

Flow Cytometric Enumeration of Monocyte and Dendritic Cell Subpopulations in IgA Nephropathy

A Study into the Innate Immune System

Maja Høegh Christensen

Master's Thesis

Aalborg University 2012

Title: Flow Cytometric Enumeration of Monocyte and Dendritic Cell Subpopulations in IgA Nephropathy – A Study into the Innate Immune System

Project Period: September 1st 2011 to May 11th 2012

Project Group: 937

Group Member: Maja Høegh Christensen

Maja Høegh Christensen

Supervisor: Ralf Agger, Associate Professor at the Department of Health Science and Technology, Aalborg University.

Numbers Printed: 6 Pages: 64 (appendices not included) Appendices: A, B, C, and D Finished: May 11th 2012

The content of this report is freely available. However, disclosure can only be effected as directed by the author.

Preface

This master thesis is written by Maja Høegh Christensen in the period of 3rd and 4th semester of the master in science in the Medicine with Industrial Specialization program, Aalborg University, Department of Health Science and Technology.

The experimental work of the thesis was performed at Laboratory of Immunology, Aalborg University, and external co-worker was The Department of Urology, Aalborg Hospital.

I would like to thank my supervisor Ralf Agger, Associate Professor, for guidance regarding theoretical and technical aspects. Thanks to Troels Ring and Birthe Thørring, Doctors of Medicine, and Charlotte Mose Skov, biomedical laboratory technician, for handling the patient related areas of this study. My thanks also go to Lotte Hatting Pugholm and Romana Maric, Ph.D. students, for their willingness to help with any question, and to Brita Holst Jensen, laboratory technician, for assisting me in the laboratory.

Abstract

Background: IgA nephropathy (IgAN), also known as Berger's nephropathy, is the most common form of glomerulonephritis worldwide. Often, the disease affects adults at ages of 20 to 30 years, and the disease occurs almost twice as often in males as in females (1). The etiology of IgAN is to date unknown, but it is clear that poorly galactosylated immunoglobulin IgA1 is the trigger in the development of nephritis. The complex pathogenesis of IgAN has been studied in attempt to unravel key abnormalities of this disease, and over the past two decades significant progress has been made. However, incomplete understanding of the origins of IgA1 molecules, the formation of circulating immune complexes, and of the cellular events of inflammation, has affected the development of specific therapeutic strategies of IgAN. Therefore, management of patients has been with generic therapies, mainly in attempt to control blood pressure. Confusion over correct and optimal treatment remains, and there is a need for studies investigating cellular events of IgAN to be able to develop specific therapies in the future (2).

Materials and Methods: Flow cytometric enumeration was made on monocyte and dendritic cell subpopulations in anti-coagulated whole blood from IgAN patients and in healthy individuals. Also, the presence of monocytes in urine from IgAN patients and healthy control subjects was investigated by flow cytometry. TLR4 and HLA-DR expression of monocyte and dendritic cell subpopulations was determined.

Results: Enumeration of dendritic cell and monocyte subpopulations in IgAN patients and in healthy controls succeeded. In all subjects the subpopulations of cells were found. The monocyte and dendritic cell subpopulations being investigated in the present study have only recently been defined (3), and to our knowledge, the distribution of subpopulations within parent population of these cells in blood has not yet been investigated in IgAN. Urine monocytes were found in one patient but not in control subjects. HLA-DR expression of dendritic cell subpopulations seemed to be consistent between all subjects with highest peak values of median fluorescence intensity seen in samples from control subjects. Intermediate monocytes expressed most HLA-DR in comparison with classical and non-classical monocytes. The intermediate monocytes of IgAN patients.

Conclusion: By this study it was confirmed, that multicolor flow cytometry offers the opportunity of analysis of intermediate, non-classical, and classical monocytes, as well as plasmacytoid CD303⁺, myeloid CD1c⁺, and myeloid CD141⁺ dendritic cells in IgAN patients. Multicolor flow cytometry provides the capability of detection of multiple colors. However, reflections should be made in order to obtain useful and high quality data. In this study, it has been clearly shown, that the choice of fluorochrome-conjugated antibodies of the experimental antibody panel should be considered carefully, particularly when using fluorochromes that may show a high degree of non-specific binding to cells. It was emphasized, that there is a need for careful quality controls when using tandem-conjugated antibodies. The number of patients and control subjects for this type of experiment should be increased in a future study, as this would provide better opportunities for evaluation of cellular events in IgAN and in health. By also implementing various improvements of the flow cytometric measurements, great opportunities would arise to achieve more information on the condition of the innate immune system in IgAN.

Table of Contents

Introduction	8
IgA Nephropathy	9
IgA1 O-glycosylation	9
Immune Complex Formation, Mesangial IgA1 Deposition and Glomerular Injury	. 11
Immune Mechanisms in Glomerular Tissue Injury	. 14
Multicolor Flow Cytometry	. 18
The Principles of Flow Cytometry	. 18
Analysis of Fluorescence	. 20
Spectral Overlap and Compensation	. 21
Securing High Quality Data	. 23
The Aim of the Study	. 26
The Course of Experiments	. 26
Materials and Methods	. 27
Subjects	. 27
Flow Cytometric Analysis	. 27
Compensation	. 27
Determination of Absolute Leucocyte Counts in Blood	. 28
Dendritic Cell Subset Enumeration in Whole Blood	. 28
Monocyte Subset Enumeration in Whole Blood	. 30
Monocyte Subset Enumeration in Urine	. 30
Gating Strategies	31
Absolute Leucocyte Differential Counts	. 35
Results	. 36
Absolute Leucocyte Counts by Flow Cytometry and Absolute Leucocyte Differential Counts	. 36
Dendritic Cell Subset Enumeration in Whole Blood	42
Distribution of CD1c+, CD303+ and CD141+ Dendritic Cells	. 43
Dendritic Cell Subclass Distribution over Time	. 44
TLR4 Expression of Dendritic Cell Subpopulations	. 45
HLA-DR Expression of Dendritic Cell Subpopulations	. 48
Monocyte Subset Enumeration in Whole Blood	. 49
Distribution of Non-Classical, Intermediate, and Classical Monocytes	50
Monocyte Subset Distribution over Time	. 51

TLR4 Expression of Monocyte Subpopulations	53
HLA-DR Expression of Monocyte Subpopulations	54
Monocyte Subset Enumeration in Urine	55
Discussion	57
Conclusive Remarks	60
Bibliography	61
Appendix A - Ethical Approval	
Appendix B - Approval by the Danish Data Protection Agency	

- Appendix C Compensation Matrix
- Appendix D Compensation: Negative and Positive Populations

Introduction

IgA nephropathy (IgAN), also known as Berger nephropathy, is the most common form of glomerulonephritis worldwide. Often, the disease affects adults at ages of 20 to 30 years, and the disease occurs almost twice as often in males as in females (1). The etiology of IgAN is to date unknown, but it is clear that poorly galactosylated immunoglobulin IgA1 is the trigger in the development of nephritis (2). The first description of IgAN was published in 1968 by Jean Berger, a Parisian pathologist (2). Since then the complex pathogenesis of IgAN has been studied in attempt to unravel key abnormalities of this disease, and over the past two decades significant progress has been made. However, incomplete understanding of the origins of IgA1 molecules and of the formation of circulating immune complexes has affected the development of specific therapeutic strategies of IgAN. Therefore, management of patients has been with generic therapies, mainly in attempt to control blood pressure. The lack of clinical trials investigating additional therapies has resulted in limited evaluation of therapies of IgAN such as immunosuppressive medications, and today confusion over correct and optimal treatment remains (2).

The aim of this master thesis is to investigate immunological cellular events of IgAN during different states of disease. More specifically, elements of the innate immune system are the focus of this study. Information about the inflammatory processes of IgAN is to date not adequate for development of more specific treatments of IgAN. The methods of investigation of this study are based on flow cytometry and by that enumeration of subpopulations of monocytes and dendritic cells in healthy individuals and in IgAN patients.

The following chapters of introduction include a further description of IgAN and its pathogenesis. The principles of flow cytometry and the issues and pitfalls associated with this method will also be described, as knowledge in these fields is of great importance in the studies of the present thesis.

IgA Nephropathy

IgA1 O-glycosylation

For years it has been known that an excess of poorly galactosylated IgA1 is present in serum and in glomerular deposits of patients with IgAN (4). O-glycosylation, hence galactosylation, of IgA1 takes place in the Golgi apparatus of B cells and is catalyzed by specific glycosyltransferases, see Figure 1. Little is known about these enzymes, and the lack of knowledge is a hindrance to a better understanding of the O-glycosylation of IgA1 molecules (5). However, it is known, that the IgA1 molecule contains a hinge region composed of 17 amino acids. These amino acids have up to nine potential sites of O-glycosylation, however, all nine sites never simultaneously carry O-glycans. O-glycan chains consist of N-acetylgalactosamine (GalNAc) that is O-linked with one of two amino acid residues, serine (usually) or threonine in the hinge region of IgA1. Additionally, galactose and/or sialic acid may be linked to GalNAc (2) (5). IgA1 monomer molecules that each consist of two α 1 heavy chains, will have significant physiochemical and immunogenic properties by means of the sites of glycosylation and thereby by the type of clustering of the O-linked sugars of the hinge region (6). It is known that by the addition of up to six O-glycan chains the hinge region of human serum IgA1 undergoes co- or post translational modification (7).



Figure 1. The human IgA1 molecule, the hinge region containing nine potential sites of O-glycosylation, and the four forms of O-glycans. A. The IgA1 molecule is composed of two light chains and two heavy chains. The O-glycan sites are in the hinge region of the heavy chains, between the CH1 and the CH2 domains, which is made up by 17 amino acids. B. Serine (Ser) and threonine (Thr) in the hinge region represents the nine possible O-glycosylation sites. C. A stepwise addition of monosaccharides to these sites results in O-glycosylation. The O-glycan chains occur in four different forms; N-acetylgalactosamine (GalNAc) alone, GalNAc extended with sialic acid by the enzyme $\alpha 2,6$ sialyltransferase, GalNAc extended with galactose by core 1 $\beta 1,3$ galactosyltransferase, or the GalNAc-galactose disaccharide can be further sialylated by $\alpha 2,3$ sialyltransferase (6) (5). Figure modified from Barratt and Feehally 2011 (5).

Production of aberrantly glycosylated IgA1 molecules of cell lines from IgAN patients have been confirmed *in vitro* and indicated a decrease in the activity and expression of β 1,3-galactosyltransferase and an increase in α 2,6-sialyltransferase activity and gene expression. This suggested that premature sialylation of GalNAc is likely and that this may contribute to aberrant galactosylation in IgAN (8).

The heterogeneity of IgA1 O-glycoforms contributes to a mixture of IgA1 antibodies present in normal serum. In IgAN, however, aberrantly galactosylated IgA1 is overrepresented in serum as well as in the mesangial deposits (5). The presence of aberrantly O-glycosylated IgA1 in the mesangial cells in IgAN suggests that IgA1 molecules in IgAN abnormally and selectively deposit in the kidneys (9). Upon investigation, a study showed significant increase in O-glycans with GalNAc as the terminal end in serum from IgAN patients compared to healthy individuals, interpreted as an increase in galactose-deficient IgA1 levels compared to the levels of normal serum (4). It has been shown that not only an increase in IgA serum levels and IgA-containing immune complexes is seen in over 50% of IgAN patients, but also an increase in

galactose-deficient IgA1 serum levels of Caucasian, Asian and Afro-American patients has been confirmed (4) (10).

It is possible that the type of O-glycans and their hinge region sites leads to differences in the 3-dimensional structure of IgAI in IgAN and thereby increases pathogenicity of the molecule. Until today, it has not been possible to characterize hinge region galactosylation to an extend that precisely defines the events of O-glycan linking in health and in IgAN. Further typing of the structures of IgA1 hinge regions in IgAN will most likely be a step towards illuminating one of the key features of IgAN (5).

One might suspect the one other O-glycosylated immunoglobulin isotype, IgD, to be influenced as well as IgA1 in IgAN patients. In healthy subjects it has been shown however, that the pattern of the IgD molecule, which is expressed before class-switching as a membrane-bound immunoglobulin on naive B-cells, is normal in patients with IgAN. The fact that IgD and IgA1 molecules are not both aberrantly O-glycosylated suggests that the cells of which enzymes are affected in IgAN do not include the entire B-cell lineage and may be a secondary due to aberrant immunoregulation (5) (11). Possibly, the microenvironment of IgA1 secreting B-cells is of significant importance by influencing post-translational modification of the IgA1 molecule (5). There seems to be high serum levels of aberrantly galactosylated IgA1 in patients after mucosal infection compared to a systemic infection. It is likely, that the events of O-glycosylation in IgAN are an abnormal systemic response to antigens that are mucosally encountered (12).

