Apoptotic cell clearance by macrophages and dendritic cells : immunoregulation in the context of innate immunity

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Dendritic cell and macrophage subsets in the handling of dying cells

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Summary

Dendritic cells and macrophages are major components of the phagocyte system and are professional antigen presenting cells. In the current review, we discuss the differential contribution of dendritic cell and macrophage subsets in the clearance of dying cells and the consequence of the process of these cells. We hypothesize that under steady-state conditions, the clearance of apoptotic cells is mostly confined to a specialized subset of phagocytes with anti-inflammatory properties.

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Introduction

Phagocytes constantly remove excessive dying cells during normal homeostasis and tissue turnover. Since 1990, significant progress has been made in understanding the immunological meaning of clearance of dying cells [1-4]. The underlying accepted paradigm is that apoptotic cells provide "eat me signals" to phagocytes to promptly and efficiently engulf apoptotic cells during the early stage of cell death, thus preventing them to release noxious substances in the local environment. In contrast, late apoptotic cells or necrotic cells might provide danger signals to activate antigen presenting cells (APCs), potentially breaking self-tolerance [2,3]. One of the well-established examples, both in mice [5,6] and men [7] of a disorder in which apoptotic cell clearance is disturbed is systemic lupus erythematosus (SLE) [8].

There are several checkpoints conceivable which together determine the immunological response towards the safe clearance of dying cells (Figure 1). I.) The different modes of cell death might determine the fate of clearance. There are at least three major types of cell death: apoptosis, necrosis and autophagy [9], which exhibit distinct biochemical and morphological changes [9-11]. Depending on the respective stimuli of death induction, these dying cells might determine phagocyte activation and antigen processing [12]. II.) Opsonization of apoptotic cells by components of the innate immune system, such as complement factors and pentraxin family members, facilitates and modulates the clearance of apoptotic cells [13]. III.) Finally, since the mononuclear phagocyte system is largely heterogeneous [14], the nature of phagocytes that phagocytose apoptotic cells might provide a defined immunological response (Fig. 1).

The concept of differences of dying cells and the differential contribution of opsonins has been extensively reviewed [12,13,15-18]. In the present review, we will concentrate on the differential contribution of phagocyte subsets, particularly subsets of dendritic cells (DCs) and macrophages (MØ) in the clearance of dying cells, as both cell types are professional phagocytes and APCs. Phagocyte subsets might be cells with intrinsic pro-inflammatory or anti-inflammatory characteristics. We suggest that the clearance of apoptotic cells is mostly confined to a specialized subset of phagocytes with anti-inflammatory properties.

DC subsets in the clearance of dying cells

DC subsets

Among the different phagocyte subsets, DCs are the most potent professional APCs [19]. Being an important component of the innate immune system, DCs have been demonstrated in almost all peripheral organs and in lymphoid tissues [20]. The heterogenity of DCs has been extensively studied. In mice, at least six DC subsets

have been described in lymph nodes, derived from two distinct pathways, myeloid and lymphoid [21]. They are distinguished by surface markers such as CD11b, CD8α, and CD11c, as well as by their function [21].

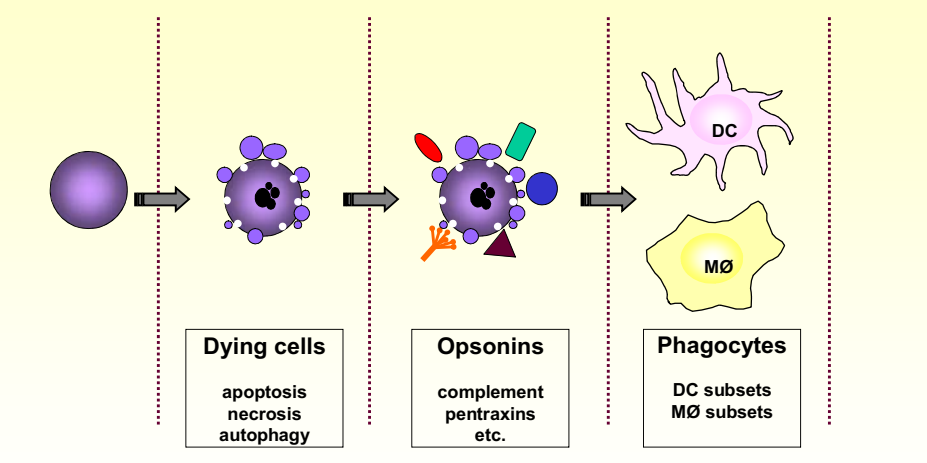

Figure 1. Three checkpoints determine the immunological response during clearance of dying cells. I.) The various modes of cell death such as apoptosis, (secondary) necrosis or autophagy, might determine the fate of dead cells. II.) Opsonization of apoptotic cells by components of the innate immune system, such as complement factors and pentraxin family members, facilitates and modulates the clearance of apoptotic cells. III.) The type of phagocytes that take up apoptotic cells might provide a defined immunological response.

