Changes in peripheral B cell subsets under immunosuppressive therapy

INAUGURAL - DISSERTATION

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To my husband, Lisi Liu,
my daughter, Yue Liu,
and my parents
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1 Introduction

1.1 General function and organization of the immune system

The immune system has two main functions: to protect the body against foreign organisms and to preserve and respect its own integrity. To achieve this goal the immune system disposes of two types of responses: the innate and the adaptive immune response. Phagocytic cells, such as the monocytes, macrophages and polymorphonuclear granulocytes, mediate the innate immune response. They bind to a variety of microorganisms via so-called “pattern recognition receptors” (toll-like receptors, complement receptors, FcIg receptors), internalize them and then kill them in their endosomal compartment. Innate immunity is nonspecific and acts as first line defence against invaders. By means of their pattern recognition receptors phagocytes are generally able to distinguish bacterial and viral structures from self antigens, but they are unable to recognize a particular invader. T and B lymphocytes are central to all adaptive immune responses, and specifically recognize and destroy antigens, whether they are inside host cells (e.g. viruses) or outside in the tissue fluids or blood. Specificity is one of the two key features of adaptive immune response. The other is immunologic memory.

Under normal conditions, lymphocytes have the capacity to discriminate between self and non-self. They achieve this ability by the expression of antigen-specific receptors on T cells (TCR) and B cells (BCR). During ontogeny these TCR and BCR carrying lymphocytes are recruited in processes termed positive and negative selection, which enables the adult immune system to react against foreign structures while remaining tolerant against self antigens. Besides their antigen-specific receptors B lymphocytes (CD19+) and T lymphocytes (CD3+) express a large number of surface molecules which are involved in differentiation, migration, activation and co-stimulation. These surface molecules can be used to identify functionally relevant subsets in healthy and diseased individuals.

B cells (CD19+) are responsible for the humoral immunity. Their BCR is encoded by special genes and is specific for a particular antigen. It represents a multimeric complex consisting of an antigen-recognition structure in form of a membrane-bound immunoglobulin (mIg), associated with two non-covalently bound signal transduction molecules, the
heterodimers Igα (CD79a) and Igβ (CD79b). The BCR plays an important role in virtually every stage of B cell development, including positive and negative selection, maturation and antigen-specific activation (DeFranco et al, 1994). Two processes are required to activate a B cell: antigen interacting with the BCR provides the first signal, which must be followed by co-stimulatory signals from T helper (Th) cells, cytokines or complement split products. This B cell activation process takes place in the germinal centers (GC) of lymph nodes, tonsils, Peyer’s patches and spleen. Once the naive B cells have been activated by antigen and Th, they engage in a terminal differentiation process, characterized by proliferation, class-switch and somatic hypermutation of their BCR. Phenotypically this “GC reaction” results in an irreversible differentiation of naive B cells (CD19⁺CD27⁻) into antibody-producing plasma cells and memory B cells (CD19⁺CD27⁺). The plasma cells (CD19lowCD38⁺) migrate to protected niches in bone marrow and lamina propria of the gut where they become long-lived and produce large amounts of protective antibodies. Memory B cells acquire during the GC reaction a new surface marker (CD27⁺) which identifies them as recirculation memory cells ready to become rapidly reactivated whenever specific antigen enters again the body.

*T cells* (CD3⁺) constitute the basis of antigen-specific cell-mediated immunity. T cell precursors are derived from hematopoietic stem cells in the bone marrow and migrate to the thymus for maturation and selection. The main functions of T cells are to exert effects on other cells, either regulating the activity of cells of the immune system or killing cells that are infected or malignant. T cells do not produce antibodies but a great variety of regulatory cytokines. T and B cells recognize different parts of antigens. While B cells use mIg to recognize epitopes of intact antigenic molecules, T cells recognize processed antigenic fragments, which are presented by MHC molecules. Helper T cells (Th:CD3⁺CD4⁺) recognize processed antigenic peptides in the context of MHC class II molecules expressed on professional antigen presenting cells (APC) while cytotoxic T cells (Tc:CD3⁺CD8⁺) recognize foreign peptides presented by MHC class I molecules.

A dysregulated or defective adaptive immunity is responsible for a variety of diseases. Thus, production of antibodies that react with self antigens may cause autoimmunity, whereas defects in T and B cell differentiation or a disturbed GC reaction lead to immunodeficiency. As the subject of this study is alterations of B cell subsets in autoimmune diseases under drug
therapy, the B cell ontogeny will be briefly summarized.

1.2 The generation, development and differentiation of B cells

Antibody responses are the culmination of a series of cellular and molecular interactions occurring in an orderly sequence between a B cell and a variety of other cells of the immune system. The antibody response not only shows different immunoglobulin classes to an antigen, but also changes in affinity and quantity of antibody produced. The primary antibody response produces serum antibodies as early as 3-5 days after the first contact with an immunogen, it peaks at days 8-10 and persists for some weeks. The produced antibodies are of low affinity and low concentration and initially of the IgM, later of the IgG isotype. The secondary antibody response following repeated exposures to the same antigen appears more rapidly and is much stronger. The antibodies produced are now of high affinity, larger amounts and contain more IgG than IgM. How does the B cell ontogeny and regulation tie into this complex humeral immune response pattern?

1.2.1 The development and differentiation of B cells in bone marrow

B cells are derived from hematopoietic stem cells by a finely tuned differentiation process which can be divided into two phases: an antigen-independent phase in the bone marrow and an antigen-dependent, terminal B cell differentiation in the secondary lymphoid organs requiring antigen and T cell help.

Within bone marrow, several precursor B-cell differentiation stages have been described: progenitor B cells (pro-B cells), precursur B cells (pre-B cells) and immature B-cells. The different B-cell differentiation stages are characterized by immunoglobulin gene rearrangement and differential expression of surface markers. These stages can be identified by the expression of stage-specific markers (CD34, CD10, CD20, CD24, CD38, terminal deoxynucleotidyl transferase (TdT), cytoplasmic (Cy) μ, VpreB, mIgM, mIgD) and B-cell lineage-specific markers (CD19, CD22, CyCD79a).

Pro-B cells are defined as CD19-, CD34+, CD38+, and CD22+. Late pro-B cells begin to express TdT+ and CyCD79a+ in the cytoplasm. In this stage, pro-B cells have not yet
completed immunoglobulin gene rearrangement.

Based on differential expression of several molecules such as CD10, CD19, CD20, TdT, CyVpreB and Cyµ, pre-B cells are divided into pre-B-I cells and pre-B-II cells. The latter are further subdivided into cycling large pre-B-II cells and non-cycling small pre-B-II cells. Pre-B-I cells upregulate recombination activating gene-1 (RAG-1) and RAG-2, and begin to express ‘surrogate’ light chains VpreB in the cytoplasm as well as the surface markers CD19+ and CD10+. In the large pre-B-II cell stage, the expression of CD34, TdT, RAG-1 and RAG-2 has been downregulated, but the µ heavy chains are now expressed in the cytoplasm. The small pre-B-II cells begin to express CD20+ and reexpress RAG-1 and RAG-2 for the subsequent light chain rearrangement. The µ heavy chain, together with the surrogate light chain and the associated Igα/Igβ heterodimers compose the pre-B cell receptor complex on the surface membrane. This receptor is critical for the early B cell differentiation but it can not yet respond to an antigen.

The final B-cell differentiation stage in the bone marrow is represented by the immature B-cell, which expresses CD10+, CD19+, CD20+, CD21+/−, CD22+ and low to high levels of mIgM+. Immature B cells have successfully produced functional light chains of either κ- or λ-type and therefore can down regulate again the RAG-1 and RAG-2 expression. The light chain becomes committed to the antigen-binding specificity of mIgM. Consequently, the immature B-cell is the first cell to express the prototype of the B-cell antigen receptor (BCR) and the first representative of the B-cell lineage to recognize and respond to antigen in a clonotypically restricted manner. The immature B-cell stage is of critical importance to the immune system; it is at this stage that antigen-specific positive and negative selection events initially operate. Such selective events exert a profound influence on the generation of the peripheral mature B-cell repertoire (King & Monroe, 2000). Immature B cells migrate to the periphery at the transitional B-cell stage, when they are still short-lived and functionally immature (Chung et al, 2001 & 2003; Carsetti et al, 2004). Transitional B cells mark the crucial link between the immature B cells of the bone marrow and mature B cells of the periphery. Only 10–20% of B cells that leave the bone marrow ever make it to the spleen where their final fitness for a GC reaction with cognate T cells is shaped (Monroe, 2004).
**Table 1  Characterization of B cells differentiation in bone marrow**

<table>
<thead>
<tr>
<th>Surface Expression</th>
<th>Marker</th>
<th>pro-B cell</th>
<th>Pre-B-I cell</th>
<th>pre-B-II cell</th>
<th>immature B cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>large</td>
<td>small</td>
<td>large</td>
<td>small</td>
</tr>
<tr>
<td>CD34</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD22</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD19</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD10</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD20</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD21</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
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<td>–</td>
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<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CD38</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>mIgM</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>mIgD</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Intrinsic Expression</td>
<td>CyCD79a</td>
<td>– / +</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TdT</td>
<td>– / +</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CyVpreB</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Cyμ</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Recombination of BCR chains</td>
<td>RAG</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>heavy chain</td>
<td>D-J</td>
<td>DJ</td>
<td>VDJ</td>
<td>VDJ</td>
</tr>
<tr>
<td></td>
<td>light chain</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>VJ</td>
</tr>
</tbody>
</table>

**1.2.2  B cell development in secondary lymphatic organs**

The generation of lymphocytes in primary lymphoid organs is followed by their migration into secondary lymphoid organs.

The transitional and immature B cells are free to exit the bone marrow, enter the circulation and travel around the blood and lymphatics through tissue and lymphoid organs. In the spleen transitional B cells develop into long-lived mature B cells (Carsetti et al, 2004), (designated also as naive B cells), which now are ripe to encounter antigen and cognate T cell help. If mature B cells do not encounter antigen, they die within a few weeks by apoptosis.
The transitional B cells express CD19\(^+\), CD21\(^+\), CD24\(^++\), CD38\(^++\), mIgM\(^++\) and mIgD\(^+\) (Carsetti et al, 2004), whereas, naive B cells express CD19\(^+\), CD21\(^+\), CD24\(^+\), CD38\(^+\), mIgM\(^+\) and mIgD\(^++\).

Following antigenic stimulation and T cell help, mature B cells migrate into the GC of lymph nodes, tonsils and spleen and undergo activation, proliferation and differentiation into plasma cells, which are the terminally differentiated form of an antibody-forming cell. Plasma cells produce and secrete antibodies of the same antigen-binding specificity as their mature B cell precursor. Surface immunoglobulins and some membrane molecules, such as mIgD, CD19, CD22 and CD24, are usually lost in plasma cells, as their receptor functions are no longer needed. Instead they re-express CD38 at high density. Plasma cells home to the bone marrow and the lamina propria of the intestine, where they become long-lived.

Alternatively, some activated B cells stop proliferation and differentiation, and become memory B cells. Their phenotype has been described as a highly antigen-specific, long-lived, small lymphocyte, generated in response to infectious agents or vaccines. In the GC they have undergone some somatic mutations and class switching (Liu et al, 1996). CD27 is their newly acquired marker of the B memory status. In addition, memory B cells express CD19\(^+\), CD24\(^++\), CD27\(^+\) but no CD38 which distinguishes them from plasma cells. Based on expression of mIgM and mIgD they can be further subdivided into four populations: IgM\(^+\)/IgD\(^+\) memory B cells (mIgM\(^+/\)mIgD\(^+\), 20% of total B cells), IgM\(^+\) only memory B cells, IgD\(^+\) only memory B cells (together perhaps 1% of total B cells) and switched memory B cells (mIgM\(^-/\)mIgD\(^-\), 20% of total B cells), (Klein et al, 1998; weller et al, 2004). IgM memory B cells have different origins and functions as compared to switched memory B cells. IgM memory B cell originates in the spleen and in extrafollicular sites, and is independent of T cell help (Krüetzmann et al, 2003). IgM memory B cells produce natural antibodies, which confer an immediate protection against many microorganisms. Switched memory B cells, on the other hand, derive from GC reactions, and depend on T cell help (Krüetzmann, et al, 2003). In the GC, B cells undergo affinity maturation by somatic hypermutation of their BCR (MacLennan et al, 1994 & 1997). Thus, switched memory B cells secrete high-affinity antibodies able to recognize previously encountered pathogens and protect the host from re-infection (Zinkernagel, 2002).

