Aus der Medizinischen Klinik mit Schwerpunkt Rheumatologie und klinischer Immunologie und dem Institut für Transfusionsmedizin der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

# Improved phenotypic analyses of peripheral blood cells allows differential diagnosis of disease activity and infection in systemic lupus erythematosus

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# Table of Contents

# Abbreviations

| | |
|---|---|
| ACC | alternating-current capacitance |
| ADCC | antibody-dependent cell-mediated cytotoxicity |
| AKT | anti-apoptotic kinases |
| ANA | antinuclear antibodies |
| ATCC | American Type Culture Collection |
| Aut. | automated |
| BAFF | B cell activation factor |
| BCMA | B-cell maturation antigen |
| BCR | B cell receptor |
| BD | Becton Dickinson |
| BLyS | B lymphocyte stimulator |
| BTK | Bruton's tyrosine kinase |
| C | Celsius |
| CD | cluster of differentiation |
| CI | confidence interval |
| CpG | Cytosin-phosphatidyl-Guanosin |
| CRP | c-reactive protein |
| Cy | cyanine |
| d | day |
| DAG | diacylglycerol |
| DC | dendritic cell |
| DCR | direct-current resistance |
| dl | deciliter |
| DNA | desoxyribonucleic acid |
| DRFZ | Deutsches Rheuma-Forschungszentrum |
| dsDNA | double stranded DNA |
| ECLAM | European Consensus Lupus Activity Measurement |
| FACS | fluorescence-activated cell-sorting |
| FcγR | receptors for the fc (fragment crystalizable) part of IgG antibodies |
| FDA | Food and Drug Administration |
| FDC | follicular dendritic cell |

| | |
|---|---|
| FITC | fluorescein isothiocyanat |
| Fl | fluorescent light |
| FSC | forward scatter detector |
| GC | germinal center |
| GCSF | Granulocyte Colony Stimulating Factor |
| GPI | glycosylphosphatidylinositol |
| GTP | guanosintriphosphat |
| HLA | human leucocyte antigen |
| IC | immune complex |
| IFN | interferon |
| Ig | immunoglobulin |
| IL | interleukin |
| Inf. | infection |
| ITAM | Immunoreceptor tyrosine-based activation motif |
| ITIM | Immunoreceptor tyrosine-based inhibition motif |
| LPS | lipopolysaccharide |
| M/F | male/female |
| MAPK | mitogen-activated-protein kinases |
| MFI | mean fluoresence intensity |
| mg | milligram |
| µl | microliter |
| ml | milliliter |
| MPS | mononuclear phagocyte system |
| N | number |
| NA | not applicable/available |
| NF-κB, | nuclear factor kappa B |
| NHS | normal healthy subject |
| NK | natural killer |
| nm | nanometer |
| Num. | number |
| OAS | oligoadenylate synthetase isoforms |
| PBMC | peripheral blood mononuclear cells |
| PBS | phosphate-buffered saline |
| BSA | bovine serum albumin |

| | |
|---|---|
| pDC | plasmacytoid dendritic cell |
| PE | phycoerythrin |
| PerCp | peridinin chlorophyll A protein |
| PKC | protein kinase C |
| PLCγ | phospholipase-Cγ |
| PtdIns | phosphatidylinositol |
| R | receptor |
| $r^2$ | coefficient of determination, |
| RA | rheumatoid arthritis |
| RF | alternating current |
| RPM | revolutions per minute |
| RTI | respiratory tract infection |
| SD | standard deviation |
| SH2 | Src homology region 2 |
| SHIP | SH2-domain containing inositol polyshosphate 5' phosphatase |
| SHP 1 /2 | SH2-domain-containing protein tyrosine phosphatase 1 /2 |
| SLE | Systemic lupus erythematosus |
| SLEDAI | Systemic Lupus Erythematosus Disease Activity Index |
| scRNP | small cytoplasmic ribonucleoproteins |
| snRNP | small nuclear ribonucleoproteins |
| SS | Sjögren's syndrome |
| SSC | side scatter detector |
| TACI | transmembrane activator and calcium-modulator and cyclophilin ligand interactor |
| TGF | Transforming growth factor |
| Th1/2 | type 1/2 helper T cell |
| TLR | Toll-like receptor |
| TNF | tumor necrosis factor |
| wk | week |

# 1. Background

## 1.1 SLE

Autoimmune diseases include a wide range of illnesses with an overall prevalence rate of 5-7% (Diamond, 2005). In autoimmune disease, the physiological mechanisms responsible for maintaining tolerance to self-antigens break down and consequentially the immune system targets an immune response against the body's own cells and tissues (Ring et al., 1999). Examples of autoimmune disease include systemic lupus erythematosus (SLE), type 1 diabetes, Hashimoto's thyroiditis, Graves' disease, multiple sclerosis, rheumatoid arthritis, Sjögren's syndrome, pemphigus and Wegner's granulomatosis. SLE is one of the more common forms of autoimmune disease with an incidence in North America, South America and Europe of 2 to 8 per 100,000 per year (Jimenez et al., 2003). Geography, sex and race affect the prevalence of SLE. The incidence is most frequent in women aged 15 to 25 with a male to female ratio of 1:8 (Lahita, 1999). However, attempts to find genetic and biological explanations for these differences have been inconclusive.

Clinical manifestations of patients with SLE are often heterogeneous and range from fatigue, fever, weight loss, skin rash, photosensitivity, arthralgia, non-erosive polyarthritis, serositis, endocarditis, vasculitis, sicca syndrome, haemolytic disorders including anemia, leukopenia and thrombocytopenia, severe renal disorders including lupus nephritis and neurological disorders including cognitive disorders and seizure (Hannahs Hahn, 2005). The wide range of clinical findings in patients with SLE suggests that the pathogenesis of the illness is multi-factorial.

The many immunological disturbances found in patients with SLE represent some of the most profound and prototypic findings in patients with autoimmune disease and include decreases in cytotoxic and regulatory T cells, disturbances of apoptosis and uptake of apoptotic material by macrophages, dysfunctional signaling in T and B lymphocytes, complement deficiency and defects in T and B cell tolerance. Moreover, hereditary factors of the innate immune system, such as distinct complement receptors and Fc receptors have been identified as predispositing factors for the manifestation of SLE (Yee et al., 1997; Arora et al., 2004; Goodnow et al., 2005; Arora et al., 2007). The

aberrations are complex and it remains unclear in many cases, which of these findings are secondary to the disease and which play a primary role in the development of the disease. Furthermore, the role of exogenous factors such as hormones, ultraviolet light and viruses has not been clearly delineated. Ultimately patients with SLE exhibit a loss of self-tolerance, with the severity of the illness depending on the organs involved. Concurrently various autoantibodies play a central role in the diagnosis and tracking of the disease. The autoantibodies found in patients with SLE include antinuclear antibodies (ANA) in 98%, anti-dsDNA antibodies in 70%, antihistone antibodies in 70%, antierythrocyte antibodies in 60% and antiphospholipid antibodies in 50% of cases (Hannahs Hanh, 2005).

Various findings suggest a central role for alterations in B cell functioning in the pathogenesis of SLE. For example, tissue deposition of immune complexes as an important pathogenic factor indicates that impaired handling of these complexes plays a role in disease manifestation (Reefman et al., 2003). Secondly, anti-dsDNA antibodies correlate with disease activity. Furthermore, B cell hyperactivity has been described in vitro and increased percentages of plasmablasts and plasma cells have been found in patients with SLE (Odendahl et al., 2000; Enyedy et al., 2001; Jacobi et al., 2003; Mok et al., 2003; Renaudineau et al., 2004). Moreover, Yan et al. (2002) have demonstrated a central role for self-reactive B cells as antigen presenting cells that consequently activate T cells in certain mouse models.

Infectious diseases have emerged as one of the leading causes of morbidity and mortality in patients with SLE, accounting for 20-55% of deaths. 50% of patients with SLE develop a major infection in the course of their disease (Fessler, 2002). Infections can mimic a lupus flare, which leads to delays in diagnosis and treatment. The pathogens vary widely in infections in patients with SLE. Most commonly infections are caused by common bacterial pathogens including Staphylococcus aureus, Streptococcus pneumonie, Escheria coli or Pseudomonas aueruginosa. Increased incidences of Salmonella infection, pneumococcal sepsis and Pneumocystis carinii pneumonia have also been reported. Furthermore, in countries where tuberculosis is endemic, Mycobacterium tuberculosis is emerging as a significant cause of morbidity and mortality in patients with SLE (Fessler, 2002). Various studies have shown that doses of more than 20 mg of prednisone per day, or equivalent, are associated with

significantly higher degrees of susceptibility to infection (Ginzler et al., 1978; Petri et al., 1992; Zonana-Nacach et al., 2001). However, immune suppression is not the only cause of increased susceptibility to infection in patients with SLE. A variety of immune defects and variations predisposing to infection have been found in patients with SLE, including complement factor deficiency, defects in chemotaxis, defective phagocytic activity, decreased immune complex clearance and genetic polymorphisms in mannose-binding lectin and Fcγ receptors (Fessler, 2002). In particular, Yee et al. (1997) found that four out of five SLE patients with invasive pneumococcal infections were homozygous for the R131 allele of FcγRIIa.

### 1.1.1 Pathogenesis of SLE

Considerable evidence is available confirming a strong and complex genetic basis of SLE. To date, strong evidence implicates HLA, Fcγ receptors, complement and complement receptors, CD95 (Fas), TNF polymorphisms, and overall B cell hyperactivity as factors that are integral to the pathogenesis of SLE (Lipsky, 2001; Kanemitsu et al., 2002; Tsao, 2002; Reefman et al., 2003; Tsao, 2004).

In general, the progression of the disease seems to require two partially independent events to take place. Initially autoimmunity needs to be initiated by breaking tolerance to self-antigens resulting in the production of autoantibodies. Subsequently, autoantibody production needs to be sustained (Ohashi et al., 2002; Arbuckle et al., 2003).

Various theories have been proposed to explain the processes by which tolerance can be broken in SLE:

1) Defects in apoptosis

It has been shown that increased loads of apoptotic cells are able to break tolerance leading to autoantibody production in mice (Mevorach et al., 1998). Consistent with this, elevated levels of apoptotic cells are present in patients with SLE. This appears to be explained by increased expression of certain apoptosis inducing molecules like Fas on lymphocytes in patients with SLE although reduced uptake of apoptotic cells may also explain this phenomenon. These mechanisms lead to prolonged exposure of the immune effector cells to autoantigens present on the surface of apoptotic cells such as

dsDNA (double stranded DNA), small cytoplasmic ribonucleoproteins (scRNP) and small nuclear ribonucleoproteins (snRNP) (Reefman et al., 2003). This increased apoptotic cell load present over a period of time may cause the apoptotic cells to go into secondary necrosis and be handled by the immune system in a pro-inflammatory manner. Alternatively, autoantigens may be modified during the apoptotic process before being translocated to the cell surface where they are recognized as foreign antigens. Subsequently, activation and proliferation of autoreactive B cells results in the production of autoantibodies.

Toll-like receptors (TLRs), which respond to pathogen-associated molecular patterns as part of the innate immune system have a significant enhancing effect on the adaptive immune response and are likely to play a central role in the development of autoimmunity. Particularly TLR9, which responds to unmethylated CpG DNA sequences, regardless of their origin, has a mitogenic effect on B cells and could predispose to autoreactive B cells as the result of confusion in self/non-self ligand (O´Neill, 2004; Means et al., 2005a; Means et al., 2005b). There is evidence that the DNA component of the immune complexes purified from SLE patients (SLE DNA-ICs) contributes to the development of the disease by inducing proliferation of self-reactive B cells and cytokine production by plasmacytoid dendritic cells (PDCs) in a TLR9 and CD32 dependent pathway. The DNA in SLE DNA-ICs is likely to be of apoptotic origin since the size consists with the apoptotic cleavage of chromatin. These immune complexes were shown to have a 5-6 times higher frequency of CpG DNA than would be expected in the genome. CD32 binds, internalize and delivers SLE-DNA-ICs to intracellular lysosomes containing TLR9, which then leads to a signal cascade via an NFκB-dependent pathway which results in the production of various cytokines and chemokines including IFN-α which exacerbate the disease. Accordingly, elevated levels of IFN-α have been found in patients with SLE in correlation with disease activity (Bave et al., 2003; Means et al., 2005a; Means et al., 2005b).

2) Intrinsic factors

B cells of patients with SLE exhibit signaling defects that may play a role in the pathogenesis of the disease and explain the B cell hyperactivity found in SLE. In freshly isolated peripheral B cells from patients with SLE, stimulation with BCR ligand led to increased calcium responses and increased tyrosine phosphorylation of intracellular

proteins compared to B cells from NHS regardless of disease activity which suggests that this aberration is primary and not secondary to the disease (Liossis et al., 1996). FcγRIIb attenuates intracellular calcium mobilization and Enyedy et al. (2001) have shown that FcγRIIb signaling is altered in B cells from patients with SLE with differences in intracellular calcium primarily in the later phases of the response. However, to verify whether this difference could be attributed to different surface expression levels of FcγRIIb, an anti-CD32 antibody from Pharmingen (San Diego, CA) was used presumably under the assumption that only CD32b was expressed on B cells as was formerly believed. Until recently, specific antibodies for CD32b were not available (Boruchov et al., 2005; Su et al., 2007). Therefore, it is likely that CD32a and CD32b expression was detected since both have been shown to be expressed by B cells, and therefore a clear statement about the expression levels cannot be made on this basis. Moreover, it has also been shown that the FcγRIIb Ile232Thr polymorphism is associated with SLE susceptibility in Asians (Kyogoku et al., 2002). Accordingly, studies of FcγRIIb1T232 transfectants suggested that defective signaling through this receptor enhances the inflammatory response (Floto et al., 2005; Kono et al., 2005). Furthermore, defective inhibitory signaling has long been suspected to play a role in the pathogenesis of SLE in the process of the selection and proliferation of IgG producing B cells in the germinal center, which is a central checkpoint for maintaining tolerance. In mice, the development of ANA-levels was shown to be inversely related to the degree of expression of the inhibitory FcγRIIb (McGaha et al., 2005).

Lyn, a B cell protein tyrosine kinase, which plays an important role as a negative regulator of BCR signaling, has also been implicated as an intrinsic factor in the development of SLE. Lyn-deficient mice exhibited enhanced activation of downstream effectors, intracellular calcium elevation and B cell hyperactivity resulting in autoantibody production (Hibbs et al., 1995; Nishizumi et al., 1995). Concordantly, decreased expression levels of Lyn were found in resting and stimulated peripheral B cells from patients with SLE regardless of disease activity (Liossis et al., 2001).

3) Extrinsic Factors

Extrinsic factors such as BAFF, prolactin, estrogen, INF-α, help from autoreactive T cells and high levels of Il-10 have been implicated in the pathogeneses of SLE. The TNF superfamily member B lymphocyte stimulator (BLyS), also referred to as BAFF, is

known to be a very effective modulator of peripheral B cell homeostasis promoting B cell survival and differentiation. BLyS binds three different receptors: BCMA (B-cell maturation antigen), TACI (transmembrane activator and calcium-modulator and cyclophilin ligand interactor) and BAFF-Receptor (BAFF-R). When coupled with simultaneous B cell antigen receptor ligation, BLyS leads to increased Bcl-2 expression and increased expression of NF-κB, which increases B cell survival, thereby potentially promoting the survival of autoimmune B cells (Schneider et al., 2005). Consistent with these findings, elevated BLyS serum levels have been found in patients with RA, SLE, and SS (Cheema et al., 2001). Furthermore, the hormones prolactin and estrogen effect the threshold of the BCR signal in the development of B cells and can favour the survival of autoimmune B cells in particular genetic settings (Peeva et al., 2004, Peeva et al., 2005).

There has been increasing evidence for INF-α having a primary role in SLE pathogenesis (Ronnblom et el., 2003). Recombinant IFN-α, which is used therapeutically in certain malignancies and hepatitis C, has been linked to the development of lupus-associated autoantibodies and even to clinical lupus. These developments were often reversible after discontinuation of the IFN-α therapy. Furthermore, elevated levels of IFN-α in SLE sera and an upregulation of genes regulated by IFN-α has been found in peripheral blood cells from patients with SLE compared to NHS. Pathogenically, it is conceivable that increased levels of IFN-α as the consequence of a virus infection or increased levels of immune complexes underlie a primary role of IFN-α in the development of SLE (Crow et al., 2004). Lastly, help from autoreactive T cells and high levels of IL-10 have been implicated in B cell hyperactivity (Grondal et al., 2000; Mok et al., 2003, Ronnelid et al., 2003).

4) Lack of censoring of autoreactive B cells

While various immunological characteristics of SLE have been defined, including a profound breakdown of tolerance in the antibody system, the precise stage where B cell tolerance is first broken is of particular relevance. Anergy and clonal deletion of autoreactive B cells are central mechanisms that lead to the maintenance of tolerance since the development of autoreactive B cells, for example, is also a physiological result of Ig gene rearrangements during early B cell development and postrecombination processes (somatic hypermutation, Ig class switching, receptor editing) during later

stages of B cell differentation. Yurasov et al. (2005a and 2005b) have shown that SLE is associated with the failure to establish self-tolerance during early B cell development resulting in increased numbers of autoreactive mature naive B cells. Their data indicates that defects in the maintenance of self-tolerance can occur both centrally and in the periphery. In SLE, a breakdown of self-tolerance centrally can result in increased numbers of polyreactive new emigrant B cells and peripherally can result in polyreactive mature naive B cells. Their results show that the defective checkpoints are not the same in all SLE patients. Although the autoantibodies found in SLE are predominantly somatically mutated, class switched IgGs, which indicates abnormalities in the late stages of B cell development, 20-50% of the mature naive B cells in SLE produce autoantibodies compared to 5-10% in controls. Although these cells do not secrete immunoglobulins, immunoglobulin secreting plasma cells and memory B cells develop from these cells and their development may concomitantly be favored in the context of the other immune alterations found in SLE such as defective signaling and increased BAFF levels (Yurasov et al., 2005a; Yurasov et al., 2005b). In addition, a reduction of regulatory T cells has been described in SLE that may contribute to the lack of control of immune activation under the conditions of the disease (Alvarado-Sanchez et al., 2006; Mudd et al., 2006).

Once tolerance is broken and stabilized for longer periods, various subsequent mechanisms contribute to sustaining autoimmunity. Autoantibodies can form immune complexes with apoptotic cells, apoptotic bodies or nucleosomes anywhere in the body. These complexes interact primarily with activating FcγR, directing the phagocyte response toward pro-inflammatory pathways. Ligation of the FcγR results in inflammation and local tissue destruction as observed in glomerulonephritis. Consequently, activating FcγR, which contribute to efficient antigen presentation also contribute to maintaining the inflammatory process.

Furthermore, FcγRIIb expression and function, which may play a role in the breaking of tolerance as previously discussed, may also be implicated in the maintainance of autoimmunity through modulation of post GC-reactions. In particular, signaling via this receptor may regulate the fate of autoreactive B cells in terms of the B cell maturation process to memory B cells or plasmablasts (Macardle et al., 2002; Nimmerjahn et al., 2006). Additionally, CD32 and TLR9, as mentioned previously, are likely to play a role in

the maintenance of the disease via the production of INF-α in response to SLE-DNA-IC. In a disease like SLE that is likely to represent a variety of entities, various factors evidently play a role to varying degrees in the manifestation of the disease.

## 1.2 B Cell Maturation and CD27

The regulated development of B cells is central to the development of self-tolerant B cells. The stages of B cell development are defined by the subsequent rearrangement and expression of the immunoglobulin genes, as well as changes in the expression of cell surface and intracellular molecules. The progressive steps of B cell development lead to the production of mature B cells that produce immunoglobulins with only one heavy chain and one light chain and ideally a single non-self specificity. The pro-B cells identified by the expression of the B cell markers CD19 and CD45R, comprise the earliest cells in the B cell lineage and are derived from pluripotent hematopoetic cells. VDJ rearrangement occurs in the heavy chain of pro-B cells resulting in the expression of the μ chain as part of the pre-B cell receptor. With expression of the pre-B receptor which contains the μ chain and surrogate light chains, the cell enters the large pre-B cell stage in which they are highly proliferative. Eventually these cells give rise to non-dividing small pre-B cells in which the μ chain is located intracellularly and the pre-B cell receptor is no longer located on the cell surface. At this stage, light chain gene rearrangement occurs. With expression of a complete IgM molecule on the cell surface, the cells enter the immature B cell stage in which the B cells are tested for self-tolerance. The B cells which survive this process in the bone marrow, emerge to the periphery and express IgD in addition to IgM. These cells are called naive B cells and circulate through secondary lymphoid organs where they may encounter their specific antigen at which point antigen dependent differentiation occurs. Activated B cells give rise to germinal centers in the secondary lymphoid tissues where clonal expansion, affinity maturation, isotype switching and differentiation of B cells into plamablasts and plasma cells as well as memory B cells occurs (MacLennan, 1994; Janeway et al., 2005).

Molecules of the TNF superfamily and their receptors have been shown to play essential roles in the development of the germinal center, and in the modulation of germinal center reactions. CD27 is a member of the TNFR family and is expressed on

some B cells as well as T cells. CD70 is the ligand for this molecule and is expressed on B and T cells after cellular activation (van Lier et al., 1987, Jung et al., 2000; Xiao et al., 2004). CD27-CD70 interaction appears to be involved in the development of the capacity to produce immunoglobulin whereby only CD27 expressing B cells produce immunoglobulins (Agametsu et al., 1997), and triggering of CD27 augments the increase in the number of plasma cells in the presence of IL-2 and IL-10 (Agametsu et al., 1998), and augments Ig production (Nagumo et al.,1998). These findings are consistent in mice and human models. Moreover, CD27-/- mice show delayed formation of germinal centers.

The expression of CD27 on B cells reveals a tight spatial and temporal regulation. Accordingly naive B cells are CD27- and induction of CD27 expression on naive B cells has been found to take place in the germinal center since most germinal center B cells are CD27+. Germinal center B cells regulate the expression of CD27 to become either CD27+ memory B cells (Klein et al., 1998; Jung et al., 2000) or, alternatively, CD27++ plasma cells (Odendahl et al., 2000).

CD27 is a transmembrane protein that contains several extracellular cysteine-rich domains that are comprised of pseudo repeats of six cysteines forming three disulphide bounds. Little is known about their structural arrangement in the cell membrane but based on other members of the TNFR family they may be present as monomers or form trimeric signaling complexes when interacting with ligand (Croft, 2003).

### 1.2.1 Diagnostic Implications of Measurements of CD27 Expression

A previous study by Jacobi et al. (2003) investigated the correlation between disease activity in patients with SLE and circulating CD27++ cells and found that the frequency of CD19+CD20-CD27++ plasma cells correlates with the SLE disease activity indices SLEDAI and ECLAM as well as with the titer of anti-double-stranded DNA autoantibodies. Patients lacking anti-double stranded DNA autoantibodies also exhibited a correlation between disease activity and peripheral CD19+CD20-CD27++ plasma cells. These findings were not influenced by age or sex, but were related to the duration of the disease and the therapeutic regimen. An inverse relationship was found regarding CD27- naive B cells with disease duration. The predictive value of the

frequency of CD19+CD20-CD27++ cells was found to be greater than that of the humoral and clinical data including anti-dsDNA antibody levels, circulating immune complexes and acute renal involvement. Subsequently, this parameter has been recommended by the FDA as the only cellular outcome parameter for clinical studies of SLE.

## 1.3 FcγR May Play Multiple Roles in Autoimmunity

Fcγ receptors are expressed on phagocytes, B lymphocytes, NK calls, and follicular dendritic cells and bind the Fc domain of IgG antibodies (Table 1). Fc stands for fragment crystalizable and forms the stem of the 'Y' shaped antibody where binding sites mediate various physiological effects of the antibodies. Consequently, FcRs provide a critical link between the humoral and cellular branches of the immune system. The IgG receptor family, called the FcγR family, consists of several activating receptors and a single inhibitory receptor, FcγRIIb. A vast structural and functional diversity exists between the Fcγ receptors and analyses have defined three major subclasses: FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16) although additional subclasses and subtypes a currently being investigated. FcγRI is the high affinity receptor able to bind monomeric IgG, the others only bind IgG when it is part of an immune complex. Most of the activating-type FcRs associate with the Fc receptor common γ-chain which contains an immunoreceptor tyrosine-based activation motif (ITAM), while FcγRIIb is a single chained molecule that contains an immunreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic tail (Reefman et al., 2003). Most cell types express both the activating and the inhibitory receptors. The ratio is influenced by the cytokine environment whereby Th1 cytokines generally upregulate the expression of the activating receptors and Th2 cytokines generally upregulate the expression of the inhibitory receptors.

***Table 1: Characteristics of Fcγ receptors***

| **Receptor** | **FcγRIa (CD64)** | **FcγRIIa (CD32a)** | FcγRIIb (CD32b) | FcγRIIIa (CD16a) | FcγRIIIb (CD16b) |
|---|---|---|---|---|---|
| **Function** | Activating | Activating | Inhibiting | Activating | Non signaling |
| **Signal Motif** | ITAM | ITAM-like | ITIM | ITAM-like | GPI link |
| **Affinity** | High affinity IgG1≥3>4>>2 | Low Affinity IgG3≥1,2>>4 | Low affinity IgG3≥1>4>2 | Low affinity IgG1,3>>2,4 | Low affinity IgG1,3>>2,4 |
| **Expression** | macrophages **monocytes** DC<br><br>inducible: **granulocytes** mast cells | macrophages monocytes DC Langerhans' cells granulocytes T cells **B cells**** Platelets | macrophages DC Langerhans' cells B cells mast cells granulocytes | macrophages monocytes DC Langerhans' cells T cells NK cells eosinophils | neutrophils<br><br>inducible: eosinophils |
| **Effect of ligation***** | Uptake, stimulation, activation of respiratory burst, induction of killing | Uptake, granule release in eosinophils | No uptake in B cells and mast cells, uptake in macrophages and granulocytes<br><br>Inhibition of stimulation | Induction of killing in NK cells | |

The cell types and receptors written in bold type indicate the cells studied in this investigation. Table adapted from Takai, 2002. **According to Rabinovitch, 2004. ***From Janeway et al., 2005.

