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DISSERTATION

Ig gene repertoire analysis in an SLE patient in clinical remission

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Abbreviations

A	**A**denine
ANA	**A**nti-**N**uclear **A**ntibodies
APC	**A**llo**P**hyo**C**yanin
B cell	**B**ursa-dependent cell, bone-marrow-derived (lymphocytes)
BCR	**B**-**C**ell antigen **R**eceptor
BSA	**B**ovine **S**erum **A**lbumin
C	**C**ytosine
°C	degree **C**elsius
cDNA	**c**omplementary **D**esoxyribo**N**ucleic **A**cid
CDR	**C**omplementary **D**etermining **R**egion
D_H	**D**iversity **H**eavy chain gene segment
D-MEM	**D**ulbecco's **M**odified **E**agle **M**edium
DNA	**D**esoxyribo**N**ucleid **A**cid
dsDNA	**d**ouble-**s**tranded **D**esoxyribo**N**ucleid **A**cid
dNTP	**d**esoxyribo**N**ucleid Tri**P**hosphate
DTT	**D**i**T**hio**T**reitol
E.coli	**E**scherichia **coli**
EDTA	**E**thylene**D**iamide**T**etraacetic **A**cid
ELISA	**E**nzyme-**l**inked **i**mmuno**S**orbent **A**ssay
ExoSap^R	**Exo**nuclease **S**hrimp **A**lkaline **P**hoshatase^R
Fab	**F**ragment **a**ntigen **b**inding
FACS	**F**luorescence **A**ctivated **C**ell **S**orting
Fc	**F**ragment **c**ristalline
FCS	**F**etal **C**alf **S**erum
FITC	**F**luorescein **I**sothyocyanate
G	**G**uanine
HEL	**H**en **E**gg **L**ysozyme
Ig	**I**mmuno**g**lobulin
IgA	**I**mmuno**g**lobulin **A**
IgD	**I**mmuno**g**lobulin **D**

IgG	**I**mmuno**g**lobulin **G**
IgH	**I**mmuno**g**lobulin **h**eavy chain
IgL	**I**mmuno**g**lobulin **l**ight chain
IgM	**I**mmuno**g**lobulin **M**
Igγ	**I**mmuno**g**lobulin **g**amma heavy chain
Igκ	**I**mmuno**g**lobulin **k**appa light chain
Igλ	**I**mmuno**g**lobulin **l**ambda light chain
IgBLAST	**I**mmuno**g**lobulin **B**asic **L**ocal **A**lignment **S**earch **T**ool
J_H	**J**oining **H**eavy chain gene segment
Jκ	**J**oining **k**appa chain gene segment
LFA	**L**upus **F**oundation of **A**merica
LPS	**L**ipo **P**oly **S**accharide
MACS	**M**agnetic **C**ell **S**orting
MHC	**M**ajor **H**istocompatibility **C**omplex
mRNA	**M**essenger **R**ibonucleic **A**cid
ORF	**O**pen **R**eading **F**rame
PBMC	**P**eripheral **B**lood **M**ononuclear **C**ell
PBS	**P**hosphate-**B**uffered **S**aline
PCR	**P**olymerase **C**hain **R**eaction
PE	**P**hyco**E**rythrin
Pre-BCR	**Pre**-**B**-**c**ell **R**eceptor
RAG	**R**ecombination **A**ctivating **G**ene
RT	**R**everse **T**ranscriptase
RT-PCR	**R**everse **T**ranscriptase- **P**olymerase **C**hain **R**eaction
SLE	**S**ystemic **L**upus **E**rythematosus
T	**T**hymine
Taq	**T**hermus **aq**uaticus
T cell	**T**hymus-derived **c**ell
TCR	**T C**ell **R**eceptor
Temp	**Temp**erature
TB	**T**errific **B**roth

U	**U**nits
UV	**U**ltra**v**iolet
V_H	**V**ariable **H**eavy Chain Gene Segment
Vκ	**V**ariable **k**appa chain gene segment
Vλ	**V**ariable **l**ambda chain gene segment

1. Introduction

Autoimmunity and SLE

Autoimmunity occurs when the immune system loses tolerance to self-antigens and reacts against self tissues. The prevalence of autoimmune diseases in western society is about 5 % [1]. Systemic lupus erythematosus is one of the major autoimmune diseases, and one of its cardinal features is the production of anti-DNA autoantibodies [2-8]. The generation of these self reactive antibodies by B cells plays a central role in the pathogenesis of SLE.

During the course of B cell development B cells undergo stringent selection at various stages in the bone marrow and the periphery, where autoreactive cells are deleted.

How and where tolerance is induced during B cell development has long puzzled researchers. Recently, three checkpoints, one central checkpoint in the bone marrow and two peripheral checkpoints have been described where selfreactive B cells are efficiently removed from the repertoire to avoid autoreactivity [2, 4, 7, 8].

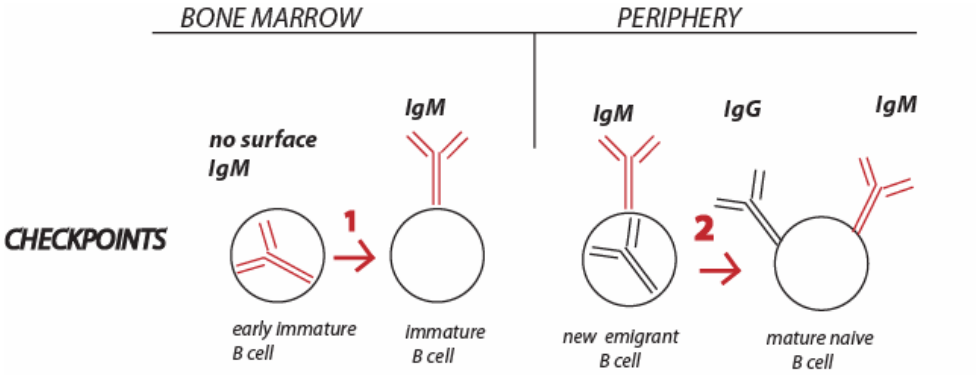


Figure 1. The illustration shows central and peripheral checkpoints during B cell development where B cell selection and maturation take place. The first checkpoint is between the transition from early immature to immature B cells in the bone marrow, the second checkpoint from the new emigrant to the mature naïve B cell compartment in the periphery and a third checkpoint occurs later during the course of B cell maturation at the transitional stage between mature naïve to antigen- experienced memory B cells.

However, these checkpoints appear to be defective in SLE patients, as they show increased levels of self- and polyreactive antibodies in the new emigrant and the mature naive B cell compartment when compared to healthy individuals [5, 8]. There appear to be certain Ig gene usage abnormalities associated with self reactivity found in the B cell pool of SLE patients.

Recent studies described alterations within the Ig gene repertoire after immunosuppressive treatment and decreased levels of polyreactive and autoreactive antibodies.

In my work, I examined one SLE patient after immunosuppressive steroid treatment and studied the Ig gene repertoire in the mature naïve B cell compartment after therapy. I compare my findings with the results of three other SLE patients before and after treatment and with two healthy controls. I show that this patient does not exhibit Ig gene usage abnormalities characteristic of SLE patients with active disease, such as long and highly positively charged CDR3 regions, or heavy and light chain gene usage biases towards the V_H4-34 and the Vκ4-1 gene families, respectively. Patient SLE21 did show more positively charged amino acids within the CDR3 region and a slightly atypical Ig light chain gene usage pattern compared to the healthy controls, with preferential usage of V_k4-1-, V_λ6 - and J_λ3 genes. In general, the resolution of this patient's symptoms after therapy correlates well with the normalization of the B cell repertoire. Based on my results, I will discuss the potential effects of immunosuppressive drugs on Ig gene usage.

The structure of an antibody molecule

1.1

Antibodies are the secreted form of the B cell receptor (BCR). They are identical to the membrane-bound BCR except for a small portion of the C-terminus of the heavy-chain constant region, which is hydrophilic and allows secretion. As antibodies are soluble and secreted in large quantities they are easily obtainable and easily studied.

All antibodies are roughly "Y"-shaped molecules composed by paired heavy (H) and light (L) polypeptide chains (Figure 1). The two heavy chains are linked to each other by disulfide bonds and each heavy chain is linked to a light chain by a disulfide bond (Figure 1). Non-covalent bonds hold together different constant domain (CH2-4) within both heavy chains. In any given immunoglobulin molecule, the two heavy chains and the two light chains are identical, giving an antibody molecule two identical antigen-binding sites (see Figure 1, Figure 2), and thus the ability to bind simultaneously to two identical structures, cross- linking them.

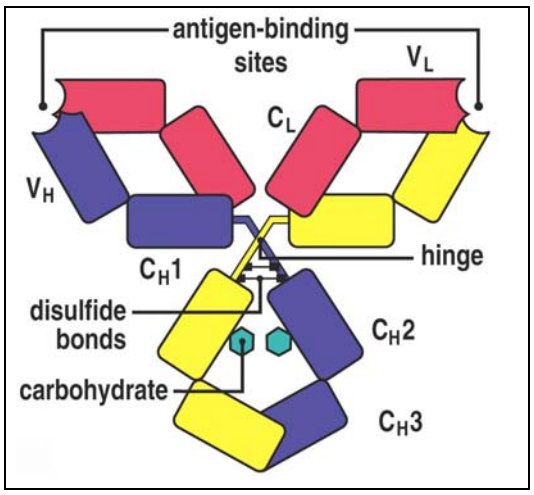


Figure 1.

The illustration shows an antibody molecule and its four- chain composition (blue, yellow, pink, pink). Each chain consists of a constant (CHs/CLs) and a variable (VH/VL) region, subdivided into several domains within one chain (e.g.CH1-CH3/VH and. CL/VL) (modified from [9]).

The term immunoglobulin (Igs) is used for all such proteins belonging to this family. Within this family, however, five different classes (isotypes) of immunoglobulins can be distinguished by the structure of their H-chains, which determines the effector function and functional activity of an

antibody molecule; the five isotypes are IgM, IgG, IgA, IgD and IgE. For the L-chain there are two types, called kappa (κ) and lambda (λ); a given antibody has either κ or λ chains. Both H and L chains have constant (C) and variable regions (V) consisting of a series of similar sequences, each about 110 amino acids long. A H-chain has an amino-terminal (N-terminal) variable domain (V_H) followed by three to four constant domains (C_H), whereas a L-chain only contains of one single constant domain [10] plus a variable region (V_L).

The association of the heavy and light chains is such that the amino-terminal variable domains of both chains (V_H and V_L, respectively) are paired, which together make up the V region of the antibody and form the antigen-binding fragment (Fab) at the end of the Y. This region binds specific antigen, while the constant domains (C domains) of the heavy and light chains (C_H and C_L, respectively) make up the C region (Figure 1) which is not involved in antigen binding, but determines the effector function of the antibody. The multiple heavy-chain C domains are numbered from the amino-terminal end (N-terminal) to the carboxy terminus, for example C_H1, C_H2, and so on. Another H to L chain association is between the C_H1 and C_L domains. The C_H3 domains pair with each other but the C_H2 domains do not interact; carbohydrate side chains attached to the C_H2 domains lie between the two heavy chains.

Two proteases, pepsin and papain, were historically used to cleave the antibody molecule in early studies of the structural components and their function. Limited digestion with the protease papain cleaves immunoglobulins in three fragments on the amino-terminal side of the disulfide bonds. This releases the two arms of the antibody as separate Fab fragments, whereas in the Fc fragment the carboxy-terminal halves of the heavy chains remain linked. The two fragments are identical and contain antigen-binding activity, termed fragment of antigen binding (Fab). The Fab contains the complete light chains paired with the VH and the CH1 domains of the heavy chains. The third fragment contains no antigen-binding activity but was originally observed to crystallize readily and was therefore called the Fragment crystallisable (Fc fragment). This part of the antibody molecule corresponds with the paired CH2 and CH3 domains and is linked to the Fabs by the hinge region (Figure 1.). The Fc fragment mainly causes the functional differences between the various isotypes by interacting with Fc receptors, complement, and other effector molecules in serum and on cells[11]. On the opposite pole of this large 150 kDa antibody molecule lie hypervariable regions within the V domains, denoted H_V1, H_V2, and H_V3. The regions between the hypervariable segments, which comprise the rest of the V domain, show less

variability and are termed the framework regions. There are four such regions in each V region (FR1, FR2, FR3, and FR4). The three hypervariable loops are shaped by both V_H and V_L chains and determine antigen specificity and affinity by forming a surface complementary to the antigen, and are thus designated the complementarity-determining regions, or CDRs (CDR1, CDR2, and CDR3) [12]. CDR-H1, H2, -L1 and –L2 create the outer border; CDR-L3 forms the base and CDR-H3 lies at the center of the Ag binding site (see Figure 2) therefore often play a critical role in the recognition of Ag [12-16].

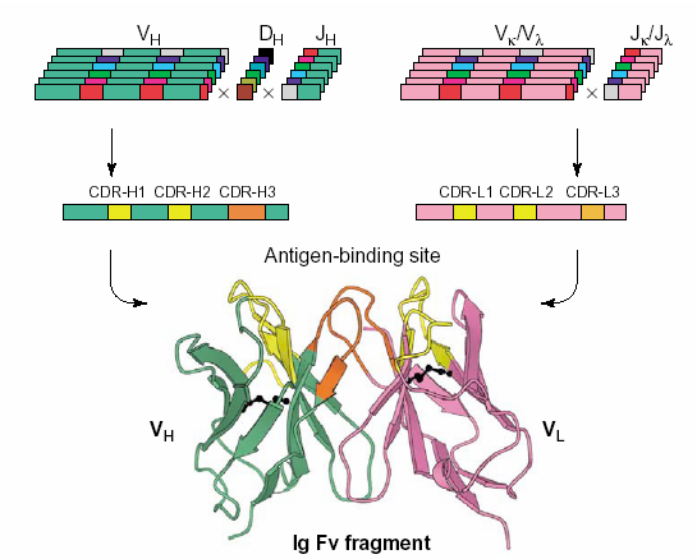


Figure 2. The three dimensional structure of the antigen binding site is composed by six CDRs. The three hypervariable loops are shaped by both VH and VL chains and determine antigen specificity and affinity by forming a surface complementary to the antigen.The CDR-H3 with its central position within the Ag binding site plays a critical role in Ag recognition. (Given by [17]).

CDRs from patients with autoimmune diseases are rich in positively charged residues and are therefore considered to play an important role in their pathogenesis, due to their increased affinity for negatively charged antigens such as dsDNA or nucleosomes [3, 18-21].

1.1.1CDR3 region

The great importance of the CDR3 region with its central position within the antibody's antigen binding pocket lies in the recognition of antigen and has been intensively studied. In humans, repertoire selection during B cell development is associated with a reduction in the distribution and the mean length of the expressed CDR3-H3 repertoire [3, 22] and loss of highly charged or

hydrophobic sequences. It has been proposed that longer and positively charged amino acid sequences within an antibody molecule increase the likelihood of self reactivity [2, 3, 23, 24] and therefore must be removed from the B cell pool during B lymphocyte development to avoid self-reactivity [3] (also see chapter 1.3).

1.2 Antibody assembly

Virtually any substance can elicit an antibody response. The total number of antibody specificities available to an individual is known as the antibody repertoire, and in humans is estimated to be at least 10^{11}. The reason for this almost unlimited number of antibody specificities lies in the gene rearrangement that takes place during B cell development in the bone marrow. Diversity is further enhanced by the process of somatic hypermutation in mature activated B cells.

In brief, there are three gene segments, termed V (variable), D (diversity) and J (joining), that undergo a strictly programmed series of gene rearrangement early in B cell development to assemble complete V region sequences [25]. The V region of the H chain is assembled from these three DNA segments, while the light chain locus has no D segments and directly recombines V to J.

In the germline configuration, the heavy chain locus has 65 V_H, 27 D_H and 6 J_H segments; recombination rearranges the locus to select one V, D, and J segment. The combinatorial rearrangement of the heavy and the light chain locus underlies the diversity of the antibody repertoire, as described above.

The recombination process is imprecise, as nucleotides are randomly added or subtracted at the joints between gene segments. For example, it is a matter of chance whether the V sequence and the J-region sequence downstream can be read in the same reading frame; each time a V gene segment undergoes rearrangement to a J segment or to an already rearranged DJ sequence (in H chains), there is a roughly two in three chance of generating an out-of-frame sequence downstream from the joint. Thus, B cell development has evolved to preserve those B cells that have made productive joints and to eliminate cells that have not. Assembly of the genes for a complete receptor requires three separate recombination events, which occur at different stages of B-cell development. These are, in the order that they occur: the joining of D to J_H and V_H to DJ_H

to produce the functional heavy-chain gene, and the joining of V_L to J_L to produce the functional light-chain gene. The kappa chain locus is usually rearranged before the lambda chain locus, the latter only initiating rearrangement if the kappa locus rearrangements have failed to productively recombine.

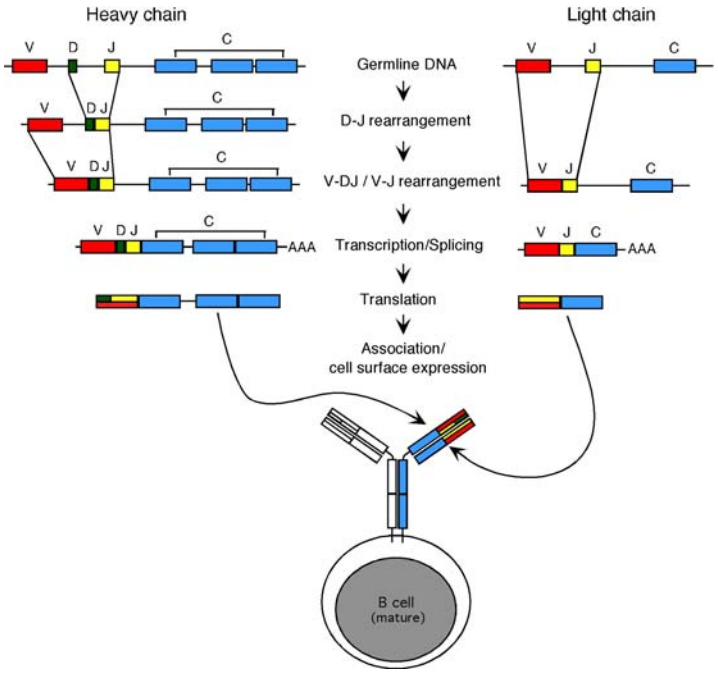


Figure 3. Formation of an antibody molecule Antibodies are composed of 2 heavy and 2 light chains. Genes segments that encode the λ and κ chains lie in germline configuration initially and gene segments must be rearranged, joined and transcribed before a mRNA is generated and translated into heavy and light chain molecules. Successful heavy and light chain gene rearrangement and antibody assembly respectively leads to a functional membrane- bound B cell receptor (modified from [17]).

Because only about one in three joinings will be successful, and three successful joinings are required to express a complete immunoglobulin molecule, a large number of developing B cells are lost because they fail to make a productive rearrangement at one of these stages. Far fewer cells are lost because of failure to make productive light chain gene rearrangements than are lost at the stage of heavy chain gene rearrangement. This is partly because there are two light chain loci—κ and λ—that can be rearranged, and partly because the opportunity for successive rearrangement attempts is much greater at each light chain locus. In the next chapter, I will focus on the stages of B cell receptor formation during early B cell development.

1.3 Antibody diversity and B cell development phases of development

B cell commitment and B cell development

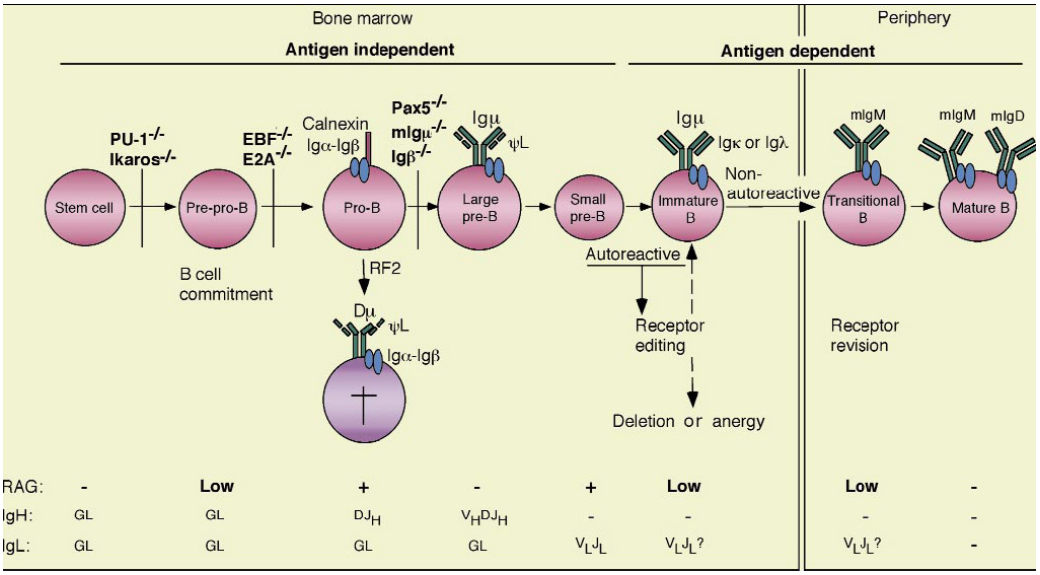


Figure 4. Picture of B cell developmental stages B cell progenitors are generated in the bone marrow and go through various developmental stages before they are released into the periphery. The different stages of B cell lymphopoiesis with cell surface expression, RAG expression on the heavy and light chain loci and the Ig gene rearrangement status are shown (modified from [26]).

