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DISSERTATION

Effects of immunosuppressive drugs and CD4+ T-cell depletion on plasma cell survival in lupus prone (NZB/W) mice

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1 Introduction

1.1 History

Immunology is the science of protective mechanisms used by the body to protect it against foreign environmental agents, including infectious pathogens. The earliest reported observation about this protective phenomenon was made in late 18th century by Edward Jenner, who found that inoculating individuals with pus from a cowpox lesion secures them from subsequent exposures to smallpox. This concept of 'immunization' was expanded almost a century later by Louis Pasteur and his associates who studied the prevention of various infectious diseases in animals and humans. Around the same time, Emil Adolph von Behring and Shibasaburo Kitasato made a monumental discovery that the serum of tetanus-immune rabbit possesses properties capable of destroying tetanus toxins, and that these toxin neutralizing substances - *'antitoxins'* could be passively transferred to other animals by means of serum transfusion. Paul Ehrlich, who also worked closely with von Behring, contributed greatly to the better understanding of these serum '*antitoxins*', their transfer to fetus *in utero* and to new born through milk. He hypothesized that these antitoxins represent '*side-chains*' that are produced and pushed off from the protoplasm and so come to exist in a free state. The elegant work of Emil Adolph von Behring and Paul Ehrlich led to the development of a concept and later to the discovery of antibodies.

1.2 Immunity

The term immunity refers to all defense mechanisms used by the body to protect it against foreign environmental agents. The immune system is a highly complex system that enables the discrimination between 'self' and 'nonself' body elements, and mounts an immune response against the 'nonself' elements. Immunity is divided into two major categories: ***innate or natural immunity*** and ***adaptive or acquired immunity***.

1.2.1 Innate immunity

Innate immunity is constituted by factors that mediate an immune response in a relatively nonspecific manner to any foreign substance that manages to break the integrity of the body. The cellular components of innate immunity include phagocytes (granulocytes and monocyte-macrophages), dendritic cells and natural killer cells (NK cells). The macrophages and dendritic cells, in addition to phagocytosis, also function as antigen presenting cells by

processing antigens intracellularly and presenting them to T lymphocytes. The NK cells recognize unique glycoproteins on cell membranes following viral infection or oncogenic transformation and release cytotoxic molecules that cause lysis or apoptosis of target cells. The elements of innate immunity also include physical or chemical barriers between the host and potential pathogen, for instance - skin, mucous membranes, low pH and enzymatic activity of secretions along gastrointestinal and pulmonary tract. The elements of innate immunity together are generally sufficient to contain low grade pathogenic infections.

Toll-like receptors (TLRs) are pattern recognition receptors that recognize microbial pathogens. Belonging to innate immune system, they also activate the adaptive immune system through nuclear factor-κB (NF-κB) [1,2]. These receptors also have potential to recognize self antigens like RNA and DNA and can consequently trigger autoimmune diseases such as lupus and rheumatoid arthritis. Stimulation of TLR4 and TLR9 on B-cells by their ligands lipopolysaccharide and CpG respectively, is sufficient to induce expression of transcription factors that cause B-cells to differentiate into plasma cells [3,4]. SLE patients have elevated levels of circulating plasma DNA which is enriched in hypomethylated CpGs (TLR9 ligand) [5]. DNA containing immune complexes in the serum of a lupus patient can also stimulate pDCs to produce cytokines and chemokines in TLR9 dependent manner [6]. Components of adaptive immunity can thus be stimulated through TLRs, a component of innate immunity, leading to pathogenesis of autoimmune diseases.

1.2.2 Adaptive immunity

In contrast to innate immunity, which is present in every living organism, adaptive immunity has developed late in evolution and is found only in vertebrates. The hallmarks of adaptive immunity are its antigen specificity and memory. They discriminate between different antigenic epitopes and respond to those for which they bear surface receptors (specificity). They also have the unique ability to remember previous challenges with a particular pathogen and mount a more rapid and larger immune response to a subsequent rechallenge with the same pathogen (memory). The elements of adaptive immunity could in turn be divided into two main categories: *Humoral immunity* and *Cell-mediated immunity*. Humoral immunity is mediated by B lymphocytes and antibodies synthesized by plasma cells (terminally differentiated B lymphocytes). Humoral immunity plays an important role in containing bacterial infections and neutralization of bacterial toxins. Cell-mediated immunity on the other hand is constituted by T-cells, mainly concerned with containing viral infections, tumors, graft rejections and delayed type hypersensitivity reactions.

1.3 B-cell ontogeny

The B lymphocytes can be divided into two independent lineages: *B-1* and *B-2* or conventional B-cells. *B1* B-cells are found mainly in peritoneum and pleural cavities. They produce mainly 'natural' IgM antibodies that recognize T-independent type 2 (TI-2), bacterial and self antigens with low affinity [7]. The predominant lineage of conventional *B2* cells, found in blood and lymphoid organs, arises from pluripotent hematopoietic stem cells of adult bone marrow and require the transcription factor – paired box protein 5 (PAX5) [8]. The earliest distinguishable cells of B lineage are from *pro-B-cell*, *pre-B-cell* and *late pre-B-cell* stages. During these stages, heavy immunoglobulin chain genes are rearranged and μ heavy chains are translocated to the cell surface in conjunction with 'surrogate' light chains. This is followed by rearrangement of genes coding for light chain and synthesis of either kappa or lambda light chain. These light chains are then associated with μ chain to form monomeric IgM molecules which are then expressed on cell surface. This stage of *immature B-cells* is the first to express B-cell receptor (BCR) on its surface. Though BCR is itself an antibody of particular antigenic specificity, B-cells at these stages are not activated and do not secrete antibodies. Moreover, a vast majority of antibodies (BCRs) expressed by these immature B-cells are self-reactive, possessing polyreactive and anti-nuclear specificities [9]. However, antigen contact with self-reactive B-cells at this stage results in receptor edition or deletion (through negative selection) rather than expansion and differentiation [10-13]. Since these stages of B-cell maturation occur in the absence of foreign antigen (in the bone marrow), tolerance to foreign antigens is avoided.

The non-reactive immature B-cells exit the bone marrow as *transitional 1 (T1) B-cells* and reach the spleen for further maturation [14]. Here, the maturation of T1 cells progresses through stages *transitional 2 (T2)* and *transitional 3 (T3) B-cells* as they finally develop into immunocompetent and fully *mature B-cells.* The mature B-cells in spleens are present as circulating IgD^{hi} IgM^{lo} *follicular B-cells* (predominant fraction) and noncirculating resident IgD^{lo} IgM^{hi} *marginal zone cells*. The follicular B-cells keep circulating in the spleen, lymph nodes and bone marrow until they find their cognate antigen and undergo further maturation or die by apoptosis. It should be noted that before maturation, all B-cells undergo two rounds of negative selection to avoid having autoreactive cells in circulation: 1) in the bone marrow at immature B-cell stage 2) in spleen at transitional B-cell stage [13].

When a mature B-cell encounters its cognate antigen in secondary lymphoid organs is gets activated. This leads to its proliferation and differentiation in either of these two pathways: antibody secreting plasmablasts or memory B-cells. While plasmablasts begin antibody synthesis and secretion immediately (discussed later), memory B-cells do not secrete antibodies. Memory B-cells are non-proliferating, generally long-lived B-cells capable of activating and mounting a much stronger immune response upon subsequent challenge by the same antigen. They possess somatically hypermutated BCRs and persist independent of antigenic stimulation [15,16]. Upon antigenic rechallenge, they react quickly by differentiating into plasmablasts that secrete class switched antibodies of high affinity [17].

1.4 B-cell/T-cell interactions

B-cells are efficient antigen presenting cells. They can process the antigen bound to its surface receptor by internalizing it, degrading it into small peptides, mounting them on MHC II molecules and expressing them on their cell surface. In the case of T-dependant antigens, the B cells after antigen processing meet with CD4+ T-cells specific for the same antigen at the boundary of B-cell and T-cell zone. The T-cell receptor (TCR) binds to the peptide on MHC II molecule of the B-cell. This is followed by binding of CD4 molecule directly to MHC class II molecule. Additional costimulatory molecular interactions required for completion of T-cell/B-cell activation include interaction of B7.1 (CD80), B7.2 (CD86) and CD40 on B-cell with CD28, CTLA-4 and CD40Ligand (CD40L) on T-cells respectively [18,19]. Appropriate stimulation of follicular B-cells by CD4+ T-helper cells result in formation of *germinal centers*. Germinal centers are histologically discrete structures in B-cell follicles where B-cells proliferate massively and their antibody encoding genes undergo somatic hypermutation, clonal selection and class switch recombination [20]. Therefore, memory B-cells and plasma cells exiting germinal center reaction express antibodies that are affinity maturated (a germinal center hallmark) and class switched. Germinal centers develop during the first week of immune response and usually persist for a further three to four weeks. Transcription factors like B-cell lymphoma-6 (BCL-6) and activation induced cytidine deaminase (AID) are important in germinal center formation and functions [21,22]. Germinal center reactions are crucial in the formation of bone marrow long-lived plasma cells [23].

1.5 Plasma cells

Plasma cells are the terminally differentiated cells of B-lineage. They play the central role in humoral immunity by producing protective antibodies. Initially, they were perceived as short-lived cells that undergo apoptosis within few days and persisting antigens were thought to drive their differentiation from activated B-cells [24]. However, evidence has emerged over the last decade that plasma cells can also survive for much longer periods without proliferation, even in the absence of persisting antigens. It has now been established that these long-lived plasma cells secrete and maintain stable levels of antigen-specific antibodies in serum [25,26]. Based on their life span, plasma cells can therefore be classified into two distinct categories: a) *short-lived plasmablasts/plasma cells* (proliferating blasts/non-proliferating short-lived plasma cells) – both having a life span of 3-5 days [27] and b) *long-lived plasma cells* (non-proliferating) with a life span of months to years [28,29]. The term - *antibody secreting cells* (ASCs) – refers to both short-lived and long-lived plasma cells.

1.5.1 Origin of plasma cells

Antibody secreting plasma cells arise from both activated mature B-cells (in marginal zones and B-cell follicles of secondary lymphoid organs) and memory B-cells after antigen encounter (Fig.1). Due to their location, non-circulating marginal zone B-cells are probably the first cells to encounter blood-borne antigens, which respond mainly to T-independent antigens and lipopolysaccharides [30]. Similarly, circulating follicular B-cells in ‘extrafollicular foci’ may also get activated in a T-independent manner by their cognate antigen. Upon activation, these naïve B-cells undergo plasmacytic differentiation into *plasmablasts* (proliferating plasma cells with short life span) and subsequently into *short-lived plasma cells* (non-proliferating plasma cells with short life span) which rapidly undergo apoptosis [4,31] (Fig.1). However, when follicular B-cells are activated in context of T-cell help, germinal center formation occurs [20], as a consequence of which large numbers of plasmablasts and memory B-cells are produced [32]. Both plasmablasts and memory B-cells exiting germinal center reactions have undergone class switch reactions and somatic hypermutations, and therefore produce class switched antibodies with high affinities for their cognate antigen [20]. Upon subsequent antigenic re-challenge, memory B-cells can also differentiate into plasmablasts which contain somatic hypermutations and produce high affinity antibodies. Most of the plasmablasts/short-lived plasma cells arising from germinal center reactions and from activated memory B-cells have short life spans and undergo apoptosis. However, some of these high-affinity plasmablasts enter blood circulation and are rescued from apoptosis in

bone marrow (and to lesser extent in secondary lymphoid organs and inflamed tissues), where appropriate survival niches are present (details in the following sections). In such an environment, these plasmablasts mature into *non-proliferating long-lived plasma cells* which have a life span of several months to several years [29] (Fig.1). The non-proliferating long-lived plasma cells have immense significance in the maintenance of antibody memory *(also referred to as plasma cell memory)* for tackling subsequent antigenic challenges.

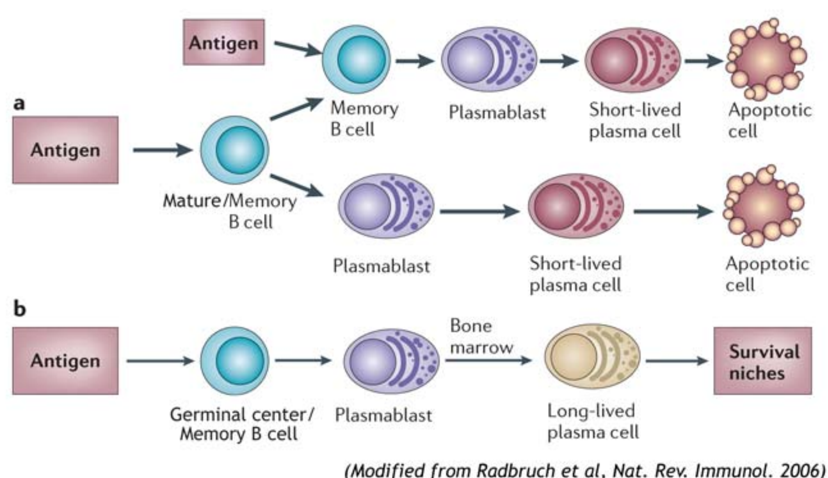

Figure 1: Differentiation of activated B-cells into short- and long-lived plasma cells.

a) Proliferating plasmablasts arising from activated mature and memory B-cells differentiate into short-lived plasma cells which undergo apoptosis after a very short period.

b) A few plasmablasts arising from germinal center reactions and memory B-cells become long-lived plasma cells which survive without proliferation for several months to years, provided they receive appropriate survival signals in bone marrow niches.

1.5.2 Regulation of plasma cell development

Transcription factors crucial in plasma cell differentiation include B-lymphocyte induced maturation protein-1 (Blimp-1), X-box-protein-1 (XBP-1) and interferon-regulatory factor 4 (IRF4) [4]. During the initial stages of B-cell maturation, transcription factors Pax5, BCL-6 and MITF (all important in commitment to and maintenance of B-cells) repress plasma cell differentiation by inhibiting Blimp-1, XBP-1 and IRF4. Blimp-1, the most important transcription factor in plasma cell differentiation, in turn represses Pax5 and BCL-6 [33,34]. In addition to plasmacytic differentiation, Blimp-1 and XBP-1 are also required for antibody secretion [35]. Additionally, XBP-1 induces unfolded-protein response in plasma cells, and thus

rescues the plasma cells from endoplasmic reticulum stress induced apoptosis (caused by massive antibody production) [36]. IRF-4 is needed for initial proliferative burst of activated B-cells. Many cytokines, in particular IL-6 and IL-21, are known to contribute greatly to Blimp-1 expression and plasma cell differentiation [37,38].

1.5.3 Plasmablast chemokinesis

Plasmablasts, the proliferating precursors of long-lived plasma cells, are prone to apoptosis [39]. However, these cells can be rescued by appropriate survival signals provided by niches that are located mainly in bone marrow, and to a lesser extent in the spleen and inflamed tissues (Fig.2). Owing to their short life span, plasmablasts have only a few days within which they should reach these survival niches, or else go into apoptosis [29]. Plasmablasts have the capability to migrate under the influence of various chemokine signals *(a property they lose upon differentiation into long-lived plasma cells)* [40,41]. They express the chemokine receptor - CXCR4, which mediates their migration towards CXCL12 expressing bone marrow stromal cells [42,43]. Plasmablasts deficient in CXCR4 have an impaired ability to home to the bone marrow [44]. Moreover, the proinflammatory cytokine - IFNγ upregulates chemokine receptor - CXCR3 on plasmablasts and its ligands CXCL9, CXCL10 and CXCL11 in inflamed tissues [45]. Therefore, under inflammatory conditions, plasmablasts can also migrate to inflamed tissues which serve as a favorable environment for plasma cell survival. Plasma cells have been found to be present in inflamed kidneys of mice and patients with SLE, and in synovium of patients with rheumatoid arthritis. Additionally, plasmablasts expressing chemokine receptors CCR9 and CCR10 migrate towards CCL25 and CCL28, respectively, which are expressed in the mucosal tissues [41], indicating the role of these chemokines in mucosal immunity.

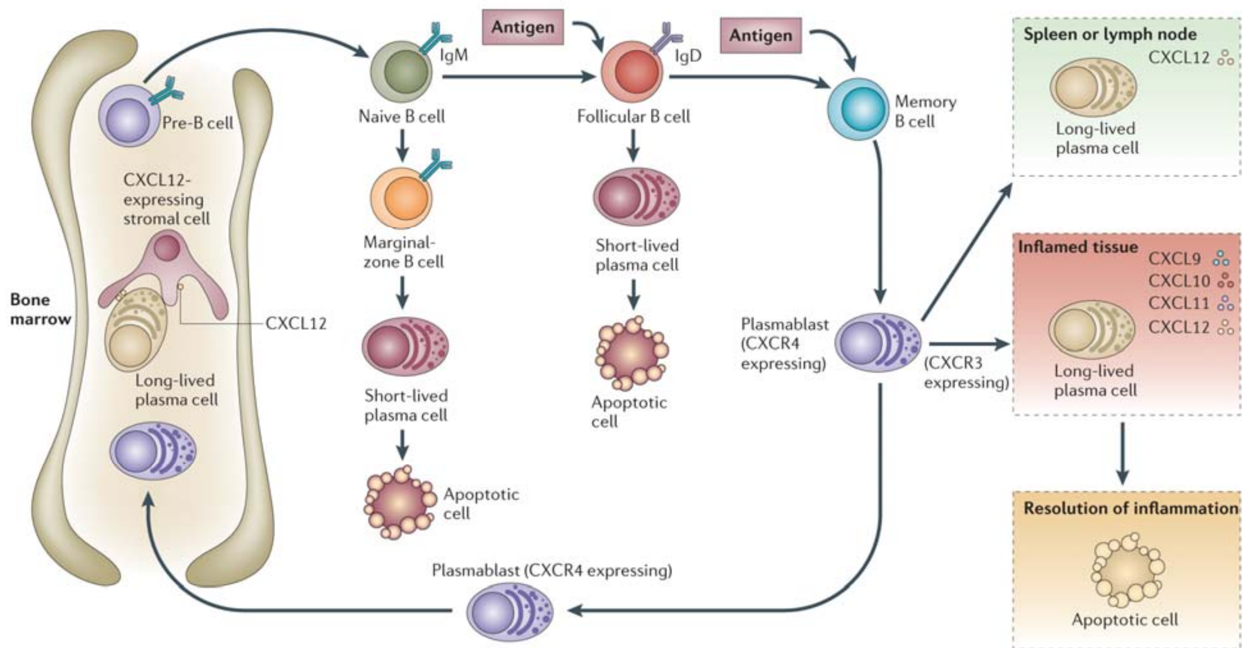

Figure 2: Plasma cell ontogeny and chemokinesis. *Naïve B-cells (marginal zone and follicular B-cells) upon activation by antigen differentiate into plasmablasts (not shown) and short-lived plasma cells, both of which undergo apoptosis rapidly. Alternatively, activation of follicular B-cells in the presence of T-cell help can result in germinal center reaction and formation of memory B-cells and plasmablasts. Memory B-cells, when activated by antigenic rechallenge can differentiate into plasmablasts. Most of these plasmablasts migrate towards CXCL12 expressing bone marrow (which bind CXCR4 on plasmablasts) and mature into long-lived plasma cells. A small portion of plasmablasts remain in the secondary lymphoid organs. Also, some CXCR3 expressing plasmablasts migrate to CXCL9, 10 and 11 expressing inflamed tissues. However, upon resolution of inflammation, plasma cells would cease to survive in inflamed tissues.*

The migratory behaviour of plasmablasts has been extensively studied in mice. The antigenic challenge and differentiation of B-cells into plasmablasts occurs in the secondary lymphoid organs. After secondary immunizations, there is a proliferative burst in the spleen, with formation of plasmablasts which reaches its peak around day 5 [46]. However, one to two weeks post immunization (depending on antigen), the antigen-specific plasma cell numbers in this organ decline significantly, coming back to levels just slightly higher than that prior to secondary immunization. This decline is associated with a parallel increase of antigen-specific plasma cell numbers in bone marrow, indicating their migration to this organ [46]. While the majority of long-lived plasma cells, under physiologic conditions, reside in bone marrow [26,46], considerable numbers of these cells can also be present in activated spleens during autoimmunity [39].

1.5.4 Long-lived plasma cells and plasma cell memory

To explain plasma cell memory (*i.e.* production and maintenance of antibodies of a given specificity in serum for a long period), three independent concepts were proposed:

a) Antibody production by short-lived plasmablasts that are continuously generated from memory B-cells as a result of persistent antigen mediated activation [24].
b) Antibodies produced by long-lived plasma cells of defined half-life, generated continuously from memory B-cells activated via cytokine receptors and Toll-like receptors (bystander activation) [48].
c) Antibodies produced by long-lived plasma cells that survive several months up to several years in appropriate survival niches in bone marrow [46,49].

The third concept, *i.e.* antibody production by the bone marrow long-lived plasma cells, is the most accepted mechanism for plasma cell memory maintenance. This was convincingly proven in experiments where complete depletion of CD20+ B-cells (which are also immediate precursors of plasmablasts) over prolonged periods did not affect antibody levels in the serum, proving that these antibodies are produced by CD20- long-lived plasma cells [50,51]. Bone marrow was already reported in 1980s as the major site of long-term antibody production [52]. In line with this, *Manz et al.* demonstrated that two months after secondary immunization, 90% of antigen-specific plasma cells in the bone marrow are long-lived and produce antibodies in the absence of antigen stimulation [46]. Similarly, *Slifka et al.* have demonstrated that these bone marrow long-lived plasma cells provide antibody memory, *i.e.* they produce and maintain stable antigen-specific antibody levels for several months to several years after immunizations [28,53]. The long-lived plasma cells thus contribute to humoral memory through antigen-specific antibody production over a long period. These memory providing long-lived plasma cells are characteristically non-proliferating and immobile (do not proliferate and do not respond to migratory chemokine signals). Only 10-20% of plasma cells generated in secondary lymphoid organs during the peak of immune response are capable of surviving for longer periods in the bone marrow [54]. Affinity maturation taking place in germinal centers is crucial for the formation of long-lived plasma cells [23]. Plasmablasts that are formed in germinal centers reactions and in memory/secondary responses are much more competent to become long-lived than those formed in extrafollicular areas or in primary immune challenge [29].

Factors in the bone marrow, that are most likely to form survival niches for memory providing long-lived plasma cells, include CXCL12 or SDF1, IL-6, TNFα, IL-5, and CD44 ligation [55]. Other potent survival factors include BAFF (B-cell activating factor) and APRIL (a proliferation inducing ligand), both of which are ligands for BCMA (B-cell maturation antigen) receptor expressed by plasma cells [56]. When competent plasmablasts generated in germinal center reactions find free survival niches in bone marrow, or manage to displace older plasma cells from their existing niches (by competition), they differentiate into mature long-lived plasma cells. Intriguingly, plasma cells survival factors like CXCL12, IL-6, BAFF, APRIL and TNF are also expressed in inflamed tissues and activated secondary lymphoid organs [40,56,57]. Consistent with this, spleens and inflamed kidneys of lupus prone NZB/W mice contain nearly 10 times higher numbers of plasma cells compared to nonautoimmune mice [47], many of which are long-lived [39].

In summary, plasma cell survival might be dependent of two factors [29]:

a) Molecular competence to respond to these signals, as shown by the likeliness of plasma cells derived from memory B-cells or germinal center reaction to become long-lived.
b) Presence of appropriate survival signals e.g. those in the bone marrow and in inflamed tissues.

1.6 Antibodies

Antibodies are soluble immunoglobulins synthesized and secreted by plasma cells. They exhibit a high degree of specificity and react with one particular antigenic structure each.

1.6.1 Basic structure of an antibody

Rodney R. Porter first proposed the structure of an antibody in 1959, describing it to be composed of three fragments. Two of these are highly identical and capable of binding antigen and are called fragment antigen binding or *Fab*. The third fragment, responsible for the biologic functions of the antibody, is called Fragment crystallisable or *Fc*. Around the same time, Gerald M. Edelman reported that a human immunoglobulin is composed of four polypeptides: two identical *Heavy chains* and two identical *Light chains*; that each light chain is linked to a heavy chain and that the two heavy chains are in turn linked to each other by disulfide bonds [58-60]. He also proposed that each chain contains a variable region (antigen binding region) and a constant region. Porter and Edelman shared a Nobel Prize in 1972 for

describing the structure of antibody.

Almost all species have two major classes of immunoglobulin light chain: κ (kappa) and λ (lambda). Almost every species can produce both classes of light chain, but the ratio of κ chains to λ chain varies with species. For instance, 95% of mouse light chains are κ light chains, while in humans only 60% of light chains are κ light chains. In contrast to the two classes of light chain, the heavy chains can belong to 5 different classes depending on the size, structure, carbohydrate content and most importantly the biologic function. The five antibody classes are IgM, IgG, IgA, IgD and IgE containing μ, γ, α, δ and ε heavy chain, respectively. All individuals can produce heavy chains of all classes; however, any single plasma cell will only produce immunoglobulin molecules that contain homologous heavy chains. Similarly, any single plasma cell can produce light chains that are always either both κ or both λ, but never one each.