As B cells grow into a clonal response to antigen, they may rearrange their
immunoglobulin heavy chain gene and change into switched B cell. This occurs as a result of recombination of the same Ig VDJ gene (the variable region of the Ig) with a different constant (C) region gene, e.g. switch from $\mu$ to $\gamma$ or $\alpha$. In other words, once a B cell makes surface IgM, it can switch to a different class of antibody (isotype), but all antibodies made by that cell recognize the same antigen. The switching mechanism enables the body to have different clones of plasma cells, which produce antibodies with different effector functions.

With the advances in technology during the last 6 years functional B-cell subpopulations (Table 2) can now be identified in peripheral blood allowing interesting insights in the complex development of the B cell lineage in certain diseases.

### Table 2  Markers of B-cell subpopulation in peripheral blood

<table>
<thead>
<tr>
<th>Marker</th>
<th>Transitional B-cell</th>
<th>Naive B-cell</th>
<th>IgM-memory</th>
<th>Switched-memory</th>
<th>Plasmablast</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>CD20</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CD21</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>CD24</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>CD27</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>CD38</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>mIgD</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>mIgM</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>-/(+)</td>
</tr>
</tbody>
</table>
B cells are derived from hematopoietic stem cells. In bone marrow (BM), the development of a B-lineage cell proceeds from pro-B cells to immature/transitional B cells through several stages marked by cell surface molecules, and the rearrangement and expression of the immunoglobulin (Ig) genes. At the pro-B cell stage the heavy-chain (H-chain) locus rearranges first. A successful VDJ<sub>H</sub> rearrangement leads to the expression of a complete Ig heavy chain as part of the pre-B-cell receptor. Pre-B-I cells express cytoplasmic VpreB/λ5 which is encoded from the light-chain (L-chain) locus. At the large pre-B-II cell stage the cytoplasmic expression of surrogate light chains occurs together with the μ heavy chain. Small amounts of both molecules combine and form the pre-B-cell receptor, which translocates to the cell surface. At the immature B cell stage a complete membrane IgM (mIgM) molecule is expressed on the cell surface. The expression of the Ig heavy and light chains are key milestones in this differentiation pathway.

Immature and transitional B cells can migrate from the BM to the spleen and develop there into mature B cells which express both mIgM and mIgD. Following antigenic stimulation and T cell help, in the germinal centers (GC) of spleen, lymph nodes or tonsils mature B cells develop into plasma cells which home back to the BM. In peripheral blood plasma cells are short-lived, in BM long-lived. Alternatively, some of activated mature B cells become memory B cells. IgM/IgD<sup>+</sup> memory B cells develop in the spleen; switched memory B cells in the GC.
1.2.3 B cell tolerance to self

The immune system of normal individuals is able to recognize, respond to, and eliminate foreign antigens (non-self) while not reacting harmfully to the own self antigens. Immunologic tolerance is defined as a state of specific unresponsiveness of an immunocompetent cell towards its antigen. B cell tolerance to self-antigens is maintained by several mechanisms. Experiments with transgenic mice have led to the concept that there are multiple “checkpoints” during B cell maturation and activation at which encounter with self antigens may abort these processes. B cells that recognize self-antigens undergo a screening and censoring process to eliminate their ability to produce autoantibodies (Hartley et al, 1993).

The immature and transitional B-cell stages define an important window in B-cell development (King & Monroe, 2000). In the bone marrow, all immature B cells, in the absence of secondary signals, undergo receptor-mediated negative selection, a process whereby autoreactive B-cell receptors are culled from the immune repertoire. Few immature B cells (ca. 20%) which are not negatively selected survive this process. They leave the bone marrow and emigrate initially to the spleen, where they are known as transitional B cells. Three mechanisms of negative selection have been described: deletion; anergy; and receptor editing (King & Monroe, 2000; Wang & Clark, 2003).

The first process, clonal deletion, is the physical elimination of B cell clones by apoptosis. Some of the immature B cells expressing a BCR with high affinity for autoantigens might be deleted, i.e. negatively selected at the transition from bone marrow to spleen (Rolink et al, 2004). The second process, clonal anergy, refers to the functional inactivation of autoreactive B cells. Immature B cells, which encounter lower affinity autoantigens, enter a state of anergy. Anergic B cells have become inactivated but remain alive (Hodgkin & Basten, 1995). The third process is receptor editing which means that autoreactive immature B cells may induce secondary immunoglobulin gene rearrangements in order to replace their autoreactive receptors. As a consequence this B cell is longer at risk to be eliminated or inactivated (Pelanda et al, 1997; Tiegs et al, 1993; Gay et al, 1993; Prak & Weigert, 1995; Hertz & Nemazee, 1997).
Peripheral mature B cells that recognize self-antigens in the absence of specific helper T cells may be rendered functionally unresponsive. These B cells lose their capacity to migrate into lymphoid follicles and thus to be activated to produce antibodies against the self-antigens.

1.3 Diseases

Normal homeostasis of the immune system is critical for the maintenance of immunological tolerance and immunocompetence. Failure of self-tolerance results in autoimmune reactions which may progress to chronic autoimmune diseases. In this study peripheral B cells subsets were studied in three established autoimmune conditions described below.

1.3.1 Systemic lupus erythematosus (SLE)

Systemic lupus erythematosus (SLE) is a prototypical human autoimmune disease (Liossis et al, 1996; Criscione & Pisetsky, 2003) characterized by the appearance of high titer IgG autoantibodies against nuclear self-antigens (ANA) leading to systemic immune complex vasculitis and organ damage.

SLE is a worldwide disease with a variable incidence (2-10/100,000/year) depending on age, sex and ethnicity (Jonsson et al, 1990; McCarty et al, 1995; Lahita, 1995; Stahl-Hallengren et al, 2000). SLE can occur at all ages, but diagnosis before puberty and after menopause is uncommon (Mok & Lau, 2003). Women in the reproductive age are 9-10 times more often affected than men.

The etiology of SLE remains unknown. It is likely that a combination of genetic defects (early complements components, apoptosis controlling genes, MHC), environmental influences (viruses, ultraviolet light, and drugs), and hormonal factors work together to cause the disease.

In addition, SLE shows a strong familial aggregation, with a much higher frequency among first degree relatives of patients. In twins the disease concordance rate is 2–5% for dizygotic twins and 24–58% for monozygotic twins (Tsao, 2003).
The pathogenesis of SLE is complex. Upon apoptosis of host cells a variety of nuclear antigens (e.g., nucleosomes, histones, dsDNA, U1 RNP, Ro/SS-A, a.o.) are released. Normally they are rapidly removed from circulation and degraded by phagocytes of the reticulo-endothelial system. In SLE patients - due to defects in early complement factors and an impaired phagocytosis - nuclear self-antigens show a prolonged persistence in blood and therefore are more readily available for B cell activation. At the same time the frequency of autoreactive B cells in peripheral blood is significantly increased due to defects in negative selection of the immature BCR repertoire (Schwab et al, 1994). This scenario facilitates the breaking of tolerance and the production of harmful anti-nuclear-antibodies, which combine with auto-antigen to form pathogenic immune complexes. Their deposition in target structures (glomeruli, vessels, brain, skin a.o.) leads to tissue damage and more release self-antigens, which perpetuate the process (Hahn, 2001).

B cells are essential in the pathogenesis of SLE (Chan et al, 1999). Several studies have demonstrated a polyclonal B cell hyperactivity (Grammer & Lipsky, 2003; Zouali 2001) with increased rates of proliferation and a spontaneous production of immunoglobulins (Kikuchi et al, 2002). The produced immunoglobulins comprise both natural antibodies and autoantibodies reactive with nuclear, cytoplasmic and cell membrane derived self-antigens (Liossis & Zouali, 2004). Besides ANAs many other autoantibodies are produced in SLE patients leading to widespread inflammation, immune complex vasculitis and multiorgan involvement of skin, joints, heart, lungs, bowel, kidneys, serosal surfaces, blood cells, vessels and central nervous system. Infections and diseases of the cardiovascular, renal, pulmonary, and central nervous systems are the most frequent causes of death in SLE patients (Koh et al, 1997; Blanco et al, 1998; Jacobsen et al, 1998; Stahl-Hallengren et al, 2000).

For a reliable diagnosis of SLE the classification criteria proposed and validated by the American College of Rheumatology (ACR) have proven very useful (Tan et al, 1982; Hochberg, 1997).

Treatment of SLE is supposed to reduce the level of autoimmunity, to decrease inflammation and to restore organ function. Patients with SLE can prevent "flares" of disease by avoiding sun exposure and infections. Drug treatment must be individualized for each patient, depending on the particular symptoms and their severity. Non-steroidal
Introduction

anti-inflammatory drugs (NSAIDs) are usually recommended for muscle and joint pain. If the symptomatology escalates corticosteroids and immunosuppressive or disease modifying antirheumatic drugs (DMARDs: hydroxyl/chloroquine, azathioprine, methotrexate, cyclosporine A, mycophenolate) need to be prescribed. Continuous use of DMARDs may prevent “flares” from recurring. In some cases, the use of DMARDs allows an individual to take lower doses of corticosteroids, thereby decreasing the risks of hypercorticism. For lupus nephritis and neuropsychiatric lupus monthly intravenous pulses of 500-1000mg cyclophosphamide (CYC) have been shown to be the treatment of choice owing its efficacy to the capacity of CYC to rapidly block transcription and translation of autoimme and proinflammatory gene products. However, as CYC is potentially very toxic for gonads, bone marrow, bladder mucosa and the immune system and since it has been proven to be carcinogeneic and teratogenic it needs a very careful treatment indication and surveillance. Whereas the cumulative CYC dose is critical for the gonadal, bladder and bone marrow toxicity, nothing is known about how CYC treatment affects the B cell lineage differentiation and the B memory compartment.

1.3.2 Wegener’s granulomatosis (WG)

Wegener’s granulomatosis (WG) is an uncommon disease characterized by necrotizing granuloma of the respiratory tract, systemic vasculitis and glomerulonephritis. The disease was first described in 1931 by Heinz Klinger, a German medical student (Klinger, 1931). In 1936 and 1939, Dr. Friedrich Wegener, a young pathologist, provided detailed information about three patients with a similar illness (Wegener, 1936 & 1939).

Several epidemiologic studies suggest that WG affects both sexes equally, occurs in patients of all ages (mean age, 41 years; range, 9 to 78years) (Calabrese & Duna, 2001) The estimated incidence is 1:100,000 per year (Berlis et al, 2003).

The etiology of WG remains unknown. However, increasing circumstantial evidence supports the concept that WG is an autoimmune disease. Antineutrophil cytoplasmic antibodies (ANCA), discovered in 1985 (Van der Woude et al, 1985), play a role in its pathogenicity and are now considered to be a useful tool for diagnosis (Girard et al, 2001).
There are two main ANCA patterns detected by indirect immunofluorescence (IIF): cytoplasmic ANCA (C-ANCA) and perinuclear ANCA (P-ANCA). C-ANCA has been found to have a high degree of sensitivity and specificity for WG (Van der Woude et al, 1985; Cohen et al, 1989). There is a strong and specific association with autoantibodies directed against proteinase 3 (PR3), a constituent of neutrophil azurophilic granules (Hewins et al, 2000). C-ANCA occur in a frequency varying from 90% in clinically active WG to 40% in remission (Van der Woude et al, 1985; Specks et al, 1989; Kerr et al, 1993). The well established association of PR3-ANCAs and WG has fostered theories about the pathogenic role of these antibodies (Calabrese & Duna, 2001). It has been suggested that these antibodies participate directly in triggering and sustaining the vascular disease activity (van der Geld et al, 2002).

