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Autoimmunity, Atherosclerosis and Apoptotic Cell Clearance

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1. Introduction

In recent years, it has been reported that there is an increased incidence of accelerated atherosclerosis among young women with systemic lupus erythematosus (SLE). Accelerated atherosclerosis has also been observed in other autoimmune diseases such as rheumatoid arthritis and systemic sclerosis. The end-organ damage most commonly observed in SLE patients is kidney failure. Recent advances in the treatment of kidney dysfunction, has led to the observation that many SLE patients also suffer from coronary heart disease and other endpoint cardiac events. As a result, studies have been designed and performed to better understand the basis for the accelerated disease progression in patients with autoimmune disease.

The development of atherosclerosis is driven, to a large extent, by inflammation. Initiation of atherosclerotic lesions can occur as a result of damage to the endothelium by a number of factors including oxidized low density lipoprotein (oxLDL), inflammatory cytokines, and immune complexes. The lesion progression involves inflammatory cell interactions with the endothelium and extravasation into the subendothelial space. Inflammation resulting from both atherosclerosis and autoimmunity is an essential, yet not well understood, factor in the initiation and progression of atherosclerosis associated with autoimmune diseases. Currently, one of the most widely studied areas among the genetic causes of SLE is decreased clearance of apoptotic bodies, which is thought to propagate the progression of the disease. There are numerous *in vivo* studies that support this hypothesis. For example, it has been shown that a long-term autoimmune response does not occur when there is efficient apoptotic body clearance. In cases where the machinery that is responsible for the clearance is disrupted in genetic mouse models, it has been shown that apoptotic bodies accumulate, resulting in lupus-like autoimmune diseases. This is evidenced by the variety of mouse models that develop autoimmunity in the absence of genes involved in apoptotic cell clearance. Apoptotic cell clearance also plays a role in atherosclerotic lesion development depending on the stage of the lesion. Our lab generated the first mouse model to study the interactions between SLE and atherosclerosis and subsequently, many new mouse models have been generated in order to further elucidate the mechanism by which the synergy between the two disease processes occurs.

The focus of this chapter will be to discuss the recognition and phagocytosis of an apoptotic cell, the machinery involved in apoptotic cell clearance, and the effects of alterations to various steps of this process. This will be demonstrated by including evidence of relevant

genetic mouse models and examples of human disease resulting from impaired clearance. We will strive to illustrate the extent to which apoptotic cell clearance can affect the progression of not only autoimmune diseases such as lupus, but also extend to other pathological conditions including interactions with cardiovascular disease.

2. Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a common autoimmune disease that affects an estimated 1.5 million Americans. Notably, the number of women of childbearing age that are affected versus men is increased 10-fold. It is also more common in the Afro-Caribbean and Asian populations (Lahita 1999). The mechanism(s) that leads to the breakdown of self-tolerance in SLE is not well understood. SLE is classified as a multi-systemic disease since the clinical presentations affect multiple organs. These signs can present with varying severity in skin, joints, kidneys, brain, heart and lungs (Lahita 1999). An abundance of autoantibody can be found in patients with SLE, and over 50 autoantibodies have been described. The major type of autoantibodies are primarily antinuclear and against DNA however, antibodies to components of RNA: anti-Sm or anti-ribonuclease, chromatin, nucleosomes, histones, and ribosomes are also commonly found (Lahita 1999).

One of the major clinical manifestations of SLE is glomerulonephritis in which blood and protein can accumulate in the urine as a result of disrupted function of the glomeruli. Decreased renal function can ultimately lead to renal failure. Most notably in SLE, autoantibody production leads to the formation of immune complexes with their specific antigens and these complexes can be deposited into the glomerular capillaries. Whether these immune complexes arise from deposition of circulating immune complexes or are formed in-situ, remains to be firmly established. Glomerulonephritis can lead to hypertension, contributing to interactions between autoimmune disease and other cardiovascular disorders. Thus, the accumulation and deposition of immune complexes made up of anti-DNA or antinuclear antibodies and their antigens are thought to spark the induction of the kidney dysfunction (Lahita 1999).

The end-organ damage most commonly observed in SLE patients is kidney failure. However, due to recent advances in the treatment of kidney dysfunction, it has been observed that there are an increased number of SLE patients with coronary heart disease (Manzi et al. 1997b; Petri et al. 1992; Urowitz et al. 1976). This has led to the study of atherosclerosis as a precursor to more advanced cardiovascular diseases in these patients. Atherosclerosis can eventually lead to major coronary heart disease and cardiovascular events, therefore it is prudent to monitor the progression of atherosclerosis in patients with SLE. In recent years, it has been reported that there is an increased incidence of accelerated atherosclerosis among young women with SLE (J.M. Esdaile et al. 2001b; Lockshin et al. 2001; Manzi 2000). These young women also have increased rates of coronary heart disease. More recently, this has also been shown to occur in other autoimmune diseases such as rheumatoid arthritis and systemic sclerosis (Lockshin et al. 2001; Riboldi et al. 2002; Van Doornum et al. 2002).

The cause of SLE remains unknown, although there are several genetic, environmental, and hormonal factors that can contribute to its initiation and progression. The presence of increased levels of autoantibodies is a hallmark of SLE, however, it is thought that simply having circulating auto-antibodies cannot cause autoimmune disease (Lahita 1999). There are environmental factors, such as ultraviolet light, that can cause an autoimmune disease

like SLE to manifest itself. When taken into account that lupus occurs more frequently in women, it is thought that hormonal factors may also play a role in disease manifestation (Lahita 1999). Thus, the genetic factors may provide a predisposition to SLE, the flares of which could be triggered by environmental or hormonal factors.

Immune dysregulation can affect disease progression if there is T-cell dysfunction which presents via a shift in the cytokines produced by these helper T-cells. T-helper 1 (T_H1) pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF α), interferon-gamma (IFN γ), and IL-2 lead to B-cell growth, differentiation and activation that will in turn result in antibody production. In contrast, secretion of anti-inflammatory T_H2 cytokines such as IL-4, IL-5, and IL-10 functions to inhibit macrophage activation and downregulate T_H1 responses (Janeway et al. 2001). Therefore, modulation of cytokine production resulting in a shift towards a more pro-inflammatory response can affect lupus progression.

In SLE patients, accumulation of autoantibodies is characteristic and these autoantibodies can form immune complexes (Lahita 1999). During an SLE flare, these immune complexes can contribute to inflammation and injury to tissue. Immune complex injury and inflammatory response can occur in two ways. The first is when the antibody binds to its antigen, thus stimulating recruitment and activation of inflammatory cells such as macrophages via complement and Fc receptors. This occurs in cells or extracellular tissues and will lead to tissue injury. Second, immune complexes that form in the circulation can deposit on the vessel wall and cause an inflammatory response on the endothelium by recruitment of inflammatory cells (Abbas and Lichtman 2003). This may be of particular interest concerning the association of accelerated atherosclerosis with SLE. Aggravation of the endothelial lining by autoantibodies and immune complexes will be discussed later in more detail with relation to promoting atherosclerotic lesion formation.

