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Apoptotic Cells at the Crossroads of Tolerance and Immunity

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Abstract Clearance of apoptotic cells by phagocytes can result in either anti-inflammatory and immunosuppressive effects or prostimulatory consequences through presentation of cell-associated antigens to T cells. The differences in outcome are due to the conditions under which apoptosis is induced, the type of phagocytic cell, the nature of the receptors involved in apoptotic cell capture, and the milieu in which phagocytosis of apoptotic cells takes place. Preferential ligation of specific receptors on professional antigen-presenting cells (dendritic cells) has been proposed to induce potentially tolerogenic signals. On the other hand, dendritic cells can efficiently process and present antigens from pathogen-infected apoptotic cells to T

cells. In this review, we discuss how apoptotic cells manipulate immunity through interactions with dendritic cells.

Abbreviations

APC	Antigen-presenting cell
CR	Complement receptor
CMV	Cytomegalovirus
DC	Dendritic cell
DTH	Delayed type hypersensitivity
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HSP	Heat shock protein
HSV	Herpes simplex virus
IC	Immune complex
Ig	Immunoglobulin
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
LPC	Lysophosphatidylcholine
MHC	Major histocompatibility complex
MBL	Mannose-binding lectin
mDC	Myeloid dendritic cells
LOX-1	Oxidized low-density receptor 1
PGE_2	Prostaglandin E_2
PAF	Platelet activating factor
PS	Phosphatidylserine
PSR	Phosphatidylserine receptor
pDC	Plasmacytoid dendritic cells
SR-A	Scavenger receptor A
SLE	Systemic lupus erythematosus
TLR	Toll-like receptor
TGF	Transforming growth factor
TNF	Tumor necrosis factor

1 Introduction

Apoptosis is a physiological form of cell death occurring in normal tissue turnover, during embryogenesis, and after infection or inflammation of tissues. Uptake of apoptotic cells by surrounding phagocytes offers their safe disposal and prevents activation of bystander cells and tissue damage after the release of dying cell contents. Cells undergoing apoptosis are characterized by morphological as well as biochemical changes such as altered distribution of membrane lipids and exposure of modified carbohydrates on the plasma membrane. These changes enable rec-

ognition of apoptotic cells by specific receptors on phagocytes and their rapid uptake. Elimination of apoptotic cells is most expeditiously mediated by resident macrophages, but immature dendritic cells (DCs) also phagocytose apoptotic cells, albeit less efficiently. Additionally, nonprofessional phagocytes such as epithelial cells [206], fibroblasts [14], glial cells in the brain [29], mesangial cells in the kidney [83], or hepatocytes in the liver [43] contribute to the elimination of apoptotic cells or their derivatives, e.g., apoptotic blebs. The failure of apoptotic cells to frequently elicit clinically significant autoimmune responses is considered to be an active process whereby phagocytes are rendered immunosuppressive. In contrast, apoptotic cells, when delivered to DCs together with inflammatory signals, are an excellent source of antigen for stimulating of effector T cells. In this review we discuss the characteristics of apoptotic cell uptake by DCs and the circumstances leading to tolerance vs. immunity.

2 Apoptotic Cells and Their Receptors

The search for receptors and molecules mediating uptake of apoptotic cells began with the identification of the $\alpha_v\beta_3$ *vibronectin receptor* on macrophages [173]. Savill and colleagues later demonstrated that $\alpha_v\beta_3$ vibronectin receptor cooperates with the scavenger receptor *CD36*, and uses a bridging molecule, thrombospondin, to bind apoptotic cells [171]. Competitive binding of apoptotic cells and oxLDL by macrophages [23, 205] led to the discovery that apoptotic, but not live cells express oxidized moieties that are structurally analogous to those that enable recognition of oxLDL by scavenger receptors [30] such as scavenger receptor A (*SRA*), macrosialin or *CD68*, and oxidized low-density receptor-1 (*LOX-1*) [145, 153, 168].

The second important group of receptors consists of members recognizing exposed phosphatidylserine (PS). PS, which is usually confined to the cytoplasmic side of the plasma membrane by a phospholipid translocase, is translocated to the outer part of the membrane because of malfunction of the enzyme in cells undergoing apoptosis [217]. Receptors recognizing PS include *PSR*, which binds it directly or possibly via an intermediate molecule, annexin I [11], β_2*-GPI receptor*, the receptor tyrosine kinase *MER*, and $\alpha_v\beta_3$ vibronectin receptor [166, 180], which use the bridging proteins β_2-GPI, Gas6, and MFGE8, respectively [13, 6, 67, 89]. Recently, serum protein S was identified as yet another

ligand of PS that expedites uptake of apoptotic cells by macrophages. Protein S is partially homologous to Gas6, but its receptor on phagocytic cells remains to be identified [6].

CD14 [39] and CD91 [143] are two additional molecules of the receptor array recognizing apoptotic cells. *CD14*, also known to bind lipopolysaccharide (LPS), has not clearly been connected to a discrete molecule on apoptotic cells; however, ICAM-3, which is expressed on apoptotic cells, has been identified as one molecule recognized by CD14 [132]. Additional candidates include PS [49] and altered carbohydrates, because CD14 possesses lectinlike activity [64]. Recognition of apoptotic cells by the α_2 macroglobulin receptor (molecule CD91) is more complex. Apparently, *CD91* associates with *calreticulin* and recognizes apoptotic cells through bridging molecules: either the C1q component of the complement system or defense collagens such as mannose-binding lectin (MBL) [143], surfactant protein A and D, and bovine conglutinin [175, 215].

