REVIEW

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Apoptosis and systemic autoimmunity: the dendritic cell connection

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SUMMARY

Much effort has been devoted in recent years to the events linking recognition and disposal of apoptotic cells to sustained immunity towards the antigens they contain. Programmed death *via* apoptosis indeed provides most of the raw material the immune system exploits to establish self tolerance, *i.e.* to learn how to distinguish between self constituents and foreign antigens, belonging to invading pathogens. In parallel, events occurring during cell death may enable a restricted array of molecules endowed with diverse structure, function and intracellular distribution to satisfy the requirement to evoke and maintain autoimmune responses. Dendritic cells (DCs), the most potent antigen presenting cells, appear to play a crucial role. Here we will discuss some of the constrains regulating the access of dying cells' antigens to DCs, as well as censorship mechanisms that prevent their maturation and the full explication of their antigen presenting function.

Apoptotic cells provide a preferential source of antigens recognised by auto-antibodies and autoreactive T cells

Few intracellular molecules become targets of autoimmune responses. The requirements remain elusive, and autoantigens have little in common in terms of function, sub-cellular localisation and structural features. Heterogeneity becomes less puzzling if auto-antigens are traced into cells undergoing apoptosis. Most autoantigens abandon their original nuclear or cytosolic localisation and segregate to membrane blebs or to rims surrounding the condensed chromatin into apoptotic blebs (Casciola-Rosen et al., 1994) and membrane blebbing is a dramatic – although in some cases dispensable (Bonanno et al., 2000) - event during programmed cell death. The plasma membrane selectively associates to plasma cofactors upon exposure of anionic phospholipids (Price et al., 1996, Manfredi et al., 1998a, 1998b), which are recognised by specific antibodies. Nucleosomes, the immunogen leading to the break of tolerance towards double-stranded DNA and histone components of the chromatin, are generated during apoptosis as a consequence of Ca++/Mg++ dependent nuclease activation (Amoura et al., 1999). The early modification of snRNA (Degen et al., 2000) and the rearrangement of small nuclear ribonuclear autoantigens in structures that are extruded from apoptotic cells may preserve their availability for internalisation by antigen present-

Abbreviations used in this paper: DCs, dendritic cells; APC, antigen presenting cells; β2-GPI β2-glycoprotein I; anti-β2-GPI, anti-β2-glycoprotein I antibodies; aPL, antiphospholipid antibodies; PS, phosphatidyl serine; SLE, systemic lupus erythemato-

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ing phagocytes, enhancing their immunogenicity (discussed in Biggiogera and Pellicciari, 2000; Pellicciari et al., 2000). Figure 1 depicts the redistribution of some cell-associated autoantigens during apoptosis. Furthermore, novel epitopes generated by serine kinases and phosphatases during apoptosis are selectively recognised in autoimmune patient (see Rovere et al., 2000a, 2000b). Granzyme B is released by the granules of cytotoxic lymphocytes and contributes to the induction of apoptosis in the target cell. Granzyme B selectively modifies autoantigens: this strongly implicates the cytotoxic cell mediated apoptosis in the initiation of autoimmune responses (Casciola-Rosen et al., 1999). Cytotoxic T cells mostly kill cells infected by viruses or other intracellular pathogens: the "assisted suicide" of infected cells by cytotoxic T cells may represent a preferential source of autoantigens.

Apoptotic cells and apoptotic cell associated antigens accumulate in autoimmune patients

I*n vivo*, the unbalance between the number of dead cells and the availability of functional scavenger phagocytes results in the persistence of dying cells or in the accumulation of the material they release. A defective clearance of apoptotic cells in patients with systemic autoimmune diseases, and in particular in patients with Systemic Lupus Erythematosus (SLE) has been convincingly proposed (Herrmann et al., 1998). Accordingly, uncleared dying leukocytes accumulate in the blood and in solid tissue parenchymas of autoimmune patients (discussed in Rovere *et al.*, 2000a). Besides the ghosts of dead cells, material generated during apoptosis and released as a consequence of the membrane disruption of unscavenged cells becomes detectable in the sera of SLE patients. This is the case of nucleosomes, which are generated during apoptosis by caspase-dependent nucleases, and that account for almost all the circulating DNA in SLE patients (Rumore and Steinman, 1990).

