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## Apoptosis of Peripheral Blood Leukocytes in Systemic Lupus Erythematosus: Studies on Serum Induction and Complement-Dependent Clearance Mechanisms

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**Apoptosis of Peripheral Blood Leukocytes in Systemic Lupus Erythematosus:  
Studies on Serum Induction and Complement-Dependent Clearance Mechanisms**

**Birgitta Gullstrand**



**LUND UNIVERSITY**  
Faculty of Medicine

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Som med vederbörligt tillstånd av Medicinska Fakulteten vid Lunds Universitet för avläggande av doktorexamen i medicinsk vetenskap i ämnet laboratoriemedicin med inriktning experimentell klinisk immunologi kommer att offentligt försvaras i Segerfalksalen, Wallenberg neurocenter BMC, onsdagen den 14 april 2010, klockan 09.00

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Abstract <p>Systemic lupus erythematosus (SLE) is an autoimmune disease involving many organ systems. The cause is not known, but a complex combination of environmental and genetic factors seems to be involved. In SLE upregulation of type I interferons, a hyperactive B-cell response, presence of autoantibodies against modified nuclear components, increased complement consumption, increased apoptosis and decreased clearance of apoptotic cells are seen. The purpose of this thesis was to investigate some of these mechanisms. The thesis is based on four papers (I-IV).</p> <p>(Papers I and II) We found that the apoptosis inducing effect was specific for sera from SLE patients when comparing with sera from various control groups. However, the apoptosis inducing effect was not related to SLE disease activity. Serum from SLE patients was demonstrated to induce classical caspase-dependent apoptosis in monocytes and lymphocytes. The apoptosis induction was not dependent on death receptors but involvement of the mitochondrial pathway was indicated.</p> <p>(Paper III) Phagocytosis of apoptotic cells by macrophages and C3 deposition on apoptotic cells were investigated in the presence of sera lacking different complement proteins. We found that complement-mediated opsonisation and phagocytosis of apoptotic cells, particularly those undergoing secondary necrosis, are dependent mainly upon an intact classical pathway. C1q was not more important than other classical pathway components, suggesting a role in other pathogenetic processes than defect clearance of apoptotic cells.</p> <p>(Paper IV) We evaluated the roles of serum complement and antibodies against histones in relation to phagocytosis of necrotic cell material by polymorphonuclear neutrophil granulocytes (PMNs). Phagocytosis of necrotic material by PMNs and high concentration of antibodies against a broad spectrum of histones correlated with active SLE disease. The specificities of these anti-histone antibodies appear to determine the complement-dependent phagocytosis.</p> <p>In conclusion, sera from SLE patients have the capacity to contribute to an increased load of apoptotic cells. An efficient clearance of apoptotic and necrotic cell material is dependent on a functional classical pathway, and autoantibodies against histones reflect the presence of apoptotic or necrotic cells contributing to the autoimmune process in SLE.</p>			
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**Till**

**Joakim och Johan**

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## LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the text by their roman numerals (I-IV)

- I. Induction of apoptosis in monocytes and lymphocytes by serum from patients with systemic lupus erythematosus - an additional mechanism to increased autoantigen load? Bengtsson AA, Sturfelt G, Gullstrand B, Truedsson L. *Clin Exp Immunol.* 2004 Mar;135(3):535-43.
  
- II. SLE serum induces classical caspase-dependent apoptosis independent of death receptors. Bengtsson AA, Gullstrand B, Truedsson L, Sturfelt G. *Clin Immunol.* 2008 Jan;126(1):57-66.
  
- III. Complement classical pathway components are all important in clearance of apoptotic and secondary necrotic cells. Gullstrand B, Mårtensson U, Sturfelt G, Bengtsson AA, Truedsson L. *Clin Exp Immunol.* 2009 May;156(2):303-11.
  
- IV. Specificity of anti-histone antibodies determines complement-dependent phagocytosis of necrotic material by polymorphonuclear leukocytes in the presence of serum from patients with SLE. The LE cell phenomenon revisited. Birgitta Gullstrand, Helena Tydén, Andreas Jönsen, Christian Lood, Sören Jacobsen, Gunnar Sturfelt, Lennart Truedsson, and Anders A. Bengtsson. *Manuscript.*



## ABBREVIATIONS

ACR	American College of Rheumatology
AIE	Apoptosis inducing effect
AIF	Apoptosis inducing factor
Apaf 1	Apoptotic protease activating factor 1
AUC	Area under curve divided by follow-up time
C4BP	C4b-binding protein
CAD	Caspase-activated deoxyribonuclease
cFLIP	Cellular Fas-associated death domain-like IL-1-converting enzyme inhibitory protein
CR	Complement receptor
CRP	C-reactive protein
DAMPs	Damage associated molecular patterns
DD	Death domain
DISC	Death inducing signal complex
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FADD	Fas associated death domain
FcR	Fc receptor
FITC	Fluoroisothiocyanate
IAPs	Inhibitors of apoptosis
ICAD	Inhibitor of caspase-activated deoxyribonuclease
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
MASP	Mannan-binding lectin-associated serine protease
MBL	Mannan-binding lectin
MDM	Monocytes to differentiate into macrophages
MHC	Major histocompatibility complex
NC	Necrotic material
NHS	Normal human serum
NK	Natural killer
NODs	Proteins carrying nucleotide-binding oligomerization domains
PAC assay	Phagocytosis of apoptotic cells assay
PAMPs	Pathogen-associated molecular patterns
PARP	Poly(ADP-ribose) polymerase
PBMC	Peripheral blood mononuclear cells
PI	Propidium iodide
PMNs	Polymorphonuclear leukocytes
PNC assay	Phagocytosis of necrotic cell material assay
PRRs	Pattern-recognition receptors
PS	Phosphatidylserine
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
SLE	Systemic lupus erythematosus
SLEDAI-2K	SLE Disease Activity Index 2000
Smac/	Second mitochondria derived activator of caspase/Direct inhibitor of apoptosis
DIABLO	binding protein with low pI
TGF- $\beta$	Transforming growth factor beta

TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRADD	TNFR-1-associated death domain
TRAIL	Tumor necrosis factor related apoptosis inducing ligand

## SUMMARY

Systemic lupus erythematosus (SLE) is an autoimmune disease involving many organ systems. The cause is not known, but a complex combination of environmental and genetic factors seems to be involved. In SLE upregulation of type I interferons, a hyperactive B-cell response, presence of autoantibodies against modified nuclear components, increased complement consumption, increased apoptosis and decreased clearance of apoptotic cells are seen. The purpose of this thesis was to investigate some of these mechanisms. The thesis is based on four papers (I-IV).

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In conclusion, sera from SLE patients have the capacity to contribute to an increased load of apoptotic cells. An efficient clearance of apoptotic and necrotic cell material is dependent on a functional classical pathway, and autoantibodies against histones reflect the presence of apoptotic or necrotic cells contributing to the autoimmune process in SLE.

# INTRODUCTION

## **The immune system**

The immune system is an integrated system of organs, tissues, proteins and cells that together protect us against foreign substances and invading organisms. The first line of defence consists of barriers such as the skin, mucous membranes, saliva, tears and the acid in the gut. Besides the physical barriers, a number of different proteins, enzymes and cell types are involved in the immune system, and these are able to differentiate between self and non-self. The immune response is composed of two parts, the innate or nonspecific immune system and the adaptive or specific immune system. The innate immune system is constitutively present and ready to be mobilised upon infection, whereas the adaptive immune system requires some time to react. These systems work together by various interactions and influence each other to create an efficient immune defence [1, 2].

The innate immune response is a defence mechanism that recognises and reacts to pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), mannose-rich glycan molecules exposed on microorganisms and DNA from bacteria or virus. However, molecules released by stressed and injured human cells, such as heat shock proteins, high-mobility group box 1 (HMGB1), S 100 proteins and DNA may also act as pro-inflammatory mediators and these molecules are termed damage associated molecular patterns (DAMPs). Pattern-recognition receptors (PRRs) recognise PAMPs and DAMPs and these receptors can be cell bound such as Toll-like receptors (TLRs), intracellular, such as proteins carrying nucleotide-binding oligomerization domains (NODs) and some TLRs or soluble such as the complement proteins [1-5]. The cells involved in the innate immune response are phagocytic cells such as neutrophils, monocytes and macrophages as well as the cytotoxic natural killer (NK) cells. Upon activation, the cells release cytokines that act as signal molecules and activate other cells involved in the immune response [2].

The function of the adaptive immune response is to destroy or inactivate foreign substances, also called antigens. The adaptive immune system is composed of specialised cells, such as B-cells and T-cells, which account for antibody and cell mediated immunity, respectively. These cells become activated when a specific epitope of the antigen binds to the B-cell or T-cell receptor. However, binding of antigen to the receptor is usually not sufficient to stimulate the cells to proliferate and differentiate into an effector cell; a co-stimulatory signal provided by

another specialised cell is often required. Major histocompatibility complex (MHC) molecules are proteins expressed on the cell surface and these molecules are recognised by T-cells. In most cases, T-cells only bind to the antigen if it is presented in complex with MHC. MHC class I molecules are expressed on the majority of nucleated cells and MHC class II are expressed on antigen presenting cells such as macrophages, monocytes and B-cells. MHC also acts as a self recognition molecule. The adaptive immune response also exhibits an immunological memory [6].

### **Leukocytes**

Polymorphonuclear leukocytes (PMNs) are the most common white blood cells in the circulation. Neutrophils, the most abundant type of PMNs, possess a multi-lobed nucleus and contain cytoplasmic granules. There are three types of granules, azurophilic (also called primary), specific and small granules. The granules are generated during cell differentiation and are used as storage for different substances. They contain cytotoxic substances, neutral proteinases, acidic hydrolases and cytoplasmic membrane receptors. Their function is to provide enzymes for hydrolytic substrate degradation and killing of bacteria, and also to regulate various processes including inflammation [7]. Neutrophils rapidly engulf foreign material that is covered with antibodies and complement fragments but they also clean up damaged cells or cellular debris.

Mononuclear blood cells include cells such as monocytes, macrophages and lymphocytes. The blood monocytes possess chemotactic, pinocytic and phagocytic abilities. During migration into the tissue the monocytes undergo further differentiation to become macrophages, and these take part in the initiation of T-cell activation by processing and presenting antigens. Activated macrophages are central effectors and regulatory cells of the immune response, and they achieve this by producing different substances such as cytokines which modulate the function of other cells. Lymphocytes circulating in the bloodstream are mostly in the resting state but the lymphocytes in the lymphoid tissues and organs can be activated directly after antigen stimulation. There are different types of lymphocytes, T-cells, B-cells and NK-cells. The T-cells are further divided into three types of cells, helper, cytotoxic and regulatory. T-helper cells are needed for activation of B-cells and they recognise antigen processed and presented by antigen presenting cells in complex with the self molecule MHC class II. The cytotoxic T-cells target and destroy tumour cells and cells infected with intracellular antigen such as virus, which are presented in complex with MHC

class I. Most nucleated cells express MHC class I, so any such cell that is infected with virus or producing tumour antigens may present these antigens together with MHC class I and be removed. The cytotoxic T-cells release perforin which forms channels in the cell membrane of the target cell and causes death by osmotic lysis. The regulatory T-cells reduce the intensity of the immune response by regulating transcription of different genes and by secreting interleukin (IL)-10. B-cells become plasma cells upon activation which then secrete antibodies or become memory cells. The NK-cells do not express a specific antigen binding receptor. They have two types of receptors that either activate or inhibit activation. NK-cells can bind antibody coated targets by immunoglobulin receptors (FcR), and they also bind to cells missing the self marker MHC class I. Also the release of interferons (IFN) or cytokines from virus-infected cells may activate these cells. NK-cells possess granules containing perforin and granzymes, which can be released upon activation and induce cell death of the target cell [6].

### **Activation of T- and B-cells**

Antigen presenting cells, such as macrophages, phagocytose antigen, degrade it and expose fragments of the antigen on the cell surface together with MHC class II molecules. The MHC-antigen complex binds to a T-cell receptor specific to the presented antigen. The binding of antigen to the T-cell receptor stimulates the expression of IL-2 receptors and secretion of IL-2 which binds to these receptors and stimulates the T-cell to proliferate. For activation of the T-cell, a second co-stimulatory signal is needed. This may be mediated by the binding of the signal protein B7 on the antigen presenting cell to the T-cell receptor protein CD28 [2]. Once activated, the T-helper cells can be divided into subpopulations such as Th1, Th2 and Th17 cells depending on the cytokine signal received or become memory T-cells. The different subsets of cells secrete different cytokines that regulate the immune response. Th1 cells stimulate the cell-mediated response by secreting cytokines such as gamma IFN and tumour necrosis factor (TNF) while Th2 cells stimulate B-cells to produce antibodies by secreting cytokines such as IL-4, IL-5, IL-6 and IL-10 (Fig. 1) [6]. Th17 cells are a recently identified subset of T-helper cells and these cells synthesise and secrete IL-17. IL-17 is involved in inducing and mediating pro-inflammatory responses. However, little is known about the function in humans but it is implicated to be an effector cell in autoimmune diseases [8].

Activation of B-cells occurs when the B-cell receptor recognises an antigen and binds to it. Each B-cell has a unique receptor expressed on its surface and this receptor is a membrane

bound immunoglobulin. In most cases T-helper cells, activated with the same antigen as the B-cell, are required for activation of the B-cell, a so-called T-cell dependent activation. The interaction between the T- and B-cells occurs between the CD40 ligand present on the surface of the activated T-helper cell and the CD40 protein present on the surface of the B-cell. In the presence of different cytokines, such as IL-4, IL-5, IL-6 and IL10, the activated B-cell differentiates into a plasma cell, producing antibodies of the same specificity as the B-cell receptor that targets the antigen, or becomes a memory cell (Fig. 1) [6]. B-cells may also be activated in a T-cell independent way. The antigen involved in this process is often polysaccharides that are able to bind multiple B-cell receptors and activate the B-cell directly to secrete IgM antibodies [6].