In contrast to well-studied mouse DCs, human DCs have been studied to a relatively lesser degree. Blood is the only readily available source and at least three subtypes of blood-driven DCs have been characterized: Langerhans DCs (LDCs), interstitial DCs (iDCs) and plasmacytoid DCs (pDCs) [21]. Most of the insights into human DC subsets are derived from *in vitro* studies, following the identification of DC precursors, i.e., CD34+ cells from umbilical cord blood or bone marrow, blood monocytes, and plasmacytoid precursors from blood. Migration into non-lymphoid organs, for example the interstitium of peripheral organs, induces differentiation of DC precursors into resident tissue DCs [22]. One of the characteristics of resident DCs is that they are able to capture self (such as cellular debris) and foreign (such as microbial pathogens) antigens by several mechanisms [20]. The maturation status of DCs after antigen-capture determines whether they prime T cells or induce immune tolerance [23]. Taken together, *in vitro* and *in vivo* studies clearly indicate that DCs play a pivotal role in both innate and adaptive immunity.

Clearance of dying cells by DCs

Although heterogeneous populations of DCs have been described in mice and humans (as discussed above), relatively few studies have addressed the question whether DC subsets contribute differentially to the uptake of dying cells. Most of the available studies have investigated dying cell clearance by DCs either using immature monocyte-derived DCs (iMoDCs) in humans [24-26] or splenic $CD8^+$ DCs [27,28] or BM $CD11c^+$ DCs [29] in mice. These data show that DC subsets from different sources can phagocytose dying cells. However, it has been suggested that phagocytosis of dying cells by DCs is restricted to certain types of DCs. For example, although both CD8+ and $CD8^-$ DCs phagocytose latex particles in culture and both DC subsets present soluble ovalbumin captured *in vivo*, only $CD8^+$ DC is specialized in the phagocytosis of dying cells [28]. Another *in vitro* study compared the subsets of splenic CD8 DCs and found that splenic $CD8\alpha^+$ DC are superior to other DC subsets in internalizing dying cells [30].

Also human DC subsets exhibit differential capacities to phagocytose dying cells. A recent study compared side-by-side the phagocytic capacity among three human DC subsets: $CD11c^+$ DCs, iMoDCs and pDCs, and found that iMoDCs are three times better in the phagocytosis of apoptotic cells than $CD11c^+$ DCs, whereas pDCs were hardly able to take up apoptotic cells [31]. This suggests that the uptake of dead material is probably confined to myeloid subsets of DCs.

Consequence of uptake by DCs

Apoptotic cells are a rich source of autoantigens [32]. Upon uptake, DCs acquire antigens from apoptotic cells and (cross-)present these antigens to class I- or class II-restricted T cells [25,33,34]. It remains controversial whether apoptotic cells indeed induce maturation of DCs and whether they stimulate or tolerize T cells. It has been shown that necrotic cells or late apoptotic cells, but not (early) apoptotic cells trigger DC maturation [35,36]. However, others showed that bystander or excessive apoptotic cells induce maturation of DCs, and present antigens in the absence of exogenous danger signals [24,34]. It should be realized that these studies have made use of different sources of DCs, including PBMC [35,36] or D1 cell lines [24,34]. Similarly, various apoptotic targets are used in different studies, such as Jurkat T cells [36], 293 cell lines [35] and OVA-RMA cells [24,34]. Thus different cells used in the studies might explain the substantial difference in the consequence of uptake of apoptotic cells by DCs.

Whether uptake of apoptotic cells leads to DC activation in the context of pro- or anti-inflammatory conditions is also depending on how the death stimuli are applied [12]. Several studies have shown that apoptosis triggered via death receptors could release bio-active lipids such as sphingosine-1-phosphate (S1P) [37], or

lysophosphatidylcholine (LPC) [38]. These agents signal through endothelial-derived G-protein-coupled (EDG) receptors and these receptors are known to signal through nuclear factor-κB (NF-κB), leading to a pro-inflammatory response [12].