Apoptotic cells that overcome the phagocytic clearance are immunogenic

The evidence discussed above suggests that: i) apoptotic cells represent privileged reservoirs of autoantigens and that ii) apoptotic cells preferentially accumulate in autoimmune patients. The two events may be linked: apoptotic cells that escape early phagocytosis by scavenger cells, or the antigens they release, may preferentially elicit autoimmunity. Accordingly, the massive apoptosis of tissue cells *in vivo*, which overwhelms the tissue scavenging ability, prompts immune responses towards previously ignored antigens (Kurts et al., 1998). Furthermore, the administration of apoptotic cells to normal mice determines the gen-

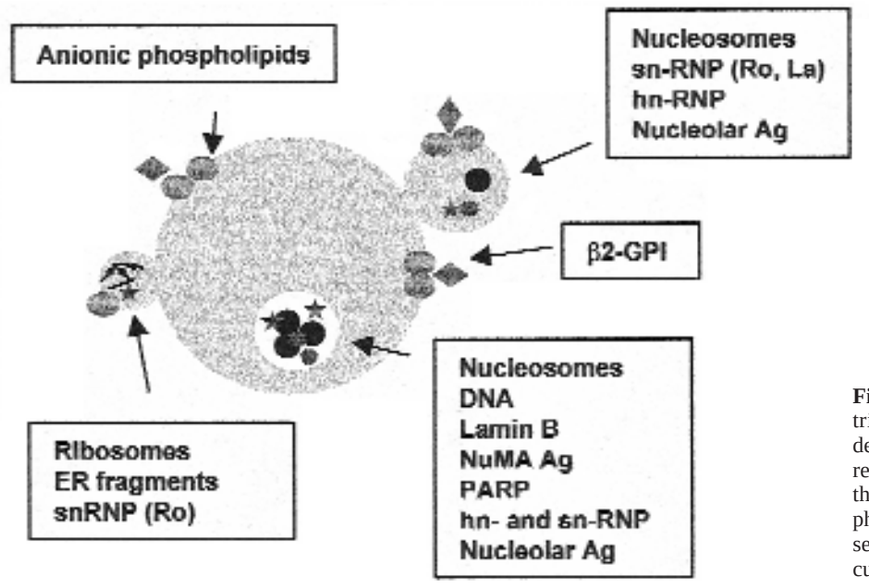

Fig. 1 - Autoantigens are redistributed during programmed death. Heterogeneous antigens redistribute in apoptotic blebs that become available for phagocytosis by antigen presenting cells. See text for discussion.

eration of antinuclear and antiphospholipid antibodies, hallmark of the prototypic autoimmune disease SLE (Mevorach et al., 1998; see below). Autoantibody production requires numbers of apoptotic cells sufficient to overcome the normal clearance pathways and is transient, suggesting the existence of censorship mechanisms (Rovere et al., 2000a and see below).

The dendritic cell phagocytic system is responsible for the immunogenicity of unscavenged apoptotic cells

The clearance of dying cells involves scavenger macrophages or neighboring amateur phagocytes (Ren and Savill, 1998; Dini, 2000). These phagocytes are devoid of the ability to initiate immune responses. Accordingly, macrophages that phagocytosed apoptotic cells fail to elicit immune responses when administered to experimental animals (Ronchetti *et al.*, 1999). In contrast, bone marrow-derived myeloid DCs are potent antigen presenting cells, able to initiate immune responses *in vivo*. DCs originate from CD34+ progenitors (discussed in Banchereau *et al.*, 2000). DC precursors enter the blood, reach peripheral organs where they develop to immature DCs. Immature DCs are capable to capture soluble antigens *via* macropinocytosis and particulate substrates, including microbes and cells dying by apoptosis or necrosis, through phagocytosis (Inaba *et al.*, 1998).

