Apoptotic molecular mechanisms implicated in autoimmune diseases

F. CACCIAPAGLIA¹, C. SPADACCIO², M. CHELLO², A. GIGANTE³, R. COCCIA⁴, A. AFELTRA¹, A. AMOROSO³

¹Department of Clinical Medicine, Immunology e Rheumatology, Campus Bio-Medico University of Rome (Italy)
²Department of Cardiovascular Sciences, Campus Bio-Medico University of Rome (Italy)
³Department of Clinical Medicine, “La Sapienza” University, Rome (Italy)
⁴Department of Biochemical Sciences, “La Sapienza” University, Rome (Italy)

Abstract. – Apoptosis is a programmed cell death that represents a normal component of the development, differentiation and health of multicellular organisms leading to an adequate cellular turnover and homeostasis.

In autoimmune diseases, the immune system recognizes various autoantigens causing damage in target organs. Dead cells represent an important source of autoantigens that, in particular conditions, can represent a stimulus for an autoimmune response.

A large number of studies reported the impairment of the apoptosis regulatory mechanisms in immune cells as a pivotal element in the pathogenesis and evolution of autoimmune disorders. Several pathogenetic pathways have been claimed to account for autoimmunity development during apoptotic processes. In fact, interestingly abnormalities potentially leading to immune disorders have been described as occurring in each step involved in apoptosis, from the very beginning to the post death phenomena.

In this extent we propose a systematic review of the molecular mechanisms strictly leading to apoptosis with particular interest to their alterations, potentially causing tissue specific and/or systemic autoimmunity.

Key Words: Apoptosis, Autoimmune diseases, Molecular pathways, LE phenomenon.

Introduction

Apoptosis or programmed cell death, is a normal component of the development, differentiation and health of multicellular organisms, warranting an adequate cellular turnover and homeostasis. A variety of stimuli has been shown to trigger a highly regulated and controlled pathway, leading to both cell death and activation of a scavenger system in order to remove cell residuals.

Upon specific instructing signals, no longer needed or “unwanted” or damaged cells enter in a genetically and epigenetically controlled pathway leading to death, in which the cells themselves play an active role in their own death.

The elimination of apoptotic cells and cell bodies by phagocytes represents an evolutionarily conserved way to prevent exposure of surrounding tissue to potentially cytotoxic, immunogenic, or inflammatory cellular contents¹-³.

Indeed, the main characteristic of apoptotic cells is that they, in contrast to necrotic cells, maintain their membrane integrity. The release of intracellular components is thereby prevented, making apoptosis distinct from necrosis in which uncontrolled cell death leads to lysis of cells, inflammatory responses and, potentially, to serious health problems⁴-⁷.

For a long time, studies on the elimination of “unwanted” cells were focused mainly on the stage of cell death⁸, while mechanisms responsible for the control of tissue fragmentation from fragments of the dying cells remained unstudied⁹,¹⁰. Only during the last decade, significant progresses have been achieved in this field due to experiments on Caenorhabditis elegans, and specific features of phagocytosis of apoptotic cells have been elucidated in many biochemical and genetic details.

The clearance of cells after necrotic death is still poorly studied, although, in contrast to im-
munologically unnoticeable apoptosis, necrosis of cells is an immunogenic and rather dangerous event. The uptake and removal of necrotic or lysed cells normally involve inflammation and an immune response.

Depending on the rate and entity of apoptotic processes required by tissutal environment, exogenous influences, cell cycle phase and differentiation state, a variety of cell types are claimed to exert scavenger activity with neighbouring cells: fibroblasts, acting during normal adult tissue turnover; macrophages, playing important roles during large scales apoptosis phenomena such as embryonic morphogenesis, ionizing radiation, or acute infections.

This complex process has been largely studied in respect of the triggering factors, molecular mechanisms and consequences.

In this extent, after a brief analysis of the molecular mechanisms involved in the apoptotic process, we propose a detailed review of the apoptotic mechanisms that can lead to organ-specific and/or systemic autoimmune disorders.

**Biological Mechanisms of Apoptosis**

Upon receiving specific signals instructing the cells to undergo apoptosis, a number of distinctive biochemical and morphological changes occur in the cell (Figure 1).

Several signalling pathways triggering apoptosis have been described and death receptors belonging to the TNF (tumor necrosis factor)/NGF (nerve growth factor) receptor superfamily have been shown to play a pivotal role in the initiation of programmed cell death\(^\text{11}\). These receptors play distinct roles, e.g. in the immune system, where they contribute to regulation of the adaptive immune response in various ways, most notably by triggering activation-induced cell death (AICD) of T cells. Thus, deregulation of death receptor signalling, either allowing too much or too little apoptosis, can lead to autoimmune disorders and also impacts on tumoral genesis or other diseases.

CD95-CD95L (Fas-FasL) death receptor system represents the best characterized among the death receptors. CD95 is a widely expressed gly-
cosylated cell surface molecule which can also occur in a soluble form generated by differential splicing. Naturally occurring mutations of the CD95 gene cause complex disorders of the immune system in mice, manifested as lymphoadenopathy and autoimmunity, similarly to human systemic lupus erythematosus (SLE)\textsuperscript{9,12}.

It was shown that CD95 receptors are expressed on cells as preassociated trimers by interaction of their PLAD (pre-ligand binding assembly domain). Expression of the CD95 gene is enhanced by interferon (IFN)-gamma and TNF and by activation of lymphocytes\textsuperscript{9}. Triggering of CD95 leads to formation of a protein complex within seconds. This so-called death-inducing signaling complex (DISC) contains the adaptor FADD/Mort1, procaspase and c-FLIP and its recruitment leads to autoproteolytic activation of caspase-8 and caspase-10 starting the execution phase of apoptosis by initiating the caspase cascade\textsuperscript{13}.

