Novel hints on the pathogenesis of lupus from *in vivo* models

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Considerable evidence supports the role of a deregulated clearance of dying cells in the pathogenesis of lupus. The dissection of this event in mouse models has provided insight into the origin and persistence of the autoantibodies, which represent a hallmark of the disease, and other processes critical for chronic inflammation and tissue damage. The comparison with animals that do not develop autoimmunity has also led to the identification of specific events in the pathway to lupus. Recent advances have provided evidence for the feasibility of rational therapeutic procedures, aimed at preventing immune-mediated damage and restoring tissue homeostasis.

Keywords: antinuclear antibodies, apoptosis, dendritic cells, immune adjuvants, nucleosomes, phagocytosis, systemic autoimmunity, systemic lupus erythematosus

future medicine

Immune outcomes of the clearance of apoptotic cells

The death of cells in the midst of tissues represents a challenge for the immune system. Indeed, antigen-presenting cells at the steady state engulf and process dying cells. Apoptotic cell antigens enter the major histocompatibility complex (MHC) class I pathway of the phagocyte via the cytosol and become available for recognition by MHC-restricted T lymphocytes. Dendritic cells (DCs) are the most potent antigen-presenting cells, specialized in eliciting both the productive activation (priming) and the tolerization of antigen-specific T cells. DCs are unusually efficient in processing dying cells for presentation to T lymphocytes. This applies to MHC class I- and class II-restricted T cells. Opsonins, similarly to autoantibodies, bind to dying cells or complement factors, possibly favoring the latter event at the site of clearance. DCs that phagocytosed virus-infected apoptotic cells productively activate (cross prime) virus-specific T cells. Macrophages, which release factors that prevent the maturation and function of DC, abrogate the cross priming of T cells by DCs [1]. In line with these seminal observations, immune silencing is the default outcome of the presentation of apoptotic cell antigens by DCs at the steady state [1,2].

This model takes into account the response to cells that die as a consequence of infection by intracellular pathogens. The cross presentation of antigens expressed by cells dying in peripheral tissues by tolerogenic DCs would allow the immune system to concentrate on the pathogen, avoiding initiation of immune responses against cell antigens that are presented along with the pathogen [1].

The deregulated responses to dying cells could play an initiating or perpetuating role in pathological settings. Systemic rheumatological diseases have been extensively studied, since autoantigens generated or selectively modified during apoptotic cell death are preferential targets [3–13].

Apoptotic cell clearance & systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a complex disease with contributions from multiple genes. Autoantibodies recognizing intracellular components, in particular nucleosomes and spliceosome constituents, are a hallmark of SLE. Autoantigens are heterogeneous in terms of function, topographical distribution and structure. However, heterogeneity abates when autoantigens are traced in cells dying via apoptosis. They cluster and concentrate in the blebs of the plasma membranes, are preferentially cleaved and undergo an array of apoptosis-related nonproteolytic modifications. These observations have focused attention on an apoptotic setting as the potential initiating stimulus for systemic autoimmunity. Specific apoptotic stimuli, in particular those related to recognition by cytotoxic lymphocytes and to granzyme B, would represent an optimal trigger for the generation of autoantigens that would be targeted preferentially in predisposed subjects [10].

Uncleared apoptotic cells and their byproducts, nucleosomes, accumulate in a fraction of SLE patients. Conversely, macrophages from SLE patients fail to clear autologous apoptotic material *in vitro* [14]. Tangible body macrophages, which control apoptotic cell clearance in the germinal centers, were strongly reduced in

some SLE patients, and antigens from apoptotic cells associated with the membrane of follicular DC in the lymph nodes, possibly facilitating the activation of autoreactive lymphocytes [15]. The molecular basis of the clearance defect is still poorly characterized. Inflammatory factors involved in apoptotic cell clearance *in vivo*, such as pentraxins and complement factors, are defective in SLE patients and representative mouse models. This defect, either directly or through the mediation of natural autoantibodies, could possibly influence the clearance of cell debris [11,16,17].

Mouse models mimicking persistent defective clearance of apoptotic cells

A defect in the clearance of dying cells may, therefore, play a role in the pathogenesis of SLE. This role could be important; for example, nucleosomes, which are only generated during apoptosis, are the major immunogens in SLE [5]. However, cells die continuously and autoimmune diseases are exceptional in contrast. This apparent paradox has led to the establishment of models linking deregulated clearance of apoptotic cells to the development of sustained inflammatory and autoimmune diseases.