### 1.3.1 Signaling Pathway of the Activating FcγR

The activating ITAM pathway is triggered by crosslinking of cell surface FcγR by Ig containing immune complexes which leads to the phosphorylation of tyrosine residues in the ITAM by receptor associated Src-family protein kinases. The resulting activation of Syk includes the stimulation of phospholipase-Cγ (PLCγ), phosphatidylinositol 3-kinase, mitogen-activated protein kinase and GTPases (Takai, 2002). Depending on the particular cell type expressing the FcγR, different kinases are involved in the signaling pathway resulting in various events including oxidative burst, cytokine release, and phagocytosis by macrophages, antibody dependent cellular toxicity by NK cells and degranulation of mast cells (Reefman et al., 2003). The activation pathway can be inhibited by co-aggregation of FcγRIIb.

### 1.3.2 Signaling Pathway of the Inhibitory FcγRIIb

In B cells, FcγRIIb ligation acts to inhibit signaling through the B cell receptor, whereby in myeloid cells, this receptor inhibits activation through activating Fc receptors. As described by Takai (2002), the initial event in the signaling of FcγRIIb is the tyrosine phosphorylation of the ITIM by the tyrosine kinase Lyn. This results in the recruitment of SH2-domain-containing phosphatases, predominantly SH2-domain-containing protein tyrosine phosphatase 1 (SHP-1), SHP-2, and SH2-domain containing inositol polyshosphate 5' phosphatase (SHIP). SHIP, which is the primary effector of FcγRIIb mediated inhibition, causes hydrolysis of phosphatidylinositol-3,4,5- triphosphate (PtdIns(3,4,5)$P_3$). Thereafter the signaling cascade is subdivided in three pathways.

In one pathway, SHIP medited hydrolysis of phosphatidylinositol-3,4,5- triphosphat hinders the membrane translocation of signal transducing molecules including Bruton's tyrosine kinase (BTK) and phospholipase-Cγ (PLCγ). BTK is required for the activation of PLCγ and the hydrolysis of PtdIns(4,5) $P_2$ which generate the second messengers Ins(1,4,5)$P_3$ and diacylglycerol (DAG). Consequentially SHIP inhibits Ins(1,4,5)$P_3$ which is required for calcium mobilization and DAG which is required for the activation of protein kinase C (PKC) and thereby inhibits cell proliferation via mitogen-activated-protein kinases (MAPK).

In a second pathway, anti-apoptotic kinases (AKT) cannot be recruited to the membrane in the presence of activated SHIP which leads to the inhibition of cell proliferation and survival.

Lastly, SHIP functions as an adaptor protein that binds Shc and p62dok that leads to Ras inhibition with downstream arrest of cell cycle progression.

### 1.3.3 Regulation and Expression of FcγR

The various FcγR show distinct expression patterns and are under the control of various regulatory mechanisms. Previous studies have quite clearly defined the regulation and expression of FcγRI (CD64). It is constituitivly expressed on macrophages, monocytes and eosinophils. It is upregulated on neutrophils as a response to LPS, IFN-γ, GCSF, complement, TNF-α, IL-12 and IL-8. The upregulation occurs in a matter of hours (Allen et al., 2002; Davis et al., 2006). Furthermore on follicular dendritic cells, IFN-γ has been shown to shift the balance of FcγR expression in favor of the activating receptors by inducing CD64 and increasing the frequency and density of CD32a while exerting the opposite effect on CD32b. However, the expression and regulation of FcγR on B cells remains disputed. The expression at various stages of B cell development remains somewhat unclear, especially regarding the expression of CD32 on germinal center B cells (Macardle et al., 2002). This can be in part explained by differences in the surface expression according to FACS analysis and the mRNA level, since the expression may be in the process of changing. Nevertheless, Rudge et al. (2002) have shown that IL-4 reduces the expression of various inhibitory receptors on B cells and abolishes CD22 and CD32 mediated suppression.

Analysis of the regulation mechanisms and expression patterns of these receptors as a way to predict and understand immune responses must consider the effects and binding characteristics of the various subclasses of antibodies on these receptors. Because the antibody subclasses bind with varying affinity to the various FcγR as shown in Table 1, the in vivo activity of a given antibody depends on the affinity to the various receptors and the expression level of the receptors. If a variety of FcγR are present on the cell surface, those FcγR with optimal affinity for the receptive isotype will engage. Consequently, changes in the expression patterns of the various receptors have larger

immunological effects for the antibodies that bind with low affinity, although the system usually works synergistically because the regulation of FcR expression and the regulation of isotypes is influenced by the same cytokines.

Inflammatory mediators like IFN-γ and C5a can upregulate the activating FcγRs, while reducing FcγRIIb. Under these circumstances antibodies with particular affinity to the inhibitory receptors will lose more activity than those which preferably bind the activating receptors. In contrast Th2 cytokines like IL-4, IL-10 and TGF-β which are produced in response to extracellular pathogens, upregulate the expression of the inhibitory FcγRIIb and downregulate the expression of the activating FcγR on innate immune effector cells. Furthermore, they enhance B cell activation including the production of IgG1 and IgE and the secretion of IgA (Nimmerjahn, et al., 2005; Nimmerjahn et al., 2006).

### 1.3.4 Functions of FcγR

Activating and inhibiting FcγR are often co-expressed on the same cell, providing an important physiological mechanism of setting thresholds for activating stimuli. As a consequence of the combination of activation and inhibitory signaling, the most prominent function of the FcγRs is the positive and negative regulation of immune cell responses. One example is the role of FcγRIIb in the maintenance of B cell tolerance. Furthermore, FcγR play an important role in the non-inflammatory uptake of immune complexes as well as in phagocytosis and enhanced antigen presentation.

FcγRIIb sets the threshold for B-cell activation such that cross-linking of immune complexes containing IgG and antigen recognized by the BCR can lead to the inhibition of antibody production (Amigorena et al., 1992; Muta et al., 1994). Furthermore, homo-aggregation of FcγRIIb on B cells has been shown to be pro-apoptotic signal mediated by an ITIM independent pathway which is blocked by the co-ligation of the BCR. Thus in the germinal center, where immune complexes are retained by FDC, FcγRIIb may be an active determinant in the negative selection of B cells whose BCR have reduced affinity for antigen as a result of somatic hypermutation, thereby maintaining peripheral tolerance (Pearse et al.,1999). However it has also been observed that in the pre-B stage, signaling by FcγRIIb diminishes the signals for apoptosis and therefore might be critical in mediating expansion of the antibody repertoire (Kato et al., 2002).

Pugh-Bernard et al. (2001) and subsequently Cappione et al. (2005) studied autoreactive B cells (9G4 B cells) using tonsil biopsies and found that in SLE 9G4 cells, unlike 9G4 cells in normal controls, progressed unimpeded through the GC reactions and are expanded in the post GC IgG memory and plasma cell compartments. Studies of the downregulation of FcγRIIb on germinal center B cells demonstrated an inverse relationship between the extent of downregulation and IgG antibody response and consequently susceptibility to autoimmune disease (Jiang et al., 2000; Pritchard et al., 2000; Fukuyama et al., 2005). Other studies demonstrated the restoration of tolerance in lupus by targeted modulation of FcγRIIb expression via retroviral vectors in mice (McGaha et al., 2005). These studies demonstrate that re-establishing tolerance in autoimmune mouse strains with diverse genetic backgrounds can be achieved by increasing the surface expression of FcγRIIb on B cells. Although only 40% of the B cell compartment was transduced, this was sufficient to re-establish the peripheral checkpoint regulated by this molecule and to prevent autoimmunity. Although the precise mechanism by which FcγRIIb expression on B cells contributes to the maintenance of tolerance is still under investigation, these findings demonstrated that FcγRIIb expression on B cells regulates the accumulation of auto-reactive plasma cells. Thus relatively small changes in the surface expression on this receptor appear to be critical for determining disease progression, and these changes provide a possible basis for therapeutic approaches. Nevertheless, it is also likely that autoreactive T cells play a role in mediating positive selection of B cells in the germinal center.

Furthermore, because of immune complex interaction with either inhibitory or activating FcγR on various cell types, these receptors can either enhance or suppress the humoral response. Clearance of immune complexes from circulation is regulated by the mononuclear phagocyte system (MPS). In this system, erythrocytes bind immune complexes through complement receptor 1, thereby transporting the complexes to mononuclear phagocytes in the liver and spleen. As an inhibitory receptor, FcγRIIb is crucial to the non-inflammatory clearance of immune complexes. Deletion of FcγRIIb leads to increased immune complex induced disease like Collagen-induced arthritis and Goodpastures's syndrome in animal models (Dijstelbloem et al., 2001). Even in non-susceptible genetic background like C57BL/6, FcγRIIb deletion sufficiently rendered mice susceptible to collagen-induced arthritis (Yuasa et al., 1999).

On the other hand, ligation of the activating FcγRI on macrophages and neutrophils leads to uptake, stimulation, respiratory burst and the induction of killing. IgG binding to activating FcγR on the cell surface leads to activation of phagocytes. Some bacteria are directly recognized, ingested and destroyed by phagocytes, but when the bacteria has a polysaccharide capsule, a coating with antibody and complement which then engages cell surface receptors including FcγR triggers bacterial uptake (Janeway et al, 2005). Furthermore, antigen uptake via FcγRs leads to enhanced antigen presentation.

In summary, these findings indicate a central role of FcγR in modulating a wide range of immune responses. Tissue deposited immune complexes can trigger activating FcγRs causing the release of pro-inflammatory mediators. FcγRIIb, on the other hand, is central to the maintenance of peripheral tolerance by modulating the activation of B cells and thus antibody production and in facilitating the non-inflammatory uptake of immune complexes.

### 1.3.5 Genetic Studies Involving FcγR and the Development of Autoimmunity

Genetic studies in mice and humans have shown that FcγRs are important risk factors for autoimmune disease (van de Winkel et al., 1993; Ioan-Facsinay et al.; 2002). Evidence in support of a role for the heterogeneity of FcγR in suseptibilty to SLE has been controversial, although several studies have reported polymorphisms in FcγRIIa, IIb, IIIa and IIIb to be associated with SLE in humans and suggest a role for these genes in the development and expression of disease (Dijstelbloem et al., 2000; Siriboonrit et al., 2003). Analysis of Japanese patients with SLE has identified a single nucleotide polymorphism in the FcγRIIb gene that results on the Ile232Thr substitution with a phenotype significantly more frequent in Japanese patients with SLE as compared to NHS. Amino acid 232 of FcγRIIb is in the transmembrane region and although the precise effect of this mutation is not known. It may be related to decreased function of FcγRIIb which results in altered apoptotic signaling allowing for the survival of B cells which produce autoantibodies. Additionally, this polymorphism may lower the threshold for activating reactions for example those via FcγRI or it may downregulate phagocytic activity of monocytes (Pricop, et al., 2001; Samuelsson, et al., 2001; Kyogoku et al., 2002). Furthermore, FcγR polymorphisms have been shown to influence

susceptibility and efficacy of anti-CD20 therapy with Rituximab in patients with non-Hodgkins lymphoma (Cartron et al., 2002) and SLE (Anolik et al., 2003).

There are various explanations for the discrepancies in the findings regarding Fcγ receptor polymorphisms and suceptibilty to disease, including differences in population based genetic differences in a multigenetic disease, in addition to linkage disequilibrium and the multi-factorial aspects of disease expression and progression. While various studies suggest that polymorphic genes are involved in SLE susceptibility and disease progression, given the multiple immunological disturbances found in SLE, further investigation of combinations of common variants of genes involved in the dysfunctional immune functions associated with susceptibility to SLE is needed.

## 1.4 Research Objectives

The primary research objective of this work was to facilitate the differentiation between a SLE flare and acute infection in patients with SLE by applying B cell phenotyping. In order to achieve this goal we investigated the possibility of doing a tandem analysis of CD27 on B cells and FcγRI (CD64) on neutrophils and monocytes using the TruCount method and additionally studied the expression of FcγRIIa (CD32a) on B cells in patients with SLE, patients with infections and NHS. Our interest in CD32a was also based on the possibility that the expression of CD32a, likely in association with CD32b expression, on B cells in SLE plays a role in the pathogenesis of the disease. Therefore, in effect, this work also investigates the expression of Fcγ receptors on peripheral blood cells of patients with SLE.

In short the first part of this study establishes the flowcytometric analysis of B cells subsets using whole blood instead of the Ficoll-Paque density gradient centrifugation method. This innovative method was required to perform a parallel analysis of (1) B cell subsets and (2) the surface expression of FcγRI (CD64) on monocytes and granulocytes. Finally in part (3), the differential expression of FcγRIIa (CD32a) was analysed on B cells subsets.

In this work several aspects of the immunphenotypical characterisation of patients with SLE were investigated. The focus was on the expression of two different Fcγ receptors

in patients with SLE compared to various other sample populations and the evaluation of a new method of studying the expression of CD27 on B cell subsets, which has been shown to be a marker of disease acivity in SLE. Since the expression of CD27 on B cell subsets has been established as an important clinical tool to evaluate lupus activity, in part 1 we investigated the possibility of simplifying the method of determining these values by using the TruCount method rather than the Ficoll-Paque density gradient centrifugation method. One reason to evaluate this method is that additional cell populations including neutrophils can be investigated using the TruCount method, providing new possibilities of tandem analyses. Furthermore we focused on FcγR expression, including the expression of FcγRI on neutrophils and monocytes in part 2 in order to investigate an additional method of diagnosing infections in patients with SLE. Lastly in part 3, we focussed on FcγIIa expression on B cells to gain further insights into the suspected dysregulation of B cells in SLE and to investigate the expression of this receptor in SLE, in cases of acute infection and in NHS.

**Research Objective 1: Evaluation of the TruCount method to analyse B cell subsets**

Previous studies of B cell subpopulations in patients with SLE using the Ficoll-Paque density gradient centrifugation method indicate a correlation between CD27++ expression and disease activity (Odendahl et al., 2000, Jacobi et al., 2003). In the past such studies calculated absolute numbers of various cell populations using the percentage of each population attained through flow cytometric analysis, in conjunction with the absolute count of lymphocytes according to an automated hematology counting system or performed the analysis based on the relative distribution of the cell populations by comparing percentages. This method of attaining cell counts for lymphocytes subpopulations is also called a dual-platform because measurements in two systems are required. This procedure, which isolates cells based on density gradients, is time consuming and the possible cell populations which can be analysed are limited to peripheral blood mononuclear cells (PBMC). Furthermore, there is a possibility of losing certain subpopulations in the process of isolating PBMC. Therefore, the possibility of using a single platform method in which the cells are stained in whole blood and cell counts are calculated based on beads that are simultaneously counted with the cells is very attractive. Whole blood methods expand the possible cell populations which can be studied beyond PBMC to include neutrophils, for example.

Additionally, this procedure, using erythrocyte lysis instead of a density gradient to isolate the cells of interest, requires fewer steps and can be performed more quickly, which is an advantage when studying short lived populations and increases the practicality for diagnostic use. However, the use of TruCount profiling as a single platform method had not been fully evaluated in this context. Therefore, we compared the previously used Ficoll-Paque method and the TruCount method in the analysis of CD27 on B cell subsets.

**Research Objective 2: FcγRI (CD64) Expression on Neutrophils and Monocytes in SLE**

Furthermore, we were searching for an additional marker that we could use in a tandem analysis to potentiate the discriminating value of the CD27 analysis in the differentiation of an SLE flare and infection on patients with SLE. Using the TruCount method, we investigated the FcγRI (CD64) on monocytes and neutrophils, which had previously been shown to be upregulated in cases of acute infection (Allen et al., 2002; Ng et al., 2002; Ng et al., 2004), in order to determine if this receptor can be used to differentiate between a flare and acute infections in patients with SLE. These patients are especially susceptible to infections and it is often difficult to differentiate between an infection and a flare although this differentiation plays a central role in making therapeutic decisions. Therefore, the expression of CD64 was investigated on monocytes and neutrophils in NHS, patients with SLE, patients with SLE and infections and patients with infections alone.

**Research Objective 3: Analysis of FCγRIIa (CD32a) Expression on B Cells in SLE**

Further studies focused on the differential expression of the activating FcγRIIa (CD32a) on B cell subsets in patients with SLE, NHS and patients with infections to investigate a potential role of this receptor in the pathogenesis of the disease and its potential use as a marker of infection. Since this was preliminarly research regarding this receptor in SLE, the Ficoll-Paque method was used.

# 2. Materials and Methods

## 2.1 Principles of Flow Cytometry

Although the cells are prepared and stained in various ways, all three aspects of this work are based on the analysis of cell surface proteins with the use of flow cytometric technology. The principle of flow cytometry is based on the simultaneous measurement of multiple characteristics while cells in a fluid stream pass through an electronically armed microscope. Flow cytometers are able to measure physical qualities of the cells (such as size and granularity) and fluorescent signals which the fluorochrome marked monoclonal antibodies bound to the cell surface give off when excited by a laser beam (Radcliff et al., 1998; Radbruch, 2000).

In the flow cytometer, cells in suspension are drawn into a stream of isotonic fluid to create a laminar flow with the objective of achieving hydrodynamic focussing whereby a single file of cells pass various interrogation points where data about the physical qualities and fluorescence is gathered (Radbruch, 2000). For these experiments the FACScalibur (Becton Dickinson, San Jose, CA, USA) was used with a 488 nm argon ion and a 635 red diode as light sources. Cells passing the measurement points scatter light in correlation to their size at an angle to the incoming laser beam. This light is detected by the forward scatter detector (FCS) and correlates to the size of the cell while the side scatter detector (SSC) detects light scattered at a 90 degree angle and correlates to the granularity of the cell surface. In addition to these characteristics, the FACScalibur can additionally measure up to four fluorescent light (Fl) parameters.

At a given interrogation point, the laser intersects the cells and the emitted light is given off in all directions and is collected via filters and mirrors that form a system capable of isolating emissions of a particular wavelength. Signals are collected and converted to digital data files for further analysis (Radcliff et al.,1998).

The spectral range of the emitted light is determined by the fluorescent label used. Nevertheless, an optimal filter setting cannot definitively prevent a certain degree of overlap between the emissions spectra. Tandem dyes, such as PerCP-Cy5.5, with

internal fluorescence resonance energy can create even longer wavelengths or give added stability to the fluorescence. Table 2 shows the fluorochromes used in this study.

***Table 2: Characteristics of fluorochromes used***

| Fluorescence Detection Channel | Fluorescence Detection Color | Fluorochrome | Excitation Peak (nm) | Emissions Peak (nm) |
|---|---|---|---|---|
| Fl-1 | Green | FITC *(Fluorescein Isothiocyanat)* | 494 | 519 |
| Fl-2 | Yellow | PE *(Phycoerythrin)* | 496/546 | 578 |
| Fl-3 | Far Red | PerCp-Cy5.5 *(Peridinin chlorophyll A protein with Cyanine dye in tandem)* | 482 | 695 |
| | Red | PerCp (*Peridinin chlorophyll A protein)* | 490 | 675 |
| Fl-4 | Red | Cy5 *(Cyanine)* | 675 | 670 |

## 2.2 Analysis of B Cell Subpopulations using the Ficoll-Paque Density Gradient Centrifugation Method in a Dual-platform and the TruCount Method

### 2.2.1 Samples and Patient Characteristics

In order to compare the Ficoll-Paque density gradient centrifugation technique used in combination with automated hematology blood counts (dual-platform) with the TruCount method to analyse B cell subsets, cell counts for various cell populations were compared in 33 samples. Whole blood (5-10 ml) was drawn in citrate from various populations including patients with infections (n = 11), patients with SLE (n = 14) and patients with SLE and an acute infection (n = 8). In the population with infections alone the infections were endocarditis, meningitis, acute pancreatitis, and multiple cases of respiratory tract infection, similarly the infections in the patients with SLE included

endocarditis, meningitis, an infection of an implanted pacemaker and multiple cases of respiratory tract infection. Patient samples were drawn at the University Hospital, Charité (Berlin, Germany) after informed consent was obtained. Various cell counts including leucocytes, lymphocytes, monocytes, neutrophils, and the B cell subpopulations were compared in order to investigate the comparability of these two methods on various levels. This was of particular interest because there are relatively few published studies comparing these methods and those that exist tend to focus on a specific subpopulation such as precursor dendritic cells (Ma et al., 2004) or CD4+ T cells (Nicholson et al, 1997; Storie et al., 2004;). Because differential blood counts were not available in all 33 cases, lymphocyte, neutrophil and monocytes were compared in 29 of these samples. B cell subsets were compared in 10 patients with SLE.

The patient populations were characterized as shown in Table 3. The mean SLEDAI of the patients with SLE was 8.6±5, the median ECLAM was 3.3±2.4. The mean duration of the illness was 6±4 years. Eighty-two percent of the patients with SLE were women. Nine of the patients had renal involvement in the past and 3 had it acutely. The current therapy regimens for the patients with SLE and SLE with acute infections are shown in Tables 4 and 5.

***Table 3: Patient population characteristics in the comparison of the TruCount with the Ficoll-Paque density gradient centrifugation method***

| | **Patients with SLE** | **Patients with SLE and infections** | **Patients with infections** |
|---|---|---|---|
| **Age*** | 33±12 | 41±18 | 64±18 |
| **CRP*** (mg/dl) (normal range <0,5 mg/dl) | 1.0±1.9 | 4.24±4 | 11.82±7.9 |
| **Leucocyte count*** (/µl) (normal range: 4500-11000/µl) | 5567±2526 | 5776±2851 | 11893±6146 |

*mean

***Table 4: Therapy regimens for the patients with SLE***

| | |
|---|---|
| 1 | Prednisolon 7 mg/ and 10 mg/d, switching each day. Cyclophosphamide 6 wks ago. |
| 2 | Prednisolon 20 mg/d, Hydroxychloroquinsulfate 20 mg /d. |
| 3 | Methylprednisolon 4 mg /d. Cyclophosphamide 4 wks ago. |
| 4 | Prednisolon 10 mg/d. Cyclophosphamide 12 wks ago. |
| 5 | Prednisolon 12.5 mg/d. |
| 6 | Cyclophosphamide 4 weeks ago. |
| 7 | Prednisolon 10 mg/d. Cyclophosphamide 4 wks ago. |
| 8 | Prednisolon 5 mg/d. Cyclophosphamide 4 wks ago. |
| 9 | Methylprednisolon 8 mg /d. Cyclophosphamide 12 wks ago. |
| 10 | No medication. |
| 11 | Prednisolon 10 mg/d, Hydroxychloroquinsulfate 400 mg/d. Cyclophosphamide 4 wks ago. |
| 12 | Prednisolon 10 mg/d. Cyclophosphamide 4 wks ago. |
| 13 | Prednisolon 5 mg/d. |
| 14 | Prednisolon 7.5 mg/d. Cyclophosphamide 4 wks ago. |

***Table 5: Therapy regimens for the patients with SLE and acute infections***

| | |
|---|---|
| 1 | Prednisolon 20 mg/d. |
| 2 | Prednisolon 15 mg/d. Intravenous immunoglobulin 4 wks ago. |
| 3 | Prednisolon 30 mg/d. Azathioprine 100 mg/d. |
| 4 | Prednisolon 7.5 mg/d. |
| 5 | Methylprednisolon 4 mg /d. Cyclophosphamide 4 wks ago. |
| 6 | Prednisolon 10 mg/d. Cyclophosphamide 1 wk ago. |
| 7 | Prednisolon 7.5 mg/d. |
| 8 | Prednisolon 7.5 mg/d. Azathioprine 150 mg/d. Plasmapheresis. |

### 2.2.2 Automatic Cells Counts

In this case the automated cell counts were generated by the Sysmex XE-2100 Automated Hematology Analyzer which determines cell counts on the basis of forward-scattered light, side-scattered light, side fluorescence and impedance. Cells are not marked with specific cell surface markers, but fluorescent staining of DNA and RNA of leucocytes is performed (Ruzicka et al., 2001).

### 2.2.3 Ficoll-Paque Density Gradient Centrifugation Method

#### 2.2.3.1 Isolation of PBMC

For the Ficoll-Paque method, peripheral blood mononuclear cells were prepared using the method described by Boyum (1976). First, the blood was diluted 1:1 with PBS/BSA (71.7 ml 0.5 M $K_2HPO_4$, 28.3 ml 0.5 M $KH_2PO_4$, 8.8 g NaCl, 2 g BSA) and transferred to form a layer above 12.5 ml of Ficoll in a 50 ml polystyrene Falcon conical bottom tube (Bacto Laboratories, Liverpool, NSW, Australia). This was centrifuged at 1800 rpm for 20 minutes at room temperature. Then the ring of monocytes and lymphocytes was transferred to another 50 ml Falcon conical bottom tube. After filling the tube up with PBS/BSA it was centrifuged for 10 minutes at 1200 rpm at 4° C. The pellet was isolated and dissolved in 500 µl PBS (71.7 ml 0.5 M $K_2HPO_4$, 28.3 ml 0.5 M $KH_2PO_4$, 8.8 g NaCl).

#### 2.2.3.2 Staining of Cells

The staining was then carried out on 100 µl of the sample containing approximately $10^6$ cells. Human Ig (Beriglobin, Aventis Behring, Marburg, Germany) was added in a dilution of 1:50 before incubation to prevent non-specific Fc- receptor binding of the antibodies. The staining was performed using the mouse anti-human antibodies shown in Table 6 for 10 minutes at 4° C in the dark. After incubation, the samples were washed with 1 ml of PBS/BSA and centrifuged at 1100 rpm for 5 minutes at 4° C. The pellet was then dissolved in 600 µl PBS. Propidium iodide (1 µg/ml, Sigma, Munich, Germany) was added just before the cytometric analysis to exclude dead cells.