1.6.2 Genetic basis of antibody structure and diversity

The antibody coding genes have a unique capability of moving and rearranging themselves within the genome to generate enormous diversity in antibody specificity. Both the heavy and light immunoglobulin chains contain a variable region for antigen binding and a highly conserved constant region which confers biological properties. The variable region of the heavy chain is coded for by three separate heavy gene segments: V_H (variable), D_H (diversity) and J_H (joining). A distinct gene region codes for the constant region of the heavy chain: C_H (constant). The variable region of the light chain is coded for by two light gene segments: V_L (variable) and J_L (joining). The B-cell rearranges its heavy chain DNA so as to join one V_H gene segment to one D_H gene segment and one J_H gene segment. The joined VDJ unit codes for the entire variable region of the heavy chain. These gene rearrangements put the VDJ unit next to the heavy chain constant region genes, Cμ and Cδ. Similarly, one V_L gene segment is joined to one J_L segment. This VJ unit is put next to the light chain constant region C_L. After this DNA rearrangement, the antigenic specificity of that particular cell is fixed and the genes are translated into *heavy* and *light* chains of IgM (μ) and IgD (δ). After antigen encounter in secondary lymphoid organs, *class switch recombination* and *affinity maturation* takes place. In class switch recombination, the heavy chain VDJ units join either Cγ, Cα or Cε resulting in synthesis of IgG, IgA, IgE class of antibodies, but with same antigenic specificity. Affinity maturation is a consequence of *somatic hypermutations* in antibody encoding genes. In this process, a high frequency of mutations is induced in the variable region of both heavy and

light chains, resulting in a highly increased binding affinity of the antibody.

Following primary immunization, IgM is the first isotype of immunoglobulin to be produced. However, late after primary immunization and upon secondary immunizations with same antigen (in the presence of CD40 ligation) plasma cells undergo class switching. As a result, the Ig heavy chain is alternatively spliced to yield a different constant region, and thus a different antibody class (IgG or IgA, IgE). Class switch recombination and somatic hypermutation are initiated by activation induced deaminase (AID) protein. AID expression in activated B-cells is restricted to germinal centers, the place where B-cells undergo class somatic hypermutation and also class switch recombination and differentiation into plasmablasts or memory B-cells.

1.7 Autoimmunity

As stated earlier, the central feature of immunity is its ability to discriminate between elements that are 'self' to the body from elements that are 'nonself', and mount an immune response only to those elements recognized as nonself. During 1950s, ideas were presented by Jerne and Burnet (both Nobel Prize winners) and by Talmage regarding cellular basis of specificity which is now collectively accepted as *clonal selection theory*. This theory proposed that T and B lymphocytes of myriad specificities exist before there is any contact with a foreign antigen, suggesting a great probability of having autoreactive cells in the repertoire of normal lymphocytes. However, the body keeps the massively proliferating lymphocytes in regular check through various control mechanism to prevent autoimmunity.

1.7.1 Regulation and pathogenesis of autoreactivity

Regulatory mechanisms to control autoreactive B and T-cell can be divided into *central* and *peripheral tolerance*. Tolerance is the state of unresponsiveness to a particular antigenic epitope. Regulation by central tolerance occurs in primary lymphoid organs when lymphocytes are still immature, while peripheral tolerance mechanisms take place in peripheral lymphoid organs in mature lymphocytes [61]. Central tolerance includes positive and negative selection, checking the responsiveness and autoreactivity, respectively, of the lymphocyte receptors. During this process, the high affinity autoreactive lymphocytes undergo clonal deletion (by apoptosis) or are rendered anergic (physically present but

functionally unresponsive). The autoreactive B-cells may also undergo receptor editing, where its BCR genes are rearranged to change its specificity [62]. In peripheral tolerance, cells escaping central tolerance are subjected to a further round of negative selection, where autoreactive lymphocytes undergo activation-induced cell death (AICD), anergy and signaling for death by Fas and TNF receptor.

Despite above mentioned check points, not all autoreactive lymphocytes are eliminated from circulatory repertoire. Susceptibility to autoimmune diseases is determined by a combination of genetic and environmental factors [62]. A large number of candidate genes and loci have been identified to be associated with development of autoimmune diseases. Moreover, autoimmune diseases may be initiated, precipitated or worsened by infections. Particularly interesting is the ability of TLRs ligands like bacterial DNA and CpG to activate B-cells to induce proliferation and cytokine secretion. Defects in FCγRIIB receptors of B-cells, linked to inhibitory signaling pathways, have also been associated with lupus disease.

1.7.2 Autoantibodies and autoreactive long-lived plasma cells in autoimmune disease

Autoantibody production is a hallmark to autoimmune diseases. Several autoimmune diseases are associated with a variety of autoantibodies that play a role in pathogenesis of autoimmunity. Such autoantibody mediated diseases include systemic lupus erythematosus with multiple organ manifestations (anti-dsDNA, anti-Sm and anti-Ro/La antibodies), rheumatoid arthritis (anti-CCP antibody, rheumatoid factor), antiphospholipid syndrome (anti-cardiolipin antibody), idiopathic thrombocytopenia (anti-GPIIa/IIIb antibody), autoimmune hemolytic anemia (antierythrocyte antibody) and myasthenia gravis (anti-acetylcholine receptor antibody). Autoantibodies in these diseases are directed against various intranuclear, cytoplasmic or surface antigens present on different cell types. In particular, lupus disease is associated with a range of intranuclear and cytoplasmic antibodies, most prominent of which is anti- dsDNA [63]. Although IgM anti-DNA may be present in circulation, the most pathogenic isotypes are considered to be IgG2a and IgG2b. The onset of lupus nephritis is actually preceded by a shift in the isotype of anti-DNA antibodies from IgM to IgG. These pathogenic forms of antibodies bear the hallmarks of T-cell dependent activation of B-cells, i.e., presence of class-switched isotypes and somatic hypermutated antibodies [64]. The autoantibodies contribute to immunopathology by forming immune complexes and complement binding. Moreover, disease flares are associated with increased titers of these autoantibodies circulating in serum.

Long-lived plasma cells can contribute significantly to autoimmunity by maintaining stable autoantibody memory during and in between disease flares [29]. It has been demonstrated that autoreactive antibodies can be produced by long-lived plasma cells in the spleens of lupus prone NZB/W mice [39]. These autoreactive long-lived plasma cells were also refractory to immunosuppressive therapy with cyclophosphamide. This study provided a direct link between long-lived plasma cells and autoimmunity. It has also been reported in other hyperimmune diseases that pathogenic antibodies persist despite treatment with immunosuppressant [65,66], suggesting that they are produced by long-lived plasma cells. Autoantibody memory provided by long-lived plasma cells is therefore an obstacle in the treatment of antibody-mediated diseases.

1.8 Systemic Lupus Erythematosus

Systemic lupus erythematosus is prototypic antibody-mediated autoimmune disease of the connective tissues affecting multiple organs. The term 'lupus erythemateux' was coined by Cazenave in 1851 and divided it into systemic and discoid form by Kaposi in 1872 [67].

1.8.1 Pathogenesis

SLE is characterized by activation of autoreactive B-cells and production of autoantibodies and immune complexes. Together with autoreactive T-cells, these autoantibodies can cause inflammatory destruction of tissue in several target organs like kidneys, blood vessels, skin and lungs. Besides the escape of autoreactive lymphocytes from central and peripheral tolerance mechanisms, defective clearance of apoptotic cells/immune complexes and dysregulation of FCγIIRB mediated inhibitory signaling pathways are also implied in development of SLE [68]. In the presence of aggravating environmental factors or inflammatory cytokines, the autoreactive B-cells of a genetically predisposed individual may be stimulated either by self-antigens or by a cross-reactive epitope of an infecting or environmental antigen [69]. This would lead to activation of the B-cell, which in the presence of T-cell help may undergo somatic hypermutation resulting in high affinity B-cells. Interestingly, autoreactive B-cells may also present foreign antigens (like viral proteins) complexed with self-antigens, and therefore receive help from non-autoreactive T-cells [70]. Thus, with its antigen presentation function, autoreactive B-cells can either break peripheral T-cell tolerance or directly activate of autoreactive T-cells [68]. Other antigen presenting cells like dendritic cells and macrophages may also activate autoreactive T-cells. Activated T-cells, in turn, help to enhance B-cells' response by cytokine production and germinal center formation, where class

switch, somatic hypermutations and affinity maturation of the antibodies takes place. Interaction of B-/T-cell leads to positive-feedback loop that causes amplification of autoimmune response. After a few cycles of this positive feedback-loop, the presence of exogenous autoantigens that initiated the response may no longer be necessary, all the lupus defects may get magnified and the disease will become self-sustained [68]. Effector T-cells may also have a direct role in tissue damage, as can be shown by T-cell infiltrates of lupus nephritis lesions. Both activated T and B-cells produce proinflammatory cytokine that can exacerbate autoimmune response by autoreactive B-cells. The cytokine profile of SLE patients consists of elevated serum levels of proinflammatory cytokines like IFN-γ, TNF-α, IL-6 and IL-10.

Although exact initiating trigger and precise series of events leading to full blown SLE are still debated on, it is clear that B-cells contribute by producing high-affinity autoantibodies as well as antigen presentation to autoreactive T-cells. Also, T-cells contribute by enhancing B-cell response and cytokine production.

1.8.2 Clinical presentation

SLE is a chronic autoimmune disorder involving multiple organs, predominantly affecting women of reproductive age [71]. The clinical manifestations are diverse and range from non-specific symptoms like fatigue, arthralgia, myalgia to potentially life-threatening renal or cerebral disease.

Renal disease: Lupus nephritis & end stage renal failure are the prime causes of morbidity and mortality of lupus patients. Deposition of anti-DNA antibodies and immune complexes occurs on the glomerular basement membrane, mesangial matrix and in subendothelial region of glomerular capillary wall [72]. These autoantibodies and immune complexes are therefore implicated as the major pathogenic mediators. Ensuing inflammatory reactions may cause cellular proliferation, necrosis and eventually fibrosis of the affected kidney [73]. These patients have proteinuria and elevated serum levels of creatinine due to impaired renal excretory function.

Neuropsychiatric disease: Presence of antineuronal and antiribosomal P protein autoantibodies has been associated with neuropsychiatric symptoms in SLE [74]. The predominant histopathologic lesions are multifocal cerebrocortical microinfarctions associated with microvascular injury and antibody-mediated neuronal cells injury. The patients may present with varying degree of memory and cognitive impairment, apathy and loss of

orientation/intellect. Slow but progressive deterioration may occur in few patients resulting in severe debilitating dementia.

Cardiac disease: SLE patients may develop lupus-related valvulopathies, coronary artery disease, myocardial dysfunction or pericardial disease. Increased risk of infectious endocarditis and accelerated atherosclerosis has been reported in SLE patients [75].

Pulmonary disease: SLE may involve pleura (pleuritis and pleural effusion), pulmonary interstitium (inflammatory alveolitis and chronic fibrosis), airways and pulmonary vasculature. Although pulmonary involvement is often not associated with any significant morbidity and may even be asymptomatic, it may become life-threatening if acute pneumonitis, hemorrhagic alveolar hemorrhage syndrome or pulmonary hypertension develops [74].

Hematologic disease: Anemia, lymphocytopenia and autoimmune thrombocytopenia occur frequently in patients with SLE. Thrombocytopenia may very rarely cause fatal hemorrhage in SLE patients.

Cutaneous lupus erythematosus: The characteristic cutaneous lesion of SLE is malar dermatitis on the face (butterfly rash on cheeks and nasal bridge, sparing nasolabial folds). It is characterized by erythema and edema with relatively sparse and superficial infiltrate without induration or scarring. Another form of skin involvement in lupus is *discoid lupus*, wherein the lesions occur on face, scalp, ears or neck. The lesions of discoid lupus, in contrast to other cutaneous lesions, undergo pigmentary changes, atrophy and scarring that have denser, deeper infiltrates. SLE patients are generally photosensitive and ultraviolet B rays (in solar radiations) are capable of inducing disease activity. Cutaneous lupus is associated with anti-Ro/SS-A antibodies. Ultraviolet light may induce the synthesis of or facilitate translocation of endogenous cytoplasmic antigens (Ro/SS-A, La/SS-B and Smith) to the plasma membrane, which could provide initial antigenic stimulus for development of autoantibodies and lead to antibody-mediated or cytotoxic cell-mediated immune response.

Pregnancy and neonatal lupus: Lupus activity is known to be precipitated or exacerbated by pregnancy, perhaps due to hormonal shifts required to maintain pregnancy [76]. Increased lupus activity, in turn, has an elevated risk for poor pregnancy outcomes like preeclampsia, still births and preterm birth. Patients with preexisting lupus nephritis frequently have worsening of hypertension and proteinuria during pregnancy. Neonatal lupus is a rare syndrome that occurs in a minority of infants delivered to anti-Ro/SS-A or anti-La/SS-B antibody positive

patients [77]. It consists of congenital heart block (CHB) and/or transient rash in the newborn period. Fetal myocardiopathy with congestive heart failure and in utero fetal death may also occur.

Drug induced lupus: Many drugs like procainamide, hydralazine, and phenytoin are linked with induction of lupus or lupus like disease. Drug induced lupus is almost always associated with the presence of antihistone antibodies. However, improvement and permanent resolution of symptoms occurs after discontinuation of the offending agent.

1.8.3 Management of SLE

Conventional treatment of SLE revolves around four main classes of drugs, often used in various combinations:

-Non-steroidal anti-inflammatory drugs (NSAIDs)
-Antimalarials
-Glucocorticoids
-Cytotoxic immunosuppressants

Patients with mild lupus can generally be maintained on a combination of NSAIDs and antimalarials (Hydroxychloroquine). However, these drugs are not very potent and are not sufficient for more severe forms of SLE that patients usually present with. The most efficient and recommended pharmacologic agents for controlling active lupus are corticosteroids and cytotoxic drugs. Though these drugs are considered to be life saving, they are associated with a vast range of serious side effects as patients often require aggressive long term therapy. Glucocorticoids are associated with a major risk of infections, hypertension, hyperlipidaemia, accelerated atherosclerosis, osteoporosis and diabetes. The use of cytotoxic drugs is also limited by more serious side effects like bone marrow suppression, malignancy and infertility.

Cytotoxic immunosuppressants: Cytotoxic drugs work by inhibiting proliferation. The most frequently used cytotoxic drugs in treating SLE are cyclophosphamide, azathioprine and mycophenolate mofetil. Cyclophosphamide is an alkylating agent, which upon metabolism by liver enzymes yields phospharamide mustard - the active metabolite. This causes DNA inter-strand and DNA-protein cross-links, which is fatal for proliferating cells [78]. Similarly, the active metabolites of azathioprine get incorporated into DNA producing antiproliferative effects. Mycophenolate mofetil is a relatively new immunosuppressive agent that has a superior side effect profile compared to cyclophosphamide and azathioprine. It inhibits

inosine monophosphate dehydrogenase, the enzyme involved in de novo purine nucleotide synthesis, thereby suppressing T and B lymphocyte proliferation.

Glucocorticoid: Glucocorticoids are anti-inflammatory immunosuppressive drugs used in treating SLE and other autoimmune diseases. Besides reducing inflammation, they induce apoptosis in lymphocytes by mechanisms irrespective of their proliferation status. Dexamethasone, a potent glucocorticoid, induces apoptosis in malignant plasma cells and is also used for treating patients with multiple myeloma [79,80].

Glucocorticoids exert their effects on lymphocytes by non-genomic and genomic mechanisms. The non-genomic mechanism of glucocorticoid action is mediated by membrane bound glucocorticoid receptors which rapidly lead to apoptosis in lymphocytes. Moreover, glucocorticoids also interact physicochemically with cellular membranes resulting in decreased intracellular Ca^{2+} concentrations [81].

The genomic mechanism of glucocorticoid action is mediated by cytosolic glucocorticoid receptors [82]. The glucocorticoids bind to their cytosolic receptors, forming 'steroid receptor complex'. This complex is translocated into cell nucleus, where it binds to specific DNA binding motifs called 'glucocorticoid-responsive element' in steroid responsive genes, leading to transactivation and transrepression of various genes. Moreover, GCs can also modulate genetic transcription through modulation of transcription factors like NF-κB and AP-1 [82]. Both transcription factors are important in the production of pro-inflammatory cytokines, including TNFα and IL-6 which provide important survival signals to plasma cells. Besides these anti-inflammatory effects, the genomic mechanisms can also induce apoptosis in lymphocytes through activation of caspase-9, which leads to a further sequential activation of caspase 6, 8, 3 and finally to mitochondrial dysfunction and cell death [83].

1.9 New Zealand Black x New Zealand White (NZB/W F1) mouse model of SLE

In the experiments described here, NZB/W F1 mouse model of SLE was chosen as it resembles human SLE very closely. It is the first generation hybrid of New Zealand black (NZB) and New Zealand white (NZW) mice. Other mouse strains developing SLE include MRL-Fas(*lpr*) and BXSB.

1.9.1 Contribution of parent strains NZB and NZW to SLE in NZB/W F1 mice

The genes contributing to development of SLE in NZB/W F1 are inherited from both parent strains, although neither strain develops SLE. The parent NZB mouse develops mild glomerulonephritis, though the primary cause of morbidity and mortality of this strain is autoimmune hemolytic anemia. The most striking abnormality of NZB mice, which is also passed on to NZB/W F1 mice, is hyperactive polyclonal B-cell and IgM hypergammaglobulinemia. In addition to anti-nuclear and anti-gp70 antibodies, these mice contain IgM and IgG anti-erythrocyte antibodies. These abnormalities may be linked to certain genes on chromosome 1 of NZB mice that influence autoantibody formation. Another region on chromosome 4 of this parent strain predisposes NZB/W F1 to glomerulonephritis.

The NZW mice, though clinically healthy, provide many SLE predisposing genes in NZB/W F1 mice. The genes contributed by parent NZW mouse to NZB/W F1 for development of SLE are categorized into two groups: 1) MHC II z genes, predisposing them to nephritis through production of anti-DNA, anti-chromatin and anti-histone antibodies and 2) non-MHC genes contributing to IgG autoantibody production. More interesting are three distinct chromosomal regions of NZW mouse labeled *Sle1* on chromosome 1 (linked to development of IgM anti-chromatin antibodies and to IgG anti-dsDNA), *Sle2* on chromosome 4 (promotes B-cell hyperactivity) and *Sle3* on chromosome 7 (promotes T-cell hyperactivity). However, NZW mice also posses another gene labeled *Sle1-suppressor* (Sle1-s), which is supposedly protective against SLE, and this is probably the reason for clinical normality of NZW mouse.

1.9.2 Characteristics of NZB/W F1 mice

The NZB/W mice develop SLE resembling the features of human disease very closely [84]. The primary cause of its morbidity and mortality is lupus nephritis. It is much more severe and occurs earlier in females than in males, with 50% mortality by 8 months. Like its parent NZB, this strain has B-cell hyperactivity with abnormally high secretion of polyclonal antibodies and autoantibodies detectable as early as 1-2 months of age. By 6-10 weeks of age, splenic plasma cells produce 20- to 40-fold more IgM than normal strains. Anti-DNA antibodies can be also detected in these mice as early as 2 months of age, however, at this point, they are mainly of IgM isotype. The isotype shift from IgM to IgG anti-DNA antibodies precedes clinical disease, with most of the pathogenic anti-DNA antibodies belonging to class IgG_{2a} and IgG_{2b} [85,86]. This isotype shift is dependent on CD4+ T-cell help and also in part on the susceptibility genes from parent strains. Other autoantibodies detected in these mice include anti-chromatin, anti-nucleosome, anti-histone, anti-RNA and also antiphospholipid and anticardiolipin antibodies. The major immune complexes circulating in NZB/W mice are that of DNA and anti-DNA and that of gp70 and anti-gp70. Kidney deposits of anti-DNA antibodies may come from either circulating immune complexes and from antibodies deposited either directly on glomerular basement membrane or on antigens embedded on it. Elevated levels of immune complexes are also associated with hypocomplementemia. The kidney disease is associated with heavy depositions of IgG and C3 in the glomerular mesangium, interstitial and glomerular capillary walls. Intravascular proteinaceous deposits, moderate proliferation of all glomerular cellular elements and crescent formation occur which are accompanied by heavy proteinuria (300-2000mg/dL, 6.6mg/day) resulting in increased blood urea nitrogen, azotemia and renal failure due to end stage kidney ensuing in death.

Another feature of NZB/W mice is lymphoproliferation. They exhibit splenomegaly and lymphadenopathy, with spleens containing higher numbers of leukocytes than a non-autoimmune mouse. There is also lymphocytic infiltration of salivary glands and DNA immune complex deposits in skin lesions. The thymus of NZB/W mouse gets atrophied with 70-90% of thymic cortex lost by 6-7 months of age. Elevated levels of cytokines, particularly of IL-6 (associated with (auto)antibody production) and IL-10 is also observed in this NZB/W mice.

The feature of NZB/W mouse that made this model most suitable for the experiments is the presence of a large (autoreactive) plasma cell population in its spleen and inflamed kidneys. Compared to non-autoimmune mice, the spleen and inflamed kidney (but not bone marrow) of NZB/W mice with manifest disease have at least 10 times higher numbers of plasma cells [47]. It should be noted that the spleens of very young NZB/W mice, which are still phenotypically healthy, do not contain such high numbers of plasma cells. Also, plasma cells are absent for the kidneys of NZB/W mice at this very young age *(Panne et al., Manuscript in preparation)*, suggesting that the increase in plasma cell numbers in these organs is associated with progressing disease.

Nearly 65% of the antibody secreting cells in the spleen of adult NZB/W mice are proliferating plasmablasts/short-lived plasma cells. They incorporate the proliferation marker Bromodeoxyuridine (BrdU) into their DNA, within 10 days of continuous feeding with this thymidine analogue. The remaining 35% of splenic antibody-secreting cells are non-proliferating, long-lived plasma cells exhibiting much longer life span [39]. Unlike short-lived plasmablasts, long-lived plasma cells do not proliferate and therefore do not incorporate BrdU into their DNA even after 3 months of continuous BrdU feeding. Despite the presence of large numbers of proliferating short-lived plasmablasts, the numbers of long-lived plasma cells in spleens of an adult NZB/W mouse remain stable. More interestingly, the long-lived plasma cell compartment in the spleens of these mice is established at a very early age (before 3 months) (Voigt, C.: doctoral thesis, Charité – Universitätsmedizin Berlin, 2008). Very few, if any, long-lived plasma cells are added to this compartment after this age, indicating that the numbers of survival niches in the spleens are limited and are occupied within the first 3 months of life. Moreover, majority of the splenic plasmablasts/short-lived plasma cells express high MHC II and bind more annexin V than long-lived plasma cells, showing that the plasmablasts are more susceptible to apoptosis than long-lived plasma cells. The splenic long-lived plasma cells, including anti-DNA antibody secreting cells, are totally resistant to immunosuppressive cytotoxic therapy with cyclophosphamide [39].

1.10 *Aim of the experiments*

The aim of this project was to study the localization of autoreactive and non-autoreactive plasma cells in lymphoid oragns and inflamed kidneys of lupus prone NZB/W mice and the effects of high dose glucocorticoids and/or cyclophosphamide on these cells. Glucocorticoids induce apoptosis in malignant plasma cells of multiple myeloma and are a part of standard therapy for both autoimmune diseases and multiple myeloma. Since they induce apoptosis in lymphocytes irrespective of their proliferation status and inhibit production of plasma cell survival factors like IL-6 and TNF-α, it was investigated if glucocorticoids, alone or in combination with cyclophosphamide, induce apoptosis in long-lived plasma cells. It was also investigated if abrogation of T-cell help at a very early age prevents the formation of long-lived plasma cells in NZB/W mice. T-cell help is critically required for the formation of germinal centers in secondary lymphoid organs, which in turn are essential for bone marrow long-lived plasma cell formation. CD4+ T-cells were therefore depleted from these mice by injecting anti-CD4 antibody at the age when the long-lived plasma cell compartment in spleens and bone marrow of these mice is still being established.

The work presented in this thesis is divided into 3 parts:
Part IA focuses on the effects of *1week* treatment with glucocorticoid and/or cytotoxic immunosuppressant on long-lived plasma cells in the lymphoid organs and inflamed kidneys of lupus mice. In **part IB**, the effects of *4 week* treatment with glucocorticoids on long-lived plasma cells were determined. In **part II**, the effect of CD4+ T-cell depletion on the formation of long-lived plasma cells in lupus mice was assessed.