The removal of dying cells requires the cells to expose appropriate signals and the scavenging phagocyte being able to respond appropriately to those signals. It is becoming clear that diverse molecules are involved in this event; they subserve a variety of functions, the importance of which may be dependent on the phase of the apoptosis program, the microenvironment and the lineage of the apoptotic cell and phagocyte. Currently, the engulfment process is viewed as involving:

- Initial sensing and recognition of apoptotic cells (involving molecules such as CD31)
- Firm tethering of apoptotic cells to phagocytes (which appears to be the role of, for example, macrophage CD14)
- Signaling for phagocytosis and anti-inflammatory mediator production (e.g., by $\alpha_v$integrins) [18]

Animals defective in phagocytic partners have been bred and analyzed for the accumulation of apoptotic cells on one hand and for the development of autoantibodies and immune-mediated tissue damage on the other. The deletion of single phagocytic partners does not always result in detectable accumulation of cell debris *in vivo*. For example, mice with a genetic deletion of the class A scavenger receptor, thrombospondin 1 or the β2-glycoprotein I plasma cofactor, cleared apoptotic cells *in vivo* with normal efficiency. However, the whole array of phagocytic receptors expressed exclusively by macrophages is redundant; embryos null for the transcription factor PU 1, which do not have macrophages, still remove apoptotic cells, relying on 'amateur' mesenchymal neighbor cells [16,19].

The expression of members of the tyrosine-based activation motif (TAM) receptors, a family of receptor tyrosine kinases, and in particular of the Mer kinases, prevents apoptotic cell accumulation and cell degeneration in peripheral tissues with clear-cut evidence of autoimmunity [20]. Other molecules play a non-redundant role in tissues where apoptosis occurs cyclically; possibly the best characterized is the milk fat globule endothelial growth factor (EGF) 8 (MGF-E8). Defective apoptotic cell clearance in MGF-E8$^{-/-}$ mice results in persistent accumulation of apoptotic cells at the periphery and in the lymph nodes, with the development of a lupus-like syndrome. Moreover, defective clearance of apoptotic cells, as well as of residual milk and milk fat in the involuting mammary glands, occurs, resulting in mammary duct ectasia with mastitis [21]. Other models reveal a key role of inflammatory molecules: apoptotic glomerular cells accumulate in the kidneys of C1q-deficient animals, which develop features of systemic autoimmunity and an immune-mediated glomerulonephritis. A substantial tissue specificity exists: C1q-deficient mice dispose of apoptotic keratinocytes efficiently after ultraviolet exposure and do not develop autoantibodies. The genetic background influences the outcome of apoptotic cell clearance in targeted mice. The defect could be corrected in mice developing autoimmune features by the transplantation of hematopoietic stem cells [22]. The data on C1q are particularly intriguing, since hereditary deficiencies of components of the classical pathways of complement activation are associated with SLE in both humans and mice, and a C1q defect is associated with the most prevalent and clinically expressive autoimmune disease [23].

Apoptotic cell membranes undergo dramatic changes, which include the exposure of altered phospholipids, such as lysophosphotidylcholine, which are recognized by natural immunoglobulin (Ig)M antibodies. Complement activation by IgM antibodies on apoptotic cells *in vivo*

was essential for C3 deposition on apoptotic cells and their uptake by peritoneal macrophages. This indicates that natural antibodies and their complement are possibly involved together in the safe clearance of cell corpses and in the prevention of autoimmunity [11,17].

Intracellular molecules, besides inducible soluble factors, apparently play a nonredundant role in the safe clearance of apoptotic cells. Mice fail to clear apoptotic cells in the absence of tissue transglutaminase Type II and develop splenomegaly, autoantibodies and glomerulonephritis [24]. This phenotype depends critically on the cytokine response elicited by apoptotic cells; uncleared apoptotic cells, although bound to the phagocytes, are not internalized and persist. Their byproducts skew the response toward the generation of proinflammatory factors [25]. Features of the DNA from apoptotic cells that escaped phagocytosis could be involved. This DNA potentially differs from the DNA that is physiologically derived from the processing of apoptotic cells, since it escaped the processing by the macrophage DNase II, whose expression is restricted to the lysosomes. Accordingly, uncleared apoptotic cell DNA has a direct proinflammatory effect, which is independent of Toll-like receptor-induced gene expression [26]. The possible role of nuclear-associated moieties endowed with adjuvant functions, such as the high-mobility group box (HMGB)-1 factors, deserves careful dissection [27,28].