B cells derive from haematopoietic precursor cells (Figure 4.)

B cell development can be distinguished into different stages: in mice, haematopoietic stem cells (HSC) become committed to the B cell lineage in the fetal liver and in the bone marrow of adult animals [27]. Several transcription factors such as E2, Pax5 and EBF have been identified as key regulators of B cell development [26]. Among these, Pax5 is crucial for maintaining B lineage commitment (Figure 4). Pax-5$^{-/-}$ pro B cells express B cell lineage markers and are able to rearrange their immunoglobulin locus but then fail to mature into B cells, instead giving rise to myeloid and T lineage cells [26].

The earliest committed B cell precursors are pre-pro-B cells [26, 28, 29]. These cells still have their immunoglobulin loci in the germline configuration but can be distinguished from other earlier B cell stages by a number of cell surface markers and because they differentiate into B cells and not into other lymphoid cells *in vitro* [30]. Pre-pro B cells express the general B cell

surface marker B220 (a splice variant of the common leukocyte antigen CD45), but do not express components of the B cell antigen receptor yet [26-28]. Pre-pro-B cells express low levels of the recombination activating genes (RAG-1 and RAG-2), two proteins that catalyse V(D)J recombination and antibody assembly [31, 32]. These very early B-lineage progenitor cells are immediate precursors of the pro-B cells and rarely have any immunoglobulin gene rearrangements. V(D)J recombination is initiated at the pro-B cell stage of B cell development, with one third of pro-B cells showing rearranged IgH genes [28].

The rearrangement of IgH genes is accomplished in two steps: (1) the recombination of a diversity segment to a joining (JH) segment, followed by (2) the recombination of a variable (VH) gene to the joined DJH gene complex [33]. Because of random nucleotide loss and addition, D segments can be joined to JH gene segments in any one of three reading frames. Most of the mature B cells in humans have DH segments in reading frame 2 (RF2) , one third of B cells produces DJH joints in RF3, and the remaining B cells carry DH segments in RF1 [34]. After DJH rearrangement, VH genes become accessible to the V(D)J recombinase and complete heavy chain transcription units are assembled [26]. It has been proposed that the switch from DJH to V(D)JH is regulated at the level of VH gene accessibility and is believed to require Pax5 and interleukin-7 (Il-7) [26].

Following translation, the IgH is transported to the cell surface where it becomes associated with the surrogate light chains VpreB and λ-like (VpreB1/2 and λ5 in mice) [35-37]. Together with the transmembrane signaling proteins Igα and Igβ, which are essential for the initiation of the IgH-dependent signal, this pre-B cell receptor controls the transition of pro-B cells into pre-B cells and activates their clonal expansion [26, 36, 38]. Signals derived from pre-BCR arrest further IgH rearrangements, a phenomenon known as allelic exclusion, and initiate IgL gene rearrangement [36, 39]. Successful light chain VJ rearrangement leads to replacement of surrogate light chains VpreB and λ-like by Igκ or Igλ that together with the IgH generate the unique B cell antigen receptor (BCR). Expression of BCR marks the transition to the antigen-dependent phase of B cell development and leads to the generation of immature B cells. Immature lymphocytes display mainly surface IgM with little or no IgD and hence are the first committed B lymphocytes that express BCRs.

B cells remain in the immature compartment for an average of 3.5 days [26, 40]. During this time B cells undergo antigen-induced BCR mediated selection that allows them to survive in the periphery.

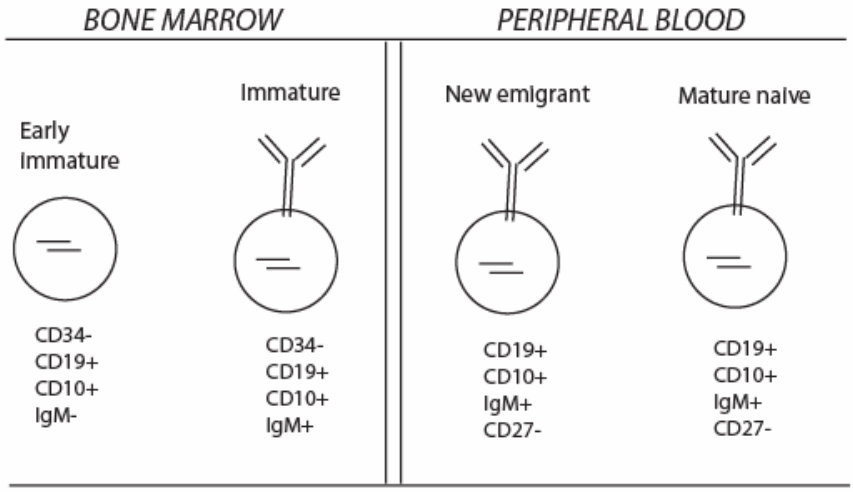


Figure 5. B cell stages during B cell development in the BM and the periphery can be characterized and distinguished by major surface marker shown in this figure (Modified from [2]).

It is been estimated that approximately only about 5% of the 15-20 million B lymphocytes formed daily in the bone marrow reach the long-lived peripheral B cell pool, presumably reflecting both negative and positive selection [40-42] (see section 1.3.2- 1.3.5 on pages 13-16). Newly emigrated B lymphocytes that are released in the periphery are short-lived and still functionally immature since they have not encountered their cognate antigen yet.

New emigrant B cells that survive in the periphery undergo further development to become mature (transitional) B cells that express an increased level of IgD and are furthermore characterized by the expression of complement receptors (CD1/CD2) [25], heat stable Ag (CD24) and B220 (CD45) [42]. New emigrant B cells are $CD19^{+}$ $CD10^{+}$ IgM^{+} $CD27^{-}$. They mark the crucial link between immature B cells in the bone marrow and peripheral mature $CD19^{+}$ $CD10^{-}$ IgM^{+} $CD27^{-}$ B cells [25, 43]. Mature, antigen experienced B cells carry the memory marker and are therefore $CD27^{+}$.

In the spleen, these immature B cells differentiate into long-lived mature B cells and recirculate through the blood stream and the lymphoid tissues where they may be activated upon encounter with cognate antigen. Following activation, B cells migrate to the germinal centres within the

lymph node where they undergo further maturation such as somatic hypermutation (SHM) and class switch recombination (CSR).

These two processes require Activation-Induced Cytidine Deaminase, AID, which specifically deaminates cytidine in ssDNA and is specifically induced in activated B lymphocytes [44-47]. AID mutation in humans is associated with absence of secondary antibody isotypes and SHM, and produces hyper-IgM syndrome, a disease associated with increased susceptibility to infections, indicating that AID is necessary for SHM and for the class switching required to generate sufficient immune responses [46, 48, 49]. Finally, affinity maturation of antibodies will permit the positive selection of high-affinity B cells to terminally differentiate either into early memory ($CD38^{-}IgD^{-}$) and memory Bm5, ($CD38^{-}IgD^{-}$) cells or into high-affinity Ab-forming cells.[33, 42, 43, 50, 51].

1.3.1
Immunological Tolerance

Hundreds of billions of different antibodies are produced to fight off invading pathogens. Due to V(D)J recombination and the millions of B lymphocytes leaving the bone marrow daily, a virtually unlimited number of antibody specificities can be produced. But a disadvantage of this antibody diversity is the generation of auto reactive and polyreactive antibodies that recognize self-antigens or harmless non-self molecules in the body and are potentially pathogenic. Polyreactive antibodies can bind various self or non-self antigens with low affinity. To avoid the generation and the release of these potentially harmful immunoglobulins into the blood stream there are several known mechanisms for silencing and shaping the immunoglobulin repertoire. In the following chapter I will discuss these tolerance mechanisms.

1.3.2
Central and Peripheral Tolerance- Counterselection at Checkpoints

Approximately $2x10^{7}$ B lymphocytes are generated daily, with millions of newly formed lymphocytes leaving the bone marrow [26, 52].

in healthy individuals, the majority (55-75 %) of newly generated immature B lymphocytes that are constantly generated by random Ig gene rearrangement show self- reactivity [4, 8]. Tolerance in the B cell lineage can only be maintained when these autoreactive B lymphocytes that produce antinuclear antibodies (ANAs) and polyreactive antibodies are purged from the B cell pool. Recently, two checkpoints have been described where self-reactive B cells are efficiently removed from the naïve repertoire [2, 4, 8, appendix 8.6].

The first checkpoint is at the early immature/immature B cell transition in the BM, where the majority of ANAs and polyreactive antibodies are eliminated. At this checkpoint the frequency of self- reactive antibodies decreases from 75.9 % to 43.1 %. The percentage of polyreactive B cell clones in the early immature stage (55.2 %)also decreases considerably, leaving a small number of polyreactive B cell clones from the immature (~7 %), new emigrant (~7 %) and mature naïve (4,3 %) B cell compartments [4, 5, 8]. These results of Hep-2 enzyme linked immunosorbent assays (Hep-2 ELISAs) suggest that more than half of the antibodies expressed in the early B lymphocyte compartment are polyreactive and that almost 90 % of polyreactive antibodies are counterselected in the immature stage at the first checkpoint [2].

The second checkpoint is in the periphery at the transitional stage where new emigrant B cells mature into naïve immunocompetent lymphocytes. The frequency of self reactive antibodies decreases notably from 43 % to 20 % at this checkpoint. Neither the anatomical correlate of the second checkpoint nor how tolerance is induced at this point are yet clear, although both positive and negative selection mechanisms have been suggested to contribute to this process [4].

A third checkpoint was recently discovered between the naïve and the IgM^+ memory B cell compartments [5], where B cells expressing self- reactive and broadly bacterially reactive antibodies are additionally sorted out from the B lymphocyte pool. This selection happens before the onset of somatic hypermutation and antibody affinity maturation is not complete at this point.

In the periphery, antigen-activated B cells proliferate and shape their BCR by somatic hypermutation in GCs by introducing mutations within V region segments of Ig genes. Multiple rounds of SHM and affinity-based selection for antigen on FDCs leads to a higher affinity of the antibody receptor for its antigen [53, 54].

Several distinct mechanisms (see below) exist to guarantee central B cell tolerance at this first checkpoint. At least three different mechanisms account for silencing potentially harmful self-reactive antibodies in the BM: *deletion, receptor editing and anergy* [2].

1.3.3

Antigen-dependent B cell selection

Deletion and anergy

Clonal deletion is defined as self antigen-induced death (apoptosis) of autoreactive B cells during early B cell development or at later stages of B cell maturation.Overall, the efficiency of clonal deletion is likely to be higher in the bone marrow than in the periphery due to the high susceptibility of immature B cells to receptor cross-linking leading to BCR-induced apoptosis [55].The number of human B cells deleted during development is not known yet, but in the mouse it has been estimated that 80 to 90% of all newly generated B lymphocytes are deleted before they enter the mature B cell compartment [41, 56, 57].

1.3.4

Anergy

Anergy is another mechanism by which self-tolerance is induced in immature B cells. Anergy describes a functionally silent stage induced in B cells, and is usually defined as non-responsiveness to BCR crosslinking [26, 36, 58, 59]. Anergic B cells are short-lived and unable to enter secondary lymphoid tissues. Furthermore, they fail to activate or respond to T helper cells and are not able to differentiate into plasma cells.

This hypo-responsive or anergic state of self-antigen specific B cells was first observed in developing B cells exposed to various concentrations of anti-μ. B lymphocytes cultured in the presence of high concentrations of anti-μ antibodies die at a high rate [60, 61]. In contrast, BCR cross-linking at intermediate levels due to lower concentrations of anti-μ does not cause B cell death but prevents B cell proliferation and antibody production upon subsequent mitogen exposure. Thus, incubation of immature B cells with anti-IgM antibodies in vitro revealed a direct correlation between the level of BCR cross-linking and the rate of cell death [60]. Thus, the state of anergy seems to be induced in B cells with a low affinity self-reactive BCR, whereas high affinity anti-self-reactivity leads to clonal deletion.

Anergy in self-antigen specific B cells has been extensively characterized in mice co-expressing the transgenic BCR specific for hen egg lyzozyme (HEL) and transgenic soluble HEL. Anergy is also induced in mice that express a BCR specific for single-stranded DNA. [26].

1.3.5
Receptor editing

Among the three mechanisms of negative selection, receptor editing, defined as a secondary Ig chain gene rearrangement, seems to be a major force in shaping the antibody repertoire under physiological conditions [3, 26]. Central receptor editing occurs in B cell clones that either failed to produce a functional BCR or have self-reactive BCRs. It is now clear that central receptor editing, particularly at light chain loci, maintains tolerance by modifying the Ag receptor specificity of immature self-reactive B cells through initiation of new Ig light chain recombination to rescue potentially autoreactive B cells from apoptosis [2, 43, 62]. Receptor editing is initiated upon BCR engagement by Ag, resulting in the upregulation of *RAG* genes, which then participate in editing the autoreactive BCR-encoding Ig VJ rearrangements. In mice, about 25% of autoreactive B cells change their specificity through secondary IgL gene rearrangement. It has been shown that Secondary VJ recombination also occurs in murine spleen and lymph node B cells in response to immunization [63]. This peripheral VJ rearrangement, termed receptor revision, might contribute to the generation of high-affinity antibodies in germinal centers (GCs) following the process of somatic hypermutation in response to stimulation by T-dependent antigens [64-67]

1.3.6
Autoreactivity- a fatal consequence of inefficient silencing mechanisms

Autoreactivity is defined by reactivity of antibodies against self molecules.
Polyreactive antibodies bind to a variety of different and unrelated self and foreign antigens [9]. Healthy human beings have about 5 % of so called natural antibodies that are poly- or self-reactive but do not cause harm [68, 69]. Most of them have a low affinity, belong to the IgM class, and are found in all immunocompetent individuals [34]. It is thought that the antigen binding pocket of polyreactive antibodies is relatively flexible, with conformational changes during antigen recognition being responsible for polyreactivity [70].
Several Ig family members have been described to be associated with autoreactivity. For instants VH4-34 gene segments encode natural autoantibodies that react against many self molecules

including the A, B, O blood group antigens [19].VH4-34 also has an enhanced role in a number of autoimmune syndromes, including lupus [2, 4, 8]. Long IgH CDR3s have been shown to be associated with self-reactive antibodies, and another feature associated with autoreactivity is the presence of positively charged amino acids within IgH CDR3 [2, 4, 19, 71].

However, neither of these attributes nor Ig gene usage itself is unique to the autoimmune antibody repertoire, and therefore CDR3 sequence alone cannot be used to predict autoreactivity [2, 8]. It has been proposed that these findings rather reflect the gigantic diversity of the Ig gene repertoire that has been selected over millions of years to protect against the immensely diverse world of pathogens while avoiding severe autoreactivity [8].

1.3.7

Defective Tolerance leads to Autoimmunity

SLE

Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterized by the production of anti- DNA, antinuclear (ANA's) and polyreactive antibodies. This leads to inflammation and damage to various body tissues and clinical involvement in multiple organ systems, including the joints, skin, kidneys, heart, lungs, blood vessels, and brain [72]. Although people with the disease may have many different symptoms, some of the most common ones include skin rashes, arthritis, extreme fatigue, vasculitis, fever, and nephritis [73] Lupus is characterized by periods of illness, called flares, and periods of wellness, or remission. The prevalence of SLE is 40-60 cases per 100,000 people with a clear dominance in women. In the 60% of SLE patients who experience onset of their disease between puberty and the fourth decade of life, the female to male ratio is 9:1. Thereafter, the female predominance again falls to that observed in prepubescents (where women and men are equally effected) suggesting that oestrogen plays an important role in the pathogenesis of SLE [74-76]. Furthermore, lupus is three times more common in African American women than in Caucasian women and is also more common in women of Hispanic, Asian, and Native American descent.

1.3.8

Treatment

At present, there is no cure for lupus. However, lupus can be effectively treated with drugs, and most people with the disease can lead active, healthy lives.

In general, the treatment of lupus depends on the severity of the disease and it can range from topical application of sun protection and steroid creams in patients with mild skin manifestation, oral steroid treatment such as prednisone in moderate disease stages up to application of stronger immunosuppressives like systemic anti malarial drugs (e.g. hydroxychloroquine) [77]. The treatment of SLE overlaps with other autoimmune diseases: besides steroids, Vitamin A (e.g. Psoriasis treatment) and retinoids (such as isotretinoids like acitretin), even stronger medication such as gold containing drugs, cyclosporine A, cyclophosphamide, methotrexate and azathioprine (e.g rheumatoid arthritis treatment) are occasionally used in lupus patients. The chimeric monoclonal antibody rituximab is one of the latest drugs used in lupus patients to stop the progression of this life long disease. It depletes B cells by targeting the pan B cell marker CD20 [78, 79]. But it is yet to be determined whether or not rituximab is useful in this disease, and controlled trial studies are necessary to clarify the effectiveness of this drug.

1.4 AIM

The aim of my thesis was to analyze the Ig gene repertoire from one lupus patient (SLE21) who had received corticosteroid therapy and was in clinical remission. We expected to find less abnormalities within the Ig gene repertoire than in SLE patients with active disease.
Therefore, I compared my findings to the Ig gene repertoire of 3 SLE patients before and after therapy and to healthy controls. I will show similarities and differences within the Ig gene usage distributions of these patients and thus be able to determine the effectiveness of steroid therapy in normalizing B cell gene repertoire. We cloned and analysed the B cell genes of 43 single mature naïve B cells derived from peripheral blood of patient SLE21.

First, we elaborated the V, D and J gene usage in this patient since a potential association between an unusual Ig gene segment representation and autoimmune disease has been reported by several groups [3, 80].

Next, we defined the Ig heavy chain (IgH) CDR3 characteristics, since some authors have proposed that positively charged and long IgH CDR3 are associated with autoreactive antibodies [3, 12, 24, 81]. Furthermore, I show the results of the Ig gene B cell repertoire and in vitro generated antibodies of 3 other SLE patients (SLE 100, 101,122) before and after corticosteroid treatment to better understand the alterations in frequency and reactivity profiles in untreated and treated SLE patients, respectively.

Finally, I turned my interest to a recently published SLE study that compared 6 patients [7] in clinical remission and show, how Ig gene abnormalities and poly- and autoreactivity change before and after therapy. Based on these results, I discuss the potential effectiveness of treatment [78, 79, 82-84] and discuss how tolerance might be induced during B cell development.
These new findings together with the previous work on SLE are very important to better understand the role B cells play in the pathogenesis of SLE, and furthermore provide useful insights into how tolerance is established and how defects in tolerance might be reset during B cell development.

1.4.1 Strategy

The first step was fluorescence-activated cell sorting (FACS) of B lymphocytes derived from peripheral blood samples from one treated SLE patient.

FACS allowed purification of single B cells from different developmental stages based on their cell surface marker expression. For each individual B cell, Ig heavy (IgH) and Ig light [29] chain variable region genes were amplified by semi-nested RT-PCR. Sequences were classified by using the National Center for Biotechnology Information (NCBI) IgBLAST database.

Suitable restriction sites were inserted during PCR amplification that allowed

the ligation of the obtained PCR products into separate expression vectors with the appropriate immunoglobulin constant regions. After amplification of these vectors in Escherichia coli (E.coli) and subsequent plasmid DNA preparation, the plasmid encoding the heavy chain and the vector with the corresponding kappa or lambda light chain sequence were co-transfected into human embryonic kidney cells (HEK 293A) by means of calcium phosphate precipitation. I used these methods for my thesis. The entire strategy of the lab allows you to generate antibodies from single B cells and test them for self- and poly- reactivity:

In vitro expression of the transfected genes in these cells leads to the production and secretion of the respective antibody molecules. The supernatants were collected and immunoglobulin proteins were biochemically purified with Protein G microbeads. To screen for polyreactivity, enzyme linked immunosorbent assay (ELISA) experiments with a defined set of antigens including dsDNA, ssDNA, LPS and insulin were performed; antibodies were defined as polyreactive if they bound more than one of these antigens. Autoreactivity was determined by HEp-2 cell lysate ELISA and indirect immunofluorescence assay (IFA).

