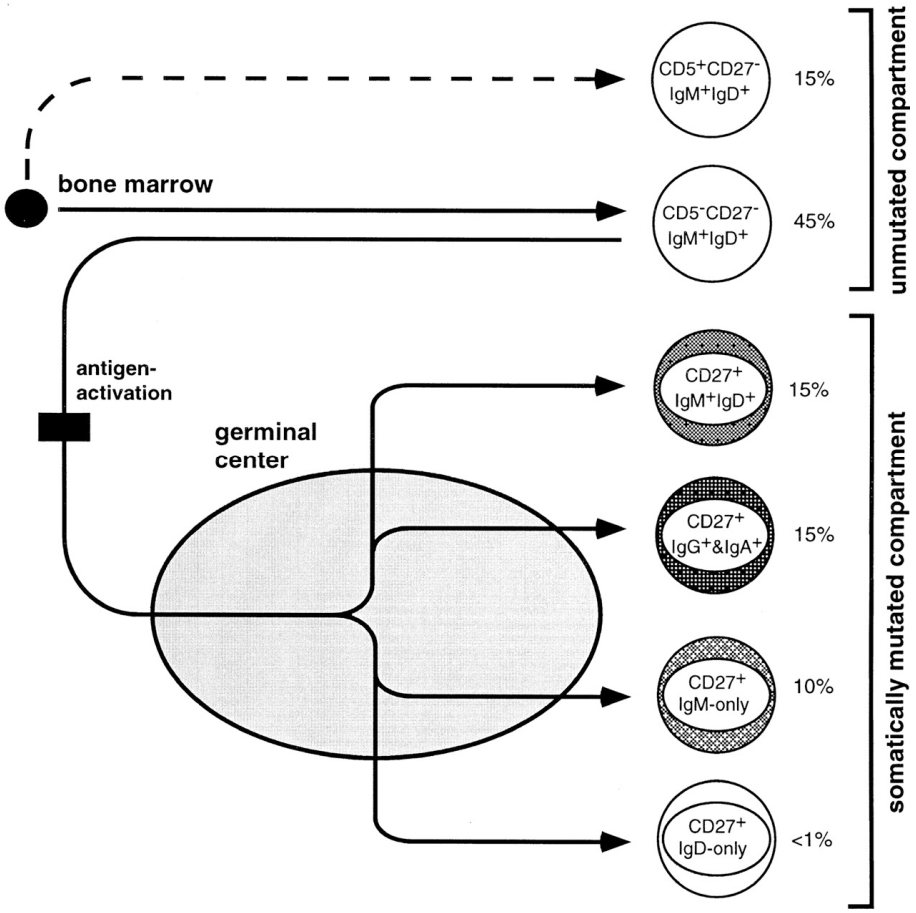


Figure 1.5 The PB B cell pool and its presumptive generation: Different subpopulations of human B cells in peripheral blood (adopted from Klein *et al*, 1998)

4) Antibodies

The main product of B cell immune responses are antibodies which circulate in blood stream which are main responsibility of plasma cells. Structurally, an antibody consists of 4 protein chains: 2 light chains and 2 heavy chains. Each chain consists of a constant portion which is involved in isotype determination and a variable part V which constitutes the antigen binding site. The figure 1.6 shows antibody structure. In human, V region from light chain could be either Vκ or Vλ while V region of heavy chain has 6 sub-genes (V_H1-V_H6) which provide diversity in VDJ recombination process and variety of antibodies .There are 5istypes of antibodies known as IgD, IgG, IgM, IgA and IgE. In human, IgA and IgG are further divided into subclasses IgA1, IgA2, IgG1, IgG2, IgG3 and IgG4 (Janeway *et al*, 2005)

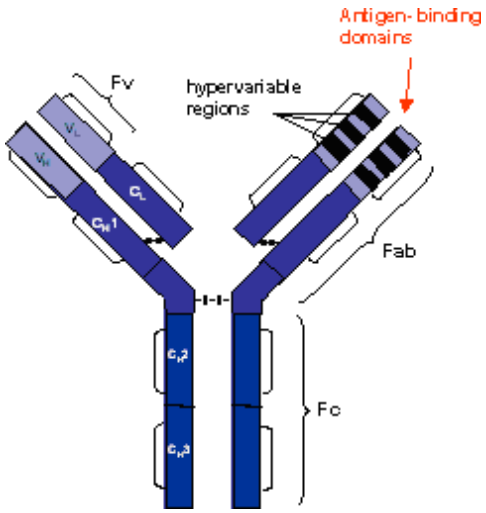


Figure 1.6 A schematic diagram of antibody: showing constant CH and variable regions V.

1.6 Regulators of B cells

Among many regulators of B cells during formation, certain cytokines are very important for B cell maturation and normal functioning .These cytokines play central role in B cells. A short description of few cytokines is described below.

BAFF

Most of cells die in absence of appropriate survival signals and growth factors. B cell activating factor is fundamental survival factor of B cells. BAFF belongs to TNF family and is crucial for B cell maturation. BAFF mRNA is present in monocytes, macrophages, dendritic cells and by growth factor-stimulated neutrophils. Very low expression is observed in T cells but completely absent in B cells (Schneider *et al*, 2003). BAFF can bind 3 TNF-α receptors i-e BAFF-R, B cell maturation antigen (BCMA) and transmembrane activator and cyclophilin ligand interactor (TACI).The mechanism by which BAFF regulates B cell survival is not known but in vitro studies have shown increased B cell proliferation and BAFF-KO mice studies have shown defect in progression of B cells (Craxton *et al*, 2005; Henley *et al*, 2008).

In human, increased BAFF level has been demonstrated in autoimmune diseases such as rheumatoid arthritis, Sjögren's disease, SLE and multiple sclerosis (Cheema *et al*, 2001; Zhang *et al*, 2001; Henley *et al*, 2008)

IL-2

IL-2 cytokine is secreted by activated T cells and has involvement in B cells differentiations which express IL-2Rα on their surface. IL-2 is also involved to increase CD23 expression on normal B cells which is mainly expressed on pre-switched memory B cells and is lost after class switching (Hivroz *et al*, 1989).

IL-4

IL-4 is produced mainly by T helper cells with pleiotropic effects on B and T lymphocytes.IL-4 acts as growth factor and induces high levels IgE and IgG4 production in human while IgG1 and IgE production in mice.IL-4 is also shown to induce expression of CD23 molecule which is low affinity receptor for IgE (Lundgren *et al*, 1989)

IL-6

IL-6 is a pleiotropic cytokine which acts as stimulator for B and T cells and promotes B cell proliferation and final maturation into Ig secreting plasma cells. IL-6 is produced by T cells, macrophages and endothelial cells. IL-6 is also inflammatory cascade player and been involved in different autoimmune diseases like RA. IL-6 produces acute phase protein production and fever (Lipsky, 2006).

Along with these cytokines IL-10 and IL-21 are also involved in B cell regulatory machinery by helping B cell survival, proliferation and differentiation. IL-10 and IL-21 are produced mainly by T cells but IL10 is also produced by NK cells, B cells and monocytes (Janeway *et al*, 2005)

1.7 Functions of B cells

B cells are important components of a normal immune responses and play a key role in maintaining cellular and humoral protective memory. In healthy individuals, B cells have also other essential functions in regulating immune responses than just being precursors of antibodies. They act in variety of ways directly and indirectly in regulation of other players in immune response (Figure 1.7). For example, lymphotoxin α/β expressed by B cells is responsible for differentiation of follicular dendritic cells. Activated B cells secrete costimulatory molecules which are essential for effector T cells and produce cytokines influence the functions of antigen-presenting dendritic cells. They are also involved in activation, anergy, differentiation and expansion of T cells. Antigen-presenting M cells can not develop in gastrointestinal mucosa in the absence of B cells (Lipsky, 2001). Additionally, they play important role in orchestration of inflammatory cascade by producing proinflammatory cytokines, such as tumour necrosis factor-α (TNF-α) and interleukin 6 (IL-6) and most importantly, they link innate, inborn and acquired adaptive immunity by using specific receptors expressed on B cells (Dörner, 2006).

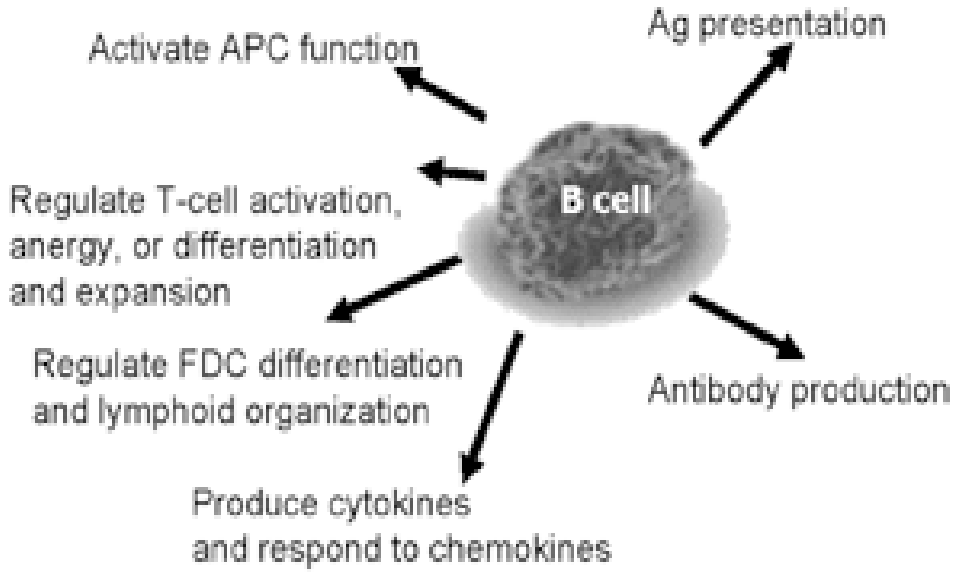


Figure 1.7 Different functions of B cells in immune system: The various functions of B cells. APC: antigen-presenting cell; FDC: follicular dendritic cell (adopted from Dörner, 2006)

1.8 Human memory B cells and marginal zone B (MZB) cells

The basic principle of acquired immunity for disease control depends on phenomenon of immunological memory. This memory is maintained by memory B cells, plasma cells and memory T cells. Different subpopulations of memory B cells have been described in section 1.5 depending on phenotypic presence of $CD27^+$ surface expression and somatic mutations in their immunoglobulin receptor comprising large circulating B cells in periphery. In secondary immune responses the memory B cells respond to lower doses of antigens facilitating quick update and presentation. These memory cells include pre and post switched memory cells depending on IgD surface expression (Figure 1.8). The majority of naïve B cells after encountering antigen under proliferation, somatic hypermutation and class switching with help of T cells in germinal centres of secondary lymphoid organs. However there is fraction of IgM^+ memory B cells which are assumed to mature in T cell independent way, particularly the marginal zone of spleen (Klein *et al*, 1998, Takizawa *et al*, 2006).

Splenic marginal zone is a unique B cell compartment which contains B cells having high surface expression of IgM and complement receptor 2(CD21) which exhibit rapid activation and Ig secretion in response to blood borne T independent (TI) antigens. These SMZ B cells are shown to carry somatic mutations. Mutated antibodies can be raised after immunization with T-independent polysaccharide vaccines. IgD^+($IgM^+CD27^+IgD^+$) memory B cells have gained specific interest in research in recent years due to their presence in peripheral blood (Weller *et al*, 2004).These memory B cells do not shift to class-switch memory by various stimuli invitro (Shi *et al*, 2003) and accumulating lines of evidences have suggested that these IgD^+ memory B cells play important role in humoral immunity and in some diseases.

It is still debated that when and where these IgD^+ memory B cells develop. These cells are suggested to be generated in spleen and control streptococcus pneumococcal infections because of their reduction in patients with inflammatory bowel disease patients related to impairment of splenic functions (Sabatino *et al*, 2005). These cells are completely absent in asplenic and splenectoy patients with an impaired immune responses to encapsulated bacteria. These studies also suggested that physiological and transient predisposition to pneumococcal infections of young children (0-2 years) is associated with lack of these IgD^+ memory B cells in spleen and of serum polysaccharide IgM (Kruetzmann *et al*,2003).All these findings induced research interest in IgD^+ memory B cells.

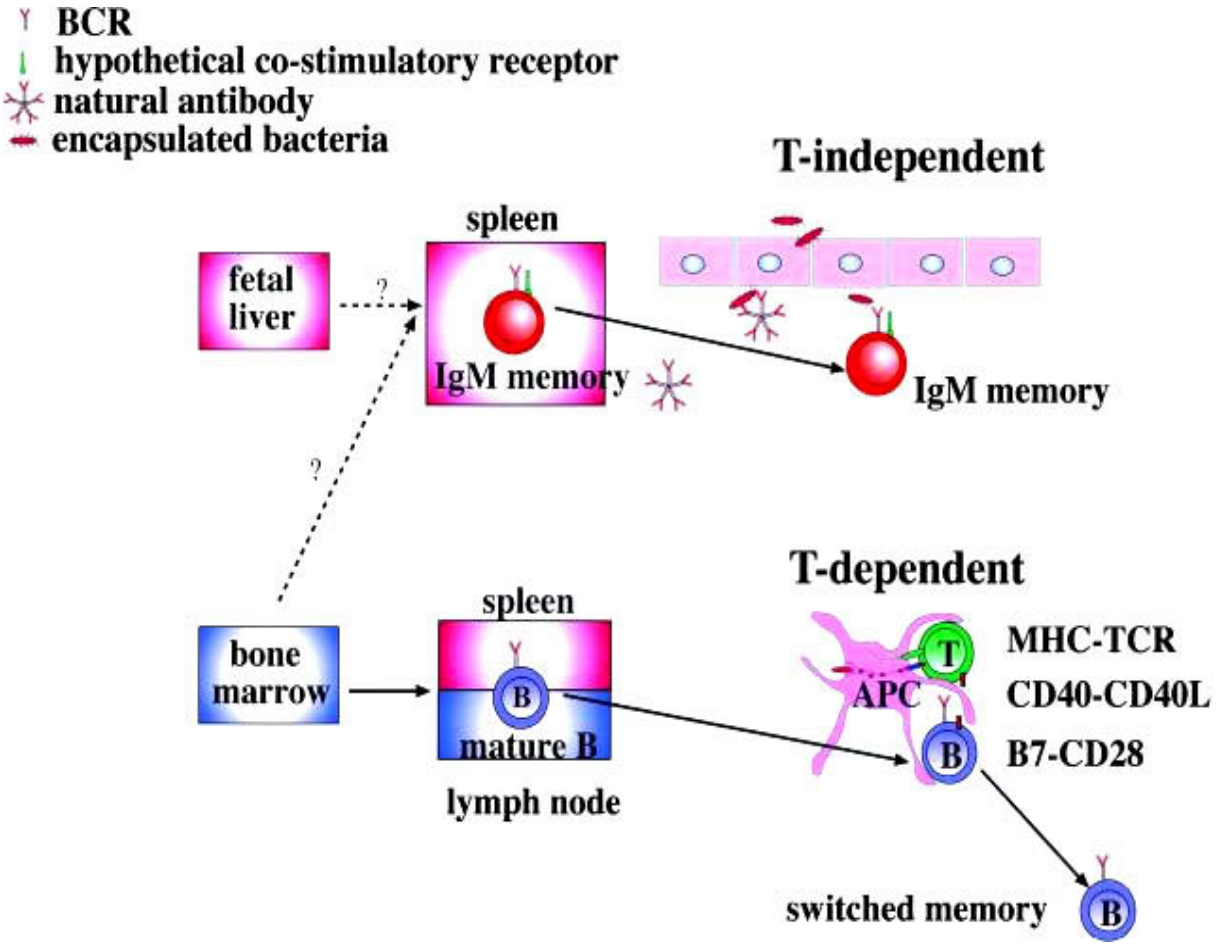


Figure 1.8 Origin and function of mature and IgD$^+$ memory B cells. IgM memory B cells might be able to migrate to sites of inflammation and recognize invading pathogens through the B cell receptor (BCR) and possibly other pathogen-binding co-receptors. Switched memory B cells are generated from bone marrow–derived mature B cells after somatic mutation and class switch in the germinal centers (adopted from Kruetzmann *et al*, 2003).

2. Autoimmunity

During lymphocyte production and maturation, they are schooled for tolerance to self antigen and cells who react to self antigen undergo programmed cell death, apoptosis but despite this strict regulatory system, immune system could be broken and leads to inflammatory reaction against own body tissues and ultimately develop autoimmune diseases which include large variety of diseases and few among them are rheumatoid arthritis, SLE and Sjögren's disease. Distinguishing self from non self is fundamental to normal immune response. Failure of deleting particular autoreactive clone can leads to emerge autoreactive condition.

Despite extensive research in this field it is still to be determined how and when this self tolerance becomes impaired and pathogenic causing autoimmune diseases.

2.1 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a complex immune-mediated inflammatory disease that particularly affects the small diarthodial joints of hands and feet. Chronic inflammation leads to progressive cartilage degradation and bone erosion which causes pain, joint deformity, and functional disability (Firestein, 2003; Siegel, 2008). RA is common chronic polyarthritis with prevalence 0.5-1.0 % (Alamanos *et al*, 2005). Two third of patients are women and the incidence of RA peaks at about 60 years of age. RA was first considered an autoimmune disease based on the identification of rheumatoid factor (RF) in the sera of patients and characterization of these factors as auto antibodies (Franklin *et al,* 1957). Further research proposed that RA represents a localized immune complex disease characterized by production of RF in synovium, the formation of RF-immune complex that fix complements with ensuing consumption of complements within the joint and recruitment of variety of cells like T cells, B cells and macrophages that become activated and contribute to inflammation and joint damage (Zvaifler ,1973). Diagnosis of patients with rheumatoid arthritis is based on these inflammatory hallmarks and in addition certain criteria based on clinical and laboratory findings should be fulfilled to classify various stages of rheumatoid arthritis. One of RA disease measurement is based on DAS (disease activity score using 28joint counts) score

based on swollen and tender joints count used by clinician in regular practice and clinical trials for response evaluation (www.das-score.nl).

Below is American college of rheumatology (formerly American rheumatism association) criteria for classification of RA which was established in 1987.

1. morning stiffness > 1hour before maximal improvement
2. arthritis of three or more joint areas
3. swelling of hand joints
4. symmetric swelling (arthritis)
5. rheumatoid nodules
6. serum rheumatic factor
7. radiological changes

Criteria 1 through 4 must have been presented for at least 6weeks (Arnett *et al*, 1987) which was further revised in 1991 by American college of rheumatology in four main classes (Hochberg et al, 1992) depending on patient activities.

Class1=able to perform usual activities, but limited in vocational activities

Class2=able to perform usual self-care and vocational activities

Class3=able to perform usual self care but limited vocational activities

Class4=limited in ability to perform self care, vocational and avocational activities.

A chronic rheumatic disease often leads to irreversible joint destruction causing permanent loss of bone architecture.

2.2 Pathophysiology of RA

The etiology of rheumatoid arthritis remains unknown but insights into pathogenic pathways have been accumulated from previous two decades indicate role of main immune system player cells including monocytes, neutrophils, macrophages, endothelial cells, fibroblasts, T cells and B cells participate in inflammatory process. In RA, CD4+ T cells, B cells and macrophages cause bone marrow infiltration by acting in variety of ways (Figure 2.1).Macrophage and fibroblast-like synoviocytes markedly increase bone hyperplasia. Locally expressed matrix metalloproteinase, serine proteinases and aggrecanases digest extracellular matrix and destroy articular structure (Firestein, 2003)

Antigen associated with RA are probably presented by APCs to T cells to entail binding of antigenic peptides to class II MHC molecules and more than 80% patients of RA carry so called shared epitope of the HLA-DRB1*04 cluster (Gregersen *et al*, 1987).These disease associated HLA-DR alleles are believed to present arthritis –related peptides which cause stimulation and expansion of autoantigen-specific T cells in joints or lymph nodes or both (Smolen *et al*, 2007)

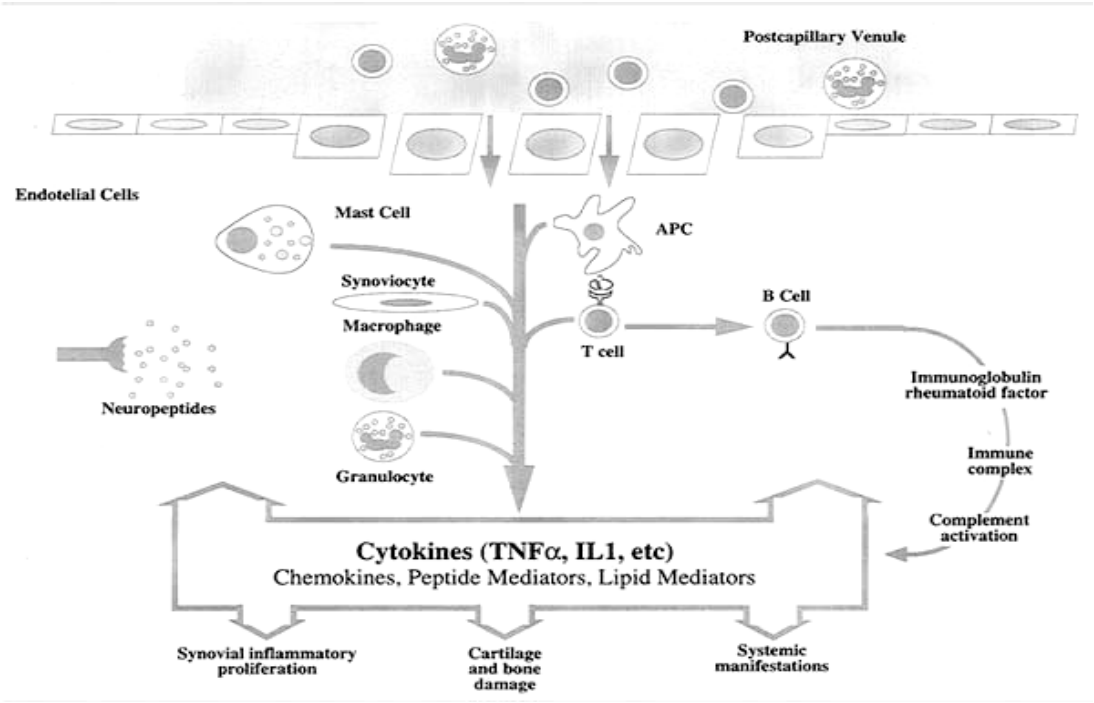


Figure 2.1 Cells involved in the immunopathogenesis of RA. Endothelial cells assume the phenotype of "high endothelial venules". Recirculating CD4+ T cells migrate into the synovium and play a crucial role in initiating the autoimmune response. Activated cells secrete cytokines and mediators which cause signs and symptoms of disease and contribute to the inflammatory self-perpetuating processes (Adopted from Afeltra, 2001)

2.2.1 Role of B cells in pathophysiology of RA

The role of B cells in RA is now well established from previous research, suggesting B cells to contribute the disease in variety of ways. B cells are involved in autoantibody production and autoantigen processing and help in the inflammatory cascade by producing different cytokines (interleukin 1,6 and 10, TNF-α and lymphotoxin α/β) and chemokines among which TNF and IL1 enhance production of IL-6 and IL-10 which stimulate B cell function via a feedback loop therefore exacerbate chronic inflammation . Activated B cells express co-stimulatory molecules such as CD154 and contribute in evaluation of T effector cells and also support activating autoreactive T cells by expressing costimulatory such as CD80/CD86 and CD40 molecule. As shown in Figure 2.2, the overall activity of B cells is observed to be enhanced in RA and they are found in synovium where they form complexes with T cells and develop tertiary lymphoid tissue structures (Kim *et al*, 2000).B cells also take part in antigen driven specific immune responses in ectopic lymphoid tissues and differentiating into plasma cells. There is evidence that chronic autoimmune disease causes changes in the repertoire of specific antigen receptors. Disturbance in B cell receptor editing and central or peripheral tolerance machinery additionally contribute to abnormal B-cell activation and proliferation (Hansen *et al*, 2007; Dörner, 2006; Tsoko *et al*, 2004).

