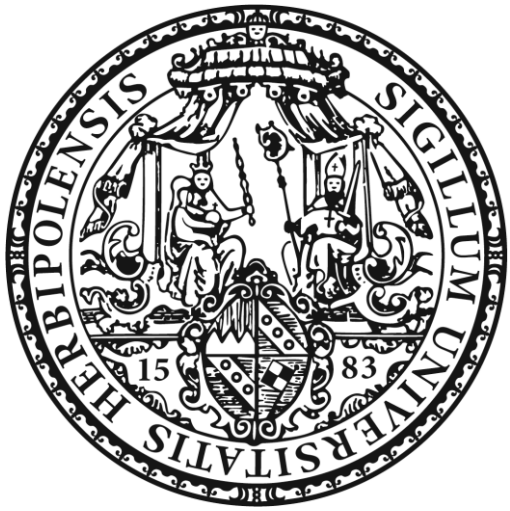

Effect of cytokine inhibition on peripheral memory B cells in patients with rheumatoid arthritis

Auswirkung der Zytokinhemmung auf periphere Gedächtnis B-Zellen in Patienten mit rheumatoider Arthritis

Dissertation towards a doctoral degree at the Graduate School of Life Sciences

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Contents

1. Introduction

Adaptive immune responses are of two types, called cell-mediated immunity and humoral immunity, which are mediated by different components of the immune system. Cell-mediated immunity is mediated by T lymphocytes (T cells) and humoral immunity is mediated by molecules in the blood and mucosal secretions, called antibodies. These molecules are produced by cells called B lymphocytes (B cells). A simple definition of B lymphocytes are cells that express clonally diverse cell-surface immunoglobulin (Ig) receptors capable of recognizing specific antigens (Ags) and with their functional end point being antibody production by terminally differentiated plasma cells (Engel et al., 2011; LeBien and Tedder, 2008). The discovery of B cells and their characteristic description were between the 1960s and 1970s based on experimental animal models and clinical evaluation of patients by cellular surface molecule characterization (Cooper et al., 1965; Cooper et al., 1966; Good and Zak, 1956; Klein et al., 1998). During the last decade it has become increasingly apparent that B cells exert important regulatory roles far more than just as passive precursors of antibody secreting cells. B cells are phylogenetically considered the most recent evolutionary development in the immune system (Engel et al., 2011; Martinez-Gamboa et al., 2006). They play an essential role in regulating immune responses and dysregulation of B cell responses may consequently lead to an attenuated immune response and development of autoimmune diseases.

1.1 B cell development in human

B cells and their antibodies are the central elements of humoral immunity and protect, as part of the adaptive immune system, against an almost unlimited variety of pathogens. Defects in B cell development, selection and function can lead to autoimmunity, malignancy, immunodeficiency and allergy (Pieper et al., 2013). From the very onset of life, B cell development is a highly regulated process whereby functional peripheral subsets are produced from hematopoietic stem cells (HSCs) in the fetal liver before birth and in the bone marrow afterward in multiple phases. Common lymphoid progenitor (CLP) cell from HSCs undergo a sequential set of differentiation events under the influence of microenvironment signals. The secreted signals from stromal cells in the bone marrow microenvironment are responsible for early B cell development (Hardy and Hayakawa, 2001). Later B cell precursor migrate to peripheral lymphoid organs till they become naïve/mature B cells (Hardy and Hayakawa, 2001; LeBien, 2000; LeBien and Tedder, 2008).

After encountering antigen, binding of antigen to B cell receptors (BCR) forms a complex (antigen:BCR). The Ag-BCR complex is internalized, processed and presented as an antigen-MHC class II complex to T cells. The encounter with antigen specific T cells causes co-stimulation of the B cell which further develops into a memory B cell or plasma cells to turn into a continuous source of antibody production (Janeway C et al., 2005; LeBien and Tedder, 2008).

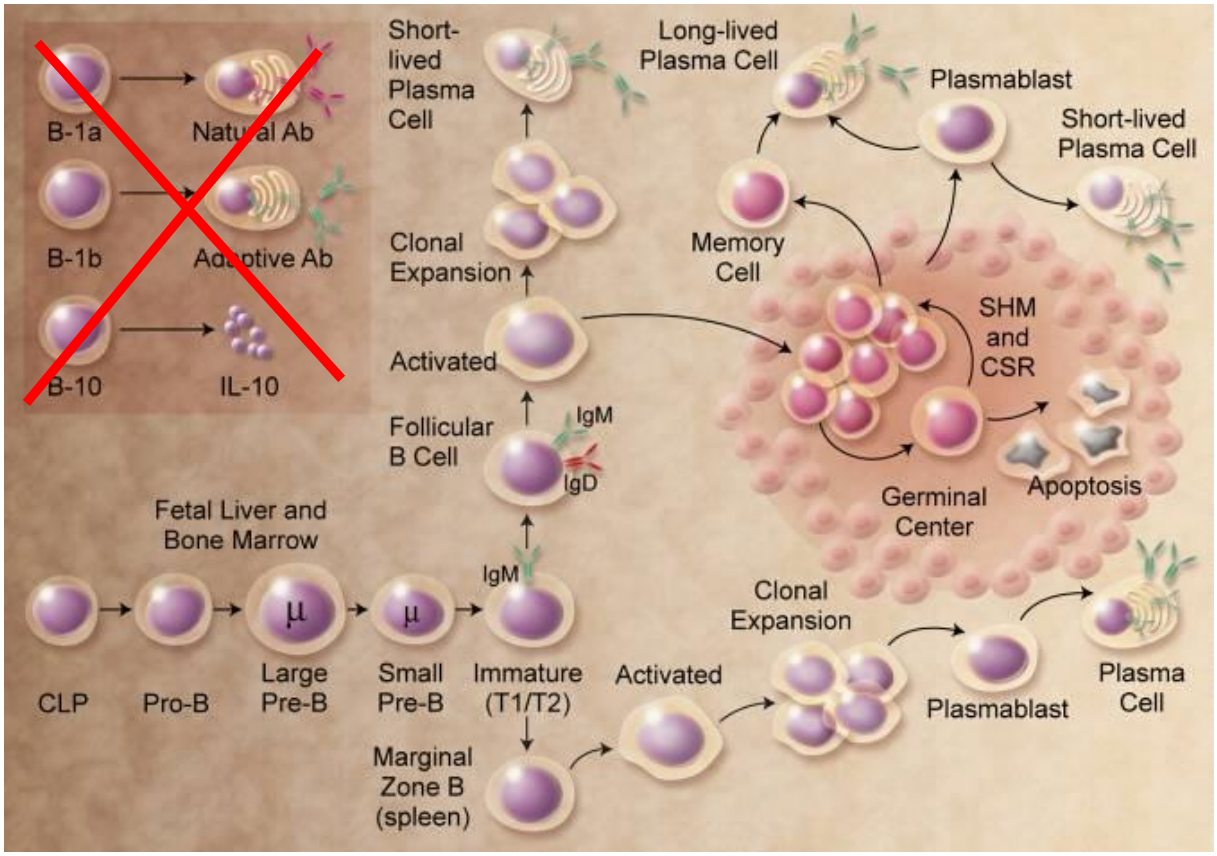

Figure 1.1 B-cell development. The figure shows the broad outline of B-cell developmental stages in mice and humans.CLP indicates common lymphoid progenitor; SHM, somatic hypermutation; and CSR, class switch recombination. (adopted from LeBen et al. 2008)

B cells pass through two main stages of development: antigen-independent (Pro B cell, Pre B cell, immature B cell and naïve B cell in the BM) and antigen-dependent (B cell activation, memory B cell and plasma B cell, in primary lymphoid organ) (LeBien and Tedder, 2008). The functional rearrangement of the Ig loci is a *sine qua non*, which occurs via an error-prone process involving the combinatorial rearrangement of the V, D, and J gene segments in the H chain locus and the V and J gene segments in the L chain loci (Brack et al., 1978).

In the antigen-independent stage the B cell repertoire is built by V(D)J rearrangement. The pro-B cell is the earliest lineage of B cells in which rearrangements of heavy chain segments takes place. During the late pro B cell stage variable V_H segment rearrangements with the D_HJ_H segment IgH chain occur. The recombination activation genes products, RAG-1 and RAG-2, play a fundamental role in these events and are very crucial for the development of BCR recombination (Kawano et al., 2006).

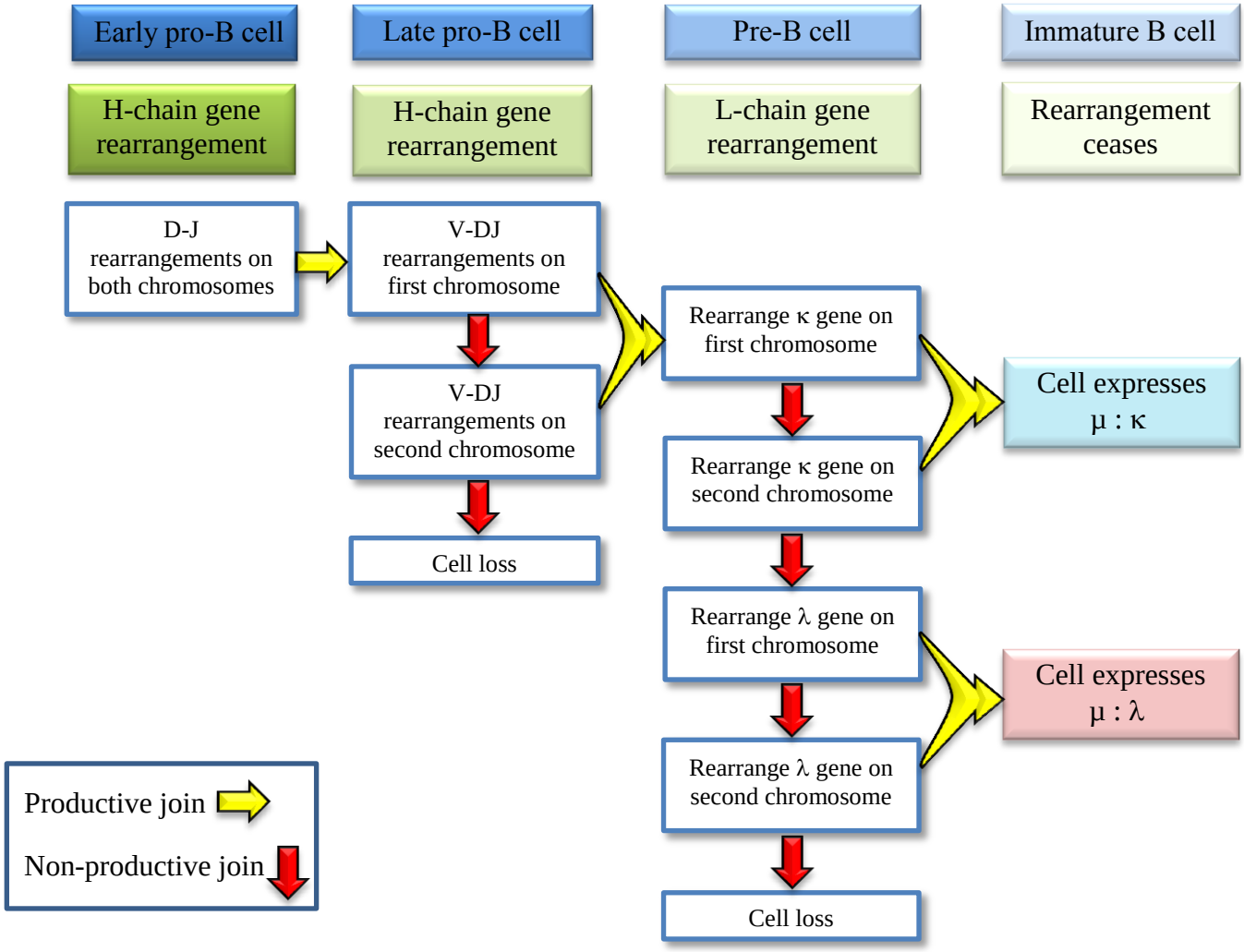

Figure 1.2 B cell development and checkpoints: The steps of B cell development involving checkpoints for generation of productive immunoglobulin gene rearrangement (adopted from immunolobiology, Janeway 2008).

A productive VDJ_H joining leads to the next B cell development stage called pre B cell stage, where μ heavy chains combine with VpreB/λ5 surrogate light chains to form the pre-B cell receptor (pre-BCR) (Kawano et al., 2006; Tsubata and Reth, 1990).

Pre BCR provides signals to proceed with light chain rearrangement. There are several control steps to make sure that B cells are not self-reactive. The pre BCR formation serves as checkpoint for positive selection of pro B cells because if the B cell fails to express the receptor, it ultimately causes cell apoptosis (Karasuyama et al., 1996; Kawano et al., 2006). Upon completion of light chain rearrangement, the IgM molecule is expressed on cell surface which defines it as an immature B cell (Fig. 1.2).

The immature B cell eventually migrates to the periphery and differentiates into mature/naïve B cell carrying IgM and IgD on the cell surface. In the peripheral lymphoid organs, the B cell encounters antigens and B cell activation takes place with further differentiation into antibody secreting plasma or memory B cells.

1.2 B cell Activation

B cells are the main component of the adaptive immune system, providing both specific and long-lasting protection from an enormously wide range of potential pathogens. B cell activation is initiated following the recognition of antigen through the B cell receptor (BCR) and results in B cell proliferation and differentiation. Activated B cells can differentiate to form either plasma cells capable of antibody secretion or memory cells that provide long-lived protection against secondary infection. The importance of these effector cells in enhancing an individual's survival requires that the processes underlying B cell activation be both highly coordinated and precisely regulated (Harwood and Batista, 2010).

1.3 Somatic hypermutation

Somatic hypermutation (SHM) is the process by which the immunoglobulin (Ig) repertoire achieves a functional diversification through alterations of the Ig locus. SHM deposits point mutations into the variable region of the Ig gene to generate higher-affinity variants. Therefore SHM fulfills a central role in antibody affinity maturation. Activation-induced cytidine deaminase (AID) is a key enzyme in initiating the hypermutation process in which error-prone versions of DNA repairs results in a diverse spectrum of point mutations (Di Noia and Neuberger, 2007; Teng and Papavasiliou, 2007).

1.3.1 Sites of somatic hypermutation

Germinal Centers (GCs) are the main sites identified to be a specialized micro-environment where antigen-driven somatic hypermutation (SHM) of the genes encoding the immunoglobulin variable region (IgV) occurs. After antigenic stimulation, B cells migrate through the helper T cell rich areas of secondary lymphoid organs (e.g. spleen and lymph nodes) to form germinal centers (Klein et al., 1998). SHM of IgV gene occurs in the dark zone of GCs where base pair mutation leads to the change in amino-acid sequence. From the paper of Klein et al 2008, a mechanistic approach to understand SHM is briefly described below.

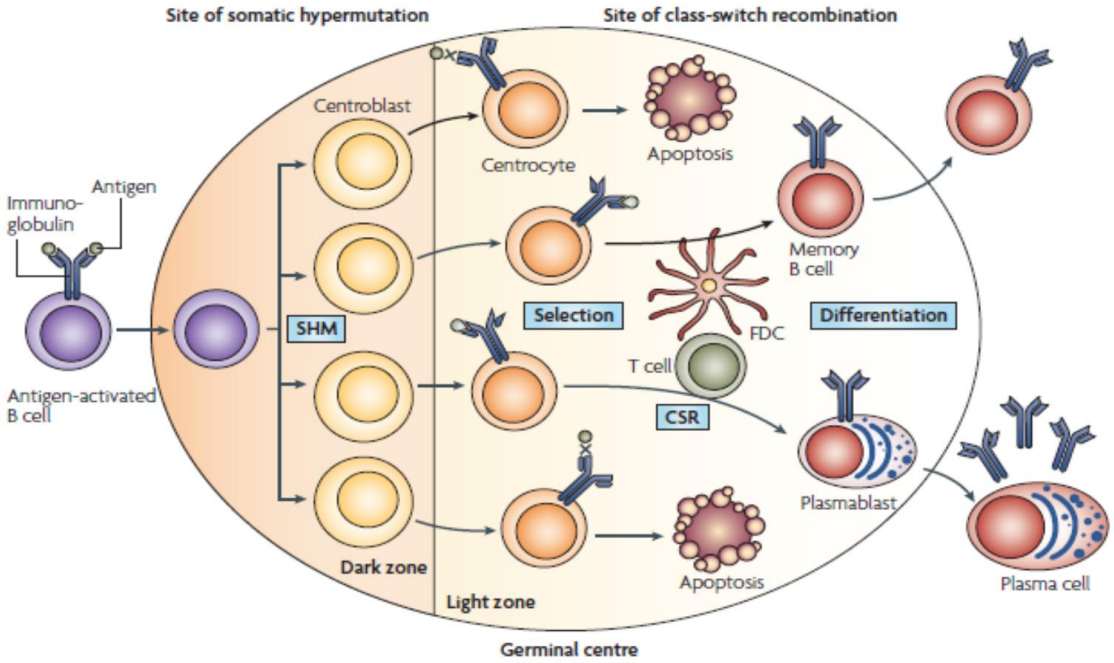

Figure 1.3 Germinal centre microenvironment. Antigen-activated B cells differentiate into centroblasts that undergo clonal expansion in the dark zone of the germinal center. During proliferation, the process of somatic hypermutation (SHM) introduces base-pair changes into the V(D)J region of the rearranged genes encoding the immunoglobulin variable region (IgV) of the heavy chain and light chain; some of these base-pair mutations lead to a change in the amino-acid sequence (Klein et al., 1998).

T cell dependent hypermutation of B cells is thought to occur in the GCs formed by follicular B cells. However, several studies also provide evidence for SHM outside GCs. Animals such as frogs and sharks that normally do not have GCs have SHM (Diaz and Flajnik, 1998). Furthermore, mice lacking TNF-receptor1 that are unable to form GCs, nevertheless produce high affinity antibodies (Kim and Berek, 2000).

1.4 Class-switch recombination

Class-switch recombination (CSR) is the process by which antibody class switching occurs in mature B cells in response to antigen stimulation and co-stimulatory signals. Ig isotype switching occurs by a unique type of a intra-chromosomal deletional recombination event within special G-rich tandem repeated DNA sequences called switch, or S, regions located upstream of each of the heavy chain constant (CH) region genes, except Cδ. The recombination is initiated by the B cell–specific AID, which deaminates cytosine in both the donor and acceptor S regions (Stavnezer et al., 2008). Class-switching is an irreversible recombination of DNA. During this process exchanges constant regions of the heavy chains are exchanged but the variable regions are left intact (Klein and Dalla-Favera, 2008).

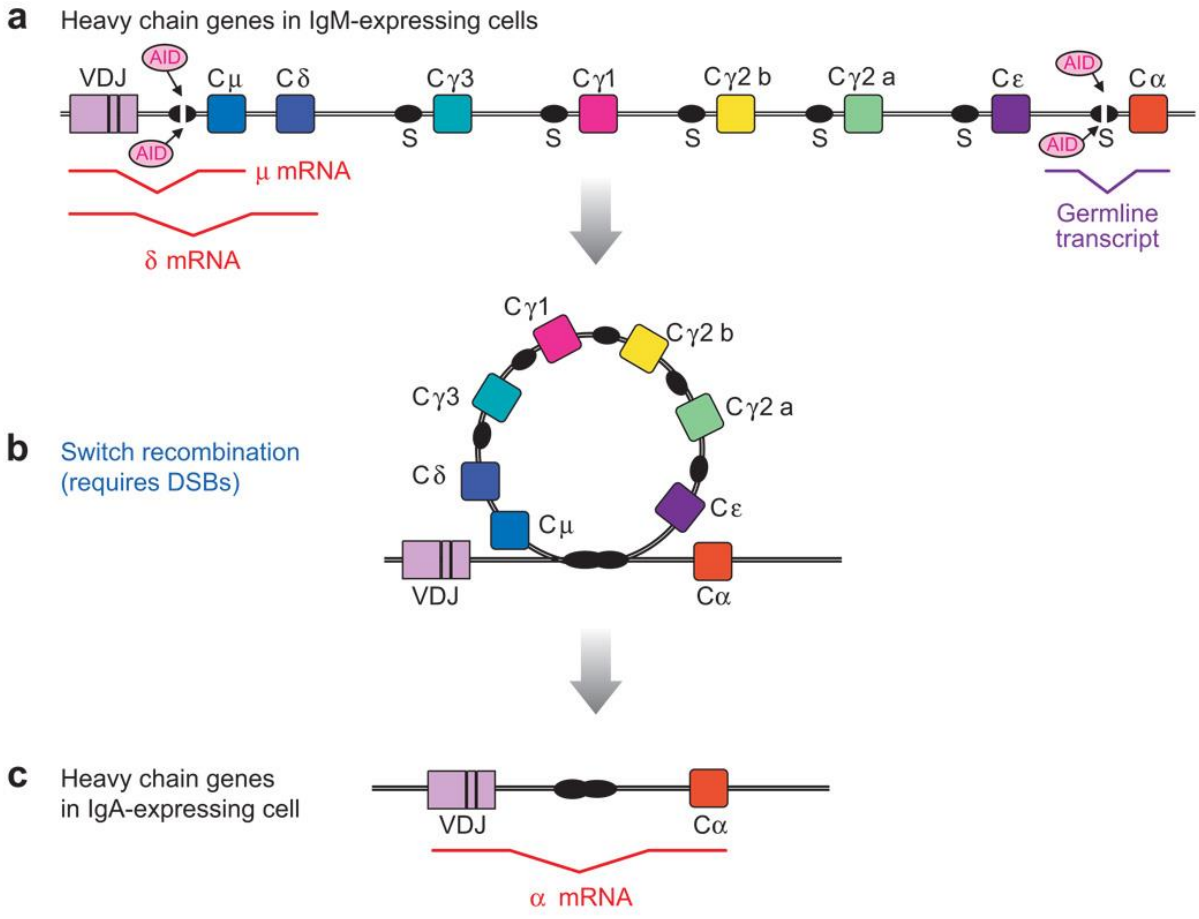

Figure 1.4 Class-switch recombination: Ig class-switch recombination CSR to IgE. Adopted from Janet Stavnezer, Trends in immunology, (Stavnezer, 2011).

1.5 Immunoglobulins

There are five different immunoglobulin classes (isotypes) of antibody molecules: IgG, IgM, IgA, IgE and IgD. They are distinguished by the type of heavy chain they contain. IgG molecules possess heavy chains known as γ-chains; IgMs have μ-chains; IgAs have α-chains; IgEs have ε-chains; and IgDs have δ-chains. The variation in heavy chain polypeptides allows each immunoglobulin class to function in a different type of immune response as effector functions, and can be either soluble or surface bound.

The Ig classes (isotypes) are briefly described here and adapted from Murphy, K. *et al.* (Janeway's Immunobiology, 8th Ed., Garland Publishing 2011)

IgD

IgD is found together with IgM on the cell membrane of naïve B cells where it functions as a receptor for antigen. Soluble IgD also exists but with unclear role and currently thought to have no function in defense.

IgM

IgM is the first Ig to be upregulated on the surface of B cells in bone marrow and is also always the first Ig to come up during the immune response. It is in a pentameric form when secreted but when attached to the cell surface it is a monomer. The ability of IgM to activate complement is strong in the classical pathway of complement activation.

IgG

IgG is the standard model antibody, appearing later during an immune response than IgM. Four subclasses of IgG exist (IgG1-IgG4), of which IgG1 and IgG3 efficiently activate complement. IgG is usually a high affinity antibody and is the only class of antibodies transported across the placenta.

IgE

IgE is present in plasma only in small amounts as most of it is tightly bound by the high-affinity Fc-ε-receptor of mast cells. It is involved in immediate hypersensitivity reactions and sits in connective tissue below outer and inner surfaces, e.g. skin, gut and bronchi.

IgA

IgA is also usually a high affinity antibody and the B cell needs to go through affinity maturation for this Ig to be produced. There is a distinct role for IgA in mucosal immunity i.e. in the gastrointestinal tract and the respiratory tract. IgA can be found as a monomer in the blood but its main function is to protect "outer" epithelial surfaces.

1.6 Peripheral B cell

After passing through all checkpoints during development, B cells finally reach the peripheral blood. On the basis of surface marker expression peripheral B cells are characterized into different subpopulations. Human peripheral memory B cells are mainly discriminated from naïve B cells by the phenotypic expression of CD27 (a member of TNFR family) and presence of somatic hypermutation (SHM) in their Ig variable genes (Goodwin et al., 1993; Klein et al., 1998). The main four subpopulations of B cells in peripheral blood are represented in a FACS plot in Figure 1.4.

a. Naïve B cells: $CD19^{+}$ IgD^{+} $CD27^{-}$
b. Pre-switch memory B cells: $CD19^{+}$ IgD^{+} $CD27^{+}$
c. Post-switch memory B cells: $CD19^{+}$ IgD^{-} $CD27^{+}$
d. Double negative memory B cells: $CD19^{+}$ IgD^{-} $CD27^{-}$

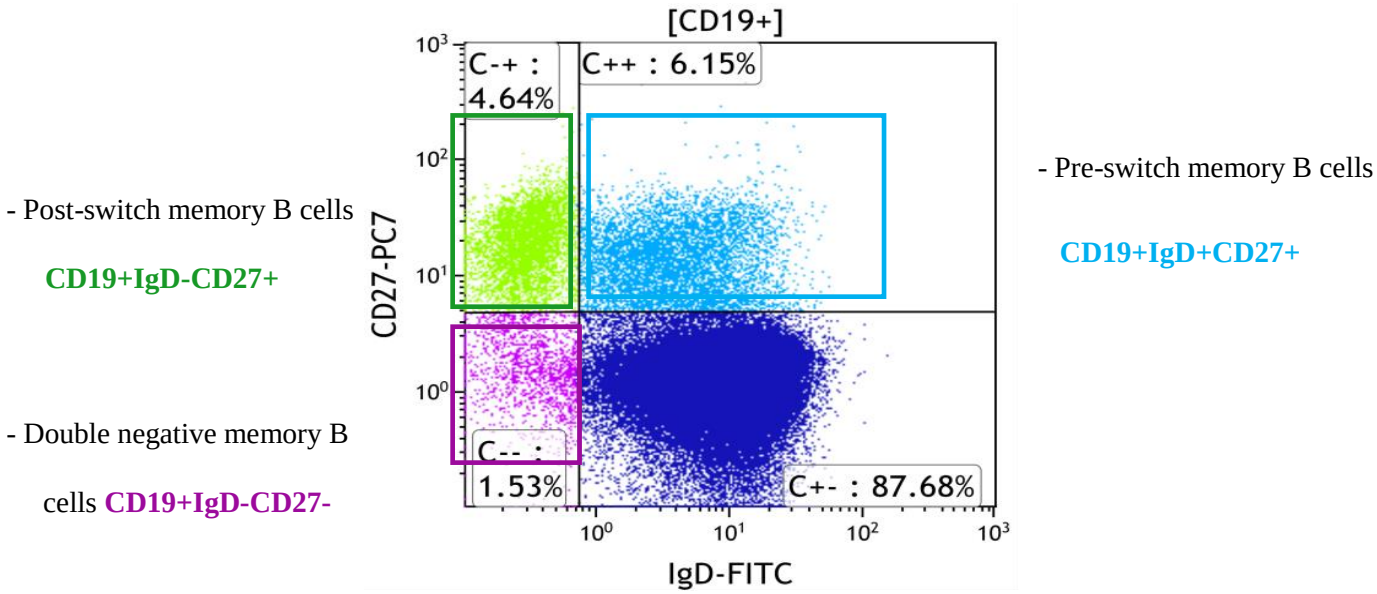

Figure 1.5 Representation of B cell subpopulations on the basis of classical markers CD27 and IgD.

Naïve mature B cells are B cells that have not encountered antigens and have an unmutated Ig receptor V gene. Memory B cells are mature B cells that have encountered antigens and have a somatically mutated Ig receptors.

CD27 surface expression is a hallmark of somatic hypermutation in memory B cells. CD27+ memory B cells are a heterogeneous population comprising mainly pre-switch (IgD+CD27+) and post-switch (IgD-CD27+) B cell subsets (Shi et al., 2003; Souto-Carneiro et al., 2009; Wang et al., 2013). A third population is the double negative (CD19 + CD27- IgD-) B cell population that bears signatures of memory B cells (Fecteau et al., 2006; Jacobi et al., 2008; Wei et al., 2007).

A very large proportion of double negative (CD27-IgD-) DN B cells express mutated Ig receptors. The evaluation of telomere length, the expression of the anti-apoptotic molecule Bcl2 and the absence of the ATP-binding cassette B1 transporter (ABCB1) have been used to distinguish them from naïve CD27- B cells and relate them to the memory B cell compartment (Colonna-Romano et al., 2009; Wirths and Lanzavecchia, 2005). Even though DN memory B cells mainly express switched Ig isotypes they have a reduced rate of SHM compared to post-switch B cells. This has been hypothesized to be due to either an impaired GC formation or resembling a distinct lineage of memory B cells (Buffa et al., 2011; Colonna-Romano et al., 2009). So far, the nature of DN B cells is still not fully delineated in general as well as in autoimmune disease.

2. Autoimmunity

Autoimmunity refers to a state of broken immune tolerance where the body's immune system fails to recognize its own cells and tissues as "self". It can appear when the mechanisms that control tolerance to self-Ags are unable to eliminate or inactivate all pathogenic auto-reactive B cells during lymphocyte production and maturation in order to prevent autoimmunity. The lack of immunological tolerance may support an attack against "self" through the production of auto-antibodies or autoimmune effectors T cells. Thus the body requires self-tolerance mechanisms to distinguish between self and non-self-determinants, in case to avoid autoreactivity (Janeway C et al., 2005). How immunological tolerance is regulated is only partially understood.

Due to the stochastic combinatorial events in the generation of specific Ag receptors, lymphocytes with receptors for self-antigens are generated in all individuals. These lymphocytes have to be controlled or eliminated in order to prevent autoimmunity. The principle mechanisms of immunological tolerance are divided into central and peripheral tolerance.

Central tolerance means a phenomenon where immature lymphocytes recognize with high affinity antigens in central (generative) lymphoid organs (thymus, bone marrow) as typically self-antigens. This results in killing of lymphocytes in the developmental stages by apoptosis and is the basis of negative selection (Goodnow et al., 2005). Other mechanisms of central tolerance render self-reactive lymphocytes harmless by receptor editing (B cells) or generation of regulatory T cells (CD4+ T cells) (Sakaguchi et al., 2008). If immature B cells recognize self-antigens that are present in high concentration in bone marrow, receptor editing is a major process to take place for eliminating self-reactivity from the B cell repertoire (Hogquist et al., 2005; Janeway C et al., 2005). If editing fails to eliminate autoreactivity, the immature B cells may die by apoptosis. The weaker recognition of self-antigens may lead to functional inactivation (anergy) of B cells rather than cell death (Giltiay et al., 2012), Abbas et al 2010).

Peripheral tolerance is most important for maintaining unresponsiveness to self-antigens that are expressed in peripheral tissues and not in the generative lymphoid organs (Janeway C et al., 2005); Abbas et al 2010). This is the phenomenon where mature lymphocytes recognize

antigens in peripheral tissues in many ways like anergy, suppression, exclusions from lymphoid follicles that lead to unresponsiveness or cell death (Muller and Nitschke, 2014). B cells that encounter self-antigens in the periphery are less able to migrate into lymphoid follicle than normal or naïve B cells (Ring and Lakkis, 1999) (Abbas et al 2010).

