Roles of ICAM-3 and CD14 in the recognition and phagocytosis of apoptotic cells by macrophages

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Introduction
Understanding the molecular mechanisms underlying phagocytic clearance of apoptotic cells is an important aim of current cell-death research. Intuitively it is expected that, in the absence of rapid, efficient, and non-phlogistic removal of apoptotic cells and bodies, the process of apoptosis would lose its physiological benefits and, through spillage of intracellular macromolecules, the dying cells would wreak havoc in a manner analogous to necrosis, causing damage to neighbouring cells, eliciting inflammation and perhaps also causing autoimmune disease (for reviews, see [1–3]). In the simplest terms, the minimal requirements for clearance of apoptotic cells without losing the value of apoptosis are: (i) changes at the surface of apoptotic cells that occur before loss of plasma membrane integrity; (ii) recognition of one or more of these changes by one or more phagocyte receptors; and (iii) a subsequent phagocytic response that results in the engulfment of the apoptotic cell without release of pro-inflammatory mediators.

We have been studying the clearance of apoptotic leucocytes by the human professional phagocyte, the monocyte-derived macrophage, and have identified: (i) the intercellular adhesion molecule, intercellular adhesion molecule (ICAM)-3, as an important marker of apoptotic leucocytes that interacts with macrophages; (ii) the glycosyl-phosphatidylinositol (GPI)-linked glycoprotein CD14 as a macrophage receptor of apoptotic leucocytes; and (iii) a dichotomy in CD14-dependent macrophage responses, a non-inflammatory response being elicited during clearance of apoptotic leucocytes and a pro-inflammatory response being activated during clearance of the CD14-binding endotoxin, lipopolysaccharide (LPS) (Figure 1).

ICAM-3: a marker of apoptotic leucocytes that is recognized by macrophages

Certain anti-ICAM-3-specific monoclonal antibodies inhibit clearance of apoptotic leucocytes

We have screened a large number of monoclonal antibodies (mAbs), raised against a number of leucocyte cell-surface antigens, to determine their ability to inhibit recognition and phagocytosis of apoptotic leucocytes by monocyte-derived macrophages in vitro. This approach has defined two ICAM-3-specific mAbs, 3A9 and BU68, which are able to inhibit significantly recognition and phagocytosis of apoptotic leucocytes ([4]; O. D. Moffatt, A. Devitt, E. D. Bell, D. L. Simmons and C. D. Gregory, unpublished work) (Figure 2). ICAM-3 is a member of the immunoglobulin supergene family that is expressed by leucocytes [6–10]. It contains five extracellular Ig-like domains and is best known for its ability to interact, via its most membrane-distal domain (domain 1), with the β2 integrin leucocyte function-associated antigen (LFA)-1 (CD11a/CD18) [11–13]. It also binds to the novel leucointegrin α5β2 [14].

Apoptotic leucocytes can interact with macrophages via domain 1 of ICAM-3

Our results ([4]; O. D. Moffatt, A. Devitt, E. D. Bell, D. L. Simmons and C. D. Gregory, unpublished work) include that 3A9 and BU68 inhibit interactions between apoptotic leucocytes and...
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Macrophages by binding to apoptotic cell-associated ICAM-3 rather than to macrophage ICAM-3. The two antibodies recognize similar epitopes, mapped using a series of recombinant ICAM-3-Ig chimeric mutant proteins, on domain 1 of ICAM-3, distinct from those of other domain 1-binding anti-ICAM-3 mAbs that failed to inhibit macrophage/apoptotic-leucocyte interactions (O. D. Moffatt, A. Devitt, E. D. Bell, D. L. Simmons and C. D. Gregory, unpublished work). We noted that the ICAM-3-dependent interaction of apoptotic cells with macrophages was a common feature of leucocyte apoptosis and occurred independently of the nature of the pro-apoptosis stimulus. Furthermore, while apoptotic non-leucocytes (not surprisingly) do not use the ICAM-3-dependent pathway in interacting with macrophages, reconstitution of the pathway is demonstrable in ICAM-3-expressing non-leucocyte transfectants. Thus, following induction of apoptosis by the protein kinase C inhibitor staurosporine, ICAM-3 transfectants of the human embryonic kidney line 293T were recognized more effectively by macrophages than their ICAM-3-negative parental counterparts. Enhanced recognition of ICAM-3-expressing 293T cells was inhibited by 3A9 and BU68, but not by other ICAM-3 mAbs (O. D. Moffatt, A. Devitt, E. D. Bell, D. L. Simmons and C. D. Gregory, unpublished work). Altogether, these results indicate that apoptotic cell-associated ICAM-3 interacts with a macrophage receptor via a specific site in domain 1.