Caspases belong to a family of proteins typically activated in the early stages of apoptosis. These proteins breakdown or cleave key cellular substrates that are required for normal cellular function, including structural proteins in the cytoskeleton and nuclear proteins, such as DNA repair enzymes. The caspases can also activate other degradative enzymes such as DNAses, which begin to cleave the DNA in the nucleus. The result of these biochemical changes is the appearance of morphological changes in the cell. Typically, the cytoplasm begins to shrink following the cleavage of laminin and actin filaments. Nuclear and chromatin condensation can also be observed following the breakdown of chromatin itself and nuclear structural proteins, and in many cases the nuclei of apoptotic cells take on a "horse-shoe" like appearance. Degradation of DNA and protein fragmentation with disassembly of organelles occur. Cells continue to shrink, packaging themselves, till collapse into small apoptotic bodies, a form that allows for easy clearance by macrophages\textsuperscript{6}.

These phagocytic cells are responsible for removing apoptotic cells from tissues in a clean and tidy fashion that avoids many of the problems associated with necrotic cell death. In order to promote their phagocytosis by macrophages, apoptotic cells often undergo plasma membrane changes that trigger the macrophage response. One such change is the translocation of phosphatidylserine (PS) from the inner leaflet of the cell to the outer surface. Membrane changes can often be observed morphologically through the appearance of membrane blebs or blisters which often appear towards the end of the apoptotic process. Small vesicles called apoptotic bodies are also sometimes observed.

The morphological and biochemical characteristics of cells dying by necrosis differ markedly from those of cells dying by apoptosis. Morphologically, necrosis is characterized by swelling of cells and their organelles leading to the disruption of the cell membrane. In contrast to apoptotic cells, the chromatin of a primary necrotic cell does not get fragmented. Mechanistically, necrotic cells die passively and messy, with loss of membrane integrity in the latter\textsuperscript{14}.

**Apoptosis and Autoimmunity**

Autoimmune diseases reflect the confluence of genetic, environmental and stochastic events\textsuperscript{15,16}. As well known, apoptotic cell death pathways are implicated in initiating and propagating autoimmune diseases.

Similarly to autoimmunity, apoptosis is a multistep process, affecting immune and target cells, integrating numerous intrinsic and extrinsic signals, and requiring the actions of multiple gene products. Of note, recent observations demonstrated that the apoptotic death might provide a primary source of self antigens or represent the target of the immune response in autoimmunity\textsuperscript{16}. Moreover, the apoptosis is both required for lymphocyte selection and immunoregulation, and is a prominent outcome of immune and inflammatory effector pathways.

The possibility to scavenge apoptotic cells in non-inflammatory setting and the effectiveness of clearing systems avoid recognition of specific epitopes leading to immune tolerance and allowing the normal turnover and repairing processes to be completed.

Large number of studies underlined the idea that dying cells are not immunologically inert, but cell death modality can define the immunological response to intracellular antigens. Early studies showed that apoptotic cells are phagocytised by macrophages and dendritic cells (DC), and this uptake is associated with secretion of antiinflammatory cytokines, such as TGF-beta and IL-10, and failure to upregulate co-stimulatory molecules\textsuperscript{17}.

Gallucci et al. demonstrated that DC fail to activate T lymphocytes if exposed to apoptotic cells, but in contact with necrotic cells they become stimulatory\textsuperscript{18}.
During normal tissue turnover apoptotic cells are captured by DC and migrate to local lymph nodes, where tolerance is induced. The failure or inability to adequately tolerate intracellular antigens in apoptotic cells may determine the susceptibility of some individuals to later development of autoimmunity. Whether such failure of tolerance induction occurs centrally or peripherally remains to be determined, as well as the mechanisms through which initial immunization to such antigens occurs in autoimmunity.

An adequate balance in the molecular repertoire supervising induction or inhibition of apoptosis has been shown to play a pivotal role in determining initiation of autoimmune phenomena. The demonstration that the autoimmune prone lpr (lymphoproliferative) and gld (generalized lymphoproliferative disease) mouse strains display a defective apoptosis due to mutations in lpr and gld, respectively, supports this concept. Moreover, mice expressing Fas (CD95) and the Fas-ligand, respectively, exhibit a defective apoptosis due to mutations in the apoptosis inhibition gene, Bcl-2 as a transgene, have an impaired apoptosis in both B- and T-cell lineages which leads to the development of autoimmune phenomenon on certain genetic backgrounds.

Several pathogenetic pathways have been claimed to account for autoimmune development during apoptotic processes and, interestingly, abnormalities potentially leading to immune disorders have been described as occurring in each step involved in apoptosis, from the very beginning to the post death phenomena.

**Loss of Self Tolerance**

Loss of self tolerance has been claimed to account for several mechanisms underlying pathogenesis of autoimmune diseases.

Besides the exogenous factors, implying initiation of immune reaction against self antigens (due to cross-reaction, antigenic sharing and/or epitopes spreading), there are two physiological phases in which the balance and control of immune response towards self-epitopes could acquire an important significance in terms of both development of autoimmune disorders and defence against exogenous agents. The early phase of positive and negative selection of self-reacting T lymphocytes and the normal aging processes have been considered crucial in this context.