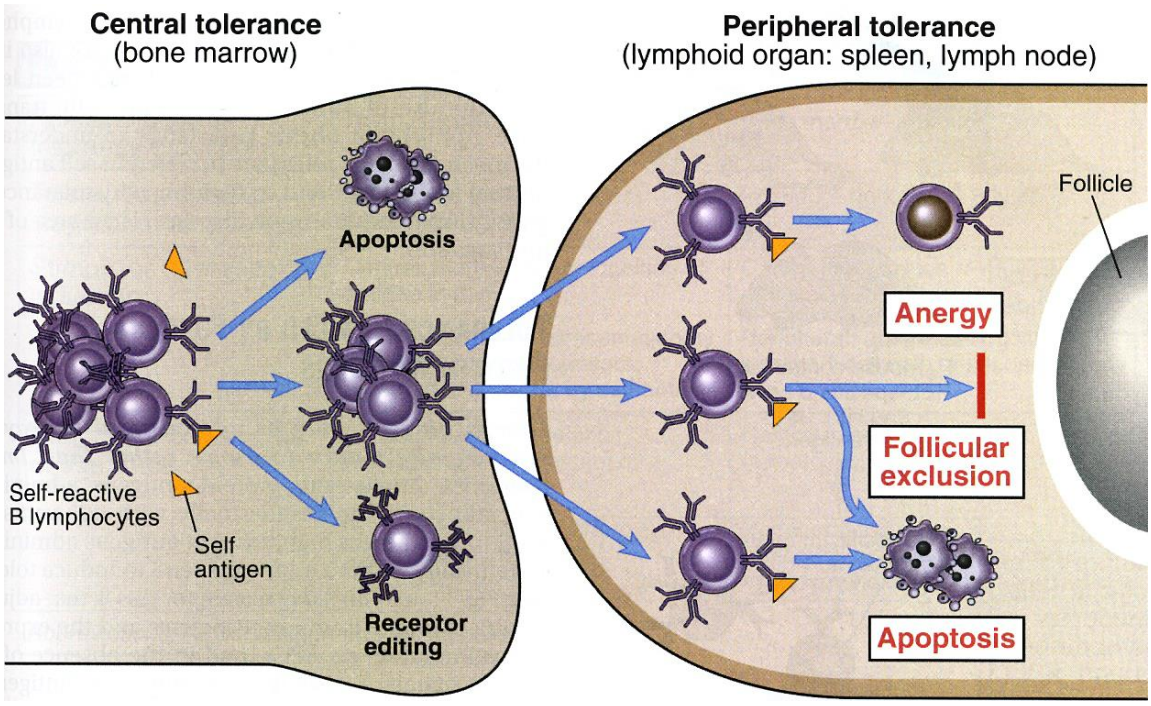

Figure 1.6 Central and peripheral tolerance in B cells. In bone marrow, immature B cells die by apoptosis or change the specificity by receptor editing on encounter with self-antigens. B cells that encounter self-antigens in the peripheral tissues become anergic, or excluded from follicles or dies by apoptosis. Adopted from Cellular and molecular immunology 6Th Edition (Abbas et al. 2010).

Failure in the mechanisms of tolerance to self-antigens results in development of autoimmune diseases caused by autoimmunity. Autoimmune disease may involve a single tissue or multiple organs and consequences of an autoimmune reaction may either be the loss of function or the hyper-activated function of the target tissue. Rheumatoid arthritis, SLE and Sjögren's disease are among some of the autoimmune diseases in human.

2.1 Rheumatoid arthritis

Rheumatoid Arthritis (RA) is a chronic, systemic, inflammatory, autoimmune disease characterized by inflammation of joints and resulting in their progressive destruction (Klareskog et al., 2009). RA affects approximately 1 % of people in the developed world, with three times as many women affected than men. It causes considerable morbidity, reduces quality of life, increases mortality and reduces life span by ~7 years (Pitzalis, 2014; Vivar and Van Vollenhoven, 2014). Joint inflammation is the hallmark of RA which comprises a syndrome of pain, stiffness, and symmetrical synovitis of diarthrodial joints (freely moveable joints such as the knee) that leads to articular destruction, functional decline. But RA is not just a disease of joints due to systemic inflammation which is also targeted in other organs. There is a substantial comorbidity in the cardiovascular, neurologic, and metabolic systems (Brennan and McInnes, 2008).

Based on the identification of rheumatoid factor in the serum of patients and identification of the rheumatoid factor as an autoantibody, RA was considered an autoimmune disease early on (Franklin et al., 1957). In 1998, it was focused that patients with RA produce other antibodies against peptides and proteins containing citrulline. These antibodies reconizing a cyclic citrullinated peptide proved to be of diagnostic value for RA (Schellekens et al., 1998; Schellekens et al., 2000). Anti-citrullinated peptide/protein antibodies (ACPA) are present in very early disease and are highly specific for RA which enables the clinician to distinguish two subclasses of patients with early RA (ACPA-positive and ACPA-negative). ACPA predict the development of more erosive disease progression as APCA-positive is strongly associated with more erosive RA (Nielen et al., 2004; van Venrooij et al., 2011). The testing for these antibodies has been included as a new serologic criteria published in 2010 RA classification citeria (Aletaha et al., 2010; Neogi et al., 2010; van Venrooij et al., 2011).

A Further research proposed that migration of inflammatory cells like T cells, macrophages and antibody producing cells to the joints will cause and propagate inflammation. The infiltration can be very extensive and many immune cells are recruited to the joint which makes it an ongoing process that causes destruction of the cartilage and bone.

The diagnosis of RA includes a list of criteria that needs to be fulfilled to have proper diagnosis (Aletaha et al., 2010). It includes swelling of both large and small joints, presence of autoantibodies in the blood and inflammatory markers like the ESR. There is a collaborative classification criteria formed by the American College for Rheumatology (ACR) and the European League Against Rheumatism (EULAR) as the year 2010.

For the classification as 'definite RA' requires the confirmed presence of synovitis in at least one joint and a total score of at least 6 from the individual scores in four domains: number and site of involved joints (range 0–5), serological abnormalities (range 0–3), elevated acute-phase response (range 0–1), and symptom duration (two levels; range 0–1) (Aletaha et al., 2010; Neogi et al., 2010; Vonkeman and van de Laar, 2013). The classification criteria are summarized in Table.

JOINT DISTRIBUTION (0-5)	
1 large joint	0
2-10 large joints	1
1-3 small joints (large joints not counted)	2
4-10 small joints (large joints not counted)	3
3 >10 joints (at least one small joint)	5
SEROLOGY (0-3)	
Negative RF **AND** negative ACPA	0
Low positive RF **OR** low positive ACPA	2
High positive RF **OR** high positive ACPA	3
SYMPTOM DURATION (0-1)	
<6 weeks	0
≥6 weeks	1
ACUTE PHASE REACTANTS (0-1)	
Normal CRP **AND** normal ESR	0
Abnormal CRP **OR** abnormal ESR	1

≥6 = definite RA

Table 2.1 The classification criteria in definite rheumatoid arthritis. The score are calculated on the basis of joint distribution, serology test (RF & ACPA), duration of symptom and acute phase reactant (CRP & ESR). The total score should be ≥6 for definite RA.

2.2 B cells in rheumatoid arthritis

Recent studies suggest that B cells play an important role in the development and progression of RA through several mechanistic pathways (Martin and Chan, 2006). By producing different inflammatory cytokines and autoantibodies they play important roles which may directly drive pathologic inflammation (Fillatreau, 2013).

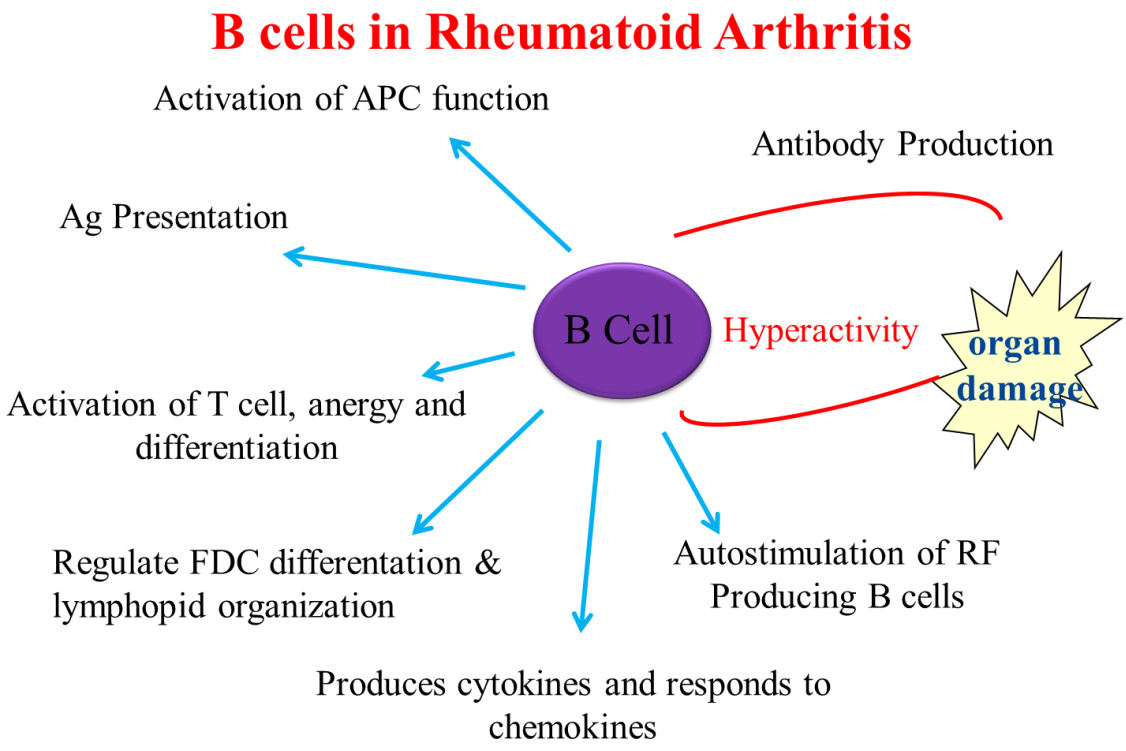

Figure 2.1 Role of B cells in rheumatoid arthritis. Major functions of B cells have been presented in graphical representation.

B cells are not limited to the production of antibodies rather they are involved directly and indirectly into many functions. B cells convey antibody production, antigen presentation, activation of antigen presenting cell (APC) function, activation of T cells and are involved in from anergy and differentiation. B cells regulate follicular dendritic cell (FDC) differentiation & lymphoid organization and produce of cytokines. Hyperactivity of B cells can be convicted to organ damage in RA patients.

2.3 Pathophysiology of RA and therapeutic targets

The pathophysiology of RA is complex and involving a lot of different cells, pro-inflammatory cytokines and chemokines are involved which allows different targets to therapy. Marked infiltration of mononuclear cells, especially T cells and macrophages into the synovial compartment leads to production of Interleukin (IL)-1, -2, -6, -8 and -10, tumor necrosis factor-α (TNF-α) and certain other factors. Another potential player from the lymphocyte pool of immune system, the B cell fraction, also infiltrates the synovium and differentiates into plasma cells producing polyclonal immunoglobulin, rheumatoid factor and ACPA (Lundy et al., 2007; Smeets et al., 1998; Weyand and Goronzy, 1997). These activities ultimately lead to pannus formation, cartilage invasion, bone erosion and destruction. Currently these factors are being targeted as therapeutics for RA and other autoimmune diseases by DMARDs (disease modifying anti-rheumatics drugs).

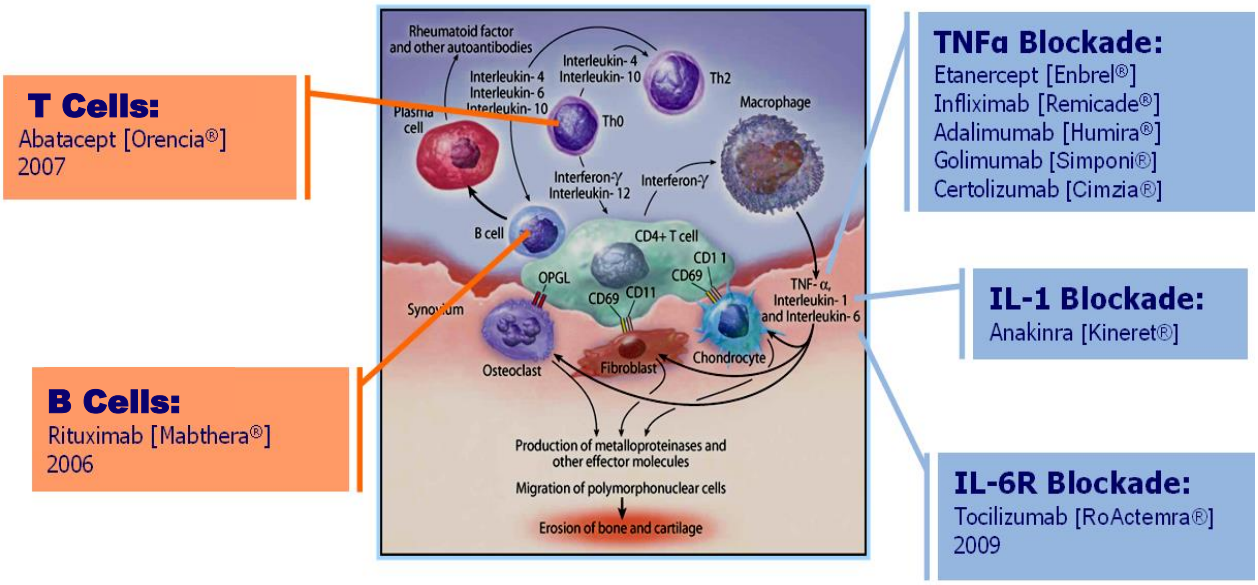

Figure 2.2 Pathophysiology: The illustrative diagram showing possible pathophysiology in RA and targeted therapies. (Adopted and modified from Choi et al, NEJM, 2008).

2.4 Therapy

So far there is no definitive cure for RA. The major goals of treatment are to relieve pain, minimize toxicity, preserve joint function and increase life expectancy**.** Early diagnosis and treatment proved to be very important to ensure for better outcome. Enhanced understanding of the pathogenesis of RA has directed to the development of targeted treatments. The current therapy management for RA is mainly based on the use of disease-modifying anti-rheumatic drugs (DMARDs).

DMARDs are those agents or drugs which reduce the disease progression or reverse the signs/symptoms, disability, inability to work and impairment of life towards better conditions. Other than glucocorticoids, DMARDs are categorized into two major classes, namely synthetic agents (sDMARDs) and biological agents (bDMARDs). sDMARDs are termed as conventional sDMARD (csDMARD) including methotrexate (MTX), leflunomide, sulfasalazine, cyclosporine and azathioprine. A new sDMARD (tofacitinib) specifically designed to target janus kinases (JAKs) is designated as a targeted sDMARD (tsDMARD) which is not yet available in German market. Biological DMARDs (bDMARDs) or biologics are at the current time the most promising drugs in the treatment of RA. Nowadays, a large variety of biological agents including TNF inhibitors, IL-1 & IL-6 inhibitors, T cell costimulation inhibition and anti-B cell agent are available for the treatment of RA. New agents are still under clinical trials and yet to be approved for treatment. Some of the major biologics are discussed in section Biologics in RA.

Glucocorticoids are also used in the treatment either alone or in combination with conventional sDMARDs. Glucocorticoids are a class of steroid hormones characterized by the ability to bind with the cortisol receptors and trigger various biological effects. They have been widely used in the treatment of RA as they have potent anti-inflammatory and immunosuppressive properties (Gabay et al., 2014; Quan et al., 2008).

With the availability of abundant variety of therapeutic options making treatment decisions in clinical practice remains still challenging and that need to be addressed (Smolen et al., 2014). The recent efficacy data were summarized in a systematic literature review by Nam et al. 2014 by addressing main areas of bDMARD use (Nam et al., 2014). They addressed bDMARD efficacy (in combination therapy with csDMARDs or as monotherapy), treatment strategies including bDMARDs, bDMARD stopping or dose reduction (Nam et al., 2014).

Some of the major recommendations for the management of rheumatoid arthritis with synthetic and biological DMARDs were 2013 updated in EULAR recommendations. There are some important points defined:

1. Therapy with DMARDs should be started as soon as the diagnosis of RA is made and should be aimed at reaching a target of remission or low disease activity in every patient.

2. There should be frequent (every 1-3 months) and strict monitoring to achieve remission otherwise therapy should be adjusted.

3. MTX should be part of the first treatment strategy in patients with active RA. Alternatively leflunomide or sulfasalazine can be considered as first treatment strategy.

4. If the treatment target is not achieved with the first sDMARD strategy then biological DMARD should be considered.

5. Current practice would be to start a TNF inhibitor which should be combined with MTX and when it has failed, patients should receive abatacept, rituximab or tocilizumab.

6. During adjusting therapy, factors apart from disease activity, such as progression of structural damage, comorbidities and safety concerns should be taken into account. (Smolen et al., 2014).

2.4.1 Biologics in RA

Currently we have cell-targeting agents (B & T cells) and cytokine-targeting agents (TNF, IL-6, IL1) available for treatment of RA.

Cell-targeting agents (B & T cells)

Abatacept

Abatacept is a fully human fusion protein comprising the extracellular domain of cytotoxic T lymphocyte associated antigen-4 (CTLA-4) linked to the Fc portion of human IgG. The antigen-specific interaction between antigen presenting cells (APCs) and T cells is normally accompanied by the binding of a ligand on the APC (CD80 or CD86) to the co-stimulatory receptor on T cells (CD28). This interaction is called co-stimulation and results in an effective T cell activation characterized by proliferation and production of inflammatory cytokines (IL-2, TNF). The inhibitory antigen CTLA-4 also binds to CD80 and CD86 with high affinity and inhibitory effect. Abatacept prevents T cell co-stimulation by binding with its CTLA-4 portion to CD80 and CD86 and blocking CD28-mediated T cell activation (Vivar and Van Vollenhoven, 2014). Abatacept is administered by infusions (once every 4 weeks) or subcutaneously (once every week). Both treatment options showed comparable efficacy and safety in patients with inadequate response to methotrexate (Genovese et al., 2011).

Rituximab

Rituximab is a chimeric murine/human monoclonal antibody directed against CD20, an antigen expressed by B cells at different stages of differentiation (pre-B cell to mature stages) but not by haematopoietic stem cells or plasma cells. It induces temporal peripheral depletion of B cells, which are involved in the production of autoantibodies as well as in the induction of T cell activation and production of pro-inflammatory cytokines (Dorner and Burmester, 2003). Its efficacy has been shown in patients who failed to respond to methotrexate or to one or more anti-TNF therapies after a single course of two infusions of rituximab (Cohen et al., 2006; Edwards et al., 2004). In addition, the presence of autoantibodies appears to favor clinical responses to rituximab, as indicated by studies showing a better effect among seropositive rheumatoid arthritis patients compared to seronegative rheumatoid arthritis patients (Chatzidionysiou et al., 2011; Lal et al., 2011). The effectiveness of rituximab was also reported in a refractory group of rheumatoid arthritis patients with long disease duration (Vivar and Van Vollenhoven, 2014).

Cytokine-targeting agents

Anti–Tumor Necrosis Factor

There are currently five TNF inhibitors licensed for the treatment of RA. Of these, three are full-length monoclonal antibodies (infliximab, adalimumab, and golimumab), one is a humanized antigen binding fragment (Fab) conjugated to polyethylene glycol (certolizumab) and one is a soluble fusion protein of TNFR p75 (etanercept).

Adalimumab

Adalimumab is a first fully human monoclonal antibody binding TNF. It is administered subcutaneously and has a longer half-life than etanercept (approximately 13 days) allowing a less frequent injection interval (every second week).

Infliximab

Infliximab is a chimeric murine/human IgG1 monoclonal antibody also directed against TNF (soluble and membrane bound), usually administered intravenously every 4-8 weeks. Randomized controlled trials have shown that infliximab in combination with methotrexate produced a rapid reduction of signs and symptoms, reduced radiographically measured disease progression and improved physical function (Lipsky et al., 2000; Vivar and Van Vollenhoven, 2014).

Etanercept

Etanercept, is a recombinant fusion protein, which links the soluble p75 TNF receptor to the Fc portion of human Immunoglobulin G (IgG). It works as a decoy receptor, binding to soluble TNF and blocking the binding to its receptor.

Golimumab

Golimumab is a human monoclonal antibody, binding to both soluble and membrane bound TNF. It is administered subcutaneously once a month and has been shown to be effective in the treatment of moderate to severe rheumatoid arthritis patients who failed to respond or were naïve to methotrexate.

Certolizumab pegol

Certolizumab pegol is a pegylated, humanized anti-TNF Fab fragment. Since it lacks the Fc portion, it does not induce apoptosis through complement activation or antibody-dependent cell-mediated cytotoxicity (ADCC). The pegylation process (addition of polyethylene glycol) delays the elimination of this small antibody-derived protein, prolonging its half-life (approximately 14 days).

Anti–IL-1

Anakinra

Anakinra, a recombinant human IL-1 receptor antagonist, has a very short half-life (4-6 hours) and must be administered subcutaneously once a day. This drug is not commonly used in adult rheumatoid arthritis due to this inconvenience, as well as indirect comparative reports showing limited success of anakinra in rheumatoid arthritis (Vivar and Van Vollenhoven, 2014).

Anti–IL-6

Tocilizumab

Tocilizumab (TCZ) is a humanized recombinant IgG1 monoclonal antibody that binds to soluble and membrane bound IL-6 receptor. The humanization of TCZ resulted in decreased antigenicity in humans additionally leading to prolonged half-life of 10-13 days and is administered intravenously every 4 weeks. A subcutaneous formulation of tocilizumab has been developed and was very recently approved. Tocilizumab has proven effective for the treatment of rheumatoid arthritis after inadequate response to conventional DMARDs (Genovese et al., 2008) and also the treatment of rheumatoid arthritis refractory to anti-TNF therapy (Emery et al., 2008). In the ADACTA trial tocilizumab monotherapy was superior to adalimumab monotherapy (Gabay et al., 2013).

IL-6 is a pleotropic cytokine that is involved in numerous biologic processes. The increased expression in synovium of RA patients and in serum levels of IL-6 have been shown to correlate with disease activity. The strategy of blocking IL-6 with the anti-IL-6R antibody is currently one of most promising treatment in RA.

Currently TNF and IL-6 are the clinically prominent targets for cytokine inhibition in RA. The drug dosages, half-life and administrative route in RA patients are shown in Table 2.2. (Siebert et al., 2015)

Table 2.2 Cytokine inhibitors licensed for the treatment of rheumatoid arthritis

Target	Generic Name	Trade Name	Administration Route	Standard Dose and Frequency	Half-Life
TNF	Adalimumab	Humira	Subcutaneous	40 mg every 2 weeks	2 weeks (mean terminal half-life)
	Infliximab	Remicade	Intravenous	3 mg/kg weeks 0, 2, 6, then 8 weekly	8–10 days
	Etanercept	Enbrel	Subcutaneous	25 mg 2×/week *or* 50 mg 1×/week	70 hours (mean elimination half-life)
	Golimumab	Simponi	Subcutaneous	50 mg monthly	12 ± 3 days
	Certolizumab	Cimzia	Subcutaneous	400 mg weeks 0, 2, 4, then 200 mg fortnightly	14 days (terminal elimination phase half-life)
IL-6R	Tocilizumab	Roactemra, actemra	Intravenous or subcutaneous	8 mg/kg every 4 weeks 162mg per week	13 days
IL-1R	Anakinra	Kineret	Subcutaneous	100 mg daily	3–6 hours

Aim of the project

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease characterized by polyarthritis with pain, swelling and inflammation of joints. Progressive destruction of bone and cartilage is a major cause of disability leading to reducede life expectancy.. Although the role of B-cells in autoimmunity is not completely understood, but with the increasing understanding of mechanistic pathways of B cell involvement of self-destruction in autoimmune diseases, there is strong eveidence that B cells play a central role in the pathogenesis of several autoimmune disease including (Roll et al., 2015). Enhanced B cell activity has been proposed in the pathogenesis of RA along with different pro-inflammatory cytokines such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α), critically involved in chronic inflammation. Biological agents targeting these cytokines IL-6 and TNF-α have considerably advanced treatment of autoimmunity. IL-6 is a multifunctional pleiotropic cytokine acting as stimulator of B cells and formerly described as late-acting B cell differentiation factor for antibody-forming cells and germinal center reactions. Elevated IL-6 levels have been associated with disease activity and progression in RA. Biological agents targeting key pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) have been substantially advanced in the treatment of autoimmunity (Carbone et al., 2013). Enhanced B cell activity, particularly memory B cells, has gained particular interest in evaluating response during therapies from biologics.

Human peripheral memory B cells can be distinguished by the phenotypic expression of CD27 and IgD defining three major B cell subpopulations: CD27+IgD+ pre-switch, CD27+IgD- post-switch and CD27-IgD- double negative (DN) memory B cells. Therefore, we analyzed different memory populations during cytokine inhibition by using tocilizumab (anti-IL-6R, TCZ) and adalimumab (anti-TNF-α, ADA), with focus on DN B cells. DN B cells lack the conventional memory marker CD27, but due to their mutational Ig repertoire (IgR) they are considered in the memory compartment. DN B cells are expanded and could be linked to autoimmunity in systemic lupus erythematosus (SLE). Other studies also suggest increased DN B cells in patients with infections and lobar pneumonia indicating their physiological role in certain immune responses (Jacobi et al., 2008). These DN B cells are also hypothesized to resemble transient effector B cells or might derive from incomplete GC or alternatively from extrafollicular reactions (Sanz et al., 2008). However, only limited data is available for these DN B cell subsets in RA, especially during treatments with biologics.

The impact of cytokine inhibition on different memory B cell subsets has not been thoroughly studied in RA. Therefore, the main aim of this thesis is to further explore peripheral memory B cell subsets in RA patients and the effect of cytokine inhibitions. In order to gain insights into the effect of cytokine inhibition on different memory B cell and their subsets, we analyzed different memory B cell compartments in RA during *in vivo* IL-6R and TNF-α inhibition in detail, specifically the DN B cell compartment by phenotypic and molecular analyses of the different isotypic DN B cell receptors, their Ig-R mutational pattern and their modulation. We also focused on the B cell activated system is modulated in different subsets and their modulation during cytokine inhibition.

With this study, we would like to discuss the following keypoints:

1. Activation status of different memory B cells subsets and their modulation by cytokine inhibition of TNF-α and IL-6.

2. Phenotype and molecular characterization of DN memory B cells in RA.

3. Modulation of DN memory B cell isotypes by anti-TNF-α and anti-IL-6R by phenotypic and molecular analysis.

4. Evaluating memory B cells as a candidate predictive marker to anti-IL-6 receptor therapy.

3. Material and Methods

3.1 Patients and healthy donors

In total 86 rheumatoid arthritis patients (RA) with a median age of 56 years (range 33-79) along with 49 age-matched healthy donors (HD) were selected for the study of cytokine inhibition therapy. All patients met the American College of Rheumatology (ACR) revised criteria for the RA diagnosis (Aletaha et al., 2010). These patients were diagnosed with active RA and were inadequate responders to classical treatment with conventional synthetic disease-modifying anti-rheumatic drugs (csDMARDs). The patients had median disease duration of 9 (range 2–33) years and 77% were female. Of these, 9 RA patients and 3 HD were selected for analysis of mutational pattern of Ig-receptors (Ig-R) by single cell PCR technique. Informed consent was obtained from all patients according to the protocol approved by the ethics committee of the University Hospital, Würzburg, Germany. Patients who failed to respond to treatment with standard pretreatment were enrolled for Tocilizumab (TCZ) therapy. A dose of 8 mg/kg was administered every 4 weeks as a 60-minute infusion in combination with ongoing methotrexate (MTX). There were 20 patients who were undergoing TNF-alpha inhibition by Adalimumab (ADA), with a dose of 40mg every 2 weeks in combination with MTX. Of these, 4 patients were selected for Ig-receptors mutational analysis. The primary endpoint was set at 12 weeks, with an extension period to 24 weeks and follow up till 1 year for TCZ and ADA.

Disease activity was regularly determined by monitoring Disease Activity Score 28 (DAS28), levels of C-reactive protein (CRP), Erythrocyte Sedimentation Rate (ESR) and rheumatoid factor (RF) values. DAS28 scores of TCZ treated patients were 5.27 ± 0.3 (mean ± SEM) and of ADA treated patients were 4.78 ± 0.4 (mean ± SEM) before therapy. CRP level were also similar in TCZ treated (0.92 ± 0.10 mg/dl) and ADA treated (0.88 ± 0.4mg/dl) patients before therapy. Table 4.1 summarizes the clinical characteristics of the patients under TCZ and ADA. During TCZ, DAS28 declined significantly from 5.27 at baseline (BL) to 3.37, 2.74 and 1.89 at week 12, week 24 and 1 yr ($p<0.0001$), respectively. The inflammatory parameters ESR and CRP declined significantly after the first infusion (as shown in table 4.1) and stayed negative during the whole subsequent study period. The clinical response was maintained up to 1 year and there were no serious adverse events or serious infections during the study. Similar effects were found in patients treated with ADA.

3.2 Peripheral blood mononuclear cells (PBMCs) isolation

Peripheral blood (50ml) was collected from RA patients and HD in EDTA vacuum tubes. PBMCs were isolated by using Ficoll-Paque PLUS separation method (GE Healthcare, Munich, Germany) described as follows:

10ml of blood was diluted with equal volume of isotonic solution (NaCl) and laid over 20ml of Ficoll followed by centrifugation at 900g for 25min without brake. Based on the density, various fractions of blood were separated into different layers. The intermediate ring-shaped white-layer, rich in PBMCs, was separated and washed with RPMI1640 medium with inactivated 10% FCS at 310g for 8 min. The PBMCs were finally suspended in PBS buffer with 0.5% BSA and determined microscopically by counting the viable PBMCs stained with trypan blue. Till further use, cells were either put on ice or frozen in cryovials using standard protocol.

3.3 Flow cytometric analysis

3.3.1 Flurochrome labeled Antibodies

Whole blood staining was used for phenotype studies and PBMCs were used for single cell PCR approach. Whole blood and PBMCs were stained with the following monoclonal anti-human antibodies used in appropriate concentrations to stain the cells. The details are as follows: anti-CD45-krome orange (Beckman Coulter, cat no. 96416), anti-CD14-PC5.5 (Beck -man Coulter, cat no. A70204), anti-CD 19-APC-Alexa fluor 750 (Beckman Coulter, cat no. A94681), anti-CD19-APC (BD Pharmingen, cat no. 555415), anti-CD27-PE (BD Pharmingen, cat no.555441), anti-CD95-APC (BD Pharmingen, cat no. 558814), anti-ki-67-PE (ebioscience Cat no. 12-5699-42), anti-IL10- PE (BioLegend Cat no. 506804), anti-CD27-PC7 (Becton Coulter cat no. A54823), anti-CD27-ECD (Becton Coulter customized), anti-IgD-FITC (BD Pharmingen, cat no. 555778), anti-IgA-FITC (Becton Coulter, cat no. 732610), anti-IgG-PECy7 (BD Pharmingen, cat no. 561298) and anti-IgM-APC (BioLegend cat no. 314510). After staining, the cells were analyzed by 10-color flow cytometry (Navios, Beckman Coulter) and atleast 20,000 CD19+ events were collected for each analysis.

3.3.2 Phenotyping by surface staining of whole blood

Whole blood (200µl) was lysed with 2ml of VersaLyse at RT for 15 minutes followed by centrifugation at 310g for 8 minutes. Cells were washed twice with FACS buffer at 310g for 8 minutes, resuspended in the appropriate antibody preparation, and incubated for 20 minutes at 4°C. Further, the stained cells were washed with FACS buffer and then resuspended in either FACS buffer for immediate analysis or kept at 4°C until further use (maximum 1 day) in FACS buffer with fixative solution.

3.3.3 Intracellular staining of PBMCs

PBMCs were first surface stained with the following antibodies for anti-CD45-krome orange, anti-CD14-PC5.5, anti-CD19-APCA750, anti-CD27-PC7 and anti-IgD-FITC at 4°C in dark for 20 minutes. After washing, the stained cells were incubated with fixation/permeabilization buffer (eBioscience cat no. 005123-43/008333-56) for 30 minutes in the dark in order to fix and permeabilize the cells, thereby allowing intracellular staining.

After incubation stained cells were washed one time with FACS buffer and one time with Perm/Wash. They were further incubated in the dark for 30 minutes at 4°C, with PE-labeled required antibody diluted in 1x Perm/Wash, washed with FACS buffer and resuspended in either FACS buffer for immediate analysis or kept at 4°C until further analysis (maximum 1 day). The intracellular expression can be made on stimulated/unstimulated B cell and their subsets for analysis.