2 Material and Methods:

2.1 Materials

A list of all the buffers, reagents, culture mediums and drugs used in carrying out the experiments and their compositions is presented here:

2.1.1 Buffers, solutions and culture medium

Buffers and culture medium	Composition	Source
Phosphate buffered saline (PBS)	8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na_2HPO_4	Roth Sigma-Aldrich Sigma-Aldrich
Phosphate buffered saline with Bovine serum-albumin (PBS/BSA)	PBS 0.5% BSA	Biomol
Elispot Blocking buffer	PBS 3% BSA	Biomol
Elispot Washing buffer	PBS 3% BSA, 0.01% Tween 20	Sigma-Aldrich
AMP-buffer for Elispot development	95 ml 2-Amino-2-methyl-1-propanol (AMP)	Sigma-Aldrich
	0.1 ml Triton X-405 150 mg/ml $MgCl_2$	Sigma-Aldrich
	900 ml Water brought to a pH of 10.25with HCl	Sigma-Aldrich
Complete cell culture medium	Rosewell Park memorial Institute Medium (RPMI) 1640	Life Technologies
	10% Fetal calf serum (FCS)	Invitrogen
	10 mM L-Glutamate	Invitrogen
	20 µM β-Mercaptoethanol	Invitrogen
	100 U/ml Penicillin	Invitrogen
	100 µg/ml Streptomycin	Invitrogen

2.1.2 Reagents to fix the cells for intranuclear BrdU staining

Reagents	**Contents**	**Source**
Bromodeoxyuridine	Bromodeoxyuridine	Sigma-Aldrich
BD Cytofix/Cytoperm Buffer (Ready to use solution)	Paraformaldehyde and Saponin	BrdU Flow Kit (BD Pharmingen)
BD Perm/Wash Buffer (10-times concentrated solution, to be diluted in deionised water)	Saponin, Fetal bovine serum, Sodium Azide	BrdU Flow Kit (BD Pharmingen)
BD Cytoperm Plus Buffer (Ready to use solution)	Paraformaldehyde, Dimethyl sulfoxide	BrdU Flow Kit (BD Pharmingen)
BD DNAse solution (3-times concentrated solution, to be diluted in PBS)		BrdU Flow Kit (BD Pharmingen)

2.1.3 Materials for Immunizations

Material	**Source**
Syringes	Braun
Cannulae	Braun
Chicken egg albumin (Ovalbumin)	Sigma-Aldrich
Inject - Alum	Pierce
0.2µm sterile micro-filters	Sarstedt

2.1.4 Materials for preparing and administering drug solutions

Material	**Source**
Dexamethasone injection (Fortecortin)	Merck Pharma GmbH
Prednisolone powder	Sigma-Aldrich
Cyclophosphamide (Endoxan)	Baxter Oncology
PBS	Described earlier
Peanut oil	Sigma-Aldrich
DMSO	Merck
Ethanol (99.98%)	Carl Roth
Syringes	Braun

Cannulae	Braun
Omnican Insulin Syringes	Braun
Dipsticks for measuring proteinuria	Bayer

2.1.5 Labware and appliances

Product	**Source**
Tubes 15/50 ml	BD Biosciences
Eppis 0.5/ 1.5/ 2 ml	Eppendorf
Pipettes	Pipetman
Pipette tips	Corning Costar
Sterile filter	Millipore
Culture flasks	Corning Costar
Cell strainers	Becton Dickinson
96 Microwell plate (flat bottom, high binding)	Corning Costar

2.1.6 ELISA Antibodies and Reagents

Product	**Source**
Coating for anti-ovalbumin antibodies	
Chicken egg albumin (Ovalbumin)	Sigma-Aldrich
ELISA Standard for determining sample antibody concentration	
Goat IgG_1 anti-ovalbumin antibody	Sigma-Aldrich
Detection antibody	
Biotinylated goat anti-mouse IgG antibody	Biozol
Development buffer	
Alkaline phosphatase development buffer	Roche

2.1.7 ELISPOT Antibodies and Reagents

Antibodies & Reagents	**Source**
Coating antibodies for total plasma cell (in PBS)	
Goat anti-mouse IgM *(polyclonal)*	Biozol
Goat anti-mouse IgG *(polyclonal)*	Biozol
Coating for DNA-specific plasma cells (in Aqua dest.)	
Methylated BSA	Sigma-Aldrich
Calf thymus DNA	Sigma-Aldrich
Coating for ovalbumin-specific plasma cells	
Chicken egg albumin (Ovalbumin)	Sigma-Aldrich
Detection antibodies	
Biotinylated goat anti-mouse IgM *(polyclonal)*	Biozol
Biotinylated goat anti-mouse IgG *(polyclonal)*	Biozol
Secondary reagent	
Streptavidin Alkaline Phosphatase	Sigma-Aldrich
Substrate	
5-Bromo-4-chloro-3-indolyl-phosphate (BCIP)	Sigma-Aldrich
Supporting medium for substrate and development buffer	
3% Agarose	Sigma-Aldrich

2.1.8 Staining reagents for flow cytometric analysis

Blocking the unspecific binding sites on leukocytes

Before staining the leukocytes with fluorochrome coupled detection antibodies, the cells were incubated with rat anti-mouse FcγReceptor antibody and rat-IgG for 5 minutes on ice. These antibodies bind and block FcγReceptors and other unspecific binding sites on the leukocytes, thereby increasing the staining specificity of the fluorochrome conjugated detection antibodies.

Antibody	Source
Blocking of FcγReceptors	
Rat anti-mouse FcγReceptor antibody (Anti-mouse CD16 and CD32)	BD Pharmingen (Clone 2.4G2)
Blocking of unspecific binding sites	
Rat IgG	Biotrend

2.1.8.1 Surface staining

For FACS analysis, surface staining of lymphocytes was done with detecting antibodies coupled to fluorochromes. These fluorochromes include Fluorescein isothiocyanate (FITC), Phycoerythrin (PE), Indodicarbocyanine (Cy5), Allophycocyanin (APC), Alexa 430 and Biotin (Bio). The biotinylated antibodies were detected on FACS through secondary reagents – Streptavidin-Peridinin chlorophyll protein (SA-PerCP) or Streptavidin-Allophycocyanin (SA-APC). In the samples where unfixed cells were to be analyzed on FACS, dead cells were excluded from analysis by staining with 4’,6-diamidino-2-phenylindole (DAPI).

The following table contains a list of specificity, clones, conjugates and sources of detection antibodies used in the experiments reported in this dissertation:

Specificity	Clone	Conjugate	Source
CD 138	281-2	PE	BD Pharmingen
MHC II	M5/114	Bio, Alexa 430	DRFZ
B220	RA3.6B2	FITC, Bio, Cy5	DRFZ
CD 3	145-2C11	Bio, FITC	DRFZ
CD4	GK1.5 YTS191	FITC, Cy5 FITC	DRFZ DRFZ
CD8	53-6.7	PE	BD Pharmingen
Secondary Reagents			
Streptavidin		PerCP APC	BD Pharmingen BD Pharmingen

2.1.8.2 Intracellular staining

The cells intended for intranuclear or intracellular staining were fixed with cytofix/cytoperm buffer (BrdU flow kit, BD Pharmingen). Intranuclear staining for BrdU was done with reagents and antibodies provided in the BrdU flow kit (BD Pharmingen). For staining ovalbumin-specific plasma cells in mice immunized with this antigen, ovalbumin (ova) coupled directly to fluorochrome-Cy5 was used. In this method, cells were fixed and stained with ova-Cy5, so that ova binds the intracellular antibodies of ova-specific plasma cells and Cy5 fluorochrome gets detected on FACS.

Reagent/Antibody specificity	Clone	Conjugate	Source
BrdU	3D4	FITC, APC	BD Pharmingen
Kappa light chain	187.1	FITC, Cy5	DRFZ
Lambda light chain	JC5-1	Cy5	BD Pharmingen
Fluorochrome coupled ovalbumin	------------	Cy5	DRFZ

2.2 Breeding and maintenance of NZB/W F1 mice

All the experiments described in this thesis were carried out on SLE prone NZB/W F1 mice. Female mice of this strain were obtained from the breeding unit of 'Bundesinstitut für Risikobewertung' (BfR, Federal institute for risk assessment) in Berlin. The mice were bred and maintained here in specific pathogen free (SPF) conditions. During the BrdU feeding and treatment phase of experiment, the mice were transferred to and maintained at the SPF mouse maintenance facility of Deutsches Rheuma Forschungs Zentrum (DRFZ, German rheumatism research center). Prior to animal experiments, an approval was obtained from the ethics committee at *'Landesamt für Gesundheit und Soziales' (LAGeSo)*. The age of mice used in various experiments is indicated in respective sections. In general, the experiments where animals with manifest lupus nephritis were required, only mice older than 20 weeks and proteinuria more than 30mg/dL (as measured by proteinuria dipsticks) were used.

2.3 BrdU feeding and control immunizations

To discriminate between proliferating short-lived plasmablasts/plasma cells and non-proliferating long-lived plasma cells, bromodeoxyuridine (BrdU) pulse labeling technique was used as described earlier [39]. BrdU is an analogue of DNA precursor – thymidine, which gets incorporated into the newly synthesized DNA of proliferating cells. This BrdU uptake by proliferating cells could later be determined by immunofluorescent staining on flow cytometry, using specific monoclonal anti-BrdU antibodies coupled to fluorochromes.

In order to control the incorporation of BrdU by proliferating plasmablasts, recently generated antigen-specific plasma cells were monitored for BrdU incorporation. For this purpose, the mice were primed and boosted with T-dependent antigen ovalbumin (ova) intraperitoneally. The BrdU feeding was started immediately after the boost immunization. Since ovalbumin is not present in the environment, the mice would not have been exposed to this antigen prior to our deliberate immunizations. And since the booster immunization induces a strong proliferation of ova-specific plasma cells and BrdU was started at this time point, all the ova-specific plasma cells generated hereafter should take up BrdU. It was shown by Manz et al. that 10 days after the booster dose, most of the ova-specific plasma cells are located mainly in the bone marrow [25]. Therefore, ova-specific plasma cells in the bone marrow of NZB/W mice were analyzed three weeks after secondary immunization and BrdU feeding.

In the second experiment where CD4+ T-cells were depleted from young NZB/W mice, the development of anti-ovalbumin IgG antibody titers in vehicle and CD4+ T-cell depleted mice was followed. Unlike the previous glucocorticoid experiments, the development of ova-specific plasma cells was not of interest here.

2.3.1 Preparation of BrdU solution for feeding

In order to achieve the labeling of proliferating cells, BrdU was fed to the mice via drinking water. For this purpose, BrdU was dissolved such that the final feeding concentration of 1mg BrdU/ml drinking water was attained. Also, 1 gram sugar was added to every 100 ml of BrdU containing water, so as to give it a sweeter taste. Since BrdU is photosensitive when dissolved in water, the feeding bottles were carefully wrapped in aluminium foil to avoid exposure to natural or artificial light, and the BrdU water was changed every 3 days.

2.3.2 Preparation of Ovalbumin for immunization

For the purpose of primary and secondary immunizations, each mouse received 100 µg of ovalbumin intraperitoneally. Ovalbumin powder was first dissolved in PBS to achieve a stock solution with a concentration of 1mg/mg. This was subsequently passed through 0.2 µm sterile microfilters to avoid contaminants or particulate matters. The stock solution was then aliquoted into 1.5 ml eppis and stored at -20°c. On the day of immunization, required volume of ova-PBS solution was thawed and mixed with equal volume of adjuvant – aluminium magnesium hydroxide (alum). The final immunization solution therefore contained immunogen (Ova) and adjuvant (alum) in 1:1 ratio. Alum also forms depot at the site of injection from where the immunogen is slowly released over a longer period of time, thereby increasing the efficiency of immunization. At each point of immunization, mice were intraperitoneally injected with 200 µl of this solution, i.e., 100 µg of ova (in 100 µl of PBS) and 100 µl of adjuvant (alum).

2.3.3 Preparation of Drugs solutions

For dexamethasone preparations, Fortecortin injection vials (Merck Pharma GmbH), containing dexamethasone at a concentration of 2mg/ml was used. Dexamethasone was diluted in sterile PBS such that 200µl of the injectable solution contained the required dose. For one month glucocorticoid treatment, prednisolone powder was dissolved in a solution containing DMSO, ethanol and peanut oil which was administered by oral route (*p.o.*). The prednisolone powder was first dissolved in an initial solution of DMSO and ethanol, which was further mixed with peanut oil. All the individual components were used in volumes such that a final concentration of 5% DMSO, 3% ethanol and 92% peanut oil was reached. Again, 200µls of the final solution contained the required dose of prednisolone for each mouse.

2.4 Treatment protocols

This section gives an overview of the age of mice, duration of BrdU feedings, immunizations, portal and techniques of drug administration, drug forms, dosages and duration of treatment of NZB/W mice in different experiments. For better understanding of different experimental strategies in different experiments, this section is divided into 3 parts, describing one experimental protocol each.

2.4.1 One week treatment of lupus mice with dexamethasone and/or cyclophosphamide and plasma cell analysis

In this experiment, the effects of one week treatment with dexamethasone and/or cyclophosphamide were determined on long-lived plasma cells of lupus suffering mice. Therefore, five month old female NZB/W mice that had developed proteinuria of atleast 30mg/dl or above were used. They were fed BrdU daily in drinking water for a period of three weeks. Also, the mice were immunized and boosted with T-dependent antigen – Ovalbumin before starting the BrdU feeding (to control the BrdU uptake by all proliferating lymphocytes). During the third week of BrdU feeding, the mice were split up into 4 treatment groups, each receiving respective treatments via intraperitoneal injections –

1) Vehicle (100 µl PBS/day on 7 days)
2) Glucocorticoids (Dexamethasone 1mg/kg/day on 7 days)
3) Cyclophosphamide (30mg/kg/day on 4 days)
4) Combination (receiving both dexamethasone and cyclophosphamide).

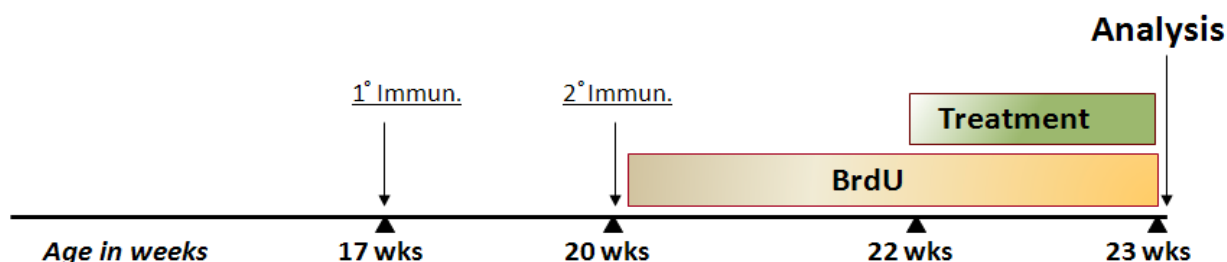

***Figure 3: Experimental layout**. NZB/W mice were immunized with ova at 17 and 20 weeks of age. They were fed BrdU for 3 weeks starting with secondary immunization and treated with immunosuppressants during the last week of BrdU feeding. The analysis was made immediately at the end of treatment.*

The doses used in this experiment are similar to those described in literature elsewhere [39,87]. Hence, with this experimental protocol, dexamethasone treated mice received a total dose of 7mg/kg and cyclophosphamide treated mice received a total dose of 120mg/kg of respective drugs during the 7 days treatment period. All the drugs were diluted in PBS to obtain the desired dose concentrations, and were injected intraperitoneally on each day. At the end of the treatment period, BrdU feeding was stopped and the mice were killed by cervical dislocation for analysis. Plasma cells and other lymphocytes were isolated from the spleens, bone marrow and kidneys of these mice and were analyzed by FACS and ELISPOT.

2.4.2 One month treatment of lupus mice with prednisolone and plasma cell analysis

With this treatment protocol, the effects of one month glucocorticoid (prednisolone) treatment on long-lived plasma cells of lupus mice were determined. BrdU feeding in this experiment was started at 22 weeks of age and carried on till the end of the experiment (28 weeks of age). The mice were treated with different doses of prednisolone beginning at the age of 24 weeks and continued daily till 28 weeks. Already at the beginning of prednisolone treatment, all the mice had developed very high proteinuria ranging between 300mg/dL and 2000mg/dL. The mice were split up into 3 treatment groups as follows, and drugs administered by intragastric gavage –

1) Vehicle (200µls of ethanol/DMSO/peanut oil)
2) Low dose prednisolone (3mg/kg/day)
3) High dose prednisolone (10mg/kg/day).

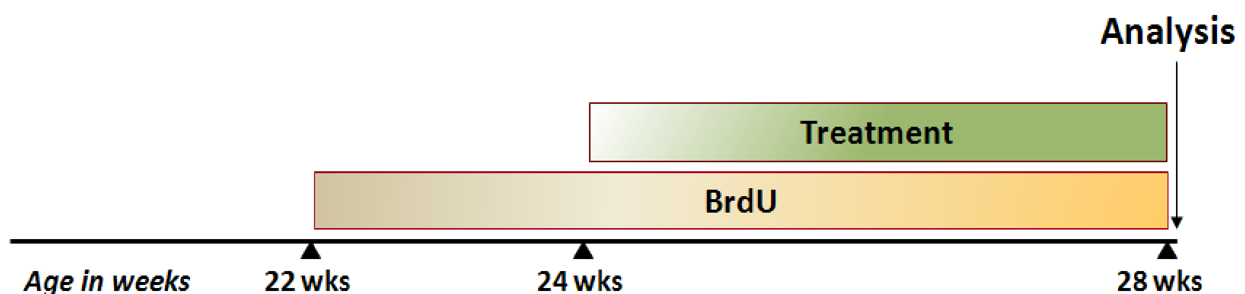

Figure 4: Experimental layout. *NZB/W mice were fed BrdU between 22 and 28 weeks of age and treated with different prednisolone doses during the last four weeks. Analysis was made immediately after.*

Powdered prednisolone was dissolved in 99.98% ethanol, DMSO and peanut oil such that the required drug dose per mouse was reached in 200µl of final solution. Hence, each mouse was administered 200µl of the drug solution or vehicle using intragastric gavages. Since the duration of treatment was relatively long for this experiment, intragastric route was preferred over intraperitoneal route to avoid daily injections.

Similar to the previous experiment, mice were killed by cervical dislocation at the end of the treatment phase. Spleens, bone marrow and kidneys were taken and lymphocytes were isolated from these organs for plasma cell analysis on FACS and ELISPOT. In addition, thymus and adrenal glands of these mice were also taken for measuring weight changes in these organs as a result of long-term prednisolone treatment.

2.4.3 CD4+ T-cell depletion in young lupus mice and plasma cell analysis

It has been shown that somatic hypermutations in the antibody encoding genes of plasma cells and resulting affinity maturation is very important in the selection of long-lived plasma cell pool. Since germinal center reactions are essential for both somatic hypermutations and class switch recombinations, and since CD4+ T-cells are crucial in the formation of germinal centers [20], CD4+ T-cells were depleted and its effect on the generation of long-lived plasma cells was analyzed. It is known from our previous experiments that nearly all the splenic long-lived plasma cells, and 50% of bone marrow long-lived plasma cells in NZB/W mice are formed by the age of 9 weeks. Therefore, CD4 cells from these young NZB/W mice were depleted between the age of 3 and 9 weeks.

For this purpose, each NZB/W mouse was injected twice weekly with 250µg anti-murine CD4 monoclonal antibody (clone GK1.5). This depleting antibody was given continuously between the age of 3 and 9 weeks through intraperitoneal route. In order to mark the proliferating short-lived plasmablasts, the mice were fed BrdU between 7 and 9 weeks of age. Thus, using this protocol, the development of long-lived plasma cell pool in these mice in the presence or absence of T-cell help before 7 weeks of age was analyzed. Additionally, it was checked if the depletion of CD4 cells from these mice caused functional abrogation of T-cell help. To this end, the mice were injected with the T-dependent antigen ovalbumin 10 days before analysis. In case of abrogation of T-cell help, the anti-CD4 injected mice should not be able to develop IgG anti-ovalbumin antibodies following ovalbumin immunizations.

For analyzing the extent of CD4 depletion, a different monoclonal anti-murine CD4 antibody was required, as all the epitopes on surviving CD4 cells were saturated by the depleteing GK1.5 monoclona antibody. These refractory CD4+ cells were detected using a different monoclonal anti-CD4 antibody (clone YTS 191). This enabled us to check the frequency of refractory CD4 cells in different organs. For analysis, the frequencies and absolute numbers of plasmablasts/short-lived plasma cells and long-lived plasma cells in different organs of CD4 depleted and non-depleted mice were compared. It was also checked if the CD4 depletion caused any changes in the CD8 and B220 cell compartments.

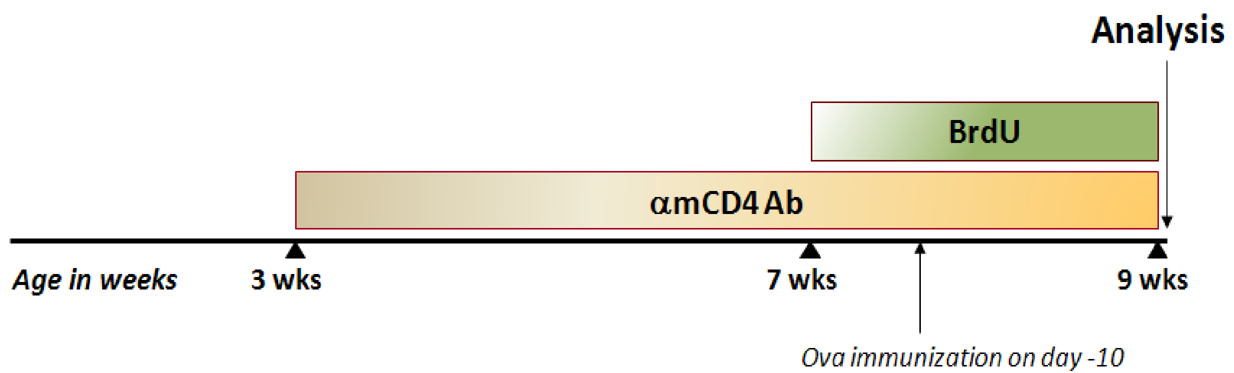

Figure 5: Experimental layout. *CD4+ T-cells were depleted from NZB/W mice between 3 and 9 weeks of age. BrdU was fed during the last two weeks and plasma cells were analyzed.*

2.5 *Preparation of cells*

2.5.1 Organ isolation and preparation of single cell suspensions

Spleens, bone marrow and kidneys were isolated from vehicle and treated NZB/W mice after killing them by cervical dislocation. However, immediately after killing, the systemic vasculature of these mice was flushed with PBS. This was done in order to prevent the contamination of resident cells in the organs from circulating cells in the blood vessels. To this end, the right auricle was first punctured, followed by injection of 5ml PBS into the left ventricle. This caused the blood from the systemic vasculature to exit through the right auricle. This was followed by removal of spleen, kidneys and bone marrow. Both femurs and both tibiae from each mouse were taken for obtaining bone marrow cells. The organs were transferred to 30 ml petri dishes and kept in RPMI-1640 medium throughout the preparation period. The spleens and kidneys were initially cut into smaller fragments using a scalpel and later pressed gently through a cell sieve of 70 μm mesh size. This gentle pressing of organs

through the mesh, using syringe stamps, was done several times until the suspension nodules were no longer visible. In order to get single cell suspensions from the bone marrow, the bones were first needed to be isolated. This was done using scalpels to detach all the muscles and tendons attached to the bones at various insertion points. The femur and tibia was then removed by displacing femur from the hip joint above the femoral head and cutting the lowest possible point on the tibia. Individual bones were then cut at both ends to get access to the bone marrow canals. Bone marrow was carefully flushed out of the bone stems and smaller bone pieces into the petri dishes using RPMI-1640 medium and 5 ml syringes attached to 25-gauge needles. The bone marrow cells were treated similar to splenic and kidney cells, in that they were maintained in RPMI-1640 medium and passed through cell sieve of 70 µm mesh size several times to achieve single cell suspensions. The cell suspensions from all the organs were washed thrice at 1200 rounds per minute (rpm) in a centrifuge, for 10 minutes in each round. After each round, the supernatant was discarded the leucocytes sediment was resuspended in fresh RPMI-1640 medium for the next wash step. At the end of the third washing step, the cells were ready to proceed for FACS stainings as described in section *2.1.8* and for ELISPOT.

2.5.2 Serum preparation

In the experiments where serum concentrations of anti-ovalbumin antibodies were to be determined, mice were bled from their lateral tail veins. The mice were first heated under heat infrared lamps to dilate the tail veins. The dilated vein was then cut with a sharp scalpel and 8-10 drops of blood was collected into Eppendorf tubes. The blood was left to coagulate at 4°C for 3 hours, after which the eppis were centrifuged at 10,000 rotations per minute for 10 minutes. The serum, which accumulates as clear supernatant after this procedure, was carefully removed to avoid contamination with pellet fragments. Serum was then frozen at
-20°C until analysis.

2.6 *Instruments and protocols for plasma cell analysis*

2.6.1 *Cell counting with the CASY*

For cell counting purposes, CASY cell counter was used. The sample cell suspension to be measured was diluted 1:1000 in ultrafiltrated FACS-flow (PBS + 0.02% N3, DRFZ) dispensed by a 10 ml dispenser (Hirschmann, Germany) and put to the Casy cell counter device. The measuring principle exploits the fact that cells of different sizes differentially alter the suspension's electrical resistance, which is measured and analyzed via a pulse field acquisition. Casy machine acquires 3×400 µl of the sample, and the average data is then presented on a 2D-diagram, plotting cell diameter against the respective counts. The cell number output can be modified by restricting the total cell count to a certain cell diameter range. For mice leucocytes, the range was set between 5 and 12 µm.

It should be noted that each femur contains 6.4% of the total bone marrow, and that the tibiae contain 0.6 times the number of cell in femurs (3.84% of total bone marrow). Therefore, to determine the number of plasma cells contained in the whole bone marrow of one mouse, the plasma cell counts in femurs and tibiae were multiplied by a factor of 4.9.

2.6.2 *Flow cytometric analysis*

To determine the frequency and absolute numbers of plasma cells in different organs, the flow cytometry technique was used. Fluorocytometry is a method for the characterization of individual cells based on their phenotypes, which are recognized by a complex set of detection mechanisms. But prior to passing the cell suspension through flow cytometer, the cells need to be stained with fluorochrome coupled monoclonal antibodies specific for surface antigens on plasma cells and other cells that are of interest. Cells thus labeled with one or more differently fluorochrome coupled antibodies can be distinguished by their emission of detectable light. The FACSCalibur (BD Bioscience) utilizes a 488-nm argon-ion laser and a 635-nm-diode laser, whereas the LSRII (BD Bioscience) is equipped with a 488-nm argon-ion laser, a 633-nm-helium-neon laser, and a 355-nm solid-state laser. Four detectors in the FACSCalibur and 15 in the LSRII allow the measurement of fluorescent light emitted from various fluorescent dyes. Fluorochromes are colored dyes, which accept light energy at a given wavelength (excitation) and emit it at a higher wavelength (emission). The excitation laser and light emitted from fluorochromes are directed via a complex set-up of mirrors, half

mirrors, lenses and prisms to their respective detection areas. The cells are exposed to the laser one after the other and depending on the amount of target molecules on an individual cell, the fluorescence intensity is measured in the respective detection channel. In the case of multiple staining, one cell event is correlated to a certain number of fluorescence values for each of the measured channels. In addition to measurements of fluorescence, the detectors of a flow cytometer are also used to detect scattering of the laser light. The fraction of light passing through the cell at an angular range of 3-10 degrees (forward scatter) correlates with the size of the cell, whereas the light dispersed at 90 degrees (sideward scatter) indicates the amount of granularity and membrane foldings. With these two detection modes, it is possible to discriminate between lymphocytic and phagocytic cell populations and also to differentiate cell events from background events caused by debris and non-biological contaminations like fluorochrome crystal.

