

RESEARCH ARTICLE

# Surfactant protein A (SP-A) binds to phosphatidylserine and competes with annexin V binding on late apoptotic cells

Anne Jäkel<sup>1</sup> (✉), Kenneth B. M. Reid<sup>2</sup>, Howard Clark<sup>3</sup>

<sup>1</sup> Department of Pharmacology, University of Oxford, Mansfield Road, Oxford OX1 3QT, UK

<sup>2</sup> MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, UK

<sup>3</sup> University of Southampton, MP 803 Level F, South Block, Southampton General Hospital, Southampton SO16 6YD, UK

✉ Correspondence: anne.jakel@mansfield.oxon.org

Received December 17, 2009; accepted December 24, 2009

## ABSTRACT

The role of surfactant protein A (SP-A) in the recognition and clearance of apoptotic cells is well established, but to date, it is still not clear which surface molecules of apoptotic cells are involved in the process. Here we present evidence that phosphatidylserine (PS) is a relevant binding molecule for human SP-A. The binding is Ca<sup>2+</sup>-dependent and is not inhibited by mannose, suggesting that the sugar-binding site of the carbohydrate recognition domain (CRD) of SP-A is not involved. Flow cytometry studies on apoptotic Jurkat cells revealed apparent inhibition of annexin V binding by increasing concentrations of SP-A in late apoptotic but not early apoptotic cells, and this was consistent for Jurkat cells and neutrophils. Supporting these data, confocal microscopy results show a co-localisation of annexin V and SP-A in late apoptotic but not early apoptotic cells. However, we cannot conclude that this inhibition is exclusively due to the binding of SP-A to PS on the cell surface, as annexin V is not wholly specific for PS and SP-A also interacts with other phospholipids that might become exposed on the apoptotic cell surface.

**KEYWORDS** surfactant proteins, phosphatidylserine, apoptotic cells

## INTRODUCTION

Apoptosis is a genetically programmed cell death conserved throughout phylogeny, and provides a counterbalance to mitosis in the regulation of tissue growth and homeostasis (Kerr et al., 1972; Wyllie et al., 1980). Engulfment of apoptotic bodies and debris represents the outcome of the death program for most cells in multicellular organisms.

Macrophages and other cells manifest their phagocytic potential by clearance of apoptotic bodies from tissues, thus preventing their lysis and the consequent release of toxic or immunogenic intracellular components. By inducing the release of mediators such as TGF- $\beta$ , IL-10, prostaglandin E2 and others, clearance of apoptotic cells also sets up an anti-inflammatory milieu within the tissue (Fadok et al., 2001; Henson et al., 2001).

Relatively recently, a role has been proposed for innate defence molecules in the recognition and clearance of dead and dying cells, which, if left *in situ*, may be as harmful to surrounding tissues as any pathogenic entry. Evidence indicates that members of the collectin family participate in apoptotic cell removal *in vivo* and *in vitro* (Schagat et al., 2001; Clark and Reid, 2002; Vandivier et al., 2002; Clark et al., 2003; Nauta et al., 2004). The collectins, which include surfactant protein A (SP-A), surfactant protein D (SP-D) and mannan-binding lectin (MBL), belong to a group of proteins that are characterized by the presence of multiple copies of a collagen-like domain and a C-type lectin domain, also

referred to as a calcium-dependent carbohydrate recognition domain (CRD) (Thiel and Reid, 1989; Holmskov et al., 1994; Hoppe and Reid, 1994; Crouch, 1998). These multimeric glycoproteins, which belong to the C-type lectin superfamily (Weis et al., 1998), can bind to specific patterns of carbohydrates found on the surface of a wide variety of microorganisms. This binding is mediated by interactions of the CRD with terminal monosaccharide residues that are characteristic for microbial surfaces, which therefore enables discrimination between self and non-self (Weis et al., 1998; Lu et al., 2002). Binding of collectins can lead to direct agglutination and neutralization of microorganisms, to opsonisation in order to present bound microbes directly to phagocytes (Malhotra et al., 1994), or, for MBL only, to complement activation via the lectin pathway (Wallis, 2002). Consequently, collectins are considered to be important sugar pattern recognition molecules of the mammalian innate immune system that can interact directly with live pathogens, and therefore play important roles in the first line defense against microbes.

It has been shown that SP-A and SP-D act as bridging molecules that bind to both apoptotic cells and phagocytes to enhance recognition and clearance, by creating a physical link between the phagocyte and the apoptotic cell to be engulfed. SP-A and SP-D promote the uptake of apoptotic cells by alveolar macrophages *in vitro* (Schagat et al., 2001; Vandivier et al., 2002) and *in vivo* (Clark et al., 2002). Uptake of SP-A and SP-D-coated apoptotic cells by macrophages may be via calreticulin-CD91 (Ogden et al., 2001; Vandivier et al., 2002).

It seems that the profound changes to cell membranes that occur during apoptotic processes are likely to cause changes in surface charge, carbohydrate, lipid or protein moieties that may present entities to which SP-A, SP-D and other putative bridging molecules bind (Hart et al., 2000). These may then act as molecular flags of apoptotic cells, alerting immune surveillance cells that engulf and digest the apoptotic cells. However, these changes in cell surface entities and the subsequent interactions of the defence mediators are as yet poorly characterised.

The lung collectin-binding site(s) on the apoptotic cell membrane are still poorly documented, due largely to the continuous modifications that take place on the surface of cells undergoing apoptosis.

A strong candidate for a role as apoptotic surface molecule marker is phosphatidylserine (PS); relocated from the inner to

the outer membrane of many cell types following the loss of membrane phospholipid asymmetry during apoptosis (Homburg et al., 1995), the anionic PS may be part of the mechanism by which phagocytic cells recognise apoptosis.

In view of the crucial role of lung-collectin-mediated phagocytosis in the maintenance of immune tolerance, this study sought to investigate PS as a possible binding molecule for SP-A on apoptotic cells.

## RESULTS

### SP-A inhibits binding of annexin V to late apoptotic but not to early apoptotic cells

Recent evidence shows that SP-A and SP-D interact with apoptotic cells in a very distinct manner. SP-A binds to viable and early apoptotic Jurkat cells and neutrophils in a predominantly  $\text{Ca}^{2+}$ -dependent fashion with lectin-site involvement, whereas its association with late apoptotic cells was largely  $\text{Ca}^{2+}$ -independent (Jakel et al., 2009). SP-D, in contrast, does not bind to viable and early apoptotic Jurkat cells at all, yet its interaction with late apoptotic Jurkat cells was  $\text{Ca}^{2+}$ -independent. The binding of SP-D to viable and early apoptotic neutrophils was inhibited by maltose and EDTA, suggesting the involvement of lectin-binding site; whereas its binding to late apoptotic neutrophils was predominantly  $\text{Ca}^{2+}$ -independent (Jakel et al., 2009).

