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Interaction of human peripheral blood monocytes with apoptotic polymorphonuclear cells

Tomasz P. Mikołajczyk,[1] Joanna E. Skrzeczyńska-Moncznik,[1] Mirosław A. Zarębski,[2] Ewa A. Marewicz,[1] Anna M. Wiśniewska,[3] Magdalena Dzięba,[1] Jerzy W. Dobrucki[2] and Juliusz R. Pryjma[1]

[1]*Department of Immunology,* [2]*Division of Cell Biophysics, and* [3]*Department of Biophysics, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa, Cracow, Poland*

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Correspondence: Tomasz P. Mikołajczyk, Department of Immunology, Jagiellonian University, Gronostajowa 7, 30-387 Cracow, Poland. Email: to.mik@interia.pl
Senior author: Juliusz R. Pryjma
email: juliusz.pryjma@uj.edu.pl

Summary

Macrophages have the potential to recognize apoptotic neutrophils and phagocytose them while the same function for monocytes is uncertain. In fact, early findings indicated that monocytes started to phagocytose neutrophils on the third day of differentiation to macrophages. Here we show, using flow cytometry and confocal microscopy, that peripheral blood monocytes phagocytose apoptotic but not freshly isolated granulocytes. Recognition of apoptotic cells is predominantly connected with CD16$^+$ monocytes (CD14^{high} CD16$^+$ and CD14^{dim} CD16$^+$) and requires CD36. Clearance of apoptotic polymorphonuclear leucocytes appears to be independent of the CD14 mechanism. Uptake of apoptotic Jurkat T cells by monocytes is CD14 and CD36 dependent. Liposomes containing phosphatidyl-L-serine reduce binding of apoptotic polymorphonuclear leucocytes. Lipopolysaccharide-activated subpopulations of monocytes while in contact with apoptotic cells produce more anti-inflammatory cytokine interleukin-10 whereas the production of pro-inflammatory cytokines, tumour necrosis factor-α and interleukin-1β is reduced.

Keywords: apoptotic Jurkat T cells; apoptotic polymorphonuclear leucocytes; CD14; CD16; macrophages; monocytes; phagocytosis

Introduction

Human polymorphonuclear leucocytes (PMN) form the first line of defence to protect the host against bacterial and fungal infections. They have a short half-life in the circulation but this can increase several-fold when neutrophils migrate into inflamed tissue.[1] The majority of infiltrating PMN become apoptotic at a later stage of inflammation. Recognition and ingestion of senescent neutrophils by macrophages protects tissue from the noxious contents of dying cells and promotes the resolution of inflammation.[2] In fact, monocyte-derived macrophages efficiently phagocytose apoptotic neutrophils.[3] Furthermore, phagocytosis of apoptotic PMN by macrophages was shown to increase secretion of transforming growth factor-β and inhibited tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-10 production.[4] This is considered an important mechanism reducing the intensity of the inflammatory response.[4–6] In contrast to macrophages, peripheral blood monocytes (MO) are usually considered as not able to engulf apoptotic cells but acquire that ability after differentiation into macrophages.[3,7,8] However, some data seem to contradict this and show that circulating MO may recognize apoptotic cells.[9–11] Monocytes express receptors that may be involved in the recognition of apoptotic cells.[12–17] Peripheral blood MO are heterogeneous and they can be separated into three distinct population subsets: CD14^{high} CD16$^-$, CD14^{high} CD16$^+$ and CD14^{dim} CD16$^+$. The MO population CD14^{dim} CD16$^+$ are regarded as pro-inflammatory because after lipopolysaccharide (LPS) stimulation they produce higher levels of TNF-α while the production of IL-10 is low or absent.[18,19] The population with high expression of CD14, CD14^{high} CD16$^+$, is the main producer of IL-10 suggesting anti-inflammatory properties for these cells.[20] Previously, it was observed that LPS-stimulated MO while in contact with apoptotic cells produce more anti-inflammatory cytokine IL-10 whereas the production of pro-inflammatory cytokines, like TNF-α and IL-1β, was somewhat reduced.[9,21] Similar results were obtained when the anti-CD36 monoclonal antibody (mAb) was used, suggesting that this receptor is involved in anti-inflammatory effects and can be engaged in apoptotic cell recognition.[15] Interleukin-10 has potent anti-inflammatory properties and may effectively impair antibacterial host defence *in vivo*.[22] It has been shown to

reduce the ability of phagocytes to engulf and kill pathogens.[23,24] Therefore, we measured the ability of freshly isolated MO to phagocytose apoptotic PMN and we also sought the potential of sorted MO to phagocytose apoptotic PMN.

Materials and methods

Monocytes

Peripheral blood mononuclear cells (PBMC) were isolated by standard Ficoll–Paque (GE Healthcare, Uppsala, Sweden) gradient centrifugation from citrate-treated blood of healthy donors. The PBMC were further subjected to counterflow centrifugation (Beckman JE-6B elutriaton system equipped with Sanderson separation chamber; Beckman Instruments Inc., Palo Alto, CA) to obtain highly purified MO as previously described.[25] The MO were suspended in the complete medium (see below) and were used immediately after isolation. In some experiments MO were labelled with vital dye PKH67 (Sigma, Saint Louis, MO), a green fluorescent lipophilic dye that stably inserts into the cell membrane.[26] Labelling procedure was performed according to the manufacturer's instructions. Washed cells were resuspended in the supplied Diluent C and immediately combined with an equal volume of PKH67 in the same diluent. Final staining conditions were 1×10^7 cells/ml in 2×10^{-6} M PKH67. After 4 min at room temperature an equal volume of fetal bovine serum (BioWest, Nuaillé, France) was added to stop the staining reaction. Labelled cells were washed three times and suspended in a complete medium.