3.3.4 Surface staining of PBMCs for cell sorting

Before single cell sorting, immunoflourescence labeling for flow cytometry was performed for differentiating different B cell subsets by incubating PBMCs with anti- CD19-APC, IgD-FITC and CD27-PE (all from Becton Dickinson, San Jose, CA) in RNAase-free sterile tubes at 4°C for 20 minutes in PBS+0.5%BSA followed by washing with FACS buffer. Finally, cells were filtered by using MACS filter columns and put on ice until sorted for single cell sequencing.

3.4 Single Cell sequencing

Single B cell sequencing from PBMCs was mainly a five steps method (Muhammad et al., 2009), described briefly as follows: First, PBMCs were stained for B cell subsets and then cells were sorted into 96 well plates filled with lysis buffer (lower reaction mix). After sorting single cells, cDNA synthesis was carried out at 50°C for 1 hour by adding reverse transcriptase reaction buffer (upper reaction mix). Using cDNA as template, a nested PCR was performed for the amplification of V_H3 gene rearrangements using family-specific primers, and in order to differentiate different isotypes, γ, μ and α specific PCRs were performed followed by purification and sequencing. Finally, sequences were analysed by matching their closest germline counterparts using the online programme JOINSOLVER (Souto-Carneiro et al., 2004).

3.4.1 Lower reaction mix

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

Titan One Tube RT-PCR kit and Oligo dT15 were purchased from Roche Diagnostics, RNAsin from Promega, and BSA and Triton-X were purchased from Sigma Aldrich. To each of the 96 wells in the plate, 30µl of lower reaction mix was added and the plate was put on ice for as long as sorting continued.

Lower reaction mix: Volume/well 30 µl

Reagents	Volume µl (100 tests)	Final conc. /well
Triton X (10%)	500 µl	1%
Oligo DT(800 ng/ul)	50 µl	400ng
BSA(10ug/ul)	5 µl	
RNAsin(40U/ul)	50 µl	20U
DTT(100mM)	250 µl	5mM
RNAse free H20	2145µl	

3.4.2 Sorting

Sorting of individual B cell stained with CD19-APC, CD27-PE and IgD-FITC was carried out with a FACS Aria-III (Beckton Dickinson, San Jose, CA) cell sorter which provides 99.9% population purity. Cells were gated by forward/-side scatter and excluded for the CD19 positive doublets. Double negative and post-switch memory B cells were sorted individually into each of the 96 wells in the plate. After sorting, the plate was spun for a very short time and cells were lysed in lysis buffer. During lysis, mRNA comes out of the lysed B cell.

3.4.3 Upper reaction mix

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

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 10 µl

Reagents	Volume µl (200 tests)	Final conc. /well
dNTPs(10mM)	100 µl	0.2mM
RT-PCR buffer (5x contains Mg)	1000 µl	1x
Reverse transcriptase (20U/µl)	100 µl	20U
H2O	800µl	

3.4.4 cDNA synthesis

cDNA was prepared at this stage from mRNA by reverse transcriptase reaction by incubating the 96 well plate at 50ºC for 1 hour in PCR machine. Plate was stored at 4ºC for further use and could be frozen at -20ºC for 3-4 years stability.

3.5 Amplification of Ig-VH3 by nested PCR

With the help of nested RT-PCR approach, the amplification of VDJ rearrangements of Ig-VH3 gene from cDNA plates after single sorting was performed. The V and J specific primers were synthesized by Metabion International AG. All primers are from '-5' to '-3' and sequences are described as follows.

3.5.1 Oligonucleotide Sequences

Heavy Chains-V_HDJ_H rearrangements

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

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=G, T; Y=C, T; B=G, T, C

3.5.2 PCR amplification reaction protocol

PCR amplification was performed on Applied Biosystem 2720 PCR system. For master mix, the AmpliTaq polymerase, 10X buffer II and MgCl2 were purchased from Applied Biosystems. Each primer's concentration was 100 pmol/µl.

External amplification round

External primers were used to Amplify VDJ rearrangement from cDNA which was performed by making master PCR mix with following ingredients:

1. Lower Reaction mix Volume/well 25 µl
2. Upper Reaction mix Volume/well 50 µl

Lower Reaction mix Volume/well 25 µl

Reagents	**volumes**
dH2O	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**
dH2O	4150 µl
10x buffer II	800 µl
TAQ polymerase	50 µl

5µl cDNA template was added to each well of a 96 well plate.

External PCR Run program

The amplification temperatures and cycles for external PCR were as mentioned below. There was always one cycle as pre-PCR with longer time for denaturation at 95°C.

Pre-PCR for 1cycle

Denaturation at 95°C for 5 min
Annealing at 50°C for 1 min
Extension at 72°C for 1 min

Followed by PCR for 35 cycles

Denaturation at 94°C for 1 min
Annealing at 50°C for 30 sec
Extension at 72°C for 1 min

This was followed by a final extension at 72°C for 5 min and the products were stored at 4°C for further amplification of internal run.

Internal amplification round

Internal primers were used to amplify VDJ rearrangement from external PCR product as template which was performed by making master PCR mix with same ingredients as external PCR.

Internal/Nested PCR Run

The nested PCR was performed in identical manner as in external amplification round except for the following changes:

- Nested primers were used as described in previous section 3.5.1.
- The template used was 5µl of external PCR product.
- Annealing temperature was 65ºC used to yield highly specific Ig-VH3 products.
- Numbers of cycles were reduced to 30.

The volume of $MgCl_2$, dNTPs, 10x Buffer and dH2O remained the same as in the case of external amplification round. Amplified DNA of Ig-VH3 family was stored at 4ºC until further use for visualization and purification as described in section 3.7

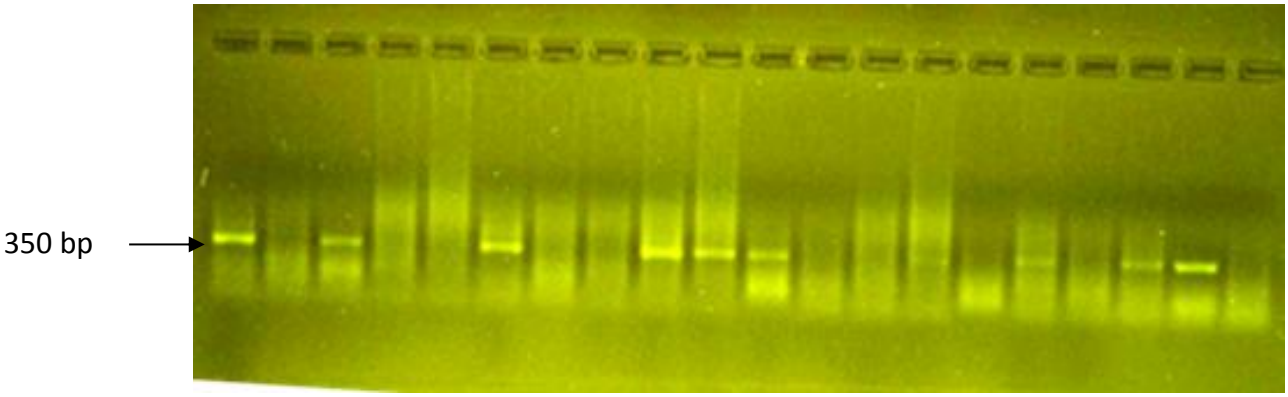

Figure 3.1. Representative gel picture showing positive band of 350 bp PCR product of Ig.

3.6 Primers used in Isotype studies

For amplification of isotypes from specific B cells, nested PCR approach was again used by using isotope-specific primers. Advantage of this technique is that to amplify all Ig-VH by combining all family specific primers along with specific primer for Isotype α/μ/g specific Ig-R. This technique was used to categorize the IgA-, IgG- and IgM- producing specific B cells in human. All the primers used in external and internal PCR rounds are described as follows. All primers were synthesized by Metabion international AG, Germany.
The concentration of each primer was 100pmol/μl.

External Primers for isotype

- ✓ 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 VH primers.

Internal Primers for isotypes

- ✓ VH1 FM: GAG GTG CAG CTG GTG CAG TCT GG
- ✓ VH2 FM: CAG GTC ACC TTG AAG GAG TCT GG
- ✓ VH3 FM: GAG GTG CAG CTG GTG GAG TCT GG
- ✓ VH4 FM: CAG GTG CAG CTG CAG GAG TCG GG
- ✓ VH5 FM: ACC AGG CAG GCG ATG ACC AC
- ✓ VH6 FM: CAG GTA CAG CTG CAG CAG TCA GG
- ✓ EcaN: ACC AGG CAG GCG ATG ACC AC
- ✓ JH Mco: CTT GGT GGA RGC TGA RGA GAC GGT GAC C

VH internal primers in combination with JHMco were used in internal PCR amplification of IgG and IgM. EcaN internal primer was used in case of IgA internal PCR.

3.6.1 Amplification rounds in isotope specific PCR

Two rounds of external and internal nested PCR were carried out in similar way as in Ig-VH3 amplification with using different set of primers, annealing temperatures and cycles. Each primer's concentration was 100 pmol/µl.

External PCR run

Lower Reaction mix Volume/well 25 µl for 100 samples

Reagents	**volumes**
dH2O	1148µl
MgCl2	800 µl
dNTPs (10mM)	160 µl
Ecµ/Ec_/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**
dH2O	4150 µl
10x buffer II	800 µl
TAQ polymerase	50 µl

5 µl cDNA template was used in external PCR run

External PCR Run program

The amplification temperatures and cycles for external PCR were the same as mentioned in section 3.5.2 and the external primers were used in combination for each isotype as mentioned in section 3.6.

Internal PCR run

The nested PCR was performed in identical manner as the external amplification round except for the following changes:

- Nested primers were used as described in previous section 3.6
- The template used was 5µl of external PCR product.
- Annealing temperature was 65ºC used during amplification of IgG and IgM specific amplification while 55ºCfor IgA specific amplification.
- Numbers of cycles were reduced to 40.

The volume of $MgCl_2$, dNTPs, 10x Buffer and dH2O remained the same as in the case of external amplification round. Amplified DNA was put at 4ºC to be used in next step to visualize PCR specific products on agarose gel and purification using miniElute kit (Qiagen).

3.7 Positive PCR product visualization and purification from Agarose gel

All internal ran nested PCR products were checked on 1.5% ultra-pure agarose electrophoresis gel (GIBCO, BRL) by running for approximately an hour at 110V and visualized with HD green (0.5µg/ml, INTAS) 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 in a very clean condition.

The positive PCR products were cut out with a clean scalpel and were kept in sterile 2.0 ml tubes for purification. MinElute Gel Extraction Kit (Qiagen) was used for the purification according to manufacturer's manual instructions. Briefly, the MinElute System uses a simple bind-wash-elute procedure, which the convenience of spin-column technology having specific binding properties of a uniquely designed silica gel membrane. DNA absorbs to the membrane in high-salt buffer and elution with low-salt buffer or water. Columns from kit are deliberate to give high-end concentration of purified DNA fragments elution in in very small volumes (as little as 10 µl) of water or elution buffer. The procedure includes dissolving agarose at 50°C, binding DNA to silica membrane at RT, washing with different buffers and finally extracting in 10µl water. Purified DNA was freezed in -20°C until further use for sequencing.

3.8 Sequence Reaction

Before sequencing, PCR was performed using purified PCR products from internal runs. ABI PRISM® BigDye™ Terminators v 3.0 Sequencing Ready Reaction Kit (Applied Biosystems) was used for performing sequence PCR. In the Ready Reaction format, the dye terminators, deoxynucleoside triphosphates, AmpliTaq DNA Polymerase, FS, magnesium chloride, and buffer are premixed into a single tube of Ready Reaction Mix and are ready to use. These reagents are suitable for performing fluorescence-based cycle sequencing reactions on single-stranded or double-stranded DNA templates, on sequence PCR.

Master Mix for Sequence reaction (each reaction)

ABI PRISM® BigDye™ Terminator	4µl
sequencing primer (10pmol/µl)	0.5µl
paint pellet blue dye	0.5µl
dH2O	11.5µl
purified DNA template	3.5µl

In case of Ig-VH3 sequence reaction the sequencing primer was internal (nested) VH3 primer and in case of Isotype studies, nested VH1-VH6 mixed primers were used at concentration 10pmol/µl.

The temperature and cycles were as follows:

Pre-PCR for 1cycle

Denaturation at 95°C for 5 min

Followed by PCR for 25 cycles

Denaturation at 95°C for 5 min
Annealing at 50°C for 30 sec
Extension at 60°C for 3 min

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

3.9 Purification of sequence PCR products

Sequence reaction products were purified using Autoseq column and isopropanol precipitation followed by sequencing in genetic analyser ABI PRISM 310 (Applied Biosystems)

3.9.1 AutoSeq Column purification

Only highly purified DNA material gives good sequencing results, so extra purification was required before proceeding to automated sequencer. The PCR products were first purified by passing through the AutoSeq G50 columns (GE Healthcare). This process was done to remove the excess dye-labelled dideoxynucleotides from the sequence reactions. Briefly, the resin columns were first vortexed gently and resin preservative was removed by spinning the columns at 2000g for 1min. The PCR products were loaded at the center of the angled surface of resin compact bed and columns were spun afterward for 1min at 2000g to collect purified DNA in a separate eppendorf tube for further step of purification.

3.9.2 Isopropanol precipitation

A further purification of PCR products was carried out by precipitating with 75% Isopropanol. 80µl of Isopropanol was added to 20 µl column purified PCR product and incubated at RT for 15min after short vortex. After incubation, samples were centrifuged at maximum speed of 13000rpm for 20min.The supernatants were discarded and pellets were resuspended in 250 µl of 75% Isopropanol and again centrifuged at same speed for 10min.The supernatant was discarded followed by drying the pellets at 40ºC for 1-2 min. Dried pellets were dissolved in 20 µl Hi-Formamide solution (Applied Biosystems) followed by denaturation at 95ºC for 3min and kept on ice for 5min or until the next step.

3.9.3 Sequencing

Genetic analyser ABI PRISM 310 (Applied Biosystems) was used for sequencing all the purified PCR products of Ig-V_H and isotype specific Ig using POP6 Polymer reagent (Applied Biosystems).

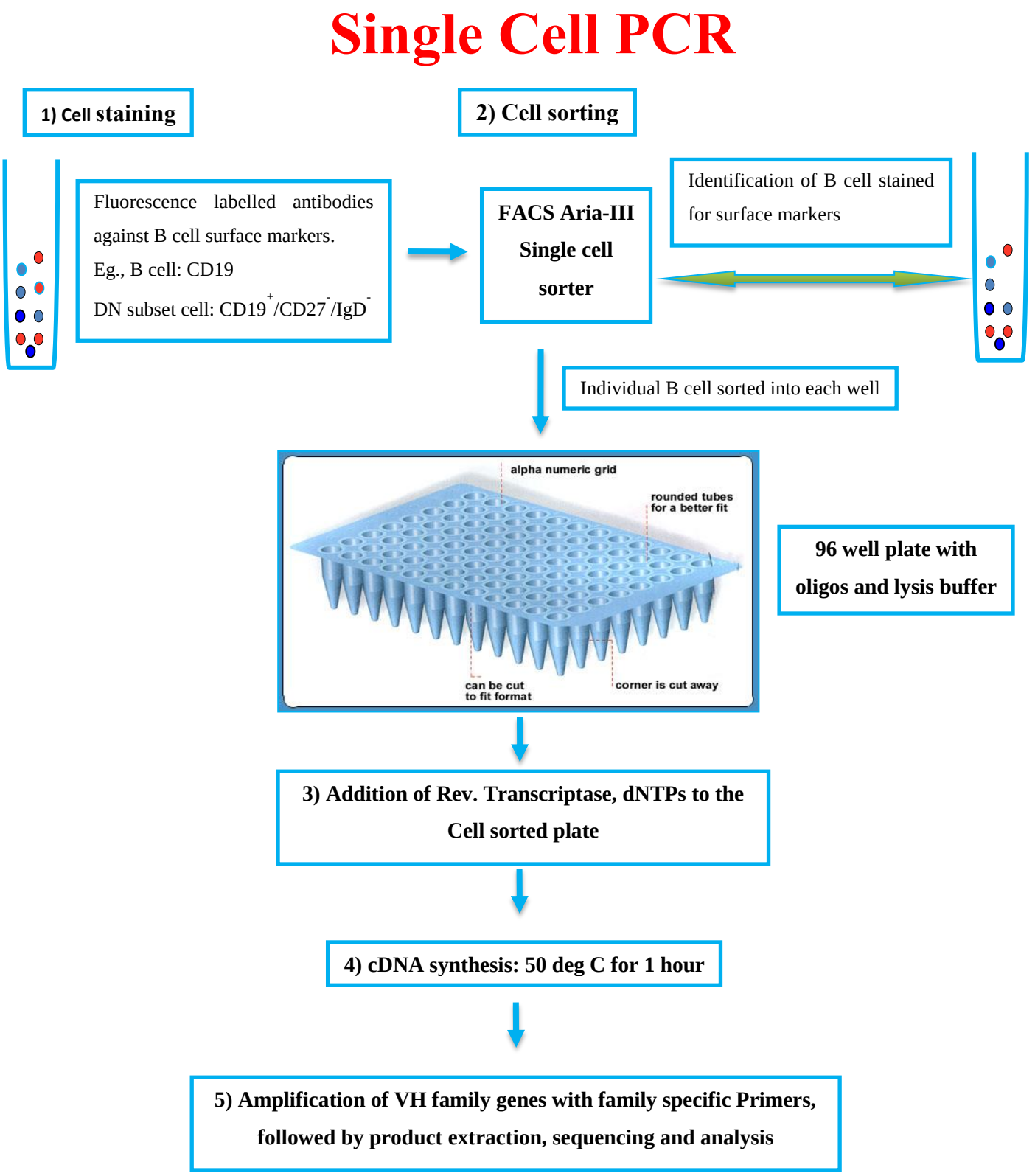

Figure 3.2 Summary of single cell PCR in illustrative diagram

3.9.4 Analysis of Sequences

Total 2438 sequences were analyzed by matching their closest germline counterparts using the online programme JOINSOLVER™ (Souto-Carneiro et al., 2004). It is a web-based software program developed for human immunoglobulin V(D)J recombination analysis created by the National Institutes of Health (NIH), National Institute of Arthritis and Musculosketelal and Skin Diseases (NIAMS) and the Center for Information Technology (CIT). It performs immunoglobulin nucleotide and amino acid alignment as well as extensive mutation and CDR3 analysis and provides results in different parameters. (http://joinsolver.niams.nih.gov)

The following parameters were analysed for Ig-V_H and isotype specific Ig:

1. **Mutational Frequency**

   The total number of mutations in variable region was determined by comparing the sequence with germline sequence. The frequency was calculated as percent of mutation in the rearrangement. It was assessed by dividing the number of mutations by total nucleaotides in the rearrangement, multiplied by 100.

2. **CDR3 length**

   The CDR3 region of the Ig gene is thought to have the most influence over antigen specificity due to actively participant of antigen binding and tended to be highly diverse in length. CDR3 length was calculated by determining number of nucleotides from residue 95-102.

3. **Mutation in hotspot motifs**

   As shown in previous studies by several groups (Muhammad *et al* 2011, Dörner *et al*, 1998; Farner *et al*, 1999), existence of mutations in RGYW and WRCY hotspot motifs. (R=purine, G=guanine, C=cytosine, Y= pyrimidine and W= A/T). Using JOINSOLVER™, the number of mutations lying in the hotspot motifs was considered and ratio of RGYW/WRCY mutations was multiplied by 100 to calculate percent of hotspot motifs for further analysis.

3.10 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, USA) and SPSS Statistics 22. Values were always compared with baseline levels by using the nonparametric Wilcoxon matched pair test and Mann-Whitney U test. Univariate logistic regression was used to calculate odd ratios and correlation using Pearson r. The values ≤ 0.05 were considered to be significant. ***$p<0.0001$, **$p<0.001$ and *$p<0.01$.

3.11 Materials

Media and reagents

Ficoll-paque (GE Biosciences)

RPMI1640 (Gibco)

Trypan Blue (Merck)

Agarose gel

1.5% Agarose Ultrapure (Gibco BRL)

1X TAE buffer

0.5μg/ml Midori green

FACS Buffer

BSA 0.1%

Sodium azide 0.01%

In PBS

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

TE(10 mM Tris,1mM EDTA) 100μl EDTA 0.5 M pH8

Chemicals and reagents

Chemical / reagent	Manufacturer
10X Permeabilization Buffer	eBioscience
Agarose	AppliChem
Bovine Serum Albumin (BSA)	Sigma
Dimethylsulfoxide (DMSO)	AppliChem
Ethanol	AppliChem
Fixation / Permeabilization Concentrate	eBioscience
Fixation / Permeabilization Diluent	eBioscience
Formamide solution	Applied Biosystems
HD green	Intas
Perm/Wash	eBioscience
Isopropanol	Roth
SDS	Roth
Sodium azide (NaN3)	Roth
Triton X	Sigma
Trypan blue	Sigma

4. Results

4.1 Characteristics of rheumatoid arthritis patients

For the study, 86 age-matched rheumatoid arthritis (RA) patients and 20 healthy donors (HD) were recruited. The patients had median disease duration of 9 (range 2–33) years and of these 77% were females. Disease activity score 28 (DAS28) in patients with active RA was high and, as expected, inflammatory parameters like C-reactive protein (CRP) and erythrocyte sedimentation rates (ESR) levels were significantly higher in patients with RA than in HD.

Of these, 40 TCZ treated-patients were followed at baseline, weeks 12 and 24 for phenotypic analysis. 33/40 patients were RF-positive and 28/40 patients were ACPA-positive. Furthermore, all the Anti–citrullinated protein antibodies (ACPA)-positive patients were positive for RF, 5/40 ACPA-negative patients were RF-positive and 7/40 patients were negative for both RF & ACPA. 4/40 patients left the study after week 12. For correlation with clinical parameters, 36 patients undergoing TCZ therapy were studied, EULAR response was calculated at week 12 of TCZ therapy.

IgA-RF and total IgA serum level were calculated using ELISA based assay for the patients with RA. During TCZ therapy, the total IgA as well as IgA-RF were reduced significantly ($p<0.05$) at week 12 and week 24. In details, total IgA were reduced from 265.4 U/ml (baseline) to 237.2 U/ml at week 12 ($p<0.05$) and 203.1 U/ml at week 24 ($p<0.05$) during TCZ therapy. IgA-RF level were also significantly ($p<0.05$) reduced from 122.3 U/ml to 99.9 U/ml at week 12 and 63.3 U/ml at week 12. The detailed numerical values are in Table 4.1.

4.1.1 DAS28 and inflammatory parameters under cytokines inhibition

Forty patients with active RA were analyzed and it was found that the clinical characteristics were significantly reduced during cytokines inhibition therapies. During TCZ therapy, DAS28 declined significantly from 5.27 at baseline (BL) to 3.37, 2.74 and 1.89 at week 12, week 24 and 1yr ($p<0.0001$), respectively. The CRP level was reduced significantly from 0.92 to 0.14, 0.09 and 0.06 at week 12, week 24 and 1yr ($p<0.0001$), respectively.

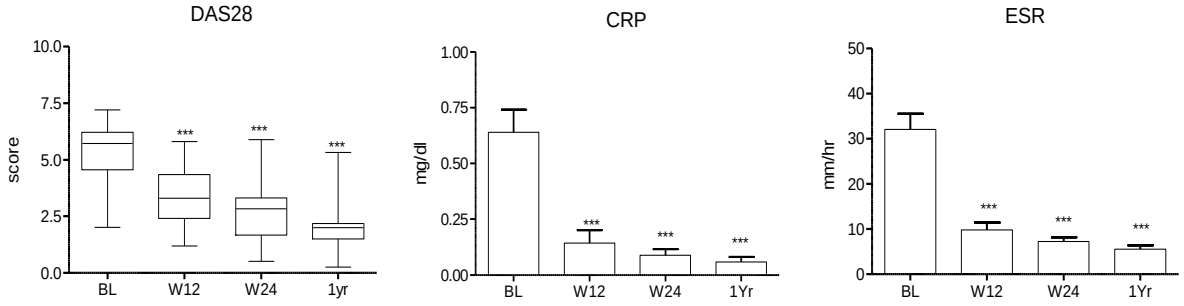

Figure 4.1 DAS28 and inflammatory parameters during TCZ therapy. Significant reduction in the values of DAS28, CRP & ESR over time span of 1 year. BL = baseline, W12 = week 12, W24 = week 24 and 1yr = year. Data shown in box-whisker plot for DAS28 score and CRP/ESR values are presented in bar graph. P values were determined by Mann-Whitney U test using GraphPad prism 5 (***P < 0.0001).

20 patients treated with ADA, also showed significant reduction in DAS28 from 4.78 at BL to 2.43, 1.91 and 1.36 at week 12, week 24 and 1 year respectively. The inflammatory parameters, ESR and CRP declined significantly after the first infusion and stayed negative during the whole subsequent study period with both therapies. The clinical response was maintained up to 1 year and there were no serious adverse events during the study.

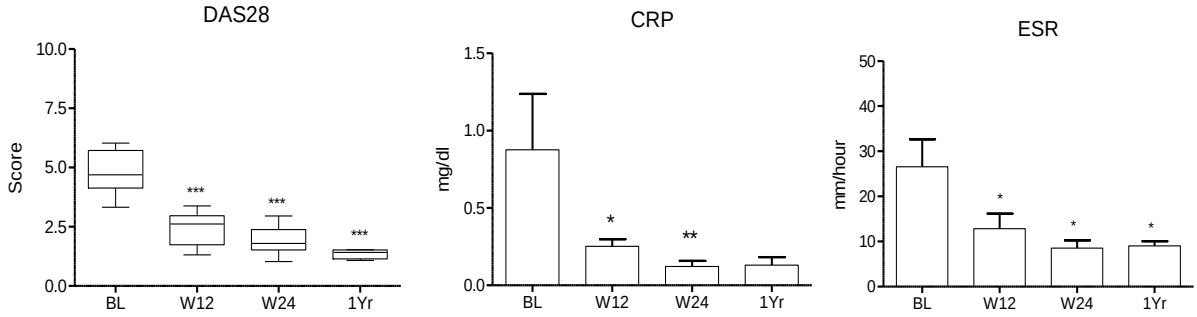

Figure 4.2 DAS28 and inflammatory parameters during TNF-alpha inhibition using ADA. Significant reduction in the values of DAS28, CRP & ESR over time span of 1 year. BL=baseline, W12=week 12, W24=week 24 and 1yr=year. Data shown in box-whisker plot for DAS28 score and CRP/ESR values are presented in bar graph. P values were determined by Mann-Whitney U test using GraphPad prism 5 (***P < 0.0001, **P < 0.001 and *P < 0.05).

From the first part of results it was concluded that RA has high disease activity, DAS28 along with elevated inflammatory conditions which are significantly reduced under cytokines inhibition therapies. Table 4.1 summarizes the clinical characteristics of the patients under TCZ and ADA.

Table 4.1 Characteristics of patients treated with tocilizumab and adalimumab.

	Baseline	Week 12	Week 24	1year
Age, Media (range) years	56 (43-79)			
% female	77%			
Disease duration, mean (range) years	9 (2-33)			
RF positive	36			
ACPA positive	28			
RF/ACPA positive	28			
Tocilizumab (n=40)	mean ± SEM			
DAS28 score	5.27 ± 0.3	3.37 ± 0.3$^{\Psi}$	2.74 ± 0.2$^{\Psi}$	1.89 ± 0.2$^{\Psi}$
CRP, mg/dl	0.92 ± 0.10	0.14 ± 0.06$^{\Psi}$	0.09 ± 0.03$^{\Psi}$	0.06 ± 0.02$^{\Psi}$
ESR, mm/hour	32.12 ± 3.4	9.82 ± 1.6$^{\Psi}$	7.23 ± 0.9$^{\Psi}$	5.58 ± 0.8$^{\Psi}$
Patient's VAS	58.31 ± 4.4	48.62 ±4.8$^{\Psi}$	34.83 ± 5.4$^{\Psi}$	21.47 ± 4.8$^{\Psi}$
IgA-RF U/ml	122.3 ± 33.3	99.9 ± 26.3ψ	63.3 ± 19.8ψ	
IgA total U/ml	265.4 ± 20.8	237.2 ± 19.5ψ	203.1 ± 18.9ψ	
Adalimumab (n=20)	mean ± SEM			
DAS28 score	4.78 ± 0.4	2.43 ± 0.3$^{\Psi}$	1.91 ± 0.2$^{\Psi}$	1.36 ± 0.1$^{\Psi}$
CRP, mg/dl	0.88 ± 0.4	0.25 ± 0.05$^{\Psi}$	0.12 ± 0.04$^{\Psi}$	0.13 ± 0.05$^{\Psi}$
ESR, mm/hour	26.67 ± 6.1	12.86 ± 3.3$^{\Psi}$	8.57 ± 1.7$^{\Psi}$	9.0 ± 1.8$^{\Psi}$
Patient's VAS	57.14 ± 7.3	26.43 ± 7.5$^{\Psi}$	19.29 ± 2.9$^{\Psi}$	11.25 ± 6.6$^{\Psi}$

The primary end point of the study was a reduction in the Disease Activity Score in 28 joints (DAS28) at 1year. Except where indicated otherwise, values are the mean ± SEM. CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; VAS = visual analog scale (100-mm). $^{\Psi}P < 0.05$ versus baseline.

4.2 Memory B cell subsets

Human peripheral memory B cells are mainly discriminated from naïve B cells by the presence of somatic hypermutation in their Ig variable genes and the phenotypic expression of CD27, a member of TNFR family. Based on surface expression of IgD and CD27, human peripheral CD19+B cells can be divided into four subsets: mature naïve B cells, pre-switch (Pre), post-switch (PS) and double negative (DN) memory B cells.

a. Mature naïve B cells : $CD19^{+}$ IgD^{+} $CD27^{-}$

b. Pre-switch (Pre) memory B cells : $CD19^{+}$ IgD^{+} $CD27^{+}$

c. Post-switch (Post) memory B cells : $CD19^{+}$ IgD^{-} $CD27^{+}$

d. Double negative (DN) memory B cells : $CD19^{+}$ IgD^{-} $CD27^{-}$

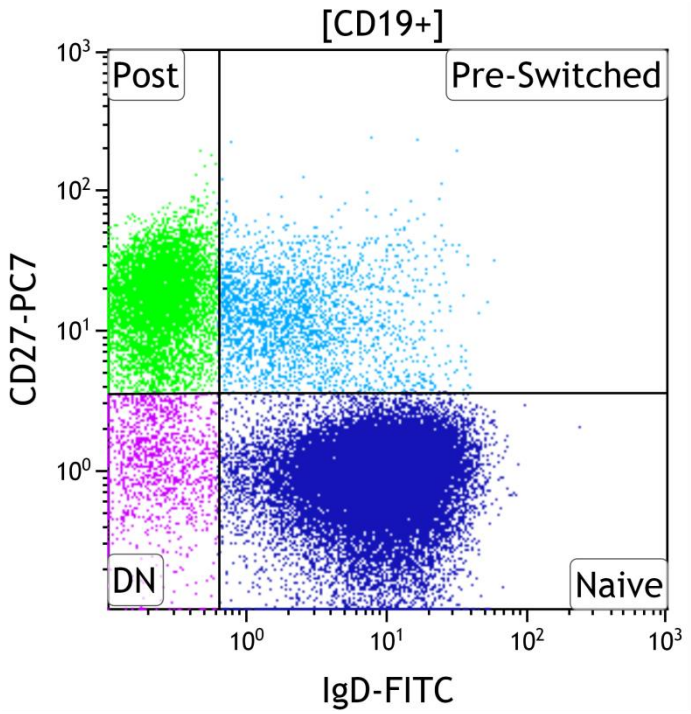

Figure 4.3 Representative FACS plot showing B cell subsets in a RA patient. Double negative (DN) B cells are defined by $CD19^{+}CD27^{-}IgD^{-}$. Pre-switch (pre), post-switch (PS) and naïve B cells as indicated.