To initiate immune responses, DCs residing in non-lymphoid tissues need to mature (Banchereau and Steinman, 1998). Differentiating DCs progressively lose the ability to capture antigens. In parallel, they upregulate the ability to process internalized antigens and the molecular machinery involved in T cell activation and co-stimulation. DC mature when challenged with primary pro-inflammatory signals including bacterial components, CD40 engagement and pro-inflammatory cytokines, like TNFα and IL-1β (Banchereau and Steinman, 1998; Lane and Brocker, 1999; Banchereau *et al.*, 2000).

Maturing DCs rearrange the actin-based cytoskeleton (Winzler *et al.*, 1997) and following chemotactic signals selectively migrate to T cell areas of lymphoid organs. DCs that present antigens to T cells receive signals that prevent their apoptosis in the lymph node and complete the differentiation process. In the absence of signals delivered by T cells, DCs die by apoptosis (Winzler *et al.*, 1997).

Immature DCs capture in physiologic condition apoptotic cells at the periphery and traffic to lymphoid organs. Indirect evidence for apoptotic cell processing by DCs comes from the study by Kurts and collaborators (1998) that showed that massive apoptosis *in vivo* elicits tissue-specific autoimmunity and that this event requires the presence of bone marrow derived antigen presenting cells. The issue has been formally proved in a recent elegant study: DCs that phagocytose apoptotic gut epithelium cells constitutively shuttle their antigens to T cell areas of draining lymph nodes (Huang *et al.*, 2000).

Few scavenger cells involved in the phagocytosis of apoptotic cells are antigen presenting cells. So far only a subset of murine peritoneal macrophages and both human and murine immature DCs have been shown to cross-activate MHC-restricted T lymphocytes specific for their intracellular antigens (Bellone *et al.* 1997; Albert *et al.*, 1998a, 1998b, Rovere *et al.*, 1998, Inaba *et al.*, 1998, Rovere *et al.*, 1999a, Russo *et al.*, 2000, Sauter *et al.*, 2000). DCs triggered sustained immunity to intracellular antigens belonging to the apoptotic cells they phagocytose (Ronchetti *et al.*, 1999).

The outcome of cross-presentation of antigens from dying cells by DCs must be tightly regulated *in vivo*, since cells die continuously during development and normal tissue turnover. DCs are somehow less proficient at phagocytosing apoptotic cells than professional phagocytes (Rovere *et al.*, 1998b) and less represented than amateur phagocytes. Therefore DCs may have limited access to dying cells *in vivo*. This could contribute to explain the higher immunogenicity of apoptotic cells in SLE patients, whose phagocytes are unable to completely clear dying cells. This event may favor the access of apoptotic cells to DCs (Hermann *et al.*, 1998). Besides being more accessible, dying cells that escaped early clearance had a chance to undergo late apoptosis *in vivo*. This may endow them with a higher ability to elicit the maturation of DCs, and to evoke autoimmunity *in vivo*.

The maturation elicited by products derived from dying cells may impinge on the outcome of DC presentation, favoring autoimmunity

Immature DCs challenged with an excess of lymphoma cells undergoing post-apoptotic necrosis *in vitro* secrete IL1β and TNF-α (Rovere *et al.*,1998a

and 1999a). These signals prompt their own maturation and antigen presenting function in an autocrine and paracrine fashion. Pro-inflammatory signals, including IL1β and TNF-α, promote the migration DCs from the periphery towards draining lymph nodes (Roake *et al.*, 1995). Unscavenged dying cells may favor the recruitment of immature DCs.