After a successful acquisition of an appropriate number of events, the obtained data can be visualized as a set of dot plots. In a dot plot, every measured event is represented by a single dot at a position in the 2D-area of the plot. The position of the event is determined by its fluorescence intensity in the channels chosen to be displayed on the plot’s x- and y-axis. The FSC/SSC–dot-plot is displayed with a linear scale, whereas all other channels per default have logarithmical scales. An invaluable tool provided by the FACS analysis software is the possibility for cell gating. Setting a gate in a dot plot defines a restrictive fluorescence condition in the 2D-area, by drawing a polygonal or ellipsoid shape, representing the set of fluorescence parameters to be included for analysis. This gate can subsequently be applied to a different dot plot, meaning that only the set of cells limited by the gate in the first dot plot will be displayed in another dot- plot showing a further fluorescence pair.

In contrast to monochromatic laser light, the fluorescence emitted by the excited fluorochrome shows somewhat broad intensity peaks. This leads to regions of fluorescence overlap between fluorochrome of close emission wave lengths. In order to prevent this, color compensation is performed, in which the amount of overlap between individual fluorochrome-pairs is determined and corrected. This color compensation is performed as an initial experiment using single stained cell samples. In order to have optimal staining with clear separation of populations, every newly used antibody has to be titrated in an initial experiment.

The flow cytometry data from all experiments was analyzed using Cell Quest ™ Re-search software (Becton Dickinson, Heidelberg).

2.6.2.1 Staining protocol for surface antigens (for FACS analysis)

- Resuspend the cell pellet in 100µl of cold staining buffer (PBS/0.3%BSA).
- Add the fluorescent antibodies to achieve appropriate staining dilution and mix well.
- Incubate cells with staining antibodies for 10 minutes in dark at 4°C.
- Wash the cells by adding 1 ml of staining buffer and centrifuge at 2000 rpm for 10 minutes.
- Discard the supernatant and resuspend the pellet in 500µl of staining buffer for acquisition.

2.6.2.2 Cell fixation and permeabilization for intranuclear and intracellular antigen staining

- Resuspend cells with 100 µl of BD Cytofix/Cytoperm Buffer.
- Incubate cells for 30 minutes at room temperature or on ice.
- Wash cells by adding 1 ml of BD Perm/Wash Buffer, centrifuge and discard supernatant.
- Resuspend the pellet with 100 µl of BD Cytoperm Plus Buffer.
- Incubate cells for 10 minutes on ice followed by washing with BD Perm/Wash Buffer.
- Refix the cell by resuspending them with 100 µl of BD Cytofix/Cytoperm Buffer.
- Incubate for 5 minutes, followed by washing with BD Perm/Wash Buffer.
- Resuspend cells with 100 µl of 300 µg/ml DNase (to expose incorporated DNA).
- Incubate cells for 1 hour at 37°C, followed by washing with BD Perm/Wash Buffer.
- Resuspend the pellet in 50µl of BD Perm/Wash Buffer containing diluted fluorescent anti-BrdU and/or antibodies specific for intracellular antigens.
- Incubate cells for 20 minutes at room temperature and wash with BD Perm/Wash Buffer.
- Resuspend the pellet with 500µl of staining buffer and analyze stained cells on FACS.

2.6.3 Enzyme Linked Immunospot (ELISpot)

The ELISpot allows specific and highly sensitive quantification of cells secreting defined molecules like antibodies or cytokines. Using this method, one can enumerate numbers of plasma cells secreting IgM and IgG antibodies as well as anti-DNA IgM and anti-DNA IgG antibodies. In this procedure, the high protein binding 96 well plate is coated with an antigen against which the antibody secretion is to be quantified. For instance, the plates could be coated with DNA when anti-DNA ASCs are to be analyzed. Alternatively, anti-IgM and anti-IgG antibodies can be used as coating agents to antibodies of these class when secreted by plasma cells. This binding of anti-IgM or anti-IgG coating antibody to IgM or IgG antibody secreted by plasma cells will leave a kind of ‘foot print’ at those particular plasma cell locations. Further treatment of these wells (after washing away the cell suspension thoroughly) with secondary - biotinylated antibodies and its subsequent development with a

series of substrates and buffers result in formation of visible colored spots at the locations where the antibody secretion by plasma cells occurred. These spots are then counted using inverted microscopes.

2.6.3.1 ELISspot protocol

Coating the 96 well plate
- Coat the plate with 55µl/well of anti-murine IgM or IgG [5µg/ml in PBS]
- Incubate the plate overnight at 4°C 'or' 2 hours at 37°C.

Blocking unspecific bindings
- wash 1x with PBS/BSA [3%]
- Add 100µl of PBS/BSA [3%]
- Incubate for 2 hours at room temperature

Sorting the cells on the plate
- wash the plate 1x with 200µl/well PBS
- flick dry and add 200µl /well RPMI 1640
- sort the cells in 300µl per well in the first row
- make step dilutions of cells from first row till the last row (1:3 or 1:5)
- culture the cells for 2 hours at 5% CO2 / 37°C

Washing the cells down
- remove the plate from the incubator
- wash thoroughly with PBS/BSA[3%]/Tween 3 times

Incubation with Biotin-labeled αmIgM and IgG detection antibodies
- add 100µl/well goat anti-mouse IgM- or IgG-Biotin (1:500 in PBS/BSA[3%]/Tween)
- incubate for 20-30 min at room temperature

Work in parallel:

Staining with SA-AP
- wash the plate 3x with PBS/BSA[3%]/Tween
- add 100µl/well SA-AP (1:3000) 2ml of PBS/BSA[3%]/Tween
- incubate for 20-30 min at RT

Creating the development buffer (1 hr before use)
- add 8ml AMP buffer to 8mg 5-BCI
- heat at 65°C for 20°C
- melt Agarose[3%] in microwave and add melted agarose to AMP buffer+5-BCIP
- incubate at 65°C for 10 min

Agarose gels on the plate
- Wash the plate 3x with PBS/BSA[3%]/Tween
- Add 100µl/well development buffer
- Put the plate in the fridge for 10 min carefully and strictly horizontal, without air bubbles
- Incubate the plate for 2hrs at 37°C

Counting the spots
- Take the plate out of the incubator and keep it in the fridge (for better spot development)
- Count the spots under the inverted microscope.

DNA ELISpots and pre-coating with methyl-BSA

The detection of antigen-specific antibody-secreting cells becomes possible by coating with particular antigen. Some antigens (e.g. dsDNA) do not bind to the plates because of adverse distribution of electric charges within the molecule; therefore a precoating of the plates with methyl-BSA is often mandatory in order to enhance the binding of DNA to the plates. Therefore, prior to coating with DNA itself, the plates were incubated with methyl-BSA for 2 hours at 37°C.

2.6.4 Enzyme Linked Immunosorbent Assay (ELISA)

To detect ovalbumin-specific IgG antibody concentrations in the sera of ovalbumin immunized mice, ovalbumin ELISA was performed. The plates were coated with 1mg/ml ovalbumin, followed by washing and blocking with 3% milk solution for 3 hours at 37°C. Plates were then incubated for 2 hours at 37°C with serial dilutions of blood sera or standards (murine anti-ovalbumin IgG antibodies of a known concentration). Sera were diluted 1:50 and titrated at 1:3 step dilutions. As detection antibody, anti-murine IgG-biotin was used, followed by development with ALP buffer. ELISA plates were analyzed in a Precision Microplate Reader (Emax).

2.6.4.1 ELISA protocol

Coating the plate with ovalbumin
- Coat the plate with 55µl/well of ovalbumin [1mg/ml in PBS]
- Incubate the plate overnight at 4°C ‘or’ 2 hours at 37°C.

Blocking unspecific bindings
- wash 1x with PBS/BSA [3%]
- Add 150µl of milk solution [3%]
- Incubate for 5 hours at room temperature

Incubation with sample, standard and control serum on the plate
- wash the plate 1x with 200µl/well 3% milk
- flick dry and add 200µl /well 3% milk
- Put sample serum (diluted 1:50), control serum and standard in 300µl per well in the first row
- make step dilutions of serum from first row till the last row (1:3)
- incubate the 96 well plate for 2 hours at 5% CO2 / 37°C

Washing the plates
- remove the plate from the incubator
- wash 3x with 3% milk

Incubation with biotin-labeled αmIgG detection antibodies
- add 100µl/well goat anti-mouse IgG-Biotin antibody (1:500 in 3% milk)
- incubate for 2 hours at room temperature

Addition of SA-AP
- Add 100µl of SA-AP solution in each well (diluted 1:3000 in PBS/BSA [3%])
- Incubate at 37°C for 20 minutes
- Wash 3 times with PBS/BSA [3%]

Staining Reaction
- Add 50µl ALP-Buffer to each well using multi-channel pipette
- Allow the development to take place at room temperature and measure the optical density (OD) of the wells using the photometric detector at wavelength of 405 nm.

3 Results

3.1 *Part IA - One week treatment of NZB/W F1 mice with dexamethasone and/cyclophosphamide*

3.1.1 *Splenic long-lived plasma cells are refractory to dexamethasone and/or cyclophosphamide*

First, the effect of dexamethasone and/or cyclophosphamide on splenic plasma cells was examined using flow cytometry. It was reported earlier that splenic plasmablast/plasma cell numbers are nearly 10 times higher in NZB/W mice compared to mice from non-autoimmune strains [47]. It was also shown that splenic short-lived plasmablasts/plasma cells, unlike long-lived plasma cells, are susceptible to cyclophosphamide depletion [39]. Although both cell types express the same surface marker CD138, it was possible to distinguish between them on FACS using BrdU incorporation technique. Therefore, the mice were fed with BrdU continuously for 3 weeks. The distinction was thus made between BrdU+ plasmablasts/plasma cells that had proliferated during the last 3 weeks and BrdU- plasma cells that were formed at least 3 weeks earlier and had not proliferated during this feeding period. The kinetics show that the plasma cells that do not incorporate BrdU during the 3 weeks of feeding can persist longer without proliferation for several more months, and could be referred to as long-lived plasma cells [39].

During the last week of BrdU feeding, mice were treated with dexamethasone (1mg/kg/day) or cyclophosphamide (30mg/kg on 4 days), or a combination of both drugs (Fig. 3). Frequencies of BrdU+ and BrdU- plasma cells in spleen and bone marrow were determined on FACS (Fig.6a) and transformed into absolute numbers per organ for better comparability Splenic BrdU+ plasmablasts/short-lived plasma cells were significantly depleted by dexamethasone *($p<0.005$)* and/or cyclophosphamide *($p<0.0001$)* (3 to 4-fold reduction), whereas BrdU- long-lived plasma cells remained largely unaffected (Fig. 6b). Dexamethasone and cyclophosphamide alone were equally effective in depleting plasmablasts/short-lived plasma cells, and the combination of two drugs was not more effective than the single drugs. Long-lived plasma cells in the spleen, on the other hand, remained refractory to all treatment protocols. These results are similar to earlier findings by Hoyer et al. [39] that splenic long-lived

plasma cells are refractory to treatment by cyclophosphamide. The current results additionally show that therapy resistance of these cells is not limited to the anti-proliferative drug cyclophosphamide, but also applies to the anti-inflammatory drug dexamethasone.

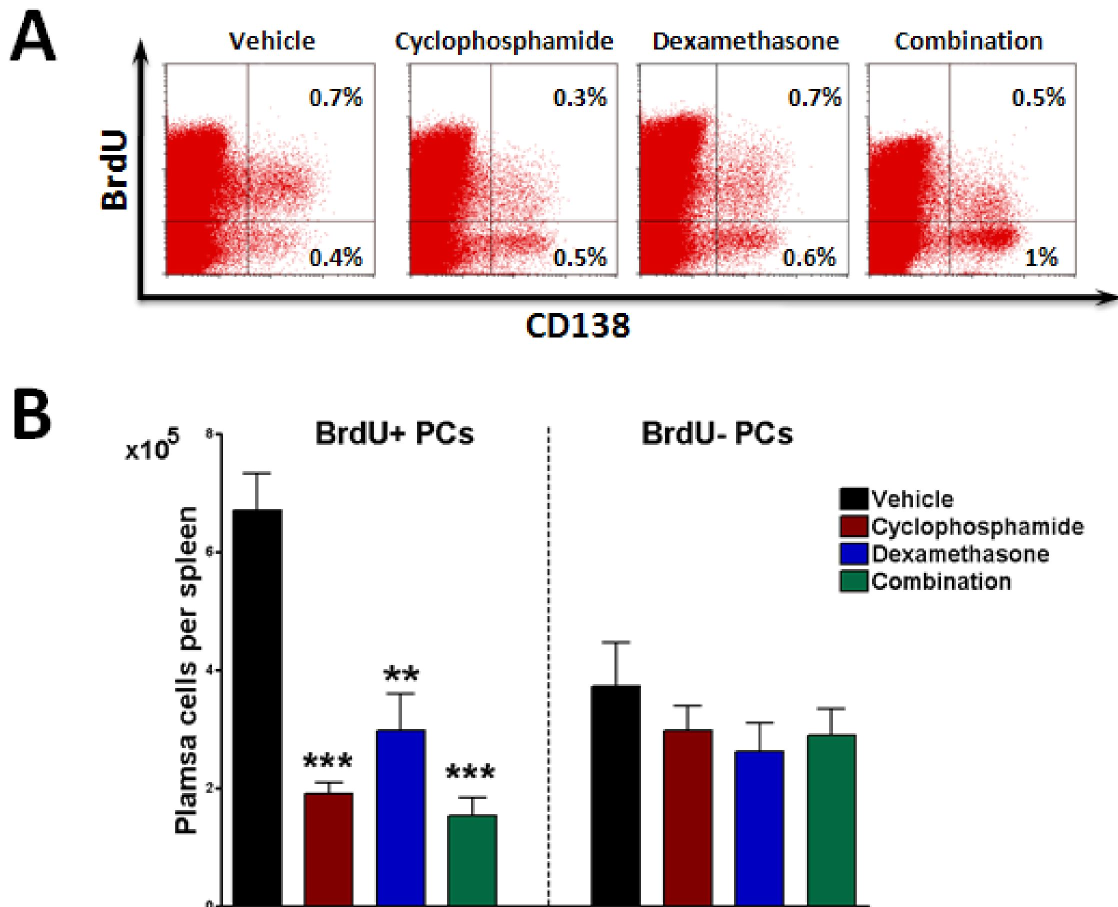

Figure 6: Splenic plasma cells after1 week immunosuppressant treatment. ***(A)*** *Representative FACS dot plots of splenic plasma cells from each treatment group. Splenic plasma cells were determined by the surface expression of CD138. The CD138+ plasma cells that have incorporated BrdU in their DNA are the recently proliferating short-lived plasmablasts/plasma cells, while the BrdU negative ones are long-lived plasma cells which have survived without proliferation for more than 3 weeks.* ***(B)*** *The total short-lived plasmablasts/plasma cells (BrdU+ PCs) and long-lived plasma cell (BrdU- PCs) numbers per organ (spleen) as obtained from their frequencies on FACS and total leukocyte on cell counter (**n**=10). The bars represent mean values with SEM of plasma cells per organ in each treatment group. The asterisk denotes values significantly lower in the corresponding group compared to vehicle treated group (*** $p<0.0001$, ** $p<0.005$). There is a significant reduction of short-lived, but not long-lived plasma cells in the spleens of immunosuppressant treated mice.*

3.1.2 *Dexamethasone and/or cyclophosphamide deplete splenic IgG ASC more effectively than IgM ASC*

ELISPOT assays were performed to determine the total number of IgM and IgG ASCs per spleen. Dexamethasone and cyclophosphamide, alone or in combination, significantly reduced the number of IgM and IgG ASCs in the spleen (Table 1). IgG ASC were more susceptible to the drugs than IgM ASC. There was only a 2-fold reduction in IgM ASCs *($p<0.05$)* compared to a 5-fold reduction in IgG ASCs *($p<0.0001$)*. Despite strong activation of the T-helper cell population in autoimmune lupus and the expected class switch to downstream antibody isotypes, the spleens of treated NZB/W mice contained more IgM than IgG ASCs. Also, most of the refractory splenic plasma cells produced IgM antibodies. This finding is in accordance with earlier observations that most of the long-lived plasma cells in NZB/W mice secrete IgM [39] and with previous reports that serum IgG antibodies are more susceptible to glucocorticoids than serum IgM [88].

Table 1: ***ASCs*** **per organ in different treatment groups compared to vehicle group**

Treatment	ASC per Spleen *($x10^5$)*		*ASC per BM ($x10^5$)*		ASC per Kidney *($x10^5$)*	
	IgM	IgG	*IgM*	*IgG*	IgM	IgG
Vehicle	6.51±1.17	4.78±1.58	*9.39±0.78*	*3.30±0.60*	0.48±0.06	4.21±1.19
Cyclophosphamide	3.16±0.39 *	0.69±0.16 ***	*8.60±0.68*	*2.40±0.37*	0.17±0.03 ***	1.42±0.25 **
Dexamethasone	3.21±0.69 *	0.95±0.16 **	*7.85±0.53*	*2.91±0.46*	0.23±0.04 **	3.45±1.43
Combination	2.74±0.39 **	0.46±0.11 ***	*9.55±1.14*	*3.15±0.73*	0.21±0.03 **	1.97±0.50

Table 1: *Mean and SEM of total IgM and IgG ASC per spleen, bone marrow and kidney obtained by ELISPOT technique (**n**=10 for IgM ASCs and 8 for IgG ASCs). The asterisks denote values that are significantly lower in the treated group as compared to vehicle group (* $p<0.05$, ** $p<0.005$, *** $p<0.0005$).*

3.1.3 *BrdU+ and BrdU- bone marrow plasma cells are not affected by immunosuppressive drugs*

Nearly 80% of the bone marrow plasma cells were BrdU-negative, i.e. did not proliferate during the 3 weeks of BrdU feeding (Fig. 7a). This result suggests that, despite chronic autoimmunity, the great majority of bone marrow plasma cells in NZB/W mice are non-proliferating cells with a relatively slow turn-over. Though this result does not allow for definitive half-live estimation, it suggests that in NZB/W bone marrow, plasma cells can survive for several weeks to months, similar to the bone marrow plasma cells in non-autoimmune mouse strains [89,90]. Non-proliferating BrdU- bone marrow plasma cells were refractory to the anti-proliferative cyclophosphamide (as expected) as well as to dexamethasone. However, unlike BrdU+ plasma cells in the spleen, which were significantly depleted in all treatment groups, those in the bone marrow were not significantly reduced by any of the treatment protocols. Cyclophosphamide alone did, however, induce a small but statistically insignificant *(P=0.08)* reduction tendency in BrdU+ bone marrow plasma cell population (Fig. 7b). However, the group receiving the combination of both drugs did not show such a tendency.

The resistance of bone marrow plasma cells to immunosuppressive drugs demonstrated by FACS analysis was also apparent in bone marrow plasma cells analyzed by ELISPOT. Both IgM and IgG ASCs remained resistant to cyclophosphamide and dexamethasone (Table 1). As in the spleen, there were more IgM ASCs than IgG ASCs in the bone marrow, and unlike the spleen, both IgM and IgG ASCs in the bone marrow were resistant (Table 1).

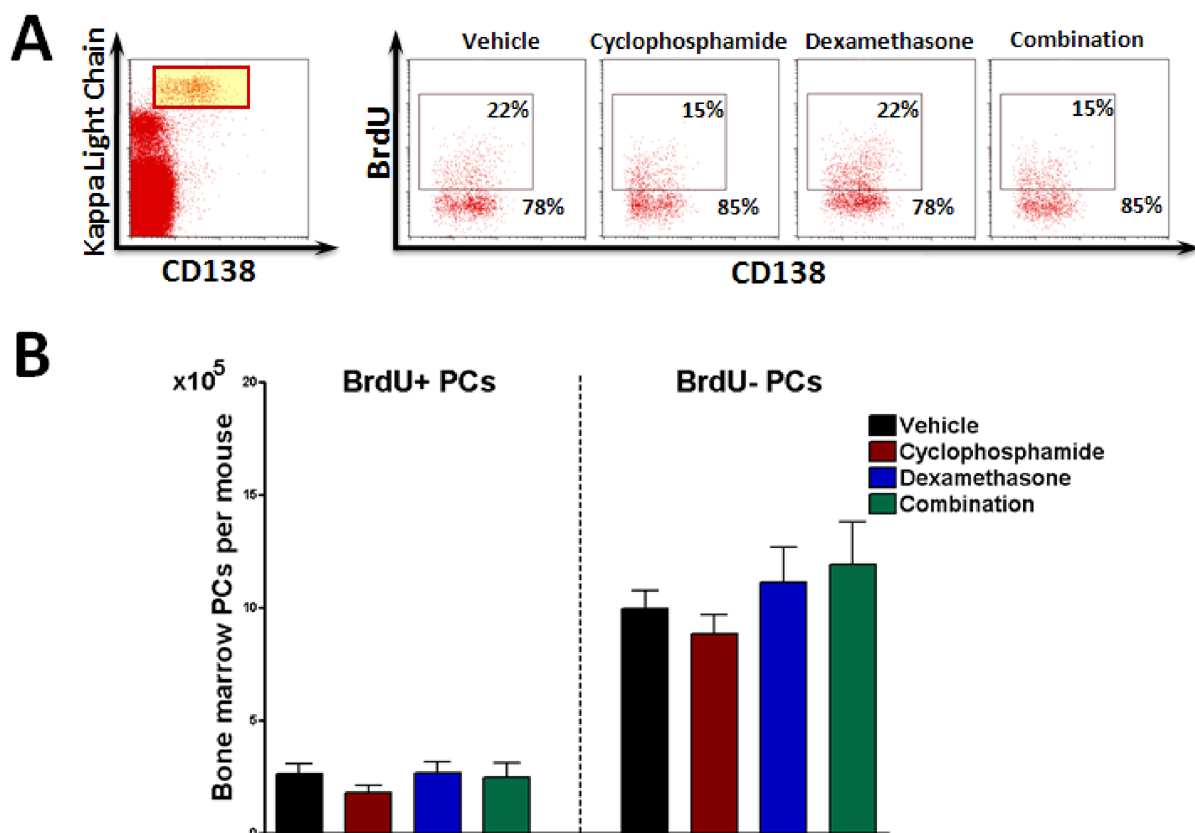

Figure 7: Bone marrow plasma cells after 1 week immunosuppressant treatment. (A) *Representative FACS dot plots of bone marrow plasma cells, as determined by CD138 and kappa light chain staining. The CD138+, Kappa light chain^{high} expressing plasma cells were analyzed for BrdU incorporation.* ***(B)*** *Absolute counts of BrdU+ and BrdU- plasma cells per mouse bone marrow. The bars represent mean and SEM of 10 mice per treatment group. Bone marrow plasma cells remain unaffected in all treatment groups.*

3.1.4 Dexamethasone and cyclophosphamide are effective in spleen and bone marrow

Dexamethasone and cyclophosphamide did not reduce bone marrow plasma cell numbers. To determine (as a control) whether the drugs were effective in the bone marrow, B220+ B-cells and CD4+/CD8+ T-cells in bone marrow and spleen were analyzed after the treatment. As expected, B and T lymphocytes in the spleen were reduced significantly after treatment with both dexamethasone and cyclophosphamide. There was approximately a 3-fold reduction in B220+ B-cells (Fig. 8c) and 2-fold reduction of CD4 T-cells in spleens (Fig. 8a), while the CD8 T-cells remained relatively unresponsive (Fig. 8b).