Currently, one of the most widely studied areas among the genetic causes of SLE is in decreased clearance of apoptotic bodies that are thought to propagate the progression of the disease. There are numerous studies of different genetic mouse models that support this hypothesis (Bickerstaff et al. 1999; Botto et al. 1998; Clynes et al. 1998; Cohen et al. 2002; Hanayama et al. 2004; Korb and Ahearn 1997; Napirei et al. 2000; Taylor et al. 2000). It has been demonstrated that short-term autoimmunity, i.e. production of autoantibodies, can be achieved by immunization with apoptotic cells. However, a long-term autoimmune response does not occur when there is efficient apoptotic body clearance (Qian et al. 2004). In cases where the machinery that is responsible for the clearance is disrupted, it has been shown that apoptotic bodies accumulate, resulting in lupus-like diseases. These studies will be discussed later in the chapter.

3. Apoptosis

Apoptosis is programmed cell death that is necessary for tissue remodeling during development. It also occurs when a cell may pose a threat to the organism. For example, cells with DNA damage that could become cancerous, or cells infected by a virus, will be recognized by cytotoxic T-cells and, in almost all cases, removed. Therefore, the role of apoptosis is not limited to development but plays a role in maintaining cellular homeostasis throughout an organism's life.

A main feature of apoptosis is its well-defined sequence of morphological changes. The cell begins by condensing both chromatin and cytoplasm resulting in nuclear blebbing and a change in cell appearance. Chromosomal DNA is digested into 200bp fragments by

endogenous nucleases that cleave at the inter-nucleosomal linker regions. This is important to note since SLE patients develop antibodies to nucleosomes that are found prior to anti-DNA antibodies. The genes involved in the digestion of DNA and chromatin from cells undergoing apoptosis are DNaseI and serum amyloid P (SAP). Deoxyribonuclease I (DNaseI) is responsible for digesting extracellular chromatin and the lack of this enzyme results in an autoimmune phenotype in mice, which develop auto-antibodies to chromatin as well as glomerulonephritis caused by immune complex deposition (Napirei et al. 2000). SAP is known to bind chromatin and perhaps act as a cover to prevent an inflammatory immune response and assist in its clearance. The absence of SAP results in auto-antibodies to chromatin which can deposit in the kidney and cause glomerulonephritis (Bickerstaff et al. 1999). The next step of apoptosis involves further shrinking of the cell and blebbing off of small membrane bound vesicles, apoptotic bodies, which will then be phagocytosed.

Apoptosis is not limited to organism development. For example, each day there are more than 10^{11} senescent red blood cells that must be eliminated, (Alberts et al. 2002) and the bone marrow produces millions of new red blood cells, monocytes, neutrophils, and lymphocytes. Regulated loss of all these blood cells occurs by apoptosis, and the dying cells are finally phagocytosed by specialized macrophages in the liver and spleen. Macrophages and dendritic cells are phagocytic cells deriving from hematopoietic stem cells. They are major players in the body's defense against infection, in addition to T-cells, B-cells, and neutrophils. The phagocytic properties which they possess are also critical to removal of dead cells. Apoptosis plays an important role in development, homeostasis, and disease. Apoptotic cell debris is efficiently removed by phagocytic cells through a process that requires a complex system of signals and receptors. Many studies show that a breakdown in the removal of apoptotic cell corpses will promote inflammation and, at its extreme, autoimmunity.

Therefore, this process is very important since the production of new cells must be balanced by an equal loss of these cells. Related to this idea, it has been suggested that the balance is maintained in a 'Yin' and 'Yang' process involving apoptosis and wound healing, respectively (Khatami 2008, 2011). With regard to inflammation, apoptotic events ('Yin') stimulate the initial responses of immune cells for the recognition and clearance of the offender. The post-inflammatory events involved in wound healing ('Yang') are important in reconstruction and repair, thereby contributing to the resolution of inflammation (Khatami 2008, 2011). In the following sections, the recognition of the apoptotic cells and their various receptors or opsonizers will be discussed.

3.1 Recognition of the apoptotic cell

Proper maintenance within the body must occur to clear the apoptotic bodies in a non-inflammatory manner. Phagocytosis is an action of engulfment that requires activation of receptors in order to initiate the process of ingestion and degradation. The final steps in the process of apoptosis occur in concert with the phagocytes. Recognition of the apoptotic cell involves a complex system of signals and receptors. This system has been the focus of intense research and has yielded evidence towards recruitment signals, including chemokines and cell surface changes, as well as the receptors responsible for physical contact with the apoptotic cells. This section will categorize and discuss phagocytic clearance, the various stages of apoptosis from initial breakdown of the cells and nucleosomes, to the eventual clearance of the apoptotic debris, and the relevant mouse models that have been useful in studying the development of SLE.

A chemoattractant that signals recruitment of macrophages to the sites of apoptotic cell death has recently been described. The apoptotic cell will release a chemokine called lysophosphatidylcholine (LPC) that signals to macrophages to engulf and digest the apoptotic body (Lauber et al. 2003). LPC is produced as a result of hydrolysis of phosphatidylcholine in LDL and cell membranes, and can be produced by phospholipase-A2 (PLA₂) or by oxidation. LPC was previously known as a chemoattractant for monocytes (Hoffman et al. 1982), however, the role in apoptotic cell clearance was not known until recently. In terms of cardiovascular disease, LPC has been shown to be a component of atherosclerotic plaques, and expression of PLA₂ is observed in the arterial wall. PLA₂ is known to be induced by proinflammatory cytokines, such as TNF α and IL-6. Since these cytokines are known to promote inflammation in both cardiovascular and autoimmune diseases, this has potential consequences not only to atherosclerosis, but also towards SLE.

A relationship between LPC and SLE has been described in several studies. First, levels of anti-LPC antibodies are elevated in patients with SLE compared to their healthy counterparts (Wu et al. 1999). Although the levels of LPC have not been analyzed in the sera of patients with SLE, it has been determined that they have increased PLA₂ activity (Pruzanski et al. 1994). Therefore it is possible that they may also have increased levels of LPC. Similar antigenic epitopes among phospholipids such as oxLDL and LPC have been identified, and these were also found to be similar to those found in endothelial cells. Further studies have demonstrated that anti-LPC antibody levels are decreased in male patients with borderline hypertension compared to normotensive controls (Wu et al. 2001). This provides evidence further linking inflammation to cardiovascular disease. Thus, the significance of LPC involvement in SLE has potential benefits to understanding the links between SLE and cardiovascular disease.

To date, the most well understood recognition signal to trigger phagocytosis is the expression of PS on the surface of the apoptotic cell membrane. When a cell undergoes apoptosis, the distribution of lipids in the plasma membrane is disrupted. Negatively charged PS will be flipped in the lipid bilayer to be exposed on the outside layer. As a consequence, this is recognized as an “eat me” signal by phagocytes (Fadok et al. 1992; Fadok et al. 2001). This is the one of the first steps that will allow recognition of the cell as apoptotic, and eventual uptake by the phagocyte. Apoptotic cells that are not cleared can undergo secondary necrosis, and this leads to the release of the intracellular components, promoting a pro-inflammatory response. Therefore, it is crucial that cells are recognized as apoptotic and cleared in a timely manner.