***Table 6: Monoclonal antibodies used in the Ficoll-Paque density gradient centrifugation cell isolation procedure surface staining to analyse B cell subsets***

| Anti-human Antibody | Supplier | Label | Clone | Dilution |
|---|---|---|---|---|
| CD27 | Provided by R. van Lier, Amsterdam | Cy5 | 7H2 | 1:500 |
| CD20 | BD | FITC | 2H7 | 1:100 |
| CD19 | DAKO | PE | HD37 | 1:100 |

### 2.2.4 TruCount Method

#### 2.2.4.1 Isolation of Cells

In the TruCount method it is not necessary to isolate cells since the staining is carried out on whole blood and then the erythrocytes are lysed with lysing solution (Fiebig et al., 1997; Nicholson et al., 1997; Ma et al., 2004)

#### 2.2.4.2 Staining of Cells

The mouse anti-human antibodies used in the TruCount method are shown in Table 7. 20 µl of staining reagent and 50 µl of well-mixed anti-coagulated citrated blood from the same samples as used for the Ficoll-Paque method were added to the TruCount tubes. The tubes were capped and gently vortexed and then incubated in the dark at room temperature for 15 minutes. 450 µl 1X FACS lysing solution (Becton Dickinson, San Jose, CA, USA) was added to the sample followed by gentle vortexing. Samples were placed on ice. Analysis was done within 30 minutes. Samples were mixed gently immediately prior to analysis.

***Table 7: Monoclonal antibodies used in the TruCount surface staining procedure to analyse B cell subsets***

| Anti-human Antibody | Supplier | Label | Clone | Dilution |
|---|---|---|---|---|
| CD27 | Provided by R. van Lier, Amsterdam | Cy5 | 7H2 | 1:500 |
| CD20 | BD | FITC | 2H7 | 1:100 |
| CD19 | BD | PerCp-Cy5.5 | 4G7 | 1:10 |

### 2.2.5 Flow Cytometry Equipment and Data Collection

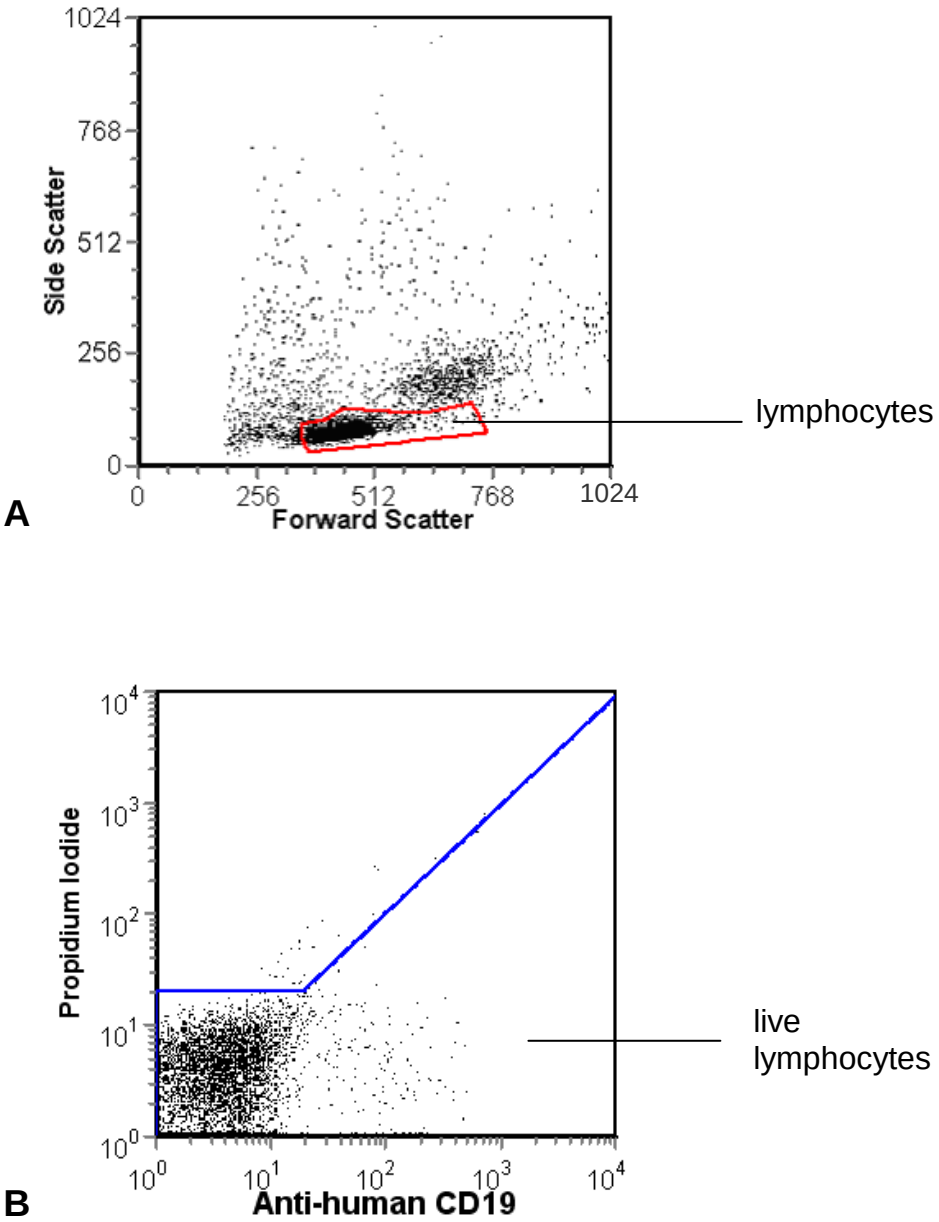
A FACScalibur flow cytometer with Cell Quest software (Becton Dickinson, San Jose, CA, USA) was used to gather the initial data. In the Ficoll-Paque method a total of 80,000 events was collected for each analysis. In the TruCount method, the number of cells analysed in each sample was normalized to the bead count. In this case, data was

collected until the number of beads counted was 30,000 which correlated to approximately a total of 80,000 events.

### 2.2.6 Gating of B cells

#### 2.2.6.1 Gating Procedure of Ficoll-Paque Density Gradient Centrifugation Method

A standard gating procedure was designed for each study in order to clearly define the population of interest. Figure 1 shows the gating strategy used for the analysis of CD27 expression on B cell subsets using the Ficoll-Paque density gradient centrifugation method.



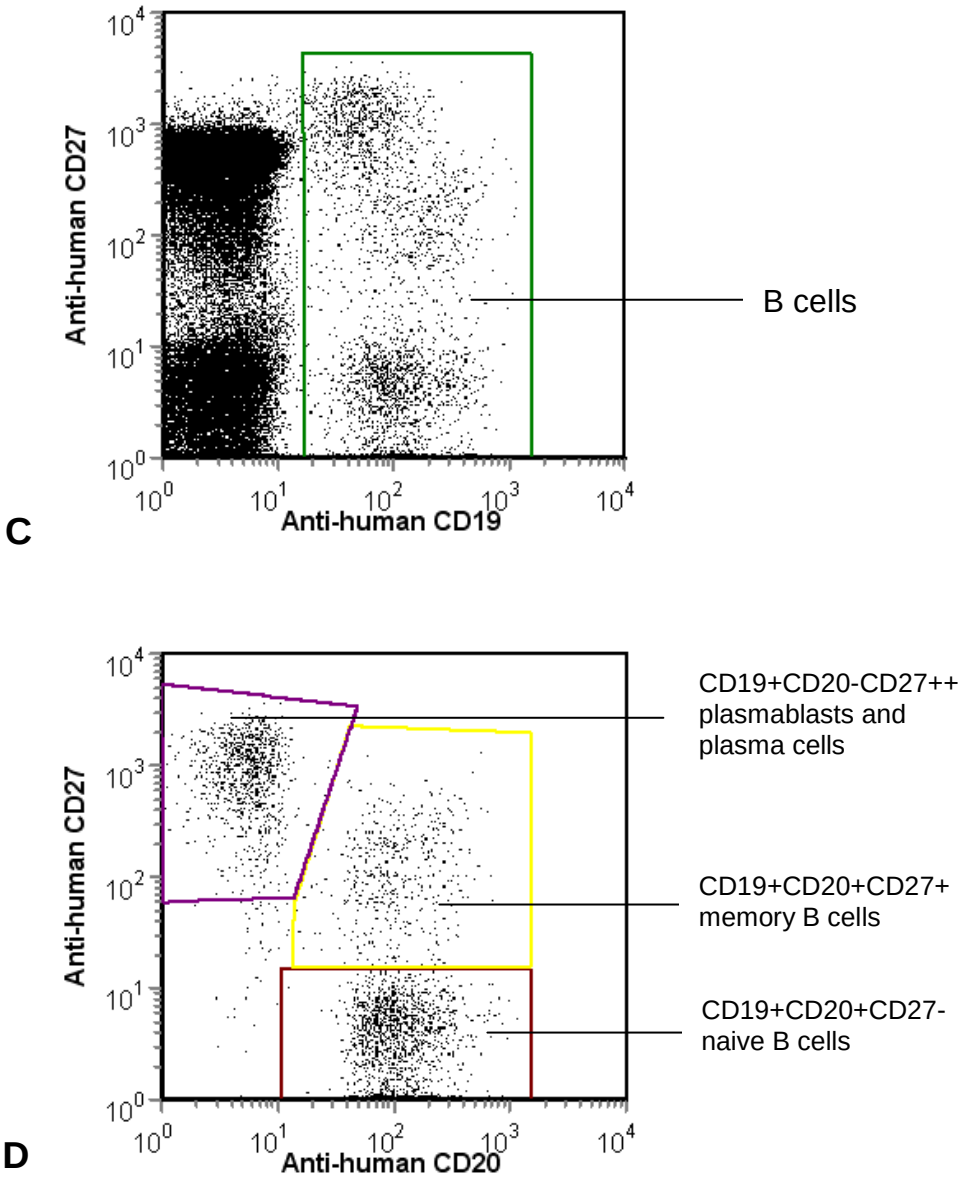
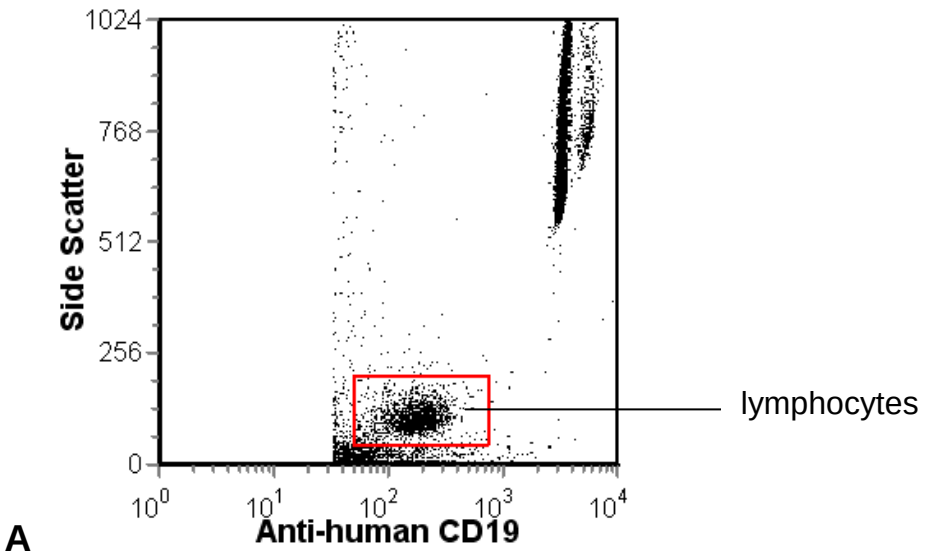


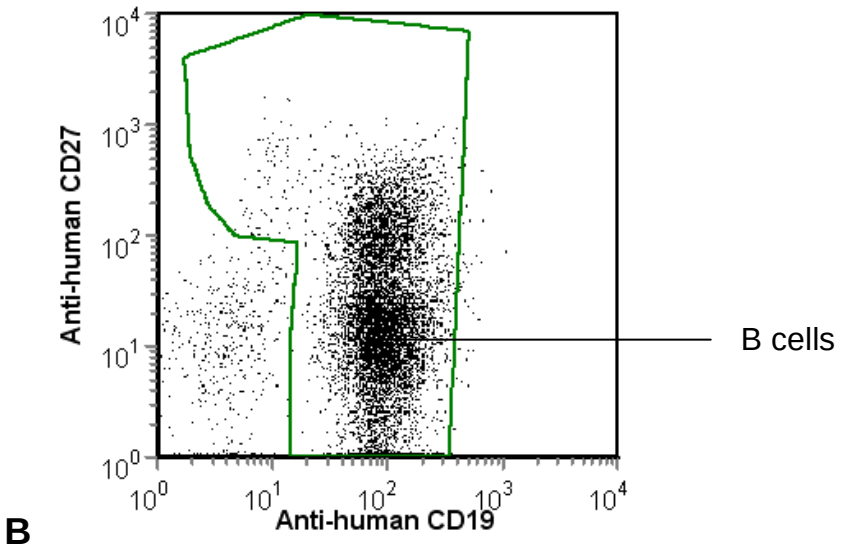

Figure 1. Gating procedure used for the analysis of B cell subsets using the Ficoll-Paque procedure. **A:** shows all events, with a gate defining lymphocytes **B:** Dotplot with a gate defining live lymphocytes **C:** Dotplot with the previously gated cells with a gate defining B cells **D:** Dotplot of the B cell subsets.

### 2.2.6.2 Gating Procedure of the TruCount Method

Figure 2 shows the gating strategy used for the analysis of CD27 expression on B cell subsets using the TruCount method.




A




B

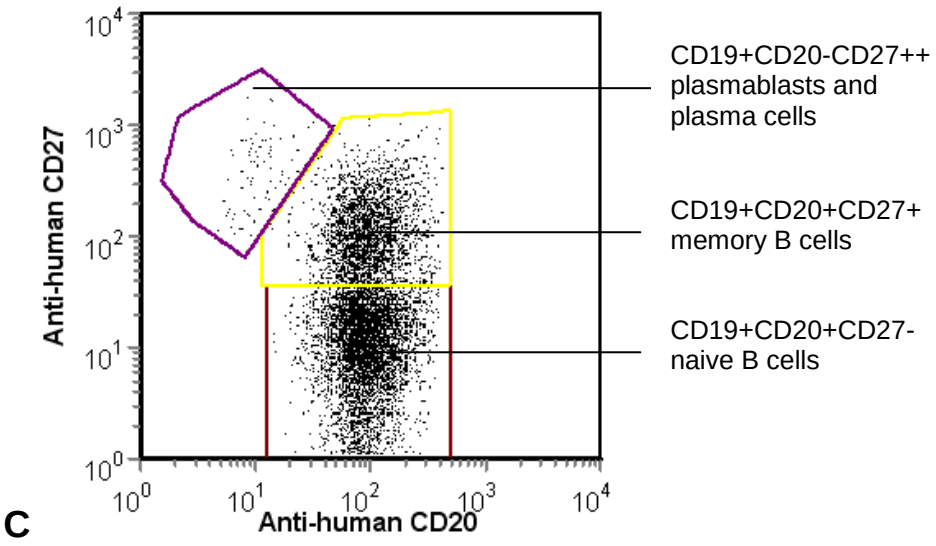



Figure 2. Gating strategy for the analysis of CD27 expression on B cell subsets using TruCount tubes. **A:** Dotplot showing all events, with a gate defining lymphocytes **B:** Dotplot of the previously gated cells with a gate defining B cells. **C:** Dotplot showing the B cell subsets.

### 2.2.7 Cell Counts

Absolute cell counts were calculated for the various cell populations in both methods.

#### 2.2.7.1 Calculation of Cell Counts in the Ficoll-Paque Method

An absolute count of B cells was calculated for the Ficoll-Paque samples using the percentage of B cell using FCS Express software (DeNovo Software, Ontario, Canada) and absolute counts of lymphoytes as determined by the Sysmex XE-2100, an automated hematology analyzer. The calculation was performed according to the following equation:

$$\frac{(\%\ \text{B cells}) \times (\text{automated absolute count of lymphocytes/ µl})}{100} = \text{absolute count of B cells /µl using dual-platform}$$

The absolute count of the B cell subsets was calculated using this absolute B cell count as follows:

$$\frac{(\%\ \text{of B cells which comprises subpopulation}) \times (\text{absolute count B cells/ µl})}{100} = \text{absolute count of subpopulation/µl using dual-platform}$$

### 2.2.7.2 Calculation of Cell Counts in the TruCount Method

The TruCount method of obtaining absolute cell numbers is based on the incorporation of a known number of lypophilized fluorescent beads in the tube during manufacturing. As shown in Figure 3, the beads occupy a light scatter position outside the normal cell populations. The absolute cell count is calculated based on the proportion of beads to cells counted for a given amount of sample added to the tube. Frequencies of the cell and bead populations were determined using FCS Express software (DeNovo Software, Ontario, Canada).

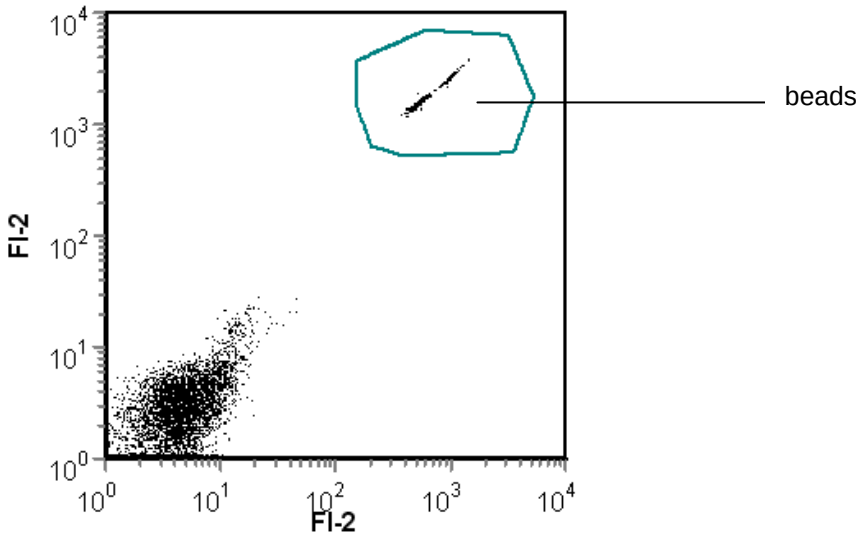



Figure 3. Determination of the bead count using Fl-1 versus Fl-2

The absolute number of cells in a particular population in was calculated using the following equation:

$$\frac{\text{Num. of cells in population of interest}}{\text{Num. of beads counted}} \times \frac{\text{Num. of beads per TruCount tube}}{\text{sample volume } (\rightarrow 50\mu l)} = \text{absolute count/ } \mu l$$

### 2.2.8 Statistical Analysis

The FACS data files were analysed using FCS-Express (DeNovo Software, Ontario, Canada), the statistical analysis was done using GraphPad Prism software (GraphPad Software, Inc. San Diego, CA, USA).

The cell counts obtained in the two systems were compared using the nonparametric Wilcoxon test, linear regression and Bland-Altman plots (Bland et al., 1986 and 1995). Additionally the Spearman correlation coefficient was calculated. For the statistical tests, significance was defined as $p < 0.05$.

## 2.3 Analysis of CD64 Expression on Monocytes and Neutrophils

### 2.3.1 Samples and Patient Characteristics

The expression of the activating FcγRI (CD64) on monocytes and neutrophils was investigated in 24 subjects using an isotype control. This sample included 7 patients with SLE, 5 patients with SLE and infection, 6 patients with acute infections and 6 NHS. The normal healthy subjects were sex matched to the study population. Further characteristics of the sample populations are shown in Tables 8-12.

***Table 8: Characteristics of patients with SLE and acute infections***

| | **Age** | **CRP (mg/dl)** | **Leucocytes/µl** | **Infection** | **Sex** |
|---|---|---|---|---|---|
| 1 | 26 | 1.46 | 10060 | RTI | F |
| 2 | 50 | 8.65 | 3720 | Endocarditis | F |
| 3 | 50 | 5.94 | 7480 | RTI | F |
| 4 | 43 | 0.71 | 3190 | Listerien sepsis | F |
| 5 | 61 | 1.02 | 4270 | Erysipelas | F |
| ***Mean*** | *46±13* | *3.6±3.6* | *5744±2934* | | *M:F 0:5* |

***Table 9: Characteristics of Patients with Infections***

| | **Age** | **CRP** | **Leucocytes/µl** | **Infection** | **Sex** |
|---|---|---|---|---|---|
| 1 | 34 | NA | NA | RTI | M |
| 2 | 79 | 4.48 | 9230 | Gastroenteritis | F |
| 3 | 81 | 9.79 | 15780 | Pneumonie | M |
| 4 | 85 | 27.59 | 12120 | Endocarditis | F |
| 5 | 65 | 16.98 | 7050 | Endocarditis | F |
| 6 | 45 | 2.29 | 14560 | Endocarditis | F |
| ***Mean*** | *65±21* | *12±10* | *11748±3630* | | *M:F 2:4* |

***Table 10: Characteristics of Patients with SLE***

| | **Age** | **ECLAM** | **SLEDAI** | **Sex** |
|---|---|---|---|---|
| 1 | 65 | 5 | 12 | M |
| 2 | 21 | 2 | 8 | F |
| 3 | 36 | 1 | 4 | F |
| 4 | 27 | 4 | 10 | M |
| 5 | 28 | 2 | 8 | F |
| 6 | 27 | 1 | 2 | F |
| 7 | 26 | 4 | 10 | F |
| ***Mean*** | *33±15* | *2.7±2* | *7.7±4* | *M:F 2:5* |

***Table 11: Immunosuppressive therapy for patients with SLE***

| | |
|---|---|
| 1 | Prednisolon 7 mg/ and 10 mg/d, switching each day. Cyclophosphamide 6 wks ago. |
| 2 | Prednisolon 20 mg/d, Hydroxychloroquinsulfate 20 mg/d. |
| 3 | Prednisolon 3 mg/d. |
| 4 | Prednisolon 10 mg/d. Cyclophosphamide 4 wks ago. |
| 5 | Prednisolon 5 mg/d. |
| 6 | Prednisolon 7.5 mg/d. Cyclophosphamide 4 wks ago. |
| 7 | Prednisolon 10 mg/d. Cyclophosphamide 4 wks ago. |

***Table 12: Immunosuppressive therapy for patients with SLE and acute infections***

| | |
|---|---|
| 1 | Prednisolon 15 mg/d. Intravenous immunoglobulin 4 wks ago. |
| 2 | Prednisolon 30 mg/d. Azathioprine 100 mg/d. |
| 3 | Prednisolon 7.5 mg/d. |
| 4 | Methylprednisolon 4 mg /d. Cyclophosphamide 4 wks ago. |
| 5 | Prednisolon 10 mg/d. Cyclophosphamide 1 wk ago. |

### 2.3.2 Preparation of Cells

Analysis of CD64 expression on monocytes and neutrophils was done using whole blood and lysis of erythrocytes with lysis solution according to the TruCount procedure.

### 2.3.3 Staining of Cells

To stain the cells in this study, 20 µl of staining reagent containing the mouse anti-human antibodies described in Table 13 and 50 µl of well-mixed anti-coagulated blood were added to the TruCount tubes. For the isotype control, the recommended mouse IgG1 isotype control (Becton Dickinson, San Jose, CA, USA) was used instead of the anti-human CD64 antibody. To investigate the role of non-specific Fc-receptor binding of the antibodies, samples were tested with and without human Ig (Beriglobin, Aventis Behring, Marburg, Germany) added in a dilution of 1:50 before incubation. The tubes were capped and gently vortexed and then incubated in the dark at room temperature for 15 minutes. 450 µl 1X FACS lysing solution (Becton Dickinson, San Jose, CA, USA) was added to the sample followed by gentle vortexing. Samples were placed on ice. Analysis was done within 30 minutes. Samples were mixed gently immediately prior to analysis.

***Table 13: Monoclonal antibodies used in CD64 surface staining of neutrophils and monocytes***

| **Anti- human Antibody** | **Supplier** | **Label** | **Clone** | **Dilution** |
|---|---|---|---|---|
| CD64 | BD/Pharmingen | FITC | 10.1 | 1:20 |
| CD45 | BD | PerCP | 2D1 | 1:10 |
| Isotype Control IgG1 | BD | FITC | X40 | 1:20 |

### 2.3.4 Flow Cytometry Equipment and Data Collection

A FACScalibur flow cytometer with Cell Quest software (Becton Dickinson, San Jose, CA, USA) was used to gather the initial data after staining. The number of cells analysed in each sample was normalized to the bead count. In this case data was collected until the number of beads counted was 15,000 which correlated to approximately 150,000 events.

### 2.3.5 Gating and Analysis of CD64 Expression

A gating strategy was developed to analyse the expression of CD64 on monocytes and neutrophils. Figure 4 illustrates this procedure.

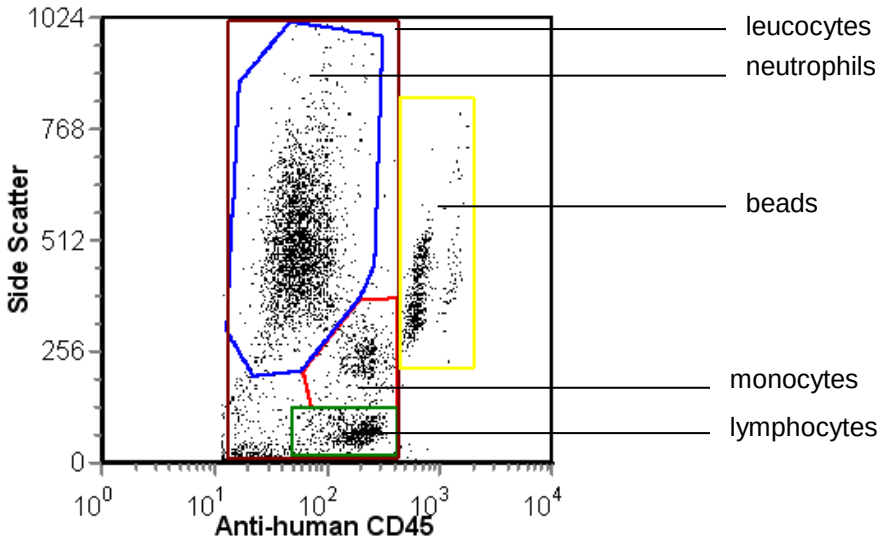
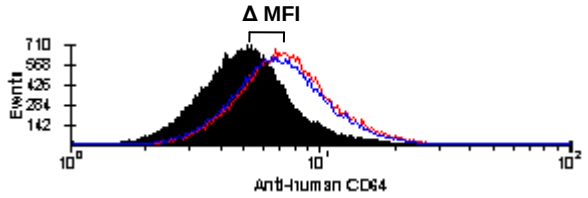



Figure 4. Gating strategy for the analysis of CD64 expression using TruCount tubes. All events are shown, the regions are labelled accordingly.