First, C-ANCA is able to activate primed neutrophils to produce oxygen radicals and release lytic enzymes, including PR3 itself (Falk et al, 1990; Charles et al, 1991; Wiik, 2000). Second, C-ANCA can interfere with the proteolytic activity of PR3 (van de Wiel et al, 1992; Daouk et al, 1995). In addition, C-ANCA induce increased adherence of neutrophils to endothelium by up-regulating cell adhesion molecules expression (Mayet et al, 1996). Accumulation of neutrophil granule enzymes adjacent to endothelium, causing localized endothelial damage (Savage et al, 1992; Mayet et al, 1994), would account for the involvement of C-ANCA in vasculitis pathogenesis.

Clinical manifestations and organ involvement of WG vary widely. A clinical triad consists of upper and lower airway disease, kidney involvement and small vessel vasculitis. Variable degrees of disseminated vasculitis, involving both small arteries and veins, may occur (Hoffman et al, 1992; Reinhold-Keller et al, 2000; Yi & Colby, 2001). Other organs involved include joints, skin, eyes, ears, heart and nervous system. The onset of WG may be gradual or rapid and severe. Patients often present with non-specific findings of fever, malaise, weight loss, arthralgias, myalgias, and chronic rhinitis or worsening sinusitis. Severe manifestations involve saddle-nose deformity, shortness of breath, bloody or purulent sputum, pulmonary hemorrhage and polyneuritis, even respiratory failure or renal failure.

In 1990, the ACR proposed criteria for the classification of WG to distinguish patients with WG from patients with other forms of vasculitis (Leawitt et al, 1990). The ACR definition highlights four selected criteria: abnormal urinary sediment, abnormal findings on
Introduction

The presence of two or more of these four criteria was associated with a WG sensitivity of 88.2% and a specificity of 92%. In addition, laboratory tests such as CRP-measurement, leukocyte count, sedimentation rate, urinalysis for hematuria and proteinuria, and serology for detection of antibodies against GBM and ANCA may be particularly helpful in confirming the diagnosis. (Stegmayr et al, 2000)

Early treatment of the disease may improve the prognosis. In former times, the outcome almost invariably was fatal. The prognosis dramatically improved once sufficient immunosuppressive regimens became available (Yi & Colby, 2001). CYC and corticosteroids are still the gold standard for the remission inducing treatment of WG, followed by immunosuppressive drugs (DMARDs) for remission maintenance. Untreated, WG carries a very poor prognosis and is usually fatal, primarily due to the associated end-stage renal failure. ANCA titers correlate well with the progression of the disease. When there is a relapse or a flare-up, the ANCA levels go up. Levels decrease when the disease is controlled by appropriate treatment.

1.3.3 Allogeneic stem cell transplantation (allo-SCT) in patients with leukemia or other hematologic malignancies

1.3.3.1 Allo-SCT in patients with hematologic disorders

Hematopoietic stem cell transplantation (HSCT) as a form of treatment began to be explored scientifically after explosion of the first atomic bomb. In the late 1960s, the results of the large animal studies began to be translated into the clinic. Since then, HSCT after high dose chemoradiation regimens has evolved into a highly effective treatment for many patients with life-threatening malignant and nonmalignant hematological diseases. In addition, HSCT could also be used to treat patients with inborn errors (e.g., sickle cell disease and immunodeficiency diseases) (Storb, 2003; Fischer 1999; Buckley et al, 1999), those with severe rheumatic autoimmune diseases (e.g., rheumatoid arthritis and SLE) (Van Laar & Tyndall, 2003), and those with neoplastic disorders.

The most common type of marrow graft is an allogeneic one from a HLA-compatible
sibling donor. Various studies have reported excellent results for HLA-identical bone marrow transplantation (BMT), with full restoration of T- and B-cell function in most patients (Fischer 1999; Buckley et al, 1999). HLA-identical family donors can be identified in approximately 30% of patients with diseases that are potentially treatable with marrow-ablative therapy and allo-SCT. Unrelated HLA-matched donors can be found for approximately 35% of patients (Beatty et al, 1988).

Allo-SCT provides a cure for various leukemias and other hematologic disorders, such as lymphomas, multiple myeloma (MM), myelodysplastic syndrome (MDS), and severe aplastic anemia. The rationale for SCT is based on the fact that all blood cells (e.g., erythrocytes, phagocytes, and platelets) and immune cells (lymphocytes) arise from the stem cells. This procedure of allo-SCT is performed to restore lymphohematopoiesis in patients with bone marrow failure, and to replace a diseased marrow with a healthy donor marrow (Tabbara et al, 2002). In addition, the studies suggested that the graft-versus-leukemia (GVL) reactions observed are thought to be due to the expression of polymorphic minor histocompatibility antigens including so-called hematopoietic antigens on normal and leukemic marrow cells, that trigger the generation of cytotoxic donor lymphocytes which kill and control the leukemia (Goulmy, 1997; Riddell & Greenberg, 1999).

Meanwhile, studies have been shown that patients receiving an unrelated HLA-identical marrow graft have higher morbidity and mortality than patients receiving a related HLA-identical marrow graft, due to an increase in the incidence of acute and chronic graft versus host disease (GvHD), graft failure, infections, and prolonged convalescence. However, these patients have a lower risk of leukemic relapse, most likely due to more intense GvHD, which translates into more significant GVL reactions (Kernan et al, 1993; Marks et al, 1993).

Despite the morbidity and mortality associated with this treatment modality, allo-SCT may provide a 20% to 90% chance of long-term, disease-free survival (DFS) to patients with a wide variety of neoplastic and abnormal marrow disorders. The studies on the long-term results of allo-SCT have been extensively evaluated for the treatment of acute and chronic leukemias (Santos et al, 1983; Chao et al, 1991; Clift et al, 1992; Biggs et al, 1992; Barrett et al, 1992; Pavletic et al, 2000). Few data were available regarding its use for MDS (Anderson et al, 1993), MM (Gahrton et al, 1995), and lymphomas (Chopra et al, 1992; Ratanatharathorn
et al, 1994).

Unfortunately, allo-SCT is associated with a high incidence of transplant related complications, including regimen-related toxicities, GvHD and opportunistic infections. Patients receiving allo-SCT are highly susceptible to infections because of immunodeficiency, neutropenia, and the immunosuppressive therapy used to prevent or treat GvHD (Tabbara et al, 2002). Thus various infectious complications (e.g., bacterial, fungal and viral infections) can be usually observed in patients after allo-SCT. Factors leading to increased susceptibility for late infections in patients with chronic GvHD include impaired mucosal defense, chemotactic defects, functional asplenia, and qualitative and quantitative B and T cell abnormalities (Tabbara et al, 2002; Siadak & Sullivan, 1994; Storek et al, 2001; Sherer & Shoenfeld, 1998; Maury et al, 2001).

Deficiencies in the cellular and humoral parts of the immune system occur to some degree in all marrow transplant recipients for a variable time period and tend to be more profound and prolonged in patients with GvHD who are receiving immunosuppressive therapy (Lum, 1990). The cellular immune deficiency consists of decreased T cell response to alloantigens and mitogens, decreased CD4+ T helper cell (Th) function, and decreased reactivity to intradermal skin tests (Tabbara et al, 2002). Humoral immune deficiency is manifested by a decrease in IgG2 and IgG4, although immunoglobulin levels may be normal. The switch from primary (IgM) to secondary (IgG) production and antigen-specific responses is abnormal, leading to impaired production of antibodies to pathogens. Nothing is known about B cell subsets in patients with stably engrafted allo-SCT. The recovery of the immune system occurs within 6 months to a year after allo-SCT in the absence of GvHD and after the discontinuance of immunosuppressive therapy (Tabbara et al, 2002).

1.3.3.2 Graft versus host disease (GvHD)

GvHD remains one of the major complications after allo-SCT. It has been divided into two forms based on timing of occurrence and clinical manifestations: acute and chronic GvHD. The acute form occurs in the first 2 to 3 months after transplantation and the chronic form manifests later in the posttransplant period (Ferrara & Deeg, 1991). The pathophysiology of GvHD
remains poorly defined. Current evidence suggests that GvHD may be due to both alloreactive and autoreactive T cells.

Acute GvHD occurs as a result of donor T cell recognition of recipient HLAs as foreign and has been reported to cause atrophy of the lymphoid system, thymic regression, delayed B cell reconstitution, and reversal of the CD4/CD8 ratio, leading to increased predisposition for infections (Ferrara & Deeg, 1991). The tissue damage occurring in acute GvHD is thought to be mediated by infiltration of the target organ by T cells. Cytokines, including interleukin-1 (IL-1), tumor necrosis factor α (TNF-α), and IL-2, are thought to be critical to the development of acute GvHD (Antin & Ferrara, 1992; Antin et al, 1994).

However, it was suggested that the pathophysiology of chronic GvHD was different from acute GvHD. In chronic GvHD, T lymphocytes were primarily autoreactive, with specificity for histocompatibility antigens shared by donor and recipient cells. These autoreactive T lymphocytes were capable of cytolysis and cytokine production. IL-4 and interferon-γ (IFN-γ) are produced by these cells and may be the cause of immunologic dysfunction associated with this syndrome, including the increased production of autoantibodies (Tabbara et al, 2002).

Chronic GvHD is the most common non-relapse problem affecting long-term survivors of allo-SCT. The median time for the diagnosis of chronic GvHD was 4.5 months after HLA-identical sibling transplant and 4 months after unrelated donor transplant (Lee et al, 2002). The majority of patients with chronic GvHD have had prior acute GvHD. The other risk factors of chronic GvHD include older age, female donors and male patients, use of mismatched or unrelated donors, infusion of donor lymphocytes, use of peripheral blood stem cell (PBSC) instead of bone marrow (BM), and lack of T-cell depletion (Higman & Vogelsang, 2004). Chronic GvHD causes profound immune dysfunction (Siadak & Sullivan, 1994; Storek et al, 1996; Sherer & Shoenfeld, 1998; Maury et al, 2001), and most chronic GvHD deaths are attributable to infection.

There is no reliable laboratory indicator of the onset or progress of chronic GvHD. The diagnosis of chronic GvHD is often based on clinical findings. The clinical presentation of chronic GvHD is similar to that of autoimmune disorders (Tabbara et al, 2002). Patients with this disease may have a wide variety of manifestations, including the sicca syndrome, skin lesions, keratoconjunctivitis, oral mucositis, esophageal strictures, bronchiolitis obliterans,
malabsorption, hepatic involvement with hyperbilirubinemia, and suppressed hematopoietic reconstitution. It is important to obtain a biopsy of the affected organ when possible. Skin and oral mucosal biopsies may be helpful in establishing the diagnosis of chronic GvHD (Tabbara et al, 2002).

1.3.3.3 Functional asplenia

Functional asplenia (FAS) is a late complication after allo-SCT and contributes to the high susceptibility to bacterial infections in patients with extensive chronic GvHD (Kalhs et al, 1988; Castagnola & Fioredda, 2003). FAS is classically defined by (1) the presence of Howell-Jolly bodies in circulating red cells and (2) the absence of reticuloendothelial splenic function, demonstrated by the disappearance of $^{99m}$Tc splenic uptake, even in the presence of a palpable spleen (Pearson et al, 1969). Recently the spleen has been identified as the place where T cell independent primary IgM antibodies to polysaccharides of bacterial membranes (e.g. Streptococcus pneumoniae and Hemophilus influenzae) are produced (Krutzmann et al, 2003; Weller et al, 2004). Splenectomy or congenital asplenia lead to the abolition of natural antibodies to pneumococcal polysaccharides and to the disappearance from peripheral blood of 90% of IgM memory B cells (CD19$^+$CD27$^+$IgM/D$^+$). One of the questions of this study was if functional asplenia following chronic GvHD would also cause a decrease in IgM memory B cells and thus explain the increased susceptibility to infections of these patients.
2 Aims and Questions of this study

1\textsuperscript{st} Aim: To analyze in SLE and WG patients the influence of immunosuppressive treatment with cyclophosphamide (CYC) and/or conventional immunosuppressive reagents (DMARDs) on peripheral B-cell subsets and outline the resulting type of immunodeficiency.

1\textsuperscript{st} Question: Differ B cell subsets in their susceptibility to immunosuppressive treatment with CYC or DMARDs?

2\textsuperscript{nd} Aim: To test the hypothesis that functional asplenia following allogeneic stem cell transplantation impairs IgM memory B cell development in a similar fashion as splenectomy in healthy individuals.