Phosphatidylserine content has been used as a measurement for circulating levels of microparticles, which are vesicles released from plasma membranes after injury or apoptosis. Increased levels of endothelial microparticles have been found in human plasma under a variety of pathological conditions and are thought to play a role in systemic cell activation. Endothelial microparticles are present in atherosclerotic plaque (Mallat et al. 1999; Nomura et al. 2000), and there is a relationship between circulating microparticles and arterial stiffness in patients with end-stage renal failure (Amabile et al. 2005). In addition, increases in endothelial microparticles have been documented in patients with severe hypertension compared to healthy controls (Preston et al. 2003). Recent work has shown a correlation between microparticles and disease activity in patients with SLE, primary Sjogren’s syndrome and rheumatoid arthritis (Pereira et al. 2006; Sellam et al. 2009).

After successful localization to the site of the apoptotic cell, further recognition signals facilitate initial attachment of the macrophage via a variety of specialized surface receptors.

Several receptors have been linked to recognizing apoptotic cells, a majority of which are known to distinguish various forms of phospholipids, including phosphatidylserine (PS). There are three types of receptors that will be discussed: those that respond to PS on the cell surface of the apoptotic cell, both directly and indirectly, and those that recognize and bind to various molecules that opsonize the apoptotic cell for ingestion.

3.2 Direct binding to PS

The presence of PS on the cell surface is known to stimulate various receptors on the macrophage in order to facilitate clearance and promote an anti-inflammatory response (Fig. 1). A macrophage receptor with a direct interaction with PS, a PS-specific receptor appears to exist (Fadok et al. 2000). Data shows macrophage binding of apoptotic cells can be mediated by a specific PS receptor (PSR). This action is associated with the production of TGF- β and the downregulation of inflammatory cytokines (Fadok et al. 1998). Another study corroborating the notion that apoptotic clearance is normally anti-inflammatory observed that tumor necrosis factor-alpha (TNF- α), a cytokine released by macrophages during a pro-inflammatory response, is downregulated by PS-liposomes (Aramaki et al. 1997). Therefore, PS is important not only for recognition by phagocytes, but also for controlling the immune response and maintaining an anti-inflammatory setting.

3.3 Bridging molecules

Other receptors exist that are linked to PS via bridging molecules (Fig. 1). These receptors include β 2-Glycoprotein I (β 2GPI), and Mer (Balasubramanian et al. 1997; Balasubramanian and Schroit 1998; Scott et al. 2001). β 2GPI is a plasma protein that binds phospholipids, in particular, it has been found to bind directly to PS. Several studies suggest that β 2GPI bound to PS on apoptotic cells contributes to clearance by then binding to its receptor (β 2GPI-R) found on macrophages. Therefore, β 2GPI is a candidate protein that could contribute to autoimmune disease if altered. Mer, a member of the receptor tyrosine kinase family, binds to the growth arrest-specific protein 6 (Gas-6). The function of Gas-6 in apoptotic clearance is to bind the exposed PS on apoptotic cells, then bind to mer on macrophages (K. Nagata et al. 1996). This action leads to phagocytosis of the apoptotic cell and, at the same time, TNF α levels decrease though the reason for this effect remains to be elucidated (Camenisch et al. 1999). This suggests that mer involvement in apoptotic cell removal may contribute to the anti-inflammatory response seen in normal clearance. Evidence of this is provided by a mouse model whereby the absence of mer results in the manifestation of autoimmunity resembling SLE (Cohen et al. 2002). Apoptotic material accumulates in lymphoid tissue, evidenced by the enlarged spleen. The autoimmunity observed in these mice is characterized by autoantibody production including anti-DNA, ANAs, and anti-phospholipid. A mild form of glomerulonephritis also occurs (Cohen et al. 2002). In addition, the liver X receptor transcription factors have been shown to be necessary for proper clearance of apoptotic bodies, by the induction of mer expression (A-Gonzalez et al. 2009). Liver X receptor-deficient mice are impaired in their ability to respond to apoptotic clearance, have dysregulated inflammatory pathway signalling, and develop lupus like disease. Taken together, these mouse models deficient in machinery necessary for apoptotic cell clearance provide further evidence to implicate impaired apoptotic cell clearance in autoimmunity.

3.4 Scavenger receptors

There are several scavenger receptors that have been implicated in apoptotic clearance: CD-36, scavenger receptor A and B1 (SR-A, SR-B), LOX -1, and CD68 (Gillotte-Taylor et al. 2001; Imachi et al. 2000; Rigotti et al. 1995; Shiratsuchi et al. 1999) (Fig. 1). Of all these receptors, CD-36 is the only one to have a known ligand. CD-36 is linked to the apoptotic body via a thrombospondin bridge. Since, these receptors also recognize and uptake oxLDL as part of macrophage foam cell formation, they are thought to function by recognizing oxidized sites on apoptotic cells.