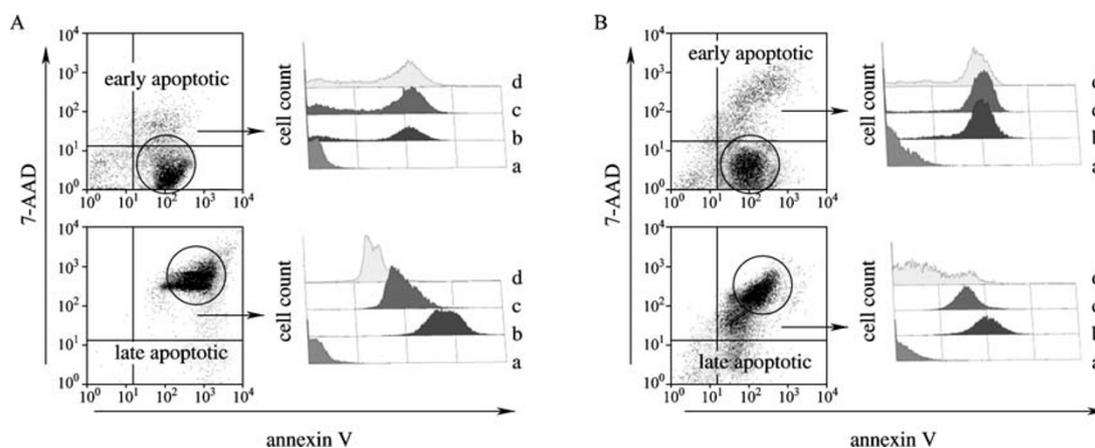
During these studies, it was observed that SP-A, but not SP-D, inhibits the binding of annexin V. and this was further investigated. Early and late apoptotic Jurkat cells and neutrophils were simultaneously incubated with FITC-labelled annexin V, 7-AAD and unlabelled SP-A in different concentrations. The inhibition of annexin V binding was monitored by flow cytometry. Cells were gated by annexin V and 7-AAD characteristics.

As shown in Fig. 1A, with increasing concentration of SP-A, the fluorescence intensity of annexin V binding to early apoptotic Jurkat cells remains relatively constant (Table 1, MFI 90–109), suggesting that SP-A does not inhibit the binding of annexin V and therefore is not binding to PS, or otherwise interfering with the binding of annexin V.

In contrast, a significant decrease by up to ~90% of annexin V-binding to late apoptotic Jurkat cells was observed with increasing concentrations of SP-A, indicating that SP-A interfered with the binding of annexin V (Table 1, MFI 81–765).

**Table 1** Summary of mean fluorescence intensity (MFI) of annexin V positive cells  $\pm$  SD as observed in Fig. 1.

SP-A concentration	early apoptotic Jurkat cells	late apoptotic Jurkat cells	early apoptotic neutrophils	late apoptotic neutrophils
(a) negative control	6 $\pm$ 3	4 $\pm$ 1	9 $\pm$ 3	5 $\pm$ 1
(b) 0	90 $\pm$ 13	765 $\pm$ 23	107 $\pm$ 18	123 $\pm$ 9
(c) 10 $\mu$ g	109 $\pm$ 8	130 $\pm$ 11	109 $\pm$ 16	50 $\pm$ 7
(d) 20 $\mu$ g	103 $\pm$ 15	81 $\pm$ 7	80 $\pm$ 9	16 $\pm$ 4



**Figure 1. Influence of SP-A on annexin V-binding.** 500,000 early and late apoptotic Jurkat cells (A) or neutrophils (B) were incubated with different concentrations of unlabelled SP-A. (a) Negative control (viable Jurkat cells incubated with annexinV-FITC and 7-AAD only); (b) without SP-A; (c) 10 µg SP-A; (d) 20 µg SP-A, respectively, in the presence of 2.5 mM CaCl<sub>2</sub>. Cells were simultaneously incubated with annexin V-FITC and 7-AAD. Binding was measured by flow cytometry. Apoptotic cells were identified and gated as shown in dot plots (encircled cell populations). Histogram data show annexin V-FITC binding.

7-AAD content of the cells remained constant with increasing concentrations of SP-A, indicating that the binding of 7-AAD to the DNA was not inhibited by SP-A (data not shown).

The influence of SP-A on annexin V-binding to early apoptotic neutrophils was also examined. As shown in Fig. 1B, with 10 µg SP-A, there is no reduction in the binding of annexin V; but with 20 µg of SP-A, an inhibition of annexin V-binding by ~30% could be observed (Table 1, MFI 80–109). This inhibition is stronger in early apoptotic Jurkat cells. Perhaps due to the spontaneous apoptosis in neutrophils over a period of 24 h, some neutrophils might represent a status between early and late apoptotic conditions.

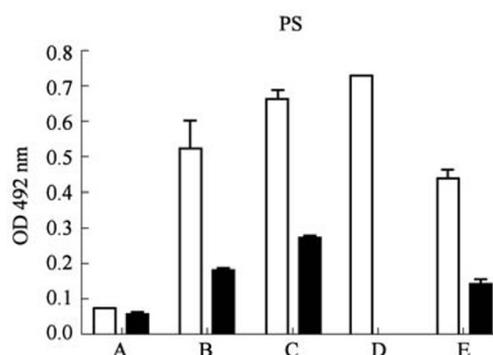
Examination of annexin V-binding with addition of SP-A to late apoptotic neutrophils showed that SP-A inhibits the binding of annexin V by ~60% at 10 µg and ~90% at 20 µg of SP-A (Table 1, MFI 16–123). These inhibition studies were performed in strict Ca<sup>2+</sup> conditions as annexin V requires Ca<sup>2+</sup> for its binding to the cell surface. It was therefore not possible to explore the Ca<sup>2+</sup>-dependence of SP-A binding to its target on the apoptotic cell.

In summary, the present data show an inhibition of annexin V-binding by SP-A to late apoptotic but not early apoptotic cells in both Jurkat cells and neutrophils. Since annexin V is generally associated with PS, direct interaction of PS and SP-A was examined.

#### SP-A binds to PS in a Ca<sup>2+</sup>-dependent manner

In order to investigate which part of SP-A might be involved in the binding to PS, direct binding analysis were performed in an ELISA approach with PS liposomes coated onto microtitreplates and incubated with different concentrations of SP-A in the presence of 2.5 mM CaCl<sub>2</sub> or 1 mM EDTA.