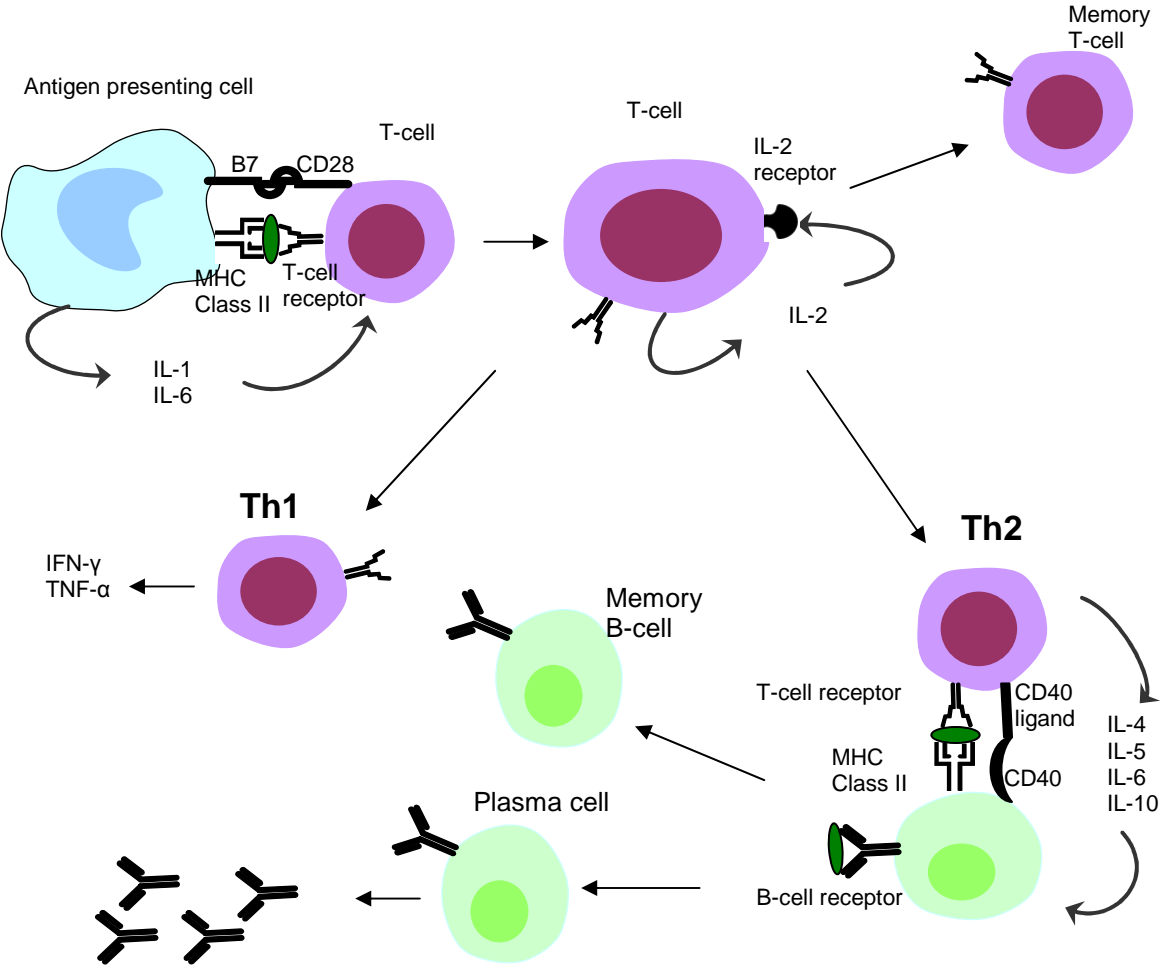


Figure 1. Activation of T-cell and T-cell dependent B-cell activation.

## Specific antibodies

Specific antibodies or immunoglobulins are produced upon B-cell activation. Their function is to target non-self substances and display them to the immune system and to neutralise antigens by binding to them. Each immunoglobulin is typically made of two identical heavy chains and two identical light chains linked together by disulfide bonds creating a molecule with a Y-like shape containing two identical antigen-binding sites (Fig. 2). There are five major classes of the heavy chains, IgM, IgG, IgA, IgD and IgE of which IgG is the most abundant in the blood and has four subclasses (IgG1, IgG2, IgG3 and IgG4) in humans. The effector function of an antibody is defined by the structure of its heavy chain. There are two types of light chains, termed kappa and lambda, and the antibody is always composed of the same variants. Both the heavy and the light chains consist of a variable and a constant region. The Fab (fragment antigen binding) part of the antibody is composed of one constant and one variable domain from each heavy and light chain. The paratope, or antigen binding site, is located in the variable region of the Fab part called the hypervariable region, which is unique to the particular antibody and determines the specificity for the ligand. The Fc (fragment crystallisable) region is the part of the antibody that interacts with cell surface receptors FcR which are expressed on most cell types and this initiates phagocytosis, release of cytokines and cytotoxic molecules. The Fc region can also initiate complement activation [9, 10].

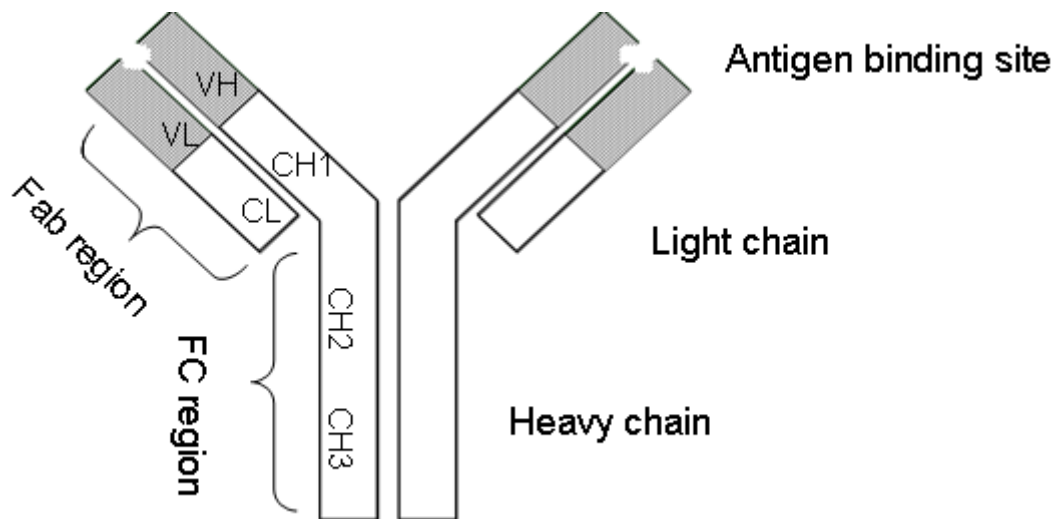


Figure 2. Schematic figure of the structure of an antibody. The heavy chain consists of three constant domains (CH1-CH3) and one variable chain (VH). The light chain consists of one constant chain (CL) and one variable chain (VL). The antigen binding site is located in the Fab region and the Fc region is responsible for interaction with effector molecules.



## **Tolerance and autoimmunity**

The immune defence is involved in detection and destruction of tumour cells and foreign invaders causing infection. For the system to function properly it must be able to discriminate between self and non-self molecules. Failure to do this may result in autoimmune diseases. All immune and blood cells develop from multi-potent hematopoietic stem cells that originate from the bone marrow and a highly diverse and random array of different specificities of T- and B-cells are produced. These cells are capable of recognising an almost unlimited number of antigens, including self-proteins. Immature T-cells undergo final maturation in the thymus where they go through an important process which enables them to distinguish between self and non-self. T-cells that recognise self-antigens are deleted by apoptosis or become inactivated. The selection that occurs in the thymus is called central selection and the cells undergo both positive and negative selection to produce T-cells that tolerate self-MHC molecules but not self-peptides. In the positive selection, T-cells with receptors that bind with neither too low nor too high affinity to surface MHC molecule on thymic epithelial cells are selected, and the other cells die. This ensures that T-cells only recognise antigen in association with MHC. The negative selection is mediated by macrophages and dendritic cells, which present self-peptides bound to MHC and the cells that recognise self-peptides bound to MHC undergo programmed cell death. The peripheral tolerance is developed after the T-cell has matured and entered the periphery. The cell is regulated by regulatory T-cells and the absence of co-stimulating signals. B-cell tolerance is not so tightly regulated. For B-cell activation to occur, a T-cell with the same antigen molecule specificity as the one that stimulates the particular B-cell is needed. This ensures the specificity of reactions to protein antigen selected by the immune system. In the bone marrow the B-cells are tested for interaction against self-antigens and the cells that recognise self-antigens are either processed for change in receptor specificity or the cells undergo programmed cell death [11, 12].

Autoimmune diseases are conditions caused by the breakdown of immune tolerance resulting in immune responses to self-antigens. Low levels of autoantibodies are found in blood from healthy individuals without causing inflammation or damage. However, an autoimmune disease occurs when a response against self-antigens involving T-cells, B-cells or autoantibodies induces damage systemically or to a particular organ. Autoantibodies could arise against novel epitopes expressed on modified self-proteins, cross-reacting antibodies if non-self molecules closely resemble self-antigens. Autoantibodies could also arise from the exposure of hidden self-molecules that would not be normally exposed to the immune system,

or as a result of hormonal component involvement, an imbalance of regulatory proteins or due to a genetic predisposition combined with environmental factors [12]. Autoimmune diseases can be organ specific and directly damage the organ target or systemic with involvement of different self-molecules and cause disease through the formation of immune complex. Under normal conditions immune complexes are rapidly cleared from the circulation by phagocytosis or by transportation. If the clearance system fails, circulating immune complex could be deposited in organs and cause inflammation or damage, such as glomerulonephritis, vasculitis and arthritis [13].

### **The complement system**

The complement system is an important part of the immune response, bridging innate and adaptive immune mechanisms. Activities of the complement system include initiation of inflammation, opsonisation of targets to promote phagocytosis, chemotaxis, lysis of cells and clearance of immune complexes and apoptotic cells [14, 15]. This complex system consists of more than 30 plasma and membrane proteins that interact and are activated through a cascade reaction. There are three main pathways by which complement activation is initiated, the classical pathway, the alternative pathway and the lectin pathway (Fig. 3). All pathways lead to cleavage of C3 and activation of the common terminal pathway leading to assembly of the membrane attack complex and eventually cell death by lysis [14, 16].

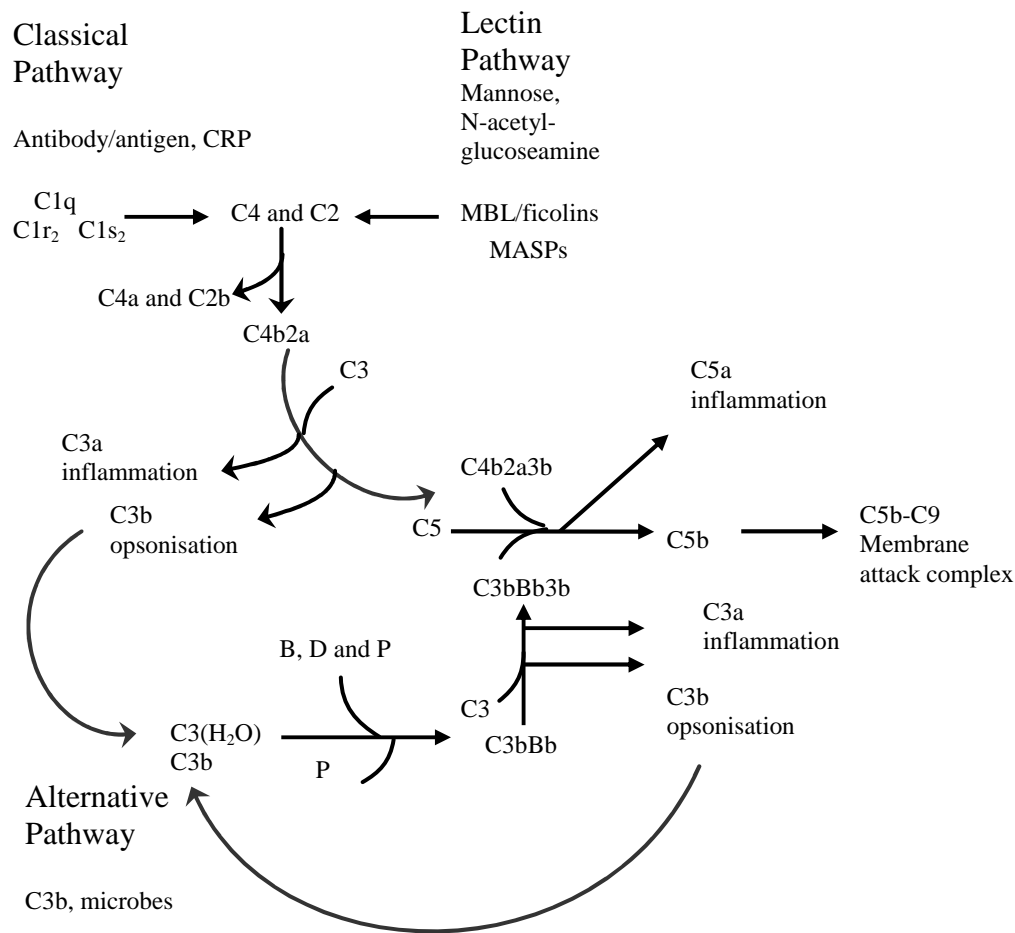


Figure 3. Activation of the classical, lectin and the alternative pathways initiate C3b opsonisation on target cells, lysis of cell by the membrane attack complex and the release of C3a and C5a, potent chemoattractants resulting in an inflammatory reaction, B (factor B), D (factor D), P (properdin).