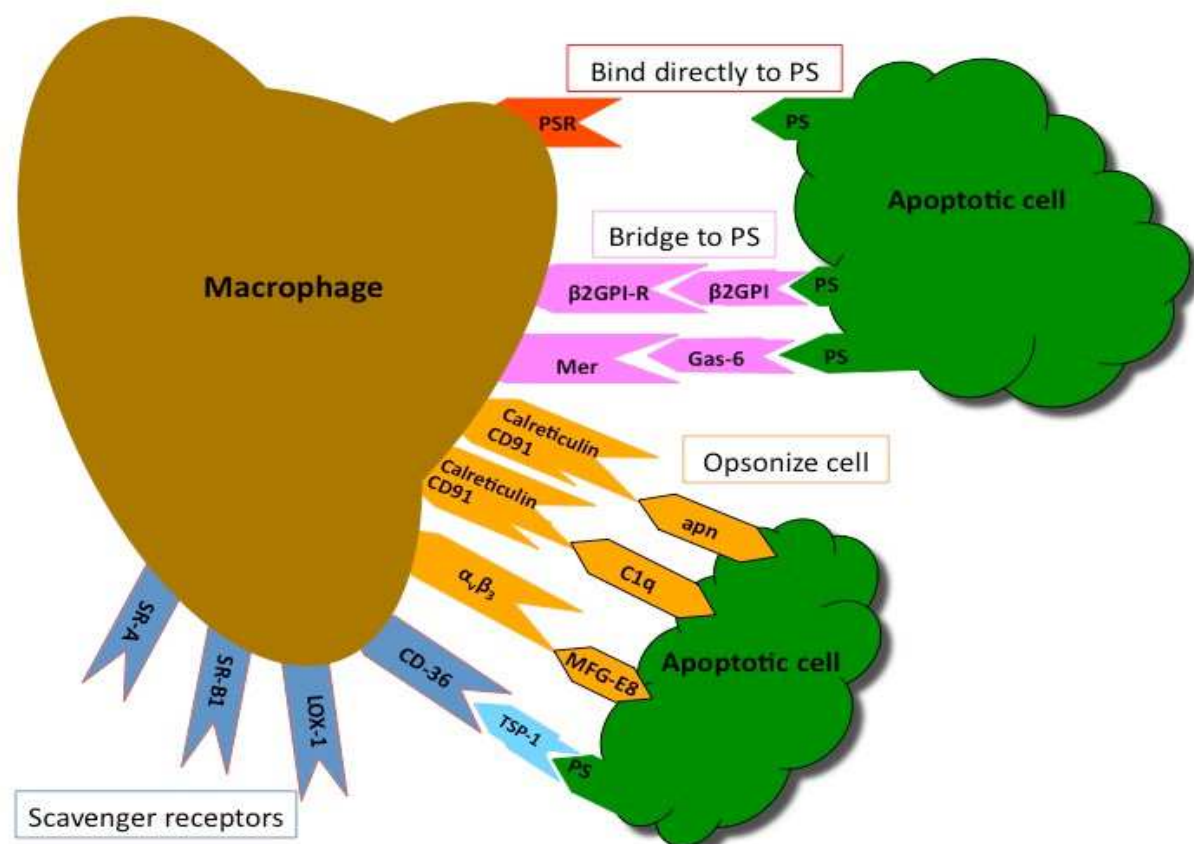


Fig. 1. Receptors associated with apoptotic cell clearance. Four types of receptors exist on macrophages to promote attachment and phagocytosis of apoptotic cells. Phosphatidylserine receptor (PSR) binds directly to phosphatidylserine (PS). Two receptors, β 2-Glycoprotein I receptor (β 2GPI-R) and Mer, bind to β 2-Glycoprotein I (β 2-GPI) and growth arrest-specific protein 6 (Gas-6) respectively; β 2GPI and Gas-6 bind PS and act as a bridge between the macrophage and the apoptotic cell. Adiponectin, complement protein C1q and milk fat globule epidermal growth factor 8 (MFG-E8) opsonize the apoptotic cell and bridge to the macrophage via calreticulin/CD91 and $\alpha_v\beta_3$ receptors, respectively. The final group includes scavenger receptor A (SR-A), scavenger receptor B1 (SR-B1), LOX1, and CD-36. CD-36 joins thrombospondin-1 (TSP-1) bound to PS on the apoptotic cell.

3.5 Opsonization of the apoptotic body

Among the genetic factors that have been implicated in the progression of SLE, is a third group that helps to regulate clearance by opsonizing the apoptotic body and facilitating

antigen clearing mechanisms (Fig. 1). Opsonization is the process of making bacteria or other cells more attractive to phagocytes; therefore, this can play a large role in the recognition and removal of apoptotic cells. Further evidence involving an opsonization mechanism is observed by a protein that is secreted by activated macrophages and dendritic cells: milk fat globule-EGF-factor 8 (MFG-E8). MFG-E8 facilitates phagocytosis of apoptotic cells by linking the apoptotic cell to the phagocyte. It binds specifically to the phosphatidylserine that is exposed on the apoptotic cells, and then binds to the $\alpha_v\beta_3$ integrin expressed on the phagocyte (Hanayama et al. 2002). Co-culture of peritoneal macrophages with a mutant MFG-E8 protein results in inhibited phagocytosis. In addition, the levels of the anti-inflammatory cytokine IL-10 are suppressed. Under normal conditions, expression of IL-10 is upregulated by macrophages that are actively engulfing apoptotic cells. This study also showed that intravenous injection of mutant MFG-E8 protein stimulated the production of autoantibodies (Asano et al. 2004). A mouse model lacking MFG-E8 revealed an autoimmune phenotype showing autoantibody production, splenomegaly, and glomerulonephritis. In addition, macrophages from MFG-E8^{-/-} mice engulfed fewer apoptotic cells than wild type macrophages which could be corrected with the addition of recombinant MFG-E8. A similar finding occurred *in vivo* where there was less co-localization of apoptotic cells with the macrophages located in the spleen (Hanayama et al. 2004). This study suggests that apoptotic cell clearance is impaired in the absence of MFG-E8, and this contributes to the propagation of autoimmune disease.

Another molecule, Complement C1q, is part of the complement system which is a major effector of the humoral immune response, but also contributes to the opsonization of apoptotic cells. The removal of apoptotic cells is facilitated by binding a portion of the globular head of C1q (independent of antibody) to the apoptotic cell (Korb and Ahearn 1997). The collagenous domain of C1q then binds to the receptor calreticulin, which is found on the macrophage (Ogden et al. 2001). In both cases, the end result is ingestion and degradation in an anti-inflammatory manner. In a mouse model, the absence of C1q results in antinuclear antibody accumulation and immune complex renal disease. In addition, disease severity related to the absence of complement decreases in relation to the placement in the pathway. C1q^{-/-} mice demonstrate a severe form of SLE compared to C4^{-/-} mice (Botto et al. 1998; Taylor et al. 2000). Complement deficiency has also been linked to SLE pathogenesis in humans. This implicates apoptotic body clearance via the complement pathway as a major factor in SLE initiation and progression.

Adiponectin is an adipose-derived cytokine known to be cardio-protective, but also opsonizes apoptotic bodies and facilitates an efficient clearance in order to promote phagocytosis that is non-inflammatory. Adiponectin has a similar structure to C1q and facilitates clearance by opsonization of the apoptotic cell body and uptake through one of its receptors, calreticulin-CD91, which is expressed on the surface of the macrophage. *In vitro* treatment of macrophages with adiponectin results in increased apoptotic body clearance. In addition, lupus-prone mice on a C57 background, deficient in adiponectin have a defect in clearance of apoptotic bodies and a worsened lupus disease phenotype (Takemura et al. 2007). Another mouse model, combining a lupus phenotype with adiponectin deficiency, on the MRL background which is in itself permissive to autoimmunity, results in exacerbated kidney morphology including crescent formation, mesangial expansion, and increased IgG and complement C3 deposits (Parker et al. 2011). Paradoxically, SLE patients with renal dysfunction have been reported to have increased

adiponectin levels compared to SLE patients with normal renal function, and adiponectin levels are also increased in the urine of SLE patients having an active renal flare (Rovin et al. 2005). This finding was one of the first that led to the finding that many chronic inflammatory diseases have increased levels of adiponectin, however this is an area of research currently being investigated.

More recently, the nuclear receptor, peroxisome-proliferator activated receptor- δ (PPAR δ) has been shown to act as an enhancer of opsonization molecules. Mouse models with ubiquitous or macrophage-specific deletion of PPAR δ have impaired clearance of apoptotic cells, resulting in increased auto-antibody production and a lupus-like phenotype. The opsonins controlled by PPAR δ in this study are C1q and MFG-E8 (Mukundan et al. 2009). Similarly, macrophage-specific deletion of other members of the nuclear receptor family, PPAR γ or retinoid X receptor- α results in autoantibody accumulation and glomerular injury (Roszer et al. 2011). In addition to the lupus phenotype, mice deficient in PPAR γ or retinoid X receptor- α are unable to efficiently clear apoptotic cells, again providing evidence that impaired clearance is important to the pathogenesis of SLE. Taken together, it would be interesting to speculate if adiponectin expression and opsonization is also maximized, since PPAR γ is known to upregulate adiponectin.

The above are genes that are involved in both the binding and clearance of apoptotic debris and immune complexes, as well as in the digestion of DNA and chromatin. These are especially interesting because they support the hypothesis, using both human and murine data, that impaired clearance of apoptotic bodies will lead to synergistic effects between atherosclerosis and autoimmune disease.