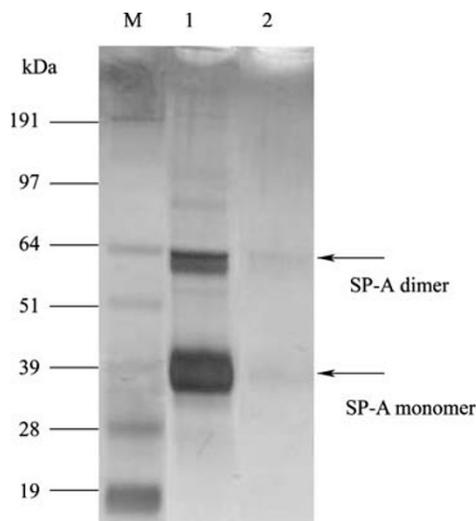
As shown in Fig. 2, SP-A binds to PS in a concentration-



**Figure 2. ELISA showing interaction of SP-A with phosphatidylserine (PS).** Liposomes of PS were coated onto microtitreplates and incubated with different concentrations of SP-A in the presence of 2.5 mM CaCl<sub>2</sub> (white bars) or 1 mM EDTA (black bars), followed by incubation with biotinylated anti-SP-A mAb and streptavidin-HRP. The optical density (OD) at 492 nm was measured. (A) Negative control (BSA was used instead of SP-A); (B) 5 µg/mL SP-A; (C) 10 µg/mL SP-A; (D) 10 µg/mL SP-A + 100 mM mannose (EDTA not tested); (E) positive control (wells coated with 20 µg/mL mannose).

and Ca<sup>2+</sup>-dependent manner. The addition of 100 mM mannose did not reduce the binding of SP-A to PS in Ca<sup>2+</sup> conditions (Fig. 2D). With addition of EDTA, the binding of SP-A was inhibited by ~60% for both tested concentrations (Fig. 2B and 2C). These data suggest that a Ca<sup>2+</sup>-sensitive site other than the sugar-binding site on the CRD region is involved in the binding of SP-A to PS.

In order to confirm these findings, PS liposomes in suspension were incubated with SP-A in the presence of 2.5 mM CaCl<sub>2</sub> and 1 mM EDTA. After washing, bound protein was analysed by SDS-PAGE. As shown in Fig. 3, SP-A binds



**Figure 3.** SDS-PAGE (reduced conditions) showing interaction of SP-A with phosphatidylserine (PS). Liposomes of PS were incubated with 1  $\mu$ g SP-A in the presence of 2.5 mM  $\text{CaCl}_2$  (lane 1) or 1 mM EDTA (lane 2). After washing, PS liposome pellets were applied to the gel. M indicates protein standard.

to PS-vesicles in a  $\text{Ca}^{2+}$ -dependent manner, and there was no detectable binding in EDTA buffer.

#### SP-A binds to other phospholipids (PLs) in a $\text{Ca}^{2+}$ -dependent but lectin-independent manner

To investigate whether the binding of SP-A to PS was specific, an ELISA with different PL liposomes (PA, PE, PI, PG, CL and DPPC) was performed. SP-A in different concentrations was incubated with immobilised PLs in  $\text{CaCl}_2$  or EDTA and with the addition of mannose. As shown in Fig. 4, SP-A binds to all tested PLs in a concentration- and  $\text{Ca}^{2+}$ -dependent manner (Fig. 4). The binding to all tested PLs was not inhibited by mannose, suggesting that the sugar-binding site of the CRD is not involved. The binding in the presence of EDTA was remarkably decreased by 40%–50% in PA and PE, whereas the binding to PG, CL and DPPC was inhibited almost down to the negative control.

#### SP-A and annexin V co-localise on late apoptotic Jurkat cells

To investigate the SP-A binding in the presence of annexin V on early and late apoptotic Jurkat cells, a collectin binding assay with SP-A and FITC-labelled annexin V on early and late apoptotic Jurkat cells in  $\text{Ca}^{2+}$  conditions was performed and the bindings were examined by confocal laser scanning microscopy. If SP-A and annexin V bind to the same targets on the cell surface, an overlap of fluorescent colours (co-localisation) is expected.

As shown in Fig. 5A, for early apoptotic Jurkat cells, the binding of annexin V indicated apoptotic status, but only little SP-A bound, mainly to visible apoptotic blebs that indicate an advanced apoptotic stage of the cell. There was no detectable colocalization except the minor overlap of fluorescence in the apoptotic blebs. This finding is consistent with previous results that SP-A bound weakly to early apoptotic Jurkat cells (Jakel et al., 2009). It is likely that the binding of SP-A in the flow cytometry assay also occurred to apoptotic blebs in early apoptotic Jurkat cells, and the SP-A binding was strictly  $\text{Ca}^{2+}$ -dependent. It was not tested here whether the specific binding of SP-A to the apoptotic blebs was inhibited by EDTA as co-localising experiments of SP-A and annexin V in microscopy must be performed in the presence of  $\text{Ca}^{2+}$  to allow the binding of annexin V.

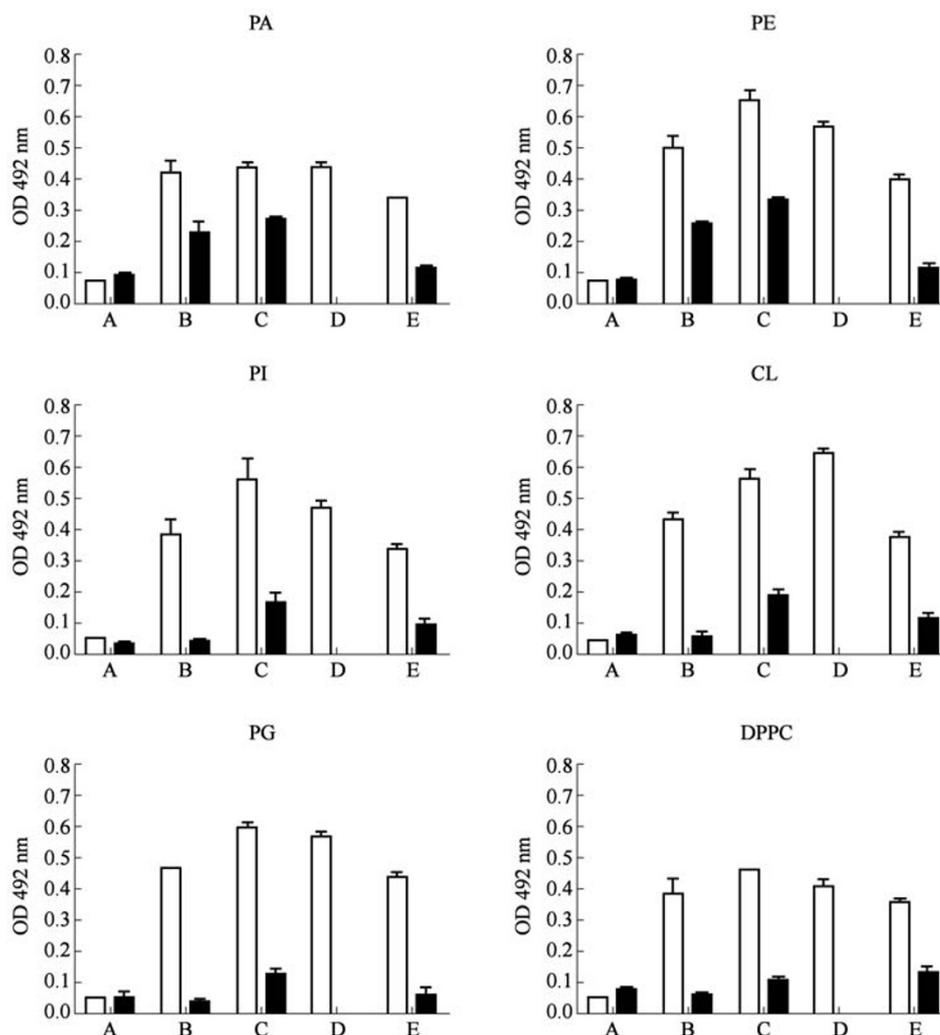
In contrast to the early apoptotic cells, late apoptotic Jurkat cells bind SP-A strongly throughout the cell membrane, particularly within the apoptotic blebs. There is also strong annexin V binding and the merged image shows good overlap of fluorescence, but complete co-localisation was not observed, possibly due to inhibition of annexin V-binding by SP-A as previously described (Jakel et al., 2009). These data confirmed a much stronger binding of SP-A to late apoptotic than to early apoptotic cells as judged by the fluorescence intensity.