Mannose-binding lectin (MBL) null mice fail to effectively clear dying cells, but do not spontaneously develop lymphoproliferation, autoimmunity or germinal center expansion [29]. A similar situation occurs in mice lacking the CD14 macrophage receptor [30]. Apoptotic cells can, therefore, persist in the absence of proinflammatory consequences. The conservation of the compensatory anti-inflammatory mechanisms elicited as a consequence of apoptotic cell recognition is probably an important factor limiting autoimmunity [31]. Tolerance or autoimmunity can both derive from apoptotic cell recognition depending on environmental factors, most of which are not yet completely characterized. For example, long-lasting protection immunity against apoptotic cell antigens *in vivo* occurs after *in vivo* depletion of regulatory T cells. Therefore, active mechanisms are recruited to control the autoimmune response induced by cross presentation [32]. The identification of the events responsible in $CD14^{-/-}$ and $MBL^{-/-}$ animal models for the maintenance of immune homeostasis is likely to prove rewarding.

Mouse models mimicking acute accumulation of apoptotic cells

Genetic models mimic a persistent defect in the clearance of dying cells. As a consequence, cells that die during normal turnover accumulate in peripheral tissues. However, the molecular machinery involved in apoptotic cell clearance is highly redundant; hence, back-up systems make up for isolated defects (see previously). Noxious effects of deregulated clearance could therefore be revealed by critical conditions only. Photosensitivity is associated with the massive death of keratinocytes in numbers that overwhelm the clearance ability of local scavenger phagocytes. This event could in turn lead to, or facilitate, the induction or relapse of clinical autoimmunity, even if the physiological death of keratinocytes at the steady state is uneventful. Massive apoptosis also occurs during acute microbial infections, which are clearly epidemiologically associated with relapses of the diseases [33].

The injection of high numbers of apoptotic cells mimics an acute overload of the clearance system *in vivo*. Alternatively, antigen-presenting cells can be propagated from bone marrow-derived precursors, functionally and phenotypically characterized *in vitro*, challenged or not with dying cells and injected into syngeneic animals. Different genetic backgrounds can be studied with relative ease. Normal laboratory mouse strains can provide information relevant to the outcome of swift accumulation of dying cells in the peripheral tissues of healthy subjects. Mouse models of lupus are also available. These animals spontaneously develop elevated antinuclear antibody (ANA) levels with variable degrees of lupus-like kidney involvement. Studies of these models have provided interesting insight into the pathogenesis of SLE. For example, the hybrids of New Zealand black (NZB) and white (NZW) mice (NZB × NZW F_1 animals) are among the best characterized strains of spontaneous SLE. The challenge of young NZB × NZW F_1 animals with dying cells, well before the development of any detectable autoimmune feature, could provide hints on the specific events preceeding massive cell death in autoimmune patients.

Injection of 10^6 syngeneic apoptotic thymocytes in itself did not result in the induction of autoantibodies or detectable immune-mediated

clinical disease [34]. This result suggests that antigens contained in apoptotic cells must be complemented with additional helper signals (so called adjuvants) to become immunogenic. Adjuvants, which are used widely for vaccine development, facilitate the trafficking of inflammatory leukocytes at the site of antigen injection. Moreover, they activate antigen-presenting cells, facilitating their migration to secondary lymphoid organs and enhancing their ability to prime antigen-specific naïve T cells. Indeed, a total of 10^6 dying cells in the presence of adjuvants efficiently induced antinuclear and antinucleosome autoantibodies and caused an accelerated and lethal renal disease [34].

Interestingly, high numbers of dying cells were both immunogenic and pathogenic in autoimmune-prone NZB × NZW F_1 [34] and MRL/MpJ-Fas(lpr) mice [35]. This suggests that dying cells that escape phagocytic clearance by scavenger cells provide appropriate accessory signals to immune cells. As discussed previously, DNAse II, a lysosomal enzyme substantially expressed in scavenger phagocytes, such as macrophages, is necessary to quench the ability of the DNA of dying cells to activate innate immune responses [26]. In the absence of an appropriate phagocytic program, this potential is intact and could contribute to sustaining inflammation and autoimmunity. Damaged cells leak abundant intracellular constituents in the environment. Some of them, such as adenosine-5'-triphosphate (ATP), uric acid, the product of the purine catabolism responsible for gouty arthritis or nonhistonic nuclear proteins, such as HMGB1, behave as primary proinflammatory signals in the extracellular milieu, directly activating antigen-presenting cells. Relocated intracellular molecules possibly represent a more sensitive signal of ongoing cytopathic infection than actual microbial components [1,36,37].