Human monocyte-derived macrophages (hMDM)

The hMDM were obtained from PBMC that were plated at a concentration 3×10^6/well in 24-well plates (Sarsted, Newton, NC) in RPMI-1640 (Gibco, Paisley, UK) supplemented with 2 mM L-glutamine, 50 μg/ml gentamycin (Sigma) and 10% heat-inactivated human autologous serum. After 2 hr, non-adherent PBMC were removed by washing and adherent cells were cultured in the same medium for 7 days. Medium was changed every 2 days.

Polymorphonuclear cells

Polymorphonuclear cells were isolated from erythrosediments (left after PBMC isolation). These were sedimented for 20 min at room temperature in 1% polyvinyl alcohol (Merck, Hohenbrunn, Germany) in NaCl. The PMN were collected from the upper part and any contaminating red blood cells were removed by hypotonic lysis (20 seconds). Isolated PMN were labelled with vital dye PKH26 (Sigma) according to the manufacturer's protocol (see above) and used immediately (fresh, non-apoptotic) or after 24 hr of incubation at 37° in a humidified atmosphere containing 5% CO_2 in complete medium (aged, apoptotic). The percentages of annexin V-positive cells within fresh and apoptotic PMN were in the ranges 1–4% and 45–85%, respectively.

Jurkat T cells

For some experiments, Jurkat T cells labelled with vital dye PKH26 (Sigma) were used. To induce apoptosis, cells were irradiated with 254-nm ultraviolet light for 10 min and cultured for 24 hr at 37° in humidified atmosphere containing 5% CO_2 in complete medium. The percentage of annexin V-positive cells was 48–66%.

Flow cytometry determination of apoptosis

The apoptosis of PMN and Jurkat T cells was evaluated by an annexin V-binding assay. The cells were suspended in annexin V-binding buffer and incubated at room temperature for 15 min with fluorescein isothiocyanate (FITC)-conjugated annexin V (Becton Dickinson, Pharmingen, San Diego, CA) to detect phosphatidylserine expression on the outer cell membrane layer. After washing cells they were analysed on the FACScan flow cytometer (Becton Dickinson).

Isolation of genomic DNA and DNA gel electrophoresis

DNA was isolated from 5×10^6 PMN pelleted from centrifugation at 360 ***g***. Lytic buffer [0·01 M Tris–HCl (pH 7·8), 0·005 M ethylenediaminetetraacetic acid (EDTA), 0·5% sodium dodecyl sulphate; 0·35 ml per cellular pellet] was added; the sample was mixed vigorously and incubated at 65° for 1 hr to obtain viscous, clear cell lysates. The lysates were then treated with 2 μl RNase A (10 mg/ml) at 37° for 1 hr and 2 μl proteinase K (20 mg/ml) at 50° for 1 hr and extracted twice with an equal volume of DNA–phenol (phenol–chloroform–isoamyl alcohol 25 : 24 : 1, pH 8·0 ± 0·2) and centrifuged at 14 000 ***g*** for 1 min at 4°. DNA in the aqueous phase was precipitated at −20° in 0·3 M sodium acetate (pH 5·2) and ice-cold 96% ethanol. Precipitates were pelleted by centrifugation (14 000 ***g***, 15 min at 4°), washed with ice-cold 70% ethanol, dried and dissolved in water. Concentration and purity were verified by a Spectrophotometer NanoDrop ND-1000 (NanoDrop, Wilmington, DE). For electrophoresis DNA samples were dissolved in gel loading buffer (25% Ficoll 400, 10 mM EDTA, 0·01% bromophenol blue, 0·01% xylene cyanol; six times concentrated) and the samples were incubated at 65° for 1 hr. Aliquots containing 8 μg DNA were loaded per slot. Samples were subjected to electrophoresis in 1·5% agarose gel containing ethidum bromide 0·5 μg/ml in Tris–HCl–acetate–EDTA buffer at 5 V/cm for 90 min. DNA was visualized

by ultraviolet light detection and then photographed (Vilber lourmat, Torcy, France) using BIOCAPTMW software. All chemicals were purchased from Sigma.

Cell culture conditions

Cells were cultured in RPMI-1640 (Gibco) complete medium supplemented with 10% fetal bovine serum (Bio-West), 2 mM L-glutamine and 50 μg/ml gentamycin (Sigma). All cultures were incubated at 37° in a humidified atmosphere containing 5% CO_2. Monocytes after purification were mixed with PMN (apoptotic or fresh) at a 1 : 1 ratio and incubated in Falcon tubes (Becton Dickinson) for up to 4 hr in complete medium. In some experiments MO were preincubated with cytochalasin D (Sigma), 30 min before mixing with PMN at a final concentration of 1 μg/ml, 0·5 μg/ml and 0·05 μg/ml. In some cases cocultures MO and PMN were incubated in the presence or absence of liposomes containing phosphatidylcholine (PC, type XVI-E from egg yolk), PC and phosphatidyl-L-serine (PS, from bovine brain) or phosphatidylglycerol (PG, dimyristoylphosphatidylglycerol) at the liposome concentrations 10 μM, 1 μM and 0·1 μM. All phospholipids were purchased from Sigma. To study the involvement of receptors in the recognition of apoptotic cells, MO were also pretreated with 100 μg/ml mAb anti-CD36 (clone SMO, Ancell, Bayport, MN), 100 μg/ml mAb anti-CD14 (MEM-18, Exbio, Praha, Czech Republic) or mAb anti-CD14 (clone RMO52; Immunotech, Marseille, France) at a 1 : 5 dilution, before mixing with apoptotic PMN. To block non-specific effects of anti-CD36 MO were preincubated before adding mAbs with human immunoglobulin G (Sigma) according to the recommendation provided by the manufacturer.

