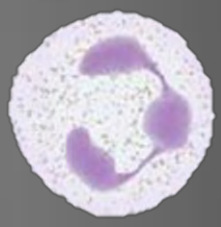
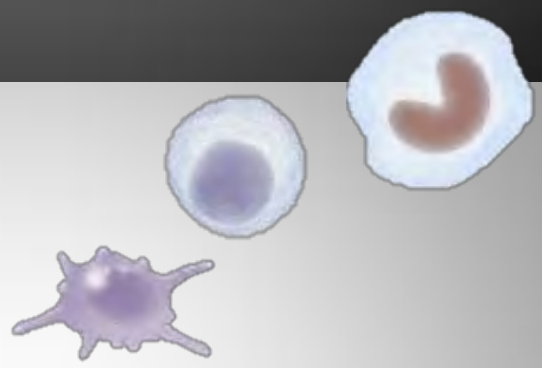
Flow Cytometric Enumeration of Monocyte and Dendritic Cell Subpopulations in IgA Nephropathy

A Study into the Innate Immune System





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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 control subjects expressed more HLA-DR than 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.

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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 α1 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).

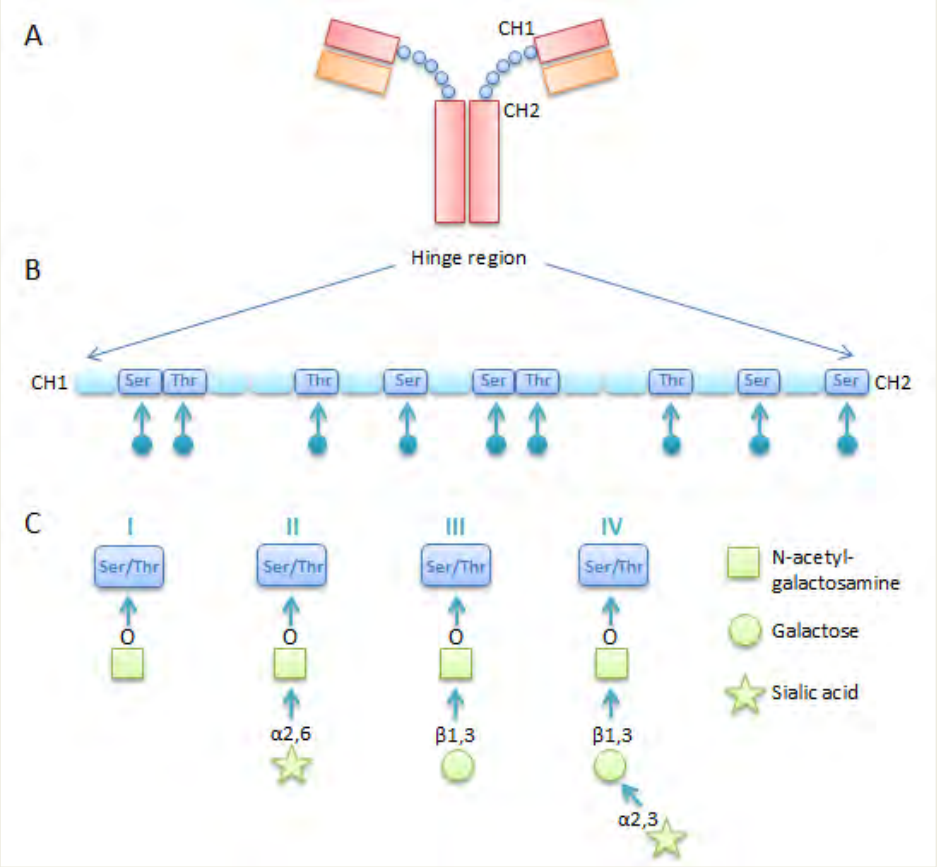

Figure 1. The human IgA1 molecule, the hinge region containing nine potential sites of O-glycosylation, and the four forms of O-glycans. A. The IgA1 molecule is composed of two light chains and two heavy chains. The O-glycan sites are in the hinge region of the heavy chains, between the CH1 and the CH2 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 α2,6 sialyltransferase, GalNAc extended with galactose by core 1 β1,3 galactosyltransferase, or the GalNAc-galactose disaccharide can be further sialylated by α2,3 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 β1,3-galactosyltransferase and an increase in α2,6-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 glycan-specific 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).