3.6 Non-inflammatory vs. pro-inflammatory phagocytosis

Macrophages have various receptors that are responsible for the uptake of apoptotic debris which typically results in an anti-inflammatory response. However, various instances of phagocytic uptake, or the inhibition of it, can cause a pro-inflammatory response. In addition, evidence exists of complications and consequential pro-inflammatory cytokine release from immune complexes and autoantibodies observed in SLE.

In general, upon ingestion of an apoptotic cell by a phagocyte, normal clearance will occur by filtration through the lymph nodes and normal degradation and digestion of the apoptotic body by the macrophage. Usually this occurs in a non-inflammatory manner (Fig. 2a) (Fadok et al. 1998). In the instance where the macrophage does not, or is unable to phagocytose the apoptotic body, other phagocytic cells called dendritic cells will proceed to take up the apoptotic body. In this case, follicular dendritic cells which reside with B-cells in the germinal center of the lymph nodes, present the apoptotic bodies as antigens to the B-cells, which will stimulate the release of antibodies. This has consequences for autoimmune diseases such as SLE by further propagating and increasing the amount of antibodies and autoantibodies being produced in the body.

Uptake and degradation of apoptotic cells through scavenger receptors does not normally stimulate an inflammatory response, however, there are instances where pro-inflammatory signaling does occur. Scavenger receptors that recognize oxidized or otherwise modified low-density lipoprotein (LDL) are also capable for the removal of apoptotic cells. Competition for binding can occur for example, in a hyperlipidemic state, with oxidized LDL (oxLDL) and inhibit uptake of the apoptotic cell (Fig. 2b). This will result in increased circulating apoptotic cells which can further degrade and go into secondary necrosis,

releasing pro-inflammatory cytokines. Therefore, it is reasonable to suggest that in hyperlipidemic environments such as those found in cardiovascular disease, there will be propagation of inflammation when apoptotic cells are not cleared from the circulation.

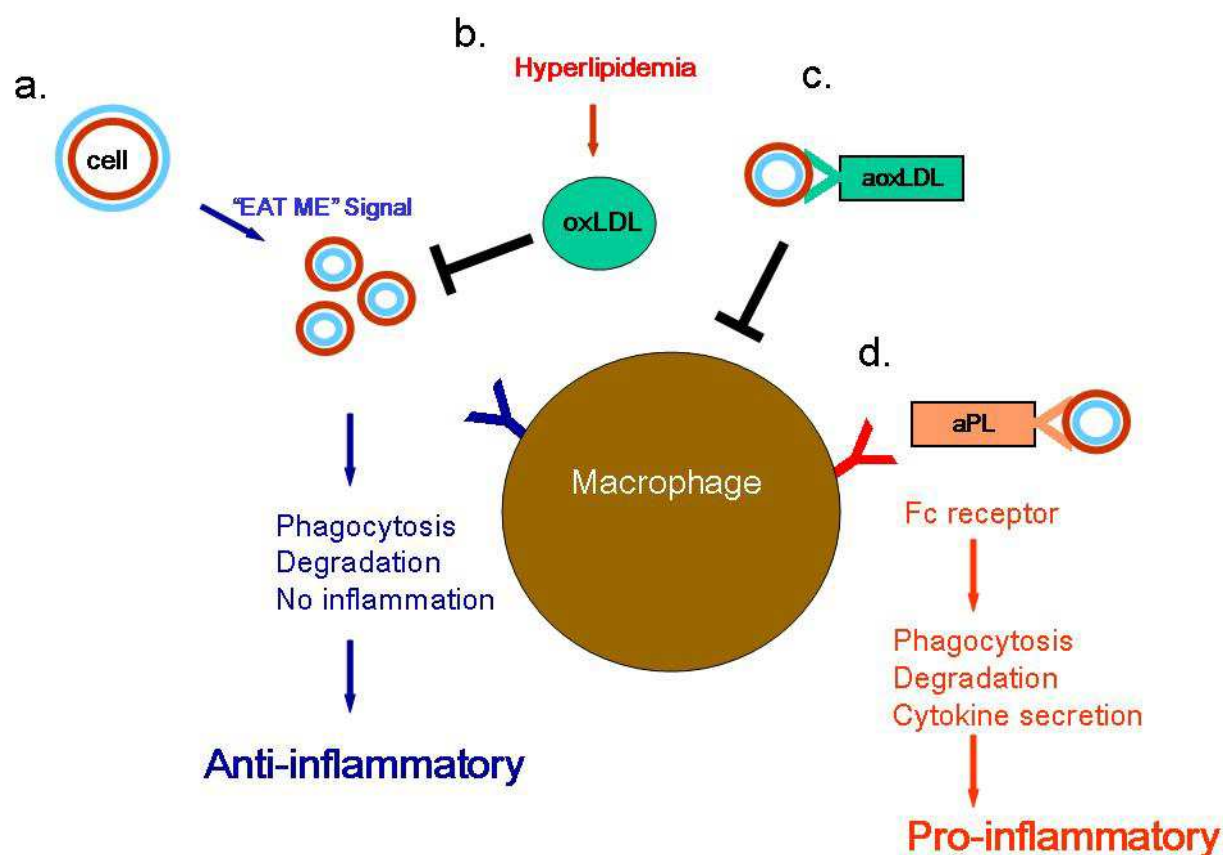


Fig. 2. Normal vs. impaired clearance of apoptotic cells. a) Apoptotic cell clearance does not promote inflammation. b) Elevated circulating lipids may contribute to increased oxidized LDL which can compete with apoptotic cells for receptor uptake. c) Apoptotic cell clearance can be inhibited by binding of anti-oxLDL. d) Opsonization of an apoptotic cell by anti-phospholipids results in uptake by macrophage Fc receptor and pro-inflammatory cytokine signaling.

Relevant to autoimmune diseases, an abundance of circulating autoantibodies to oxidized LDL for example, provides an environment suitable to a pro-inflammatory response with regard to apoptotic clearance. Anti-oxLDL can bind the apoptotic cells by forming an immune complex which inhibits phagocytosis by macrophages and has the potential to further damage tissue (Fig. 2c). In addition, antibodies such as antiphospholipid and anti- β 2GPI have been shown to bind to apoptotic cells and opsonize them for recognition by the macrophage Fc receptor (Manfredi et al. 1998b; Manfredi et al. 1998a) (Fig 2d). Apoptotic cell opsonization by β 2GPI results in dendritic cell stimulation and presentation to T-cells. Activation of B-cells occurs as a result of T-cell signaling, and subsequent autoantibody production ensues (Manfredi et al. 2005). Cross-linking of Fc receptors by IgG immune complex opsonized molecules results in pro-inflammatory cytokine secretion (Fig. 2d). Specifically, Fc γ IIA on apoptotic neutrophils is bound by

immune complexes, and this increases phagocytosis of the apoptotic cell by macrophages. However, phagocytosis by the macrophage in this context results in secretion of TNF α and IL-6, both of which are known to promote inflammation (Hart et al. 2004). Therefore, in an environment where autoantibodies are produced, such as in SLE, this can serve to exacerbate the disease.