Preparation of liposomes

Liposomes made of PC, PC and PS (mixed at 50 : 50 molar ratio) or PC and PG (mixed at 50 : 50 molar ratio) were prepared by the method of Subczynski *et al.*[27] Briefly, chloroform solutions of lipids were mixed to attain the desired compound concentrations, chloroform was then evaporated with a stream of nitrogen, and the lipid film on the bottom of the test tube was thoroughly dried under reduced pressure (about 0·1 mmHg) for 12 hr. Then, 0·5 ml of phosphate-buffered saline (PBS, pH 7·0) was added to the dried film and vortexed vigorously. The final concentration of lipids in PBS was 1 mM. Unilamellar liposomes were then prepared by extrusion of multilamellar liposomes according to MacDonald *et al.*[28] The multilamellar liposome suspension was freeze–thawed several times and then extruded 13 or 15 times through a LiposoFast extruder equipped with polycarbon filters of 100-nm diameter (both from Avestin, Inc., Ottawa, ON, Canada).

Cell sorting

To isolate MO subpopulations, cells were stained with mAbs anti-CD14-PC5 (clone RMO52; Immunotech) and anti-CD16-FITC (clone DJ130c; DAKO, Glostrup, Denmark) and using a MoFlow cell sorter (Cytomation, Denver, CO) were sorted into $CD14^{high}$ $CD16^{-}$, $CD14^{high}$ $CD16^{+}$ and $CD14^{dim}$ $CD16^{+}$ subpopulations. The purity of sorted subpopulations was approximately > 96%, > 80% and > 80%, respectively. In some experiments MO were stained with mAb anti-CD16-FITC and sorted into $CD16^{-}$ and $CD16^{+}$ subsets.

Induction of cytokine production by monocytes

Monocytes and their subpopulations obtained by cell sorting were cultured in 96-well flat-bottom plates (Sarstedt) with 1×10^5 cells/well in complete medium. In some cultures apoptotic PMN were added at a ratio of 1 : 1. Cells were stimulated with LPS from *Escherichia coli* (Sigma) at a final concentration of 1 ng/ml. Supernatants from 18-hr cultures were collected, centrifuged to remove particulate debris and stored at −20° until measurement.

Measurement of cytokine concentration

The levels of IL-10, TNF-α and IL-1β released by subpopulations of MO were determined by an enzyme-linked immunosorbent assay using OptEIA Sets (BD, Pharmingen) according to the instructions provided with each set of antibodies.

Flow cytometry analysis of interaction between hMDM and PMN

Human monocyte-derived macrophages were overlaid with freshly isolated or apoptotic PKH26 labelled PMN at a ratio of 1 : 5. Cocultures were incubated for varying periods of time at 37° in 5% CO_2 in humidified atmosphere. Non-ingested PMN were removed by washing the cultures three times in cold PBS. Macrophages were detached by a routine trypsinization procedure with trypsin/EDTA solution (Sigma) and collected for flow cytometry analysis.

Flow cytometry analysis of interaction between MO and PMN or Jurkat T cells

The ability of MO and their subsets to interact with PMN labelled with PKH26 was evaluated by flow cytometry. The analysis was performed using the FACScan flow cytometer and CELLQUEST software (Becton Dickinson). The number of PKH26-stained PMN within the MO gate defined by forward and side scatter signals or within PKH67-labelled MO was considered as an

indication of firm binding or engulfment. If PKH26-labelled apoptotic Jurkat T cells were used, the MO were labelled with PKH67 and the proportion of double-positive cells within the green population was measured.

Fluorescence confocal microscopy

Images of fluorescence of PKH67 and PKH26 in membranes of MO and PMN were recorded using a Bio-Rad MRC1024 confocal system, interfaced with a Nikon Diaphot (Nikon, Amsterdam, Netherlands) inverted microscope, 100 mW argon ion laser (ILT, Salt Lake City, UT), 25 mW krypton/argon laser (ALC, Salt Lake City, UT), and equipped with three detection channels, a Nikon PlanApo 60 × oil immersion lens (NA 1.4) and a microscope stage microincubator (Life Science Resources, Cambridge, UK). The fluorescence detection conditions for PKH67 and PKH26 were as follows: simultaneous excitation at 488 nm, primary dichroic 510DCLP (VHS filter block), secondary dichroic 565DRLP (A2 filter block), green emission filter 540DF30, red emission filter RG630. eight-bit, 512 × 512 images were collected at a rate of 1/second or 0·3/second. Images were processed using LASERSHARP software (Bio-Rad Microscience, Hemel Hempstead, UK) and IMAGEJ (http://rsb.info.nih.gov/ij/). Cocultures of MO with apoptotic or freshly isolated PMN were incubated for 3–4 hr in a culture medium under standard growth conditions (humidified air with 5% CO_2). Subsequently, the cell suspension was transferred into a custom-built chamber, placed in a microincubator and cells were allowed to settle on the surface of a coverslip for 30 min. In some experiments cells were fixed with 1% formaldehyde.

Statistical analysis

Differences of means were compared using Student's *t*-test and were considered significant when $P < 0{\cdot}05$.