Association with CD91 is not the only occasion where the complement system is involved in the uptake of apoptotic cells. Two complement receptors, *CR3* and *CR4*, that belong to the β_2 integrin family and recognize complement fragment iC3b have been identified on macrophages and/or DCs. iC3b enhances the clearance of apoptotic cells by macrophages and DCs, by coating apoptotic cells [216]. The primary role of complement receptors still remains to be shown, as it is not yet clear whether they contribute significantly to the internalization of apoptotic cells or whether they assist other receptors in increased uptake, perhaps by docking apoptotic cells to phagocytes ([216] and Škoberne and Bhardwaj, unpublished data).

Finally, *ABC*, an ATP-binding cassette transporter, has been proposed to play a role in capturing apoptotic cells [123]. However, little is known about the mechanism by which this transporter mediates phagocytosis.

Apoptotic cell-phagocyte interactions are facilitated by the deactivation of CD31 (PECAM-1). CD31 is expressed on both viable and phagocytic cells and provides a mutual "repulsion" signal that normally prevents ingestion of viable cells. However, during the process of apoptosis, CD31 is modified so that it no longer mediates detachment, thereby permitting apoptotic cells to dock to scavenger receptors [26].

Recently it was shown that phagocytic cells reach the sites where extensive apoptotic cell death occurs by chemotaxis to lysophosphatidylcholine (LPC) [110], a molecule that is first exposed on the surface of apoptotic cells in a PLA_2 -dependent manner [99] and then released by apoptotic cells to function as a chemoattractant. LPC is recognized by

G2A, a recently identified receptor on phagocytes [96]. It is tempting to speculate that LPC-G2A receptor interaction may also function to take up apoptotic cells, as G2A was shown to be internalized after binding LPC [96]. Without doubt, LPC is only one of the factors that facilitate phagocyte-apoptotic cell interactions, and several more examples will become apparent in the future.

Human DCs express several of the receptors discussed above, but so far only a few have been examined for their role in the phagocytosis of apoptotic cells. Rubartelli et al. [165] showed that $\alpha_v\beta_3$ mediates engulfment of apoptotic cells, and Albert et al. [3] could achieve up to 50% inhibition of apoptotic cell uptake by DCs previously exposed to specific antibodies against the receptors $\alpha_v\beta_5$ and CD36 . DC scavenger receptor Lox-1 binds heat shock proteins (HSPs) and cross-presents antigens bound to HSPs to T cells [36]; thus this receptor may take up not only apoptotic cells but also the cell-associated debris released from necrotic cells. This dual ability of Lox-1 may partly account for the capacity of DCs to present antigens from both apoptotic and necrotic cells [106]. In macrophages, CD91 binds HSPs and mediates the cross-presentation of associated antigens, in addition to mediating the binding of apoptotic cells [16]. Indirect recognition of apoptotic cells via Fc receptors might also play a role in vivo, as IgG-opsonized tumor apoptotic cells have been shown to be a good source of antigens in a vaccination study when charged to DCs [2]. Recently, complement receptors have also been associated with recognition of apoptotic cells by DCs, although characterization of their role needs further investigation [134, 190, 216].

Mice that lack certain receptors or factors that mediate uptake of apoptotic cells such as Mer [33], PSR [117], G2A [113], C1q [203], or IgM [25, 47] exhibit profound defects in phagocytosis of apoptotic cells and develop syndromes resembling lupus (Mer, C1q, and IgM) or are rapidly fatal (PSR). Such findings are not surprising, as failure to adequately remove apoptotic cells would have serious consequences, such as secondary necrosis and consequent inflammation. On the other hand, a loss of other receptors that also contribute to removal of C1q-opsonized apoptotic cells (e.g., CD93) does not result in development of autoimmune disease [141], nor does the loss of $\alpha_v\beta_5$, $\alpha_v\beta_3$, or CD36 receptors. In fact, these mice remain capable of cross-presenting antigens encoded by apoptotic cells to T cells [21, 179]. A multiple receptor-based system ensures a fail-safe elimination of apoptotic cells, but it seems that certain receptors are essential for the prevention of autoimmunity.

3 Inhibitory Effects of Apoptotic Cells

Several mechanisms have evolved to prevent autoimmunity to self-antigens that are contained within apoptotic cells. Most self-reactive T cell responses are eliminated early in life by central tolerance. Remaining autoimmune T cells are controlled by peripheral tolerance, through deletion, anergy or by induction of regulatory CD4+ or CD8+ T cells that actively suppress self-reactive responses. In vivo, the tissue-resident antigen-presenting cells (APCs) take up the surrounding apoptotic cells during physiological cell turnover and migrate to lymph nodes where self-antigens are processed and presented to T cells [88, 130]. In the absence of inflammation, apoptotic cells may interfere with DC maturation as well as macrophage activation [172, 210]. Therefore, DCs may reach T cell areas of lymph nodes in an immature state and induce tolerance rather than immune responses [40, 82, 161, 177] (Fig. 1).