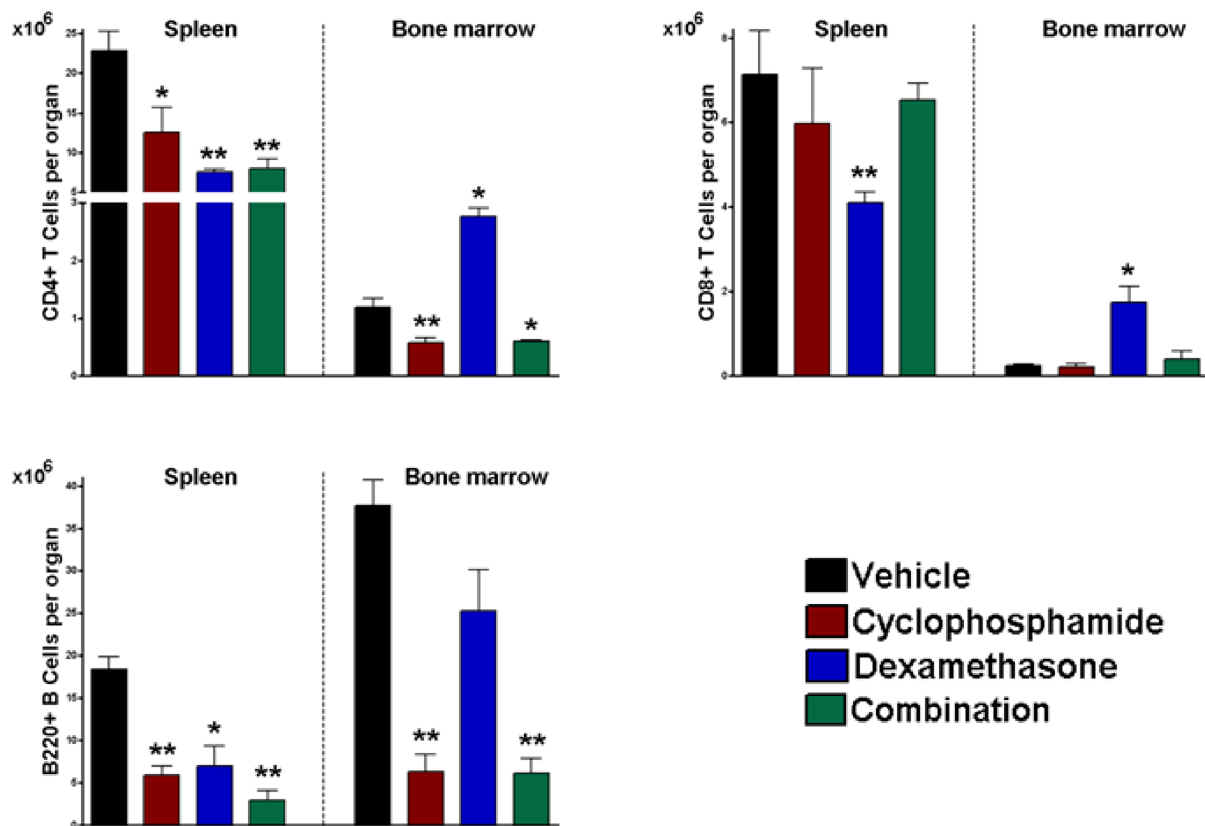

Figure 8: T- and B- lymphocytes in spleen and BM after 1 week immunosuppressant treatment. *The figure shows total numbers of* ***(A)*** *CD4,* ***(B)*** *CD8 and* ***(C)*** *B220 cells per spleen and bone marrow vehicle, cyclophosphamide and/dexamethasone treated mice. The bars represent mean and SEM values of T lymphocytes (n=5, Mann-Whitney test) and B lymphocytes (n=3, Unpaired t test) per spleen and bone marrow in each treatment group. Asterisk denotes values significantly lower/higher in corresponding treatment group compared to the vehicle group. (* $p<0.05$, ** $p<0.005$, *** $p<0.0005$).*

In the bone marrow, cyclophosphamide was strongly effective on the lymphocytes, causing an approximately 6- to7-fold reduction of B220+ cells *($p<0.005$)* (Fig. 8c) and 2-fold reduction of bone marrow CD4+ T-cells *($p<0.005$)* (Fig. 8a). However, bone marrow CD8+ T-cells were resistant to cyclophosphamide (Fig. 8b). Mice treated with dexamethasone alone showed an insignificant reduction of B220+ cells *($p=0.08$)* in this organ. Moreover, dexamethasone caused a significant increase in numbers and frequencies of CD4+ and CD8+ T-cell in bone marrow (Fig. 8a and 8b). The effect of combination drug therapy on bone marrow B and T lymphocytes was similar to that of cyclophosphamide alone. These results demonstrate that cyclophosphamide and dexamethasone were active in the bone marrow.

3.1.5 Inflamed kidneys contain mainly IgG ASCs that are partly susceptible to cyclophosphamide but not to dexamethasone

With increasing age and inflammation, large numbers of ASCs accumulate within the kidneys of NZB/W mice *(Panne et al., Manuscript in preparation)*. Interestingly, in contrast to the spleen and bone marrow, the inflamed kidneys of 6 month old NZB/W mice suffering from full blown disease contain much higher frequency of IgG ASCs than IgM ASCs. There was approximately a 2-fold reduction of IgM ASCs *($p<0.05$)* in the kidneys of mice treated with cyclophosphamide, dexamethasone or a combination of both drugs (Table 1). However, IgG ASCs were affected only in kidneys treated with cyclophosphamide alone, which caused a 2-fold reduction in its numbers. Dexamethasone had no significant effect on IgG ASCs in this inflamed organ. Notably, nearly half of the kidney plasma cells were resistant to all immunosuppressive treatment regimes indicating their longevity.

3.1.6 Bone marrow as the major site and inflamed tissues as the neglected site for immunosuppressant resistant anti-DNA antibody production

Antibodies binding to double stranded (ds) DNA are a hallmark of human and murine SLE. ELISPOT analysis was performed to determine the numbers of anti-DNA ASCs per organ of mice in the vehicle and immunosuppressive treated groups. Equal numbers of anti-DNA ASCs reside in the spleens and bone marrow of 5 month old NZB/W F1 mice (Table 2), while the inflamed kidneys contained approximately 10 times fewer anti-DNA ASCs. Nearly all renal anti-DNA ASCs were IgG producing cells, but huge inter-individual variations in total counts were observed. IgM anti-DNA ASCs were nearly absent from the kidneys.
Within the spleen and bone marrow, the frequency of IgM anti-DNA ASCs was similar to that of IgG anti-DNA ASCs (Table 2). All the treatment protocols used in this study significantly depleted splenic autoreactive ASC of both isotypes to the same extent (5 to6 fold). However, bone marrow IgM and IgG anti-DNA ASCs remained refractory to cyclophosphamide and/or dexamethasone. These results are in accordance with the observation that BrdU+ splenic plasma cells are affected by the treatments while bone marrow plasma cells are completely resistant.

In contrast to the bone marrow and spleen, the numbers of IgG anti-DNA ASCs in the kidneys were about 9 times higher than the IgM anti-DNA ASCs (Table 2). Cyclophosphamide led to a considerable decrease in both anti-DNA ASC populations in the kidney (Table 2). However, the renal anti-DNA ASC populations showed huge variations between individual mice within each treatment group, making an interpretation of these results difficult. In any case, it is worth noting that a substantial population of anti-DNA ASCs in this inflamed organ was refractory to dexamethasone treatment. These results demonstrate that inflamed tissues can maintain autoreactive anti-DNA ASCs, many of which survive glucocorticoid therapy.

Table2. αDNA ASCs per organ in different treatment groups compared to vehicle group

Treatment	αDNA ASC per Spleen		*αDNA ASC per Bone marrow*		αDNA ASC per Kidney	
	IgM	IgG	*IgM*	*IgG*	IgM	IgG
Vehicle	5282 ±1318	7815 ± 3640	*9381 ± 1166*	*5934 ± 537*	63 ± 26	1477 ± 1060
Cyclophosphamide	1345 ± 287 **	1000 ± 185 ***	*8240 ± 1158*	*5410 ± 818*	13 ± 5	38 ± 19
Dexamethasone	1255 ± 163 ***	1275 ± 325 **	*9378 ± 1329*	*5793 ± 526*	29 ± 11	317 ± 226
Combination	850 ± 135 ***	563 ± 107 ***	*6757 ± 1051*	*4509 ± 676*	17 ± 9 *	70 ± 40

Table 2: *Mean and SEM of anti-DNA IgM and IgG ASCs per spleen, bone marrow and kidneys are shown (n=10 for each group). The asterisks denote significant depletion of ASCs in corresponding treatment groups compared to vehicle injected group (* p<0.05, ** p<0.005, *** p<0.0001).*

3.2 *Part I B – One month treatment of NZB/W F1 mice with prednisolone*

Background

Since one week glucocorticoid treatment with 1mg/kg dexamethasone did not affect long-lived plasma cells in NZB/W mice, it was investigated if an extended period of treatment with glucocorticoids would exert stronger effects on these cells. To this end, the NZB/W F1 mice that had developed heavy proteinuria were treated with two different doses of prednisolone. The mice received either 3mg/kg or 10mg/kg of prednisolone for a period of 4 weeks. Powdered prednisolone was dissolved in ethanol/peanut oil such that the desired dose of 3 or 10mg/kg was obtained in 200µl of the final solution. 200 µl of drug solution was then administrated per oral, with a gastric gavage, every morning for a period of 4 weeks. The prednisolone treatment was started when the mice were 23-24 week of age and continued till they reached 27-28 week of age. BrdU feeding was started 2 weeks before the beginning of prednisolone treatment and continued through the period of treatment till the end of experiment (Fig. 4). BrdU, as stated earlier, helps us to discriminate between short-lived and long-lived plasma cells on FACS. All the selected mice had high proteinuria, ranging from 300mg/dL to more than 2000mg/dL. At the end of the 4 weeks of treatment, the proteinuria was recorded before sacrificing them by cervical dislocation and their lymphoid organs were analyzed to assess the effects of prolonged glucocorticoid treatment.

It should be noted that the experiment was started with 5 mice per treatment group. However, by the end of the experiment, some mice died due to progressing disease and also probably because of prolonged treatment with extremely high doses of glucocorticoids. Though each treatment group contained 5 mice at the beginning of experiment, towards the end, 3 mice remained alive in the control group, 4 mice in prednisolone 3mg/kg group and only 2 in prednisolone 10mg/kg group *(n=3, 4 and 2 for vehicle, pred 3mg/kg and 10mg/kg respectively)*. Because of increased mortality in vehicle and high dose prednisolone group, sufficient numbers of mice were not available for determining statistical significances.

3.2.1 Long-lived plasma cells in spleen are refractory to both 3mg/kg and 10mg/kg prednisolone treatment

The plasma cells in spleens of NZB/W mice treated with 3mg/kg and 10mg/kg prednisolone for 4 weeks were analyzed by FACS and ELISPOT. As mentioned earlier, BrdU incorporation into CD138+ plasma cells was measured on FACS in order to discriminate between short-lived and long-lived plasma cells. Since the mice were fed BrdU continuously for a period of 6 weeks before analysis, all the proliferating plasmablast and short-lived plasma cells should stain positive for BrdU+ on FACS and non-proliferating long-lived plasma cells negative. The class of antibodies produced by these plasma cells was also determined using ELISPOT technique.

3.2.1.1 FACS analysis:

Prednisolone depleted splenic short-lived plasmablasts/plasma cells by approximately 2-fold and 3-fold in 3mg/kg and 10mg/kg treatment groups, respectively. However, BrdU- long-lived plasma cells remained stable in both treatment groups (Fig. 9). This result is similar to the result in the previous experiment where 1mg/kg dexamethasone treatment for a duration of 1 week reduced short-lived but not long-lived plasma cells. The results obtained here show that prolonging the duration of high dose glucocorticoid treatment up to 4 weeks still does not affect long-lived plasma cells.

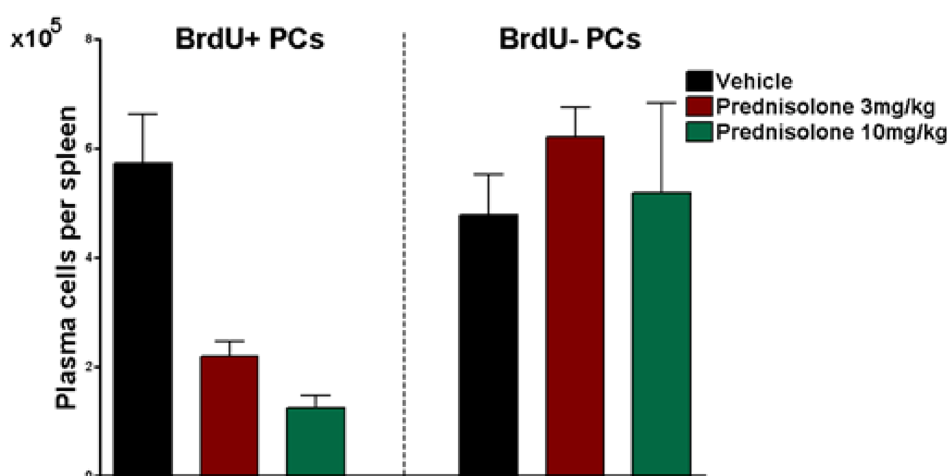

Figure 9: Splenic plasma cells after 4 week prednisolone treatment*. The total numbers of short-lived plasmablast/plasma cells (BrdU+ PCs) and long-lived plasma cell (BrdU- PCs) per spleen as obtained from their frequencies on FACS and leukocyte count on CASY cell counter. The bars represent mean with SEM values of plasma cells per spleen in each treatment group. (n=3 for vehicle, 4 for prednisolone 3mg/kg and 2 for prednisolone 10mg/kg injected mice)*

3.2.1.2 ELISPOT analysis:

10mg/kg prednisolone caused a 3-fold reduction of IgM ASCs, while 3mg/kg prednisolone did not reduce IgM ASCs considerably. However, both doses were extremely effective in depleting class switched IgG ASCs (Fig. 10). Compared to vehicles, 3mg/kg and 10mg/kg prednisolone treated groups contained 20 and 50 times lower counts of IgG ASCs in their spleens (Fig. 10). This also confirms the earlier observation that in NZB/W mice, IgG ASCs are more susceptible to immunosuppressive treatment than IgM ASCs. These results imply that most of the long-lived plasma cells in spleens of this hyperimmune NZB/W mouse strain produce IgM antibodies.

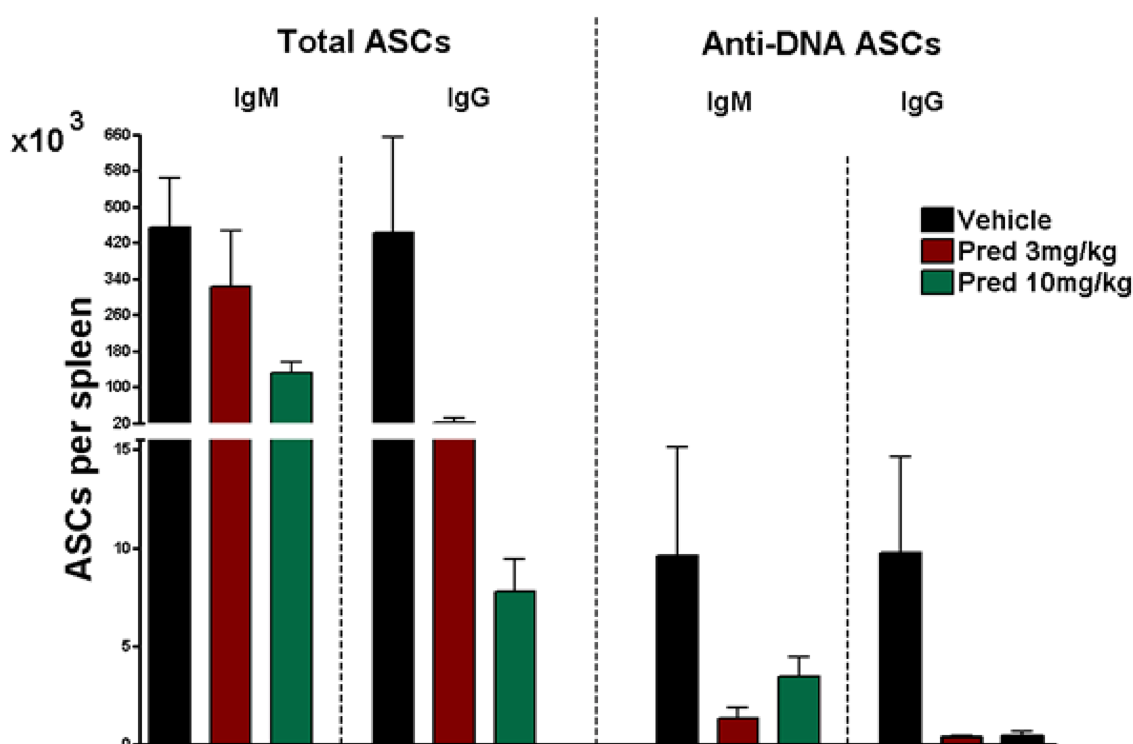

Figure 10: Total and anti-DNA ASCs per spleen after 4 week prednisolone treatment. *The bars represent mean and SEM of total and anti-DNA IgM/IgG ASCs per spleen as obtained by ELISPOT technique (n=3 for vehicle, 4 for prednisolone 3mg/kg and 2 for prednisolone 10mg/kg injected mice).*

Anti-DNA autoantibody secreting plasma cells were also analyzed. Similar to total IgG ASCs in the spleen, IgG anti-DNA ASCs were more strongly reduced than IgM anti-DNA ASCs. There was a 3-5 fold reduction of IgM anti-DNA producing ASCs, while IgG anti-DNA producing ASCs were reduced 15-fold in both treatment groups (Fig. 10).

3.2.2 Bone marrow plasma cells are refractory to 4 week treatment with 3 and 10mg/kg prednisolone

3.2.2.1 FACS analysis:

The bone marrow BrdU+ and BrdU- plasma cells were analyzed on FACS to study their response to prolonged prednisolone treatment. Again, similar to one week dexamethasone experiment, neither BrdU+ nor BrdU- bone marrow plasma cells were affected by either dose of prednisolone after 4 weeks of treatment (Fig. 11). This is in contrast to BrdU+ plasma cells in the spleens of the same mice that were reduced upon prednisolone treatment. Together with the results of one week dexamethasone experiment, the results obtained here demonstrate the refractory nature of bone marrow plasma cells to both prolonged and short term treatment with glucocorticoids.

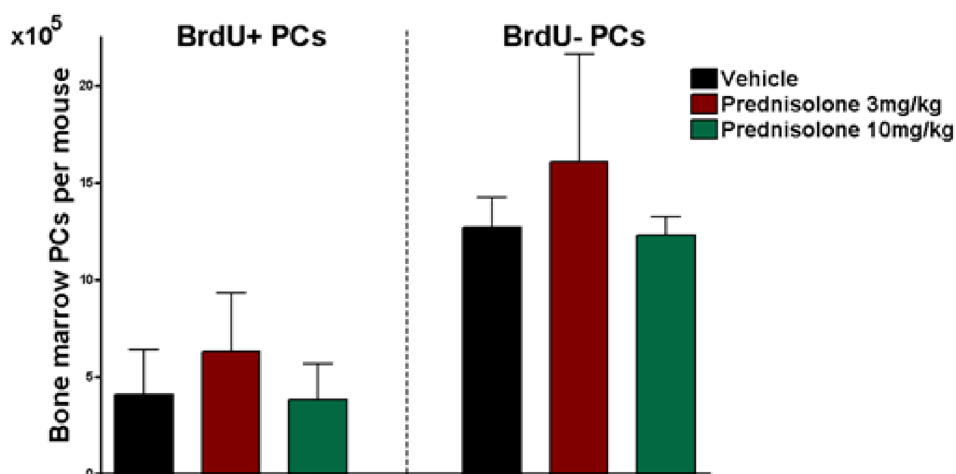

Figure 11: Bone marrow plasma cells after 4 week prednisolone treatment. *Bone marrow plasma cells were determined by CD138 and kappa light chain staining. The CD138+, Kappa light chain^{high} expressing plasma cells were analyzed for BrdU incorporation. The bars represent mean and SEM of BrdU+ and BrdU- plasma cells per mouse bone marrow. (n=3 for vehicle, 4 for prednisolone 3mg/kg and 2 for prednisolone 10mg/kg injected mice).*

3.2.2.2 ELISPOT analysis:

Reflecting the bone marrow plasma cell results from FACS, the bone marrow plasma cells in ELISPOT analysis were not affected either. Neither 3mg/kg nor 10mg/kg prednisolone treatment affected IgM and IgG ASCs, despite 4 weeks of treatment (Fig. 12). Interestingly, and in accordance with the previous results, the findings presented here confirm that the bone marrow of NZB/W mice contain approximately 3 times higher numbers of IgM ASCs than IgG ASCs.

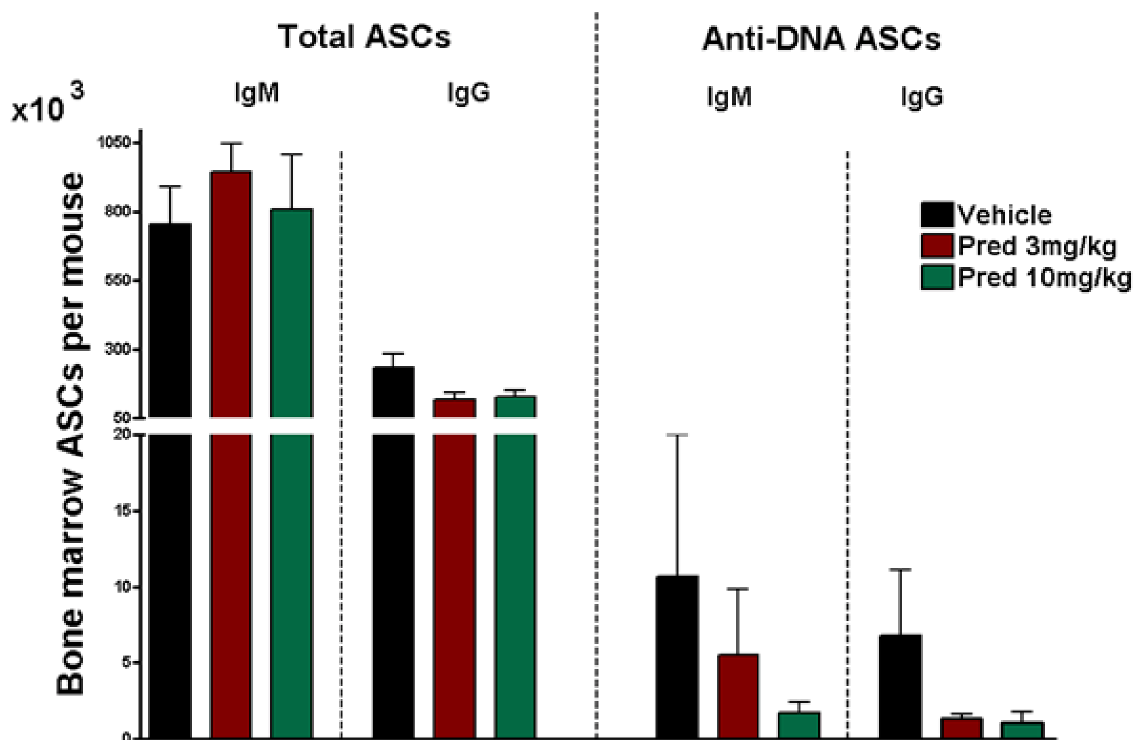

Figure 12: Total and anti-DNA ASCs per bone marrow after 4 week prednisolone treatment. *Bars representing mean and SEM of total and anti-DNA IgM/IgG ASCs per bone marrow as obtained by ELISPOT technique (n=3 for vehicle, 4 for prednisolone 3mg/kg and 2 for prednisolone 10mg/kg injected mice).*

The bone marrow also contained similar numbers of anti-DNA IgM and IgG producing ASCs (Fig. 12). There is an apparent reduction in the mean counts of anti-DNA ASCs of both classes after treatment with prednisolone. Although it could not be excluded here that high dose glucocorticoids are effective on autoantigen specific ASCs in the bone marrow, the more likely reason for this particular observation is one outlier in the vehicle group that varied extremely from the remaining mice. This outlier alone is also responsible for the big variation present in the vehicle group (of both antibody classes). Without this outlier, the bone marrow

anti-DNA ASC count in vehicle group is similar to prednisolone treated groups. Moreover, it is also in strong contrast to the total IgM and IgG ASCs in the same organ which remained unaffected in both treatment groups. It was observed in the previous experiment that autoreactive plasma cells are extremely stable in the bone marrow. As stated earlier, very few mice were available for analysis in each group. A conclusive interpretation of this data is not possible due to insufficient numbers of mice surviving till the end of treatment period.

3.2.3 Prednisolone reduced splenic B-T lymphocytes and bone marrow B lymphocytes in a dose dependent manner

The effect of 4 week prednisolone treatment on total leukocyte count and on B- and T-lymphocyte count in spleen and bone marrow was analyzed. There was a 2-4 fold decrease in leukocyte counts of spleen, the strongest being in 10mg/kg prednisolone group (data not shown). Also, a strong reduction of B lymphocytes and CD4+ T lymphocytes was observed (Fig. 13). The higher dose caused stronger reduction, with approximately 6-fold reduction in B220+ B-cell count and 3-fold reduction in CD4+ T-cells count. However, CD8+ T-cells were not affected by these treatment protocols (Fig. 13). The results are in accordance with the previous experiment with 1 week dexamethasone treatment, where both B220+ and CD4+ cells were affected by glucocorticoids but CD8+ T-cells remained resistant.

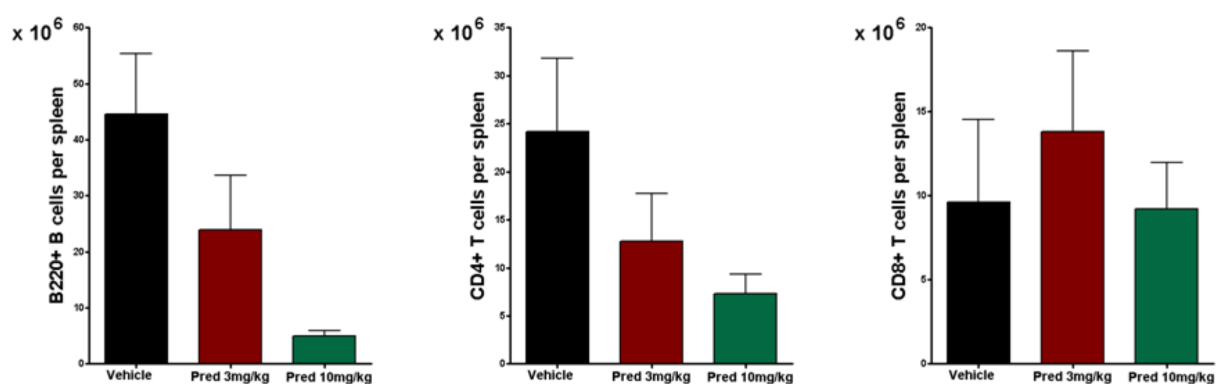

Figure 13: Splenic B- and T- lymphocytes after 4 week prednisolone treatment. *The figure shows total numbers of B220+, CD4+ and CD8+ T-cells per spleen in different treated groups. The bars represent mean and SEM values of indicated cell types in vehicle (n=3), prednisolone 3mg/kg (n=4) and prednisolone 10mg/kg (n=2) injected mice.*

In the bone marrow, there was strong dose dependent depletion of B220+ cells. There was a 2-fold and 10-fold reduction of bone marrow B-cells in 3mg/kg and 10mg/kg prednisolone treatment group respectively (Fig. 14). This is a much stronger degree of reduction of bone marrow B-cells, compared to 1 week dexamethasone experiment. Also, both CD4+ and CD8+ T-cells in the bone marrow remain unaffected regardless of the prednisolone dose administered (Fig. 14). This observation is contrary to the previous experiment and other reports, where treatment with glucocorticoids caused significant increase in bone marrow CD4 and CD8 counts as a result of T-cell accumulation in this organ.