To validate the immune regulatory role of apoptotic cells, also *in vivo* studies have been performed. Mouse $CD8^+$ DC were shown to actively capture apoptotic cells and induce immune tolerance [27,28]. These findings are supported by another *in vivo* study showing that a subset of rat DCs, $CD4^-/OX41^-$ DCs, constitutively endocytoses and transports apoptotic cells to the T cell areas of mesenteric lymph nodes [39], suggesting that DCs carrying apoptotic material can silence T cells against self-antigens [40].

If under normal conditions, apoptotic cell removal by DCs is an immunologically silent process, the question remains how apoptotic cell-loaded DCs break peripheral tolerance, as observed in SLE. One possibility is that when early apoptotic cells are not removed promptly, the remaining cells might become late apoptotic (or secondary necrotic), thus converting immune tolerance to autoimmunity. In lupus-prone mice, only DCs loaded with necrotic cells, but not apoptotic cells, induce lupus-like disease [29], suggesting that the intracellular contents released from necrotic cells provide additional danger signals and lead to activation of DCs. A second possibility is that the presentation of autoantigens derived from apoptotic cells by DCs to T cells could be triggered by the presence of ligands for TLRs. A very recent paper showed that only DCs captured apoptotic cells in the presence of TLR4 triggering by LPS could present antigens to CD4 T cells and induce IL-2 production [41]. However, simultaneous phagocytosis of apoptotic cells and microbial pathogens does result in DC maturation, but under these conditions only antigens from bacteria, but not the ones from apoptotic cells, were presented to CD4 T cells [41]. Thus extra danger signals provided by TLR ligands could make a substantial contribution in the initiation of autoimmunity by apoptotic cell-loaded DCs.

Another possible explanation could be that one specialized DC subset contributes exclusively to initiate autoimmunity. Recently, much attention has focused on understanding of how DNAs and RNAs containing autoantigens, that are derived from dying cells, activate DCs in the setting of SLE. Although a previous study showed that *in vitro* pDCs do not take up apoptotic cells [31], they do take up immune complexes (ICs) containing DNAs [42]. It was shown that ICs derived from patients with SLE , upon intracellular delivery via CD32, were able to activate pDCs through toll like receptor 9 (TLR9) [42]. This observation was extended in another study showing that small nuclear RNAs within ribonucleoprotein particles activate pDCs through TLR7 [43], suggesting a link between pDCs and autoimmmunity to both DNA- and RNA-containing autoantigens. These data have provided novel insights in the mechanisms of loss of peripheral tolerance to autoantigens in SLE. The

suggestion that SLE is a pDC-driven disease seems to be confirmed by the identification of IFN-α as an important pathogenic factor [44,45].

MØ subsets in the clearance of dying cells

MØ subsets

It has been proposed that at least two types of MØ exist in vivo: resident (tissue) MØ and inflammatory elicited MØ [46]. Most resident MØ are derived from circulating bone marrow-derived monocytes. Human and mouse studies have indicated that a large heterogeneity exists in MØ populations in lymphoid organs and non-lymphoid organs such as the lung (alveolar MØ), liver (Kupffer cells), spleen (white and red pulp MØ), peritoneum (peritoneal MØ) and nervous system (microglia) [46,47]. These MØ subsets are phenotypically and functionally different, but all of them play broad roles in tissue remodeling and homeostasis [48]. Depending on the cytokine environment, MØ can be activated classically (IFN-γ) or alternatively (IL-4 and IL-13), as reviewed elsewhere [49,50]. Similarly, in mice these subsets have been defined as type 1- (i.e., classically activated) or type 2- (i.e., alternatively activated) activated MØ [51], which are characterized as pro-inflammatory and anti-inflammatory cells, respectively. However, it is not yet fully defined whether resident MØ are anti-inflammatory.

The growth and differentiation of MØ depends on lineage-determining cytokines such as granulocyte/macrophage colony-stimulating factors (GM-CSF) and M-CSF (also termed CSF-1) [50,52]. Mice lacking M-CSF develop a general MØ deficiency [53], whereas GM-CSF-knockout mice have no major deficiency of MØ [54,55]. Importantly, in humans, M-CSF, but not GM-CSF, is a ubiquitous cytokine circulating in the human body [56,57]. Therefore, it is likely that M-CSF is the default cytokine to drive MØ differentiation under steady-state conditions.