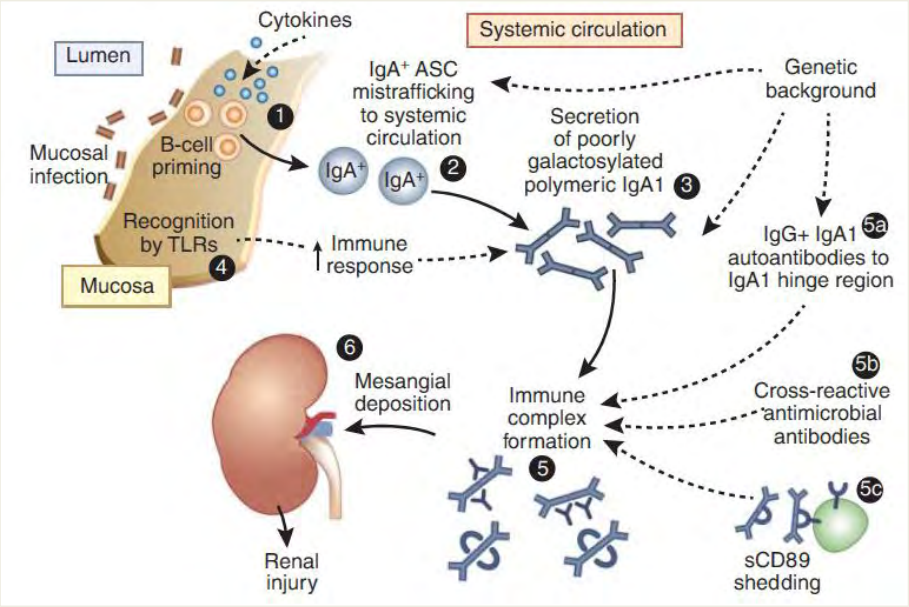

Figure 2. The proposed pathogenesis of IgA nephropathy. B-cells are primed by a mucosal infection to class switch and secrete IgA1. This priming of the B-cells can be induced by stimulation of Toll-like receptors on the B-cell membrane or by cytokines from T-cells (1). After stimulation some of the antigen 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.

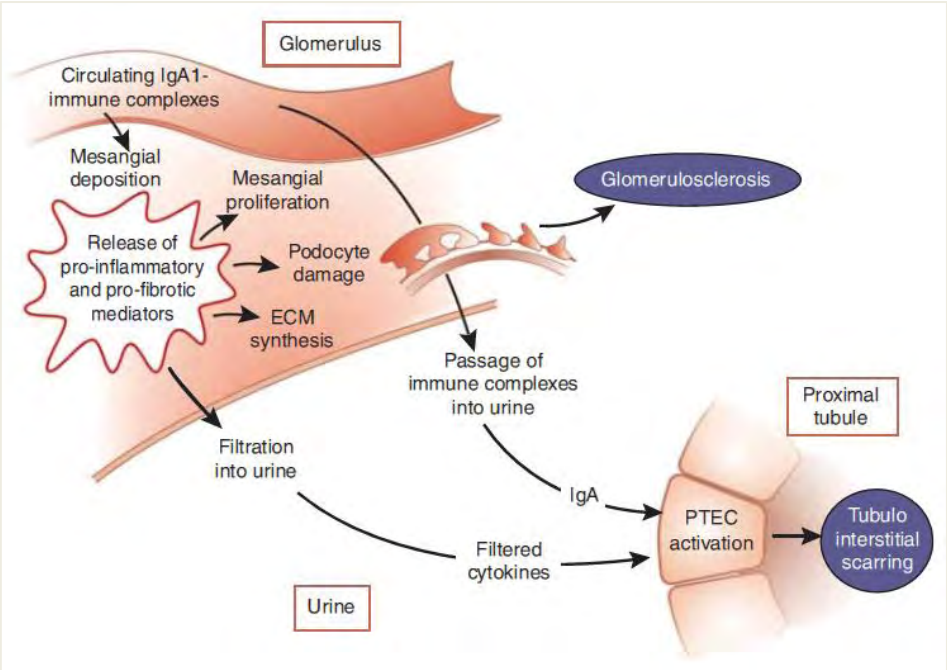

Figure 3. Glomerular and tubuluinterstitial injury in IgAN. IgA1-containing immune complexes in the systemic circulation deposit in the mesangial cells. Immune complex deposition activates meangial cells and trigger release of pro-inflammatory and pro-fibrotic mediators. These mediators cause mesangial 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).

