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Opsonization with C1q and Mannose-Binding Lectin Targets Apoptotic Cells to Dendritic Cells

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Deficiencies of early components of the classical complement pathway, particularly C1q, are strongly associated with susceptibility to systemic lupus erythematosus. Recent data link this predisposition to autoimmunity to an inappropriate clearance of apoptotic cells, which could lead to a loss of self-tolerance. In the present study, we demonstrate that opsonization of apoptotic cells with C1q and mannose-binding lectin allows and facilitates their uptake not only by macrophages but also by human immature dendritic cells (DCs). Both C1q and mannose-binding lectin enhance the uptake of apoptotic cells by DCs in a dose-dependent way. The uptake of C1q-opsonized apoptotic cells, but not nonopsonized apoptotic cells, by DCs stimulated the production of IL-6, IL-10, and TNF-α, without an effect on IL-12p70. We conclude that these recognition molecules of the complement system do not sequester apoptotic cells from DCs, but rather promote their uptake by immature DCs. Therefore, we propose that early complement components support safe clearance of cellular debris by facilitating phagocytosis and possibly by immunomodulatory mechanisms, thus preventing autoimmunity. The Journal of Immunology, 2004, 173: 3044–3050.

A poptotic cell death and the removal of cells dying by apoptosis is an essential process during development in the maintenance of normal tissue homeostasis and in the resolution of inflammation (reviewed in Refs. 1 and 2). A redundant system of receptors, soluble bridging molecules, and apoptotic cell surface molecules has been implicated in the recognition and removal of apoptotic cells. However, for only a few candidates, including the pattern recognition molecule C1q, their importance in apoptotic cell clearance could be confirmed in vivo (3–7).

Pattern recognition molecules, including C1q and mannose-binding lectin (MBL), are part of the innate immune system and are thought to be primarily important in host defense by their capability to specifically recognize pathogens and facilitate their removal. Recent evidence extends the role of these pattern recognition molecules to the recognition and clearance of dying cells (reviewed in Refs. 8 and 9). C1q and MBL have been shown to bind directly to apoptotic cells and apoptotic cell-derived blebs (10–14). Furthermore, binding to cells dying via necrosis has also been demonstrated for MBL. Inherited deficiencies in early complement components, particularly C1q, are associated with an elevated risk for the development of systemic lupus erythematosus (SLE) (15). The vast majority (~95%) of individuals with C1q deficiency develop SLE-like disease (15). Deficiency in the collectin MBL is also associated with an increased susceptibility to SLE (16). Moreover, C1q-deficient mice spontaneously develop autoantibodies and glomerulonephritis associated with the presence of multiple apoptotic cells in the kidney (4), consistent with demonstrable defects in the clearance of apoptotic cells by their macrophages (6). The strong correlation between deficiency in early complement components and the development of SLE, along with the observation that autoantigens targeted in SLE are present within apoptotic cells (17), has led to the development of the waste disposal hypothesis (18). In this hypothesis, it is proposed that massive apoptosis and a failure in the clearance by macrophages result in the recruitment of dendritic cells (DCs), engulfing apoptotic cells and presenting autoantigens to autoreactive T cells, potentially leading to the onset of an autoimmune response. In this respect, it has been shown that purified C1q and MBL directly facilitate the phagocytosis of apoptotic cells by macrophages (12, 14). Complement components therefore are proposed to be essential for the adequate uptake of apoptotic cells by macrophages, thereby sequestering apoptotic cells from DCs. However, recent data indicate that DCs are not only important for immune activation, but also play an active role in the maintenance of tolerance (19).

Therefore, we have investigated the role of C1q and MBL in the uptake of apoptotic cells by DCs. Previously, we and others have demonstrated that MBL can facilitate the uptake of apoptotic cells by macrophages (12, 14). We now show that opsonization of apoptotic cells with C1q and MBL targets these cells to DCs as well as to macrophages and increases the efficiency of their uptake. An extended waste disposal hypothesis is proposed in which complement ensures safe removal of apoptotic cells and additional signals determine whether an immune response is induced.