As outlined below, several lines of evidence suggest strongly that ICAM-3 appears in a form on apoptotic cells that is qualitatively different from that on viable cells. Consequently, 'apoptotic' and 'viable' ICAM-3 are recognized by distinct phagocyte receptors.

**Apoptotic cell-associated ICAM-3 fails to bind to LFA-1**

In an *in vitro* model using B cells (derived from a Burkitt lymphoma line) interacting with monocyte-derived macrophages, we have found that whereas viable B cells fail to bind to these macrophages, apoptotic B cells bind effectively and are subsequently phagocytosed (Figure 3). Binding is mediated in significant part by ICAM-3 on the apoptotic cell but occurs independently of

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**Figure 1**

**Schematic view of the roles of ICAM-3 and CD14 in non-phlogistic ('quiet') clearance of apoptotic leucocytes by human monocyte-derived macrophages**

For simplicity, the qualitative change in apoptotic cell-associated ICAM-3 is designated by a change in shading.

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its receptor, LFA-1, on the macrophage. Specifically, mAbs that block interactions between ICAM-3 and LFA-1, be they directed at LFA-1 or ICAM-3, fail to inhibit interactions between apoptotic B cells and macrophages (O. D. Moffatt, A. Devitt, E. D. Bell, D. L. Simmons and C. D. Gregory, unpublished work). On the other hand, ICAM-3/LFA-1 binding is facilitated by 'activation' of LFA-1 and viable B cells bind effectively to LFA-1 after treatment of macrophages with LFA-1-activating mAbs (e.g. KIM 127 [15]). Significantly, viable B cells are inhibited from macrophage-binding by the aforementioned anti-LFA-1 and anti-ICAM-3 mAbs and the bound B cells are not phagocytosed (O. D. Moffatt, A. Devitt and C. D. Gregory, unpublished work).

ICAM-3 appears to be down-regulated at the cell surface during apoptosis

Intriguingly, analysis of cell-surface expression of ICAM-3 by immunofluorescence staining and flow cytometry indicates that ICAM-3 is down-regulated (or at least is rendered inaccessible to mAbs) following activation of apoptosis. The down-regulation of ICAM-3 coincides with loss of plasma membrane phospholipid asymmetry as assessed by binding of annexin V to externalized phosphatidylserine (PS) (O. D. Moffatt, A. Devitt, E. D. Bell, C. L. Simmons and C. D. Gregory, unpublished work).

3A9 and BU68 epitopes are accessible on 'viable' and 'apoptotic' ICAM-3

Immunocytochemical detection of ICAM-3 on viable cells is as efficient with the blocking mAbs 3A9 and BU68 as with non-blockers (e.g. CAL 3.38) (O. D. Moffatt, A. Devitt, E. D. Bell, D. L. Simmons and C. D. Gregory, unpublished work). This suggests that on viable ICAM-3 either the
epitopes recognized by 3A9 and BU68 are not recognized directly by macrophage receptors involved in apoptotic leucocyte clearance, or they are not in a conformation that permits recognition by such receptors.