The expression was analysed as mean fluoresence intensity (MFI) of the anti-human CD64 antibody minus the MFI of the isotype control.

Histograms were used to determine the mean fluorescence intensity, MFI, enabling the calculation of the Δ MFI as shown in Figure 5.

Neutrophils




Monocytes

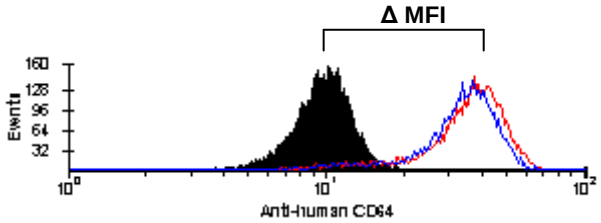



Figure 5. Histograms of CD64 expression on neutrophils and monocytes showing determination of the ΔMFI
Legend:
Black filled in curve: isotype control
Blue line: CD64 fluorescence after pre-incubation with Beriglobin
Red line: CD64 expression without pre-incubation

### 2.3.6 Statistical Analysis

The FACS data files were analysed using FCS-Express (DeNovo Software, Ontario Canada). Statistical analysis was done using GraphPad Prism software (GraphPad Software, Inc. CA, USA).

In comparing of the expression of CD64 on monocytes and neutrophils in patients with infections, patients with SLE, patients with SLE and infections and normal healthy subjects, the Mann-Whitney test was used. $P<0.05$ was considered to be statistically significant.

## 2.4 Analysis of the Expression of FcγRIIa (CD32a) on B cells

### 2.4.1 Samples and Patient Characteristics

In order to investigate the expression of the activating FcγRIIa (CD32a) on B cell subsets, whole blood was drawn in citrate from patients with SLE, from patients with infections and from NHS. The expression of CD32a on B cell subsets was examined using the Ficoll-Paque method in 14 patients with SLE, 8 NHS and 5 patients with infections. Characteristics of these populations are shown in Table 14.

***Table 14: Characteristics of patients with SLE used in the study of CD32a expression on B cell subsets***

| | n | Mean Age | Mean SLEDAI | Mean Leucocyte Count (/µl) | CRP (mg/dl) | % Female |
|---|---|---|---|---|---|---|
| **Patients with SLE** | 14 | 36±13 | 9±7 | NA | NA | 93% |
| **NHS** | 8 | 39±11 | NA | NA | NA | 63% |
| **Patients with Infections** | 5 | 60±16 | NA | 12236±8488 | 9.1±5 | 60% |

NA: not applicable/available

### 2.4.2 Isolation of Cells

For the Ficoll-Paque method, peripheral blood mononuclear cells were prepared using the method described by Boyum (1976).

### 2.4.3 Staining of Cells

The staining of cells for the analysis of CD32a expression on B cell subsets was performed as follows. PBMC were incubated with the monoclonal antibodies listed in Table 15. In order to evaluate the effect of Beriglobin on the binding of the fluorescence marked anti-human CD32a antibodies to their binding sites, which are Fc receptors, samples were processed with and without Beriglobin which binds Fc receptors and is normally used in FACS analysis to reduce non-specific binding of monoclonal

antibodies. The staining was performed using the antibodies shown in Table 15 for 10 minutes at 4° C in the dark. After incubation, the samples were washed with 1 ml of PBS/BSA and centrifuged at 1100 rpm for 5 minutes at 4° C. The pellet was then dissolved in 600 µl PBS. Propidium iodide (1 µg/ml, Sigma, Munich, Germany) was added just before the cytometric analysis to exclude dead cells.

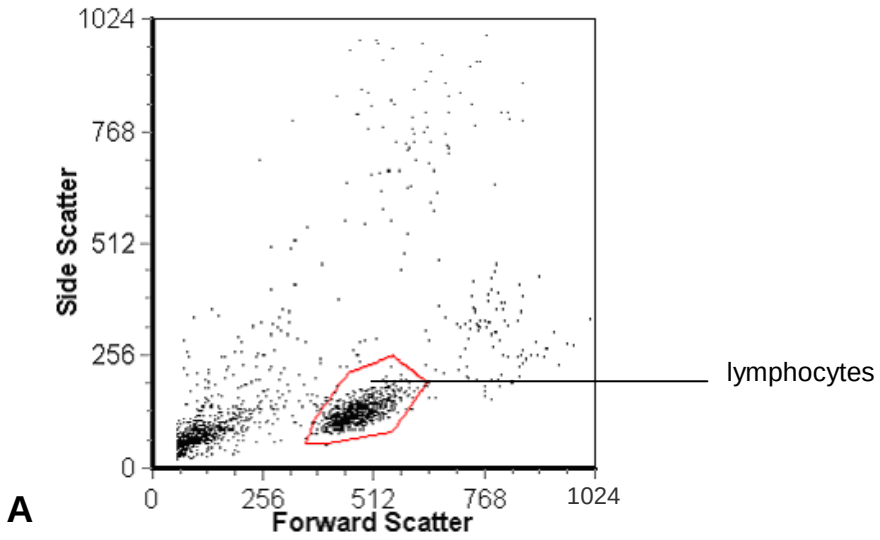
***Table 15: Antibodies used in the analysis of CD32a expression on B cell subsets***

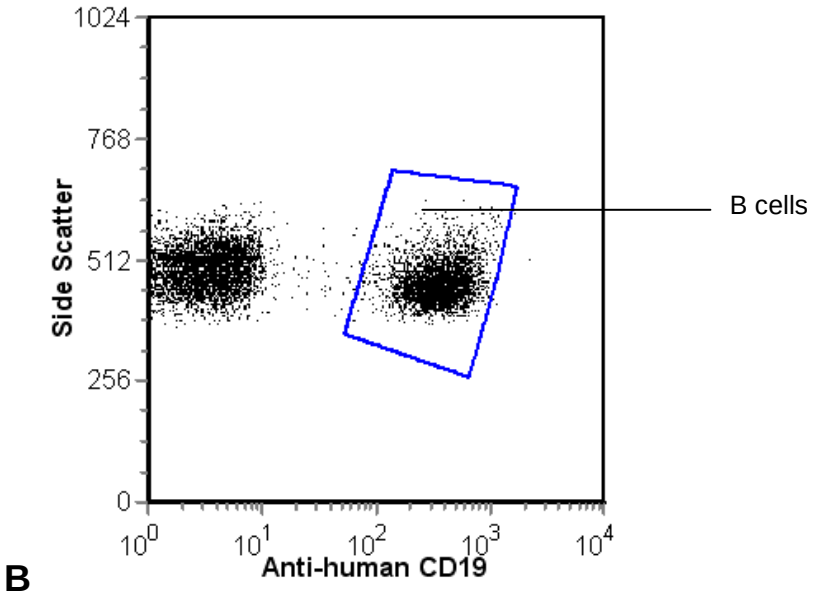
| **Anti-human Antibody** | **Supplier** | **Label** | **Clone** | **Dilution** |
|---|---|---|---|---|
| CD27 | Provided by R. van Lier, Amsterdam | Cy5 | 7H2 | 1:200 |
| CD32a* | ATCC (American Type Culture Collection) | FITC | IV.3 | 1:20 |
| CD19 | DAKO | PE | HD37 | 1:100 |

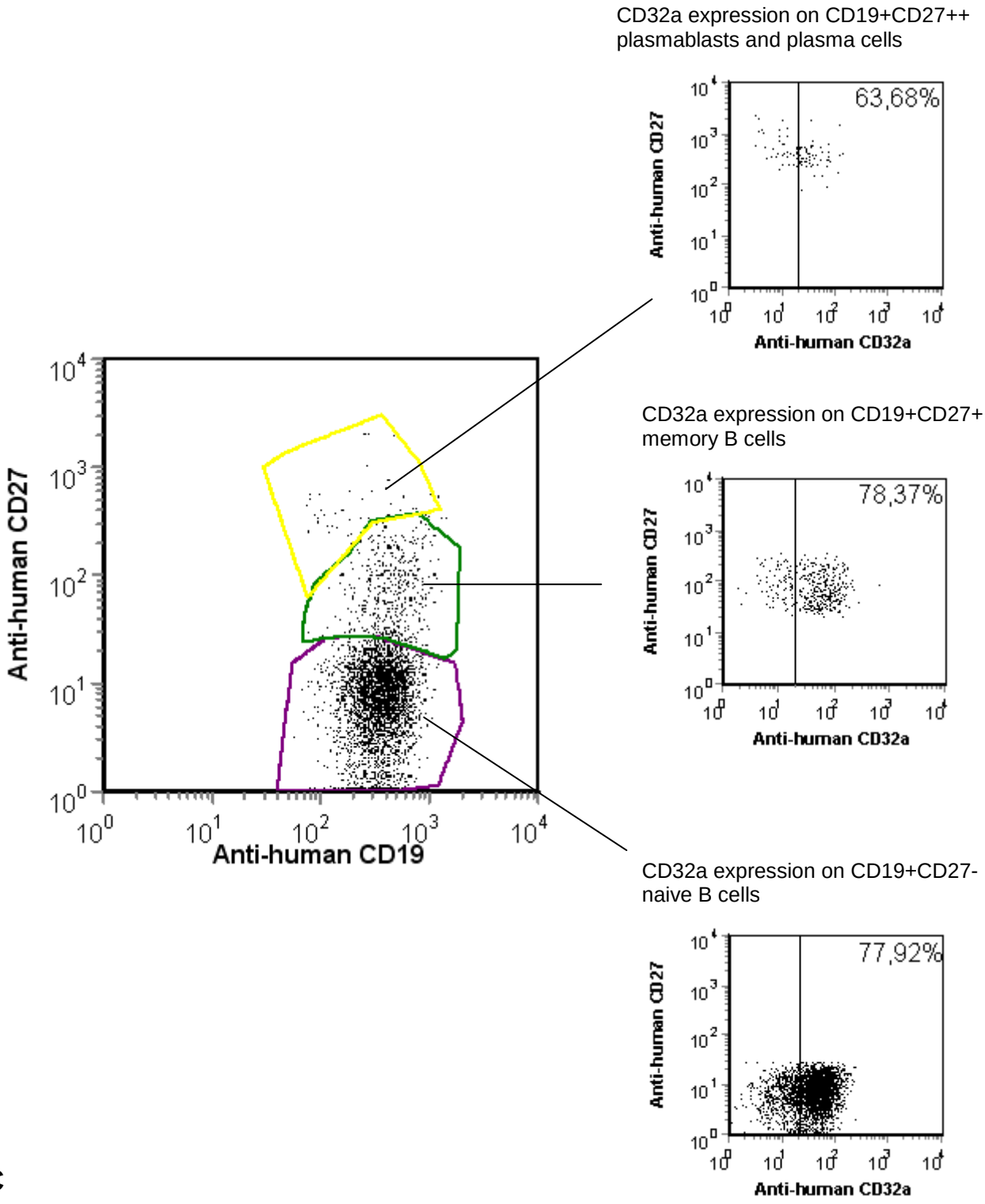
*This antibody shows a very, very strong preferential binding to CD32a, with trace amounts of binding to CD32b (Rabinovitch et al., 2004)

### 2.4.4 Gating Procedure of the CD32a Analysis

A gating procedure was designed in this case, as well, in order to focus on the cells of interest and to determine positive and negative populations for the expression of CD32a. Figure 6 illustrates the gating procedure.






**C**

Figure 6. Gating procedure for CD32a analysis of B cell subsets. **A**: Dotplot of all events with a gate defining lymphocytes. **B**: Dotplot showing lymphocytes as defined in A with a gate defining B cells. **C:** Diagram showing the B cell subsets and CD32a expression on the respective populations.

### 2.4.5 Statistical Analysis

The FACS data files were analysed using FCS-Express (DeNovo Software, Ontario Canada), the statistical analysis was done using GraphPad Prism software (GraphPad Software, Inc. San Diego, CA, USA).

The statistical evaluation of the frequencies of CD32a positive and negative B cells in the B cell subpopulations in patients with SLE compared to normal healthy subjects was done using the Mann-Whitney test. Additionally we tested whether the addition of Beriglobin (human immunoglobulin) which also binds Fc receptors affected the binding of the monoclonal antibodies using the Wilcoxon matched pairs signed rank test.

# 3. Results

## 3.1 Cell counts from the TruCount Method in Comparison to the Automated Hematology Analyser or the Dual-platform Method

### 3.1.1 Overview of Cell Count Comparison Studies

Our objective in this analysis was to evaluate the TruCount method of determining B cell subset cell counts in order to evaluate whether the TruCount method is a viable substitute for the Ficoll-Paque method regarding the determination of plasma cell/-blast counts in patients with SLE. In this analysis, we also compared other cell populations beyond B cell subsets because they, in part, play a role in the calculation of the B cell subset cell counts and the comparison of multiple cell populations using both methods is indicative of the likelihood that a single population will yield comparable results in the two systems.

Therefore, we analysed the leucocyte, lymphocyte, neutrophil and monocyte counts in patients with SLE, patients with SLE and acute infections and patients with infections alone in a total of 33 samples. This analysis compared the cell counts attained by the automated hematology system with the TruCount cell counts. Thereafter, building on these cell counts, in particular regarding the lymphocyte counts which are needed for the dual-platform calculation of the B cell subsets in combination with the Ficoll-Paque method, B cell subset counts were compared in 10 patients with SLE.

### 3.1.2 Comparison of Cell Counts Attained with the TruCount Method and the Automated Hematology Analyzer in Patients with SLE, Patients with SLE and Infections, and Patients with Infections

To statistically compare the absolute numbers of cells determined by the TruCount and the automated hematology counter, we calculated the Spearman correlation coefficient, performed the Wilcoxon matched pairs signed rank test, drew regression lines and plotted Bland-Altman diagrams (Figure 7 and 8 and Table 16) The two methods showed a high degree of correlation, with Spearman correlation coefficients, $r_s$, of 0.9464 for the leucocyte counts ($p < 0.0001$), 0.7803 for the lymphocyte counts ($p < 0.0001$), and 0.9205 for the neutrophil counts ($p < 0.0001$). In monocytes the correlation was lower, 0.5371 ($p < 0.0013$). The median leucocyte, lymphocyte, neutrophil and monocyte values were higher when determined by the automated hematology analyzer but the difference was not significant in all cases. In the case of leucocytes and neutrophils, the median values did statistically differ from each other. The median values with range and Wilcoxon matched pairs signed rank test p values are shown in Table 16.

The linear regression lines show the correlation graphically and provide similar coefficient of determination values, $r^2$, to the correlation coefficients $r_s$. The $r_s$ values differ from the square root of the coefficient of determination because the Spearman correlation does not assume a Gaussian correlation while the $r^2$ coefficient of determination of the linear regression does. According to convention, p values are not given for $r^2$ because these are not calculated in GraphPad Prism and would not provide additional information. The p values of $r_s$ answer the question whether the correlation is due to chance. If the p value is small the correlation is not due to random sampling. The $r^2$ values are shown here primarily to quantify the apparent variation in the linear regression diagrams which provide an informative graphical presentation of the data.

The results of the Spearman correlation are therefore, as expected, similar to the coefficients of determination in the linear regression analysis. Overall, the comparison of the two methods resulted in high correlation coefficients, $r^2$, in the linear regression for the leucocyte, lymphocyte, and neutrophil values (0.9300, 0.8043 and 0.9012 respectively). The average $r^2$ for these cell populations was 0.8785 (SD ± 0.0658). The monocyte counts however resulted in an $r^2$ of 0.3689.

***Table 16:** Median values with range, Wilcoxon matched pairs signed rank test and Spearman correlation coefficients for the comparison of the two methods in patients with SLE, patients with SLE and infections and patients with acute Infections **

| | **Median and Range (/µl)** | | | |
|---|---|---|---|---|
| | **Automated Hematology** | **TruCount** | **Wilcoxon p value **** | **Spearman Correlation Coefficient** |
| **Leucocytes** | 7050 (860 - 25880) | 5913 (621-21261) | $p < 0.0001$ | $r_s = 0.9464$ $p < 0.0001$ |
| **Lymphocytes** | 860 (331 - 3051) | 821 (228 – 2751) | $p = 0.9096$ | $r_s = 0.7803$ $p < 0.0001$ |
| **Neutrophils** | 5100 (800 - 21800) | 3758 (823 – 18727) | $p < 0.0001$ | $r_s = 0.9205$ $p < 0.0001$ |
| **Monocytes** | 310 (37 – 1910) | 306 (77 – 980) | $p = 0.1857$ | $r_s = 0.5371$ $p < 0.0013$ |

*cell counts determined by the TruCount procedure and the automated hematology analyzer, n= 33 for leucocytes, n= 29 for lymphocytes, neutrophils and monocytes

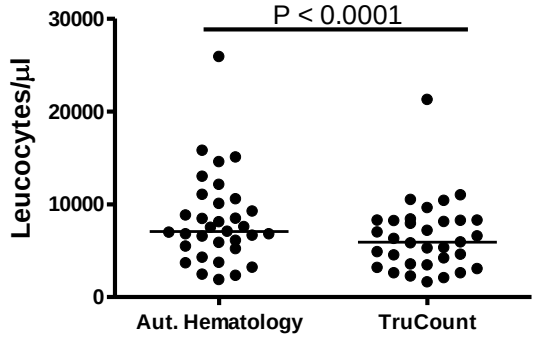
** Wilcoxon matched pairs signed rank test

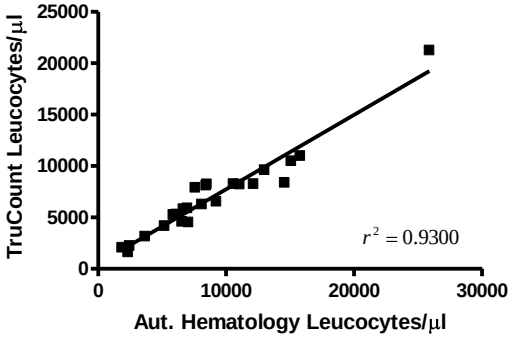
Bland-Altman plots provide an additional method of comparative analysis. Here a graphical presentation of the relationship between the values goes beyond the linear regression in that the difference between the values obtained by the two systems is placed in the context of the absolute values, allowing an analysis of whether higher counts diverge more in the two methods than lower counts, in addition to the calculation of the bias. The leucocyte and neutrophil populations showed a strong bias. The difference in the values obtained from the two systems as depicted on the Bland-Altman plots for these two polulations shows a relationship between the absolute cell count and the discrepancies in the counts from the two systems. When the cell counts are higher, the difference between the two systems is larger.

The bias for leucocytes was 1789.52/µl with a 95% confidence interval of -1538 to 5117.38. The neutrophils had a bias of 1692.45/µl and a 95% confidence interval of 1140.64 to 4525.54. The monocytes, which showed the lowest coefficient of

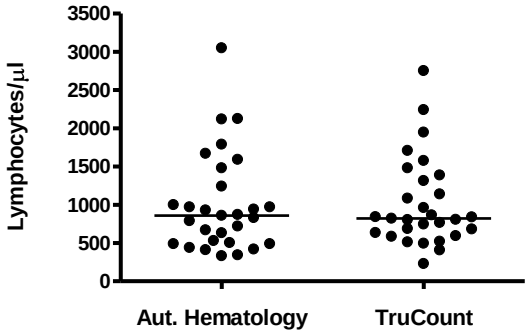
determination in the linear regression as mentioned above, also had a unique finding in the Bland-Altman plots. Here, compared to the other populations there was the strongest relationship between number of monocytes counted and the difference between the values from the two methods. When the absolute value increased, the difference also increased. Here the bias is 188.79/µl with a 95% confidence interval of -697.67 to 1075.27. An overview of the bias values is shown in Table 17.

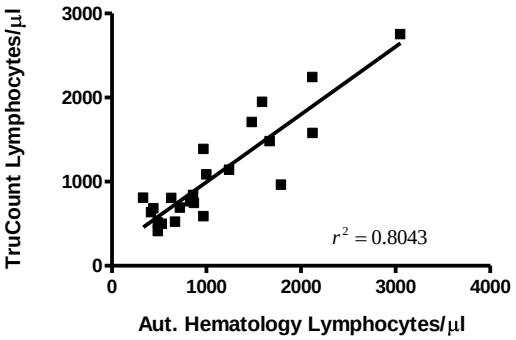
**Leucocytes**



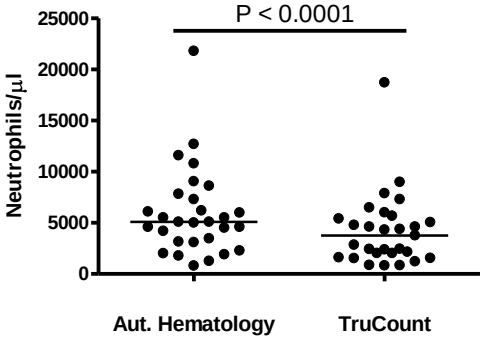



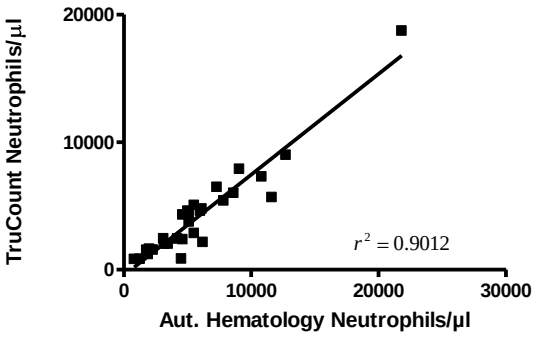

**Lymphocytes**




**Neutrophils**



## Monocytes

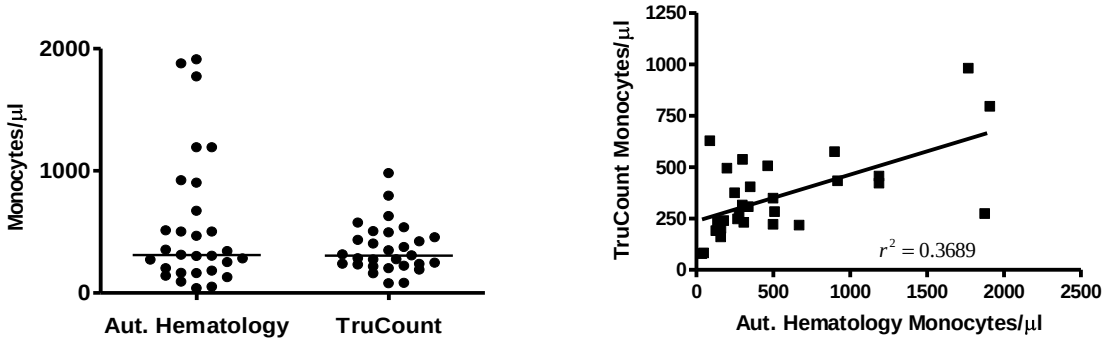
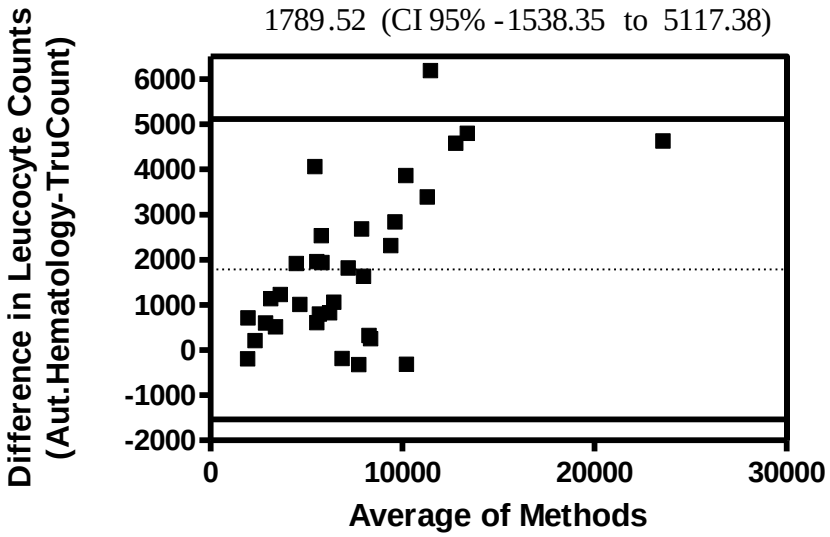
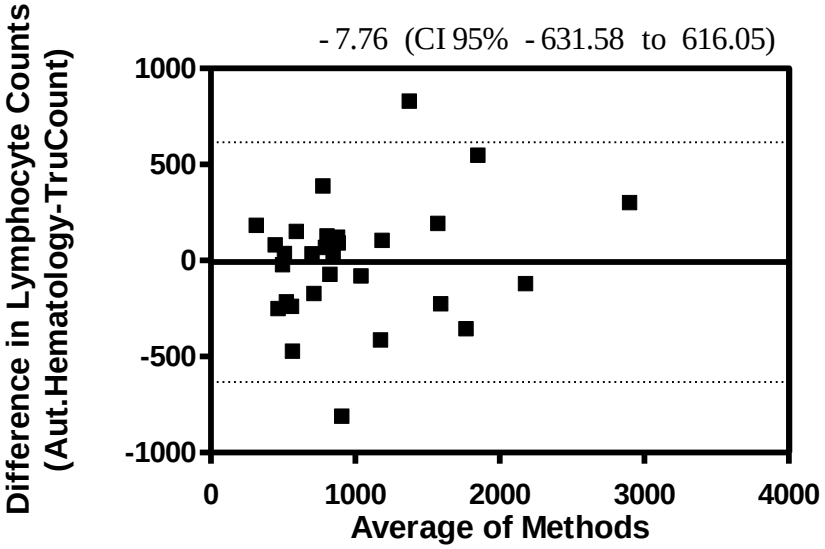



Figure 7. Scatter plots with medians and linear regression of the comparison of cell counts determined by the TruCount procedure and the automated hematology analyzer (referred to here as "Aut. Hematology") for the various cell populations studied in pooled samples from patients with SLE, patients with SLE and infections and patients with infections (n= 33 for leucocytes, n = 29 for lymphocytes, neutrophils and monocytes). p values of < 0.05 indicate a statistical difference between the paired samples as determined by the Wilcoxon matched pairs signed ranks test and are shown. The coefficient of determination,$r^2$ ,indicates, when expressed as a percent, to what percent the variation in the X axis can be explained by variation in the Y axis and vice versa.

