Human CD14 mediates recognition and phagocytosis of apoptotic cells

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Cells undergoing programmed cell death (apoptosis) are cleared rapidly *in vivo* by phagocytes without inducing inflammation[1]. Here we show that the glycosylphosphatidylinositol-linked plasma-membrane glycoprotein CD14 (refs 2, 3) on the surface of human macrophages is important for the recognition and clearance of apoptotic cells. CD14 can also act as a receptor that binds bacterial lipopolysaccharide (LPS), triggering inflammatory responses[4]. Overstimulation of CD14 by LPS can cause the often fatal toxic-shock syndrome[5,6]. Here we show that apoptotic cells interact with CD14, triggering phagocytosis of the apoptotic cells. This interaction depends on a region of CD14 that is identical to, or at least closely associated with, a region known to bind LPS. However, apoptotic cells, unlike LPS, do not provoke the release of pro-inflammatory cytokines from macrophages. These results indicate that clearance of apoptotic cells is mediated by a receptor whose interactions with 'non-self' components (LPS) and 'self' components (apoptotic cells) produce distinct macrophage responses.

We have shown previously that the monoclonal antibody 61D3 binds to the surface of human monocyte-derived macrophages and markedly inhibits their capacity to interact with various leukocytes undergoing apoptosis[7]. We now use transient expression cloning in COS cells[3] to identify the molecule bearing the epitope that binds to 61D3. The nucleic acid sequence of the complementary DNA identified with this monoclonal antibody (61D3 cDNA) was identical to that of human CD14 (data not shown). Several anti-CD14 mouse monoclonal antibodies and a rabbit polyclonal anti-CD14 antiserum became reactive in cell-binding (not shown) or western blot (Fig. 1) assays when COS cells were transfected with 61D3 cDNA. The protein identified with the 61D3 monoclonal antibody has a relative molecular mass of ~55,000 (M_r 55K), the reported size of CD14, and 61D3 also reacts specifically with purified recombinant CD14 (Fig. 1). These results indicate that the 61D3-binding

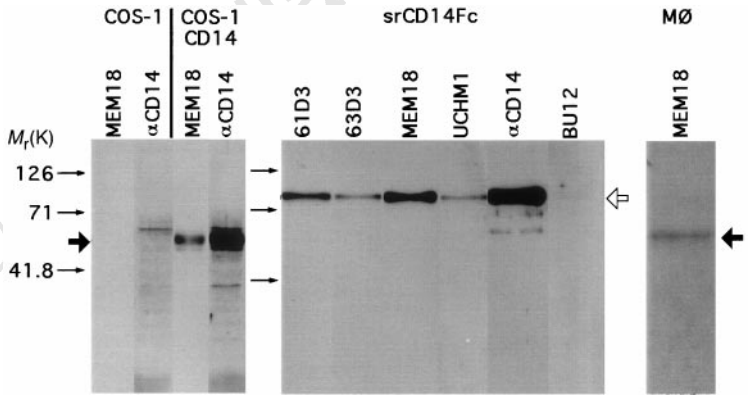


Figure 1 CD14 carries the epitope defined by monoclonal antibody 61D3. Western blot analysis of COS-1 cells transiently transfected with 61D3 cDNA (COS-1/CD14) or control vector (COS-1) (left-hand panel), soluble recombinant CD14-Fc fusion protein (srCD14-Fc) (middle panel), and macrophage (seven-day-old monocyte-derived) lysate (MØ) (right-hand panel). Blots were probed with the indicated monoclonal antibodies or with a polyclonal rabbit anti-human CD14 antiserum (αCD14). The reported M_r of CD14 is 55K (thick black arrows) and that of CD14-Fc is 80K (open arrow).