Immune Complex Formation, Mesangial IgA1 Deposition and Glomerular Injury

Excessive amounts of poorly galactosylated IgA1 is fundamental to the formation of immune complexes in IgAN. However, since complex formation seems to require a second hit by means of formation of glycanspecific IgG or IgA autoantibodies, these IgA1 O-glycoforms probably do not cause IgAN alone. One theory of immune complex formation is that these hinge-region reactive autoantibodies are cross-reactive antimicrobial mucosal IgA and IgG antibodies in serum, that are generated against microbial cell wall carbohydrates (6) (5). These IgA and IgG antibodies recognize the poorly galactosylated hinge region, and immune complexes with IgA1 are formed in situ within the glomerulus or within the circulation (5). It is also hypothesized that IgA1 against mucosal infections appearing in serum further contribute to the amount of autoantibodies by their characteristics of poorly galactosylated hinge regions compared to those of serum IgA1. This is supported by the fact that visible hematuria is associated with episodes with mucosal infections in IgAN patients (6). The clinical onset of IgAN often occur in connection to an upper-respiratory tract infection, and it is therefore suspected that aberrant IgA1 molecoles and the following immune complex formation reflect an abnormal mucosal response to upper-respiratory tract infections (13) (14).

The appearance and overrepresentation of mucosal-type poorly galactosylated IgA1 in IgAN may reflect a displacement or *mis-homing* of mucosally IgGA1-committed B-cells in a way that locates these in systemic compartments. In case of this mis-homing, mucosally primed B-cells secrete their polymeric IgA1 antibodies directly into the circulation instead of into the submucosa (6) (5) (14). Mis-homing may be due to alterations in surface homing receptors on lymphocyte subpopulations that results in the residence of B-cells in the bone marrow (6). Additionally, it is possible, that these B-cells receive different cytokine signals than those of the mucosal-associated lymphoid tissue which promotes the aberrant galactosylation of serum IgA1 molecules in IgAN (6) (5). Findings of decreased numbers of polymeric IgA1-secreting plasma cells at mucosal sites, and increased numbers in systemic sites support the theory of mis-homing (6).

The myeloid Fc-receptor for IgA, CD89, is another potential contributory factor to the formation of immune complexes. Myeloid cells express this receptor in membrane-bound and soluble form. One of the larger soluble isoforms of CD89 has been found in only serum from patients with IgAN and is suspected of promoting immune complex formation. The process of complex formation and the following mesangial deposition is hypothesized to start with binding of polymeric IgA1 to membrane bound CD89 resulting in proteolytic shedding and thereby in circulating immune complexes that accumulates in the kidneys (6) (15). This shedding may explain the previously observed decrease in monocyte expression of CD89 in IgAN patients (15) (16). Currently, mesangial deposits of immune complexes containing CD89 have not been demonstrated, and so it remains unclear whether or not this Fc-receptor is significant in damage of kidney tissue induced by deposition in the mesangial cells (5).



Figure 2. The proposed pathogenesis of IgA nephropathy. B-cells are primed by a mucosal infection to class switch and secrete IgA1. This priming of the B-cells can be induced by stimulation of Toll-like receptors on the B-cell membrane or by cytokines from T-cells (1). After stimulation some of the antigen secreting cells (ASC) mis-traffick into the systemic compartment (2) where secretion of mucosal-type poorly galactosylated polymeric IgA1 takes place (3). Secretion of IgA1 can be augmented if mucosal-derived pathogen-associated patterns enter circulation binds to Toll-like receptors (4). Immune complexes are formed in the circulation and are composed of poorly galactosylated IgA1 combined with IgG and IgA autoantibodies (5a), cross-reactive anti-microbial antibodies (5b) or CD89 that is shed from the surface of myeloid cells (5c). Finally, mesangial immune deposits cause renal injury (6) by a series of events of inflammation. Figure from Boyd et al. 2012 (6).

Mesangial IgA1-immune complex deposition impacts kidney function by induction of several events of inflammation that is followed by histo-pathologic lesions. Also, the IgA1 molecule itself has increased affinity to fibronectin and type IV collagen, and contributes by that to glomerular damage. The lesions occur by several pathways that ultimately can lead to progressive renal failure (6) (5) (17).

According to the Oxford Classification of IgAN, key pathologic events are caused by immune complex deposition (18). These include activation and proliferation of mesangial cells, podocyte injury, and tubulo-interstitial scarring (5). Figure 2 gives an overview of the main pathologic events in IgAN.



Figure 3. Glomerular and tubuluinterstitial injury in IgAN. IgA1-containing immune complexes in the systemic circulation deposit in the mesangial cells. Immune complex deposition activates meangial cells and trigger release of pro-inflammatory and pro-fibrotic mediators. These mediators cause mesangial cell proliferation, damage of podocytes, and synthesis of extracellular matrix (ECM). Cytokines are filtered into the urine and activate, together with immune complexes that pass due to glomerulosclerosis, proximal tubular epithelial cells and promote tubulointerstitial scarring. Figure from Boyd et al. 2012 (6).

Activation of mesangial cells takes place on exposure to immune complexes. Usually, immune complexes bind to the transferrin receptor, CD71, on mesangial cells, and upon activation, the mesangial cells release pro-inflammatory and pro-fibrotic mediators (6) (5). Mesangial cell proliferation and apoptosis is triggered, and upregulation of interleukin-6, interleukin-8, transforming growth factor- β , tumor necrosis factor- α takes place. It is suspected, that different constellations of immune complexes have different effects on mesangial cell function; some may stimulate proliferation while others are inhibitory. Large complexes seem to be more likely to activate the mesangial cells (5).

Most likely podocytes and proximal tubular epithelial cells are constantly exposed to filtered IgA immune complexes in IgAN once glomerular injury is initiated. This may accelerate cellular injury. It is known that both cell types can bind IgA1 immune complexes, however, at this point in time little is known about the specific interaction with immune complexes and the effect of the pro-inflammatory and pro-fibrotic mediators secreted by activated mesangial cells on podocytes and tubular epithelial cells (6).

One key factor that contributes to the progression of glomerular scarring is that podocytes are unable to proliferate upon damage. When podocyte injury occurs, due to the mediators secreted by mesangial cells (see Figure 3) and direct contact with filtered IgA, the foot processes retract and broadens and possibly detaches from the glomerular basement membrane. The integrity of the glomerular filtration barrier is lost, allowing passage of e.g. proteins due to increased barrier pore size. Also, epithelial cells can come into contact with the basement membrane, which may result in glomerulosclerosis (6) (5). Consistent with this, IgAN patients have increased excretion of podocytes in the urine, and the number of podocytes excreted in the urine can provide information about the severity of active glomerular injury and thereby the degree of glomerulosclerosis (19).

Tubulointerstitial scarring takes place when the proximal tubular epithelial cells interact with filtered immune complexes and the mediators derived from mesangial cells. Apparently, exposure of albumin on the proximal tubular epithelial cells activates different signaling pathways, and ultimately matrix proteins and pro-inflammatory and pro-fibrotic mediators are secreted (5).

As shown in Figure 2, Toll-like receptors may be significant in the pathogenesis of IgAN. The IgA1 molecules that deposit in the glomeruli of the kidneys and trigger inflammation have as described features in common with mucosal IgA1. The molecules are mainly polymeric and are directed against microbial and environmental antigens. It is suspected that there is some sort of hyper-responsiveness against this aberrantly galactosylated IgA1 in IgAN and that altered activity of Toll-like receptors may contribute to this (5). The following section describes the basic immune mechanisms involved in glomerular inflammation and injury, including the role of Toll-like receptors in IgAN.

Immune Mechanisms in Glomerular Tissue Injury

Understanding the immunopathogenesis of IgAN may offer the opportunity of development of specific and effective treatments in the future. Not just mesangial cells and podocytes are affected by direct contact with immune complexes in IgAN; the glomerulonephritis and glomerular injury that occurs in IgAN is also the result of activation of innate and adaptive immunity (20) (21). This activation is shown in Figure 4.

The complement system is most likely to become activated in IgAN by pathogen-associated molecular patterns (PAMPS), endogenous cell-derived host ligands called danger-associated molecular patterns (DAMPS) and/or immune complexes, which leads to cleavage of C3 and C5. This cleavage results in release of chemotactic factors, including C5b-9 and C5a. C5a is a factor that attracts circulating inflammatory cells such as neutrophils, macrophages, and platelets that release mediators like growth factors, chemokines cytokines, and eosanoids, and contribute to glomerular damage. C5b-9 is the terminal membrane attack complex, and can initiate pathways in resident glomerular cells, e.g. mesangial cells that become effector cells. These cells produce upon activation, as previously described, cytokines, oxidants and proteases, and upregulate synthesis of extracellular matrix (20).

Stimulation of signaling receptors on innate immune cells by pathogens results in the production and release of cytokines and chemokines. One important group of these signaling receptors is the Toll-like receptors (TLRs). TLRs are one group of four classes of pattern-recognition receptors and are expressed by several cells of the innate and adaptive immune system, including macrophages, dendritic cells and B cells (22) (23). These receptors represent by their induction of antimicrobial peptides an evolutionarily ancient host defense system of the body (22). Activation of mammalian TLRs is based on binding of PAMPs and

DAMPs (see Figure 4). In humans 13 TLRs have been discovered, each devoted to recognizing a distinct set of PAMPs. The repetitive structure of microbial components such as proteins, carbohydrates and lipids are recognizable by mammalian TLRs. One example of this is lipopolysaccharide of the outer membrane of Gram-negative bacteria that is recognized by the innate immune system by means of TLR4 (22) (23).

The location of TLRs varies depending on the type of TLR – some TLRs are expressed on the cell surface while others are located intracellularly in endosome membranes. All TLRs are microbe sensors, but their location has an impact on the route of detection of PAMPs (22) (21) (20). Cell surface receptors bind foreign substances directly from the extracellular space, whereas phagocytosis, receptor-mediated endocytosis or macropinocytosis is required for detection by the intracellular TLRs. TLR-1, TLR-2, TLR-4, TLR-5 and TLR-6 are cell-surface receptors, and TLR-3, TLR-7 and TLR-9 are intracellular receptors (22). Ligand recognition of TLRs is followed by an intracellular signal transduction by adaptor molecules and activation of kinase cascades resulting in translocation of transcription factors to the nucleus. Thereby gene expression is induced, and the production of cytokines takes place. Activation of TLRs not only plays a major part in the innate immune system; they also have an impact on the adaptive immunity by their activation and coordination of T- and B-lymphocyte responses (23).

In evolution, antimicrobial peptides seem to be the earliest defense mechanism against infections and hence, by their induction of just antimicrobial peptide production after pathogen recognition, TLRs seem to be the earliest receptors involved in the defense against infections (22). The relationship between infection and development of IgAN indicates, that TLRs are relevant in the investigation of IgAN pathogenesis (14).



Figure 4. Schematic overview of the major pathogenic sequences in human glomerulonephritis. Both innate and adaptive immune mechanisms are involved in glomerular tissue injury caused by stimulation of the complement system, Toll-like receptors (TLRs), Nod-like receptors (NLRs), and antigen presenting cells by pathogen-associated molecular patterns (PAMPs), e.g. from an upperrespiratory tract infection, and danger-associated molecular patterns (DAMPs). Complement is most likely to become activated in IgAN by PAMPS, DAMPS and/or immune complexes, which leads to cleavage of C3 and C5 resulting in release of chemotactic factors, including C5b-9 and C5a. C5a is a factor that attracts circulating inflammatory cells such as neutrophils, macrophages, and platelets. These cells release mediators (growth factors, chemokines and cytokines, and eicosanoids) that contribute to glomerular damage. C5b-9 is the terminal membrane attack complex, and can initiate pathways in resident glomerular cells, e.g. mesangial cells that become effector cells. These cells produce upon activation, as previously described, cytokines, oxidants and proteases, and upregulate synthesis of extracellular matrix. PAMPS/DAMPS as well as TLRs also stimulate and activate antigen presenting cells. This promotes CD4⁺ helper cell differentiation and antibody production by B cells. The production of antibodies leads to formation of immune complexes in the circulation or in situ, which can further activate the innate immune system by means of complement and TLRs. Helper T cells ($T_H 17$) can damage glomerular tissue directly and attract circulating inflammatory cells ($T_H 1$ and T_H2) (20). Figure modified from Couser 2012 (20).

A study by Coppo et al. in 2009 (21) investigated the expression of TLR-3, TLR-4 and TLR-7 in circulating mononuclear cells of patients with IgAN. TLR-3 and TLR-7 expression and mRNA transcriptional levels were not modified significantly when comparing IgAN patients with healthy controls. However, TLR-4 expression and mRNA levels were significantly elevated in IgAN patients. Also, expression of TLR4 and mRNA levels seemed to be correlated to the state of disease; the levels were significantly higher in patients with very active IgAN (proteinuria and severe microscopic hematuria) compared to patients with inactive IgAN (low proteinuria and absent or minimal hematuria). An association between TLR10 gene polymorphisms and IgAN in Korean children was found in a study by Park et al, 2011 (24). This indicates that genetic variations of TLRs may contribute to the development of IgAN.

The adaptive immune response involved in glomerulonephritis involves antigen presenting cells, helper T cells, and B cells. PAMPS/DAMPS as well as TLRs stimulate and activate antigen presenting cells. This promotes CD4⁺ helper cell differentiation and antibody production by B cells. The production of antibodies leads to formation of immune complexes in the circulation or *in situ*, which can further activate the innate immune system by means of complement and TLRs. Helper T-cells can damage glomerular tissue can be damaged directly by Th17 helper cells whereas Th1 and Th2 helper cells attract circulating inflammatory cells, as shown in Figure 4 (20).