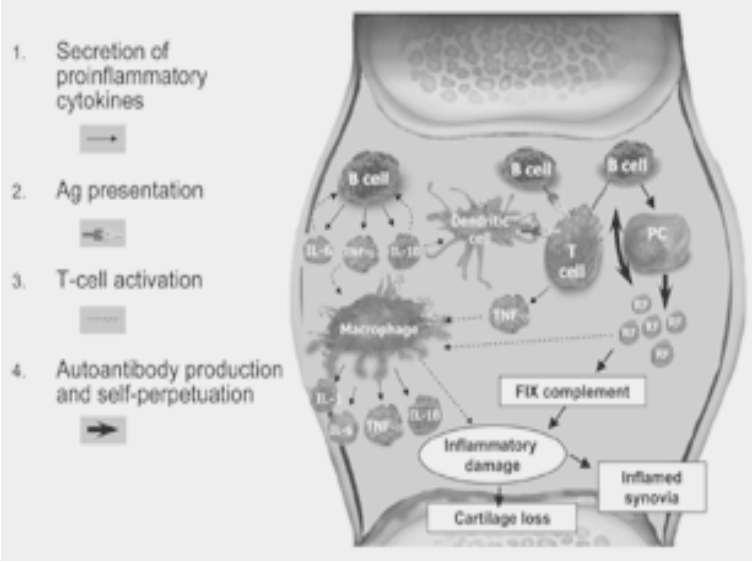


Figure 2.2 Potential roles of B cells in the immunopathgensis of RA: Different mechanistic approach by B cells in cartilage loss (adopted from Dörner, 2006)

2.3 Therapeutic Targets in RA

Therapy to autoimmune diseases is a challenging task and none of the available drugs for rheumatoid arthritis can not cure the disease but only offer means to control flares and suppress symptoms and help to prevent disease at certain extent. Unfortunately, short or longterm intake of these medications exerts some adverse effects. The drugs selection and efficacy depend on personal responses and severity of disease manifestations. Traditionally, rheumatoid arthritis has been treated with anti-inflammatory non-steroidal drugs, glucocorticoids and disease-modifying antirheumatic drugs. Therapeutic options for rheumatoid arthritis have tremendous increase in past decade with the introduction of biological agents. A large variety for cellular and cytokine targets have been identified with specific inhibitors being approved to treat (Bingham, 2008).

To cope with mild RA, among routinely used drugs include glucocorticoids and disease modifying antirheumatic drugs. Glucocorticoids drugs include *prednisolone, dexamethasone and hydrocortisone*. Glucocorticoid is powerful drug with capability to suppress both inflammatory and immune responses and help in halting destructive disease processes (Weinblatt, 2003; Vallerskog, 2007).Unwanted effects of these drugs could include osteoporosis, suppression of immune reactions against infections and iatrogenic Cushing's syndrome.

Disease modifying antirheumatic drugs (DMARD)

The most commonly used small molecule disease modifying antirheumatic drug is *methotrexate* which is believed to be a cornerstone of most treatment regimes in rheumatoid arthritis with highest retentions rate as compared with other agents (Weinblatt, 1995). Methotrexate is a folate antagonist being a metabolite disturbing the DNA synthesis and cell division.

Alternative drugs are *azathioprine* and *cyclophosphamide*. All these drugs effect cellular proliferation, so unwanted effects could be usual outcome in shapes of anaemia, and decreased immune responses. So longterm and devoted research in the field of drug development offered different biological agents which proved to be very efficient in autoimmune diseases.

2.3.1 Biologics in RA

Now a days a large variety of biological agents are available in treatment of RA and many are still under clinical trials and yet to be approved for treatment.

1) Anti TNF-α therapy

As TNF is involved in enhancing inflammation it was chosen as targeting therapy to block and ultimately reduce TNF in blood. Three TNF antagonists are currently approved for treating RA; 1) Adalimumab (ADA), a fully human monoclonal antibody against TNF-α. 2) Etanercept (ETN) the TNF receptor (p75): FcIgG construct.3) Infliximab (IFX), a chimeric monoclonal antibody. Major difference in these available compounds is their methods of delivery and frequency of administration. Two newly introduced compounds which also inhibit TNF are in late stages of clinical development, Certolizumab (CZP) and Golimumab (GLM). Certolizumab pegol is construct of 'Fab' of humanized monoclonal anti-TNF antibody bound with polyethylene glycol (PEG) that do not interfere with TNF binding properties while Golimumab is fully human monoclonal antibody to TNF(Bingham,2008).

Most of these drugs have been shown to be effective in combination with disease modifying drugs like methotrexate (Smolen *et al*, 2003; Smolen *et al*, 2007).

2) IL-6 receptor inhibition

An alternative target for treatment of RA is use of cytokine receptor inhibitions which are involved in inflammatory cascade. So different cytokines including IL-6, IL-1, IL-15 and RANKL have been chosen as suitable therapeutic targets. Tocilizumab is a humanised anti IL-6 R monoclonal antibody which binds with soluble and membrane-expressed IL-6 R preventing IL-6 mediated proinflammatory activity (Emery *et al*, 2008,Genovese *et al*,2008). Tocilizumab has been approved in February 2009 for the treatment of active RA inadequately treated with at least methotrexate.

Pleiotropic cytokine IL-6 blockade is shown to be beneficial in rheumatic diseases. IL-6 is not only stimulator of B and T cells functions but also promotes maturation of plasmablasts during their final maturation stage as well as triggering immune system in playing pathogenic role development of inflammatory manifestation in RA(Lipsky,2006).

3) CTLA-4 Ig molecule

CTLA-4 is a molecule that can out-compete CD28 for ligation of the B7 molecule. Its affinity for the B7 is 10-20 times greater than that of CD28. Inhibition of CD28 mediated co-stimulation signals is a potent means of immunosuppression that can be achieved by blocking either CD28 or CD80 and CD86. So biologically these differences in affinity result in limitation and subsequently down regulation in T cell responses. Abatacept (CTLA-4 Ig) is the first in a new class of drugs targeted at T-cell function in autoimmune disease: the co-stimulation blockers. In Clinical trials, Abatacept has favorable effects in patients with psoriasis vulgaris and in patients with RA. This drug was also being used for avoiding graft rejection after organ transplantation. It has shown safety and efficacy in rheumatoid arthritis and in December 2005 FDA approved Abatacept for treatment of rheumatoid arthritis (Acuto *et al*, 2003; Malmström *et al*, 2005).

2.4 B cell targeted therapies in RA

1) Anti CD20 molecule

CD20 molecule is a B cell surface marker expressed on major B cells populations from pre-B cell stage to memory B cells except pro-B cells and plasma cells. CD20 is 33-37kd membrane associated phosphoprotein which functions as a calcium channel subunit. Due to this property of CD20 molecule, it affects B cell activation; differentiation and cell cycle progression from G1 to S phase (Silverman *et al*, 2003).

Rituximab is a chimeric anti CD20 monoclonal antibody and have been effective in treating patients with RA and other autoimmune diseases (Keystone, 2005). Rituximab has shown promising clinical results and suitable treatment for RA patients refractory to therapy with tumour necrosis factor blockers (Leandro *et al*, 2002; Edwards *et al*, 2004)

2) Anti CD22 molecule

CD22 is a membrane protein expressed on B cell surface during maturation stages of differentiation and appears to upregulate the B-cell receptor and serves as an adhesion molecule. CD22 deficient mice study suggests its key role in B cell development and survival. Epratuzumab is a humanized antiCD22 monoclonal antibody. The results from open label study showed that Epratuzumab may be effective and safe in patients with moderately active SLE (Dörner *et al*, 2006). Currently Epratuzumab is under clinical trials for studies in SLE, NHL, lymphoma, B cell NHL and leukemia (Hawker, 2008)

3) Anti BAFF

BAFF (also known as BlyS) is critically involved in B cell maturation and development. Several lines of evidence implicate BAFF signalling in autoimmunity because autoantigen binding B cells may have increased dependency on it and circulating level of increased soluble BAFF are increased in autoimmune diseases such as rheumatoid arthritis (Cheema *et al*,2001), SLE (Zhang *et al*,2001) and Sjögren's disease (Mariette *et al,*2003). So inhibition of BAFF signalling is therapeutic option in B cell medicated diseases. Belimumab is anti-BAFF monoclonal antibody and during clinical trials have shown biological activity in humans including moderate decrease in peripheral blood B cells (Vugmeyster *et al*, 2005; Wei *et al*, 2007)

2.5 Transient B cell depletion by anti-CD20 antibody Rituximab

Rituximab is a chimeric monoclonal antibody which is a construct of human IgG1 and Kappa constant regions and of mouse variable regions from a hybridoma directed at human CD20. Rituximab was developed to treat non-Hodgkin lymphomas but was also approved for treatment of rheumatoid arthritis in 2006. It selectively depletes $CD20^+$B cells from peripheral blood, secondary lymphoid organs and bone marrow. Only pro-B cells and plasma cells are spared as they do not express CD20 molecule (Rouziere *et al*, 2005; Silverman, 2003; Silverman *et al*, 2007)

Rituximab follows four possible mechanisms to deplete B cells from RA patients which are summarized in Figure 2.3.

1) **Antibody dependent cellular cytotoxicity**

Anti CD20 antibody may permit antibody dependent cell mediated cytotoxicity which occurs after recognition of Fc portion of the antibody by appropriate receptors on cytotoxic cells.

2) **Complement dependent cytotoxicity**

It occurs after binding of antibody to extracellular domain of CD20 antigen resulting complement activation and lyse the targeted cell.

3) **Altering B cells response**

Another possible way is to alter the ability of B cells to respond to antigen or other stimuli.

4) **Apoptosis**

Anti CD20 may initiate programmed cell death by shifting CD20 into a lipid-raft environment thereby altering calcium flux and inducing apoptotic events.

Fc receptor mediate cytotoxicity is prominent in B cell destruction in peripheral blood whereas complement activation kills B cells in lymphoid organs (Tsokos, 2004; Browning, 2006)

Rituximab has been investigated intensively in wide range of autoimmune diseases including rheumatoid arthritis, SLE, Sjögren's disease, idiopathic thrombocytopenia, IgM-mediated polyneuropathy, Factor VIII deficiency, inflammatory myositis and ANCA associated vasculitis (Cohen, 2005; Edwards *et al*, 2006; Browning, 2006; Martin *et al*, 2006; Keogh *et al*, 2006)

Rituximab results in selective loss of virtually all $CD20^+$ B cells in the periphery and in the majority of patients this level remains greatly depressed with very few detectable $CD19^+$ cells for 4-5 months after single dose of rituximab. The extent of depletion is variable in different B cell subsets. Regeneration data have revealed that the peripheral B cell

repopulate with newly emerging populations leading to a new naïve B cell pool (Leandro *et al*, 2002; Keystone, 2005; Roll et al, 2006).

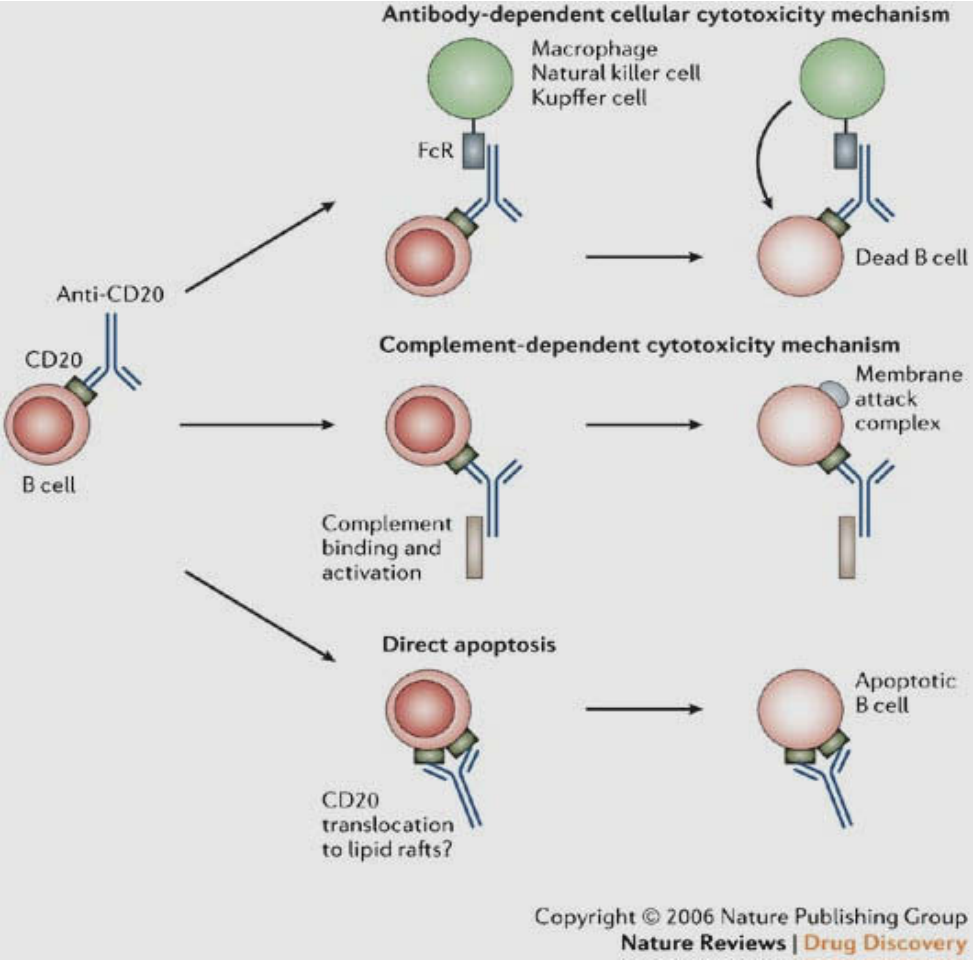


Figure 2.3 Mechanism of action of anti-CD20 antibodies: B cells coated with anti-CD20 molecule are killed by different mechanisms (adopted from Browning, 2006)

Objectives of Project

The efficacy of therapeutic B cell depletion using rituximab has gained specific interest in understanding the role of B cells in autoimmune diseases like RA. Autoimmunity is characterized by significant changes in the lymphoid system. Regarding the B cell system there is evidence for a shift in the expressed B cell repertoire of specific B cell receptors. Disturbance in B cell receptor editing and central or peripheral tolerance contribute to abnormal B cell activation in chronic immune mediated diseases (Leandro *et al*, 2006; Silverman, 2006). Therefore resetting the immune system by transient B cell depletion seems to be an interesting goal. Rituximab, a B cell depleting anti-CD20 antibody has shown profound efficacy and promising results in recent years. Rituximab holds good safety record in all studies except for mild infusion reactions.

Intravenous application of 2x1g anti-CD20 antibodies (Rituximab) results in selective loss of vast majority (>97%) of B cells expressing CD20 molecule from peripheral blood and secondary lymphoid organs. B cell numbers in blood remain low for about 6-12 months (Anolik *et al*, 2004). Clinical efficacy of rituximab in treating RA patients has been well documented in clinical trials (Edwards *et al*, 2004; Cohen *et al*, 2006; Emery *et al*, 2006). As plasma cells being CD20 negative are not depleted during rituximab therapy therefore serum immunoglobulin levels derived from long lived plasma cells are maintained (Browning, 2006). Disease activity score and RF levels have been reported to be reduced in an open label study with RA patients (Kneitz *et al*, 2004). In another study increased BAFF level after rituximab therapy have been observed(Vallerskog *et al,* 2006) along with other serological changes including reduction in IgA-RF, IgG-RF and anti-CCP antibodies have been documented(Cambridge *et al*,2003;2006).

During regeneration phase, B cells showed a characteristic pattern in peripheral blood with elevated numbers of immature B cells which differentiate into mature/naïve B cells and dominate during later time points (Leandro *et al*, 2006; Roll *et al*, 2006). Repopulation data have revealed a delayed recovery of memory B cells after rituximab, particularly of pre-switched (IgD^+CD27^+) memory B cells studied over a period of two years (Roll *et al*,2006). Memory B cells seem to be particularly important targets for treatment since disease driving cells seem to be part of this populations. Regeneration of pre-switched memory B cells could be correlated with response and non response to rituximab therapy in RA (Roll *et al*, 2008). In this study good responders were shown to have diminished recovery of IgD^+ memory B cells

as compaired to non responders who recoverd these cells more numerously. In addition, patients with lower number of memory B cells prior to treatment had significantly more durable response to rituximab.

Memory B cells are also associated with additional disease conditions. An overall memory B cell deficiency is observed in patients with graft-versus-host disease after allogeneic hemopoietic stem cell transplantation where distribution of B, T and natural killer cell numbers is normal (Greinix *et al,*2008). Similar findings were observed in patients with common variable immunodeficiency (Agematsu *et al*, 2002). A skewed distribution of peripheral memory B cells is also observed in patients with Sjögren's disease (Hansen *et al*, 2002) and SLE (Odendahl *et al,* 2000). These observations formed the basis of studying memory B cells after B cell depletion therapy in this thesis.

A precise role of memory B cells in autoimmunity still remains unclear. Since, IgD^+ memory cells are particularly susceptible to rituximab therapy and their extent of repletion is connected to clinical efficacy in treated patients, we undertook a detailed molecular analysis and comparison of memory B cells and particularly IgD^+ memory B cells on the single cell level in patients who underwent rituximab mediated B cell depletion. We prospectively analyzed Ig-VH_3 gene rearrangement of B cell subsets with regard to their mutational pattern and frequency before B cell depletion and during repletion phase over a period of six years. The mutational pattern of memory B cell subsets was also compared with patients who underwent allogeneic and autologous stem cell transplantation (SCT). In addition to the study of variable genes, Ig receptors of class switched memory B cells were analyzed for expression of IgG and IgA during rituximab mediated B cell depletion therapy.

Aims of study

The goal of the thesis was to study the longterm effect of Rituximab on the memory B cell pool. We addressed mainly the following questions

- ***Regeneration pattern of memory B cells after rituximab therapy.***
- ***Acquisition of B cell receptor (IgR) mutations in IgD^+ memory B cells after rituximab mediated B cell depletion***
- ***Development of mutationally class-switched Ig Receptors during regeneration***
- ***Detection of IgG and IgA specific $CD27^+$ memory B cells before and after anti-CD20 antibody treatment***
- ***Acquisition of IgR mutations in memory B cells after Allogeneic and autologous stem cell transplantation.***

3. Patients, Materials and Methods

3.1 Patients

18 Patients with a median age 52years (31-67years) diagnosed with active rheumatoid arthritis were included in study of monoclonal anti-CD20 antibody (rituximab) treatment. The patients were refractory to previous treatment with Disease Modifying Anti-Rheumatic Drugs (DMARD) including methotrexate and /or TNF-α-antagonists. All Patients met the ACR (American College of Rheumatology) revised criteria for RA diagnosis. They were on stable doses of methotrexate before treatment with 1000 mg rituximab (Mabthera ®, Hoffmann La – Roche, Grenzach – Whylen, Germany) twice, two weeks apart. They stayed on standard methotrexate therapy during the whole study.

Informed consent was obtained from patients and normal donors according to the protocol approved by ethical committee of University of Würzburg, Germany. 16 patients were studied for pre-switched memory ($CD19^+CD27^+IgD^+$) B cells at single cell level and amplified Ig-VH3 family gene rearrangements by using single sorting technique followed by nested RT-PCR approach. Patients were studied at baseline prior to initiation of rituximab in 7 patients and categorically during 6 years post-treatment B cell repletion phase in 9 patients Three patients were studied prospectively before and one year after RTX. For disease control, one patient with SLE after RTX treatment, 2 patients one year after autologous stem cell transplantation and 3 patients 1 year after allogeneic stem cell transplantation (ASCT) were studied for comparison. Among allogeneic SCT patients, two patients were suffered from acute myeloid leukemia and one with myelodysplastic syndrome. Conditioning regimen included TBI (12 Gy) with cyclophosphamide (60 mg/kg x 2) in 2 patients or fludarabin (30 mg/ m^2) and melphalan (75 mg/m^2) in 1 patient. GvHD prophylaxis was done with cyclosporine A and mycophenolate mofetil. As control, 4 healthy donors were analyzed. On the otherhand, 2 RA patients and 2 ASCT patients were studied for class-switched memory ($CD19^+CD27^+IgD^-$) B cells prior and 1 year post-treatment period.