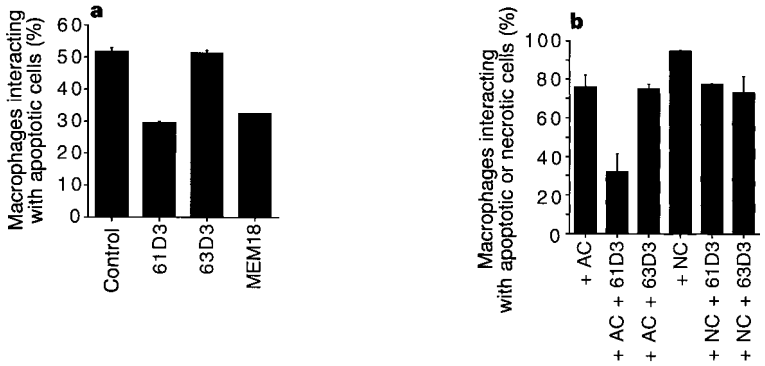
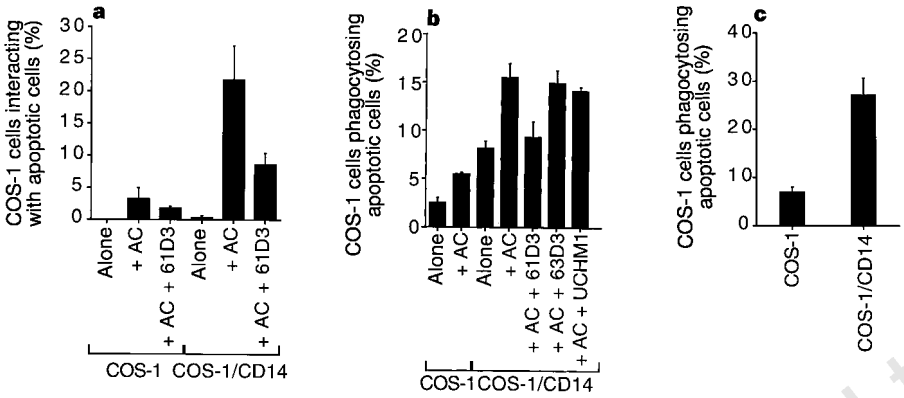
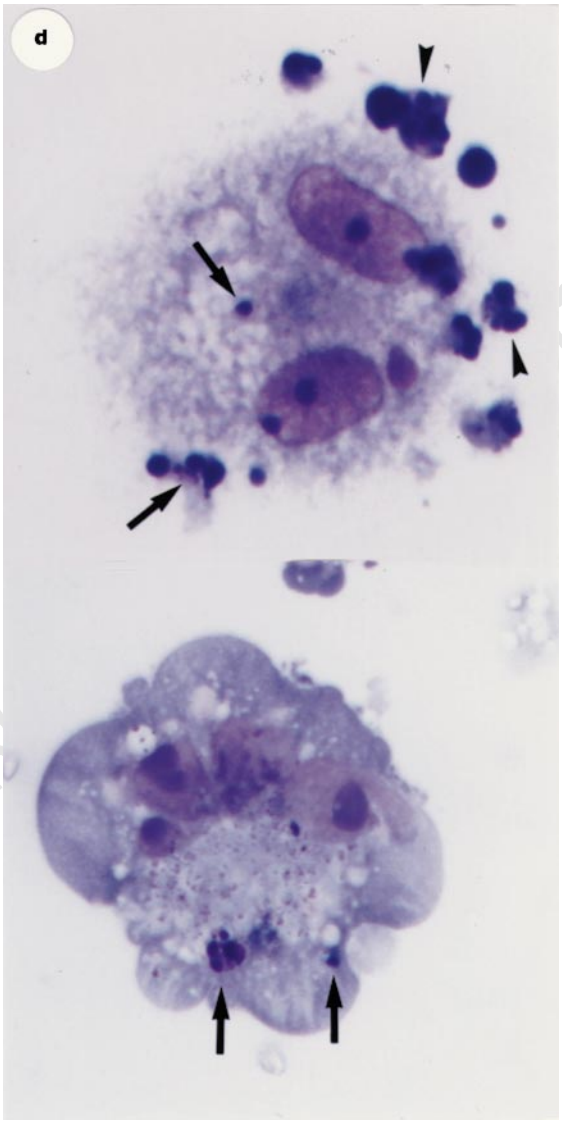


Figure 2 CD14-mediated recognition and phagocytosis of apoptotic cells by macrophages. **a**, Interaction (binding and phagocytosis) of apoptotic Burkitt lymphoma cells with seven-day-old monocyte-derived macrophages, and inhibition of the interaction by monoclonal antibodies 61D3 and MEM-18. **b**, Comparison of the interaction of apoptotic lymphoma cells (AC) and their necrotic counterparts (NC) with macrophages. Inhibition of interaction of AC, but not NC, with macrophages by 61D3.