Multicolor Flow Cytometry

Flow cytometric measurements have during the last decades become part of clinical and diagnostic pathology, and are used in research as well, for interrogating the phenotype and characteristics of different cells (25) (26). Currently, this method of analysis provides diagnostic and therapeutic support for clinicians in the treatment of several diseases, malignant as well as nonmalignant. Often, flow cytometry is used for e.g. immunophenotyping of leukemia, counting of leukocytes and monitoring of lymphocyte subpopulations (26). Multicolor flow cytometry can reveal a large amount of biological information regarding identification and functional characterization of complex cell populations, and it is therefore an effective method to analyze several characteristics of cell populations within the immune system (27). In this thesis the main goal is to monitor dendritic cell and monocyte subpopulations and to analyze their differentiation markers in IgAN patients and healthy individuals. In order to insure high quality of the data acquired from flow cytometric measurements proper handling of samples and correct use of the flow cytometer is a necessity. The following section deals with the issues and pitfalls in flow cytometry.

The Principles of Flow Cytometry

Flow cytometry is a technique that allows analysis of individual particles and subpopulations within a heterogeneous suspension. The word "particles" often refer to cells, but may be used for any object of suitable size that flows through the flow cytometer. As these particles flow through the interrogation point of the flow cytometer they are illuminated by lasers. Each cell is registered by detectors that detect size, granularity, and fluorescence (25) (28). Modern flow cytometers are comprised of lasers and a sensing system including optics and detectors/photomultiplier tubes, and a fluidics system plus electronics and computer system, see Figure 5 A (25) (29). The optics are composed of lenses, mirrors and filters that gather and direct light, and the photomultiplier tubes detect the emitted light that enter the filters (28) (29).

Light of specific wavelengths is generated from lasers. The most commonly used is the argon ion laser with an emission wavelength of e.g. 488 nm. Early flow cytometers contained one single laser. However, the demand for staining of many markers in one single experimental setup has led to requirement for distinguishing more colors simultaneously, and hence, to an increasing number of lasers in today's flow cytometers. The more lasers, the greater the capacity will be of detecting different wavelengths of fluorescence. Today, the argon ion laser is often supplemented with lasers such as the red helium neon laser with an emission wavelength of 633 nm. Other lasers are diode lasers or fiber lasers (28).



Figure 5. Instrument and interrogation point overview. A. The components of a flow cytometer are the lasers, the fluidics system, the optics, the detectors, and the electronics and computer system. B. The interrogation point is where the laser beam and the fluidics system meet and provide forward and side scatter characteristics for each cell passing by due to hydrodynamic focusing of the sample. Figures modified from Invitrogen 2012 (29).

The interrogation point of the flow cytometer is where the laser beam and the fluidics system meet. At this point each particle is illuminated, one at a time, due to hydrodynamic focusing of the sample. This hydrodynamic focusing occurs by use of a sheath flow technique in which cells are confined to the center of the sample flow stream by use of sheath fluid that draws the sample and its contents into a stream. This ensures accurate and precise positioning of sample contents and allows cells to pass one by one through the interrogation point (25) (29). Each particle spends approximately 0.2-4 μ s in the interrogation point, and for each laser incorporated in the flow cytometer there is one interrogation point (28). Forward scatter (FSC) and side scatter (SSC) characteristics are provided for each cell passing by the interrogation point, see Figure 5 B (28) (29). Forward scatter is a measure of size; as a particle passes through the laser beam it is illuminated, and light is scattered in a forward direction proportional to the size of the particle, and registered by the photomultiplier tube and translated into a voltage pulse. A lens/obscuration bar located between the interrogation point and the photomultiplier tube block the laser beam itself and allows only scattered light to be detected. The granularity of the cell is reflected by side scatter perpendicular to the direction of the laser beam. When light enters the cell the nucleus and other cell contents it is reflected and refracted in a 90° angle, which is proportional to cell granularity (25) (28) (29). Granulocytes scatter more light to the side than do lymphocytes, partly due to a high extent of granularity, partly because of their irregular nuclei compared to the more spherical nuclei of lymphocytes (28). The distribution of monocytes, lymphocytes and granulocytes in a FSC/SSC plot is shown in Figure 6.



Figure 6. Distribution of leukocytes into lymphocytes, monocytes and neutrophils in a forward scatter/side scatter plot that shows size and complexity (granularity), respectively. Figure from Invitrogen 2012 (29).

Analysis of Fluorescence

Multicolor flow cytometry and measurements of e.g. marker expression, enzyme activity and DNA content can be obtained by analysis of fluorescence emissions (25). Fluorescent dyes conjugated to monoclonal antibodies can be used in the analysis of receptor expression on the cell surface or on extracellular components, and in this manner be a read-out for the amount of antigen on or within a non-fluorescent cell (25). In this way individual characteristics of a large number of cells can be obtained from one suspension, and in a relatively short amount of time (28).

Selection of fluorochromes for an experimental setup should be based on the type and number of lasers in the flow cytometer. Different fluorochromes are excited at different wavelengths, and so the lasers are of great importance in the selection of proper fluorochromes. Also, the combination of mirrors and filters have to be compatible with the choice of colors to ensure detection of all fluorochromes (28) (30). The optical filters that are located between the lasers and the photomultiplier tubes ensure that only appropriate wavelengths are detected by the photomultiplier tubes. In this way, different colors of emitted light will be detected by specific photomultiplier tubes due to different filters inside the instrument (28). The laser emission wavelengths, filters and fluorochromes of the BD FACSCanto[™] flow cytometer used in the experimental setup of this thesis are pictured in Figure 7.



Figure 7. Overview of the laser emission wavelengths, filters and fluorochromes of the BD FACSCanto[™] flow cytometer used in the experimental setup of this thesis. Blue light of 488 nm is emitted from an argon ion laser, which excites the fluorochromes FITC, PE, PE-Cy5 and PE-Cy7. Red light of 635 nm from a second laser excites APC and APC-Cy7 fluorochromes. The filters are marked as pillars in colors matching the color of the emitted light from the fluorochromes that are passed to the photomultiplier tubes. Figure from BD Biosciences 2012 (45).

In the last decade great advances has been made in the analysis of fluorescence; partly due to availability of high-performance instrumentation by means of additional laser and detector options, partly due to advances in biochemistry regarding the increased number of fluorochromes available. This has led to the opportunity to simultaneously analyze an increased number of parameters in one single experiment, and by that increased the usefulness of flow cytometry. However, these advances have also led to higher demands to ensure proper experimental setup and quality of the results generated from multicolor flow cytometry (27). The following chapter deals with spectral overlap and compensation, as this is essential to acquisition of high quality data.

Spectral Overlap and Compensation

For each fluorochrome used in multicolor flow cytometry there is an emission spectrum. In an experimental setup with multiple fluorochromes physical spectral overlaps occur because parts of their emission spectra are at the same wavelengths. One example of this is the emission spectrum for fluorescein isothiocyanate (FITC) that overlaps with the emission spectrum of phycoerythrin (PE), see Figure 8. This means that, without compensation in which the FITC spillover is subtracted from the PE emitted light, some of the light emitted by FITC will be transmitted to the PE detector and enter the photomultiplier tube for PE, hence be registered as light emitted by PE. Likewise, the small amount of PE spillover into the FITC detector will have to be subtracted from the emitted light from FITC (25) (27).



Figure 8. Spillover of FITC into the PE channel. Figure from BD Biosciences 2012 (27)

Compensation of all fluorochromes in an experimental setup is crucial for achieving proper visualization and analysis of data. Without a proper compensation setup data interpretation becomes difficult or impossible, as visualization and gating of populations will be influenced by the spillover of fluorochrome emission spectra (25) (27) (30). Figure 9 illustrates the impact of proper compensation, under-compensation, and over-compensation of FITC spillover on the median fluorescence intensity of PE.



Figure 9. An example of properly compensated and incorrectly compensated samples. The left graph is a visual demonstration of proper compensation calculated by FACSDiva software; the unlabeled population is negative for both FITC and PE, whereas the FITC labeled population is positive for FITC only. Median fluorescence intensities of PE for the unlabeled and the FITC labeled population are equal. The middle graph is an example of under-compensation seen by elevated FITC labeled population, hence elevated median fluorescence intensity of the PE (245) compared to the properly compensated graph. The right graph shows how over-compensation influences the FITC labeled population and decreases median fluorescence intensity of PE to -54. Figure from BD Biosciences 2012 (27).

To correct for spillover and to create a compensation matrix the spectral overlap values must be measured for every fluorochrome used and in all detectors. This is done via single-color control samples by which every single spillover value is obtained in comparison to an unstained, negative control. These single-color control samples may be with cells of the experiment that bind the fluorochrome-conjugated antibody or by commercially available beads that bind antibodies independently of antigen expression. Since compensation is fluorochrome dependent, and not cell type dependent, compensation controls do not have to be based on the tissue used in the experiment. However, to ensure a similar auto-fluorescence of positive and negative populations it is important to use the same cell type/bead type in the positive and negative control samples (27).

Securing High Quality Data

Besides proper compensation to ensure high quality of the results generated from flow cytometric measurements it is important to have guidelines and quality controls regarding instrument handling and methodological procedures (26). In this chapter the main issues of the use of a flow cytometer and the potential problems in the processing of specimens for immunophenotyping will be described.

Calibration of the Instrument and Processing of Samples

It is recommended to calibrate the flow cytometer on a daily basis. Usually, this calibration is performed using commercially available fluorescent beads. Calibration allows monitoring of laser and detector function and sensitivity (26).

The way samples for analysis are processed has great impact on the acquired flow cytometric data. First of all, specimens should be as fresh as possible, especially when quantitative expression of antigens of lymphocyte subpopulations is made (31). The choice of anticoagulant is of great importance. For analysis of lymphocytes sodium heparin is recommended, since other anticoagulants compromise the functional capacity of this cell type (32). Lysis of red cells and/or cell separation procedures must be the same if data from different studies are to be compared since different approaches cause analytical variation. Furthermore, over-lysis can lead to altered FSC and SSC cell properties, affect fluorochrome emission spectra, and lead to a loss of nucleated cells, while consistent identification of populations and gating becomes difficult in poorly lysed specimens. FSC and SSC characteristics may also be changed in cases of excessive centrifugation. Conversely, too gentle centrifugation will increase cell loss during washing steps (26).

Proper vortexing of samples can prevent tube-to-tube variation. Over-vortexing may cause an increase in cell debris. Cell doublets may occur in under-vortexed samples, and these doublets will, due to their characteristics, most likely be excluded from the populations on FSC/SSC plots during flow cytometric analysis. Due to the escape from gates these doublets are also named "escapees" (26) (33). During preparation of bead-based samples for absolute counting an electrostatic charge on the tube can be induced by excessive vortexing. This results in beads sticking to the wall of polystyrene tubes, and thereby this method of absolute cell counting becomes void. Addition of proteins to the tubes before vortexing can prevent this phenomenon and secure credibility of counting beads (34).

Reagent Selection

The binding site of monoclonal antibodies to antigens varies between clones, hence, different monoclonal antibodies can react with different epitopes on the antigen molecule. For this reason each antibody of an experimental setup panel for immunophenotyping in flow cytometry must throughout the experiment period be from the same clone. Otherwise the detected expression of antigen may vary between analyses and cannot be compared properly (26). Isotype controls are often used as negative control samples of irrelevant specificity that allow establishment of a reference point of nonspecific fluorescence intensity of the samples. In this way variations in nonspecific binding of the antibodies can be taken into account, and the extent of Fc receptor binding of antibodies on cells can be evaluated. The isotype control antibody must have the same properties of the antibody with binding specificity by means of subclass, fluorochrome

conjugate, concentration, and manufacturer to provide a proper measure of baseline background fluorescence (35). In multicolor flow cytometry, the all-minus-one method is recommended when defining negative populations. In the all-minus-one approach, negative cell populations are defined in samples that are stained with all antibodies except for the one in question. This is done for each antibody of the complete panel. The theory behind this approach is that background fluorescence of unstained cells is different from that of cells stained with multiple antibodies, despite of proper compensation (36).

Polystyrene tubes have properties that make monocytes adhere to the surface of this material (37). Hence, for the purpose of flow cytometric enumeration of monocytes the use of this type of material for sample preparation should be minimized.

In order to reduce spillover the choice of color/fluorochrome should be matched to each antibody of the setup panel. Some antigens may be dimly expressed while others are brightly expressed. It is therefore critical to the analysis of data that dimly expressed antigens are stained with bright colors and vice versa to ensure proper detection of the antigen. A bright color (e.g. FITC) used for staining of a highly expressed antigen may cause spillover that prevents definition of a population with weakly expressed antigens (e.g. stained with PE). Even with proper compensation this must be taken into account when designing the antibody panel of an experimental setup. The use of a more dim color (e.g. PerCP) to stain the highly expressed antigens may reveal the dimly expressed PE stained population and is thereby a more proper choice of color (26) (27) (30).