4.3 High prevalence of activated B cells in peripheral blood of patients with RA

To evaluate the activity of B cell and their subsets, the surface expression of CD95 and intracellular expression of ki-67 staining on B cells and their subsets were analyzed. RA patients showed a significantly higher of surface expression of CD95 (P<0.0001) and intracellular expression of ki-67 (P<0.001) ki-67 on B cells than healthy donor (HD) (Figure 4.5). RA patients had a median (range) of 12.9% (4.5-33.3) expressing CD95 B cells as compared with 5.4% (2.5-11.1) in healthy individuals. Similarly, RA patients had a median (range) of 3.2% (1.7-8.3) intracellular expression of ki-67 as compared with 1.8 (0.9-4.9) in HD.

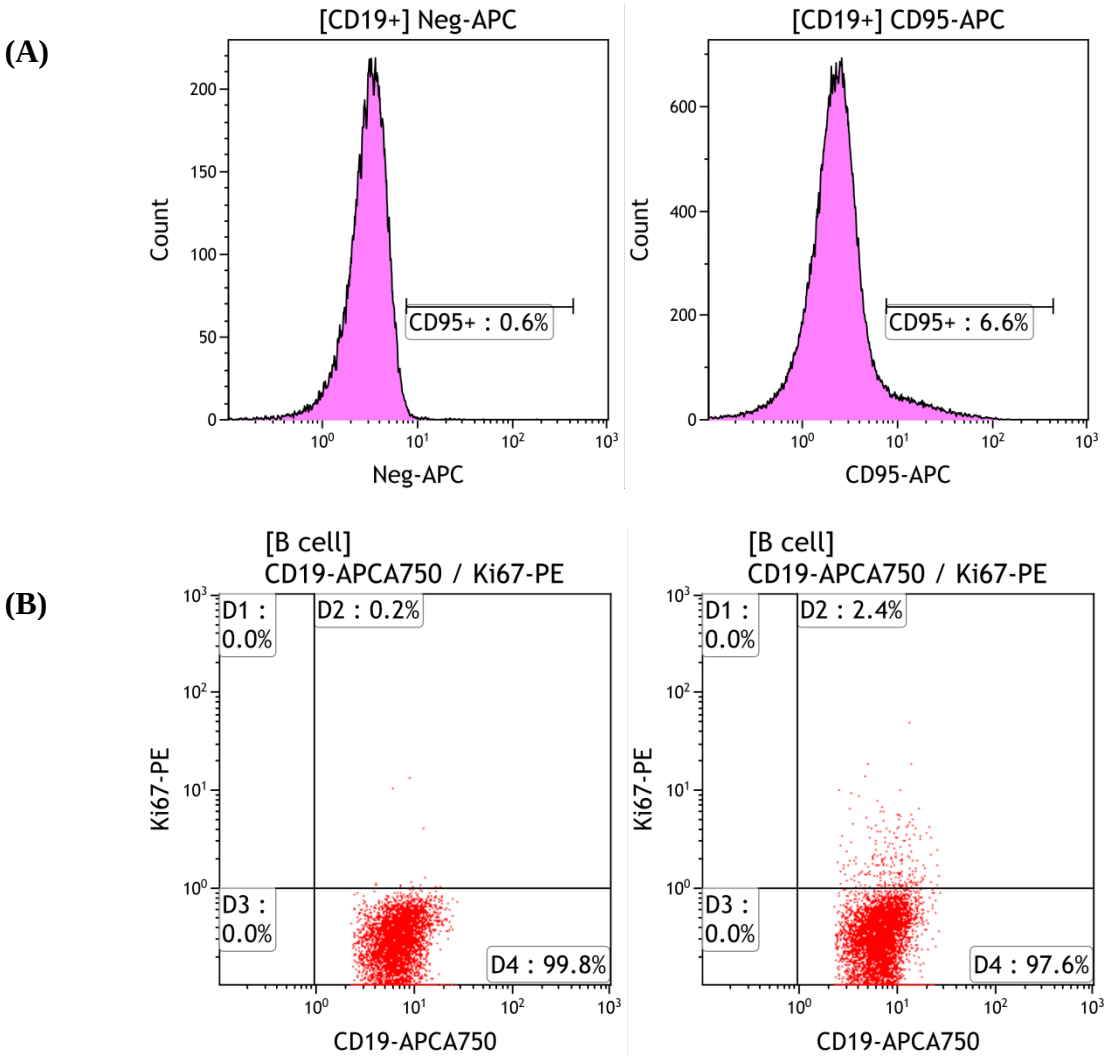

Figure 4.4 Representative FACS plot showing CD95 and ki-67 expression. **(A)** Surface expression of CD95 and **(B)** intracellular expression of ki-67 of B cells are shown with their isotype control.

The surface expression of CD95 and intracellular expression of ki-67 of B cells in patients with active RA were significantly higher than the expressions in healthy donor (HD).

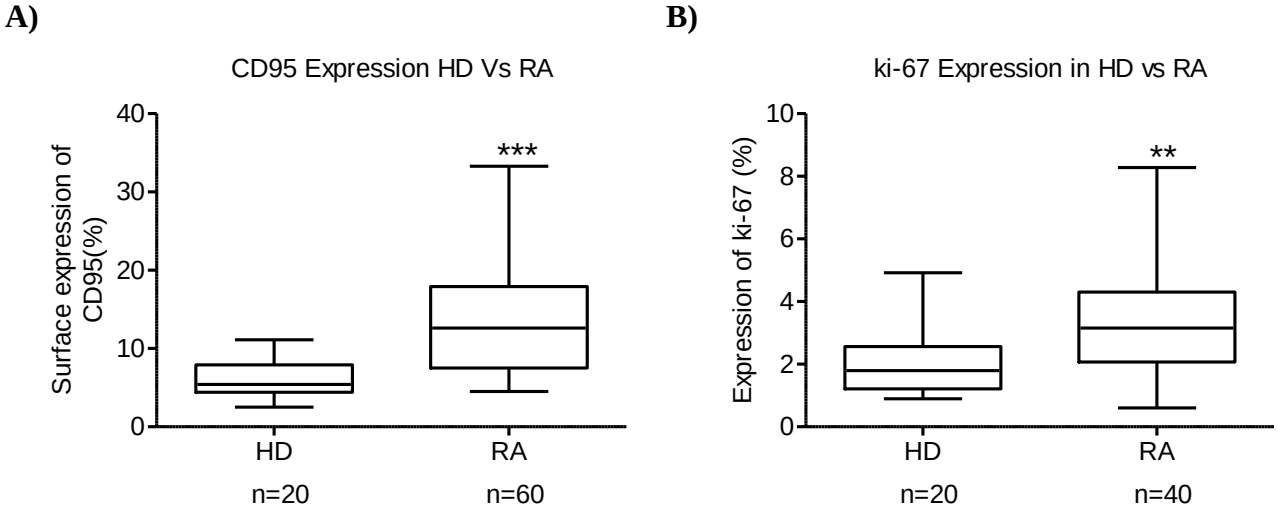

Figure 4.5 Activated B cells in RA and HD. The phenotypically analyzed B cells showed a significantly higher percentage of surface CD95 and intracellular ki-67 expression on B cells in RA individuals (P<0.0001**)** than HD. Data shown in box-whisker plot where boxes represent 25th to 75th percentiles and the lines within the boxes represent the median. P values were determined by Mann-Whitney U test using Graph pad prism 5 (***P < 0.0001 and **P < 0.001). n= number of individuals.

4.3.1 Comparison of CD95 and ki-67 expression in B cell subsets (RA vs HD)

The expression of CD95 and ki67 in each subset was significantly higher in RA as compared to HD. The CD95 expression was significantly higher in DN B cells ($p<0.0001$, median percent in RA=20.6% & HD=12.8%); post-switch B cells ($p<0.0001$, median percent in RA= 38.5% & HD=19.0%); pre-switch B cells ($p<0.0001$, median percent in RA=18.2% & HD=8.5%) and in naïve B cells ($p=0.034$, median percent in RA=1.1% & HD=0.5%). The details are shown in figure 4.6.

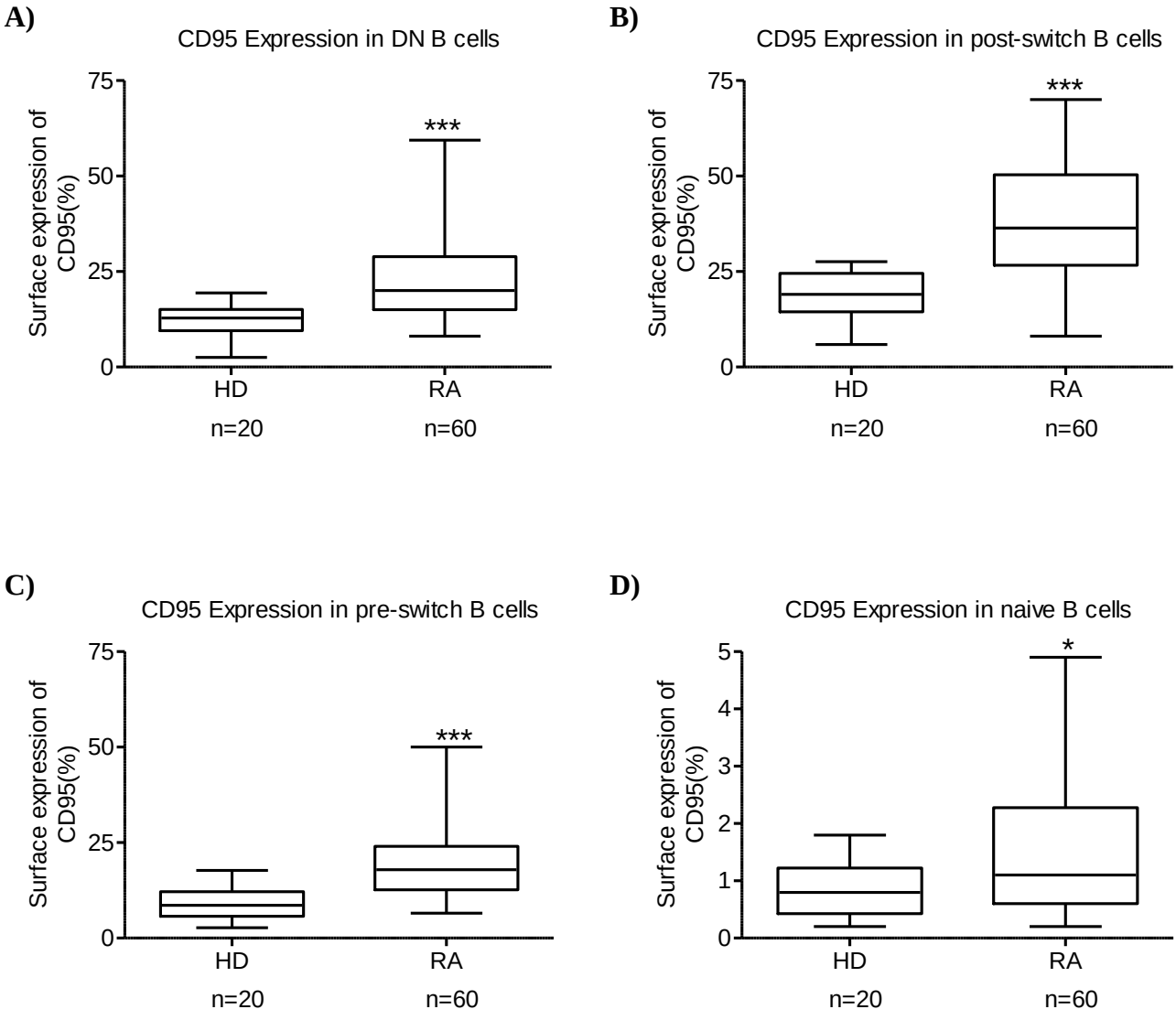

Figure 4.6 CD95 expressions in B cell subsets (RA vs HD). **A)** DN B cells. **B**) Post-switch. **C)** Pre-switch and **D)** naïve B cell subsets. Data shown in box-whisker plot where boxes represent 25th to 75th percentiles and the lines within the boxes represent the median. P values were determined by Mann-Whitney U test using Graph pad prism 5 (***$P < 0.0001$ and *$P < 0.05$). n= number of individuals.

The ki-67 expression was significantly higher in all three memory B cell subsets except in naïve B cells. In details, the intracellular expression of ki-67 in DN B cells (p=0.0014, median percent in RA=3.9% & HD=1.8%); post-switch B cells (p=0.012, median percent in RA= 7.1% & HD=4.9%); pre-switch B cells (p=0.038, median percent in RA=4.5% & HD=2.8%) and in naïve B cells (p=0.64, median percent in RA=0.5% & HD=0.6%). The graphical representation is shown in box-whisker plots (Figure 4.7).

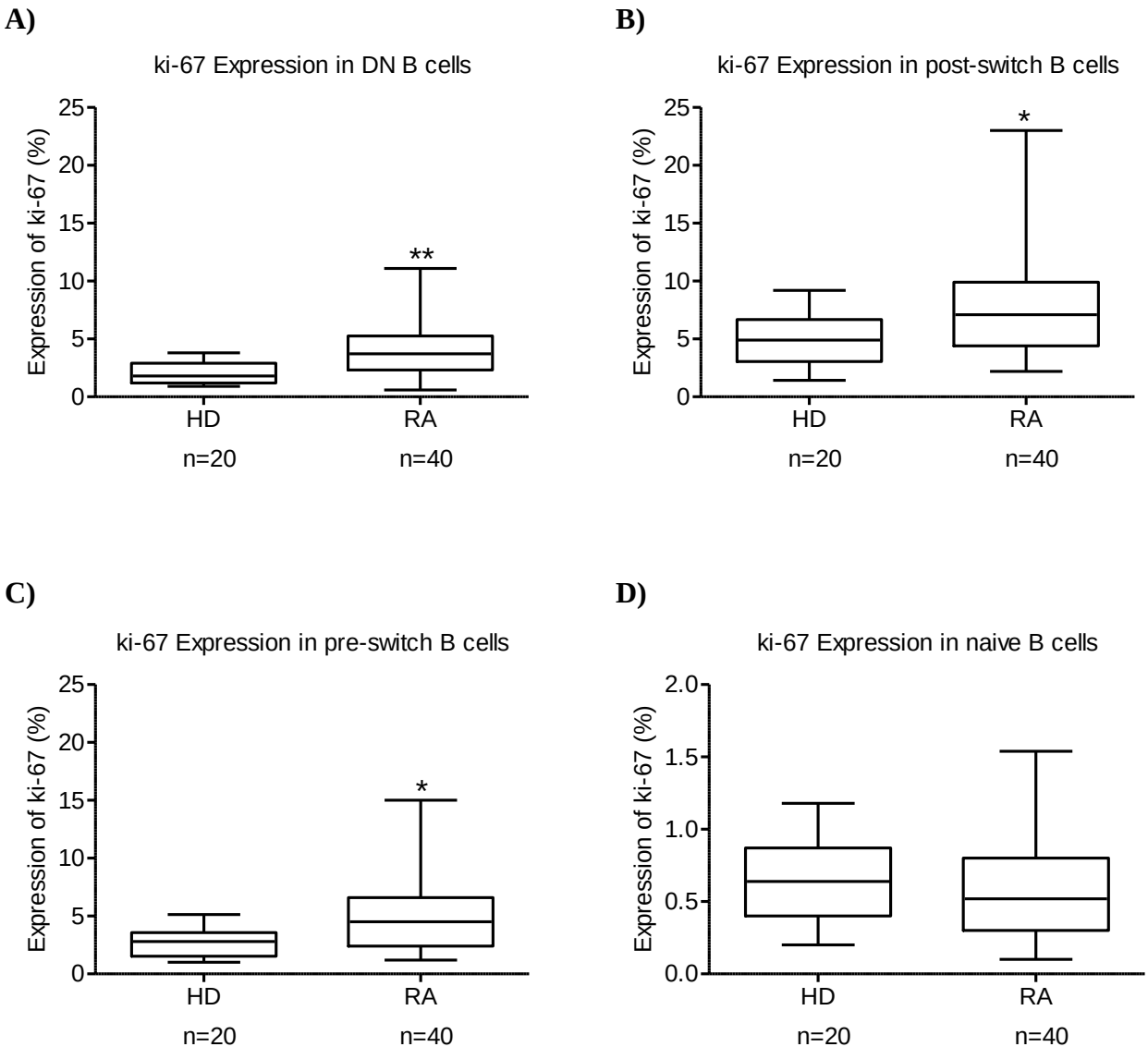

Figure 4.7 ki-67 expressions in B cell subsets (RA vs HD). **A)** DN B cells. **B)** Post-switch. **C)** Pre-switch and **D)** naïve B cell subsets. Data shown in box-whisker plot where boxes represent 25th to 75th percentiles and the lines within the boxes represent the median. P values were determined by Mann-Whitney U test using Graph pad prism 5 (**P < 0.001 and *P < 0.05). n= number of individuals.

4.3.2 CD95 and ki-67 expression on B cell subsets in peripheral blood of RA patients

Analysis of each subset of B cell showed higher activity in RA compared to HD. The CD95 and ki-67 expressions on B cell subsets were highest in post-switch memory B cells followed by DN and pre-switch B cell subsets (Figure 4.8). Post-switch subset has the highest CD95 surface expression of median of 38.5% as compared with 20.6% in DN and 18.2% in pre-switch memory B cells. Naïve B cell subset has the significantly lower surface expression of CD95 with respect to all three memory B cell subsets. The DN and pre-switch B cells have similar expressions of CD95 which is significantly lower than post-switch B cell subset. The statistical graph is shown in box-whisker plots below.

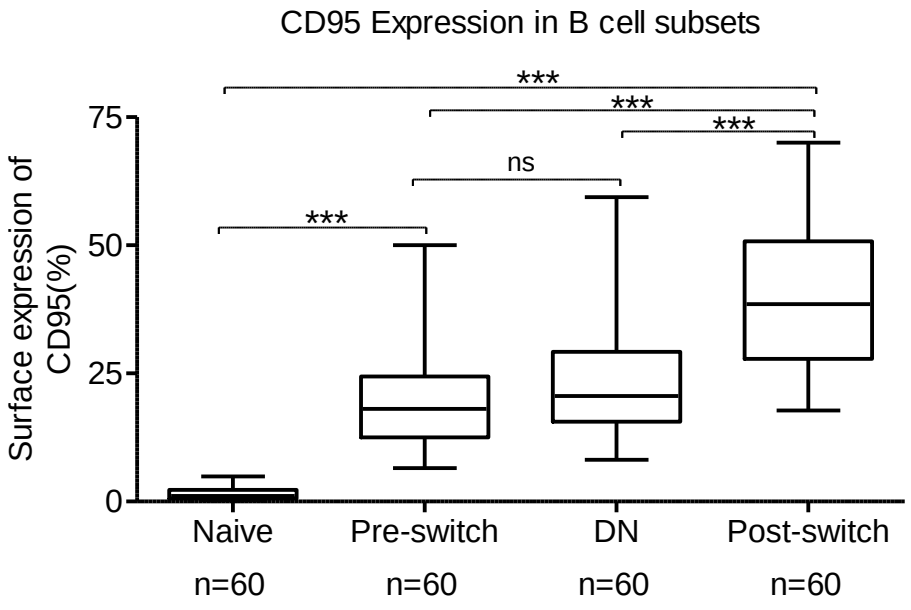

Figure 4.8 CD95 expressions of B cell subsets in RA patients. CD95 surface expression in double negative (DN), pre-switch, post-switch and naïve cell of B cell subsets shown in box-whisker plot where boxes represent 25th to 75th percentiles and the lines within the boxes represent the median. P values were determined by Mann-Whitney U test using Graph pad prism 5 (***P < 0.0001, ns=non-significant). n= number of individuals.

Similarly, intracellular ki-67 expression was highest in post-switch memory B cells followed by DN and pre-switch memory B cells (Figure 4.9). Post-switch subset has the highest intracellular ki-67 expression of median of 7.1% as compared with 3.9% in DN and 4.5% in pre-switch memory B cells. Naïve B cell subset has significantly lower expression of ki-67 with respect to all three memory B cell subsets. The DN and pre-switch B cells have similar expressions of ki-67 which is significantly lower than post-switch B cell subset. The statistical graph is shown in box-whisker plots below.

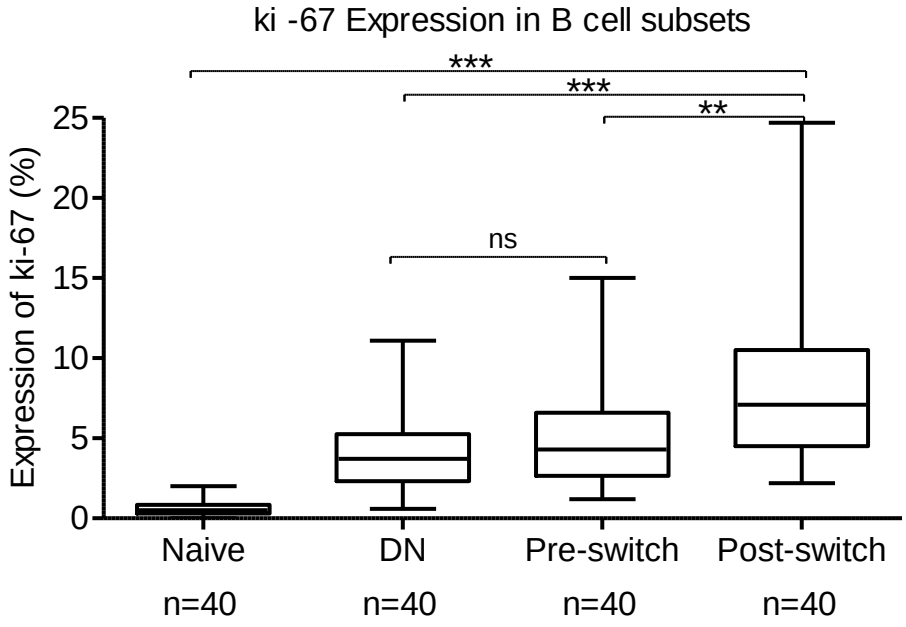

Figure 4.9 Intracellular ki-67 expressions of B cell subsets of RA patients. ki-67 surface expression in double negative (DN), pre-switch, post-switch and naïve cell of B cell subsets shown in box-whisker plot where boxes represent 25th to 75th percentiles and the lines within the boxes represent the median. P values were determined by Mann-Whitney U test using Graph pad prism 5 (***P < 0.0001, **P < 0.001, ns=non-significant). n= number of individuals.

4.4 Double Negative Memory B cells

4.4.1 Relatively expanded double negative (CD19+IgD-CD27-) B cell population in RA

Based on expression of CD19+IgD-CD27-, we performed phenotypic analysis of peripheral DN B cells in RA patients (n=86; median age ~56 years) and healthy donors (n=49; median age ~52 years). Interestingly, we found significantly enhanced frequencies of DN B cells in RA patients compared to HD (Figure 4.10). Here, RA patients had a median (range) 8.4 (2.0-18.1) percent DN B cells as compared with the 3.3 (1.0-7.9) percent in HD. Notably, the absolute numbers of DN B cells of RA with 10.7 (1.9-32.9) cells/µl were comparable to HD with 9.6 (1.7-29.5) cells/µl. The relative and absolute numbers of DN B cells of RA were not influenced by either of the cytokine inhibitions.

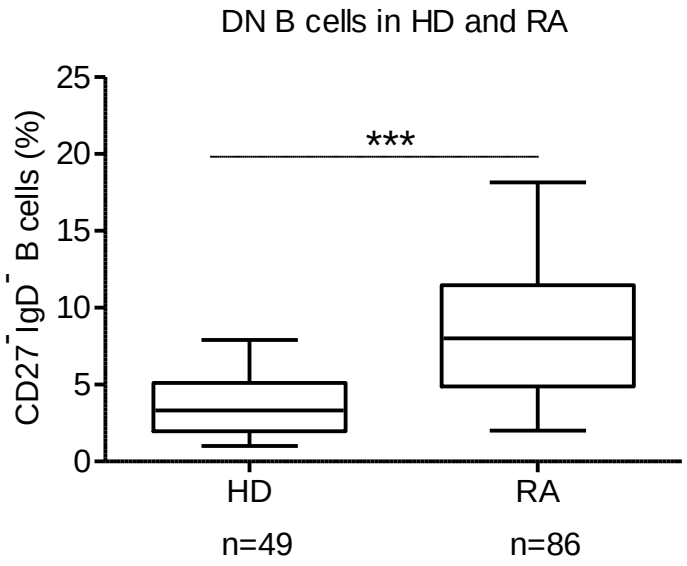

Figure 4.10 Phenotype analysis of CD27-IgD- (DN) B cells in RA patients and comparison of DN B cells in RA and HD. DN B cells in RA patients show a significantly higher percentage of the frequency of DN B cells in RA patients. The data shown in box-whisker plot where boxes represent 25th to 75th percentiles and the lines within the boxes represent the median. P values were determined by Mann-Whitney U test using Graph pad prism 5 (***P < 0.0001). n= number of individuals.

4.5 Isotypes profile of DN and post-switch memory B cells

In order to reveal the isotypic distribution of memory B cells, DN and post-switch B cells were analyzed for the surface expression of IgG, IgA and IgM isotypes. The representative FACS plot for IgA, IgG and IgM expressing cells in memory B cells is given figure 4.11.

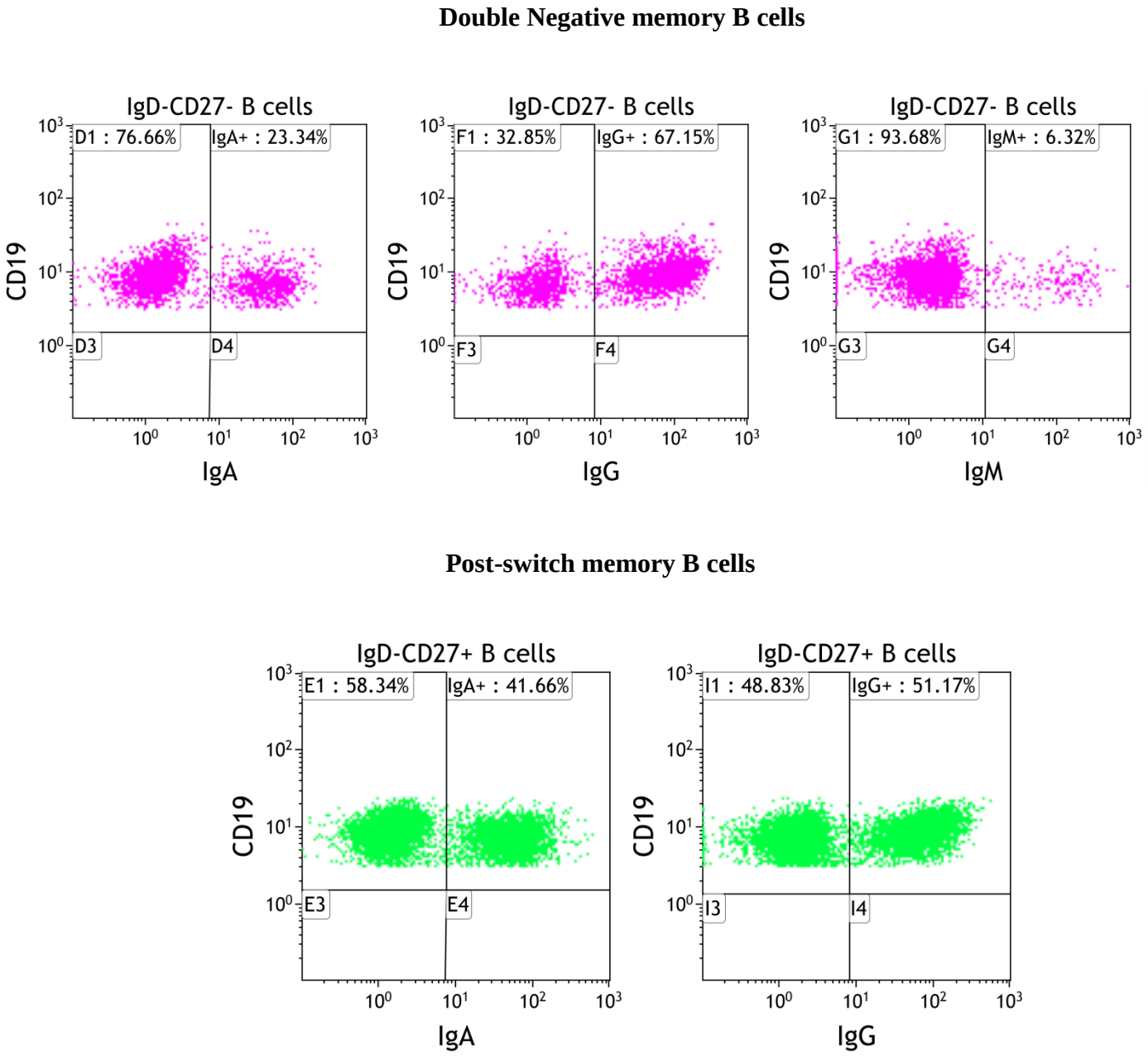

Figure 4.11 Representative FACS plot for isotype-specific immunoglobulins. Double negative is heterogeneous mixture of IgG, IgA and IgM whereas post-switch has equal distribution of IgG and IgA. The isotype expressions of IgG, IgA and IgM were gated on DN and post-switch memory B cells. DN= CD19+IgD-CD27- and post-switch= CD19+IgD-CD27+.

4.5.1 Heterogeneous Ig isotypic distribution

From the isotype-specific immunophenotyping, DN B cells revealed a heterogeneous mixture of cells containing IgA+, IgG+ and IgM+ expressing B cells with a dominance of IgG+ B cells. IgG+ B cells contained a median (range) of 63.6 (16.0-95.1) percent of total DN B cells followed by 25.1 (8.0-54.2) percent of IgA+ and 7.3 (2.1-30.1) percent of IgM+ B cells. For comparison, post-switch memory B cells displayed an almost equal distribution of IgG+ and IgA+ cells; 45.3% (26.2-67.3) IgG+ and 42.7% (24.5-57.4) IgA+ (Figure 4.11-4.12). The distribution of IgG+, IgA+ and IgM+ DN B cells in HD were comparable to RA patients.

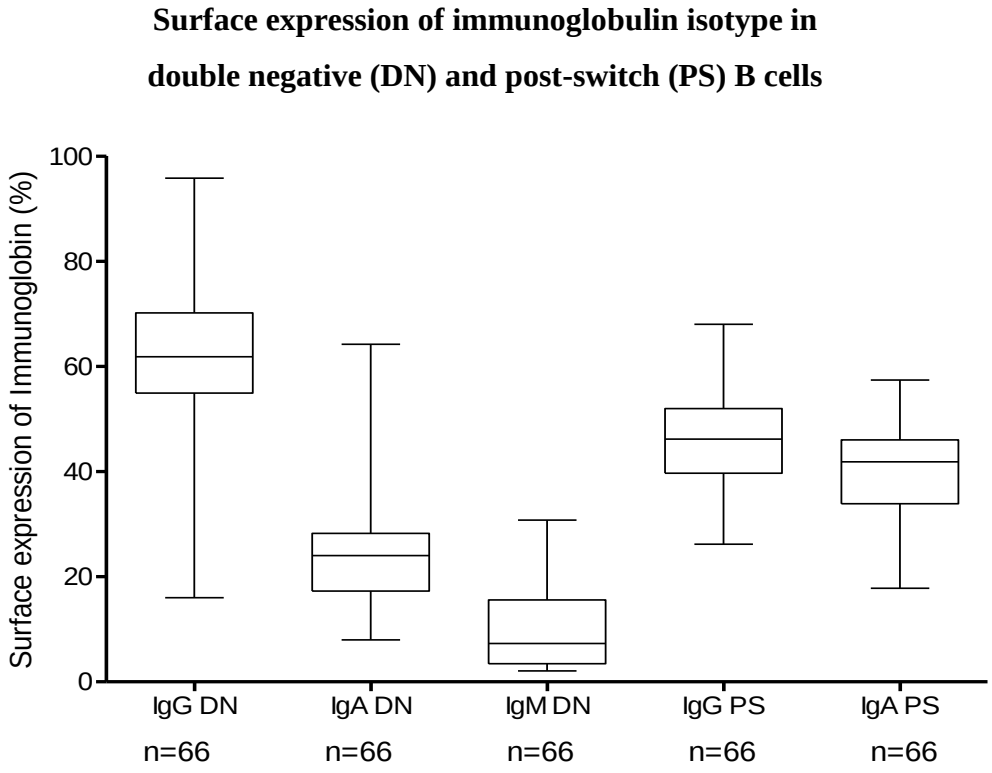

Figure 4.12 Composite graph for isotype-specific immunoglobulins. IgG is predominant in double negative B cell followed by IgA and IgM. In contrast, post-switch B cells have equal expression of IgG and IgA. The data shown in box-whisker plot where boxes represent 25th to 75th percentiles and the lines within the boxes represent the median. n= number of individuals, IgG DN= IgG expressing DN memory B cells, IgA=IgA expressing DN memory B cells, IgM DN= IgM expressing DN memory B cells, IgG PS= IgG expressing post-switch memory B cells, IgA PS= IgA expressing post-switch memory B cells.