### The classical pathway

The classical pathway is activated by immune complexes containing IgM or IgG but other molecules such as LPS, C-reactive protein (CRP), apoptotic cells and nucleic acids are also able to activate this pathway (Fig. 4) [17]. The components involved in the classical pathway are C1, C4 and C2. C1 is a  $\text{Ca}^{2+}$ -dependent complex consisting of C1q, C1r<sub>2</sub> and C1s<sub>2</sub>. When the recognition molecule C1q binds to a target, a conformational change occurs, leading to activation of C1r which, in turn, activates C1s. Activated C1s cleaves C4 into a small C4a fragment and a larger C4b fragment. The small C4a fragment diffuses away while the larger C4b fragment may attach covalently to the activator or other molecules in the vicinity. C2 is also cleaved into two fragments by activated C1s; in the presence of  $\text{Mg}^{2+}$  the larger C2a

fragment can bind to C4b and form the classical pathway C3 convertase C4b2a. The C3 convertase cleaves C3 into two major fragments (C3a and C3b), and C3b molecules can bind to the C3 convertase forming the C5 convertase C4b2a3b which cleaves C5 and activates the terminal pathway (Fig. 3) [16].

Oponisation of the target by C3b or C4b supports uptake and clearance of antigen, apoptotic cells and immune complexes by phagocytic cells. The smaller fragments released during complement activation, C3a and C5a are potent chemoattractants and their release results in an inflammatory reaction and the recruitment of phagocytic cells to the damage site by increasing permeability of the capillary beds [14].

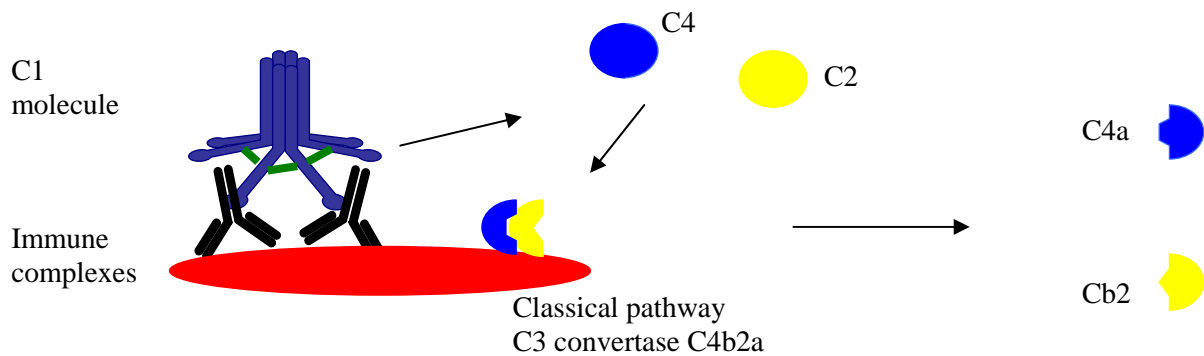


Figure 4. Complement activation of the classical pathway.

### The alternative pathway

The alternative pathway, described as an antibody-independent pathway, includes the complement components C3, factor B, factor D and properdin. This pathway is initiated by spontaneous hydrolysis of the internal thioester in C3, to form C3(H<sub>2</sub>O). Factor B binds to C3(H<sub>2</sub>O) in the presence of Mg<sup>2+</sup> and factor D then cleaves factor B into the fragments Bb and Ba. The complex produced, C3(H<sub>2</sub>O)Bb, is also known as a fluid phase C3 convertase and can cleave C3 into C3a and C3b. Various cell surfaces bind C3b which then can form a complex with factor B and after cleavage by factor D generate the alternative pathway C3 convertase C3bBb, which is able to cleave C3 into C3a and C3b. In this way more C3b is generated which can activate the alternative pathway creating an amplification loop. The C3b formed by activation of the classical or lectin pathway also initiates activation of the

alternative pathway [16]. Properdin binds and stabilises the alternative C3 convertase but has recently been described to also act as an initiator of the alternative pathway as well [18-20]. Activation of C3 via a C2 bypass pathway initiated by C1 or Mannan-binding lectin (MBL) but independent of C2 or MBL-associated serine protease (MASP)-2 has also been described [21-24].

### **The lectin pathway**

The lectin pathway is similar to the classical pathway and is initiated by the binding of MBL or ficolins in complex with MASP molecules (MASP 1, 2 and 3), to sugar structures like N-acetylglucosamine and mannose present on many microorganisms [25]. The binding is  $\text{Ca}^{2+}$ -dependent and activates the MASP-2 which then can cleave C4 and C2, generating the C3 convertase C4b2a, the same C3 convertase as in the classical pathway.

### **The terminal pathway**

The C5 convertases (C4b2a3b and C3bBbC3b) generated by the different pathways cleave C5 into its active form C5b and this remains bound to the C5 convertase. This cleavage initiates the assembly of the membrane attack complex, with the binding of C6 and finally C7 to C5b, forming a C5b67 complex which dissociates from the C5 convertase and binds to the membrane surface. Thereafter C8 is incorporated, then C9 molecules bind to form the final membrane attack complex which leads to the formation of pores into the cell causing osmotic swelling and cell rupture [26]. The C5a fragment released after cleavage acts as a potent anaphylatoxin and chemoattractant for phagocytotic cells [27].

### **Regulation of complement activation**

Activation of the complement system needs to be carefully regulated to protect host cells and tissue from damage and both fluid phase and membrane bound regulatory proteins are present at high concentrations. The balance between activation and inhibition determines the outcome. C1 esterase inhibitor (C1INH) controls activation of both the classical and the lectin pathway by binding reversibly to C1 and MASP-2, but it can also bind to activated C1r and C1s and inhibit their activity [28]. Factor I regulates complement activity by cleavage of C3b and C4b using C4b-binding protein (C4BP) and factor H as soluble cofactors. C4BP also regulates the classical pathway by dissociating the subunits of the C3 convertase and the alternative pathway C3 convertase is regulated in the same way by factor H. Membrane bound molecules acting as inhibitors of complement activation include membrane cofactor protein

(MCP, CD46) and complement receptor (CR) 1 (CD35), which regulates the C3 activation by its function as a cofactor protein for factor I mediated cleavage of C3b. Decay accelerating factor (DAF, CD55) prevents the assembly of C3 and C5 convertases and may also accelerate the disassembly of preformed convertases. CR1, which binds to C1q, C3b, C4b and MBL on opsonised targets, mediates transport of immune complexes by erythrocytes and promotes phagocytosis [14]. The terminal pathway is regulated by clusterin and S-protein (vitronectin), which binds to fluid phase C5b67 and inhibits the assembly of the membrane attack complex. Protectin (CD59) prevents binding of C9 to the C5b678 complex [29]. To date, properdin is the only positive regulator described for complement activation, by binding to and stabilising the alternative pathway C3 convertase.

Complement receptors expressed on different cell types play a role in the regulation of complement, and binding of complement to the receptor influences other cellular function such as phagocytosis of immune complexes and apoptotic cells. CR2 (CD21) expressed on B-cells binds to iC3b or C3d and binding of C3d opsonised antigen to CR2, when antigen is also bound to B-cell receptor, results in B-cell activation and proliferation [30]. The complement receptors CR3 (CD11b/CD18) and CR4 (Cd11c/CD18) belong to the family of integrins and these receptors are also involved in the waste disposal of apoptotic cells by mediating phagocytosis of iC3b opsonised targets [31]. There are also several receptors described for C1q. Calreticulin in complex with CD91 binds to the collagen-like region of C1q and this interaction is involved in clearance of apoptotic cells [32]. The gC1q receptor binds to the globular region of C1q [33].

### **Complement deficiency**

Deficiency of complement proteins can be inherited or acquired. Deficiencies are rare but impaired complement function is associated with infections and autoimmunity, especially systemic lupus erythematosus (SLE). MBL-deficiency is the most common inherited defect among the complement proteins resulting in increased susceptibility to infections especially in early childhood [34]. Homozygous deficiency of the complement components in the classical pathway (C1, C4 and C2) is associated with pyogenic infections, but also with risk of development of SLE [35]. Alternative pathway deficiency states and deficiencies of components in the terminal pathway are all associated with an increased risk of invasive infections caused by predominantly *Neisseria* species. Acquired deficiencies may result from

decreased production or increased consumption of complement components caused by activation, but autoantibodies against complement components can also contribute [35, 36].

## **Programmed cell death**

Programmed cell death is a physiological process essential for the development of all organisms and for maintenance of homeostasis by removing unwanted cells, but is also involved in pathological conditions including immunological diseases and cancer [37, 38]. Programmed cell death is a complex, controlled, active process involving both biochemical and morphological changes that are dependent on signals and activities within the dying cells. During this process, the plasma membrane remains intact until the dying cell is phagocytosed [39]. Apoptosis is a form of programmed cell death, but cell death can occur in a non-apoptotic way and still be a physiological response. For the cell to be defined as apoptotic, morphological features such as cell shrinkage, chromatin condensation, nuclear fragmentation and membrane blebbing, with the maintenance of membrane integrity, should be fulfilled [40-43]. Another form of cell death is necrosis, a premature death caused by physical or chemical damage to cells, such as infections, toxins or trauma. It occurs suddenly without the actions of enzymes, and includes cell rupturing and displays pro-inflammatory properties (Fig. 5) [44]. A cell is considered dead when it has lost the integrity of its plasma membrane, or the cell, including its nucleus, has undergone complete fragmentation or has been engulfed by a nearby cell [43].

The term apoptosis and its morphological characteristics were described in 1972 by Kerr, Wyllie and Currie [40]. Apoptosis can be divided into different phases such as initiation, regulation, execution and clearance. The initiation phase is dependent on cell type and the apoptotic stimuli. The two major initiation pathways of apoptosis are the external death receptor pathway and the internal mitochondrial pathway, which are dependent on death inducing signals. In the regulation phase, proteases such as caspases, a group of proteases that become activated in response to cell death stimuli, participate in an enzymatic cascade leading to termination of the life of the cell [42, 45, 46]. Caspases are synthesised as inactive zymogens with little or no protease activity. They can be divided into two groups according to

their active function; the initiator caspases 2, 8, 9, 10, and 12 and the effector caspases 3, 6 and 7. If the ‘point of no return’ is reached, the cell is executed and undergoes an organised degradation. Apoptotic cells express phagocytic markers on their cell membrane and are rapidly phagocytosed without induction of an inflammatory response [47-49]. This is in contrast to necrosis which is a passive process characterised by cellular and nuclear swelling and cell rupture leading to an inflammatory response.

The nucleus, endoplasmic reticulum (ER), Golgi apparatus and lysosome also play an important role in the initiation and regulation of programmed cell death [50]. Once a cell gets a death signal the most likely outcome is a programmed cell death, however, there are options to arrest the process.

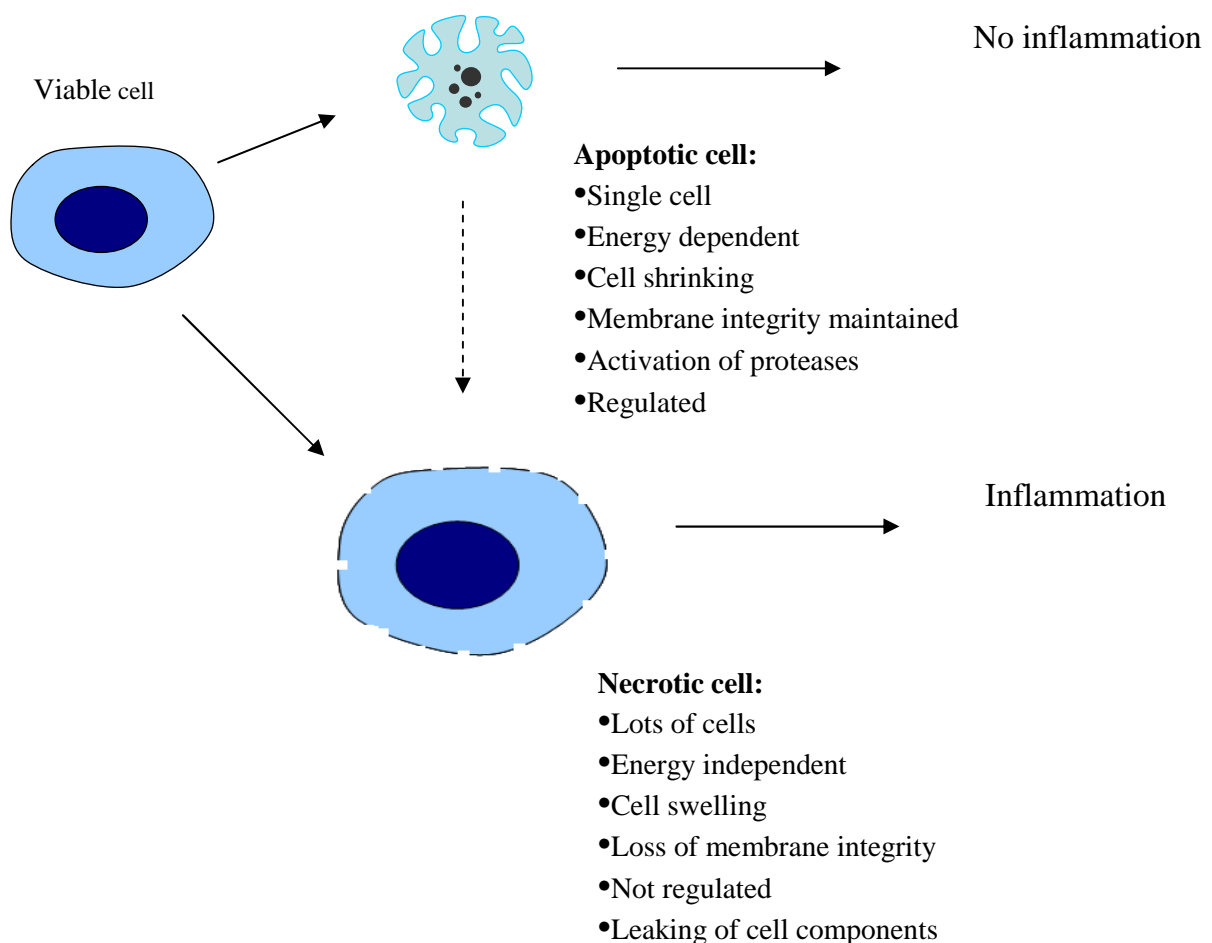


Figure 5. Apoptosis versus necrosis.