#### SP-A does not interact directly with immobilised annexin V

In order to investigate whether SP-A interacts directly with annexin V and thereby inhibit the binding to apoptotic cells, a microtitre plate binding assay in 2.5 mM  $\text{CaCl}_2$  or 1 mM EDTA was performed. The positive controls ensured the detection of SP-A (Fig. 6D) and the coating of annexin V (Fig. 6E). However, there was no binding of SP-A to immobilised annexin V as compared to the negative control (Fig. 6B and 6C).

## DISCUSSION

PS is an aminophospholipid carrying a phosphoserine head-group, which gives this phospholipid a negative charge at pH 7.4. It is a major constituent of the plasma membrane. In normal conditions, the phospholipids of the plasma membrane are distributed asymmetrically between the outer and inner layers of the membrane. Choline-containing lipids, such as PC and sphingomyelin, are concentrated on the outer leaflet, whereas the aminophospholipids, including PE and PS, are present in higher abundance on the inner leaflet. Loss of phospholipid asymmetry and exposure of phosphatidylserine was first demonstrated for apoptotic lymphocytes (Fadok et al., 1992b; Schlegel et al., 1993) and these findings have been confirmed for a number of cell types, including neutrophils, tumor cell lines, smooth muscle vascular cells,

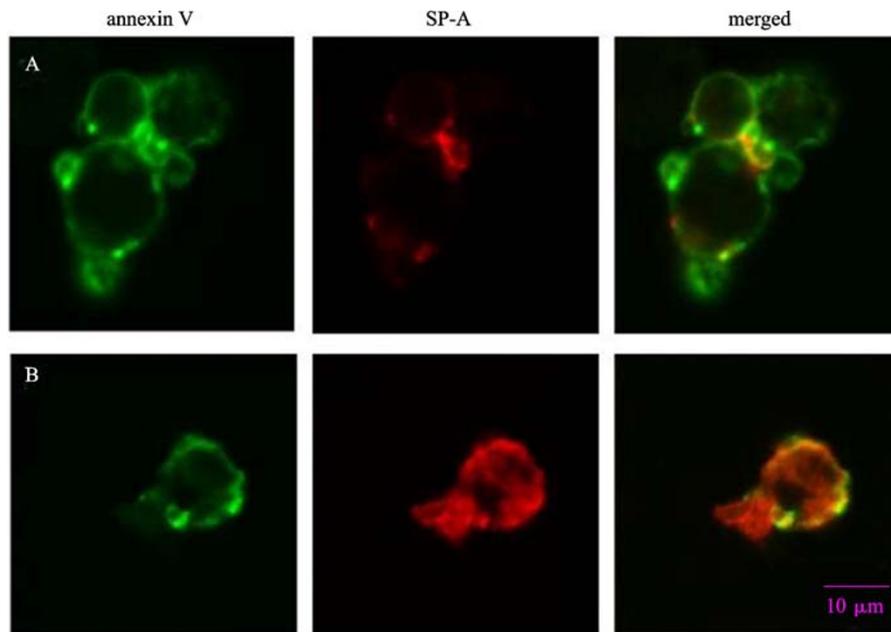


**Figure 4. ELISA showing interaction of SP-A with phospholipids (PLs).** Liposomes of phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylglycerol (PG), cardiolipin (CL) and dipalmitoylphosphatidylcholine (DPPC) were coated onto microtitreplates and incubated with different concentrations of SP-A in the presence of 2.5 mM CaCl<sub>2</sub> (white bars) or 1 mM EDTA (black bars), followed by incubation with biotinylated anti-SP-A mAb and streptavidin-HRP. The optical density (OD) at 492 nm was measured. (A) Negative control (BSA was used instead of SP-A); (B) 5 µg/mL SP-A; (C) 10 µg/mL SP-A; (D) 10 µg/mL SP-A + 100 mM mannose (EDTA not tested); (E) positive control (wells coated with 20 µg/mL mannose).

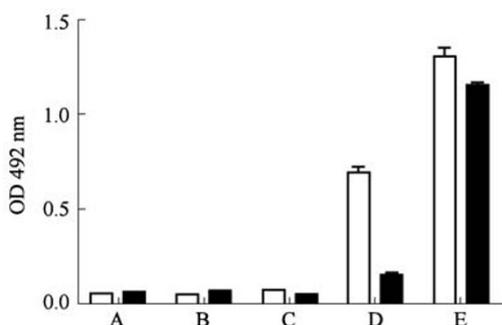
spermatogonia and Jurkat T-cells (Fadok et al., 1992a; Bennett et al., 1995; Homburg et al., 1995; Martin et al., 1995; Shiratsuchi et al., 1997). Because exposure of PS appears to be a universal feature of apoptotic cells, it is now used as a marker in flow cytometry assays utilising fluorochromelabelled annexin V, which binds to PS in a Ca<sup>2+</sup>-dependent manner (Vermes et al., 1995). It has previously been shown that human MBL, a C-type lectin which is structurally closely related to SP-A, binds to PS in a concentration- and Ca<sup>2+</sup>-dependent manner: the binding was inhibited by various monosaccharides, indicating that the CRD region of the protein is involved in the binding (Kilpatrick, 1998). The binding of another innate immune recognition molecule, C1q, to PS and other anionic phospholipids

adsorbed to microtitre plates or incorporated into liposomes has been shown in our laboratory (Sim et al., 1998). Using recombinant globular heads of C1q, it was demonstrated that the binding of C1q to phospholipids occurred via its globular heads. These data were confirmed by Paidassi et al. (2008), who investigated the binding behaviour of C1q to PS and concluded that C1q recognizes PS specifically through multiple interactions between its globular domain and the phosphoserine group of PS.