HMGB1 released in the extracellular environment orchestrates a complex homeostatic response. It attracts vessel-associated stem cells into damaged tissue allowing their passage through endothelia, thus promoting tissue repair and immune activation. The last effect is mediated through the adaptation of the ability of DCs to mature and present soluble and corpuscolate antigens *in vitro* and *in vivo* [28,38]. Accordingly, low numbers of apoptotic cells, which *per se* did not elicit any immune effect, became immunogenic when complemented with recombinant HMGB1 or with HMGB1 released by dying cells, formally proving its role in the extracellular environment as an immune adjuvant [27].

Besides DNA and nuclear protein, membrane moieties modified during apoptosis play a role in the inflammatory responses elicited by apoptotic cells. This has been elegantly demonstrated for biologically active phospholipids, which are spontaneously generated during programmed cell death; oxidized lipids contribute to their immunogenic and proinflammatory potential when apoptotic cells are injected into normal mice [39]. Annexin V, an anionic phospholipid-binding protein that limits coagulation and fibrin deposition, behaves as a potent signal increasing the immunogenicity of apoptotic cells. This effect is possibly achieved via interference with the immunosuppressive clearance of apoptotic cells driven by the recognition of exposed phosphatidylserine [40].

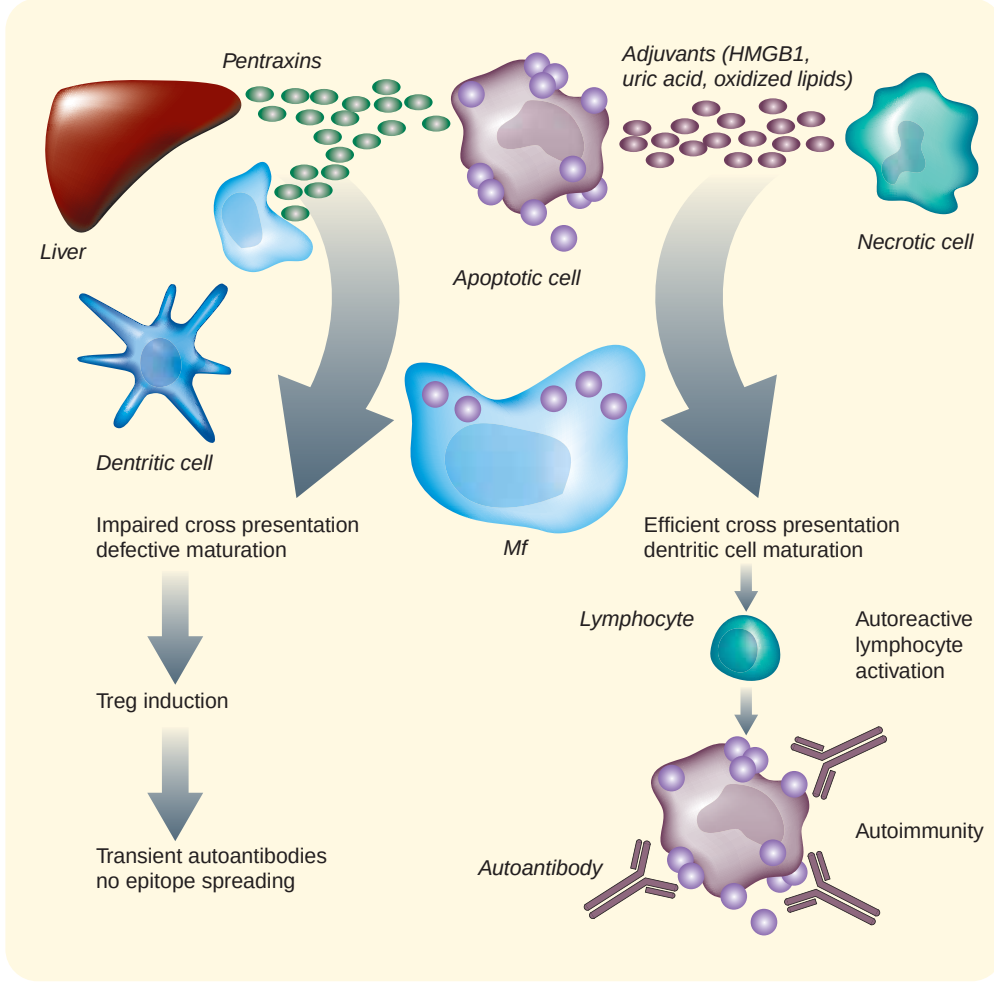
Genetic contributions to disease maintenance