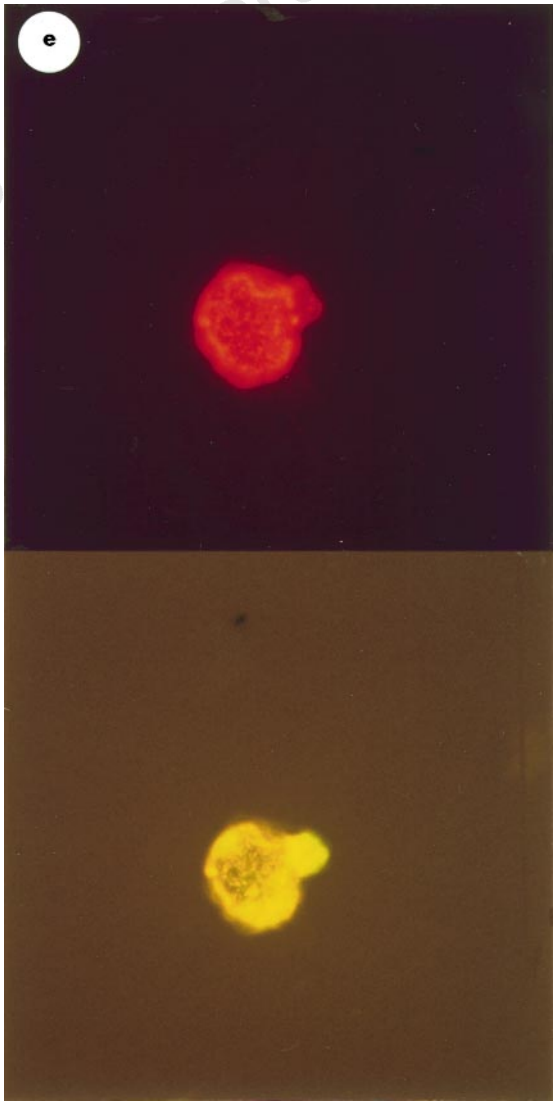


Figure 3 Role of CD14 in recognition and phagocytosis of apoptotic cells. **a**, Increased interaction (binding and phagocytosis) of apoptotic lymphoma cells (AC) with COS-1 cells transiently transfected with CD14 (COS-1/CD14) (31% CD14-positive by immunofluorescence). Inhibition of this interaction by monoclonal antibody 61D3. **b**, Increased phagocytosis of apoptotic cells by COS-1/CD14 (22% CD14-positive) and inhibition of phagocytosis by 61D3. **c**, Fluorescence assay showing enhanced phagocytosis of apoptotic cells by COS-1/CD14 cells. Means ± s.e.m. of two separate experiments. **c**, COS-1/CD14 cells (Jenner-Giemsa stain). Upper panel, binding and phagocytosis of apoptotic lymphoma cells; lower panel, phagocytosis only. Arrowheads indicate surface-bound apoptotic cells; arrows indicate probable phagocytosed apoptotic cells and debris. **e**, CD14-expressing (red immunofluorescence, upper panel) COS-1 cell engaged in phagocytosis of PKH2-labelled apoptotic lymphoma cells (appearing yellow, lower panel).

epitope is carried by CD14 and that no extra cell-surface molecules, macrophage-associated or otherwise, make a necessary contribution to the epitope.