The immunosuppressive effects of apoptotic cells were first identified on macrophages, where production of several proinflammatory factors such as TNF-α, IL-1β, IL-8, GM-CSF, and thromboxane B_2 was inhibited on their ingestion [50]. In addition, ligation of PSR by apoptotic cells stimulated TGF-β1 secretion and inhibited LPS-induced TNF-α release in macrophages [51]. Binding of CR by specific antibodies or natural ligands also inhibited IL-12 and IFN-γ production by monocytes [125] or even induced IL-10 production [226]. Similarly, ligation of CD36 induced IL-10 production and inhibited TNF-α and IL-12 production in response to LPS [219].

It is interesting that pathogens often mimic apoptotic cells and take advantage of the same receptors to enter phagocytes and to promote immunosuppressive effects. For example, infectious stages of *Leishmania major* bind CR3 and suppress IL-12 production by macrophages [63, 124, 128]. Also, *Bordetella pertussis* binds CR3, induces IL-10, and inhibits IL-12 production by a macrophage cell line on exposure to LPS and IFN-γ [129].

DCs were long believed to stay immunologically inert after ingestion of apoptotic cells, but data now suggest that they also play an active role in immunosuppression [200]. In this respect, the possible involvement of complement receptors, CD36 and PSR has received the greatest attention.

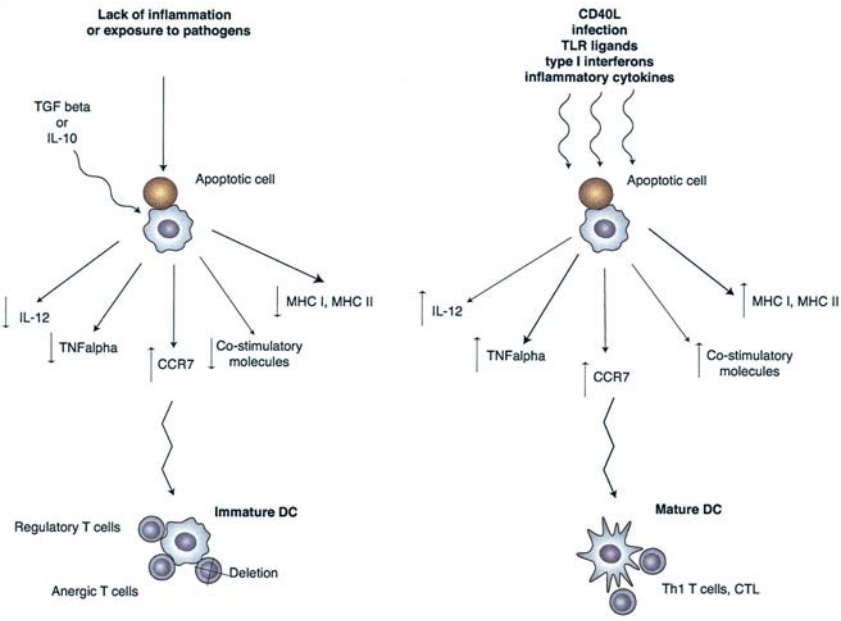

Fig. 1 Maturation state of DC influences T cell responses. In the periphery, DCs continuously capture physiologically dying apoptotic cells and process their antigens. In the absence of maturation signals, these DCs retain low expression of MHC and costimulatory molecules while they upregulate their expression of CCR7. They can then migrate to the draining lymph nodes, present antigens derived from apoptotic cells to T cells, and induce anergy, deletion, or tolerance of antigen-specific T cells. In addition, these DCs can be further skewed to a tolerizing activity by the cytokines, e.g., IL-10 or TGF-β, that are produced by neighboring macrophages that have ingested apoptotic cells, by tumor cells, or by microbes. In contrast, when DCs undergo maturation, the presentation of antigens derived from apoptotic cells results in the priming of effector T cells. Factors that contribute to full maturation of DCs include impaired clearance of apoptotic cells that is followed by secondary necrosis and release of inflammatory cell contents, presence of microbial components, inflammatory cytokines produced by infected cells or by components of preexisting immunity, such as opsonization by antibodies or activation by CD40 molecule on activated T cells

3.1 Complement Receptors CR3 and CR4

Myeloid DCs express high levels of two complement receptors, CR3 (CD11b/CD18) and CR4 (CD11c/CD18), which both recognize iC3b, a breakdown product of complement and an opsonin for apoptotic cells. Apoptotic cells activate complement via the classical pathway [101] or

the alternative pathway [126, 207]. On activation, C3 breaks into C3a and C3b. The latter is deposited on the surface of apoptotic cells and is probably rapidly converted into iC3b by serum factor H or I [126, 131, 149]. Molecules on apoptotic cells responsible for activation of complement have not been identified, but exposure of PS and deposition of C3b fragments have been positively correlated [133]. In addition, IgM antibodies that activate the classic pathway of complement, bind LPC, a recently defined "find and eat me" marker of apoptotic cells (The "find and eat me" concept is nicely reviewed in [111]). In support of the role of IgM antibodies in noninflammatory removal of apoptotic cells, $IgM^{-/-}$ mice were found to develop SLE-like disorders [25, 47].