**Conclusion: mechanisms underlying ICAM-3-dependent interaction between apoptotic leucocytes and macrophages**

ICAM-3 therefore acts as an apoptotic cell marker that is involved in recognition and clearance of apoptotic leucocytes by macrophages, at least in vitro. As yet, these features are shared only with PS, which becomes externalized in the plasma membrane during apoptosis [16]. In common with that of PS, the macrophage receptor for 'apoptotic' ICAM-3 remains elusive. The putative receptor for apoptotic leucocyte-associated ICAM-3 is clearly not LFA-1 and our preliminary evidence indicates that it is not \( \alpha_4\beta_7 \) either. Further studies are required to determine whether any of the other known macrophage receptors for apoptotic cells interact with ICAM-3. In this respect, ICAM-3 is not required for CD14-dependent clearance of apoptotic cells (see below). Studies of the roles of \( \alpha_4\beta_7 \) and CD36 are in progress. Additional work is also required to define the properties of apoptotic ICAM-3 that permit its recognition by macrophages. As it is a heavily glycosylated molecule (it is the most glycosylated of the ICAM family), one possibility is that 'apoptotic' and 'viable' ICAM-3 are differentially glycosylated forms of the molecule that have different receptor preferences.

**CD14: a multifunctional macrophage receptor involved in innate immunity and in clearance of apoptotic cells**

*mAb 61D3 defines CD14 as a macrophage receptor for apoptotic cells*

Earlier studies from this laboratory [17] identified a mAb, 61D3, as an effective inhibitor of the recognition and phagocytosis of apoptotic B cells, T cells and neutrophils by macrophages in vitro (see Figure 2). Using this antibody as a probe, we carried out transient expression cloning in COS cells, and identified the GPI-linked protein CD14 as the 61D3-reactive molecule [18]. When CD14 was expressed in COS cells, which are highly inefficient at phagocytosis of apoptotic cells, such phagocytic activity was improved significantly, although CD14-expressing COS cells remained poorly phagocytic in comparison with macrophages [18]. These results demonstrated that macrophage CD14 mediates interactions with apoptotic cells but that other molecules(s) are likely to play a facilitatory role. The discovery of the participation of CD14 in phagocytic clearance was initially surprising because, at that time, the only well-characterized function of CD14 was as a receptor for LPS in pro-inflammatory anti-bacterial responses [19–21].

**LPS and apoptotic cell-associated ligand(s) interact with similar regions of CD14**

Of several anti-CD14 mAbs tested, we found that 61D3 and MEM-18 were potent inhibitors of the interaction between apoptotic cells and macrophages [17, 18]. Crude epitope mapping studies suggested that these two mAbs recognize similar or identical epitopes on soluble recombinant CD14, binding to regions distinct from those of other, non-inhibitory anti-CD14 mAbs [18] (e.g. 63D3, Figure 2). Previous work has shown that MEM-18-binding to CD14 is dependent on a region spanning amino acids 57–64 [22]. Our results suggest that this same region of CD14 is also required for interaction with apoptotic cells. 61D3 and MEM-18, but not 63D3, were found to inhibit pro-inflammatory cytokine [tumour necrosis factor (TNF)-\( \alpha \)]-release from macrophages in response to LPS [18]. Therefore, similar regions of CD14 are involved in interactions with LPS and with apoptotic cells.

**Differential responses of macrophages to CD14-mediated clearance of LPS and apoptotic cells**

The finding that CD14 apparently interacts with LPS and apoptotic cell-associated ligand(s) through similar regions of the molecule is intriguing, particularly in view of the pro-inflammatory effects of LPS binding to CD14. However, while CD14-dependent macrophage responses to LPS were found to be potently pro-inflammatory, with high levels of TNF-\( \alpha \) being released, the same macrophages failed to produce TNF-\( \alpha \) during CD14-dependent clearance of apoptotic cells [18]. These results indicate that the mode of macrophage signalling initiated at CD14 is ligand-dependent.