To understand further how CD14 interacts with apoptotic cells, we investigated the ability of additional anti-CD14 monoclonal antibodies to inhibit the interaction of apoptotic lymphocytes with macrophages (Fig. 2). We also tested the ability of CD14 to support binding and phagocytosis of apoptotic cells when transiently expressed in COS cells (Fig. 3). As shown in Fig. 2a, the anti-CD14 monoclonal antibodies 61D3 and MEM-18, but not 63D3, substantially inhibited binding and phagocytosis of apoptotic lymphocytes by seven-day-old monocyte-derived macrophages. The CD14-mediated interaction of lymphocytes with macrophages was specific for apoptotic cells, as viable lymphocytes did not interact with macrophages in this assay (data not shown; see ref. 7) and interaction of macrophages with necrotic cells was not inhibited by 61D3 (Fig. 2b). When transiently expressed on the surface of COS cells, CD14 was found to impart an increase in both binding (Fig. 3a, d) and phagocytosis (Fig. 3b–e) of apoptotic lymphocytes added to the cultures. CD14 also promoted the phagocytosis of apoptotic COS cells present in the cultures (Fig. 3b). Both binding and phagocytosis of apoptotic lymphocytes by CD14-transfected COS cells were markedly inhibited by the 61D3 monoclonal antibody (Fig. 3a, b). In contrast, phagocytosis by CD14 transfectants was unaffected by the anti-CD14 monoclonal antibodies 63D3 and UCHM-1.

A two-colour fluorescence assay using anti-CD14 monoclonal antibody and fluorescent apoptotic cells showed that enhanced phagocytosis occurred specifically in CD14-expressing COS cells (Fig. 3c, e). These results indicate that CD14 supports both binding and phagocytosis of apoptotic cells, and that this activity is not dependent on a macrophage context. As only 25–30% of CD14-expressing COS cells were observed to phagocytose apoptotic cells (despite overexpression of the molecule on COS cells as compared with macrophages; data not shown), we suggest that additional molecules contribute to this activity in the context of the professional phagocyte.

The observation that both 61D3 and MEM-18 inhibited the interaction of apoptotic cells with macrophages indicated that these antibodies may recognize neighbouring or identical regions of CD14. To address this question, we used a competitive enzyme-linked immunosorbent assay (ELISA) of the binding of monoclonal antibodies to recombinant CD14 (Fig. 4). Monoclonal antibodies 61D3, 63D3 and MEM-18 were all strongly reactive with recombinant CD14 in the ELISA (Fig. 4a). However, whereas 61D3 and MEM-18 strongly inhibited the binding of further 61D3 to the recombinant molecule, 63D3, an antibody that lacked blocking activity in the macrophage assay, did not (Fig. 4b). Conversely, 63D3, but not 61D3 or MEM-18, strongly inhibited the binding of further 63D3 to recombinant CD14 (data not shown). Therefore, the activity of CD14 in regulating the interaction of apoptotic cells with macrophages depends on a molecular site that is identical to, or maps close to, the epitope(s) defined by 61D3 and MEM-18.

MEM-18 is known to inhibit potently the binding of CD14 to its prototypic ligand, LPS[8]. Thus the binding sites on CD14 for LPS and for apoptotic cells are either identical or closely associated. Apoptotic cells are thought to be cleared by macrophages without the induction of pro-inflammatory responses. However, LPS clearance through CD14 activates macrophages to release pro-inflammatory mediators. We therefore determined whether pro-inflammatory cytokine release was triggered in macrophage populations that interact with apoptotic cells through CD14. Figure 5 shows the production of tumour-necrosis factor-α (TNF-α) by seven-day-old monocyte-derived macrophages, which use CD14 to interact with apoptotic cells (Fig. 2a). These macrophage cultures did not produce TNF-α in response to apoptotic cells. In contrast, exposure of the same seven-day-old macrophage cultures to LPS resulted in significant TNF-α release (Fig. 5). TNF-α production, like uptake of apoptotic cells, was inhibited by 61D3 and MEM-18 but not by 63D3 (Fig. 5), indicating that apoptotic cells and LPS interact with closely associated or identical regions of CD14. Nevertheless, LPS promotes pro-inflammatory cytokine release whereas apoptotic cells do not.