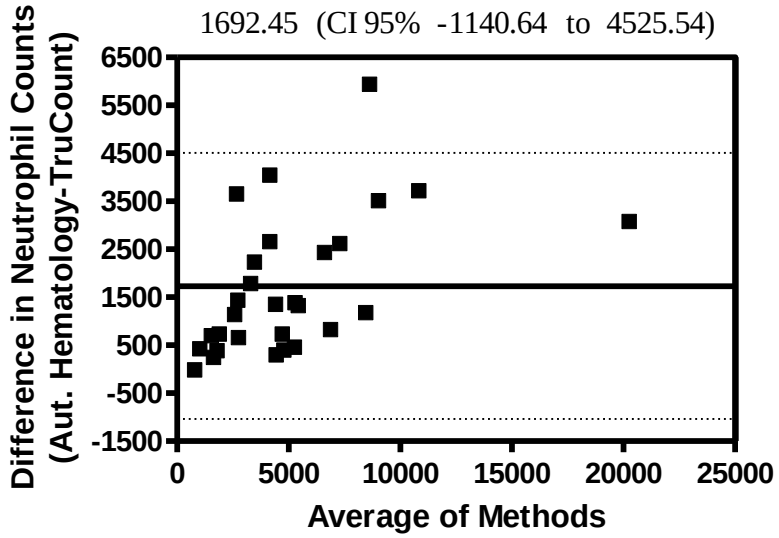
## Leucocytes




## Lymphocytes



**Neutrophils**




**Monocytes**

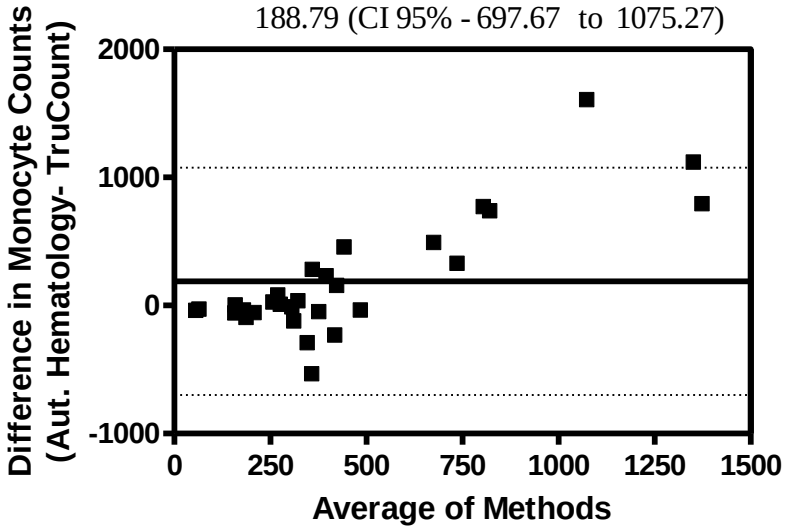



Figure 8. Bland-Altman Bias plots of the comparison of cell counts determined by the TruCount procedure and the automated hematology analyzer (referred to here as “Aut. Hematology”) for the various cell populations studied in pooled samples from patients with SLE, patients with SLE and infections and patients with infections (n= 33 for leucocytes, n = 29 for lymphocytes, neutrophils and monocytes). The plots graphically display the difference between the two systems as a function on their average and additionally this analysis gives a value for the bias (shown above each graph) between the two systems. Cell counts are per microliter.

***Table 17: Bias and 95% confidence intervals of the cell counts determined by the TruCount procedure and the automated hematology analyzer****

| Population | Bias and 95% Confidence Interval |
|---|---|
| Leucocytes | 1789.52 (CI95% -1538.35 to 5117.38) |
| Lymphocytes | -7.76 (CI95% -631.58 to 616.05) |
| Neutrophils | 1692.45 (CI95% -1140.64 to 4525.54) |
| Monocytes | 188.79 (CI95% -697.67 to 1075.27) |

* anaylzed in pooled samples from patients with SLE, patients with SLE and infections and patients with infections, n= 33 for leucocytes, n = 29 for lymphocytes, neutrophils and monocytes

### 3.1.3 Comparison of Cell Counts Attained with the Automated Hematology Analyzer or the Ficoll-Paque Density Gradient Centrifugation Procedure as a Dual-platform for B cell Subsets and the TruCount Method in Patients with SLE

#### 3.1.3.1 Comparison of Trucount and Automated Hematology Analyzer data

To compare the absolute numbers of cells determined by the TruCount method and automated hematology counter in patients with SLE, we again calculated the Spearman correlation coefficient, performed the Wilcoxon matched pairs signed rank test to test for similarity, drew regression lines and plotted Bland-Altman diagrams as shown in Figure 9 and Table 18. The two methods showed a high degree of correlation for the leucocyte, lymphocyte and neutrophil counts, with Spearman correlation coefficients of 0.9197 ($p < 0.0001$) for the leucocyte counts, 0.9341 ($p < 0.0001$) for the lymphocyte counts, 0.9449 ($p < 0.0001$) for the neutrophil counts. For monocytes the Spearman correlation coefficients was 0.2484 ($p = 0.1960$). The median leucocyte, neutrophil and monocyte values were higher when determined by the automated hematology analyzer, the median lymphocyte count was slightly higher in the TruCount system. In this sample of patients with SLE, the Wilcoxon matched pairs signed ranks test again showed that the leucocyte and neutrophil populations are significantly different. See Table 18 for more details.

***Table 18: Median values with range, Wilcoxon matched pairs signed rank test and Spearman correlation coefficients for the comparison of the two methods in patients with SLE****

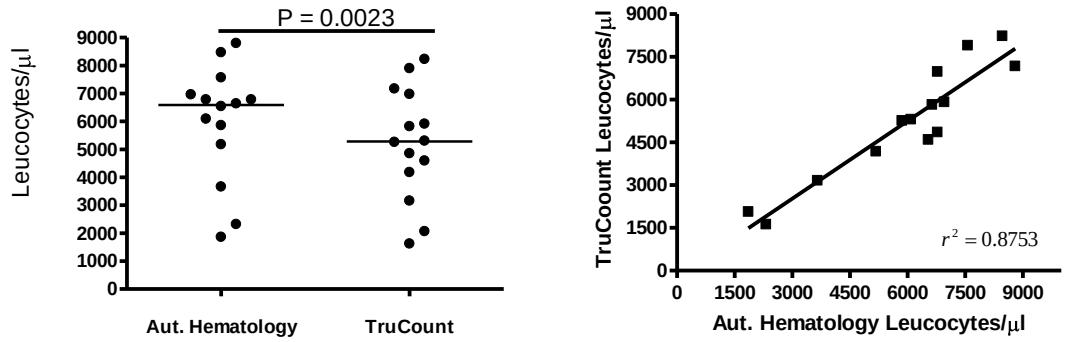
| | **Median and Range (/µl)** | | | |
|---|---|---|---|---|
| | **Automated Hematology** | **TruCount** | **Wilcoxon p value **** | **Spearman Correlation Coefficient** |
| **Leucocytes** | 6590 (1860 - 8800) | 5285 (1621 - 8231) | p = 0.0023 | $r_s$ = 0.9197<br>p < 0.0001 |
| **Lymphocytes** | 915 (417 - 3050) | 960 (408 - 2751) | p = 0.5016 | $r_s$ = 0.9341<br>p < 0.0001 |
| **Neutrophils** | 4850 (800 - 7832) | 3304 (823 – 6481) | p = 0.0002 | $r_s$ = 0.9449<br>p < 0.0001 |
| **Monocytes** | 295 (139 – 670) | 278 (158 – 504) | p = 0.9032 | $r_s$ = 0.2484<br>p = 0.1960 |

*cell counts determined by the TruCount procedure and the automated haematology analyzer, n = 14

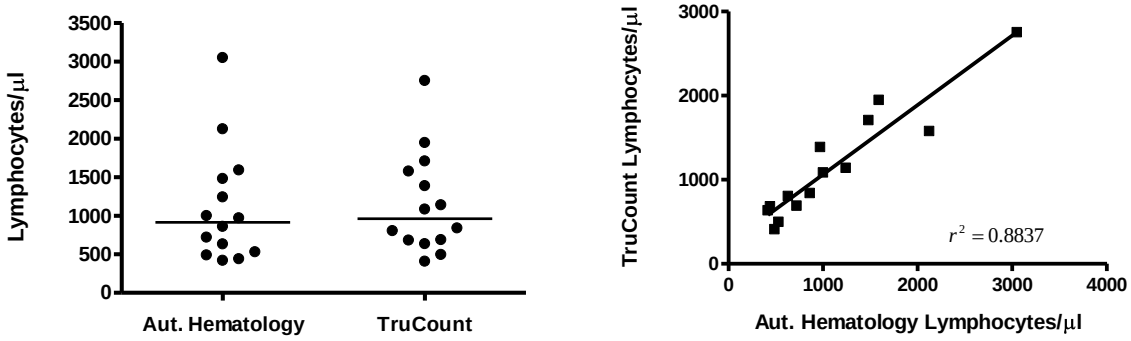
** Wilcoxen matched pairs signed rank test

As shown in Figure 9, the SLE samples alone yielded similar regression analysis results to those of the combined sample populations. The coefficient of determination, $r^2$, again was high, with an average of 0.8769 SD ± 0.0062 for the leucocyte, lymphocyte and neutrophil populations. Specifically, the $r^2$ values are: 0.8753 for leucocytes, 0.8837 for lymphocytes, and 0.8716 for neutrophils. The comparison of monocytes values ($r^2$ = 0.0160) was lower than when the combined samples were considered.

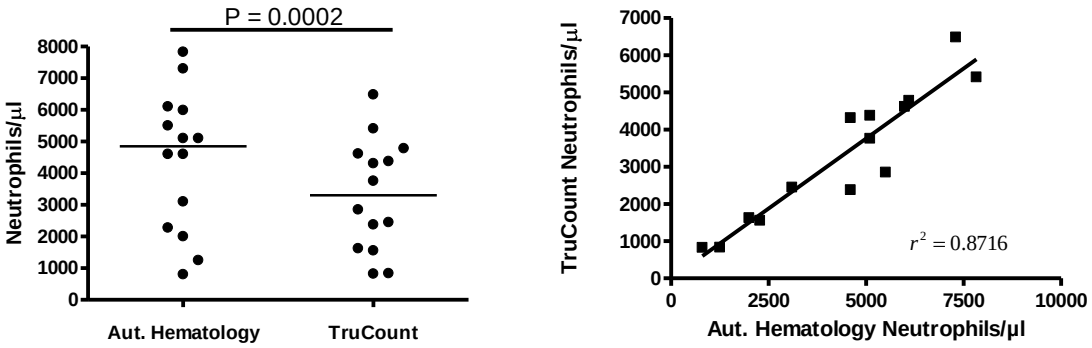
**Leucocytes**



## Lymphocytes




## Neutrophils




## Monocytes

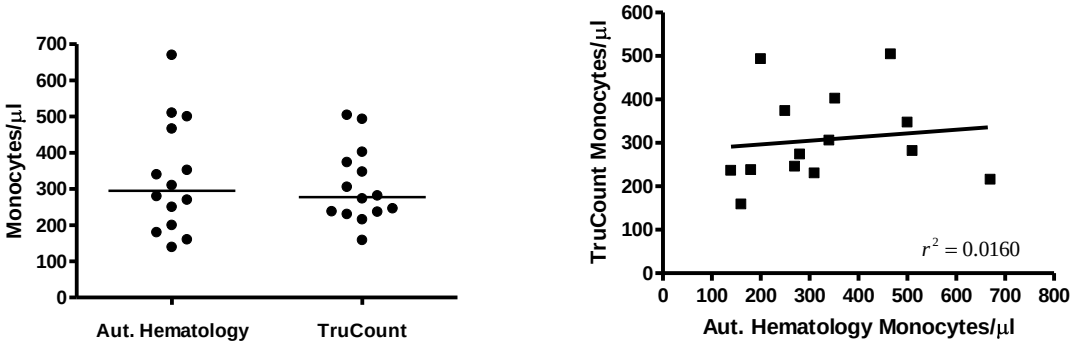
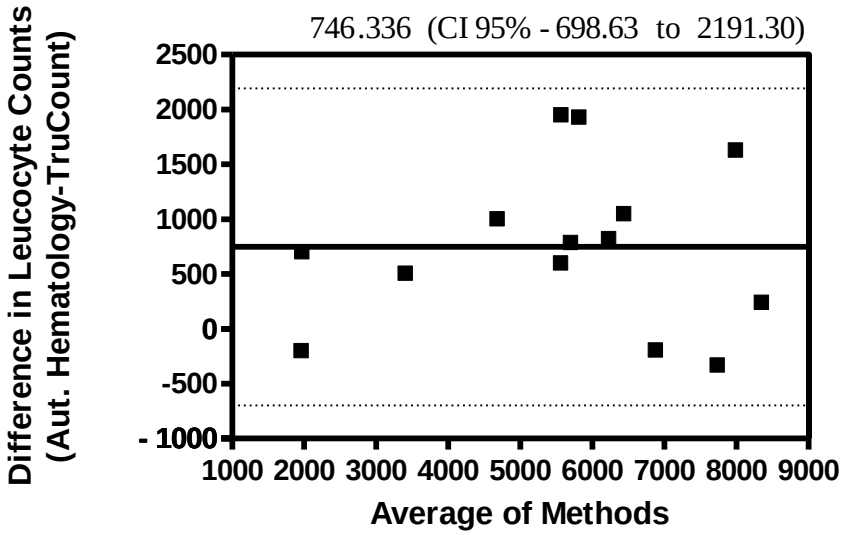


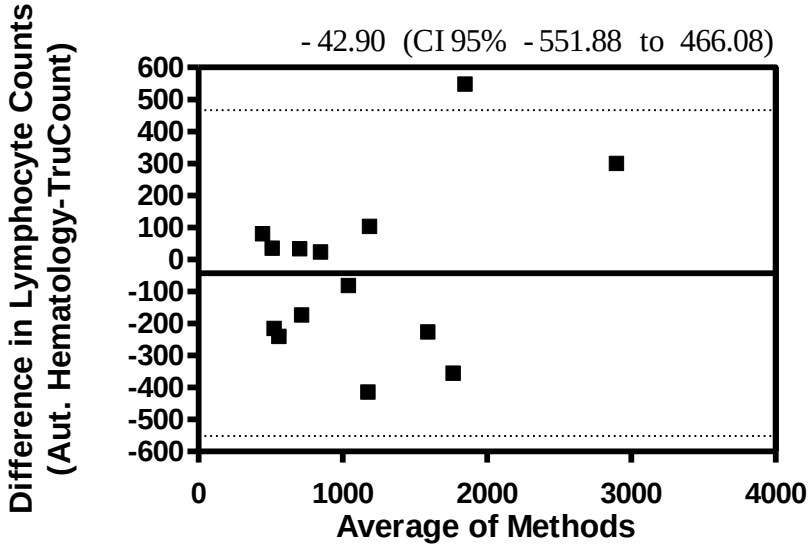

Figure 9. Scatter plots with median and linear regression of the comparison of cell counts determined by the TruCount procedure and the automated hematology analyzer (referred to here as "Aut. Hematology") for the various cell populations in patients with SLE (n = 14). P < 0.05 indicates statistical significance and are shown. The Wilcoxon matched pairs signed ranks test tests for significant differences between populations. p < 0.05 indicates statistical significance and in cases of statistical significance the p values are shown in the scatter plots. See Table 16 for details. The $r^2$ values indicate, when expressed as a percent, to what percent the variation in the X axis can be explained by variation in the Y axis and vice versa.

The Bland-Altman plots (Figure 10 and Table 19) showed the greatest correlation between the absolute cell counts and the differences in the cell counts from the two systems for the monocytes, which again show larger differences with higher cell numbers.

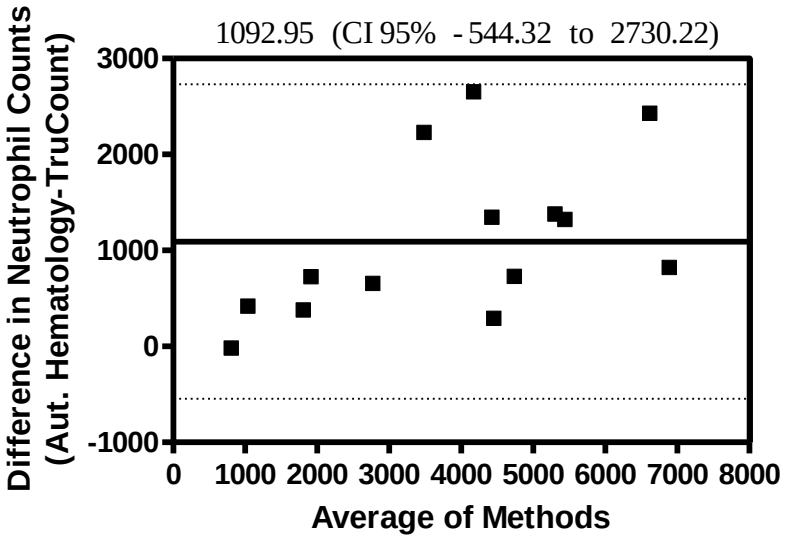
**Leucocytes**




**Lymphocytes**



**Neutrophils**




**Monocytes**

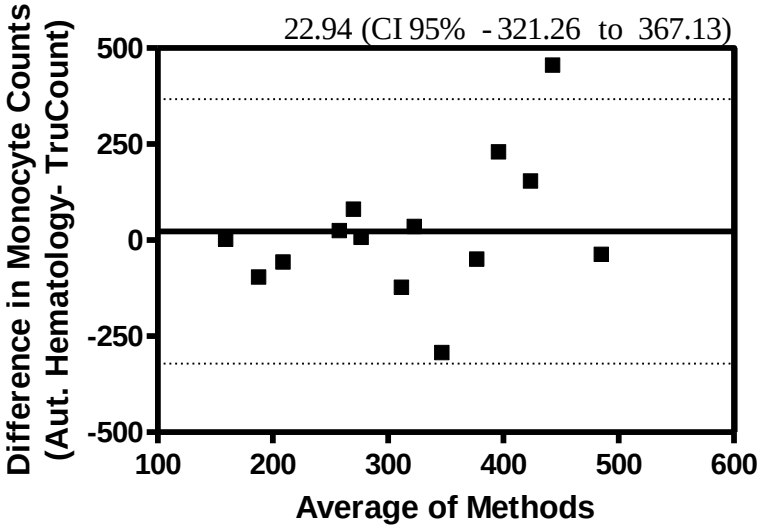



Figure 10. Bland-Altman Bias plots of the comparison of cell counts determined by the TruCount procedure and the automated hematology analyzer (referred to here as “Aut. Hematology”) for the various cell populations in patients with SLE (n = 14). The plots graphically display the difference between the two systems as a function on their average and additionally this analysis gives a value for the bias (shown above each graph) between the two systems. Cell counts are per microliter.

***Table 19: Bias and 95% confidence intervals of the cell counts determined by the TruCount procedure and the automated hematology analyzer in patients with SLE****

| Population | Bias and 95% Confidence Interval |
|---|---|
| Leucocytes | 746.336 (CI 95% - 698.63 to 2191.30) |
| Lymphocytes | - 42.90 (CI 95% - 551.88 to 466.08) |
| Neutrophils | 1092.95 (CI 95% - 544.32 to 2730.22) |
| Monocytes | 22.94 (CI 95% - 321.26 to 367.13) |

*n = 14

### 3.1.3.2 Comparison of Absolute Numbers of B Cells and B Cell Subpopulations in Patients with SLE using the TruCount with Dual-platform Methods

To compare the absolute numbers of the B cell subsets in patients with SLE determined by the TruCount and dual-platform method (the combined system using percentages of B cells and their subsets from FACS analysis and lymphocyte counts from automated hematology), we carried out a subpopulation analysis. The two methods showed a high degree of correlation with Spearman correlation coefficients of 0.8788 ($p = 0.0004$) for the total B cell counts, 0.8903 ($p = 0.0003$) for the naive B cell counts, 0.9273 ($p < 0.0001$) for the memory B cell counts and 0.7818 ($p = 0.0038$) for the plasma cell/-blast counts. The medians of the total B cell counts and the B cell subpopulations did not differ from each other significantly according to the Wilcoxon signed ranks matched paired test. See Table 20 for more details

As shown in Figure 11, in patients with SLE, high coefficients of determination were found for the B cells and the B cell subset counts when using the TruCount and the dual-platform systems with a $r^2$ of 0.9378 for the B cells and an average $r^2$ for the B cell subsets of 0.9142 SD ± 0.0579. Specifically the $r^2$ values of the B cell subsets are, 0.9154 for CD19+CD20+CD27- naive B cells, 0.9716 for the CD19+CD20+CD27+ memory B cells, and 0.8557 for the CD19+CD20-CD27++ plasma cells/-blasts.

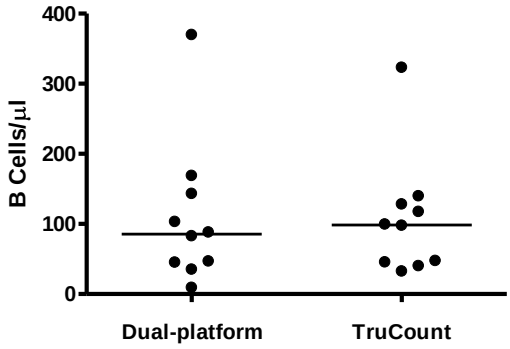
***Table 20: Median values with range, Wilcoxon matched pairs signed rank test and Spearman correlation coefficients for the comparison of the two methods in patients with SLE****

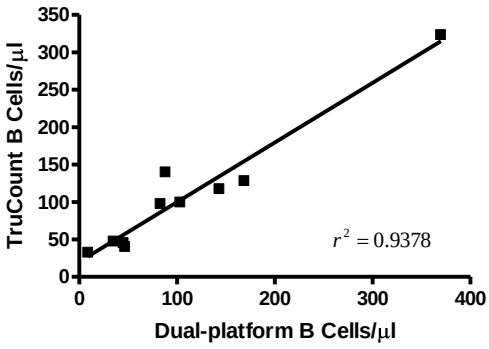
| | **Median and Range (/µl)** | | | |
|---|---|---|---|---|
| | **Dual-platform** | **TruCount** | **Wilcoxon p value **** | **Spearman Correlation Coefficient** |
| **Total B cells** | 86 (9 – 370) | 99 (32 -323) | p = 0.9219 | $r_s$ = 0.8788 p = 0.0004 |
| **Naive B cells** | 72 (6 – 278) | 79 (24 – 240) | p = 0.6953 | $r_s$ = 0.8903 p = 0.0003 |
| **Memory B cells** | 12 (3 – 85) | 12 (4 – 70) | p = 0.2245 | $r_s$ = 0.9273 p < 0.0001 |
| **Plasma Cells/ -blasts** | 6 (0.5 – 22) | 3 (2 – 14) | p = 0.0840 | $r_s$ = 0.7818 p = 0.0038 |

*cell counts determined by the TruCount procedure and the dual-platform method, n = 10

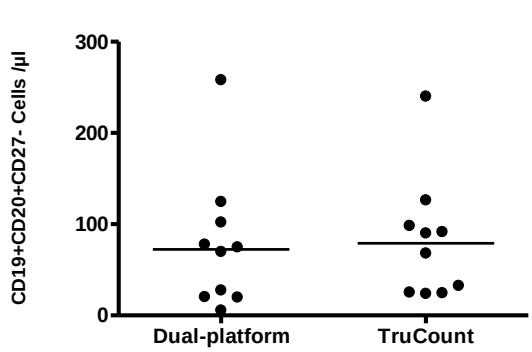
** Wilcoxon matched pairs signed rank test