Initially, the binding studies of SP-A to cells in different viability stages showed that the binding of annexin V to late apoptotic cells was significantly reduced with increasing concentrations of SP-A (Fig. 1, Table 1), whereas this did not happen with SP-D (data not shown). This suggested that



**Figure 5. Confocal images of SP-A and annexin V binding on the surface of apoptotic Jurkat cells.** Early (A) and late (B) apoptotic Jurkat cells were incubated with SP-A followed by biotinylated anti-SP-A mAb, and then simultaneously incubated with streptavidin-PE and annexin V-FITC.



**Figure 6. ELISA showing no interaction of SP-A with annexin V.** Annexin V was coated onto microtitreplates (10 µg/mL) and incubated with SP-A in different concentrations in the presence of 2.5 mM CaCl<sub>2</sub> (white bars) or 1 mM EDTA (black bars), followed by incubation with biotinylated anti-SP-A mAb and streptavidin-HRP. The optical density (OD) at 492 nm was measured. (A) Negative control (BSA was used instead of SP-A); (B) 10 µg/mL SP-A; (C) 20 µg/mL SP-A; (D) positive control 1 (wells were coated with 20 µg/mL mannose and incubated with 20 µg/mL SP-A); (E) positive control 2 (5 µg/mL anti-annexin V polyclonal Ab (Abcam, Cambridge, UK), followed by incubation with 1000-fold diluted HRP-labelled goat anti-rabbit antibody (Sigma)).

SP-A may compete with annexin V for the binding to PS. To confirm this hypothesis, a competition assay was designed to investigate the direct inhibition of annexin V binding to the cell surface by addition of increasing concentrations of SP-A.

These experiments were performed on early and late apoptotic Jurkat cells and human neutrophils.

Direct binding of SP-A to PS liposomes was then demonstrated using liposomes in suspension or coated on ELISA plates (Fig. 2 and 3). Binding occurred in both conditions and was Ca<sup>2+</sup>-dependent. However, SP-A also bound to other PLs in similar conditions (Fig. 4). To confirm co-localisation of annexin V and SP-A, laser scanning confocal microscopy was performed on early and late apoptotic Jurkat cells (Fig. 5). Finally, an ELISA was performed to exclude a direct interaction of SP-A with annexin V (Fig. 6).

The results show the novel finding that SP-A interacts with PS. The binding is Ca<sup>2+</sup>-dependent and is not inhibited by the addition of mannose, suggesting that the sugar-binding site of the CRD region is not involved. Studies using early and late apoptotic Jurkat cells and neutrophils showed an inhibition of annexin V-binding by SP-A on late apoptotic but not on early apoptotic cells, and these data were supported by the observation that SP-A does not co-localise with annexin V on early apoptotic but does on late apoptotic cells.

Annexin V is commonly considered highly specific for PS. However, previous results suggested a relatively unimportant role for the phospholipid headgroup as a determinant for the annexin V phospholipid-binding sites based on the similarities of PS and PC binding (Meers and Mealy, 1993). Further studies using various PLs with different headgroups showed that annexin V interacted comparably in a Ca<sup>2+</sup>-dependent manner with PS, PC, PA, PE and PG and that only the binding

to PI was markedly weaker than that to the other PLs. With addition of EDTA, the interaction of annexin V with all tested PLs was inhibited (Meers and Mealy, 1994). Therefore, the binding of annexin V to PS can be regarded as a feature of a broader specificity.

Interactions of SP-A with other PLs are well established. Early studies by Kuroki and Akino (1991) reported that SP-A binds DPPC. Their proposal was derived from interaction studies between labelled  $^{125}\text{I}$ -SP-A and phospholipids immobilized on thin layer chromatography silica gel plates (Kuroki and Akino, 1991). In another study, measurements of the intrinsic tryptophan fluorescence showed that the interaction of SP-A with DPPC was stronger than with other phospholipid vesicles (Casals et al., 1993). In contrast to other studies, this interaction appeared to be independent of  $\text{Ca}^{2+}$ . A detailed analysis of the lipid binding properties of SP-A showed that liposomes with different phospholipid compositions containing saturated and/or unsaturated fatty acids undergo similar  $\text{Ca}^{2+}$ -dependent interactions with SP-A (Meyboom et al., 1999). Five different derivatives of PC with saturated and unsaturated fatty acids as well as PG, PE and PI were used and the binding behaviour of SP-A to these PLs was examined. SP-A bound to PC and PI in similar manners, while the interactions with PE and PG were weaker. However, SP-A did not exhibit high specificity for either the polar or the apolar moiety of phospholipids. Thus, both head group and fatty acid composition are relevant to SP-A phospholipid interaction.

Our results show that SP-A binds to different PLs in a  $\text{Ca}^{2+}$ -dependent manner, which was not inhibited by mannose, suggesting involvement of a non-sugar binding-site of the CRD region. These results are consistent with the observations made with PS (Fig. 2) and with previous findings that did not include PS in studies (Meyboom et al., 1999). As shown in the SP-A/annexin V inhibition assay, the impact of SP-A on annexin V-binding is most remarkable in late apoptotic cells, but for these cells, SP-A binding is largely  $\text{Ca}^{2+}$ -independent (Jakel et al., 2009). Therefore, most binding of SP-A to late apoptotic cells is not to PLs. If annexin V and SP-A are competing for the same target, it could well be a phospholipid (not necessarily only PS). However, PL may be the major (most abundant) binding site for annexin V, but not for SP-A.

## MATERIALS AND METHODS

All chemicals used were purchased from Sigma unless otherwise stated.

### Protein purification

Native human SP-A was purified from bronchiolar lavage fluid (BALF) obtained from alveolar proteinosis patients as described previously (Suwabe et al., 1996). Purity of the protein was verified by SDS-PAGE analysis and was >95%. The purified protein was dialysed into

TBS-buffer (20 mM Tris, 150 mM NaCl, pH 7.4) and concentrated up to 1 mg/mL using Amicon filter units (cut off 100 kDa, Amicon, Denver, CO, USA). Endotoxin was removed from purified SP-A by passing the protein solution through a 10 mL Polymyxin B column (Pierce, Rockford, IL, USA) in sterile TBS with 0.02% (w/v) sodium azide. Remaining levels of endotoxin were assayed using a Limulus Amoebocyte Lysate kit according to the manufacturer's instructions (BioWhittaker, Gaithersburg, MD, USA). An endotoxin level of < 10 pg per microgram of protein was judged acceptable to use in cell-based assays.