Results

Human MDM efficiently bind apoptotic neutrophils

Human MDM are well known as cells able to recognize and phagocytose apoptotic PMN.[2,3] To evaluate the ability of macrophages to interact with apoptotic cells, we employed PKH26-labelled PMN. Seven-day cultured hMDM were overlaid with freshly isolated or apoptotic PMN (Fig. 1b). At different times the non-adherent cells in the coculture were washed out and hMDM were detached by trypsinization and analysed by flow cytometry. As shown, 42% of hMDM contained PKH26 label after 3 hr of coculture with apoptotic PMN while freshly isolated neutrophils were only weakly bound by macrophages (Fig.1a). This finding is in agreement with previous reports.[3–6]

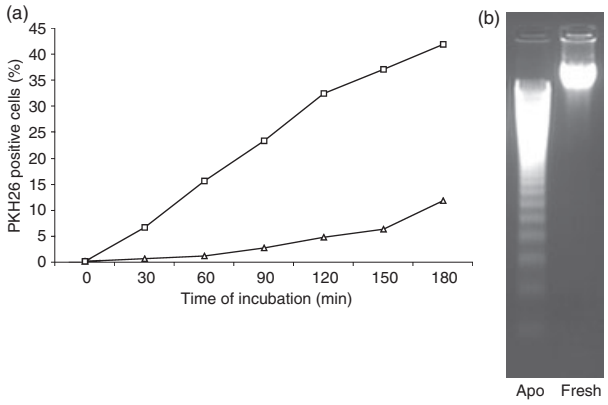

Figure 1. Ingestion of apoptotic or fresh polymorphonuclear cells (PMN) by human monocyte-derived macrophages (hMDM). (a) The hMDM were incubated with PKH26 labelled fresh (Δ) or apoptotic (□) PMN. Percentage of PKH26-positive cells was measured by flow cytometry at the indicated time of coculture. Data represent one typical experiment. (b) Electrophoretic ladder patterns. The PMN were incubated overnight at 37° in a humidified atmosphere in complete medium to induce apoptosis. After 24 hr, DNA was isolated from aged neutrophils (Apo) and was compared with DNA obtained from freshly isolated PMN (Fresh).

Monocytes bind/engulf apoptotic but not freshly isolated PMN

To analyse interaction between MO and PMN, freshly isolated MO were cocultured with apoptotic or fresh PMN which were labelled with PKH26. To confirm the apoptotic stage of aged PMN, DNA was isolated and run on an agarose gel to demonstrate the typical nucleosome ladder profile as shown in Fig.1(b). At different times the coculture cells were analysed by flow cytometry. As shown, the pattern of MO and PMN location was distinct and populations were well separated (Fig. 2a,i and ii). After 4 hr of coculture with apoptotic PMN 31·5% of MO contained PKH26 label (Fig. 2a, iv) whereas association of freshly isolated PMN with MO was negligible (Fig. 2a, iii). The kinetics of interaction of MO and PMN is shown in Fig. 2(b). The MO associate with or engulf apoptotic but not fresh PMN. In the presented experiments MO and freshly isolated PMN were usually obtained from one donor whereas apoptotic PMN were from a second donor. However, MO bound heterologous apoptotic PMN identically as autologous (data not shown). To learn more about this interaction we used cytochalasin D. It has been shown that cytochalasin D was able to abolish the uptake of apoptotic cells by macrophages.[29] Cytochalasin D impairs the maintenance of long-term potentiation of actin filaments and promotes conditions favourable for their depolymerization. This may prevent reorganization of the actin cytoskeleton, which precedes active phagocytosis.[30]

To find out whether treatment with cytocholasin D blocks the reaction we treated MO for 30 min, before the

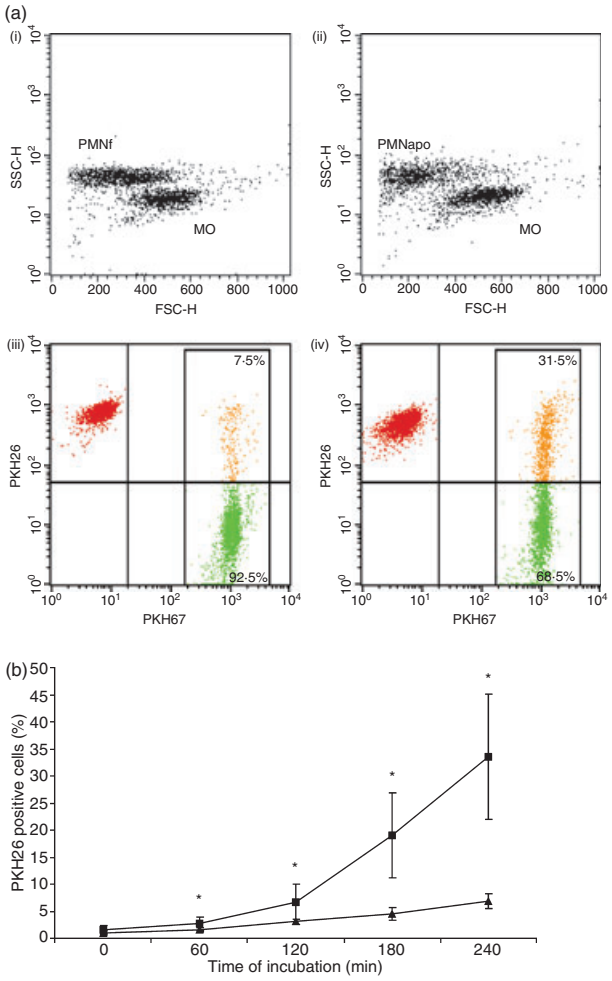

Figure 2. Flow cytometry analysis of interaction between monocytes (MO) and polymorphonuclear cells (PMN). (a) Scatter patterns (i, ii) and two-colour dot-plots (iii, iv) of cocultures of MO and PKH26-labelled PMN. The MO were incubated with freshly isolated (i, iii) or apoptotic (ii, iv) PMN for 4 hr. The percentage of double-positive cells (yellow dots) was analysed within PKH67-stained MO. Please note that apoptotic PMN have a reduced FSC-H signal (i, ii). (b) MO were incubated with fresh (▲) or apoptotic (■) PKH26-stained PMN. Percentage of PKH26-positive cells was measured at the indicated time of coculture. Data represent mean ± SD of eight experiments. Asterisks indicate $P < 0{\cdot}05$ in comparison to control (Student's *t*-test).