When working with tandem dyes special consideration must be taken. The spectral overlap values of tandem dyes vary from lot to lot, and this leads to the need for each lot of a particular tandem dye to have its own compensation control (27). Furthermore, tandem dyes are subject to degradation when exposure to light, high temperatures and formaldehyde-based fixatives (27) (30). When tandem dyes degrade they will emit light mainly from the parent dye (e.g. PE in PE-Cy7) which may result in false positive events in the detector of the parent dye (30). For these reasons samples must be analyzed soon after staining and be exposed to a minimal amount of light, and exposure to fixatives should be minimized to increase stability of the dye (27) (30).

Data Acquisition and Data Analysis

When acquiring data, the FSC/SSC plot should be adjusted to facilitate visualization of the cells of interest. The number of events required varies depending on the type of cell population to be analyzed. For lymphocyte analysis at least 5,000 events should be acquired. Given that data derive from gating of different populations the gate should be precise and consistent between samples. Relevant cells will be excluded if the gate is too tight, and falsely low results may be obtained when the gate is too big (26).

The type of display chosen during analysis should match the type of marker in the analysis. Linear displays show a limited range of fluorescence intensities, while logarithmic graphs axes display a larger range. When analyzing DNA content linear displays of fluorescence intensity is appropriate as the increase in intensity is small relative to e.g. an increase in protein expression, which may require the larger range of a logarithmic display (28).

In summary, the great advances that have been made over time in the field flow cytometry have led to great opportunities for analysis of a wide range of cellular molecules in one single sample. As described

above, there are many variables to consider in immunophenotyping by flow cytometry, all of which are essential for acquisition of reliable data. However, correct instrument use and sample preparations provide fast and efficient analysis of cells compared to other methods.

The Aim of the Study

Since the production of affinity matured autoantibodies against aberrantly glycosylated IgA1 is supposed to be an important part of the etiology of IgAN (6) (5), dendritic cells are mostly likely involved in the activation of T helper cells supporting the B cells producing the antibodies, or perhaps even directly implicated in activating these B cells. Furthermore, it is quite clear that cells from the monocyte/macrophage system are heavily involved in the pathogenesis of the disease. It therefore seems rational to carry out a detailed analysis of the subpopulations of dendritic cells and monocytes in the blood of IgAN patients. This is an investigation which to our knowledge has not been done before.

Inspired by a nomenclature published in 2010 (3) for dendritic cells and monocytes in human, enumeration was based on staining of the following cells;

- Myeloid CD1c⁺ dendritic cells in blood
- Plasmacytoid CD303⁺ dendritic cells in blood
- Myeloid CD141⁺ dendritic cells in blood
- Non-classical CD14⁺ CD16⁺ monocytes in urine and blood
- Classical CD14⁺⁺ CD16⁻ monocytes in urine and blood
- Intermediate CD14⁺⁺ CD16⁺ monocytes in urine and blood

As recent investigations suggest that the expression of TLR receptors, especially TLR4 (21), on immune cells are important, and since it could also be very interesting to evaluate the state of activation/maturation of the monocytes and dendritic cell subpopulations, investigation of TLR4 and HLA-DR expression of the listed cell types was also aim of present study.

Since very little information on the listed subpopulations of monocytes and dendritic cells exist, it was intended to study a group of healthy individuals in parallel to the investigations of patients. In present study, blood and urine samples from outpatients were analyzed, in part to investigate whether or not patient samples showed different cellular events than control subjects, in part to investigate if the activity of disease correlates with observations from the study.

The Course of Experiments

The first months of experiments included optimization of protocols based on blood samples from volunteers. Considerations were made regarding the setup of a proper compensation for experiments, washing steps, and incubation periods. Compensation was influenced by the fact that dendritic cell enumeration was achieved by use of a commercially available antibody kit that contained an antibody cocktail of all antibodies of interest. The use of compensation beads allowed compensation of all colors of the experimental setup antibody panel, without being dependent of cells with binding capacity for each antibody. Unspecific binding of isotype controls for TLR4 antibody was evaluated throughout the course of the experimental period. Blood samples and urine samples of patients were obtained in the period of April to June, 2012.

Materials and Methods

Subjects

Peripheral blood was obtained by venipuncture from two IgAN patients and two control subjects and collected into sodium heparin vacuum tubes (Greiner Bio-One #456080 or Sarstedt #01.1613.100, respectively). Urine samples were transferred into sodium heparin vacuum tubes as well immediately after collection.

Patient 1 was a 20 year old man with a medical history of microscopic hematuria and proteinuria at the time of diagnosis and persisting proteinuria. Blood pressure of patient 1 was normal at the time of sample collection, and he received treatment with ACE inhibitors. Patient 2 was a 27 year old woman, diagnosed in 2006 with symptoms of hypertension, proteinuria (2 g/day) and a serum creatinine level of 170 µmol/l. Despite treatment with ACE inhibitors and angiotensin II receptor antagonists, kidney function declined in time. One episode of urinary tract infection had occurred. Patient 2 were tonsillectomized. Both patients were diagnosed by kidney biopsy. Exclusion criteria of patients were diabetes, dialysis treatment, and malignant disease. Control subject 1 was a 33 year old woman, while control subject 2 was a man of the age of 24. Both control subjects were healthy individuals and had no medical history of hypertension, or kidney problems.

The study was approved by the North Denmark Region of The National Committee on Health Research Ethics (Appendix A) and by The Danish Data Protection Agency (Appendix B). Signed informed consent was obtained from all participants.

Patient 1 was tested four times in the course of the experimental period, more specifically at day 1, day 21, day 36, and day 68. Patient 2 was tested at day 21, 36, and day 68 of the period. Control subjects were tested three times during the period, more specifically at day 42, 49, and 62.

Flow Cytometric Analysis

Data acquisition and flow cytometric analysis of blood and urine dendritic cells and monocytes were performed on a BD FACSCanto[™] flow cytometer (Becton Dickinson, USA). The flow cytometer was equipped with two lasers; a red laser with excitation laser line of 633 nm, and a blue laser with excitation laser line of 488 nm, providing the capability of 6-color analysis. The software used for data acquisition was FACSDiva[™] 5.0.3, and for further data analysis FlowJo 7.5 software was used. Instrument calibration was performed daily by use of BD FACS 7-Color Setup Beads (BD Biosciences #335775) according to the manufacturer's instructions.

Compensation

In order to compensate for spectral overlaps of the antibody panel in the experimental setups BD[™] CompBeads (BD Biosciences #552843) and FACSDiva[™] Software 5.0.3 was used. According to manufacturer's protocol both antibody-binding beads and negative control beads were added to the same tube and stained with the appropriate fluorochrome. This was done for each fluorochrome included in the setup (PE, FITC, APC, APC-Cy7, PE-Cy7, PE-Cy5), see Figure 7.The FACSDiva[™] Software compensation program then allowed us to create a compensation matrix for future use by means of gating on positive and negative populations for each fluorochrome. The compensation matrix generated from the setup is

shown in Appendix C, and positive and negative populations from the compensation tubes can be seen in Appendix D.

When possible, staining of CompBeads was made with the antibodies used in the experimental setups to prevent variations in fluorescence emission values between the compensation and the experimental setups. However, due to limitations of commercially available single antibodies (compared to commercially available antibody cocktails), some of the fluorochromes included in the compensation was conjugated to monoclonal antibodies that were not used in the following experiments. The fluorochrome conjugated antibodies used in the compensation are listed in Table 1.

Antibodies for Staining of BD[™] CompBeads HLA-DR APC-Cy7 (BD Biosciences #335831) TLR4 Pe-Cy7 (eBioscience #25-9917) CD14 FITC* (Thermo Scientific Pierce Products #MA1-19561) CD16 PE* (BD Biosciences #560995) CD80 Pe-Cy5** (BD Biosciences #559370) CD83 APC** (BD Biosciences #551073)

Table 1. Overview of the monoclonal antibodies used in the compensation of the different spectral overlaps of antibodies. *Manufacturer of antibody different from antibody used in dendritic cell enumeration in whole blood. ** Manufacturer of antibody different from antibody used in experimental setups.

Determination of Absolute Leucocyte Counts in Blood

For determination of absolute counts of leucocytes in blood BD TruCountTM Tubes (BD Biosciences #340334) were used. According to the manufacturer's protocol 50 μ L of well-mixed anticoagulated blood was transferred to the TruCountTM Tubes with reverse pipetting technique to ensure a precise amount of volume. Smearing of blood down the side of the tube was avoided. Some tubes were stained with 10 μ L anti-CD14-FITC (Thermo Scientific Pierce Products #MA1-19561) and 7.5 μ L CD16-PE (BD Biosciences #560995). Some tubes were left unstained. The tube was then vortex'ed gently to mix and incubated for 15 minutes in the dark at room temperature. After incubation, 450 μ L of lysing solution was added and incubated for 15 minutes. In one setup ACK lysing buffer (4.15 g NH₄Cl, 0.5 g KHCO₃, 18.63 mg EDTA-Na₂*2H₂O, and 50 mL Milli-Q H₂O) was used, in another BD FACS lysing solution (BD Biosciences #349202) (recommended by BD Biosciences for BD TruCountTM Tubes) was used. The BD FACS lysing solution had expired. The sample was then analyzed on the flow cytometer.

Dendritic Cell Subset Enumeration in Whole Blood

Samples for dendritic cell subset enumeration were prepared by use of a kit for enumeration of human dendritic cells (Miltenyi Biotec #130-091-086). Whole anti-coagulated blood was transferred to polypropylene tubes and stained with 20 µL anti-BDCA cocktail (from kit) containing anti-CD303-FITC, anti-CD1c-PE, anti-CD14-PE-Cy5, anti-CD19-PE-Cy5 and anti-CD141-APC antibody. This anti-BDCA cocktail was supplemented with anti-HLA-DR-APC-Cy7 antibody (BD Biosciences #335831) and anti-TLR4-PE-Cy7 antibody (eBioscience #25-9917), in total named "DC antibody cocktail". Likewise, an isotype control tube was prepared with control cocktail (from kit) containing FITC-, PE-, PE-Cy5-, and APC-conjugated isotype control antibodies. The control cocktail was supplemented with APC-Cy7- (BD Biosciences #557751) and PE-

Cy7-conjugated isotype control (eBioscience #25-4724) antibodies. Dead Cell Discriminator (from kit) was added, each sample was mixed gently and samples were incubated on ice for 10 minutes in a horizontal position under a 60 W light bulb (distance of 3-5 cm). After incubation on ice, red blood cells were lysed by addition of 4 mL 1X Red Blood Cell Lysis Solution (from kit) to each sample followed by incubation for 10 minutes in the dark. After lysing, cells were washed twice by centrifuging the samples (300g, 5 minutes, room temperature), removing supernatant completely and resuspending in 4 mL washing buffer (1xPBS buffer (Life Technologies #70011) with 0.5% bovine serum albumin (Sigma-Aldrich #A2153) and 0.01% sodium azide). Finally, samples were spun down (300g, 5 minutes, room temperature), supernatant was removed and cells were resuspended in 300 μ L washing buffer. Additionally, 150 μ L fixing Solution and 5 μ L Discriminator Stop Reagent (both reagents from kit) was added to each sample. Samples were analyzed within 2 hours and stored in the dark at 4-5°C until analysis. Immediately before analysis samples were vortexed and transferred to polystyrene tubes appropriate for the BD FACSCantoTM instrument.

Dendritic Cell Subset Enumeration in 300 μ l Whole Blood		
DC antibody cocktail:	Control Cocktail:	
20 μL Anti-BDCA Cocktail (kit)	20 µL Control Cocktail (kit)	
- CD303 FITC	- Mouse IgG1 FITC	
- CD1c PE	 Mouse IgG2a PE 	
- CD14 PE-Cy5	- CD14 PE-Cy5	
- CD19 PE-Cy5	- CD19 PE-Cy5	
- CD141 APC	 Mouse IgG1 APC 	
10 μL anti-HLA-DR APC-Cy7	10 μL isotype APC-Cy7	
10 μL anti-TLR-4 PE-Cy7	10 μL isotype PE-Cy7	

Table 2. Overview of the monoclonal antibodies used in the dendritic cell subset enumeration of whole blood. Prior to analysis each antibody was titrated in order to find optimal concentration for staining of cells.

Monocyte Subset Enumeration in Whole Blood

For monocyte subset enumeration, whole anticoagulated blood was transferred to polypropylene tubes. One tube was incubated with mouse anti-CD14-FITC (Thermo Scientific Pierce Products #MA1-19561), CD16-PE (BD Biosciences #560995), HLA-DR-APC-Cy7 (BD Biosciences #335831), and TLR4-PE-Cy7 (eBioscience #25-9917) antibodies (named Monocyte antibody cocktail) at 4 °C in the dark for 30 minutes, and one was incubated with FITC- (Thermo Scientific Pierce Products #SA1-12183), PE- (BD Biosciences #555749), APC-Cy7- (BD Biosciences #557751) and PE-Cy7-conjugated (eBioscience #25-4724) isotype antibodies (control cocktail) at 4 °C in the dark for 30 minutes. After incubation with antibodies red blood cells were lysed with 2 mL 1X ACK lysing buffer for 8 minutes at room temperature. The cells were then spun down (300g, 5 minutes, 4°C), supernatant were completely removed, and cells were resuspended in 4 mL washing buffer (1xPBS buffer (Life Technologies #70011) with 0.5% bovine serum albumin (Sigma-Aldrich #A2153) and 0.01% sodium azide). This washing step was repeated once. Cells were then spun down (300g, 5 minutes, 4°C), supernatant was removed, and cells were resuspended in 450 μ L fixing buffer (PBS with 1% formaldehyde). Samples were analyzed within two hours and stored in the dark at 4-5°C until analysis. Immediately before analysis samples were vortexed and transferred to polystyrene tubes appropriate for the instrument.