Recently it has been shown that human MØ can be polarized *in vitro* into pro-inflammatory (MØ1) and anti-inflammatory cells (MØ2) by GM-CSF and M-CSF, respectively [57-59]. GM-CSF-driven MØ1 are characterized by high production of pro-inflammatory cytokines such as IL-6, IL-12 and IL-23, whereas M-CSF-driven MØ2 are characterized by high production of IL-10 in the absence of pro-inflammatory cytokines. It should be noted that M-CSF-driven MØ2 do not completely resemble alternatively activated MØ or type 2-activated MØ with respect to their surface marker expression and cytokine production [50,60]. For example, alternative activated MØ showed increased expression of mannose receptors (MR) [50], and type 2-activated MØ secrete TNF-α upon stimulation [60]. However, in the case of M-CSF-driven MØ2, low MR expression was found and these cells fail to secrete TNF-α [59,61]. Since MØ2 express the unique surface marker CD163 [61], it is tempting to speculate that MØ2 reflect $CD163^{+}$ highly phagocytic resident MØ *in vivo* [62].

Clearance of dying cells by MØ subsets

The role of MØ in the uptake of apoptotic cells has been studied extensively. In mice, among various MØ subsets, bone marrow-derived MØ [63,64] and peritoneal MØ [65-68] have been widely used. Recently, also microglia [69] and liver macrophages [70,71] have been investigated for their roles in the apoptotic cell removal, suggesting that tissue MØ are active in the removal of dying cells in various anatomical locations. Besides tissue MØ, murine MØ cell lines [72-74] are also able to actively phagocytose apoptotic cells. The data above clearly show that various MØ subsets actively recognize and ingest cells that underwent apoptosis.

In human system, most studies were carried out *in vitro* by using peripheral blood monocyte-derived MØ, differentiated in the presence of GM-CSF [75], M-CSF [76], or in the absence of growth factors [77-80]. These MØ can be activated by various factors. For example, activation of MØ by glucocorticoid augments phagocytosis of apoptotic cells [81,82]. Similarly, IL-10-activated MØ show an enhanced capacity for the uptake of apoptotic cells [83]. These data indicate that the micro-environment influences the differentiation process of MØ and thereby modifies their functions as well.

Recently, we compared the capacities for the uptake of apoptotic cells among three types of phagocytes iMoDCs, MØ1, and MØ2 generated from the same monocyte population, and found that MØ2 have the unique capacity to preferentially take up early apoptotic cells [59]. We found that MØ2 have the capacity to take up early apoptotic cells more efficiently than late apoptotic or necrotic cells (four fold increase), and that this uptake was superior compared to that of MØ1 and iMoDCs. Thus we hypothesize that under steady-state conditions, scavenging of apoptotic cells is largely confined to a specialized subset of phagocytes with anti-inflammatory properties. Other subsets of phagocytes might act as backups, or even be more involved in the resolution of inflammation and /or immune regulation. We propose that in the case of MØ, the anti-inflammatory MØ2 are the default phagocytes that take up early apoptotic cells in a silent manner, whereas excessive apoptotic cells progress to a late stage of cell death and might provide "eat me signals" to the non-resting phagocytes such as the pro-inflammatory MØ1. In the latter case, appropriate opsonization may determine the consequence of the uptake (Fig. 2). It is important to note that most opsonins, including C1q, MBL, PTX3 and SAP, preferentially bind to late apoptotic cells [13].

Consequence of uptake by MØ

A substantial number of *in vitro* studies have shown that MØ that have ingested apoptotic cells are inhibited in their production of pro-inflammatory cytokines [63,65,74,79], consistent with the prevailing believe that apoptotic cells are removed by

MØ in a silent manner. Since both pro-inflammatory and anti-inflammatory MØ exist, a relevant question is then: do they respond to apoptotic cells in different ways? Our studies showed that upon ingestion of apoptotic cells, pro-inflammatory MØ1 down-regulate the production of pro-inflammatory cytokines, whereas anti-inflammatory MØ2 retain high level of IL-10 production in the absence of pro-inflammatory cytokines [59].