These findings establish a previously unrecognized function for

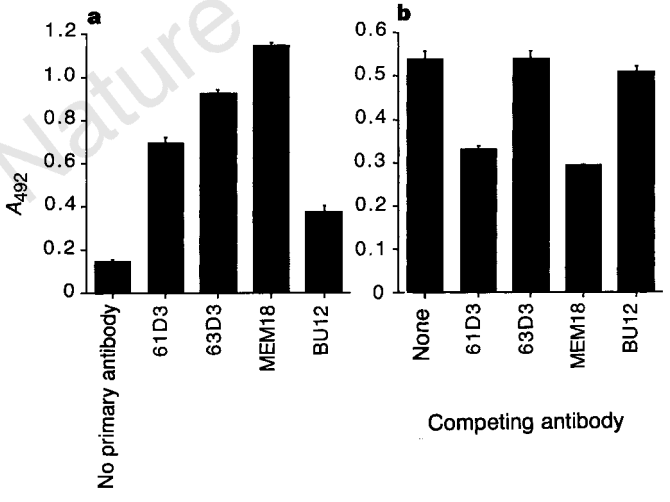


Figure 4 Epitope mapping of the 61D3 monoclonal antibody by ELISA, using immobilized soluble recombinant CD14-Fc fusion protein. **a**, CD14-binding of murine anti-CD14 monoclonal antibodies 61D3 (10 μg ml^{-1}), 63D3 (10 μg ml^{-1}), MEM-18 (1:50) and BU12 (CD19, 10 μg ml^{-1}), visualized using anti-mouse-Ig-HRP. **b**, Competition for binding to CD14 of biotinylated 61D3 (5 ng ml^{-1}) by the indicated monoclonal antibodies. Binding was visualized using streptavidin-HRP. In the experiment shown, binding of 61D3 to CD14 was reduced by 39% and 46% in the presence of 61D3 and MEM-18, respectively.

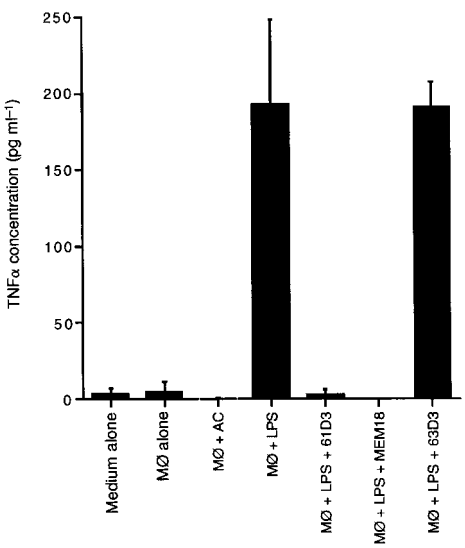


Figure 5 Production of TNF-α by macrophages stimulated with LPS but not apoptotic cells. Macrophages (MØ) were incubated either alone or in the presence of apoptotic lymphoma cells (AC) or LPS (5 ng ml^{-1}) or LPS with monoclonal antibodies 61D3, MEM-18 or 63D3, for 3 h before assaying the concentration of TNF-α present in the culture supernatants. Background levels of TNF-α present in the assay medium (medium alone) are shown for comparison.

CD14 and extend to five, with the vitronectin receptor $\alpha_v\beta_3$ integrin[9], CD36 (ref. 10), class A scavenger receptor[11] and the ATP-binding-cassette transporter ABC-1 (ref. 12), the number of known macrophage surface molecules involved in the removal of apoptotic cells. The apparent abundance of phagocyte receptors for apoptotic cells may illustrate the vital importance of the clearance process. In the absence of clearance, or when it is overwhelmed[13], secondary necrosis occurs, with the catastrophic consequences of inflammation and damage to normal tissue.