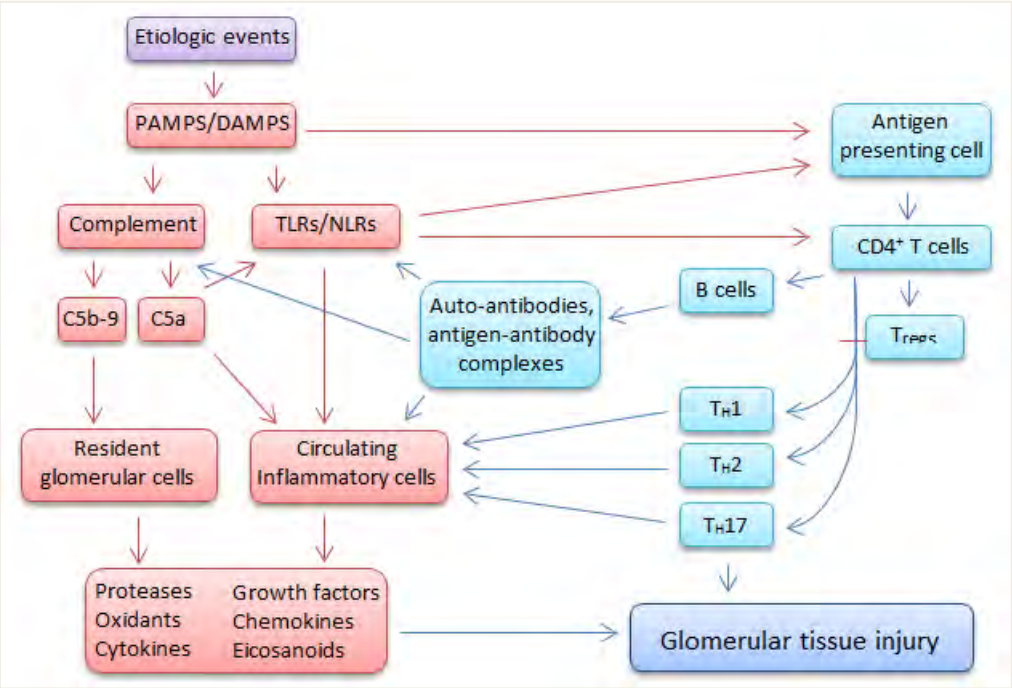

Figure 4. Schematic overview of the major pathogenic sequences in human glomerulonephritis. Both innate and adaptive immune mechanisms are involved in glomerular tissue injury caused by stimulation of the complement system, Toll-like receptors (TLRs), Nod-like receptors (NLRs), and antigen presenting cells by pathogen-associated molecular patterns (PAMPs), e.g. from an upper-respiratory 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_H17) can damage glomerular tissue directly and attract circulating inflammatory cells (T_H1 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).