addition of neutrophils. The addition of cytochalasin D to MO cultures inhibited phagocytosis of apoptotic PMN, in a dose-dependent manner (from 37% at dose 0·05 μg/ml to 82% at dose 1 μg/ml, after 4 hr of coculture – data not shown). The data presented above indicated that freshly isolated MO can bind and probably engulf apoptotic PMN. To visualize the interaction between MO and PMN, cells were also analysed by fluorescence confocal microscopy. In this case PMN were labelled with vital dye PKH26 (red) and MO were labelled with PKH67 (green). The confocal microscopy clearly showed that MO recognize apoptotic PMN (Fig. 3b,c) but not freshly isolated PMN (Fig. 3g,h). After 4 hr of coculture, internalized apoptotic PMN were found within MO (Fig. 3a, d–f). Our findings support previous observations that MO can phagocytose apoptotic cells.[10,11] We examined the function of CD36 and CD14 in phagocytosis of apoptotic PMN and apoptotic Jurkat T cells. CD36 was shown to be involved in the binding of oxidized low-density lipoprotein.[31] It has been reported that macrophage CD36 can also associate with oxidized PS.[32] CD36, together with the $\alpha_v\beta_3$ integrin, has been identified as one of the adhesion molecules on the surface of macrophages implicated in the clearance of apoptotic PMN.[33] Our data show that recognition of apoptotic granulocytes and apoptotic Jurkat T cells by peripheral blood MO requires CD36. As shown in Fig. 4(a) phagocytosis of apoptotic PMN was blocked by approximately 65% when the anti-CD36 mAb (clone SMO) was used. Similar results (50% inhibition of phagocytosis) were obtained with apoptotic Jurkat T cells (Fig. 4b). Uptake of apoptotic PMN was also decreased when oxidized low-density lipoproteins were used as ligands for CD36 (data not shown).

The CD14 receptor for lipopolysaccharide was also shown to be involved in intercellular adhesion molecule-3-dependent clearance of apoptotic cells.[34,35] The anti-CD14 mAb (clone MEM-18) was a poor inhibitor of phagocytosis of apoptotic neutrophils. Another anti-CD14 mAb (clone RMO52) had no effect on the inhibition of apoptotic PMN phagocytosis, which suggests that CD14 is not involved in apoptotic neutrophil uptake by peripheral blood MO. Previous observations have shown that macrophage CD14 mediates the binding and uptake of apoptotic lymphocytes but not apoptotic neutrophils.[36,37] Our data indicated that phagocytosis of apoptotic Jurkat T cells by MO was inhibited 50% when the anti-CD14 mAb (clone RMO52) was used (Fig. 4b). Our results are consistent with previous reports.[38] As shown in Fig. 4(b), MEM-18 had no effect on apoptotic Jurkat T cells uptake.

Phosphatidylserine exposed on the surface on apoptotic cells is proposed as a general 'eat signal' for phagocytes.[39] We evaluated the engulfment of apoptotic PMN by MO in the presence of liposomes containing various phospholipids. The PS liposomes significantly reduced binding of apoptotic PMN, in a dose-dependent manner, from 18% to 44% after 4 hr of coculture. The inhibition was restricted to the PS. Other anionic (PG) and neutral (PC) liposomes had no effect on the phagocytosis of apoptotic cells (Fig. 5). Our data suggest that exposure of PS is an important prerequisite to the engulfment of apoptotic cells.

Recognition of apoptotic cells is predominantly connected with CD16$^+$ monocytes

Peripheral blood MO after labelling with anti-CD14 and anti-CD16 mAbs can be separated into three distinct

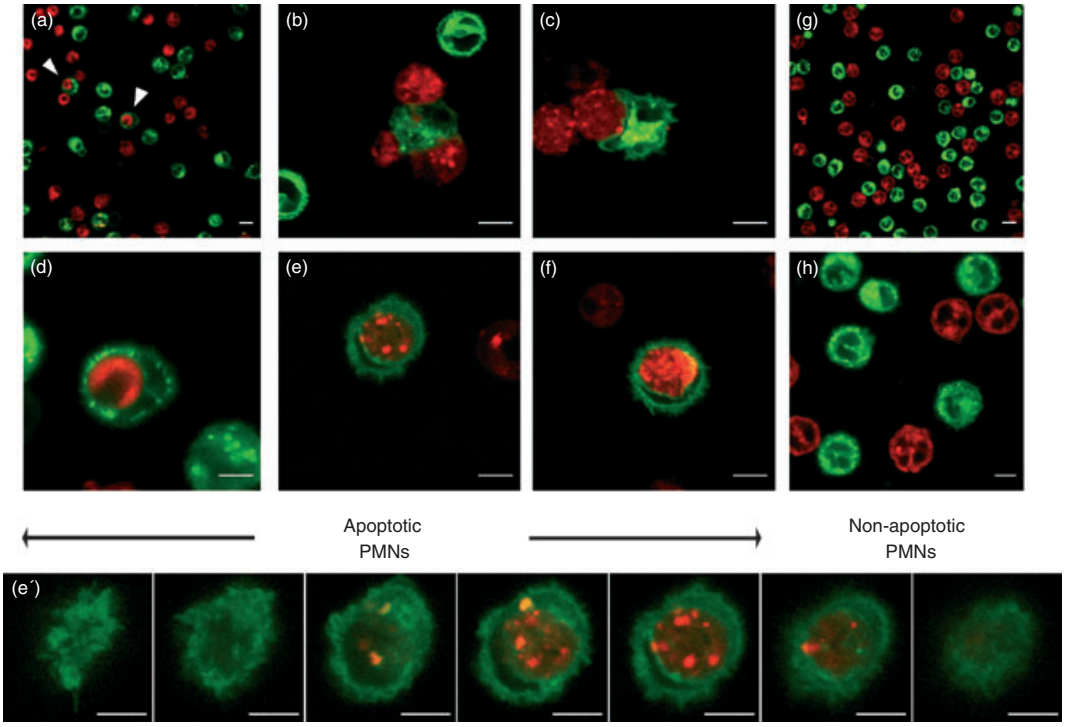

Figure 3. Fluorescence microscopy detection of phagocytosis of apoptotic polymorphonuclear cells (PMN) by monocytes (MO). The MO were labelled with green vital dye PKH67 and PMN with red PKH26. Confocal images show the process of target cell binding (b, c) and ingestion (a, d–f). Closed arrowheads in (a) indicate internalized apoptotic cells. Monocytes do not interact with freshly isolated PMN (g, h). The images demonstrate central cross-sections of the cells and are representative of five identical experiments performed. (e') represents series of optical sections (Z-series) of the cell, previously shown in (e). (a, d, g, h) represent fixed cells; (b, c, e, f) represent live cells. (a, g) Scale bars 10 μm; (b–f, h) scale bars 5 μm.