To observe rituximab mediated changes in Isotype switching, Post-switched memory B cells were studied in 2 patients before and 1 year after rituximab therapy to make Isotype (IgG and IgA production) analysis during treatment.

3.2 Peripheral blood mononuclear cells (PBMCs) preparation

Peripheral blood mononuclear cells were prepared from peripheral blood (60ml) by ficoll-paque plus separation (GE Healthcare, Munich, Germany).

Equal volumes (10ml) of blood were diluted with isotonic solution (NaCl) and were laid over 20ml of Ficol followed by centrifugation at 900g for 25min without brake. As a result, differential layers are formed based on cellular density having red blood cells at the bottom and whitish ring like layer rich in PBMCs between fluids which is separated and washed twice with RPMI 1640 medium containing penicillin-streptomycin and L-glutamine with centrifugation speed 1300g for 10min.PBMCs were counted microscopically stained with trypan blue on a Neubar chamber. Finally PBMCs were suspended in PBS+0.5% BSA buffer and put on ice till further use.

3.3 Flow cytometry

Before single cell sorting, Immunoflourescence labelling for flow cytometry was performed for differentiating different memory B cell subpopulations by incubating PBMCs with a variety of Immunoflourescence labelled antibodies. These were APC-labelled anti-CD19, FITC-labelled anti-IgD, APC labelled anti-CD23, PE-labelled anti-CD27, APC labelled anti-CD21 and PerCP-labelled anti-IgM (Becton Dickinson, San Jose, CA., USA). Cell incubation with antibodies was performed at 4°C for 15 minutes in PBS/0.5%BSA followed by subsequent washing. Data acquisition and flow cytometric analysis was performed by FACSCalibur flow cytometer using Cell Quest software (Beckton Dickinson, San Jose, CA).

3.4 Cellular preparation for single cell sorting

Memory B cell subpopulations are easily distinguishable between pre and post switched memory B cells on the basis of IgD surface expression. So for single B cell sorting we stained our cells as described in above section by using RNAase free sterile tubes and following 3 fluorescence colours were used: APC labelled CD19, PE labelled CD27 and FITC labelled IgD. Finally cells were filtered by using MACS filter columns and put on ice until sorted.

3.5 Single Cell sorting

3.5.1 Lower reaction mix

A lower reaction mix which is cocktail of different ingredients(shown in table below) is prepared and put into 96 well plate which would be used for sorting. Low reaction mix is a lysis buffer.

Titan One Tube RT-PCR kit and Oligo dT15 was purchased from Roche, RNAsin from Promega while BSA and Triton were purchased from Sigma Aldrich.

To each of 96 well, 30µl is added and plate is put on ice until sorting proceeds.

Lower reaction mix: Volume/well 30 µl

Reagents	Volume µl (100 tests)	Final conc. /well
• Triton X (10%)	2145µl	5mM
• Oligo DT(800 ng/ul)	500 µl	20U
• BSA(10ug/ul)	50 µl	1%
• RNAsin(40U/ul)	5 µl	400ng
• DTT(100mM)	50 µl	0.5ug
• RNAse free H_20	250 µl	

3.5.2 Sorting

Individual B cell sorting was carried out in a FACSDiVa (Beckton Dickinson, San Jose, CA) cell sorter which was outfitted with an automated cell deposition unit which provides 99.9% population purity. Pre and post switched memory B cells were sorted individually into each of 96 well plate. After sorting plate was spun for a very short time and cell were lysed in lysis buffer. During lysis action the B cell is lysed and mRNA comes out of cell.

3.5.3 Upper reaction mix

Upper reaction mix, which is a combination of RT-PCR buffer, dNTPs and reverse transcriptase enzyme in water, added to the sorted B cells. The composition of upper reaction mix is shown in table below.

Reverse transcriptase and RT-PCR buffer was provided in Titan One Tube RT-PCR kit and dNTPs were purchased from PeqLab.

Upper reaction mix: Volume/well 20 µl

Reagents	Volume µl (100 tests)	Final conc. /well
• H_20	800 µl	
• RT-PCR buffer (5x contains Mg)	1000 µl	1x
• Reverse transcriptase (20U/µl)	100 µl	20U
• dNTPs(10mM)	100 µl	0.2mM

3.5.4 cDNA synthesis

The plate was incubated at 50ºC for 1 hour and cDNA is prepared in this stage from mRNA by reverse transcriptase reaction. Plate was stored at 4ºC for further use and could be frozen at -20ºC for 3-4 years stability.

3.6 Amplification of Ig-VH3 by nested PCR

Nested RT-PCR approach was employed for the amplification of VDJ rearrangements of Ig-V_H3 gene from cDNA plates after single sorting. Primer details of Ig-V_H3 family were followed as described previously (Brezinschek *et al*, 1995; Ruzickova *et al*, 2002).The V and J specific primers were synthesized by MWG Biotech AG.

Primer sequences are detailed below. All primers are from '5' to '3'.

3.6.1 Oligonucleotide Sequences

Heavy Chains-V_HDJ_H rearrangements

3.6.2 External Primers

VH3 E– CCATGGAGTTTGGGCTGAGC

JH1, 2, 4, 5 E – TGA GGA GAC GGT GAC CAG GGT

JH3 E – TAC CTG AAG AGA CGG TGA CC

JH6 E – ACC TGA GGA GAC GGT GAC C

3.6.3 Internal Primers

VH3 N– GAGGTKCAGCTGGTGGAGTCTGGGGG

JH1, 3, 4, 5 N – CGA CGG TGA CCA GGG TBC CYT GGC C

JH2 N – CGA CAG TGA CCA GGG TGC CAC GGC C

JH6 N – CGA CGG TGA CCG TGG TCC CTT GCC

IUB codes used in primers correspond to following mixes K=GT, Y=CT, B=GTC

3.7 PCR amplification rounds

PCR amplification was performed on geneAmp PCR system 9700(Perkin Elmer)

3.7.1 External amplification round

External primers were used to Amplify VDJ rearrangement from cDNA which was performed by making master PCR mix with following ingredients:
Primers concentration was 100 pmol/µl

Lower Reaction mix Volume/well 25 µl

Reagents	**volumes**
dH_2O	1428µl
MgCl2	800 µl
dNTPs	160 µl
VH3 (E)	56 µl
JH mix E (1,2, 3, 4, 5,6)	56 µl
(JH mix =40 µl JH1, 2,4,5E +10 µl JH3E + 10 µl JH6E)	

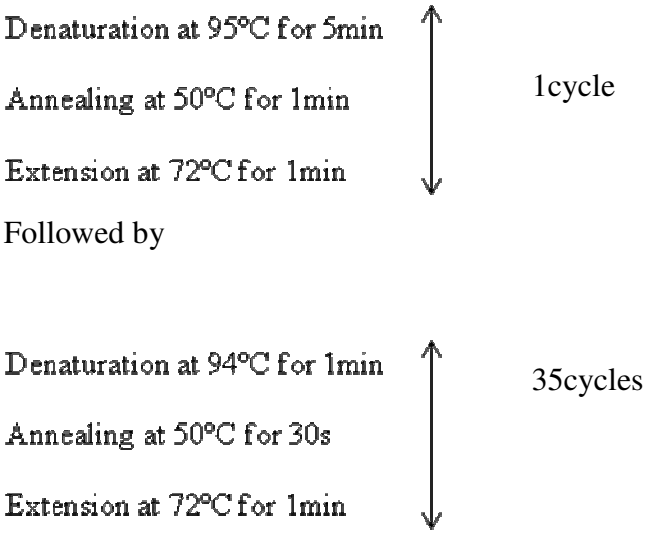
Upper Reaction mix Volume/well 50 µl

Reagents	**volumes**
dH_2O	4150 µl
10x buffer II	800 µl
TAQ polymerase	50 µl

5 µl cDNA template was adding to each of 96 well plate.
The AmpliTaq polymerase, 10X buffer II and $MgCl_2$ were purchased from Applied Biosystems.

External PCR Run

The amplification temperatures and cycles were as following:



Followed by a final extension at 72ºC for 5min and stored at 4ºC to process for internal amplification round.

3.7.2 Internal amplification round

Internal/Nested PCR Run

The nested PCR was performed in identical manner as in external amplification round except following changes.

- Nested primers were used as described in section 3.6.3.
- The template used was 5ul of external PCR product.
- Annealing temperature was 65ºC used to yield highly specific Ig-VH3 products.
- Numbers of cycles were reduced to 30.

Amplified DNA of Ig-VH3 family was stored at 4ºC until further use as described in section 3.10.

3.8 Primers used in Isotype studies

Nested PCR approach was also employed for amplification of IgG, IgA and IgM producing specific B cells by using Isotype α/μ/g specific primers. Beauty of this technique is to amplify all Ig-V_H families from V_H1-V_H6 by combining all family specific primers in same PCR amplification instead of amplifying all families separately. We mainly used this technique to categorically separate IgG and IgA producing B cells in Post-switched memory B cell compartment during rituximab therapy.

Set of Ig-V_H primers used in external and internal PCR rounds are described below. All primers were synthesized by Metabion AG.

3.8.1 External Primers

Following external primers were used:

VH1 LC: CCA TGG ACT GGA CCT GGA

VH4 LC: ATG AAA CAC CTG TGG TTC TT

VH6 LC: ATG TCT GTC TCC TTC CTC AT

VH2 LC: ATG GAC ACA CTT TGCTMC AC

VH3 LC: CC ATG GAG TTT GGG CTG AGC

VH5 LC : ATG GGG TCA ACC GCC ATC CT

Ecμ: TCA GGA CTG ATG GGA AGC CC

Ecg : CAG GCC GCT GGT CAG AGC G

Eca: GGA AGA AGC CCT GGA CCA GGC

Isotype specific primers were used separately in combination with all V_H primers.

3.8.2 Internal Primers

Detailed sequences of all primers used are described below.

VH3 FM: GAG GTG CAG CTG GTG GAG TCT GG

VH4 FM: CAG GTG CAG CTG CAG GAG TCG GG

VH6 FM: CAG GTA CAG CTG CAG CAG TCA GG

JH Mco: CTT GGT GGA RGC TGA RGA GAC GGT GAC C

VH1 FM: GAG GTG CAG CTG GTG CAG TCT GG

VH5 FM: ACC AGG CAG GCG ATG ACC AC

VH2 FM: CAG GTC ACC TTG AAG GAG TCT GG

EcaN: ACC AGG CAG GCG ATG ACC AC

V_H internal primers in combination with J_HMco were used in internal PCR amplification except in case of IgA internal PCR α internal primer was used instead of J_HMco.

3.9 PCR amplification rounds in Isotype studies

Nested PCR amplification rounds were carried out in similar way as in Ig-VH3 amplification with some difference described below.

3.9.1 External PCR run

External PCR run was carried out by using following master mix.

Primers concentration was 100 pmol/µl

Lower Reaction mix Volume/well 25 µl

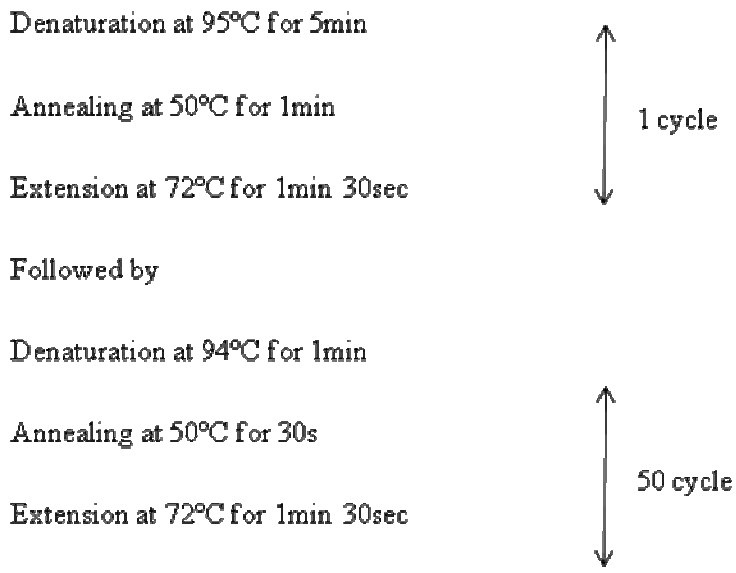
Reagents	**volumes**
dH_2O	1148µl
$MgCl_2$	800 µl
dNTPs (10mM)	160 µl
Ecµ/Ecα/Ecg	56 µl
VH1LC	56 µl
VH2 LC	56 µl
VH3 LC	56 µl
VH4 LC	56 µl
VH5 LC	56 µl
VH6 LC	56 µl

Upper Reaction mix Volume/well 50 µl

Reagents	**volumes**
dH_2O	4150 µl
10x buffer II	800 µl
TAQ polymerase	50 µl

5 µl cDNA template was used in external PCR run.

The amplification temperatures and cycles were as following:



Followed by a final extension at 72ºC for 8min and stored at 4ºC to process for internal amplification round.

3.9.2 Internal/nested PCR run

Internal PCR was carried out in identical manner except that internal set of primers were used and few other modifications in PCR amplification programme.

- Internal primers were used as described in section 3.8.2
- Annealing temperatures was used 65ºC during amplification of IgG and IgM specific amplification while 55ºCfor IgA specific amplification
- External PCR product was used as template
- Numbers of cycles were reduced to 40.

Amplified DNA was put at 4ºC to be used in next step to visualize PCR specific products on Agarose gel and purification.

3.10Visulization of PCR product and DNA isolation from Agarose gel

All nested PCR products were run on 1.5% Ultra pure agarose electrophoresis gel(GIBCO,BRL) for an hour at 100V and visualized with ethidium bromide (0.5µg/ml, sigma) under UV light at wavelength of 254nm. As shown in Fig.3.1, positive bands (around 350bp) were selected for further purification and cut from agarose gel.

The products excision was carried out with a clean scalpel and purified by using MinElute Gel Extraction Kit (Qiagen) according to manufacturer's manual instructions. MinElute Gel extraction combines the convenience of spin-column technology having specific binding properties of a uniquely designed silica gel membrane. DNA absorbs to the membrane. These columns are designed to give high-end concentration of purified DNA fragments elution in water. Impurities are efficiently washed away.

The procedure includes dissolving agarose, binding DNA to silica membrane, washing with different buffers and finally extracting in 10µl water.

Figure 3.1 Agarose gel; showing 350bp region corresponding to Ig-V_H3 specific products.

3.11 Sequence Reaction

Purified PCR products were processed for sequence reaction prior to sequencing. Sequence reaction was performed by using ABI PRISM® BigDye™ Terminators v 3.0 Sequencing Ready Reaction Kit (Applied Biosystems).

This kit contains premixed deoxynucleotide triphosphates, AmpliTaq DNA polymerase, FS, dT*th* pyrophosphatase (a component in AmpliTaq DNA Polymerase, FS), dye terminators, magnesium chloride and buffers which provide a fluorescent-based cycle sequencing reaction.

Sequence reaction mixture

The sequence reaction mixture contains

4µl of ABI PRISM® BigDye™ Terminator

3.5µl of purified DNA template

0.5µl of paint pellet blue dye

11.5µl dH_2O

0.5 µl of sequencing primer (10pmol/µl)

In case of Ig-V_H3 sequence reaction the sequencing primer is nested V_H3 primer and in case of Isotype studies V_H1-V_H6 mixed primer was used at concentration 10pmol/µl.

The cycling parameters were

Annealing temperature 95°C for 5min	↑	1 cycle
Annealing at 50°C for 1min	↓	
Extension at 60°C for 1min		
Followed by		
Denaturation at 95°C for 30s	↑	25cycles
Annealing at 50°C for 1min	↓	
Extension at 60°C for 3min		

Followed by a final extension at 72ºC for 5min and stored at 4ºC

3.12 Purification of sequence reaction products

3.12.1 AutoSeq Column purification

Sequencing needs a highly purified DNA material so a lot of care is taken with PCR products after sequence reaction to extra purify them from different debries. So the PCR products were first purified by passing through the AutoSeq G50 columns (GE Healthcare).This process removes the excess dye-labelled dideoxynucleotides from the sequence reactions.

Procedure: First resin columns were vortexed gently and resin preservative was removed before spinning columns at 2000g for 1min followed by loading the PCR products at the centre of the angled surface of resin compact bed and columns were spun subsequently for 1min at 2000g to collect purified DNA in a separate eppendorf tube.

3.12.2 Isopropanol precipitation

A further purification of PCR products was carried out by precipitating with 75% Isopropanol. For this purpose 75µl of Isopropanol was added to total 25 µl column purified PCR product and put in room temperature for 15min after gentle mixing. After incubation, samples were centrifuged at maximum speed of 14000rpm for 20min.The supernatants were discarded and pellets were suspended again in 250 µl of 75% Isopropanol and centrifuged at same speed for 10min.The supernatant was discarded followed by pellets drying at 40ºC for 1-2 min after air-dry. These pellets were dissolved in 20 µl Template Suppression Reagent (Applied Biosystems),denatured at 95ºC for 3min and place on ice for 5min or until further processing.

3.13 An overview of Single B cells sorting

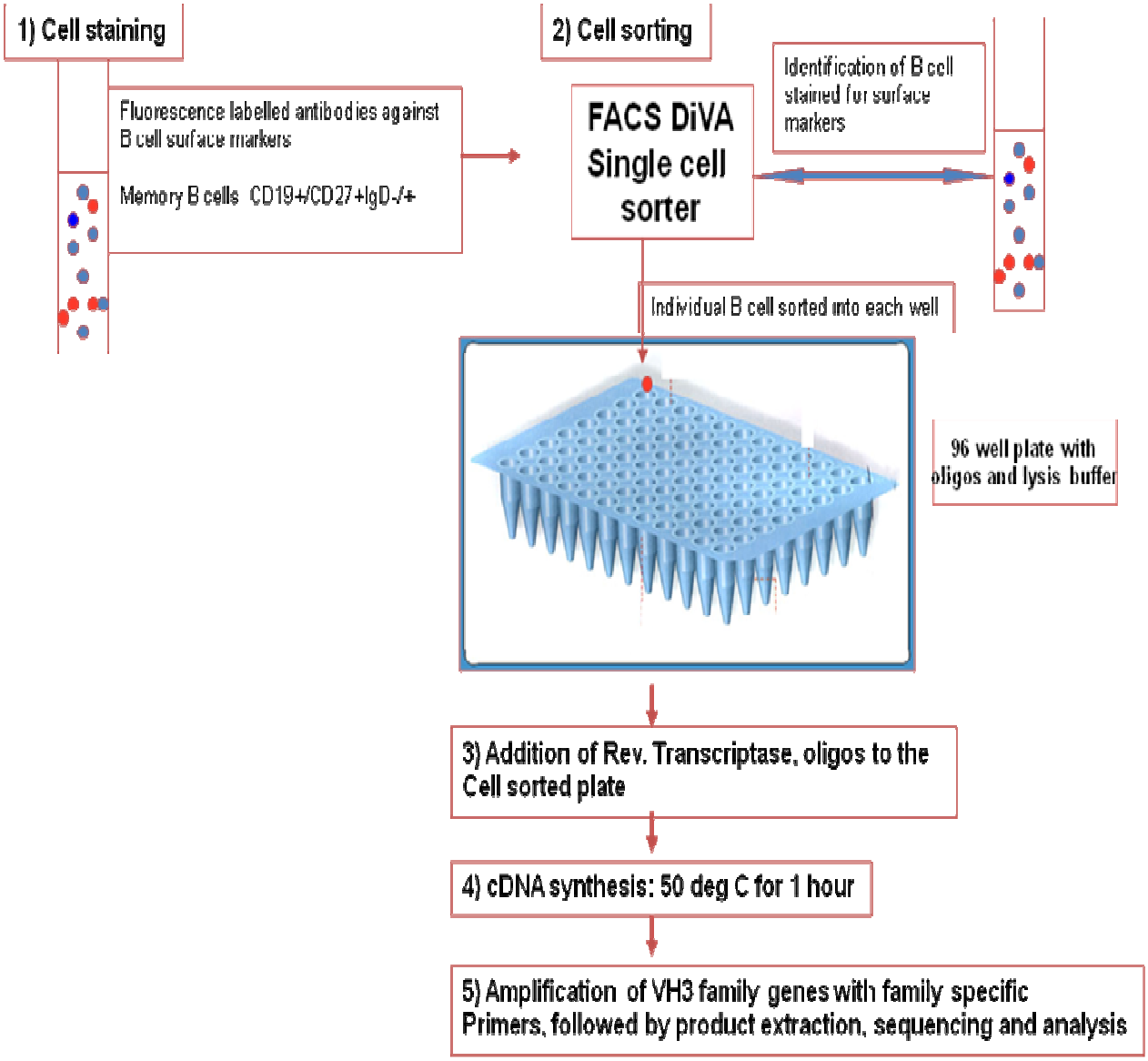


Figure 3.2 A schematic representation of single cell sorting technique

3.14 Sequencing

Finally samples were sequenced on an automated ABI PRISM 310 Genetic Analyser (Applied Biosystems).Sequencing was carried out by using POP6 Polymer reagent.

3.15 Sequence analysis of Ig sequences

Sequence analysis was carried out by using online web-based software programme JOINSOLVER (http://joinsolver.niams.nih.gov/index.htm) which was created by National Institute of Health (NIH), National institute of Arthritis and Musculoskeletal and Skin Disease (NIAM) and centre for information technology (CIT). It is used for human immunoglobulin V(D)J recombination, their nucleotide and amino acid alignment, extensive mutational analysis and CDR3 analysis and summarizes results in different forms.
Following parameters were analyzed in our study.

1. Mutational frequency

Total number of mutations in the variable region was determined during sequence analyzed by comparing the germline sequence. Mutational frequency was calculated by dividing the number of mutations by total nucleotides in the rearrangement, multiplied by 100.