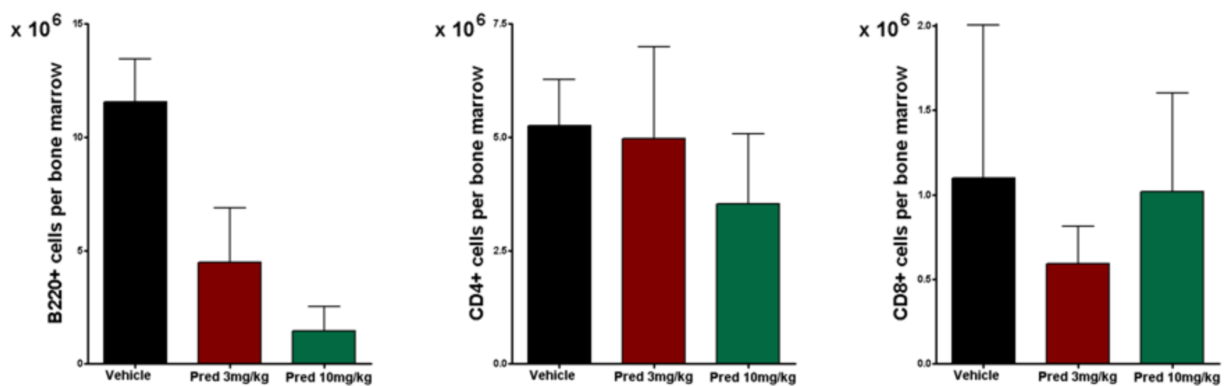

Figure 14: Bone marrow B- and T-lymphocytes after 4 week prednisolone treatment. *The figure shows total numbers of B220+, CD4+ and CD8+ T-cells per bone marrow in different treated groups. The bars represent mean and SEM values of indicated cell types in vehicle (n=3), prednisolone 3mg/kg (n=4) and prednisolone 10mg/kg (n=2) injected mice.*

3.2.4 Long term prednisolone causes a reduction in lymphoid organ weight and size

Heavy dose glucocorticoids are known to cause reduction in weight and size of lymphoid organs [91]. The analysis shows a dose dependent decrease in splenic and thymic weights of prednisolone treated mice. The weight reduction in these lymphoid organs was 2-3 folds and correlated with reduced organ size and cell count as well (Fig. 15). A reduction in the weight of adrenal glands was also noticed. However, majority of mice treated with 10mg/kg prednisolone got sicker and died before the end of experiment. Importantly, in spite of all the strong effects on the parameters observed in this experiment, there was no effect on the proteinuria after treatment with both prednisolone doses for 4 weeks (data not shown).

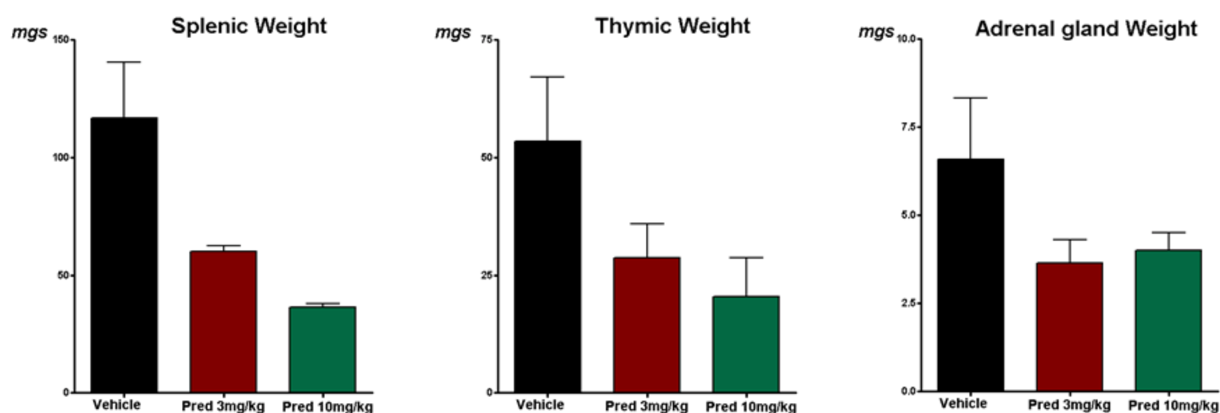

Fig 15: Lymphoid organ and adrenal gland weights after 4 week prednisolone treatment. *The figure shows differences in the weights of spleen, thymus and adrenal glands of NZB/W mice from different treatment groups. The bars represent mean and SEM of weights of organs indicated.*

3.3 *Part II – Depletion of CD4+ T-cells and its effects on plasma cells in NZB/W F1 mice*

Background: Activation of mature B-cells by antigens in peripheral lymphoid organs results in B-cell differentiation into plasmablasts/short-lived plasma cells, which produce low affinity IgM antibodies. However, if the B-cells are activated in the context of CD4+ T-cell help, they form germinal centers [20]. The hallmark of germinal center reaction is rearrangement of antibody encoding genes and somatic hypermutations, resulting in production of affinity matured antibodies that bind with high affinity to their cognate antigens [20,92,93]. Plasma cells exiting germinal center reactions may also have undergone class switch recombination reaction to produce class switched/downstream antibodies [94]. Bone marrow long-lived plasma cells are such high affinity selected antibody secreting cells, suggesting that they arise in germinal centers [29]. Since the evidence indicates that majority of bone marrow long-lived plasma cells are formed in germinal center reactions, and since CD4+ T-cell help is a prerequisite for germinal center formation, the effect of CD4+ T-cell depletion on production of both splenic and bone marrow long-lived plasma cells in NZB/W mice was analyzed.

Before proceeding with CD4 depletion and analyzing its effect on long-lived plasma cell formation, it was required to know the age at which long-lived plasma cells are formed in NZB/W mice. Experiments to determine the kinetics of long-lived plasma cell formation in NZB/W mice were done earlier in our group. In these experiments, NZB/W mice aged 3, 5, 7 and 9 weeks were fed BrdU for a 2 week period, at the end of which spleen and bone marrow plasma cells (BrdU+ and BrdU-) were analyzed. The results from these experiments demonstrated that majority of splenic long-lived plasma cells are formed by 9 weeks of age (Voigt, C.: doctoral thesis, Charité – Universitätsmedizin Berlin, 2008), after which their numbers remain stable (comparable to long-lived plasma cell count in older mice) [39]. In the bone marrow, however, accumulation of long-lived plasma cells is much slower and takes place over a longer period of time. At the age of 9 weeks, when majority of splenic long-lived plasma cells are already formed, bone marrow still has only half as many long-lived plasma cells as 23 week old NZB/W mouse.

Interestingly, in both spleen and bone marrow, the numbers of long-lived plasma cells increased 4-5 times between the age of 3 and 9 weeks. Since the majority of splenic and nearly 50% of bone marrow long-lived plasma cells are formed by 9 weeks of age, and since there is at least 4 times increase in long-lived plasma cell numbers between 3 and 9 weeks of age, this age was chosen for depletion of CD4+ T-cells and for checking its affects on long-lived plasma cells.

3.3.1 In vivo depletion of CD4+ T-cells from NZB/W F1 mice

In order to assess the role of T-cell help in long-lived plasma cell formation, CD4+ T-cells were depleted from young NZB/W F1 mice using a monoclonal anti-murine CD4 antibody (clone GK1.4). Within 2 days after a single injection of 1mg of anti-CD4 antibody, CD4+ T-cells are depleted by over 90% from the blood, spleen and lymph nodes [95]. However, same results were obtained after injecting 250µg of anti-CD4 antibody, which was as effective as injecting 1mg in depleting CD4+ T-cells (data not shown). Over 93% of splenic (Fig. 16) and 98% of lymph node CD4 T-cells were depleted with this protocol. The remaining 7% and 2% of spleen and lymph node CD4+ T-cells, respectively, remained refractory irrespective of the dose and duration of anti-CD4 treatment. These cells were not refractory due to an inadequate therapy, as the used doses caused saturated binding of all target epitopes on the surface of CD4 cells. This was demonstrated by inability of fluorochrome conjugated GK1.5 monoclonal antibody (depleting antibody) to detect any CD4 cells during analysis, while another fluorochrome conjugated monoclonal anti-CD4 antibody (YTS 191) recognized the free CD4 epitopes available on these refractory cells. In contrast to spleen and lymph nodes, CD4 depletion is much less effective in bone marrow, where 35% of CD4+ cells were still detectable with monoclonal YTS 191 (Fig. 16). However, the presence of refractory CD4 cells in the bone marrow does not affect the experimental outcome, since the intention is to analyze the effect of CD4 cells on induction and formation of long-lived plasma cells. It is known that the plasma cells found in bone marrow are actually produced by activated or memory B-cells in spleen and lymph nodes. Since the formation of (long-lived) plasma cells occurs in secondary lymphoid organs [4,29] and not in the bone marrow, CD4 cells in the bone marrow should not affect the results.

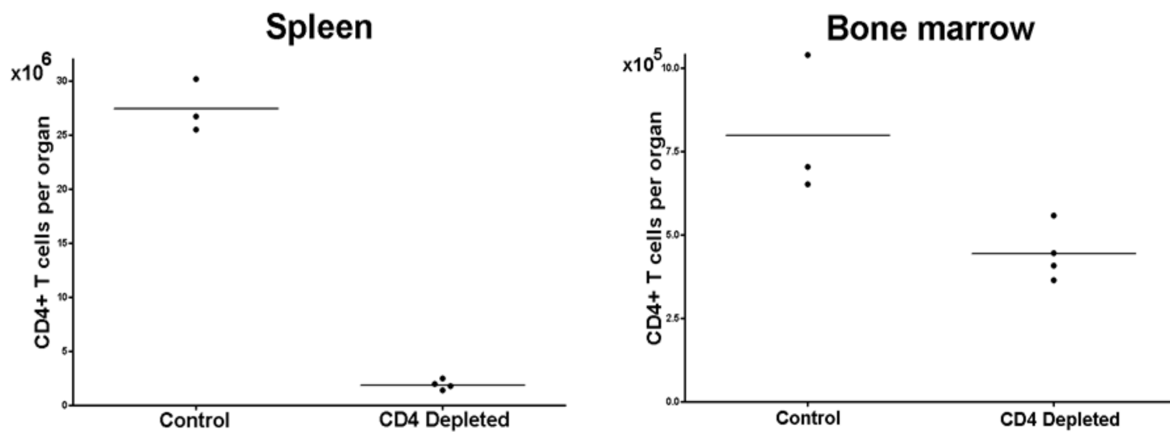

Figure 16: Depletion of CD4+ T-cell from spleen and bone marrow. *The figure shows total CD4+ T-cell count per organ in vehicle and anti-CD4 mAb injected mice. There is a marked reduction of CD4+ T-cells in the spleens, while CD4+ T-cells in the bone marrow are reduced to a lesser extent.*

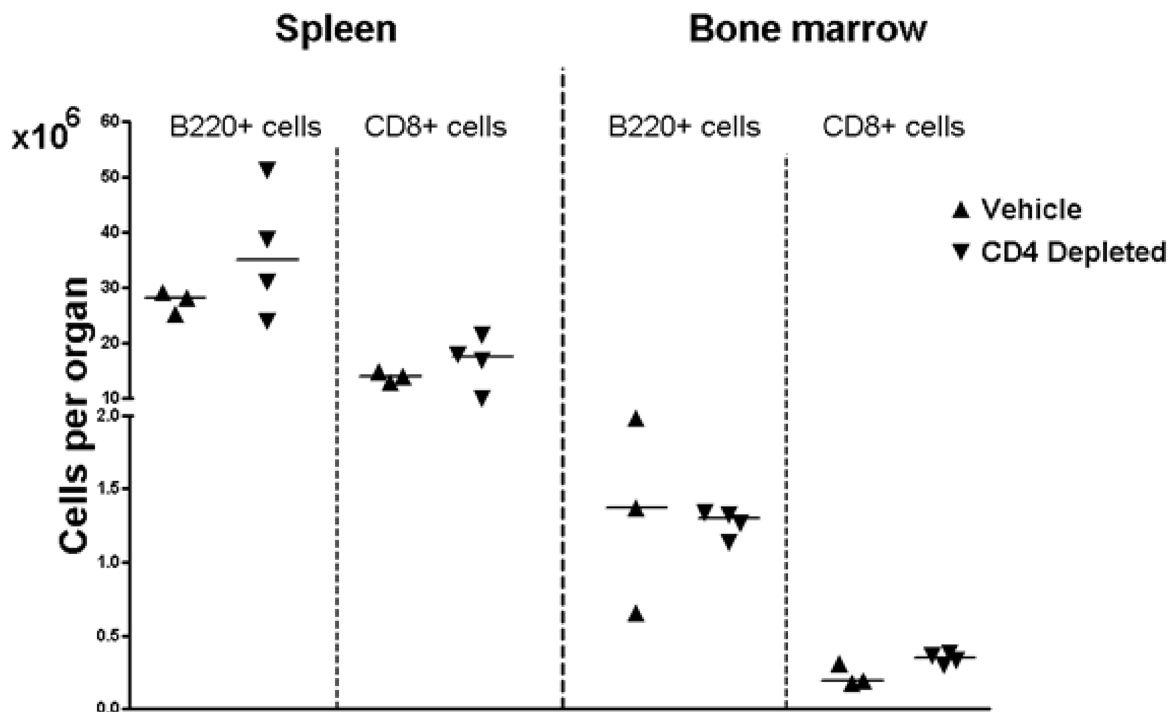

Figure 17: B-cells and CD8+ T-cells after CD4 depletion. *The figure shows total B220+ cell and CD8+ cell count per organ in vehicle and anti-CD4 mAb injected mice. Non-CD4+ lymphocytes were not affected by anti-CD4 mAb injections.*

The B220+ B-cell count and CD8+ T-cell count in the spleen and bone marrow remained totally unaffected after 6 weeks of continuous CD4 depletion (Fig. 17). Therefore, it could be fairly stated that the injected anti-CD4 antibody did not affect other, CD4 non-expressing lymphocytes of the adaptive immune system.

3.3.2 Formation of long-lived plasma cells in very young NZB/W mice is independent of T-cell help

Since the majority of splenic long-lived plasma cells and a considerable proportion of bone marrow long-lived plasma cells in NZB/W mice are formed before 9 weeks of age, CD4+ T-cells were depleted in these mice from 3 till 9 weeks of age. Beginning at the age of 3 weeks, the mice received twice weekly either 250µg of anti-CD4 depleting antibody (GK1.5) in 200 µl of PBS or 200 µl of vehicle (PBS) through intraperitoneal injections. In order to discriminate between short-lived plasmablasts/plasma cells and long-lived plasma cells, the mice were fed BrdU for a period of 2 weeks before analysis. In this protocol, all the long-lived plasma cells that were formed before 7 weeks of age will remain BrdU-negative at the time of analysis, while the recently proliferating plasmablasts and newly formed long-lived plasma cells during age 7-9 weeks will have taken up BrdU. Therefore, in this experimental setup, only the long-lived plasma cells that formed before the age of 7 weeks are focused upon. In order to control the abrogation of T-cell help after CD4 depletion, the mice were immunized with T-dependent antigen ovalbumin (100µg ovalbumin in 100µl alum) 10 days before analysis. If the T-cell help is efficiently abrogated, these mice should not develop serum anti-ovalbumin IgG immunoglobulins in response to the ovalbumin immunizations.

Depletion of CD4 cells had no influence on the development of plasma cell compartment in spleens and bone marrow. Neither the total leukocyte count nor total plasma cell count in spleen and bone marrow of CD4 depleted mice changed after anti-CD4 antibody injections. BrdU+ and BrdU- plasma cells of the spleen and bone marrow are comparable in vehicle and CD4 depleted mice, except for a tendency for the bone marrow BrdU+ plasma cells to decrease *(p=0.06)*(Fig. 18).

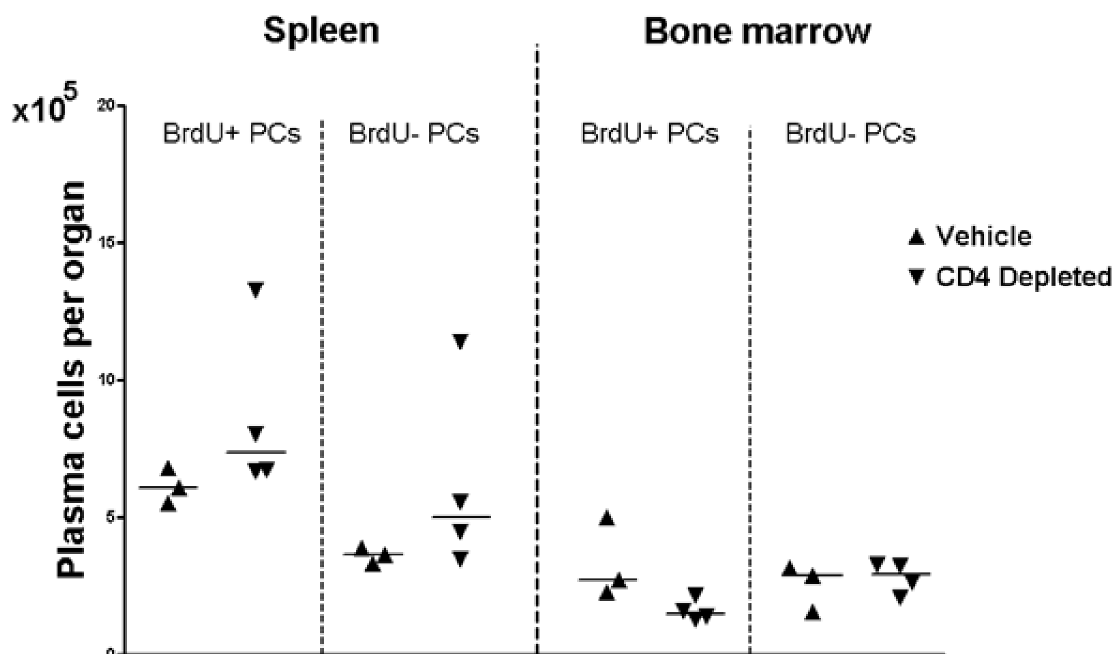

Figure 18: BrdU+ and BrdU- plasma cells in spleen and bone marrow after CD4 depletion. *The figure shows total numbers of BrdU+/BrdU- plasma cells in spleen and bone marrow of vehicle and anti-CD4 mAb injected mice. Except for a reduction tendency of BrdU+ plasmablasts in bone marrow (p=0.06), there are no differences in vehicle (n=3) and anti-CD4 mAb (n=4) injected mice.*

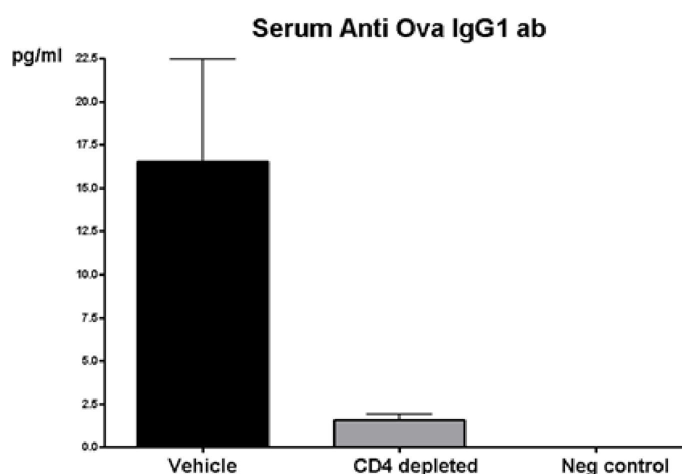

Figure 19: Serum levels of anti-ova IgG1 antibody after CD4 depletion. *The figure shows concentration of anti-ovalbumin IgG antibodies in serum of vehicle injected, anti-CD4 mAb injected and unimmunized NZB/W mice determined by ELISA technique. The bars represent mean and SEM of antibody concentrations. Clearly, CD4 depleted mice failed to develop an optimum anti-ovalbumin IgG antibody response.*

Abrogation of T-cell help in CD4 depleted mice was confirmed by following the IgG antibody response to T-dependent antigen ovalbumin, which is the major class of antibody responding to this antigen. The CD4 depleted mice failed to develop optimal antibody response to immunization with this T dependent antigen (Fig. 19). The serum IgG titers in CD4 depleted mice were 10 times lower than vehicle injected mice.

Since the depletion of CD4 T-cells was started at an age when long-lived plasma cell compartment in these mice just begins to form and was continued till it is fully and partially established in spleen and bone marrow respectively, the observation of no obvious influence on the formation of long-lived plasma cells leads to the conclusion that the development of long-lived plasma cell compartment at this initial stage (early life) of NZB/W mice is independent of T-cell help. This is in contrast to the formation of long-lived plasma cells in older NZB/W mice where T-cell help, like in other non-autoimmune mouse models, is critically required.

4 Discussion

Autoantibodies play a major role in the pathogenesis of various autoimmune diseases, including SLE. They are important parameters in determining disease activity and exacerbations. Autoantibodies, in particular anti-DNA antibodies, are linked to lupus nephritis, which is the prime cause of mortality in SLE. Autoreactive plasma cells, which produce these pathogenic antibodies, should therefore be considered targets for the treatment of autoimmune diseases. Autoreactive plasma cells could be detected in spleens and bone marrow of NZB/W mice even before the onset of lupus symptoms (Hoyer, B.F.: doctoral thesis, Charité – Universitätsmedizin Berlin, 2006). Plasma cells were also detected in the inflamed tissues. Many reports have claimed the persistence of (auto)antibodies in patients with autoimmune diseases, despite cytotoxic and CD20 *(B-cell)* depletion therapies [65,66,51]. Patients in remission or patients responding well to cytotoxic therapies still maintained reduced but stable levels of circulating autoantibodies, and these levels spike again during disease flares and relapses. These observations strengthened the concept of *plasma cell memory* (antibody memory provided by long-lived plasma cells). Since cytotoxic drugs inhibit proliferation, they affect only plasmablasts but not long-lived plasma cells. Autoreactive long-lived plasma cells thus provide autoantibody memory which is refractory to cytotoxic drugs. Also, unlike other cells of B-lineage, plasma cells do not express CD20 [148]. Hence, they are refractory to B-cell depletion therapy with anti-CD20 antibody *(Rituximab)* too.

How long-lived plasma cells would respond to glucocorticoids had not been explored. Glucocorticoids induce apoptosis in lymphocytes irrespective of their proliferation status. They cause both activation and repression of several transcription factors involved in cell survival, leading finally to cell death [82,83]. They also inhibit production of pro-inflammatory cytokines like IL-6 and TNFα, which facilitate plasma cell survival [82]. For these reasons, glucocorticoids are used in treating both autoimmune diseases and various hematological malignancies in combination with other chemotherapeutic agents. It is also used effectively in treating plasma cell malignancy – multiple myeloma [79,80]. The idea therefore was to determine how long-lived plasma cells respond to glucocorticoids. This report suggests that both drugs are ineffective in depleting long-lived plasma cells irrespective of their organ of localization. Moreover, these refractory long-lived plasma cells include autoreactive anti-DNA plasma cells that could contribute to the maintenance of drug resistant autoimmunity.

4.1 *Why are long-lived plasma cells refractory to dexamethasone?*

It has been demonstrated here that dexamethasone, cyclophosphamide and a combination of both effectively induced apoptosis in splenic lymphocytes and plasmablasts. However, the splenic BrdU- long-lived plasma cells remained refractory to both drugs. Similarly, BrdU- plasma cells in the bone marrow were resistant to these drugs too. It should be noted that cyclophosphamide, an alkylating agent, is an anti-proliferative drug. This may be the reason for refractoriness of non-proliferating long-lived plasma cells in spleens of NZB/W mice demonstrated earlier [39]. However, dexamethasone induces apoptosis by other mechanisms that are proliferation-independent, and the results presented in this work show that these mechanisms do not induce apoptosis in long-lived plasma cells either.

Understanding the unresponsiveness of long-lived plasma cells to dexamethasone may require a deeper insight into factors mediating plasma cell survival and the mechanism of dexamethasone action. It is known that most of the dexamethasone effects are mediated by inhibiting DNA binding of NF-κB and AP-1 transcription factors [82]. NF-κB can activate transcription of genes involved in production of inflammatory cytokines like TNF-α, IL-1β, IL-6, IL-8, granulocyte-macrophage colony stimulating factor (GM-CSF), and genes that encode adhesion molecules like intercellular adhesion molecule-1 (ICAM-1), vascular-cell adhesion molecule-1 (VCAM-1) and E-selectin. Many of these factors, in particular TNF-α, IL-6, and adhesion molecules are very important for plasma cell survival – both *in vivo* and *in vitro*. Principally, dexamethasone should have decreased plasma cell survival by inhibiting these factors. However, this effect effect was not observed, and there are many possible explanations for this.