Monocyte Subset Enumeration in 100 µl Whole Blood		
Monocyte Antibody Cocktail:	Control Cocktail:	
15 μL anti-CD14 FITC	15 μL isotype FITC	
20 μL anti-CD16 PE	20 μL isotype PE	
5 μL anti-HLA-DR APC-Cy7	5 μL isotype APC-Cy7	
5 μL anti-TLR4 PE-Cy7	5 μL isotype PE-Cy7	

Table 3. Overview of the monoclonal antibodies used in the monocyte subset enumeration of whole blood. Prior to analysis each antibody was titrated in order to find optimal concentration for staining of cells.

Monocyte Subset Enumeration in Urine

Different approaches were made to make the optimal sample preparation for analysis of urine leukocytes, including monocytes. The first approach was as follows; 6 mL of urine was spun down and (300 g for 5 minutes, room temperature), supernatant was removed, and cells were resuspended in 2 mL of human AB serum in a polypropylene tube. This suspension was then centrifuged (300 g for 5 minutes, room temperature), supernatant removed, and cells resuspended in the residual amount of serum. Afterwards, cells were stained with the same monoclonal antibodies and in same concentrations as for the whole blood monocyte enumeration (see Table 3). The sample was transferred to a polystyrene tube and then analyzed on the flow cytometer.

The second approach to staining urine leukocytes was more extensive than the first approach. 12 mL of urine was concentrated by centrifugation and pellet was resuspended in 8 mL media (RPMI 1640 (Invitrogen #52400) with and 20 μ g/mL penicillin-streptomycin (Ampliqon #AMPQ40133.0005) and 10% AB-serum. Mononuclear cells were then isolated by use of 5 mL LymphoprepTM (Axis-Shield #NYC-1114544).

After gradient centrifugation, the interface containing mononuclear cells were harvested by use of a Pasteur pipette. The harvested fraction was then washed twice in 1xPBS (Life Technologies #70011) with x 1 mM EDTA, and once in washing buffer. Subsequently, cells were stained in polypropylene tubes with stained with the monocyte antibodies for 30 minutes (as in Table 3). Cells were then washed twice in washing buffer (1xPBS buffer (Life Technologies #70011) with 0.5% bovine serum albumin (Sigma-Aldrich #A2153) and 0.01% sodium azide).

In the third approach, additionally to the steps of the second approach, anti-CD45 PE-Cy5 antibody (BD Biosciences #560974) was used to stain cells. Table 4 gives an overview of the antibodies used in the third approach of urine cell flow cytometric analysis.

Monocyte Subset Enumeration in 12 mL Urine Sample		
Monocyte Antibody Cocktail:	Control Cocktail:	
15 μL anti-CD14 FITC	15 μL isotype FITC	
20 μL anti-CD16 PE	20 μL isotype PE	
5 μL anti-HLA-DR APC-Cy7	5 μL isotype APC-Cy7	
5 μL anti-TLR4 PE-Cy7	5 μL isotype PE-Cy7	
10 μL anti-CD45 PE-Cy5	10 μL anti-CD45 PE-Cy5	

Table 4. Overview of the monoclonal antibodies used in the second approach of the monocyte subset enumeration of urine samples.

Gating Strategies

For Determination of absolute leucocyte counts in blood by use of BD TruCountTM Tubes the gating strategy was to determine the number of beads acquired and the number of leukocytes by means of a bead gate and a monocyte, leukocyte and granulocyte gate. According to protocol, beads would locate as a separate population to the right of granulocytes in a FSC/SSC plot. Absolute count (number of cells per μ L of blood) could then be calculated by the equation;



For blood dendritic cell enumeration several gating steps were performed as described by the manufacturer. An acquisition treshhold was set to exclude the majority of debris and blood platelets. Firstly, the region P1 included all visible leucocytes in the FSC/SSC plot, see Figure 10. Acquisition was performed till 1,000,000 evens were acquired in the P1 region. Gated on P1, T cells including dendritic cells were found by a SSC/Pe-Cy5 plot and defined by the P2 region. By this step B cells, granulocytes and monocytes were excluded. Lastly, and CD141⁺ dendritic cells was found within the P2 region by gating on a FITC/PE plot (CD303⁺ and CD1c⁺ dendritic cells) and a FITC/APC plot (CD141⁺ dendritic cells).



Figure 10. The gating strategy of blood dendritic cell subtype analysis. At the top left: Leukocyte gate (P1) defined by FSC/SSC plot. At the top right: Granulocytes, dead cells, monocytes and B cells were excluded by a SSC/CD14 CD19 Pe-Cy5 plot by means of a lymphocyte gate (P2) including T cells and dendritic cells. At the buttom left: CD1c⁺ (P3) and CD303⁺ (P4) cell populations were found by the CD303 FITC/CD1c PE plot. At the buttom right: The CD141⁺ subset (P5) of dendritic cells were defined by gating on a CD303 FITC/CD141 APC plot. Plots acquired from FACSDiva[™] Software.

For use in calculations of the blood concentration of dendritic cell subpopulations, a gate around the lymphocytes with B cells included was needed. This was done in a FSC/SSC plot and ensured, that B cells were included in the lymphocyte population used for calculations of blood dendritic cell concentrations, see Figure 11.



Figure 11. Gating strategy for lymphocyte gate used in blood concentration calculations. To ensure proper calculations of blood dendritic cell concentrations, a lymphocyte gate including B cells was used.

The blood monocyte enumeration was performed by a leukocyte gate (P1 region) and a monocyte gate (P2 region), both defined in the FSC/SSC plot, see Figure 12. The three subtypes of monocytes were found by changing the parameters of the P2 region to a FITC/PE plot, allowing division of the cells according to CD14 and CD16 expression. An acquisition threshold was set, and acquisition was performed until 10,000 evens or more were acquired in the P2 region.



Figure 12. The gating strategy of blood monocyte subtype analysis. Left plot: Monocyte gate (P2) defined by FSC/SSC plot. Right plot: Nonclassical (Q1), intermediate (Q2) and classical monocytes (Q4) were found by their expression of CD14 and CD16 in a CD14 FITC/CD16 PE plot. Plots acquired from FACSDiva[™] Software.

For investigation of monocytes in urine samples the first gate (P1) was set on CD45 positive cells in a FSC/CD45 plot (possible with the third approach of urine cell sample preparation). Subsequently, the
monocyte population was identified (P2) in a FSC/SSC plot of the P1 region. In cases of low numbers of cells in the P1 region monocytes could not be defined. Provided that the samples were prepared without the addition of CD45 (second approach) the P2 monocyte gate was made from the FSC/SSC plot directly, see Figure 13.



Figure 13. The gating strategy of urine monocyte analysis. Monocyte population (right picture) was located in a FSC/SSC plot (left picture) when samples was prepared by the second approach. Plots acquired from FlowJo[™] software.

The populations from the flow cytometric analysis (dendritic cells and monocytes in whole blood and monocytes in urine) allowed analysis of their expression of HLA-DR and TLR4. Histogram overlays of APC-Cy-7and PE-Cy7, respectively, was made (see Figure 14 and Figure 15), and the difference between median fluorescence intensity for antibody and its isotype control was calculated.





Figure 14. Histogram overlay of APC-Cy7 fluorescence intensity of monocytes stained with the monocyte antibody cocktail (orange curve) and monocytes stained with the isotype cocktail (blue curve). Y-axis: percent of total monocyte count in the sample. Data from control subject 1, day 21, FlowJo[™] software.

Figure 15. Histogram overlay of PE-Cy7 fluorescence intensity of monocytes stained with the monocyte antibody cocktail (orange curve) and monocytes stained with the isotype cocktail (blue curve). Y-axis: percent of total monocyte count in the sample. Data from control subject 1, day 21, FlowJo[™] software.

Absolute Leucocyte Differential Counts

White blood cell differential counts were performed at The Department of Urology, Aalborg Hospital.

Results

Absolute Leucocyte Counts by Flow Cytometry and Absolute Leucocyte Differential Counts

One of the main objectives of the present study was to quantitate subpopulations of dendritic cells and monocytes in the blood of IgAN patients and control subjects. In addition to the relative quantity of these cell types, provided by standard flow cytometry, it was also an aim to determine the exact number of each cell type per ml of blood. For this purpose, a defined volume of blood was added to a tube containing a known number of BD TruCount[™] beads prior to analysis. However, the use of BD TruCount[™] beads to obtain absolute leucocyte counts was not successful. The problem was that normal, well defined populations of leucocytes could not be identified in FSC/SSC dotplots when using the BD TruCount[™] tubes. Assuming this could be due to a problem with the lysing of erythrocytes, two different lysing solutions were tried, but this did not solve the problem, see Figure 16.



Figure 16. FSC/SSC plots of BD TruCount[™] tubes. A. Red blood cells lysed with ACK lysing buffer. B. Red blood cells lysed with BD FACS Lysing Solution recommended by the manufacturer of the TruCount[™] tubes. Graphs are generated from FlowJo[™] software.

As an alternative, absolute differential cell counts of patient samples were provided by the Department of Urology, Aalborg Hospital (see Table 5), and percent wise distribution within parent gate, the concentration of monocyte and dendritic cell subpopulations in whole blood was calculated (see Table 6).

Patient 1					Patient 2				
Visit no.	Leucocyte count (x10 ⁹ /L)	Lymphocyte count (x10 ⁹ /L)	Monocyte count (x10 ⁹ /L)	Visit no.	Leucocyte count (x10 ⁹ /L)	Lymphocyte count (x10 ⁹ /L)	Monocyte count (x10 ⁹ /L)		
1	5.9	1,91	0,44	1	5.9	1,19	0,24		
2	7.8	2,54	0,52	2	6.8	1,51	0,1		
3	6.2	2,57	0,42	3	8.6	3,08	0,17		
4	8.2	3,37	0,63	-	-	-	-		

Table 5. Absolute leucocyte differential counts (leucocytes, lymphocytes and monocytes) provided for the each visit of the two patients.

Subject Visit no.	CD1c ⁺ DC (x10^7/L)	CD303 ⁺ DC (x10^7/L)	CD141 ⁺ DC (x10^7/L)	Non-classical mo. (x10^7/L)	Intermediate mo. (x10^7/L)	Classical mo. (x10^7/L)
Patient 1 Visit 1	3,262	0,302	0,013	2,455	4,302	35,786
Patient 1 Visit 2	3,985	0,109	0,023	2,170	6,621	41,645
Patient 1 Visit 3	5,996	0,149	0,008	2,194	1,434	37,249
Patient 1 Visit 4	6,558	0,192	0,007	4,436	1,928	51,271
Patient 2 Visit 1	3,739	0,048	0,007	0,157	2,303	20,370
Patient 2 Visit 2	3,532	0,068	0,003	0,159	0,547	8,948

Table 6. The calculated concentrations of dendritic cell and monocyte subtypes in whole blood from patient 1 and patient2. Concentrations are calculated from the values of the absolute leucocyte differentiation count and the values of distribution within the parent gate (lymphocyte gate with B-cells included and monocyte gate, respectively).

The absolute leucocyte differential count showed the concentration of leucocytes, lymphocytes, and monocytes in blood of the patients. Graph 1 illustrates the blood concentration of leucocytes. Patient 1 had an increase in leucocyte blood concentration at the time of the second measurement, then the concentration decreased at third measurement. Both patients had an increase in leucocyte blood concentration towards the end of the period. The greatest difference between patients was seen at day 21.



The blood lymphocyte concentration was higher in patient 1 compared to patient 2, see Graph 2.



Graph 2. Blood lymphocyte counts of the two patients (absolute leucocyte differential count). Data only available for the time points indicated by symbols. Dashed lines connect the symbols of each patient for the sake of clarity, only.

The blood concentration of monocytes was low in patient 1, see Graph 3. The monocyte concentration of patient 1 decreased between the first and the last measurement. The lowest value was seen at the second measurement. Patient 2 showed an overall increase in the concentration of blood monocytes during the period. At the third day of testing the lowest value was seen.



Graph 3. Blood monocyte counts of the two patients (absolute leucocyte differential count). Data only available for the time points indicated by symbols. Dashed lines connect the symbols of each patient for the sake of clarity, only.

Blood concentrations of non-classical, intermediate, and classical monocytes of patient 1 was all higher than those of patient 2 in all the days of testing (see Graph 4 to Graph 6). The non-classical monocyte blood concentration was increased in both patients at the time of the final test with the greatest increase seen for patient 1. Until day 36 the values were rather stable in both patients.

In patient 1 a decrease in intermediate monocyte concentration occurred at day 36 compared to the value of day 21. Patient 2 also had a lower value of day 36 compared to day 21. The difference between patients was greatest at day 21, and smallest at day 36, see graph 5.