2. VDJ segments usage

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

4. CDR3 length

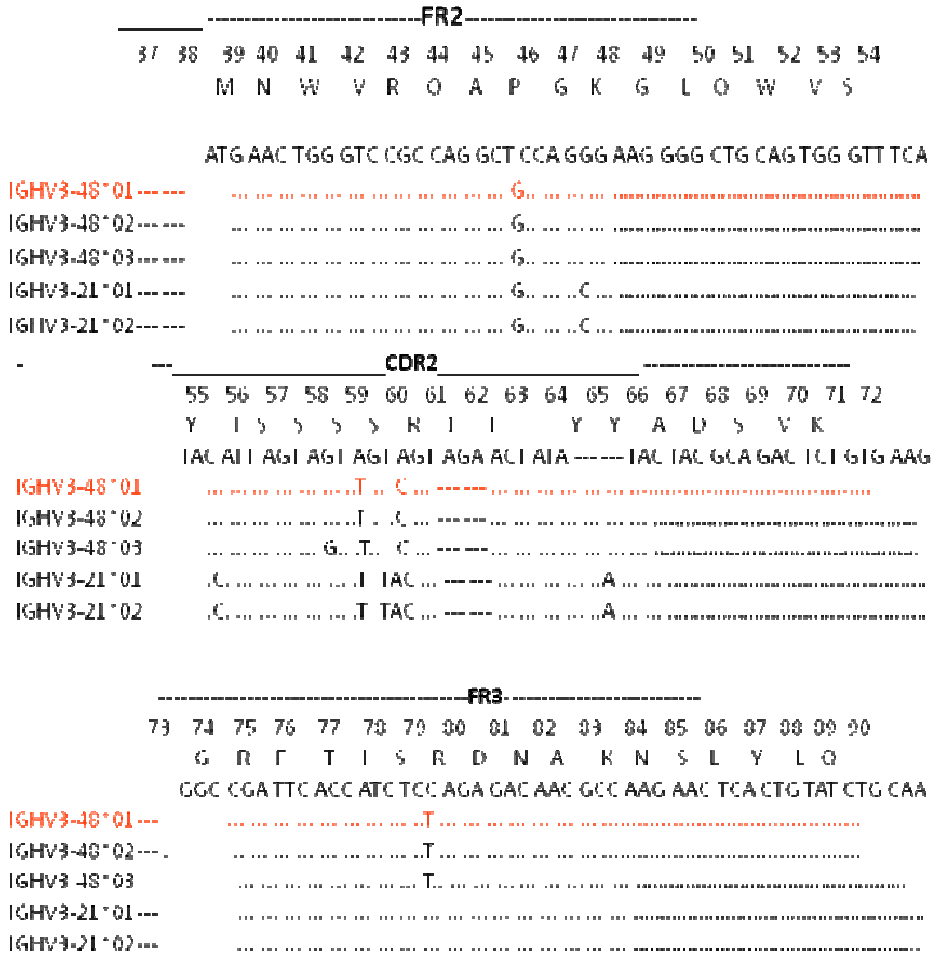
CDR3 region is actively participant of antigen binding. CDR3 length was calculated by determining number of nucleotides from residue 95-102. CDR3 was calculated by using Joinsolver only software.

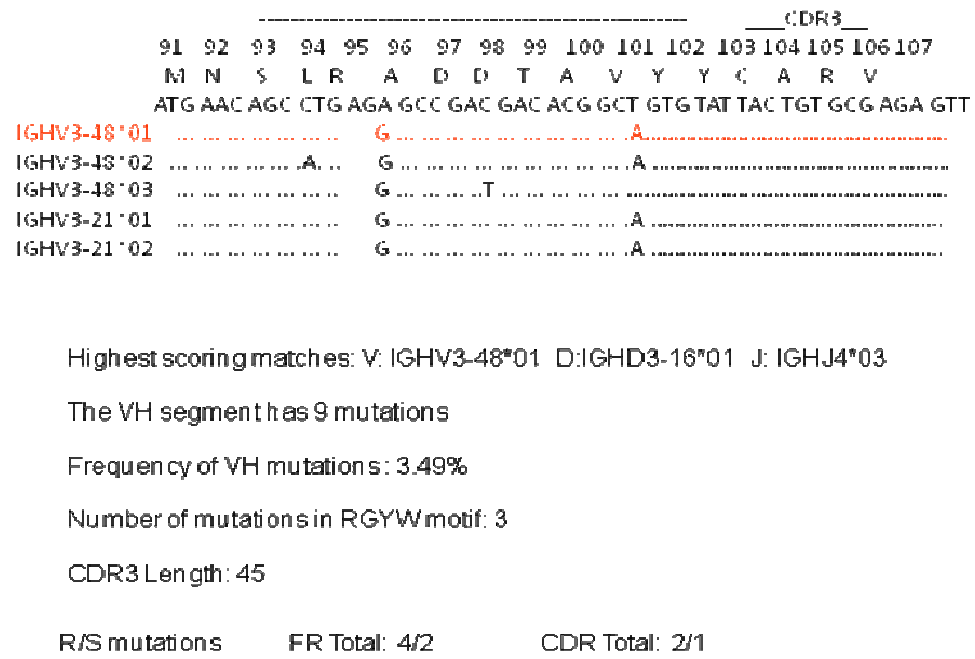
3. Mutations in RGYW and WRCY motifs

As shown by previous studies presence of predefined RGYW/WRCY (R = purine, Y= pyrimidine and W= A/T) mutational hotspot motifs (Dörner *et al*, 1998; Farner *et al*, 1999) which are believed to undergo somatic hypermutation particularly during T-B cell interactions

in germinal center. The number of mutations lying in the hotspot motifs was assessed and the percentage of RGYW/WRCY mutations was calculated.

An example of such results is shown down.





A representative sequence analyzed on Joinsolver showing FR/CDR regions and summarized results

3.16 Statistical analysis

Statistical analysis was performed by using GraphPad Prism 3.03 (GraphPad Software, San Diego, CA, USA). Analysis of FACS data, mutational differences and CDR3 length differences were calculated by non parametric Mann-Whitney U test. Mutational frequencies were calculated by using online GraphPad quick calculator (www.graphpad.com). *P* values <0.05 was considered to be significant.

3.17 Materials

Media and reagents

Ficoll-paque (Amersham Biosciences)

HBSS (Gibco BRL)

RPMI 1640(Sigma)

Trypan Blue (Merck)

Penicillin-streptomycin (Biochrom AG)

L-glutamine (Gibco BRL)

Kanamycin (USB Corporation)

Agarose gel

1.5% Agarose Ultrapure (Gibco BRL)

1X TAE buffer

0.5µg/ml ethidium bromide

Buffers

TAE electrophoresis buffer(50X)	242g Tris base 57.1ml glacial acetic acid 100 ml 0.5M EDTA(pH 8.0)
Loading dye(6X)	0.25% bromophenol blue 0.25% xylem cyanol 30% glycerol in water
LoTE(3mM Tris,0.2 mM EDTA)	20µl EDTA 0.5M pH 8 made upto 50ml with water
TE(10 mM Tris,1mM EDTA)	100µl EDTA 0.5 M pH8

500µl Tris 1M

Made upto 50ml with water

Sequencer

Genetic analyzer buffer (Applied Biosystems)

Pop-6 gel (Applied Biosystems)

4. Results

4.1 Identification of peripheral blood memory B cell subsets

Human peripheral blood B cells can be segregated into two populations on basis of surface expression of IgD and CD27 as follows: 1) a large population of $IgD^{+}CD27^{-}$ fraction represents mature naïve B cells(D4) and 2)$CD27^{+}$ memory population which can be separated into IgD^{+} memory B cells (pre/non-class switched memory)and IgD^{-} (class-switched memory) subsets. The FACS plot (Figure 4.1) below shows different memory subpopulations based on 3 colour staining showing D1 as class-switched memory B cells and D2 as pre-switched memory B cells.

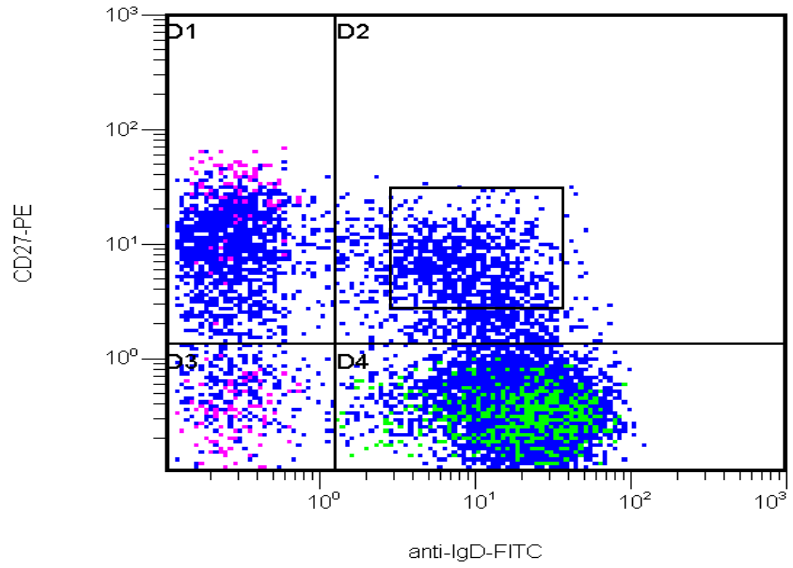
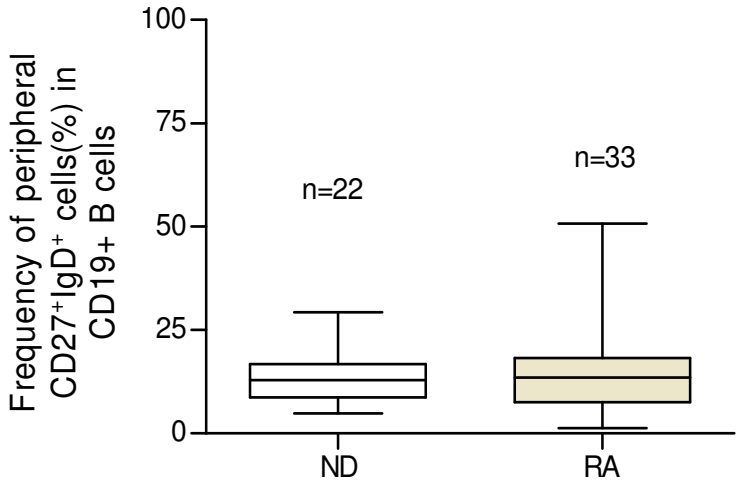


Figure 4.1 Different B cell subsets. Showing naïve B cells in D4 while pre and post-switched memory B cells in compartment D1 and D2 respectively.

4.2 Peripheral blood CD27$^+$ memory B cells in patients with RA

To assess any significant changes in memory B cells pool in patients with rheumatoid arthritis, we analysed memory B cell subpopulations in 33 patients with rheumatoid arthritis as well as in 22 normal donors. We find no significant differences between mean percentages of IgD$^+$CD27$^+$ memory populations in CD19$^+$B cell pool in patients with RA and ND (Figure 4.2a). %Mean CD27$^+$IgD$^-$ memory B cells population was observed 21.3±1.85% which were non-significantly increased in RA patients (Figure 4.2b) than normal donors 17.2±1.9% which confers a similarity in memory B cell pools in rheumatic patients and ND.

A)



B)

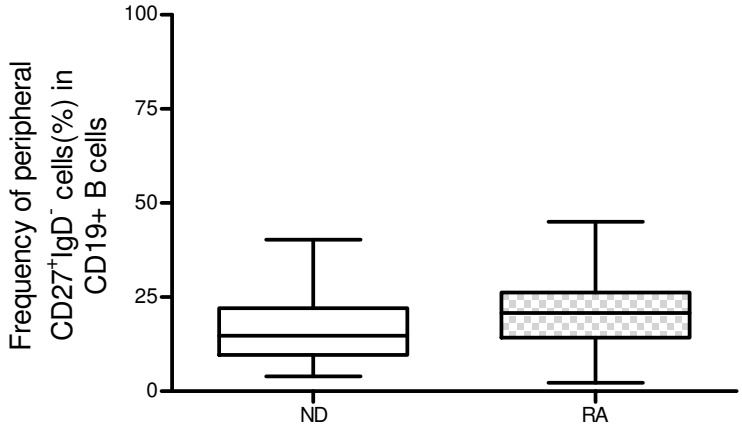


Figure 4.2 Frequency of pre and post-switched memory B cells. Peripheral CD19$^+$ gated comparative frequency of (A) IgD$^+$CD27$^+$ and (B) IgD$^-$CD27$^+$ memory B cells in patients with rheumatoid arthritis and healthy donors.

4.3 CD21 surface expression of IgD^+ memory B cells

Human splenic marginal zone B cells are distinguished from naïve and class-switched memory B cells on basis of different surface markers $IgM^{high}IgD^{low}CD21^{high}CD23^{low}$.Previous research has shown that a population of $CD27^+IgD^+$ memory B cells in peripheral blood represents circulating marginal zone B cells and these cells could also be recognized on basis of $CD21^+$, $CD23^-$ and CD1c ^{high} surface expression (Weller *et al*, 2004).

Therefore we addressed whether all $CD27^+IgD^+$ memory B cells belong to circulating marginal zone B cell subset on the basis of CD21 surface expression. We analyzed IgD^+CD27^+ B cells from 13 healthy donors and 22 RA patients for expression of CD21 surface molecule by 4 colour FACS staining and found majority (> 90%)of IgD^+CD27^+ B cells were $CD21^+$ and very small population was CD21 negative. There was no difference between RA patients and healthy donors.

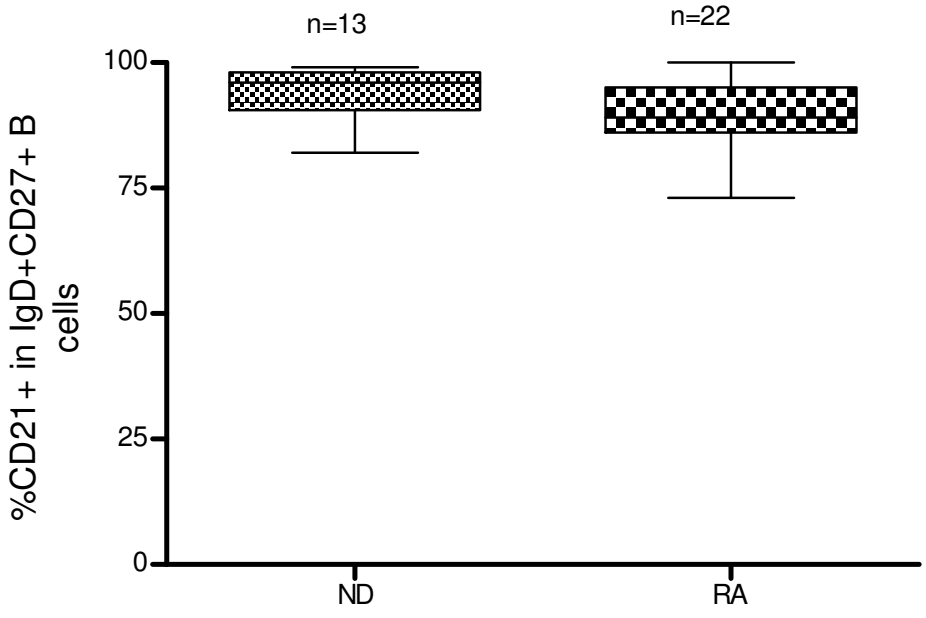


Figure 4.3 Percent expression of $CD21^{+/-}$ population. Normal donors and RA patients gated on IgD^+CD27^+ memory B cells.

4.4 Mutational pattern of $IgD^+CD27^+CD21^{+/-}$ memory B cells

As we found majority of IgD^+ memory B cells expressing CD21 surface marker, so addressed the question whether low and high mutated pattern of IgD^+ memory B cells could be segregated on the basis of CD21 expression as seen in healthy donors and RA patients. For this purpose, we sorted $IgD^+CD27^+CD21^{+/-}$ memory B subsets from a healthy donor and looked in Ig-V_H3 mutational pattern.

As shown in figure 4.4, no significant difference was observed in these populations on basis of mutational load in their IgR indicated highly homogenous population. These findings indicate that acquisition of mutations in IgD^+ memory B cells is not dependent on CD21 molecule.

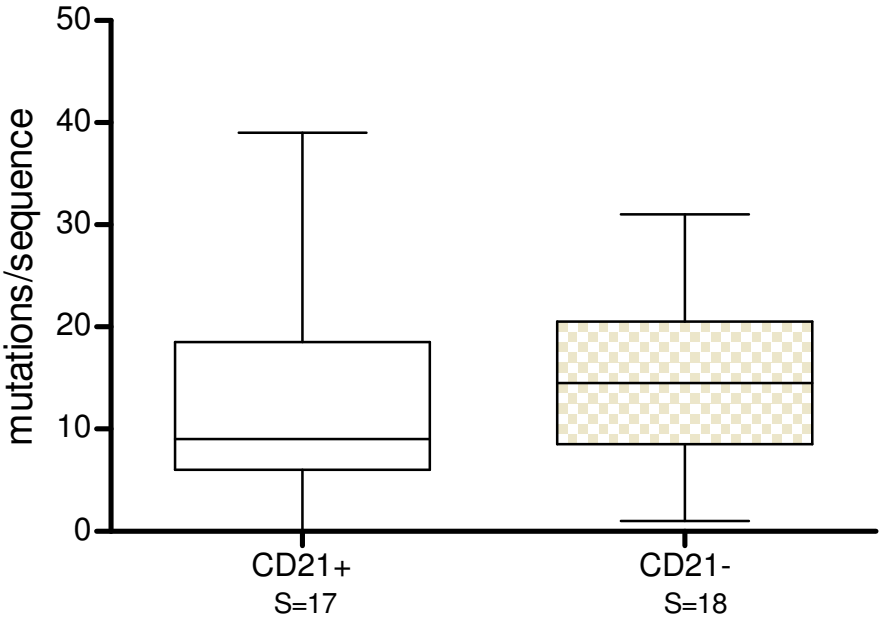


Figure 4.4 Mutational patterns of $IgD^+CD27^+CD21^{+/-}$ memory B cells. No significant changes in their Ig receptor (S= number of analyzed sequences) mutations were observed.

4.5 Mutational pattern of $IgD^+CD27^+CD23^{+/-}$ memory B cells

Similar to CD21 marker, we also checked IgD^+ memory B cell expression of CD23 and sorted $CD23^+$ and negative $CD27^+IgD^+$ B cells and looked in their mutational pattern as shown in figure 4.5. We found no difference in acquisition of mutations in IgR depending on CD23.From these findings we could conclude that low and high mutational pattern of IgD^+ memory B cells in independent of surface markers specific for circulating marginal zone B cells.

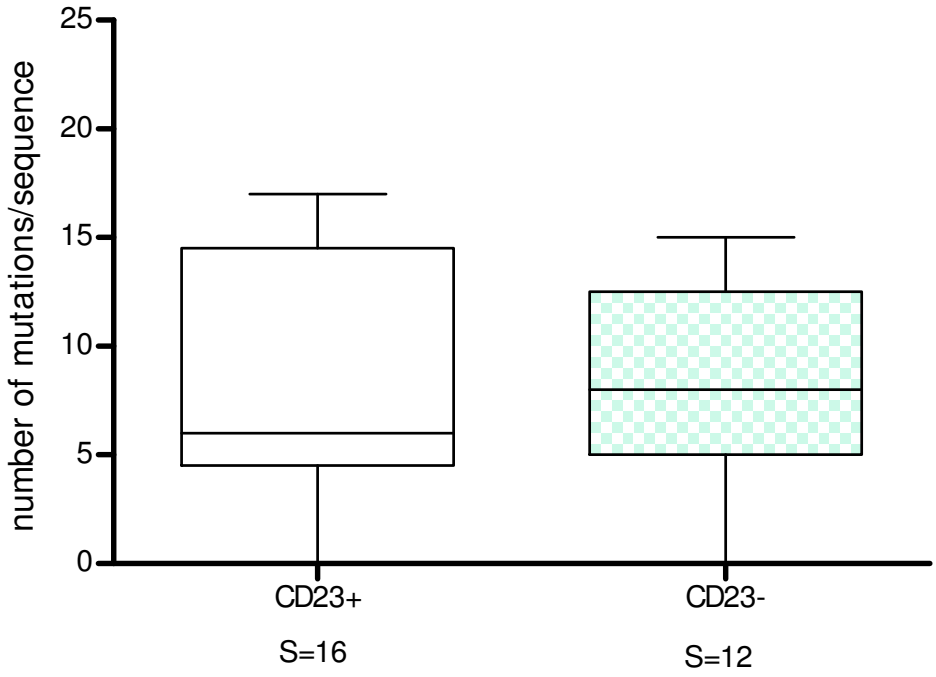


Figure 4.5 Mutations/sequence of $IgD^+CD27^+CD23^{+/-}$ B cells. Mutational pattern of $IgD^+CD27^+CD23^{+/-}$ memory B cells sowing similarity in their Ig receptor (S= number of analyzed sequences) mutations.

4.6 A diversified IgR mutational pattern of IgD^+ memory population

To assess the immunoglobulin receptor (IgR) mutational behaviour of $IgD^+/CD27^+$ memory B cells, we studied V_H3 family genes in IgD^+ memory B cells sorted from 7 patients with active rheumatoid arthritis before undergoing rituximab therapy along with 4 healthy individuals chosen to make comparative study for assessment of disease induced changes in IgR of IgD^+ memory B cell subset. Ig-V_H3 family was amplified by using single B cell sorting technology followed by nested PCR. Single cell sorting is most reliable technique to study single cell behaviour in more accurate way. We analysed 248 rearranged Ig-V_H3 gene sequences from individual IgD^+ memory B cells population (shown in Figure 4.6) collected from RA patients and healthy donors (HD). Ig-V_H3 family was selected for our study based on the fact that it is largest and dominant subfamily among variable genes of IgR heavy chain (Brezinschek *et al*, 1995; Rouziere *et al*, 2005). Only productive sequences were included in study for further analysis. The frequency of IgD^+CD27^+ memory B cells was on average 15% of total CD19 positive B cells in both RA and ND.