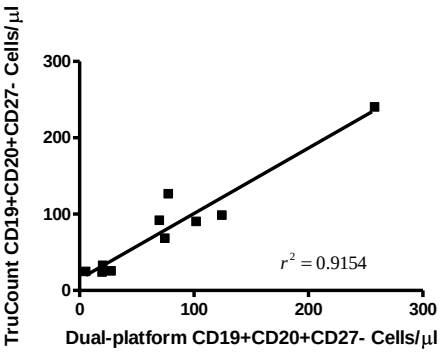
**Total B cells**



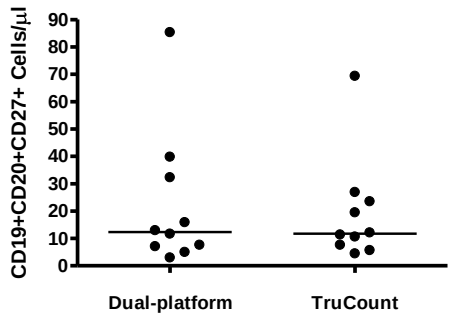



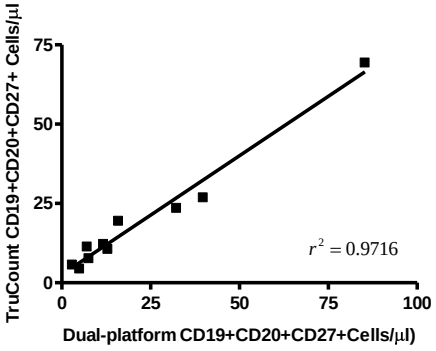
## Naive B cells



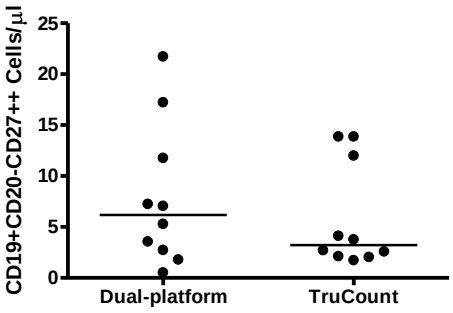




## Memory B cells




## Plasma Cells/-blasts



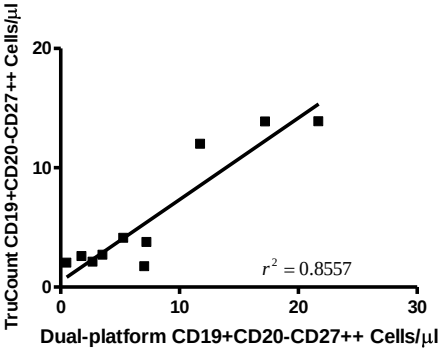
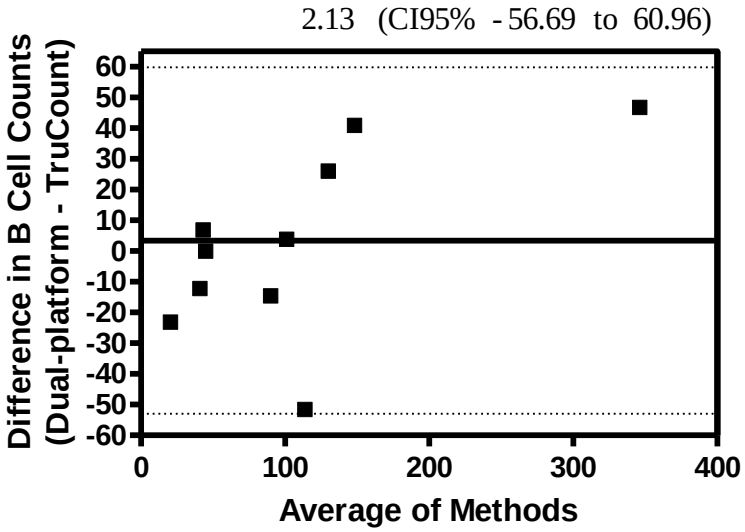



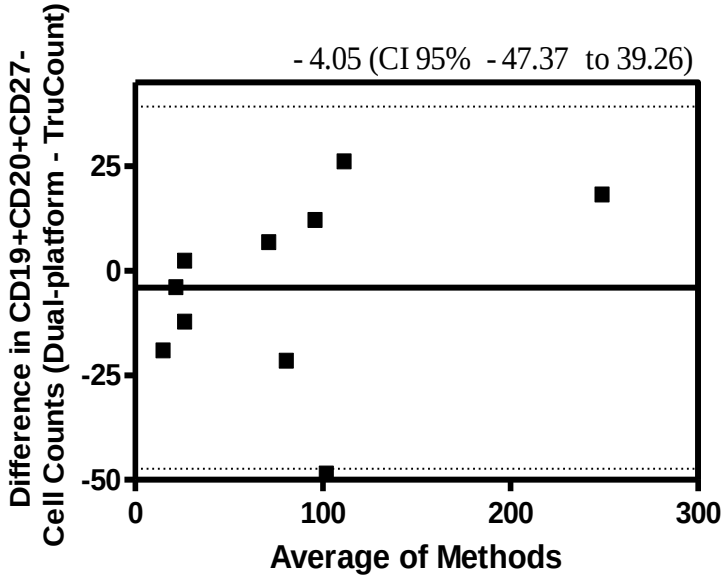

Figure 11. Scatter plots with medians and linear regression of the comparison of results determined by the Trucount and dual-platform procedure for the various B cell populations studied in patients with SLE (n = 10). The Wilcoxon signed ranks matched pairs test did not result in any significant differences between the populations. p values are given in Table 20. The $r^2$ values indicate, when expressed as a percent, to what percent the variation in the X axis can be explained by variation in the Y axis and vice

The Bland-Altman plots (Figure 12 and Table 21) show the highest correlation between variation and absolute cell count for the CD20+CD27- naive B cells and the CD20+CD27+ memory B cells.

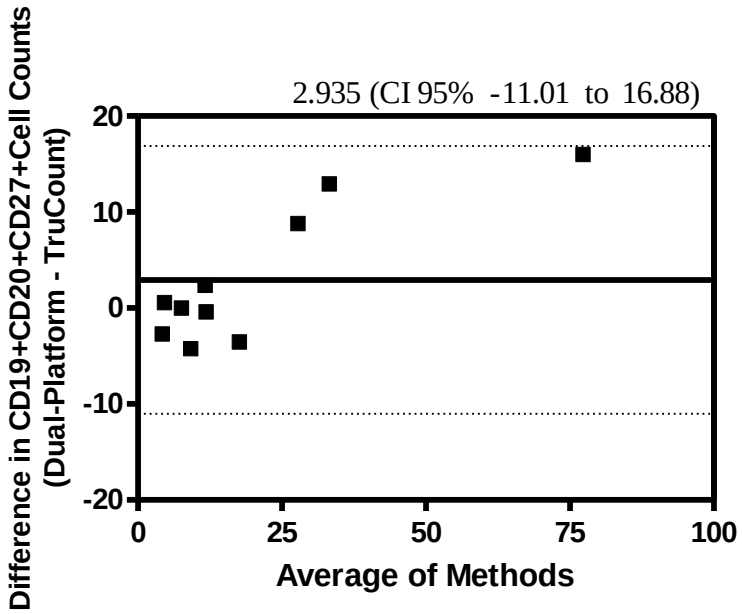
**Total B cells**




**Naive B cells**



**Memory B cells**




**Plasma Cells/-blasts**

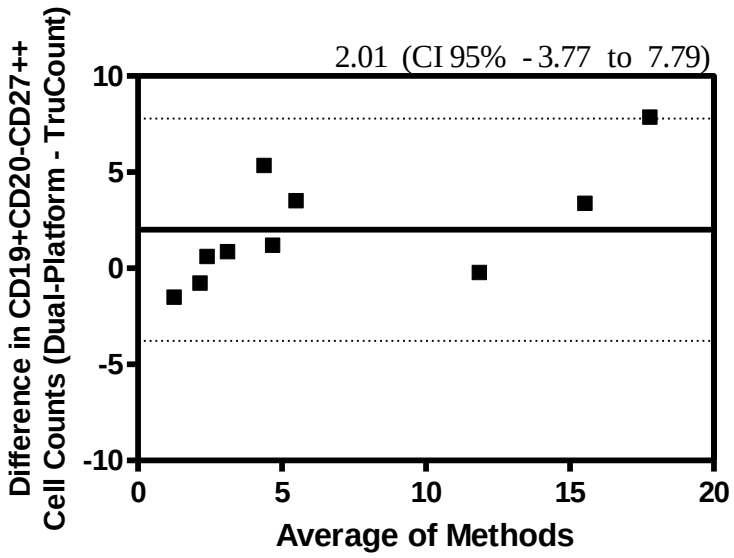



Figure 12. Bland-Altman Bias plots of the comparison of cell counts determined by the TruCount procedure and the automated hematology analyzer (referred to here as “Aut. Hematology”) for the B cells and the B cell subpopulations in patients with SLE (n = 10). The plots graphically display the difference between the two systems as a function on their average and additionally this analysis gives a value for the bias (shown above each graph) between the two systems. Cell counts are per microliter.

***Table 21: Bias and 95% confidence intervals of the cell counts determined by the TruCount procedure and the dual-platform system in patients with SLE****

| Population | Bias and 95% Confidence Interval |
|---|---|
| **Total B cells** | 2.13 (CI95% -56.69 to 60.96) |
| **Naive B cells** | -4.05 (CI 95% -47.37 to 39.26) |
| **Memory B cells** | 2.935 (CI 95% -11.01 to 16.88) |
| **Plasma Cells/-blasts** | 2.01 (CI 95% -3.77 to 7.79) |

* n = 10

## 3.2 FcγRI (CD64) Expression on Neutrophils and Monocytes

CD64 expression on monocytes and neutrophils was studied in 24 different subjects using an isotype control. This sample includes 7 patients with SLE, 5 patients with SLE and infection, 6 patients with acute infections and 6 NHS. Figure 13 and Table 22 show the results for CD64 expression on neutrophils and monocytes without pre-incubation with Beriglobin. Since the use of Beriglobin did not alter the differences in the MFI, the findings without Beriglobin were used for further analysis and will be discussed here. The expression of CD64 on neutrophils of patients with SLE and infections differed significantly from the expression in patients with SLE alone ($p = 0.0051$) when tested with the Mann-Whitney test (Table 22). This difference occurred although the patients with SLE analysed have high disease activity scores as shown in Table 10. Furthermore, a difference is seen in the expression of CD64 when comparing patients with infections and normal healthy subjects ($p = 0.0087$). The expression of CD64 on monocytes also demonstrated a significant upregulation of CD64 in patients with infections compared to normal healthy subjects ($p = 0.0043$) but not in the comparison between SLE patients with and without infections ($p = 0.3434$). When comparing patients with SLE and NHS, the expression of CD64 on the neutrophils and monocytes did not differ from each other significantly in any case ($p = 0.2949$ and $0.2343$ respectively).

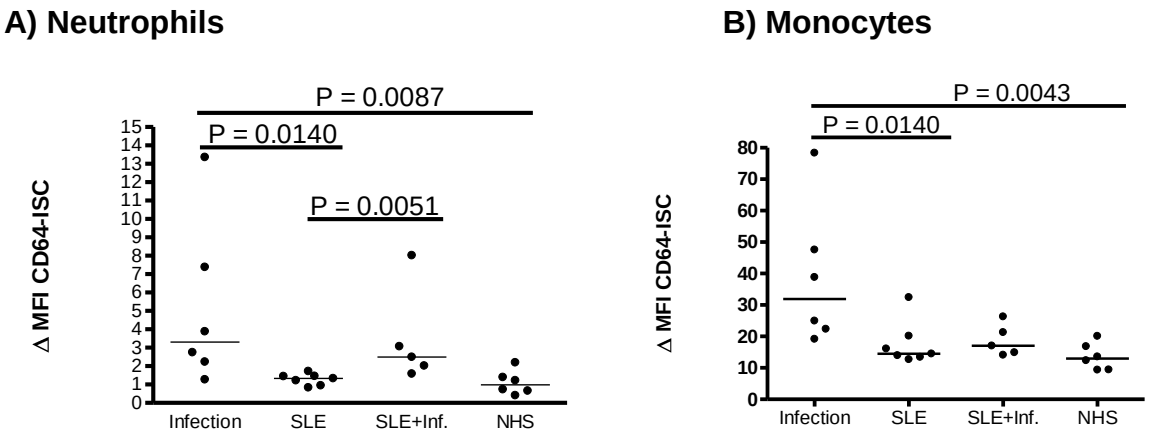



Figure 13. ΔCD64 MFI without pre-incubation with Beriglobulin in monocytes and neutrophils. Medians and significant p values according to the Mann-Whitney test are shown.

***Table 22: Statistical difference (p values) of the Mann-Whitney test comparing the Δ CD64 MFI in distinct cell populations****

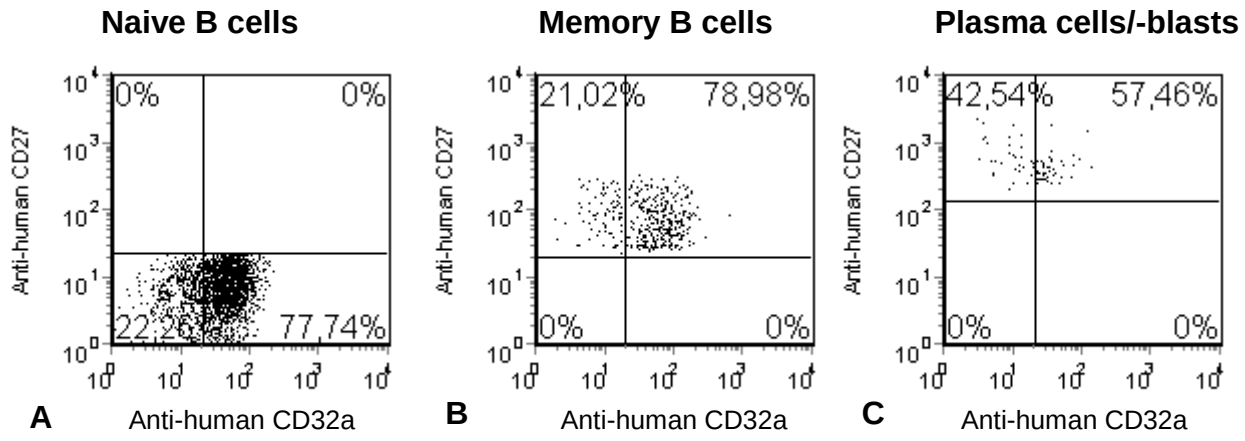
| Sample population compared | Neutrophils | Monocytes |
|---|---|---|
| **SLE versus SLE+Inf.** | **0.0051** | 0.3434 |
| **NHS versus Infection** | **0.0087** | **0.0043** |
| **SLE versus NHS** | 0.2949 | 0.2343 |
| **SLE versus Infection** | **0.0140** | **0.0140** |

*Bold type designates statistical significance

## 3.3 FCγRIIa (CD32a) Expression on B Cells

The third research objective of this study was to investigate the expression of FcγRIIa (CD32a) on B cells of patients with SLE because alterations in the expression of this receptor may in part explain the B cell hyperactivity and immunoglobulin production against self-antigens found in patients with SLE and may also be a distinguishing marker of acute infection as a function of its level of expression. The uniquely human Fcγ receptor FcγRIIa has not been studied in as much detail as the inhibitory Fcγ receptor IIb and its expression on human B cells has been disputed (Ravetch et al., 1991; Takai, 2002; Hogarth, 2002; Rabinovitch et al., 2004; Su et al., 2007). We performed this analysis in 14 patients with SLE, 8 NHS and 5 patients with infections. A difference was found in the frequency of FcγRIIa in CD27+ memory and CD27++ plasmablasts and plasma cells of patients with SLE compared to NHS as shown descriptively in Figure 14. The frequency of FcγRIIa+ cells was lower in patients with SLE or infections compared to NHS for all the subpopulations, whereby the frequency of FcγRIIa expression among CD27++ plamablasts and plasma cells was significantly lower in patients with SLE and patients with infections compared to NHS (Figure 15). Because the median values are of interest, beyond the significant differences, for the analysis of similarities and differences in the frequency of FcγRIIa+ cells in the various cell populations in the various groups, the median values and ranges are shown in Table 23. Analysis of the median values reveals that the median frequency of FcγRIIa positive cells in SLE and infection are very similar in CD27+ memory cells, 63.4% and 65.7% respectively, although a significant difference compared to the expression in NHS, 80.9%, was not attained.

**NHS**




**Patient with SLE**

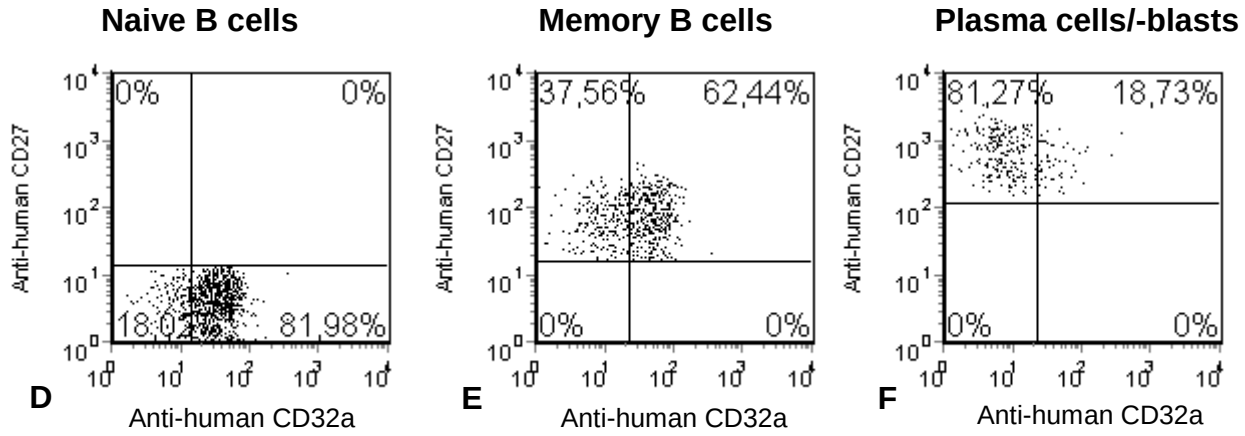



Figure 14. Dotplots showing the frequency of CD32a (FcγRIIa) expression on B cell subsets in a NHS (A-C), and in a patient with SLE (D-F) **A**: CD32a expression of CD19+CD27- naive B cells of a NHS **B**: CD32a expression of CD19+CD27+ memory B cells of a NHS **C**: CD32a expression of CD19+CD27++ plasmablasts and plasma cells of a NHS **D**: CD32a expression of CD19+CD27- naive B cells of a patients with SLE **E**: CD32a expression of CD19+CD27+ memory B cells of a patient with SLE **F**: CD32a expression of CD19+CD27++ plasmablasts and plasma cells of a patients with SLE showing a representative decrease in CD19+CD27++CD32a+ cells compared to NHS

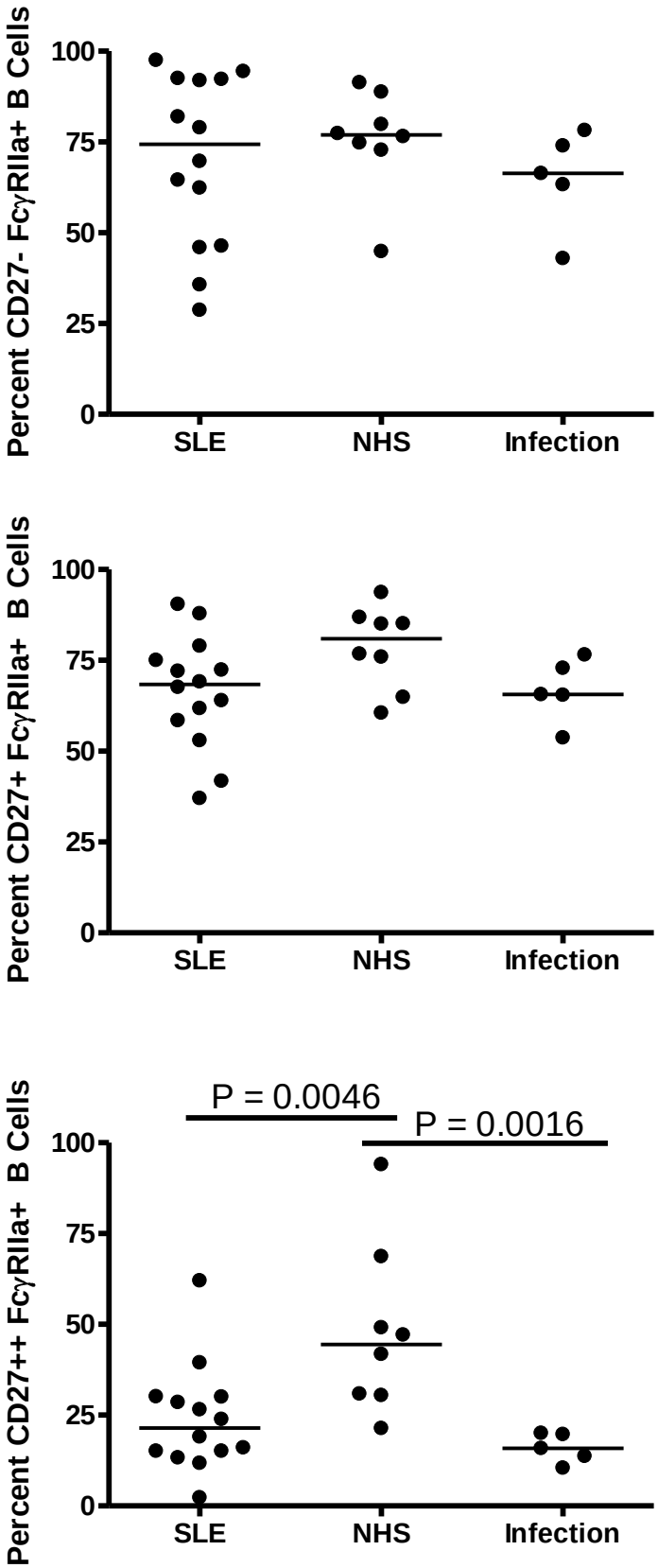



Figure 15. Scatter plots of frequencies FcγRIIa positive cells in the B cells subsets showing a significantly lower frequency of CD19+CD27++ FcγRIIa+ plasmablasts and plasma cells in patients with SLE and patients with infections compared to NHS. Medians and significant p values according to the Mann-Whitney test are shown.

***Table 23: Medians and range for the expression of CD32a (FcγRIIa) on the B cell subsets***

| | **Naive CD19+ CD27- B cells** | | |
|---|---|---|---|
| | SLE (n=14) | NHS (n=8) | Infection (n=5) |
| **Median** | 74.4 | 77.0 | 53.2 |
| **Range** | 29-98 | 45-91 | 43-78 |

| | **Memory CD19+ CD27+ B cells** | | |
|---|---|---|---|
| | SLE | NHS | Infection |
| **Median** | 63.4 | 80.9 | 65.7 |
| **Range** | 37-90 | 61-94 | 54-77 |

| | **Plasma CD19+ CD27++ cells/-blasts** | | |
|---|---|---|---|
| | SLE | NHS | Infection |
| **Median** | 21.4 | 44.5 | 15.9 |
| **Range** | 2-62 | 21-94 | 10-20 |

Beriglobin is commonly added during cell staining for FACS analysis in order to reduce non-specific binding of monoclonal antibodies to Fc receptors. However, in the case where the surface structure of interest is an Fc receptor, the use of Beriglobin is particularly interesting and debated. Therefore, we analysed the fluorescent expression of the monoclonal antibodies with and without the use of Beriglobin in 6 samples. There were no significant differences in the expression (Table 24).

***Table 24: Medians with range and p values from the comparison of frequencies of CD32a+ cells with and without pre-incubation with Beriglobin****

| | **Median without Beriblobin** | **Median with Beriglobin** | **p value**** |
|---|---|---|---|
| **Naive B cells** | 76 (73 - 89) | 62 (55 – 70) | 0.0625 |
| **Memory B cells** | 79 (61 – 94) | 75 (58 – 93) | 0.0625 |
| **Plasma cells/ -blasts** | 47 (22 – 94) | 48 (11 – 95) | 0.8125 |

**n =6,* **Wilcoxon matched pairs signed rank test

# 4. Discussion

## 4.1 Analysis of the TruCount Method for Determining Cell Counts in Comparison to the Automated Hematology Analyser or the Dual-platform Method in Patients with SLE, Patients with SLE and Infections, and Patients with Infections

### 4.1.1 Comparison of TruCount with Automated Hematology Cell Counts

The central question addressed in this analysis was how cell counts attained with the TruCount method compare to those of the automated hematology analyser used for routine blood counts. The Spearman correlation coefficients indicate statistically significant pairing for all of the populations and therefore provide evidence for reliable detection by the rapid TruCount method. The correlation coefficient was lowest however for the monocytes which could be due to higher fragility of these cells to the lysis solution used in the TruCount method, the natural longevity of the population ex vivo or that the automatic hematological analyser counts certain CD19+CD20-CD27++ plasmablasts and plasma cells as monocytes on the basis of their large size. In accordance with this hypothesis, the median and range was higher according to the automated hematology counter although this difference was not statistically significant. The correlation coefficient for monocytes was even lower when only SLE patients were considered which is a population with a consistent tendency toward a relative increase in plasma cells and plasmablasts. It is conceivable that according to the Wilcoxon

matched pairs signed ranks test the populations did not differ significantly from each other because the additional plasma cells/-blasts counted as monocytes did not make a major difference compared to the overall size of the monocyte population, but since the miscounted cells occurred on an inconsistent basis since the size of the plasma cells and plasmablasts varies, the correlation was effected. The Bland-Altman plots support this hypothesis by showing a bias 188.79/µl for the monocyte population with the automated hematology counter resulting in higher values for this population.

Furthermore, the Bland-Altman plots allow for an analysis of the cell count discrepancies in relation to the absolute number of monocytes. This shows that the difference between the values from the different systems increases with increasing cell counts suggesting that when there are unusually high numbers of cells in this population, the automated hematology counter shows increasingly higher values in comparison with the TruCount system, likely since plasma cells/-blasts are being counted in addition to monocytes in the automated system which does not use cell surface markers but uses forward and side scatter, side fluorescence and impedance.

The medians of the monocyte and the CD19+CD20-CD27++ plasmablasts and plasma cell counts did not differ significantly from each other according to the Wilcoxon matched pairs signed ranks test since the difference in the measurement of these values was not consistent but seems to be multi-factorial including being the consequence of cell size and how this was interpreted by the automated hematology counter in addition to possible other factors such as susceptibility to lysis and natural longevity. Nevertheless, in cases where cell counts were in the normal range, the values from the two systems were very consistent with each other as shown in the Bland-Altman plots.

The Wilcoxon matched pairs signed ranks test showed that the neutrophil and leucocyte populations have significantly different medians but relatively high correlation coefficients, suggesting that neutrophils were consistently lost to some extent in the TruCount method. In this situation where the Wilcoxon matched pairs signed ranks test results in significant difference but there is good correlation, the most plausible explanation is that the cells are consistently lost in one method or cells are consistently

miscounted. Here it is plausible that the neutrophils are consistently lysed in the TruCount method.