2\textsuperscript{nd} Question: Do patients with functional asplenia after allogeneic stem cell transplantation develop a normal IgM memory B cell compartment and is there a difference between patients with and without Howell-Jolly bodies in circulating erythrocytes?
3 Materials and methods

3.1 Patients and controls

3.1.1 The patient groups of SLE and WG

45 patients suffering from systemic lupus erythematosus (SLE) and 19 from Wegener’s granulomatosis (WG) were analysed in the study. All patients fulfilled the diagnostic criteria proposed by the American College of Rheumatology (ACR) for SLE or the classification criteria for WG (Tan et al, 1982; Hochberg, 1997; Leawitt et al, 1990). SLE patients were subdivided into 3 groups according to their therapeutic regimen: 1st. Newly diagnosed and untreated SLE patients. 2nd. Patients who had never received CYC but were treated with conventional immunosuppressive treatment such as chloroquine, azathioprine, methotrexate, leflunomide or cyclosporine A (DMARDs). 3rd. Patients who had received pulse cyclophosphamide (CYC) between 1 and 108 months prior to testing. In between they received conventional DMARDs. All WG patients had completed CYC treatment (orally 2mg/kg or monthly pulses 10-15mg/kg) one to three months prior to testing. In between they received maintenance therapy with methotrexate, leflunomide or azathioprine (Table 3).

Table 3 Characterization of the patient groups of SLE and WG

<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>Mean age (yr)</th>
<th>Gender (M:F)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st No therapy</td>
<td>5</td>
<td>30.7</td>
<td>0:5</td>
</tr>
<tr>
<td>SLE 2nd DMARD*</td>
<td>24</td>
<td>45</td>
<td>3:21</td>
</tr>
<tr>
<td>3rd CYC** +DMARD</td>
<td>16</td>
<td>42.1</td>
<td>3:13</td>
</tr>
<tr>
<td>WG CYC+DMARD</td>
<td>19</td>
<td>58.6</td>
<td>14:5</td>
</tr>
</tbody>
</table>

* Disease modifying antirheumatic drug  ** Cyclophosphamide

3.1.2 The patients after allo-SCT

In addition we studied a group of 12 patients with hematologic malignancies in stable remission 2 to 8.5 years after allo-SCT. This group comprised 4 patients with acute myeloid leukemia (AML), 2 patients with chronic myeloid leukemia (CML), 2 patients with acute...
lymphoblastic leukemia (ALL), 2 patients with myelodysplastic syndrome (MDS) and 2 patients with multiple myeloma (MM). 10 patients presented with a mild to moderate graft-versus-host reaction (GvHD), 5 had a small spleen with Howell-Jolly bodies in circulating erythrocytes. The detailed characterization of these patients is shown in Table 4.

<table>
<thead>
<tr>
<th>Pat.</th>
<th>Age (yr.)</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Allo-SCT</th>
<th>GvHD*</th>
<th>Howell-Jolly&amp;</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pat.1</td>
<td>37</td>
<td>F</td>
<td>CML</td>
<td>6y8m*</td>
<td>++</td>
<td>+</td>
<td>small**</td>
</tr>
<tr>
<td>Pat.2</td>
<td>57</td>
<td>M</td>
<td>MDS</td>
<td>2y5m*</td>
<td>++</td>
<td>+</td>
<td>small**</td>
</tr>
<tr>
<td>Pat.3</td>
<td>59</td>
<td>M</td>
<td>MDS</td>
<td>2y0m*</td>
<td>++</td>
<td>+</td>
<td>small**</td>
</tr>
<tr>
<td>Pat.4</td>
<td>48</td>
<td>M</td>
<td>Myelom</td>
<td>5y6m*</td>
<td>++</td>
<td>+</td>
<td>small</td>
</tr>
<tr>
<td>Pat.5</td>
<td>28</td>
<td>F</td>
<td>CML</td>
<td>8y6m</td>
<td>++</td>
<td>+</td>
<td>Splenectomy</td>
</tr>
<tr>
<td>Pat.6</td>
<td>55</td>
<td>F</td>
<td>AML</td>
<td>3y4m</td>
<td>++</td>
<td>–</td>
<td>normal**</td>
</tr>
<tr>
<td>Pat.7</td>
<td>58</td>
<td>M</td>
<td>Myelom</td>
<td>4y5m*</td>
<td>++</td>
<td>–</td>
<td>normal</td>
</tr>
<tr>
<td>Pat.8</td>
<td>32</td>
<td>F</td>
<td>AML</td>
<td>4y10m</td>
<td>–</td>
<td>–</td>
<td>normal**</td>
</tr>
<tr>
<td>Pat.9</td>
<td>41</td>
<td>F</td>
<td>ALL</td>
<td>7y8m</td>
<td>++</td>
<td>–</td>
<td>normal**</td>
</tr>
<tr>
<td>Pat.10</td>
<td>29</td>
<td>M</td>
<td>ALL</td>
<td>2y8m</td>
<td>–</td>
<td>–</td>
<td>normal**</td>
</tr>
<tr>
<td>Pat.11</td>
<td>73</td>
<td>F</td>
<td>AML</td>
<td>3y3m*</td>
<td>++</td>
<td>–</td>
<td>normal</td>
</tr>
<tr>
<td>Pat.12</td>
<td>37</td>
<td>M</td>
<td>AML</td>
<td>4y0m</td>
<td>++</td>
<td>–</td>
<td>normal**</td>
</tr>
</tbody>
</table>

* Familial donor
** Donor recipient sex mismatch
& Howell-Jolly bodies indicating asplenia

3.1.3 The normal control group

The normal control group comprised 26 healthy subjects, 16 men and 10 women, ranging in age from 29 to 53 years, with an average of 40.1 years.

Informed consent was obtained from all subjects included in this study.
3.2 Materials

The reagents, media and equipment used in this study are listed in the tables below.

3.2.1 Reagents and media

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Composition</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>FCS-fetal calf serum</td>
<td>PAN Biotech.,</td>
<td>Aidenbach</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aidenbach</td>
</tr>
<tr>
<td>R0-complete medium</td>
<td>RPMI 1640</td>
<td>Biochrom, Berlin</td>
</tr>
<tr>
<td></td>
<td>+100U/ml Penicillin</td>
<td>Biochrom, Berlin</td>
</tr>
<tr>
<td></td>
<td>+100μg/ml Streptomycin</td>
<td>Biochrom, Berlin</td>
</tr>
<tr>
<td>R10</td>
<td>R0 + 10%FCS</td>
<td>Biochrom, Berlin</td>
</tr>
<tr>
<td>Ficoll Separating Solution</td>
<td>Density 1.077g/cm³</td>
<td>Biochrom, Berlin</td>
</tr>
<tr>
<td>FACS Solution</td>
<td></td>
<td>Becton Dickinson, Heidelberg</td>
</tr>
<tr>
<td>Optilyse B Lysing Solution</td>
<td></td>
<td>Immunotech, France</td>
</tr>
<tr>
<td>Aqua dest. Solution</td>
<td></td>
<td>Delta Select, Pfullingen</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>3%</td>
<td>Roth, Karlsruhe</td>
</tr>
</tbody>
</table>
3.2.2 Equipments and subsidiary

<table>
<thead>
<tr>
<th>Apparatuses</th>
<th>Mark</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow cytometer</td>
<td>FACS Calibur</td>
<td>Becton-Dickinson, Heidelberg</td>
</tr>
<tr>
<td>Sterile workbank</td>
<td>UVF 6.12S(DIN 12950)</td>
<td>BDK, Sonnenbühl-Genkingen</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>Rotixa/P</td>
<td>Hettich, Tuttlingen</td>
</tr>
<tr>
<td>Vortex</td>
<td>REAX 2000</td>
<td>Heidolph, Schwabach</td>
</tr>
<tr>
<td>Counting Chamber Neubauer</td>
<td>0.0025mm²</td>
<td>Brand, Wertheim</td>
</tr>
<tr>
<td>Microscope</td>
<td>Standard 20</td>
<td>Zeiss, Oberkochen</td>
</tr>
<tr>
<td>Centrifuge tubes</td>
<td>Polypoplylen 15ml, 50ml</td>
<td>Greiner, Frickenhausen</td>
</tr>
<tr>
<td>FACS-tube</td>
<td>Polystyrol, 5ml</td>
<td>Falcon, Heidelberg</td>
</tr>
<tr>
<td>Pipettors</td>
<td>0.5-10μl</td>
<td>Ratiolab, Dreieich</td>
</tr>
<tr>
<td></td>
<td>50-200μl</td>
<td>Ratiolab, Dreieich</td>
</tr>
<tr>
<td></td>
<td>200-1000μl</td>
<td>Biohit, Köln</td>
</tr>
<tr>
<td>Pipettes</td>
<td>5ml, 10ml</td>
<td>Greiner, Frickenhausen, Costor, Corning</td>
</tr>
<tr>
<td>CELL Quest Software</td>
<td></td>
<td>Becton-Dickinson, Heidelberg</td>
</tr>
</tbody>
</table>
### 3.2.3 Staining antibodies

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Clone</th>
<th>Fluorochrome</th>
<th>Company</th>
<th>Expression / function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotype control</td>
<td>Goat F(ab)₂</td>
<td>PE</td>
<td>SB Biozol, Eching</td>
<td>immature and mature B cells bear surface mIgM; B cell receptor</td>
</tr>
<tr>
<td>Isotype control</td>
<td>Goat F(ab)₂</td>
<td>Cy5</td>
<td>Dianova, Hamburg</td>
<td>mature B cells bear surface mIgD; B cell receptor</td>
</tr>
<tr>
<td>Isotype control</td>
<td>Mouse IgG1</td>
<td>FITC</td>
<td>Beckman-Coulter, Krefeld</td>
<td>mature class switched B cells bear surface mIgA; B cell receptor</td>
</tr>
<tr>
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<td>Cy5</td>
<td>Dianova, Hamburg</td>
<td>immature and mature B cells bear surface mIgM; B cell receptor</td>
</tr>
<tr>
<td>Anti-IgD</td>
<td>Goat F(ab)₂</td>
<td>PE</td>
<td>SB Biozol, Eching</td>
<td>mature B cells bear surface mIgD; B cell receptor</td>
</tr>
<tr>
<td>Anti-IgA</td>
<td>Goat F(ab)₂</td>
<td>PE</td>
<td>SB Biozol, Eching</td>
<td>mature B cells bear surface mIgA; B cell receptor</td>
</tr>
<tr>
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<td>mature B cells; light chain</td>
</tr>
<tr>
<td>Anti-lambda</td>
<td>JDC-12</td>
<td>PE</td>
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<tr>
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<td>PC7</td>
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<tr>
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<td>BL13</td>
<td>FITC</td>
<td>Beckman-Coulter, Krefeld</td>
<td>mature B cells, FDC; receptor of complement and EBV idem</td>
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<tr>
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<tr>
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<td>M-T271</td>
<td>FITC</td>
<td>DakoCytomation, Hamburg</td>
<td>binds CD70</td>
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3.3 Methods

To investigate the distribution of the B cell subset in healthy donors and patients with autoimmune diseases peripheral blood mononuclear cells (PBMC) were isolated, stained with monoclonal reagents and analysed by flow cytometry. After informed consent was obtained from each tested person 8ml of venous blood were drawn into an anticoagulant EDTA tube. The samples were tested within 24 hours.

3.3.1 Isolation of peripheral blood mononuclear cells (PBMC)

PBMC were isolated from EDTA blood by density gradient centrifugation. During centrifugation, cells in the blood sample sediment towards the interface between blood and the Ficoll Separating Solution. Differential migration during centrifugation results in the formation of layers containing different cell types. Several factors, such as the different density of peripheral blood cells, contribute to the success of this separation. Erythrocytes have a high density due to their iron content and therefore sediment readily to the pellet at the bottom of the tube. Granulocytes also sediment into the Ficoll Separating Solution but not as deep as erythrocytes. This process is facilitated by an increase in density caused by contact with the slightly hypertonic Ficoll medium. Because of their lower density, lymphocytes and monocytes can not penetrate into the Ficoll layer and therefore stay at the plasma/Ficoll interface from where they can be recovered by careful aspiration (Figure 2)
In detail the following procedure was applied:

1. Mix 8ml EDTA blood with an equal volume of medium R0 (RPMI1640 supplemented with + Hepes+Glutamin+100U/ml Penicillin+100μg/ml Streptomycin).