The signals that dying cells deliver that promote the autocrine release of maturative factors are still elusive. However, recent studies provide several hints:

i) Low numbers of dying cells, which are swiftly engulfed *in vitro*, fail to promote DC maturation (Rovere *et al.*, 1998a, 1999a, Gallucci *et al.*, 1999; Sauter *et al.*, 2000).

ii) Early apoptotic cells fail to promote DC maturation. Apoptotic cells must largely overwhelm DCs, and be allowed to undergo extensive disruption of plasma and intracellular membranes.

iii) Cells killed by primary necrosis promote DC maturation. The event is therefore independent of caspase activation (Gallucci *et al.*, 1999; Sauter *et al.*, 2000 and our unpublished results). Maturation requires repeated cycles of freezing and thawing (one cycle in hypotonic solution, that breaks plasma membranes of most cells, is insufficient - Sauter *et al.*, 2000 and our unpublished results).

iv) Once released, signal(s) are soluble (Sauter *et al.*, 2000 and our unpublished results).

Different laboratories are devoting much energy to the study of these "pre-packaged signals": soluble molecule(s), stored in intracellular compartments in living cells, released only after extensive disruption of intracellular membranes and able to elicit DC maturation. It is likely that a molecular identification will be achieved in the next future.

Factors that associate to the membrane of apoptotic cells that escaped phagocytosis may control immunogenicity by apoptotic cells

C1q is the first component of the classical pathway of complement activation. It interacts with immunoglobulins and other moieties, including membranes of apoptotic cells. The deficiency of human C1q almost invariably associates with SLE. In a consistent fraction of C1q deficient mice apoptotic cells accumulate in renal parenchyma and antinuclear antibodies develop (Botto et al., 1998). Unscavenged apoptotic bodies accumulate as a consequence of the failure of phagocytosis dependent by C1q receptors on scavenger cells. Their availability may facilitate the release of pre-packaged signals and the access of the antigenic material to antigen presenting DCs.

During inflammation extensive cell death occurs at sites in which pro-inflammatory cytokines promote DC maturation. The control over apoptosis-elicited autoimmunity must therefore be stringent. Besides C1q, other inflammatory molecules play a role. Pentraxins are acute phase proteins characterised by cyclic pentameric structure, which are highly conserved during the phylogenesis. Short, classical, pentraxins, like C reactive protein (CRP) and serum amyloid P component (SAP), are produced in the liver in response to inflammatory mediators. They bind to potential autoantigens, like chromatin components or small nuclear ribonucleoproteins (Du Clos, 2000). The function of pentraxins includes amplification of innate resistance against microbes and regulation of the scavenging of DNA released from apoptotic or necrotic cell. Both SAP and CRP delay the clearance of chromatin injected in mice and prevent its deposition in the spleen and in the kidneys. Mice with targeted deletion of the SAP gene degrade chromatin in vivo more rapidly and they spontaneously develop SLE. SLE features are exacerbated after immunization with chromatin (Bickerstaff et al., 1999).

These results indicate that pentraxins play a divergent role. They control and exalt the immune responses elicited by microbial components, opsonizing pathogens and favouring the establishment of effective anti-infectious responses (Du Clos, 2000). Their selective binding to dying cells contribute to shield self components - and possibly whole dying cells (Rovere et al., submitted for publication), from uptake from antigen presenting phagocytes, contributing to censor autoimmunity. This double-edged role is shared by the CD14 receptor, which is involved in the recognition and in the pro-inflammatory response to invading microbes and in the non-phlogistic clearance of autologous dying cells (Gregory, 2000). The biochemical bases of these divergent responses need further elucidation.

The cross talk between scavenger phagocytes and antigen presenting DCs

The lack of inflammatory reaction during normal

apoptotic cell clearance (Ren and Savill, 1998) is achieved via different, probably co-operating, pathways:

i) Phagocytes swiftly engulf cells undergoing apoptosis, preventing their secondary necrosis.

ii) Phagocytes release immunoregulatory factors, which in turn quench inflammation and possibly exert an immunosuppressive role.