### **The external pathway**

The death receptors are transmembrane proteins belonging to the TNF receptor superfamily. They contain an intracellular death domain (DD) which can activate the death cascade or initiate a kinase pathway that turns on gene expression prolonging the life of the cell [51, 52]. The TNF receptor family contains several members that trigger apoptosis, the mechanisms of action of Fas and TNF receptor 1 (TNFR1) are the best elucidated. Binding of soluble or membrane bound ligands to the extracellular domain of the death receptors causes receptor trimerisation and aggregation of the cytoplasmic DDs. When apoptosis is triggered by Fas ligand, Fas becomes activated and forms a complex with the adaptor protein FADD (Fas associated death domain) [53, 54]. This recruits the apoptosis initiating protease procaspase 8 or 10 and the death-inducing signalling complex (DISC) is formed [55, 56]. This complex triggers the intracellular signalling cascades that induce apoptosis. This step can be regulated by cFLIP (cellular FADD-like interleukin-1-converting enzyme inhibitory protein), which is an inactive homologue of caspase 8 and prolongs the survival of the cell [57]. By proteolytic cleavage of procaspase 8 at a specific aspartic acid residue, a large and a small subunit are released which associate to form heterodimers containing two active sites. Caspase 3, the substrate for active caspase 8, is activated in two ways. The first mechanism involves direct cleavage of procaspase 3 to yield activated caspase 3, which, in turn, stimulates other effector caspases resulting in cleavage of structural and regulatory intracellular proteins and DNA fragmentation, and finally cell death. The second mechanism is indirect activation where caspase 8 cleaves the pro-apoptotic protein Bid which then can translocate to the mitochondria where it triggers cytochrome c release, eventually leading to activation of caspase 3 (Fig. 6).

TNF produced by activated T-cells and macrophages in the inflammatory response, is able to activate the TNFR1 receptor. The TNFR1 receptor acts in a similar way as the Fas pathway but it needs the adaptor molecule TRADD (TNFR-1-associated DD) [58] before the FADD complex is formed, and procaspase 8 or 10 is recruited (Fig. 6).

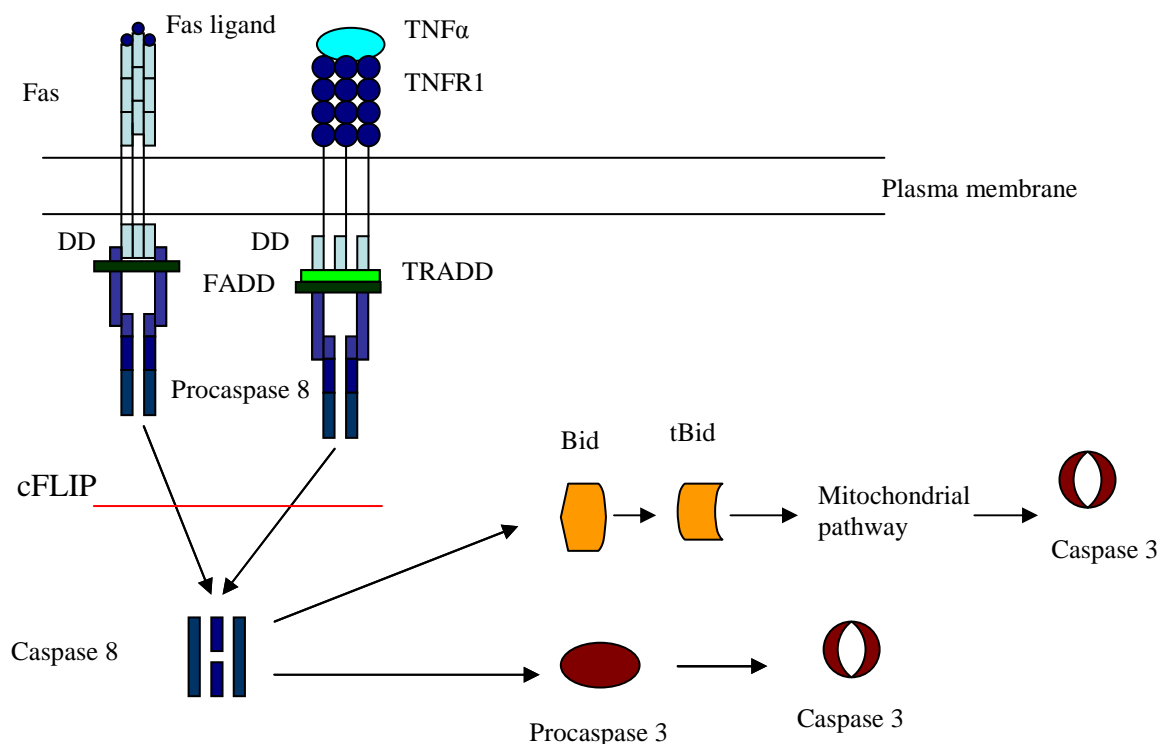


Figure 6. Apoptosis induced by the death receptors Fas and TNF- $\alpha$ .

The interaction of TNF with its receptors can also inhibit apoptosis by activation of nuclear factor  $\kappa$ B (NF- $\kappa$ B), causing an up-regulation of the expression of several anti-apoptotic proteins, which results in a prolonged cell survival [37].

TNF-related apoptosis inducing ligand (TRAIL) is able to activate five different receptors. Two of the receptors, TRAIL-R1 and TRAIL-R2, contain an intracellular DD and they are capable of inducing apoptosis but are mainly expressed on transformed cells. The other three receptors (TRAIL-R3, TRAIL-R4 and TRAIL-R5) are decoy receptors with no or non-functional DD and cannot induce apoptosis [59].

### The internal pathway

The mitochondrial pathway is a central regulator of apoptosis and acts as a local sensor of cellular stress, growth factor deprivation, irradiation and for the absence of survival signal.

The mitochondrial pathway is triggered and regulated by proteins belonging to the Bcl-2 family, which possesses both anti-apoptotic and pro-apoptotic activity [60, 61]. Bcl-2 family members control cell death by regulating the release of cytochrome c and other proteins from the mitochondrial intermembrane space into the cytosol [62, 63]. The Bcl-2 family members contain specific homologous regions, called Bcl-2 homology (BH) domains and all members have one to four of these domains. They have been classified on the basis of their structural similarities, resulting in four categories of BH domains (BH1 to BH4) and according to functional criteria as either pro- or anti-apoptotic [64, 65]. Pro-apoptotic proteins contain BH1, BH2 and BH3 type domains such as Bax and Bak. They act by perturbing the mitochondria outer membrane allowing release of intermembrane space proteins and ions. The proteins with only BH3 type domains, such as Bid, Bad and Bim are also pro-apoptotic; they inhibit the activity of the anti-apoptotic proteins and promote activity of the BH1-BH3 pro-apoptotic proteins. The proteins with anti-apoptotic properties contain all four BH type domains. Examples are Bcl-2 and Bcl-x<sub>L</sub> and they protect cells from death by stabilising the mitochondrial outer membrane. The pro-apoptotic Bcl-2 family proteins are located in the cytosol or associated with the cytoskeleton. After a death signal, the pro-apoptotic Bcl-2 family members undergo a conformational change that enables them to target and integrate into membranes [66]. The anti-apoptotic Bcl-2 members are initially integral membrane proteins found in the mitochondria, ER or nuclear membrane. They can inhibit the activation of the pro-apoptotic Bcl-2 family members through dimerisation with them [67]. The pro-apoptotic Bcl-2 family members are inserted into the outer mitochondrial membrane where they form channels probably together with other proteins and mitochondria lipids. This formation of specific pores in the outer membrane of the mitochondrion is reversible in respect to mitochondrial function but it triggers other death signals by the release of proteins and ions [63]. Another pathway results in the loss of mitochondrial membrane potential by opening permeability transition pores (PT). There is a collapse in the electrochemical gradient across the mitochondrial membrane which results in equilibration of ions between the matrix and cytoplasm, osmotic swelling of the mitochondrial matrix, rupture of the mitochondrial outer membrane and the release of proteins and ions. The PT pathway occurs mainly in response to the release of Ca<sup>2+</sup> from ER, and Bcl-2 members are also involved in the regulation of Ca<sup>2+</sup> release from the ER.

Upon release of cytochrome c from the mitochondrion [68] it forms a complex with apoptotic protease activating factor 1 (Apaf-1) in combination with either ATP or dATP. Apaf-1

oligomerises and associates with procaspase 9, via the caspase activation and recruitment domain (CARD). This complex, called the apoptosome, is able to activate caspase 3 and cell death occurs [69, 70]. The formation of the apoptosome is regulated by inhibitors of apoptosis (IAPs) that prevent the formation of the complex. There are at least five different IAPs which are released from the mitochondria and they act by directly inhibiting the caspases. They are removed by Smac/DIABLO (Second mitochondria activator of caspase/Direct IAP binding protein with low pI) a protease also released from the mitochondria in a coordinated fashion release with cytochrome c before the formation of the apoptosome takes place. Bid is activated through the death receptor pathway and can activate the mitochondrial pathway by translocation to the mitochondria and through complexation with Bax induce pores in the outer membrane [71, 72]. An additional activator of apoptosis is ceramide. Ceramides are lipid molecules and are found in high concentrations within the cell membrane and they are pro-apoptotic molecules which act to induce apoptosis by initiating cytochrome c release from the mitochondria (Fig. 7) [73].

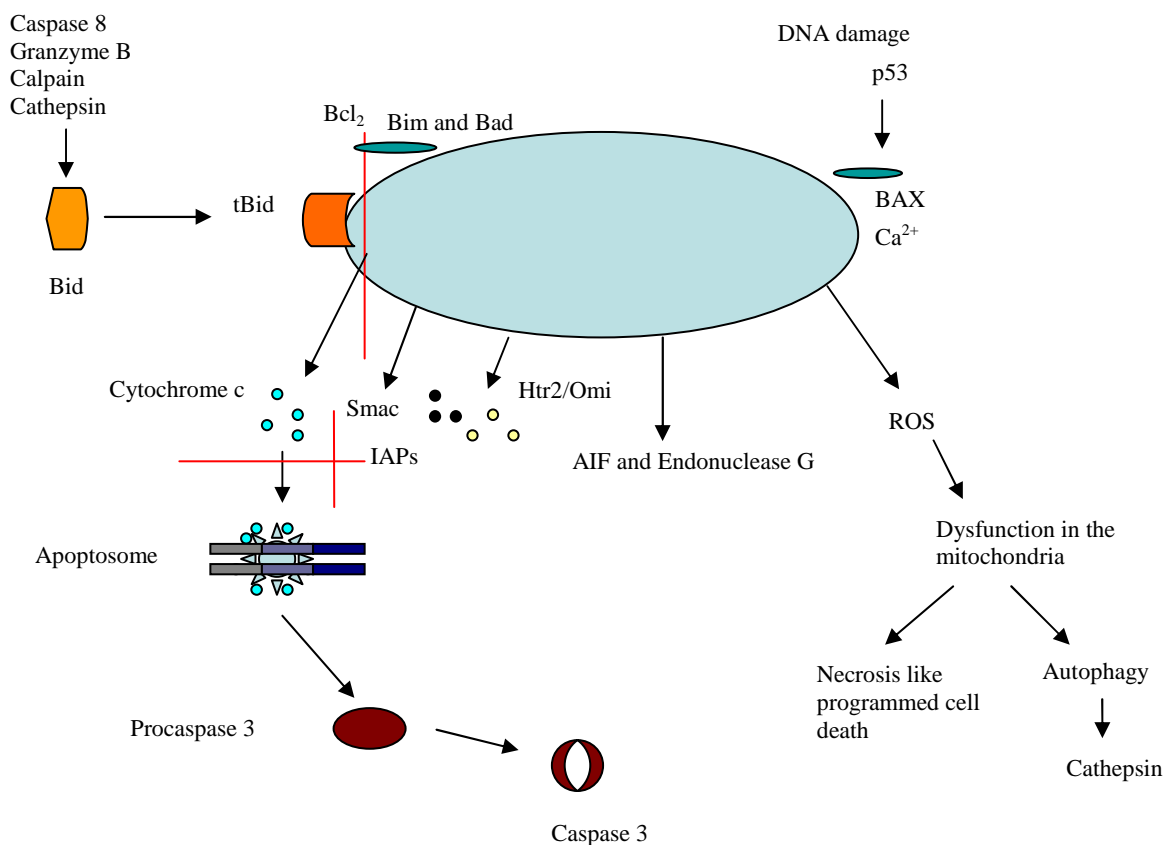


Figure 7. Apoptosis via the internal mitochondrial pathway.