4.6 Do activation markers and B cell subsets correlate with the disease activity?

4.6.1 Linear correlation between DAS28 and activation markers

Activated B cell with higher CD95 surface expression and intracellular ki-67 expression were positively correlated with the disease activity as of DAS28 score of RA patients. The graph below is showing the positive correlation between the DAS28 and surface CD95 expression (r^2=0.329, p=0.0042). The linear correlation between DAS28 and intracellular ki-67 expression in RA was positive and significant (r^2=0.357, p=0.0088).

Correlation between DAS28 and CD95/ki-67 expressing B cells

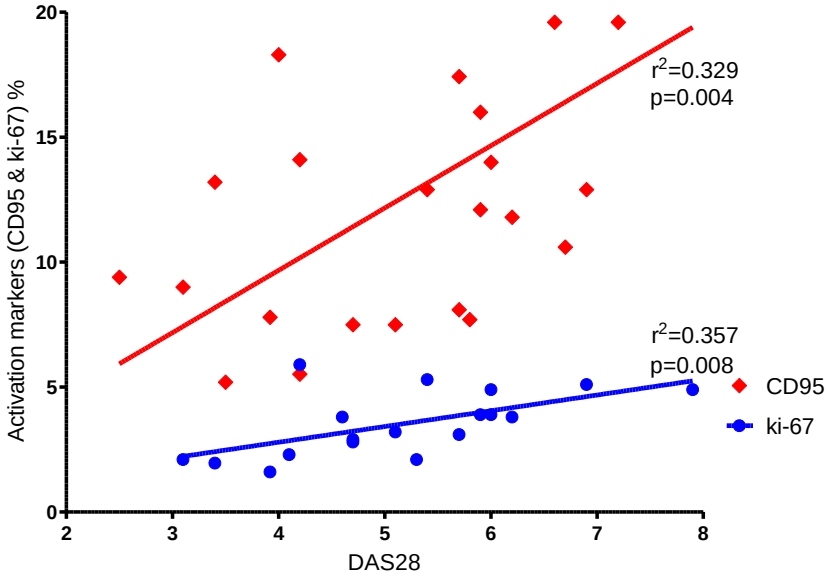

Figure 4.13 Correlation between activated B cell and DAS28. There is a significant positive linear correlation between activated B cells by CD95+ or ki-67+ expression and DAS28 as measure disease activity. The relationship between variables was evaluated using Pearson linear correlation test. A two-side p value of <0.05 was considered statistically significant.

4.6.2 Correlation between DAS28 and memory B cell subsets

Human memory B cell subsets were correlated with the disease activity of the RA patients using linear Pearson's correlation. The post-switch B cell subset was positively correlated with the DAS28 of the RA patients (r^2=0.04, p=0.061) while pre-switch B cell subset was negatively correlated with DAS28 of RA patients (r^2=0.208, p=0.022). Active RA patients with higher post-switch memory B cells are likely to have higher disease activity due to higher DAS28 and vice-versa with pre-switch memory B cells.

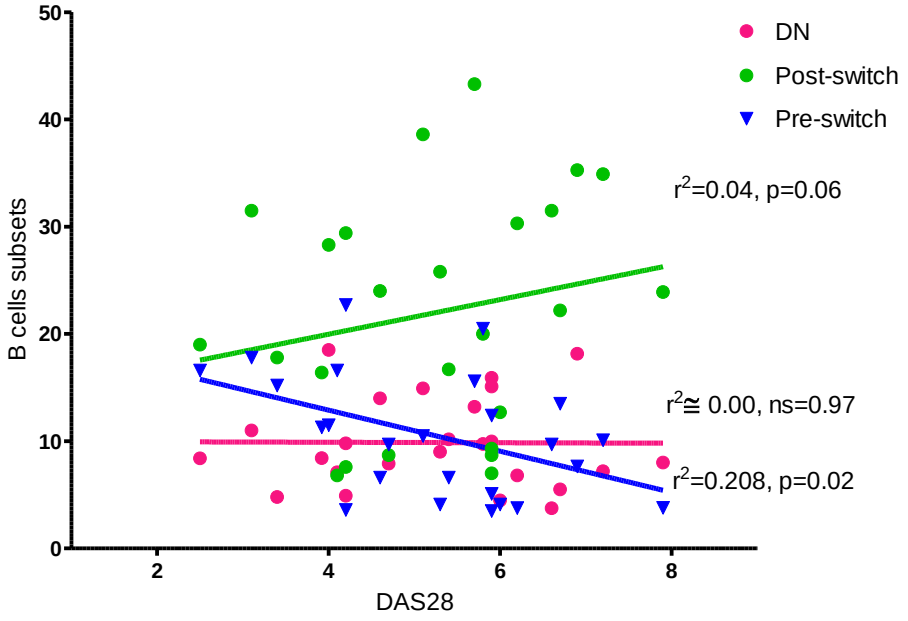

Figure 4.14 Correlation between DAS28 and B cell subsets. DN B cells do not have any correlation while post-switch have positive and pre-witch have negative correlation with DAS28 score of RA patients.

Interestingly, there was no significant correlation between frequency of DN B cells with DAS28 (R = -0.007; p = 0.97) and CRP levels (R = 0.07; p = 0.70). The percentage of DN B cells was independent of DAS28 and CRP level of active RA patients which leads to think that whether DN is a predictive marker for disease or therapy.

4.7 The frequency and number of DN B cells correlate to clinical response to tocilizumab

During TCZ therapy, DAS28 of patients declined significantly from 5.2 at baseline (BL) to 3.3, 2.7 and 1.9 at week 12, 24 and 1 year ($p < 0.0001$). The inflammatory parameters ESR and CRP were also significantly reduced (Table 4.1). At week 12, during IL-6 inhibition with TCZ therapy 14 out of 36 patients reached a EULAR good response, 20 patients reached a moderate response and 2 patients were non-responders. Interestingly, we found that good EULAR responders to TCZ at week 12 had a significantly lower frequency of DN B cells compared to moderate responders, at baseline. The same correlation was also found for absolute numbers of DN B cells at baseline (Figure 4.15). Also there was no significant correlation between frequency of DN B cells with DAS28 ($R = -0.007$; $p = 0.97$) and CRP levels ($R = 0.07$; $p = 0.70$) at baseline.

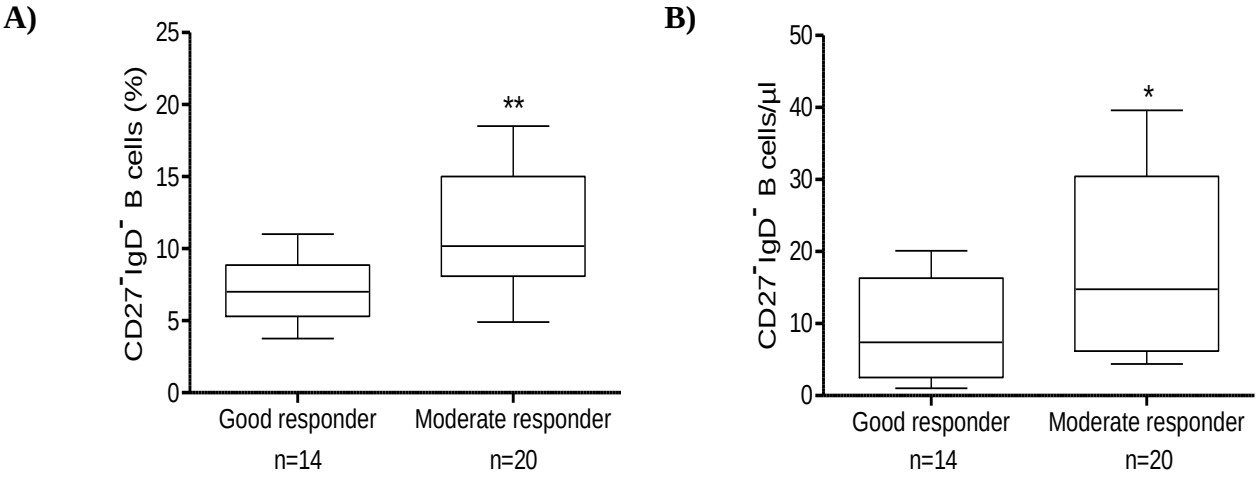

Figure 4.15 EULAR responses to IL-6R inhibition. A) Week 12 EULAR good responders (BL DAS28 = 5.1 ± 0.3) to TCZ have significantly (**p = 0.006) lower frequency of DN B cells at baseline compared to EULAR moderate responders (BL DAS28 = 5.3 ± 0.3). **B)** Week 12 EULAR good responders have significantly (*p = 0.05) lower absolute DN B cell numbers at baseline compared to moderate responders.

Univariate logistic regression analysis revealed that the frequency of DN B cells at baseline is inversely correlated with a subsequent EULAR good response with a significant ($p = 0.024$) odds ratio of 1.48 (95% CI as 1.05-2.06) favoring EULAR good response. This indicates that the percentage of DN B cells is elevated in RA and correlated with EULAR response to TCZ.

4.8 Double negative memory B cells during cytokine inhibition

Sixty six TCZ and 20 ADA treated patients were selected to analyze the DN B cells during cytokine inhibition. There were no significant changes in the DN memory B cells under cytokines inhibition. The relative as well as absolute number remains stable during course of therapy using TCZ and ADA. The patients were analyzed at baseline, week 12 and week 24. The primary end point of the activation expression study was at week 24 and the values for analysis are shown in median (range) (Figure 4.16).

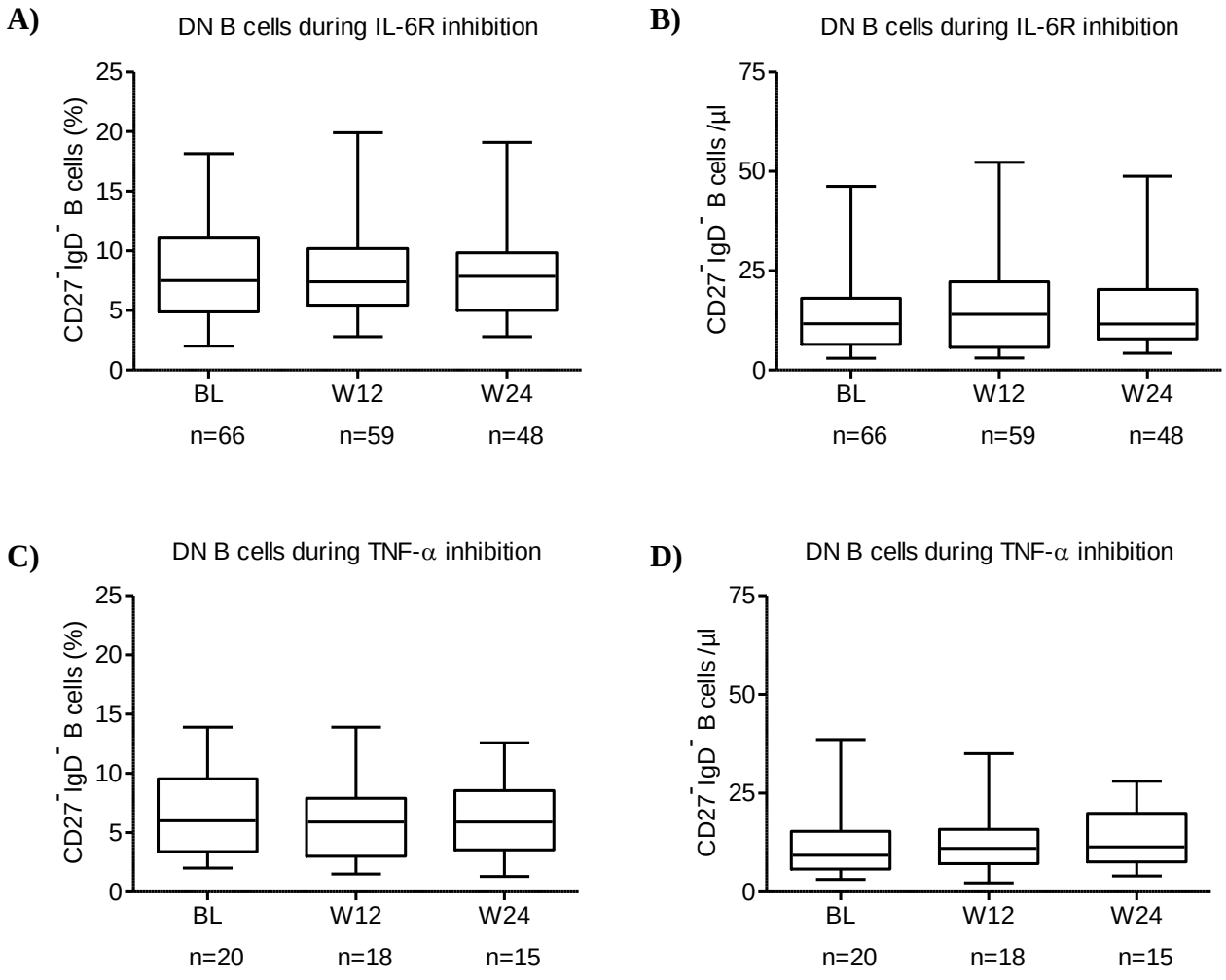

Figure 4.16 Relative and absolute DN B cells during IL-6R & TNF-α inhibition. A) Relative DN B cells during IL-6R inhibition at BL, week12 and week 24. **B**) Absolute DN B cells per microliter during IL-6R Inhibition**. C)** Relative DN B cells during TNF-α therapy. **D)** Absolute DN B Cells per microliter during TNF-α therapy. N=number of patients treated with respective therapy.

4.9 Modulation of CD95 and ki-67 expression in B cells during cytokines inhibition

The effect of cytokine inhibition using IL-6R and TNF-α inhibitors were analysed on CD95 and ki-67 expressions of B cells and their subsets. During TCZ therapy, CD95 expression on B cells was significantly reduced from a median (range) of 12.9% (4.5-33.3) to 8.5% (2.4-21.1) at week 12 ($p=0.016$) and 7.0% (1.0-19.6) at week 24 ($p<0.0001$). The ki-67 expression significantly reduced from a median 3.2% (1.7-8.3) to 1.5% (0.3-3.6) at week 12 ($p<0.0001$) and 1.0% (0.9-4.9) at week 24 (<0.0001). The primary end point of the activation expression study was at week 24 and the values for analysis are shown in median (range) (Figure 4.17).

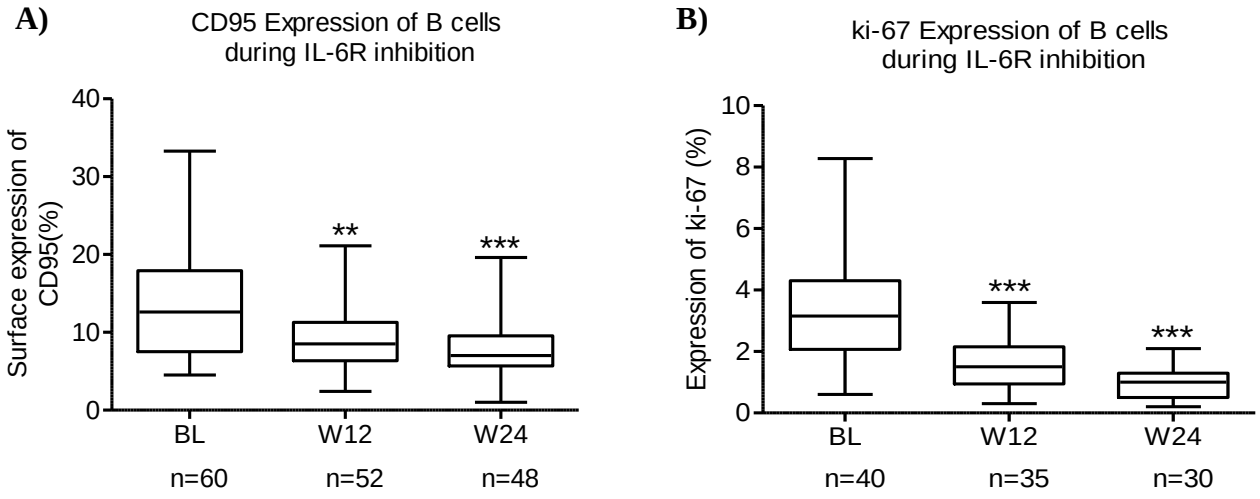

Figure 4.17 Modulation of CD95 and ki-67 expression in B cells during IL-6R inhibition. A) Surface CD95 expressions on CD19+ B cells were significantly reduced during IL-6R inhibition using TCZ at week 12 and week 24. **B)** Intracellular ki-67 expressions on CD19+ B cells were significantly reduced during IL-6R inhibition using TCZ at week 12 and week 24.Values were always compared with baseline levels by using the nonparametric Wilcoxon matched pair test. The values ≤0.05 were considered to be significant (***$p<0.0001$ and **$p<0.001$). n= number of individuals.

Likewise, during ADA therapy, CD95 expression on B cells significantly reduced from a median (range) 11.9% (4.5-23.3) to 6.8% (2.4-18.0) at week 12 (p=0.039) and 6.4% (1.0-15.0) at week 24 (p=0.018), and ki-67 expression declined from median (range) 3.6% (1.7-8.2) to 2.4% (1.0-5.1) at week 12 (p=0.02) and 1.1% (0.5-4.2) at week 24 (p<0.0001). The primary end point of the activation expression study was at week 24 and the values for analysis are shown in median (range) (Figure 4.18).

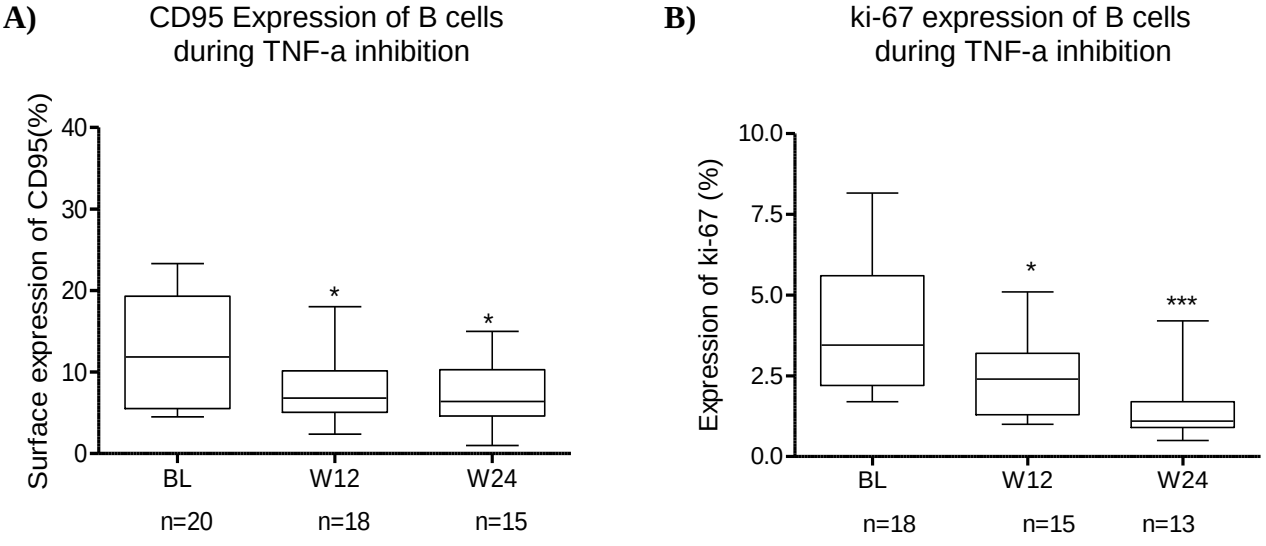

Figure 4.18 Modulation of CD95 and ki-67 expression in B cells during TNF-α inhibition. A) Surface CD95 expressions on CD19+ B cells were significantly reduced during TNF-α inhibition using ADA at week 12 and week 24. **B)** Intracellular ki-67 expressions on CD19+ B cells were significantly reduced during during TNF-α inhibition using ADA at week 12 and week 24. Values were always compared with baseline levels by using the nonparametric Mann-Whitney U test. The values ≤0.05 were considered to be significant (***p<0.0001 and *p<0.01). The primary end point of the activation expression study was at week 24 and the values for analysis are shown in median (range). n= number of individuals, BL = baseline, W12 = week 12 and W24 = week 24.

4.10 Modulation of CD95 and ki-67 expression in B cell subsets during cytokine inhibitions using TCZ

Surface expression of CD95 and intracellular ki-67 expression of B subsets were analysed during cytokine inhibition using IL-6R and TNF-α inhibitors. The expressions of both CD95 and ki-67 were significantly reduced on each B cell subsets during cytokine inhibitions.

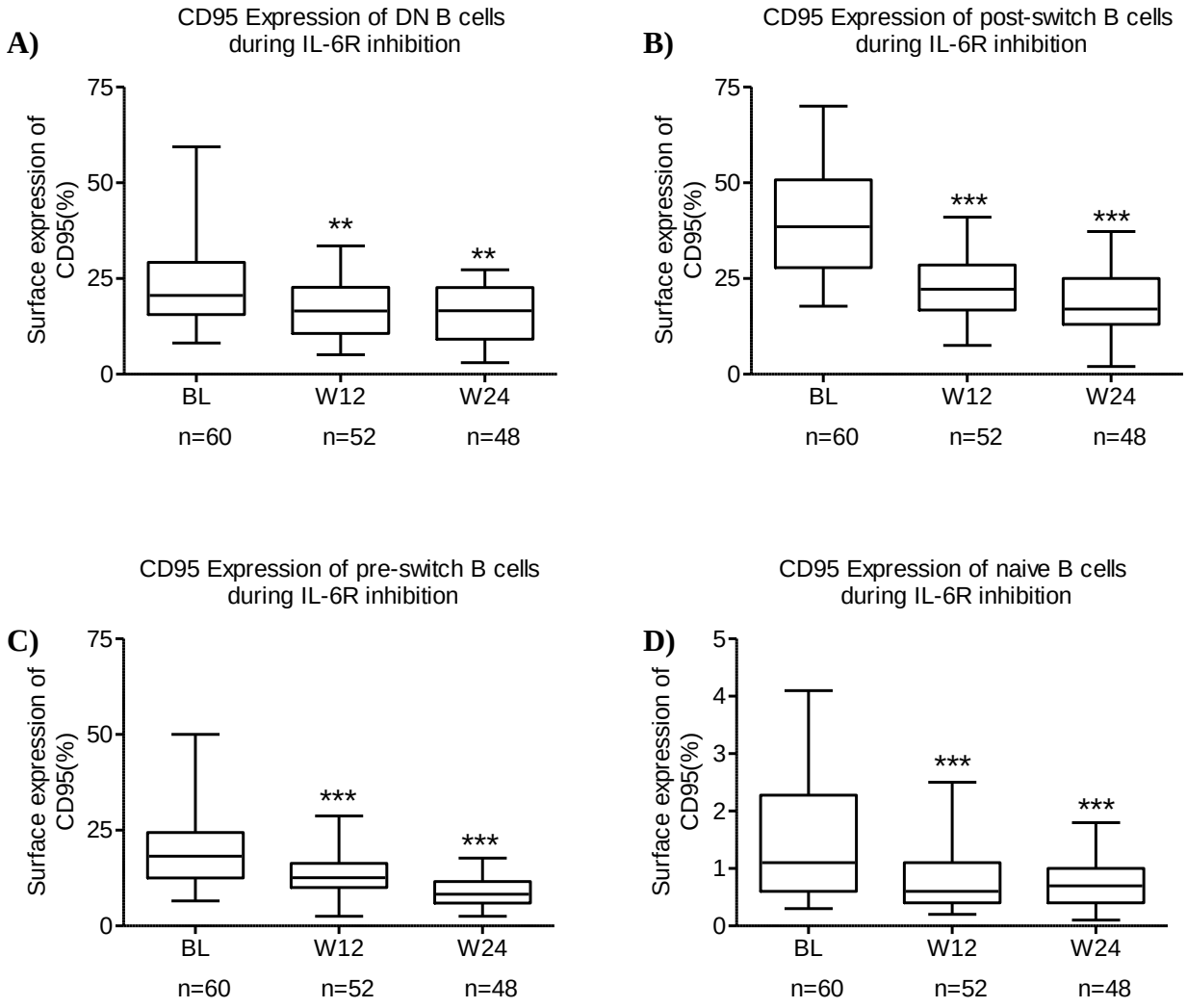

Figure 4.19 Modulation of CD95 expressions in B cell subsets during IL-6R inhibition. CD95 expressions on B cell subsets were significantly reduced during IL-6R inhibition. **A)** DN B cells **B)** post-switch **C)** pre-switch **D)** naïve B cells during IL-6R inhibition.Values were always compared with baseline levels by using the nonparametric Mann-Whitney U test (***p<0.0001, ***p<0.001 and *p<0.01). n= number of individuals, BL = baseline, W12 = week 12 and W24 = week 24.

Similarly, the intracellular expressions of ki-67 of all B cell subsets were significantly reduced during IL-6R inhibition using TCZ (Figure 4.20).

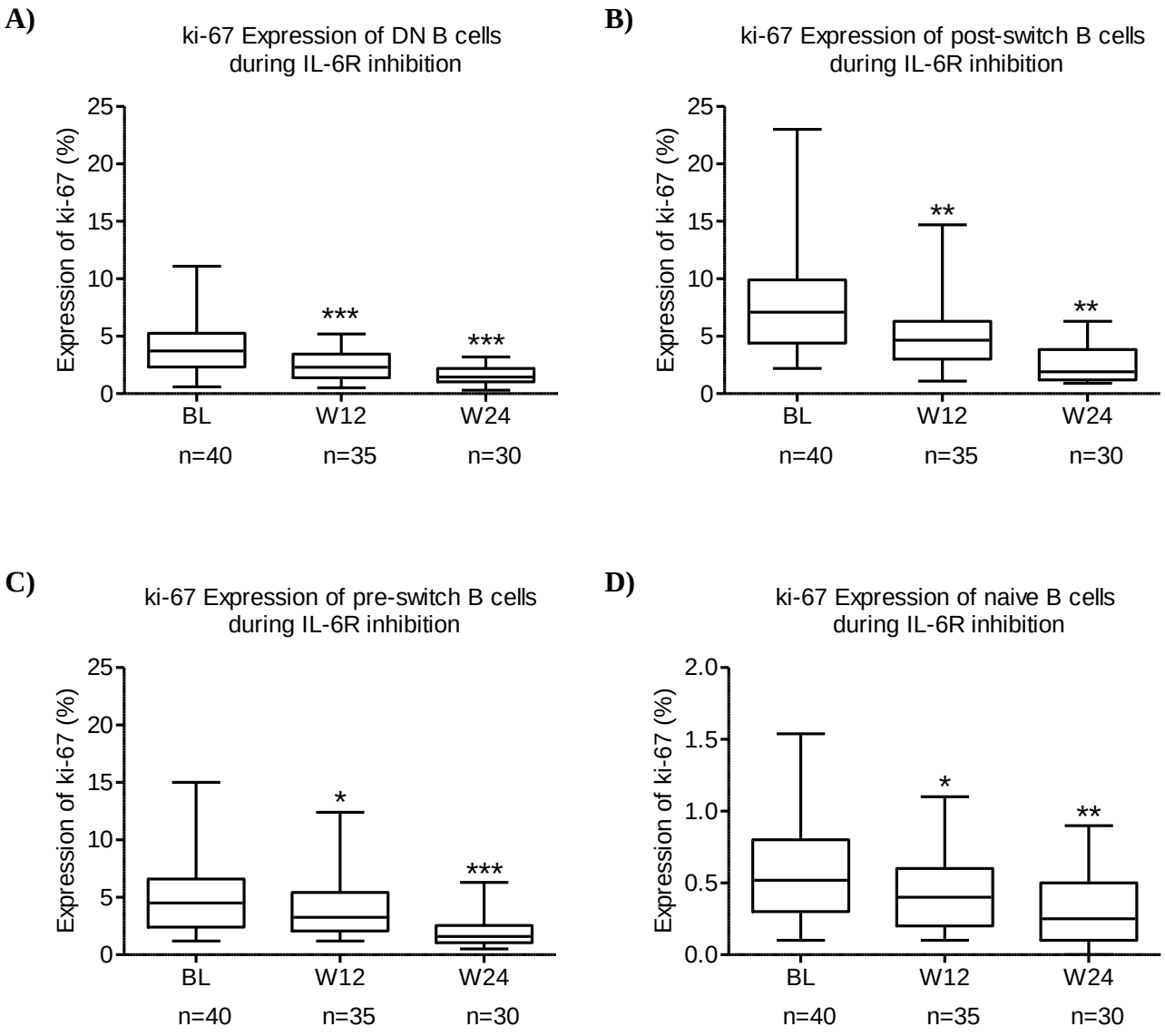

Figure 4.20 Modulation of ki-67 expressions in B cell subsets during IL-6R inhibition. ki-67 expressions on B cell subsets were significantly reduced during IL-6R inhibition. **A)** DN B cells **B)** post-switch **C)** pre-switch **D)** naïve B cells during IL-6R inhibition. Values were always compared with baseline levels by using the nonparametric Mann-Whitney U test (***$p<0.0001$, ***$p<0.001$ and *$p<0.01$). n= number of individuals, BL = baseline, W12 = week 12 and W24 = week 24.

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4.11 Modulation of CD95 and ki-67 expression in B cell subsets during cytokine inhibitions using ADA

Twenty RA patients were treated with anti-TNF-α therapy and expression of activated B cells subsets were analysed (Figure 4.21).