Immunoregulatory factors include: transforming growth factor-beta (TGF-β1, which in turn recruits prostaglandin E2 and platelet activating factor (Fadok et al., 1998a), IL-10 (Voll *et al.*, 1997) and soluble Fas-ligand (Brown and Savill, 1999). The latter event, which leads to Fas-mediated apoptosis of bystander leukocytes, may be involved in the termination of the inflammatory response *in situ*. Such factors mediate their effect in both an autocrine and justacrine fashion: macrophages challenged with a *bona fide* inflammatory signal like LPS release high amounts of pro-inflammatory cytokines, like TNF-α and IL-1β. The challenge with apoptotic cells will facilitate the release of TGF-β by LPS stimulated macrophages. TGF-β will quench the autocrine release of proinflammatory cytokines. Furthermore it will down regulate their release by phagocytes that did not engulf apoptotic cells, acting in a paracrine fashion (Fadok *et al.*, 1998b).

A similar autocrine/paracrine regulation has been proposed for IL-10 (Voll *et al.*, 1998). IL-10 quenches inflammation and interferes with T cell activation. It also inhibits the maturation of antigen presenting DCs (Allavena *et al.*, 1998) and apoptotic tumor cells are more immunogenic in mice bearing a genetically disrupted IL-10 gene (Ronchetti *et al.*, 1999). Interference with the local recruitment