**Self reacting T Lymphocytes Selection**

Apoptosis is important to maintain peripheral lymphocyte homeostasis and self tolerance and to minimize the accumulation of autoreactive lymphocytes. Disruption of apoptotic pathways has been linked to lymphadenopathy, breakdown of peripheral tolerance and the development of autoimmune diseases. Normally selection of autoreactive clones concerns anergy, clonal ignorance and apoptosis. Nevertheless, inhibiting apoptosis in lymphocytes is not sufficient to break self-tolerance by itself, suggesting the involvement of other cell types as dendritic cells and several complementary molecules.

During the development of T cells in the thymus, those cells that do not recognize MHC molecules are not positively selected and die thereafter. T cells that are positively selected will next be subjected to negative selection. T cells with high affinities for self-MHC molecules are deleted to avoid maturation and systemic migration of autoreactive T cells. T cells with low affinity for self-MHC or self-antigens bound to their own MHC molecules evade negative selection. This last group of T cells goes to constitute the repertoire of mature T cells. Although the negative thymic selection, low affinity T cells, that populate the peripheral lymphoid organs, are potentially cross-reactive to self antigens and must be controlled by extra-thymic tolerance mechanisms.

Lymphocyte apoptosis has emerged as a principal system for peripheral tolerance. In the regulation of lymphocyte fate in the periphery, there are two general pathways to yield cell death. First, a lymphokine withdrawal death in which lymphocytes, especially activated T cells that proliferate in the presence of cytokines, will undergo apoptosis if antigen stimulation and/or concomitant lymphokine production ceases. This pathway physiologically may be useful to remove the expanded population of lymphocytes after an antigen has been successfully cleared.

Second, a propriocidal regulation: antigen-induced death of activated T cells, and possibly B cells. This is achieved by the death receptors such as CD95 (Fas/Apo-1) and other molecules related to TNF and TNF receptors (TNFRs). Propriocidal regulation is a powerful negative feedback mechanism that limits or reverses clonal expansion in the case of persistent encounters with antigen.

A great deal of attention has been focused on the molecular functions of the TNFR family as defects in these receptors or their downstream signalling pathways have been linked to uncontrolled lymphocyte proliferation in peripheral lymphoid organs, and the development of autoimmune diseases in mice and humans.
Errors in central or peripheral tolerance at the T- or B-cell level have also been suggested as causes for autoimmunity. These mechanisms would have more applicability to explain systemic rather than organ-specific autoimmune diseases, since it is difficult to understand how this defect could be confined to a single, organ restricted, antigen.

A possible experimental example of autoimmunity induced by faulty T-cell tolerance may be the development of multi-organ disease in irradiated bone-marrow reconstituted mice, treated with cyclosporine. This effect is presumably due to the interference of cyclosporine with apoptosis during negative selection of self reactive clones.

Apoptosis of mature lymphocytes represents another step of peripheral protection against autoimmunity. In fact, deficiency in mature lymphocyte apoptosis has been correlated with the development of disease: studies in lpr and gM mice, defective in CD95 and CD95L respectively, confirmed this hypothesis. Moreover, genetic deficiencies of IL-2 or its receptor, crucial elements for T cell susceptibility to antigen-induced apoptosis, lead to lymphoid hyperplasia and autoimmunity. Moreover, it has been demonstrated that patients with autoimmune lymphoproliferative syndromes present mutation in death receptor machinery concerning abnormalities of CD95/CD95L.

Clinical development of organ-specific autoimmune diseases is caused by conventional immunological responses against self-antigens for which, normally, T-cell tolerance has not been established. This lack of tolerance towards tissue-specific antigens may be attributed to antigens being unavailable under normal conditions, which may be a consequence of anatomic sequestration, inadequate presentation due to the cryptic nature of the determinant and/or lack of costimulatory factors. Events such as trauma, inflammation by a tissue-specific or cross-reactive pathogen, may provide the means for initiation of such organ-specific autoimmune responses. Nevertheless, recent elucidations of the role of central tolerance in preventing organ-specific autoimmunity have changed the view of self/nonself discrimination.

In this context, the discovery of promiscuous expression of tissue-restricted self-antigens by medullary thymic epithelial cells (mTEC) has been crucial. This micro-environment emulates virtually all tissues of the body, independently from spatial or temporal expression patterns. This claims to carefully evaluate the role of mechanisms of deletion and anergy induction of self reactive cells. As mTEC present potentially all body tissues antigens, failure in maintaining tolerance will result in development of autoimmunity even independently by the introduction of potentially cross reacting exogenous epitopes or sequestreated antigens.

With regard to systemic autoimmune diseases such as SLE, neither exogenous polyclonal B or T-cell activators nor immunoregulatory disturbances appear to be satisfactory explanations for pathogenic mechanism. Moreover, the poor association with environmental factors underlines the involvement of an ubiquitous antigen or pathogen and the widespread autoimmune response against a variety of dissimilar self-antigens also seems in contrast with an exogenous mimicry theory. Probably, this condition could be due to an above-threshold levels engagement of a large set of non-tolerant T cells, that recognize different self-peptides displayed on MHC class II molecules.

**Ageing Processes**

Biological ageing phenomena have been demonstrated to be caused by free radicals, protein glycosylation as well as several factors causing DNA malfunctions.

Other theories are based on the assumption of genetic determination of aging, resulting in a limited number of possible cell divisions. Changes in cell cycle control and telomere shortening lead to the cessation of replication in senescent cells.

A major aspect of individual human aging is the decline of immune function in the elderly, also known as “immunosenescence”, characterized by changes in T cell subsets, cellular and molecular alterations and thymic atrophy, resulting in a decline of T and B cell function.

Aging of immune system may also result in a loss of the ability of the discrimination of “self” and “non-self” antigens.