4. Apoptotic clearance in autoimmune disease

Currently, one of the most widely studied areas among the genetic causes of SLE is in decreased clearance of apoptotic bodies which are thought to propagate the progression of the disease. It is thought that immune complexes play a role in the pathogenesis of SLE. Evidence to support this idea is provided in a study investigating cytokine production in SLE patients. Both *in vitro* and *in vivo* data has demonstrated that material released from necrotic and apoptotic cells will combine with IgG found in serum from SLE patients. This results in production of IFN α , a pro-inflammatory cytokine that is elevated in SLE patients (Lovgren et al. 2004). This study suggests that the autoantibodies present in serum from SLE patients can form immune complexes with nucleic acids released by apoptotic cells. This will stimulate IFN α production, thus contributing to disease pathogenesis. In addition, administration of IFN α to patients for diseases unrelated to SLE revealed that about 25% of these subjects developed serum anti-nuclear antibodies (ANAs), and a small number of these then went on to develop subsequent autoimmunity (Baechler et al. 2004). From these studies, it is reasonable to suggest that increased IFN α production is, in part, a result of impaired clearance of apoptotic cells.

Evidence exists of autoimmune disease in humans with gene defects of key players in apoptotic clearance machinery. For example, recent evidence has shown that circulating levels of Gas6 and soluble Axl, involved in the bridging of apoptotic cells to the macrophage, correlate to disease activity in SLE especially with the involvement of lupus nephritis (Ekman et al. 2011). In addition, complement deficiency has been noted to occur and result in SLE. In a healthy individual, complement C1q opsonizes antigen-antibody complexes for ingestion and degradation in an anti-inflammatory manner. In addition, a second function of C1q is its ability to bind apoptotic debris through a portion of its globular head independent of antibody (Korb and Ahearn 1997). This is made more efficient if it occurs in conjunction with simultaneous activation of the Fc γ receptors by IgG molecules that have also bound to the antigen or immune complex (Abbas and Lichtman 2003). Adding complement proteins *in vitro* to a phagocytosis assay using human monocyte-derived macrophages from C1q deficient humans resulted in a three-fold increase in phagocytosis of apoptotic cells (Mevorach et al. 1998a). Complement deficient humans follow similar patterns in terms of what is observed in experimental mice: the more upstream in the pathway, the more severe SLE that will develop (Botto and Walport 1993; Botto et al. 1998). The most common deficiencies and also the ones that present the most severe signs of SLE occur with proteins in C1 and C4. In contrast, deficiencies in C2 and C3 have lower occurrences in humans and are also less associated with the development of SLE. Thus, a trend leading towards varying flares of SLE is observed in humans and related to decreased apoptotic cell clearance (Walport 2002). Therefore, it is reasonable to suggest that a hierarchy exists among the proteins of the complement pathway with regard to anti-inflammatory phagocytic clearance.

Another group of defects implicated in autoimmunity involves genes that normally bind immune complexes and aid in their removal. Polymorphisms predisposing people to SLE are known to occur in the receptors FcγRIIA and FcγRIII, also known as CD32 and CD16, respectively. These genes are part of a family of receptors that bind to the Fc domains of many IgG isotypes. FcγRIIA and FcγRIII are low-affinity receptors, so IgG monomers are unable to bind and activate the receptors. These receptors are found on macrophages as well as dendritic cells and neutrophils and mediate the phagocytosis of opsonized particles, and stimulate other leukocytes to degrade the phagocytosed particles. In a lupus mouse model where the Fcγ receptor is disrupted, immune complexes still deposit in the glomeruli but do not contribute to mortality of the animal (Clynes et al. 1998). Therefore, Fcγ receptors play a role in inflammatory cytokine signaling that can contribute to autoimmunity.

An *in vitro* study utilizing monocyte-derived macrophages from SLE patients revealed a significantly lower ability of these macrophages to phagocytose apoptotic cells, compared to healthy controls (Herrmann et al. 1998). In addition, Baumann and colleagues have provided *in vivo* clinical evidence that human patients with SLE have impaired clearance of their apoptotic debris. They showed that apoptotic cells accumulated within the germinal centers of the lymph nodes of SLE patients. They studied macrophages within the lymph nodes and found that they not only have an abnormal morphology, but also that there is a decreased amount of apoptotic body co-localization, suggesting that macrophage phagocytosis is disrupted in these patients. Further, the apoptotic bodies were found to colocalize more frequently with follicular dendritic cells (DCs). This association could lead to presentation by the DCs of the apoptotic body as an antigen, and promote auto-antibody production by the B-cells (Baumann et al. 2002). Thus, there are several lines of evidence that implicate impaired apoptotic body clearance in the progression of autoimmune diseases such as SLE.

5. Cardiovascular disease- conventional vs. SLE-specific risk factors

Clinical studies have largely examined the relationship between SLE and endpoint cardiac events including myocardial infarction and stroke. More recently, attention has shifted towards the *causes* of advanced cardiovascular diseases; the focus now being on the contribution of accelerated atherosclerosis in SLE patients (J.M. Esdaile et al. 2001b; Lockshin et al. 2001; Manzi 2000). Conventional risk factors for development of atherosclerotic vascular and coronary artery disease include age, circulating levels of high-density lipoprotein (HDL) and total cholesterol, blood pressure, smoking, and diabetes mellitus. This list of risk factors continues to expand since further study has revealed that systolic and diastolic blood pressure levels can be considered separate risk factors; independent roles for obesity and specific adipose tissue distribution has stemmed from studies of diabetes mellitus associated with coronary artery disease. Additions that are actively being researched include C-reactive protein, lipoprotein(a), fibrinogen, and homocysteine (Hackam and Anand 2003). The list of risk factors is constantly being updated as studies continue to search for new markers to predict disease.

Risk factors have also been identified specifically for SLE patients with cardiovascular disease (CVD), in addition to the conventional risk factors discussed above. These are a distinct set of risk factors that separate these patients from SLE patients without CVD as well as their healthy counterparts (Svenungsson et al. 2001). Using the risk factors defined by the Framingham study, a significant proportion of CVD associated with SLE was shown

to occur based on other unknown risk factors. In this study, when traditional Framingham risk factors were accounted for, it was observed that there was an 8- to 17-fold increase in nonfatal myocardial infarction, stroke, death from CHD and overall occurrences of CHD, in patients with SLE (J. M. Esdaile et al. 2001a). In young, pre-menopausal women, this increase was as high as 50-fold. In another study, women with SLE and no evidence of CVD were studied with regards to carotid plaque, intima-media wall thickness (IMT), and aortic stiffness. It was determined that the risk factors for carotid plaque and IMT were the same for cardiovascular disease in the absence of SLE. However, other risk factors with respect to aortic stiffness were found to be specific to SLE patients. For example increased C3 complement levels were observed in SLE patients with higher vascular stiffness. This suggests that immune dysregulation and complement metabolism play a role in the interactions between these two disease processes (Selzer et al. 2004). It has also been shown that patients with SLE have significant endothelial dysfunction that occurs at rates higher than those predicted after taking traditional CHD risk factors into account (El-Magadmi et al. 2004). Several other studies evaluating the role of traditional CVD risk factors in patients with SLE have been performed, but have excluded SLE patients with various existing risk factors.