Materials and Methods

Cell culture

Jurkat cells were cultured in serum-free AIMV medium supplemented with penicillin (90 U/ml) and streptomycin (90 μg/ml) (P/S) (all from Invitrogen Life Technologies, Breda, The Netherlands). Human monocytes were...
isolated on a Percoll gradient as described before (20). The cells were allowed to mature into macrophages over a 7-day period in RPMI 1640 containing 15% heat-inactivated FCS and P/S and was supplemented with 5 ng/ml GM-CSF (Leucomax; Novartis Pharma, Arnhem, The Netherlands). Monocyte-derived DCs were generated by culturing monocytes in RPMI 1640 with 10% heat-inactivated FCS, P/S, 5 ng/ml GM-CSF, and 10 ng/ml IL-4 (PeproTech, Rocky Hill, NJ) for 7 days.

**Flow cytometric analysis of cell surface molecules**

Macrophages and DCs were analyzed by flow cytometry using Abs against the following surface molecules: CD1a (Leu-6) and CD14 (Leu-M3) (both from BD Biosciences, San Jose, CA), HLA-DR (clone B8.11.2), DC-SIGN (CD209; kindly provided by Dr. Y. van Kooyk, Vrije Universiteit Medical Center, Amsterdam, The Netherlands), CR1 (CD35) (generated in our own laboratory), CD91 (American Diagnostics, Pendleton, IN), collagenous tail C1qR (cC1qR) (mouse monoclonal IgG1; Calbiochem, San Diego, CA), cC1qR1 (rabbit antisera against the recombinant N domain of cC1qR), cC1qR2 (rabbit antisera against the recombinant P domain of cC1qR), and cC1qR3 (rabbit antisera against native cC1qR; all three kindly provided by Dr. B. Sim, Oxford University, Oxford, U.K.) (21, 22). Staining was visualized by using PE-conjugated polyclonal goat anti-mouse Ig or PE-conjugated goat anti-rabbit Ig (DakoCytomation, Glostrup, Denmark). The cells were assessed for fluorescence using a FACSCalibur (BD Biosciences). For negative control staining, irrelevant isotype-matched mouse mAb and nonimmune rabbit serum were used.

**Induction of apoptosis**

Apoptosis of Jurkat T cells was induced by incubation with 40 μM etoposide (Sigma-Aldrich, St. Louis, MO) for 20 h in AIMV culture medium (Invitrogen Life Technologies). Apoptosis was confirmed by double staining with FITC-labeled annexin V (Nexins Research, Hoeven, The Netherlands) and propidium iodide (Molecular Probes, Leiden, The Netherlands) and analyzed by confocal laser scanning microscopy with a Bio-Rad MRC1024 ES krypton-argon ion laser scanning imaging system (Hercules, CA) using appropriate filter settings.

**Cytokine detection**

DCs were cultured with apoptotic cells or C1q-opsonized apoptotic cells at a 1:1 ratio for 24 h in AIMV culture medium. Cytokines were detected in cell-free supernatant using ELISA. The measurements of IL-6 and TNF-α were performed as described before (26). The analysis of IL-10 and IL-12p70 was performed according to the instructions provided by the manufacturer (Sanquin Research and R&D Systems, Minneapolis, MN, respectively).

**Statistical analysis**

Statistical analysis was performed by two-way ANOVA using GraphPad PRISM (GraphPad Software, San Diego, CA). Differences were considered statistically significant when p values were <0.05.

**Results**

C1q increases the phagocytosis of apoptotic cells by immature DCs

Strong binding of C1q to apoptotic Jurkat cells was observed following induction of apoptosis in the presence of C1q (Fig. 1A).
The uptake of apoptotic cells by DCs was analyzed by flow cytometry. Incubation of apoptotic Jurkat cells with DCs at 37°C resulted in the formation of double-positive cells (Fig. 1B) representing DCs with ingested and/or surface-bound apoptotic cells. Opsonization of apoptotic cells with purified C1q resulted in an increased number of DCs that bound and ingested apoptotic cells both at 30 min and at 2 h following start of coculture (Fig. 1B), while there was no effect at 4°C. Similarly, and in accordance with the literature (12), C1q facilitated the phagocytosis of apoptotic cells by macrophages (Fig. 1C).