Firstly, bone marrow stromal cells, besides expressing glucocorticoid labile plasma cell survival factors, also express BAFF and APRIL. BAFF and APRIL are a very strong plasma cell survival factor, and its receptor - BCMA is expressed on long-lived plasma cells [56,96]. Interestingly, BAFF is a strong activator of NF-κB binding activity, and has been shown to protect malignant plasma cells from dexamethasone induced apoptosis [96]. The binding of BAFF to BCMA receptors on plasma cells causes activation of NF-κB and mitogen-activated protein kinase (MAPK), induction of antiapoptotic protein Mcl-1expression, and reduced

expression of proapoptotic protein Bak. Also, injection of TACI-Ig *in vivo* causes strong reduction of plasma cell numbers [56]. TACI-Ig binds and blocks BAFF and APRIL *in vivo*, leaving inadequate levels of these cytokines available to plasma cells. Immunized mice were shown to have nearly 65% fewer antigen specific long-lived plasma cells in bone marrow after TACI-Ig injections. Moreover, $BCMA^{-/-}$ mice have a reduced capacity to sustain long-lived plasma cells [56]. Thus, BAFF mediated mechanisms can contribute to the enhancement of antiapoptotic protein expression by long-lived plasma cells, which are in turn capable of rescuing them from dexamethasone induced apoptosis efficiently.

Dexamethasone resistance in malignant plasma cells has also been attributed to interleukin-6 signaling [97-100]. IL-6 is a very strong and essential plasma cell survival factor. IL-6 levels are known to be elevated in autoimmune diseases and NZB/W mice, increasing the likelihood of observed dexamethasone resistance to be contributed in part to elevated IL-6 levels [101,102]. However, it is not likely that IL-6 alone is the reason for dexamethasone resistance, as it is not elevated in serum under normal/healthy conditions. Although the effects of this factor on long-lived plasma cell resistance cannot be completely excluded in the current experiments with NZB/W mouse model, it is harder to explain similar effects reported by other groups on bone marrow plasma cells of non-autoimmune mice. Moreover, it should be noted that IL-6 production is an event occurring downstream of NF-κB (dexamethasone target).

Another speculative explanation for the resistance of long-lived plasma cells could be the involvement of proteasomes in dexamethasone induced apoptosis; and inhibition of this dexamethasone induced apoptosis by Bcl-2 expression. Proteasomes are multicatalytic protease complexes that degrade proteins targeted for destruction by polyubiquitination [103,104]. Glucocorticoids can induce apoptosis in a proteasomes dependent manner, where dexamethasone induces proteasomes to cause targeted degradation of a variety of prosurvival factors [105]. It has been demonstrated that blocking of proteasome function by the proteasome inhibitors – lactacystin or MG132 can inhibit dexamethasone induced apoptosis in thymocytes [106]. However, the cells expressing the antiapoptotic protein Bcl-2 can stabilize several proteasome targets like AP-1, NF-κB and c-Fos [107,108], all of which are implicated in glucocorticoid induced apoptosis. Therefore, Bcl-2 overexpression (in thymocytes) can inhibit proteasome activation and apoptosis caused by dexamethasone [105].

Interestingly, Bcl-2 expression has also been shown to inhibit dexamethasone induced apoptosis in malignant plasma cells of multiple myeloma too [109,110]. Thus, expression of antiapoptotic protein Bcl-2 can decrease dexamethasone-induced proteasome-mediated apoptosis [83]. It has been demonstrated by our group and other groups that nonproliferating long-lived plasma cells, particularly in the bone marrow express high levels of Bcl-2 , (*Yoshida et al., Manuscript in preparation)* [111-113], along with other survival prolonging proteins like Blimp-1 and XBP-1. Therefore, modulation of dexamethasone mediated apoptosis by high expression of Bcl-2 in long-lived plasma cells could be a possible explanation for dexamethasone resistance in these cells.

It must however be noted that another novel proteasome inhibitor – Bortezomib (Velcade) induces apoptosis in myeloma cells (even in dexamethasone resistant myeloma cells) and is presently being used in treating this disease. It has also been demonstrated very recently that Bortezomib depletes both short- and long-lived plasma cells from NZB/W mice [161]. Why certain proteasome inhibitors like lactacystin and MG132 inhibit dexamethasone induced apoptosis, while others like bortezomib synergize with dexamethasone [114,115] in inducing apoptosis is not yet explained. It is possible that different binding properties of these drugs can contribute to different outcomes in different cells [116-119].

4.2 Why are bone marrow BrdU+ plasma cells resistant to immunosuppressive drugs?

The work presented here shows that there was a strong reduction of BrdU+ plasma cells in spleens and also in the lymph nodes (data not shown) after treatment with dexamethasone, cyclophosphamide and combination of both drugs. However, none of these drugs depleted BrdU+ plasma cells in the bone marrow. The BrdU+ plasma cells in bone marrow remained totally unaffected by dexamethasone therapy. Although a reduction tendency was seen after treatment with cyclophosphamide alone *($p=0.08$)*, it did not reach significance. It should be noted that the same treatment depleted BrdU+ plasma cells in spleen *($p<0.0001$)* and in lymph nodes.

One likely explanation for this resistance could be that the BrdU+ plasma cells in the bone marrow represent a different stage of plasma cell maturation than BrdU+ plasma cells in spleen and other secondary lymphoid organs. While the majority of splenic BrdU+ cells are proliferating plasmablasts, the bone marrow BrdU+ plasma cells reflect cells that have recently entered the bone marrow as BrdU+ plasmablasts and differentiate here in this microenvironment into mature plasma cells. They remain BrdU+ since they incorporated BrdU during a last cell division just prior to their entrance into the bone marrow. This would mean that BrdU+ plasma cells in the bone marrow are newly formed long-lived plasma cells and are therefore resistant to immunosuppressants. Since germinal center formation and therefore resulting plasma cell generation does not occur in the bone marrow [120], it is highly unlikely that the BrdU+ plasma cells here are locally generated proliferating plasmablasts. There is good evidence that bone marrow plasma cells originate from plasmablasts of secondary lymphoid organs as a consequence of T-dependent immune reactions or chronic inflammation, and become long-lived in bone marrow microenvironment after receiving appropriate survival signals [29]. There is also sufficient proof that somatic hypermutations occurring in germinal center reactions are important in the selection of bone marrow plasma cells. These plasmablasts migrate from their site of production in secondary lymphoid organs to the bone marrow under the influence of migratory chemokine signal CXCL12. Alternatively, they can also migrate to the inflamed tissues under the influence of inflammatory migratory chemokine signals CXCL9, CXCL10 and CXCL11. The plasmablasts that reach the bone marrow, and are competent of becoming long-lived, are

somatically hypermutated and produce high affinity antibodies. These competent plasmablasts, upon receiving appropriate survival signals or finding empty survival niches in the bone marrow, are capable of upregulating antiapoptotic and prosurvival proteins like Bcl-2, Bcl-XL, XBP-1 and Blimp-1 and becoming long-lived plasma cells [29]. Moreover, the bone marrow is home for mainly BrdU- non-proliferating long-lived plasma cells. Therefore, the BrdU+ plasma cells appearing in the bone marrow are very likely to be either recently migrated long-lived plasma cell precursors competing for appropriate survival signals, or the newly formed long-lived plasma cells, thereby explaining their resistance to immunosuppressive drugs.

In support of this idea, data generated in our lab also shows that the phenotype of bone marrow BrdU+ plasma cells, in context of MHC class II expression, resembles the phenotype of BrdU- plasma cells in the same organ closely. It is known that the short-lived plasmablasts express high levels of MHC class II and that this expression is lost during the course of its differentiation into more mature long-lived plasma cells [39]. Interestingly, majority of BrdU+ plasma cells in bone marrow had down regulated their MHCII expression (personal communication). This is unlike the BrdU+ plasma cells from the spleens of the same mice where majority of them express high levels of MHCII, thereby suggesting that BrdU+ plasma cells in the bone marrow are plasmablasts that have undergone some degree of maturation recently. Moreover, recent data also shows that these BrdU+ plasma cells from bone marrow are not migratory (Hoyer et al., manuscript in preparation). Using transwell migration assays, it was demonstrated here that MHCII expressing plasma cells from the spleens migrate in response to chemokine signals SDF1 and IP10/CXCL10, while the bone marrow plasma cells are totally unresponsive. This also confirms the hypothesis that the BrdU+ plasma cells in the bone marrow represent either recently formed long-lived plasma cells or cells in an intermediate stage on their way to becoming mature plasma cells.

However, it cannot be totally excluded that the bone marrow provides niches for dividing BrdU+ plasmablasts and that the bone marrow microenvironment mediates drug resistance for these cells. The growth of plasmablasts and survival of plasma cells is crucially dependent on specific cytokines and cell-contact mediated stimulation [121]. The bone marrow plasma cells that are long-lived *in vivo* rapidly undergo apoptosis *ex vivo* upon isolation, unless rescued by stimulation with specific survival factors, in particular IL-5, IL-6, SDF-1/CXCL12, TNF-α

and CD44 signaling [55]. These survival factors are highly expressed in the bone marrow, which could provide supportive microenvironmental niches for long-term plasma cell survival. Consistent with this, plasma cells survive for months, even years in the bone marrow despite the absence of antigenic stimulation [25,122]. Furthermore, bone marrow plasma cells have been shown to co-localize with SDF-1/CXCL12 expressing stromal cells [43], which also produce cytokines and factors that promote plasma cell survival, in particular IL-6 and TNF-α. These interactions between cells in the bone marrow microenvironment have been implicated in mediation of drug resistance in plasma cells malignancies [123], and the same factors could also play a role in mediating drug resistance in long-lived plasma cells as well.

Environment mediated drug resistance (EM-DR) is a phenomenon well known from tumor biology and plasma cell malignancy – multiple myeloma. EM-DR could be conferred by soluble factors like cytokines produced and made available in the microenvironment (soluble factor mediated-drug resistance, SM-DR). Alternatively, it could be conferred by direct cell adhesion between tumor cells and the cells in its microenvironment (cell adhesion mediated-drug resistance, CAM-DR) [123]. Notably, factors responsible for mediating drug resistance in plasma cell malignancy by both these mechanisms are available under regular circumstances to bone marrow long-lived plasma cells as well. The bone marrow stromal cells, in close proximity of which long-lived plasma cells lie, contribute to SM-DR by producing not only SDF-1, IL-6 and TNF-α, but also BAFF and APRIL. As discussed earlier, BAFF and APRIL signal through BCMA on long-lived plasma cells, and result in activation of NF-κB. Furthermore, this signaling is known to rescue malignant plasma cells from dexamethasone induced apoptosis. IL-6 and TNF-α are equally potent stimulators of plasma cell survival. The second mechanism of drug resistance - CAM-DR - is mediated through the interaction of adhesion molecules on malignant plasma cells with those on stromal cells and fibronectin in its microenvironment. These adhesion molecules include VLA-4 and LFA-1 on plasma cells which bind to their receptors VCAM-1 and ICAM-1 on bone marrow stromal cells [123,124]. Both these adhesion molecules, in addition to E-selectin and P-selectin, are known to be present on plasma cells and mediate plasma cell survival in bone marrow. Together, these mechanisms could mediate chemotherapeutic and glucocorticoid resistance, not only in BrdU+ plasma cells, but also in BrdU- plasma cells in the bone marrow microenvironment. The bone marrow could thus provide a highly stable and drug resistant microenvironment for long-term plasma cell memory.

4.3 Is long term treatment with high dose glucocorticoids superior to pulse treatment in eliminating plasma cell memory?

In the experiments described in Part IA, continuous treatment of the mice with 1 mg/kg/day dexamethasone for 1 week or 30 mg/kg cyclophosphamide on 4 days, or a combination of both drugs did not affect splenic and bone marrow long-lived plasma cells. However, the same treatment was effective in depleting other cell types, showing that the direct cytolytic effect of dexamethasone is exerted within this period. However, these experiments did not exclude the possibility that a longer treatment with glucocorticoids can result in a reduction of long-lived plasma cells. Since a longer treatment, in addition to its apoptotic affects, was expected to diminish the production of proinflammatory cytokines that support plasma cell survival, NZB/W mice were treated with glucocorticoids for 4 weeks. In this experiment, the mice were treated with 3 mg/kg/day or 10mg/kg/day prednisolone. It should be noted that 1mg dexamethasone is as potent as 5mg methylprednisolone in mediating genomic effects and 1.2 mg methylprednisolone in mediating nongenomic effects [125]; and that 1mg methylprednisolone is equivalent to 1.25 mg prednisolone [126]. This would mean that 1mg dexamethasone is equivalent to 6-7 mg prednisolone approximately. Therefore, in terms of drug equipotency ratios, the dose of glucocorticoids used in 1 month prednisolone experiment (Part IB) was even higher than the dose used in the 1 week dexamethasone experiment. It should also be noted that the doses used here are comparable to those used in glucocorticoid *pulse* therapy for treating autoimmune diseases or graft rejections in transplant recipients [126,127]. In pulse therapy however, patients receive these high glucocorticoid doses *only* on 3 days (per month), after which they receive moderate or low doses of glucocorticoids. Therefore, the doses used in 1 month glucocorticoid experiment are extremely high as compared to clinical doses.

The results demonstrate that despite prolonged treatment with extremely high doses of glucocorticoids, long-lived plasma cells in spleens and bone marrow are not reduced. Since these doses are sufficient to saturate glucocorticoid receptors and to mediate specific and non-specific nongenomic affects [127,128], and since long-lived plasma cells are not affected by both 1 week and 1 month of glucocorticoid treatment, we believe that plasma cell memory is

refractory to glucocorticoids irrespective of drug dose and duration of treatment. This data is consistent with earlier reports that bone marrow maintains dexamethasone refractory antibody response following immunizations [129,130]. In these experiments, Benner et al. immunized mice of various strains with either T-dependent or T-independent antigen followed by booster doses. Daily dexamethasone treatment was then started either on 1 day before or day 5 following the booster injection. The doses varied from 0.25 mg/kg to 16 mg/kg dexamethasone and treatment duration was between 10 and 20 days. Both T-dependent and T-independent antigen specific plaque-forming cells (antibody secreting cells) in the spleens, but not in the bone marrow, were reduced after the treatment. There was even a tendency for increase of antigen specific plaque forming cells in the bone marrow following T-independent immunization. Moreover, a single injection of 144 mg/kg dexamethasone did not affect bone marrow plasma cells while depleting plasma cells in other peripheral lymphoid organs enormously [88]. In addition to confirmation of this data, the results presented here also show that glucocorticoid resistant plasma cells in peripheral lymphoid organs are mainly non-proliferating long-lived plasma cells. Moreover, with advanced techniques available today, it is demonstrated here that bone marrow contains both recently proliferated and non-proliferating long-lived plasma cell, both of which are equally resistant to glucocorticoid therapy. More importantly, the results show that high dose glucocorticoid treatment for a period of 4 weeks is not more beneficial in depleting memory plasma cells than 1 week treatment. Treatment with high doses for 1 week is similar to glucocorticoid pulse treatment in clinics, where patients with active or fulminant disease receive similar doses of steroids for 3-5 days in the beginning of regular treatment.

It was observed that treatment with these high doses of glucocorticoids was not suitable for prolonged periods in these mice. The condition of mice treated with 10mg/kg prednisolone worsened and more mice died in this group before the end of experiment (compared to mice give vehicle or 3 mg/kg prednisolone). Moreover, lower doses of prednisolone are known to improve disease and prolong survival in NZB/W mice [131,132], suggesting that the very high doses used here are perhaps not tolerable for longer treatment periods.

4.4 Maintenance of immunosuppressive drug resistant plasma cell memory by bone marrow: Pros and Cons

The results presented demonstrate that the immunosuppressive drug resistant plasma cells are located mainly in the bone marrow and to some extent in peripheral lymphoid organs. The refractoriness of plasma cells in bone marrow to immunosuppressive drugs has its own advantages and disadvantages.

Positive aspects: Protective immunity of non-autoimmune individuals, requiring immunosuppressive therapy for malignancies or prevention of transplant rejections, will remain unhampered after immunosuppressive treatment. In normal, clinically healthy individuals, bone marrow plasma cells maintain persistent antigen-specific antibody titers in serum for several months to decades following immunizations, even in the absence of corresponding antigen [25,26,49]. The bone marrow thus contains a plasma cell population with heterogeneous antigenic specificities, all encountered by that individual during its life time [48,54,133]. These protective antibody titers are required for immediate pathogen neutralization during recurring infections. Protective antibodies from bone marrow plasma cells therefore help in maintaining a relatively high threshold for activation of immune system after trivial recurrent infections. Thus, following anti-inflammatory/immunosuppressive therapy, protective plasma cell memory of a non-autoimmune individual will remain uncompromised.

Negative aspects: In case of autoimmunity and allergic diseases, autoreactive and pathogenic plasma cells in the bone marrow would remain unaffected by these drugs. The results demonstrate that the autoreactive antibody secreting plasma cells are extremely stable in the bone marrow. The results show that that approximately same numbers of anti-DNA antibody producing cells reside in the spleen and bone marrow of NZB/W mice. There was a very strong reduction of anti-DNA antibody secreting cells from the spleens, but bone marrow anti-DNA antibody secreting cell remained refractory to immunosuppressive treatment (Table 2). This suggests that the splenic autoreactive ASCs are mainly plasmablasts, and that the bone marrow autoreactive ASCs are either long-lived in nature or protected in bone marrow microenvironment. By their survival through immunosuppressive treatments, the autoreactive plasma cells in bone marrow can contribute to drug refractory disease, or to episodes of disease flares and relapses, and thereby prevent cure of disease.

4.5 *Do inflamed tissues contribute significantly to maintenance of plasma cell memory?*

It is interesting to note that plasma cell infiltration into the kidneys is seen only under inflammatory conditions, for example inflamed kidney of lupus nephritis prone NZB/W mice. The inflamed kidneys of these autoimmune mice contain nearly 50 times more plasma cells than non-inflamed kidneys of age matched non-autoimmune mice [47]. The migration of plasma cells to inflamed kidneys and inflamed tissues occurs under the influence of interferon inducible chemokines CXCL9, 10 and 11 that are expressed in these inflamed organs/tissues [40]. Such tissues are likely to support plasma cell survival for prolonged periods, particularly with its inflammatory environment and cytokines [29].

Results from these experiments demonstrate that inflamed kidneys are an important site for anti-DNA autoantibody production. The role of inflamed tissues in the production of autoreactive antibodies was previously unidentified, and has therefore been neglected as a therapeutic target. The results also show that nearly half the plasma cells in these inflamed organs (including anti-DNA ASCs) survive immunosuppressive treatment, suggesting that remaining cells are mature, possibly long-lived or are protected in this inflamed environment by inflammatory or pro-survival signals (similar to the observation made in bone marrow). It can also not be excluded that the drugs have restricted access to some kidney plasma cells. Unlike B-cells in kidneys, which are localized in distinct foci, plasma cells are scattered across the whole organ on kidney cross sections *(Panne et al., Manuscript in preparation)*. Therefore it can be speculated that the plasma cells located in not well perfused areas cannot be successfully eliminated by the treatment. This might explain why autoantibodies of certain specificities like scleroderma-specific autoantibodies [134] or anti-Ro/SSA in SLE [135] are detectable even after drastic immunoablative regimens including antithymocyte globulin followed by autologous stem cell transplantation. In any case, the results show that inflamed tissues can contribute significantly to the maintenance of both autoreactive and total plasma cell memory. The importance of inflamed tissues in maintenance of plasma cell memory should therefore not be downplayed.

Since plasma cells are encountered in these tissues only under inflammatory conditions, hypothetically, the resolution of inflammation should cause disruption of this supportive microenvironment. As a consequence, plasma cells would cease to survive there, resulting in the waning of plasma cell memory provided by those previously inflamed tissues. Similarly, autoreactive plasma cells present in such tissues would also cease to survive after the resolution of inflammation. It should however be comprehended that the autoreactive plasma cell memory provided by bone marrow is a bigger challenge in the treatment of autoimmunity. Although autoantibody production from inflamed tissues cannot be ignored, we can still expect it to diminish after resolution of inflammation. In contrast, the autoantibody producing plasma cells that have entered stable bone marrow environment would still persist, even in the absence of inflammation.

4.6 *What factors cause the inflamed kidneys to contain predominantly IgG producing ASCs?*

Interestingly, the kidneys contained abundant IgG producing ASCs, which formed 90% of all ASCs in this organ (Table 1). This was in contrast to the spleens and bone marrow where IgM producing ASCs dominated plasmablast/plasma cell compartment. The ASCs are accumulated in the inflamed kidneys with increasing age and progressing disease *(Panne et al., Manuscript in preparation)*. Though the reasons for these differences between antibody classes produced by plasma cell in spleen, bone marrow and kidneys remain unclear, it is likely that the inflamed tissue specific survival factors in kidneys play a role in migration and maintenance of class switched IgG producing plasma cells.

An alternative explanation could be the age at which formation of plasma cell compartment takes place. Majority of long-lived plasma cells in spleens and bone marrow of NZB/W mice are formed very early in life, during which their formation is independent of T-cell help (discussed below). Moreover, IgM is the predominant class of antibody produced by splenic B-cells of young NZB/W mice [136]. Therefore, IgM producing ASCs are more likely to occupy empty plasma cell survival niches in the spleen and bone marrow of NZB/W mice at this age. Theofilopoulos et al. also demonstrated that splenic B-cells from older and disease bearing NZB/W mice start producing class switched IgG antibodies [136]. This is in accordance with current findings that immunosuppressive drugs deplete IgG ASCs in the spleens much stronger than IgM ASCs (Table 1), suggesting that the IgG ASCs are mainly recently formed proliferating plasmablasts. It is at this age that inflamed kidneys start harboring plasma cells [47]. Therefore, it is likely that ASCs homing to kidneys are mainly IgG producing.

Similar to total IgG ASCs, kidneys of NZB/W mice contained almost exclusively IgG anti-DNA ASCs. Interestingly, even the spleens contained more IgG anti-DNA ASCs (spleens contained more total IgM ASCs) (Table 1 and 2). In short, both spleens and kidneys contained more IgG than IgM anti-DNA ASCs as opposed to bone marrow that contained more IgM anti-DNA ASCs. This could be explained by earlier reports demonstrating that young NZB/W mice produce predominantly IgM anti-DNA antibodies, and that the class switch to pathogenic IgG anti-DNA antibodies occurs with progressing age and disease [86,137]. Since it is in spleen where induction and formation of plasma cells occur, it is possible that the isotype switch of autoreactive plasma cells is first observed in this organ. Moreover, as discussed

earlier, the appearance of plasma cells in kidneys is a relatively late event, as plasma cells appear here only during or after inflammatory damage in this organ. Therefore, the accumulation of autoreactive plasma cells in kidneys should have taken place recently, probably during the switch of autoreactive plasma cells from IgM to IgG isotype, leading to the presence of more IgG than IgM anti-DNA ASCs in this organ. On the other hand, it is likely that autoreactive plasma cells in the bone marrow, like other plasma cells here, have been formed relatively early and have survived here for a longer period. Therefore, it is possible that accumulation of IgG anti-DNA ASCs in this organ may occur later than in spleens and kidneys.

Unfortunately, the number of these anti-DNA IgG ASCs in the kidneys of both treated and untreated mice varied hugely, making it difficult for us to make concrete conclusions regarding their response to immunosuppressants (Table 2).

4.7 Increased T-cell counts in bone marrow of dexamethasone treated mice

There have been reports earlier that one week treatment with high dose glucocorticoids causes redistribution of lymphocytes from peripheral lymphoid to the bone marrow [138,139]. Particularly affected are CD3+ T lymphocytes, which are significantly increased in the bone marrow, while the total BM lymphocyte counts remain unaffected [140]. In the 1 week dexamethasone treatment experiment, thre was a similar accumulation of CD4+ and CD8+ T-cell in the bone marrow of dexamethasone treated mice, although B220+ B-cell count remained relatively stable. However, this increase was not observed in one month treated mice. Dexamethasone has been shown to increase the expression of migratory chemokine receptor - CXCR4 on lymphocytes and monocytes in a dose dependent manner *in vitro* [141], which could be inhibited by mifepristone - a glucocorticoid receptor antagonist. Also, uveitis patients treated locally with dexamethasone show an increased expression of CXCR4 on aqueous humor derived T-cells, compared to peripheral blood T-cells [142]. This glucocorticoid induced CXCR4 is functional, as is shown by the increased migration of dexamethasone treated cells in response to the ligand CXCL12.

4.8 Impact of T-cell depletion on long-lived plasma cell formation

It has already been established that under physiologic conditions, memory providing long-lived plasma cells reside primarily in the bone marrow; and that the activated spleens can harbor increased numbers of long-lived plasma cells in the course of an autoimmune disease [47]. Following immunization with non-self antigens, antigen-specific bone marrow long-lived plasma cells show strong affinity maturation [32] - a hallmark of germinal center reaction. Although a few splenic long-lived plasma cells can be formed in extrafollicular reactions after T-independent immunizations [27], bone marrow long-lived plasma cells were shown to be stringently selected for high affinity antibody production even in Bcl-2 transgenic mice [23]. In these transgenic mice, Bcl-2 - an anti-apoptotic protein, inhibited the death of low-affinity antibody producing cells in germinal centers. Under regular circumstances, these cells with low affinity for their cognate antigen would have undergone apoptosis in germinal centers, while cells with high affinity survive the selection process. However, in Bcl-2 transgenic mice, memory B-cells and plasma cells with both low- and high-affinity antibodies survived in germinal center reactions. As a result, cells with low-affinity antibodies infiltrated the memory B-cell compartment and splenic plasma cell compartment. Interestingly, the ability of bone marrow to select/maintain high-affinity antibody secreting cells was still not altered, indicating that bone marrow (long-lived) plasma cells are stringently affinity selected, even in the presence of excessive non-apoptotic antibody secreting cells. Together, these results indicate the important role of germinal center reactions in the formation of bone marrow long-lived plasma cells. The development of germinal centers is in turn critically dependent on the presence of T-cell help [20]. It was therefore studied how the formation of LPCs proceeded in the absence CD4+ T-cells.