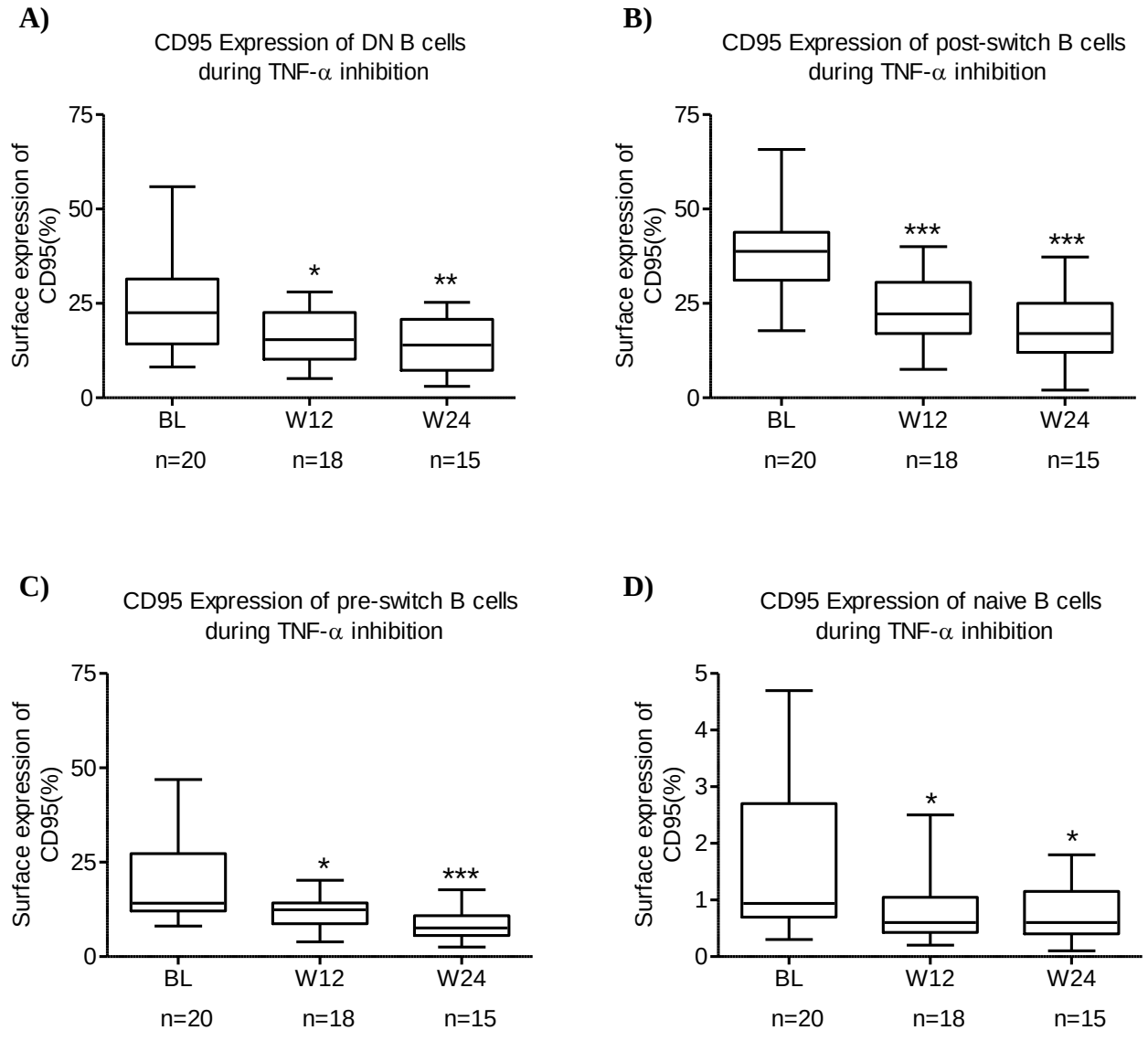


Figure 4.21 Modulation of CD95 expressions in B cell subsets during TNF-α inhibition. CD95 expressions on all B cell subsets **A)** DN B cells **B)** post-switch **C)** pre-switch **D)** naïve B cells were significantly reduced during TNF-α inhibition. Values were always compared with baseline levels by using the nonparametric Mann-Whitney U test (***$p<0.0001$, ***$p<0.001$ and *$p<0.01$). n= number of individuals, BL = baseline, W12 = week 12 and W24 = week 24.

Likewise, the intracellular expressions of ki-67 of all B cell subsets were significantly reduced during TNF-α inhibition using ADA (Figure 4.22).

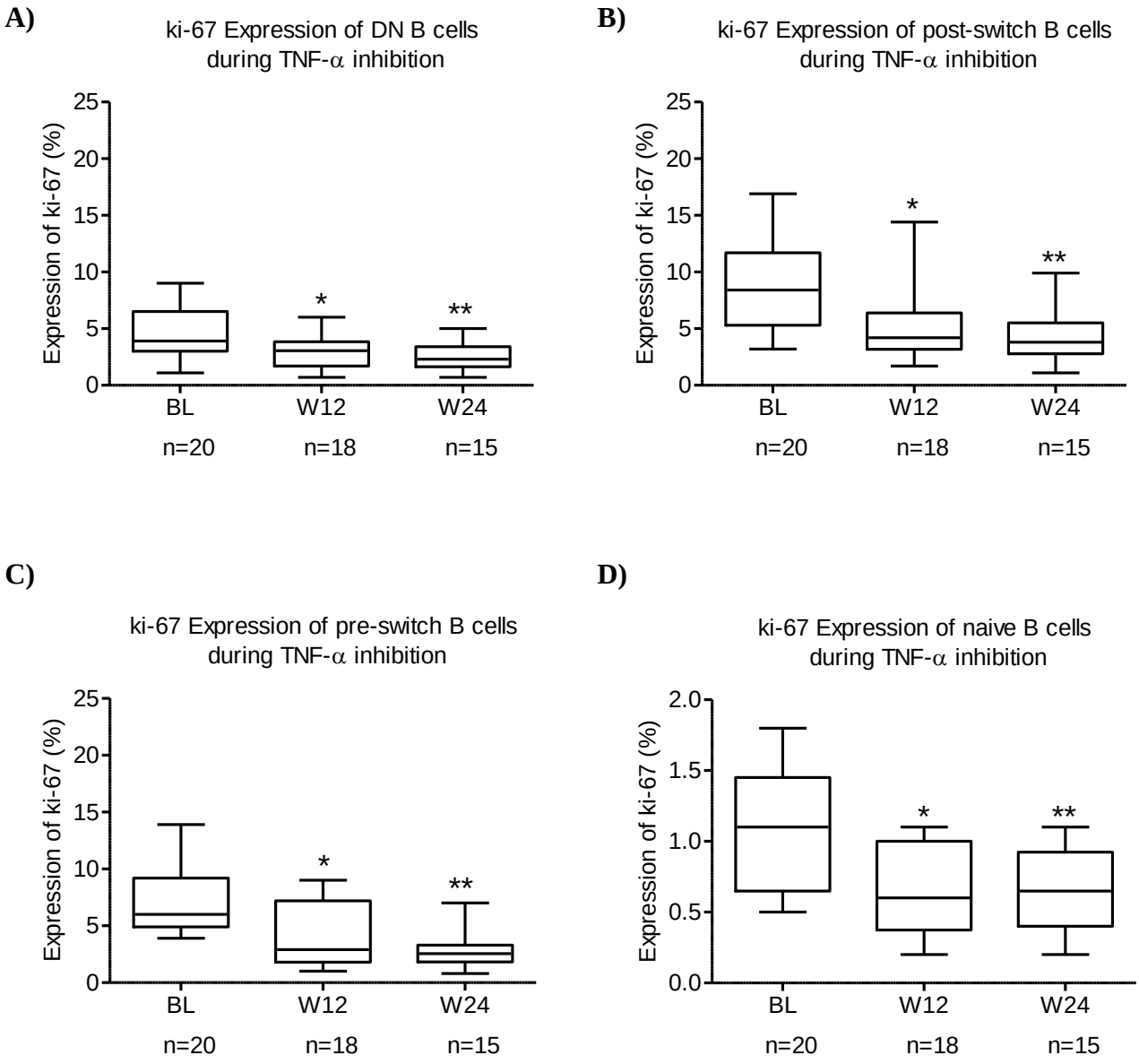


Figure 4.22 Modulation of ki-67 expressions in B cell subsets during TNF-α inhibition. ki-67 expressions on B cell subsets **A)** DN B cells **B)** post-switch **C)** pre-switch **D)** naïve B cells were significantly reduced during TNF-α inhibition. Values were always compared with baseline levels by using the nonparametric Mann-Whitney U test (***p<0.0001, ***p<0.001 and *p<0.01). n= number of individuals, BL = baseline, W12 = week 12 and W24 = week 24.

4.12 Modulation of isotypic Ig class in memory B cells during cytokine inhibition

The next question which was asked if the distribution of IgG+, IgA+ and IgM+ isotypes in the DN and post-switch B cell compartment can be influenced by IL-6R inhibition. IgA, IgG and IgM expressing cells of DN and post-switch memory B cells were analyzed during cytokine inhibition. Since IL-6 inhibition has been shown to influence B cell maturation, we studied DN B cells during TCZ therapy. TCZ did not significantly change the enhanced frequency of the overall population of CD27-IgD- DN B cells. However, by analyzing the Ig isotypes in CD27-IgD- DN B cells we found a significant decrease of relative IgA+ cells from a median (range) 25.1 (8.0-54.2) percent to 19.0 (4.8-51.1) percent at week 12 with P= 0.0008 and 20.5 (4.6-33.8) percent at week 24 with P= 0.0016 respectively. During TCZ therapy, data did not show any remarkable changes in IgG+ or IgM+ B cells (Figure 4.23).

Surface expressions of immunoglobulins in DN B cells during IL-6R inhibition

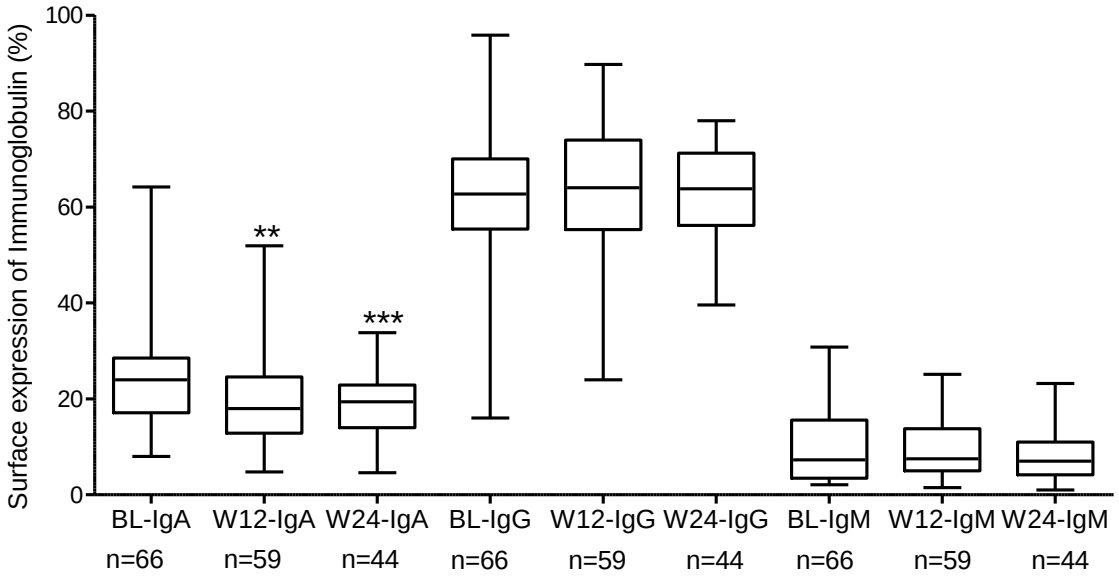


Figure 4.23 Surface expression of Immunoglobulin in DN B cells during IL-6R inhibition. Significant reduction in relative IgA+ B cell whereas no significant changes were observed with IgG and IgM during TCZ therapy. Patients were followed at week 12 and week 24. BL = baseline, W12 =week 12, W24 = week 24, n = number of patients, IgA = IgA expressing cells, IgG = IgG expressing cells, IgM = IgM expressing cells. The Mann-Whitney U test were used for statistics using GraphPad prism 5 (***p<0.0001 and **p<0.001).

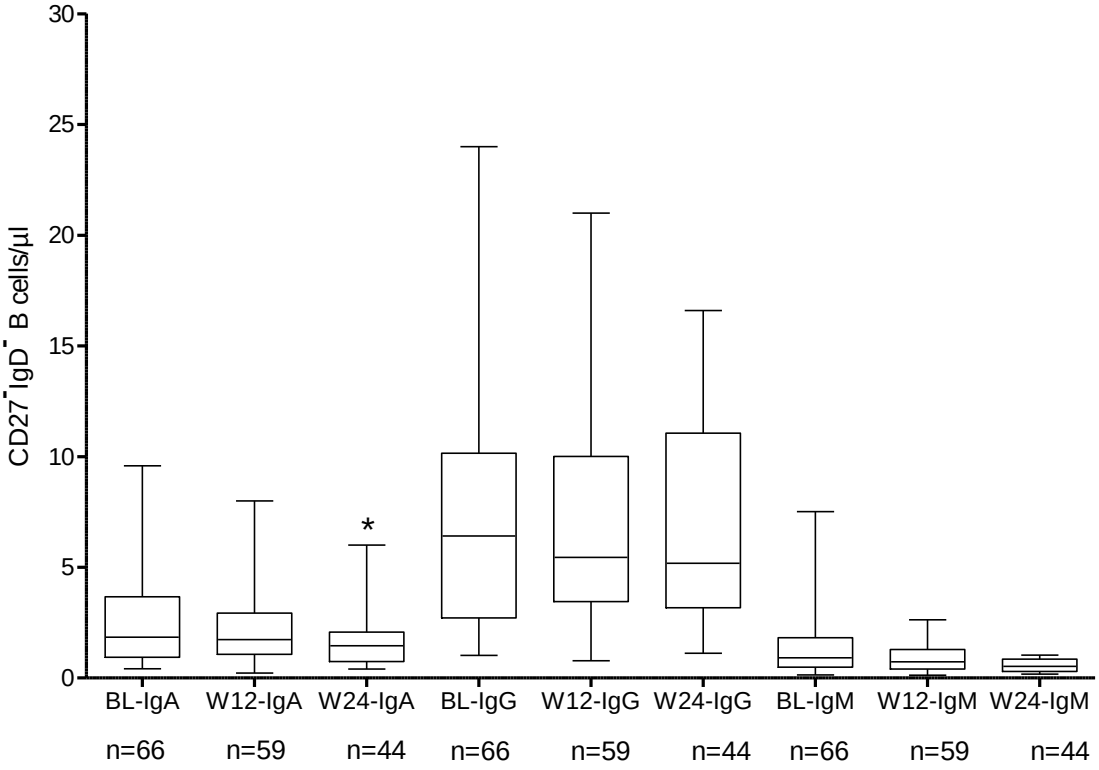


Figure 4.24 Absolute cell numbers of Immunoglobulins expressions in DN B cells. Significant reduction in relative IgA+ B cell Values were always compared with baseline levels by using the nonparametric Mann-Whitney U test (*p<0.01). n= number of individuals, IgA = IgA expressing cells, IgG = IgG expressing cells, IgM = IgM expressing cells, BL = baseline, W12 = week 12 and W24 = week 24.

Moreover, the absolute cell numbers of all three isotype-specific DN B cells declined, especially IgA+, which was significant (P<0.05). The reduction in IgG+ and IgM+ DN B cells were not significant with respect to baseline. In contrast, post-switch memory B cells were observed to be a mixture of equal IgA+ and IgG+ expressing B cells. The frequency of both IgA+ and IgG+ in the post-switch B cells compartment was not influenced during TCZ therapy (Figure 4.25), indicating a more dynamic IgA response in the DN compartment. Interestingly serum IgA levels as well as RF-IgA both declined significantly during TCZ therapy (Table 4.1). Patients were followed at week 12 and week 24 time points during cytokine inhibition therapy using TCZ.

Surface expression of immunoglobulins in post-switch memory B cells during IL-6R inhibition

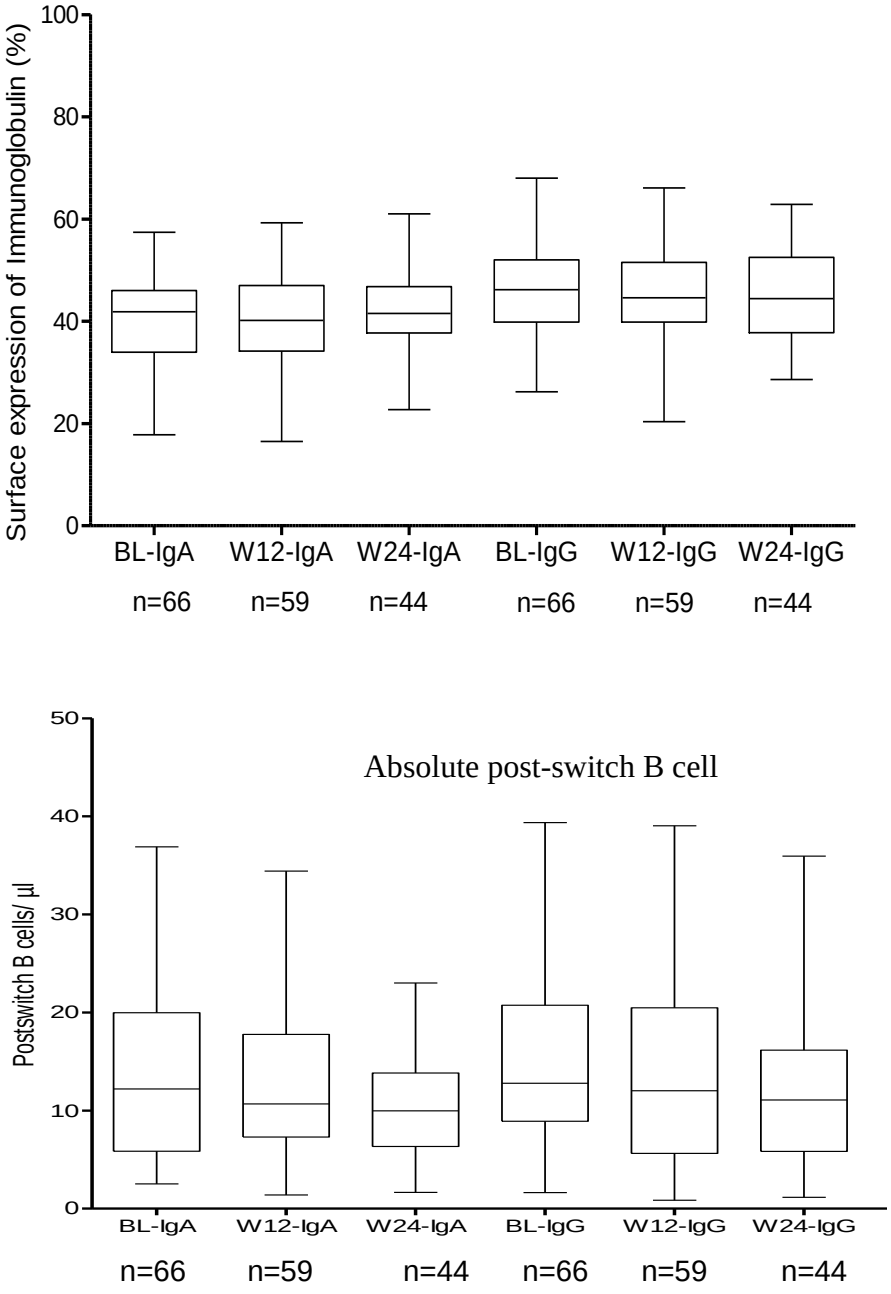
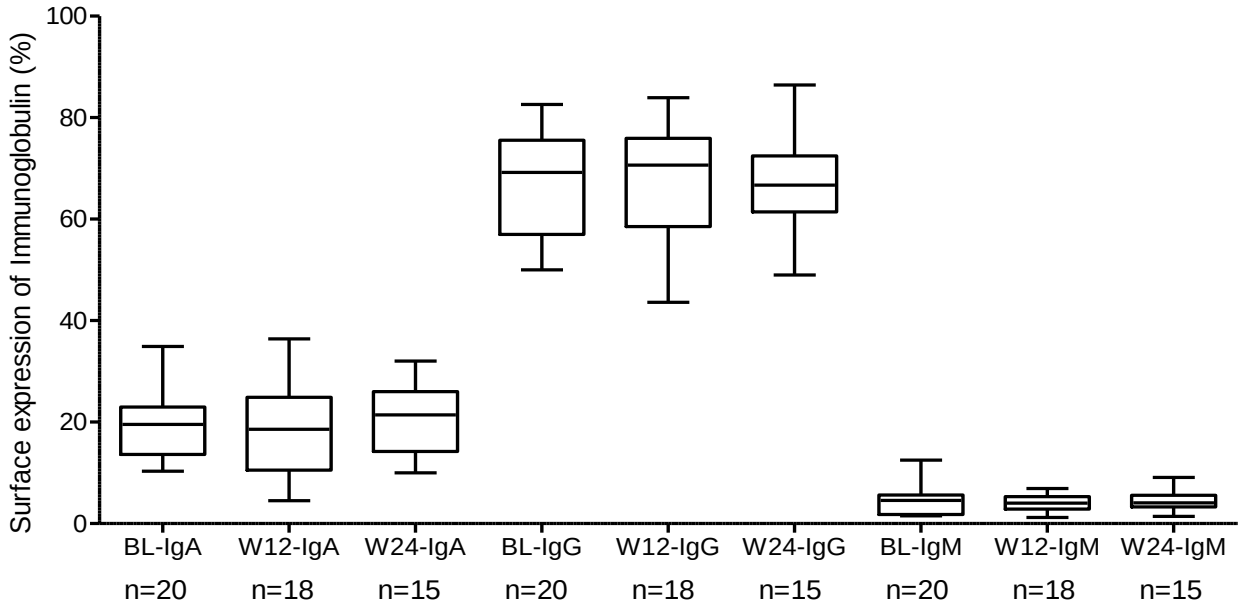


Figure 4.25 Surface expression of Immunoglobulin in post-switch B cells during IL-6R inhibition. No changes were observed in relative as well as IgA+ and IgG+ expression in post-switch memory B cell compartment during TCZ therapy.

There is no remarkable change in the absolute cell numbers of IgA+ and IgG+ expressing post-switch memory B cells during the TCZ therapy.

Surface expression of isotype Ig in DN and post-switch B cells during TNF-α inhibition

Twenty patients were analyzed for surface of expression of isotype Ig class in DN and post-switch memory B cells during anti-TNF-α therapy.



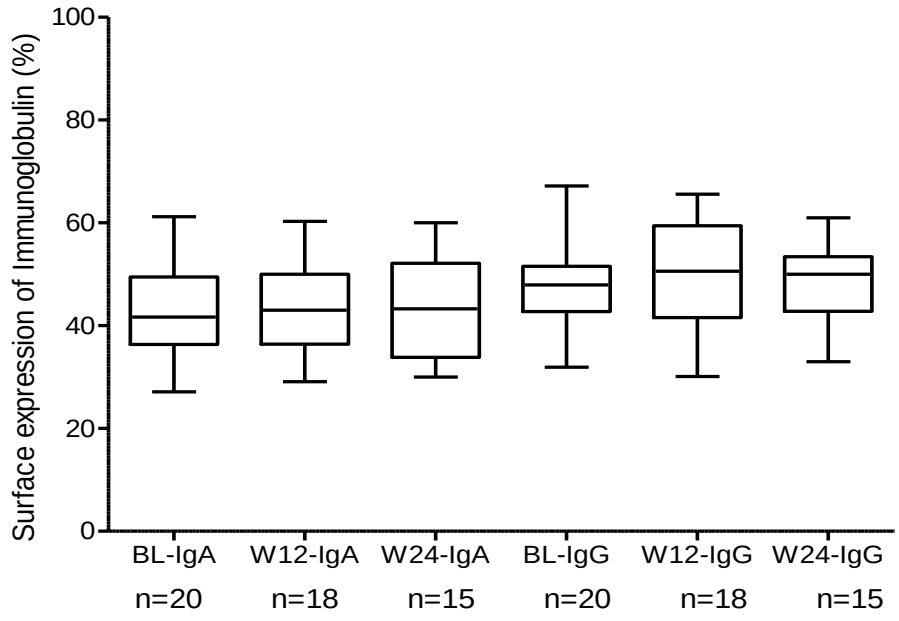


Figure 4.26 Surface expression of Immunoglobulin in DN and post-switch B cells during TNF-α inhibition. No significant changes were observed with IgA and IgG during ADA therapy. Patients were followed at week 12 and week 24. BL = baseline, W12 =week 12, W24

= week 24, n = number of patients, IgA = IgA expressing post-switch B cells, IgG = IgG expressing post-switch B cells.

Moreover, phenotypically analyzed data from 20 patients undergoing ADA therapy did not reveal any remarkable changes in their isotype distribution, relative as well as absolute. Relative data of DN and post-switch B cells expressing isotype immunolglobulin has been shown in Figure 4.25-4.26

4.13 Somatically mutated Ig-receptors in memory B cell subsets

In a subsequent study we performed molecular analysis of DN B cells and analyzed in detail the immunoglobulin receptor of individually sorted cells from healthy donors (n = 3) and from RA patients during TCZ (n = 9) or anti-TNF-α therapy (n = 4) using single cell sequencing. Ig-VH3 gene rearrangements of DN B cells Ig-R showed less mutation compared to pre-switch and post-switch B cells. Specifically, mutational frequency of DN B cells was 4.0 ± 0.2% compared to 4.5 ± 0.2% for pre-switch B cells, and 6.2 ± 0.3% for post-switch B cells. Moreover, the mutational frequency of DN B cells was comparable in RA and HD. DN B cells largely containing memory-like B cells had the least mutational frequency amongst the three memory B cell subsets.

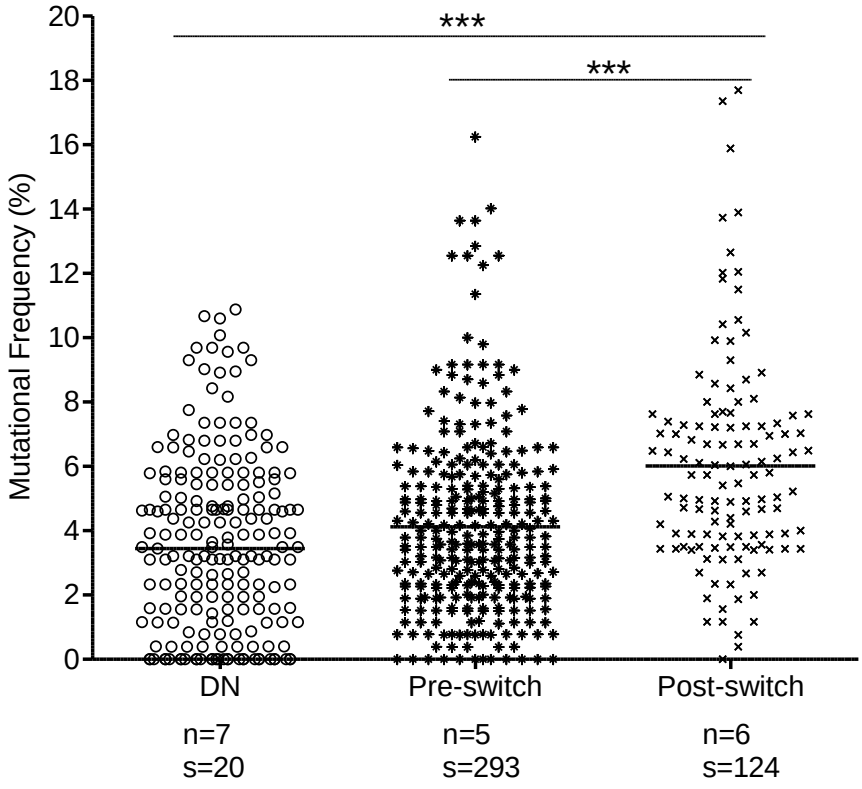


Figure 4.27 Ig-receptor somatic hypermutation of gene rearrangements. Ig-VH3 gene rearrangements in memory B cell subsets namely DN, pre-switch and post-switch. n=number of individuals. S=number of sequences, n= number of individuals

4.14 Modulation of somatically mutated Ig-receptor of DN B cells during IL-6R Inhibition

Modulation in somatic hypermutation.

Prior to tocilizumab (TCZ) therapy, IgR of Ig-VH3 gene rearrangements of DN B cells were mutated containing, both low and high mutated sequences. No fundamental difference was observed in mutational frequency of these cells between RA and HD. To find a therapy-induced modulation in the Ig receptor of DN B cells, we treated these patients with TCZ and performed a longitudinal analysis of these cells at different time intervals (week 12, week 24 and 1 year). Foremost, mutational frequency of Ig-R of DN B cells was significantly reduced at week 12, week 24 and 1 year during TCZ therapy which correlates with the decrease in inflammatory activity (CRP, ESR and DAS28), as shown in table 4.1. In detail, mutational frequency of DN B cells decreased from 4.04±0.19% (baseline) to 2.52±0.16% at week 12 ($p<0.0001$), 1.98±0.3% at week 24 ($p<0.0001$) and 1.89±0.3% ($p<0.0001$) at 1 year, respectively, during TCZ therapy.

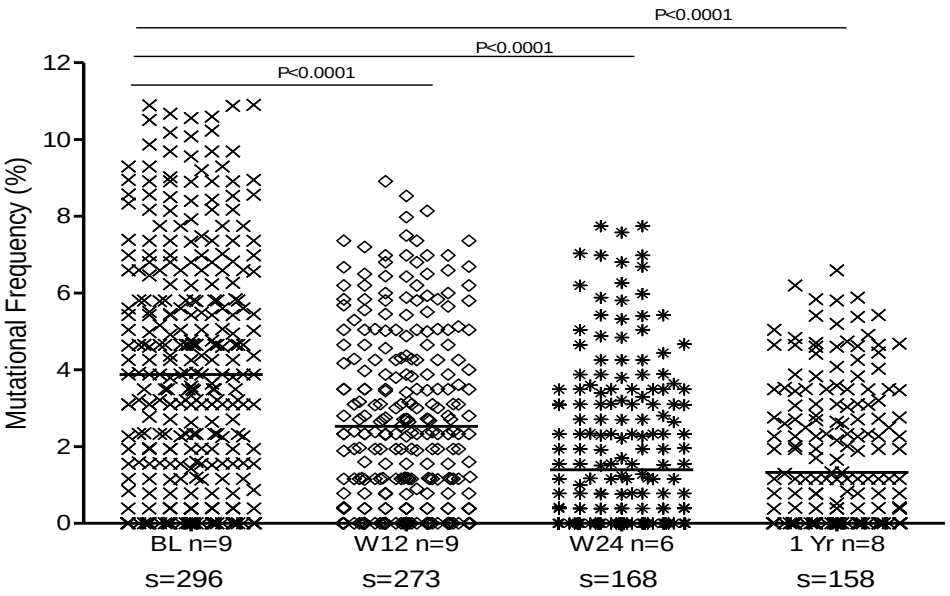


Figure 4.28 Ig-receptor somatic hypermutation of gene rearrangements during IL-6R in DN B cells. Reduction in mutational frequency of Ig-VH3 gene rearrangements of DN B cells from the peripheral blood of RA patients during TCZ therapy. The mutational frequency was significantly reduced at week 12, 24 and 1 year (***$p < 0.0001$ compared to baseline). In a scatter plot, the line represents mean of all values and each dot depicts the mutational

frequency of a single sequence. P values were determined by Wilcoxon test using Graphpad prism 5. (BL = baseline, W12 = week 12, W24 = week 24, n = number of individuals, s = number of sequence analyzed).

Based on mutational frequency of individual sequences, we observed a significant decrease in the number of highly mutated sequences during the course of therapy (Figure 4.29). Highly mutated sequences decreased from 46.2±5.1% (baseline) to 28.7±5.5% at week 12 (p= 0.018), 23.1±6.8% at week 24 (p= 0.035) and 17.3±3.9% at 1 year (p=0.0003) during TCZ therapy. The percentage of low mutated sequences changed remarkably at week 12 (p=0.05) and week 24 (p=0.05). Significant increase was observed in the non-mutated sequences from 15.1±3.4% (baseline) to 22.7±3.7% at week 12 (p= 0.05), 32.1±4.6% at week 24 (p=0.005) and 37.1±4.6% at 1 year (p= 0.004). Overall, these findings indicate a very protracted acquisition of mutations in DN B cells during TCZ therapy.