Recently, ligation of the complement receptors CR3 and CR4 has been implicated in the induction of DCs with regulatory properties [190, 216]. Verbovetski et al. used serum-deprived apoptotic Jurkat cells opsonized with complement fragment iC3b to show that engagement of CR3 and CR4 prevents DC maturation on stimulation with either LPS or CD40L. Interestingly, despite the inhibition of upregulation of various maturation markers, complement-treated DCs still heightened the expression of CCR7 (a receptor for the chemokines CCL19 and 21 expressed in the lymph node), so conceivably these DCs could migrate and induce tolerogenic T cells in the secondary lymphoid organs [216].

Further confirmation of the importance of CR in DC modulation comes from animal experimental models. Morelli et al. [134] showed that iC3b fragments exert immunomodulary functions in vivo. They demonstrated that in mice splenic marginal zone DCs use complement receptors CR3 and CR4 to phagocytose circulating apoptotic cells. Interaction of apoptotic cells with these complement receptors reduces the production of inflammatory cytokines such as IL-1α, IL-1β, and TNF-α but does not interfere with production of TGF-β1.

Sohn and colleagues used a rat model to show complement involvement in systemic tolerance induced in an immunoprivileged site. OVA-loaded APC pretreated with either polymeric iC3b or iC3b-opsonized erythrocytes induced suppression of DTH to OVA when injected into rats. These observations were supported by in vitro studies demonstrating that after exposure to iC3b-opsonized erythrocytes, OVA-loaded APCs produced TGF-β2 and IL-10. However, the authors used peritoneal exudate cells that represent a nonhomogeneous population as APCs [190].

Altogether, these studies provide support for the hypothesis that engagement of complement receptors CR3 and CR4 interferes with DCs

maturation. Furthermore, they imply that such DCs could be involved in tolerance induction.

3.2 The Scavenger Receptor CD36

Plasmodium falciparum is a parasite causing malaria in humans. During its intraerythrocytic stages it expresses the protein PfEMP-1 in erythrocyte membranes that mediates binding to host cells [138]. All variants of PfEMP-1 analyzed so far bind the scavenger receptor CD36 [15]. *P. falciparum*-infected erythrocytes bind CD36 directly and the CD51/α_v integrin chain indirectly (via thrombospondin) and consequently inhibit LPS-induced expression of maturation markers on human DCs [209, 211]. Engagement of these receptors with antibodies inhibited DC maturation [210], and therefore CD36 and $\alpha_v\beta_3$ or $\alpha_v\beta_5$ are among candidates for receptors with immunosuppressive activity. Interestingly, it was recently shown that $CD8^+$ T cell responses are impaired during malaria infection [142], although the mechanisms leading to this impairment have not been identified.

3.3 The Phosphatidylserine Receptor

Although not yet shown in DCs, in macrophages the recognition of PS by PSR is crucial for a switch to an anti-inflammatory response [75] and also to resolve an already established inflammation through production of TGF-β, PGE_2, and PAF and inhibition of IL-12 production [85]. In addition to its immunosuppressive role, PSR is better known for mediating phagocytosis of apoptotic cells via a "tether-tickle mechanism." This theory proposes usage of different sets of receptors for docking the apoptotic cell to the phagocyte ("tether" signal) or providing a "tickle" signal resulting in phagocytosis of apoptotic cells. The "tickle" signal is provided by PSR as simultaneous binding of PSR converts adhesion into ingestion even by receptors that are normally not involved in phagocytosis [79]. Ligation of PSR is necessary for activation of Rac1 and Cdc42, stimulation of Arp2/3 complex-dependent actin polymerization, and phagosome formation [79]. In contrast to activated macrophages, immature DCs express Cdc42 and are constitutively macropinocytic and phagocytic [61]. As mature DCs downregulate expression of PSR, it is unlikely that PSR plays a "tickling" role [191] and receptors such as $\alpha_v\beta_5$ integrin may be self-sufficient for phagosome formation [5].

4 Cross-Presentation

Apoptotic cells were first appreciated not for their role in tolerance induction, but as a source of antigen in priming of T cell immune responses. $CD8^+$ T cells generally recognize major histocompatibility complex (MHC) class I molecules presenting peptides of 8–10 amino acids. These cells are crucial for immune responses to tumors and to pathogens such as viruses and intracellular bacteria and are primed when naive T cells encounter professional APC bearing an antigenic peptide. Two mechanisms are responsible for loading of peptides onto MHC class I molecules: the first is the endogenous or classic pathway in which the antigens are derived from proteins produced or existing in the cytosol of an APC. The second mechanism, also referred to as "cross-presentation," involves an exogenous pathway in which antigens are derived from exogenous sources (such as apoptotic cells) that have been taken up by the APC. The term "cross-priming" discriminates the priming of $CD8^+$ T cell responses to antigen derived from such sources. Cross-presentation when associated with maturation of DCs results in priming of effector $CD4^+$ and $CD8^+$ T cell responses (cross-priming) but when associated with immature or partially mature DCs can lead to "cross-tolerance" (reviewed in [20, 136, 137]).