The Ig-V_H3 gene study showed that IgD^+ memory B cells display a diversified mutational pattern (Figure 4.6) including non-mutated, low mutated and high mutated sequences. It collectively revealed 88% of mutated rearranged Ig-V_H3 sequences with an overall mutational frequency 3.83±0.19% (mean±SEM) and only 12% non-mutated sequences. Depending on the number of mutations/sequence as proposed by Palanichamy (Palanichamy *et al*, 2008), we can further categories mutated sequences into low and high mutated sequences (median= 9mutations/sequence). Among all mutated sequences, 52% of IgR had high mutations i-e >9 mutations/sequence while 48% were low mutated i-e ≤ 9 mutations/sequence. A similar mutational load was observed in IgR in 4 healthy donors as shown in Figure 4.6. In total 199 rearranged sequences showed 90% mutated sequences with an overall mutational frequency 3.77±0.27% while 10 % non-mutated IgV_H3 sequences. These results indicate a similar mutational frequency of IgD+ memory B cells in patients with rheumatoid arthritis and healthy donors.

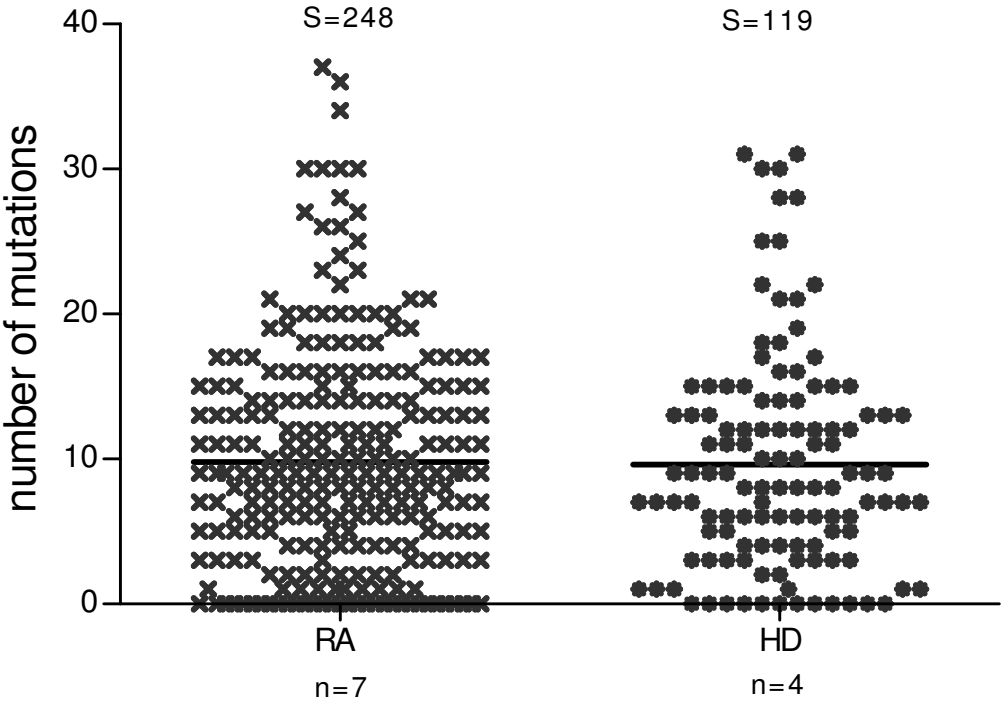


Figure 4.6 IgD$^+$ memory B cells in HD and RA. Showing a diverse expression of mutations in Ig-VH3 Family. A comparative expression of RA patients and healthy donor (HD) showing similar mutational behaviour (S=number of analyzed IgR sequences, n= number of studied individuals).

4.7 Rituximab mediated modulation of Ig-V_H3 receptors of IgD$^+$ memory B cells

Rituximab causes selective depletion of CD20$^+$ B cells from periphery and secondary lymphoid organs. B cells in periphery remain diminished over a period from 6-12 months while a marked reduction of memory B cells have been reported previously among which IgD$^+$ memory B cells repopulate specifically slow in different memory B cell populations during regeneration in rheumatic patients (Roll *et al*, 2006). So our specific interest developed to observe changes in their IgR during regeneration , we investigated nine patients in detail at specified time points during B cells repletion phase over a period of six years and find interesting dramatic changes in mutational behaviour in Ig-V_H3 receptors. During the first

year after rituximab therapy IgD^+CD27^+ B cells are generally not detectable. After one year IgD^+ memory B cells could be found in periphery. Figure 4.11 shows in detail summary of mutational load in IgR of individual IgD^+ memory cells studied a 6 years (results a-d) time course after anti-CD 20 mediated B cell depletion.

a) One year after rituximab

Among 7 patients treated with rituximab, 3 patients were followed one year after B cells depletion. During first year of regeneration phase IgD^+ memory B cells showed highly different mutational load in their IgR in comparison to pre-depletion phase. Total 82 sequences analysed from 3 patients showed that 84% of rearranged Ig-V_H3 sequences harboured no mutations at all which were 12% before therapy (*P*=0.0001) as shown in figure 4.7. Further discrimination showed only low mutated sequences (16%) while highly mutated sequences were completely absent during 1st year of regeneration phase (52% before therapy, *P*=0.0001). This marked absence of highly mutated sequences was further assessed in patient treated two years before with rituximab.

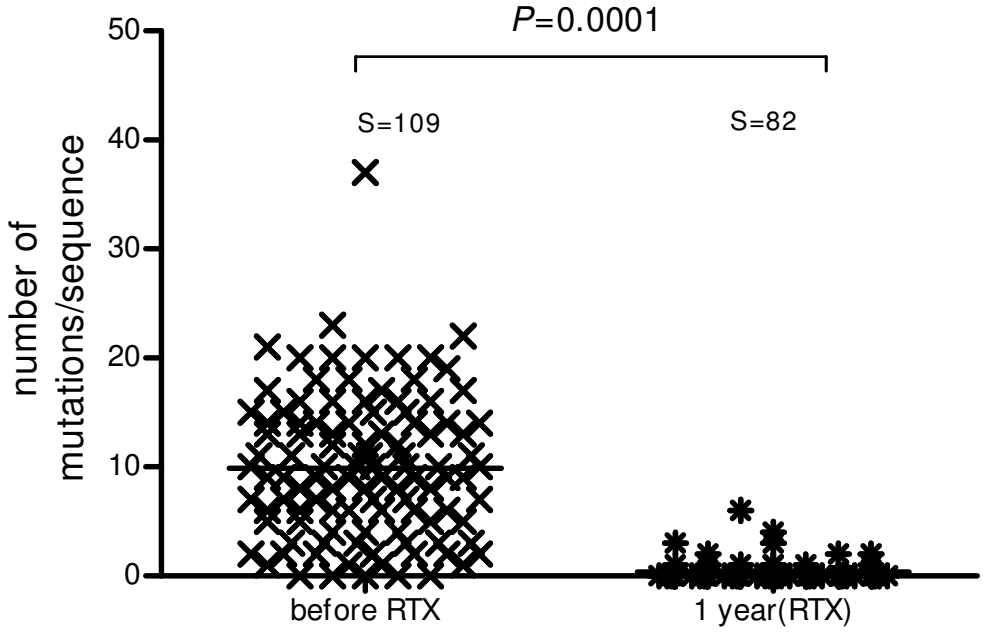


Figure 4.7 Acquisition of mutations in single IgR one year after rituximab. Mutational pattern of single IgD^+ memory B cells in comparison to pre-therapy showing marked reduction of mutational load (S=number of analyzed IgR sequences).

b) Two years after rituximab

We analyzed one patient two years after B cell depletion. As shown in Figure 4.8; IgD^+ memory B cells acquired few mutations in second year of repletion phase. Figure shows individual sequences harbouring mutations in their IgR in this time point but still showing very few high mutated sequences. In total 40 analysed sequences, we could observed only 7.8% highly mutated (*P*=0.0001 as compared to before therapy) sequences. Rest of sequences comprised 79.2% non-mutated while 13% were low mutated sequences. This time point was quite interesting because total B cell numbers were normalized in periphery but IgD^+ B cells were still reduced.

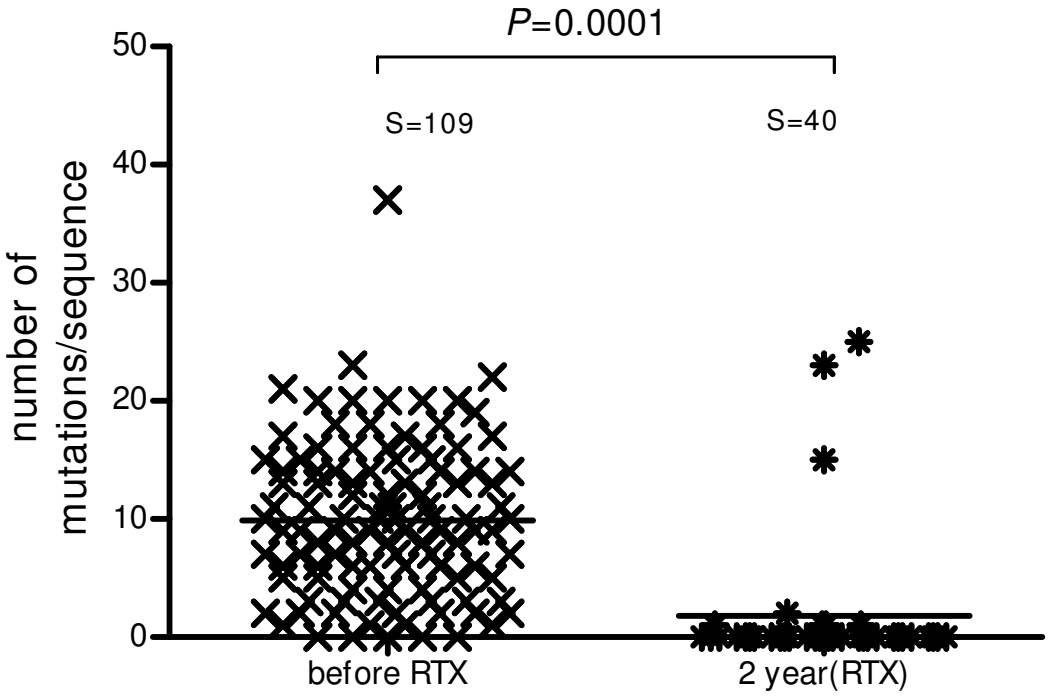


Figure 4.8 Acquisition of mutations in single IgR two years after rituximab. Majority of low or non mutated sequences were recovered two years after RTX(S=number of analyzed IgR sequences).

c) Four years after rituximab

A longitudinal follow up of two patients after 4 years treatment with RTX again indicated that IgD^+ memory cells did not get their pre-treatment IgR expression (as before therapy) and Ig-V_H3 family sequences (50 sequences in total) showed that only 14% of highly mutated sequences were recovered even 4 years (Figure 4.9) after B cell depletion (*P*=0.0001).But on the other hand, frequency of non-mutated sequences decreased to 40.5% but still higher than pre-treatment while frequency of low mutated sequences got normal i-e 45.5% (as before therapy).This showed that rituximab directed B cell depletion not only hampered regeneration of IgD^+ memory B cells but also acquisition of mutations in their Ig receptors lead us to further explore this phenomenon in patients treated with rituximab in later time point.

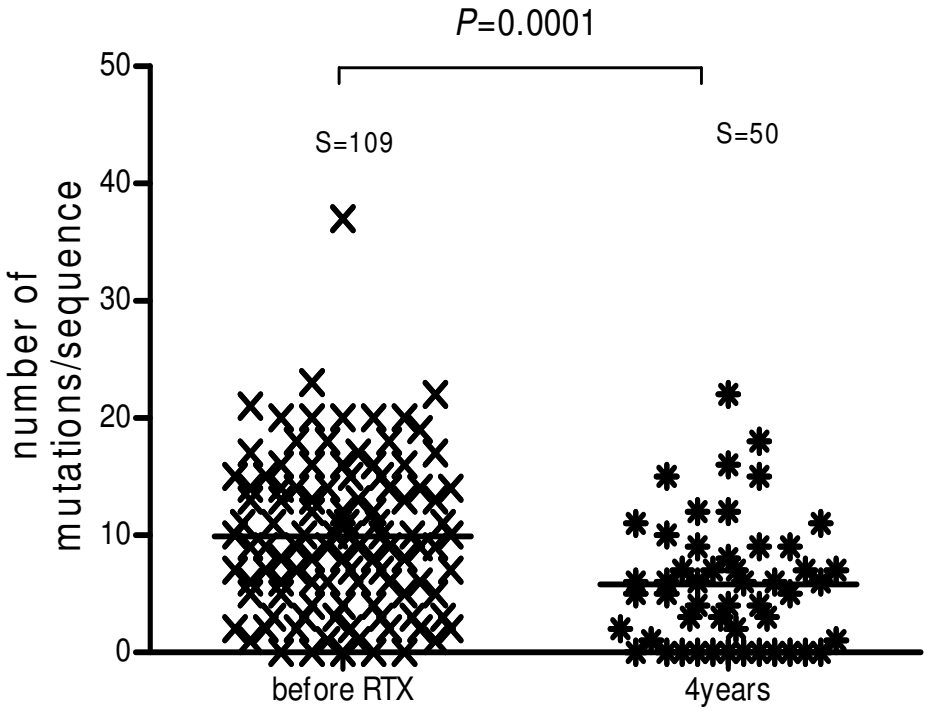


Figure 4.9 Acquisition of mutations in single IgR 4 years after rituximab.14% high mutated sequences (S=number of analyzed IgR sequences) were recovered (52% pre-treatment, *P*=0.0001)

d) Six years after rituximab

As we find already that IgD^+ memory B cells show a skewed mutations in their IgR, so we proceeding to study these cells in patients treated long ago with rituximab. So we studied mutational pattern in 3 patients treated with rituximab 6 years ago. Even long after treatment, not only IgD^+ memory population was diminished on basis of cell numbers in overall memory B cell pool but also significant reduction in highly mutated sequences in Ig-V_H3 receptors was observed. In detail, out of 74 analyzed (Figure 4.10) sequences, total 75% mutated sequences were recovered (88% pre-treatment, *P*=0.0001) in which only 25% highly mutated sequences were acquired which were still diminished than before therapy (*P*=0.0001). Overall B cell count of CD19 positive B cells was normalized 2years after rituximab, IgD^+CD27^+ B cells were still below 5% of total $CD19^+$ B cells 6 years after therapy. These results clearly indicate that rituximab directly B cell depletion has great impact on the mutational acquisition of IgD^+ memory B cells.

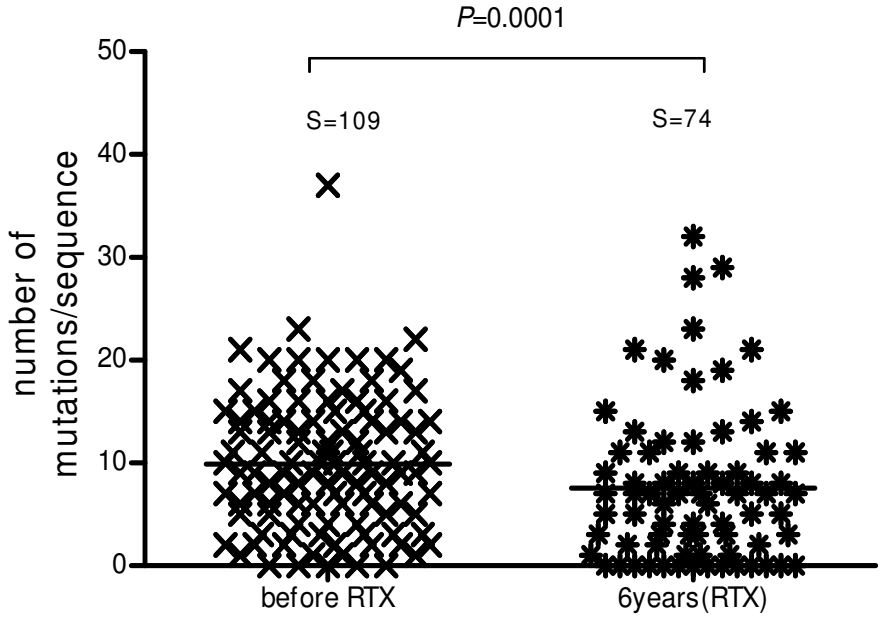


Figure 4.10 Acquisition of mutations in single IgR 6 years after rituximab. IgD^+ memory B cells showing recovery of low mutated sequences while high mutated sequences were still not normalized(S=number of analyzed IgR sequences).

Summary

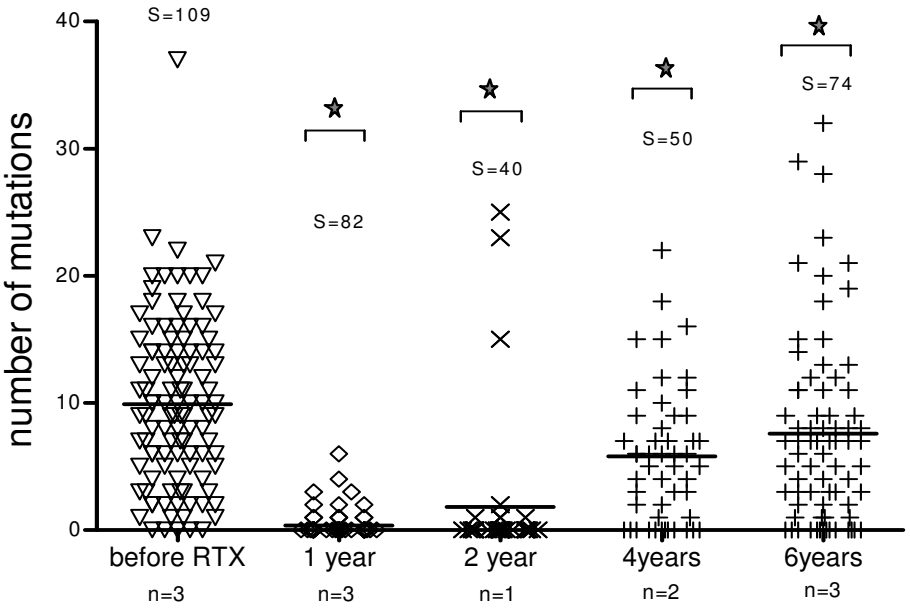


Figure 4.11 Mutations/sequence of V_H3 gene rearrangements expressed by individual IgD^+ memory B cells after rituximab therapy: showing a reduction of highly mutated sequences (as compared to pre-depletion) over a course of 6 year follow up(S= number of analyzed IgR sequences, n= number of studied individuals) in RA patients treated with rituximab. (✯P = 0.0001 versus before therapy)

4.8 IgD$^+$ memory B cells in SLE patient after rituximab

To evaluate effect of anti-CD20 mediated B cell depletion on IgD$^+$ memory B cells in a disease independent manner, we analyzed one patient with systemic lupus erythematosus (SLE) treated with rituximab. Results shown in Figure 4.12 indicate clearly that rituximab treated RA and SLE patient showed no difference in acquisition f mutations in their IgR and showed similar mutational frequency. The affected reduced mutational rate of IgD$^+$ memory B cells after anti-CD-20 mediated B cell depletion by itself seems not to be related to RA or ongoing methotrexate therapy as in SLE patient no additional immunosuppressive treatment was given and brought same pattern of mutations in IgD$^+$ memory B cells. These results clearly indicated that rituximab hinders these cells in acquiring not only normal peripheral pool but also acquisition of mutations in their IgR.

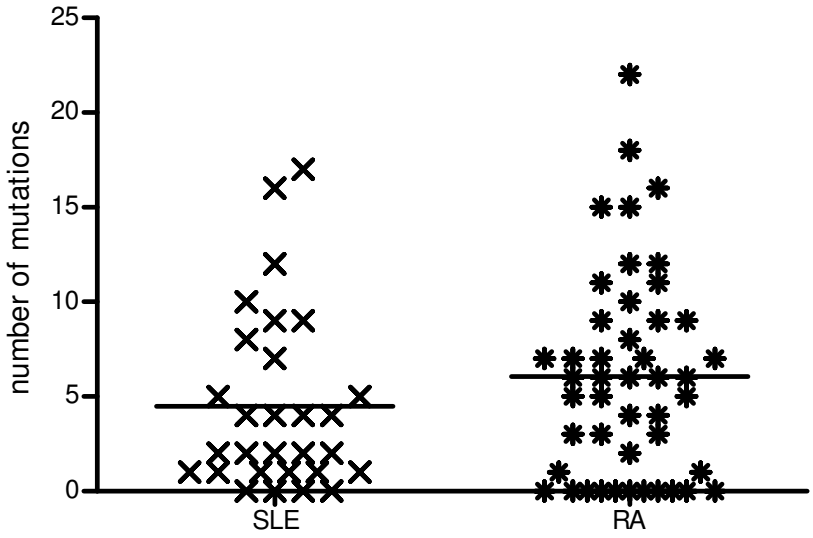


Figure 4.12 Number of mutations in single IgR 4 years after rituximab in SLE and RA. Mutational pattern of IgD$^+$ memory B cells in patients with RA and SLE showing similar mutational acquisition in their IgR at same time span.

4.9 Significant increase in CDR3 length after rituximab

By using IgR sequence analysis approach to study IgD^+ memory B cells during rituximab therapy, other parameters were also checked and found interesting changes in them as well, CDR3 length comparison was one of them. CDR3 region is actively participant of antigen binding. CDR3 length was calculated by determining number of nucleotides from residue 95-102. CDR3 lengths were compared at above described Ig-V_H3 receptor study time points by using Mann-Whitney U test. A mean length of CDR3 region before therapy (Figure 4.13) was 43±0.89 bp which increased to 49±1.17bp during early repletion phase (P=0.0002) and consistently elevated in IgD^+ memory B cells during 2 years (mean 49±1.61, P=0.0014) and 4 years (mean 50±1.54bp, P=0.0010) follow up.CDR3 length was even not normalized after 6 years (mean 48±1.3bp, P=0.0080).These results indicate that transient B cell depletion therapy have selective pressure on the IgD^+ memory cell's CDR3 length which is major antigen binding site. These results may also develop the hypothesis that after B cell depletion phase, these regenerated IgD^+ memory B cells particularly represents ontogeny.