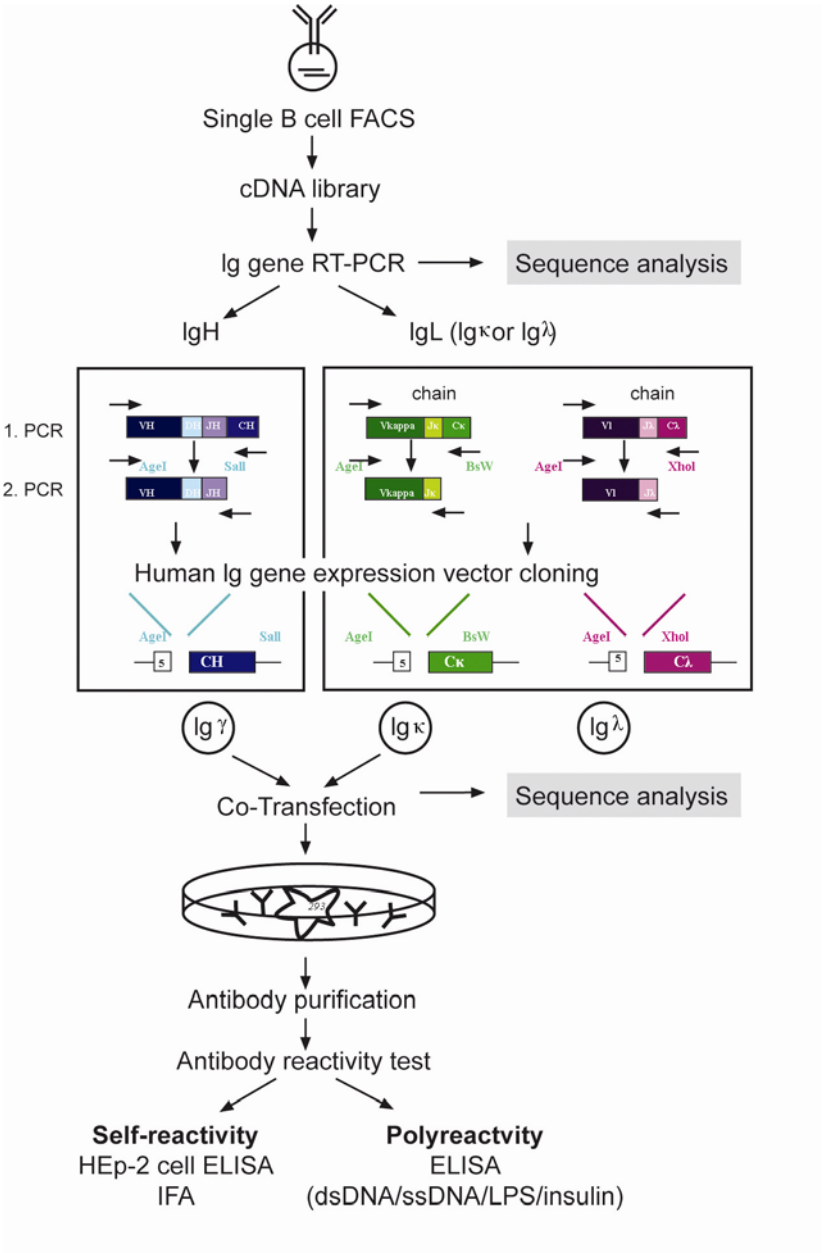


Figure 6. Strategy

Single B cells from the mature naïve B cell compartment are isolated by fluorescent activated cell sorting (FACS) into 96 well plates. A cDNA library is generated and IgH and corresponding Igk or Igl light chain genes are amplified by two successive rounds of RT-PCR. The obtained Ig genes are characterized by sequence analysis, cloned, then analyzed again to confirm identity with the original PCR product amplified from the cDNA. Antibodies are expressed by in vitro cells of the human 293A cell line, co-transfected with plasmids encoding the IgH and IgL chain originally amplified from the same B cell. The cells secrete the antibodies into the tissue culture supernatant. Each individual purified antibody can then be tested in an unlimited number of different enzyme linked immunosorbent assays (ELISA) and by indirect immunofluorescence assay (IFA) to determine its reactivity profile with self- and non-self antigens (modified from http://www.mpiib-berlin.mpg.de).

2. Materials

2.1Antibodies and antigens

ELISA capture antibody

Goat anti-human IgG, Fcγ	Jackson, ImmunoResearch Laboratories, West Grove, USA

ELISA secondary antibody

Goat anti-human IgG, Fcγ (HRP)	Jackson, ImmunoResearch Laboratories, West Grove, USA

ELISA standard and control antibodies

Human IgG1,kappa	Sigma, St. Louis, USA
ED38 (high positive polyreactive Ig)	see reference [48, 69]
JB40 (low positive polyreactive Ig)	
mGO53 (non polyreactive Ig)	

FACS sorting

The following antibodies are monoclonal mouse anti-human antibodies linked with the specified fluorochromes; as fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) or biotin. Biotinylated Igs are detected with Streptavidin- PerCP.

Anti-human CD10 (PE)	BD Biosciences, Pharmingen San Diego, USA
Anti-human CD 19 (APC)	BD Biosciences, Pharmingen San Diego, USA
Anti-human CD27 (FITC)	BD Biosciences, Pharmingen San Diego, USA
Anti-human IgM (Biotin)	BD Biosciences, Pharmingen San Diego, USA

Streptavidin (PerCP)	BD Biosciences, Pharmingen, San Diego, USA

Antigens for ELISA

DNA sodium salt	Sigma, St. Louis, USA
Human recombinant insulin	Fitzgerald Industries International Concord, USA
LPS, E.coli 055:B5	Sigma, St. Louis, USA

2.2 Bacteria and vectors

E.coli DH 10B	Clontech, Palo Alto,USA
Vectors (include heavy-,κ- and λ-chain C regions)	Kind gift of Dr. J. Ravetch Rockefeller University 1230 York Avenue, NYC, USA

2.3 Cell lines

HEK 293A	Invitrogen, Carlsbad, USA

2.4 Chemicals, buffers and solutions and special reagents

Acetic acid (wash buffer)	
Calcium Chloride	
Cresol Red 5x Loading buffer	1 mM cresol red (Sigma) 60 % sucrose
EDTA	
Ethidium bromide	Sigma
Glycerol	
Glycine buffer 0.1M, pH3	
HEPES	
Isopropanol	Fisher Chemicals
Potassium Chloride, Potassium Phosphate, Sodium Chloride, Sodium Phosphate	
RNAse free PBS	Gibco
RNAse free water	QIAGEN

Sucrose, Trizma ®base, Tween ®20

1x PBS pH=7.4	137 mM NaCl 2.7 mM KCl 4.3 mM Na2HPO4.7H20 1.4 mM KH2PO4
2x HBSS pH=7.05	280 mM NaCl 50 mM HEPES 10 mM KCl 12 mM Dextrose 1.5 mM Na2HPO4.7H20
50x TAE	2 M Tris 0.05% (v/v) glacial acetic acid 0.05 M EDTA (pH=8)
ELISA blocking buffer	1x PBS 0.05% (v/v) Tween® 20 1 mM EDTA
Elution buffer pH=3 (antibody purification)	0.1 M glycine

2.5 Commercial kits

ANA Immunofluorescence Kit	Bion Enterprises, Des Plaines, USA
HiSpeed® Plasmid Maxi Kit	Qiagen, Valencia, U.SA
HRP substrate Kit	Bio-Rad, Hercules,USA
QIAprep® Spin Miniprep Kit	Qiagen,Valencia, CA, USA
QUANTA LiteTM ANA ELISA Kit	Inova Diagnostics, San Diego, USA
Quick Ligation Kit	New England Biolabs, Beverly, USA

2.6 Tissue **Culture media**

Ampicillin	SIGMA
Antibiotic-Antimycotic	GibcoBRL
Dulbecco's Modified Eagle Medium (DMEM)	GibcoBRL
HEPES Buffer Solution (1M)	GibcoBRL
L-Glutamine (200mM, 100x)	GibcoBRL
Low IgG Fetal Calf Serum (FCS), heat inactivated	GibcoBRL
Luria Broth Media [83]	Gibco BRL
MEM Sodium Pyruvate 100mM	GibcoBRL
MEM Non-essential amino acids solution 10 mM (100x)	GibcoBRL
Nutridoma-SP	Roche
Penicillin/Streptomycin	GibcoBRL
Sodium Pyruvate (100mM)	SIGMA
Terrific Broth (TB)	Difco Laboratories, Becton Dickinson
Trypsin-EDTA	GibcoBRL
Ultra-low IgG FBS	GibcoBRL

2.7 Enzymes, Proteins and Additives

Ligation

T4-DNA Ligase	New England Biolabs, Beverly, USA

Purification

ExoSAP-IT®	USB Corporation Cleveland, USA

Restriction enzymes

AgeI Restriction Enzyme	New England Biolabs, Beverly, USA
BsiWI Restriction Enzyme	New England Biolabs, Beverly, USA
SalI Restriction Enzyme	New England Biolabs, Beverly, USA
SacI Restriction Enzyme	New England Biolabs, Beverly, USA

RT and PCR

HotStarTaq DNA polymerase	Qiagen, Valencia, USA
10x PCR buffer	Qiagen Valencia, USA
SuperScriptTM II RT	GibcoBRL Rockville, USA
Taq DNA polymerase	Roche Molecular Biochemicals Indianapolis, USA
RNAsin®	Promega, Madison, USA
DTT Gibco	BRL, Rockville, MD, USA
5x First strand buffer	GibcoBRL, Rockville, USA
Non-Idet NP40	Sigma, St. Louis, USA
SeaKem® LE Agarose	Cambrex Rockland, USA

ELISA

1-StepTM ABTS	Pierce, Rockford, USA

2.8 Laboratorial equipment

Allegra 6KR Centrifuge (rotor: GH-3.8A)	Beckman Coulter Fullerton, CA, U.S.A.
Alpha ImagerTM 1220	Alpha Innotech Corporation San Leandro, CA, U.S.A.
BioPhotometer	Eppendorf, Hamburg, Germany
CO2 Incubator Model2450	VWR Scientific Bridgeport, USA

GeneAmp® 9700 PCR machine	Applied Biosystems Foster City
GS-6R Centrifuge (rotor: GH-3.8)	Beckman Coulter Fullerton, USA
J-6M Centrifuge (rotor: JS-4.2)	Beckman Coulter Fullerton, USA
Multitron Shaker	Infors AG Bottmingen, Switzerland
Plate Wash Plus	PerkinElmer Life Sciences Boston, USA
Sterilguard Hood Class II	The Baker Company, Inc. Sanford, USA
VersaMax Microplate Reader	Molecular Devices Sunnyvale, USA
Waterbath	VWR Scientific Bridgeport, USA
1.5 ml reaction tubes	Sarstedt, Newton, USA
250 ml polypropylene centrifuge tube	Costar, Corning Acton, USA
Bio-Spin® chromatography columns	Bio-Rad Hercules, USA
ELISA plates (96 well, flat bottom)	Costar, Corning Acton, USA
Inoculating loops/needles, polystyrene	VWR International, Bridgeport, USA
MicroAmp® reaction tubes	Applied Biosystems Foster City, USA
Petri dishes (100 mm)	Falcon, Becton Dickinson Labware Franklin Lakes, USA
Polypropylene tubes (15 ml, 50 ml)	Falcon, Becton Dickinson Labware Franklin Lakes, USA
ThermoGridTM PCR plates	Denville Scientific Inc.

	Denville, USA
Tissue culture plates (150 mm)	Falcon, Becton Dickinson Labware
	Franklin Lakes, NJ, U.S.A.

Nucleotides and nucleic acids

1 Kb Plus DNA ladder	Promega, Madison, USA
Desoxynucleotide Triphosphates	Roche Molecular Biochemicals
(dNTPs)	Indianapolis, USA
Oligonucleotides	Integrated DNA Technologies, Coralville,

(the sequences of the primers used in the work are listed in Table 1 in the appendix)

Random Hexamer Primer	GibcoBRL, Rockeville, USA

2.9 Software

Adobe Photoshop	Adobe, San José, USA
CellQuest	Becton Dickinson
EditSeq ™	DNASTAR, Madison, USA
EndNote ® 8	Thomson Scientific, Philadelphia, USA
IgBLAST	NCBI
DNAStar Software	NCBI
Microsoft ® Office	Microsoft, Stuttgart, Germany
Microsoft Excel	Microsoft
Microsoft Word	Microsoft

3. Methods

3.1 FACS and Flow Cytometry

A B cell sort was done prior to the start of my thesis and thus it is mentioned fairly briefly.
B cell subpopulations were purified from peripheral blood of the patient SLE21. $CD10^{+}$PE cells were pre-enriched after staining with anti-human CD10-PE antibody followed by purification with anti-PE magnetic microbeads. CD10- and the CD10+ fractions were stained with anti-human CD19-APC, anti-human IgM-Biotin and anti-human CD27-FITC. Biotinylated antibodies were detected using Streptavidin-PeCy7. Single B cells were sorted on a FACSVantage automated cell sorter into 96- well PCR plates containing 4 µl Lysis solution (0.5 PBS containing 10 mM DTT, 8 U RNAsin, 0.4 U 5'-3' Prime RNAse Inhibitor) and immediately frozen on dry ice. All samples were stored at -80°C.

The following steps including cDNA synthesis and single cell polymerase chain reaction (PCR) were performed in RNA and DNA free area to avoid contamination.

3.2 RT-PCR and cDNA synthesis

Reverse transcription-polymerase chain reaction (RT-PCR) is a method for analyzing gene expression. RT-PCR combines complementary DNA (cDNA) synthesis with PCR and provides rough quantitation of the expression of a particular gene. Due to the high sensitivity of PCR, RT-PCR is used to study expression in single cells. Furthermore, RT-PCR is used for identifying and studying RNA and its different isoform transcripts.

The complementary DNA (cDNA) is made by the enzyme reverse transcriptase, a RNA dependent DNA polymerase that catalyses the conversion of RNA into cDNA. This enzyme needs a hybridized primer to attach the next complementary desoxyribonucleoside triphosphate (dNTP). Therefore, hexamer primers can be used to anneal randomly to the RNA template. In addition, RNA inhibitors are used to inactivate RNA degrading enzymes and a buffer, to maintain ideal conditions. Further components of the mix are Dithiotreitol (DTT), a reducing agent that helps remove RNA secondary structures and the detergent Nonidet - P40 used for the lysage of cell remnants.
To synthesize cDNA, RNA from 4.0 µl single cell lysates was reverse transcribed
in a total volume of 14 µl in the original 96-well plate using

Concentration	*Reagents*
3.6	µl H20
3	µl 5x Buffer
150 ng	random hexamer primer
0.5 ng	dNTP-Mix (10mM each nucleotide)
1 µl	0.1 M DTT
0.5%	NP-40
4 U	RNAsin
6 U	Prime RNAse Inhibitor
50 U	SuperScript II Reverse Transcriptase

The cDNA synthesis was accomplished by of 4 steps following the protocol

Step	Temperature	Time
1. Denatuaration	42°C	5’
2. Annealing of Hexamers	25°C	10’
3. Reverse transcription	42°C	55’
4. Reaction stop	94°C	5’

3.3 Polymerase chain reaction (PCR)

The *Polymerase chain reaction* is a technique for in vitro amplification of a specific DNA fragment by use of two short single stranded primers flanking this fragment. First steps implicate mixing of template DNA, two appropriate oligonucleotide primers outlining the region of interest, thermostable Taq DNA polymerase, dNTPs and a buffer. The mixture undergoes then repeated cycles of heat denaturation, primer annealing, primer extension that allow the synthesis of a new DNA strand, respectively. Due to the increase in temperature the most effective amplification by the Taq DNA polymerase is achieved. The fragment of interest is amplified exponentially up to a million fold and is specific in size and sequence. PCR products are further separated by agarose gel electrophoresis and stained with the intercalating

fluorescent dye ethidium bromide. To examine the yield and specificity of PCR products the gel is examined under UV light.

3.5 µl of cDNA were used to amplify IgH, Iglambda (λ) and Igkappa (κ) sequences in the first round of PCR with a total volume of 40µl containing

31.4 µl	H2O
4 µl	10x Buffer
0.5 µl	dNTP-Mix (10mM each nucleotide)
250 pm	5'primer
250 pm	3'primer
1.2 U	Taq DNA polymerase

After the first round of PCR (that was only due to amplification of the specific transcripts) a second run was performed under almost the same conditions. This time, 3.5 µl of the first PCR product as starting material and a different set of primers to insert restriction sites were used. Primer sequences for specific amplification of VH and L-chain (VL) regions are described in the appendix in table 1. The forward primers for the first PCR are family based and overspan parts of the two leader exons of the V region sequences. As reverse primer the conserved C region primer CHµ1 was used to amplify Igµ sequences. For amplification of k or λL-chain sequences, sets of family-based leader forward primers were used in conjunction with C region primers complementary to the C-terminal portion of the L-chain coding sequence. For IgH and Igλ genes restriction sites were introduced during the second PCR with primers containing restriction sites. Igκ 2nd PCR reactions were performed with a consensus kappa primer and after sequencing a 3rd round of PCR with restriction-site containing specific kappa gene primers was performed (Fig.7). To increase the specificity, the PCR was carried out as a ´hot start´ reaction, with an initial temperature at 94°C.
Each round of PCR was performed for 50 cycles

Step	Temperature	Time

Denaturation	94°C	30 ‘’
Annealing	57°C (for IgH and Igκ)	
	60°C (for Ig λ)	
		30 ‘’
Elongation	72°C	55 sec (1st round)
	94°C	45 min (2nd round)

6 µl aliquots of the second PCR products were run on a 2% agarose gel with ethidium bromide, an intercalating fluorescent dye to identify successful amplification products.

3.4 DNA Purification and DNA Sequencing

Prior to sequencing the PCR products were purified with Exonuclease I and Shrimp Alkaline Phosphatase (ExoSAP*) necessary for degrading unused dNTPs and oligonucleotides. The sample was in a total volume of 12µl according to the requirements of the Genewiz Company.
One sample consisted of

3 µl	digested PCR product
1 µl	ExoSap*

and incubated for 30 minutes

Step	**Temperature**	**Time**
‘ExoSap* purification	37°C	15 min
‘ExoSap* inactivation	80°C	15 min

Secondly,

6pmol primer (5′ AgeI VH-Mix for heavy chain sequences)
(3′ XhoI Cl for lambda gene sequences)
(3′ Ck494 for kappa gene sequences)

and sterile water was added to the now purified PCR product to yield a total volume of 12 µl.

Sequence analysis was performed using NCBI's database IgBlast on the computer. This is a useful tool to get detailed information about the VH, JH and Vkappa, Jkappa or Vlambda, Jlambda gene families used in the Ig repertoire.
D segments and D reading frame were determined and further Ig heavy chain characteristics like amino acid [85] number of the complementary determining region (CDR3) and the amount of positive and negative charges in this CDR3 region were carefully analysed. Arginine (Arg, R) Histidin (His, H) and Lysine (Lys, K) are positively charged, in difference to Glutamate (Glu, E) and Aspartate (Asp, D) that carry a negative charge. IgH CDR3 length was determined by counting the amino acids between the end of VH framework 3 and the conserved amino acid Tryptophan (Trp, W) at position 103 in JH segments as indicated by IgBlast. Rare non- germline sequences that contained high numbers of mutations that could not be attributed to sequence polymorphisms were excluded from further analysis.

3.5 DNA Sequencing and Analysis

DNA sequencing is a method for deduction of the precise amino acid sequence in a DNA sample and it additionally allows for the analysis of mutations.
Our samples were sequenced by Genewiz (http://www.genewiz.com) and further analysed by using IgBlast (http://www.ncbi.nlm.nih.gov/igblast).
DNA sequencing can be carried out in many different ways and the presumably most popular approach for doing this is the dideoxy or chain termination method introduced by Sanger in 1977. It is named after the synthetic 2,3'-dideoxynucleotide 5'-triphospate (ddNTPs) that lack the hydroxyl residue at the 3' carbon atom necessary for DNA elongation and therefore plays a crucial role within the whole process. Naturally, four nucleotides (dNTPs) synthesizing the DNA bind separately to the 3' – position of the last nucleotide added.
In the sequence reaction the template DNA is supplied with

- a mixture of all four deoxynucleotides (dATP, dGTP, dCTP, dTTP) in equal quantities
- a mixture of all four dideoxynucleotides (ddATP, ddGTP, ddCTP, ddTTP) each present in limiting quantities and each labeled with a fluorescence dye of a different colour
- DNA polymerase
- a primer for determining the starting point as well as the direction of the sequencing reaction

DNA sequencing shows similarities to the PCR reaction in regard to its 3 major steps: denaturation of the DNA fragment to be sequenced, annealing of the single primer and elongation of the new DNA strand that are repeated in 30 or 40 cycles.