Graph 4. Blood concentration of non-classical monocytes of the two patients. Data only available for the time points indicated by symbols. Dashed lines connect the symbols of each patient for the sake of clarity, only.

Graph 5. Blood concentration of intermediate monocytes of the two patients. Data only available for the time points indicated by symbols. Dashed lines connect the symbols of each patient for the sake of clarity, only.

As seen in Graph 6, the concentration of classical monocytes of patient was elevated compared to patient 2. The development of concentrations of patient 2 was similar to that of the intermediate monocytes.



Graph 6. Blood concentration of classical monocytes of the two patients. Data only available for the time points indicated by symbols. Dashed lines connect the symbols of each patient for the sake of clarity, only.

Dendritic cell subset concentrations of blood are illustrated in Graph 7 to Graph 9. As seen with the monocyte subpopulations, the values of CD303⁺ and CD141⁺ subpopulationss in patient 1 were higher than those of patient 2. The number of CD303⁺ dendritic cells was at day 1 of patient 1 compared to the values of the rest of the tests and of patient 2, see Graph 7.

Regarding the concentration of $CD1c^+$ dendritic cells the values of the two patients did not differ much, except for an elevated value of patient 2, day 68, in comparison with the rest of the concentrations of both patients, see Graph 8.





Graph 7. Blood concentration of CD303⁺ dendritic cells of the two patients. Data only available for the time points indicated by symbols. Dashed lines connect the symbols of each patient for the sake of clarity, only.

Graph 8. Blood concentration of CD1c⁺ dendritic cells of the two patients. Data only available for the time points indicated by symbols. Dashed lines connect the symbols of each patient for the sake of clarity, only.

The concentration of $CD141^+$ dendritic cells differed only little within the measurements of patient 2. Patient 1, however, showed a concentration that differed in a larger scale between measurements, see Graph 9.



Graph 9. Blood concentration of CD141⁺ dendritic cells of the two patients. Data only available for the time points indicated by symbols. Dashed lines connect the symbols of each patient for the sake of clarity, only.

Dendritic Cell Subset Enumeration in Whole Blood

The populations and gating of dendritic cells are illustrated in Figure 17 in which the data from one representative sample is shown. The corresponding plots obtained from the same sample with isotype controls are shown in Figure 18.



Figure 17. Populations and gates of the dendritic cell subset enumeration. P1: Leucocyte gate. P2: Lymphocyte gate. P3: CD1c⁺ dendritic cell gate. P4: CD303⁺ dendritic cell gate. P5: CD141⁺ dendritic cell gate. Data from one representative sample is shown. Plots are generated from FACSDiva[™] software.



Figure 18. Populations and gates of the dendritic cell subset enumeration of the isotype control sample. P1: Leucocyte gate. P2: Lymphocyte gate. P3: CD1c⁺ dendritic cell gate. P4: CD303⁺ dendritic cell gate. P5: CD141⁺ dendritic cell gate. Data from one representative sample is shown. Plots are generated from FACSDiva[™] software.

Distribution of CD1c+, CD303+ and CD141+ Dendritic Cells

Dendritic cell subpopulation distribution can be seen in Graph 10 to Graph 13. The graphs illustrate mean distribution of dendritic cell subpopulations for each subject. In general, the distribution of dendritic cells was as follows; CD141⁺ dendritic cells represented as the smallest subpopulation followed by CD1c⁺ dendritic cells. The CD303⁺ dendritic cells comprised the largest subpopulation. The exception was seen in patient 1 with a slightly higher percentage of CD1c⁺ dendritic cells than CD303⁺ dendritic cells.



Graph 10. Mean distribution of dendritic cell subtypes in patient 1. The numbers indicate mean percent of leucocyte gate. Standard deviation between the days of testing is shown. DC: dendritic cell.



Graph 11. Mean distribution of dendritic cell subtypes in patient 2. The numbers indicate mean percent of leucocyte gate. Standard deviation between the days of testing is shown. DC: dendritic cell.



Graph 12. Mean distribution of dendritic cell subtypes in control subject 1. The numbers indicate mean percent of leucocyte gate. Standard deviation between the days of testing is shown. DC: dendritic cell.



Graph 13. Mean distribution of dendritic cell subtypes in control subject 2. The numbers indicate mean percent of leucocyte gate. Standard deviation between the days of testing is shown. DC: dendritic cell.

Dendritic Cell Subclass Distribution over Time

The development in distribution of the dendritic cell subpopulations in the experimental period is shown in Graph 14 to Graph 19. Between patients there was a difference of approximately 0.4 percentage points in the size of CD1c⁺ subpopulation up to day 36, see Graph 14. At the last day of testing, the subpopulation was rather similar in the two patients. Samples from the two control subjects differed less by a maximum of approximately 0.2 percentage points regarding the CD1c subpopulation, see graph 15. Patient 1 seemed to reach the highest value compared to patient 2, and control subject 1 and 2 during the period of testing.

1

0,8

0,6

0,4

0,2

0

40

50

% of leucocyte gate



Graph 14. Distribution of CD1c⁺ dendritic cells in patients. Data only available for the time points indicated by symbols. Dashed lines connect the symbols of each patient for the sake of clarity, only.

Graph 15. Distribution of CD1c⁺ dendritic cells in control subjects. Data only available for the time points indicated by symbols. Dashed lines connect the symbols of each control subject for the sake of clarity, only.

CD1c+ Dendritic Cells

: 😒

60

Control 1

•••ו• Control 2

Days

70

The CD303⁺ subtype was, as mentioned, the largest of the dendritic cell subtypes. Between all subjects the values differed by approximately 0.4 percentage points (see Graph 16 and Graph 17), and the greatest change during the period of testing was seen in control subject 1.



Graph 16. Distribution of CD303⁺ dendritic cells in patients. Data only available for the time points indicated by symbols. Dashed lines connect the symbols of each patient for the sake of clarity, only.



Graph 17. Distribution of CD303⁺ dendritic cells in control subjects. Data only available for the time points indicated by symbols. Dashed lines connect the symbols of each control subject for the sake of clarity, only.

The difference in the CD141⁺ dendritic cells between subjects reached a maximum of approximately 0.55 percentage points; see Graph 18 and Graph 19. Patient 1 reached the highest percentage of CD141⁺ dendritic cells at the second testing, and as with the CD1c⁺ dendritic cells the value decreased from that point with approximately 0.5 percentage points towards the end of the period. The difference between days of testing of patient 2 and control subjects 1 and 2 was less distinct compared to patient 1.



Graph 18. Distribution of CD141⁺ dendritic cells in patients. Data only available for the time points indicated by symbols. Dashed lines connect the symbols of each patient for the sake of clarity, only.



Graph 19. Distribution of CD141⁺ dendritic cells in control subjects. Data only available for the time points indicated by symbols. Dashed lines connect the symbols of each control subject for the sake of clarity, only.

TLR4 Expression of Dendritic Cell Subpopulations

In order to monitor TLR4 expression, cells were stained with anti-TLR4 antibody. TLR4 expression of the dendritic cell subclasses is shown in Graph 20 to Graph 23. The highest TLR4 expression of patient 1 was seen at day one with CD1c⁺ cells expressing most TLR4. This level of TLR expression by dendritic cells was the highest seen between subjects. At day 21 an overall decrease was seen for all three subtypes and a small increase of CD303⁺ and CD141⁺ expression occured at day 68. At day 36 and 68 the TLR4 expression of CD1c⁺ dendritic cells was negative, indicating higher isotype fluorescence than that of the anti-TLR4 antibody sample.



Graph 20. TLR4 expression of dendritic cell subtypes in patient 1.

Values of TLR4 expression in patient 2 also appeared to be negative for CD1c⁺ and CD141⁺ cells, respectively, at day 21 and 36 of the period. The highest value of TLR4 expression of CD303⁺ dendritic cells was seen at day 68. At this time, low TLR4 expression of CD1c⁺ dendritic cells occurred, see Graph 21.



Graph 21. TLR4 expression of dendritic cell subtypes in patient 2.

Control subject 1 showed a decrease in TLR4 expression by $CD1c^+$ dendritic cells in time, and an increase in $CD303^+$ expression. The expression level of $CD141^+$ cells was stable in general. At day 62 the value of $CD1c^+$ cells was negative.



Graph 22. TLR4 expression of dendritic cell subtypes in control subject 1.

Several TLR4 expression values of control subject 2 subpopulations were negative, see Graph 23. These included that of $CD1c^+$ expression, day 42 and 49, and of $CD303^+$ day 42. In general, values were raised for the expression of all three subtypes at day 62.



Graph 23. TLR4 expression of dendritic cell subtypes in control subject 2.

HLA-DR Expression of Dendritic Cell Subpopulations

HLA-DR expression of dendritic cell subtypes showed to be consistent between all samples. CD1c⁺ dendritic cells expressed most HLA-DR, see Graph 24 to Graph 27. Except for patient 1, CD303⁺ cells showed an expression level in between that of CD1c⁺ cells and CD141⁺ cells in general, with CD141⁺ cells being the subpopulation having the lowest expression. CD1c⁺ expression was high in the control subjects compared to the two patients.



Graph 24. HLA-DR expression of dendritic cell subtypes in patient 1.



Graph 26. HLA-DR expression of dendritic cell subtypes in control subject 1.



Graph 25. HLA-DR expression of dendritic cell subtypes in patient 2.



Graph 27. HLA-DR expression of dendritic cell subtypes in control subject 2.

Monocyte Subset Enumeration in Whole Blood

The populations and gating of non-classical, intermediate, and classical monocytes are illustrated in Figure 19 in which the data from one representative sample is shown. The corresponding plots obtained from the same sample stained with isotype controls are shown in Figure 20.



Figure 19. Populations and gates of the monocyte subset enumeration. P1: Monocyte gate. Q1: Non-classical monocytes. Q2: Intermediate monocytes. Q4: Classical monocytes. Data from one representative sample is shown. Plots are generated from FACSDiva[™] software.



Figure 20. Populations and gates of the monocyte subset enumeration of the isotype control sample. P1: Monocyte gate. Q1: Non-classical monocytes. Q2: Intermediate monocytes. Q4: Classical monocytes. Data from one representative sample is shown. Plots are generated from FACSDiva[™] software.

Distribution of Non-Classical, Intermediate, and Classical Monocytes

The distribution of non-classical, intermediate, and classical monocytes within the monocyte gate is shown in Graph 28 to Graph 31. The graphs illustrate mean distribution of monocyte subpopulations for each subject. Undetermined cells composed some of the cells of the monocyte gate, which explains why the percentages of the three subpopulations do not in total give a value of 100. The overall result was low numbers of non-classical and intermediate monocytes in comparison with the classical monocytes. Both patients had slightly higher average percentage of classical monocytes than control subjects. Patient 2 had a smaller frequency of non-classical monocytes than the other subjects.



Graph 28. Mean distribution of monocyte subtypes in patient 1. The numbers indicate mean percentage of monocyte gate. Standard deviation between the days of testing is shown. Mo: monocyte.



Graph 30. Mean distribution of monocyte subtypes in patient 1. The numbers indicate mean percentage of monocyte gate. Standard deviation between the days of testing is shown. Mo: monocyte.



Graph 29. Mean distribution of monocyte subtypes in patient 2. The numbers indicate mean percentage of monocyte gate. Standard deviation between the days of testing is shown. Mo: monocyte.



Graph 31. Mean distribution of monocyte subtypes in patient 1. The numbers indicate mean percentage of monocyte gate. Standard deviation between the days of testing is shown. Mo: monocyte.

Monocyte Subset Distribution over Time

The frequence of non-classical monocytes was in most instances higher in control subjects than in patients, see Graph 32 and Graph 33. Control subject 1 showed the greatest difference of approximately 3.5 percentage points between measurements. The non-classical monocyte subpopulation differed approximately 2.9 and 2.5 percentage points between samples of patient 1 and patient 2, respectively. The values of control subject 2 differed the least.





Graph 32. Distribution of non-classical monocytes in patients. Data only available for the time points indicated by symbols. Dashed lines connect the symbols of each patient for the sake of clarity, only.

Graph 33. Distribution of non-classical monocytes in control subjects. Data only available for the time points indicated by symbols. Dashed lines connect the symbols of each control subject for the sake of clarity, only.

Regarding the classical monocytes, all samples showed a high frequency of this cell type. Control subjects had slightly lower values than did those of the patients, see Graph 34 and Graph 35.





Graph 34. Distribution of classical monocytes in patients. Data only available for the time points indicated by symbols. Dashed lines connect the symbols of each patient for the sake of clarity, only.

Graph 35. Distribution of classical monocytes in control subjects. Data only available for the time points indicated by symbols. Dashed lines connect the symbols of each control subject for the sake of clarity, only.

Great variations were seen in the distribution of intermediate monocytes - both between subjects and between samples of each subject. As with the CD1c⁺ and CD141⁺ dendritic cells, patient 1 had a decreasing frequency of intermediate monocytes when comparing early to late measurements. Patient 2 also showed a low number of intermediate monocytes in the last sample. Control subject 1 showed higher values than control subject 2 in two out of three measurements, see Graph 36 and Graph 37.