Confocal microscopy confirmed that DCs and macrophages had internalized apoptotic cells (Fig. 2A). Incubation of apoptotic Jurkat cells with C1q significantly increased the ingestion of these cells by DCs in a concentration-dependent manner (Fig. 2B). Similarly, increasing concentrations of C1q augmented the ingestion of apoptotic cells by macrophages up to 2-fold (Fig. 2C).

Effect of MBL on the uptake of apoptotic cells by DCs

We next examined the ability of the structurally related molecule MBL to opsonize apoptotic cells for phagocytosis. The binding of MBL to apoptotic cells was verified by flow cytometry (Fig. 3A). Opsonization of apoptotic cells with MBL significantly increased

**FIGURE 2.** C1q enhances the phagocytosis of apoptotic cells by DCs and macrophages. A, CFSE-labeled apoptotic cells were incubated for 2 h at 37°C with DCs or macrophages. The cells were stained with anti-HLA-DR (DCs) or anti-CD14 (macrophages) and visualized with confocal laser microscopy. Confocal images of the cells in the red channel (a and d), in the green channel (b and e), or the merge of two channels (c and f). B, Apoptotic cells, generated in the presence of different concentrations of C1q, were incubated with DCs (B) or macrophages (C) for 2 h at 37°C. The results are presented as the mean fold increase of phagocytosis ± SD of three independent experiments performed in duplicate. For each experiment, the percentage of double-positive cells obtained in the presence or absence of C1q was calculated. Differences between presence and absence of C1q were evaluated by two-way ANOVA. C, Similar experiment, as described in B, performed with macrophages. Results represent mean ± SD of four independent experiments performed in duplicate. *, p < 0.05 and **, p < 0.001.

**FIGURE 3.** MBL facilitates the uptake of apoptotic cells by DCs and macrophages. A, Apoptotic cells were generated in the presence (1 μg/ml) or absence of MBL. Binding of MBL was by flow cytometry using a mAb directed against MBL. B, Apoptotic cells, generated as described in A, were incubated for 30 and 120 min at 37°C, and as a control at 4°C, with DCs, and the percentage of DCs that bound/ingested apoptotic cells was assessed by flow cytometry. C, Apoptotic cells, cultured in the presence of different concentrations of MBL, were incubated with DCs for 2 h. The results are expressed as the mean ± SD of three independent experiments performed in duplicate. D, Apoptotic cells opsonized with MBL were incubated with macrophages for 2 h. The percentage of macrophages that bound/ingested apoptotic cells was assessed. Data are presented as the mean ± SD of three experiments performed in duplicate. *, p < 0.05 and **, p < 0.001.
ingestion of these cells by DCs (Fig. 3, B and C) and macrophages (Fig. 3D) in a dose-dependent manner. Expression of C1q and MBL receptors Several molecules have been described to function as receptors for the structurally and functionally similar collagen-like tails of C1q and MBL (27). Substantial evidence has been provided for a role of calreticulin (cC1qR), present on the cell surface in association with CD91, in the clearance of C1q- and MBL-opsonized apoptotic cells by macrophages (7). Another potential clearance mechanism may be through interaction with CR1, since CR1 has been described to be a functional receptor for both C1q and MBL (28, 29). Because we now show that C1q and MBL also enhance the uptake of apoptotic cells by DCs, experiments were performed to examine the expression of cC1qR, CD91, and CR1 on DCs and macrophages. DCs are characterized by a high expression of CD1a (CD1a\(^+\)), absence of CD14 (CD14\(^-\)), and clear expression of DC-SIGN, whereas macrophages are CD1a\(^-\), CD14\(^+\), and DC-SIGN\(^-\) (Fig. 4A). Although monocytes and macrophages express both candidate molecules, DCs only express CR1 and are negative for cC1qR and CD91 (Fig. 4B). A recent study demonstrated that DCs express a different form of cC1qR on their cell surface (30). Therefore, several Abs recognizing different domains on cC1qR were used to examine whether cC1qR is expressed on DCs. A relatively low expression of cC1qR on DCs was demonstrated with one of the anti-cC1qR Abs recognizing the N-terminal domain of cC1qR (Fig. 4C). Macrophages, on the other hand, show positive staining with all three anti-cC1qR Abs.