It has been hypothesized that, consecutively to thymus involution, there is a reduction of naive T cell output from the thymus and memory T cells compensatory proliferation for the loss of thymic output which are reactive to “neoantigens” formed during the aging process. This autoimmune-prone lymphocyte arrangement with “neoantigens” acquired during the aging process may cause the development of autoimmune conditions.
Moreover, it has been emphasized that reduced apoptosis and impaired T cell homeostasis could promote a chronic inflammatory state and it has lead to the concept of an autoimmune-risk phenotype.

The most striking changes in “immunosenescence” are a shift in the expression of CD45 isoforms from the CD45RA+/CD45RO- to the CD45RA-/CD45RO+ subsets in human CD4+CD8+ T lymphocytes, mostly attributed to chronic antigenic stimulation and depletion of naive T cells, secondary to the involution of the thymus.

This condition determines reduced interleukin-2 (IL-2) production and IL-2 receptor expression, and consecutively poor response to IL-2 and lower IL-7 expression levels in T cells of aged humans.

The loss of CD28 expression together with a telomere shortening have been well documented in CD4+ as well as CD8+ T cells in the elderly and it is typical for T cells that have excessively replicated. CD28 mediates the major co-stimulatory signal that coupled to those derived from T cell receptor amplifying T cell proliferation, IL-2 and IFN-gamma production, thus affecting the functional profile of T cells. Clonal CD28-T cells have been shown to be autoreactive and displayed longer survival and increased resistance to apoptosis. However, even if deprived of a major co-stimulatory signal, CD4+/CD28- T cells produce large amounts of IFN-gamma and therefore have proinflammatory capacities.

Clinical reflex of this autoimmune-risk phenotype could be a weak and shorter primary antibody response with lower affinity, and the decline of the immune response to vaccinations in the elderly. Moreover, increased autoantibody levels, oligoclonal expansion of CD8+ T cells (such as during chronic viral infections), changes in cytokine profile, towards a chronic inflammatory cytokine state (i.e., TNF-alpha, IL-1, and IL-6), and increased susceptibility to infections are present as well.

On the other hand, it has yet to be proved which kind of mechanism turn the immune system to manifest autoimmune disease and how the supposed defects in T cell differentiation and interaction, leading to premature aging of the immune system, may contribute to the development of autoimmune diseases.

Besides the above mentioned decreasing in naive T cell rate and accumulation of memory T cells, the shift from Th1 to Th2 cytokine profiles seems to be primarily associated with autoimmune response. Several experimental models of SLE supported this hypothesis: mice with Th1 predominance, induced by IL-12 administration, were susceptible to present SLE-related symptoms. As well-known, autoimmunity may increase with ageing, probably by the “neoantigens” formation during life and consequent activation of memory B cells. But, despite higher autoantibodies levels in elderly, this condition, commonly, do not reach clinical relevance because of tolerance mechanism. The shift to Th2 cytokines has been postulated to permit autoreactive antibodies to obtain clinical significance.

Interestingly, this imbalance in lymphocyte types repertoire can occur as a result of normal ageing process, but can also represent the primary abnormality in autoimmune diseases.

Alterations in T cell apoptosis may be an important mechanism to promote autoimmune diseases by accumulation of clonal cells, as not avoided expansion of unwanted cell lines.

Chronic antigenic stimulation is supposed to cause oligoclonal expansion of CD8+ T cells and their senescence. In this extent chronic viral infections, as long term cytomegalovirus (CMV) infection, provide an additional source of risk. In fact, it has been shown that CMV drives CD8+ T cell differentiation, inducing premature immunosenescence and leading to significant changes in the CD8+ T cell repertoire. This condition represents a potential basis for the imbalance in the cytokine production profile in elderly people. Moreover, life-long latent CMV infection seems to diminish the size of both naive and early memory T cell pool and to promote a cytokine polarization within the immune system, leading to a reduced diversity of the CD8+ responses and to chronic inflammatory processes.

Typical late-onset diseases, manifestations of self-tolerance loss, include rheumatoid arthritis (RA), Hashimoto’s thyroiditis, pulmonary fibrosis and Sjogren’s syndrome. The pathophysiological mechanism of these diseases involves decreased apoptosis and increased oligoclonal activation of T cells, due to a defect in the activation-induced cell death.

In RA, T cell immunosenescence occurs prematurely, probably due to deficiency in the ability to generate sufficient numbers of novel T cells. Autoimmunity in RA has been proposed as a consequence of immunodegeneration associated with age-inappropriate remodeling of the T cell pool and loss of telomers observable in pa-
tients with very early disease and remaining unaffected by progressive disease, suggesting that it is not an epiphenomenon over the course of the disease.

**Recognition and Clearance**

The recognition and clearance of apoptotic cells by phagocytes involves multiple components on the surface of the apoptotic cell and the phagocytic cell, as well as a variety of ‘bridging’ molecules frequently derived from plasma constituents. These bridging molecules, including milk fat globule EGF-factor 8 (MFGE8), annexin I, protein S, Gas6, and others, bind to the apoptotic cell surface, thus enabling recognition by the phosphatidylserine (PS) receptor and other phagocytic receptors on macrophages and DCs.

Early studies demonstrated that PS was an important surface determinant on apoptotic cells, and its exposure on the apoptotic cell surface permitted recognition, phagocytosis and tolerance-inducing clearance. Botto et al. demonstrated that interaction, between phagocytic cells and apoptotic cell surface plays a central role in mediating non-inflammatory clearance, tolerance induction, and autoimmunity prevention. In fact, they first observed, in vitro and in vivo, that C1q deficiency (which is strongly associated with development of SLE in humans and mice) was associated with diminished clearance of apoptotic cells.