Graph 36. Distribution of intermediate monocytes in patients. Data only available for the time points indicated by for the sake of clarity, only.

Graph 37. Distribution of intermediate monocytes in control subjects. Data only available for the time points indicated by symbols. Dashed lines connect the symbols of each patient symbols. Dashed lines connect the symbols of each control subject for the sake of clarity, only.

TLR4 Expression of Monocyte Subpopulations

The TLR4 expression of patient 1 did not show any negative values. The day 1 measurement showed higher values of TLR4 expression of all cell types compared with the following three measurements, see Graph 38. However, in the fluorescence intensity of patient 2 and both control subjects, negative values were revealed, see Graph 39 to graph 41. The intermediate monocytes of patient 1 showed highest TLR4 expression amongst monocyte subtypes at day 21, 36, and 68. This was also the case with patient 2, all measurements, and control subject 1, last two measurements. In control subject 2 there was higher TLR4 expression of the non-classical monocytes, than of the intermediate monocytes (Graph 41).



Graph 38. TLR4 expression of all monocyte subtypes in patient 1.



Graph 40. TLR4 expression of all monocyte subtypes in control subject 1.





Graph 41. TLR4 expression of all monocyte subtypes in control subject 2.

HLA-DR Expression of Monocyte Subpopulations

Intermediate monocytes of all subjects expressed most HLA-DR, see Graph 42 to Graph 45. The maximum values of the intermediate monocytes differed between subjects, and the patients had lower maximum values than the control subjects. Compared to the other subtypes, HLA-DR expression was lowest for the classical monocytes, in general. One value, the one of day 42 of the classical monocytes was negative for control subject 2, see Graph 45.





Graph 42. HLA-DR expression of all monocyte subtypes in patient 1.



Graph 44. HLA-DR expression of all monocyte subtypes in control subject 1

Graph 43. HLA-DR expression of all monocyte subtypes in patient 2



Graph 45. HLA-DR expression of all monocyte subtypes in control subject 2.

Monocyte Subset Enumeration in Urine

For flow cytometric analysis, the cells of urine samples were first concentrated by centrifugation and then the mononuclear cells in the concentrated sample were isolated by density gradient centrifugation. However, in samples from control subjects, no typical cell populations could be identified in FSC/SSC dotplots, see Figure 21.



Figure 21. FSC/SSC plot for urine monocyte enumeration. The plot represents all samples of control subjects. 100% events are shown. Plot generated from FACSDiva[™] software.

Urine monocytes were not found in patients when samples were not prepared with LymphoprepTM and gradient centrifuged, which was the case of the first patient samples.

When changing the method of preparation, cell populations were seen. In the sample from patient 1, day 36, leucocyte subpopulations were located in a FSC/SSC plot, see Figure 22. When gating on the monocytes, the classical monocytes constituted the majority of monocytes. No sample was analyzed for patient 2 that day.



Figure 22. FSC/SSC plots for urine cells of patient 1, day 36, revealed the presence of leucocytes (left and middle plot). Classical monocytes seemed to be present in a CD14/CD16 plot (right plot). Sample preparation was according to approach no. 2. 100% of events are shown. Plots generated from FACSDiva[™] software.

Classical monocytes was also observed in the urine of patient 1, day 68, see Figure 23. No monocyte population could be defined for patient 2, day 68. See Figure 24.



Figure 23. Upper left plot: FSC/SSC plot of urine cells of patient 1, day 68. Sample preparation was according to approach no. 3. Upper right plot: CD45-positive cells were localized. Lower left plot: FSC/SSC plot of CD45-positive cells revealed leucocyte populations, and a gate was set around monocytes. Lower right plot: CD14/CD16 plot revealed prescence of mainly classical monocytes. 100% events are shown. Plots generated from FACSDivaTM software.



Figure 24. Upper left plot: FSC/SSC plot of urine cells of patient 2, day 68. Sample preparation was according to approach no. 3. Upper right plot: CD45-positive cells were localized. Lower left plot: FSC/SSC plot of CD45-positive cells revealed no leucocyte populations. 100% events are shown. Plots generated from FACSDiva[™] software.

Discussion

The etiology of IgAN is still unknown, however immune mechanisms are crucially involved in the pathogenetic processes, and possibly the cause of the disease is also to be found within the immune system (5). The aim of the present project was to investigate monocyte and dendritic cell subpopulations in a group of IgAN patients and a group of controls.

Due to an unexpected delay in the approval process of the plans for the research project, a lower than expected number of suitable patients, and the fixed time allowed for a research project for the master's degree, only one third of the number of patients and controls anticipated, has been enrolled in the project. Obviously, this severely limits the conclusion that can be drawn on the basis of the results obtained.

Regarding enumeration of dendritic cell and monocyte subpopulations in IgAN patients and in healthy controls, the study has succeeded. In all subjects the subpopulations of cells were found. The monocyte and dendritic cell subpopulations being investigated in the present study have only recently been defined (3), and to our knowledge, the distribution of subpopulations within parent population of these cells in blood has not yet been investigated in IgAN. This makes the experimental setup of this study unique, and hence the results of it interesting.

The distribution of plasmacytoid CD303⁺, myeloid CD141⁺, and myeloid CD1c⁺ dendritic cells, was consistent within control subjects. The CD303⁺ dendritic cells constituted the largest group of the dendritic cell subpopulations, and CD141⁺ cells accounted for the smallest fraction of subpopulations. In patient 1, the mean CD1c⁺ subpopulation was increased in relation to the other subjects, see Graph 10 to Graph 13. However, based on the number of measurements it was not possible to establish a strong basis for describing tendencies of distribution in patients and in controls. In future studies the number of patients should therefore be increased and the period measurements prolonged.

This also applied to the distribution of monocytes. However a tendency of increased proportions of classical monocytes was seen in patients compared to control subjects. Mean percent wise distribution of the non-classical monocytes was decreased in both IgAN patients (Graph 28 to Graph 31). These tendencies may only be confirmed by experiments of a larger number of patients.

Ideally, it had been useful to have data from the BD TruCount[™] tubes. In that way, it would have been possible to obtain data for the absolute concentration of the subpopulations of dendritic cells and monocytes directly from the flow cytometric data. Information on blood leucocyte concentrations, including monocyte and lymphocyte concentrations, was of interest, as the percent wise distribution within parent the population (e.g. percentage of classical monocytes within the monocyte gate), could not itself reveal information on the level of blood concentrations. It is of interest to know, whether or not e.g. an increase in a monocyte subpopulation percentage was to be seen in the total blood concentration of monocytes and leucocytes in general. Given that no such data was obtained, data from the absolute leucocyte differential count was used in the calculations.

Since our attempt to determine absolute counts by the TruCount[™] bead method failed, we unfortunately cannot provide absolute counts for the subpopulations of dendritic cells and monocytes from the control subjects. This is due to the fact that an absolute differential count was not performed on blood from the control subjects, only on blood from patients. However, in the literature, reference values for these

subpopulations can be found. The blood of healthy human adults contains approximately 0.45 x10⁹ monocytes per litre (38). According to Graph 3, the blood monocyte concentration of the two patients differed in that patient 2 showed a lower blood monocyte concentration in comparison with patient 1. Patient 2 showed a concentration ranging closely to the reference value of 0.45 x10⁹ monocytes/L at the first three measurements. Only the last measurement was elevated with reference to normal monocyte concentration. The same peak in blood concentration was seen for the non-classical and for the classical monocytes in Graph 4 and Graph 6. This indicated that the overall increase in monocytes and classical monocytes. The highest peak of monocyte blood concentration for patient 2 was seen at the first day of measurement, see Graph 3. When looking at Graph 4 to Graph 6, classical and intermediate monocytes seemed to be the contributing cell types for this overall blood monocyte concentration. It is noteworthy, that the peaks in blood monocyte concentrations of the two patients were, in part, due to an increase in classical monocytes.

The blood monocyte concentration of patient 2 seemed low at each measurement in comparison with the reference value (Graph 3). Likewise, lymphocyte concentration was lower in patient 2 than in patient 1 (Graph 2). Despite these values of concentration, total leucocyte blood concentration of patient 1 was similar to that of patient 2 (Graph 1). This indicated that granulocytes of the total leucocyte population were present in a higher amount in patient 2 compared to patient 1. Both patients had total leucocyte concentrations around the reference value of 6.19×10^9 cells/L (39). When evaluating the monocyte and dendritic cell subpopulations and blood concentrations found in this experiment, it should be considered that the number of patients over a longer period of time would be preferred, as this would probably provide greater insight into the development over time and differences between subjects. It would have been interesting to see concentrations of the dendritic cell subpopulations in healthy individuals, since this is a necessity for evaluation of differences in IgAN patients and in control subjects.

Clear tendencies were observed in all subjects regarding the expression of HLA-DR on the different dendritic cell subpopulations (Graph 24 to Graph 27). CD1c⁺ dendritic cells clearly had the highest expression of HLA-DR. CD303⁺ cells showed the second level of HLA-DR expression, and the CD141⁺ dendritic cells expressed least of the marker compared with the other subpopulations. Interestingly, peak levels of HLA-DR on CD1c⁺ dendritic cells were higher for the control subjects than for the patients. Amongst the monocytes, HLA-DR expression was not as clearly defined between subpopulations as with dendritic cell suppopulations. In general, the intermediate monocytes had high expression of HLA-DR. Peak levels of HLA-DR expression on intermediate monocytes were slightly higher for control subjects than for the two patients. These results of HLA-DR expression indicate the possibility, that HLA-DR expression may be associated with IgAN. This result is in line with the findings of others that have demonstrated a genetic association with HLA and IgAN (40).

TLR4 expression of dendritic cells and monocytes did not reveal any clear difference between and within subjects. In several instances the TLR4 expression was found to be negative, both regarding monocyte and dendritic cell expression, see Graph 20 to Graph 23, and Graph 38 to Graph 41, respectively. Monocyte expression of TLR4 revealed increased values of intermediate and non-classical monocytes of patient 1, day

1, (see Graph 38) in comparison with the rest of the measurements of patient 1. In patient 2 and control subject 1 intermediate monocytes also showed the greatest TLR4 expression between subpopulations.

The negative values of TLR4 expression indicated, that non-specific binding of the isotype control antibody was higher than the binding of the anti-TLR4 antibody. It was also clear that the PE-Cy7 isotype control used with the PE-Cy7 conjugated anti-TLR antibody exhibited a stronger binding than did the other isotype controls. Indeed, binding of PE-Cy5 conjugates has been demonstrated in a study by van Vugt et al., 1996 (41). It appears that when using PE-Cy5 conjugated monoclonal antibodies there is an effective binding of the PE-Cy5 conjugate of the antibody to the human CD64 receptor. In this way there is a risk that the fluorescence from PE-Cy5-conjugated antibodies does not reflect binding specificity to antigens of interest, but rather the binding of PE-Cy5 fluorochrome to CD64 on cells (41). It could be suspected, that there was a difference in the degree of PE-Cy7-fluorochrome-conjugation between the anti-TLR4 antibody and the isotype control antibody in this study, and that this contributed to a higher extent of non-specific binding of the isotype control antibody. In a situation like that, samples of very low values of fluorescence intensity caused by low TLR4 expression would probably be highly influenced by non-specific binding of the isotype control antibody. Median fluorescence intensities of an isotype control antibody with a high level of nonspecific binding may, when retracted from the median fluorescence intensity of cells with little TLR4 expression, result in negative values of fluorescence. Indeed, the binding of the anti-TLR4 antibody does seem to be low in those instances where the value becomes negative after subtraction of the isotype value when looking on the original data (data not shown).

The undesirable binding of tandem PE-Cy5 conjugates was by others shown to be mediated by the CD64 receptor. A study of van Vugt et al. demonstrated that this binding could be inhibited by the addition of an anti-CD64 antibody (41). Since the PE-Cy7 fluorochrome is also a tandem dye (42), we suspect that the two fluorochromes are chemically related and hence, expect that the binding of the PE-Cy7 conjugates is also mediated by CD64. We therefore investigated the effect of adding an unconjugated anti-CD64 antibody to the antibody panel. No major changes were seen on the median fluorescence intensity of the PE-Cy-7 conjugated isotype antibody when staining with the anti-CD64 antibody. A study by Jahrsdörfer et al. in 2005 demonstrated, that phosphorothyoate oligonucleotides effectively suppress nonspecific binding of Cy5 conjugates to monocytes, more effectively than do anti-CD64 antibodies and Fc receptor blocking reagents (43). It may be suspected, that the binding epitope targeted by the anti-CD64 antibody used in present study was different from that of the PE-Cy7 conjugate. This would explain why the CD64 receptor blocking did not have any effect on the non-specific binding of the PE-Cy7 conjugated isotype control antibody.

The compensation of the present study was based on the use of BD CompBeads[™]. The staining of these beads was partly based on monoclonal antibodies of the experimental setup, partly on monoclonal antibodies of different specificity and manufacturer than in those of the experiment. Ideally, only the specific antibodies of the experiment should be used, since lot. numbers of antibodies may have slightly different fluorescence spectra (27). This was not possible, however, since dendritic cell enumeration was based on a commercially available antibody cocktail from which each single antibody could not be isolated and used in the compensation. The manufacturer of the antibodies of the cocktail did not provide separately antibodies like those of the cocktail, and as a consequence, other antibodies had to be used in the compensation. A future compensation setup should be based on each of the antibodies of the

experiment, which may eliminate the option of using commercially available antibody cocktails, but in return allow optimal compensation of the spectral overlaps of fluorescence.