Sanger's method is based on the use of small amounts of ddNTPs that are, as described above as well added to the growing DNA strand. Hence, DNA elongation proceeds normally until the polymerase inserts a dideoxy nucleotide instead of a normal dNPT. Once a ddNTP is incorporated in the nascent DNA
strand, chain elongation will be terminated because there is no 3' position available for the next nucleotide to be attached to. If the ratio of normal nucleotide to dideoxy substrate is sufficient enough, some DNA strands succeed in growing several hundreds nucleotides long before insertion of the dideoxy substrate will finally stop the process. At the end of the incubation period, the synthesized fragments are separated by length by acrylamide gel electrophoresis. The resolution is so good that a difference of one single nucleotide is enough to separate that specific strand from the next shorter and next longer strand.
The fluorescently labelled ddNTPs help to detect the colour of the last base of each fragment on an automated sequencer since the different dyes with which each base is marked make it possible to determine which base has been added lastly.

3.6 Transformation

Transformations were accomplished by

9 µl	competent bacteria
3 µl	ligation product (µ-chain, lambda-kappa-chain ligation)

Per transformation, 9 µl of competent bacteria were given into 1.5 ml Eppendorf tubes and then 3 µl of ligation product were added to the tubes and immediately kept on ice followed by an incubation time of 30 min. A heat shock was performed by 42°C for 45-60 seconds and hence bacterial cells were allowed to grow in 100 µl warm LB medium w/o Ampicillin for 30 min under moderate shaking. 100 µl were then plated on selective plates containing 100 µg/ml ampicillin and incubated overnight at 37°C.

3.7 Insert screening

Next morning the plates were examined for bacterial growth. Hence, bacterial colonies were screened for the presence of the insert by PCR.
PCR conditions were performed as described above except that the amplification was carried out for 27 cycles and with a different set of primer. The 5´primer (Ab sense) used for all inserts was complementary to a sequence in the vector upstream of the insert site. The 3´primer (3´ Cg1) for heavy chain inserts was binding to the constant g region of the vector. For kappa and lambda light chain insert amplification the same 3´primer were used as for the second nested PCR [Primer list, Table 1, Appendix]. The PCR products were analyzed by gel electrophoresis to confirm the expected sizes and after ExoSAP- purification sequenced by Genewiz Company to check for correct and unmutated insert sequences. The settings were the same as in the previous sequencing reactions.

3.8 Plasmid preparation

After confirmation of the right insert the appropriate bacterial colonies were used for plasmid preparation. Plasmid DNA was prepared using the commercially available QIAprep Spin Miniprep Kit from QIAGEN. All steps were carried out as instructed by the manufacturer. Briefly, single colonies were grown in 3 ml TB media containing 75 µg/ml ampicillin overnight. After alkaline lysis of the cells and neutralization the sample fluid was loaded on silica-gel membrane resins and washed twice with the supplied washing buffer. After elution with 70 µl elution buffer the DNA concentration was determined spectro-photometrically at a wavelength of 260 nm. The plasmids were stored at -20°C.

3.9 Cell culture

The human embryonic kidney 293A (HEK 293A) fibroblast cell line was used for expression of antibody molecules. The cells were cultured at 37 °C and 5% CO2 in 25 ml complete Dulbeccos modified eagle medium (DMEM) containing 10 % heat-inactivated ultra-low IgG fetal bovine serum (FBS), 2.4 mmol/l sodium pyruvate, 5.6 mmol/l Lglutamine and 1.3 % antibiotic-antimycotic mix. Cells were split every 2-4 days at a confluency of 70-80 %. After removing the medium from the plates the cells were washed with 10ml serum-free DMEM and following a very short treatment with 7 ml trypsin-EDTA the cells were resuspended in 25-30 ml complete DMEM and distributed in a ratio of 1:6-1:10 depending on the desired splitting frequency.

3.10 Statistics

P-values for Ig gene repertoire analysis, analysis of positive charges in IgH CDR3, and antibody reactivity were calculated by 2 x 2 or 2 x 5 Fisher Exact test or X^2 test. P-values for IgH CDR3 length were calculated by Student's t-test.

4. Results

In this chapter, I will present the results of patient SLE 21 regarding Ig gene usage, CDR3 length and charges within this region. I will compare my findings with the results of 2 healthy donors whose genes were previously characterized in our lab [appendix 8.5, table 4-5]. Later on, I will show the previous findings from patient SLE 100, 101, and 121 before and after treatment [appendix, 8.3] and compare the results with my newly obtained data.

Analyses of all DNA sequences were performed by using the NCBI database IgBlast; information on V_H, D and J_H gene segment usage for heavy chains, and V_L and J_L gene segments for L-chains was obtained. All lupus patient we examined in our group were diagnosed based on the Revised Criteria of the American College of Rheumatology [73] and all samples were obtained after signing informed consent at the Division of Pediatric Rheumatology of UT South West Medical Center. Remission was defined by normalization of clinical symptoms and laboratory findings [7].

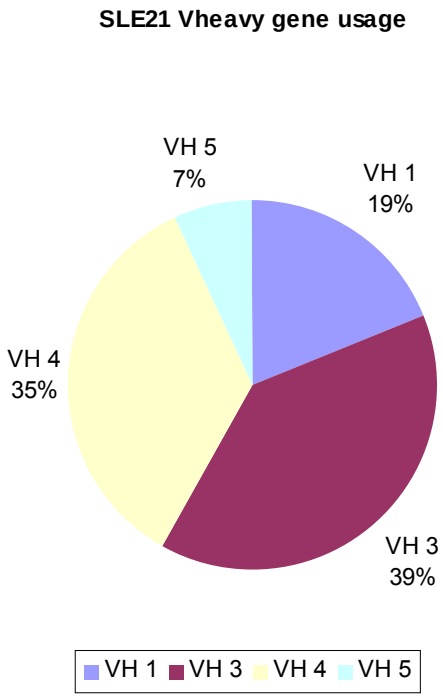
4.1 No Ig heavy chain gene abnormalities in SLE21

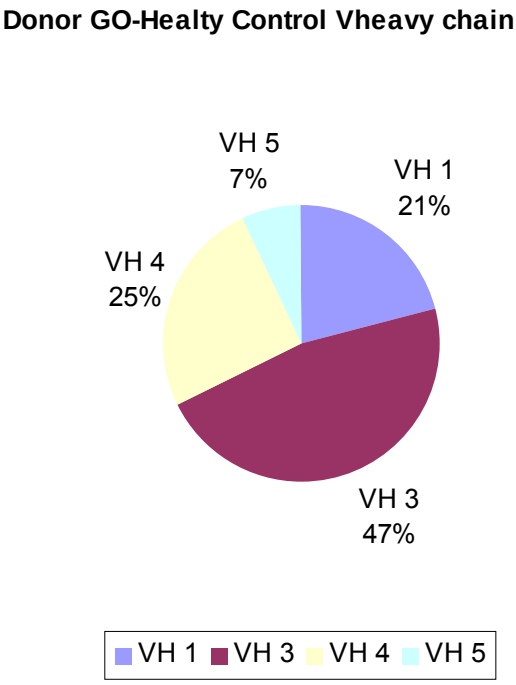
SLE 21 in clinical remission shows a similar Ig gene repertoire to healthy individuals

V_H gene proportions in SLE21 [appendix 8.2, Table 2] are similar compared to healthy controls (Figure7). There is no overrepresentation of V_H4 and V_H3 genes observed in SLE21, with similar proportions of V_H4 genes (35 %) used as compared to the healthy controls (JB 39%). Thirty-nine percent of VH3 genes were used in SLE21; that is more than the representation of this gene in GO (27%), but less when compared to donor JB (47%). There is a trend towards an underrepresentation of the V_H1 family genes in SLE21 (19% vrs. 24% in JB), however this result is not statistically significant.

The distribution of the heavy and light chain gene usage among the tested mature naive B cells in SLE patient 21 and in 2 healthy controls are indicated in the following pie charts:

Ig gene usage normalities and abnormalities in SLE patients





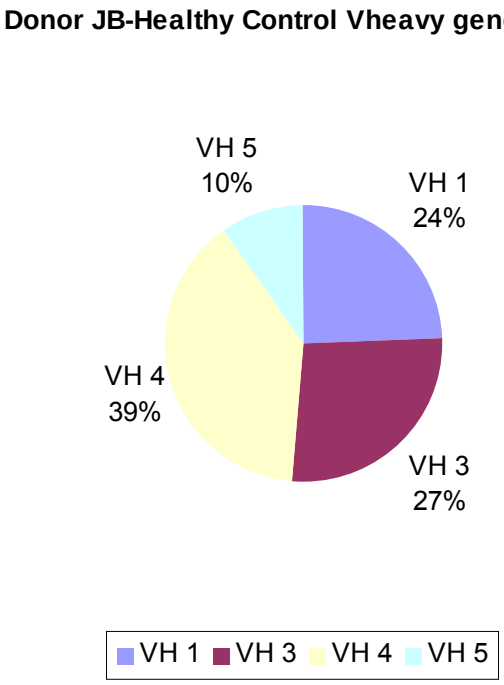


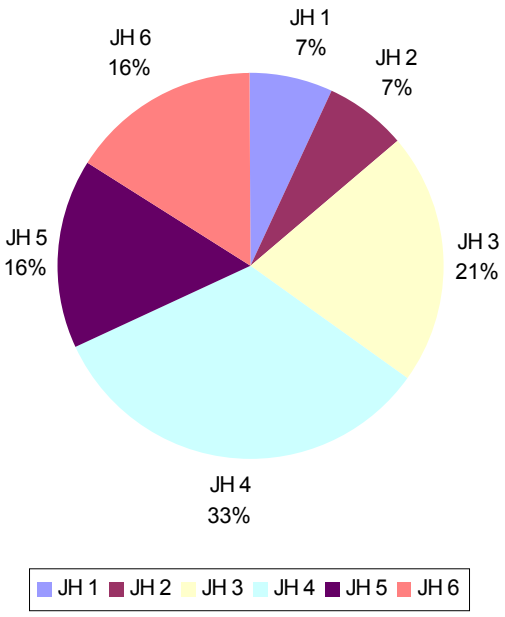
Figure 7a-c
a) The total number of 43 mature naive B cells were analysed in this study. 19 % of VH1, 39 % VH3, 35 % VH4 and 7% of VH5 genes were used in the B cells tested by single cell RT-PCR.
b-c) The VH gene family usage of the healthy donors Go and JB do not differ significantly ($p>0,25$) from the VH gene repertoire of SLE21.

4.1.1

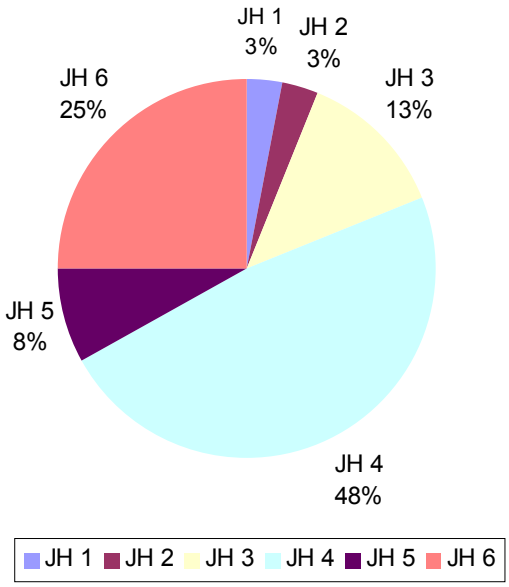
In terms of J_H gene usage, notable alterations were found when SLE21 was compared to the healthy control GO ($p<0,001$, Figure 8). A slight increase in the J_H1 gene usage (7% vs. 4% and 3 % in GO/JB) and underrepresentation of the J_H4 gene (33 % vrs. 48% in GO and 46% in JB) could be detected. This gene usage alteration is not observed when comparing SLE 21 with healthy control JB ($p>0,35$).

The distribution of JH chain gene usage among 43 tested single B cells in patient SLE 21 shows the following results:

SLE21 J heavy gene usage

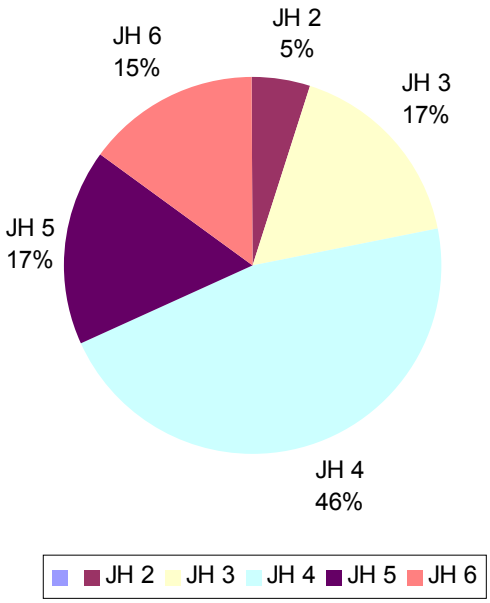


Donor GO- Healthy Control Jheavy gene usage



p<0.001 compared to SLE21

Donor JB-Healthy Control Jheavy gene usage



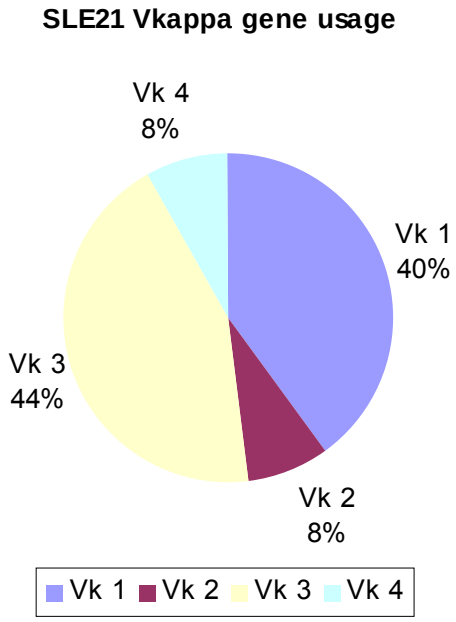
p>0.35 compared to SLE21

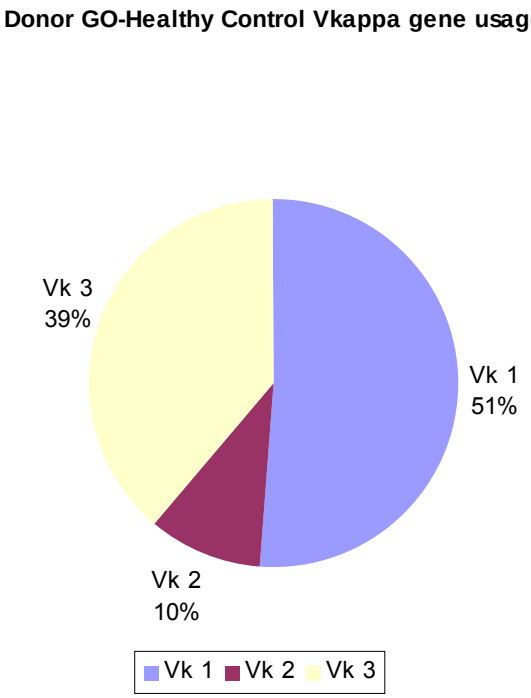
Figure 8a-c
a) The total number of 43 B cells of the mature naïve compartment were analysed to investigate the JH gene usage. J_H 1 and J_H2 genes were both used in 7 %, the J_H 3 gene locus was represented in 21% of all B cells tested, J_H 4 gene sequences were used in 33 %, J_H5 genes in 16%, and the J_H 6 gene is present in 16 % of all 43 B cells tested by single cell RT- PCR.
b) Healthy control GO displays a different gene distribution ($p<0,001$) compared to SLE21 as well as to JB. J_H6 is overrepresented (25% vrs. 15% in JB and 16% in SLE21) while the J_H3 gene is underrepresented (13% vrs 17% in JB and 21% in SLE21). The J_H4 genes in SLE21 are underrepresented (33%) when compared to the healthy controls (48% in GO and 46% in JB).
c) The JH gene repertoire in donor JB shows similar percentages ($p>0,35$) than SLE21 JH gene usage.

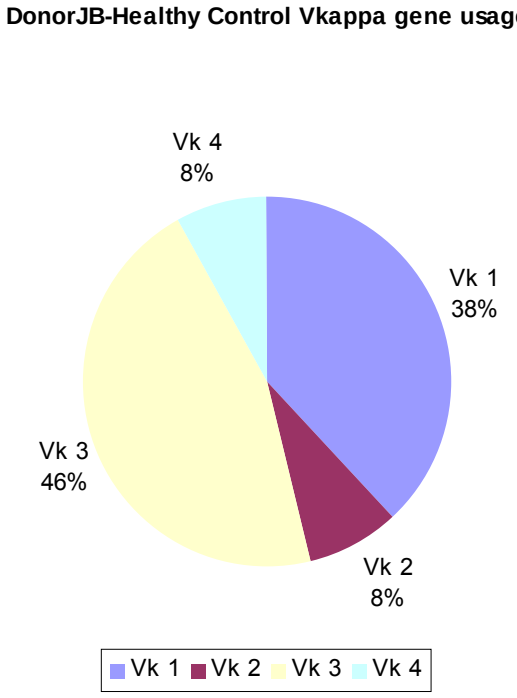
4.2 Ig light chain gene usage in SLE patients and in healthy controls

We detected significant differences within the Vκ gene usage of patient SLE21 when compared to healthy control GO, but not when compared to JB. The major difference between patient SLE21 and GO was the Vκ4 gene usage:
Vκ4 genes in the mature naïve B cells of patient SLE 21 were used significantly more (8 %) compared to donor GO, who did not show Vκ4 genes in the repertoire ($p<0,001$). The other major difference between the two patients was increased Vκ1 gene usage in GO (51%) when compared to SLE21 (40%).

Further findings regarding the kappa chain genes show the following characteristics:





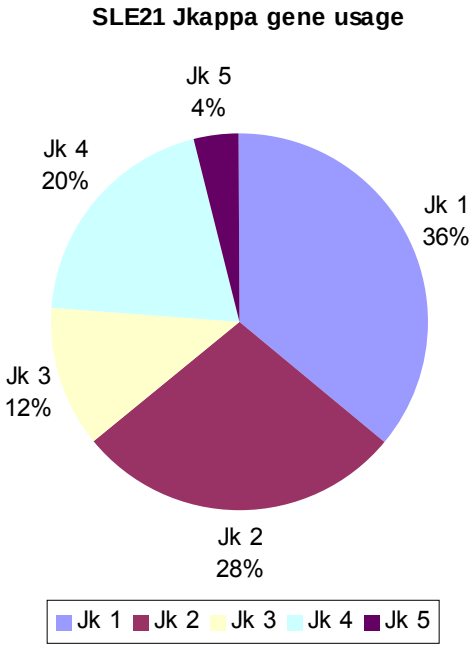


p<0.001 compared to SLE21

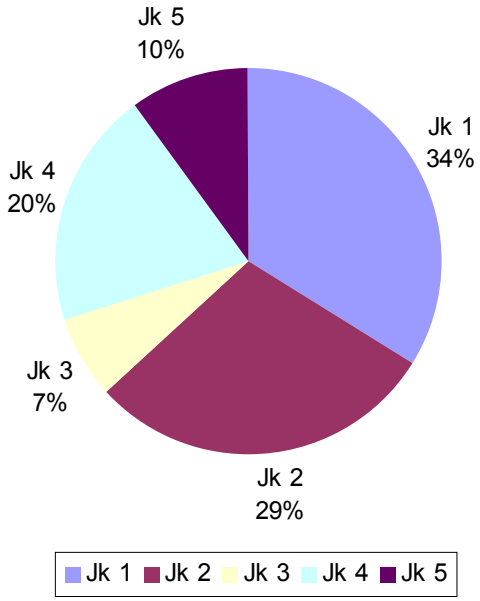
Figure 9a-c

a) Vk gene usage of SLE patient 21 is shown in this pie chart. 25 among 43 B cells show kappa gene usage. V_k 1 genes were used in 40 % of the 25 B cells, V_k2 were used in 8 %, V_k 3 were present in 44 %, whereas the V_k 4 loci were only used in 8 % of the 25 kappa genes B cells of SLE patient 21. b-c) The Vkappa gene usage of healthy donor Go deviates significantly from donor JB and SLE21 (p<0,0001) in that Go does not use V_k 4 genes, whereas the Vkappa gene usage of JB and SLE21 are almost alike (p=0,9789). V_k 1 genes are used in 40% of mature naïve B cells of SLE21, 51% V_k 1 genes in GO and 38% in JB.