Furthermore, Kim et al. extended these findings by demonstrating that C1q binding to the apoptotic cell surface is, at least in part, IgM dependent, with IgM binding to the lysophospholipid form of phosphatidylcholine, which is generated by the activity of the caspase-activated phospholipase-A2.

Hanayama et al. reported that MFG-E8, an RGD-containing protein secreted by activated macrophages, binds to apoptotic cells in a PS-dependent manner. Cells transfected with the avb3 integrin phagocyte apoptotic cells in the presence, but not in the absence, of MFG-E8. Moreover, MFG-E8 knockout mice developed a lupus-like autoimmune phenotype, including splenomegaly, anti-double-stranded DNA antibodies, and immune-complex-mediated glomerulonephritis. In this animal model, the macrophages retained the ability to bind, but not internalize, apoptotic cells: a phenotype similar to that observed by Baumann et al. in lymphonodes from patients with SLE. It has been also shown that the polymorphism glutamic acid/aspartic acid in the RGD motif plays a crucial role in MGF-E8 activity and mice injected with Asp89Glu, mutant form of MGF-E8, developed anti-cardiolipin and antinuclear antibodies, and renal immune complex deposition.

A large series of studies have also highlighted the importance of recognition pathways on the apoptotic cell surface in determining the destiny of apoptotic cell clearance, particularly with regard to autoimmunity. It has been shown the importance of the membrane-bound tyrosine kinase c-mer in the ability to clear apoptotic cells. Interestingly, failure to express this receptor was associated with development of a constellation of lupus-like features, including anti-chromatin antibodies. Its ligand protein Gas6 can also bind PS, suggesting that Gas6 may function as another bridging molecule mediating apoptotic cell clearance. Of note, protein S, another ligand for c-mer known as a circulating phospholipid-binding protein with anticoagulant properties, was found to be responsible for stimulating macrophage phagocytosis of apoptotic target cells in a Ca++ and PS dependent manner. This condition underscores the significant redundancy and interaction among the different pathways that mediate apoptotic cell clearance.

Bondanza et al. reported the potential of modulating the PS-mediated anti-inflammatory response by the soluble PS-binding protein annexin V. Addiction of soluble recombinant annexin V reversed resistance to macrophage phagocytosis of apoptotic tumor cells in vitro.

This provides an excellent example of the importance of a balanced immune response to apoptotic cells: an overly robust response is detrimental in the setting of autoimmune disease, whereas a weak response may be detrimental in the setting of cancer.

Another important contributing factor in apoptotic setting is represented by phagocytic receptors and the downstream signaling mechanisms for apoptotic cell clearance.

On macrophages and DCs there are a variety of receptor systems that are responsible for silent clearance of apoptotic cells, marked with apoptotic surface markers and bridging molecules, as discussed above. The apoptosis recognition system is characterized by both cell surface markers and/or their associated bridging molecules which may be considered as apoptosis-associated molecular patterns (AAMPs), and their cognate clear-
ance receptors known as apoptosis recognition receptors (ARRs). By a sort of analogy to the innate immune mechanisms, recognition by ARRs is relatively nonspecific and redundant, and several ARRs may recognize PS and its binding molecules on the surface of apoptotic cells, thus driving to the activation of several transduction pathways. It has been demonstrated in vitro how the binding of PS with its receptor on activated human macrophages induces expression of a novel “zinc finger”-containing transcription factor, GC-BP, which, in turn, causes a marked reduction in production of the proinflammatory cytokine IL-12, a key regulator of immunological homeostasis.

The PS receptor is implicated in direct PS-mediated signaling. Hoffmann et al. demonstrated to act through a reduction in inflammatory cytokine production and an increase in anti-inflammatory cytokine production in response to an ovalbumin antigenic challenge.

### Apoptotic Material as a Source of Autoantigens

Although abnormal apoptotic cell clearance can play a role in rendering an individual susceptible to systemic autoimmunity, it is clear that this is a highly complex and redundant system, and that a defect in apoptotic cell clearance alone is not sufficient to generate an autoimmune phenotype. Double knockout mice for a single gene implicated in clearance and scavenge of apoptotic cells failed to show associated anti-nuclear antibodies, or changes in secretion of pro- or anti-immune cytokines or proteinuria.

Besides abnormalities in clearance and apoptotic processes, material from inside apoptotic cells may be immunogenic in certain circumstances too, even if it is not clear how it must be presented to generate a response.

Chang et al. showed that apoptotic thymocytes generate increased levels of lysophosphatidylcholine and other oxidized lipids, and that immunization with these cells in mice leads to production of IgM and antibodies recognizing oxidized lipids. If adjuvant is included, anti-oxidized lipid IgG antibodies are also produced. Mice immunized with apoptotic cells plus adjuvant have increased production of both IFN-γ and IL-10, and endothelial cells exposed to apoptotic cells in vitro exhibit increased IL-8 production and enhanced capacity for monocyte adhesion.

The apparent immunogenicity of these oxidized epitopes highlights the importance of clearance mechanisms to prevent the priming of an autoreactive immune response.

Leadbetter et al. showed that chromatin-IgG complexes could activate B cells through ligation of both the B cell antigen receptor and TLR9. In this study the authors showed that lupus-prone mice lacking TLR9 do not produce antinuclear and anti-dsDNA antibodies, thus providing strong support to the notion that TLR co-ligation of immune complexes can facilitate anti-self lymphocyte activation. TLR9 is a pattern recognition receptor for prokaryotic CpG DNA, but apparently host chromatin-IgG complexes contain sufficient hypomethylated CpG sequences to activate the receptor. It is not yet clear whether TLR-mediated binding/phagocytosis of apoptotic material elicits downstream effects distinct from those generated by non-self ligands.