The autoantibodies induced upon immunization with dying cells share several characteristics with spontaneous autoantibodies, including the ability to recognize intracellular antigens with high affinity. Isotype switching, somatic hypermutation and affinity maturation are under the control of activated, autoreactive T cells, which in turn depend on antigen-presenting cells [41]. To directly address the role of cross-presenting DCs in the induction of autoimmunity, independent laboratories investigated the outcome of the direct immunization of syngeneic DCs *in vivo*. DCs from normal BALB/c mice or NZB × NZW F_1 upon phagocytosis of apoptotic thymocytes were sufficient to induce the development of antinuclear and anti-dsDNA Abs [42]. The immunogenic potential of DCs in normal mice was strictly dependent on their maturation state [43]. Immunization of nonautoimmunity-prone mice failed to induce clinical or histological features of autoimmunity or tissue involvement [34,42,43]. This suggests that the anti-inflammatory or direct regulatory circuits normally limit the effects of cross presentation (see previously and [32]). The lack of clinical effect is associated, as expected, with the lack of intra- and intermolecular epitope spreading and with the progressive disappearance of the autoantibodies [44]. By contrast, autoimmunity was maintained in susceptible mice that died precociously as a consequence of renal failure [42].

The events that quench, in normal subjects, an established autoimmune response, preventing the development of a full-blown clinical disease, are not yet completely understood. Tissue-generated factors, such as pentraxin (PTX)3, which is generated under the control of interleukin (IL)-1β and tumor necrosis factor (TNF)-α in peripheral tissues, or acute phase proteins produced in the liver under the control of pro-inflammatory signals, such as C-reactive protein (CRP) [45,46], could be involved.

A defect in the production of CRP during systemic inflammation has been known for a long time in SLE patients. NZB × NZW F_1 mice, which are exquisitely sensitive to apoptotic cell-induced autoimmunity (see previously), fail to produce the murine equivalent of the CRP, the serum amyloid P (SAP) component. Conversely, CRP actively protects the kidney from experimental immune-mediated tissue damage [47,48]. CRP, PTX3 and SAP bind to apoptotic cells and play nonoverlapping roles in controlling their perception by scavenger and antigen-presenting phagocytes [45,49]. PTX3 is a flexible adaptor of DC function, regulating the maturation program and the secretion of soluble factors. PTX3 is enriched at the phagocytic synapse between dying cells and DCs and actively restricts the cross presentation of self-, viral- and tumor-associated model antigens expressed to

Figure 1. Apoptotic cell accumulation (defective clearance, overload).



The clearance of dying cells has different outcomes *in vivo*. The active induction of tolerance, or the default induction of persistent autoimmunity, depends on the balance between endogenous and exogenous factors that promote the activation and function of antigen-presenting phagocytes (adjuvants) or that restrict apoptotic cell uptake and cross presentation, including tissue-generated pentraxins.
HMGB: High-mobility group box; Mf: Macrophage; Treg: Regulatory T cell.

T cells, possibly contributing to limiting tissue damage under inflammatory conditions and the activation of autoreactive T cells [49].