Programmed cell death can take many forms but not all fulfil the criteria of apoptosis. Htr2/Omi, a serine protease released from the mitochondria, participates in both caspase dependent and independent programmed cell death by virtue of its ability to function as an inhibitor of IAPs and as a general protease. Apoptosis inducing factor (AIF) and Endonuclease G, both released from the mitochondria, are able to translocate to the nucleus and cause programmed cell death by a caspase independent DNA-fragmentation of chromatin, resulting in high molecular weight DNA fragments [74]. Mitochondria outer membrane permeabilisation can be induced by reactive oxygen species (ROS),  $\text{Ca}^{2+}$  release, and an accumulation of misfolded proteins from the ER. Calpains are cysteine proteases that naturally occur as inactive pro-enzymes. However, in the presence of high  $\text{Ca}^{2+}$  concentration they become activated and participate in apoptosis initiated by glucocorticoids or irradiation [75].

In normal cells, the p53 tumour suppressor protein is present at very low levels. The main function of p53 is to regulate the cell cycle, but in response to cellular stress, such as DNA damage, hypoxia and oncogene activation, the p53 protein is up-regulated and the cell undergoes cell-cycle arrest. This allows for DNA to be repaired, or if that fails the cell dies by apoptosis. The pro-apoptotic Bcl-2 family member protein, Bax, is up-regulated in response to DNA damage and increased p53 levels. Furthermore, the anti-apoptotic Bcl-2 family members are transcriptionally repressed by p53. Transcription of genes that increase production of ROS, an activator of the mitochondrial apoptotic pathway, is induced by p53 and p53 may also up-regulate Fas, inducing Fas mediated programmed cell death.

Granzyme B and perforin (see page 13) is able to induce apoptosis in target cells by entering the cell through a non-specific ion pore composed of perforin [76]. Granzyme B can also initiate apoptosis by cleavage of Bid, caspase 3 and caspase 7.

Cathepsins are proteases involved in the digestion of apoptotic cells. Cathepsins are located in the lysosome which is responsible for proteolysis of endocytosed and autophagocytosed proteins at low pH. Destabilisation of the lysosomal membranes by oxidants, pore formation through the Bcl-2 family members or shingosine, a lysosomotropic detergent, results in release of cathepsins to the cytosol where they induce cell death through proteolytic effects in the cytoplasm and the nucleus, or cleavage of Bid. Cathepsin is also able to activate caspases by direct cleavage and shares many substrates with DNA repair enzyme poly(ADP-ribose)

polymerase (PARP), Bid and caspases [77-80].

### **Autophagy**

In healthy cells, autophagy is a process where the cell degrades old and damaged organelles within the cell, acting like a cell survival pathway but under certain circumstances it acts as an alternative cell-death pathway [81]. During starvation or in hormone deprived cells, the material can be recycled. The damaged organelles are engulfed by autophagosomes created by donated membranes from the ER that surrounds the organelle. The autophagosomes fuse with the lysosomes and form autophagic vacuoles. Lysosomes contain digestive enzymes and are responsible for degrading old and damaged organelles within the cell. Autophagy can be triggered by the same signals as apoptosis and is described as a backup system of apoptosis.

### **The execution**

Caspase-activated deoxyribonuclease (CAD) is the nuclease that degrades the genomic DNA between the nucleosomes into approximately 180 base pair fragments, which, when DNA is analysed, appears as a DNA ladder and is a marker for apoptosis. The nuclease CAD exists as an inactive complex (ICAD) with no DNase activity in living cells. However, ICAD becomes activated upon caspase 3 mediated cleavage and can enter the nucleus and degrade the chromosomal DNA [82]. The nuclear shrinkage and budding is caused by caspase mediated cleavage of laminin, a network of protein filaments surrounding the nuclear periphery that maintains the shape of the nucleus and mediates interactions between chromatin and the nuclear membrane [83-87]. Cleavage of PAK2, a member of the p21-activated kinase family mediates the active blebbing observed in apoptotic cells [88]. Caspases also cleave the cytoskeleton proteins fodrin and gelsolin causing loss of cell shape [89]. The DNA repair enzyme PARP is cleaved by caspases with the subsequent loss of its DNA repair activity [90]. DNA topoisomerase II, a nuclear enzyme essential for DNA replication and repair, could also be inactivated by caspases leading to DNA damage.

## **Phagocytic clearance of dying cells**

Phagocytosis of apoptotic cells is a very complex procedure, but under normal conditions it is a fast and a non-inflammatory process preventing exposure of self-molecules. The uptake of apoptotic cells actively suppresses the release of pro-inflammatory molecules and promotes the release of anti-inflammatory molecules [91, 92]. The cell that is about to die sends out so-called “find-me” signals such as lysophosphatidylcholine. For the recognition by the phagocyte, the apoptotic cell displays so-called “eat-me” signals on its surface. These can be pre-existing molecules, modified existing molecules, as well as the appearance of molecules on the cell surface such as phosphatidylserine (PS), which is normally located on the inner leaflet of the plasma membrane. At the same time the so-called “do not eat-me” signals are down regulated, shed or internalised [48, 93, 94]. Many different recognition molecules on the phagocyte orchestrate the clearance of apoptotic cells such as scavenger receptors (SR-A, LOX-1, CD68, CD36, CD14), lectin receptors (CD91/calreticulin), integrin receptors (vitronectin receptor  $\alpha v/\beta 3$ , CR3 and 4) and ATP-binding cassette transporter. Different bridging molecules such as trombospondin, Gas-6, MFG-E8 and complement factors such as C1q, MBL, ficolins, properdin and C3 have also been implicated as significant players in this process [31, 94]. The binding of complement components to apoptotic cells can take place directly to the apoptotic cell surface, but they can also act as bridging molecules between the phagocyte and the apoptotic cell (Fig. 8) [95]. The complexity in studying and defining apoptotic cells as early apoptotic or secondary necrotic cells has led to some lack of clarity in the understanding regarding the role of complement components in the phagocytosis of apoptotic cells. C1q, MBL and properdin bind to apoptotic cells late in the death process and enhance their phagocytosis [31, 96-103], but binding of C1q, MBL and properdin to early apoptotic cells is also described [104, 105]. During apoptosis the membrane bound regulatory proteins are either internalised, down-regulated or detached from the cell surface, which makes it favourable for C3b binding to the cell surface [16, 106], but at the same time fluid phase C4BP and factor H regulate complement activation from extensive lysis of the apoptotic cell [100].

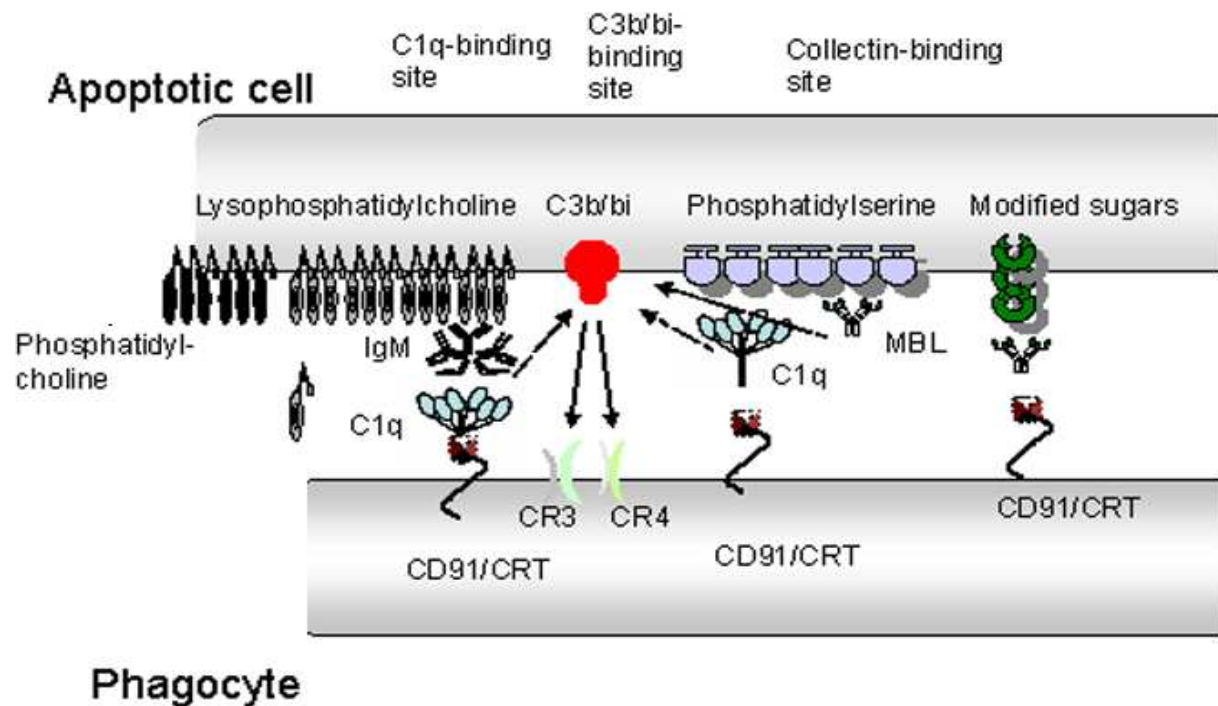


Figure 8. Complement components can bind directly to apoptotic cells or act as bridging molecules between the phagocyte and the apoptotic cell.

## Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a chronic inflammatory, relapsing, autoimmune disorder. Manifestations are diverse and affect many organ systems such as the skin, joints, kidneys, nervous system, heart and lungs by causing inflammation and organ damage. The disease may vary from mild to severe and fatal and common symptoms include fatigue, fever, arthritis, rash, vasculitis and sensitivity to sunlight. SLE is 6-8 times more common among women than among men and onset usually occurs between the ages of 10-50 years and with an incidence of 4.8/100 000 per year in southern Sweden [107]. Both the innate and the adaptive immune systems contribute to the pathology seen in the disease.

The cause is not known, but a complex combination of genetics and environmental factors seems to be involved. UV-light, virus infection or other infectious agents, hormones and drugs may result in the disordered immune response that typifies SLE. Environmental influences on the expression of disease manifestations are clearly seen in SLE but no clear evidence has been found that an environmental trigger is involved in the initiation of the disease [108, 109]. SLE is influenced by many genetic factors and the MHC class II and class



III genes show strong association with SLE. The MHC genes located on chromosome 6 are involved in the immune response by participating in the recognition of self and non-self but also the complement genes C2, C4 and factor B are encoded by this region. C4 is encoded by two closely linked genes, which have minor differences, producing two isotypic variants, C4A and C4B and null alleles of C4A are associated with SLE. A single nucleotide polymorphism, identified in the gene of integrin alpha M (ITGAM), a subunit of the CR3 receptor (CD11b) involved in binding of iC3b, has shown strong association with SLE. Several other genetic variations are seen in SLE such as a general up-regulation of IFN-inducible genes, termed the type I IFN signature [110]. Genetic factors seem to play a role in the development of SLE and environmental factors may trigger the disease in genetically susceptible subjects.

The diagnosis of SLE is based on clinical manifestations together with immunological abnormalities. For case definition in clinical research, classification criteria, American College of Rheumatology (ACR) are used [111]. The 11 manifestations included in the ACR criteria are malar rash, discoid rash, photosensitivity, oral ulcers, arthritis, serositis, renal disease, neurological disorder, haematological manifestation (leukopenia, lymphopenia and thrombocytopenia), immunologic disorder (anti-DNA antibodies, anti-Smith autoantibodies and anti-cardiolipin antibodies) and anti-nuclear antibodies. The presence of anti-cardiolipin antibodies is seen in the antiphospholipid syndrome which may occur in isolation or in association with connective disease, particularly SLE [112]. The most common clinical manifestations of this syndrome are venous or arterial thrombosis and recurrent fetal loss. For diagnosis of SLE, the presence of four or more of the ACR criteria is required. Disease activity can be measured by the validated SLE Disease Activity Index 2000 (SLEDAI-2K) [113].

There is no cure for the disease; treatment of SLE is to relieve symptoms and protect from organ damage by decreasing inflammation in the body. The disease is heterogeneous and varies from person to person as well as in person with periods of flare followed by periods of remission. The symptoms and organ system involved and severity of the disease decide which drug to be used. Anti-inflammatory drugs, glucocorticoids and immune suppressive treatment may be used.

The production of autoantibodies is antigen-driven and dependent on T-cell and hyperactive B-cell responses, generating circulating immune complexes causing damage by deposition in

organs and activation of the complement system. The autoantibodies produced are directed to components of the cell nucleus including dsDNA, RNA, histones, nucleosomes and small nuclear ribonucleoproteins (snRNPs) [109, 114, 115]. One of the first described laboratory abnormalities associated with the SLE disease is the LE cell phenomenon. In 1948 Hargraves *et al* observed PMNs in bone marrow preparations that had engulfed large masses of material containing cell nuclei and autoantibodies against the nuclei [116]. It has later been recognised that autoantibodies against histone 1 are involved and the presence of complement is required for the formation of LE cells [117, 118].