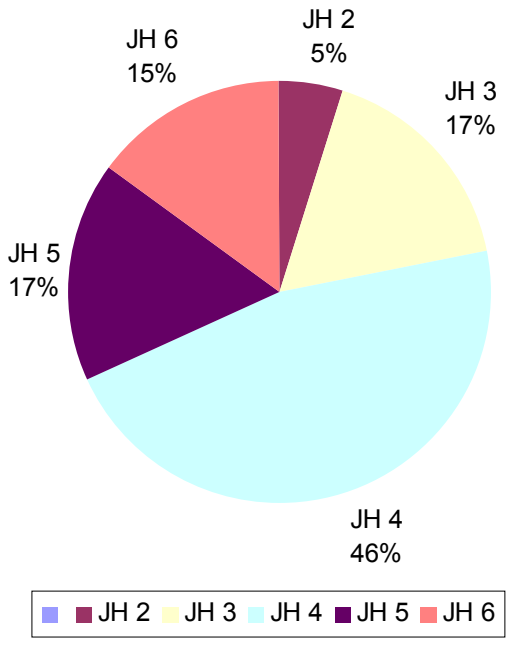
4.2.1 In terms of Jkappa gene usage, the distribution among the different gene loci showed the following results:



Donor GO-Healthy Control Jkappa gene usage



Donor JB-Healthy Control Jheavy gene usage



p<0.01 compared to SLE21

Figure 10a-c

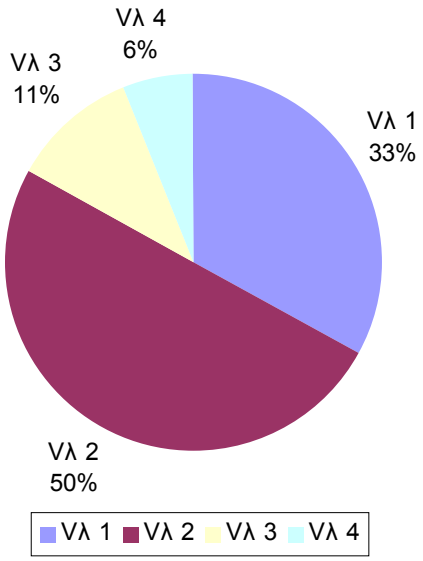
a) 25 B cells (58.1%) among the total number of 43 B cells investigated in this study used Jkappa genes. The J_k1 gene was used in 36 %, the J_k2 in 28 %, the J_k3 gene is present in 12 %, the J_k4 gene was used in 20 % and the J_k5 gene was present in 4 % of the 25 B cells in SLE patient 21 that used Jkappa genes.

b-c) SLE21 display a similar J kappa gene usage when compared to the healthy control JB ($p>0,01$), whereas healthy control GO shows significantly different J kappa distributions than SLE21 ($p<0,01$) with an overrepresentation of the J_k5 gene.

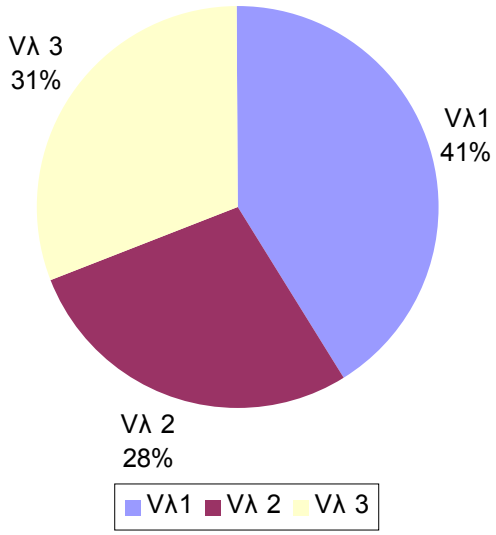
4.3 Significant Ig lambda chain gene usage differences in SLE21

Regarding V_λ and J_λ gene usage in SLE21, significant differences could be observed when compared to healthy controls ($p<0,001$; Figure 11). Unlike in SLE21, the distal$V_\lambda6$ gene family was not used in both of the healthy controls. Furthermore, $V_\lambda2$ and $V_\lambda3$ appear to be differently used when compared to healthy controls.$V_\lambda2$ genes seem to be increased in SLE21 (50% vrs. 28% and 29% in GO/JB), whereas $V_\lambda3$ gene subgroups seem to be slightly underrepresented in SLE21 (11% vrs. 31% and 24%).

SLE21 Vlambda gene usage

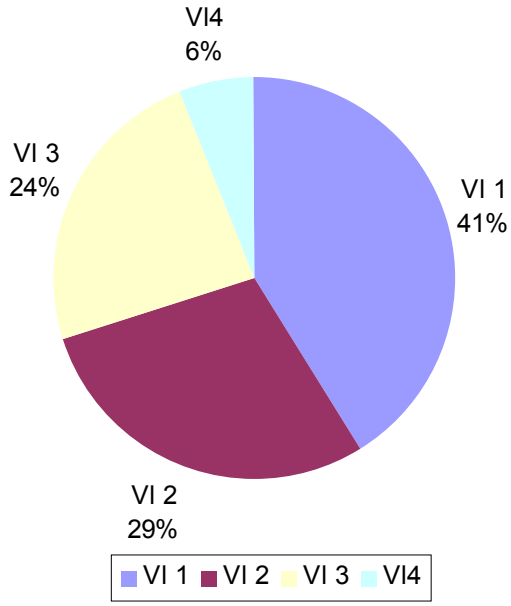


Donor GO-Healthy Control Vlambda gene usage



p<0.0001 compared to SLE21

Donor JB-Healthy Control Vlambda gene usage



p< 0.0001 compared to SLE21

Figure 11a-c

a) V lambda gene usage in SLE21 is indicated in the above pie chart. 18 B cells (41, 9 %) among 43 B lymphocytes analysed in this study used V lambda genes.

Fifty percent of the 18 B cells used $V_\lambda 2$ genes, 33 % used $V_\lambda 1$ genes, 11 % show $V_\lambda 3$ usage and 6 % used $V_\lambda 6$ genes.

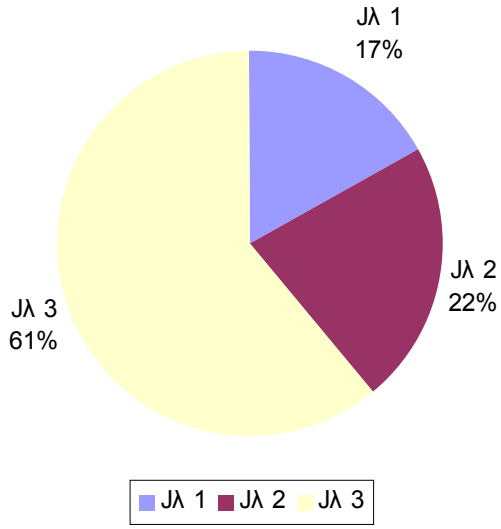
b-c) We see differences in the V lambda gene usage in patient Go and JB in comparison to SLE 21. The $V_\lambda 6$ gene family is not used in both of the healthy controls, whereas $V_\lambda 4$ gene is only found in patient JB.

An overrepresentation of $V_\lambda 2$ genes is found in SLE21 (50% vrs. 28% and 29% in GO/JB) and an underrepresentation of the $V_\lambda 3$ genes (11% vrs. 31% and 24%) when compared to the healthy controls The p values of $p<0,0001$ in GO and JB indicate the deviation of V_λ gene usage when compared to SLE21.

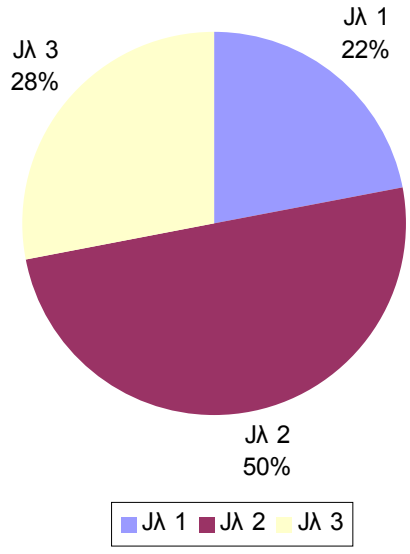
With respect to J_λ gene repertoire, the $J_\lambda 2$ gene is decreased in SLE21 (22% vrs. 50%), whereas the distal $J_\lambda 3$ gene appears to be overrepresented (61% vrs. 28%) when compared to the healthy donors.

4.3.1

SLE21 Jlambda gene usage

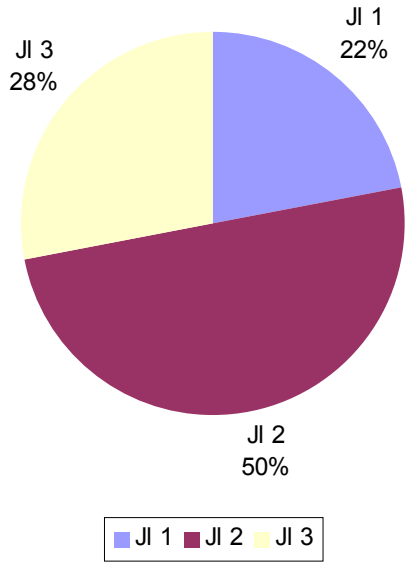


Donor GO-Healthy Control Jlambda gene usage



$p<0.0001$ compared to SLE21

Donor JB-Healthy Control Jlambda gene usage



$p<0.0001$ compared to SLE21

Figure 12a-c
a) J lambda chain sequence features are shown in the above pie chart. J_λ3genes were used in 61 %, J_λ2 genes in 22 % and J_λ1 genes in 17 % among the 18 B cells that used genes of the J_λ gene family.
b-c) J_λ gene usage of the healthy controls are seen in these 2 pie charts.
SLE 21 show significantly different J lambda gene usage than both healthy controls GO and JB ($p < 0.001$). The J_λ2 gene is decreased in SLE21 (22% vrs. 50%), whereas the J_λ3 gene appear to be overrepresented (61% vrs. 28%) when compared to the two healthy donors.

4.4 No abnormalities in CDR3 length in SLE21 and healthy controls

The average CDR3 length of the antibodies in SLE patient 21 was 13,0 amino acids, similar to the two healthy donors (GO 13,9 aa, JB 12,6 aa; Figure 13). SLE21 shows a higher proportion of short CDR3 (<9aa ~24%) than GO and JB (16%,17%) and does not have CDR3 longer than 20aa. In contrast to SLE patient 21, the two healthy controls both show CDR3s longer than 20aa (~8%, ~2% in GO/JB).

23, 8 % (10) of all antibodies had a CDR3 length of 9 or fewer amino acids. 38, 1 % (16) of the antibodies had 10 up to 14 amino acids and the same percentage (38, 1 %) of antibodies had a CDR3 length between 15-19 amino acids. No antibody had a CDR3 region longer than 20 amino acids.

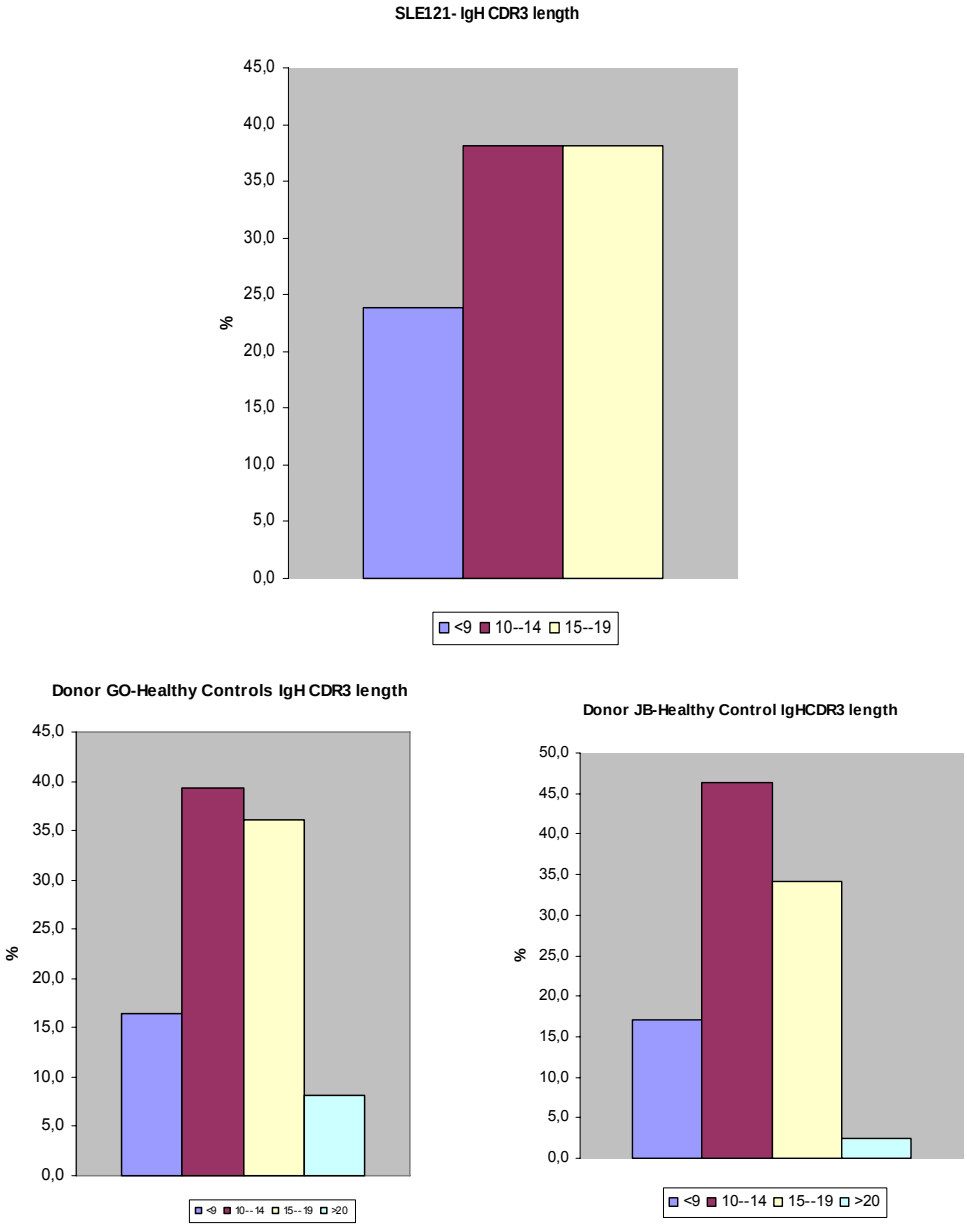
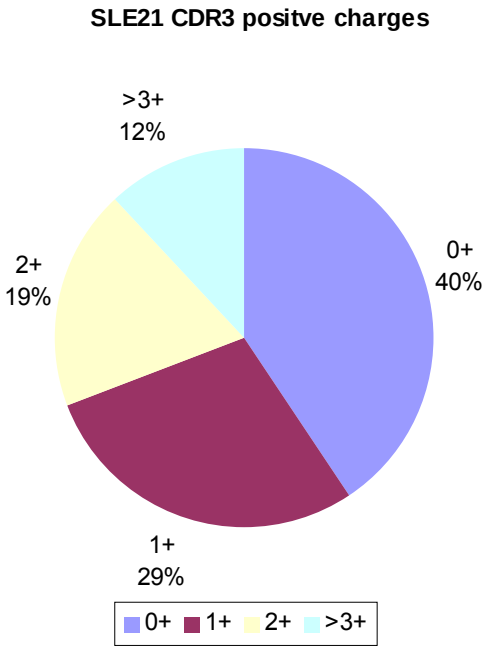
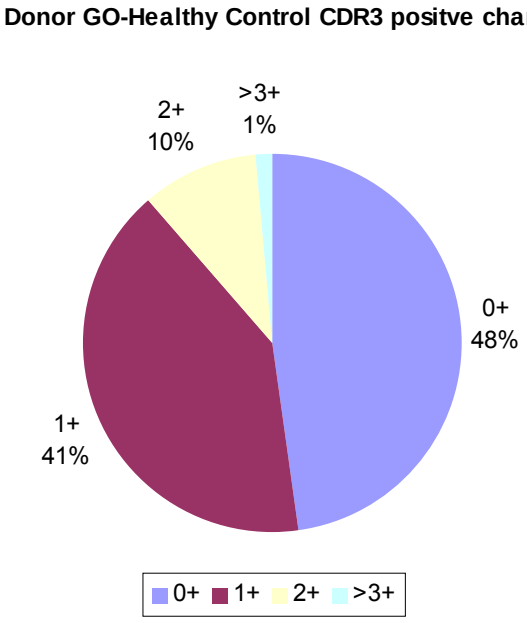


Figure 13a-c)
The CDR3 length of SLE21 (in a) and the Healthy controls GO (b) and JB (c) are shown in these graphs. The CDR3 length of SLE21 does not show significant differences in length compared to the healthy donors (GO p>0,39 vrs. JB p>0,47) with the following deviation; SLE21 uses a higher proportion of short CDR3 (<9aa ~24%) than GO and JB (16% vrs. 17%), but nearly the same proportion of CDR3 (~37%) composed of 15-19aa as GO (~35%). SLE 21 has no CDR3 longer than 20aa in contrast to the healthy controls (aa>20 aa,~8% vrs ~2% in GO/JB).

4.4.1 No differences in the proportion of positive charges within the CDR3 between SLE21 and healthy controls

SLE21 shows more CDR3s with two or three positive charged aa (31% vrs. 11% and 8% in GO/JB) than the 2 healthy controls, and correspondingly fewer CDR3s with a single positive charge (19% vrs 41% and 47% in GO/JB; Figure 14). However, these results were not statistically significant and can be only interpreted as a tendency.





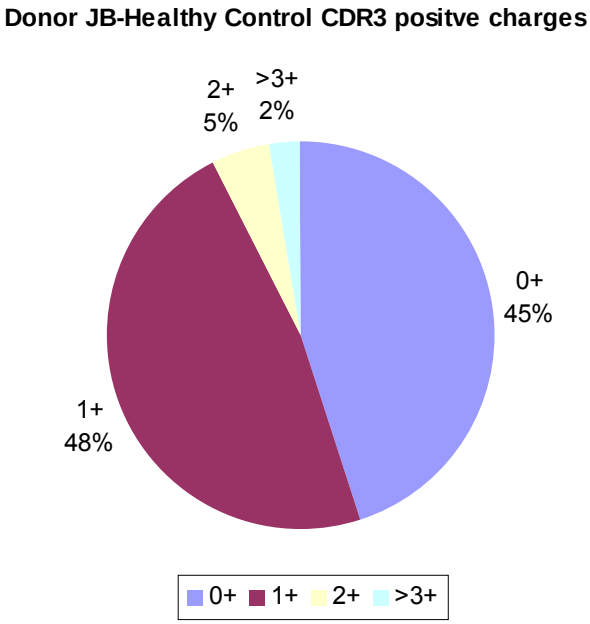


Figure14a-c

These pie charts show the proportions of positive charges within the IgH CDR3 region of SLE21, GO and JB patient. All 3 patients show nearly the same percentage of neutrally charged aa (SLE 40% vrs. 45 in JB and 48% in GO), but SLE21 uses less single positive aa (19% vrs 41% and 47% in GO/JB) and more double and triple positively charged aa (31% vrs. 11% and 8% in GO/JB) within the CDR3 than the two healthy controls.

4.5 Patients chart summary of SLE100, 101, 122 before and after treatment

The next two tables show the Immunoglobulin gene repertoire alterations of three other SLE patients analysed before and after therapy.

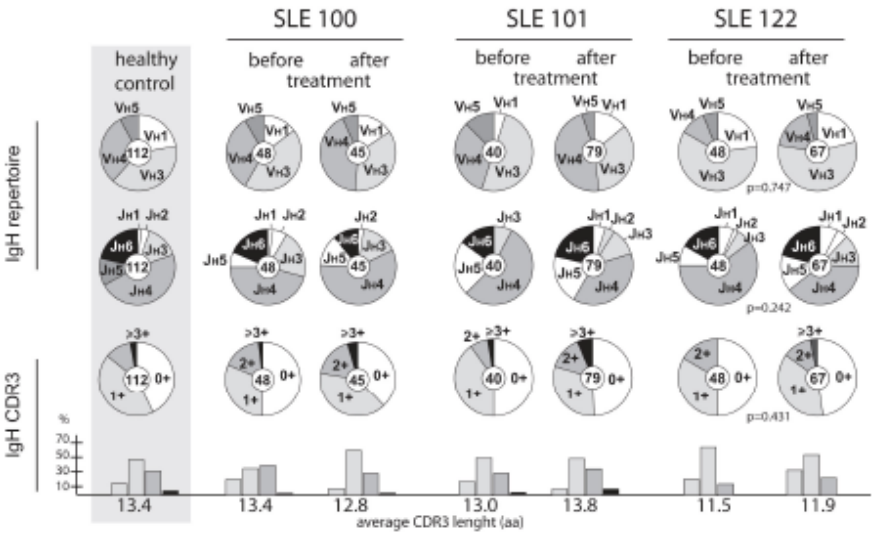


Figure 15. IgH chain gene characteristics These diagrams show the IgH gene repertoire from the mature naïve B cell compartment of three lupus patient (SLE100, 101, 122) before and after medication, the number of positively charged residues within the CDR3 region and the CDR3 length. The results are compared to healthy controls (left). The total number of sequences analyzed is indicated in the center of all pie charts. Pie charts show V_H and J_H gene usage and the proportion of IgH CDR3s with 0, 1, 2 or >3 positive charges. Bar graphs reflect frequencies of IgH CDR3 with 9 or less aa (white), 10-14 aa (light grey), 15-19 aa (dark grey) and 20 or more (black) aa. P-values are indicated for SLE122 (modified from [99]).