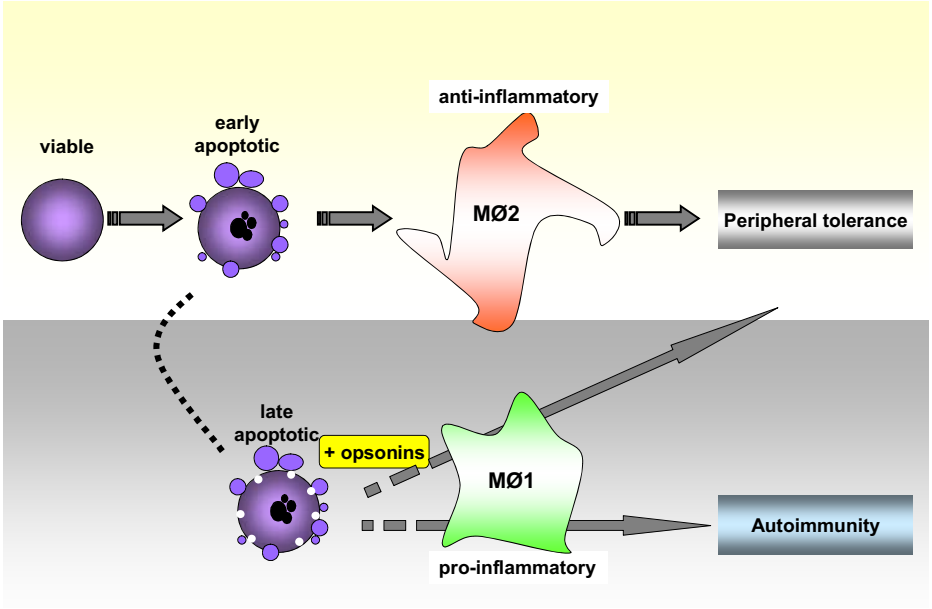

Figure 2. Apoptotic cell uptake by MØ subsets. We propose that concerning MØ, at least two subtypes exist, i.e., the pro-inflammatory MØ1 and the anti-inflammatory MØ2. MØ2 preferentially bind and ingest early apoptotic cells in a non-inflammatory fashion. Excessive apoptotic cells progress to a late stage of cell death and might provide "eat me signals" to the non-resting phagocytes such as pro-inflammatory MØ1. In this case, appropriate opsonization of apoptotic cells may determine the consequence of the uptake.

Similar to DCs, not only apoptotic cells, but also necrotic cells are taken up by MØ [84,85]. Like for apoptotic cells, several studies have addressed the question whether necrotic cells activate MØ to trigger immunity. It has been shown that phagocytosis of necrotic cells by MØ does not induce inflammation [84,85]. However, these findings were challenged in a recent publication showing that exposure of MØ to early and late apoptotic cells induces identical signal transduction in these cells in terms of inhibition of ERK 1/2 and induction of JNK and P38, whereas necrotic cells induced an opposite signal transduction [86]. Taken together, these data provide an

ambiguous image on whether necrotic cells are much more dangerous than apoptotic cells. Further studies will be required to understand the interaction of MØ with the intracellular contents released from necrotic cells such as HSP [87], HMGB1 [88] and uric acid [89].

Like DCs, MØ are also professional APCs [47]. It would be of interest to investigate how these intracellular molecules modulate MØ function including the presentation of autoantigens. Indeed, most available studies have not investigated the role of dying cell-derived antigen presentation on MØ. It has been suggested that MØ fail to cross-present antigenic material contained within the engulfed apoptotic cells [33]. In contrast to DCs that can retain antigen for at least 2 days [90], MØ robustly degrade the ingested antigens, and therefore may fail to promote T cell priming [91]. Thus it remains to be investigated how MØ process antigens derived from dying cells and subsequently present these to Class I- or II-restricted T cells.

Concluding remarks

As discussed so far, there are different professional phagocyte subsets that are actively involved in the clearance of dying cells. Presumably, also neighboring non-professional phagocytes might actively participate in the removal of dying cells. Therefore, especially under steady-state conditions, the fate of dying cells will ultimately be determined by local conditions and the composition of the tissue. Excessive apoptosis leads to the release of intracellular signals and therefore alert the immune system in various aspects. Again, the local environment will determine 1) the composition of attracted phagocytes; 2) the presence of innate molecules that help in opsonization and clearance; or 3) presence of TLR ligands that might activate immunity. The sole presence of high amounts of apoptotic cells as a consequence in deficiencies in clearance, as shown in $CD14^{-/-}$ mice [92] or C1q-/- mice [5] on a non-autoimmune background, does not always lead to a break of immunological tolerance. *In vivo* dissection of phagocyte subsets with distinct functional properties will be of particular importance to understand how the clearance of apoptotic cells by phagocytes is regulated and how this may lead to induction or loss of peripheral tolerance.

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