5.1 Atherosclerosis

Atherosclerosis is the underlying cause of most cardiovascular disease accounting for the majority of death in the Western world. It is a disorder in which intimal thickening and lipid deposition occur in the elastic arteries such as the aorta, and places of turbid flow, as well as in the larger arteries such as the coronary arteries. There are six levels of atherosclerotic lesion progression; the last three are considered complex and occlusive, having a thinner cap and a very cholesterol-rich core, making it more susceptible to rupture. Plaque deposition and rupture can lead to a cardiovascular event such as a myocardial infarction or stroke. Initial events that lead to lesion development occur when LDL is allowed to migrate into the vessel wall. This can occur in one of two ways: a diffusion like-mechanism if the serum LDL level is extremely high; or injury to the endothelial lining, occurring from hypertension, toxins, bacteria, viruses, or immune complexes (Ross 1993).

About half of all the circulating LDL within the body is cleared daily. Although two-thirds of LDL particles are taken up through the LDL receptors during normal clearance of lipids from the body, the remaining LDL is cleared by other mechanisms. For example, within the interstitium, LDL can become modified to various oxidized forms. This allows the LDL to be engulfed by type A scavenger receptors on macrophages and/or smooth muscle cells which are then termed "foam cells." These foam cells contribute to the initiation and progression of atherogenesis.

Accelerated atherosclerosis is believed to be a critical factor contributing to stroke and coronary heart disease (CHD), which is one of the leading causes of death among young women with SLE (Manzi et al. 1997a; Petri et al. 1992; Urowitz et al. 1976). Clinical studies have largely examined the relation between SLE and endpoint cardiac events including myocardial infarction and stroke. Attention has shifted towards the causes of advanced cardiovascular diseases; the focus now being on the contribution of accelerated atherosclerosis in SLE patients (J. M. Esdaile et al. 2001a; Lockshin et al. 2001; Manzi 2000).

As mentioned earlier, progress in elucidating the feedback interactions between atherosclerosis and autoimmune disease has been impaired by the lack of appropriate animal models, and further research is necessary to determine the mechanisms in order to

provide more beneficial treatment to patients. A mouse model developed by our lab has demonstrated a synergy between atherosclerosis and SLE (Aprahamian et al. 2004). This model combined an autoimmune phenotype, *gld*, (due to FasL deficiency) with an atherosclerotic background, *apoE*^{-/-}, (due to apoE deficiency). Fas ligand (FasL) is a type II membrane protein that induces apoptotic cell death in cells that bear the Fas (CD95/Apo-1) receptor (S. Nagata and Golstein 1995). Mice lacking Fas or FasL have a marked deficiency in apoptosis, leading to the accumulation of lymphocytes. The *gld.apoE*^{-/-} double mutant mouse was subjected to a high cholesterol Western-type diet and compared to the wild type, *gld*, and *apoE*^{-/-} mice controls. Through analysis of cholesterol levels and atherosclerotic lesion area, it was found that the atherogenic phenotypes were exacerbated in the presence of inflammatory autoimmune disease. In addition, analysis of autoantibody levels, splenomegaly, and lymphadenopathy revealed that the autoimmune phenotypes were exacerbated when subjected to an atherogenic background. Although to a lesser extent, these results were also significant when mice were maintained on normal diet. Next, the mechanism by which this observed synergy occurred was dissected by first examining the number of apoptotic cells within the lymph nodes of *gld* and *gld.apoE*^{-/-} mice. A significant number of TUNEL positive stained cells in *gld* mice compared to wild type and *apoE*^{-/-} mice was observed, and this number was further increased in *gld.apoE*^{-/-} mice. In addition, examination of apoptotic bodies within the circulation corroborates this finding. Histological analysis in lymph nodes revealed that fewer macrophages colocalized with TUNEL positive material in the *gld.apoE*^{-/-} mice, indicating impaired uptake of the apoptotic bodies by macrophages. Disruption of the chemoattractant gradient for macrophage clearance in *gld* mice resulted in an increase in apoptotic bodies within the lymph nodes. Taken together, these data suggest that the synergy between the two disease processes observed in the *gld.apoE*^{-/-} mouse may occur in part from impaired clearance of apoptotic bodies.

Other studies followed, utilizing mouse models to further elucidate the interaction between atherosclerosis and lupus. Using the lupus-susceptible *Sle*1.2.3 mouse model and creating LDLr bone marrow chimeras resulted in accelerated atherosclerosis associated with increased T and B cell activation when maintained on a high cholesterol Western diet (Stanic et al. 2006). In addition, the same group has shown that high fat fed LDLr.SLE chimeras have increased mortality and are significantly more hypertensive, indicating a synergy between the lupus disease and vascular complications (Braun et al. 2008). Another bone marrow chimera experiment transplant using *gld* bone marrow to LDLr^{-/-} mice was shown to accelerate plaque progression (Gautier et al. 2007). Ma et al., has demonstrated that induction of cGVH in B6.*ApoE*^{-/-} mice, breeding a Fas null gene onto these B6/*lpr.ApoE*^{-/-} mice, and breeding the *ApoE*^{-/-} defect onto MRL/*lpr* mice all caused a modest increase of atherosclerosis at 24 weeks of age compared to B6.*ApoE*^{-/-} controls, as well as increased lupus like symptoms (Ma et al. 2008). The involvement of adiponectin has not been examined in these models, however given the importance of this adipokine in regulating SLE as well as inflammatory processes involved in atherosclerosis, it would be of great interest to study not only the levels of adiponectin, but also to administer exogenous adiponectin to determine if the phenotypes could be rescued. The paradox of low levels of adiponectin during flares of SLE is an interesting area that requires further exploration.

In addition, it is known that chronic inflammation can affect bone metabolism and that the pro-inflammatory cytokines TNF, IL-1 β , and IL-6 may play a major role. To this end, *lpr*

mice, which have a mutation in the fas receptor, were crossed to apoE^{-/-} mice and this double mutant was reported to develop lupus nephritis, atherosclerosis and decreased bone mineral and volume density, which may be helpful to study the role of osteopenia in lupus (Feng et al. 2007). In a further study, these mice were treated with pravastatin and an apo1 mimetic, and results showed beneficial effects. This is particularly interesting since pravastatin has been shown to increase adiponectin expression at both the mRNA and protein level, as well as enhance insulin sensitivity (Sugiyama et al. 2007). Although there is little data about the role of adipokines in degenerative bone disease, decreased adiponectin levels have been observed in the bone marrow supernatant fluid of women with osteoporosis when compared to non-osteoporotic (Pino et al. 2010). In addition, Oshima et al demonstrated that exogenous overexpression of adiponectin in wild type mice resulted in increased bone mass and decreased number of osteoclasts. Further study in vitro showed that adiponectin can prevent development of osteoclasts by inhibiting differentiation of mouse bone marrow macrophages as well as human mononuclear cells (Oshima et al. 2005). In addition to facilitating apoptotic clearance, adiponectin inhibits the expression of TNF α , and there is a feedback loop whereby both TNF α and IL-6 inhibit the production of adiponectin in adipocytes. It is therefore interesting to speculate that varying levels of adiponectin can affect inflammatory processes that could result in degenerative bone disorders found in some inflammatory conditions such as obesity, inflammatory bowel disease, and diabetes.

Based on these data, it is reasonable to suggest that the presence of two disease states involving inflammation promotes impaired apoptotic cell clearance and thus provides a positive feedback mechanism which drives the progression of the two diseases. Therefore, the two disease processes result in a vicious cycle that catalyzes the progression of atherosclerotic lesion formation and autoimmune disease.