Some SLE patients in clinical remission show similar Ig gene repertoire abnormalities as in active disease

The IgH chain repertoire did not change significantly after treatment in SLE100 and SLE122. For SLE101, there was a 2-fold increase in theV_H4 gene segment usage in clinical remission (Figure 15). The V_H3 gene overrepresentation in this patient before therapy normalized in clinical remission, and returned to the level seen in healthy individuals. In SLE122, we observed a decreased V_H4 gene usage before therapy (10, 4 % vs. 30, 4 % in healthy controls) and this gene segment was still underrepresented in clinical remission (17, 1 % vs. 30, 4 % in healthy controls). Likewise, the increase in V_H3 usage for SLE122 before corticosteroid application (60.4 % vs.

39.3 % in controls) did not change after drug application (57, 1 % vs. 39, 3 % in controls). SLE21 displays a similar VH gene repertoire compared to SLE100 and SLE 101 after therapy and only SLE122 showed increased V_H3 gene usage (57, 1 %) in clinical remission (vs. ~38 % for SLE21, 100, 101).

The J_H gene usage features of SLE21 reflect the J_H gene usage characteristics of SLE patient 101 and 122 in that they show a similar decrease in the J_H4 gene in clinical remission. SLE patient 100, who presented with an increase of this gene after therapy, was an exception. There were no other significant JH gene segment usage changes observed between remission and active disease status in all three patients.

CDR3 length and positive charges of SLE patients do not significantly change before and after therapy and show similar features as healthy controls

The contribution of positively charged amino acids to IgH CDR3 was comparable in all 3 patients (SLE100, 101, 122) before and after therapy (Figure 15). There were no alterations as compared to the healthy control group with no positively charged IgH CDR3 in the majority of analyzed antibodies. SLE21 showed no significant alterations in positive residues within the CDR3 region when compared to SLE100,101,122 after treatment.

In SLE100, the average IgH CDR3 length of 13,4 aa seen before treatment dropped to 12,8 aa after therapy; which is similar to the average CDR3 length of SLE21 (13,0). Antibodies analyzed from SLE101 showed an average CDR3 length of 13,0 aa before and 13,8 aa after treatment. For SLE122, IgH CDR3 length was found to be unusual before treatment in that the mature naive B cells displayed short CDR3. The average length was 11,5 aa vs. 13,2 aa in controls ($p=0.003$). No difference in the average CDR3 length was observed after treatment (11, 9 aa).

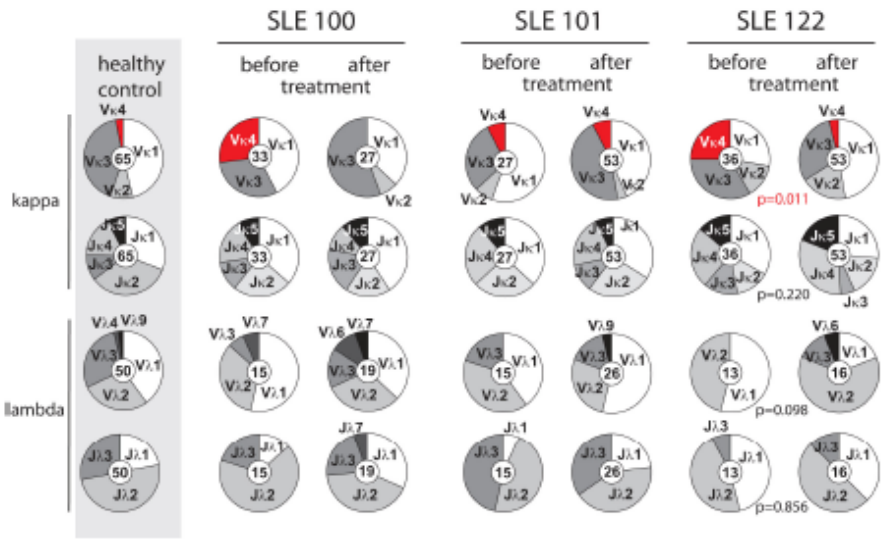


Figure 16. Ig light chain gene sequence features from the mature naïve B cell compartment of SLE100, 101 and 122 before and after treatment The proportions of Vκ, Jκ, Vλ and Jλ gene family usage are indicated in the pie charts and compared to healthy controls. Pie charts depict VL and JL gene family usage for kappa (first row) and lambda (last row) light chains. P-values are shown for SLE122. Vκ4 proportions are indicated in red. The total number of sequences analyzed is indicated in the center of all charts (modified with permission of [99]).

Vκ gene usage changes significantly after therapy

With regard to the light chain repertoire a notable change in Vκ4 gene usage after treatment was observed in SLE100 and SLE122. We observed an overrepresentation of the Vκ4 gene in patient SLE 100 and SLE122 before therapy (Figure 29, red filling). The appearance of the Vκ4 gene segment was 5-6 fold decreased in clinical remission and reached normal gene usage levels when compared with the healthy control group. After therapy, the Vκ4 gene segment representation in all patients (SLE21, SLE100, SLE101, and SLE 122) were comparable to healthy controls. The interpretation of this result remains unclear since no correlation between Vκ4-1 and autoreactivity was found in our study.

No changes were observed for V_λ and J_λ gene usage in SLE patient 100, 101 and 122, except a slight increase of $J_\lambda 1$ gene segments in mature naive B cells from SLE101 (Figure 29).

The results of SLE 21 show similar features to previous studies done by my colleagues. Their findings about SLE patient 100,101 and 122 with respect to Immunoglobulin Heavy and Light chain gene usage after treatment are comparable to SLE patient 21.

In the next section, I will discuss my newly obtained data with the results of these three SLE patients and the two healthy controls, by showing similarities and differences in the Ig gene repertoire and characteristics.

5. Discussion

5.1

In the current work I analysed the Ig gene repertoire of patient SLE21 in clinical remission and compared my findings with the results from three different SLE patients before and after treatment and with the Ig gene repertoire of two healthy controls.

There are no consistent abnormalities and only few molecular differences apparent in the peripheral mature naïve B cell repertoire of SLE21; including the tendency of high distribution of positively charged aa within the CDR3 region. Significant differences in the gene repertoire were only observed in the Ig Light gene loci; the enhanced presence of the Vkappa 4-1 and distal Vλ6 gene as well as the preferential $J_{\lambda}3$ gene usage suggests receptor editing, or secondary light chain rearrangement events were important in shaping the B cell repertoire in patient SLE21 [2, 3, 62, 71, 117, 118]. In other respects, the Ig gene repertoire in SLE patient 21 shows similar results to healthy controls.

Previous studies described Ig gene abnormalities in SLE. A bias towards V_H3, V_H-34 and Vκ1- and Vκ4 gene family usage has been reported to be associated with autoimmune diseases [86-88]. Moreover, a bias towards the V_H5 family and an underreprensention in the V_H1 family has recently been described [24, 89].V Light chain gene repertoire abnormalities are indicated by a greater representation of the Jlambda distal cluster C, Vlambda genes and the Vlambda distal Jlambda7 element in SLE patients [76, 88, 90] that might be derived from enhanced Light chain editing processes during B cell development.

It has been proposed by other groups that Vκ4-1 is associated with anti-DNA antibodies [90-92]. Vκ4-1 is the most downstream Vκ4 gene segment and can be rearranged in secondary gene recombination events. An increased usage of this particular gene has been shown in one untreated patient [93]. Due to secondary gene rearrangement, potential autoreactive antibodies can be silenced through receptor editing. This could be true for SLE patient 21, who used 8 % Vk4 genes in the mature naïve B cells compared to donor GO, who did not show Vκ4 genes in the

repertoire (p<0,001). This finding is consistent with the idea that secondary gene rearrangement might play a role in SLE patients. However, significant Vk4 gene usage is not unique to SLE patients, as healthy control JB also showed V_k4 genes in the repertoire.

Regarding V_λ and J_λ gene usage in SLE21, significant differences could be observed when compared to healthy controls (p<0,001). Unlike in SLE21, the distal$V_\lambda6$ gene family was not used in both of the healthy controls. Furthermore, the clusterA genes ($V_\lambda2$ and $V_\lambda3$) within the IgVL locus appear to be differently used when compared to healthy controls [94].$V_\lambda2$ genes seem to be increased in SLE21 (50% vrs. 28% and 29% in GO/JB). This finding goes consistent with the observation that $V_\lambda2$ genes are overrepresented in SLE patients [94-96]. $V_\lambda3$ gene subgroups seem to be slightly underrepresented in SLE21 (11% vrs. 31% and 24%), which does not match the previous finding where an increase in this gene was observed [94, 96].

With respect to J_λ gene repertoire, the Jλ2 gene is decreased in SLE21 (22% vrs. 50%), whereas the distal Jλ3 gene appear to be overrepresented (61% vrs. 28%) when compared to the healthy donors. The interpretation of these findings are not totally clear, but they might reflect the preferential usage of distally located V lambda genes of cluster C (as Vλ6 in SLE21) located near the Jλ-Cλ cluster in SLE patients, suggesting that there was receptor editing within the V lambda locus [82, 112].

5.2 CDR3 characteristics in SLE patients

The association between autoreactivity and positively charged and long amino acids (>20aa) in heavy chain CDR3 has been defined well [2-4, 7, 8, 18, 19, 81, 97, 98]. It is thought that an antibody with relative richness of positive charges within the antigen binding pocket is more likely to interact with the negatively charged phosphate residues in the DNA backbone.
However, my work did not show all of the associations described in previous studies.
The CDR3 lengths of SLE21 were not significantly different from other SLE patients in clinical remission and healthy donors, but interestingly, my patient displayed a trend towards more positively charged amino acid residues within this region (31% 2+/3+) compared to healthy controls (11% and 8% GO/JB). This would increase the likelihood to bind negatively charged molecules, consistent with the association between positive charges and autoimmunity observed

previously [3]. One patient, SLE 122 showed unusually short CDR3 length, which did not change after treatment; this abnormality was not found in other patients.

Ig gene usage normalities and abnormalities in SLE patients

We found no consistent abnormalities in IgH or IgL gene usage repertoires in SLE patients in clinical remission or with active disease. Although some specific features such as long CDR3s are associated with increased self-reactivity, they are not predictive.

Thus, CDR3 length, positive charges and IgH or IgL gene usage itself is not sufficient enough to predict the reactivity of an antibody molecule. We rather think that this reflects the enormous diversity of immunoglobulins that are important to ensure reactivity against a vast array of pathogens, while avoiding severe autoimmunity.

In terms of auto- and polyreactivity, we found alterations in the mature naïve compartment of SLE patient 100, 101 and 122 before and after treatment. According to previous and recently published studies, poly- and self-reactivity appear to differ in untreated and treated patients [8, 99]. All 3 patients showed high numbers of Hep-2 ELISA reactive antibodies (20, 4 % in controls versus 44, 7 % in SLE100, 50, 0 % in SLE101 and 41, 5 % in SLE122) under untreated conditions. After therapy, the numbers of self reactive antibodies in the mature naïve B cell compartment decreased to levels lower than in those observed in active disease. In SLE101, the frequencies of self reactive antibodies dropped down to the level of healthy controls (SLE101 with 19, 4 % versus 20, 4 % in healthy controls).This finding correlates with the improved clinical status of the patient after treatment. In SLE122, the frequency of Hep-2 reactive antibodies was 31, 2 % and SLE101 displayed 36, 1 % self reactive antibodies. These results, although lower than before treatment, remain above the level of healthy controls.

Consistent with these findings is a recently published study [7] extended to 6 SLE patients compared in clinical remission. Here, they report a persistence of elevated numbers of self reactive and polyreactive circulating antibodies found in the mature naïve B cell compartment, but that the number of B cells expressing these antibodies is lower than in patients with active disease [7]. They conclude that abnormal levels of self reactive mature B cells present in the majority of patients in clinical remission indicate that early checkpoint abnormalities are a fundamental characteristic of SLE.

It is important to mention that these mature naïve B cells are not antibody secreting cells and are therefore not directly involved in the pathogenesis of SLE. And a third checkpoint in the periphery helps to maintain self-tolerance by further selection against selfreactivity during IgM^+ memory B cells development in humans [5]. But keeping in mind that mature naïve B cells can differentiate into memory B cells and plasma cells, they are thought to be precursors of the cells that produce pathogenic lupus antibodies [7]. The pathogenic autoantibodies found in SLE patients are somatically mutated and class- switched immunoglobulins, indicating that T cell self-tolerance is at least partially lost as well [100, 101].

5.3 What effects do immunosuppressive drugs have on B cells?

As shown with SLE 21, Ig gene repertoire abnormalities can be largely reset with immunosuppressive therapy.

The overrepresentation of circulating plasma cells in SLE patient with active disease decreases significantly with treatment [100].

There are several known immune regulatory mechanisms of corticosteroids and other immunosuppressive drugs (e.g.antimalarials) that might influence B cell development and tolerance induction, crucial for avoiding autoimmunity. Some of them are described in the next section.

5.3.1. Effectiveness of Immunosuppressive therapy in SLE

Cytokines in SLE- and the role of the transcription factor NF-κB

Corticosteroids regulate the expression of many genes, with a clear anti-inflammatory effect. First, they reduce the production of inflammatory mediators, including cytokines, prostaglandins, and nitric oxide. Second, they inhibit inflammatory cell migration to sites of inflammation by inhibiting the expression of adhesion molecules. Third, corticosteroids promote the death by apoptosis of leukocytes and lymphocytes.

One of the many known effects of corticosteroids is the inhibition of the NF- κB pathway. The transcription factor NF-κB activates transcription of immunoglobulin-κ light chains in B lymphocytes (Figure 30).

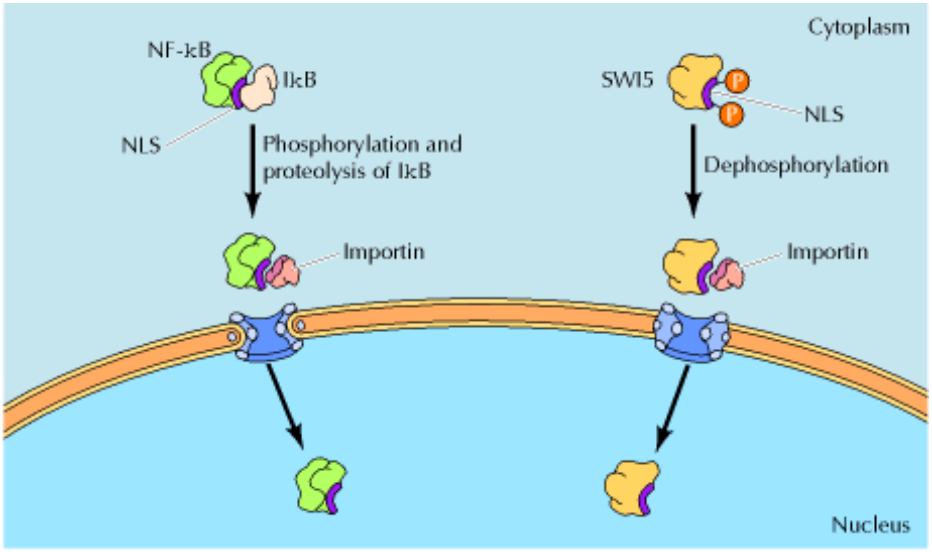


Figure 30. In unstimulated cells (left pathway), NF-κB is maintained as an inactive complex with an inhibitory protein (IκB) in the cytoplasm. Binding to IκB appears to mask the NF-κB nuclear localization signal (NLS), thus preventing NF-κB from being transported into the nucleus. In stimulated cells (right pathway), IκB is phosphorylated and degraded by ubiquitin-mediated proteolysis, allowing NF-κB to enter the nucleus and activate transcription of its target genes (given by [17]).

NF- κB plays a crucial role in transcriptional regulation of many cytokine genes (such as TNF-α, IFN-γ, IL-3, IL-4, IL-6, IL-8, IL-10) in lymphocytes. IL-4, IL-6 and IL-10 are the major cytokines that determine the Th2 immune response, leading to humoral immunity [25]. Thus, corticosteroids alter the cytokine milieu, affecting B cell development and B cell tolerance as well as the direction of the cytokine-dependant lymphocyte development (Th1 versus Th2 and antibody production, respectively). Autoimmune diseases can be generally classified into two major groups according to their effector mechanisms; Insulin dependent diabetes mellitus (IDDM), for instance, is mainly driven by pathogenic T cells, whereas the other major group of autoimmune disorders is caused by defective B cell selection and function and the production of autoantibodies. This is thought to be the case for SLE pathogenesis, although some defect in T cell tolerance is clearly present as well, given that the pathologic antibodies are class-switched and hypermutated – features of mature B cells that require T cell help.

As mentioned above, corticosteroids decrease the expression of adhesion molecules and thus reduce the emigration of lymphocytes from blood vessels into body tissue. These immunosuppressive drugs do not only reduce the expression of vascular addressins on the surface of vascular endothelial cells, they also downregulate their leukocyte counterparts, namely the

selectins (L-selectin, CD62L) and integrins (leukocyte function- associated antigen-1 ,LFA-1) found on the surface of naive and effector T cells, macrophages and neutrophils that enable them to exit the blood and enter into peripheral lymphoid tissue. This leads to reduced emigration of lymphocytes from the blood vessels into peripheral tissue, and thus reduces the degree of inflammation and tissue damage.

In SLE, B cells display an increased expression of co-stimulatory molecules like CD40 or molecules derived from the B7 family (B7.1 and 2 isoforms of CD80, CD86) [25, 69, 82]. The increased production and secretion of survival and differentiation factors like the TNF family members B cell activating factor of the TNF family (BAFF) and its homolog A proliferation-inducing ligand (APRIL) may account for abnormal B cell proliferation in SLE [103-107]. These two factors seem to be important during B cell development as they provide survival factors crucial for further development, maturation and activation of B lymphocytes. It has been shown that a selective blockage of BAFF via BAFF- receptor Ig, or a non- selective blockage via transmembrane activator or calcium modulator ligand interactor- Ig, which blocks both BAFF and APRIL in a murine model, lead to prolonged survival in NZB/W F1 mice when either given before or after disease onset [83, 102, 104, 106, 107]. Selective BAFF blockage further decreases the production of B cells and reduces the progressive T cell activation and dendritic cell accumulation that occurs with age in NZB/W mice. In addition, the blockage of both survival factors leads to decreased serum IgM levels, reduces the frequency of plasma cells in the spleen, and inhibits the IgM response to T cell dependent antigen in this model [102]. Human lupus patients also show elevated BAFF blood levels suggesting treatment with BAFF neutralizing agents such as monoclonal antibodies or decoy receptors should delay or slow down the disease [83, 102]. It has been proposed that corticosteroids, together with other immunosuppressive drugs might interfere with BAFF and APRIL production in a way that leads to a decreased serum level in a murine SLE model, but the exact mechanism still remains elusive [103].

Antimalarial drugs' (chloroquine, hydroxychloroquine) most important mechanism appears to be interference with normal physiologic function of subcellular compartments which depend upon an acid milieu [108]. Antimalarials are weak bases which not only enter lysosomes, but all acidic compartments, where they are protonated, raise the pH, and interfere with functions dependent upon an acidic pH [109]. It has also been suggested that decreased secretion of

monocyte-derived proinflammatory cytokines [110] may be due to non-lysosomotrophic effects of antimalarials. Such effects are seen in a decrease in secretion of tumor necrosis factor alpha (TNF-alpha) by stimulated human peripheral blood derived mononuclear cells in response to chloroquine. This was associated with decreased amounts of TNF-messenger RNA [111]. Secondly, Cytosine phosphate diester-guanine (CpG) DNA stimulates cells through toll-like receptor (TLR)-9, which is found in lysosomes. Chloroquines may block costimulation of the B cell antigen receptor and TLR9 pathways and thus act as an antiinflammatory agent[112]. Furthermore antimalarials can inhibit activation of intracellular TLR-3, 7, and 9, and thus may block activation of the interaction of nucleic acids and TLR [113]. Further secondary effects on immune function are decreased productions of cytokines and other inflammatory mediators, decreased lymphocyte proliferation and possibly alteration of autoantibody production. Antimalarials may influence the binding of autoantigenic peptides to MHC class II molecules, thereby interfering with antigen processing and ultimately the immune response to autoantigens [114]. Antiinflammatory effects may include inhibition of phospholipases, antagonization of prostaglandin, stabilization of lysosomal membranes, decreased fibronectin release by macrophages, blocking of superoxide release, and decreasing metalloproteinase production by synoviocytes [108, 114, 115]. With regard to lupus patients these drugs also block ultraviolet light absorption, an effect which may protect against lupus skin lesions [116].