Several studies are consistent with the idea of apoptotic cells as important natural resources of both antigen and anti-immune “self” contextual signals, thereby actively inducing tolerance to frequently encountered intracellular antigens.

Although incomplete tolerance induction might be one prominent mechanism underlying susceptibility to autoimmunity, it is also possible that unique “unconventional” forms of apoptotic death can occur, by the generation of autoantigenic not previously tolerated, and thus capable of initiating a primary immune response.

One form of death that appears to be particularly relevant in this regard is cytotoxic lymphocyte granule-induced death, which targets virally infected or transformed cells, and in which induction of tolerance towards contained antigens is unlikely to be a primary outcome.

Of note, in systemic autoimmune diseases autoantigens are specifically cleaved by granzyme B and other granule proteases during granule-induced death, generating unique fragments that are not encountered in other forms of homeostatic cell death.

Blanco et al. strongly suggests that this pathway is activated in patients with active SLE, because during flares SLE patients have increased frequency of granzyme B+ activated CD8+ lymphocytes, which produce large amounts of soluble nucleosomes, and granzyme-B-specific fragments of the autoantigen U1-70K. Furthermore, sera from SLE patients stimulate the production
of DCs that drive production of the granzyme B+/CD8+ lymphocytes, thereby potentially establishing a positive feedback loop.

Moreover granzyme B pathway is similarly activated in patients with RA, where it correlates with severe and erosive disease\textsuperscript{90}. However, RA-specific autoantigens cleaved by granzyme B remain to be defined.

Understanding whether modifications of autoantigens occur in a tissue-specific manner is therefore an important aim as it could clarify the mechanism underlying the development of generalized autoimmune phenomena, arising from a local and tissue-specific reactions, as well as why some conditions remain circumscribed.

**LE Phenomenon**

Considering the association between apoptosis and autoimmunity induction, it is not to be forgotten the LE phenomenon, that probably had represented the first demonstration of apoptosis in vivo.

In fact, in the 1948 the American clinical haematologists Malcolm Hargraves and Robert Morton, working with laboratory technician Helen Richmond, were the first to describe an unusual phenomenon that they called LE cell\textsuperscript{91-94}. They had observed two particular conditions in several bone marrow preparations, which they termed “tart cell” and “LE cell”: the huge significance of their observations was unclear to them at the time. The former had been observed in most of the histological preparations they had examined, but in increased numbers in certain patients, such as those with lymphoblastoma and metastatic carcinoma. An apparent secondary nucleus (and a third in some cases) was seen in histiocytes, and occasionally in eosinophils and polymorphs. The appearance of part of this structure outside the cell suggested that it may have been an abortive nucleus in the process of being extruded, and they later termed the phenomenon “nucleophagocytosis”.

In the “LE cell”, nuclei are phagocytosed by mature polymorphonuclear leucocytes and digested.

“Tart cells” are monocytes and occasionally polymorphonuclear leucocytes with one or two round inclusions in their protoplasm owing to the phagocytosis of leukocyte nuclei. In contrast with “LE cells”, these inclusions are not homogeneous containing chromatin and nuclear membrane material and often having a dark ring of hyperchromic material.

Hargraves et al had two hypotheses for the appearances they observed: i) that there was phagocytosis of free nuclear material, leading to the development of homogeneous round vacuoles containing lysed nuclear material; ii) there was autolysis of one or more lobes of the polymorph nucleus.

The vacuolated area containing partially digested nuclear material resembled that of the “tart cell”, but due to the variability in the appearance of chromatin in the “LE cell”, the authors were able to distinguish them clearly.

Interestingly, they also observed that once the nuclear material had been phagocytised, surrounding polymorphs rapidly moved away, suggesting that the chemotactic attractiveness of the material had been lost.

The LE cell was so termed because of its exclusive presence in the bone marrow of 25 patients with confirmed or suspected SLE\textsuperscript{93,95}, but a more conclusive link with SLE came after that the “LE cell” phenomenon was observed in the marrow of several patients with classical SLE\textsuperscript{92}.

“LE cells” were not usually found in peripheral blood, although Sundberg and Lick\textsuperscript{96} observed in 1949 that the “LE cell” phenomenon could form in the buffy coat of peripheral blood, after a period of incubation. “LE cells” have since been found in synovial fluid, cerebrospinal fluid and pericardial/pleural effusions from SLE patients\textsuperscript{97-101}.

In 1949, Haserick and Bortz\textsuperscript{102} addressed the important question of whether the “LE cell” phenomenon was a primary cytological alteration or secondary to a constituent of the plasma of these patients. They added plasma from patients with SLE to bone marrow preparations from normal subjects and compared the results with control preparations from the same subjects. Plasma from patients with SLE induced the “LE cell” phenomenon in these marrows, with the formation of clumps of polymorphs around amorphous masses of nuclear material. The highest number of “LE cells” developed when plasma from the sickest patient was used. Furthermore, plasma from a patient with discoid lupus failed to induce the phenomenon. Thus, the formation of LE cells appeared to be secondary to a plasmatic factor of SLE patients\textsuperscript{102}. Haserick and Sundberg emphasized the value of bone marrow examination in the diagnosis of SLE, and the “LE cell” phenomenon was considered the most specific test available for the diagnosis of SLE, supporting the autoimmune theory for its pathogenesis.
even before the nature of this factor in the plasma became apparent\textsuperscript{103}. A number of workers eventually discovered the ability of the “LE factor” to bind to nuclei and ribonucleoprotein. Now we know that the autoantibodies that lead to the “LE cell” phenomenon bind histones, consecutively to a defective clearance of apoptotic material\textsuperscript{104}.