2. With a sterile pipette, place 15ml Ficoll Separating Solution into a 50ml conical centrifuge tube.

3. Carefully layer the diluted blood on Ficoll Separating Solution by gently pipetting. Centrifugate at 900 xg for 20 min. at room temperature.

4. Using a sterile pipette, carefully remove the mononuclear cells, located at the interface between the plasma (upper layer) and the Ficoll Separating Solution (bottom).

5. Transfer the aspirated mononuclear cells to a 50ml conical centrifuge tube.

6. First wash - Add 20 ml R0 and mix thoroughly. Centrifuge at 900 xg for 10 min. at room temperature.

7. Discard the supernatant. Gently resuspend the pellet in 1ml R10 and transfer the cell suspension to a 15ml conical centrifuge tube.

8. Second wash - Add 5 ml R10 and mix thoroughly. Centrifuge at 200 xg for 10 min. at room temperature.
9. Discard the supernatant and resuspend cells in 1 ml R10.

10. Count the cell density and adjust the cell concentration to $10 \times 10^6 / \text{ml}$ with R10.

### 3.3.2 Immunofluorescence Staining of PBMC

The surface markers of B-lymphocytes represent receptors that relate with their differentiation, activation and function. B-cell subpopulations can be identified and characterized on the basis of surface marker expression, using the techniques of immunofluorescence. Four different fluorescent-labelled monoclonal antibodies were employed simultaneously. The antibodies were mixed and appropriately diluted as delineated in Table 5.

#### Table 5  The mix of fluorescent-labelled antibodies

<table>
<thead>
<tr>
<th>No. tubes</th>
<th>FITC</th>
<th>PE</th>
<th>PC7</th>
<th>APC/Cy5</th>
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<tr>
<td>1</td>
<td>CD27</td>
<td>Goat F(ab)2</td>
<td>CD19</td>
<td>Goat F(ab)2-Cy5</td>
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<td>1:5 /100 μl</td>
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<td>25 μl</td>
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<td>Anti-IgD</td>
<td>CD19</td>
<td>CD45APC</td>
</tr>
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<td>1:40 /100 μl</td>
<td>25 μl</td>
<td>25 μl</td>
</tr>
<tr>
<td>3</td>
<td>CD27</td>
<td>Anti-IgD</td>
<td>CD19</td>
<td>Anti-IgM-Cy5</td>
</tr>
<tr>
<td></td>
<td>1:5 /100 μl</td>
<td>1:40 /100 μl</td>
<td>25 μl</td>
<td>1:20 /25 μl</td>
</tr>
<tr>
<td>4</td>
<td>Anti-kappa</td>
<td>Anti-lambda</td>
<td>CD19</td>
<td>Anti-IgM-Cy5</td>
</tr>
<tr>
<td></td>
<td>1:2 /100 μl</td>
<td>1:10 /100 μl</td>
<td>25 μl</td>
<td>1:20 /25 μl</td>
</tr>
<tr>
<td>5</td>
<td>CD21</td>
<td>Anti-IgA</td>
<td>CD19</td>
<td>Anti-IgM-Cy5</td>
</tr>
<tr>
<td></td>
<td>100 μl</td>
<td>1:40 /100 μl</td>
<td>25 μl</td>
<td>1:20 /25 μl</td>
</tr>
<tr>
<td>6</td>
<td>CD38</td>
<td>CD24</td>
<td>CD19</td>
<td>Anti-IgM-Cy5</td>
</tr>
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<td></td>
<td>100 μl</td>
<td>100 μl</td>
<td>25 μl</td>
<td>1:20 /25 μl</td>
</tr>
<tr>
<td>7</td>
<td>CD38</td>
<td>CD27</td>
<td>CD19</td>
<td>Anti-IgM-Cy5</td>
</tr>
<tr>
<td></td>
<td>100 μl</td>
<td>100 μl</td>
<td>25 μl</td>
<td>1:20 /25 μl</td>
</tr>
<tr>
<td>8</td>
<td>CD38</td>
<td>CD21</td>
<td>CD19</td>
<td>Anti-IgM-Cy5</td>
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<tr>
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<td>100 μl</td>
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<td>1:20 /25 μl</td>
</tr>
<tr>
<td>9</td>
<td>CD27</td>
<td>CD21</td>
<td>CD19</td>
<td>Anti-IgM-Cy5</td>
</tr>
<tr>
<td></td>
<td>1:5 /100 μl</td>
<td>100 μl</td>
<td>25 μl</td>
<td>1:20 /25 μl</td>
</tr>
</tbody>
</table>
Immunofluorescence labelling procedure:

1. Place 10μl appropriate mixture of specific fluorescent-labelled monoclonal antibodies into each FACS-tube.

2. Add 50μl of cell suspension containing 10 X 10⁶/ml cells to the antibodies. Mix on vortex, incubate at 4 ºC in dark for 20 minutes.

3. Fix the cells by addition of 30 μl Optilyse B Lysing Solution, vortex, incubate at room temperature in the dark for 15 minutes.

4. Add 500 μl of the Aqua dest. Solution. Vortex, incubate at room temperature in dark for 5 minutes.

5. Then add 500 μl of the FACS Lysing Solution. Centrifuge at 500 xg for 2 min. at room temperature and discard supernatant.

6. Resuspend the stained cell pellet in 500 μl FACS Solution for flow cytometry.

3.3.3 Flow cytometric analysis of PBMC

In this study, B cells at different stages of development were characterized through the use of fluorescent-labelled monoclonal antibodies against surface markers. Flow cytometric analysis was performed using a multicolor flow cytometer (FACS Calibur, Becton Dickinson) for 4-colour fluorescence analysis. The instrument uses two spatially separated laser beams: an air-cooled Argon-ion laser (488-nm) and a red diode laser (635-nm). While individual cells are passing through the path of the laser beams, fluorescent antibodies bound to specific cell surface receptors are excited. The different fluorescent dyes subsequently emit light of specified wave length, which is filtered and reflected by several wave length-specific light filters and finally converted to electrical signals by 4 photomultiplier tubes (PMTs). In addition, the laser beam passes through the cells (forward scatter) or reflects off it (side scatter). The side scatter and forward scatter are measured by photodetectors. The light scattered from each cell can produce information related to cell size and density. All of these data are transferred to a computer, and plotted onto the computer screen (Figure 3).
Materials and methods

Following the proper calibration by standard fluorescent beads the acquisition of B-cells was performed by gating on CD19$^+$ events and lymphocyte characteristics using both forward and sideward scatter and the CellQuest® software (Becton Dickinson). 5000 events of CD19$^+$ B cells were collected for each analysis.

3.3.4 Statistical analyses

The unpaired Student’s $t$ test was used to make statistical comparisons of data. The mean±SD was used for data expression. Differences between groups were considered significant at $P<0.05$. 

Figure 3  Schematic mechanism of FACS Calibur

Following the proper calibration by standard fluorescent beads the acquisition of B-cells was performed by gating on CD19$^+$ events and lymphocyte characteristics using both forward and sideward scatter and the CellQuest® software (Becton Dickinson). 5000 events of CD19$^+$ B cells were collected for each analysis.
4 Results

Based on characterizations of human B cell subsets, we devised a protocol enabling the identification of peripheral B cell subsets (Table 5). According to the various surface marker expression pattern the peripheral B cell subsets were defined as follows: total B cells: CD19⁺; naive B cells: CD19⁺IgD/M⁺CD27⁻; IgM memory B cells: CD19⁺IgD/M⁺CD27⁺; switched memory B cells: CD19⁺IgD⁺CD27⁺; plasma cells: CD19⁺CD38⁺CD27⁺CD24⁺.

4.1 Expression of peripheral B cell subsets in the patients with systemic lupus erythematosus

A total of 45 SLE patients were enrolled. The patients were classified into three treatment groups:

1st, newly diagnosed, untreated SLE patients (n=5).

2nd, patients who are currently on conventional immunosuppressive therapy (DMARDs: chloroquine, azathioprine, methotrexate, cyclosporine A) but never had received CYC (n=24).

3rd, patients who have received aggressive therapy with CYC in the past and are currently on immunosuppressive maintenance therapy with DMARDs (n=16).

In the 3rd group B cell subset analysis was performed between 1 and 108 months (mean 39 months) after termination of CYC therapy (Figure 4). The patients had received 5 CYC pulses on an average and the mean cumulative CYC dose was 4.1 g (Figure 5).
Results

Figure 4  Time in months of B cell subset analysis after the last pulse CYC therapy in the 3rd SLE treatment group.

Figure 5  Cumulative dose and number of CYC pulses in the 3rd treatment group. There was a lack of information on cumulative CYC dose in the charts of patients 4th, 11th and 12th.
In the 2\textsuperscript{nd} group, the mean duration of immunosuppressive therapy before measuring B-cell subsets was 34.4 months and the average number of different immunosuppressive drugs was 1.66 per patient (Figure 6).

![Figure 6](image_url)

**Figure 6** Mean number and duration of immunosuppressive therapy before measuring B-cell subsets of the 2\textsuperscript{nd} SLE group.
4.1.1 Total lymphocytes

Total lymphocyte counts were measured in peripheral blood of three SLE groups and a healthy control group (Figure 7). The mean total lymphocyte count as percent of white blood cells amounted to 31% (range 20.8-39.3%) in normal controls. A decrease in the percentage of lymphocytes was noted in all three SLE groups. In the 1\textsuperscript{st} group, the percentage of lymphocytes (27%) was slightly, but not significantly diminished. However, in the 2\textsuperscript{nd} and 3\textsuperscript{rd} group the percentages of lymphocytes were significantly decreased compared with the control group (20%, P<0.05 and 13.8%, P<0.05, respectively) (Figure 7a). The mean absolute lymphocyte count in healthy controls was 1952/ul (range 1286-2896/ul). Similarly, in the 1\textsuperscript{st} SLE group, the absolute lymphocyte counts (mean=1609/ul) were slightly diminished, whereas they were significantly decreased in the 2\textsuperscript{nd} and 3\textsuperscript{rd} group (1229/ul, P<0.05 and 816/ul P<0.05, respectively) (Figure 7b).

![Figure 7](image)

Figure 7  Total lymphocyte counts of SLE patients and normal controls. The arithmetic statistical mean values are indicated for each group. (a) Relative frequency of lymphocytes as percent of leukocytes in three SLE groups and a healthy control group. (b) Corresponding absolute numbers of total lymphocytes.
4.1.2 Total B cells

B cells were identified by the expression of the surface molecule CD19$^+$ on peripheral blood mononuclear cells (PBMC). The mean percentage of CD19$^+$ B lymphocytes in normal controls was shown to be 12.5% (range 4.7-13.9%). We detected an increased percentage of total B cells in the 1$^{st}$ and 2$^{nd}$ group (15.9% and 14.0%, respectively) and a decreased percentage in the 3$^{rd}$ group (8.8%); however, all differences lacked statistical significance (Figure 8a). The absolute number of CD19$^+$ B cells in normal controls amounted to 177.7/ul (range 88.2-383.2/ul); it was not significantly different from the B cells counts in the 1$^{st}$ and 2$^{nd}$ patient group (241.3/ul and 161.9/ul, respectively). However, in the 3$^{rd}$ group the absolute number of total B cells was with 73.4/ul severely depleted (P<0.05) (Figure 8b).

Figure 8 Mean CD19$^+$ B cell counts of SLE patients. (a) Relative frequency of B cells as percent of total leukocytes in three SLE groups and a healthy control group. (b) Corresponding absolute numbers of total B cells.
4.1.3 Naive B cells

The surface markers expressed on naive B cells are CD19\(^+\)IgD/M\(^+\)CD27\(^-\). The mean percentage of naive B lymphocytes amounted in normal controls to 62.4% of total B cells (range 37.5-80.7%) corresponding to 135/ul (range 52.7-303.6/ul) in absolute numbers (Figure 9 a and b). All three SLE groups exhibited an insignificant increase of naive B cell in percent (1\(^{st}\), 2\(^{nd}\) and 3\(^{rd}\) SLE group: 67.0%, 67.0% and 68.0%, respectively) compared with normal controls. Expressed in absolute numbers naive B cells were significantly decreased only in the 3\(^{rd}\) group (57.0/ul). Untreated patients (156/ul) and patients treated by immunosuppressive drugs (110.0/ul) did not significantly deviate from the normal range (52.7-303.6/ul).