Gaseous messengers are also involved in the pathogenesis of systemic autoimmune diseases. This issue has been studied in detail in an independent mouse model of SLE, in which the exaggerated production of nitric oxide (NO) correlates with renal involvement. The defective control of the inducible NO synthase (iNOS) inflammatory pathway is a potential cause of chronically enhanced NO production [50]. Defects in the function or expression of the peroxisome proliferation activated receptor (PPAR)-γ are possibly involved and may result in unrestrained activation of the iNOS synthase in involved kidneys. Modulation of these events could be involved in the beneficial effects of mycophenolate mofetil, a drug whose efficacy in SLE nephritis is well established [50]. Of interest, NO-mediated kidney involvement is independent of autoantibody generation or immune complex deposition, suggesting that NO is involved mainly in maintenance and amplifying the immune-mediated tissue damage [51]. However, NO possibly plays a more complex role in inflamed tissues. Long-term exposure to low concentrations of NO induces mitochondrial biogenesis. This process is mediated by the activation of soluble guanylate cyclase and involves increased expression of PPARγ, nuclear respiratory factor 1 and mitochondrial transcription factor A, and is important for cell and tissue metabolism, as demonstrated by studies in mice deficient in endothelial NO synthase (eNOS) [52].

Interestingly, persistent mitochondrial hyperpolarization and ATP depletion, associated with enhanced mitochondrial mass, are hallmarks of the T lymphocytes of SLE patients [53]. These data agree well with the observation of a key role of NO-induced, cyclic guanosine 3',5'-monophosphate-mediated events in the control of diverse leukocyte programs, including activation-dependent apoptosis and DC maturation and function [52,54–56].

Executive summary

Immune outcomes of the clearance of apoptotic cells

- Cells die continuously during development and normal tissue turnover. Usually, this event is not associated with bystander inflammation or productive autoimmunity. Induction of tolerance by innate cells involved in the clearance of dying cells, in particular by antigen-presenting dendritic cells, actively prevents these events.

Apoptotic cell clearance & systemic lupus erythematosus

- A failure in the clearance of apoptotic cells has been studied carefully in systemic lupus erythematosus (SLE) models, and shapes both the autoantigen repertoire of SLE patients and the diverse features of the disease, including clinical relapses in particular.

Mouse models mimicking persistent defective clearance of apoptotic cells

- Important efforts in the last decade have led to the establishment of informative models linking deregulated clearance of apoptotic cells to the development of sustained autoimmune diseases. They include mice deficient in the Mer kinase, the soluble factor milk fat globule endothelial growth factor 8, fractions of the classical cascade of complement activation, in particular C1q, for the tissue transglutaminase Type II.
- Interestingly, two models have been described in which the deficiency of molecules relevant for the correct clearance, such as the mannose-binding lectin and the CD14 macrophage receptor, is not associated with autoimmunity, suggesting that these two molecules are not required for immune silencing against apoptotic cell constituents.

Mouse models mimicking acute accumulation of apoptotic cells

- The injection of high numbers of apoptotic cells or of antigen-presenting cells loaded with apoptotic cells mimics an acute overload of the clearance system *in vivo*.
- The preimmune hybrids of New Zealand black and white mice challenged with dying cells antigens developed autoantibodies and a lethal accelerated disease only if appropriate adjuvants were provided.
- Endogenous molecules that fulfill the requirements for immune adjuvants include high-mobility group box 1, uric acid and oxidized lipid moieties.

Genetic contributions to disease maintenance

- Strain-specific factors control the outcome of cross presentation *in vivo*. Nonautoimmune-prone mice, although producing autoantibodies, did not undergo clinical or histological features of autoimmunity or tissue involvement; the outcome was strictly dependent on their maturation state.
- The lack of clinical effect was associated with the lack of intra- and inter-molecular epitope spreading and with the progressive disappearance of the autoantibodies. The molecules involved in restricting autoimmunity and immune-mediated tissue damage are poorly characterized; acute phase proteins, in particular pentraxins, are promising candidates.

Conclusions

Before the full implication of deregulated clearance *in vivo* of dying cells for autoimmunity can be appreciated, a much more sophisticated understanding of the systems involved in any given single patient, and of their relative hierarchy, is needed. However, the original clues that deregulated clearance of apoptotic cells and autoimmunity are actually linked have now been supported convincingly in diverse informative *in vivo* models (Figure 1). The efforts to characterize at the molecular level the checkpoints regulating the outcome of apoptotic cell clearance will be facilitated by the availability of these models.

Future perspective

The therapy of autoimmune diseases still depends largely on nonspecific immunosuppression, with important side effects and questionable efficacy. The identification of certain molecular events that 1) promote the initiation of clinical autoimmunity, 2) are required for the maintenance of autoimmunity and 3) actively limit immune-mediated tissue damage, will provide novel tools for effective and less toxic targeted intervention in patients with SLE. The study of pentraxins on the one hand, and immune adjuvants on the other, seems to be particularly promising.

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