### Purification of neutrophils and induction of apoptosis

Human neutrophils were isolated and the spontaneous apoptosis was induced by incubating the cells over a time course of 48 h as previously described (Jakel et al., 2009).

Early apoptotic cells were harvested after 24 h, and late apoptotic cells were obtained after 48 h. Confirmation of early and late apoptosis was assessed by double staining cells with FITC-labelled annexin V (Immunotools, Friesoythe, Germany) and 7-AAD (BD Pharmingen, San Diego, CA, USA) and subsequent detection by flow cytometry using a FACScan instrument (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) as previously described (Jakel et al., 2009). Briefly, 500,000 cells were resuspended in 50  $\mu\text{L}$  TBS containing 20 mM  $\text{CaCl}_2$ , 1% (w/v) BSA, 20-fold diluted FITC-labelled annexin V and 7-AAD for 1 h at room temperature (RT). After incubation, cells were centrifuged for 10 min at 800 g and washed once in the same buffer before fixing in 200  $\mu\text{L}$  TBS containing 20 mM  $\text{CaCl}_2$  and 1% (v/v) formaldehyde for 15 min. Acquisition and processing of data from 10,000 cells per sample was carried out with the CellQuest Software (Becton Dickinson). Early apoptosis corresponds to annexin V binding without 7-AAD uptake while late apoptosis was judged by binding of annexin V and 7-AAD uptake (Jakel et al., 2009).

### Induction of apoptosis in Jurkat cells

Jurkat E6.1 cells were cultured in RPMI 1640 (BioWhittaker) supplemented with 2 mM L-glutamine, 100 U/mL penicillin and 100  $\mu\text{g}/\text{mL}$  streptomycin (one vial containing all three components, Sigma, St. Louis, IL, USA), 10% (v/v) FCS (PAA Laboratories, Linz, Austria), 1% non-essential amino acids (Invitrogen, Carlsbad, CA, USA) and 1% Na-pyruvate (Invitrogen) at 37°C, 5%  $\text{CO}_2$  and 95% humidity. Apoptosis was induced as previously described (Jakel et al., 2009) by adding a 1 mM staurosporine stock solution to a final concentration of 1  $\mu\text{M}$ . Cells were incubated over a time course of 0 to 15 h. Early apoptotic cells were obtained after 6 h while late apoptotic cells were harvested after 15 h. After incubation, cells were harvested and centrifuged at 800 g for 10 min. After washing the cells twice in TBS containing 1% (w/v) BSA, confirmation of early and late apoptosis was performed as described above.

### Flow-cytometry annexin V binding assay

500,000 cells in 50  $\mu\text{L}$  of TBS containing 1% (w/v) BSA and 2.5 mM  $\text{CaCl}_2$  were incubated simultaneously with 0–20  $\mu\text{g}$  of unlabelled SP-A (1 mg/mL concentration), FITC-labelled annexin V (20-fold diluted from suppliers stock solution) and 7-AAD (20-fold diluted from suppliers stock solution). After 1 h incubation at RT in the dark, the

cells were centrifuged and washed once in 200  $\mu$ L TBS containing 1% (*w/v*) BSA and 2.5 mM  $\text{CaCl}_2$ , and fixed with 200  $\mu$ L TBS containing 2.5 mM  $\text{CaCl}_2$  and 1% (*v/v*) formaldehyde for 15 min before being measured by flow cytometry. A decrease of fluorescence in comparison to the sample without collectin indicated inhibition of binding to the cell-surface receptor(s). Viable cells were incubated with annexin V-FITC and 7-AAD as negative control.

### Preparation of phospholipid liposomes

To generate multilamellar phospholipid vesicles, 30  $\mu$ mol of lyophilised powder of dipalmitoyl-phosphatidylcholine (DPPC, Cat# 850457), phosphatidylethanolamine (PE, Cat# 850757), phosphatidic acid (PA, Cat# 840857), phosphatidylserine (PS, Cat# 840039) or phosphatidylglycerol (PG, Cat# 840465), all purchased from Avanti Polar Lipids, Alabaster, AL, USA, and phosphatidylinositol (PI, Cat# P6636-1G, Sigma) as well as cardiolipin (CL, Cat# C-1649, Sigma) were dissolved in 200  $\mu$ L chloroform followed by vigorous vortexing to ensure complete mixing of the lipid components. The resulting solution was dried under a stream of oxygen-free nitrogen in a glass scintillation vial. The lipid cake was then rehydrated with 300  $\mu$ L TBS at 34°C for 1 h with vigorous vortexing for 20 s every 15 min followed by a further rehydration for 2 h at RT.

### ELISA

The following general procedure was used to examine the binding of SP-A to immobilised phospholipid liposomes, annexin V and mannose. 96-well Maxisorp microtitre plates (Nunc, Denmark) were coated with 10  $\mu$ g/mL annexin V (ImmunoKontakt, Abingdon, UK), 20  $\mu$ g/mL mannose or 25  $\mu$ L (5  $\mu$ mol) of the prepared liposome suspension in 100  $\mu$ L TBS, and left overnight at 4°C. Wells were then washed three times with 200  $\mu$ L TBS containing 0.05% Tween-20 (TBS/Tween) and blocked with 200  $\mu$ L TBS containing 1% (*w/v*) BSA (TBS/BSA) for 30 min at RT. After washing three times again with TBS/Tween, SP-A (10 or 20  $\mu$ g/mL) in 100  $\mu$ L TBS/BSA was added to each well. Addition of these ligands to uncoated, blocked wells served as background controls. After incubating for 1 h at RT and washing three times with TBS/Tween, bound SP-A was detected by incubation for 1 h at RT with 100  $\mu$ L of 1  $\mu$ g/mL biotinylated monoclonal anti-SP-A antibody (Antibodyshop, Gentofte, Denmark) in TBS/BSA. After washing with TBS/Tween, 100  $\mu$ L of 200-fold diluted streptavidin-HRP (Sigma, Cat# S5512) in TBS/BSA was added to each well, and plates were incubated at RT for 1 h prior to washing. Finally, substrate and corresponding buffer were prepared by dissolving one Sigma FAST™ *o*-phenylenediamine dihydrochloride tablet set in 20 mL water and 100  $\mu$ L of this solution was added to each well. The reaction was stopped with 100  $\mu$ L 1M  $\text{H}_2\text{SO}_4$ . Absorbance values were measured at 492 nm, and background control values were subtracted from sample values.