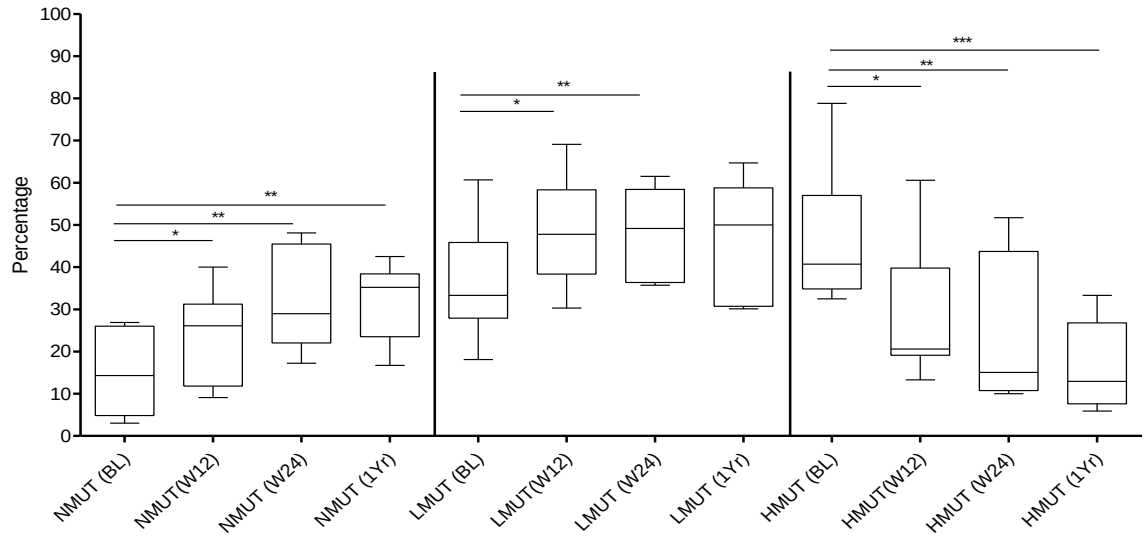


Figure 4.29 Distribution of mutations, classified as unmutated (NMUT), low (LMUT) and high mutated (HMUT) sequences within DN B cells. Under TCZ therapy, a significant reduction in the high mutated sequences and increase in the unmutated and low mutated sequences were observed.

Reduced targeting of mutational hotspots motifs.

It is known that somatic hypermutation favours defined mutational hotspots. In particular, RGYW/WRCY motif mutations (R, purine; Y, pyrimidine; W, A/T) are preferentially targeted by T-cell-dependent signals including CD40–CD40 ligand interactions (Muhammad et al., 2011). Analysis of the frequency of targeted RGYW/WRCY mutations (R, purine; Y, pyrimidine; W, A/T) showed that it decreased significantly from median (range) 24.6 (21.1-38.9) at baseline to 20.5 (17.7-26.1) at week 12 (p = 0.046), 20.7 (14.9-23.4) at week 24 (p = 0.004) and 19.2 (12.6-24.5) at 1 year (p = 0.004) during TCZ therapy in all patients (Figure 4.30).

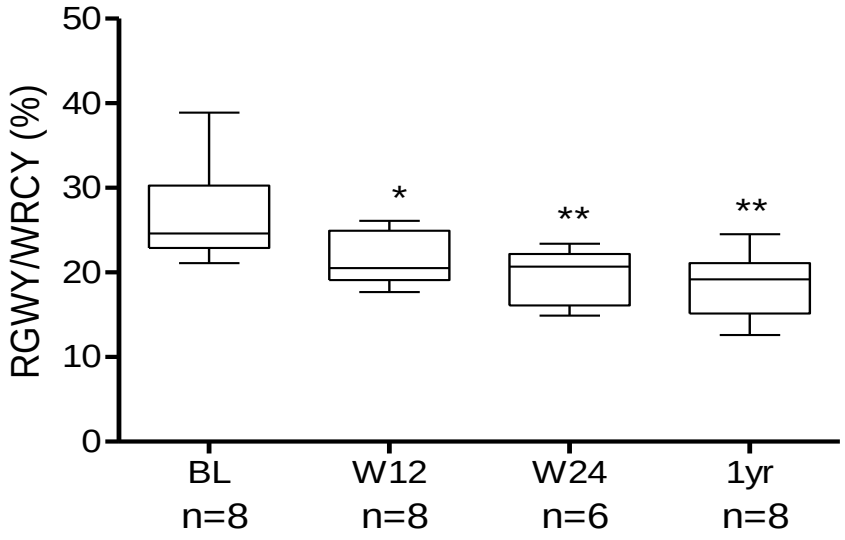


Figure 4.30 Frequency of targeted mutations within hotspot motifs during IL-6R inhibition. Significant reduction in frequency of targeted mutations within RGWY/WRCY is found in Ig-VH gene rearrangements of DN B cells during TCZ therapy (*p = 0.046, **p = 0.004).

Increased third complimentary determining region (CDR3) length during TCZ therapy.

The CDR3 region of the Ig gene is thought to have the most influence over antigen specificity; therefore we also studied CDR3 characteristics of Ig-receptors of DN B cells. In Figure 4.31, CDR3 lengths of VH gene rearrangements at different time points are shown. In detail, CDR3 length of DN B cells increased from (mean±SEM) 44.7±0.6 bp (baseline) to 48.2±0.7 bp at week 12 ($p=0.0004$), 50.6±1.1 bp at week 24 ($p<0.0001$) and 50.9±1.1% ($p<0.0001$) at 1 year respectively during TCZ therapy (Figure 4.31).

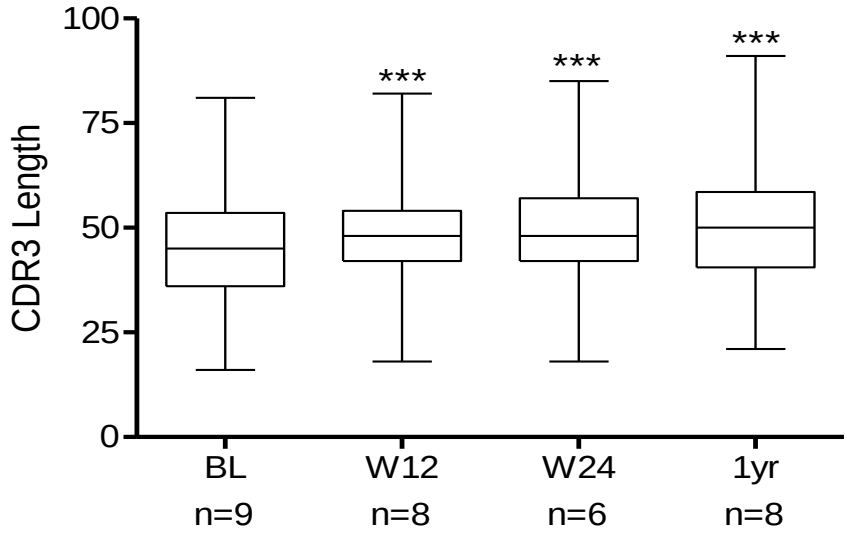


Figure 4.31 CDR3 length during IL-6R inhibition. Significant increase in the CDR3 length of Ig-VH gene rearrangements of DN B cells from the peripheral blood of RA patients during IL-6R inhibition (***$p< 0.0001$). P values were determined by Wilcoxon test using GraphPad prism 5.

Overall these findings indicate that TCZ therapy not only modulates acquired somatic hypermutations but also exerts effects on the length of CDR3 in DN B cells. Thus, functionality of antigenic stimulation and/or selection seems to be less on these cells.

4.15 Ig-specific isotypes are modulated during TCZ therapy in DN B cells

Modulation in mutational frequency.

Since we observed modulation in overall Ig-receptor of DN B cells during TCZ therapy, we asked ourselves if such modulation might also be present in isotypic composition which might reveal the possible cause of decreased mutational frequency of these DN B cells during TCZ therapy. For this purpose, we performed isotype-specific PCRs from single sorted DN cells and amplified the Cγ,Cμ,Cα specific immunoglobulin gene rearrangements (Figure 4.32). Interestingly, at the baseline, the isotype-specific IgR analysis revealed that mutational frequencies of IgA+ were higher than IgG+ and IgM+ DN B cells. However, as expected IgM+ cells contained low mutational frequency both in HD and RA patients. During TCZ therapy, all isotypes IgA+, IgG+ and IgM+ DN B cells showed a significantly reduced mutational frequency.

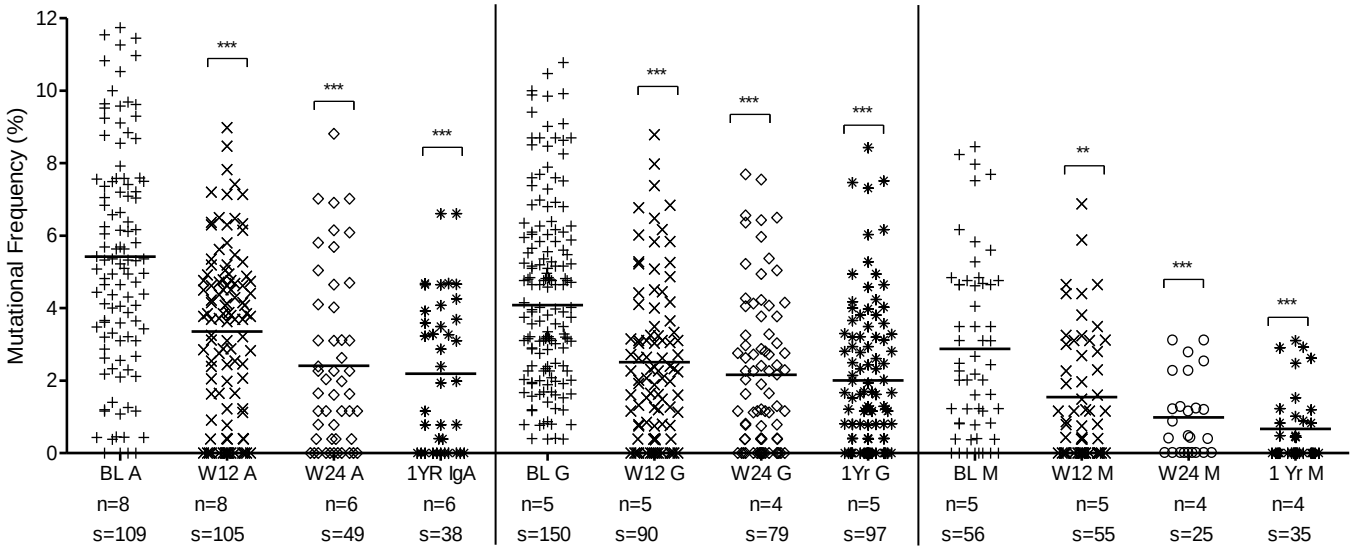


Figure 4.32 Ig-receptor somatic hypermutation of isotype specific IgA+, IgG+ and IgM+ gene rearrangements during IL-6R Inhibition in DN B cells. At the baseline, the mutational frequency of IgA+ DN B cells is significantly higher compared to IgG+ and IgM+ DN B cells. During TCZ therapy, all isotypes IgA+, IgG+ and IgM+ DN B cells showed a significantly reduced mutational frequency (***$P < 0.0001$,**$P < 0.001$).

Mutational frequency of IgA+ cells reduced from percent (mean±SEM) 5.42±0.30% (baseline) to 3.35±0.22% at week 12 (P<0.0001), 2.41±0.34 % at week 24 (P<0.0001) and 2.26±0.33 % (P<0.0001) at 1 year. Mutational frequency of IgG+ cells reduced from 4.45±0.20% (baseline) to 2.51±0.22% at week 12 (p<0.0001), 2.16±0.23 % at week 24 (P<0.0001) and 2.00±0.20 % (P<0.0001) at 1 year. Similarly, mutational frequency of IgM+ cells also decreased during TCZ therapy from 2.87±0.32% (baseline) to 1.55±0.23% at week 12 (p=0.0016), 0.98±0.21% at week 24 (p=0.0008) and 0.67±0.17 % (P<0.0001) during 1 year TCZ treatment. This data of reduced mutational frequency of IgA+, IgG+ and IgM+ is consistent with overall decreased mutational frequency observed during TCZ treatment (Figure 4.28). Furthermore, the isotype-specific IgR anaylsis in post-switch B cells came out with no remarkable change in mutational frequencies of IgA+ and IgG+ B cells during TCZ therapy in three patients. These findings show that mutational frequencies in DN B cell are modulated by TCZ therapy but not in post-switch cells.

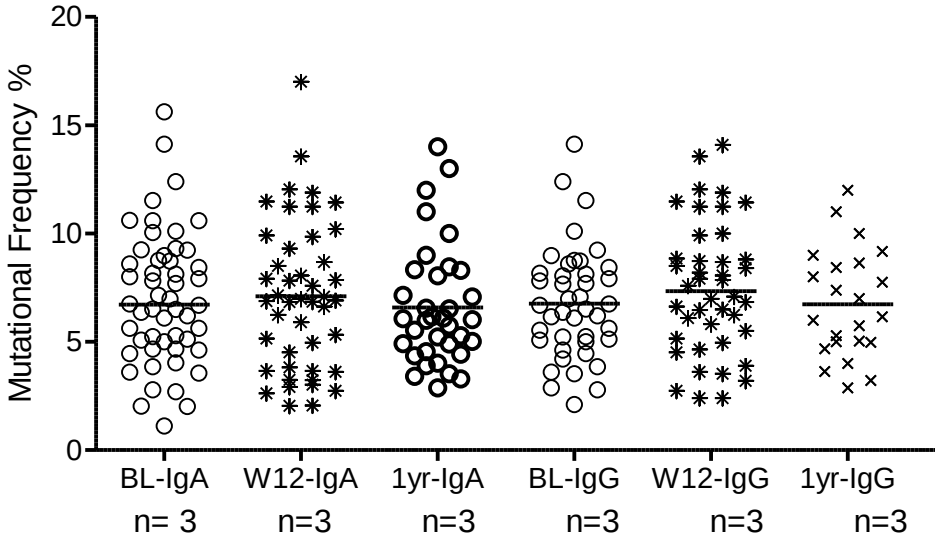


Figure 4.33 Ig-receptor somatic hypermutation of of isotype specific IgA+ and IgG+ gene rearrangements during IL-6R Inhibition in post-switch memory B cells. No changes in mutational frequency were observed during TCZ therapy.

Reduced targeting of mutational hotspots motifs during TCZ therapy.

The frequency analysis of targeted mutations were found to decrease significantly from baseline to week 12, week 24 and 1 year in all isotype specific IgA, IgG and IgM positives DN B cells during TCZ therapy.

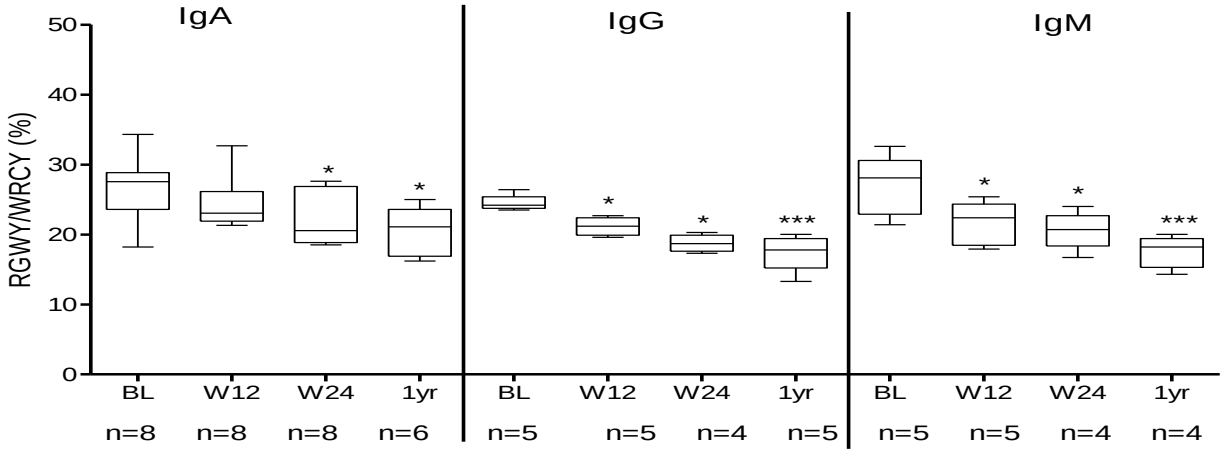


Figure 4.34 Frequency of targeted mutations within hotspot motifs during IL-6R inhibition.

Increased CDR3 length during TCZ therapy.

Likewise, the CDR3 lengths of isotype specific IgA, IgG and IgM gene rearrangements at different time points were calculated. CDR3 length of isotype specific DN B cells increased from baseline to week 12, 24 and 1 year during TCZ therapy (Figure 4.35).

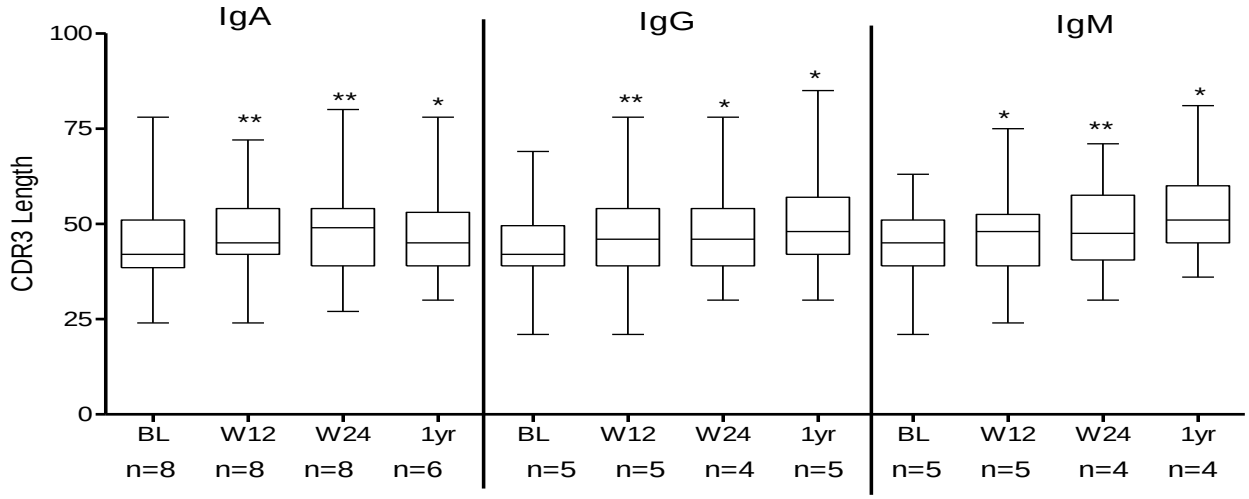


Figure 4.35 CDR3 length during IL-6R inhibition. Significant increase in the CDR3 length in all 3 isotypes during TCZ therapy.

4.16 Anti-TNF-α therapy failed to modulate the mutational frequency in DN B cells

To evaluate more specific TCZ modulation in the somatic hypermutation of Ig gene arrangement, we analyzed the mutational frequency under anti-TNF-α therapy. As an alternative anti-inflammatory therapy, we studied patients who were treated with tumor necrosis factor-alpha (TNF-α) inhibition using adalimumab (n=4) at three different time intervals: baseline, week 12, week 24 and 1 year (Figure 4.36). Notably, contrary to the results with IL-6R inhibition, results from 4 patients undergoing adalimumab therapy revealed that the percentage of mutational frequencies of Ig-R was not changes at all time-points. The mutational frequencies of IgR of DN B cells were comparable to baseline values of pre-IL-6R inhibition patients. During anti-TNF-α therapy, the inflammatory activity (CRP, ESR and DAS28) was significantly reduced and got normalized and remains within range during theeapy.

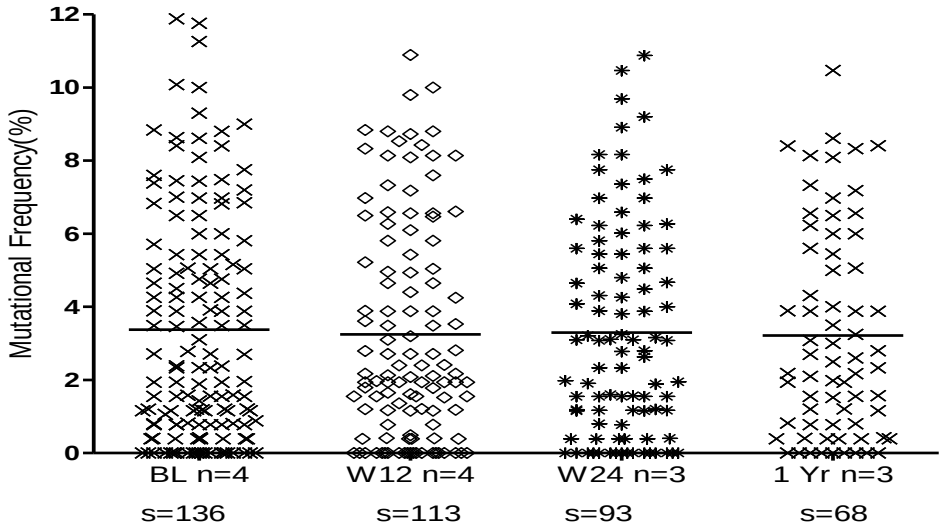


Figure 4.36 Ig-receptor somatic hypermutation of VH gene rearrangements during TNF-α inhibition. During TNF-α inhibition by adalimumab (ADA), a comparable mutational frequency of VH gene rearrangements of DN B cells from the peripheral blood of RA patients were observed at all-time points. In a scatter plot, the line represents mean of all values and each dot as a mutational frequency of a single sequence.s=total number of sequences, n=number of individuals, BL=baseline, W12=week12 and W24= week 24.

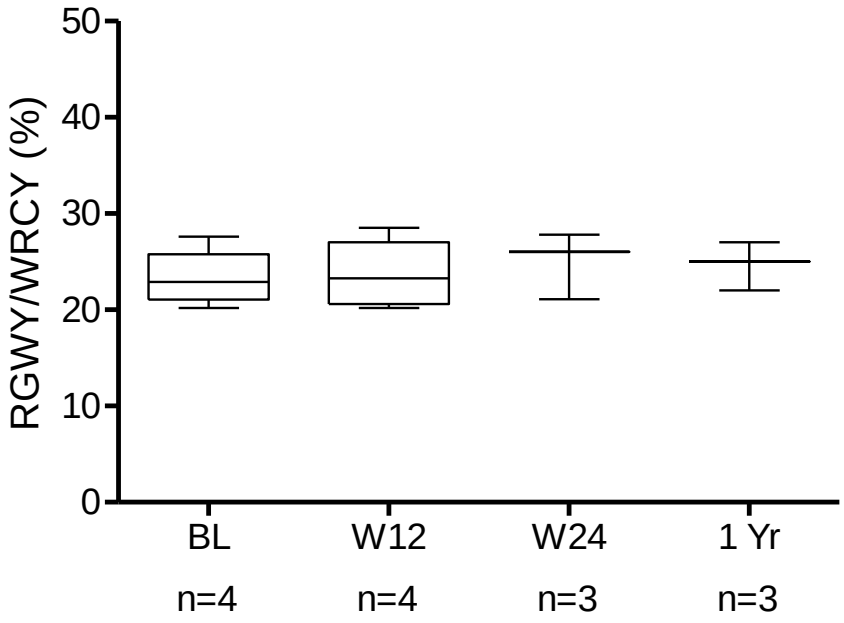


Figure 4.37 Frequency of targeted mutations within hotspot motifs during TNF-α inhibition. These results indicate that anti-TNF-α therapy does not lead to the decrease in mutational frequencies and influences the process of somatic hypermutation of DN B cells.

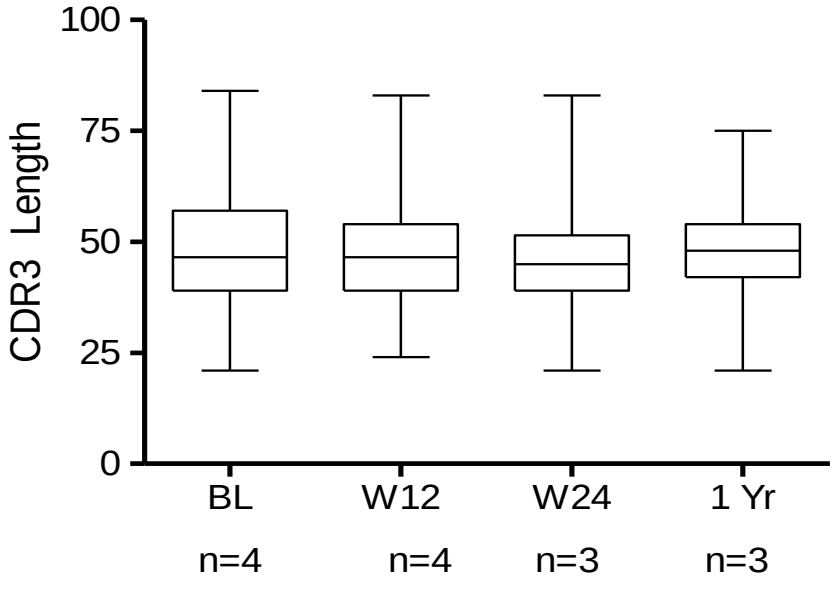


Figure 4.38 CDR3 length during TNF-α inhibition. No significant changes in CDR3 length during TNF-α inhibition using ADA.

The mutational frequency, mutational hotspot targeting and CDR3 length analysis revealed no change in DN B cells Ig-receptor during TNF-α inhibition using ADA. These results indicate that anti-TNF-α therapy does not influence the process of somatic hypermutation of DN B cells.

5. Discussion

Rheumatoid arthritis (RA) is a chronic, systemic, autoimmune disease characterized by inflammation of the joints, which results in their progressive destruction and is a major cause of disability. Being the most common inflammatory arthritis affecting about 1% of the population, RA is a complex disease that involves many cells of the immune system. In disease process inflammatory cells are recruited to the joints and different cell types play different roles (Firestein, 2003; Klareskog et al., 2009). With the increasing understanding of the mechanistic pathways of B cell involvement of self-destruction in autoimmune diseases, there is strong confirmation that B cells play a central role in the pathogenesis of several autoimmune disease including RA (Roll et al., 2015). The role of B cell is not only restricted to autoantibody production but rather also includes B cells as antigen presenting cells to autoreactive T cells or production of inflammatory cytokines. Extensive RA studies indicate chronic B cell activation and memory B cells accumulation in peripheral blood and synovial membrane (Wang et al., 2013). Therefore, an enhanced B cell activation and expansion of specific B cell subsets might play major role in RA pathogenesis (Henneken et al., 2005).

Memory B cells, in particular, seem to be important in driving chronic inflammation (Edwards et al., 2004; Muhammad et al., 2009). Memory B cells have substantial phenotypic heterogeneity and in human CD27 can serve as a good marker for memory B cells with mutated IgR. Apart from the classical CD27+ post-switch (CD27+IgD-) and pre-switch (CD27+IgD+) memory B cells, studies have shown a population (CD27-IgD-) DN B cells that bears all signatures of memory B cells (Fecteau et al., 2006; Jacobi et al., 2008; Wei et al., 2007) (Figure 4.3). Since the role of B cell revealed several approaches targeting B cells (anti-B cell therapy) directly and indirectly are in practice and provide powerful tools in the treatment of RA (Mariette, 2004).

Targeting pre-switch and post-switch memory B cells has been found to be important for the response to rituximab (Brezinschek et al., 2012). However the impact of different memory B cell subsets has not been thoroughly studied in RA. Moreover, memory B cells have gained particular interest in evaluating response to therapies with biologics that have

shown promising results in the treatment of RA. Biological agents (biologics) targeting key proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) have substantially advanced the treatment of autoimmunity (Carbone et al., 2013). Particularly, treatment with TNF blockers ameliorates the signs, symptoms and disease progression of RA (Feldmann, 2002; Lipsky et al., 2000; Souto-Carneiro et al., 2009). Also, IL-6 is a multifunctional pleiotropic cytokine which act as stimulator of both B and T cell functions. It has been previously described as a late-acting B cell differentiation factor that is involved in *in vitro* differentiation of B cells into antibody-forming cells and germinal center reactions. Dysregulation of IL-6 production and its pathological role in different autoimmune diseases have been well documented and highlight IL-6 and its signaling cascade as a potential target for autoimmune therapy. Elevated IL-6 levels have been associated with disease activity and progression of RA. IL-6 inhibition using IL-6R blockade (TCZ) has also shown convincing clinical efficacy by reduction of signs/symptoms and a marked inhibition of radiological progression (Dorner and Lipsky, 2014; Garnero et al., 2010; Souto-Carneiro et al., 2009; Tanaka et al., 2012).

The impact of cytokine inhibitions on different memory B cell subsets has not been thoroughly studied in RA. Therefore, we analyzed different memory B cell compartments in RA during *in vivo* IL-6R and TNF-α inhibition in detail, specifically the DN B cell compartment by phenotypic and molecular analyses of the different isotypic DN B cell receptors, their Ig-R mutational pattern and their modulation. Although enhanced B cell activation has been acknowledged in autoimmunity but only scarce data are available regarding B cell activation in different memory subsets and their modulation during cytokine inhibition. Therefore, the main aim of this thesis is to further explore peripheral memory B cell subsets in RA patients and the effect of cytokine inhibition.

5.1 Activated B cells and their subsets in RA

Enhanced B cell activation has already been reported in autoimmune diseases like SLE and RA and might play a major role in pathogenesis (Henneken et al., 2005; Wang et al., 2013). A previous study had shown that the expression of CD86 and CD95 were up-regulated in B cells of patients with new-onset RA and the percentages of CD86+CD19+ B cells were positively correlated with the values of DAS28 in those patients (Wang et al., 2013). Earlier reports in SLE also showed that the percentage of CD95 expression by B cells were increased, related to their activation and correlate to disease activity in patients (Bijl et al., 2001). SLE patients showed higher expression of CD95 in CD27+ B cells (Odendahl et al., 2000), and CD95+ CD27−IgD− B cells were shown to be associated with active disease (Jacobi et al., 2008). We analyzed B cell activation by surface expression of CD95 and intracellular expression of ki-67 and extended our study for activation in different B cell subsets and their modulation during cytokine inhibition. Our first data set revealed that an activated phenotype has been observed in the B cells and memory B cell subsets including DN, pre-switch and post-switch B cells of RA patients. The higher activity of B cell and their subsets were due to the high expressions of surface CD95 (n=60) and intracellular ki-67 (n=40) in RA as compared to healthy donors (HD, n=20) (Figure 4.5-4.7). The median of activation markers of B cells (CD95, ki-67) were significantly higher ($p<0.0001$) as compared to HD. In active RA patients, disease activity measures like DAS28 score and other inflammatory factors (ESR & CRP levels) are elevated. From regression analysis of activated B cells and DAS28, there is positive correlation with B cell expressing higher CD95 ($r^2=0.329$, $p=0.004$) & ki-67 ($r^2=0.357$, $p=0.008$) as shown in Figure 4.13.

Among all the B cell subsets, the expression of CD95 and ki-67 of post-switch B cell was significantly higher with all subsets while pre-switch and DN had no significant difference in their expressions (CD95 & ki-67). This implies that overall B cell activation might have more dependence on post-switch and less dependence on DN B cells (Figure 4.8-4.9).

Next we wanted to ask whether activated B cells serve as markers for disease activity or response to therapy. We reported in active RA patients DAS28 score and other inflammatory factors like ESR, CRP levels to be highly elevated which is common in this disease condition. During anti-IL-6R and anti-TNF-α the higher activity of B cells and their subsets analysed by expression of CD95 and ki-67 were significantly reduced at week 12 and week 24 (Figure 4.18 - Figure 4.21). DAS28 score and other inflammatory factors like ESR, CRP levels were also significantly (<0.0001) reduced during both the anti-cytokine therapy (Table 4.1, Figure 4.1-4.2).

Under both kinds of cytokine inhibition therapies the B cell and B cell subset activation reduced significantly along with disease activity and inflammatory parameters like ESR, CRP.

5.2 Phenotype of B cell subsets in active RA

These results led us to explore more in detail about the phenotype of B cell memory subsets in active RA patients and focussed our attention on unique population of memory B cells. Since DN B cells carry the features of memory B cells like somatic hypermutation, we were particularly interested in studying their behaviour in active RA. So far these cells have not been thoroughly studied in RA with generally scarce information available in the literature. DN B cells are a heterogeneous mixture of IgG-, IgA-, IgM- isotype expressing cells with a mutated IgR and confer a notable component of the peripheral blood B cell compartment in RA patients (Figure 4.3). We found a relatively higher frequency of the DN B cell subset in RA (n=86) patients (p<0.0001) compared to healthy donors (n=45) (Figure 4.10). Due to lymphopenia, the absolute numbers of DN B cells were comparable to HD in RA patients. An expansion of DN B cells has been described in systemic lupus erythematosus (SLE) and could be linked to autoimmunity by analysis of the specific autoantibodies including 9G4 expression (Wei et al., 2007). Other studies also suggest increased DN B cells in patients with infections indicating their physiological role in certain immune responses (Jacobi et al., 2008). Elevated DN memory B cells have been described to be related to aging and persistent antigen stimulation and seem to reflect overall B-cell hyperactivity (Colonna-Romano et al., 2009; De

Jong et al., 1992; Kai et al., 1999; Swaak et al., 1995; Wei et al., 2007). A recent report suggested that Syk++ B cells lacking CD27 expression represent a unique atypical memory-like B cell which may be relevant for IgG+ plasmablast generation (Fleischer et al., 2014). However, only limited data are available for these DN B cell subsets in RA, especially during treatments with biologics.