**Conclusion: mechanisms underlying CD14-dependent interaction between macrophages and apoptotic cells**

CD14 therefore plays a significant role in the clearance of apoptotic cells by macrophages and
directs the phagocytic response via a non-physiologic route. Amongst the many important questions remaining are: what are the apoptotic cell-associated ligands for CD14, how does CD14 direct macrophage signals along pro-inflammatory or non-inflammatory pathways and can the non-inflammatory pathway(s) actively suppress the inflammatory response [23,24]? The role of CD14 as a pattern-recognition receptor [25] and its proven ability to interact with numerous classes of microbial molecules including, in addition to LPS, muramyl dipeptide [26], soluble peptidoglycan [26,27], uronic acid polymers [28], streptococcal rhamnose-glucose polymer [29], mycobacterial lipoarabinomannan [25], lipoteichoic acid [30] and the yeast cell-wall protein, WI-1 [31], implies that multiple CD14 ligands, amongst several different classes of molecule, may be available on apoptotic cells. Availability of individual ligands may be related to cell lineage and/or of apoptosis. PS is an obvious ubiquitous candidate apoptotic cell-associated CD14 ligand as CD14 has lipid transfer capabilities [32,33] and may be able to interact with cell-associated PS under certain conditions [34]. As yet, we have been unable to demonstrate any role for PS in CD14-dependent clearance of apoptotic cells by human monocyte-derived macrophages. An attractive possibility is that these macrophages make use of the lectin-like activity of CD14 [35] to interact with carbohydrate determinants on apoptotic cells. This would accord with the original studies of macrophage recognition of apoptotic cells, which concluded that altered carbohydrate moieties exposed on the cell surface during apoptosis are recognized by lectin-like macrophage receptors [36]. Others have implicated related mechanisms [37]. Alterations in the carbohydrate composition of the cell surface during apoptosis is likely to be varied and dependent upon multiple molecules. One candidate on leucocytes is the heavily glycosylated ICAM-3. However, like leucocytes, apoptotic non-leucocytes also interact with macrophages by CD14-dependent mechanisms (O. D. Moffatt, A. Devitt, E. D. Bell, D. L. Simmons and C. D. Gregory, unpublished work) indicating that ICAM-3 is not necessary for CD14-dependent clearance.

What is the molecular basis for the differential macrophage responses that follow CD14 ligation? CD14 is GPI-linked and there is much evidence to suggest that signal transduction following CD14 ligation is mediated through its association with 'partner' proteins possessing transmembrane and intracytoplasmic domains [38]. Thus signal transduction following LPS binding to CD14 can be initiated by a membrane complex involving CD14 and the leucointegrin, Mac-1 (CD11b/CD18, CR3) [39]. Apart from NFκB activation [40], the downstream events that signal pro-inflammatory cytokine production following interaction of CD14 with LPS are not yet clear but it seems likely that the signalling pathways that follow interaction of CD14 with either LPS and apoptotic cells diverge at an early stage. An attractive possibility is that the CD14 ligand dictates the constitution of the initial signalling complex at the cell membrane. It will be important to determine whether other molecules on the macrophage surface that are implicated in clearance of apoptotic cells (e.g. α,β3, CD36 [41,42] and ABC-1 [43]) are involved in a putative CD14 signalling complex. Similarly, future studies should be directed towards dissecting the intracellular signalling pathways that direct macrophage phagocytosis of apoptotic cells following their interaction with CD14. It will be of particular interest in this context to determine whether DOCK 180, a homologue of the cell corpse engulfment protein CED-5 of Caenorhabditis elegans [44], plays any role. DOCK 180 interacts with the cytoskeleton through the adaptor CRK, an SH2/SH3 domain-containing protein [45]. It is tempting to speculate that this CED-5 homologue (or a close relative) plays a key role in the phagocytosis of apoptotic cells in higher organisms. Answers to some of the many questions outstanding in this area should be forthcoming in the near future.

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