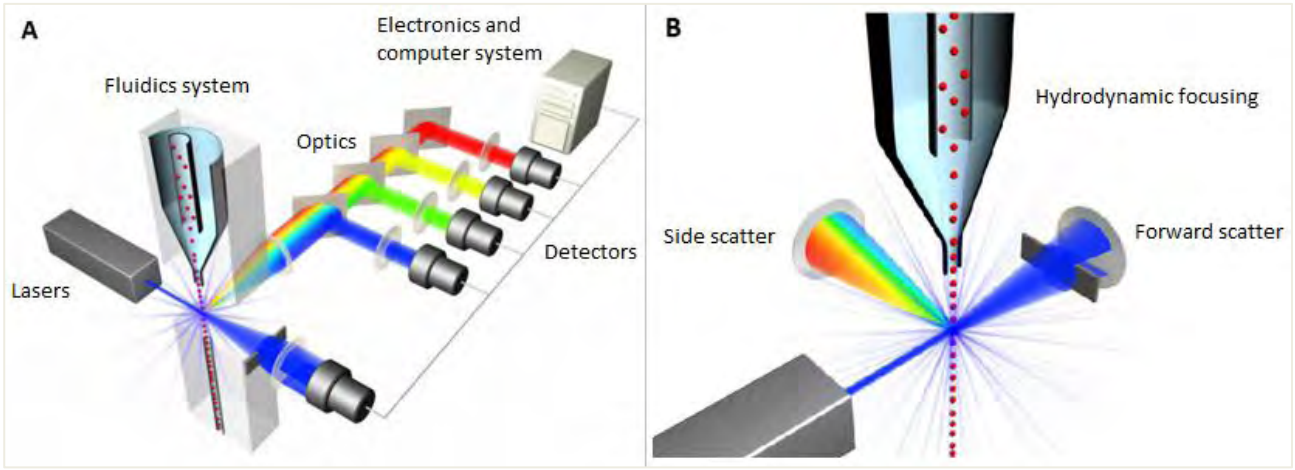

Figure 5. Instrument and interrogation point overview. A. The components of a flow cytometer are the lasers, the fluidics system, the optics, the detectors, and the electronics and computer system. B. The interrogation point is where the laser beam and the fluidics system meet and provide forward and 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 µs 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.

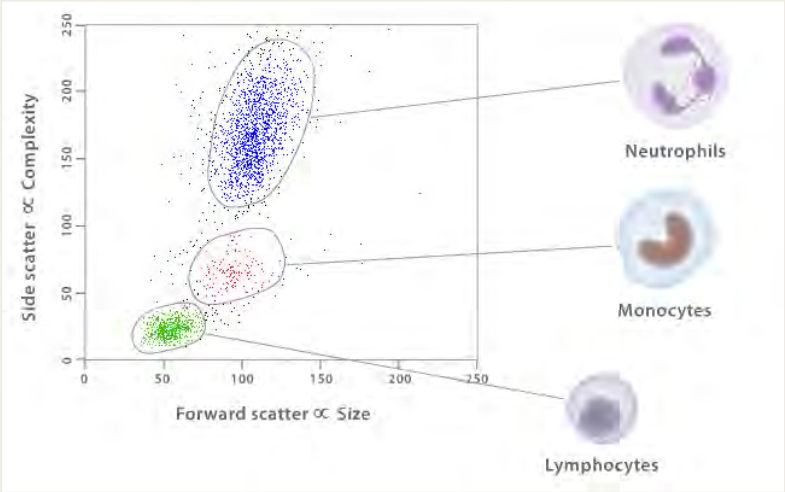

Figure 6. Distribution of leukocytes into lymphocytes, monocytes and neutrophils in a forward scatter/side scatter plot that shows size and complexity (granularity), respectively. Figure from Invitrogen 2012 (29).

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.