The phenotypic analysis of heterogeneous DN B cells of RA patients (n=66), showed that this population contains IgG+, IgA+ and IgM+ DN B cells with pre-dominance of the IgG+ which is clearly different from post-switch memory B cells (Figure 4.11-4.12). The frequency of IgG expressing cells comprises more than ~60% in the DN compartment followed by IgA and IgM expressing DN B cells. This is different to the post-switch compartment where an almost equal distribution of IgG and IgA expressing post-switch B cells is observed (Figure 4.12). Study from Buffa et al. reported similar to our results that in elderly people, elevated DN B cells contained about 60% IgG+, 20% IgA+ and less than 10% IgM+ cells (Buffa et al., 2011). These expanded cells in aging are discussed to be late memory or exhausted cells that have down regulated CD27 or might represent a distinct lineage of memory B cells (Colonna-Romano et al., 2009). It has been hypothesized that they resemble transient effector B cells, might be derived from an incomplete GC reaction or from extrafollicular responses (Sanz et al., 2008). Enhanced activity of B cells during autoimmunity plays a role in expansion of DN B cell subset and might play major role in RA pathogenesis (Wang et al., 2013).

We further elaborated our findings on phenotype and molecular changes in DN B cells in RA under cytokine inhibition during therapy.

5.3 Modulatory effect of IL-6R inhibition on heterogeneous DN isotype and DN B cells as baseline predictive marker of EULAR response

During *in vivo* IL-6R inhibition by TCZ, there was moderate influence on the phenotypic composition of the DN compartment. During TCZ therapy, there was no significant difference in neither relative nor in the absolute number of elevated DN B cells with little influence on DN isotype composition.

Interestingly, the IgA-DN B cell phenotype seems to be particularly amenable to IL-6R inhibition. The percentage and absolute numbers of IgA+ DN B cells were reduced significantly during TCZ therapy whereas other isotypes were not changed (Figure 4.23, Figure 4.24). Though the overall DN B cells composition with predominance of IgG was not influenced during TCZ therapy, we found the mutational frequency of IgA but in this case also of IgG and IgM significantly reduced under IL-6R inhibition (discussed later). Moreover corresponding to the cellular data, we saw a significant reduction of serum IgA (Table 4.1, (Roll et al., 2011)) as well as RF-IgA levels (Table 4.1).

This highlights the IL-6 susceptibility of the IgA isotype. However it should be pointed out that our data provide no direct relation of IgA-DN B cells to declining IgA serum levels. Nevertheless IL-6 has been ascribed a critical player in the development of local IgA antibody responses and a remarkable reduction of mucosal IgA–producing cells has been reported in mice with targeted disruption of the gene that encodes IL-6 (Ramsay et al., 1994). Future experiments need to address how peripheral IgA+ DN B cells may be related to the mucosal immune system.

During TCZ therapy, clinical disease activity measured by DAS28 declined significantly along with other inflammatory parameters like ESR and CRP (Table 4.1). At week 12, out of 36 RA patients during TCZ therapy 14 patients reached an EULAR good response, 20 patients reached an EULAR moderate response and 2 patients were non-responders. Our study did not show a direct correlation between the elevation of DN B cells and the clinical disease activity (r^2=0.00, ns=0.97). The percentage DN B cells were independent of DAS28 of active RA patients. This led us to explore DN as a predictive

marker for disease activity under therapy. Interestingly, we found that good EULAR responders to TCZ at week 12 had a significantly lower frequency of DN B cells compared to moderate responders at baseline. Moreover, the same correlation was also found for absolute numbers of DN B cells at baseline (Figure 4.15). Therefore DN B cells may serve as a predictive marker for EULAR response to TCZ therapy. There was no significant correlation between the frequency of DN B cells with DAS28 (p= 0.97) and CRP levels (p= 0.70). Univariate logistic regression analysis revealed that the frequency of DN B cells at baseline is inversely correlated with a subsequent EULAR good response. A significant (p=0.024) odds ratio of 1.48 (95% CI as 1.05-2.06) favoring EULAR good response could be calculated. This indicates that the percentage of DN B cells is elevated in RA and correlated with EULAR response to TCZ which may serve as candidate for a predictive marker to therapy. Patients with lower frequency of DN B cells are more likely to be EULAR good responder to TCZ therapy.

5.4 SHM as a hallmark of memory and its modulations by IL-6R inhibition

The susceptibility of IgA+ cells to TCZ, led us to further explore these cells also at the molecular level in more details. We have previously shown a molecular impact of in vivo IL-6R inhibition on peripheral B cells in RA where the status of somatic hypermutation of pre-switch memory B cells opposite to the post-switch compartment could be modulated. SHM of immunoglobulin gene rearrangements is a hallmark of B cell maturation into memory cells after antigen encounter. In this study we performed molecular analysis of DN B cells and analyzed in detail the immunoglobulin receptor of individually sorted cells from healthy donors (n=3) and from RA patients during TCZ (n=9) or anti-TNF-α therapy (n = 4) using single cell sequencing.. Despite their mainly switched phenotype we also found the mutational frequency Ig-VH3 gene rearrangements of DN B cells IgR to be significantly lower as compared to post-switch B cells and similar to pre-switch B cells. Moreover, the mutational frequency of DN B cells was comparable in RA and HD. DN B cells largely containing memory-like B cells had the lowest mutational frequency amongst the three memory B cell subsets. Interestingly, IgA+ DN B cells (5.42%) harboured the highest mutational frequency compared to IgG+ (4.45%) and IgM+ (2.87%) DN B cells. This was

which was evident in RA as well as healthy donors. We now extended our studies to the analysis of mutational frequency in all 3 isotypes of DN B cells. Under *in vivo* IL-6R inhibition we found a significant ($p<0.0001$) decrease in mutational frequency of the BCR at week 12, 24 and over a year (Figure 4.28). Furthermore, TCZ treatment induced a profound change with reduced mutational status in all 3 isotypes (Figure 4.32). The reduced mutational frequency in the DN memory B cell compartment is due to a reduction in highly mutated IgR of individual B cells and a relative increase of B cells with low or unmutated IgR (Figure 4.29).

It is known that somatic hypermutation (SHM) favors defined mutational hotspots. In particular, RGYW/WRCY motif mutations are preferentially targeted by T-cell-dependent signals including CD40–CD40 ligand interactions (Monson et al., 2001). Therefore, we determined dynamic changes in SHM in specific RGYW/WRCY, which were previously identified to be targeted (Dorner et al., 1998). We also analyzed for the CDR3 region which 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 SHM (Souto-Carneiro et al, 2005). In our study, the decrease in frequency of targeted mutations within RGYW/WRCY and increase in CDR3 during IL-6R inhibition indicate that during anti-IL-6R therapy, the DN B cell population shifts to overall less antigen-experienced B cells. This effect seems to be influenced only by *in vivo* IL-6R inhibition since we did not observe any change in these parameters during anti-TNF-α therapy using adalimumab (Figure 4.36-Figure 4.38). In a previous study looking at the pre-switch B cell compartment, we observed a similar pattern with adalimumab failing to influence IgR mutation (Muhammad et al., 2011). Therefore, it seems likely that IL-6R blockade does affect B cell maturation *in vivo* to a substantial extent and DN B cells have a dependence on the IL-6/IL-6R system for differentiation *in vivo*, which can be modulated by therapy. However, at the moment it is not clear how direct and indirect effects on B cells contribute to the observed effects since TCZ is also reported to reduce activated CD4+ T cells as early as 12 weeks after treatment (Shirota et al., 2012). Our data still leaves these questions open.

Since mutational frequencies of DN and pre-switch B cells are significantly lower than typical post-switch B cells, it might be suggested that either of these cells are pre-germinal centre or leave the germinal centres quickly by shedding CD27. On the other hand, DN B cells still could fail to up-regulate CD27 expression before leaving germinal centres since CD27 negative memory B cells have also been found and isolated from human tonsils (Ehrhardt et al., 2005). Since shedding or down-regulation of CD27 have been reported as an indication of continuous antigen stimulation and T-cell exhaustion, our observed connection of DN B cells numbers to response to IL-6R inhibition may reflect a state of chronic B cell hyperactivity closely linked to IL-6 (De Jong et al., 1992; Kai et al., 1999; Swaak et al., 1995). Furthermore, the data are consistent with the idea that DN memory B cells are distinctly generated or have a shorter half-life than conventional post-switch memory B cells resulting in a measurable impact of IL-6R blockade on DN B cells.

In conclusion, this study has demonstrated the phenotype of activated B cells and their memory subsets in active RA patients. The higher expressions of surface CD95 & intracellular ki-67 reflects disease activity and is positively correlated to it. A significantly higher proportion of CD27-IgD- DN B cells is present in RA which is independent of disease activity but is related to response to therapy. Elevated DN B cells are a mixture of somatically mutated IgG, IgA and IgM isotype expressing cells with a pre-dominance of IgG isotype. TCZ therapy results in a decrease in the frequency of IgA+ DN B cells in particular. Along with this a decrease of serum IgA and RF-IgA levels is observed. On the molecular level, mutated DN B cells are less mutated compared to pre-switch and post-switch B cells. Interestingly IgA+ DN B cells are highest mutated among the all 3 isotypes. TCZ therapy reduces the mutational frequency and RGYW hotspot targeting in all DN B cell isotypes which is ongoing over a period of 1 year of treatment. Lower baseline values of DN B cells were related to a higher EULAR good response in patients treated with *in vivo* IL-6R inhibition by tocilizumab and may serve as a candidate marker for response to TCZ therapy.

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Summary

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory autoimmune disease. Enhanced B cell activity has been proposed in the pathogenesis of RA along with different pro-inflammatory cytokines such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α), critically involved in chronic inflammation. Biological agents targeting these cytokines IL-6 and TNF-α have considerably advanced treatment of autoimmunity. Enhanced B cell activity, particularly memory B cells, gained particular interest in evaluating response during therapies from biologics. Human peripheral memory B cells can be distinguished by the phenotypic expression of CD27 and IgD defining three major B cell subpopulations: CD27+IgD+ pre-switch, CD27+IgD- post-switch and CD27-IgD- double negative (DN) memory B cells. Therefore, we analyzed different memory populations during cytokine inhibition by using tocilizumab (anti-IL-6R, TCZ) and adalimumab (anti-TNF-α, ADA), with focus on DN B cells. DN B cells lack the conventional memory marker CD27. But due to their mutational Ig receptor (IgR) they are considered to belong to the memory compartment. However, only scare data are available for this DN subpopulation in RA.

The phenotype analyses of activation markers (CD95 and ki-67) of B cell and their subsets were compared in RA patients (median age ~56 years) and in HD. DN memory B cells were phenotypically analyzed from RA patients during IL-6R or TNF-α inhibition at baseline week 12, week 24 and 1 year. Single B cell PCR approach was used to study Ig-receptors VH genes and isotype specific genes. Nonparametric Wilcoxon matched pair test and Mann-Whitney U test was used for statistical analysis by using GraphPadPrism 5. Univariate logistic regression was used to calculate odd ratios and correlation using Pearson r using SPSS statistics 22.

The analysis of surface and intracellular staining of B cells showed a significantly higher percentage of CD95 and ki-67 expressions in RA, which was highest in post-switch memory B cells followed by pre-switch and DN memory B cells. During cytokine (IL-6R & TNF-α) inhibition, both CD95 and ki-67 expression were significantly reduced at week 12 and 24 along with reduction in clinical parameters like DAS28, CRP, ESR. Furthermore, phenotypic analysis in 86 RA patients and 49 healthy donors (HD) showed a significantly expanded population of DN B cells in RA which contain a heterogeneous mixture of IgA, IgG and IgM expressing cells with a clear dominance of IgG+ cells. Pre-therapy analysis of

rearranged IgR sequences from patients (n=9) revealed that DN B cells carry rearranged heavy chain gene sequences with a diversified mutational pattern consistent with memory B cells. In contrast to tumor necrosis factor alpha (TNF-α) inhibition, a significant reduction in mutational frequency of BCR gene rearrangements at week 12, 24 and 1 year ($p<0.0001$) was observed by *in vivo* IL-6R inhibition. These changes were observed for all BCR isotypes IgG, IgA and IgM at week 12, 24 and 1 year ($p<0.0001$). IgA-RF, IgA serum level and IgA+ DN B cells decreased significantly ($p<0.05$) at week 12 and week 24 during TCZ. Patients with a good European League Against Rheumatism (EULAR) response to TCZ had less DN B cells at baseline as compared to moderate responders ($p=0.006$). Univariate logistic regression analysis revealed that the frequency of DN B cells at baseline is inversely correlated to a subsequent good EULAR response ($p=0.024$) with an odds ratio of 1.48 (95% confidence interval as 1.05-2.06).

In conclusion, the study has demonstrated the phenotype of activated B cells and their subsets in active RA patients. The higher expressions of surface CD95 & intracellular ki-67 reflects disease activity and is positively correlated to DAS28 levels. Significantly higher proportion of CD27-IgD- DN B cells is found in RA. These elevated DN B cells are a mixture of somatically mutated IgG, IgA and IgM isotype expressing cells with a pre-dominance of IgG isotype. TCZ therapy results in a decrease in the frequency of IgA+ DN B cells in particular. Along with this, a decrease of serum IgA and RF-IgA levels is observed. On the molecular level, mutated DN B cells are less mutated compared to pre-switch and post-switch B cells. Interestingly IgA+ DN B cells are the most mutated among the all 3 isotypes. TCZ therapy reduces the mutational frequency and RGYW hotspot targeting in all DN B cell isotypes (IgA, IgG and IgM) over a treatment period of 1 year. The amount of DN B cells is not correlated to baseline disease activity but is correlated to the achieved EULAR response in patients started on anti-IL6R therapy. Based on these current findings, DN memory B cells may serve as a candidate biomarker for response to TCZ therapy as lower baseline values of these cells were related to higher proportions of EULAR good responders.

Zusammenfassung

Die rheumatoide Arthritis (RA) ist eine chronische, systemische, und entzündliche Autoimmunerkrankung. Es wird angenommen, dass neben verschiedenen inflammatorischen Zytokinen wie beispielsweise Interleukin-6 (IL-6) und Tumor Nekrose Faktor alpha (TNF-α) auch eine verstärkte Aktivität von B-Zellen in chronischen Entzündungsprozessen involviert ist. Biologische Agenzien, die gezielt gegen die Zytokine IL-6 und TNF-α gerichtet sind, haben die Behandlungsmöglichkeiten der Autoimmunerkrankungen wesentlich vorangetrieben. Derzeit rückt eine verstärkte B-Zell Aktivität, insbesondere die von Gedächtnis B-Zellen, bei der Evaluierung von Reaktionen auf biologische Therapien in den Focus des Interesses. Humane periphere Gedächtnis B-Zellen können anhand der Expression von CD27 und IgD phänotypisch charakterisiert werden und lassen sich dadurch in drei Subpopulationen einteilen: CD27+IgD+ prä-switch, CD27+IgD- post-switch und CD27-IgD- doppelt negative (DN) Gedächtnis B-Zellen. Wir untersuchten die verschiedenen Gedächtnis B-Zell-Populationen während der Zytokininhibierung mittels Tocilizumab (anti-IL-6R, TCZ) und Adalimumab (anti-TNF-α, ADA), wobei der Focus auf DN B-Zellen lag. DN B-Zellen fehlt der konventionelle Gedächtniszellen-Marker CD27, sie werden aber aufgrund ihres mutierten Ig-Repertoires (IgR) den Gedächtniszellen zugeordnet. Für diese DN B-Zell Subpopulation gibt es allerdings bislang nur wenige Daten im Zusammenhang mit RA.

Die B-Zell Subpopulationen von RA Patienten (durchschnittliches Alter ~56 Jahre) und gesunden Spendern (englisch: healthy donors, HD) gleichen Alters als Kontrolle, wurden hinsichtlich der Aktivierungsmarker, CD95 und ki-67, phänotypisch analysiert und verglichen. DN Gedächtnis B-Zellen von RA Patienten wurden phänotypisch zu Beginn der IL-6R oder TNF-α Inhibition, nach 12 und 24 Wochen sowie 1 Jahr nach Behandlungsbeginn bestimmt. Es wurden Einzel-PCR Analysen von B-Zellen durchgeführt, um die VH Gene der Ig-Rezeptoren und spezifische Gene zu typisieren. Für die statistischen Auswertungen wurde in GraphPad Prism 5 der ‚nonparametric Wilcoxon matched pair test' und der ‚Mann-Whitney U test' verwendet. Die eindimensionale logistische Regression wurde angewandt, um Odd-Ratio und Korrelationen mittels Pearson r in SPSS Statistik 22 zu berechnen.

Die phänotypische Analyse der Oberflächen und intrazellulären Färbungen der B-Zellen ergab einen signifikant erhöhten Prozentsatz der CD95 und ki-67-Expression in RA-Patienten, wobei die Expression am höchsten in post-switch Gedächtnis B-Zellen, gefolgt von prä-switch und DN Gedächtnis B-Zellen, war. Während der Zytokin-Inhibierung (IL-6R & TNF-α) war sowohl die CD95 als auch ki-67-Expression zum Zeitpunkt Woche 12 und Woche 24 signifikant reduziert, einhergehend mit der Verringerung klinischer Parameter wie DAS28, CRP und ESR. Weiterhin zeigte die phänotypische Analyse von 86 RA-Patienten und 49 HD eine deutlich expandierte DN B-Zell Population in RA-Patienten. Diese setzte sich aus einer heterogenen Mischung von IgA, IgG und IgM exprimierenden Zellen zusammen, wobei IgG+ B-Zellen deutlich dominierten. Die Analyse von neu-gruppierten IgR Sequenzen von B-Zellen aus RA Patienten (n=9) vor Therapiebeginn zeigte, dass DN B-Zellen umgruppierte Gensequenzen der schweren Ketten mit diversen Mutationsmustern tragen, wie es bei Gedächtnis B-Zellen der Fall ist. Im Gegensatz zur TNF-α-Hemmung ist bei der *in vivo* IL-6R-Inhibierung eine signifikante Reduktion der Mutationsrate innerhalb der umgruppierten der Gene der B-Zell Rezeptoren (BCR) nach 12 und 24 Wochen sowie nach 1 Jahr ($p<0,0001$) zu beobachten. Diese Änderungen waren bei allen BCR Isotypen IgG, IgA und IgM bei Woche 12, 24 und 1 Jahr ($p<0.0001$) zu sehen. IgA-RF, IgA-Serum Spiegel sowie IgA+ DN B Zellen nahmen 12 und 24 Wochen nach Beginn der Behandlung mit TCZ signifikant ab ($p<0,05$). Patienten mit einer guten klinischen Besserung (EULAR Antwort) auf TCZ hatten weniger DN B-Zellen vor Therapiebeginn im Blut als Patienten, die eine moderate Reaktion zeigten. Univariate logistische Regressionsanalysen zeigten, dass die Frequenz von DN B-Zellen vor Therapiebeginn invers mit einer sich anschließenden guten EULAR Antwort korreliert. Die Odds Ratio war 1,48 (95% Konfidenz Intervall wie 1,05-2,06).

Im Ergebnis hat die Studie Phänotypen von aktivierten Gedächtnis B-Zellen und deren Untergruppen in Patienten mit aktiver RA nachgewiesen. Die höhere Expression des Oberflächenmarker CD95 und des intrazellulären Marker ki-67 spiegelt die Krankheitsaktivität wieder und korreliert mit dieser positiv. Signifikant höhere Populationen von CD27-IgD- DN B-Zellen in der RA stehen jedoch unabhängig von der Krankheitsaktivität in Bezug zum Ansprechen auf die Therapie. Diese erhöhten DN B-Zellen sind eine Mischung aus somatisch mutierten Isotyp IgG, IgA und IgM exprimierenden Zellen,

wobei IgG Isotypen dominieren. Die TCZ Therapie bewirkt insbesondere eine Abnahme der Frequenzen von IgA+ DN Gedächtnis B-Zellen, zusammen mit einer Abnahme des Serum-IgA und RF-IgA-Spiegels. Auf molekularer Ebene ist zu sehen, dass mutierte DN B Zellen geringere Mutationen aufweisen als prä-switch und post-switch B-Zellen. Interessanterweise sind IgA+ DN B-Zellen am meisten unter allen 3 Isotypen mutiert. Die TCZ Therapie reduziert die Mutationsrate, auch innerhalb der RGYW Hotspots, in allen DN B-Zell Isotypen über einen Zeitraum von einem Jahr.

Eine geringe Anzahl von DN Gedächtnis B-Zellen vor Therapiebeginn mit TCZ (anti-IL-6R) war mit einer verbesserten klinischen Antwort korreliert. Auf Grundlage dieser aktuellen Ergebnisse erscheinen DN Gedächtnis B-Zellen als möglicher Biomarker Kandidaten zu sein, um eine Reaktion auf eine TCZ Therapie anzuzeigen.

Substantial parts of this thesis were published in following articles:

1. **Mahmood Z**, Muhammad K, Schmalzing M, Roll P, Dörner T, Tony H-P.

   CD27-IgD- memory B cells are modulated by in vivo interleukin-6 receptor (IL-6R) blockade in rheumatoid arthritis

   Arthritis Research & Therapy (2015) 17:61 March 14 (Highly accessed article)

2. Roll P, **Mahmood Z**, Muhammad K, Feuchtenberger M, Dörner T, Tony H-P.

   Long-term repopulation of peripheral B cell subsets after single and repeated rituximab infusions in patients with rheumatoid arthritis

   Clinical and Experimental Rheumatology 2015 April 16

3. IL-6R inhibition reduces activation, modulate migration and influence cytokine profile of different peripheral memory B cell subsets in RA. (Under preparation)

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Abbreviations

ACPAs	Anti-citrullinated protein antibodies
ACR	American College of Rheumatology
APC	Antigen presenting cells
ADA	Adalimumab
AID	Activation-induced cytidinedeaminase
Anti-CCP	Anti-cyclic citrullinated peptide
APCs	Antigen-presenting cells
BAFF	B cell activating factor
BAFF-R	BAFF-receptor
Bcl-2	B-cell lymphoma 2
BCMA	B cell maturation antigen
BCR	B cell receptor
Blimp-1	B lymphocyte-induced maturation protein
BLyS	B lymphocyte stimulator
bp	Base pairs
CCP	Cyclic citrullinated peptides
CD	Cluster of differentiation
CDR3	Length of the third complementary-determining Regions
CIA	Collagen-induced arthritis
CRP	C-reactive protein
CSR	Class-switch recombination
DAS28	Disease activity score of 28 joints
DCs	Dendritic cells
DMARDs	Disease modifying anti-rheumatic drugs
DN	Double negative
EA	Early arthritis
ELISA	Enzyme linkedimmunosorbentassay
ERA	Early rheumatoid arthritis
ESR	Erythrocyte sedimentation rate
EULAR	European League Against Rheumatism
FCS	Fetal calf serum
Fcγ-R	Fc gamma-receptor
FDC	Follicular dendritic cell

GC	Germinal center
HAQ	Health assessment questionnaire
HD	Healthy donor
HLA	Human leukocyte antigen
ICOS	Inducible Costimulator Molecule
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
ITP	Idiopathic thrombocytopenic purpura
MHC	Major histocompatibility complex
MS	Multiple sclerosis
MTX	Methotrexate
NSAIDs	Nonsteroidal anti-inflammatory drugs
OA	Osteoarthritis
Pax5	Paired box 5
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
RA	Rheumatoid arthritis
RAG	Recombination-activating gene
RANKL	Receptor activator for nuclear factor κB ligand
RF	Rheumatoid factor
RTX	Rituximab
SF	Synovial fluid
SHM	Somatic hypermutation
SLE	Systemic lupus erythematous
TCZ	Tocilizumab
TFH	Follicular helper T cell
Th	T helper
TLRs	Toll-like receptors
TNF-α	Tumor necrosis factor-alpha
TNFR	TNF receptor
W12/W24	Week 12/week 24
VAS	Visual Analogue Scale
β2m	β2-microglobulin

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Curriculum Vitae

Name **:** Zafar Mahmood

Date of birth **:** 13th November 1983

Nationality **:** Indian

Education & Research Experiences

Oct 2010-till date **Ph.D. dissertation**

Thesis topic: Effect of cytokine inhibition on peripheral memory B cell in patient with rheumatoid arthritis.

Primary supervisor: Prof. Hans-Peter Tony

Co-supervisors: Prof. Thomas Hünig and Dr. Ingolf Berberich

Oct 2010-Sep 2013 **Member of the Graduate College 520**

"Immunomodulation", Würzburg, under the coordination of Prof. Thomas Hünig

Jan 2009-Sep 2010 **Project Assistant** at Indian Institute of Toxicology Research, India.

Topic I: Proteomics approach for developing novel biomarkers for cancer and assessment of toxic responses by 2-Dimensional gel electrophoresis.

Topic II: Chemopreventive effect of tea polyphenols on cancer cells

Jul 2008-Dec 2008 Successful preparation for national level test for **Lecturership** at Indian university conducted by Council of Scientific & Industrial Research (CSIR), India.

Jul 2006-Jun 2008 **Master of Science (Biosciences)** from Jamia, New Delhi, India.

Master thesis: "Detection of High Risk Human Papillomavirus 16 and 18 Infection by Multiplex PCR in Cervical Cancer lesions patients" at Institute of Cytology and Preventive Oncology (ICPO), India under supervision of Dr B.C.Das (Director).

Jul 2003-Jun 2006 **Bachelor of Science** (Biosciences) from Jamia, New Delhi, India.

List of publications

Publications coming out of doctoral thesis

- **Mahmood Z**, Muhammad K, Schmalzing M, Roll P, Dörner T, Tony H-P. "CD27-IgD- memory B cells are modulated by in vivo IL-6R blockade''. Arthritis Research & Therapy, March 2015 (Highly accessed article).

- Roll P, **Mahmood Z**, Muhammad K, Feuchtenberger M, Dörner T, Tony H-P. "Long-term repopulation of peripheral B cell subsets after single and repeated rituximab infusions in patients with rheumatoid arthritis''. Clinical and Experimental Rheumatology, April 2015 (cited by 1)

Publications prior to the PhD (google scholar)

- Arora D, **Mahmood Z**, George J, Yadav AK, Kumar S, Shukla Y. "Plasma Protein Profiling of Breast Cancer Patients of North Indian Population: A Potential Approach to Early Detection''. Journal of Proteomics & Bioinformatics 2013; 6: 088-098. doi:10.4172/jpb.1000267.

- Singh M, Singh R, Bhui K, **Mahmood Z**, Shukla Y. "Tea polyphenols induce apoptosis through mitochondrial pathway and by inhibiting nuclear factor-kappaB and Akt activation in human cervical cancer cells". Oncology Research. 2011; 19(6):245-57 (35 citations).

- **Mahmood Z** and Shukla.Y. "Death receptors: Targets for cancer therapy". Experimental Cell Research 2010; 316(6):887-99 (123 citations).

- George J, Prasad S, **Mahmood Z**, Shukla Y. "Studies on glyphosate-induced carcinogenicity in mouse skin: a proteomic approach". Journal of Proteomics. 2010;73 (5):951-64 (37 citations).

- George J, Singh R, **Mahmood Z**, Shukla Y. "Toxicoproteomics: new paradigms in toxicology research". Toxicology Mechanism and Methods. 2010; 20(7):415-23 (13 citations).

Published abstracts in international journals

- **Mahmood Z**, Schmalzing M, Dörner T, Tony H (2014). Memory B Cell Subtype Modulation in Patients with Rheumatoid Arthritis. [abstract] Arthritis & Rheumatology 2014; 66: S1–S1402. doi: 10.1002/art.38914.
- **Mahmood Z**, Muhammad K, Schmalzing M, Roll P, Dörner T, Tony H (2013). IL-6R Inhibition Reduces Activation Of Different Peripheral Memory B Cell Subsets In RA. [abstract]. Arthritis & Rheumatism 2013; 65 Suppl 10:915 doi: 10.1002/art.2013.65.issue-s10.
- **Mahmood Z**, Muhammad K, Roll P, Schmalzing M, Dörner T, Tony H (2013). In vivo IL-6 inhibition using anti-IL-6 receptor antibodies modulates double negative($CD19^{+}IgD^{-}CD27^{-}$) B cells in RA patients. Frontier of Immunology. Conference abstract: 15th International Congress of Immunology (ICI). doi: 10.3389/conf.fimmu.2013.02.01055
- **Mahmood Z**, Muhammad K, Roll P, Kleinart S, Dörner T, Tony H (2012). IL-6 Receptor Inhibition by Tocilizumab Modulates Double Negative (CD19+CD27-IgD-) B cells in RA. [abstract]. Arthritis & Rheumatism, 64: S1–S1216. doi: 10.1002/art.37735.

Active Participations in International Workshops & schools

- RIKEN Research Center for Allergy and Immunology (RCAI) International Summer Program, Yokohama, Japan 2013 (prestigious program in immunology, full scholarship by RIKEN, Japan).
- Basic Immunology: Fundamental Concepts and Relevance to Human Disease and Therapeutics, Genoa, Italy 2013 (travel grant by European Federation of Immunological Societies).
- International School on innovative approaches in regenerative medicine, Berlin-Brandenburg School for Regenerative Therapies (BSRT), Berlin, Germany 2012 (travel grant by BSRT). Focused on GMP for T cell therapy in patient and regenerated therapy.
- International Advanced Course "Innate Immunity: From Evolution to Revolution", Sorrento, Italy 2012 (EFIS-EJI grant).

Active participation in international meetings and conferences

- Poster presented at American college of Rheumatology (ACR-2014) meeting in Boston, MA, USA.
- Poster presented at American college of Rheumatology (ACR-2013) meeting in San Diego, CA, USA (travel grant provided by Deutsche Gesellschaft für Immunologie, DGfI).
- Poster Presented in International Congress of Immunology (ICI-2013), Milan, Italy (travel grant from European Federation of Immunological Societies, EFIS).
- 8th RCAI-JSI International Symposium on Immunology 2013 "Interface between Immune System and Environment", Japan (travel grant by RIKEN society, Japan).
- Poster presented at American college of Rheumatology (ACR-2012) meeting in Washington DC, USA (travel grant by Graduate School of Life Sciences, University of Würzburg).
- Poster Presented in 8th, 7th and 6th in International Doctoral Students Symposium, Würzburg 2013, 2012 and 2011 respectively.
- Oral and poster presentations in 7th and 6th Annual Meeting Würzburg, Erlangen and Tubingen Immunology Training Network, 2012 and 2011 respectively.

Additional certificate courses

- Two days summer school on innate and adaptive immune system, 2011, Cologne.
- Certificate for participating in weekly seminar on Cellular and Molecular Immunology, 2011.
- Certificate for participating in workshop on writing strategies for Publishing Research.
- Certificate for participating in workshop on Statistics, 2011, Wurzburg.
- Certificate for participating in workshop on poster design and presentation.
- Certificate for German language level B1+ (Intermediate level).

Place, date Signature

AFFIDAVIT

The experimental work described in this thesis was carried out in the Department of Rheumatology & Clinical immunology, Centre for Internal Medicine, University Hospital, Würzburg, Germany, from October 2010 to May 2015, under the supervision of Prof. Dr. Hans-Peter Tony.

I hereby declare that my thesis entitled ***Effect of cytokine inhibition on peripheral memory B cells in patients with RA*** is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis.

Furthermore, I verify that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg……
Date Signature

(*Eidesstattliche Erklärung*)

Hiermit erkläre ich an Eides statt, die Dissertation

‘‘*Auswirkung der Zytokinhemmung auf periphere Gedächtnis B-Zellen in Patienten mit rheumatoider Arthritis*“ eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg ……
Datum Unterschrift