subpopulations: $CD14^{high}$ $CD16^{-}$, $CD14^{high}$ $CD16^{+}$ and $CD14^{dim}$ $CD16^{+}$.[20] Little is known about the interaction of these cells with apoptotic PMN. To investigate the ability of MO subsets to recognize apoptotic PMN, sorted MO subpopulations were cocultured with PKH26-labelled apoptotic cells. As shown in Fig. 6(a), $CD14^{high}$ $CD16^{+}$ and $CD14^{dim}$ $CD16^{+}$ MO efficiently phagocytosed apoptotic PMN. After 180 min of coculture $CD16^{+}$ MO were much more efficient in the phagocytosis of apoptotic PMN than $CD16^{-}$ cells (Fig. 6b). Flow cytometry analysis shows an approximately 3·5- to 5-fold increase of binding PKH26-labelled PMN by CD16-positive cells in comparison to control MO. Reduced phagocytosis, although not significant, was observed in the case of $CD14^{high}$ $CD16^{-}$ cells in comparison to control MO (Fig. 6a). Monocytes labelled with anti-CD14 and anti-CD16 antibody engulfed apoptotic granulocytes identically like control (unstained) MO (data not shown).

Apoptotic PMN regulate pro- and anti-inflammatory cytokine production by LPS-activated subpopulations of MO

It was observed that LPS-stimulated MO while in contact with apoptotic cells produce more anti-inflammatory cytokine IL-10 whereas the production of pro-inflammatory cytokines, like TNF-α and IL-1β, was reduced.[9,21] Based on the above we have asked whether subsets of MO react identically to apoptotic PMN. As shown in Fig. 7(a) LPS-stimulated $CD14^{high}$ $CD16^{+}$ MO produce more IL-10 in comparison to $CD14^{high}$ $CD16^{-}$ and $CD14^{dim}$ $CD16^{+}$ MO. The result supports our previous findings.[20] Exposure of these subsets to apoptotic PMN similarly augmented the production of IL-10 (Fig. 7a). In contrast, LPS-stimulated $CD14^{dim}$ $CD16^{+}$ MO produce high levels of pro-inflammatory cytokine TNF-α but very little anti-inflammatory IL-10.[18–20] Our data show that $CD14^{dim}$ $CD16^{+}$ MO produce more TNF-α and IL-1β than $CD14^{high}$ $CD16^{-}$ and $CD14^{high}$ $CD16^{+}$ subsets. Addition of apoptotic PMN reduced the production of pro-inflammatory cytokines in each LPS-stimulated subpopulation of MO (Fig. 7b,c).

Discussion

The available data show that acute inflammation starts with mostly neutrophilic exudation, which from the third day become monocytic.[40,41] Whether this also offers the time necessary for MO to mature to macrophages is not known but is rather unlikely because phagocytosis starts

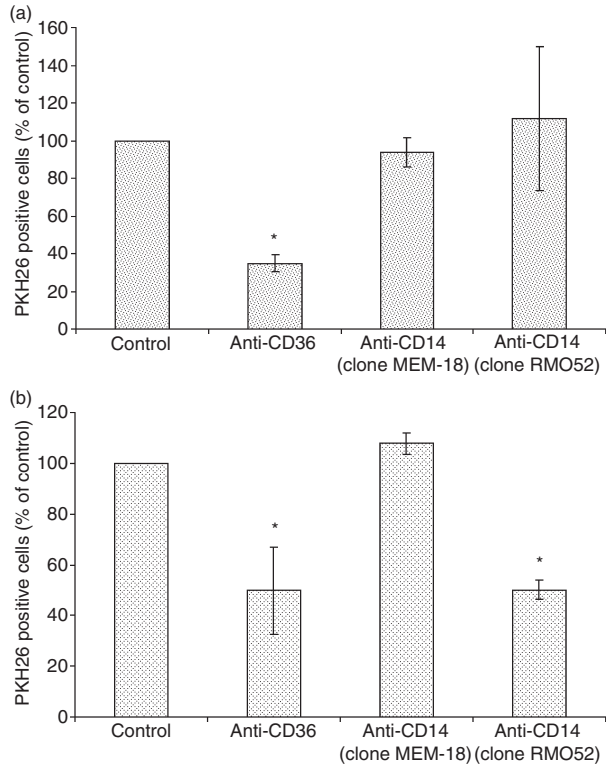

Figure 4. Binding of apoptotic polymorphonuclear cells (PMN) and apoptotic Jurkat T cells by monocytes (MO) pretreated with monoclonal antibody (mAb). The MO were incubated with mAb anti-CD36 (clone SMO) or mAb anti-CD14 (clone MEM-18 or RMO52) 30 min before addition of PMN (a) or Jurkat T cells (b). Phagocytosis was measured by flow cytometry after 4 hr of coculture. Data represent mean ± SD of four experiments performed for PMN or three experiments for Jurkat T cells. Asterisks indicate $P < 0{\cdot}05$ in comparison to control (not treated with mAb).