4.1 Cell Types Responsible for Cross-Presentation

In contrast to macrophages that can cross-present but cannot cross-prime, DCs are efficient at both cross-presentation and cross-priming [27, 102, 158]. Cell types responsible for cross-presentation in vivo have been extensively studied in mouse models. DCs were always the prime candidate and their role was formally confirmed in the studies by den Haan et al. [37], where the authors showed that the $CD8^+$ subset of DCs presents cell-associated antigens to cytotoxic T cells. Further confirmation comes from Jung et al. [95], who have shown that by short-term depletion of CD11c+ DCs in mice, they could abrogate cross-priming of $CD8^+$ T cells . However, classification of DCs in mice is complex and not directly comparable to humans. In humans fewer DC subsets are known so far [the predominant are myeloid DCs (mDCs), plasmacytoid DCs (pDCs) and the Langerhans cells], whereas in mice at least six subsets of DCs have been characterized on the basis of their phenotype [72, 77, 155]. These murine DC subsets display differential and complex capaci-

ties to cross-present antigens derived from cellular sources vs. immune complexes (ICs). For example, $CD8^+$ DCs but not $CD8^-$ DCs cross-present antigens from ovalbumin (OVA)-loaded spleen cells to $CD8^+$ T cells [38]. Furthermore, $CD8^+$ DCs also present soluble OVA in vivo to $CD8^+$ T cells but $CD8^-$ DCs preferentially present soluble OVA to $CD4^+$ T cells [38]. However, the efficiency of cross-presentation of cell-associated antigens is several hundredfold greater than cross-presentation of soluble antigen [116]. Finally, both $CD8^+$ and $CD8^-$ DCs cross-present immune complexes (ICs) to $CD8^+$ T cells. However, only the $CD8^-$ DCs cross-present ICs to $CD4^+$ T cells. The loss of all three FcRs leads to loss of presentation to $CD8^+$ T cells by $CD8^-$ DCs but there is no effect on $CD8^+$ DCs—perhaps because these DCs may acquire ICs via complement fixation [38]. Another intriguing and open question is whether antigen acquisition in the peripheral tissues and transport to the draining lymph node can be segregated from the presentation of antigen to T cells. In support, recent studies by Belz et al. suggest that in addition to DCs that have migrated from the periphery, the lymph node-resident, non migrating DCs are responsible for antigen presentation after transfer of antigen from the migrating DCs [19].

Less is known about the role of pDCs in cross-presentation. Although pDCs are generally more appreciated for the production of type I interferons, studies by Jung et al [95] cannot exclude their role in cross-priming (in mice CD11c is expressed by the pDC and the mDC). Interestingly, pDCs lack (mouse) or express low levels (human) of CR3—one of the primary receptors identified so far that negatively influences DC maturation on apoptotic cell internalization [65, 77, 134, 190, 216]. However, pDCs might be less specialized for endocytosis compared to mDCs as cord blood and blood pDC are not very efficient in uptake of proteins [56, 193] and studies of tonsil or blood human pDC show that they are not very efficient in endocytosis of latex or dextran beads [65, 199]. They may be better in the transfer of membranes from live, necrotic, or apoptotic targets, although still less efficient compared to mDCs [199]. Moreover, the profile of expression of cathepsins in human mDC and pDCs might suggest that they differ in their capacity to process and present antigens [54]. Indeed, murine pDCs are less potent than mDCs in assembly of viral peptide-MHC class II complexes after in vivo or in vitro exposure to virus [104]. In addition, after intravenous injection into female mice, both CpG-matured male pDCs and mDCs were able to induce direct CTL priming against the male-specific transplantation antigen. However, contrary to mDCs, CpG-matured male pDC prepulsed with soluble OVA injected into female animals failed to cross-prime

OVA-specific $CD8^+$ T cells or prime OVA-specific $CD4^+$ T cells [167]. Further studies are required to determine whether pDCs have a direct role in cross-priming or whether they promote cross-presentation and cross-priming ability of "bystander" mDC through production of type I IFN.

4.2 Mechanism of Cross-Presentation

Presentation of exogenous antigens onto MHC class I by APCs occurs by at least two distinct mechanisms [8, 103, 116, 140, 159]. Antigens derived from various sources, e.g., soluble proteins, ICs, and protein-coated latex beads, can be conveyed from the endocytic compartment into the cytosol of APCs. In the cytosol, antigens are degraded into oligopeptides and are transported via the transporter associated with antigen processing (TAP) into the endoplasmatic reticulum (ER) for loading onto newly synthesized MHC class I molecules [57, 68, 103, 116, 140, 152, 159, 160]. In addition, cell-associated viral antigens also appear to require TAP for cross-presentation [57, 186]. Alternatively, antigens can be processed in endosomal compartments where peptides are generated with participation of cathepsin S, loaded on recycling MHC class I molecules, and transported to the cell surface for presentation [68, 152, 182]. This pathway is TAP-independent and is used by soluble proteins, multibranched lysine with attached peptides, and, under some conditions, proteins chaperoned by HSPs [8, 103, 148].