The question of whether the 95% confidence intervals of the Bland-Altman plots result in values which are tolerable are, according to Bland and Altman, questions of judgement and depend on the clinical situation. In some cases 95% confidence intervals will be tolerable while others may require 99% confidence intervals. Consequently Stöckl et al. (2004) found that the Bland-Altman plot had become a popular tool for the presentation of method comparisons but was rarely used to judge the quality of the method. Furthermore, the confidence intervals require a Gaussian distribution of the differences. Bland and Altman recommend using histograms to analyse the distribution, given that if it is skewed or has very long tails the assumption of normality will not be valid. According to the Kolmogorov-Smirnov test for normality, the differences between the two methods for all of the populations showed a Gaussian distribution, however, in many cases the histograms showed long tails and were skewed. Accordingly, Stöckl et al. (2004) demonstrated the role of sample size on the range of the 95% confidence intervals. In summary, therefore, in order to answer specific questions about the exchangeability of the two systems, the tolerable range of the variation between the two systems needs to be determined clinically.

Our findings show that the two systems of blood analysis result in comparable cell counts, except for the case of the monocytes, as shown in the weak coefficient of correlation, and the overall leucocyte and neutrophil counts which differed significantly although the correlation was good. This pilot feasabilty study in patients and NHS suggests that both methods provide comparable results and the TruCount method can be used for more rapid diagnostic testing in further studies.

### 4.1.2 Comparison of Trucount with Automated Hematology Data in Patients with SLE

When only samples from patients with SLE are considered, the results were similar to those from the mixed sample populations. The correlation remained high for the values attained from the two systems. The Spearman correlation coefficients indicate statistically significant pairing for all of the populations except for the monocytes, $r^2$ =

0.0160, which was even lower than when the samples from patients with infections were also considered. The Wilcoxon signed ranks matched paired test shows that the medians differ significantly for the leucocyte and neutrophil populations. Therefore, one can conclude that for the leucocytes, lymphocytes and neutrophils, the medians may differ but the pairing is effective as shown by the correlation coefficients and the linear regression. As noted previously, the lack of correlation for the monocyte counts are consistent with the hypothesis that in samples with high numbers of CD19+CD20-CD27++ cells, as is the case in patients with increased SLE activity, the automated analyser counts these cells as monocytes and therefore the two systems result in different findings. Accordingly, the median of the monocytes was even higher according to the automated counter when only samples from patients with SLE are considered, although the difference here was also not significant. The Bland-Altman plot for the monocytes shows an increase in the discrepancies with increased cell numbers. The variation in this case as compared to the variation when all the samples were considered is somewhat more inconsistent. This could be because the CD19+CD20-CD27++ cells are counted as monocytes because of their large size, but not all of these cells, especially in patients with SLE, are equally large. Therefore, a somewhat inconsistent pattern of variation can result. The bias in this case is not as high as when all the samples were considered reflecting that the variation was less consistent.

Furthermore, it has been observed in the clinical setting that patients with high plasma cell/-blast counts were found to have an unexplained monocytosis when measured in an automated system. Figure 16 is a schematic scatterplot for the Sysmex XE-2100 which is produced by staining the DNA and RNA after permeabilizing the cell membrane and plotting the side scattered light against the fluorescence. In a further step, using an agent to protect the cell membranes of immature cells from disruption by an additional reagent in order to investigate only immature cells, the populations are differentiated into blasts and immature granulocytes according to their direct-current resistance (DCR) and alternating current capacitance (ACC). As demonstrated in Figure 16, there is an overlapping population among blasts and monocytes. In contrast to the case of multiple myeloma, for example, where the blasts might appear as atypical lymphocytes or as large population of blasts in the DCR/ACC scattergram, the plasmablasts and plasma cells in SLE would not be flagged in any particular way and could mistakenly fall in to the population of monocytes. These limitations were circumvented in the non-automated

systems by applying a widened lymphocyte gate and using CD27 as a marker of memory and plasma cells/-blasts (Odendahl et al., 2000).

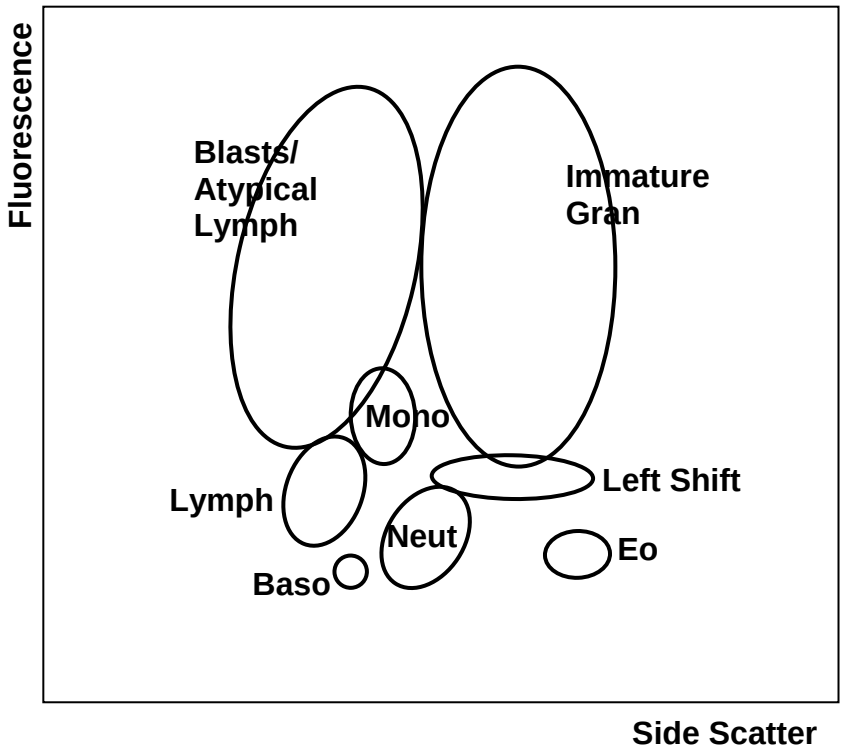



Figure 16. A schematic scattergram showing areas of normal and abnormal WBC populations from the Sysmex XE-2100 automated hematology analyzer according to the product information manual. Lymph = lymphocytes, Gran = granulocytes, Mono = monocytes, Neut = neutrophils, Eo = eosinophils, Baso = basophils.

### 4.1.3 Comparison of B Cells and B Cell Subpopulation Counts in Patients with SLE using the TruCount and Dual-platform Methods

The total B cell counts and the B cell subpopulations attained in the two systems all resulted in high Spearman correlation coefficients. Furthermore, there were no significant differences in the medians of the cell populations according the Wilcoxon matched pairs signed rank test. The linear regression diagrams and the corresponding coefficients of correlation also show effective pairing. The high Spearman correlation coefficients, coefficients of determination and the fact that the Wilcoxon matched pairs signed rank test showed no significant differences in the medians of the B cells and the B cells subpopulations when comparing the two methods, indicates that these two methods result in comparable results when analysing B cell subsets. In this case the Bland-Altman plots result generally in non-polarized findings for all of the cell populations except the memory B cells which indicates that the variation in the results is

not dependent on the magnitude of the measurements. These findings suggest that the TruCount method of determining B cell subsets can be also be used, providing a more rapid and practical method of determining this clinically important parameter.

These findings are relevant in conjunction with the findings of Jacobi et al. (2003) which identified a correlation between plasma cells and disease activity in patients with SLE. This clinical context has the most relevance for the application of the Bland-Altman plots. In the case of the CD20-CD27++ plasma cells and plasmablasts the bias is 2/µl but the 95% confidence interval is relatively large. As demonstrated on Stöckl et al. (2004) the 95% confidence interval can decrease with increased sample size. Our findings indicate that the results of the two methods are similar enough to warrant further investigation of the TruCount method in a clinical setting to see if the variation in the results is of clinical relevance. Our preliminary findings suggest that the TruCount method could be a useful tool but ultimately needs to be tested in larger studies. Thereby ideally, both methods would be performed side-by-side and then the results analysed retrospectively according to the clinical situation. Furthermore, this analysis could be enhanced with a simultaneous analysis of the expression of CD64 on neutrophils as a marker of acute infection, as discussed below. In a sample of this size with a wide range of activity scores including an ECLAM of 0, it was not possible to correlate disease activity and CD19+CD20-CD27++ plasma cells for either method, therefore an analysis on this basis cannot be made. Additional studies studies are now underway in order to evaluate the clinical application of this method.

The TruCount method has been compared to the dual-platform method in numerous studies with various types of cells. Nicholson et al. (1997) found that lymphocyte subsets values obtained by the TruCount method were higher than those obtained by a dual-platform method. They found that the TruCount method had a significant advantage in that much less blood was needed and the lymphocyte subset counts could reliably be determined more than 24 hours after blood was drawn. Furthermore, it can be assumed that once the TruCount method is established and is routine, there will be much less variability in a method using a single platform than in a method utilizing numerous platforms. Single platform methods have been shown to have less intra- and interlaboratory variation (Storie et al., 2004). On the other hand, the TruCount method is absolutely dependent on measuring exactly 50 µl of whole blood. Therefore, the pipettes need to be carefully and frequently calibrated. Our study did not address the question of intra-test variability. However, after establishing that the TruCount method

can be used to determine plasma cell/-blast counts in patients with SLE as a diagnostic tool, assessment of the intra-assay variability will be investigated. Furthermore, other systems of determining cell counts in whole blood are available and possibly another system like CytoCount by Dako may be less operator sensitive since the beads are also pipetted by the investigator and therefore any error in pipetting or calibration would potentially be reproduced for the sample and the beads, thereby cancelling the effects.

### 4.1.4 Synopsis: Evaluation of Cell Counts Attained by the Trucount and the Automated Hematology Analyzer/ Dual-platform Method

In evaluating the TruCount method for the study of plasma cells/-blasts in patients with SLE, we compared various populations to gather insights into the general comparability of the two systems. In general, this comparison revealed that the two methods yield comparable results for the populations studied except for monocytes. In regard to our specific objective of evaluating whether the TruCount method can be used as a more practical substitute instead of the dual-platform method using the Ficoll-Paque method to determine plasma cell/-blast counts, our findings show a good correlation of the results. The coefficient of determination, $r^2$=0.8557, is lower than for the other B cell populations but is within a tolerable range for good correlation and the Wilcoxon matched pairs signed rank test shows no significant difference between the paired samples. Nevertheless, the 95% confidence interval of the Bland-Altman plot is relatively wide. However, the test for normality, while positive, reveals wide tails. Therefore, it would be important to investigate if a larger sample size leads to a narrower 95% confidence interval while simultaneously focusing on the clinical application. Our results suggest that reliable plasmablast and plasma cell counts can be achieved using this method to track disease activity and an additional tandem analysis of the expression of CD64 on neutrophils can be used to diagnose infections in patients with SLE. Thus, discriminative co-analysis of both phenotypic parameters has the potential for improved differential diagnosis.

## 4.2 Analysis of FcγRI (CD64) Expression on Neutrophils and Monocytes

Using the isotype control and the resulting difference in MFI, the expression of CD64 on neutrophils of patients with SLE and infections differed significantly when tested with the

Mann-Whitney test from the expression in patients with SLE alone (p = 0.0051) as shown in Table 22 with an overview as Table 25. Furthermore, a difference is seen in the expression of CD64 when comparing patients with infections and normal healthy subjects (p = 0.0087). The expression of CD64 on monocytes also demonstrate a significant upregulation of CD64 in patients with infections compared to normal healthy subjects (p = 0.0043) but not in the comparison between SLE patients with and without infections (p = 0.3434). When comparing patients with SLE and NHS, the expression of CD64 on the neutrophils and monocytes did not differ from each other significantly in any case (p = 0.2949 and 0.2343 respectively) although the median expression was higher in patients with SLE which is in accordance with the findings of Hepburn et al. (2004) who did similar investigations in monocytes without using an isotype control. Interestingly, the presence of an acute infection resulted in increased expression of CD64 on monocytes and neutrophils for either NHS or patients with SLE and there was no significant difference between SLE patients without infections and NHS. A statistically significant increase in the expression comparing patients with SLE and these patients with an additional acute infection was found only in neutrophils.

***Table 25: Overview table of significant findings regarding the expression of CD64 as a marker of acute infection***

| **Sample population compared** | **Neutrophils** | **Monocytes** |
|---|---|---|
| **SLE versus SLE+Inf.** | SLE+Inf. sign. higher | |
| **NHS versus Infection** | Infection sign. higher | Infection sign. higher |
| **SLE versus NHS** | | |
| **SLE versus Infection** | Infection sign. higher | Infection sign. higher |

The studies of the expression of CD64 on monocytes and neutrophils showed an increase in the expression of CD64, an activating Fcγ receptor, on neutrophils of

patients with infections. This finding applies to patients with infections without other known illnesses as well as to SLE patients with acute infections. The study of cell counts attained with the automated haematology analyzer compared to the TruCount method revealed that the cell counts in the TruCount method for neutrophils where lower than those in the automated system, suggesting that some of the neutrophils were lost. Since it is possible that a certain subpopulation is preferentially lost, it is possible that out findings regarding CD64 are skewed because of lost cells or subpopulations. Nevertheless, this is unlikely since we found that the overall expression of CD64 went up in the patients with infections. In order to be related to the lost cells, neutrophils with low CD64 expression would have to be preferentially lost in the samples from patients with infections, which is unlikely.

This finding is relevant for the frequently posed clinical question of whether a patient with SLE is suffering from a flare or an acute infection. Markers of acute infection have generally been the focus of intense research with the goal of improving sensitivity overall and in particular patient groups such a newborns or in febrile neutropenia and with the goal of establishing markers of severe infectious disease with impending deterioration in critically ill patients. These investigations have frequently focussed on IL-6 and procalcitonin (Dollner et al., 2001; Persson et al., 2005; Stryjewski et al., 2005; Khassawneh et al., 2007; Secmeer et al., 2007). In SLE no single definitive marker has been established to differentiate between an SLE flare and acute infections. Patients with SLE have been shown to have a very variable CRP levels in the course of their disease which do not always correlate with diasease activity (Hesselink et al., 2003). Procalcitonin was shown to be increased in the early febrile period in fungal and bacterial infections in patients with SLE with a decrease with defervescence (Shin et al., 2001). However, the sensitivity of procalcitonin levels in signalling acute infections in SLE remains disputed (Eberhard et al., 1997; Espinosa-Morales et al., 1998; Moosig et al., 1998). Furthermore, whereas IL-6 has been established as a useful marker of infection in neonatal sepsis and febrile neutropenia, in the case of SLE, IL-6 levels have been shown to correlate with disease activity and with the haematologic manifestation of the disease (Dollner et al., 2001; Persson et al., 2005; Ripley et al., 2005; Sabry et al., 2006). Ye al al. (2007) propose using 2’5’-oligoadenylate synthetase isoforms (OAS) to differentiate between SLE flares and acute infection. In a pilot study they found that OASL expression was a predictor of acute infections. The analysis was performed using

real-time PCR and the authors argue that enzymatic and immunologic assays at a protein level are needed to analyse these isozymes in a larger study group. Currently, a diagnostic void exists regarding the diagnosis of acute infections in patients with SLE. Our findings suggest that the analysis of CD64 on neutrophils can be used as a marker to determine if a patient with SLE is suffering from an acute infection and thereby provides a practical diagnostic tool to clarify a very relevant clinical question. In further studies, it would be interesting to investigate the expression of CD64 in febrile neutropenia and other cases where the diagnosis of acute infections is of critical importance but has as of yet remained elusive to definitive lab parameters with the consequence that the assessment is made based on clinical judgement using various parameters.

CD64 has very low rates of basal expression and its upregulation occurs in a matter of hours in response to the cytokines of acute inflammatory responses including IL-12, IFN-γ and GCSF. Therefore, Davis et al. (2006) also argue for the use of an isotype control because the distribution is a shift in expression on the entire population and therefore it is inappropriate to quantify the increase in CD64 expression as the percent of positive cells. Furthermore, it lends itself as a diagnostic marker because the expression is stable at room temperature for more than 36 hours.

In support for the use of CD64 as a marker of acute infection, Fjaertoft et al. (2005) describe the possibility of using the degree of expression of CD64 on neutrophils and monocytes in patients with complicated influenza A infections and to differentiate Influenza A infections from bacterial infections. Furthermore, the absence of increased expression of CD64 on neutrophils excluded almost to 100% any ongoing infection caused by either bacteria or influenza A virus. Therefore, they conclude that neutrophil expression of CD64 is a very sensitive marker of any kind of infection since all subjects with either bacterial or influenza infections showed increased expression (Fjaertoft, et al., 2005). Likewise Herra et al. (1996) found significantly increased levels of CD64 in patients with systemic or localized infection compared to a non-infected patient group. Infections with gram-negative bacilli induced higher CD64 responses than infections with streptococci and staphylococci. They also concluded that CD64 measurements on neutrophils can we used a rapid indicator of bacterial infection. Furthermore, CD64 has

been shown to be useful in identifying late-onset nosocomial infections in very low birth weight infants and for early-onset neonatal infection (Ng et al., 2002; Ng et al., 2004).

The expression of CD64 has been investigated in various studies with particular regard to the expression in systemic lupus erythematosus. Szücs, et al. (1995) reports an increase, though lacking statistical significance, in the expression of CD64 on granulocytes in patients with SLE compared to NHS. Allen et al. (2002), show an increased expression of CD64 on neutrophils in patients with systemic infection compared to patients with SLE. Of particular interest is a study, which is only available in English as an abstract, by Yang et al. (2003) which showed an increased expression of CD64 on neutrophils in patients with SLE and acute infections compared to the non-infected SLE patient cohort. In contrast to our findings, Szücs et al. (1994) found a significant difference in the expression of CD64 on monocytes in patients with SLE compared to NHS using multiple isotype controls in the calculation of the relative mean fluorescence intensity.

The central role of CD64 in immune processes is evident in the studies of Barnes, et al. (2002) in FcγRI-deficient mice. These studies showed the importance of CD64 in endocytosis of monomeric IgG, phagocytosis of immune complex, in macrophage antibody-dependent cell-mediated cytotoxicity (ADCC), and in immune complex dependent antigen presentation. Overall, FcγRI-deficient mice have reduced inflammatory responses. Given these functions of FcγRI, it is fitting that this receptor is rapidly upregulated in cases of acute infection.

### 4.2.1 Synopsis: Analysis of FcγRI (CD64) Expression on Neutrophils and Monocytes

This study of the expression of CD64 on monocytes and neutrophils shows an increase in the expression of CD64 on neutrophils in patients with infections. This finding applies to patients with infections without other known diseases as well as to patients with SLE and acute infections. CD64 is known to have very low rates of basal expression and a rapid upregulation in response to acute inflammatory cytokines including IL-12, IFN-γ and GCSF. Furthermore, it plays a central role in neutrophil function. Subsequently,

CD64 expression on neutrophils represents a promising marker to differentiate between a flare and an infection in patients with SLE in the clinical context.

## 4.3 Analysis of FCγRIIa (CD32a) Expression on B Cells

The expression of the activating receptor FcγRIIa is significantly decreased on CD27++ plasmablasts and plasma cells of patients with SLE compared to NHS and the expression is similar to that in patients with infections, which is also significantly lower than in NHS. Since the expression was not increased in patients with infections alone, in contrast to the expression of CD64, it is unlikely that CD32a will be a good marker of infection in patients with SLE.

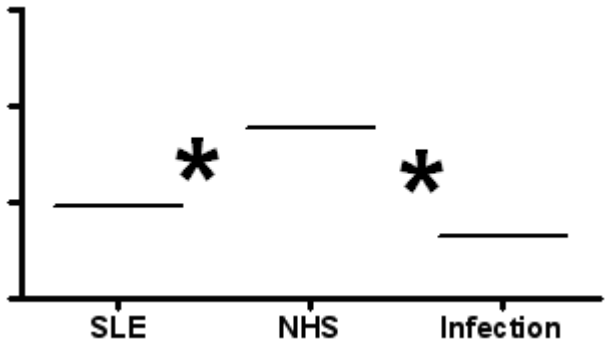



Figure 17. Schematic overview diagram of median frequencies of FcγRIIa+ expression on CD27++ plasmablasts and plasma cells, * demarks significant differences

Interestingly, the addition of Beriglobin did not statistically significantly affect the binding of the monoclonal antibody. This suggests that the monoclonal antibodies have a higher binding affinity or a distinct binding site which is not blocked by Beriglobin. Therefore, it is unlikely that the increased amount of immune complexes and circulating immunoglobulin in patients with SLE leads to a masking of CD32a and therefore the detected decreased levels of expression that we see here. The decreased expression levels of FcγRIIa on plasmablasts and plasma cells of patients with SLE and patients with infections compared to NHS can be interpreted in various ways. One physiological explanation for this finding is that a downregulation of this receptor is a response to previous and persisting immune stimulation as is the case in patients with SLE and infections. Possibly certain activation processes have already be launched and via a feed-back loop this receptor, in contrast to CD64 on monocytes and lymphocytes, has been downregulated. Another possibility is that only B cells with particular antigen

specificities have retained the receptor at higher levels and on others, the expression has been downregulated. Secondly, it is possible that the distribution of various subpopulations in the body differ in these different circumstances and that in the case of SLE and infection, a different cross-section of the total cell population is found in the periphery. To further investigate this possibility it would be helpful to investigate the spleen and lymph nodes, but in the human model these samples are rarely available. Thirdly, it is possible that the expression of this receptor is polymorphogenic in SLE which has not been delineated so far. Fourthly, increased amounts of circulating immunoglobulins in the case of SLE and infection, may shield the monoclonal antibody from the receptor. However, the addition of Beriglobin, which mimics a surplus of immunoglobulins, did not systematically reduce the median expression levels and therefore it can be assumed that the binding sites differ.

As described in the introduction, the actual consequence of the expression of a given receptor is the result of the overall expression of the FcγRs on the cell surface and the levels of various circulating immunoglobulins which have varying affinity levels to these receptors. Our findings show a downregulation of the activating receptor of FcγRIIa on plasma cells/-blasts of patients with SLE and infections. In this context, the expression levels of the other FcγRs is also of interest, particulary of of FcγRIIb since it is the inhibitory receptor and such systems of agonism/antagonism are known to work in tandem. In October of 2005 a mAb was described for the first time which exclusively binds FcγRIIb (Boruchov et al., 2005). The use of such a monoclonal antibody should play a significant role in clarifying the regulation of these two receptors and their functional relevance. Although many aspects of positive signaling in the generation of effective immune responses has been described, the role of the pairing of activation and inhibition is likely to play a central and as yet underestimated role in modulating immune responses. The availability of discriminating monoclonal antibodies will facilitate further studies to elucidate this phenomenon in the case of the FcγRIIa and FcγRIIb.

There are a number of controversies regarding our current understanding of these receptors. The expression of FcγRIIa on B cells has been disputed (Gamberale et al., 2002; Rabinovitch et al., 2004). Although it is believed to play a role in B cell development there is no reported evidence for a functional relevance of FcγRIIa in B cells (Gamberale et al., 2002). Various studies suggest that the control of the

expression of FcγRIIb in the germinal center is of pathogenetic relevance in the development of SLE (Jiang et al., 1999; Macardle et al., 2002). Most pathogenic autoantibodies in autoimmune diseases like SLE are class-switched and of high affinity and therefore they are the product of germinal center reactions (Ferry et al., 2006). In the germinal center where immune complexes are retained by FDC, FcγRIIb may play a role in the negative selection of B cells whose BCR have reduced affinity for antigen as the result of somatic hypermutation (Pearse et al., 1999). This in combination with a reduced expression of FcγRIIb on germinal center B cells allow for affinity maturation. Therefore the expression of the activating and inhibitory of FcγRs in GC cells is of particular interest and needs to be further investigated in patients with SLE. The timing of this expression is of particular interest since inhibitory signaling via FcγRIIb would hamper affinity maturation which is a necessary part of the GC reaction.

### 4.3.1 Synopsis: Analysis of FCγRIIa (CD32a) Expression on B Cells

The expression of the activating receptor FcγRIIa is significantly decreased on CD27++ plasmablasts and plasma cells of patients with SLE compared to NHS and the expression is similar to that in patients with infections, which is also significantly lower than in NHS. There was no apparent increase in the expression in the case of infection. Since FcγRIIa is an activating receptor, the decreased regulation may represent a response to the high levels of immune activity found in SLE or be the result of a redistribution of subpopulations in SLE in terms of cells which are found in the periphery. A shielding of the receptor by increased amounts of circulating immunoglobulin and immune complexes is unlikely since adding Beriglobin did not significantly change the detected expression levels. Lastly, this difference may result from a polymorphism found in SLE.

## 5. Summary

These studies explore a number of aspects of improved diagnostics using FACS analysis relevant to treatment decisions in SLE while also exploring possible mechanisms that may play a role in the pathogenesis of the disease.

Practicality and speed of diagnostics play a large role in the usefulness of a given diagnostic marker. The expression of CD27 on plasmablasts and plasma cells in

patients with SLE has proven to be an important marker of disease activity. Therefore, the exploration of new methods to determine the expression of CD27 on the B cells subsets more easily is essential to improving diagnostics in the context of SLE. In the first part of the study we compared cell counts of various populations as determined by the TruCount method and the automated hematology analyzer or dual-platform method in various sample populations. In summary, the TruCount method allows reliable and comparable results to the Ficoll-Paque method in determining B cell subsets. The statistical analysis employing the nonparametric Wilcoxon test, linear regression, Bland-Altman plots and the Spearman correlation coefficient reveal that while the two methods produce comparable cell counts for these populations, the next step in evaluating the TruCount system is embedded in the clinical context since that is the only place where the tolerable range for variations in cell counts can be determined.

Secondly, the TruCount method makes the FACS analysis of neutrophils possible and this is very useful in the context of SLE. We have shown here that the expression of the activating receptor FcγRI (CD64) on neutrophils is higher in patients with SLE and acute infections than in patients with SLE alone. Thus, CD64 testing on neutrophils candidates as a biomarker for differentiating infection from lupus activity.

In the third part of the study, we investigated the expression of the activating receptor FcγRIIa on the B cells subsets and compared the expression in patients with SLE, NHS and patients with infections alone. The expression of the activating receptor FcγRIIa is significantly decreased on CD27++ plasmablasts and plasma cells of patients with SLE compared to NHS and the expression is similar to that in patients with infections, which is also significantly lower than in NHS. Therefore, this marker does not lend itself to the differential diagnosis of a lupus flare or an acute infection in patients with SLE. Further investigation of the expression of FcγRIIb on B cell subsets in SLE as an inhibitory counterpart to FcγRIIa are needed to better understand the possible significance of this difference in SLE.