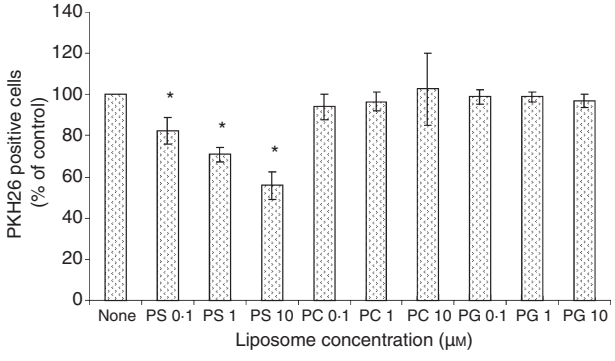

Figure 5. Binding of apoptotic polymorphonuclear cells (PMN) by monocytes (MO) in the presence of various phospholipids. Monocytes were incubated with PKH26-labelled apoptotic PMN in the presence of phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylglycerol (PG) liposomes. Phagocytosis was measured by flow cytometry after 4 hr of coculture. Data represent mean ± SD of four experiments. Asterisks indicate $P < 0{\cdot}05$ in comparison to the control (not treated with liposomes).

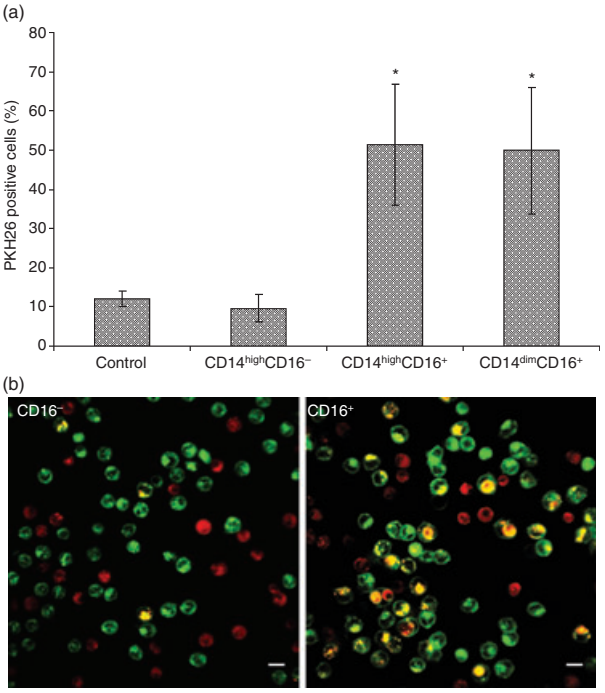

Figure 6. Phagocytosis of apoptotic polymorphonuclear cells (PMN) by subpopulations of monocytes (MO). (a) MO subpopulations ($CD14^{high}$ $CD16^{-}$, $CD14^{high}$ $CD16^{+}$ and $CD14^{dim}$ $CD16^{+}$) obtained by fluorescence-activated cell sorting were incubated in coculture with PKH26-labelled apoptotic PMN. The percentage of PKH26-positive MO was determined by flow cytometry after 180 min of coculture. Data represent mean ± SD of four independent experiments. The percentage of MO subpopulations were respectively: $CD14^{high}$ $CD16^{-}$ 85 ± 5·5, $CD14^{high}$ $CD16^{+}$ 4·2 ± 2·6 and $CD14^{dim}$ $CD16^{+}$ 2·5 ± 1·6. Asterisks indicate $P < 0{\cdot}05$ in comparison to control (unstained MO). (b) Fluorescence microscopy detection of phagocytosis of apoptotic PMN by $CD16^{+/-}$ subpopulations. MO were labelled with monoclonal antibody anti-CD16 and were sorted in to $CD16^{-}$ and $CD16^{+}$ subsets. After sorting cells were stained with green vital dye PKH67 and were cocultured 180 min with PMN labelled with red PKH26. The images demonstrate central cross-sections of these cells and are representative of one typical experiment performed. Scale bars 10 μm.

from the first day.[41,42] There are strong suggestions that IL-6 is a possible regulator of the influx of MO to the inflammatory site.[43] The complex of IL-6–sIL-6Rα favours the transitions of exudates from PMN to MO in inflammation[44,45] and it has been shown that activation of PMN with IL-8 induces IL-6Rα shedding.[46]

Our present results clearly indicate that peripheral blood MO can engulf apoptotic PMN. This was shown by flow cytometry and confocal microscopy. We showed that the kinetics of engulfment of apoptotic PMN by hMDM is higher than that by peripheral blood MO, although the method of assay was different. It is possible that macrophages recognize apoptotic cells using multiple phagocyte receptors, including PS receptors (stabilin-2, BAI1 and Tim4)[47–49], vitronectin receptor ($\alpha_v\beta_3$)[33,36], CD36[33,36],