5.2 Hypercholesterolemia and impaired clearance

Apoptotic cell ingestion by macrophages normally induces a non-inflammatory response. Conversely, phagocytosis of many bacteria and foreign antigens normally results in a pro-inflammatory response by macrophages which could include the generation of reactive oxygen species, proteolytic enzyme release, and the production of numerous inflammatory cytokines and growth factors (Fadok et al. 1998). If the immune system is disrupted, apoptotic cell phagocytosis may result in a pro-inflammatory response. This is not limited to conventional immune disorders since there is evidence that hyperlipidemia can disrupt proper phagocytosis of apoptotic cells.

Aggravated autoimmune disease may result from interference with the signal gradients that are required for the normal recruitment of phagocytes to dying cells. As discussed earlier, apoptotic bodies release LPC, facilitating macrophage recruitment to the dying cell. LPC is a major component of oxLDL, which is a proatherogenic form of LDL cholesterol (Lauber et al. 2003). Thus, hyperlipidemic conditions may provide elevated levels of oxLDL, thereby disrupting the LPC chemoattractant gradient. This would render the apoptotic cell "lost" as far as macrophage recognition. It has previously been shown that increased levels of LPC disrupts phagocytic uptake of apoptotic bodies, which is further exacerbated in mice on an atherogenic background (Aprahamian et al. 2004). Therefore, atherosclerosis may compound the impaired clearance of apoptotic cells in patients with SLE.

Similarly, it has been shown that apoptotic bodies are recognized and taken up through many of the same receptors that bind oxLDL, a modified form of LDL that has been shown

to be proatherogenic (Oka et al. 1998; Platt et al. 1996; Sambrano and Steinberg 1995). Therefore, an atherogenic environment may provide an increased amount of oxLDL that can compete with the apoptotic cell for binding to the scavenger receptor. This would interfere with the recognition of apoptotic bodies by macrophages and contribute to further inflammation. Increased levels of both oxLDL and anti-oxLDL have been proposed as risk factors for cardiovascular disease in female patients with SLE (Wu et al. 1999). In addition, antibodies to other phospholipids are risk factors that are involved in the progression of atherosclerosis and advanced cardiovascular outcomes such as myocardial infarction (Puurunen et al. 1994; Salonen et al. 1992; Wu et al. 1997). Anti-oxLDL antibodies are capable of binding to apoptotic cells and this inhibits phagocytosis by macrophages (Chang et al. 1999). Therefore, a number of reasons suggest that hypercholesterolemia may contribute to macrophage disruption and the impaired clearance of apoptotic cells leading to a more severe manifestation of SLE outbreak.

It is interesting to note that another phospholipid, cardiolipin, and its autoantibodies, are found at increased levels in patients with SLE, and also in patients with cardiovascular disease (Vaarala 2000). It has been shown that antinuclear antibodies and anti-cardiolipin antibodies can arise after *in vivo* administration of apoptotic cells to healthy wild-type mice (Mevorach et al. 1998b). Of note, antibodies to cardiolipin cross-react with oxLDL in patients with SLE (Vaarala et al. 1993). Another interesting feature of anti-phospholipids is that they also cross react with anti-endothelial cell antibodies (Hasselaar et al. 1990). Since antibodies directed towards negatively charged phospholipids can bind to endothelial cells, they could therefore be a driving force in the initiation of endothelial activation and dysfunction, ultimately leading to atherosclerosis.

Activated endothelial cells are characterized by changes in the vascular integrity and expression levels of adhesion molecules and cytokines (Hunt 2000). Human anti-cardiolipin antibodies have been implicated in the activation of endothelial cells, resulting in expression of E-selectin, vascular cell adhesion molecule-1, and intracellular adhesion molecule-1, all of which facilitate monocyte adhesion to the vessel wall (Simantov et al. 1995; Simantov et al. 1996). This suggests that anti-cardiolipin may facilitate extravasation of these monocytes and contribute to inflammation within the vessel wall, whether in atherogenesis, or autoimmune disease. In addition, patients with SLE and in particular, those with cardiovascular disease, have enhanced levels of lipid peroxidation (Frostedgard et al. 2005). It is likely that hyperlipidemic conditions contribute to the severity of autoimmune disease by promoting the accumulation of apoptotic debris.

6. Conclusion

Published data provides mounting evidence that accelerated atherosclerosis and cardiovascular disease is a growing problem in patients with SLE and other autoimmune diseases. This chapter has focused on the role that clearance of apoptotic bodies plays in the progression of both the autoimmune and cardiovascular components of disease. Specifically, *in vitro* and *in vivo* data have been presented to demonstrate the roles of the various individual molecules involved in the machinery of the apoptotic and phagocytic processes. These studies clearly show an intricate connection between impaired apoptotic clearance and the development or progression of autoimmune disease. Further, the role of inflammation in atherosclerosis, accelerated in the presence of autoimmune disease, has

been shown to be important to the progression of both diseases. The driving mechanisms of impaired clearance of apoptotic cells and precise etiology of these results in driving disease development are currently an intense area of research.

7. References

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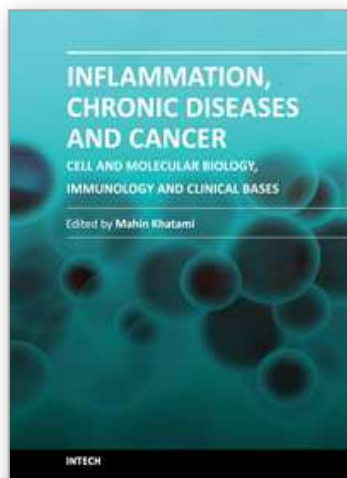
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Inflammation, Chronic Diseases and Cancer - Cell and Molecular Biology, Immunology and Clinical Bases

Edited by Dr Mahin Khatami

ISBN 978-953-51-0102-4

Hard cover, 430 pages

Publisher InTech

Published online 09, March, 2012

Published in print edition March, 2012

This book is a collection of excellent reviews and perspectives contributed by experts in the multidisciplinary field of basic science, clinical studies and treatment options for a wide range of acute and chronic inflammatory diseases or cancer. The goal has been to demonstrate that persistent or chronic (unresolved or subclinical) inflammation is a common denominator in the genesis, progression and manifestation of many illnesses and/or cancers, particularly during the aging process. Understanding the fundamental basis of shared and interrelated immunological features of unresolved inflammation in initiation and progression of chronic diseases or cancer are expected to hold real promises when the designs of cost-effective strategies are considered for diagnosis, prevention or treatment of a number of age-associated illnesses such as autoimmune and neurodegenerative diseases as well as many cancers.

How to reference

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Tamar Aprahamian (2012). Autoimmunity, Atherosclerosis and Apoptotic Cell Clearance, Inflammation, Chronic Diseases and Cancer - Cell and Molecular Biology, Immunology and Clinical Bases, Dr Mahin Khatami (Ed.), ISBN: 978-953-51-0102-4, InTech, Available from: <http://www.intechopen.com/books/inflammation-chronic-diseases-and-cancer-cell-and-molecular-biology-immunology-and-clinical-bases/autoimmunity-apoptosis-and-accelerated-atherosclerosis>

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