In summary, the TruCount method is a viable method to determine B cell subsets in SLE providing a valuable method of tracking diasease activity. CD64 can be used to diagnose acute infections in patients with SLE, which is relevant since acute infections are critical factors in morbitity and mortality in patients with SLE. Finally, the expression

of the activating receptor FcγRIIa is significantly decreased on CD27++ plasmablasts and plasma cells of patients with SLE compared to NHS and further investigations are needed to clarify the biological significance of this difference. These findings confirm that enhanced phenotyping in patients with autoimmune diseases, such as SLE, facilitate the tracking of disease activity and the identification of acute infections and will serve as an important method for the identification of biomarkers and targets of therapy.

## 6. Erklärung

Ich, Julia Huebner, erkläre, dass ich die vorgelegte Dissertationsschrift mit dem Thema: „Improved phenotypic analyses of peripheral blood cells allows differential diagnosis of disease activity and infection in systemic lupus erythematosus“ selbst und ohne die Hilfe Dritter verfasst habe. Andere als die angegebenen Quellen und Hilfsmittel wurden nicht benutzt und ggf. Zitate aus anderen Veröffentlichungen sind unter Angabe der Quelle entsprechend gekennzeichnet.

07.06.2007 Julia Huebner

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# 8. References


Agematsu K, Nagumo H, Yang FC et al. B cell subpopulations separated by CD27 and crucial collaboration of CD27+ B cells and helper T cells in immunoglobulin production. Eur J Immunol 1997; 27(8):2073-2079.

Agematsu K, Nagumo H, Oguchi Y et al. Generation of plasma cells from peripheral blood memory B cells: synergistic effect of interleukin-10 and CD27/CD70 interaction. Blood 1998; 91(1):173-180.

Allen E, Bakke AC, Purtzer MZ et al. Neutrophil CD64 expression: distinguishing acute inflammatory autoimmune disease from systemic infections. Annals of the Rheumatic Diseases 2002; 61(6):522-525.

Alvarado-Sanchez B, Hernandez-Castro B, Portales-Perez D et al. Regulatory T cells in patients with systemic lupus erythematosus. J Autoimmun 2006; 27(2):110-118.

Amigorena S, Bonnerot C, Drake JR et al. Cytoplasmic domain heterogeneity and functions of IgG Fc receptors in B lymphocytes. Science 1992; 256(5065):1808-1812.

Anolik JH, Campbell D, Felgar RE et al. The relationship of FcgammaRIIIa genotype to degree of B cell depletion by rituximab in the treatment of systemic lupus erythematosus. Arthritis Rheum 2003; 48(2):455-459.

Arbuckle MR, McClain MT, Rubertone MV et al. Development of autoantibodies before the clinical onset of systemic lupus erythematosus. N Engl J Med 2003; 349(16):1526-1533.

Arora V, Verma J, Dutta R et al. Reduced complement receptor 1 (CR1, CD35) transcription in systemic lupus erythematosus. Mol Immunol 2004; 41(4):449-456.

Arora V, Mondal AM, Grover R et al. Modulation of CR1 transcript in systemic lupus erythematosus (SLE) by IFN-gamma and immune complex. Mol Immunol 2007; 44(7):1722-1728.

Barnes N, Gavin AL, Tan PS et al. FcgammaRI-deficient mice show multiple alterations to inflammatory and immune responses. Immunity 2002; 16(3):379-389.

Bave U, Magnusson M, Eloranta ML et al. Fc gamma RIIa is expressed on natural IFN-alpha-producing cells (plasmacytoid dendritic cells) and is required for the IFN-alpha production induced by apoptotic cells combined with lupus IgG. J Immunol 2003; 171(6):3296-3302.

Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1986; 1(8476):307-310.

Bland JM, Altman DG. Comparing methods of measurement: why plotting difference against standard method is misleading. Lancet 1995; 346(8982):1085-1087.

Boruchov AM, Heller G, Veri MC et al. Activating and inhibitory IgG Fc receptors on human DCs mediate opposing functions. J Clin Invest 2005; 115(10):2914-2923.

Boyum A. Isolation of lymphocytes, granulocytes and macrophages. Scand J Immunol 1976; Suppl 5:9-15.

Cappione A, III, Anolik JH, Pugh-Bernard A et al. Germinal center exclusion of autoreactive B cells is defective in human systemic lupus erythematosus. J Clin Invest 2005; 115(11):3205-3216.

Cartron G, Dacheux L, Salles G et al. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. Blood 2002; 99(3):754-758.

Cheema GS, Roschke V, Hilbert DM et al. Elevated serum B lymphocyte stimulator levels in patients with systemic immune-based rheumatic diseases. Arthritis Rheum 2001; 44(6):1313-1319.

Croft M. Costimulation of T cells by OX40, 4-1BB, and CD27. Cytokine Growth Factor Rev 2003; 14(3-4):265-273.

Crow MK, Kirou KA. Interferon-alpha in systemic lupus erythematosus. Curr Opin Rheumatol 2004; 16(5):541-547.

Davis BH, Olsen SH, Ahmad E et al. Neutrophil CD64 is an improved indicator of infection or sepsis in emergency department patients. Arch Pathol Lab Med 2006; 130(5):654-661.

Diamond B. Autoimmunity. Immunological Reviews 2005; 204:5-8.

Dijstelbloem HM, Bijl M, Fijnheer R et al. Fcgamma receptor polymorphisms in systemic lupus erythematosus: association with disease and in vivo clearance of immune complexes. Arthritis Rheum 2000; 43(12):2793-2800.

Dijstelbloem HM, van de Winkel JG, Kallenberg CG. Inflammation in autoimmunity: receptors for IgG revisited. Trends Immunol 2001; 22(9):510-516.

Dollner H, Vatten L, Austgulen R. Early diagnostic markers for neonatal sepsis: comparing C-reactive protein, interleukin-6, soluble tumour necrosis factor receptors and soluble adhesion molecules. J Clin Epidemiol 2001; 54(12):1251-1257.

Eberhard OK, Haubitz M, Brunkhorst FM et al. Usefulness of procalcitonin for differentiation between activity of systemic autoimmune disease (systemic lupus erythematosus/systemic antineutrophil cytoplasmic antibody-associated vasculitis) and invasive bacterial infection. Arthritis Rheum 1997; 40(7):1250-1256.

Enyedy EJ, Mitchell JP, Nambiar MP et al. Defective Fc gamma RIIb1 signaling contributes to enhanced calcium response in B cells from patients with systemic lupus erythematosus. Clinical Immunology 2001; 101(2):130-135.

Espinosa-Morales R, Escalante A. Use of procalcitonin as a diagnostic test: comment on the article by Eberhard et al. Arthritis Rheum 1998; 41(3):567-568.

Ferry H, Leung JC, Lewis G et al. B-cell tolerance. Transplantation 2006; 81(3):308-315.

Fessler BJ. Infectious diseases in systemic lupus erythematosus: risk factors, management and prophylaxis. Best Pract Res Clin Rheumatol 2002; 16(2):281-291.

Fiebig EW, Johnson DK, Hirschkorn DF et al. Lymphocyte subset analysis on frozen whole blood. Cytometry 1997; 29(4):340-350.

Fjaertoft G, Pauksen K, Hakansson L et al. Cell surface expression of FcgammaRI (CD64) on neutrophils and monocytes in patients with influenza A, with and without complications. Scand J Infect Dis 2005; 37(11-12):882-889.

Floto RA, Clatworthy MR, Heilbronn KR et al. Loss of function of a lupus-associated FcgammaRIIb polymorphism through exclusion from lipid rafts. Nat Med 2005; 11(10):1056-1058.

Fukuyama H, Nimmerjahn F, Ravetch JV. The inhibitory Fcgamma receptor modulates autoimmunity by limiting the accumulation of immunoglobulin G+ anti-DNA plasma cells. Nat Immunol 2005; 6(1):99-106.

Gamberale R, Geffner JR, Giordano M. Immune complexes and apoptosis in B-cell chronic lymphocytic leukemia. Leuk Lymphoma 2002; 43(2):251-255.

Gamberale R, Fernandez-Calotti P, Sanjurjo J et al. Signaling capacity of FcgammaRII isoforms in B-CLL cells. Leuk Res 2005; 29(11):1277-1284.

Ginzler E, Diamond H, Kaplan D et al. Computer analysis of factors influencing frequency of infection in systemic lupus erythematosus. Arthritis Rheum 1978; 21(1):37-44.

Goodnow CC, Sprent J, Fazekas de St GB, Vinuesa CG. Cellular and genetic mechanisms of self tolerance and autoimmunity. Nature 2005; 435(7042):590-597.

Grondal G, Gunnarsson I, Ronnelid J et al. Cytokine production, serum levels and disease activity in systemic lupus erythematosus. Clin Exp Rheumatol 2000; 18(5):565-570.

Hannahs Hahn B. Systemic Lupus Erythematosus. In: Harrison's Principles of Internal Medicine. 16th ed. (Accessed October 12, 2006, at http:gateway.ut.ovid.com/gw1/ovidweb.cgi.) New York: McGraw-Hill, 2005:300.

Hepburn AL, Mason JC, Davies KA. Expression of Fcgamma and complement receptors on peripheral blood monocytes in systemic lupus erythematosus and rheumatoid arthritis. Rheumatology (Oxford) 2004; 43(5):547-554.

Herra CM, Keane CT, Whelan A. Increased expression of Fc gamma receptors on neutrophils and monocytes may reflect ongoing bacterial infection. J Med Microbiol 1996; 44(2):135-140.

Hesselink DA, Aarden LA, Swaak AJ. Profiles of the acute-phase reactants C-reactive protein and ferritin related to the disease course of patients with systemic lupus erythematosus. Scand J Rheumatol 2003; 32(3):151-155.

Hibbs ML, Tarlinton DM, Armes J et al. Multiple defects in the immune system of Lyn-deficient mice, culminating in autoimmune disease. Cell 1995; 83(2):301-311.

Hogarth PM. Fc receptors are major mediators of antibody based inflammation in autoimmunity. Curr Opin Immunol 2002; 14(6):798-802.

Ioan-Facsinay A, de Kimpe SJ, Hellwig SM et al. FcgammaRI (CD64) contributes substantially to severity of arthritis, hypersensitivity responses, and protection from bacterial infection. Immunity 2002; 16(3):391-402.

Jacobi AM, Odendahl M, Reiter K et al. Correlation between circulating CD27high plasma cells and disease activity in patients with systemic lupus erythematosus. Arthritis Rheum 2003; 48(5):1332-1342.

Janeway C, Travers P, Walport M, et al. Immunobiology: the immune system in health and disease. 6th ed. New York: Garland Science Publishing; 2005: 258-264 and 399.

Jiang Y, Hirose S, Sanokawa-Akakura R et al. Genetically determined aberrant down-regulation of FcgammaRIIB1 in germinal center B cells associated with hyper-IgG and IgG autoantibodies in murine systemic lupus erythematosus. Int Immunol 1999; 11(10):1685-1691.

Jiang Y, Hirose S, Abe M et al. Polymorphisms in IgG Fc receptor IIB regulatory regions associated with autoimmune susceptibility. Immunogenetics 2000; 51(6):429-435.

Jimenez S, Cervera R, Font J et al. The epidemiology of systemic lupus erythematosus. Clinical Reviews in Allergy & Immunology 2003; 25(1):3-11.

Jung J, Choe J, Li L et al. Regulation of CD27 expression in the course of germinal center B cell differentiation: the pivotal role of IL-10. Eur J Immunol 2000; 30(8):2437-2443.

Kanemitsu S, Ihara K, Saifddin A et al. A functional polymorphism in fas (CD95/APO-1) gene promoter associated with systemic lupus erythematosus. J Rheumatol 2002; 29(6):1183-1188.

Kato I, Takai T, Kudo A. The pre-B cell receptor signaling for apoptosis is negatively regulated by Fc gamma RIIB. J Immunol 2002; 168(2):629-634.

Khassawneh M, Hayajneh WA, Kofahi H et al. Diagnostic markers for neonatal sepsis: comparing C-reactive protein, interleukin-6 and immunoglobulin M. Scand J Immunol 2007; 65(2):171-175.

Klein U, Rajewsky K, Kuppers R. Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. J Exp Med 1998; 188(9):1679-1689.

Kono H, Kyogoku C, Suzuki T et al. FcgammaRIIB Ile232Thr transmembrane polymorphism associated with human systemic lupus erythematosus decreases affinity to lipid rafts and attenuates inhibitory effects on B cell receptor signaling. Hum Mol Genet 2005; 14(19):2881-2892.

Kyogoku C, Dijstelbloem HM, Tsuchiya N et al. Fcgamma receptor gene polymorphisms in Japanese patients with systemic lupus erythematosus: contribution of FCGR2B to genetic susceptibility. Arthritis Rheum 2002; 46(5):1242-1254.

Lahita RG. Emerging concepts for sexual predilection in the disease systemic lupus erythematosus. Ann N Y Acad Sci 1999; 876:64-69.

Liossis SN, Kovacs B, Dennis G et al. B cells from patients with systemic lupus erythematosus display abnormal antigen receptor-mediated early signal transduction events. J Clin Invest 1996; 98(11):2549-2557.

Liossis SN, Solomou EE, Dimopoulos MA et al. PP. B-cell kinase lyn deficiency in patients with systemic lupus erythematosus. J Investig Med 2001; 49(2):157-165.

Lipsky PE. Systemic lupus erythematosus: an autoimmune disease of B cell hyperactivity. Nat Immunol 2001; 2(9):764-766.

Ma L, Scheers W, Vandenberghe P. A flow cytometric method for determination of absolute counts of myeloid precursor dendritic cells in peripheral blood. J Immunol Methods 2004; 285(2):215-221.

Macardle PJ, Mardell C, Bailey S et al. FcgammaRIIb expression on human germinal center B lymphocytes. Eur J Immunol 2002; 32(12):3736-3744.

MacLennan IC. Germinal centers. Annu Rev Immunol 1994; 12:117-139.

McGaha TL, Sorrentino B, Ravetch JV. Restoration of tolerance in lupus by targeted inhibitory receptor expression. Science 2005; 307(5709):590-593.

Means TK, Luster AD. Toll-like receptor activation in the pathogenesis of systemic lupus erythematosus. Ann N Y Acad Sci 2005a; 1062:242-251.

Means TK, Latz E, Hayashi F, Murali MR, Golenbock DT et al. Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9. J Clin Invest 2005b; 115(2):407-417.

Mevorach D, Zhou JL, Song X et al. Systemic exposure to irradiated apoptotic cells induces autoantibody production. J Exp Med 1998; 188(2):387-392.

Mok CC, Lau CS. Pathogenesis of systemic lupus erythematosus. Journal of Clinical Pathology 2003; 56(7):481-490.

Moosig F, Reinhold-Keller E, Csernok E. Limitations on the usefulness of procalcitonin as a marker of infection in patients with systemic autoimmune disease: comment on the article by Eberhard et al. Arthritis Rheum 1998; 41(3):566-567.

Mudd PA, Teague BN, Farris AD. Regulatory T cells and systemic lupus erythematosus. Scand J Immunol 2006; 64(3):211-218.

Muta T, Kurosaki T, Misulovin Z et al. A 13-amino-acid motif in the cytoplasmic domain of Fc gamma RIIB modulates B-cell receptor signalling. Nature 1994; 368(6466):70-73.

Nagumo H, Agematsu K. Synergistic augmentative effect of interleukin-10 and CD27/CD70 interactions on B-cell immunoglobulin synthesis. Immunology 1998; 94(3):388-394.

Ng PC, Li K, Wong RP et al. Neutrophil CD64 expression: a sensitive diagnostic marker for late-onset nosocomial infection in very low birthweight infants. Pediatr Res 2002; 51(3):296-303.

Ng PC, Li G, Chui KM et al. Neutrophil CD64 is a sensitive diagnostic marker for early-onset neonatal infection. Pediatr Res 2004; 56(5):796-803.

Nicholson JK, Stein D, Mui T et al. Evaluation of a method for counting absolute numbers of cells with a flow cytometer. Clin Diagn Lab Immunol 1997; 4(3):309-313.

Nimmerjahn F, Bruhns P, Horiuchi K et al. FcgammaRIV: a novel FcR with distinct IgG subclass specificity. Immunity 2005; 23(1):41-51.

Nimmerjahn F, Ravetch JV. Fcgamma receptors: old friends and new family members. Immunity 2006; 24(1):19-28.

Nishizumi H, Taniuchi I, Yamanashi Y et al. Impaired proliferation of peripheral B cells and indication of autoimmune disease in lyn-deficient mice. Immunity 1995; 3(5):549-560.

O'Neill LA. Immunology. After the toll rush. Science 2004; 303(5663):1481-1482.

Odendahl M, Jacobi A, Hansen A et al. Disturbed peripheral B lymphocyte homeostasis in systemic lupus erythematosus. J Immunol 2000; 165(10):5970-5979.

Ohashi PS, DeFranco AL. Making and breaking tolerance. Curr Opin Immunol 2002; 14(6):744-759.

Pearse RN, Kawabe T, Bolland S et al. SHIP recruitment attenuates Fc gamma RIIB-induced B cell apoptosis. Immunity 1999; 10(6):753-760.

Peeva E, Venkatesh J, Michael D et al. Prolactin as a modulator of B cell function: implications for SLE. Biomed Pharmacother 2004; 58(5):310-319.

Peeva E, Zouali M. Spotlight on the role of hormonal factors in the emergence of autoreactive B-lymphocytes. Immunol Lett 2005; 101(2):123-143.

Persson L, Soderquist B, Engervall P et al. Assessment of systemic inflammation markers to differentiate a stable from a deteriorating clinical course in patients with febrile neutropenia. Eur J Haematol 2005; 74(4):297-303.

Petri M, Genovese M. Incidence of and risk factors for hospitalizations in systemic lupus erythematosus: a prospective study of the Hopkins Lupus Cohort. J Rheumatol 1992; 19(10):1559-1565.

Pricop L, Redecha P, Teillaud JL et al. Differential modulation of stimulatory and inhibitory Fc gamma receptors on human monocytes by Th1 and Th2 cytokines. J Immunol 2001; 166(1):531-537.

Pritchard NR, Cutler AJ, Uribe S et al. Autoimmune-prone mice share a promoter haplotype associated with reduced expression and function of the Fc receptor FcgammaRII. Curr Biol 2000; 10(4):227-230.

Pugh-Bernard AE, Silverman GJ, Cappione AJ et al. Regulation of inherently autoreactive VH4-34 B cells in the maintenance of human B cell tolerance. J Clin Invest 2001; 108(7):1061-1070.

Rabinovitch N, Gelfand EW. Expression of functional activating and inhibitory Fcgamma receptors on human B cells. Int Arch Allergy Immunol 2004; 133(3):285-294.

Radbruch A, Ed. Flow Cytometry and Cell Sorting. 2nd ed. Berlin: Springer-Verlag; 2000.

Radcliff G, Jaroszeski MJ. Basics of flow cytometry. Methods Mol Biol 1998; 91:1-24.

Ravetch JV, Kinet JP. Fc receptors. Annu Rev Immunol 1991; 9:457-492.

Reefman E, Dijstelbloem HM, Limburg PC et al. Fc gamma receptors in the initiation and progression of systemic lupus erythematosus. Immunology and Cell Biology 2003; 81(5):382-389.

Renaudineau Y, Pers JO, Bendaoud B et al. Dysfunctional B cells in systemic lupus erythematosus. Autoimmun Rev 2004; 3(7-8):516-523.

Ring GH, Lakkis FG. Breakdown of self-tolerance and the pathogenesis of autoimmunity. Semin Nephrol 1999; 19(1):25-33.

Ripley BJ, Goncalves B, Isenberg DA et a. Raised levels of interleukin 6 in systemic lupus erythematosus correlate with anaemia. Ann Rheum Dis 2005; 64(6):849-853.

Ronnblom L, Alm GV. Systemic lupus erythematosus and the type I interferon system. Arthritis Res Ther 2003; 5(2):68-75.

Ronnelid J, Tejde A, Mathsson L et al. Immune complexes from SLE sera induce IL10 production from normal peripheral blood mononuclear cells by an FcgammaRII dependent mechanism: implications for a possible vicious cycle maintaining B cell hyperactivity in SLE. Ann Rheum Dis 2003; 62(1):37-42.

Rudge EU, Cutler AJ, Pritchard NR et al. Interleukin 4 reduces expression of inhibitory receptors on B cells and abolishes CD22 and Fc gamma RII-mediated B cell suppression. J Exp Med 2002; 195(8):1079-1085.

Ruzicka K, Veitl M, Thalhammer-Scherrer R et al. The new hematology analyzer Sysmex XE-2100: performance evaluation of a novel white blood cell differential technology. Arch Pathol Lab Med 2001; 125(3):391-396.

Sabry A, Sheashaa H, El-Husseini A et al. Proinflammatory cytokines (TNF-alpha and IL-6) in Egyptian patients with SLE: its correlation with disease activity. Cytokine 2006; 35(3-4):148-153.

Samuelsson A, Towers TL, Ravetch JV. Anti-inflammatory activity of IVIG mediated through the inhibitory Fc receptor. Science 2001; 291(5503):484-486.

Schneider P. The role of APRIL and BAFF in lymphocyte activation. Curr Opin Immunol 2005; 17(3):282-289.

Secmeer G, Devrim I, Kara A et al. Role of procalcitonin and CRP in differentiating a stable from a deteriorating clinical course in pediatric febrile neutropenia. J Pediatr Hematol Oncol 2007; 29(2):107-111.

Shin KC, Lee YJ, Kang SW et al. Serum procalcitonin measurement for detection of intercurrent infection in febrile patients with SLE. Ann Rheum Dis 2001; 60(10):988-989.

Siriboonrit U, Tsuchiya N, Sirikong M et al. Association of Fcgamma receptor IIb and IIIb polymorphisms with susceptibility to systemic lupus erythematosus in Thais. Tissue Antigens 2003; 61(5):374-383.

Stockl D, Rodriguez CD, Van UK. Interpreting method comparison studies by use of the bland-altman plot: reflecting the importance of sample size by incorporating confidence limits and predefined error limits in the graphic. Clin Chem 2004; 50(11):2216-2218.

Storie I, Sawle A, Goodfellow K et al. Perfect count: a novel approach for the single platform enumeration of absolute CD4+ T-lymphocytes. Cytometry B Clin Cytom 2004; 57(1):47-52.

Stryjewski GR, Nylen ES, Bell MJ et al. Interleukin-6, interleukin-8, and a rapid and sensitive assay for calcitonin precursors for the determination of bacterial sepsis in febrile neutropenic children. Pediatr Crit Care Med 2005; 6(2):129-135.

Su K, Yang H, Li X et al. Expression profile of FcgammaRIIb on leukocytes and its dysregulation in systemic lupus erythematosus. J Immunol 2007; 178(5):3272-3280.

Szucs G, Kavai M, Suranyi P et al. Correlations of monocyte phagocytic receptor expressions with serum immune complex level in systemic lupus erythematosus. Scand J Immunol 1994; 40(5):481-484.

Szucs G, Kavai M, Kiss E et al. Correlation of IgG Fc receptors on granulocytes with serum immune complex level in systemic lupus erythematosus. Scand J Immunol 1995; 42(5):577-580.

Takai T. Roles of Fc receptors in autoimmunity. Nat Rev Immunol 2002; 2(8):580-592.

Tsao BP. An update on genetic studies of systemic lupus erythematosus. Curr Rheumatol Rep 2002; 4(4):359-367.

Tsao BP. Update on human systemic lupus erythematosus genetics. Curr Opin Rheumatol 2004; 16(5):513-521.

van de Winkel JG, Capel PJ. Human IgG Fc receptor heterogeneity: molecular aspects and clinical implications. Immunol Today 1993; 14(5):215-221.

van Lier RA, Borst J, Vroom TM et al. Tissue distribution and biochemical and functional properties of Tp55 (CD27), a novel T cell differentiation antigen. J Immunol 1987; 139(5):1589-1596.

Xiao Y, Hendriks J, Langerak P et al. CD27 is acquired by primed B cells at the centroblast stage and promotes germinal center formation. J Immunol 2004; 172(12):7432-7441.

Yan J, Mamula MJ. B and T cell tolerance and autoimmunity in autoantibody transgenic mice. Int Immunol 2002; 14(8):963-971.

Yang XY, Lin J, Li YM. [The expression of peripheral blood neutrophil CD64 in systemic lupus erythematosus with infection or disease activation]. Zhonghua Nei Ke Za Zhi 2003; 42(12):854-856.

Ye S, Guo Q, Tang JP et al. Could 2'5'-oligoadenylate synthetase isoforms be biomarkers to differentiate between disease flare and infection in lupus patients? A pilot study. Clin Rheumatol 2007; 26(2):186-190.

Yee AM, Ng SC, Sobel RE et al. Fc gammaRIIA polymorphism as a risk factor for invasive pneumococcal infections in systemic lupus erythematosus. Arthritis Rheum 1997; 40(6):1180-1182.

Yuasa T, Kubo S, Yoshino T et al. Deletion of fcgamma receptor IIB renders H-2(b) mice susceptible to collagen-induced arthritis. J Exp Med 1999; 189(1):187-194.

Yurasov S, Hammersen J, Tiller T et al. B-cell tolerance checkpoints in healthy humans and patients with systemic lupus erythematosus. Ann N Y Acad Sci 2005a; 1062:165-174.

Yurasov S, Wardemann H, Hammersen J et al. Defective B cell tolerance checkpoints in systemic lupus erythematosus. J Exp Med 2005b; 201(5):703-711.

Zonana-Nacach A, Camargo-Coronel A, Yanez P et al. Infections in outpatients with systemic lupus erythematosus: a prospective study. Lupus 2001; 10(7):505-510.

# LEBENSLAUF

Aus Datenschutzgründen wurde der Lebenslauf entfernt.