Recently, it was proposed that the process facilitating cross-presentation is carried out in a special compartment, an early ER-derived phagosome [1] that has the characteristics of both the ER and the phagosomes. After endocytosis exogenous antigens are transported from this compartment into the cytosol, possibly via the Sec61 complex, where degradation by proteasomes occurs [1, 80]. The peptides produced by proteasomes are then transported back via TAP complex into the lumen of this distinctive compartment, where antigens are finally loaded on MHC class I molecules [66, 80]. The presence of MHC class I molecules in this compartment can be explained by a target signal that they bear in their cytoplasmic domain, which directs them to endosomal compartments [120]. Conceivably, peptides generated in the cytosol through this mechanism could also access MHC class I peptides in the ER. This novel mechanism has been studied in the cross-presentation of antigen-coated latex beads by mouse DCs, and involvement of a similar process in cross-presentation of dying cells by human DCs is plausible [57].

4.3 Sources of Antigen for Cross-Presentation

In vitro, several forms of antigens can access the exogenous pathway for MHC class I presentation (reviewed in [106]), including remainders of dying cells such as apoptotic cells, apoptotic bodies or necrotic cells, HSPs, DNA- or RNA-encoded antigens, organisms, e.g., bacteria, viruses, viruslike particles, exosomes, immune complexes, and soluble proteins [135, 224] and even "bits" of live cells which "are nibbled off" by phagocytes [158, 69, 70].

Whereas in vitro data clearly show that apoptotic cells are an important source of antigens [3, 105, 107] fewer data are available regarding their role in vivo. Huang et al. [82] have shown that mouse DC subsets can acquire apoptotic cells in the intestine and transport the ingested material to mesenteric lymph nodes. Bevan's group [37] showed that the $CD8^+$ subset of murine DCs can ingest OVA-loaded cells and prime OVA-specific T cells. Although not shown, these cells were presumably dying. Iyoda et al. [90] have confirmed the above studies and state that the majority of the cells ingested by DCs were apoptotic before injection. Further confirmation for cross-presentation of apoptotic cells by DCs comes from Hugues et al. [84]. In their studies, induction of apoptosis of pancreatic β-cells led to the access of islet antigens to $CD11c^+$ and $CD11b^+$ DCs and induced the development of regulatory $CD4^+$ T cells [84]. Studies by Scheinecker et al. [177] do not address the viability of cross-presented cells but clearly show that cell-associated antigens are taken up and processed by $CD11c^+$ DCs that in turn migrate to draining lymph nodes where antigens are recognized by specific T cells. The identity of the cellular fraction that has the predominant role in cross-priming of cell-associated antigens (whether these are HSP-associated proteins, native proteins, peptides, or some other component) has only recently begun to be explored. Some answers have come from the group of Shen and Rock [183], who show that the fully processed peptides are not a major source for cross-presentation but that rather native proteins released from the dying cells are taken up by the surrounding APCs. Partially supporting these observations Norbury et al. [139] demonstrated that proteasomal substrates are the prime form of antigens transferred from donor to recipient cells. However, the contribution of HSP-peptide complexes should not be excluded on the basis of these results.

5
Cross-Priming Versus Cross-Tolerance

Under noninflammatory conditions apoptotic cells serve as a source of antigen for DCs; however, DCs fail to upregulate MHC and costimulatory molecules [118, 177] but upregulate the chemokine receptor CCR7 associated with migration to lymph nodes [216]. After uptake of apoptotic cells in the steady state, DCs reach lymph nodes in an immature state and are presumably responsible for induction of "cross-tolerance." The DCs most probably express some degree of costimulation to sufficiently activate T cells but not to generate long-term effector and memory cells [71, 118, 197]. When steady-state conditions are altered, e.g., during infection with pathogens, DCs can undergo activation, present antigens from dying cells, and prime effector T cells instead. We discuss below cross-priming to microbial antigens during infection and factors that might be involved in the switch from tolerance to immunity.

5.1
Cross-Priming to Microbial Antigens

It is unlikely that all microbes are able to infect professional APCs. In addition, microbial immune escape mechanisms involve abrogation of presentation on MHC class I molecules but nevertheless efficient long-term immunity is established, as in the case of cytomegalovirus (CMV) or herpes simplex virus (HSV) [225]. Therefore, alternative mechanisms such as cross-presentation are essential for priming of CD8+ T cells at least in the above-mentioned circumstances. In vitro, antigens of numerous viruses have been shown to be presented via cross-presentation of dying infected cells (for review see [55]), starting with pioneering studies on influenza virus by Albert and colleagues [4]. Later, the repertoire was extended to vaccinia virus [107], human CMV [9, 10, 202], Epstein-Barr virus [76, 201], HIV-1 [108, 7], canarypox virus [87], HSV [154], and measles virus [178, 181]. Apoptosis of infected cells is also a prerequisite for cross-presentation of bacteria such as *Salmonella* [227] or mycobacteria [176], whereas some other bacteria such as *Klebsiella* [93] or *Listeria* may utilize different sources of antigens [187, 208, 91]. Interestingly enough, cross-presentation of bacterial antigens not only occurs in the context of MHC class I molecules but also in the context of CD1b molecules as shown for the mycobacterial antigens [176]. Bearing in mind that, in addition to the better-studied viruses, numerous bacteria and parasites also induce apoptosis of infected cells [122, 220], the list of

microorganisms to which immune responses develop via cross-presentation of apoptotic cells is expected to increase substantially.