Figure 9  Mean naive B cell counts of SLE patients. (a) Relative frequency of naive B cells as percent of total B cells in three SLE groups and a healthy control group. (b) Corresponding absolute numbers of naive B cells.
4.1.4 IgM memory B cells

The IgM memory B cells express CD19^{+}IgD/M^{+}CD27^{+} on their cell surface. We found that all SLE groups exhibited a marked reduction of IgM memory B cells in percentages and absolute numbers (Figure 10 a and b). In the newly diagnosed and untreated patients (1\textsuperscript{st} group) the percentage and absolute number of IgM memory B cells was only slightly decreased (11.9\%, 29.4/ul) compared with the controls (18.9\%, range 7.8-37.2\%; 36.1/ul, range 10.8-60.4/ul). However, the patients who were treated with DMARDs (2\textsuperscript{nd} group) only exhibited a profound decrease of IgM memory B cells (9.9\%, 14.0/ul); even more so the 3\textsuperscript{rd} group of SLE patients who were previously treated with CYC followed by DMARDs (7.2\%, 3.9/ul). Both treatment groups showed highly significant differences compared with healthy controls (P<0.05).

![Figure 10](attachment:image.png)

Figure 10  Mean IgM memory B cell counts in SLE patients. (a) Frequency of IgM memory B cells as percent of total B cells in three SLE groups and healthy controls. (b) Corresponding absolute numbers of IgM memory B cells.
4.1.5 Switched memory B cells

Compared with class-switched memory B cells of normal controls (CD19+IgM/D CD27+: mean 13.8%, range 6.5-33.8%) the mean percentage of this B cell subset was in all SLE groups slightly, but not significantly altered: 15.8% in the 1st group, 18.2% in the 2nd and 19.2% in the 3rd group (Figure 11a). In contrast, the absolute numbers of switched memory B cells exhibited differences between the three SLE groups. Whereas in untreated SLE patients switched memory B cells were slightly increased (35.7/ul), they showed an insignificant decrease in the DMARD treated patients (20.6/ul) and a significant decrease (7.9/ul) in the CYC treated patients compared with healthy controls (28.3/ul, range 11.4-65.9/ul, P<0.05) (Figure 11b).

Figure 11  Mean switched memory B cell counts in SLE patients. (a) Frequency of switched memory B cells as percent of total B cells in three SLE groups and healthy controls. (b) Corresponding absolute numbers of switched memory B cells.
4.1.6 Plasma cells

Within peripheral blood, plasma cells are the terminally differentiated form of antibody-forming B cells (AFCs). They are characterized by the surface expression of CD19+CD38+CD27+CD24-. The mean value and the normal range of plasma cells in healthy controls amounted to 1.9% of the peripheral B compartment (range 0.5-4.2%); in absolute counts this corresponded to 3.4 cells/ul (range 1.1-6.9 cells/ul) (Figure 12 a and b). Interestingly, the percentages of plasma cells were elevated in the three SLE groups with 2.6% in the 1st group, 4.8% in the 2nd and 7.0% in the 3rd reaching statistical significance in the 2nd and 3rd group when compared with normal controls (1.9%, range 0.5 to 4.2%; P<0.05). In absolute numbers plasma cells showed in the 3rd group a slight decrease (2.5/ul) and in the 1st and 2nd group an insignificant increase (4.6/ul and 5.0/ul, respectively).

Figure 12 Mean plasma cell counts of SLE patients. (a) Relative frequency of plasma cells in three SLE groups and healthy controls. (b) Corresponding absolute numbers of plasma cells.
4.2 Expression of peripheral B cell subsets in the patients with Wegener’s Granulomatosis

A total of 19 WG patients were enrolled in the WG group. All the patients have received aggressive therapy with CYC in the past and were currently on immunosuppressive maintenance therapy with DMARDs. B cell subset analysis in the WG group was performed between 1 and 71 months (mean 23.7 months) after termination of CYC therapy (Figure 13). The mean cumulative CYC dose of the patients was 35 g (Figure 14).

Figure 13  Time of B cell subset analysis in months after CYC therapy of WG patients.

Figure 14  Cumulative CYC dose of WG patients.
4.2.1 Total lymphocytes

The population of lymphocytes was detected in the peripheral blood of WG group patients and normal controls (Figure 15). The percentage of total lymphocytes in normal controls was 31.0% (range 20.8-39.3%). The percentage of lymphocytes was significantly decreased in WG patients (14%, P<0.05) (Figure 15a). The mean absolute lymphocyte count in healthy controls was 1952/ul (range 1286-2896/ul) compared with 1192/ul in WG patients (P<0.05) (Figure 15b).

![Figure 15](image_url)  Mean lymphocyte counts of WG patients. (a) Relative frequency of lymphocytes as percent of total leukocytes in the WG group and in healthy controls. (b) Corresponding absolute numbers of total lymphocytes in WG patients and a control group.

4.2.2 Total B cells

In WG patients who were all treated with CYC and DMARD, we detected a reduction of CD19+ B cells (Figure 16). The percentage and absolute number of total B cells were similarly decreased (7.3% and 89.0/ul, respectively) compared with the normal control group (12.5%, P<0.05 and 177.7/ul, P<0.05, respectively).
4.2.3 Naive B cells

WG patients exhibited divergent values for relative and absolute numbers of naive B cells (CD19\(^+\)IgD/M\(^+\)CD27\(^-\)) (Figure 17 a and b). Whereas the percentage of naive B cells was insignificantly increased (71.3%) compared with normal controls (62.4%, range 37.5-80.7%), the absolute cell counts were decreased (68.3/ul, P<0.05) compared to the control group (mean: 135/ul, range 52.7-303.6/ul).

Figure 17  Mean naive B cell counts in WG patients. (a) Relative frequency as percent of total B cells, (b) corresponding absolute numbers.
4.2.4 IgM memory B cells

IgM memory B cells run at 18.9% (range 7.8-37.2%) in normal controls corresponding in absolute numbers to 36.1/ul (range 10.8-60.4/ul) (Figure 18 a and b). Similarly to the 3rd SLE group (Figure 10), all the WG patients who were treated with CYC and DMARD exhibited a severe depletion in percentage (7.5%) and absolute number (3.6/ul) of IgM memory B cells. The differences were highly significant compared with normal controls (P<0.05, P<0.05, respectively).

![Figure 18](image)

Figure 18  Mean IgM memory B cell counts of WG patients. (a) Relative frequency of IgM memory B cells in WG patients and control group, (b) corresponding absolute numbers.

4.2.5 Switched memory B cells

As can be seen in Figure 19a and b, switched memory B cells (CD19^+IgD^-CD27^+) reached a mean of 13.8% (range 6.5-33.8%) in healthy controls corresponding to 28.3 cells/ul (range 11.4-65.9/ul) in absolute counts. In the WG group switched memory B cells tended to diverge between relative and absolute counts. While the absolute count of switched memory B cells was significantly decreased (7.9/ul) compared with normal controls (P<0.05), the percentage showed a slight increase (19.2%).
4.2.6 Plasma cells

In our study the mean percentage of CD19⁺CD38⁺CD27⁻CD24⁻ plasma cells in normal controls was 1.9% (range 0.5-4.2%); the WG group showed an insignificant increase (2.9%) (Figure 20a). In contrast, the absolute number of plasma cell was significantly decreased (1.5/ul, P<0.05) compared with normal controls (3.4/ul, range 1.1-6.9/ul) (Figure 20b).
4.3 Peripheral B cell subsets in allogeneic stem cell transplant recipients

We analyzed peripheral B cell subsets in 12 patients after allogeneic stem cell transplantation (Table 4). According to an existence of Howell-Jolly bodies in erythrocyte of peripheral blood the patients were classified in two groups: the **Howell-Jolly body group** comprised 5 patients, 3 males and 2 females; 4 patients had familial stem cell donors and 3 patients were donor recipient sex mismatches, 1 patient was previously splenectomized, the other 4 patients had small spleens and all patients exhibited a graft versus host disease (GvHD); the **Non-Howell-Jolly body group** included 7 patients, 3 males and 4 females, 2 patients had familial stem cell donors and 5 patients were donor recipient sex mismatches, 5 patients exhibited some GvHD and all patients had normal sized spleens.

B cell subset analysis was performed between 2 and 8.5 years (mean 4.7 years) after allogeneic stem cell transplantation.

4.3.1 Total B cells: CD19+

Compared with normal controls, it was demonstrated that the percentage of total B cells in both patient groups with and without Howell-Jolly bodies lacked significant differences (Figure 21a). The absolute number of total B cells in the **Howell-Jolly body group** was not different from the control group with the exception of one splenectomized patient who showed unusually high total B cells (1200/ul). The **Non Howell-Jolly body group** exhibited an increase of relative and absolute B cell counts but only the latter one was significant (P=0.023) (Figure 21b).
Results

4.3.2 Naive B cells: CD19^+IgD/M^+CD27^-

The expression of naive B cells in *Howell-Jolly body group* did not show any statistically significant difference in relative and absolute cell numbers compared with controls. Only the splenectomized patient who exhibited a high total B cell count showed also an unusually high number of naive B cells. Interestingly, the relative and absolute numbers of naive B cells in the *Non-Howell-Jolly body* patient group were significantly elevated compare with controls (P<0.0001 and P=0.005, respectively) (Figure 22 a and b).
4.3.3 IgM memory B cells: CD19⁺IgD⁻/⁺M⁺CD27⁺

When compared with healthy controls both patient groups showed a highly significant decrease of their relative and absolute numbers of IgM memory B cells irrespective of the Howell-Jolly body status (Figure 23a,b).

**Results**

Figure 22
Naïve B cells (CD19⁺CD27⁻IgM/D⁺) in the patients after allogeneic stem cell transplantation.
(a) Naïve B cells as percent of total B cells in the patient groups and the control group.
(b) Absolute number of naïve B cells in the patient groups and the control group.
&  Splenectomized patient with unusually high total and naïve B cell counts.

Figure 23
IgM memory B cells in the patients after allogeneic stem cell transplantation.
(a) IgM memory B cells as percent of total B cells in the patient groups and the control group.
(b) Absolute number of IgM memory B cells in the patient groups and the control group.
&  Splenectomized patient with unusually high total, naïve and IgM memory B cells.
4.3.4 Switched memory B cells: CD19^+IgD^-CD27^+

The percentage of switched memory B cells was shown to be reduced in both patient groups, however, only for the *Non-Howell-Jolly group* was the difference significant compared with controls (P=0.001) (Figure 24a). The absolute numbers of switched memory B cells did also show a decrease in both patient groups, but the differences to the healthy controls were less impressive and not significant (Figure 24b).

![Figure 24](image)

Switched memory B cells in the patients after allogeneic stem cell transplantation.
(a) Switched memory B cells as percent of total B cells in the patient groups and the control group.
(b) Absolute number of switched memory B cells in the patient groups and the control group.
(c) Splenectomized patient with unusually high total, naive, IgM memory and switched memory B cell compartments.
5 Discussion

5.1 Change in peripheral B cell subsets in patients with SLE

SLE is a prototypic autoimmune disease characterized by multi-organ involvement in association with production of numerous autoantibodies, most prominently those directed to components of the cell nucleus. Recent studies suggested that B cells play an important role in the pathogenesis of autoimmune diseases and influence autoimmunity through multiple pathways such as defective T-cell-dependent B lymphocyte activation, altered expression of B-cell surface molecules, and impaired B-cell maturation and differentiation (Porakishvili et al, 2001). In addition, active countering of pro-apoptotic mechanisms may cause a continuous survival of autoreactive B cells (Tsubata & Honjo, 2000). B cell overactivity is considered to be responsible for the hypergammaglobulinemia and the production of a large variety of autoantibodies which contribute to the autoimmune disease. Recent evaluation of the role of B cells in the immune system has indicated that they are more than just the precursors of antibody-secreting cells. B cells have more essential functions in regulating immune responses than had previously been appreciated (Porakishvili et al, 2001; Lipsky, 2001; Zouali, 2002).