### Binding of lung collectins to soluble PS liposomes

One hundred microliter of the prepared solution of PS liposomes (10  $\mu$ mol) was spun down at 9000 g for 15 min. The pellet was incubated with 1  $\mu$ g SP-A in 50  $\mu$ L TBS containing 2.5 mM  $\text{CaCl}_2$  or 1 mM EDTA for 1 h at 37°C. The liposomes were then washed twice with 400  $\mu$ L TBS (containing  $\text{CaCl}_2$  or EDTA) and the resulting pellet was analysed by SDS-PAGE to determine the binding of SP-A.

### SDS-PAGE

Protein samples were prepared by diluting with 1/3 volume of 4 $\times$  NuPAGE® SDS sample buffer (Invitrogen) and 1/9 volume of reducing agent (Invitrogen). The samples were incubated at 95°C for 5 min. Pre-stained low molecular marker (Invitrogen) was used as protein standard. NuPAGE® 4%–12% Bis-Tris-HCl gels (Invitrogen) were run at 200 V and 400 mA in 1 $\times$ NuPAGE® MES SDS running buffer (Invitrogen) for approximately 30 min. After electrophoresis, gels were stained with SilverQuest™ Silver Staining Kit (Invitrogen) according to the manufacturer's instructions.

### Confocal laser scanning microscopy

Visualisation of fluorescently-labelled proteins on the surface of human cells was achieved using a confocal laser Scanning Microscope Zeiss LSM 510 Meta and LSM software (Carl Zeiss Ltd, Welwyn Garden City, UK).

Binding of SP-A to early and late apoptotic Jurkat cells was detected with biotinylated mouse monoclonal anti-human SP-A antibody followed by simultaneous incubation with streptavidin-PE and annexin V-FITC, as previously described (Jakel et al., 2009). Briefly, 500,000 cells were suspended in 20 mM Tris, 150 mM NaCl, pH 7.4 (TBS) plus 1% (*w/v*) BSA (TBS/BSA) and centrifuged at 800 g for 10 min. The pellet was resuspended in 50  $\mu$ L of the same buffer containing 2.5 mM  $\text{CaCl}_2$  and SP-A (10  $\mu$ g in 10–20  $\mu$ L) was added before incubating the samples for 1 h at RT. After spinning at 800 g for 10 min, the pellet was washed once in 200  $\mu$ L TBS/BSA containing 2.5 mM  $\text{CaCl}_2$ , resuspended in 50  $\mu$ L of the same buffer containing biotinylated anti-SP-A monoclonal antibody (1  $\mu$ g/mL final concentration, Antibodyshop) and incubated for 1 h at RT. After spinning at 800 g for 10 min and washing with TBS/BSA containing 2.5 mM  $\text{CaCl}_2$ , phycoerythrin (PE)-labelled streptavidin (200-fold diluted, Immunotools, Cat# 31274244) was added to the pellet in a total volume of 50  $\mu$ L TBS/BSA containing 2.5 mM  $\text{CaCl}_2$  and incubated for 1 h at RT in dark. After washing and fixation of the cells in 200  $\mu$ L TBS containing 2.5 mM  $\text{CaCl}_2$  and 1% (*v/v*) formaldehyde for 15 min, cells were centrifuged again for 10 min at 800 g. The pellet was dissolved in 20  $\mu$ L ProLong® Gold antifade reagent with DAPI mounting medium (Invitrogen). Cells were mounted on glass slides, covered with cover slips and cured for 24 h prior to imaging. Serial optical sections were taken at 1  $\mu$ m intervals throughout the thickness of all cells. Analysis and image overlay was performed using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>).

### Statistics

Data are presented as means  $\pm$  standard deviation, unless otherwise stated. All experiments have been performed at least twice on different days and with different donors if neutrophils have been involved.

### ACKNOWLEDGMENTS

We thank Dr. Katja Rietdorf, Department of Pharmacology, University of Oxford, for technical assistance in the generation of confocal images and Dr. Maurits de Planque, School of Electronics and

Computer Science, University of Southampton, for technical advice on the generation of phospholipid liposomes.

This work was financially supported by the Medical Research Council, UK.

## ABBREVIATIONS

7-AAD, 7-Amino-actinomycin D; BALF, bronchiolar lavage fluid; CL, cardiolipin; CRD, carbohydrate recognition domain; DPPC, dipalmitoyl phosphatidylcholine; FCS, foetal calf serum; MBL, mannan-binding lectin; MFI, mean fluorescence intensity; OD, optical density; PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL(s), phospholipid(s); PS, phosphatidylserine; SP-A, surfactant protein A; SP-D, surfactant protein D