A fast and efficient removal of dying cells and their remnants are of importance for protection against exposure to these autoantigens. Cells that die by apoptosis are rapidly taken up by neighbouring cells or by specialised phagocytes such as macrophages [119]. The uptake of apoptotic cells by the macrophages leads to release of anti-inflammatory substances like IL-10 and transforming growth factor beta (TGF- $\beta$ ), and pro-inflammatory mediators like IL-2 and TNF- $\alpha$  are suppressed [91, 120]. SLE patients show an impaired clearance of apoptotic cells [121, 122], an accelerated apoptosis of cells [123-126] and, furthermore, an apoptosis inducing factor is present in the serum of SLE patients [127-130]. The waste and disposal theory of SLE postulate that ineffective clearance of apoptotic cells and cell debris leads to the initial break in self tolerance. Apoptotic cell material is most likely the main source of autoantigens in SLE; the increased amount of potential autoantigens could be an important disease mechanism (Fig. 9) [109, 131, 132]. In knockout mouse models involving molecules in the clearance of apoptotic cells, such as DNaseI, Serum Amyloid P component (SAP), C1q and IgM all showed development of classical symptoms of SLE [133-135].

Type I IFNs have many effects on the immune system and most of them promote immune response. In SLE, besides the presence of hyperactive B-cell response, autoantibodies against modified nuclear components, increased complement consumption, decreased clearance of apoptotic cells, an ongoing production of type I IFNs is seen. Elevated levels of serum IFN- $\alpha$ , a cytokine belonging to the family of type I IFNs, have been shown in SLE and the elevated levels correlate with both disease activity and severity. Immune complexes containing RNA or DNA may activate plasmacytoid dendritic cells (natural IFN-producing cells) to produce IFN- $\alpha$ , this is mediated by the involvement of Fc $\gamma$  receptor, TLR-7 or TLR-9 [136]. C1q is shown to inhibit the immune complex induced IFN- $\alpha$  production by the plasmacytoid dendritic cells [137].

Deficiency of proteins in the classical pathway of complement activation, C1q, C4 and C2 are associated with the development of SLE. A clear hierarchy exists where more than 90% of C1q deficient individuals, 75% of C4 deficient individuals and about 20% of C2 deficient individuals develop SLE. Among the patients deficient in C1q and C4 an equal gender distribution of disease is seen but in the C2 deficient individuals, SLE is more common among women [35, 138, 139]. Acquired deficiency associated with antibodies against C1q or due to complement activation is also commonly seen among patients with SLE [140]. The components in the classical pathway are all needed for an efficient phagocytosis of apoptotic cells [102] but C1q is also important for the regulation of cytokines and IFN- $\alpha$  production induced by DNA containing immune complexes [137]. The complement system mediates two sides in the pathogenesis of SLE; complement is needed for an efficient phagocytosis of immune complexes and apoptotic cells but at the same time it mediates inflammation and tissue damage. This is called the Lupus Paradox [141].

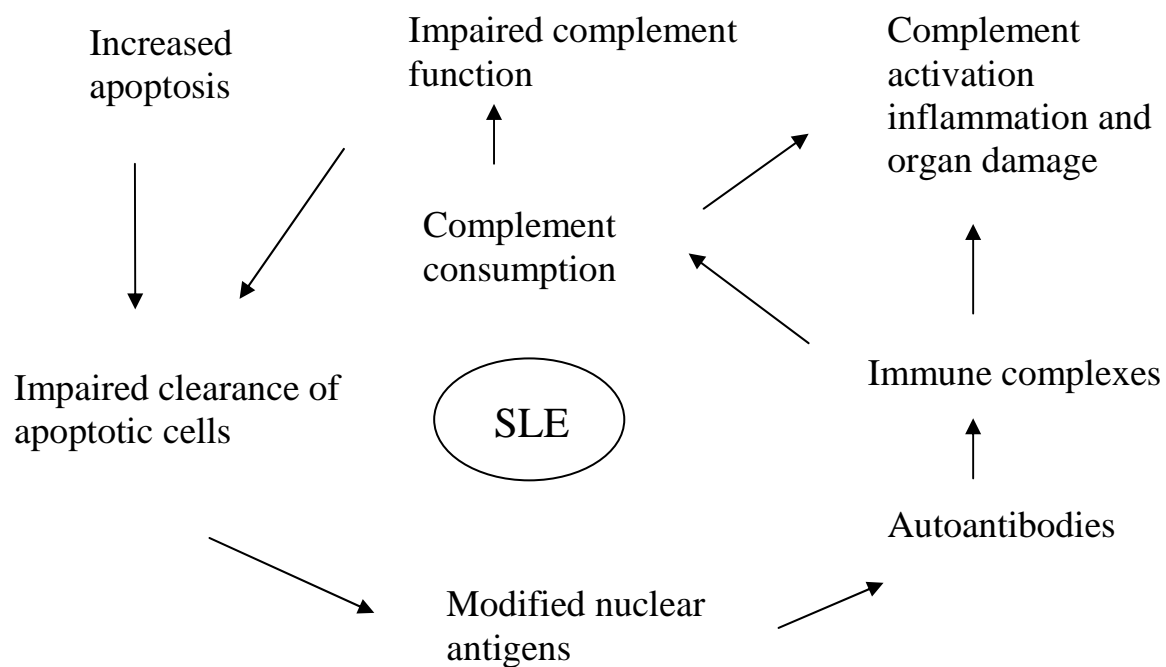


Figure 9. Impaired complement function contributes to a decreased clearance of apoptotic cells leading to exposure of autoantigens, such as modified nuclear components and formation of immune complexes causing complement activation, inflammation and organ damage. UV-light and virus infection can cause increased apoptosis.

# PRESENT INVESTIGATION

## Aims

Paper I: To confirm and extend observations of soluble factors in serum from SLE patients with capacity to induce apoptosis in normal cells and cell lines.

Paper II: To characterise mechanisms involved in the apoptosis induced by serum from SLE patients.

Paper III: To investigate the relative importance of the different complement activation pathways regarding clearance of apoptotic cells.

Paper IV: To analyse the presence of autoantibodies against histones in serum from SLE patients and measure phagocytosis of necrotic material in the presence of serum from SLE patients by polymorphonuclear leukocytes.

## Papers I and II

A most likely source of autoantigens in SLE is apoptotic cells, and due to decreased clearance of such cells increased immune response is seen. When this study was started, evidence suggested that an apoptosis inducing factor was present in serum from SLE patients [127]. It was originally observed that sera from SLE patients affect monocytes, resulting in decreased phagocytosis, spreading and cell adherence [142]. In Papers I and II we study the ability of serum from SLE patients to induce apoptosis on different cells and the mechanisms behind it. Serum induced apoptosis was studied by using serum from SLE patients with both active and inactive disease. Serum from patients with other autoimmune diseases such as rheumatoid arthritis (RA) and primary systemic vasculitis, infectious diseases such as mononucleosis (Epstein-Barr virus infection) and *Streptococcus pneumoniae* septicaemia and sera from healthy individuals were also used.

Routine laboratory tests included serum concentrations of CRP, complement proteins (C1q, C3 and C4), and anti-dsDNA antibodies. In addition, full blood counts were performed on the SLE patients.

Peripheral blood mononuclear cells were obtained from fresh heparinised blood samples from different healthy donors, and purified monocytes and lymphocytes were used. The monocytoid U937 cell line and lymphocytoid Jurkat T-cell lines E6-1, A3, I 2.1 (FADD mutant of A3), SFFV-*neo* and SFFV-*bcl-2* (overexpressing Bcl-2) were used. The cells were incubated with serum from patients and from the controls, and the serum induced apoptosis was detected by measuring binding of Annexin V-fluoroisothiocyanate (FITC) and propidium iodide (PI) to the cells with flow cytometry. Two SLE sera with a high apoptosis inducing effect were depleted of IgG by absorption on protein G sepharose and two sera were heat inactivated at 56°C for 30 minutes to inhibit complement activation.

Monoclonal antibodies against Fas (ZB4) and TNFR were used in experiments designed to block apoptosis via the death receptor pathway and cells were also pretreated with pan-caspase and caspase-8 inhibitors when evaluating the involvement of caspases in the serum induced apoptosis. The apoptosis inducing effect (AIE) was defined as percentage of apoptotic cells induced by the test serum minus the percentage of apoptotic cells induced by the negative control. Caspase activity during serum induced apoptosis was measured by flow cytometry using the kits CaspaTag™Caspase-3 (DEVD), CaspaTag™Caspase-8 (LETD), CaspaTag™Caspase-9 (LEHD) (Intergen Company, Norcross, GA, USA) and Caspase-8 FLICA (FAM-LETD-FMK), Caspase-9 FLICA (FAM-LEHD-FMK) (Immunochemistry Technologies, LLC, Bloomington, MN, USA).

## **Results**

This study showed that apoptosis was induced in a time dependent manner in monocytes and lymphocytes from healthy donors by supplementing the growth medium with 20% of serum from SLE patients. The Annexin V binding to the cells preceded PI reactivity supporting the idea that serum from SLE patients induces apoptosis and not primary necrosis. Morphological studies with light and confocal microscopy showed that serum induced apoptosis on normal cells incubated with serum from SLE patients displayed a classic apoptosis with shrunken cells, nuclear condensation and formation of apoptotic bodies. This was confirmed by flow cytometry by distribution of the cells in forward and side scatter properties (reflecting size

and structure, respectively) and by Annexin V binding. Caspase 8 and caspase 9 activity measured over time mirrored each other with an increased activity already apparent after 4h. The caspase activity could be reduced significantly ( $p < 0.05$ ) by the caspase inhibitors after 6h incubation of cells with SLE serum, but no effect by the caspase inhibitors was seen after 16h incubation. No decrease in the AIE of serum from SLE patients was seen when using antibodies blocking the death receptor pathway, indicating that death receptor independent pathways are more important than death receptor dependent ones.

Serum induced apoptosis was also seen when using Jurkat, U937 and FADD-deficient cell lines, but with the cell line over-expressing Bcl-2, a significant decrease ( $p < 0.05$ ) of serum induced apoptosis was seen, indicating the involvement of the mitochondrial pathway. Depletion of IgG from serum and heat inactivation of serum did not have any effect on the AIE, demonstrating that it was independent of IgG and complement activation.

The observed serum induced apoptosis was not seen when using sera from other autoimmune diseases, infectious diseases or healthy individuals. Furthermore, no differences were seen between sera from SLE patients with active or inactive disease.

There was no correlation between SLEDAI and the AIE on monocytes or lymphocytes using serum from SLE patients. Patients with low C1q and/or low C4 levels had notably higher AIE on both monocytes and lymphocytes. This was also seen for serum with low C3 levels, but in this case the correlation was less pronounced. Levels of C5a in cell culture supernatants correlated with the AIE on monocytes but much less with AIE on lymphocytes. The serum concentration of anti-dsDNA antibody correlated weakly with AIE on monocytes and between anti-cardiolipin antibody levels and lymphocyte apoptosis a correlation was also found. Levels of TNF- $\alpha$  in cell culture supernatants, but not in the sera, correlated with the AIE on both monocytes and lymphocytes.

### **Paper III**

Inherited deficiencies in complement components of the classical pathway are associated with a high risk for development of SLE. Therefore, the pathways involved in complement activation by apoptotic cells were studied using serum from individuals deficient in the

complement proteins C1q, C2, C4, C3, properdin and MBL. Normal human serum and a pool of sera from ten different healthy individuals were also used. Serum reagents lacking C1q and factor D or factor D only were prepared as previously described [143]. The serum used for C1q and factor D depletion was also deficient of MBL. Purified complement proteins C1q, C2, C4, factor D and properdin were available in the laboratory and published methods for purification had been used [144-148]. Recombinant MBL (rMBL) was kindly provided by Professor J. C. Jensenius (Aarhus, Denmark).

For the generation of macrophages, peripheral blood monocytes were obtained from fresh heparinised blood samples from healthy donors. The monocytes were cultured for 5 days in RPMI 1640 media, containing 10% normal human serum, gentamycin and amphotericin allowing the monocytes to differentiate into macrophages (MDM). Apoptotic cells were obtained by treating Jurkat cells with staurosporine; yielding approximately 50% early apoptotic cells and 50% late apoptotic cells, and then the cells were labelled with CFS-E. The labelled apoptotic cells were allowed to interact with the MDMs in the presence of different serum. The MDMs were collected and RPE-cy5 anti-CD14 was added. Engulfment of labelled apoptotic Jurkat cells by the MDMs was analysed by flow cytometry. Cells positive for both CD14 and CFS-E were considered as apoptotic cell-engulfing MDMs and the number of those cells were calculated as a percentage of the total number of CD14-positive cells. For measuring complement deposition on apoptotic cells, the cells were incubated with different deficient sera or reagents. Complement activation was assessed by measuring C3 deposition using antibodies against C3 fragment, with specificity for C3d, followed by incubation with specific antibodies (anti-mouse) conjugated with phycoerythrin before analysed by flow cytometry.

## **Results**

Sera from individuals deficient in classical pathway components (C1q, C2 and C4) all showed a decreased phagocytosis of apoptotic cells. When adding back the missing complement component, the phagocytosis increased to a level similar to that of normal human serum. The lectin and the alternative pathway did not influence phagocytosis of apoptotic cells, since sera lacking MBL, properdin or factor D showed equal levels of phagocytosis as normal human serum. The C3 deficient serum showed decreased levels of phagocytosis of apoptotic cells but increased after reconstitution with C3 albeit not to the same level as normal human serum.

Sera from healthy controls and sera with low MBL levels supported C3 deposition on apoptotic cells over a wide range of serum concentrations (2.5-40%). The effect increased with increasing serum concentration and was more pronounced on secondary apoptotic cells. Serum deficient in the classical pathway only showed C3 deposition in high serum concentrations, indicating involvement of the alternative pathway. Experiments with MBL-deficient serum further depleted of C1q and factor D supported the predominance of the classical pathway regarding C3 deposition on apoptotic cells.