As shown in studies of patients with SLE, as well as in animal models, functional abnormalities of various immune cell types promote B cell hyperactivity that culminates in pathogenic autoantibodies production (Criscione & Pisetsky, 2003). B cells can regulate many aspects of immune reactivity and differentiate into antibody-producing cells. In SLE, B cells showed increased rates of proliferation and spontaneously produced increased amounts of immunoglobulins (Ginsburg et al, 1979; Blaese et al, 1980). In animal model of lupus, has been shown that B cell deprived MRL lpr/lpr mice do not develop glomerulonephritis or vasculitis, which implies an essential role for B cells (Shlomchik et al, 1994).

One hypothesis proposes that the defining pathogenic event in human SLE is enhanced B cell function (Lipsky, 2001). SLE patients are known to display peripheral blood lymphopenia. Despite the low circulating lymphocyte levels, the numbers of B cells that produce increased amounts of immunoglobulins correlate rather accurately with SLE activity (Zouali et al, 1991). It is suggested that an intrinsic tendency of B cells to respond excessively
to immune stimulation may be an essential feature of SLE (Lipsky, 2001). However, abnormalities of B cells have not been fully explored especially the relation between changes of peripheral B cell subsets and different treatments in patients with SLE.

In this study, we analysed peripheral B cell subsets of 45 SLE patients divided into three groups according to their therapeutic regimen (Table 3). In the 1\textsuperscript{st} group which comprised untreated patients with active disease the relative and absolute lymphocyte counts were decreased. Although this change had no statistical significance, the results were similar to prior studies (Potter et al, 2002). But both total CD19\textsuperscript{+} B cells and CD27\textsuperscript{-} naive B cells showed an increase in percentage and absolute counts. The 2\textsuperscript{nd} and 3\textsuperscript{rd} group comprised SLE patients under immunsuppressive therapy (2\textsuperscript{nd} group) or following pulse CYC therapy (3\textsuperscript{rd} group). Percentages and absolute counts of lymphocytes, notably in the 3\textsuperscript{rd} group, were significantly decreased, as were absolute counts of total CD19\textsuperscript{+} B cells and CD27\textsuperscript{-} naive B cells.

Following antigenic stimulation and T cell help, in the germinal centers (GC) of spleen and lymph nodes, mature B cells undergo activation, proliferation and differentiation into plasma cells. Alternatively, some activated B cells stop their proliferation and differentiation, and become memory B cells. CD27 has been identified as surface marker for human memory B cells (Klein et al, 1998; Agematsu et al, 1998). Memory B cells are resting and long-lived, and play a central role in antibody responses. In peripheral blood, memory B cells have been identified as IgM\textsuperscript{+}IgD\textsuperscript{-}, IgM-only, class-switched Ig isotypes and a minor IgD-only subpopulation (Klein et al, 1998; Weller et al, 2004). The IgM memory B cells develop in the spleen and produce IgM antibodies which are of low affinity (Krüetzmann et al, 2003; Carsetti et al, 2004). However, IgG antibodies derive from switched memory B cells generated and selected in GC (Carsetti et al, 2004).

As the antibodies characteristic of SLE are mostly high-affinity IgG antibodies, they are very likely to result from T cell dependent responses (Salmon & Gordon, 1999). Abnormal memory effector cells in SLE express surface immunoglobulin with specificity for autoantigen. The produced immunoglobulins are usually of high avidity due to class switching to IgG and IgA and somatic hypermutation of immunoglobulin variable gene regions (Grammer & Lipsky, 2003). Recently, it has been demonstrated that the CD27/CD70

Studies have shown that a reduction in the absolute numbers of memory B cells was found in both active and inactive SLE (Odendahl et al, 2000; Potter et al, 2002). We detected that in untreated SLE patients (1st group) the percentage and absolute number of IgM memory B cells were slightly reduced, whereas the relative and absolute numbers of switched memory B cells were increased. Following treatment with CYC and/or conventional immunosuppressive reagents (2nd and 3rd group) IgM memory B cells showed the most significant reduction in absolute and relative numbers, suggesting that this B cell subset is most sensible to immunosuppressive treatment. It has recently been shown, that the great majority of IgM memory B cells are generated in the spleen independent of T cell help. These cells represent the first line response to new antigens and link the innate to the adaptive immune system (Krützmann et al 2003, Weller et al 2004). The fact that this compartment is hit most severely by CYC and immunosuppressive drugs sheds new light on the susceptibility to infections in SLE.

The abnormal expression of B cell subsets is a hallmark of SLE. It was showed that patients with high disease activity had an increased frequency of both CD19+B cells and plasma cells. The number and frequency of plasma cells were significantly correlated with the SLE disease activity indices and with the titer of anti–double-stranded DNA autoantibodies (Jacobi et al, 2003). In our study plasma cells were found at high relative percentages in the all three groups. Under the immunosuppressive therapy the plasma cells tended to show a further increase suggesting that they are more resistant to CYC and immunosuppressive therapy. Regarding the absolute counts of plasma cells they were highest in the 2nd group compared with 1st group and normal controls. After pulse CYC the absolute counts of plasma cells showed a slightly, but not significant decrease.

Previous studies have demonstrated the efficacy of intravenous CYC (Dinant et al, 1982; Steinberg & Steinberg, 1991; Neuwelt et al, 1995; Bansal & Beto, 1997), and this therapy has been established as the main therapy for steroid and DMARD resistant lupus patients (Amano et al, 2000). CYC is a potent suppressor of B cell activity and antibody formation (Cupps et al, 1982; Takeno et al, 1993). Our results indicated profound abnormalities of various peripheral
Discussion

B cell subsets in SLE patients. CYC seems to influence peripheral B cell subsets more than DMARDs. However, both have profound and long-lasting negative effects on the peripheral pool of naive B cells and notably the IgM memory B cells. Switched memory B cells, in contrast, are less affected by immunosuppressive therapy and plasma cells hardly at all. These findings may explain why our current therapeutic regimens are usually not curative and why autoantibody titers hardly ever normalize. On the other hand, primary antibody responses, which rely on a functional IgM memory compartment often give unsatisfactory results in SLE patients. Thus, 12 of the SLE patients examined here have been vaccinated 3 times against hepatitis B after completion of CYC pulse therapy. Only three patients showed an antibody response (Hu et al submitted; Miehle et al 2004). Taken together our findings suggest, that current immunosuppressive regimen in SLE are much more efficient in suppressing primary than anamnestic immune responses.

5.2 Change in peripheral B cell subsets in patients with Wegener’s granulomatosis

WG is an autoimmune disease of unknown etiology. It typically starts with necrotizing granulomas in nose, sinuses, pharyngo-larynx und lungs. Progression to systemic vasculitis with kidney involvement is significantly associated with the occurrence of antineutrophil-cytoplasma antibodies (cANCA) directed against proteinase-3 (PR3), a neutrophil azurophilic granule antigen. A minority of patients do not have antibodies to PR3 but have, instead, autoantibodies against an alternative granule antigen, myeloperoxidase (MPO) (Hewins et al, 2000). ANCA are highly sensitive and specific markers for WG and are produced by B cells and/or plasma cells. ANCA titers correlate with clinical disease activity and predict relapses. ANCA are thought to be involved in the pathophysiology of Wegener’s vasculitis by activating neutrophilis to the production of oxygen radicals which may damage the vascular endothelium. Still elusive remains the pathophysiology of Wegener’s granuloma although staphylococcal superantigens have been incriminated in its initiation (Hewins et al, 2000).

Recently Popa et al. (1999) investigated the relationship between lymphocyte activation and disease activity in patients with WG. They found that percentages of CD38bright activated
B cells were higher in patients with active WG than in patients experiencing disease remission or in healthy control subjects. T-cell activation starts early in disease (Guttleisch et al, 1993) and persists during remission pointing to an intrinsic disorder of the immune system. The authors also found a positive correlation between ANCA levels and active disease, but not between percentages of activated B cells and ANCA levels (Popa et al, 1999).

Prior to the introduction of corticosteroids and CYC (Fauci et al, 1973) into the treatment of WG the disease had a mortality rate of 93% within 2 years. CYC, an alkaling agent, interferes with transcription and translation of genetic message and is thereby the most rapidly acting immunosuppressive drug. Cumulative doses >30g favor the development of myelodysplastic syndromes or myelo-monocytic leukemias. Corticosteroids are most effective inhibitors of proinflammatory cytokines by inducing macroglobin which interferes with phospholipase A2 and inducible nitrogen oxide synthetase (INOS). The susceptibility of peripheral B cell subsets to immunosuppressive therapy has so far not been fully explored in patients with WG.

In order to obtain more insights in peripheral B cell subsets of WG patients, we analyzed in 19 WG patients the influence of immunosuppressive treatment on peripheral B cell subsets. All WG patients had completed CYC treatment (orally 2mg/kg or monthly pulses 10-15mg/kg) one to three months prior to testing. The treatment was usually instituted for rapid progressive glomerulonephritis. After remission induction maintenance therapy consisted of DMARDs (methotrexate, leflunomide or azathioprine). We found that both, relative percentages and absolute counts of, CD19+ B cells and IgM memory B cells were significantly decreased compared with normal controls. Similarly, a significant decreases of absolute counts in naive B cells, switch memory B cells and plasma cells were observed in the WG group. It was suggested that there is a profound and long lasting impact on lymphocytes and peripheral B cell subsets in WG patients after induction of remission with CYC. It was attempted to correlate these changes to a score for infectious risk (Miehle et al, ongoing study).

Recent studies have seen an upsurge of interest in the notion that B cells play an integral part in the pathophysiology of autoimmunity and that blocking them may be beneficial (Gorman et al, 2003). Based on this notion several targeted approaches for the treatment of
patients with autoimmune diseases are being explored. One attempt to deplete B cells was done with rituximab an anti-CD20 chimeric monoclonal antibody. A case report described the successful use of rituximab in Wegener’s granulomatosis. Following rituximab, circulating B-cells became undetectable and ANCA-titres decreased significantly. Remission was achieved and maintained as long as B-cells were absent (Specks et al, 2001).

Other approaches to B cell inhibition have been suggested during the 2003 rheumatology symposium in Edinburgh. The modulator of B lymphocyte stimulator (Blys) (also known as BAFF, TALL-1, zTNF4, THANK, and TNFSF13B), seems to plays a critical role in maintaining B cell homeostasis. Excessive BLys signalling may lead to B cell-mediated immunopathology. Recent studies have confirmed the presence of elevated levels of Blys in WG and SLE.(Khare et al, 2000; Dörner & Putterman, 2001; Zhang et al, 2001; Stone, 2003; Stohl et al, 2003). However, controlling Blys levels as a new approach to induce remission in WG and SLE is far from being reality. It requires additional studies to learn more about the short and longterm effects of therapeutic Blys targeting.

5.3 Alterations of peripheral B cell subsets in patients treated with allogeneic stem cell transplantation for hematological malignancies

Immune reconstitution is one of the major factors which determines the outcome after allo-SCT. The reconstitution of immunological competence in patients following allo-SCT depends on distinct cellular components present in the donor grafts. These include (1) reappearance of functional B cells, (2) thymic and extra-thymic T-cell development, (3) reconstitution of effector cells including cytotoxic T cells and natural killer (NK) cells, and (4) efficient antigen presentation to reconstitute the pretransplantation immune repertoire (Guillaume et al, 1998). After transplantation, reconstitution of bone marrow (BM) consists of two distinct phenomena, numerical recovery of BM cellular elements on the one hand and functional recovery of cellular interactions on the other.

While allo-SCT provides the chance for a cure of hematologic malignancies and a longterm, disease-free survival the patients are also at risk for severe complications such as acute or chronic GvHD, infections and recurrence of the primary malignancy. Functional
asplenia is known to represent a late complication of allo-SCT and might be a consequence of chronic GvHD with severe immune dysfunction (Castagnola & Fioredda, 2003). It is associated with an increased susceptibility to encapsulated bacteria, particularly Streptococcus pneumoniae. Patients are also at higher risk for invasive fungal infections and Pneumocystis carinii pneumonia (Kulkarni et al, 2000; Chen et al, 2003). Commonly functional asplenia can be diagnosed by the presence of Howell-Jolly bodies in peripheral blood smears (Styrt, 1990; Brigden, 2001).