## REFERENCES

- Bennett, M.R., Gibson, D.F., Schwartz, S.M., and Tait, J.F. (1995). Binding and phagocytosis of apoptotic vascular smooth muscle cells is mediated in part by exposure of phosphatidylserine. *Circ Res* 77, 1136–1142.
- Casals, C., Miguel, E., and Perez-Gil, J. (1993). Tryptophan fluorescence study on the interaction of pulmonary surfactant protein A with phospholipid vesicles. *Biochem J* 296 (Pt 3), 585–593.
- Clark, H., Palaniyar, N., Hawgood, S., and Reid, K.B. (2003). A recombinant fragment of human surfactant protein D reduces alveolar macrophage apoptosis and pro-inflammatory cytokines in mice developing pulmonary emphysema. *Ann N Y Acad Sci* 1010, 113–116.
- Clark, H., Palaniyar, N., Strong, P., Edmondson, J., Hawgood, S., and Reid, K.B. (2002). Surfactant protein D reduces alveolar macrophage apoptosis in vivo. *J Immunol* 169, 2892–2899.
- Clark, H., and Reid, K.B. (2002). Structural requirements for SP-D function in vitro and in vivo: therapeutic potential of recombinant SP-D. *Immunobiology* 205, 619–631.
- Crouch, E.C. (1998). Collectins and pulmonary host defense. *Am J Respir Cell Mol Biol* 19, 177–201.
- Fadok, V.A., de Cathelineau, A., Daleke, D.L., Henson, P.M., and Bratton, D.L. (2001). Loss of phospholipid asymmetry and surface exposure of phosphatidylserine is required for phagocytosis of apoptotic cells by macrophages and fibroblasts. *J Biol Chem* 276, 1071–1077.
- Fadok, V.A., Savill, J.S., Haslett, C., Bratton, D.L., Doherty, D.E., Campbell, P.A., and Henson, P.M. (1992a). Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. *J Immunol* 149, 4029–4035.
- Fadok, V.A., Voelker, D.R., Campbell, P.A., Cohen, J.J., Bratton, D.L., and Henson, P.M. (1992b). Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol* 148, 2207–2216.
- Hart, S.P., Ross, J.A., Ross, K., Haslett, C., and Dransfield, I. (2000). Molecular characterization of the surface of apoptotic neutrophils: implications for functional downregulation and recognition by phagocytes. *Cell Death Differ* 7, 493–503.
- Henson, P.M., Bratton, D.L., and Fadok, V.A. (2001). The phosphatidylserine receptor: a crucial molecular switch? *Nat Rev Mol Cell Biol* 2, 627–633.
- Holmskov, U., Malhotra, R., Sim, R.B., and Jensenius, J.C. (1994). Collectins: collagenous C-type lectins of the innate immune defense system. *Immunol Today* 15, 67–74.
- Homburg, C.H., de Haas, M., von dem Borne, A.E., Verhoeven, A.J., Reutelingsperger, C.P., and Roos, D. (1995). Human neutrophils lose their surface Fc gamma RIII and acquire Annexin V binding sites during apoptosis in vitro. *Blood* 85, 532–540.
- Hoppe, H.J., and Reid, K.B. (1994). Collectins—soluble proteins containing collagenous regions and lectin domains—and their roles in innate immunity. *Protein Sci* 3, 1143–1158.
- Jakel, A., Clark, H., Reid, K.B., and Sim, R.B. (2009). The human lung surfactant proteins A (SP-A) and D (SP-D) interact with apoptotic target cells by different binding mechanisms. *Immunobiology*. In press.
- Kerr, J.F., Wyllie, A.H., and Currie, A.R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 26, 239–257.
- Kilpatrick, D.C. (1998). Phospholipid-binding activity of human mannan-binding lectin. *Immunol Lett* 61, 191–195.
- Kuroki, Y., and Akino, T. (1991). Pulmonary surfactant protein A (SP-A) specifically binds dipalmitoylphosphatidylcholine. *J Biol Chem* 266, 3068–3073.
- Lu, J., Teh, C., Kishore, U., and Reid, K.B. (2002). Collectins and ficolins: sugar pattern recognition molecules of the mammalian innate immune system. *Biochim Biophys Acta* 1572, 387–400.
- Malhotra, R., Lu, J., Holmskov, U., and Sim, R.B. (1994). Collectins, collectin receptors and the lectin pathway of complement activation. *Clin Exp Immunol* 97 Suppl 2, 4–9.
- Martin, S.J., Reutelingsperger, C.P., McGahon, A.J., Rader, J.A., van Schie, R.C., LaFace, D.M., and Green, D.R. (1995). Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med* 182, 1545–1556.
- Meers, P., and Mealy, T. (1993). Calcium-dependent annexin V binding to phospholipids: stoichiometry, specificity, and the role of negative charge. *Biochemistry* 32, 11711–11721.
- Meers, P., and Mealy, T. (1994). Phospholipid determinants for annexin V binding sites and the role of tryptophan 187. *Biochemistry* 33, 5829–5837.
- Meyboom, A., Maretzki, D., Stevens, P.A., and Hofmann, K.P. (1999). Interaction of pulmonary surfactant protein A with phospholipid liposomes: a kinetic study on head group and fatty acid specificity. *Biochim Biophys Acta* 1441, 23–35.
- Nauta, A.J., Castellano, G., Xu, W., Woltman, A.M., Borrias, M.C., Daha, M.R., van Kooten, C., and Roos, A. (2004). Opsonization with C1q and mannose-binding lectin targets apoptotic cells to dendritic cells. *J Immunol* 173, 3044–3050.
- Ogden, C.A., deCathelineau, A., Hoffmann, P.R., Bratton, D., Ghebrehiwet, B., Fadok, V.A., and Henson, P.M. (2001). C1q and mannose binding lectin engagement of cell surface calreticulin and CD91 initiates macropinocytosis and uptake of apoptotic cells. *J Exp Med* 194, 781–795.
- Paidassi, H., Tacnet-Delorme, P., Garlatti, V., Darnault, C., Ghebrehiwet, B., Gaboriaud, C., Arlaud, G.J., and Frachet, P. (2008). C1q binds phosphatidylserine and likely acts as a multiligand-bridging molecule in apoptotic cell recognition. *J Immunol* 180, 2329–2338.

- Schagat, T.L., Wofford, J.A., and Wright, J.R. (2001). Surfactant protein A enhances alveolar macrophage phagocytosis of apoptotic neutrophils. *J Immunol* 166, 2727–2733.
- Schlegel, R.A., Stevens, M., Lumley-Sapanski, K., and Williamson, P. (1993). Altered lipid packing identifies apoptotic thymocytes. *Immunol Lett* 36, 283–288.
- Shiratsuchi, A., Umeda, M., Ohba, Y., and Nakanishi, Y. (1997). Recognition of phosphatidylserine on the surface of apoptotic spermatogenic cells and subsequent phagocytosis by Sertoli cells of the rat. *J Biol Chem* 272, 2354–2358.
- Sim, R.B., Moffatt, B.E., Shaw, J.M., Ferluga, J. (2008). Complement control proteins and receptors: from FH to CR4. *Molecular aspects of innate and adaptive immunity*, pp 84–104. RSC Publishing, Cambridge, UK.
- Suwabe, A., Mason, R.J., and Voelker, D.R. (1996). Calcium dependent association of surfactant protein A with pulmonary surfactant: application to simple surfactant protein A purification. *Arch Biochem Biophys* 327, 285–291.
- Thiel, S., and Reid, K.B. (1989). Structures and functions associated with the group of mammalian lectins containing collagen-like sequences. *FEBS Lett* 250, 78–84.
- Vandivier, R.W., Ogden, C.A., Fadok, V.A., Hoffmann, P.R., Brown, K. K., Botto, M., Walport, M.J., Fisher, J.H., Henson, P.M., and Greene, K.E. (2002). Role of surfactant proteins A, D, and C1q in the clearance of apoptotic cells in vivo and in vitro: calreticulin and CD91 as a common collectin receptor complex. *J Immunol* 169, 3978–3986.
- Vermes, I., Haanen, C., Steffens-Nakken, H., and Reutelingsperger, C. (1995). A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods* 184, 39–51.
- Wallis, R. (2002). Structural and functional aspects of complement activation by mannose-binding protein. *Immunobiology* 205, 433–445.
- Weis, W.I., Taylor, M.E., and Drickamer, K. (1998). The C-type lectin superfamily in the immune system. *Immunol Rev* 163, 19–34.
- Wyllie, A.H., Kerr, J.F., and Currie, A.R. (1980). Cell death: the significance of apoptosis. *Int Rev Cytol* 68, 251–306.