Interaction with opsonized apoptotic cells modulates cytokine production by DCs

Recent studies have suggested that the clearance of apoptotic cells can influence immune responses by enhancing or suppressing inflammation (reviewed in Ref. 31). Therefore, the effects of ingestion of C1q-opsonized apoptotic cells on cytokine production by DCs was examined. Fig. 5 shows that interaction with apoptotic cells reduced the level of TNF-\(\alpha\) secreted by DCs. The levels of IL-6, IL-10, and IL-12p70 secreted by immature DCs were not measurable. Interaction with C1q-opsonized apoptotic cells stimulated the production of IL-6, TNF-\(\alpha\), and IL-10, but did not induce a measurable effect on IL-12p70 production (Fig. 5). Stimulation of DCs with CD40L did up-regulate IL12-p70 production (data not shown).

Discussion

Phagocytosis of apoptotic cells is thought to be important in the resolution of inflammation, not only by preventing the release of toxic and immunogenic intracellular contents, but also by active ingestion of these cells by DCs (Fig. 3, B and C) and macrophages (Fig. 3D) in a dose-dependent manner.

Expression of C1q and MBL receptors

Several molecules have been described to function as receptors for the structurally and functionally similar collagen-like tails of C1q and MBL (27). Substantial evidence has been provided for a role of calreticulin (cC1qR), present on the cell surface in association with CD91, in the clearance of C1q- and MBL-opsonized apoptotic cells by macrophages (7). Another potential clearance mechanism may be through interaction with CR1, since CR1 has been described to be a functional receptor for both C1q and MBL (28,
suppression of inflammation (32). Several receptors and ligands have been reported to be important in the uptake of apoptotic cells. The present study demonstrates that opsonization of apoptotic cells with C1q and MBL directly facilitates clearance of apoptotic cells by both DCs and macrophages.

Defects in the clearance of apoptotic cell debris are important in the pathogenesis of SLE (18, 33). Molecules of the innate immune system, including complement and pentraxins, have been implicated in the rapid and efficient clearance of apoptotic cells (6, 12, 34, 35). A deficit in this function may explain the high occurrence of SLE among individuals deficient in complement. The emerged waste disposal hypothesis has proposed that in situations with impaired clearance of apoptotic cells, DCs might be recruited, engulfing apoptotic cells and presenting apoptotic cell-derived intracellular Ags to T cells (18). Because of the great immunostimulatory potential of DCs, the uptake of apoptotic cells by DCs might favor the onset of an autoimmune response. However, our findings demonstrate that opsonization of apoptotic cells with C1q and MBL also favors the uptake of apoptotic material by immature DCs. Thus, opsonization with complement is important in mediating rapid and efficient uptake of apoptotic cells, rather than having a critical role in targeting apoptotic material to specific cells. Therefore, an extended waste disposal hypothesis is proposed to account for the association of complement deficiency with autoimmunity. Opsonization with complement ensures the rapid and efficient removal of apoptotic material by DCs and macrophages and may have a critical role in controlling the immune response to apoptotic cell-derived Ags.

DCs in the steady state, constantly sampling and presenting Ags in a tolerogenic way, are proposed to play an important role in the induction of peripheral tolerance (19). Recent studies demonstrated that the uptake of apoptotic cells by DCs in the absence of maturation signals contributes to the maintenance of tolerance to self-tissue (36). However, in the presence of proinflammatory signals, maturation of DCs is stimulated, which will induce T cell activation and will switch the response from tolerance to immunity. Progression of the cell death process into a later phase (37) or the opsonization of apoptotic cells with autoantibodies (38) attributable to impaired clearance may provide such a proinflammatory context leading to the maturation of DCs.

Recent studies suggested that the uptake of apoptotic cells can actively suppress inflammatory responses, inhibiting production of proinflammatory mediators and stimulating production of anti-inflammatory mediators (32, 39–41). However, phagocytic clearance of apoptotic cells can potentially also stimulate a proinflammatory response (reviewed in Ref. 31). In the present study, we demonstrate that after interaction with C1q-opsonized apoptotic cells, DCs produced increased levels of IL-6, TNF-α, and IL-10, with no effect on the production of IL-12p70. It is striking that C1q-opsonized apoptotic cells induce a cytokine response of DCs, rather than having a critical role in targeting apoptotic material to specific cells. Therefore, an extended waste disposal hypothesis is proposed to account for the association of complement deficiency with autoimmunity. Opsonization with complement ensures the rapid and efficient removal of apoptotic material by DCs and macrophages and may have a critical role in controlling the immune response to apoptotic cell-derived Ags.