While analyzing CDR3 expression by different sequence subsets categorically based on acquisition of mutations, an increased CDR3 length was only contributed by non-mutated Ig receptors and a decreased CDR3 length was observed in low and high mutated sequences. An increased CDR3 (Figure 4.14) length was participated by due to majority of non-mutated sequences contribute in 6 years follow up after rituximab.

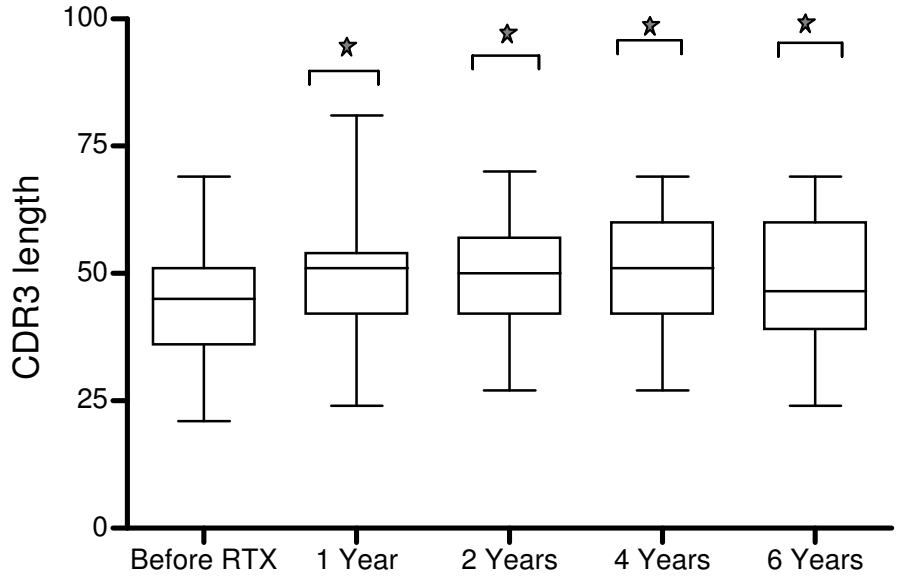


Figure 4.13 Significantly increased CDR3 length in regenerated IgD$^+$ memory B cells: (✯ $P \leq 0.0025$ versus before therapy). The CDR3 length did not normalize even after 6 years compared to the values prior to therapy.CDR3 length was analyzed from sequences shown in Fig.4.11.

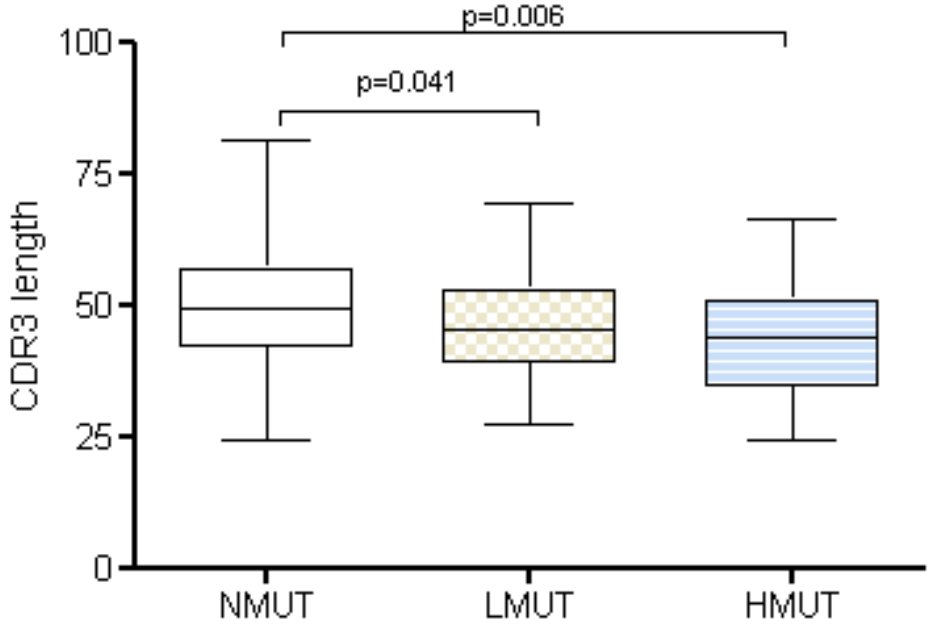


Figure 4.14 Distribution of CDR3 length in non-mutated (NMUT), low mutated (LMUT) and high mutated (HMUT) sequences. Significantly increased CDR3 length is only associated with non- mutated sequences.

4.10 V, D and J gene segments usage during repletion of IgD^+ memory B cells

To access any prominent shift in V, D and J gene usage in V_H gene recombination phase during repletion of IgD^+ memory B cells, we studied pre and post-treatment IgV_H3 sequences in same patients. Before therapy, a variety of ten V_H3 mini-genes were expressed while two of them comprised 50% of all V_H3 rearrangement (V_H3-23, 35%; V_H3-30, 15%). During regeneration phase, a significant increased use of V_H3-23 was observed (50%, P=0.0449) while most of V_H3 mini-genes were observed unaffected in regenerated IgD^+ memory B cells.

No significant changes were observed in D gene usage during repopulation phase and large varieties of D genes were present before and after treatment without any prominent usage. Analysis of J_H segments showed unaffected distribution of these segments during regeneration.J_H4 gene was represented most frequently.

Table 4.1 Difference in VH_3 minigene usage during rituximab

VH3 subfamily	Before rituximab (%)	After rituximab (%)
VH3-23	35	50
VH3-30	15	12

4.11 Class-switched memory B cells during rituximab therapy

During memory B cells development, class-switching is an important process. These class-switched $IgD^{-}CD27^{+}$ memory B cells are believed to undergo somatic hypermutation in pre-defined germinal centres. Previous studies showed an overall reduction of memory B cells after rituximab therapy (Roll *et al*, 2006; Anolik *et al*, 2007) and as we find interesting changes in mutational pattern of pre-switched memory B cells during anti-CD20 medicated B cell depletion so class-switched memory B cells became our great interest to study. To elucidate effect of rituximab on mutational pattern of these cells, we studied them pre and 1 year post treatment in two patients and results are shown in figure 4.15. Although overall memory B cells were diminished after anti-CD20 therapy but single $IgD^{-}CD27^{+}$ memory B cell analysis showed that in contrary to IgD^{+} memory B cells, $IgD^{-}CD27^{+}$ memory B cells harboured quantitatively similar mutations (an average 18 mutations/sequence) in their IgR during repletion phase (median 18mutations/sequence in $IgD^{-}/CD27^{+}$ vs. median 9 mutations/sequence in $IgD^{+}/CD27^{+}$; P=0.0001) and no expressional difference was observed in pre and post-treatment repopulations. These results not only gave comparative results but also indicated that in class-switched memory B cells, the process of somatic hypermutation is not affected by B cell depletion and clearly indicate that these two memory B cell population mature in different sites.

Similar to the mutational frequency, class-switched memory B cells did not exhibit an increase in their CDR3 length as shown in figure 4.16. We observed 43±1.63bp CDR3 length in class-switched memory B cells which was non-significantly decreased to 40.5±1.84bp after anti-CD20 mediated B cell depletion therapy.

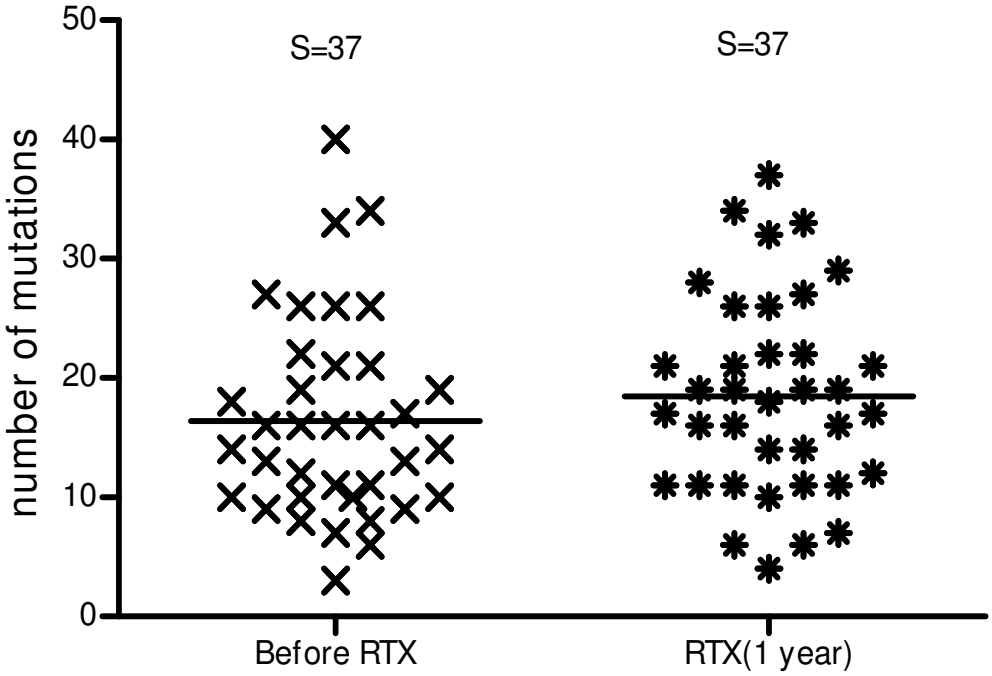


Figure 4.15 Mutational pattern of IgD$^-$CD27$^+$ memory B cells during rituximab therapy. Results show a similar IgR mutational acquisition(S=number of analyzed sequences).

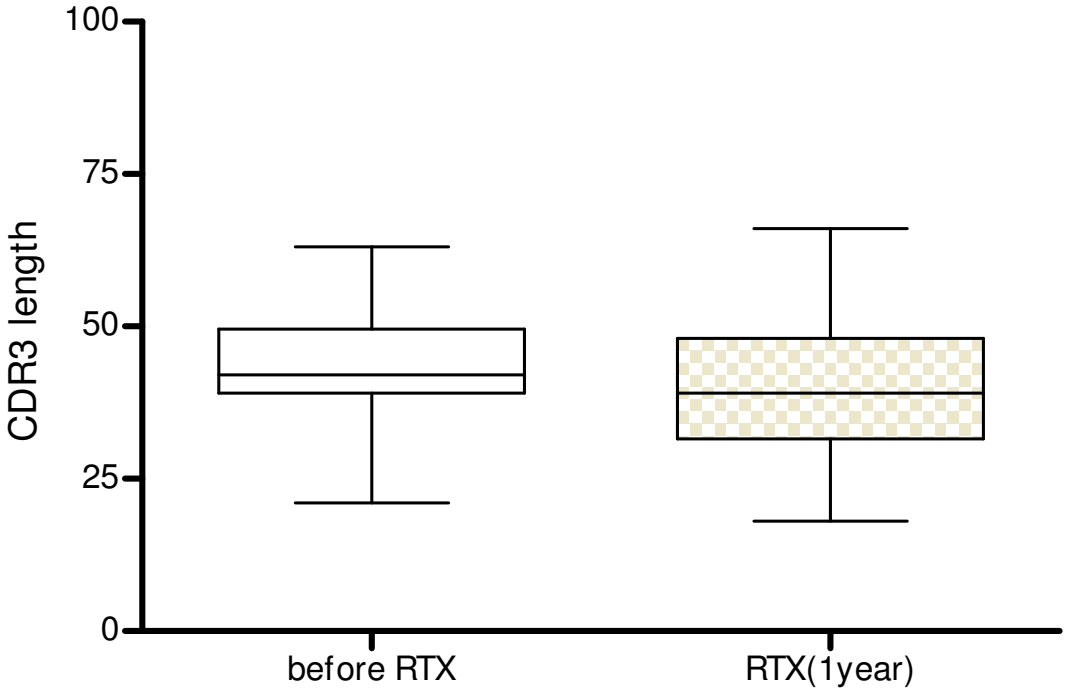


Figure 4.16 CDR3 in class-switched memory B cells during rituximab. In accordance with mutational pattern of class-switched memory B cells in fig. 4.15, no significant change of CDR3 length was observed during rituximab therapy

4.12 V, D and J gene usage in class-switched memory B cells

To access any prominent shift in V, D and J gene usage in V_H gene recombination phase during repletion of class-switched memory B cells, we studied pre and post-treatment IgV_H3 sequences in same patients. Before therapy, a variety of eleven V_H3 mini-genes were expressed which showed consistency during repletion phase and no prominent use of particular mini-gene was observed.

No significant changes were observed in D gene usage during repopulation phase and large varieties of D genes were present before and after treatment without any prominent usage. Analysis of J_H segments showed unaffected distribution of these segments during regeneration. J_H4 gene was represented most frequently.

4.13 Mutations in hotspot motif RGYW/WRCY during rituximab

To evaluate modulation of class-switched memory B cells, we focused on mutations into pre-defined hotspot motifs RGYW and WRCY (R= purine, Y= pyrimidine, W=A/T) along Ig rearrangements(Dörner *et al*, 1998; Farner *et al*, 1999) was assessed in these cells circulating before therapy and one year after rituximab therapy. As shown in the figure 4.17, class-switched memory B cells showed a reduced mutational targeting of RGYW/WRCY motifs before therapy in comparison to after therapy status. A significantly higher number of mutations were confined to the RGYW/WRCY motifs during repletion phase. Of note, class-switched memory B cells showed 27% mutations in RGYW/WRCY motifs before treatment which was significantly increased to 43% after therapy (*P*=0.0003)

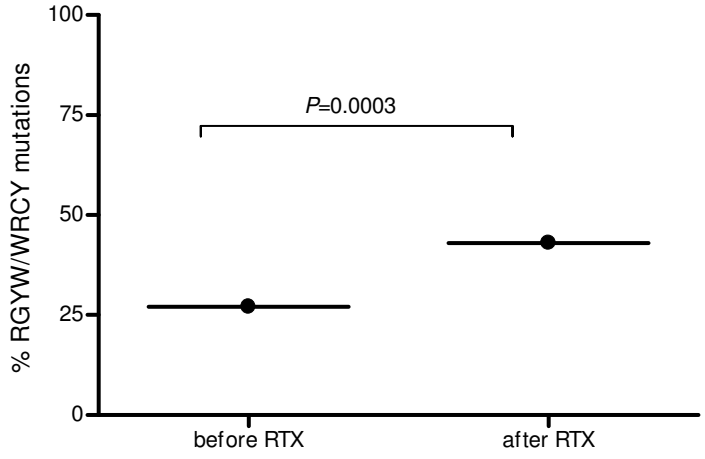


Figure 4.17 Average RGYW/WRCY targeting in one patient before and after therapy. Significantly RGYW/WRCY increased mutational targeting was observed. *P* value calculated by chi square test.

4.14 IgD$^+$ memory B cells after allogeneic stem cell transplantation

As slow acquisition of mutations in individual IgR was observed in IgD$^+$ memory B cells only after transient B cell depletion, so we addressed how acquisition of mutations developed in these cells after allogeneic stem cell transplantation? So we selected 3 patients treated with high dose chemotherapy including total body irradiation followed by allogeneic stem cell transplantation (ASCT).

Figure 4.18 illustrates Ig-V_H3 family studied in 3 allogeneic SCT patients included in study one year after treatment. In detail, we analyzed 85 productive V_H3 sequences and found that 41% non-mutated sequences (35 out of 85 sequences) which were significantly lower than RA (84% in RA verses 41%, *P*=0.0001).In rest of mutated sequences, 17.5% sequences acquired higher mutation and 41.5% low mutation sequences were present which clearly indicates that in allogeneic stem cell transplant patients these IgD$^+$ memory cells repopulate in normal pattern. This data concludes that temporal B cell depletion by rituximab has more pronounced effect on IgD$^+$ memory B cell arm among CD19$^+$ B cells during repletion phase.
In line to mutational data, CDR3 length was significant between two groups i-e ASCT patients showed a normal (in accordance to HD) CDR3 length than RA patients (Figure 4.19). CDR3 length in IgD$^+$ memory B cells from ASCT patients had mean length of 45.86 ± 1.04 bp significantly lower than RA patients 49.30 ± 1.16 bp (*P*=0.0280) but in line with healthy donors.

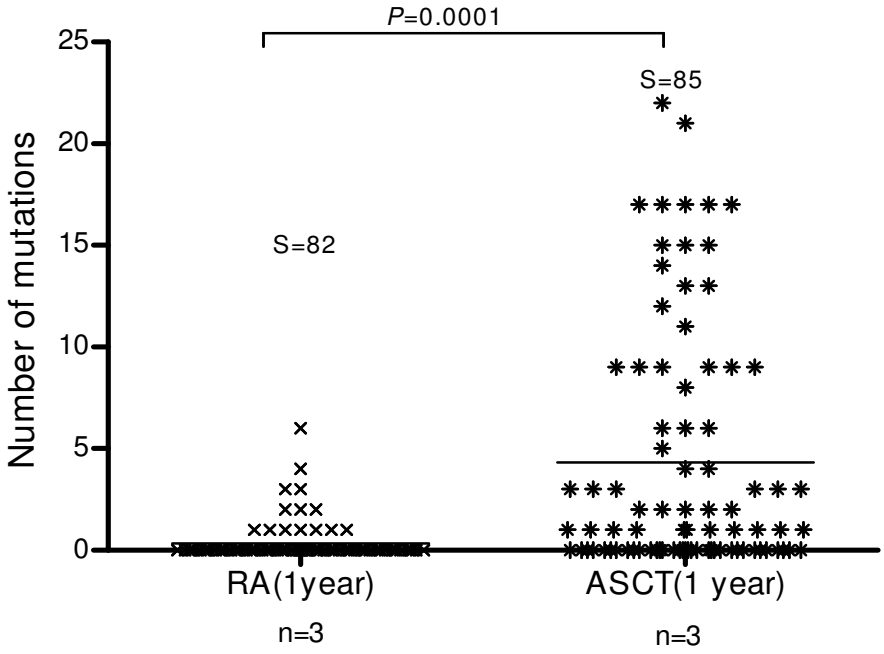


Figure 4.18 Individual IgR of IgD$^+$ memory B cells in Allogenic SCT vs. RA. 3 patients after RTX and 3 patients after ASCT (S=number of analyzed IgR sequences, n= number of studied individuals) showing significant different in IgR mutational pattern.

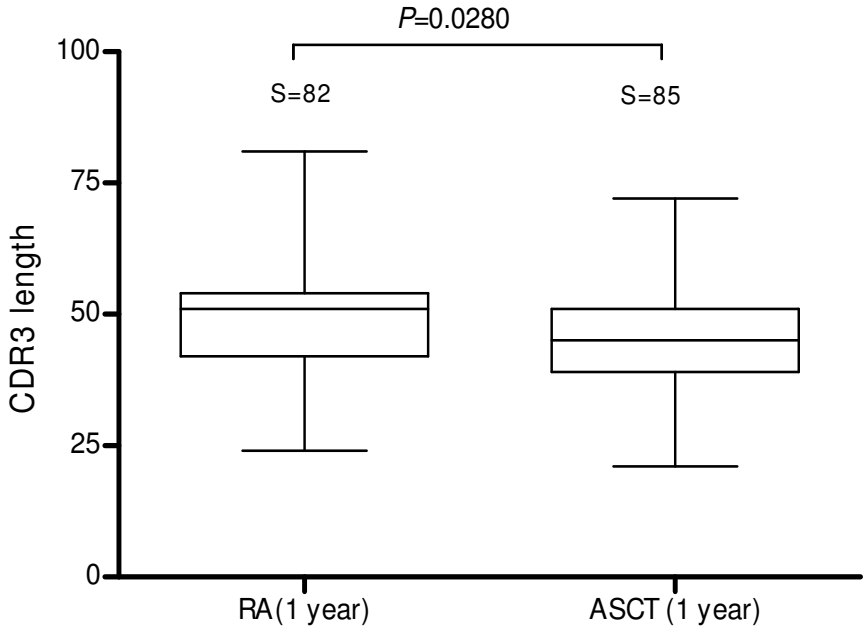


Figure 4.19 CDR3 length in allogenic SCT vs.RA.CDR3 length was shorter in ASCT regenerated IgD^+ memory cells in comparison to those from RA patients.

4.15 Class-switched memory B cells after ASCT

As seen previously in RA patients after rituximab (Figure 4.15), Ig receptors harboured unaffected quantitatively similar mutational pattern in patients after allogeneic stem cell transplantation emphasizing our previous findings (Figure 4.20).

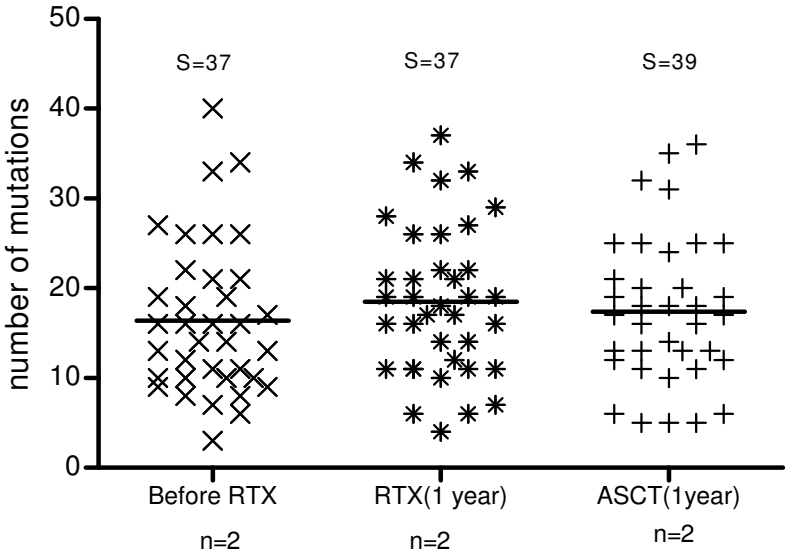


Figure 4.20 Mutations/sequence of class-switched memory B cells after rituximab and after ASCT. No quantitative difference in the number of mutations were in allogeneic stem cell transplant patients as seen previously in RA (S=number of analyzed IgR sequences, n= number of studied individuals).