New treatment in SLE

Another promising therapy in SLE is B cell depletion with anti-CD 20 antibodies (rituximab, epratuzimab). It has been shown that administration of rituximab intravenously is followed by profound depletion of B cells from peripheral blood to levels of less than 5 B cells/ μl^3 within one week of a single dose. Long term B cell depletion up to 2 years have been reported in many patients [79], although relapses of autoimmunity may still occur.

It has been suggested that the fundamental value of B cell depletion may be less for any downregulation of autoantibody production than for the suppression of the immunoregulatory and inflammatory roles that B cells play within the pathogenesis of SLE [79, 82, 104, 107]. And although rituximab's mechanisms of action are incompletely understood, the effects of rituximab are likely mediated by antibody-dependent cell-mediated cytotoxicity and the induction of

apoptosis. The resultant repopulation of B cells, alteration of abnormal B cell homeostasis and down-regulation of co stimulatory molecules on both B and T cells and the downregulation of inflammatory cytokines like INF-γ all likely contribute to the clinical efficacy of anti-CD20 antibodies.

Finally, it needs to be asked what immunosuppressive drugs do to the B cell repertoire. In autoimmunity, we can hope that the pathological B cell repertoire is targeted and ablated by anti-CD20 antibodies and other immunosuppressive drugs.

Since B cell precursors develop in the bone marrow from CD20 cells throughout life, and central selection happen at two checkpoints in the bone marrow, one could predict that B cell repertoire should return towards the normal state when B cell numbers return.

6. Conclusion

Former studies show that B cell abnormalities in SLE patients before treatment are reversible and can be reset to a large extent, indicating that immunosuppressive drugs can largely reset defective central tolerance. How corticosteroids, antimalarial and cytotoxic drugs might fix defective tolerance checkpoints is not completely understood yet and further investigations are required to answer these questions. As seen in SLE 100,101 and 122 frequencies of autoreactive B cells of the mature naïve compartment in patients in clinical remission normalized and were comparable to healthy controls. SLE21 in clinical remission did not show major Ig gene abnormalities as seen in patients with active disease. Significant differences were only present in the Ig Light chain gene usage, in that distal V_L and distal J_L genes were preferentially used; this characteristic, which is suggestive of receptor editing, has been previously described in SLE patients [94, 119].

A newly published SLE study [7] shows that the majority of patients in clinical remission continued to have persistent low level abnormalities in antibody tolerance in their mature naïve B cell compartments, suggesting that early checkpoint defects are partly independent of active disease and a fundamental characteristic of SLE. These findings are consistent with the data of SLE21, where persistent minor Ig gene abnormalities in clinical remission were found when compared to healthy controls. However, since the pathogenic antibodies are produced not by mature naïve IgM^+ B cells but by class-switched IgG^+ B cells, the persistence of low-level

abnormalities in the mature naïve B cell compartment of patients in clinical remission is not inconsistent with a partial restoration of a normal B cell repertoire by treatment. As the treatments these patients received (including steroids, chloroquine) have many effects on different cell types, including later stages of development than mature naïve B cells, a comprehensive study must examine antigen-experienced B cells as well, including the IgG^+ B cells that produce the pathogenic antibodies in SLE.

There are no datas of SLE21 before therapy.

7. References

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8.Appendix

8.1
Primer list

IgH	Sense		Antisense	
First PCR	5´ L-VH 1 5´ L-VH 3 5´ L-VH 4/6 5´ L-VH 5	ACAGGTGCCCACTCCCAGGTGCAG AAGGTGTCCAGTGTGARGTGCAG CCCAGATGGGTCCTGTCCCAGGTGCAG CAAGGAGTCTGTTCCGAGGTGCAG	3´ Cμ CH1	GGGAATTCTCACAGGAGACGA
Second PCR	5´ AgeI VH 1 5´ AgeI VH 1/5 5´ AgeI VH 3 5´ AgeI VH 4 5´ AgeI VH 3-23 5´ AgeI VH 4-34	CTGCAACCGGTGTACATTCCCAGGTGCAGCTGGTGCAG CTGCAACCGGTGTACATTCCGAGGTGCAGCTGGTGCAG CTGCAACCGGTGTACATTCTGAGGTGCAGCTGGTGGAG CTGCAACCGGTGTACATTCCCAGGTGCAGCTGCAGGAG CTGCAACCGGTGTACATTCTGAGGTGCAGCTGTTGGAG CTGCAACCGGTGTACATTCCCAGGTGCAGCTACAGCAGTG	3´ SalI JH 1/2 3´ SalI JH 3 3´ SalI JH 4/5 3´ SalI JH 6	TGCGAAGTCGACGCCTGAGGAGACGGTGACCAG TGCGAAGTCGACGCTGAAGAGACGGTGACCATTG TGCGAAGTCGACGCTGAGGAGACGGTGACCAG TGCGAAGTCGACGCTGAGGAGACGGTGACCGTG
Igκ	Sense		Antisense	
First PCR	5´ L Vκ 1/2 5´ L Vκ3 5´ L Vκ4	ATGAGGSTCCCYGCTCAGCTGCTGG CTCTTCCTCCTGCTACTCTGGCTCCCAG ATTTCTCTGTTGCTCTGGATCTCTG	3´ Cκ 543	GTTTCTCGTAGTCTGCTTTGCTCA
Second PCR	5´Pan Vκ	ATGACCCAGWCTCCABYCWCCCTG	3´ Cκ 494	GTGCTGTCCTTGCTGTCCTGCT
Specific PCR	5´ AgeI Vκ 1-5 5´ AgeI Vκ 1-9 5´ AgeI Vκ 1D-43 5´ AgeI Vκ 2-24 5´ AgeI Vκ 2-28 5´ AgeI Vκ 3-11 5´ AgeI Vκ 3-15 5´ AgeI Vκ 3-20 5´ AgeI Vκ 4-1	CTGCAACCGGTGTACATTCTGACATCCAGATGACCCAGTC TTGTGCTGCAACCGGTGTACATTCAGACATCCAGTTGACCCAGTCT CTGCAACCGGTGTACATTGTGCCATCCGGATGACCCAGTC CTGCAACCGGTGTACATGGGGATATTGTGATGACCCAGAC CTGCAACCGGTGTACATGGGGATATTGTGATGACTCAGTC TTGTGCTGCAACCGGTGTACATTCAGAAATTC CTGCAACCGGTGTACATTCAGAAATAGTGATGACGCAGT TTGTGCTGCAACCGGTGTACATTCAGAAATTGTGTTGACGCAGTCT CTGCAACCGGTGTACATTCGGACATCGTGATGACCCAGTC	3´ BsiWI Jκ 1/4 3´ BsiWI Jκ 2 3´ BsiWI Jκ 3 3´ BsiWI Jκ 5	GCCACCGTACGTTTGATYTCCACCTTGGTC GCCACCGTACGTTTGATCTCCAGCTTGGTC GCCACCGTACGTTTGATATCCACTTTGGTC GCCACCGTACGTTTAATCTCCAGTCGTGTC
Igλ	Sense		Antisense	
First PCR	5´ L Vλ 1 5´ L Vλ 2 5´ L Vλ 3 5´ L Vλ 4/5 5´ L Vλ 6 5´ L Vλ 7 5´ L Vλ 8	GGTCCTGGGCCCAGTCTGTGCTG GGTCCTGGGCCCAGTCTGCCCTG GCTCTGTGACCTCCCTATGAGCTG GGTCTCTCTCSCAGCYTGTGCTG GTTCTTGGGCCAATTTTATGCTG GGTCCAATTCYCAGGCTGTGGTG GAGTGGATTCTCAGACTGTGGTG	3´ Cλ	CACCAGTGTGGCCTTGTTGGCTTG
Second PCR	5´ AgeI Vλ 1 5´ AgeI Vλ 2 5´ AgeI Vλ 3 5´ AgeI Vλ 4/5 5´ AgeI Vλ 6 5´ AgeI Vλ 7/8	CTGCCCTACCGGTTCCTGGGCCCAGTCTGTGCTGACKCAG CTGCTACCGGTTCCTGGGCCCAGTCTGCCCTGACTCAG CTGCTACCGGTTCTGTGACCTCCTATGAGCTGACWCAG CTGCTACCGGTTCTCTCTCSCAGCYTGTGCTGACTCA CTGCTACCGGTTCTTGGGCCAATTTTATGCTGACTCAG CTGCTACCGGTTCCAATTCYCAGRCTGTGGTGACYCAG	3´ XhoI Cλ	CTCCTCACTCGAGGGYGGGAACAGAGTG

Table1: Oligonucleotides used as primers in single cell PCR
Restriction sides are shown in red

samples	HEAVY				
mSLE21	VH	D	JH	CDR3 (aa)	Length
mSLE21 68	4-4	/	2	ASQGYFDL	8
mSLE21 70	4-34	2-2	3	GWYCSSTSCYGDAFDI	16
mSLE21 75	3-23	7-27	4	PPGGSPAFFDT	11
mSLE21 76	4-28	/	4	RTYYDSSGYSFGNT	14
mSLE21 77	4-34	/	4	GRGLRWQNQENDY	13
mSLE21 79	3-15	3-22	4	EDSKSHYYDSSGYQDY	15
mSLE21 81	3-7	/	4	DLRAVVVITGSGYYFDY	8
mSLE21 89	4-34	2-8	4	DSGTMVYADFDY	12
mSLE21 92	1-69	/	1	DREDYYDSSGTTHHG	15
mSLE21 94	3-48	/	3	LSRDDAEDI	9
mSLE21 102	3-30	/	4	ASFRSFDT	8
mSLE21 105	3-20	4-17	6	VLGGSGTGMDD	11
mSLE21 111	3-48	4-23	6	VRGYYGMDD	9
mSLE21 139	5-51	/	3	QRRGAFDI	8
mSLE21 141	4-39	6-19	1	ELPYSSGWVRRVAEYFQH	19
mSLE21 144	4-31	2-2	3	FVVVAAFDI	9
mSLE21 153	3-33	/	6	DELHQGIYYYGMDD	14
mSLE21 155	4-59	1-26	2	SVGATSRDFDL	11
mSLE21 162	4-61	5-18	3	DTVDTAMAPDAFDI	14
mSLE21 164	3-7	2-8	6	VDVLDSLRVDDDDGMDV	17
mSLE21 167	5-51	6-6	3	QKDQLAKEDAF	13
mSLE21 173	1-2	2-2	5	VGDFIAAAGSKGKFDP	15
mSLE21 186	1-18	/	4	DPQLGDFDY	9
mSLE21 188	3-7	2-2	6	VFEYCSSTSCFDYYYGMDV	19
mSLE21 193	4-34	1-26	3	SWELLSPDAFDI	12
mSLE21	VH	D	JH	CDR3 (aa)	Length
mSLE21 59	1-69	/	6	GLTKRPHYYYYMDV	14
mSLE21 64	3-15	4-17	4	DRIGRGPELAATETYKVDY	19
mSLE21 78	5-51	1-26	4	HSEGVGATPTFDTDY	15
mSLE21 82	4-34	/	4	RRRAKIIDY	9
mSLE21 86	3-23	6-13	1	DRNRIIAAAGTAEYFQH	17
mSLE21 87	4-34	3-16	4	VGSYPVDTAMVPI	13
mSLE21 107	1-2	/	5	GGSTLTNWFDP	11
mSLE21 108	3-23	3-22	3	YYYDSSPNGNDAFDI	15
mSLE21 126	1-3	/	6	NGQLLFGGNYYYYMDV	16
mSLE21 154	4-31	6-31	5	GAEQQLDNNWFDP	13
mSLE21 156	3-33	2-2	2	stop	-
mSLE21 158	3-7	1-26	5	ATGSYYRIRGNWDP	17
mSLE21 168	4-28	4-17	4	ANPPVDY	7
mSLE21 174	1-2	2-2	5	GGARQLLWTLANWFDP	16
mSLE21 183	3-48	/	3	APRFGSWFASDAFDI	15
mSLE21 190	4-34	6-6	4	RIAARPGNFDY	11
mSLE21 192	3-23	3-3	5	DLNHAYYDFWSGYHA	15
mSLE21 194	1-24	3-10	5	EGLLLWFLGGFDP	13

Table3. Repertoire of Ig heavy chain gene usage, CDR3 lengths and positive and negative charges (blue/red) are indicated

LIGHT				samples
Vk	Jk	CDR3 (aa)	Length	mSLE22
1-5	1	QQYNSYWT	8	mSLE21 68
1-39	1	QQSYSTLRT	9	mSLE21 70
2-28	2	MQALQYPLDF	9	mSLE21 75
1-9	3	QQLNSYPLS	9	mSLE21 76
1-5???	1	QQY?DL?WPP?T	9	mSLE21 77
3-11	2	QQRSNWPPYT	10	mSLE21 79
3-15	1	QQYNNWPPLGT	11	mSLE21 81
1-39	2	QQSYSTLLYT	10	mSLE21 89
3-20	5	QQYGSSPSR	9	mSLE21 92
3-11	4	QQRSNWLT	8	mSLE21 94
3-20	1	QQYGSSPWT	9	mSLE21 102
1-39	1	QQSYSTLRT	9	mSLE21 105
1-16	3	QQYNSYPLT	9	mSLE21 111
1-6	1	LQDYNYPRT	9	mSLE21 139
3-15	1	QQYNNWPPLGT	11	mSLE21 141
2-40	2	MQRPESRVLRR	12	mSLE21 144
3-20	4	QQYSGSSLT	8	mSLE21 153
4-1	4	QQYYSTLLT	8	mSLE21 155
3-11	3	QQRSNWPPV	9	mSLE21 162
3-11	4	QQRSNWPRGLT	11	mSLE21 164
3-15	2	QQYNNWPTT	9	mSLE21 167
3-15	4	QQYNNWPLT	9	mSLE21 173
1-5	2	QQYNSYT	7	mSLE21 186
1-39	1	QQTYSTPWT	9	mSLE21 188
4-1	2	QQYYSTLYT	9	mSLE21 193
VL	Jl	CDR3 (aa)	Length	mSLE22
2-14	2	SSYTSSSTLVV	11	mSLE21 59
3-22	3	LSGDLDNPAV	10	mSLE21 64
3-21	3	QSYDSSLSGVY	11	mSLE21 78
1-51	3	GTWDSSLSAGV	11	mSLE21 82
1-51	3	GTWDSSLSVWV	11	mSLE21 86
6-57	3	QSYDSSN?????	7	mSLE21 87
2-11	1	CSYAGSYTSYV	11	mSLE21 107
1-47	3	AAWDDSLSGWV	11	mSLE21 108
1-44	3	AAWDDSLNGPV	11	mSLE21 126
2-14	3	CYYSCCCSWV	10	mSLE21 154
2-11	3	CSYAGSYYPG	10	mSLE21 156
1-40	2	QSYDSSLSGVV	11	mSLE21 158
2-14	3	SSYTSSSTWV	10	mSLE21 168
2-14	1	SSYSSSTLNYV	12	mSLE21 174
1-47	2	AAWDDSLSGPCVV	13	mSLE21 183
2-14	2	SSYTSSSTLVV	11	mSLE21 190
2-23	1	CCSYAGYWNV	10	mSLE21 192
2-23	3	CSYAGSCSRV	10	mSLE21 194

Table3. Repertoire of Ig light chain gene usage, CDR3 lengths and positive and negative charges (blue/red) are indicated

8.3

Patient information

SLE21

Sample Date	7/21/2005
Clinical Feature	Clinical remission
Gender	Female
Age at diagnosis	17 years
Treatment	Initially IV steroids, stopped 5 years ago, continued on PL
Haematology	normal
$CD19^+$ cells $x10^3/ml$	145

	SLE 100		SLE101		SLE122	
Sample Date	**10/10/02**	**02/21/04**	**10/24/02**	**03/03/04**	**02/22/03**	**09/14/04**
Clinical Feature	**arthritis**	**remission**	**myocarditis**	**remission**	**chorea**	**remission**
Gender	**Female**		**Male**		**Female**	
Age at diagnosis	**15 years**		**15years**		**9 years**	
Family History	**Maternal SLE**		**IDMM, parental MS**		**-**	
Treatment	**-**	**PDN 5mg/d MPiv,HCQ, ASA**	**-**	**CPH, MPiv, MMF,HCQ,ASA**	**-**	**PDN 10mg/d, MPiv, HCQ**
Serology	**D,P, Sm**	**D**	**P**	**NL**	**D**	**D**
ANA	**+**	**+**	**+**	**+**	**+**	**+**
Haematology	**L,A,T**	**NL**	**A**	**NL**	**L,A**	**NL**
C3/C4	**low**	**low**	**low**	**NL**	**low**	**NL**

Table1. Patient characteristics

A, anaemia; ASA; Acetyl salicylic acid, D, anti-dsDNA antibody; HCQ; hydrochloroquine, IDMM; Insulin dependent diabetes mellitus, MMF; Mycophenolate mofetil, MP;Methylprednisolone, MS;

Multiple Sclerosis, leukopenia; P, antiphospholipid antibody; PL, Plaquenil; PND; Prednisolone, RNP, anti-RNP antibody, SLE; Systemic lupus erythematosus, Sm; anti-Smith antibody, T; thrombocytopenia,

8.3

Patient information

SLE patient 100

This patient is a15 years old African-American girl who was diagnosed in September 2002. She presented with arthritis and nephritis and generalized lymphadenopathy. Her past medical history is uneventful. The family history is positive with one SLE case on her mother's side.

Complete blood count (CBC) on admission:
White Blood Cells (WBC): 1.9x106/ml (normal 4 to 9x106/ml);
neutrophils: 78%
Lymphocytes: 17%
Eosinophils: 1%
Basophils: 1%
Monocytes: 3%
hemoglobin: 9.3 g/dl
platelets, 133x106/ml
Reticulocytes, 0.8%.

Urine analysis: moderate blood with 10–25 RBC/hpf and 2+ protein. Protein in 24h urine: 3.7 g.
Renal biopsy: lupus nephritis WHO class IIb
Serology:
ANA by immunofluorescence on HEp-2 slides: >1:2,560 (normal<1:40)
anti-dsDNA antibodies: 961 IU/ml (normal <25)
anticardiolipin antibodies, IgM, 36 IU/ml (normal <10)
IgG, 37 IU/ml (normal <11)
antiphosphatidylserine/inositol, negative
anti-Smith, positive
C3 complement: 30 mg/dl (normal: 70 – 206 mg/dl)
C4 complement, 2 mg/dl (normal: 11 – 61 mg/dl)

The 15 year old patient was treated with intravenous methylprednisolone pulses, oral hydroxychloroquine, and prophylactic doses of aspirin. Her clinical status improved during the course of treatment with a resolution of the arthritis, lymphadenopathy, and

pancytopenia. However, she continued to present minimal proteinuria and hematuria with normal renal function during 2 years of follow-up.

8.3

SLE patient 101

A 15 years old Caucasian boy who was diagnosed in October 2002 with cardiomyopathy, nephritis, coagulopathy, and hemolytic anemia. His Past medical history was unremarkable. In his family a maternal IDDM and a paternal MS case is reported.

CBC on admission:
WBC, 10.9x106/ml (normal 4 to 9x106/ml);
Neutrophils: 65%;
Lymphocytes: 19%
Eosinophils: 5%
Monocytes: 11%
hemoglobin: 5.8 g/dl
platelets: 171x106/ml
Reticulocytes: 4%
Urine analysis: blood with 5 RBC/hpf, 2+ protein, protein in 24 h: 1.5 g
Renal biopsy, lupus nephritis WHO class III.
Serology:
ANA <1:640 (normal <1:40)
anti-dsDNA antibodies, 53 IU/ml (normal <25)
anticardiolipin antibodies, IgM 15.2IU/ml (normal <10)
IgG 65 IU/ml (normal <11)
IgA: 16 (normal <13)
antiphosphatidylserine, IgM 32.5 IU/ml (normal <20)
IgG 74 IU/ml (normal <10)
IgA 36.3 mg/dl (normal <6)
anti-Smith, negative
C3 complement, 95 mg/dl (normal: 70–206 mg/dl)
C4 complement: 6mg/dl (normal: 11–61 mg/dl)

Patient was treated with iv pulses of methylprednisolone and cyclophosphamide as well as oral hydroxycloroquine and prophylactic doses of aspirin During the next 2 years, the patient's clinical status significantly improved with resolution of the cardiomyopathy, coagulopathy, anemia, and proteinuria. The renal function remained within normal limits.