**Apoptotic Mechanisms Lead to Tissue Specific Autoimmune Disorders**

To date is well known from early studies that apoptosis plays a key role in the homeostasis of the immune system, as a mean of lymphocyte repertoires selection both in primary lymphoid organs and in the periphery\textsuperscript{105-108}. Autoimmune diseases are characterized by aberrations in the repertoire of lymphocytes, and distortions in the apoptotic process may, therefore, be implicated in AD pathogenesis.

Apoptosis, necrosis and the different modalities leading to cell death, together to infective or neoplastic processes can trigger autoimmunity with the local release of antigenic materials, which in turn may initiate a non-selective inflammatory reaction with autoantibodies production\textsuperscript{109}.

Characteristics of the self antigen, recognised by the immune system, can determine the localization of pathogenic phenomena, but the exact mechanisms allowing antigenic spreading and generalization have to be defined. However, an initial systemic autoimmune movement, sustained by acquired or genetic alteration of the immune balance, can circumstantiate in a specific organ\textsuperscript{110}.

In this context, two main physiological models can be reported: the nucleolar protein B23, a tumor autoantigen, frequently detected in human hepatocellular carcinoma (HCC), and the pathogenesis of murine autoimmune diabetes.

Ulanet et al\textsuperscript{111} found that, although B23 in normal tissue is not a substrate for granzyme B, while in HCC liver is efficiently and specifically cleaved by granzyme B, generating novel fragments. A conformation-specific antibody demonstrated that in HCC tumors B23 exists in a different conformation in tumor tissue and has different oligomerization properties when compared to B23 in healthy tissue. These data suggest that many ubiquitously expressed autoantigens may acquire novel conformations in specific microenvironments, including alterations in splicing, post-translational modifications, folding, oligomerization and complex formation, and that these conformations, under certain circumstances, may initiate autoimmunity.

In light of the well known relation between genotype aberrations and autoimmunity development\textsuperscript{19-24,63,112}, Colucci et al\textsuperscript{113} investigated the possible association between apoptosis-related genes knock out and the induction of autoimmune diabetes in Non Obese Diabetic (NOD) mice, testing the hypothesis that impairments in the apoptosis mechanisms can have a role in the disease development.

The lymphocytes of NOD mice have been early described as resistant to both spontaneous and induced apoptosis\textsuperscript{114,115} and the distal part of chromosome 6 has been shown to map a gene for resistance to dexamethasone induced apoptosis in immature CD4+/CD8+ thymocytes\textsuperscript{116}.

Apoptosis resistance of NOD lymphocytes was also observed in tetraparental chimeras (NOD×B6), an experimental system in which cells of distinct genetic origin coexist in the same individual. Thus, the resistance of NOD lymphocytes to spontaneous apoptosis is likely to reflect an intrinsic cellular property rather than a feature conferred by systemic factors. Interestingly, the diabetogenic effect of cyclophosphamide, drug known for its immunodepressive effect related to lymphocyte apoptosis induction, is reversed in these mice\textsuperscript{117}. Its diabetogenic effect has to be ascribed to an action on chimeric lymphoid cells, thus to an immune system alteration rather than to abnormalities in metabolic pathways. Moreover, it has been demonstrated that splenocytes and bone marrow stem cells from diabetic chimeras can adoptively transfer the disease to NOD rag2\textsuperscript{−/−} mice\textsuperscript{118}.

Furthermore, the induction of the disease in the chimeras is analogous to the preferential depletion in lymphoid cells of B6 origin. Thus, cyclophosphamide constitutes an apoptosis signal to peripheral lymphocytes, but NOD immune system including B cells, as well as both CD4+ and CD8+ T cells, display resistance to cyclophosphamide induced apoptosis\textsuperscript{114}. From these observations in this experimental model of type 1 diabetes emerges the idea that the selection and maintenance of the immune repertoires could be conditioned by impairments in the apoptotic pathways, which in turn can contribute to the pathogenesis of the disease.

The apoptosis dysfunction reported here may have implications for the T cell selection in the periphery and raises the possibility that the NOD
mouse presents a reduced ability to delete potential self-reactive cells. In several different circumstances the lymphocytes of the NOD mouse show an abnormal resistance to apoptosis processes. The genetic basis of this resistance is now being determined and it may concern beside the B6 locus mapping the resistance to dexamethazone-induced apoptosis in immature NOD thymocytes, other loci potentially acting as cofactors to some of these apoptosis subphenotypes116.

**Apoptotic Mechanisms Lead to Systemic Autoimmune Diseases**

Recognition and removal by phagocytes is the final common event of the death programs. Apoptotic and necrotic cells are strong candidates as sources of autoantigens that drive the autoantibody response in autoimmune diseases. The fast and efficient uptake of dying cells is of main importance to prevent contact of the immune system with intracellular autoantigens119. Several authors reported that defects in the clearance of dying cells may be crucial in the etiopathogenesis of SLE: increased apoptosis or impaired clearance of apoptotic material has been the most investigated events in human SLE61,79,109,120-122.

The early recognition and incorporation (“engulfment”) of apoptotic cells is necessary to avoid that cells enter late stages of apoptosis. During these late stages cells can often not maintain their plasma membrane integrity, releasing intracellular contents, and reaching the secondary necrotic state. Deficiencies in molecules, implicated in this part of the apoptotic process, may promote the development of lupus: these may be divided into two categories: i) digested and masked autoantigens, such as DNaseI and Serum Amyloid P (SAP)123,124; ii) promoting the clearance of dying cells, such as C1q and IgM125,126. A reduced activity and levels of DNaseI in serum127, and complement defects128 has been described in SLE patients.