Although studies by Sigal et al. [185] confirmed that cross-priming to viral antigens can indeed take place in vivo during infection, the relevance of cross-priming in relation to direct priming in vivo remains an open question. Yewdell and colleagues [31] have recently shown in a convincing study that cross-priming indeed plays an important role in vivo for induction of antiviral immunity. When DCs are directly infected and infection results in partial apoptosis of the APC, presumably both direct and cross-presentation will take place. However, when DCs are not infected or when the infection kills APC rapidly, cross-presentation would be the prime mechanism for activating T cells. This opinion is to some extent opposed by studies of Zinkernagel and colleagues [58] in which the authors claim that in the priming of T cell responses to tumor and viral antigens, cross-presentation is only of minor importance.

5.2 Factors That Influence the Priming Capability of Dendritic Cells

What factors distinguish the cross-presentation of microbial antigens that lead to effector cell responses and cross-presentation of self-antigens that induce tolerogenic responses? We consider the state of DCs during encounter of the antigen to set the stage. To avoid autoimmune responses, by default DCs do not mature on encounter of apoptotic cells and therefore immature DCs could induce tolerance [127, 170, 198]. In contrast, when signs of danger (e.g., infection) are present, DCs undergo maturation and switch to an active immunostimulatory role [127].

In vitro, several factors defining "danger" were shown to induce maturation and enhance cross-presentation by DCs that have ingested apoptotic cells [105, 106, 107]. However, in vivo signals responsible for the switch from tolerance to immunity of apoptotic cells are still poorly characterized. We would expect that in vivo the final outcome would depend on multiple factors, including stimulation through Toll-like receptors (TLRs), presence of proinflammatory cytokines, engagement of CD40, and presence and inhibitory activity of antigen-specific regulatory T cells, to name only a few. Below we discuss some of the factors that might be involved in turning on the cross-priming ability of DCs.

5.2.1 Microbial Factors

Microbial molecules influence the cross-priming capability of DCs on several levels. First, molecules derived from pathogens such as dsRNA, ssRNA, unmethylated CpG containing DNA (CpG DNA), LPS, or peptidoglycans, which bind an array of TLRs transmit signals that induce DCs maturation (reviewed in [97]). Second, certain TLR ligands when added to culture together with soluble OVA induce cross-presentation by bone marrow-derived $CD11c^+$ $CD4^-$ $CD8\alpha^-$ DCs [35]. The importance of TLR9 in activation of DCs after endocytosis of antigen was implied by experiments in TLR9-deficient mice in which injection of OVA covalently linked to CpG DNA induced cross-presentation but not cross-priming [74]. How stimulatory signals mediated through TLR9, and possibly other TLRs, combine with signals induced by cell-bound antigen, namely apoptotic cells, remains to be determined. Studies in DCs and macrophages indicate that this mechanism is complex, as the production of cytokines by these cells when cultured in the presence of apoptotic cells and TLR ligands, differs from that when they are exposed to either TLR ligands or apoptotic cells alone [121, 200, 216]. TLR ligation might also influence the efficiency of phagocytosis of apoptotic cells. Although not yet shown for DCs, murine macrophages upregulate the expression of apoptotic cell receptors, e.g., SR-A, Lox-1, and CD36, after triggering of certain TLRs [44]. Additionally, two groups have shown that ligation of TLR increases the uptake of antigens [24, 221]. However studies by Blander and Medzhitov [24] demonstrate that while ligation of TLR by bacteria increases their uptake and influences the maturation of phagosomes, no influence was seen on the uptake and digestion of simultaneously phagocytosed apoptotic cells. Notably, while uptake of infected apoptotic cells was not studied, the bacteria and uninfected apoptotic cells were confined to distinct compartments.

Finally, pathogen-derived TLR ligands such as LPS and CpG DNA were shown to induce DC maturation and overturn the inhibitory capacity of regulatory T cells in vitro, in a costimulatory molecule-independent and IL-6-dependent fashion [151]. However, this reversal of the suppressive effects of regulatory cells in vivo was either not observed [147] or was shown to require persistent TLR signaling [223]. In their paper [223], Yang and colleagues compared infection with hemagglutinin (HA)-encoding recombinant virus with injection of TNF-α-matured and HA-loaded DC in the ability to activate HA-specific $CD8^+$ T cells. Lentiviruses or vaccinia viruses succeeded to break CD8 tolerance and

to protect against tumoral challenge. DC-based vaccines failed unless regulatory $CD4^+$ T cells were removed, TLR ligand was constantly added, or mice were concomitantly infected with an irrelevant virus (not encoding HA). However, ex vivo TLR-ligand-matured DCs could not break tolerance and after withdrawal of LPS, the production of proinflammatory cytokines by DCs diminished rapidly [223].