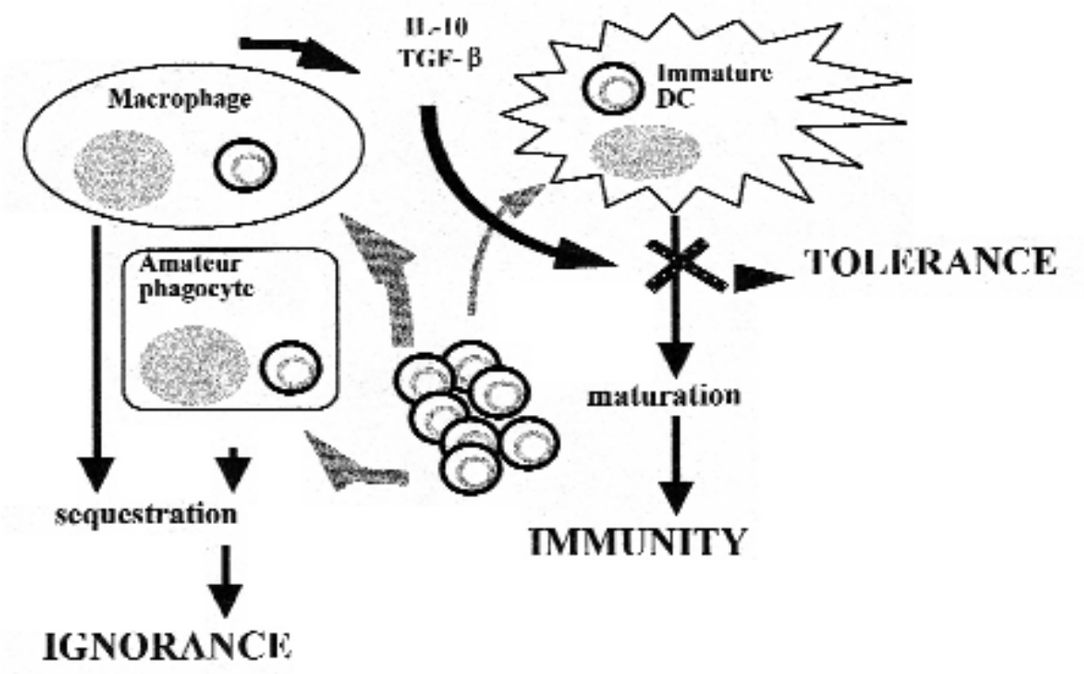

Fig. 2 - Diverse outcomes of the interaction between dying cells and phagocytes. *Ignorance*: In normal conditions, other phagocytes compete with immature DCs for the clearance of dying cells, preventing T cell activation. The antigens will be ignored. *Immunity*: immature DCs contribute to the clearance of dying cells that escaped scavenger phagocytes. In the presence of pro-inflammatory cytokines or unidentified signals released from dying cells themselves, DCs mature and migrate to draining lymphoid organs. There they activate T lymphocytes specific for antigens of the dying cell. *Tolerance*: In the absence of maturative signals, or in the presence of signals that interfere with DC maturation, DCs that phagocytosed apoptotic cells do not mature. Presentation of epitopes from internalized apoptotic cells by immature DCs blocks the activation of autoreactive T lymphocytes. Bystander macrophages internalizing apoptotic cells secrete IL-10 and TGF-β, which obstacle DC maturation and favor the induction of tolerance.

and the function of antigen presenting DCs at the site of inflammation is shared by TGF-β1, which also inhibits their migration in response to chemotactic stimuli (Ogata *et al.*, 1999).

The scheme in Figure 2 depicts a view of the interaction between scavenger and antigen presenting phagocytes. The outcome of the cross-talk among soluble factors generated and released as a consequence of the recognition of apoptotic cells may determine the outcome of the cross–presentation of apoptotic cell antigen by DCs. Antigen presentation by immature DCs blocks T cell activation, favoring tolerance versus autoimmunity.

Dying cells recognized by antibodies

The exposure of anionic phospholipids is an early feature of programmed cell death. Human autoantibodies capable to bind anionic phospholipids (aPL) have been described in patients with autoimmune or infectious diseases. Autoimmune aPL have been associated to a syndrome characterised by thrombosis, recurrent abortions, thrombocytopenia, and possible neurological involvement. The β2-GPI cofactor is required for the binding of aPL to anionic phospholipids *in vitro* (discussed in Roubey, 1996). β2-GPI has a highly positively charged sequence region that is involved in anionic phospholipid binding and in aPL recognition of the complex. β2-GPI also buffers exposed anionic charges on apoptotic cells and human aPL bind to PS stabilised β2-GPI on the surface of apoptotic cells (Price *et al.*, 1996, Manfredi *et al.*, 1998a, 1998b, Pittoni *et al.*, 2000).

Since the 1950s, Moore and collaborators stressed the notion that "false positive" serologic tests for syphilis, which are due to aPL, were associated with an increased risk for autoimmune diseases. Macrophages phagocytose aPL-bound apoptotic cells with higher efficiency and secrete substantial amounts of TNF-α (Manfredi *et al.*, 1998a, 1998b). The clearance of apoptotic cells may be skewed in patients with circulating aPL, which have persistent "recalls" of antigen (the apoptotic cell) in the presence of potential adjuvants (the opsonizing antibodies), with the generation of TNF-α *in situ.*

Immature DCs express receptors for the Fc fragment of immunoglobulins, which they use to concentrate antigens for presentation to T cells (Banchereau and Steinmann, 1998). Antibody binding favours the internalisation of apoptotic cells by immature DCs, which efficiently presented antigens derived from their intracellular processing to class II-restricted T lymphocytes and secreted TNF-α and IL-1β (Rovere *et al.*, 1999b). Circulating aPL therefore short circuit the clearance of apoptotic cells towards a dangerous outcome, leading to initiation of autoimmunity and possibly continuously re-boosting an established autoimmune response. The existence of such amplificatory loops may contribute to the spreading of autoimmunity towards cellular components.

Autoantibodies frequently develop in subjects recovering from acute infections or during chronic infectious diseases. In the vast majority of the subjects, still non identified endogenous censorship mechanisms prevent autoimmunity. A better understanding on the molecular control on these events will allow us to consider both sides of the coin, the physiologic establishment of self tolerance and the pathogenesis of systemic autoimmunity.

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