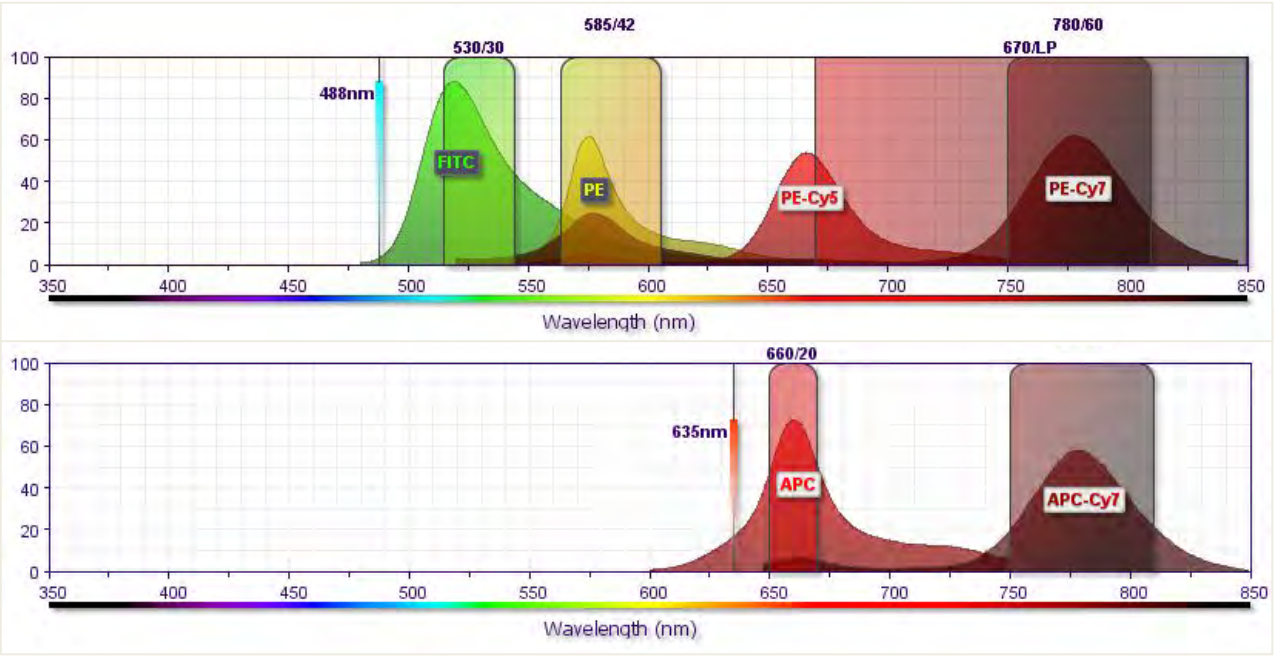

Figure 7. Overview of the laser emission wavelengths, filters and fluorochromes of the BD FACSCanto™ flow cytometer used in the experimental setup of this thesis. Blue light of 488 nm is emitted from an argon ion laser, which excites the fluorochromes FITC, PE, PE-Cy5 and PE-Cy7. Red light of 635 nm 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).

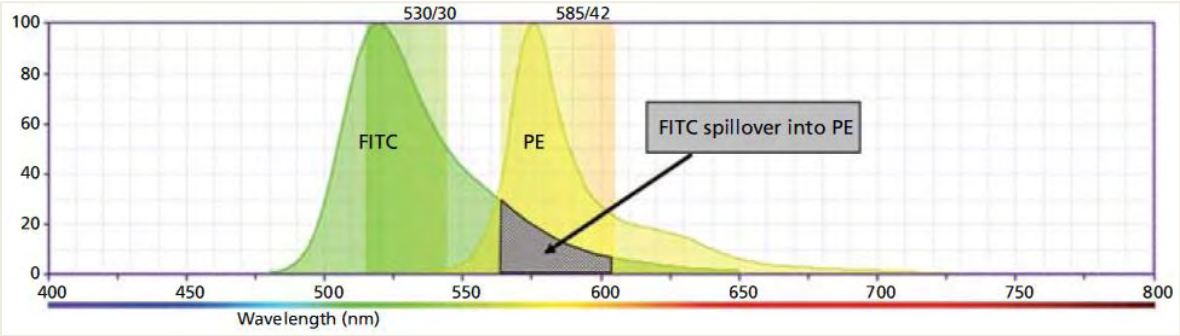

Figure 8. Spillover of FITC into the PE channel. Figure from BD Biosciences 2012 (27)

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.