The binding of complement components and pentraxins occurs during the late phase of the apoptotic process: C1q binding to late apoptotic cells was much stronger than that to early apoptotic cells and binding of MBL was exclusively demonstrated to late apoptotic cells (13, 14). Therefore, C1q and MBL are probably primarily important in the clearance of apoptotic cells in later stages of the cell death process. In the clearance of early apoptotic cells, other mechanisms may prevail (9). However, complement-mediated clearance might be especially relevant in situations with high local rates of apoptosis and low or impaired phagocytic capability. Furthermore, MBL (14) does not only directly bind to late apoptotic cells but also to necrotic cells. Therefore, it is tempting to speculate that similar mechanisms may also be involved in the clearance of necrotic material.

Although our data indicate that the direct effect of MBL on phagocytosis by DC and macrophages is stronger than that of C1q, the role of C1q in apoptotic cell clearance might still be more important in vivo. The concentration of circulating C1q is around 100-fold higher than that of MBL. Furthermore, C1q is able to bind to apoptotic cells not only directly, but also via a number of intermediate molecules, such as the pentraxin family members C-reactive protein, serum amyloid P component, and pentraxin 3, as well as natural IgM (reviewed in Ref. 9). These various levels of interaction can strongly enhance the amount of C1q bound to apoptotic cells. Furthermore, binding of C1q (13) but not MBL (14) can induce activation of the complement cascade, leading to secondary deposition of activated C4 and C3. Indeed, a role for C1q in apoptotic cell clearance has been demonstrated in vivo (4), whereas for MBL, this remains to be established. The recent generation of MBL knockout mice (46) can facilitate this type of study.

Recent studies demonstrate that C3b opsonization enhances the uptake of apoptotic cells by DCs and promotes the maintenance of tolerance (47–49). C-reactive protein has also been demonstrated to enhance clearance of apoptotic cells in an anti-inflammatory context (34). However, the described interactions of other important opsonins of the innate immune system, surfactant protein A with TLR4, and the pentraxins C-reactive protein and serum amyloid P component with FcγRs (8), strongly suggest that opsonin-mediated clearance of apoptotic cells might also evoke a proinflammatory response. Therefore, we hypothesize that the recognition mechanism used for engulfment of apoptotic cells will determine the response of the phagocyte.

The cytokine response of DCs after interaction with C1q-opsonized apoptotic cells is consistent with a recent study that demonstrated that interaction of the collagen-like tails of C1q and MBL with calreticulin/CD91 can stimulate a proinflammatory response by macrophages (50). Several receptors have been postulated to play a role in C1q- or collectin-mediated uptake of apoptotic cells, including C1qR (calreticulin) and CR1 (27). Substantial evidence has been provided that C1q and MBL mediate clearance of apoptotic material by macrophages via calreticulin in association with CD91 (12). However, evidence for the involvement of C1qR and CD91 in the uptake of opsonized apoptotic cells by DCs is not available. Because the expression of C1qR and CD91 on DCs is very low, an alternative mechanism might be involved. A recent study reported scavenger receptor A as a second receptor involved in binding and internalization of calreticulin (51). The scavenger receptor A is prominently expressed not only on macrophages but also on DCs and therefore is a potential candidate to be involved in the uptake of apoptotic material opsonized with C1q and MBL. Furthermore, CR1 could be involved since CR1 is present on DCs and has been described to be a receptor for both C1q and MBL (28, 29). Further studies are required to identify the uptake mechanisms
for Clq- and MBL-enhanced engulfment of apoptotic cells by DCs, as well as the receptors involved in the modulation of cytokine production by Clq-opsinized apoptotic cells.

In conclusion, Clq and MBL promote the clearance of apoptotic cells by both macrophages and DCs and opsonization with Clq enhances cytokine production by DCs. Opsonization of apoptotic cells by molecules of the innate immune system may have a critical role in determining the context and consequences of apoptotic cell removal.

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