Although interactions between apoptotic cells and phagocytes are most simply envisaged as receptor–ligand pairing at the surface of apposed cells, intermediate molecules such as thrombospondin, which binds $\alpha_v\beta_3$ integrin and CD36 through separate epitopes and appears to form a molecular 'bridge' between macrophages and apoptotic cells[10], may also be involved. CD14 interacts with several constituents of microorganisms, including, in addition to LPS, muramyldipeptide and soluble peptidoglycan[14], uronic acid polymers[15], streptococcal cell-wall polysaccharides[16], mycobacterial lipoarabinomannan[17], and a yeast cell-wall protein, WI-1 (ref. 18). Cell-wall components of both Gram-positive and Gram-negative bacteria[17,19] bind to CD14. Potentially, therefore, several categories of molecule may become available as CD14 ligands on the surface of apoptotic cells. None of the known cell-surface components of apoptotic cells has been linked specifically with any of the known macrophage receptors for apoptotic cells, although carbohydrate[20,21] and phospholipid[22] changes at the cell surface during apoptosis may yield important ligands. The anionic phospholipid phosphatidylserine is redistributed to the outer leaflet of the plasma membrane during apoptosis and is involved in the recognition of apoptotic cells by certain classes of macrophage[22]. Phosphatidylserine may provide an apoptotic-cell-associated ligand for CD14, as suggested by a study in which the 61D3 monoclonal antibody blocked the (phosphatidylserine-dependent) interaction of lipid-symmetric erythrocytes with macrophages[23]. CD14 interacts with anionic phospholipids[24] and acts as a lipid-transfer protein that may play a role in lipid-A-dependent LPS responses[25]. In addition, the ability of CD14 to bind polysaccharide-containing molecules[26] indicates that its interaction with apoptotic cells may occur through a lectin-like activity. This would concur with the proposal[20] that the recognition of apoptotic cells by phagocytes involves interactions between sugars on the altered apoptotic-cell surface and novel macrophage-surface-associated lectins.

We conclude that CD14 is a multifunctional receptor which, in addition to its known role as an innate immune receptor for 'infectious non-self' components[17], also interacts with 'apoptotic self' components. Further work is required to find the important CD14 ligand(s) on apoptotic cells and to define the mechanism underlying the different macrophage responses—non-inflammatory apoptotic-cell clearance versus pro-inflammatory cytokine release—to CD14 ligation. It will also be interesting to determine whether apoptotic cell/CD14 interactions generate signals that actively suppress pro-inflammatory signals, as suggested by recent work[27,28]. The differential signalling following ligation of CD14 by LPS and apoptotic cells is consistent with the proposal that ligand-induced association of this glycosylphosphatidylinositol-linked receptor with transmembrane proteins[4,29] is a likely mechanism of initiation of intracellular signal-transduction cascades. Our finding that CD14 does not appear to function as efficiently in the COS cell as it does in the macrophage (Figs 2 and 3) also indicates that additional macrophage molecules may cooperate with CD14 to effect the binding and phagocytosis of apoptotic cells. □

Methods

Expression cloning. Transient expression cloning was carried out as described[3] using a cDNA library in plasmid pCDM8, from the human promyelocytic leukaemia cell line HL60 stimulated with dibutyryl cAMP. After three rounds of panning using the 61D3 monoclonal antibody[30], plasmid DNA was purified from 12 individual clones and transiently expressed in COS-1 cells. Reactivity with 61D3 monoclonal antibody was detected in one clone by indirect immunofluorescence, and complete sequencing of this clone (61D3 cDNA) was carried out by the Biopolymer Synthesis and Analysis Unit, University of Nottingham Medical School, using a Perkin Elmer/ABI 373A sequencer. The 61D3 cDNA sequence was identical to that of human CD14 cDNA, as determined using the GCG package to search the GenBank and EMBL nucleotide databases (GCG: http://ictsg10.unil.ch:8080/w2h/; EMBL: gopher://biox.embnet.unibas.ch:13021/77/.index/embl/index; GenBank: http://www.ncbi.nlm.nih.gov/web/genbank/index.html).

Immunofluorescence staining, flow cytometry and immunoblotting. The following monoclonal antibodies were used: 61D3, 63D3 (ref. 30), MEM-18 (Monosan), UCHM-1 (Sigma), TÜK4 (Dako) (all anti-CD14) and BU12 (anti-CD19). Indirect immunofluorescence staining was carried out on COS-1 cells in suspension and flow-cytometric analysis was performed using a FACScan (Becton Dickinson). For immunoblotting, cell lysates or protein preparations were separated using one-dimensional 10% SDS–PAGE. Proteins were blotted to PVDF membrane (Immobilon P, Millipore) and detected using the ECL system (Amersham).