4.16 Autologous stem cell transplantation

As we observed improved regeneration and mutational pattern of IgD^+ memory B cells in allogeneic SCT patients (figure 4.18) in comparison to B cell depletive therapy, we analyzed IgD^+ memory B cells in patients undergone autologous stem cell transplantation (SCT). With this type of transplant, the patient's stem cells are obtained prior to high-dose chemotherapy, frozen, and stored and are then given back afterward.

We analyzed IgD^+CD27^+ memory B cells from two patients one year after autologous SCT. The results are shown in figure 4.21. We found a non-significant correlation between acquisition of mutations in IgD^+ cells from patients undergoing autologous SCT and allogeneic SCT. But on the otherhand, the number of mutations in IgD^+ memory B cells in autologous SCT was significantly high compared to rituximab treated RA patients studied at same time point. They showed mutational frequency 6.44 ± 2.41% vs. 0.35 ± 0.11% in RA (*P*=0.0001). All these results collectively indicated that rituximab mediated B cell depletion exerts strong impact on somatic hypermutation in IgD^+ memory cells.

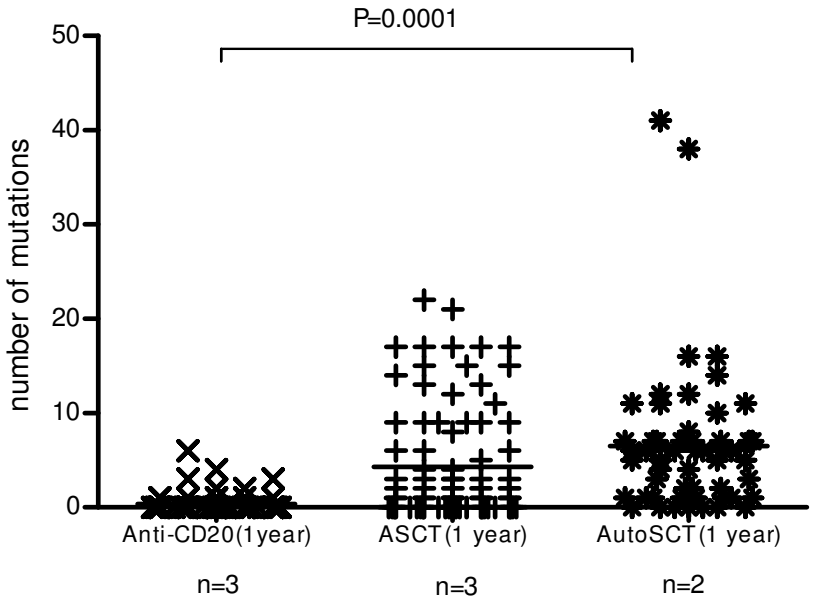


Figure 4.21 Number of mutations/sequence in IgD^+ memory B cells 1 year after autologous SCT in comparison to anti-CD20 treatment and ASCT. Individual IgR of IgD^+ memory B cells were analyzed from 3 patients after RTX,3 patients with allogeneic SCT and 2 patients after autologous SCT showing significant different in IgR mutational pattern.

4.17 Modulation of Ig-Isotypes during rituximab

Memory B cells expressing CD27 surface molecule and undergoing somatic hypermutation in immunoglobulin variable (V) region classically switch from initial expression of IgM to that of other Ig classes resulting in surface expression of IgG, IgA or IgE and lack surface expression of IgD and CD38 (Shi *et al*, 2003) . In healthy adults, more than half of $CD27^+$ B cells are class-switch cells (mainly IgG or IgA surface expression), while the remaining are IgD^+ memory cells. Therefore we addressed the question whether anti-CD20 mediated B cell depletion induce any change in process of classical isotype switching in IgD^- $CD27^+$ memory B cells in two patients undergoing rituximab therapy. We focused mainly all IgG and IgA by using single cell sorting technique to assess their percentages in memory B cells after amplified V_H1-V_H6 families by nested PCR.

As shown in figure 4.22& 4.23 IgA and IgG expressing single memory B cells are equally distributed before therapy. However after rituximab therapy class-.switched memory B cells were predominantly of IgA isotype. We find 50% of IgA expressing B cells before therapy which increased to 76% during first year of B cell repletion phase (P=0.0001).

On the otherhand, IgG expressing class-switched memory B cell decreased from 50% to 24% (P=0.0001). These results indicated that rituximab also modulated isotype switching in memory B cells resulted increase in IgA producing cells.

We closely observed immunoglobulin variable (V) gene usage during rituximab therapy in these IgA and IgG producing B cells and find no characteristic changes in usage of VH sub-families during B cell repletion phase as shown in table 4.2.

Table 4.2: Usage of VH subfamilies during pre and post-RTX treatment

VH subfamilies	Before rituximab (%)	After rituximab (%)
VH1	11	19
VH2	7	3
VH3	50	54
VH4	25	20
VH5	7	4

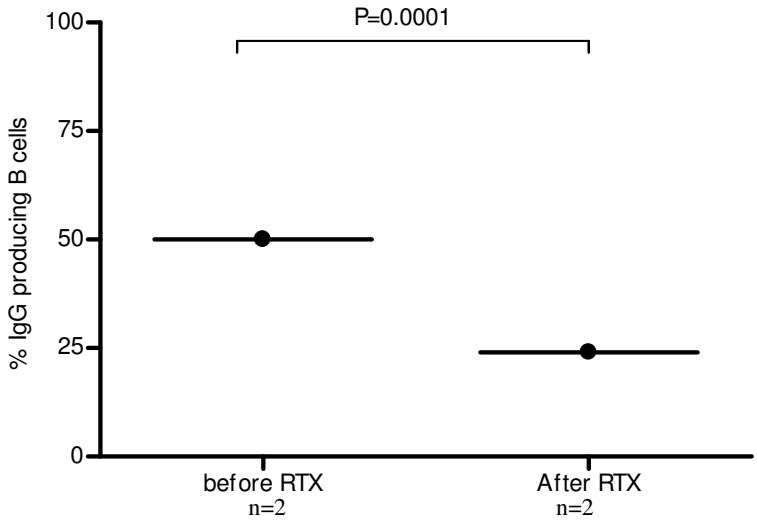


Figure 4.22 Modulation of IgD$^-$CD27$^+$ memory B cells producing IgG. A reduced number of IgG producing memory B cells recovered one year after rituximab.

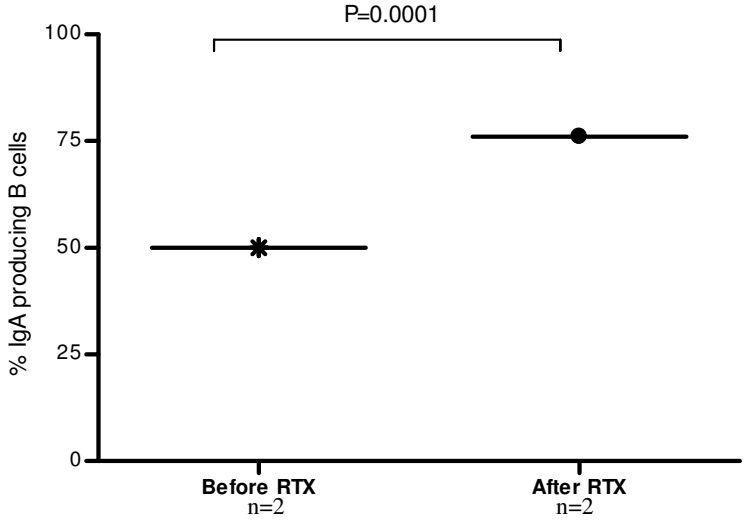


Figure 4.23 Modulation of IgD$^-$CD27$^+$ memory B cells producing IgA. A significantly increased number of IgA producing memory B cells recovered one year after rituximab.

5. Discussion

Human B cells play a critical role in the pathophysiology of different autoimmune diseases. In addition to secretion of autoantibodies and proinflammatory cytokines (interleukin 1, 6 and 10, TNF-α and lymphotoxin α/β), they efficiently take up, process and present autoantigens and influence the formation of T cell dependent niches (Silverman *et al*, 2003; Hansen *et al*, 2007). Therefore approaches to target B lymphocytes directly or indirectly are developed for clinical practice. In this context, transient B cell depletion by the anti-CD20 specific monoclonal antibody (rituximab) has shown promising results in treating patients with autoimmune diseases including rheumatoid arthritis, SLE and systemic vasculitis (Leandro *et al*, 2006; Browning, 2006; Cohen, 2008).

B cell regeneration data after therapeutic B cell depletion with rituximab revealed a characteristic repletion pattern that seems to recapitulate B cell ontogeny. In the early stages of peripheral B cell repopulation increased numbers of transitional B cells are followed by B cells with naïve phenotype (Roll *et al*, 2006&2008; Anolik *et al*, 2007). Although peripheral B cell numbers normalize in most patients between one and two years after a single course of rituximab a longterm reduction of memory B cells, particularly the IgD^+CD27^+ memory subset, has been observed (Roll *et al*, 2006). In a prospective clinical study our laboratory has shown previously that the overall number of memory B cells correlates well to the duration of clinical response to rituximab. Moreover the early regeneration of IgD^+CD27^+ memory B cells could be linked to nonresponse to rituximab therapy (Roll *et al*, 2008). These findings developed our specific interest for the molecular study of B cell receptors in memory B cells study during B cell repletion after rituximab. Therefore the prime aim of the current study was to search for BCR gene modulations in memory B cell subsets after B cell recovery which may provide insights into a longterm molecular impact of rituximab. We explored in detail the somatic mutational frequency and pattern of Ig-V_H3 gene rearrangements among pre- and post-switched memory B cells before and up to 6 years after rituximab therapy.

The phenotypic analysis of peripheral pre-switched (IgD^+CD27^+) and post-switched (IgD^-CD27^+) memory B cells did not reveal any quantitative differences in RA patients (n=33) prior to B cell depletion therapy compared to healthy donors (n=22) (Figure 4.2). In a previous study overall increase in IgD^-CD27^+ memory B cells were described in RA patients (Fekete *et al*, 2007) but no change in IgD^+ memory B cells was observed. Our study showed

non-significant increase in class-switched memory B cells as well. This difference may be attributed to different treatment methods in patients' cohort and disease duration. We extended those studies in directly analysing the B cell Ig receptor in single B cells of RA patients and healthy controls. The mutational analysis of individual Ig receptors in pre-switched memory B cells in rheumatic patients (n=7) showed no difference in acquisition of mutations in comparison to healthy donors (n=4) (Figure 4.6). In these studies no obvious selective pressure could be identified on these cells by disease itself or by the stable concomitant low dose methotrexate (MTX) treatment.

However comparing pre-switched and post-switched memory B cells a highly significant difference in the amount of mutations can be seen. The population of IgD^+CD27^+ memory B cells is comprised of non-mutated, low and highly mutated (median= 9 mutations/sequence) rearranged Ig receptors while the IgD^-CD27^+ memory B cell compartment shows quite uniformly highly mutated sequences (median 18mutations/sequence) indicating a significant difference between these two groups of memory B cells (mutational frequencies 3.83±0.19% vs. 7.1±0.53%; P=0.0001). The presence of a significant proportion of ~12% of non-mutated Ig sequences in IgD^+CD27^+ memory B cells (Figure 4.6) in both healthy and RA patients suggests that these cells are still under a diversification process which is not seen in post-switched CD27 expressing B cells. These findings indicate that the two memory subpopulations may have different sites of origin and diversification. For class switched memory B cells it is known that they are generated in germinal centres directly involving T cell dependent responses against antigen (Klein *et al*, 1998). The origin of IgD^+ memory B cells is still debated. Due to their phenotype ($IgD^+CD27^+IgM^+CD23^-CD21^{high}CD1c^+$) they have been correlated as a circulating counterpart of marginal zone B cells (Weller *et al*, 2004). In our analyses > 90% of these cells express $CD21^{high}$ $CD23^{low/-}$. With regard to the Ig receptor mutations, non- and mutated-Ig receptors were non-selectively distributed among $CD21^{+/-}$ $CD23^{+/-}$ IgD^+ memory B cells. A recent study (Weller *et al*, 2008) has provided clear evidence for the presence of IgD^+ memory B cells already in infants below 2 years of age. With increasing age Ig receptor mutations increase from 60% to 83% in IgD^+ memory B cells. These data correspond well with our findings where in adults 88% mutated sequences were found within the IgD^+ / $CD27^+$ subset. This indicates continuously slowly ongoing somatic hypermutation with age. In contrast, within the class-switched IgD^-CD27^+ memory B cells more than 95% showed mutated sequences with high mutational frequency.

So far the development and process of somatic hypermutation in IgD^+ memory B cells are not so clear. Previous studies have suggested that development of somatically mutated IgD^+ memory B cells does not require CD40 or CD40L interactions (Razanajaona *et al*, 1996) as the presence of these cells in patients with germinal centre (GC) development defects have been observed (Salzer *et al*, 2006;Tangye *et al*, 2007;Weill *et al*, 2004). Hyper-IgM patients possess IgD^+ memory B cells but lack class switched memory B cells (Weller *et al*, 2001). Also the mutational frequency of IgD^+ /$CD27^+$ cells in hyper IgM patients was similar to that seen in healthy donors. It has been suggested that mutated IgD^+ memory B cells are developmentally dedicated to T cell independent responses because these cells were found to be localized in the marginal zone of spleen, a site where in rodents typically thymus independent (TI) responses develop (Martin *et al*,2000). This idea is also supported from studies where IgD^+ memory B cells were completely diminished in splenectomised and congenital asplenic whereby switched memory B cells were present at normal frequency (Kruetzmann *et al*, 2003; Martinez-Gamboa *et al*, 2009). Therefore IgD^+ memory B cells are likely generated outside GCs and potentially are under less stringent negative selection (Dörner, 2006). Currently, in-vivo mouse model in which human hemopoietic stem cells repopulation was studied clearly showed that development and induction of somatic hypermutation of human IgD^+ memory B cells can occur in a T cell-independent manner (Scheeren *et al*, 2008).

In our study the repopulation of pre-switch memory B cells (IgD^+/ $CD27^+$) after transient B cell depletion with rituximab showed significantly retarded repopulation of this B cell subtype. Even 6 years after therapy the relative amount was diminished. At that time peripheral IgD^+ memory B cells comprised still less than 5% of regenerated B cells compared to ~15% before rituximab therapy (P=0.0051). This longterm reduction of memory B cells particularly IgD^+ memory B cells has been reported previously after rituximab in various diseases including SLE (Anolik *et al*, 2007), RA (Roll *et al*, 2006) and NHL (Nishio *et al*, 2007). However, analysing the acquisition of mutations in these repopulated pre-switch memory B cells brought unexpected results. After a single course of treatment newly emerging IgD^+ memory B cell showed a much delayed mutational behaviour. 80% of their Ig receptor showed no mutations at all during the first year after rituximab (Figure 4.11). In a prospective analysis of 3 patients before and 1 year after rituximab treatment we could not find any Ig receptor sequence with higher number (> 9 mutations/sequence) of somatic mutation (52% before therapy, P=0.0001) in the IgD^+/$CD27^+$ memory B cells. Patients with a

longer follow up after a single course of rituximab still had a remarkable delayed acquisition of somatic hypermutation in their IgD^+ memory B cells Ig receptors which were significantly less even 6 years after treatment with only 27% highly mutated cells (Figure 4.10). We did find a time dependent increase in highly mutated sequences i-e 7.8% during 2^{nd} year of regeneration (P=0.0001), 14% after 4 years (n=2) and 27% highly mutated sequences after 6years (n=3) rituximab treatment (P=0.0001) which argues for a retarded but continuously re-establishing IgD^+ memory compartment. These findings indicated that anti-CD20 mediated B cell depletion seems not only to delay the production of pre-switch memory B cells but also significantly affects the acquisition of mutations in the IgD^+ memory B cell pool. These data argue for different requirements to undergo somatic hypermutations in comparison to class switched memory B cells.

Post-therapy analysis of CDR3 length of regenerated IgD^+ memory B cells revealed increased CDR3 length which also correlates well with elevated number of non-mutated VH gene rearrangements observed during repletion phase. A mean length of CDR3 region before therapy (Figure 4.13) was 43±0.89 bp which increased to 49±1.17bp during early repletion phase (P=0.0002). We observed a gradual decrease in CDR3 length along with mutational acquisition in these cells over a period of 6 years. Nevertheless, the mean CDR3 length was still significantly elevated (P=0.0080). Close observation of CDR3 lengths between unmutated and mutated sequences in our study showed that increased CDR3 length was restricted only to unmutated sequences which were more numerous after RTX (Figure 4.14). The CDR3 region is an active participant of antigen binding and the area of greatest diversity in the human Ig V region. Increased CDR3 length is particularly reported during ontogeny and decreases during antigen selection and somatic hypermutation (Souto-Carneiro *et al*, 2005). Therefore our data may indicate that these cells are still under diversification and the impact of molecular mechanisms including TdT activity and selection process later on restricts a shorter CDR3 length.

Reduced mutational frequency of IgD^+ memory B cells after rituximab seems not to be related only to patients with rheumatoid arthritis or ongoing methotrexate therapy because we observed similar mutational pattern in a patient with SLE who had received no additional immunosuppressive treatment after rituximab directed B cell depletion (Figure 4.12). Therefore the effect of rituximab on pre-switch memory B cells seems to be a general effect and does not show any disease correlation.

To get more insight in mechanism regulating B cell recovery, we studied patients undergoing high dose chemotherapy followed by allogeneic and autologous stem cell transplantation. It was chosen because particular allogeneic stem cell transplantation inflicts broad impact on mesenchymal cells which can provide important environmental signals for optimal B cell development. Previous studies in stem cell transplant patients also have shown delayed recovery of overall memory B cells (Avanzini *et al*, 2005). Again we studied mutational acquisition in individual memory B cell receptors. Under these conditions class-switched memory B cells also recovered with a normal high mutation rate, whereas the IgD^+ memory B cell compartment showed again decreased mutations one year after stem cell transplantation compared to healthy donors. Interestingly, pre-switch B acquired more mutations after 1 year in these patients compared to patients after a single course of rituximab (figure 4.20). This argues for an additional unique effect of anti-CD20 antibodies on pre-switch memory formation.

The exact mechanism of action of rituximab mediated B cell depletion remains uncertain. Although the reactive B-cell memory is depleted to below the detection level in peripheral blood, changes in total Ig levels are minimal. B cell trafficking between RA lesions and secondary lymphoid organs are intrinsically involved in pathophysiology of rheumatoid arthritis (Voswinkel *et al*, 1999). Recently an ‘‘immune complex decoy hypothesis’’ and a ‘‘road block hypothesis’’ (Taylor *et al*, 2007; Silverman *et al*, 2008) have been proposed to explain the effects of rituximab. ‘The immune complex decoy hypothesis’ suggests that rituximab-opsonized B cells may serve as decoys by engaging FcγR on effector cells to improve treatment efficacy. During Immune thrombocytopenic purpura (ITP) in which IgG anti-platelet antibodies promote platelet clearance via FcγR on macrophages, infusion of rituximab generates IgG-opsonized cells which bind to macrophages ultimately sparing platelets. On the otherhand in rheumatoid arthritis or SLE where immune complexes are present in joints and synovium, these rituximab-opsonized B cell decoys in blood stream and tissues may divert monocytes or macrophages from pathogenic interactions with tissue associated immune complexes and ultimately reduce disease activity without affecting autoantibody levels (Tayler *et al,* 2008). Rituximab affecting cellular trafficking lead to the formation of the so called ‘‘road block hypothesis’’ which postulates impaired trafficking of pathologic memory B cells and inflammatory cells in the joints by rituximab. The histological inspection of synovia of RA patients receiving rituximab has shown that some B cells persist in synovial tissue along with depletion of $CD68^+$ macrophages and other invasive cells

affecting trafficking in synovia possibly by 'road blocking'. In line with this idea of negative feedback, our data suggest that remnants of depleted B cells, including Ig receptors may have capacity to act as negative regulators on newly emerging B cells and may be particularly impeding IgD^+ memory B cells formation and Ig receptor mutation acquisition in our rheumatoid arthritis patients' cohort. Contrary to RTX, in allogeneic and autologous stem cell transplant patients, an additional eradication of pro-B cells takes place which affects subsequently different timings of repopulating B cells subsets. This may influence the less pronounced effect of delayed recovery of somatic hypermutation in IgD^+ memory B cells in these patients.

Contrary to the mutational pattern of IgD^+ memory B cells after rituximab in our study, IgD^-CD27^+ memory B cells showed different results. Although the numeric replenishment of these recirculating class-switched memory B cells was also reduced after rituximab, we found no delay in quantitative acquisition of mutations in this memory B cell subset (Figure 4.15). These results indicate a normal development process with an unimpaired mechanism of mutational acquisition in class-switched memory B cells. The results of mutational pattern of class-switched memory B cells also indicated that the two memory subpopulations may have different regeneration sites with different requirements of cellular structures for mutational differentiation. However it cannot be excluded that the IgD^+ memory B cell pool derives from early B cell progenitors whereas the class-switched memory B cell pool may derive partly from a memory pool reseeding from protected niches.