8.3

SLE patient 122

A 9 years old Hispanic girl who was diagnosed in February 2004 with weight loss, nephritis, and chorea as an isolated neurological manifestation. Her past medical history was normal. So was her family history.

CBC on admission:
WBC, 4.1x106/ml (normal 4 to 9 x106/ml)
Neutrophils: 77%
Lymphocytes: 22%
Monocytes: 1%
hemoglobin: 11.2 g/dl
platelets, 155x106/ml
Infectious disease work- up (including ASO/anti-DNaseB): negative.
Ceruloplasmin, copper, zinc levels: normal.
Urine analysis: blood with 3–5 RBC/hpf. Protein in 24h urine: 670 mg
Renal biopsy: lupus nephritis WHO class IIb.
Serology:
ANA, 1:1,280 (normal <1:40)
anti-dsDNA antibodies, <500 IU/ml (normal <25)
anticardiolipin antibodies, negative
anti-Smith: negative
antiphosphatidylserine/inositol, negative
C3 complement, 66mg/dl (normal: 70–206 mg/dl)
C4 complement, 7 mg/dl (normal: 11–61 mg/dl)

This 9 year old patient was treated with iv methylprednisolone pulses and oral hydroxychloroquine.
Over the course of the observation time, the patient's clinical condition notably improved with resolution of the chorea, hematuria, and proteinuria.

8.5

Table 4: Ig gene Repertoire from mature naive Bcells of donor GO-Healthy control
antibodies could that not be expressed,RF reading frame

Ig	HEAVY						LIGHT			
	VH	D	RF	JH	CDR3 (aa)	Length	Vκ	Jκ	CDR3 (aa)	Length
m-GO2	4-39	3-22	2	4	HVYYYDSSGYYPQYFDY	17	3-15	1	QQYNNWPWT	9
m-GO4	3-7	/	/	6	DKGGIYYYYYGMDV	14	2D-28	1	MQALQTPRT	9
m-GO6	4-59	5-12	2	6	AGYDKATYYYYGSDV	15	3-20	1	QQYGSSLPWT	10
m-GO13	3-43	/	/	3	DRLGFGPGLGNAFDI	15	1-5	1	QQYNSYSRT	9
m-GO15	3-53	4-23	2	4	EADYGGNSGLFNY	13	1-5	2	QQYNSFQYT	9
m-GO21	3-15	1-26	1	1	ELH	3	1-5	4	QQYNSYPLT	9
m-GO25	1-24	1-26	2	6	VSRGGSYPYGSDV	13	1-39	5	QQSYSTPIT	9
m-GO26	3-48	6-13	3	5	VPIAAAPNWFDP	12	3-11	5	QQRSNWPPIT	10
m-GO29κ	4-39	3-9	2	3	GGYYDILTGYYTEPRDDASKGTFDI	25	3-15	4	QQYNNWLT	8
m-GO40	4-34	6-19	2	6	GTYSSGWPDYYYYGMDV	17	3-20	2	QQYGSSPPCT	10
m-GO43	4-34	/	/	6	GRISWAFNRHGMDV	14	3-20	3	QQYGSSPPIT	10
m-GO50#	3-43	6-6	3	3	DTIAAAFDI	9	1-5	5	QQYNSYSSIT	10
m-GO53	4-39	2-15	2	4	FGYCSGGSCLPFDY	14	3-15	1	QQYNNWPRGT	10
m-GO56	3-15	2-2	3	6	DPPPRIVVVPAPGDYYYYYGMDV	23	3-20	2	QQYGSSSYT	9
m-GO62	1-3	5-5	2	4	DFSLGYSYGLQNPTNFDY	18	1-39	4	QQSYSTPLT	9
m-GO64	3-30	3-22	2	4	DLSYYYDSSGLDY	13	1-39	2	QQSYSTPHT	9
m-GO66	3-48	3-22	2	4	SPGYYYDSRGIFDY	14	1-39	1	QQSYSTQWT	9
m-GO73	3-48	1-26	2	6	DNSGSYFPANYYYYYGMDV	19	1-9	2	QQLNSYPHT	9
m-GO74	1-69	5-5	3	6	AGDTAAPPTSTYYYYGMDV	19	2-30	4	MQGTHWSLT	9
m-GO75	1-18	3-10	3	3	EPLPMVRGVSVAFDI	15	1-33	2	QQYDNLPYT	9
m-GO79	3-30	4-23	3	3	TTVVTVVDAFDI	12	1-9	4	QQLNSYPLT	9
m-GO85	3-21	/	/	6	DPYGMDV	7	3-11	2	QQRSNWPPYT	10
m-GO86	4-34	4-23	2	4	GPHDYGAFPNDY	12	3-20	5	QQYGSSPRIT	10
m-GO88	3-23	/	/	6	EARSGATGSLYYYYGMDV	18	2D-28	1	MQALQTPRT	9
m-GO116#	1-24	5-24	3	4	FLTIVDY	7	3-11	1	QQRSNWPLT	9
m-GO119	3-49	3-22	2	4	HYYDSPFDY	9	3-20	1	QQYGSSPT	8
m-GO121#	5-51	5-18	2	4	SYRGYSYDPGY	11	2D-28	3	MQALQTLFT	9
m-GO126	1-46	3-10	2	4	VGGSGSYYNRYFDY	14	1-39	1	QQSYSTPWT	9
m-GO132	4-59	3-22	2	3	NSYYYDSSGYFDAFDI	16	1-39	1	QQSYSTPQT	9
m-GO136#	1-69	5-18	3	4	TNTAMVTSFFDY	12	1-39	2	QQSYSTPRT	9

8.5

	VH	D	RF	JH	CDR3 (aa)	Length	Vκ	Jκ	CDR3 (aa)	Length
m-GO139	3-15	4-23	2	4	GKRDYGGNSFDY	12	1-33	4	QQYDNLLV	8
m-GO154#	4-34	3-16	2	5	VAHPGGYVWGSYRYNWFDP	19	3-15	1	QQYNNWPPWT	10
m-GO161	3-48	6-19	3	4	ESGVAVAGTFADY	13	1-39	1	QQSYSTSWT	9
m-GO162	1-69	/	/	6	NLAASGGYYGMDV	13	1-39	2	QQSYSTPYT	9
m-GO166	4-39	/	/	4	WVGETDY	7	3-15	1	QQYNNWPPT	9
m-GO175κ#	3-74	2-21	3	4	VTATLDY	7	1D-37	3	QRTYNALT	8
m-GO180#	3-23	3-10	1	4	DWFGELTMEGTFDY	14	1-39	2	QQSYSTLRT	9
m-GO186	1-46	4-17	3	4	GGMTTVTTGFDY	12	1-9	4	QQLNSYPLT	9
m-GO188	3-23	/	/	4	KDTSPVGTRGADY	12	3-15	2	QQYNNWPMYT	10
m-GO191	3-15	3-16	1	3	VELRLGELSLLAI	13	1-39	2	QQSYSTLYT	9
m-GO193	3-48	6-19	2	4	VDSSGWYGY	9	3-15	4	QQYNNWPLT	9
	VH	D	RF	JH	CDR3 (aa)	Length	Vλ	Jλ	CDR3 (aa)	Length
m-GO1#	1-24	1-26	2	4	GGDSGSEGFVDY	12	1-44	3	AAWDDSLNGREV	12
m-GO3	4-28	4-17	2	4	DRYGFYGDYDGGDY	14	1-44	3	AAWDDSLNGRV	11
m-GO16	4-34	3-10	3	4	GRPAITMVRGVMNRDDY	17	2-14	1	SSYTSSSTGV	10
m-GO19	3-74	3-9	1	6	DRYFDWLLAGMDV	13	3-21	2	QVWDSSSDHGV	11
m-GO20	5-51	3-22	2	2	GYYDVWYFDL	10	3-21	1	SSYTSSSTHV	10
m-GO28	3-23	3-22	3	4	DRSHSITMIVVAPWED	16	3-1	2	QAWDSSTGVV	10
m-GO29λ							3-21	3	QVWDSSSDRV	10
m-GO49	3-15	1-26	1	4	DLLRWELLDY	10	1-44	2	AAWDDSLNGVV	11
m-GO65	4-39	6-13	1	2	SASQQLVWGWWYFDL	15	2-14	1	SSYTSSSTLYV	11
m-GO67	3-48	2-21	3	6	RGIVVVGEGYYYYYGRDV	18	2-14	2	SSYTSSSTVV	10
m-GO76	1-69	5-18	1	6	DLRIQLWSTKLPDYYYYYGMDV	22	2-14	1	SSYTSSSTHNYV	12
m-GO77	4-39	6-13	3	1	HSAAAPPPNWYFNL	14	1-40	2	QSFDSSPSGYVV	12
m-GO87#	5-51	6-19	2	3	KGSSGWYEEPGAFHI	15	3-25	2	QSADSSGTYDVV	12
m-GO91#	3-64	6-19	2	4	GGSSGRWRILDY	12	3-21	2	QVWDSSSDVV	10
m-GO102	4-28	/	/	4	CGVVVKVAYFDL	12	1-36	3	ATRDDSLNGWV	11
m-GO103#	4-59	6-13	2	5	GPISSSWAPNTLRQIDNWFDP	21	3-1	2	QAWDSSTVV	9
m-GO113	1-2	3-10	1	6	ELWFGEVRNGMDV	13	1-40	2	QSYDSSLTEGVV	12
m-GO115	5-51	2-15	2	5	LQRPNHCSGGSCYWFDP	17	1-40	3	QSYDSSLSAWV	11
m-GO130#	3-7	4-17	2	4	DSYDYGDPL	9	3-21	3	QVWDSSSDPWV	11
m-GO140#	1-69	/	/	5	DGGGTFRPNWFDP	13	1-51	2	GTWDSSLSAVV	11
m-GO142#	3-9	1-26	3	4	SFIVGDPGGFDC	12	2-14	1	SSYTSSSTPYV	11
	VH	D	RF	JH	CDR3 (aa)	Length	Vλ	Jλ	CDR3 (aa)	Length
m-GO152	3-9	1-26	2	4	DIGRGSYWGMDY	12	1-44	1	SSYTSSSTPYV	11
m-GO156#	3-33	/	/	4	DQAKLGLDY	9	2-14	2	SSYTSSSTPYV	9
m-GO157	5-51	3-10	1	6	HVGFGEFSQTDYYYYGMDV	19	1-36	2	SSYTSSSTPYV	11
m-GO163	4-34	2-21	3	4	NPPGHVVVTASPNFDY	16	2-23	3	SSYTSSSTPYV	11
m-GO165	3-30	1-26	2	6	DKSPNSGSYLLTYGMDV	17	3-21	2	SSYTSSSTPYV	10
m-GO167	1-69	2-21	3	5	VSLVTAEDWDNWFDP	15	1-40	2	SSYTSSSTPYV	10
m-GO173	3-43	2-21	3	4	DGVVVVTATYFDY	13	1-51	2	SSYTSSSTPYV	10
m-GO175λ#							3-27	3	SSYTSSSTPYV	10
m-GO176	3-11	3-10	1	4	DEGWFGELSCDY	12	1-47	1	SSYTSSSTPYV	11
m-GO187	4-34	2-2	2	6	EDAYCSSTSCYGGYYYGMDV	20	2-8	3	SSYTSSSTPYV	10
m-GO189	1-8	3-22	2	3	SYYDSSGYYSPTFFDI	16	2-14	2	SSYTSSSTPYV	10

8.5

Table 5: Repertoire of the mature naive B cell compartment of donor JB- Healthy control
antibodies could that not be expressed,RF reading frame

Ig	HEAVY						LIGHT			
	VH	D	RF	JH	CDR3 (aa)	Length	Vk	Jk	CDR3 (aa)	Length
m-JB 2	1-2	1-20	2	4	DNWNDAYYFDY	11	4-1	1	QQYYSTPPT	9
m-JB 4	4-34	/	/	4	GLLGDEWSSPLPFDY	15	3-20	2	QQYGSSPDT	9
m-JB 6	1-18	3-10	1	6	NLLLWFGELGYNGMDV	16	3-20	3	QQYGSSLFT	9
m-JB 9	3-30	/	/	4	EHNWAGEIFDY	11	1-39	1	QQSYSTPPT	9
m-JB 10#	4-39	3-10	1	4	LIAAGEFDY	9	1-39	2	QQSYSTPYT	9
m-JB 30#	4-59	4-17	3	2	DGGAATVTDYWYFDL	15	4-1	2	QQYYTTPHT	9
m-JB 39κ	4-39	1-26	1	4	VFNRMWELSPLYYFDY	16	3-15	4	QQYNNWPSLT	10
m-JB 40	4-39	3-22	2	4	NYYDRPFDY	9	3-20	3	QQYASSGF	8
m-JB 41	1-24	3-3	2	5	GLGTYYDFWSGYP	13	2-30	2	MQGTHWPYS	9
m-JB 49	1-2	/	/	5	ATSDWPRRKGEFDP	14	3-20	1	QQYGSSHRT	9
m-JB 52	3-9	6-6		5	DGIAARPVVPWFDP	14	1-16	2	QQYNSYPYT	9
m-JB 57	4-59	6-19	1	4	LEQWLATFDY	10	3-20	4	QQYGSQVT	8
m-JB 59	4-31	3-9	1	4	DFDY	4	2-28	1	MQALQTPRT	9
m-JB 61#	4-59	/	/	5	ELLSGRWFDP	10	1-16	1	QQYNSYPRT	9
m-JB 63	4-39	5-5	1	4	GEEWGIQLWLRLGYDY	16	3-20	2	QQYGSSHS	8
m-JB 65#	3-53	3-22	2	3	GNGYYDSSGYWQRWDAFDI	19	1-17	3	LQHNSYPFT	9
m-JB 73	3-48	4-23	2	6	LFRGGATPYYYYGMDV	16	3-11	4	QQRSNWPLT	9
m-JB 74	3-48	3-22	3	6	GHMIVVVIWTDPGMDV	16	3-20	2	QQYGSSPPYS	10
m-JB 76	4-59	4-17	2	2	DRHPYGDYGYWYFDL	15	3-20	5	QQYGIPT	7
m-JB 77	5-51	2-2	3	6	HVWSVVPAAQDPYYYYGMDV	20	1-39	2	QQSYSTPHT	9
m-JB 81	3-9	1-26	3	4	DVVGATTAFDY	11	1-5	1	QQYNSLGT	8
m-JB 86	4-39	5-24	3	4	DQEPLEMAAVGGVDY	15	3-15	2	QQYNNWPLYT	10
m-JB 87	3-30	1-20	3	4	DPGTTGFFDY	10	1-9	3	QQLNSYPPFT	10
m-JB 89	4-34	5-12	2	4	VGGYSGYARGGY	12	1-39	2	QQSYSTPRT	9
	VH	D	RF	JH	CDR3 (aa)	Length	Vλ	Jλ	CDR3 (aa)	Length
m-JB 8	1-69	5-5	2	6	NQRYSYGYGYYYYGMDV	17	2-8	1	STYAGSNNL	9
m-JB 12	5-51	3-22	2	4	GLLGSGYLDY	10	1-44	2	AAWDDSVVV	9
m-JB 17	3-30	4-23	2	4	HDYGGDY	7	9-49	3	GADHGSGSNFAWV	13
m-JB 25	5-51	3-10	2	3	YYRYDAFDI	9	1-44	2	AAWDDSLNVV	10
m-JB 29	4-39	2-15	2	4	DIIYHSEPCYFDY	13	3-1	1	QAWDSSTFYV	10

Table 4-5: (copied from www.sciencemag.org/cgi/content/full/301/5638/1371/DC1)

B lymphocyte compartments
Reactivity profiles

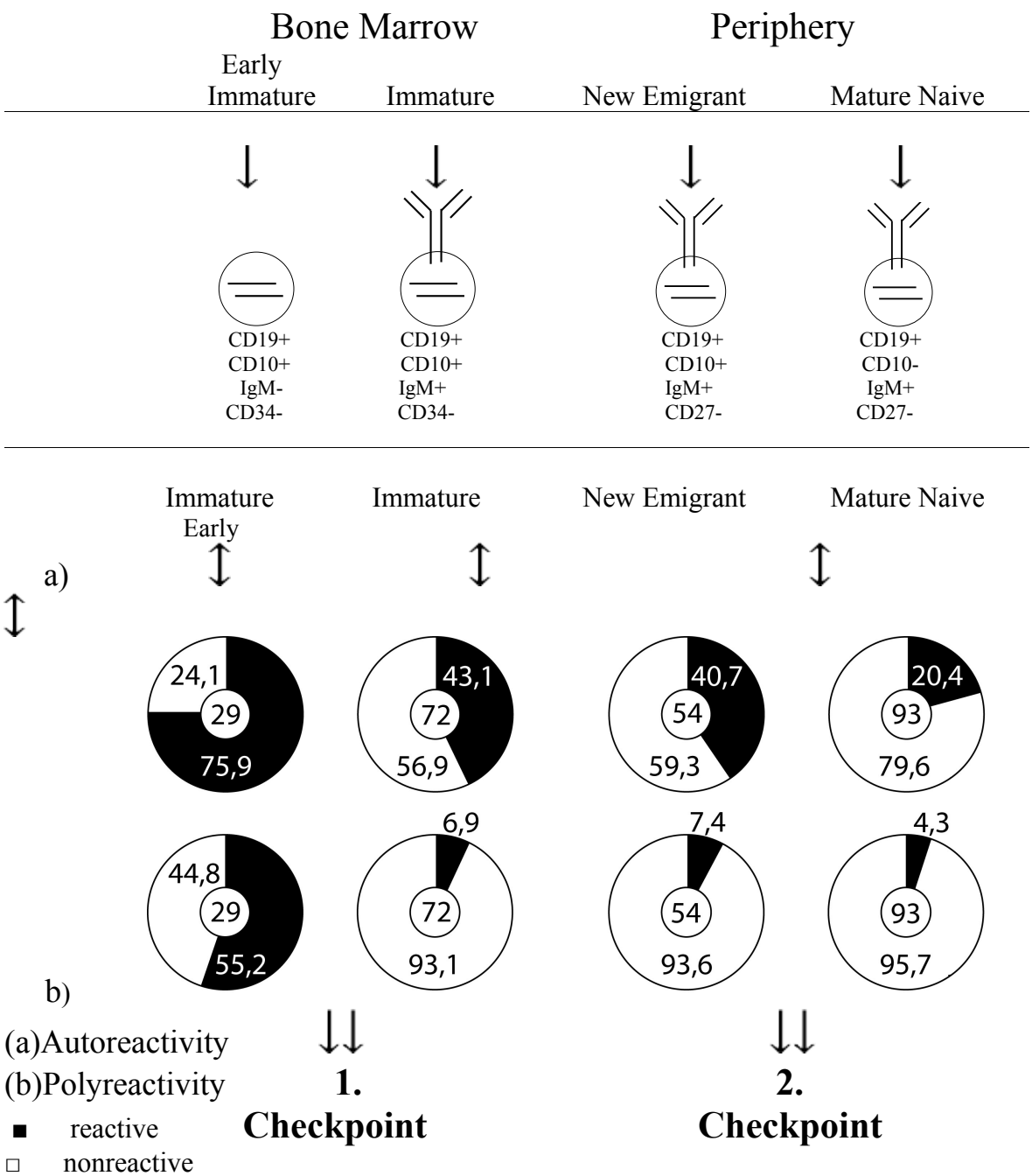

Figure 2. 4 B cell compartments (Early Immature, Immature, New Emigrant, Mature Naïve) during B lymphocyte development are shown. Early Immature and Immature B cells develop and stay in the Bone Marrow whereas New Emigrant and Mature Naïve B lymphocytes are released in the Periphery after successful Ig gene rearrangement (upper table). The black lines within the B cells outline the Ig gene transcripts, which are assembled to a functional receptor on the surface of the B cells in compartment 2-4 (upper table). Several surface marker shown in the table define B cell stages.

The lower table shows B cell tolerance checkpoints during early B cell developing stages in the BM and the periphery in healthy donors. The frequency (%) of self- and polyreactive antibodies within 4 B cell compartment are shown in *Pie charts*. The Pie chart center illustrates the total number of antibodies analyzed for each B cell fraction. Polyreactive (b) antibodies are efficiently counterselected at the first B cell tolerance checkpoint in the BM. Self-reactive (a) antibodies are eliminated at two of the checkpoints: in the BM and the Periphery (modified from [4,8]).

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Declaration

I, Lisa Freude, hereby declare that I completed the submitted thesis work entitled
'Ig gene repertoire analysis in an SLE patient in clinical remission' and that I did not use any other sources or materials than indicated in the references. All sources are listed and appropriately acknowledged in the references.
This work was not written with the aid of a third party and also does not include parts or copies of other dissertations.