Apoptotic cells trigger complement and their impaired uptake by human macrophages has been early observed in human serum depleted of specific complement components (C1q and C3)129,130. Complement and DNaseI play a pivotal role in the clearance process of dying cells and subcellular fragments: the complement binding represents an early event in the case of primary necrotic cells and serves as opsonisation molecules for the recognition of late apoptotic cells131,132. Furthermore, C1q and DNaseI cooperate in the degradation of chromatin from necrotic cells. The complement component C1q was found to be necessary for an effective uptake of degraded chromatin by monocyte-derived phagocytes132. A cooperation of DNaseI with the plasminogen system was also suggested to contribute to a fast and effective breakdown of chromatin during necrosis133.

Sera of SLE patients showed a reduction in DNaseI activity with impaired capability to degrade necrotic-cell-derived chromatin and, most interestingly, SLE sera showed a strongly reduced degradation capacity of necrotic-cell-derived chromatin in comparison to rheumatoid arthritis and normal sera134-136. Thus, impaired cleared apoptotic cells become secondary necrotic, resulting in the release of antigenic material and of a variety of “danger” signals, which contribute to inflammation and consecutively to the initiation of an autoimmune reaction136.

These impaired clearance functions for dying cells may explain the accumulation of apoptotic and subsequently necrotic cells in various tissues of SLE patients66,137,138. During necrotic as well as apoptotic cell death, autoantigens are cleaved or otherwise modified88,139. These changes may expose “new” antigenic fragments (cryptic epitopes) to the immune system identification140-142. Dendritic cells may then acquire modified autoantigens, like apoptotic nuclei and chromatin, and consequently start an immune reaction.

In fact, in a subgroup of SLE patients it has been shown that apoptotic cells were accumulated in the germinal centers of the lymph nodes66. Notably, the apoptotic material was observed in the exterior follicular dendritic cells (FDC) and the numbers of macrophages, usually containing engulfed apoptotic nuclei, was significantly reduced. These findings were the first observation in humans of the accumulation of free apoptotic cells in germinal centres of the lymph nodes over the course of SLE. Moreover, it was disclosed that, a deficient cellular debris clearance in germinal centres of lymph nodes may be a pivotal event in the etiopathogenesis of autoimmunity in SLE66. Further in vivo demonstrations revealed that differentiated macrophages from SLE patients stem cells presented a reduced uptake ability and survival143, indicating that the defective clearance of dying cells in SLE patients could be an intrinsic defect.

Interestingly, plasma of SLE patients has been demonstrated to contains larger amounts of circulating DNA than the plasma of healthy
individuals in early studies. This circulating DNA is present in the form of oligonucleosomal DNA fragments (180–200 bp) complexed with histones (dsDNA and histones). In murine models of SLE, it was previously demonstrated that the initial immune response is directed against nucleosomes, as a large family of antinucleosome antibodies, and spreads later to its individual components dsDNA and histones. 

Co-incubation of autologous apoptotic material with freshly isolated peripheral blood mononuclear cells (PBMC) led to the expansion of histone-specific T-cell clones which could provide help to B-cells for the formation of dsDNA antibodies and nucleosome-specific T-cells were detected in SLE patients as well. However, increased serum levels of nucleosomes is not a SLE specific characteristic with dialysis and sepsis being other conditions associated with this finding. Thus, other factors, as the previously mentioned Dnase1 activity, could be claimed as a contributor in the complex SLE pathogenesis. In this extent, it has been recently pointed out the importance of not only Dnase I concentration but also of its accessibility to DNA. It has been previously reported that phosphatidylserine (PS) and phosphatidylcholine PC interact with chromatin and that PS containing multilamellar vesicles can induce unfolding of the nucleosome core, thereby sensitizing chromatin to Dnase I and affecting gene expression, while PC has an opposite effect on Dnase I accessibility. Moreover, phospholipids strongly bind all histones, in particular H2A, contributing to the surface exposure of histones/nucleosomes and to the generation of histone-containing apoptotic blebs in the membrane that may in turn foster autoimmunity towards nuclear compounds. Thereby, autoimmunity could be triggered not only in the early phases but even in the late stage of apoptosis, in which phospholipids might be involved in the ordered release of apoptotic nuclear material from dying cells. In pathological conditions with impaired clearance such as SLE, phospholipid bound histones may increase the immunogenicity of nuclear autoantigens.

Moreover SLE patients often have elevated levels of IFN-α, and there has been considerable recent interest in “IFN signature” gene expression patterns in SLE. Lovgren et al. showed that IgG purified from SLE patients, when combined with apoptotic cells, stimulate peripheral blood mononuclear cells to produce IFN-α, IFN-α induction requires an intact Fc portion, and is inhibited by normal IgG. This effect appears to be mediated through FcγRIIa receptors on plasmacytoid DCs, the cells primarily responsible for IFN-α, secretion. Further studies showed that necrotic cells and SLE IgG have the same effect, and that nucleic acids are required for the secretion of IFN-α, suggesting that the IFN-inducing activity of the dying cells may be nucleoprotein particles, which combine with autoreactive SLE IgG to produce stimulatory immune complexes.

Thus altered mechanisms for the clearance of dying cells represent a central pathogenic process in the development and acceleration of autoimmune diseases, like SLE. Clearance deficiencies in SLE are in part intrinsic and heterogeneous since phagocytes from different SLE patients showed in part different phagocytic defects. Non controlled scavage of apoptotic material triggers normal immune responses which in turn result in the production of inflammatory mediators eventually leading to initiation or worsening of the disease.

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