Production of soluble recombinant CD14-Fc and anti-CD14 antiserum. CD14 cDNA[3] subcloned into the plg vector (Invitrogen) was transfected into COS-1 cells. Expression and secretion of the CD14-Fc fusion protein was allowed to proceed for 5 days before affinity purification, using a protein A Sepharose column (Pharmacia Biotech), from the culture supernatant. Polyclonal anti-CD14 antibody was raised in rabbits immunized with CD14-Fc fusion protein. Anti-Fc reactivity was removed by absorption with human IgG.

Macrophage and COS-1 recognition/phagocytosis assays. Macrophages were obtained from peripheral blood monocytes of normal healthy donors. Seven-day-old monocyte-derived macrophages cultured on multiwell glass slides (Hendley, Essex, UK) were incubated with apoptotic cells for 1 h in the presence or absence of monoclonal antibodies as described[7]. Macrophages were washed extensively to remove unbound apoptotic cells, fixed in methanol and stained with Jenner-Giemsa. Cultures were scored by light microscopy for the percentage of macrophages interacting with apoptotic cells, both surface-bound and phagocytosed, using established criteria[7]. Interaction of apoptotic leukocytes with macrophages from donors used in these experiments was routinely inhibited by 61D3 monoclonal antibody (>90% of donors).

COS-1 cells were transfected as described[3] with 61D3 cDNA in plasmid pCDM8 and cultured on multiwell glass slides for 3 days. Mock-transfected COS-1 cells were used as controls. Co-incubation with apoptotic cells, removal of unbound cells and Jenner-Giemsa staining were carried out as for the macrophage recognition/phagocytosis assay. COS cells interacting (that is, binding and/or phagocytosing) with two or more apoptotic cells were scored as positive.

To assess phagocytosis of apoptotic cells separately from cell–cell binding, COS-1 cells with associated apoptotic cells were treated with 0.05% trypsin/0.02% EDTA for 2 min. Cytocentrifuge smears of the resultant cell suspensions were made and stained with Jenner-Giemsa. In these preparations, no apoptotic cells bound to the surface of COS-1 cells were apparent. Simultaneous assessment of phagocytic activity and CD14 expression by COS-1 cells was achieved using a fluorescence assay. Before the induction of apoptosis, lymphocytes were labelled using the PKH2 General Cell Linker Kit (Sigma). Labelling was carried out using 5 μM PKH2 for 5 min. COS-1 cells transfected with 61D3 cDNA were exposed to PKH2 (green)-labelled apoptotic lymphocytes, subjected to trypsin/EDTA treatment as described above, and stained by immunofluorescence for surface CD14 (red) using 63D3 and phycoerythrin-conjugated goat anti-mouse IgG (Sigma). Assays were scored by fluorescence microscopy of 1,000 CD14-positive and 1,000 CD14-negative cells from individual transfections.

In recognition/phagocytosis assays, the group I Burkitt lymphoma cell line Mutu I, induced into apoptosis by 16 h incubation with 1 μg ml^{-1} of the calcium ionophore ionomycin (Calbiochem)[7], was used as the source of apoptotic cells. Viable cells from this line fail to interact with macrophages[7] or COS-1 cells (data not shown) in these assays. Necrosis was induced by heat treatment of Mutu I cells at 56 °C for 40–45 min until they were 60–70% trypan-blue-positive. All binding and phagocytosis assays were carried out in the presence of human serum. Where indicated, monoclonal antibodies were

included at the following concentrations: 61D3 and 63D3, 80 μg ml^{-1}; MEM-18 and UCHM-1, 1:20 dilution of manufacturer's preparations.

CD14 epitope mapping. Binding of monoclonal antibodies to CD14 was assessed by ELISA using CD14-Fc (5 μg ml^{-1}) captured with immobilized sheep anti-human IgG (The Binding Site, 10 μg ml^{-1}). Bound antibody was visualized using sheep anti-mouse immunoglobulin (The Binding Site) absorbed against normal human serum and conjugated with horseradish peroxidase (HRP). In competition assays, binding of biotinylated 61D3 or biotinylated 63D3 to captured CD14-Fc in the presence or absence of competing monoclonal antibodies was detected using streptavidin–HRP (Southern Biotechnology), *o*-phenylenediamine dihydrochloride substrate and an A_{492} read-out.