As previously described, in multicolor flow cytometry, the flourochrome emission spectrum of a single fluorochrome can be affected by fluorescence spectra of additional colors in the same sample, despite proper compensation (36). The all-minus-one method is therefore preferable when staining cells with multiple colors, as it is the case of present study. By this approach of defining a negative control, it is possible to take into account that emission spectra may change with the presence of several colors. However, non-specific binding of e.g. tandem conjugates to cells must still be evaluated. All-minus-one plus isotype control is a way of combining the two ways of defining a negative population. This method includes the isotype control antibody of the antibody that is left out of the all-minus-one sample, and may be the optimal way of defining negative populations in studies involving multicolor flow cytometry.

Several optimizations of the protocol for urine monocyte enumeration were made in the course of this study, and this was essential to be able to analyze the presence of cells urine. Limited information is to be found on analysis of cells in urine (44), and the results of this study regarding monocytes in urine of IgAN patients seems to be unique to our knowledge. In healthy individuals, kidney function would be expected to be normal and glomerular damage non-existing. In present study no populations of granulocytes, monocytes, and lymphocytes were seen in the urine of control subjects. However, in one of the IgAN patients, leucocytes including monocytes were clearly present and could be analysed by flow cytometry. When looking at the subpopulations of these monocytes, it was revealed that classical monocytes comprised the majority of the monocytes. This result suggests further investigation of urine monocytes over time and during different states of disease in IgAN.

Conclusive Remarks

By this study it has been confirmed, that multicolor flow cytometry offers the opportunity of analysis of intermediate, non-classical, and classical monocytes, as well as plasmacytoid $CD303^+$, myeloid $CD1c^+$, and myeloid $CD141^+$ dendritic cells in IgAN patients.

Multicolor flow cytometry provides the capability of detection of multiple colors. However, reflections should be made in order to obtain useful and high quality data. In this study, it has been clearly shown, that the choice of fluorochrome-conjugated antibodies of the experimental antibody panel should be considered carefully, particularly when using fluorochromes that may show a high degree of non-specific binding to cells. In this study it was emphasized, that there is a need for careful quality controls when using tandem-conjugated antibodies.

The number of patients and control subjects for this type of experiment should be increased in a future study, as this would provide better opportunities for evaluation of cellular events in IgAN and in health. By also implementing the various improvements of the technique suggested above, great opportunities would arise to achieve more information on the condition of the innate immune system in IgAN.

Bibliography

1. McCance, Kathryn L. and Huether, Sue E. *Pathophysiology - The Biologic Basis for Disease in Adults and Children.* 5th edition. Missuori : Elsevier Mosby, 2006. 0-323-03507-8.

2. Boyd, Joanna K., et al., et al. An update on the pathogenesis and treatment of IgA nephropathy. 2012.

3. **Ziegler-Heitbrock, Loems, et al., et al.** Nomenclature of monocytes and dendritic cells in blood. *Blood.* 116, 2010, 16.

4. **Moldoveanu, Z., et al., et al.** Patients with IgA nephropathy have increased serum galactose-deficient IgA1 levels. *Kidney International.* 2007, 71, pp. 1148-1154.

5. Barratt, Jonathan and Feehally, John. Primary IgA Nephropathy: New Insights Into Pathogenesis. *Seminars in Nephrology*. 2011, 4, pp. 349-360.

6. Boyd, Joanna K., et al., et al. An update on the pathogenesis and treatment of IgA nephropathy. *Kidney International.* 2011.

7. **Tarelli, Edward, et al., et al.** Human serum IgA1 is substituted with up to six O-glycans as shown by matrix assisted laser desorption ionisation time-of-flight mass spectrometry. *Carbohydrate Research*. 2004, 339, pp. 2329-2335.

8. **Suzuki, Hitoshi, et al., et al.** IgA1-secreeting cell lines from patients with IgA nephropathy produce aberrantly glycosylated IgA1. *The Journal of Clinical Investigation*. 2008, 2, pp. 629-639.

9. Allen, Alice C., et al., et al. Mesangial IgA1 in IgA nephropathy exhibits aberrant O-glycosylation: Observations in three patients. *Kidney International.* 2001, 60, pp. 969-973.

10. Hastings, M. Colleen, et al., et al. Galactose-Deficient IgA1 in African Americans with IgA Nephropathy: Serum Levels and Heritability. *Clinical journal of the American Society of Nephrology.* 2010, 5, pp. 2069-2074.

11. Smith, Alice C., et al., et al. O-Glycosylation of Serum IgD in IgA Nephropathy. *Journal of the American Society of Nephrology*. 2006, 17, pp. 1192-1199.

12. **Smith, Alice C., et al., et al.** O-Glycosylation of Serum IgA1 Antibodies against Mucosal and Systemic Antigens in IgA Nephropathy. *Journal of the American Society of Nephrology.* 2006, 17, pp. 3520-3528.

13. **Novak, Jan, et al., et al.** Aberrant Glycosylation of IgA1 and Anti-Glycan Antibodies in IgA Nephropathy: Role of Mucosal Immune System. *Advances in Oto-Rhino-Laryngology.* 2011, 72.

14. **Coppo, Rosanna, et al., et al.** Innate immunity and IgA nephropathy. *Journal of Nephrology.* 23, 2010, 6, pp. 626-632.

15. Launay, Pierre, et al., et al. Fc a Receptor (CD89) Mediates the Development of Immunoglobulin A (IgA) Nephropathy Berger's Disease): Evidence for Pathogenic Soluble Receptor-IgA Complexes in Patients and CD89 Transgenic Mice. *The Journal of Experimental Medicine*. 191, 2000, 11, pp. 1999-2009.

16. **Grossetête, Béatrice, et al., et al.** Down-regulation of Fcα receptors on blood cells of IgA nephropathy patients: Evidence for a negative regulatory role of serum IgA. *Kidney International*. 1998, 53, pp. 1321-1335.

17. Narita, Ichiei and Gejyo, Fumitake. Pathogenic significance of aberrant glycosylation of IgA1 in IgA nephropathy. *Clinical and Experimental Nephrology*. 2008, 12, pp. 332-338.

18. **Cattran, Daniel C., et al., et al.** The Oxford classification of IgA nephropathy: rationale, clinicopathological correlations, and classification. *Kidney International.* 2009, 76, pp. 534-545.

19. Hara, Masanori, Yanagihara, Toshio and Kihara, Itaru. Cumulative Excretion of Urinary Podocytes Reflects Disease Progression in IgA Nephropathy and Schönlein-Henoch Purpura Nephritis. *Clinical Journal of the American Society of Nephrology*. 2007, 2, pp. 231-238.

20. **Couser, William G.** Basic and Translational Consepts of Immune-Mediated Glomerular Diseases. *Journal of the American Society of Nephrology.* 2012, 23.

21. **Coppo, R., et al., et al.** Toll-like receptor 4 expression is increased in circulating mononuclear cells of patients with immunoglobulin A nephropathy. *Clinican and Experimental Immunology.* 2009, 159, pp. 73-81.

22. **Myrphy, Kenneth.** *Janeway's Immunobiology.* 8th edition. New York : Garland Science, Taylor & Francis Group, 2012. 978-0-8153-4243-4.

23. **Netea, Mihai G. and van der Meer, Jos W.M.** Immunodeficiency and Genetic Defects of Pattern-Recognition Receptors. *The New England Journal of Medicine.* 2011, 364, pp. 60-70.

24. **Park, Hae Jeong, et al., et al.** Association between toll-like receptor 10 (TLR10) gene polymorphisms and childhood IgA nephropathy. *European Journal of Pediatrics*. 2011, 170, pp. 503-509.

25. **Macey, Marion G.** Principles of Flow Cytometry. *Flow Cytometry: Principles and Applications.* Totowa, New Jersey : Humana Press, 2007, 978-1-59745-451-3.

26. Barnett, David and Reilly, John T. Quality Control in Flow Cytometry. [book auth.] Marion G. Macey.
Flow Cytometry: Principles and Applications. Totowa, New Jersey : Humana Press, 2007, 978-1-59745-4513.

27. **BD Biosciences.** An Introduction to Compensation for Multicolor Assays on Digital Flow Cytometers. *BDbiosciences.com.* [Online] 2009.

http://www.bdbiosciences.com/eu/support/resources/flowcytometry/index.jsp#techspecs.

28. **Givan, Alice L.** Flow cytometry: An Introduction. [book auth.] Teresa S. Hawley and Robert G. Hawley. *Flow Cytometry Protocols - Methods in Molecular Biology.* 3rd edition. s.l. : SpringerLink, 2011, 978-1-61737-950-5.

29. **Invitrogen.** Introduction to Flow Cytometry. *Invitrogen.com.* [Online] [Cited: May 15, 2012.] http://probes.invitrogen.com/resources/education/tutorials/4Intro_Flow/player.html.

30. **Maecker, Holden and Trotter, Joe.** Selecting Reagents for Multicolor Flow Cytometry. *BDBiosciences.com.* [Online] 2012. http://www.bdbiosciences.com/eu/support/resources/flowcytometry/index.jsp.

31. **Ekong, Theresa, et al., et al.** Technical influences on immunophenotyping by flow cytometry - The effect of time and temperature of storage on the viability of lymphocyte subsets. *Journal of Immunological Methods.* 1993, 164, pp. 263-273.

32. **BDbiosciences.** Techniques for Immune Function Analysis - Application handbook. *BDBiosciences.com.* [Online] 2nd edition, 2009.

http://www.bdbiosciences.com/ecat/documentSearch.do?key=Handbook&prodCount=0&charset=utf-8&MatchAllTerms=true.

33. **Gratama, Jan W.**, et al., et al. Analysis of Factors Contributing to the Formation of Mononuclear Cell Aggregates ("Escapees") in Flow Cytometric Immunophenotyping. *Cytometry*. 1997, 29, pp. 250-260.

34. **Brando, Bruno, et al., et al.** The "Vanishing Counting Bead" Phenomenon: Effect on Absolute CD341 Cell Counting in Phosphate-Buffered Saline-Diluted Leukapheresis Samples. *Cytometry.* 2001, 43, pp. 154-160.

35. **O'Gorman, Maurice R.G. and Thomas, Joanne.** Isotype Controls - Time to Let Go? *Cytometry.* 1999, 38, pp. 78-80.

36. **Baumgarth, Nicole and Roederer, Mario.** A practical approach to multicolor flow cytometry for immunophenotyping. *Journal of Immunological Methods.* 2000, 243, pp. 77-97.

37. **Battiston, Kyle G., et al., et al.** Differences in protein binding and cytokine release from monocytes on commercially sourced tissue culture polystyrene. *Acta Biomaterialia.* 2012, 8, pp. 89-98.

38. **Robbins, Clinton S. and Swirski, Filip K.** The multiple roles of monocyte subsets in steady state and inflammation. *Cellular and Molecular Life Sciencens.* 2010, 67, pp. 2685-2693.

39. Sennels, Henriette P., et al., et al. Diurnal variation of hematology parameters in healthy young males:
The Bispebjerg study of diurnal variations. *Scandinavian Journal of Clinical and Laboratory Investigation*.
2011, 71, pp. 532-541.

40. **Feehally, John, et al., et al.** HLA Has Strongest Association with IgA Nephropathy in Genome-Wide Analysis. *Journal of the American Society of Nephrology.* 2010, 21, pp. 1791-1797.

41. van Vugt, M.J., van den Herik-Oudijk, I.E. and van de Winkle, J.G. Binding of PE-CY5 conjugates to the human high-affinity receptor for igG (CD64). *Blood.* 1996, 88.

42. **Bishop, James E., et al., et al.** A Setup System for Compensation: BD CompBeads plus BD FACSDiva Software. *BDBiosciences.com.* [Online]

http://www.bdbiosciences.com/ecat/documentSearch.do?key=A+Setup+System+for+Compensation%3A& prodCount=0&charset=utf-8&MatchAllTerms=true.

43. Jahrsdörfer, Bernd, Blackwell, Sue E. and Weiner, George J. Phosphorothyoate oligodeoxynucleotides block nonspecific binding of Cy5 conjugates to monocytes. *Journal of Immunological Methods.* 2005, 297, pp. 259-263.

44. **Beatty, John D., et al., et al.** Urine dendritic cells: a noninvasive probe for immune activity in bladder cancer? *British Journal of Urology International.* 2004, 94, pp. 1377-1383.

45. **BD Biosciences.** BD Fluorescence Spectrum Viewer - A Multicolor Tool. *BDBiosciences.com.* [Online] 2012. http://www.bdbiosciences.com/eu/research/multicolor/spectrum_viewer/index.jsp.

Appendix A - Ethical Approval
Appendix B - Approval by the Danish Data Protection Agency

Appendix C - Compensation Matrix

Appendix D - Compensation: Negative and Positive Populations