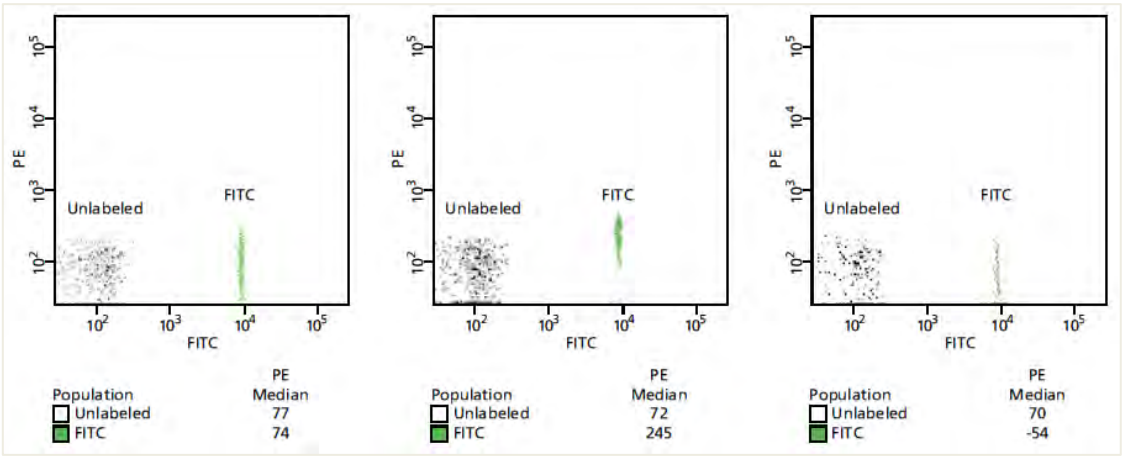

Figure 9. An example of properly compensated and incorrectly compensated samples. The left graph is a visual demonstration of proper compensation calculated by FACSDiva software; the unlabeled population is negative for both FITC and PE, whereas the FITC labeled population is positive for FITC only. 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 TruCount™ Tubes (BD Biosciences #340334) were used. According to the manufacturer's protocol 50 µL of well-mixed anticoagulated blood was transferred to the TruCount™ 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 µL anti-CD14-FITC (Thermo Scientific Pierce Products #MA1-19561) and 7.5 µL 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 µL of lysing solution was added and incubated for 15 minutes. In one setup ACK lysing buffer (4.15 g NH_4Cl, 0.5 g $KHCO_3$, 18.63 mg EDTA-Na_2*$2H_2O$, and 50 mL Milli-Q H_2O) was used, in another BD FACS lysing solution (BD Biosciences #349202) (recommended by BD Biosciences for BD TruCount™ 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 µL washing buffer. Additionally, 150 µL fixing Solution and 5 µL 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 FACSCanto™ instrument.

Dendritic Cell Subset Enumeration in 300 µl Whole Blood	
DC antibody cocktail: 20 µL Anti-BDCA Cocktail (kit) - CD303 FITC - CD1c PE - CD14 PE-Cy5 - CD19 PE-Cy5 - CD141 APC 10 µL anti-HLA-DR APC-Cy7 10 µL anti-TLR-4 PE-Cy7	Control Cocktail: 20 µL Control Cocktail (kit) - Mouse IgG1 FITC - Mouse IgG2a PE - CD14 PE-Cy5 - CD19 PE-Cy5 - Mouse IgG1 APC 10 µL isotype APC-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 µL 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: 15 µL anti-CD14 FITC 20 µL anti-CD16 PE 5 µL anti-HLA-DR APC-Cy7 5 µL anti-TLR4 PE-Cy7	Control Cocktail: 15 µL isotype FITC 20 µL isotype PE 5 µL isotype APC-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 µg/mL penicillin-streptomycin (Ampliqon #AMPQ40133.0005) and 10% AB-serum. Mononuclear cells were then isolated by use of 5 mL Lymphoprep™ (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 20 µL anti-CD16 PE 5 µL anti-HLA-DR APC-Cy7 5 µL anti-TLR4 PE-Cy7 10 µL anti-CD45 PE-Cy5	15 µL isotype FITC 20 µL isotype PE 5 µL isotype APC-Cy7 5 µL isotype PE-Cy7 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 TruCount™ 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 µL of blood) could then be calculated by the equation;

$$\frac{\text{Number of events in cell containing gate}}{\text{Number of events in bead containing gate}} \times \frac{\text{Number of beads per test}}{\text{Test volume (50 µL)}} = \text{Absolute count of cell}$$

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).