## **Paper IV**

Autoantibodies directed against autoantigens, such as nucleic acid and nucleic acid associated proteins, are common in SLE, and apoptotic and necrotic cells are known to expose these autoantigens. Phagocytosis of necrotic material (NC) and apoptotic cells by polymorphonuclear leukocytes (PMNs) were studied in the presence of serum samples collected consecutively over several years from 19 SLE patients (n=798). Routine laboratory testing of complement proteins (C1q, C3 and C4) and anti-dsDNA antibodies had previously been performed on serum from these SLE patients.

Sera from 64 SLE patients selected for time points at high and low disease activity estimated by SLEDAI-2K [113] and sera from individuals deficient in the complement proteins C1q, C2, C4 and properdin were also used. Purified complement proteins C1q, C2, C4, and properdin were available in the laboratory and were used to restore deficient sera. These proteins had previously been purified according to published methods [144-148]. As controls, sera from 100 healthy blood donors were used.

Peripheral blood mononuclear cells (PBMC) were obtained from fresh heparinised blood samples from healthy donors and PMNs were isolated by density gradient centrifugation according to the manufacturer's protocol. Engulfment of NC or apoptotic cells by the PMNs in the presence of serum was analysed by flow cytometry and two assays for phagocytosis were used. One of these assays measured phagocytosis of necrotic cell material (PNC assay) and the other assay measured phagocytosis of apoptotic cells (PAC assay). The NC material was generated by incubating PBMC for 10 min at 70°C and apoptotic cells were generated by treatment with staurosporine. Autoantibodies against histones were measured by ELISA. Two



different ELISAs were developed, one detecting antibodies against only histone 1, the linker histone, and a second in which a mix of histones (1, 2, 3 and 4), of which 2, 3 and 4 belong to the core histones, was used as antigen.

## **Results**

Using flow cytometry, PMNs containing necrotic cells and apoptotic cells could be detected. Increased levels of phagocytosis of NC and apoptotic cells were seen in the presence of serum from SLE patients. Of the 19 SLE patients, 17 showed increased levels of phagocytosis of NC at some time point and this was not seen in the healthy controls. The phagocytosis of NC was dependent on antibodies and a functional complement system, shown by decreased levels in the PNC assay after depletion of IgG and heat inactivation of sera.

To further establish the importance of the complement system for an efficient phagocytosis of NC, different sera from complement deficient individuals were used, and anti-histone antibodies were added to promote phagocytosis of NC. Sera with deficiencies within the classical pathway (C1q, C2 or C4) all showed a clear decrease in phagocytosis of NC in the presence of anti-histone antibodies when compared to normal human serum (NHS) supplemented with anti-histone antibodies. After reconstitution of the missing complement protein in the presence of anti-histone antibodies, the phagocytosis of NC equalled that of anti-histone antibody-supplement NHS. The serum deficient of properdin showed an equal capacity to promote phagocytosis of NC as the anti-histone antibody-supplemented NHS and no change was seen after reconstitution with properdin.

Anti-histone antibody levels measured by ELISA showed a good correlation with the ability to phagocytose NC. Classification of the anti-histone antibodies in the 19 SLE patients' sera by SDS-PAGE and Western blot showed that 10 sera contained autoantibodies against more than one histone, 5 sera contained autoantibodies against only histone 1, and the remaining 4 patient sera did not have detectable levels of autoantibodies against any of the histones tested.

Among the patients who were positive in the PNC assay, with antibodies against more than one histone, we found a trend of decreased concentrations of the complement proteins C1q, C3 and C4 not seen in sera with only antibodies against histone 1. A similar trend was seen in the correlation between antibodies against histones and the complement components C1q, C3 and C4.

Individual values of area under curve divided by follow-up time (AUC) in each of the 19 longitudinally followed patients were calculated and the patients were divided into groups based on the presence and specificity of antibodies to histones. Sera containing antibodies against more than one histone had increased activity in the PNC assay as compared to serum containing antibodies against only histone 1 ( $p=0.01$ ). Sera containing antibodies against more than one histone were also higher in the PNC assay as compared to sera without detectable anti-histone antibodies ( $p=0.008$ ). No differences in AUC values were seen when comparing phagocytosis of NC in serum containing antibodies against histone 1 with serum negative in the PNC assay. A trend towards decreased concentrations of C3 and C4 was seen in the group containing antibodies to more than one histone as compared to sera containing antibodies to only histone 1 or without anti-histone antibodies, but this was not statistically significant. Patients with antibodies to a broader spectrum of histones also had autoantibodies against DNA. In the 64 patients where serum samples had been selected for time point of low and high disease activity, a clear relation of increased phagocytosis of NC and high levels of autoantibodies against histones was seen at time point of high disease activity.

## **Discussion and future perspectives**

SLE is a complex disease and the cause is unknown, as in most other autoimmune diseases. Multiple genetic factors interacting with environmental factors seem to play a role in development of SLE and both the innate and the adaptive immune systems contribute to the pathology seen in the disease. Apoptotic cells have been suggested to be a major source of autoantigens in SLE since clustering and concentration of lupus autoantigens in the surface blebs of apoptotic cells have been demonstrated [114]. SLE patients show an impaired clearance of apoptotic cells, which may result in an increased exposure of autoantigens [149]. The formation of autoantibodies gives rise to circulating immune complexes causing damage by deposition in many organ systems and activation of complement.

In Paper I we showed that the apoptosis inducing effect in sera from SLE patients was not related to non-specific inflammatory events and it seems to be specific for SLE and still no difference was seen between active and inactive disease. Others have shown accelerated Fas-dependent apoptosis of monocytes and macrophages from SLE patients [124], and increased apoptosis of CD34<sup>+</sup> stem cells exposed to SLE serum has also been reported [128]. We have

further investigated the correlation between serum induced apoptosis and expression of Fas on freshly prepared cells from SLE patients and no correlation was found. However, when using serum from SLE patients, with the capacity to induce apoptosis, and autologous cells, the serum also induced apoptosis on these cells (unpublished data). The presence of a pro-apoptotic mechanism in serum from SLE patients could increase the load of autoantigens and influence the efficiency of clearance of apoptotic cells. The apoptosis inducing effect was not dependent on IgG and complement activation. This indicates that IgG containing immune complexes are not directly involved and cell death is not caused by complement activation but still split products of complement activation could be involved. The relationship seen between the concentrations of the complement protein in the classical pathway and serum induced apoptosis could not be seen when we extended the number of samples analysed (unpublished data). It has been described that purified autoantibodies such as anti-dsDNA and anti-cardiolipin antibodies do induce apoptosis *in vitro* [150, 151]. Even if only a weak correlation was seen between the presence of these autoantibodies and serum induced apoptosis, and removal of IgG by protein G absorption did not influence the serum induced apoptosis, further studies regarding the presence of autoantibodies in serum are of interest. Ongoing studies with a larger cohort regarding correlation between serum induced apoptosis and different diseases manifestation are in progress.

In Paper II we showed that serum from SLE patients induces a classical caspase dependent apoptosis, independent of death receptors but most likely involving the mitochondrial pathway. Classical apoptosis is characterized by distinct morphological changes such as compact chromatin condensation. Other criteria such as caspase activity and maintenance of membrane integrity should also be fulfilled [43]. The serum induced apoptosis could be reduced when the pan-caspase inhibitor Z-VAD fmk was used together with serum, indicating a caspase dependent apoptosis. Activity of caspase 8 indicates involvement of the death receptor pathway and activity of caspase 9 indicates involvement of the mitochondrial pathway, but caspase 8 also has the possibility to activate caspase 9 via tBid-mediated cytochrome c release. However, we saw both caspase 8 and caspase 9 activity occurring simultaneously. These unexpected findings have also been described when anti-cancer drugs are targeting the mitochondrial pathway where caspase 8 is activated in the absence of death receptors [152]. In this situation caspase 8 is thought to function as an executioner caspase in the mitochondrial pathway [153] and it has also been described that caspase 3 mediates feedback activation on upstream caspases such as caspase 8 [154]. A reduction of the

apoptosis inducing effect was seen when using a Bcl-2 over-expressing cell line as compared to corresponding vector control cells, indicating the involvement of the mitochondrial pathway. No reduction of the serum induced apoptosis was seen when using a FADD-deficient cell line or when blocking the death receptors Fas and TNF- $\alpha$ . This indicates that the death receptor pathway is not involved in SLE serum induced apoptosis, but it cannot be ruled out that a novel FADD-independent death receptor is involved. Furthermore, the balance between pro- and anti-apoptotic stimuli may decide the destiny of the cell and the different death pathways may co-exist. The factor in serum inducing apoptosis has not yet been identified, current findings suggest involvement of a negatively charged protein, and further investigations are ongoing.

In Paper III we investigated the role of complement in opsonisation of apoptotic cells. Homozygous deficiency of the classical pathway components C1q, C4 or C2 is associated with an increased susceptibility to SLE. Nearly all individuals with C1q deficiency develop SLE, often at a young age, whereas C4-deficient individuals develop SLE less often, with individuals deficient in C2 even less so [35]. We showed that the complement components of the classical activation pathway are all important for an efficient phagocytosis of apoptotic cells and the main contributor to opsonisation of C3 fragment on these cells. Thus, the strong association between C1q-deficiency and SLE is only partly explained by the role of C1q in the clearance of apoptotic cells. We studied the uptake of apoptotic cells by monocyte derived macrophages in the presence of complement-deficient sera. The clearance of apoptotic cells was shown to be dependent upon the classical pathway of complement activation, whereas neither a functional lectin nor alternative pathway was necessary. These findings are in accordance with most other reports within this research area [31, 155]. However, in our experimental setup, we could not see any difference between serum from individuals deficient in C1q, C4 or C2. This finding indicates that components of the classical pathway appear to be equally important in the clearance of apoptotic cells, which contradicts the hypothesis of a hierarchical role for classical pathway complements proteins with regard to their role in clearance of apoptotic cells [156]. Elevated levels of serum IFN- $\alpha$  seen in SLE patients have been proposed to have a significant role in the pathogenesis of the disease and our group recently described a novel function of C1q in the regulation of immune complex induced production of IFN- $\alpha$ . These findings suggest another role for C1q and contribute to the explanation of why C1q-deficiency is such a strong risk factor for the development of SLE [137].

The results obtained in this study using complement-deficient sera and sera depleted of C1q and factor D showed that activation of complement by apoptotic cells resulted in deposition of C3 fragments, which was dependent mainly upon the classical pathway. Deposition of C3 fragments through classical pathway activation was seen at low serum concentrations, and was most pronounced on secondary necrotic cells. This is in agreement with results from other investigators [96, 98]. The partly discrepant findings compared with previous studies with regard to complement pathways in opsonisation and phagocytosis of apoptotic cells could be due to the serum concentration used, but also to different cell types and cells at different stages of apoptosis. In our experiments, human sera from complement-deficient individuals were used, which could be another important difference. Nevertheless, our experiments strongly indicate that activation of the classical pathway is beneficial for the phagocytosis of cells undergoing apoptosis, especially of those also being secondarily necrotic.

The major source of autoantigens in SLE, as mentioned above is thought to be apoptotic cells. These autoantigens may be processed in a way that neo-epitopes will emerge to which the immune system is not tolerant and autoantibodies are therefore formed against nuclear antigens which are frequently seen in SLE patients. The presence of PMNs containing phagocytosed nuclear material is also seen in SLE. This is described as the LE cell phenomenon and was one of the first described laboratory findings associated with SLE [116]. In Paper IV we evaluate the roles of complement and antibodies against histones in relation to phagocytosis of necrotic cell material (NC) by PMNs. We found that autoantibodies against histones were associated with high capacity of PMNs in phagocytosis of NC and also that the classical complement pathway contributed to an efficient phagocytosis. Serum deficient of C1q, C2 or C4 all showed decreased capacity to promote phagocytosis of NC in the presence of antibodies against histones but no differences were seen when using serum deficient of properdin as compared to normal serum with antibodies histones added. In SLE patients a broad anti-histone, reactivity was associated with complement consumption and clinical manifestations such as vasculitis and glomerulonephritis. It has been suggested that histone 1 is the major autoantigen in SLE for the generation of LE cells [118, 157]. However, our findings suggest that the LE cell phenomenon is dependent on antibodies against histone 3 especially in combination with antibodies against histone 1 or histone 2. The patients with antibodies to more than one histone also had antibodies against dsDNA indicating a broad spectrum of autoantibody

specificities. This suggests that not exclusively autoantibodies against histone 1 but also autoantibodies against other nuclear proteins, DNA, and perhaps autoantibodies against nuclear ribonucleoproteins could be able to induce phagocytosis by PMNs. It has been described that antibodies are able to penetrate into living cells and interact with their respective antigen, which may affect intracellular functions as well as induction of apoptosis [158]. This could lead to exposure of more autoantigens and generation of a broader spectrum of autoantibodies if the apoptotic cells are not properly cleared from the system.

Possibly, the activation of the complement system seen in the patients with a broader autoantibody profile reflects a higher density of bound antibodies making it more favourable for C1 to bind resulting in complement activation. An Fc receptor-mediated phagocytosis of antibody-coated nuclear material seems not to be sufficient; instead a cooperative binding of complement fragments to receptors expressed on PMNs seems to be necessary for an efficient phagocytosis. Further studies will be carried out regarding this issue.