It has already been demonstrated that the NZB/W mice can develop spontaneous germinal centers in their spleens as early as 1-2 months of age [143]. These germinal centers appear in absence of purposeful immunizations and adventitious infections. Abrogation of T-cell help (by injecting anti-CD40L mAb) resolved these germinal centers from spleens [143]. Moreover, earlier experiments have revealed that in NZB/W mice, the bulk of splenic long-lived plasma cells and nearly 50% of bone marrow long-lived plasma cells are formed by the age of 9 weeks (Voigt, C.: doctoral thesis, Charité – Universitätsmedizin Berlin, 2008). Since germinal

centers are implicated in the generation of long-lived plasma cells, and since CD4+ T help is essential for germinal center formation, CD4+ T-cells were depleted from NZB/W mice using anti-murine CD4 mAb from 3 till 9 weeks of age. The generation of plasma cells at this early age however was not affected by the absence of CD4 T-cells. Long-lived plasma cell formation in both spleens and bone marrow of CD4 depleted mice proceeded with the same kinetics as age-matched controls, demonstrating that long-lived plasma cells in NZB/W mice are not derived from germinal centers alone. Lack of response to the T-cell dependent antigen – ovalbumin in CD4 depleted mice was suggestive of a successful abrogation of T-cell help in these mice.

These experiments demonstrate that both bone marrow and splenic long-lived plasma cells can be formed in T-independent immune responses as well. However, it cannot be excluded that high-affinity plasma cells originating in germinal centers would have been preferentially selected, had they been present. This observation could be logically explained by the hypothesis of ‘competition’ for limited plasma cell survival niches in lymphoid organs [29] (Fig. 20). The lymphoid organs are known to have a finite capacity for sustaining long-lived plasma cells [27]. The long-lived plasma cell compartment in spleens and bone marrow increase till it reaches a certain size, after which it remains stable [39,54,89,144]. Since the survival niches for long-lived plasma cells are limited, the newly formed plasmablasts would have to compete directly for survival signals with the pre-existing resident long-lived plasma cells. In this process, only a more competent plasmablast could dislocate a resident long-lived plasma cell to replace it, based on its affinity and expression of transcription factors like Blimp-1, XBP-1 etc. [29]. Since these niches are not likely to be occupied at birth, it would be easier for plasmablasts of all origins to become long-lived at this young age (as in current experimental setup). In older mice on the other hand, plasmablasts would have to compete more strongly with pre-existing resident long-lived plasma cells in those survival niches (Fig. 20).

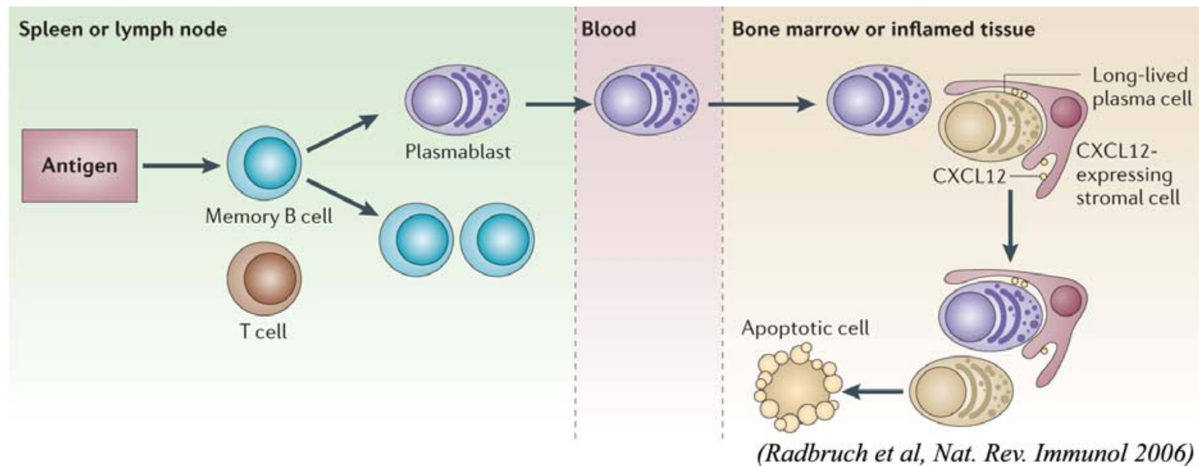

Figure 20: Competition between plasmablast and plasma cells for survival niches. *The newly generated plasmablasts have a migratory potential and circulate via blood towards CXCL12 expressing bone marrow stromal cells. These plasmablasts can dislocate and replace resident long-lived plasma cells. The successful plasmablasts differentiate into plasma cells and lose their migratory potential, while the dislocated immobile plasma cell undergoes apoptosis. Only as many plasma cells of old specificities are dislocated from their niches as the plasmablasts with new specificities are recruited to the pool of memory plasma cells.*

These experiments were carried out in very young NZB/W mice and the results suggest that long-lived plasma cells at this age can be formed even in the absence of T-cell help. However, the results *do not* imply that the long-lived plasma cell formation is in general a T-help independent process. The importance of T-cell help in formation of bone marrow long-lived plasma cells has already been outlined. The results only suggest that at this young age in NZB/W mice, when long-lived plasma cell compartments in lymphoid organs are empty, plasmablasts formed even in the absence of T-cell help can become long-lived. The results may however vary with the age of mice. Moreover, the B-cells in NZB/W mice are known to be hyper-reactive and easily activated. It remains to be explored if plasma cells in non-autoimmune mice possess the same kinetics of longevity.

4.9 *Future perspectives of SLE therapy*

Biological therapies: Recent advances in the knowledge of molecular mechanisms involved in pathogenesis of SLE lead to development of a new class of drugs targeting specific molecules associated with disease development. Promising drugs of this class are Rituximab (monoclonal anti-human CD20 antibody), tocilizumab (monoclonal anti-human IL6R antibody) and anti-TNF-α therapy (infliximab, adalimumab, etanercept). The most successful of these – Rituximab, binds and depletes peripheral blood CD20+ B-cells by various mechanisms, including complement mediated and antibody-dependent cytotoxicity [145,146]. FcγR-mediated phagocytosis by macrophages has also been implied [147]. CD20 is a B-cell specific molecule expressed during intermediate stages of B-cell development and lost upon its terminal differentiation into plasma cells [148]. However, tonsillar plasma cells, obtained from patients undergoing tonsillectomy (due to chronic tonsillitis), express CD20 [149]. Rituximab was initially approved for treatment of relapsed low grade B-cell follicular non-Hodgkin's lymphoma [150]. However, trials on treatment of autoimmune disorders like idiopathic thrombocytopenic purpura and resistant rheumatoid arthritis showed encouraging results [151-153]. Rituximab trials in SLE patients showed rapid and effective induction of disease remission [154,155], indicating that B-cell functions like – regulation of T-cell activity, cytokine production and antigen presentation - can directly be involved in the disease progression. Since plasma cells express drastically low levels of CD20, rituximab has no direct impact on plasma cell numbers or immunoglobulin levels [148]. However, by depleting B-cells (immediate precursors of plasmablasts) rituximab can cause a temporary reduction in peripheral blood plasmablast count. Moreover, rituximab trials showed no effect on protective antibody titers and some autoantibody titers [51], confirming the existence of CD20-negative long-lived plasma cells that can produce and maintain stable antibody levels even in the absence of B-cells. Unlike short-lived plasmablast, long-lived plasma cells do not require continuous differentiation from B-cells and are therefore spared from the depletory effects of rituximab.

A very interesting recent study has demonstrated that blockade of plasma cell adhesion molecules using a combination of anti-LFA-1 and anti-VLA-4 monoclonal antibodies reduced antigen specific plasma cells from the bone marrow drastically, perhaps due to their dislocation from survival niches in bone marrow [148]. However, this did not prevent the newly

generated or peripheral plasmablasts from relocating to and repopulating bone marrow plasma cells. Interestingly, simultaneous administration of mAbs blocking adhesion molecules LFA-1/VLA-4 and anti-CD20 mAb prevented repopulation of bone marrow plasma cells [148]. This indicates that only dislocation of plasma cells from bone marrow is not sufficient to prevent regeneration of plasma cell memory, which could be prevented by simultaneous rituximab mediated depletion of plasma cell precursors (activated or memory B-cells).

Also, inhibition of B-cell/T-cell interaction by blocking CD40-CD40L or CD80/CD86-CTLA4/CD28 costimulatory signals has been studied. These signals are required for lymphocyte activation and antibody production. The blockade was achieved by recombinant CD40L or CTLA-4 Ig, consisting of immunoglobulin Fc region linked to CTLA4 or CD40L domain, respectively. These soluble ligand-immunoglobulin fusion proteins bind and block their cognate receptors on B-cells, thereby depriving the B-cells of activation stimuli from T-cells. CTLA-4 Ig showed promising results in trials with murine models of lupus nephritis. It not only ameliorated the renal disease and prolonged survival in lupus mice, but also prevented the increase in anti-dsDNA antibody titers [156]. Unfortunately, costimulatory molecule blockings have not been shown to be very successful in treating SLE during early phase clinical trials [155].

Autologous hematopoietic stem cell transplant (ASCT) has been explored in patients with severe autoimmune diseases who were unresponsive to conventional therapies or suffered intolerable side effects [157-159]. In this procedure, stem cells are mobilized with cyclophosphamide and granulocyte colony stimulating factor (G-CSF). This is followed by immunoablation with high-dose cyclophosphamide and antithymocyte globulin (ATG), with or without methylprednisolone as conditioning. This procedure depletes mature and activated (autoreactive) immune cells, while the CD34+ progenitors are reinfused back into the body. ATG has multiple specificities and depletes plasma cells as well [160]. Interestingly, bone marrow autoreactive plasma cells have been shown to be depleted by ASCT in patients suffering from severe refractory SLE [135]. All the patients included in this study had high anti-nuclear antibody (ANA) and anti-dsDNA serum antibody titers before therapy. Following immunoablation and ASCT, autoantibodies either disappeared totally from circulation or were reduced to clinically insignificant levels for the remaining observation period of 8 years [135]. This correlated with clinical remission in disease activity. It should however be noted that this

procedure is currently reserved only for patients with persistent organ-threatening SLE despite aggressive standard therapy.

Recently, another interesting report has demonstrated that the proteasome inhibitor Bortezomib (Velcade) efficiently depletes short- and long-lived plasma cells from the bone marrow, and to a lesser extent, from the spleen of NZB/W mice [161]. The renal disease was ameliorated and survival prolonged in mice treated with this drug. These promising results have prompted new clinical studies of Bortezomib in patients with refractory SLE.

Since autoreactive and therapy resistant long-lived plasma cells are likely to contribute to treatment refractoriness and flares of lupus, preventing the establishment of autoreactive plasma cell memory by therapeutic intervention at early stages of disease development may prove beneficial. With the advent of promising biologicals and therapeutic procedures in lupus treatment, we are much closer to achieving the cure for SLE than perceived in previous years.

5 Summary

Plasma cells can be classified into short-lived and long-lived. While short-lived plasma cells undergo apoptosis within few days of formation, long-lived plasma cells survive for months up to several years. Unlike memory B-cells which do not secrete any antibodies in resting state, long-lived plasma cells secrete antibodies continuously, contributing directly to the maintenance of humoral antibody memory. While antibodies produced by long-lived plasma cells are protective under physiologic conditions, they can be pathologic under the circumstances of autoimmunity. In this work, it was investigated if glucocorticoids, conventional drugs for the treatment of autoimmune diseases and multiple myeloma, deplete (autoreactive) long-lived plasma cells. Experiments were performed in the NZB/W mouse model of SLE. Glucocorticoids were given alone or in combination with cyclophosphamide. To mark and differentiate short- from long-lived plasma cells on flow cytometry, incorporation of BrdU into the DNA of proliferating cells was analyzed. The results show that BrdU+ short-lived plasma cells were depleted from spleen, but BrdU- long-lived plasma cells were refractory to both drugs. An unexpected observation was made in bone marrow, where both BrdU+ and BrdU- plasma cells were unaffected. Further intriguing was the finding that most autoreactive (anti-DNA) plasma cells resided in the bone marrow, almost all of which were resistant to both drugs. Bone marrow plasma cells are therefore the major sources of immunosuppressant resistant (autoreactive) antibody production. Another interesting finding was that inflamed kidneys of these lupus manifested mice contained autoantibody-secreting cells, many of which were resistant to immunosuppressants. These results demonstrate the important role of bone marrow and inflamed tissues in prolongation of plasma cell survival. These organs also provide niches for the maintenance of autoreactive plasma cells in autoimmunity. Future therapeutic strategies should therefore focus on elimination of autoreactive plasma cell memory from these organs. The second focus of this work was to analyze the effect of CD4+ T-cell depletion on the formation of long-lived plasma cells in spleen and bone marrow of young lupus mice. CD4+ T-cells are critically required for germinal center reactions, which in turn are essential for long-lived plasma cell formation. Despite CD4-depletion, no changes were observed in the kinetics of long-lived plasma cell formation. Together, these results underscore bone marrow and inflamed tissues as important sources of therapy resistant autoreactive antibody memory. They also suggest that T-cell depletion therapy is not helpful in preventing the formation of long-lived plasma cell compartment.

6 Zusammenfassung

Plasmazellen werden in kurzlebige und langlebige unterteilt. Kurzlebige Plasmazellen haben eine Halbwertszeit von wenigen Tagen, während langlebige Plasmazellen mehrere Monate bis Jahre überleben. Im Gegensatz zu Gedächtnis-B-Zellen, die im Ruhezustand keine Antikörper sezernieren, sezernieren langlebige Plasmazellen kontinuierlich Antikörper und tragen dadurch direkt zum humoralen Antikörpergedächtnis bei. Unter physiologischen Bedingungen sezernieren langlebige Plasmazellen protektive Antikörper, können aber bei Autoimmunerkrankungen Autoantikörper sezerniert. In dieser Arbeit wurde untersucht ob Glucocorticoide, die konventionell zur Behandlung von Autoimmunerkrankungen und des multiplen Myeloms eingesetzt werden, (autoreaktiven) langlebige Plasmazellen depletieren. Hierfür wurden autoimmune NZB/W-Mäuse mit Glucocorticoid allein oder in Kombination mit Cyclophosphamid behandelt. Zur Unterscheidung von kurz- und langlebigen Plasmazellen wurde die Inkorporation von BrdU in die DNA analysiert. In der Milz erwiesen sich BrdU- langlebige Plasmazellen im Gegensatz zu BrdU+ kurzlebigen Plasmazellen refraktär auf beide Medikamente. Im Knochenmark waren sowohl die BrdU+ als auch BrdU- Plasmazellen resistent auf die Therapien. Außerdem waren die meisten autoreaktiven, Anti-DNA-Antikörper-sezernierenden Plasmazellen im Knochenmark lokalisiert. Das Knochenmark ist somit ein Hauptort für therapieresistente Plasmazellen. Daneben fanden sich auch in den entzündeten Nieren von Lupus-Mäusen therapierefraktäre, autoreaktive Plasmazellen. Die Ergebnisse unterstreichen die Bedeutung von Knochenmark und entzündeten Geweben für das Überleben von Plasmazellen. Bei Autoimmunerkrankungen bilden sie die Nischen für die Aufrechterhaltung eines autoreaktiven Plasmazellgedächtnisses. Zukünftige therapeutische Strategien sollten deshalb auf die Eliminierung dieses autoreaktiven Plasmazellgedächtnisses ausgerichtet sein.

Ein zweiter Schwerpunkt der Arbeit lag auf der Untersuchung der Beteiligung von CD4+ T-Zellen bei der Generation langlebiger Plasmazellen in Milz und Knochenmark junger NZB/W-Mäuse. CD4+ T-Zellen sind essentiell für die Ausbildung von Keimzentren aus denen unter physiologischen Bedingungen langlebige Plasmazellen hervorgehen. Die Depletion von CD4+ T-Zellen hatte überraschenderweise keine Auswirkungen auf die Entstehung der Plasmazell-Kompartimente in Milz und Knochenmark von NZB/W-Mäusen *in vivo*.

Zusammenfassend zeigen diese Ergebnisse die wichtige Rolle von Knochenmark und entzündetem Gewebe als Quelle eines autoreaktiven, therapieresistenten Antikörper-Gedächtnisses. Sie deuten darauf hin, dass eine T-Zell depletierende Therapie die Entwicklung eines langlebigen Plasmazellkompartments nicht verhindern kann.

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8 Abbreviations

AICD	Activation-Induced Cell Death
AID	Activation Induced cytidine Deaminase
ANA	Anti-Nuclear Antibodies
AP-1	Activator Protein - 1
APRIL	A Proliferation Induced Ligand
ASCs	Antibody Secreting Cells
ASCT	Autologous Stem Cell Transplantation
ATG	Anti-Thymocyte Globulin
BAFF	B-cell Activation Factor
BCL-6	B-cell Lymphoma - 6
BCMA	B-Cell Maturation Antigen
BCR	B-cell receptor
Bio	Biotin
BLIMP-1	B-Lymphocyte Induced Maturation Protein - 1
BM	Bone Marrow
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CXCL	CX-Chemokine Ligand
CXCR	CX- Chemokine Receptor
Cy5	(indodicarbo)Cyanine-5
DAPI	4', 6-Diamidino-2-Phenylindole
DNA	Deoxyribonucleic Acid
DNAse	Deoxyribonuclease
DRFZ	Deutsches Rheuma-Forschungszentrum
dsDNA	Double Stranded DNA
ELISA	Enzyme Linked Immunosorbent Assay
ELISPOT	Enzyme Linked Immunospot
Fab	Fragment Antigen Binding
FACS	Fluorescence Activated Cell Sorting
Fc	Fragment Crystallisable
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
GC	Germinal Centre
Ig	Immunoglobulin
IL	Interleukin
INF	Interferon
IRF-4	Interferon-Regulatory Factor-4
mAb	Monoclonal Antibody
MHC	Major Histocompatibility Complex
NF-κB	Nuclear Factor-kappa B
NSAIDs	Nonsteroidal Anti-inflammatory Drugs
NZB/W	New Zealand Black/White
Ova	Ovalbumin
PAX – 5	Paired Box Protein - 5
PBS	Phosphate Buffered Saline
PCs	Plasma Cells
PE	Phycoerythrin
RNA	Ribonucleic Acid
RPMI	Rosewell Park Memorial Institute
SA-PerCP	Streptavidin – Peridinin Chlorophyll Protein
SDF	Stromal Derived Factor
SEM	Standard Error of the Mean
SLE	Systemic Lupus Erythematosus
TACI	Transmembrane Activator and Calcium modulator ligand Interactor
TCR	T-cell Receptor
TNF	Tumor Necrosis Factor
XBP-1	X Box Protein - 1

9 Acknowledgement

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10 *Curriculum Vitae*

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

11 Publications and Conference Participations

Publications

- Plasma cells in inflamed tissues besides bone marrow survive treatment with dexamethasone and cyclophosphamide.
  Mumtaz I. M., Hoyer B. F., Panne D., Moser K., Winter O., Cheng Q. Y., Yoshida T., Radbruch A., Manz R. A. and Hiepe F. *(Manuscript in preparation)*

- Establishment of long-lived plasma cell compartment in young NZB/W mice is independent of CD4+ T-cell help.
  Mumtaz I. M., Hoyer B. F., Voigt C., Panne D., Cheng Q ., Wondossen S., Radbruch A., Manz R. A., Hiepe F. *(Manuscript in preparation)*

- How to cope with pathogenic long-lived plasma cells in autoimmune diseases.
  Hoyer B. F., Mumtaz I. M., Yoshida T., Hiepe F. and Radbruch A. (*Ann Rheum Dis 2008; 67 (3): p. iii87-iii89)*

- HLA-DR^{high} plasmablasts indicate active disease in patients with SLE.
  Jacobi A. M., Mei H., Hoyer B. F., Mumtaz I. M., Theile K., Radbruch A., Burmester G.R., Hiepe F. and Dörner T. *(Ann Rheum Dis 2009; Ahead of print)*

- B cell and plasma cell homing to inflamed kidneys of NZN/W mice through distinct CXCR3/CXCR5-mediated mechanisms.
  Panne D., Moser K., Mumtaz I. M., Winter O., Voigt C., Enghard P., Hoyer B.F., Radbruch A., Riemekasten G., Fillatreu S., Manz R. A. and Hiepe F. *(Manuscript submitted in Arthritis Rheum.)*

- Short-lived plasmablasts from spleen, but not long-lived plasma cells from spleen and bone marrow of NZB/W mice migrate to IP10 and SDF1α.
  Hoyer B.F., Mumtaz I. M., Voigt C., Radbruch A., Manz R. A. and Hiepe F. *(Manuscript in preparation)*

- IVIG-ID is ineffective on plasma cell compartment of young and old NZB/W mice.
  Hoyer B.F., Mumtaz I. M., Radbruch A., Manz R. A., Shoenfeld Y. and Hiepe F. *(Manuscript in preparation)*

Published Congress Abstracts

- Autoreactive plasma cell memory in murine lupus is refractory to both glucocorticoids and cyclophoshphamide.
  Mumtaz I. M., Hoyer B. F., Panne D., Moser K., Cheng Q. Y., Wondossen S., Winter O., Radbruch A., Manz R. A. and Hiepe F. *(Ann Rheum Dis 2008; 67 (2S): p.471)*

- Bone marrow plasma cell memory is extremely refractory to immunosuppressive therapy.
  Mumtaz I. M., Hoyer B. F., Panne D., Moser K., Cheng Q. Y., Wondossen S., Winter O., Radbruch A., Manz R. A. and Hiepe F. *(Arthritis Rheum 2008; 58 (9S): p.444)*

- Impact of CD4+ T-Lymphocyte depletion on generation of long-lived plasma cells.
  Mumtaz I.M., Hoyer B.F., Panne D., Cheng Q., Wondossen S., Radbruch A., Manz R.A., Hiepe F. *(Lupus 2008; 17 (5): p.501)*

Scientific Conference Participations

1. "AIDS Awareness - Working with high school students". (November 2003 – Pleven, Bulgaria)

2. "Study on the prevalence of Chlamydia trachomatis infections among infertile couples in Pleven region - Immune mechanisms in Chlamydia infections" *Oral presentation* - Immunological Days in Pleven (20.-23. May 2004 – Pleven, Bulgaria)

3. "Diagnostic screening for vision abnormalities in pre-school children" *Oral presentation* - 15th European Students' Conference (ESC) (19.-23. October 2004 – Berlin, Germany)

4. "Glucocorticoids and/or cyclophosphamide do not affect long-lived plasma cell compartment in lupus mice" *Poster presentation* - Keystone Symposia - Biology of B cells in health and disease. (06.-12. February 2007 - Banff, Canada)

5. "Effects of glucocorticoids and/or cyclophosphamide on spleen and bone marrow plasmablasts/plasma cells" *Poster presentation* - 5th German B cell (30. April -02. May 2007 - Bad Bevensen)

6. "Impact of plasma cell memory on autoimmunity" *Oral presentation* - DGRh 2007(19.-22. Sept. 2007 – Hamburg, Germany)

7. "Long-lived plasma cells in lupus mice survive high-dose glucocorticoid and cyclophosphamide therapy" *Poster presentation* - 18th ESC 2007 (7.-11. Oct. 2007 – Berlin, Germany)

8. "Glucocorticoid resistant autoreactive plasma cell memory in lupus mice is contributed mainly by bone marrow plasma cells" *Oral presentation* - 7th European Lupus Meeting (7.-10. May 2008 – Amsterdam, Netherlands)

9. "Impact of CD4+ T lymphocyte depletion on generation of long-lived plasma cells" *Poster presentation* 7th European Lupus Meeting (7.-10. May 2008 – Amsterdam, Netherlands)

10. "Autoreactive plasma cell memory in murine lupus is refractory to both glucocorticoids and cyclophosphamide" *Poster presentation* EULAR 2008 meeting (11.-14. June 2008 – Paris, France)

11. "Depletion of CD4+ T-cells in lupus prone young NZB/W mice does not prevent generation of long-lived plasma cells" *Poster presentation* DGRh 2008 (24.-27. September 2008 – Berlin, Germany)

12. "Bone marrow plasma cell memory is extremely refractory to immunosuppressive therapy" *Poster presentation* ACR 2008 scientific meeting (24.-29. October 2008 – San Francisco, USA)

12 Declaration/Erklärung

I, Imtiaz Mohammed Mumtaz, confirm that my dissertation entitled: 'Effects of immunosuppressive drugs and CD4+ T-cell depletion on plasma cell survival in lupus prone (NZB/W) mice' represents my own work and no part of it is has been reproduced from other works. Any use made within it of works of other authors in any form (ideas, figures, text, and tables) is properly acknowledged with references at their point of use.

Ich, Imtiaz Mohammed Mumtaz, erkläre an Eides statt, dass ich die vorgelegte Dissertationsschrift mit dem Thema: 'Effects of immunosuppressive drugs and CD4+ T-cell depletion on plasma cell survival in lupus prone (NZB/W) mice' selbst verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt, ohne die (unzulässige) Hilfe Dritter verfasst und auch in Teilen keine Kopien anderer Arbeiten dargestellt habe.

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