TNF-α assay. TNF-α was measured in macrophage-culture supernatants after treatment of macrophages for 3 h with either LPS (*Escherichia coli* O111:B4, Sigma) or apoptotic cells in the presence or absence of monoclonal antibodies. TNF-α was assayed by ELISA using matched-pair capture and biotinylated detection antibodies (R&D Systems).

Unless otherwise stated, all data shown are means ± s.e.m., with experiments representative of at least three similar experiments.

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Inhibition of oxytocin receptor function by direct binding of progesterone

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The steroid hormone progesterone (P_4) is essential for establishing and maintaining pregnancy in mammals[1–3]. One of its functions includes maintenance of uterine quiescence by decreasing uterine sensitivity to the uterotonic peptide hormone oxytocin[3–5]. Although it is generally held that steroid hormones such as P_4 act at a genomic level by binding to nuclear receptors and modulating the expression of specific target genes[6], we show here that the effect of P_4 on uterine sensitivity to oxytocin involves direct, non-genomic action of P_4 on the uterine oxytocin receptor (OTR), a member of the G-protein-coupled receptor family. P_4 inhibits oxytocin binding to OTR-containing membranes *in vitro*, binds with high affinity to recombinant rat OTR expressed in CHO cells, and suppresses oxytocin-induced inositol phosphate production and calcium mobilization. These effects are highly steroid- and receptor-specific, because binding and signalling functions of the closely related human OTR are not affected by P_4 itself but by the P_4 metabolite 5β-dihydroprogesterone. Our findings provide the first evidence for a direct interaction between a steroid hormone and a G-protein-coupled receptor and define a new level of crosstalk between the peptide- and steroid-hormone signalling pathways.

Because P_4-induced reduction in uterine oxytocin binding occurs in the absence of protein synthesis[5] and is not accompanied by a significant decrease in OTR gene expression[7], we investigated whether P_4 could affect OTR function through a non-genomic mechanism. As shown in Fig. 1a, addition of P_4 *in vitro* to membranes derived from a parturient rat uterus inhibited binding of the OTR-specific ligand OTA[8] (inhibition constant, $K_i = 16 \pm 2$ nM; maximal inhibition was 59 ± 6% at 1 μM). As circulating P_4 concentrations in the rat reach 500 nM during pregnancy[9], our effective P_4 concentrations were within physiological range. Inhibition was due to a decrease in binding capacity ($B_{max} = 0.80 \pm 0.02$, compared with 0.39 ± 0.01 pmol mg^{-1} protein) without an effect on binding affinity ($K_D = 0.23 \pm 0.07$ compared with 0.20 ± 0.03 nM) (Fig. 1b).

P_4 also induced a dose-dependent reduction of specific oxytocin binding to recombinant rat OTR expressed in CHO cells ($K_i = 15 \pm 2$ nM; Fig. 1c and d). By contrast, P_4 had no effect on binding of the V_{1a} vasopressin-receptor-specific ligand LVA[10] to recombinant V_{1a} receptor ($V_{1a}R$) (Fig. 1c). Similar results were obtained with ^{3}H-labelled natural agonists ($K_i = 19 \pm 3$ nM for [^{3}H]oxytocin; no inhibition of [^{3}H]vasopressin binding). P_4 coupled to bovine serum albumin also reduced oxytocin binding

Table 1 Effects of P_4 on [^{3}H]AVP binding to OTR and $V_{1a}R$

Receptor	P_4	B_{max} (pmol mg^{-1} protein)	Inhibition by P_4 (%)
OTR	–	0.96 ± 0.08	
	+	0.37 ± 0.03	61 ± 6
$V_{1a}R$	–	1.32 ± 0.05	
	+	1.31 ± 0.04	1 ± 3

Results are the means ± s.e. of triplicate determinations.