The spleen is the largest lymphoid organ in the body. Besides degradation of old erythrocytes it filters the blood for pathogens and immune complexes and contributes to a functional immune system by supporting extramedullary late B cell maturation. Recently Krüetzmann et al have (2003) reported that the spleen is indispensable for the generation of circulating IgM memory B cells and a humoral immune response to pneumococcal polysaccharides. Splenectomy or congenital asplenia lead to a lifelong defect of IgM memory B cells. Similar findings were observed in spleenless Hox11⁻ mice (Wardemann et al, 2002). In contrast to IgM memory, switched memory B cells were only transiently depleted after splenectomy and were present at normal frequencies in asplenic patients. IgM memory B cells are thought to originate in the spleen, in extrafollicular sites, and independent of T cell help, whereas switched memory B cells depend on T cell help and originate from the germinal centers (Krüetzmann et al, 2003).

By studying patients with functional asplenia after allo-SCT we wanted to see whether chronic GvHD disturbs the IgM memory B cell development and whether there is a difference between patients with and without Howell-Jolly bodies. To this end we analyzed the B cell subsets of a group of 12 patients with hematologic malignancies in stable remission 2 to 8.5 years (mean 4.7 years) after allo-SCT. 10 patients presented with a mild to moderate GvHD, 5 had a small spleen with Howell-Jolly bodies in circulating erythrocytes (Table 4). Howell-Jolly bodies indicate asplenia or functional asplenia. According to absence or presence of Howell-Jolly bodies the patients were classified in 2 groups.

To our surprise we did not find a difference in peripheral B cell subsets between patients with and without functional asplenia (Howell-Jolly bodies positive). The absolute counts of CD19⁺ total B cells and naive B cells were significantly increased compared with normal
controls whereas, a profound decrease of IgM memory B cells was observed in both groups. Switched memory B cells were also decreased in both groups but less significantly. Among the 12 patients treated with allo-SCT 10 patients exhibited still an ongoing mild to moderate GvHD. We assume therefore, that chronic GvHD may be instrumental in impeding IgM memory formation, whereas the presence of Howell-Jolly bodies indicates an additional disturbance of the splenic function to degrade erythrocytes.

Following allo-SCT mature donor CD19⁺ B cells can provide sustained antibody production upon booster immunization or reinfection of the transplant recipient. Transfer of humoral immunity has been documented from donor to recipient, including immunity to tetanus, diptheria, varicella, influenza virus, cytomegalovirus, hepatitis B virus, and human immunodeficiency virus (Saxon et al, 1986; Gratama et al, 1986; Wimperis et al, 1986; Engelhard et al, 1993; Ilan et al, 1993; Lane et al, 1990). It is suggested that functional B cells are passively transferred by transplantation (Guillaume et al, 1998). However, Allo-SCT involves GvHD and the use of immunosuppressive therapy to control this disease, both of which interfere with the early developmental stages of immune reconstitution (Guillaume et al, 1998). Storek et al (2001) have studied factors influencing B lymphopoiesis after allo-SCT in 93 recipients. They found that the number of B-cell precursors on day 365 was 18-fold lower in patients with extensive chronic GvHD compared with patients with no or limited chronic GvHD. The number of B-cell precursors was not related to CD34 cell dose, type of transplant (marrow versus blood stem cells), donor age, or patient age. It is suggested that post-transplantation B-cell deficiency results in part from inhibition of B lymphopoiesis by GvHD and/or its treatment (Storek et al, 2001).

The pathophysiology of chronic GvHD is poorly understood because of the lack of highly satisfactory animal models and basic studies in patients (Kansu, 2004). Chronic GvHD causes profound immune dysfunction (Sidak & Sullivan, 1994; Storek et al, 1996; Sherer & Shoenfeld, 1998; Maury et al, 2001), and most chronic GvHD deaths are attributable to infections. The clinical presentation of chronic GvHD is similar to that of autoimmune disorders (Tabbara et al, 2002). It is shown that deficiencies in the cellular and humoral aspects of the immune system occur to some degree in all marrow transplant recipients for a variable duration and tend to be more profound and prolonged in patients with GvHD (Lum,
1990). In experimental and clinical studies of chronic GvHD, thymic atrophy, lymphocyte depletion, and autoantibody formation have been described. Disruption of thymic apoptosis and failure to eliminate the majority of self-reactive lymphocytes may lead to impairment of lymphocyte homeostasis and self tolerance. Expansion and effector functions of autoreactive T-cells will then promote autoreactive B-cell activation and the production of autoantibodies with target-organ damage (Kansu, 2004).

Taken together our results show that chronic immunosuppressive therapy of SLE and WG as well as chronic GvHD in allo-SCT treated patients cause profound disturbances of the peripheral B cell compartment with a profound decrease of spleen-derived IgM memory B cells. These cells reside in the marginal zone of the spleen and produce independent of T cell help primary antibodies to bacterial polysaccharides. They represent a link between the innate and adaptive immune system. From our findings we would predict that these patients are more at risk for novel primary infections than for pathogens against which exist already antibodies and anamnestic responses.
Summary

Normal homeostasis of the immune system is critical for the maintenance of immunological tolerance and immunocompetence. Failure of self-tolerance results in autoimmune reactions which may progress to chronic autoimmune diseases. B cells play an important role in the pathogenesis of autoimmune diseases and influence autoimmunity through multiple pathways. With the availability of informative B cell surface markers it is now possible to identify within the circulating B cell compartment (CD19+) at least 4 subsets: naive B cells (CD27-), IgM memory B cells (CD27+IgM/D+), switched memory B cells (CD27+IgM/D-) and plasma cells (CD27+CD38+CD24-). For the first time this B cell subset analysis was performed in three established autoimmune conditions (systemic lupus erythematosus (SLE), wegener’s granulomatosis (WG), chronic graft versus host disease (GvHD)) with special emphasis on changes induced by immunosuppressive therapy (disease modifying antirheumatic drugs (DMARDs)) and cyclophosphamide (CYC) treatment.

There was a significant impact of immunosuppressive treatment on peripheral B cell subsets in SLE and WG patients. Absolute lymphocyte numbers and CD19+ B cell counts were significantly decreased, as were naive B cells and switched memory B cells. Plasma cells tended to show a relative but not an absolute increase. In SLE patients the most significant decrease was observed for IgM memory B cells after CYC and DMARD therapy: absolute and relative cell counts were very low and remained so for a mean period of over 3 years after the last CYC treatment. Untreated SLE patients showed no significant decrease of IgM memory B cells. It is suggested that remission inducing therapy with CYC has a profound and long-lasting impact on total lymphocytes and peripheral B cell subsets in SLE and WG patients. Notably the IgM memory B cells are seriously hit. In contrast, plasma cells which can hide in protected niches of bone marrow and lamina propria intestini appear to be quite resistant to conventional immunosuppressive therapy. As IgM memory B cells provide the first T cell independent humoral immune response to bacterial polysaccharides from Streptococcus pneumoniae and Hemophilus influenzae it may be predicted that primary immune responses against these bacterial antigens are severely compromised in CYC and DMARD treated patients.

It has recently been shown that splenectomy and congenital asplenia cause a profound and long-lasting loss of IgM memory B cells together with an impairment of the immune response to pneumococcal polysaccharides. As successful allogeneic stem cell transplantation (allo-SCT) patients with stable chimerism often show signs of functional asplenia with appearance of Howell-Jolly bodies in erythrocytes we were interested to study B cell subsets in those patients. After a mean follow-up of 4.7 years 12 clinically stable allo-SCT recipients (five with positive Howell-Jolly bodies) exhibited a profound decrease of IgM memory B cells similar to splenectomized or CYC treated SLE and WG patients. There was no difference in IgM memory B cells numbers between allo-SCT recipients with and without functional asplenia as evidenced by the occurrence of Howell-Jolly bodies. Ten of the 12 patients have an ongoing mild to moderate GvHD, suggesting that this process may impede IgM memory formation in the spleen. In any case the presented findings underline that the generation of IgM memory B cells is critically dependent on a functional spleen and is highly susceptible to CYC and DMARD treatment.
Zusammenfassung


8 References


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## 9 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>ACR</td>
<td>American College of Rheumatology</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukaemia</td>
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<tr>
<td>allo-SCT</td>
<td>Allogeneic stem cell transplantation</td>
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<tr>
<td>AML</td>
<td>Acute myeloid leukaemia</td>
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<tr>
<td>ANA</td>
<td>Antinuclear antibody</td>
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<td>ANCA</td>
<td>Antineutrophil cytoplasmic antibody</td>
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<tr>
<td>a.o.</td>
<td>and other</td>
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<tr>
<td>APC</td>
<td>Allophycocyanin</td>
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<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
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<td>BAFF</td>
<td>B cell-activating factor</td>
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<td>BCR</td>
<td>B-cell antigen receptor</td>
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<td>Blys</td>
<td>B lymphocyte stimulator</td>
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<td>BM</td>
<td>Bone marrow</td>
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<tr>
<td>BMT</td>
<td>Bone marrow transplantation</td>
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<td>℃</td>
<td>Grad Celsius</td>
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<td>CD</td>
<td>Cluster of differentiation</td>
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<td>CML</td>
<td>Chronic myeloid leukemia</td>
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<td>CRP</td>
<td>C reactive protein</td>
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<td>Cy</td>
<td>cytoplasmic</td>
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<td>Cy</td>
<td>Cyanine succinimidylester</td>
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<td>CYC</td>
<td>Cyclophosphamide</td>
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<tr>
<td>DFS</td>
<td>disease-free survival</td>
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<tr>
<td>DMARD</td>
<td>Disease modifying antirheumatic drug</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetat</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<td>FAS</td>
<td>Functional asplenia</td>
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<td>Fc</td>
<td>Fragment</td>
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<td>FCS</td>
<td>Fetal calf serum</td>
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<td>FSC</td>
<td>Forward Scatter</td>
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<td>GC</td>
<td>Germinal centers</td>
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<td>GvHD</td>
<td>Graft versus host disease</td>
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<td>GVL</td>
<td>Graft-versus-leukemia</td>
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<tr>
<td>HLA</td>
<td>Histocompatibility leukocyte antigen</td>
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<td>HSCT</td>
<td>Hematopoietic stem cell transplantation</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<td>H-chain</td>
<td>heavy chain</td>
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<td>light chain</td>
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<td>IIF</td>
<td>Indirect immunofluorescence</td>
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<td>IL</td>
<td>interleukin</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>mIg</td>
<td>membrane-bound immunoglobulin</td>
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<td>ul</td>
<td>microliter</td>
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<td>MDS</td>
<td>Myelodysplastic syndrome</td>
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<td>ml</td>
<td>milliliter</td>
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<td>min.</td>
<td>minute</td>
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<td>MM</td>
<td>Multiple myeloma</td>
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<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
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<tr>
<td>PC7</td>
<td>PE-Cy7</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
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<td>PBSC</td>
<td>Peripheral blood stem cell</td>
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<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<tr>
<td>PMT</td>
<td>Photomultiplier tube</td>
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<td>PR3</td>
<td>Proteinase 3</td>
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<tr>
<td>pre-B cell</td>
<td>Precursor B cell</td>
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<td>pro-B cell</td>
<td>Progenitor B cell</td>
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<tr>
<td>RAG</td>
<td>Recombination activating gene</td>
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<td>SSC</td>
<td>Side Scatter</td>
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<td>SLE</td>
<td>Systemic lupus erythematosus</td>
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<tr>
<td>TALL-1</td>
<td>TNF- and apoptosis ligand-related leukocyte-expressed ligand-1</td>
</tr>
<tr>
<td>Tc</td>
<td>Cytotoxic T lymphocyte</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<tr>
<td>TdT</td>
<td>Terminal deoxynucleotidyl transferase</td>
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<tr>
<td>Th</td>
<td>T helper cell</td>
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<tr>
<td>THANK</td>
<td>TNF homologue that activates apoptosis, nuclear factor-kappaB, and c-jun NH2-terminal Kinase</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TNFSF13</td>
<td>Tumor nerosis factor ligand superfamily member 13</td>
</tr>
<tr>
<td>WG</td>
<td>Wegener’s granulomatosis</td>
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