In that context we analysed the distribution of mutations within the Ig gene more carefully in class switched memory B cells under rituximab treatment. Mutational targeting in characteristic RGYW/WRCY hotspot motifs (Dörner *et al*, 1998; Farner *et al*, 1999) has been described. This analysis was performed to address question whether any qualitative modulation occurred in the class-switched memory B cells during rituximab therapy. In accordance with previous findings by Palanichamy *et al* (Palanichamy *et al*, 2008), our data showed that post-therapy mutational targeting in RGYW/WRCY motifs were significantly increased as compared with that of pre-treatment (27% before rituximab vs. 43% after therapy, P=0.0003). This indicates that affinity maturation may operate differently in class-switched memory B cells before and after B cell depletion. RGYW/WRCY motifs are believed to undergo somatic hypermutation particularly during T-B cell interactions in germinal center. The dependency of RGYW/WRCY motifs on T-B cell interaction was

further proven by studies of hyper-IgM patients who showed reduced mutational frequency in these motifs (Brezinschek *et al*, 2000). A possible explanation of reduced mutational targeting in RGYW/WRCY motifs before rituximab could be that T-cell independent B cell activation seems to be more dominant before therapy indicating that these processes are active in longstanding autoimmune diseases.

The expressed Ig isotypes in class-switched memory B cells are mainly IgG and IgA. In rheumatoid patients before rituximab mediated transient B cell depletion the proportion of IgA and IgG expressing memory B cells was equal. However, in the repletion phase the percentage of IgA expressing memory B cells was strongly increased (Figure 4.22, 4.23). Previous studies monitoring serum Ig levels after rituximab therapy have indicated that IgG and particularly IgM serum levels were decreased after single and repeated courses of rituximab (Cambridge *et al*,2005; Roll *et al*, 2008) whereas serum IgA levels were shown to be maintained and only rarely decreased in one out of 34 studied patients (Popa *et al*, 2007). The fall in serum IgM and IgG levels was related to decrease in memory B cell formation after rituximab. Although it is still not clear why IgA expressing B cells are increased in our studied patients it may indicate that they derive from cells residing in secondary lymphoid organs where B cells depletion was not complete. Mechanistically, IgA class-switch recombination (CSR) occurs in Payer's patches (PP) which are specialized clusters of lymphoid cells located in small intestine and also in non-lymphoid sites in the lamina propria (Stavnezer *et al*, 2009). IgA CSR in germinal centres within PP is T cell dependent. Additionally, different factors were studied to be involved in IgA CSR. Interestingly TGF-β is shown to be a key factor in IgA class-switching (Coffman *et al*, 1989; Sonoda *et al*, 1989). Activated B cells including plasma cells, T cells and dendritic cells are main source of TGF-β production (Stavnezer *et al*, 2009).

In summary, the study has demonstrated a previously unknown delayed acquisition of somatic hypermutations in single Ig receptor V_H gene rearrangements of IgD^+ memory B cells after transient B cell depletion with anti-CD20 therapy in patients with rheumatoid arthritis. The results show a longterm effect of a single cycle of rituximab which was still evident even 6 years after treatment. Rituximab seems to be particularly effective in targeting the development of IgD^+ memory B cells and mutational acquisition in their Ig receptor since patients exposed to intensive chemotherapy and subsequently allogeneic/autologous SCT recover higher numbers of somatic hypermutations already within the first year after

treatment in that compartment. On the otherhand, class-switched memory B cells showed a regular mutational pattern after rituximab directed B cell depletive therapy which was similar to stem cell transplant patients. However IgA expressing B cells dominated the class-switched memory B cell compartment during the B cell recovery phase. In addition antigenic pressure and/or selection are substantially reduced by rituximab therapy which is basically not seen in the class-switched memory B cell compartment. Taken together, these data are in line with the hypothesis that IgD^+ memory B cells have distinct requirements for activating their mutational machinery whereas class-switched memory B cells recover with normal mutation rates early on. These results have implications in understanding the pathophysiology of memory B cell in rheumatoid arthritis and may be helpful in designing new targeted therapies.

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7. Summary

Diverse roles of B cells in the pathophysiology of rheumatoid arthritis are now well established. B cells contribute to autoimmunity by producing autoantibodies, processing autoantigen and the production of different cytokines which are involved in the inflammatory cascade. Therefore approaches to target B lymphocytes directly or indirectly are developed for clinical practice to treat autoimmune diseases including rheumatoid arthritis. Transient B cell depletion by rituximab (anti-CD20 antibody) has gained prime importance in recent years. Meanwhile anti-CD20 mediated transient B cell depletion therapy is now used with clinical efficiency in the treatment of patients with rheumatoid arthritis.

Rituximab induces noteworthy changes in the homeostasis of peripheral B cell subpopulations during the repletion phase with emerging immature B cells in peripheral blood followed by normalization of the naïve B cell pool and a longterm delay in memory B cell subsets in patients with rheumatoid arthritis. Particularly IgD^+CD27^+ memory B cells repopulate very slowly during B cell regeneration. In a prospective clinical study, our laboratory has shown that the overall number of memory B cells correlates well to the duration of clinical response to rituximab. Little is known about the particular molecular changes in the memory B cell repertoire after rituximab therapy. To better understand peripheral memory B cell subsets, we explored in detail the somatic mutational frequency and pattern of Ig-V_H3 gene rearrangements by using a single B cell sorting technique followed by nested PCR before and up to 6 years after rituximab therapy in 18 RA patients. We compared rituximab inflicted dynamics of mutational acquisition to memory B cell repopulation in 4 healthy donors and 6 non RA patients undergoing high dose chemotherapy followed by autologous or allogeneic stem cell transplantation (SCT).

Firstly we analyzed the peripheral composition of memory B cell subsets. The phenotypic analysis of peripheral pre-switch (IgD^+CD27^+) and post-switch (IgD^-CD27^+) memory B cells did not reveal any quantitative differences in RA patients prior to B cell depletion therapy compared to healthy donors. However extending those studies in directly analysing the B cell immunoglobulin receptor from individual B cells of RA patients and healthy controls brought interesting results. Pre-switched and post-switched memory B cells showed a highly significant difference in the amount of mutations/sequence. The population of IgD^+CD27^+ memory B cells is comprised of non-mutated, low and highly mutated

(median= 9 mutations/ sequence) rearranged Ig receptors whereas the IgD^-CD27^+ memory B cell compartment shows quite uniformly highly mutated (median 18 mutations/ sequence) sequences indicating a significant difference between these two groups (mutational frequencies 3.83±0.19% vs. 7.1±0.53%; *P*=0.0001).

Profound changes were noted in the re-emerging pre-switch memory B cells (IgD^+/ $CD27^+$) after transient B cell depletion with rituximab. These cells showed over a time period of 6 years after treatment with rituximab significantly delayed acquisition of mutations in Ig receptors on the single B cell level. One year after a single course of rituximab 84% of single repopulating $IgD^+/CD27^+$ B cells were unmutated and no highly mutated Ig-V_H gene rearrangements were found(*P*=0.0001). Over time increasing numbers of mutations could be detected i-e 7.8% during 2^{nd} year of regeneration (*P*=0.0001), 14% after 4 years (n=2). Nevertheless even 6 years after rituximab, V_H mutations in IgD^+ memory B cells were still reduced with 27% highly mutated sequences compared to 52% pre therapy(*P*=0.0001). Post-therapy analysis of CDR3 length of regenerated IgD^+ memory B cells revealed increased CDR3 length which also correlates well with elevated number of non-mutated VH gene rearrangements observed during repletion phase. In comparison patients undergoing high dose chemotherapy followed by allogeneic stem cell transplantation repopulated IgD^+ memory cells earlier with higher numbers of mutations in IgD^+ memory B cells. One year after transplantation Ig receptors showed already 22% highly mutated and 42 % unmutated V_H rearrangements. These findings indicated that anti-CD20 mediated B cell depletion seems not only to delay the production of pre-switch memory B cells but also significantly affects the acquisition of mutations in the IgD^+ memory B cell pool.

In contrary to the mutational pattern of IgD^+ memory B cells after rituximab class switched memory B cells repopulate in the periphery with quantitatively normal mutations in their Ig receptors. Although the numeric replenishment of these recirculating class-switched memory B cells was also reduced after rituximab, we found no delay in quantitative acquisition of mutations also an increased proportion of IgA expressing B cells in this memory B cell subset was detected. Our data showed that post-therapy mutational targeting in RGYW/WRCY motifs were significantly increased as compared with that of pre-treatment (27% before rituximab vs. 43% after therapy, *P*=0.0003) indicating that affinity maturation may operate differently in class-switched memory B cells before and after B cell depletion. These results indicate a normal development process with an unimpaired mechanism of

mutational acquisition in class-switched memory B cells. These data argue for different requirements to undergo somatic hypermutations in IgD^+ memory B cells in comparison to class switched memory B cells.

To conclude, our work has demonstrated for the first time a delayed acquisition of somatic hypermutations at single Ig receptor V_H gene rearrangements of IgD^+ memory B cells in comparison to class-switched memory B cells. These results demonstrate that IgD^+ memory B cells are particularly susceptible to anti-CD20 treatment in patients with rheumatoid arthritis. In addition antigenic pressure and/or selection are substantially reduced by rituximab therapy which is basically not seen in the class-switched memory compartment. These data are in line with the hypothesis that IgD^+ memory B cells have distinct requirements for activating their mutational machinery compared to class-switched memory B cells which recover normal mutations during regeneration phase. The results have implications in understanding the pathophysiology of memory B cell in rheumatoid arthritis and may be helpful in designing new targeted therapies.

Zusammenfassung

B-Lymphozyten leisten unterschiedliche Beiträge zur Pathophysiologie der Rheumatoiden Arthritis. Sie produzieren Autoantikörper, präsentieren Autoantigene und schütten verschiedene Zytokine, die am proinflammatorischen Prozess beteiligt sind, aus. Aufbauend auf diesen Ergebnissen wurden in den letzten Jahren Therapien entwickelt, die gezielt B-Lymphozyten ansteuern um direkt oder indirekt in den autoimmunen Krankheitsverlauf einzugreifen. Die zeitlich begrenzte B-Zell-Depletion mit Rituximab (anti CD20-Antikörper) hat dabei in den letzten Jahren einen hohen Stellenwert erlangt und wird im klinischen Alltag insbesondere bei der Behandlung von Patienten mit rheumatoider Arthritis angewandt.

Rituximab induziert im peripheren Blut bemerkenswerte Veränderungen in der Homöostase der B-Zell-Subpopulationen. Nach Therapie mit dem anti-CD20 Antikörper Rituximab beginnt die Repletionsphase mit der peripheren Aussaat von transitionalen unreifen B-Zellen. Im weiteren Verlauf kommt es zu einer Normalisierung des naiven B-Zell-Pools. Das B-Zell Gedächtnis und in besonderem Maße die IgD^+CD27^+ Gedächtniszellen erholen sich nach Therapie nur langsam. In einer prospektiven klinischen Studie hat unsere Arbeitsgruppe gezeigt, dass die Gesamtzahl der Gedächtniszellen gut mit der Dauer der klinischen Antwort auf Rituximab korreliert. Es ist wenig über die speziellen molekularen Veränderungen innerhalb der Gedächtnis B-Zellen nach Rituximab Therapie bekannt. Um die Veränderungen im peripheren Blut zu verstehen untersuchten wir die somatische Mutationsfrequenz und das Muster der Ig-VH3 Gen Rearrangements, indem wir prä- und posttherapeutisch bei 18 Patienten einzelne B-Zellen isolierten und den individuellen B-Zellrezeptor durch eine Einzelzell RT-PCR amplifizierten und sequenzierten. Wir verglichen das Mutationsmuster nach erfolgreicher B-Zelldepletion in den neu rezirkulierenden Gedächtnis B-Zellen mit dem Mutationsmuster von vier Gesunden Blutspendern und sechs nicht-RA Patienten, die eine Hochdosis Chemotherapie mit anschließender autologer oder allogener Stammzelltransplantation erhalten hatten.

Zunächst haben wir die Zusammensetzung der Gedächtniszellen im peripheren Blut analysiert. Der Phänotyp der peripheren prä-switch (IgD^+CD27^+) und post-switch (IgD^-CD27^+) Gedächtniszellen zeigte keine quantitativen Unterschiede in RA-Patienten im Vergleich zu Gesunden. Bei der direkten Analyse des B-Zell Immunglobulin Rezeptors fanden sich jedoch zwischen klassengeswitchten und ungeswitchten Gedächtnis B-Zellen

signifikante Unterschiede in der Anzahl der Mutationen in der variablen Region der Ig Rezeptors. Die Population der IgD^+CD27^+ Gedächtniszellen beinhaltete sowohl nicht mutierte, wenig mutierte und stark mutierte (Median= 9 Mutationen pro Sequenz) rearrangierte Ig- Rezeptoren, wohingegen die IgD^-CD27^+ Gedächtniszellen einen durchgehend hoch mutierten (Median = 18 Mutationen pro Sequenz) Rezeptor aufwiesen. Der Unterschied zwischen beiden Gruppen war signifikant (Mutationsfrequenzen 3.83±0.19% vs. 7.1±0.53%; *P*=0.0001).

Grundlegende Veränderungen wurden bei den rezirkulierenden ungeswitchten Gedächtniszellen (IgD^+CD27^+) nach vorübergehender B-Zell Depletion mit Rituximab festgestellt. Diese Zellen wurden bis 6 Jahre nach Rituximab beobachtet und zeigten eine stark verzögerte Zunahme an Mutationen im Ig-Rezeptor. Ein Jahr nach einmaliger Gabe von Rituximab waren 84% der einzelnen zirkulierenden $IgD^+/CD27^+$ B-Zellen unmutiert. Zu diesem Zeitpunkt fanden sich keine stark mutierten Ig-VH3 Gen Rearrangements (*P*=0.0001). Mit zunehmendem Abstand zur B-Zell depletierenden Therapie konnten in der Repopulationsphase zunehmende Zahlen an Mutationen in den B-Zell Ig Rezeptoren festgestellt werden. Beispielsweise waren während des 2. Jahres der Regeneration (*P*=0.0001) 7.8%, sowie nach 4 Jahren nur 14% der Ig Rezeptoren mutiert. Sogar 6 Jahre nach Behandlung, waren VH Mutationen in IgD^+ Gedächtniszellen noch deutlich vermindert. Selbst nach dieser Zeit fanden sich in der prä-switch Gedächtnispopulation nur 27% hochmutierte Sequenzen während vor der passageren B-Zelldepletion 52% ein hohe Zahl an Mutationen trugen (*P*=0.0001). Die posttherapeutische Analyse der CDR3 Länge der regenerierten IgD^+ Gedächtniszellen ergab eine erhöhte CDR3 Länge, die signifikant mit der Anzahl der nicht mutierten VH Genrearrangements während der Repletionsphase korreliert. Interessanterweise regenerierten Patienten nach Hochdosis Chemotherapie und allogener Stammzelltransplantation ihre IgD^+ Gedächtniszellen mit einer deutlich höheren Anzahl an Mutationen. Ein Jahr nach Transplantation zeigten die Ig Rezeptoren schon 22% hoch mutierte und 42% unmutierte VH Rearrangements. Das zeigt, dass eine gegen CD20 gerichtete Behandlung nicht nur eine Verzögerung der Produktion der ungeswitchten Gedächtniszellen zur Folge hat, sondern darüber hinaus einen signifikanten Effekt auf die Mutationsrate im präswitch Gedächtnis B-Zellpool besitzt.

Im Gegensatz zum Mutationsmuster der IgD^+ Gedächtniszellen regenerierten die klassengeswitchten Gedächtniszellen nach anti-CD20 Depletion im peripheren Blut mit

quantitativ normalen Mutationen im Ig Rezeptor. Interessanterweise fand sich allerdings eine Änderung der exprimierten Isotypen mit deutlicher Dominanz IgA exprimierender B Zellen. Weitere Analysen der klassengeswitchten Gedächtnis B-Zellen zeigen außerdem eine Therapie induzierte qualitative Veränderung dieses B-Zellpools. So waren posttherapeutisch die Mutationen in bestimmten T-Zell abhängigen Mutationshotspots, dem RGYW/WRCY Motiv, signifikant vermehrt (Mutationstargeting vor Therapie 27% vs. 43% nach Rituximab, P=0.0003). Dies weist darauf hin, dass die Mechanismen der Affinitätsreifung im klassengeswitchten B-Zellgedächtnis vor und nach B-Zelldepletion unterschiedlich funktionieren. Der Mutationsmechanismus selbst ist allerdings in diesen Zellen quantitativ nicht eingeschränkt.

Zusammenfassend zeigt unsere Arbeit zum erstem mal, dass es nach einer passageren B-Zelldepletion mit anti-CD20 Antikörpern zu einer über Jahre hinweg nachweisbaren ausgeprägten Verzögerung in der Aquisition von somatischen Mutationen in rearrangierten VH Genen der IgD^+ Gedächtniszellen kommt. Demgegenüber erholt sich das klassengeswitchte B-Zellgedächtnis mit uneingeschränkter Zahl von Mutationen im Ig Rezeptor. Diese Resultate zeigen, dass anti-CD20 gerichtete Therapien in besonderem Maße IgD^+ Gedächtniszellen beeinflussen. Der Selektionsdruck durch Antigene und/oder die Selektion der Ig Rezeptoren erscheint unter diesen Bedingungen speziell bei IgD-Gedächtnis B-Zellen reduziert. Die Daten unterstützen die Hypothese, dass prä-switch Gedächtnis B-Zellen im Vergleich zu post-switch Gedächtnis B-Zellen andere Bedingungen für die Aktivierung der Mutationsmaschinerie benötigen. Die Resultate eröffnen neue Wege für das Verständnis der Pathophysiologie der B-Zell Gedächtnisentwicklung und können helfen neue zielgerichtete Therapien zur Behandlung von Autoimmunerkrankungen zu konzipieren.

Abbreviations

ACR	american college of rheumatology
ADCC	antibody dependent cellular cytotoxicity
ADA	Adalimumab
AID	activation induced cytidine deaminase
ANCA	anti-neutrophils cytoplasmic antibody
APC	antigen presenting cell
BAFF	B cell activating factor
BCR	B cell receptor
BCMA	B cell maturation antigen
BLyS	B lymphocyte stimulator
bp	base pair
C	constant
cDNA	complementary DNA
CDC	complement dependent cytotoxicity
CDR	complementary determining region
CSR	class switch recombination
CZP	Certolizumab
D	diversity gene
DAS	disease activity score
DMARD	disease modifying anti rheumatic drug
DNA	deoxyribonucleic acid
DSB	double strand break
ETN	Etanercept
FCS	fetal calf serum
FR	framework region
FACS	fluorescence activated cell sorting
GC	germinal centre
GLM	Golimumab
HLA	human leukocyte antigen
IL	interleukin
IFN-γ	interferon gamma
IFX	Infliximab
Ig	immunoglobulin

Ig-H	immunoglobulin heavy chain
IgL	immunoglobulin light chain
IgR	immunoglobulin receptor
IL	interleukin
ITP	immune thrombocytopenia purpura
J	joining gene
Jk	kappa light chain joining gene
Jλ	lambda light chain joining gene
MTX	Methotrexate
mRNA	messenger RNA
PB	peripheral blood
PBMCs	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PEG	polyethylene glycol
R	purine
RA	rheumatoid arthritis
RAG	recombination activating gene
RF	rheumatoid factor
R/S	replacement to silent mutation
RT-PCR	reverse transcriptase polymerase chain reaction
RTX	Rituximab
SCT	stem cell transplant
SHM	somatic hypermutation
SLE	systemic lupus erythematosus
TD	T cell dependent
TI	T cell independent
TLR	toll like receptors
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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Publications

1. Original articles

- **Delayed acquisition of somatic hypermutations in repopulated IgD^+CD27^+ Memory B cell receptors after rituximab**
  Muhammad K., Roll P., Hermann E., Dörner T. and Tony H-P
  (Manuscript accepted in Arthritis&Rheumatism)

2. Published abstracts

- **Muhammad K.,** Roll P., Palanichamy A., Dörner T. and Tony H-P. Repopulation of IgD^+CD27^+ memory B cells after Rituximab: Delayed acquisition of mutations in single B cell Ig receptors
  Annual meeting American College of Rheumatology October 24-29, 2008 San Francisco, USA

- **Muhammad K.,** Dörner T. and Tony H-P. Different molecular impact of anti-CD20 mediated B cell depletion versus allogeneic stem cell transplant on Ig receptors expressed by IgD^+ memory B cells
  5^{th} spring School in Immunology March 1-6, 2009, Ettal, Germany

3. Posters and oral presentations at congresses and symposia

- **Muhammad K., Palanichamy A., Roll P. and Tony H-P**. Development of memory B cells after anti CD20 mediated B cell depletion in patients with Rheumatoid arthritis (poster presentation). 6th Joint retreat "Immunmodulation meets lymphocyte activation'' July 24-26, 2006 Markttaschendorf, Germany.

- **Muhammad K., Roll P. and Tony H-P**. Development of memory B cells after anti CD20 mediated B cell depletion in patients with Rheumatoid arthritis (oral presentation). Annual retreat 2007 Graduate College Immunmodulation, 14-16 may 2007, Kloster Banz, Germany.

- **Muhammad K., Roll P and Tony H-P.** Development of Ig D memory B cells after anti CD20 mediated B cell depletion in patients with Rheumatoid arthritis (poster presentation). Annual retreat 2008 Graduate college Immunmodulation, July 6-8, 2008, wildbad, Germany.
- **Muhammad K., Roll P., Palanichamy A., Dörner T. and Tony H-P**. Repopulation of IgD^+CD27^+ Memory B Cells after Rituximab: Delayed Acquisition of Mutations in Single B cell Ig Receptors (poster presentation). Annual Scientific ACR meeting October 24-29, 2008, San Francisco, USA.
- **Muhammad K.,** Dörner T. and Tony H-P. Different molecular impact of anti-CD20 mediated B cell depletion versus allogeneic stem cell transplant on Ig receptors expressed by IgD^+ memory B cells (poster presentation). 5^{th} spring School in Immunology March 1-6, 2009, Ettal, Germany
- **Muhammad K.** and Tony H-P. Different molecular impact of anti-CD20 mediated B cell depletion versus stem cell transplant on BCRs expressed by IgD^+ memory B cells (poster presentation). 4^{th} international symposium March 26-27, 2009, Würzburg, Germany