5.2.2 Endogenous Adjuvants

DCs also undergo maturation in the absence of foreign substances. Massive apoptotic cell death can result in failure of clearance of apoptotic cells and secondary necrosis [164]. Necrotic, in contrast to apoptotic cells mature DCs and lead to initiation of immune responses [22, 60, 170]. Several reports have described the endogenous activation of DCs via engagement of TLRs. Endogenous factors that are released from or are associated with necrotic cells induce DC activation (reviewed in [188]). They include immunostimulatory self DNA that binds TLR9 [48], self ssRNA that may stimulate TLR7 and TLR8 [41, 73], secondary structures of mRNA that activate TLR3 [98], HMGB-1 that signals through TLR2, TLR4 [150], or RAGE [174], and HSPs that stimulate TLR4 [144, 212–214]. HSPs are one of the prime candidates to contribute to conversion of a tolerogenic to a priming signal. They induce innate immune responses [214], they are released after necrosis, and the amount of expression correlates with maturation of DCs [192]. In addition, increased expression of HSPs by apoptotic cells also increases the immunogenicity of the latter [53]. Interestingly, feverlike thermal conditions enhance HSP expression on cell membranes and promote DC maturation and the priming of specific T cells [52, 53]. Recently, uric acid was identified as another factor associated with cell death and activation of DCs [184]. Finally, the immune system is alerted to massive cell death not only by factors released from dying cells, but also by factors emanating from disruption of tissue architecture, e.g., fibrinogen [189], oligosaccharides of hyaluronan [204], EDA-containing fibronectin [146], and heparan sulfate proteoglycan that stimulate TLR4.

5.2.3 Cytokines in Dendritic Cells' Milieu

The milieu in which DCs encounter apoptotic cells may regulate their stimulatory capacity. For example, DCs exposed in vitro to IL-10, TGF-

β, vascular endothelial growth factor (VEGF), or IL-10 homolog have diminished IL-12 production, expression of costimulatory molecules, and capacity to stimulate T cells [18, 34, 100, 194–196]. These factors can be produced by macrophages on ingestion of apoptotic cells, by tumor cells, or by microbes [46, 59, 62, 109, 195]. On the other hand, recognition of microbes by macrophages and neutrophils results in the production of inflammatory cytokines such as TNF-α, IL-6, or IL-1β, which mature DCs and contribute to their activation and priming capabilities [115]. For example, *Shigella flexneri* and *Salmonella* induce macrophage apoptosis, activation of caspase 1, and release of IL-1β and IL-18 [92, 169].

The role of type I IFNs, especially IFN-α in cross-priming has also gained substantial attention. In mice, type I IFNs, secreted either on TLR9 triggering [32] or viral infection [112], when coadministered with antigens were shown to be sufficient for cross-priming. IFN-α is produced in great amounts after interaction of pDCs with virus and not only influences other cells (e.g., NK cells), but can also induce maturation and perhaps cross-priming by bystander mDCs [56, 112]. Involvement of proinflammatory cytokines in overturning the tolerogenic potential of apoptotic cells has been demonstrated in studies by Zimmermann et al. By using an artificial model of TNF-α conjugated to apoptotic melanoma cells they could achieve full maturation of DCs on apoptotic cell ingestion and priming of partially protective CTL responses [228].

5.2.4 Opsonization of Apoptotic Cells with Antibodies

Sole cross-linking of FcγRI or FcγRII leads to NF-κB translocation and production of TNF-α by monocytes [45]. In addition, opsonization with antibodies and targeting FcγR was shown to mediate internalization and to overcome initial inhibitory effects of apoptotic cells [2]. Ligation of FcγR also interferes with expression of IL-10-inducible genes [94], showing the importance of this receptor as a "switch" to immunostimulatory DCs. Defective clearance of apoptotic cells in susceptible individuals was linked to initiation of autoimmunity (reviewed in [222]). Additionally, autoreactive antibodies were shown to target self-antigens located in apoptotic blebs or the chromatin released from apoptotic cells [28, 157, 162]. Apoptotic cells combined with lupus IgG induce generation of immune complexes that contain DNA. These complexes can simultaneously bind TLR9 (with the chromatin part) and the FcR (with the antibody) on pDC and B cells. Such binding induces IFN-α production by pDC

[17] and could also further activate B cells ([114] and reviewed in [218]), resulting in amplified autoimmune responses.

5.2.5 Ligation of CD40

Ligation of CD40 on DCs is one of the better-explored mechanisms that can override inhibitory effects of apoptotic cells. CD40 interacts with CD40 ligand (molecule CD154) that is expressed on immune cells such as activated B and T cells, but also on mononuclear phagocytes and activated platelets and during an inflammatory response on epithelial cells and smooth muscle cells among others (reviewed in [156]). Stimulation of CD40 was shown to override immune unresponsiveness induced by immature DCs [42, 86, 163]. In addition, tolerance induced by dying OVA-loaded cells in mice was abolished when agonistic anti-CD40 antibody was coinjected. Studies in mice show that CD40-deficient DCs are only capable of inducing a transient immune response that is followed by unresponsiveness to subsequent challenge with the same antigen [78]. A pivotal role of CD40 is supported by several therapeutic approaches in which blocking of CD40-CD154 interaction results in silencing of autoimmune disease in mouse models and induction of tolerance [12, 81, 119].

6 Concluding Remarks

Apoptotic cell death as a consequence of normal tissue turnover inhibits DC maturation and leads to tolerance. However, the concurrent presence of microbes, stress, or massive cell death presumably prevents the inhibitory effects of apoptotic cells and leads to DC maturation and induction of immune responses. Several receptors have already been associated with inhibition or stimulation of DC maturation. Molecular mechanisms leading to different outcomes after apoptotic cell ingestion are being evaluated. In the future, it will be necessary to study the signaling pathways triggered and to evaluate the hierarchy and cooperation of signals transduced via different receptors. This information will lead to new strategies to develop “tolerogenic” or “immunostimulatory” DCs.

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