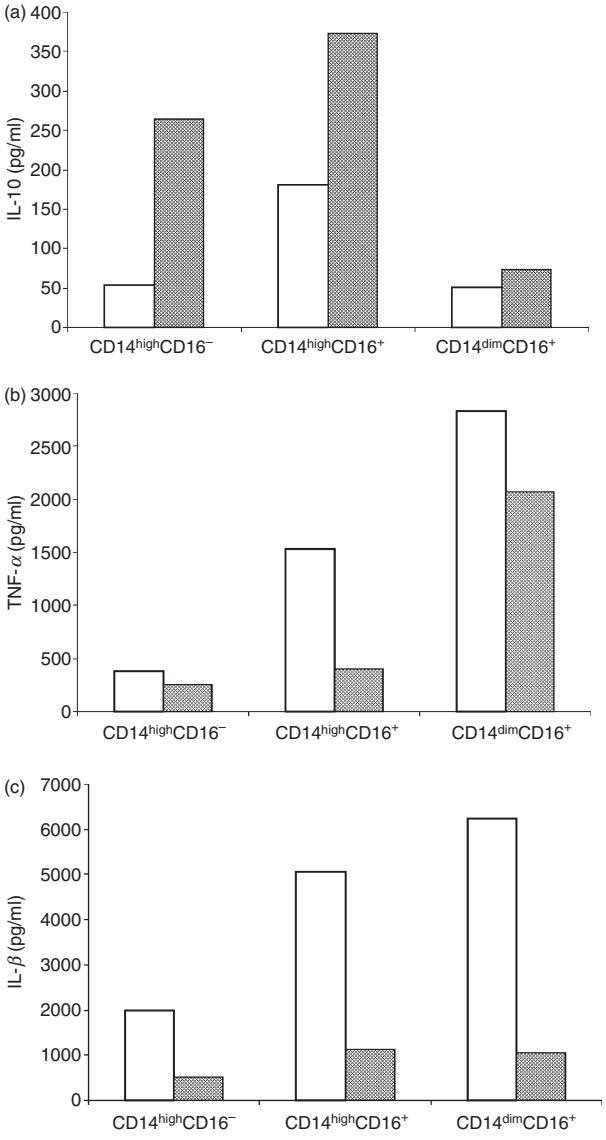

Figure 7. Production of interleukin-10 (IL-10), tumour necrosis factor-α (TNF-α) and IL-1β by subpopulations of monocytes (MO) stimulated with lipopolysaccharide (LPS). Subpopulations of MO stimulated with LPS were cultured alone (open histograms) or with apoptotic PMN (shaded histograms). The concentrations of IL-10 (a), TNF-α (b) and IL-1β (c) in 18 hr supernatants were measured by an enzyme-linked immunosorbent assay. Please note that production of IL-10, TNF-α and IL-1β by unstimulated cells was not detectable (below 7·8 pg, data not shown).

CD14[50], CD91[51], CD68[52] and SR-A[53]. Circulating human blood MO do not express stabilin-2[54], Tim4[55] and SR-A[53] but they express BAI1[12], $(\alpha_v\beta_3)$[13], CD36[14,15], CD14[16] and CD91[17]. It cannot be excluded that these receptors are involved in the recognition of apoptotic cells by peripheral blood MO. Our data show that CD36 is a general receptor involved in the recognition of apoptotic PMN and apoptotic Jurkat T cells. This is in agreement with previous reports where macrophages were analysed.[32,33,36] The liposomes containing phosphatidyl-L serine inhibited, in a dose-dependent manner, apoptotic PMN uptake. This supports previous observations that PS exposed on the surface of apoptotic cells function as an 'eat signal' for phagocytes.[39] It cannot be excluded that MO CD36 plays an important role in the recognition of PS.[56] Anti-CD36 mAb (clone SMO) inhibited phagocytosis of apoptotic cells. The other PS receptor, BAI1, was not tested. To evaluate the role of CD14 in apoptotic cell uptake we used two anti-human CD14 mAbs MEM-18 and RMO52. MEM-18 recognizes an epitope in the region between amino acids 57 and 64[57]. RMO52 recognizes an epitope which partially overlaps amino acids between 34 and 44.[58,59] Our data indicated that anti-CD14 mAb RMO52 was a good inhibitor of apoptotic lymphocyte uptake. That is in agreement with the report of Lingnau *et al.*[38] Clone RMO52 had no effect on apoptotic PMN engulfment. Recently it has been shown that MEM-18 inhibited the phagocytosis of apoptotic lymphocytes but not of apoptotic neutrophils.[36] Our present data indicated that MEM-18 was a poor inhibitor of apoptotic cell uptake (both PMN and Jurkat T cells). It cannot be excluded that MO used different epitopes of CD14, in comparison to macrophages, to recognize apoptotic cell ligands. Clearance of apoptotic PMN appears independent of the CD14 mechanism.

We also examined the capacity of MO subsets to recognize apoptotic PMN. Flow cytometry and confocal microscopy data show that recognition of apoptotic cells is connected with $CD16^+$ MO (both $CD14^{high}$ $CD16^+$ and $CD14^{dim}$ $CD16^+$). The $CD14^{high}$ $CD16^-$ MO had a lower ability to interact with apoptotic PMN. Recently it has been shown that $CD16^+$ monocytes preferentially differentiate into dendritic cells in a model of transendothelial trafficking.[60] This suggests that $CD16^+$ MO represent dendritic cell precursors. The high capacity of CD16-positive MO to interact with apoptotic PMN may be related to the more mature phenotype of these cells.[60,61] Peripheral blood MO recruited into inflammatory sites do not die but migrate to the local lymph nodes[62] and eventually become dendritic cells after up-regulating their human leucocyte antigen class II antigen and acquiring costimulatory molecules.[63]

$CD16^+$ MO were also shown to increase in the course of sepsis.[64] It is interesting that in the sepsis of neonates and small children the IL-1 was down while IL-10 was up (although not significantly).[65] It is conceivable that MO (especially CD16-positive MO) can recognize apoptotic cells in the blood vessels before entering into inflamed tissue because PMN are the most common type of apoptotic leucocytes observed in the blood.[66] This paper supports the previous observation that LPS-stimulated MO, as well as their subsets in contact with apoptotic PMN, produced more IL-10 while the production of TNF-α and IL-1β was reduced.[9,21] The $CD14^{high}$ $CD16^+$

monocytes produced a large amount of IL-10 and represented a population with anti-inflammatory properties.[20] In contrast, $CD14^{dim}$ $CD16^{+}$ MO produce more TNF-α and much less IL-10, which makes them a pro-inflammatory subpopulation.[18,19]

In conclusion, our data provide evidence that human peripheral blood MO efficiently phagocytose apoptotic PMN, in particular, $CD16^{+}$ MO are especially active in this process.

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