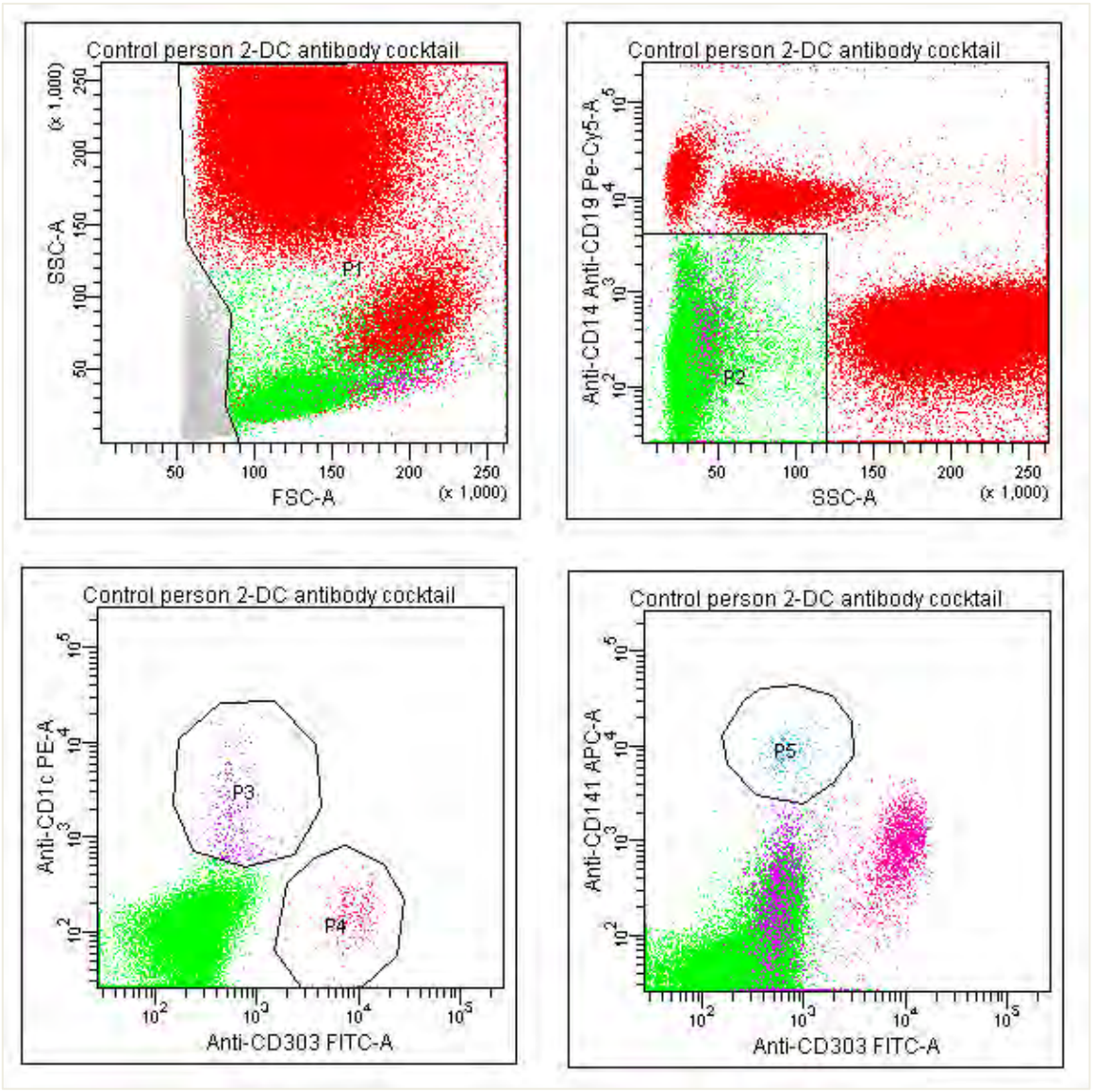

Figure 10. The gating strategy of blood dendritic cell subtype analysis. At the top left: Leukocyte gate (P1) defined by FSC/SSC plot. At the top right: Granulocytes, dead cells, monocytes and B cells were excluded by a SSC/CD14 CD19 Pe-Cy5 plot by means of a lymphocyte gate (P2) including T cells and 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.