Influence on phagocytosis of apoptotic cells by PMNs in the presence of autoantibodies with different specificities will be studied. To further clarify the association between the specificities of autoantibodies against histones and clinical findings, more patients will be included in the study.

In this thesis, some of the mechanisms believed to be important in the pathogenesis of SLE have been addressed. The ability of serum from SLE patients to induce apoptosis could contribute to impaired clearance of apoptotic cells, an increased load of autoantigens, and the formation of autoantibodies. Also low concentration of the complement classical pathway components could cause impaired clearance of apoptotic cells and as a consequence, more apoptotic cells may expose autoantigens. Autoantibodies against histones are important for the uptake of the damaged cells in PMNs and may reflect the presence of apoptotic cells in the circulation. The autoantibodies may form circulating immune complexes, and deposition of these complexes in various organs may propagate an inflammatory response with complement activation leading to complement consumption. The complement consumption which leads to low concentrations of the components of the classical pathway may, in turn, contribute to impaired clearance of apoptotic cells.

## Conclusions

- Sera from SLE patients induce apoptosis in monocytes and lymphocytes from normal healthy donors, as well as in the monocytoïd cell line U937 and the T-cell line Jurkat.
- The apoptosis inducing effect is not related to non-specific inflammatory events and seems to be specific for SLE but is not a marker for disease activity.
- The apoptosis inducing effect is not dependent on IgG complexes or complement activation.
- Sera from SLE patients induce a classical caspase dependent apoptosis, independent of death receptors but most likely involving the mitochondrial pathway.
- Deficiency of the factors C1q, C4 or C2, of the classical complement activation pathway, resulted in an equally decreased ability to phagocytose apoptotic cells.
- Activation of the classical complement pathway seems to be the most important contributor of opsonisation of C3 fragments on apoptotic cells and is required for an efficient phagocytosis of apoptotic and necrotic cells.
- The strong association between C1q-deficiency and SLE is only partly explained by the role of C1q in the clearance of apoptotic cells.
- Phagocytosis of necrotic material by PMNs is dependent on the presence of IgG and on the classical complement activation pathway.
- Broad anti-histone antibody reactivity is strongly associated with increased phagocytosis of necrotic material by neutrophils, disease activity and with complement consumption.

# POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Systemisk lupus erythematosus (SLE) är en kronisk autoimmun sjukdom, det vill säga en sjukdom där immunförsvaret angriper kroppens egen vävnad och förorsakar inflammation. SLE kan drabba många av kroppens organsystem så som hud, leder, njurar, hjärta, kärl, lungor och det centrala nervsystemet. Sjukdomsorsaken är okänd men tros bero på en samverkan mellan gener och miljöfaktorer. Immunsystemet är involverat i sjukdomsprocessen på många sätt, bl. a. finns en ökad produktion av antikroppar mot många naturligt förekommande strukturer i kroppens celler däribland mot DNA, så kallade autoantikroppar. Granulocyter vilka aktivt tagit upp delar av cellkärnor, i regel i komplex med antikroppar riktade mot cellkärnornas komponenter, bland annat mot histoner kan beskrivas som LE-celler. Detta LE-cellsfenomen är associerat med SLE och redan 1948 beskrev M. Hargraves en mikroskopbaserad metod för att detektera dessa celler.

Komplementsystemet består av ett 30-tal lösliga eller membranbundna proteiner, vilka utgör en viktig del av immunsystemet. Vissa komplementfragment fungerar som opsoniner, d.v.s. märker ut främmande ämnen (antigen), vilket underlättar för fagocyter (celler som äter upp) att ta upp (fagocytera) dessa ämnen och oskadliggöra dem, vidare kan komplement också vid aktivering göra hål på cellen så att den förstörs. Komplementsystemet kan aktiveras via tre vägar, den klassiska vägen, den alternativa vägen och lektinvägen. Bristfällig funktion av komplementsystemet är associerad med sjukdomen SLE, speciellt defekter av proteiner involverade i den klassiska vägen. Vid brist av något av dessa proteiner ökar risken för att utveckla SLE och en rangordning finns där ca 90 % av personer med brist av C1q utvecklar SLE, 75 % vid brist av C4 och 10 % vid brist av C2. En ökad aktivering av komplementsystemet kan också ses vid sjukdomen SLE framförallt vid sjukdomsskov.

Apoptos, en form av programmerad celldöd, är nödvändig för utvecklande och upprätthållande av multicellulära organismer och som försvarsmekanism. Det är en aktiv process som är beroende av signaler eller aktiviteter i den döende cellen och framkallar inte något inflammatoriskt svar, i motsats till nekros som är en passiv celldöd orsakad av skada vilken ger upphov till ett inflammatoriskt svar. Apoptos kan aktiveras externt genom receptorsignal eller internt via mitokondrien som då frisätter pro-apoptotiska ämnen. Kaspaser



är en grupp enzymer som normalt finns i inaktiv form, men som aktiveras vid apoptos och då kan bryta ner eller aktivera andra proteiner och cellen förbereds för död. Vid programmerad celldöd förändras cellen, nya molekyler exponeras i cellmembranet, så att fagocyterande celler känner igen och omedelbart kan ta upp den. Vid ett snabbt omhändertagande av de apoptotiska cellerna undviks att de intracellulära komponenterna exponeras för immunsystemet. Patienter med sjukdomen SLE har en försämrad förmåga att ta bort döende celler, vilket leder till en ökad mängd av kvarvarande apoptotiska celler. Dessa apoptotiska celler blir då tillgängliga för immunsystemet och de döende cellerna är troligtvis huvudkällan för exponering av autoantigen som bidrar till bildandet av autoantikroppar.

Avsikten med detta projekt var att utvidga och beskriva det tidigare fyndet att serum från patienter med sjukdomen SLE inducerar apoptos hos celler från friska givare. Vi ville även studera komplementsystemets påverkan vid borttagandet av apoptotiska eller döda celler genom att använda sera med komplementbrist och undersöka deponering av C3 på de döende cellerna. Vidare var avsikten att undersöka relationen mellan autoantikroppar riktade mot histon och förmågan att fagocytera döda celler.

Metoder: Flödescytometri är en teknik där man enkelt kan mäta och identifiera olika celler genom att låta cellerna skickas en och en genom en laserstråle. Det reflekterande och avböjande laserljuset registreras och omvandlas till elektriska signaler som ger ett mått på cellens storlek och form. Cellerna kan även märkas med olika cellspecifika markörer till vilka det är kopplat olika ämnen, fluorokromer som utsänder ljus vid olika våglängder och på så sätt kan olika subpopulationer identifieras. Celler kan också undersökas med konfokalmikroskop, här utnyttjas laserljus för att konstruera tredimensionella bilder. Laserljuset flyttas över provet och endast det ljus som är i fokus skickas till detektor och övrigt ljus filtreras bort, detta gör det möjligt att ta bilder i flera optiska plan. Förekomst av antikroppar kan mätas med ELISA (enzyme-linked immunosorbent assay), principen för denna metod är att klä en plastplatta med antigen. Därefter tillsätter man det prov man vill undersöka och med hjälp av en enzymatisk reaktion kan man mäta inbindning av antikroppar till den beklädda plattan.

Delarbete I: *Induction of apoptosis in monocytes and lymphocytes by serum from patients with systemic lupus erythematosus – an additional mechanism to increase autoantigen load?* I detta arbete studerade, bestyrkte och utökade vi observationen av att det i serum från SLE-

patienter finns en löslig faktor som har förmåga att inducera apoptos i celler från friska givare och denna förmåga att förorsaka apoptos relaterades till kliniska och immunologiska data. Annexin V (AV) binder till fosfatidylserin exponerat på cellytan på apoptotiska celler och propidiumjodid (PI) binder till DNA. Levande, apoptotiska och nekrotiska celler kan särskiljas genom att mäta AV- och PI-inbindning till celler med flödescytometri. Vi odlade celler från friska givare i närvaro av serum från SLE-patienter och inbindning av AV och PI mättes. Resultaten visar att serum från SLE-patienter förorsakar primärt apoptos och inte nekros av dessa celler. För att undersöka om detta fenomen även fanns vid andra sjukdomstillstånd undersökte vi sera från patienter med andra autoimmuna sjukdomar så som reumatoid artrit, vaskulit och infektionssjukdomar samt friska kontroller. Förmåga att inducera apoptos i celler från friska givare var unikt för sjukdomen SLE, men ingen skillnad kunde ses mellan aktiv och inaktiv sjukdom. Således kunde vi visa att sera från SLE-patienter har förmåga att inducera apoptos i celler och att denna förmåga verkar vara specifik för SLE-sjukdomen. Denna egenskap skulle kunna bidra till en ökad exponering av autoantigen.

*Delarbete II: SLE serum induces classical caspase-dependent apoptosis independent of death receptors.* I detta arbete gick vi vidare och försökte karakterisera på vilket sätt serum från SLE-patienter initierar apoptos. Med konfokalmikroskopi kunde vi konstatera att de apoptotiska cellerna hade ett typiskt klassisk kaspasberoende apoptotiskt utseende, det vill säga att kromosomerna vilka innehåller DNA och protein hade sammanpressats och apoptotiska cellkroppar hade börjat bildas. Andelen apoptotiska celler minskades vid blockering av kaspasaktivitet. Däremot påverkades inte den apoptosinducerande effekten vid blockering av dödsreceptorvägen. Bcl-2 är ett anti-apoptotiskt protein vilket hämmar apoptos via mitokondrievägen. Vi undersökte om en cell-linje som överuttrycker Bcl-2 kunde hämma apoptos förorsakad av sera från SLE-patienter. En signifikant minskning kunde ses vilket indikerade att denna väg är inblandad. Således kunde vi konstatera att sera från SLE-patienter inducerar en klassisk kaspasberoende apoptos oberoende av dödsreceptorer på friska celler.

*Delarbete III: Complement classical pathway components are all important in clearance of apoptotic cells.* Här studerade vi fagocytos av apoptotiska celler i närvaro av komplementdefekta sera. Genom att sätta upp en flödescytometerbaserad metod där makrofager (fagocyterande celler) inkuberades med apoptotiska celler i närvaro av serum kunde vi mäta antalet apoptotiska celler som fagocyterats av makrofagerna. Resultaten visade att den fagocyterande förmågan försämrades lika mycket vid frånvaro av C1q, C2, C4 eller

C3 i serum, vilket indikerar att aktivering av alla komponenter i den klassiska vägen är av lika stor betydelse för en effektiv fagocytos av apoptotiska celler. Detta innebär att den starka kopplingen mellan brist på C1q och SLE inte enbart kan förklaras med den minskad förmåga att fagocytera apoptotiska celler. Vi undersökte då deposition av C3-fragment på de apoptotiska cellerna i närvaro av komplementdefekt serum. I dessa försök möjliggjordes mätning av C3-deposition både på tidigt apoptotiska celler (AV-positiva/PI-negativa) och sent apoptotiska celler (både AV- och PI-positiva). Resultaten visade inbindning av C3-fragment på apoptotiska celler och denna inbindning var huvudsakligen beroende av aktivering via den klassiska vägen. Inbindning av C3-fragment kunde ses på både tidigt och sent apoptotiska celler men var mest uttalat på sent apoptotiska celler. En viss inbindning av C3 till apoptotiska celler kunde detekteras i närvaro av komplementdefekta sera i hög koncentration, vilket indikerar att C3-deposition via alternativa vägen mest fungerar som en förstärkning av aktivering via den klassiska vägen.

Någon skillnad i brist på den klassiska vägens olika komponenter och utvecklandet av sjukdomen SLE kunde inte ses i dessa försök utan här var alla komponenterna i den klassiska vägen lika viktiga. Dessa experiment visade att komplementberoende opsonisering med C3-fragment på döende celler är viktig för att få ett effektivt borttagande av dessa celler och därmed en minskad exponering av autoantigen. Sålunda, den starka associationen mellan SLE och brist av C1q kan inte enbart förklaras av den roll C1q har i borttagandet av apoptotiska celler utan även andra förklaringar som exempelvis reglering av cytokiner via C1q skulle kunna påverka denna association.

Delarbete IV: *Specificity of anti-histone antibodies determines complement-dependent phagocytosis of necrotic material by polymorphonuclear leukocytes in the presence of serum from patients with SLE. The LE cell phenomenon revisited.* Syftet med detta arbete var att mäta förekomst av autoantikroppar riktade mot histoner i serum från patienter med sjukdomen SLE och att undersöka om granulocyter fagocyterar necrotiskt material i närvaro av serum från SLE-patienter eller serum från komplementdefekta individer. Två olika ELISA-metoder utvecklades för att kunna mäta antikroppar mot histon 1 och antikroppar mot en mix av histoner (1, 2, 3 och 4) och en flödescytometerbaserad metod användes för att mäta fagocytos av nekrotiskt material. Dessa metoder utvärderades och jämfördes med varandra och med kliniska data. Resultaten visade en god korrelation mellan de olika metoderna och att förekomst av antikroppar mot histon framför allt antikroppar mot flera olika histoner främjar

fagocytos. Förutom närvaro av antikroppar behövdes ett fungerande komplement system, där den klassiska vägen aktiveras, för att på ett effektivt sätt fagocytera det antikroppsklädda nekrotiska materialet. Antikropps-koncentration visade ett starkt samband med sjukdomsaktivitet. Således kunde vi här visa att ett brett spektrum av autoantikroppar mot flera histoner främjar fagocytos av nekrotiskt material i närvaro av den klassiska vägen. Att mäta antikroppar riktade mot histoner i serum från SLE-patienter skulle kunna vara ett sätt att påvisa närvaron av döende celler, vilka kan bidra till ett ökat autoimmunsvar.

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