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Review

Interactions of surfactant protein A with epithelial cells and phagocytes

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Abstract

Surfactant protein A (SP-A) has been shown to bind to and regulate the functions of both alveolar type II cells and immune cells including alveolar macrophages. The interaction of SP-A with type II cells has been shown in vitro to inhibit lipid secretion and to promote the uptake of lipid by these cells and these observations led to the hypothesis that SP-A plays an important role in regulating surfactant turnover and metabolism. The finding that mice made deficient in SP-A by homologous recombination (SP-A −/− mice) have relatively normal surfactant pool sizes has raised the possibility that either redundant mechanisms function in vivo to keep pool sizes normal in the absence of SP-A or that the in vitro findings are not significant in the context of the whole, unstressed animal. The interaction of SP-A with immune cells has been shown to affect a variety of responses which, in general, function to promote host defense against infection. Although SP-A receptors have been identified, additional studies will be required to elucidate the mechanism of interaction of SP-A with these cells and the relative importance of the different receptors in SP-A mediated regulation of cell function.

Keywords: Pulmonary surfactant; Surfactant protein A; Alveolar epithelium; Alveolar macrophage; Monocyte; Phagocytosis

Contents

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1. Introduction

Surfactant protein (SP) A was initially identified in surfactant purified from lavage fluid by King and Clements [1–3]. Although SP-A is only one of four surfactant-associated proteins identified thus far, in retrospect it is easy to understand why it was identified first. It is a very abundant protein and, unlike the hydrophobic proteins SP-B and SP-C, it resolves and stains easily on SDS-PAGE gels.

Because SP-A co-isolates with surfactant lipids, its extracellular functions in mediating surface tension reducing properties and participation in the organization of surfactant lipids were the focus of early investigations. These concepts are discussed in other chapters (see chapters by Goerke and McCormack in this issue for details). The concept that SP-A may interact with cells and regulate cell responses is a newer one about which less is known.

The purpose of this chapter is to review the available information on the interaction of SP-A with epithelial and phagocytic cells and the functional consequences of those interactions. The chapter will begin, however, with a brief discussion of the cellular sites and sources of action of SP-A and a review of the methods and some problems associated with purification of SP-A.

2. Cellular sources and sites of action of SP-A

Both protein and message for SP-A have been localized to the alveolar epithelial type II cell and the Clara cell of the bronchiolar region [4,5]. Therefore, it seems likely that SP-A secreted by these cells would be available to interact with cells of both alveolar origin and bronchiolar origin. Although macrophages have been shown to contain immunoreactive protein [4,6], they do not contain mRNA for SP-A. These data suggest that macrophages do not synthesize or secrete SP-A and, as discussed below, it appears that macrophages actively internalize and degrade SP-A.

3. Purification and properties of isolated and labeled SP-A

Many of the studies reported here were conducted with SP-A purified by different methodologies and it is important to note that there is clear evidence that the method of purification can have profound effects on the functions of SP-A.

One of the most common methods of purification is butanol extraction followed by detergent solubilization or column chromatography (e.g. [7]). This butanol extraction appears to have adverse effects on SP-A isolated from some species, such as rats and dogs, but SP-A isolated from the lavage of patients with alveolar proteinosis, a disease of unknown etiology that is characterized by an accumulation of surfactant, seems resistant to denaturation by butanol [8]. Alternative methods of SP-A purification include extraction of surfactant by chelation of calcium [9], ion exchange chromatography [8], and treatment of surfactant with reducing and denaturing agents followed by isoelectric focusing [10]. Although an exhaustive comparison of the effects of SP-A isolated by these various methods has not been reported, a comparison of the functions of SP-A isolated from rat and canine lavage by butanol extraction and ion exchange chromatography indicated that there are significant differences in these preparations. These studies raise a cautionary flag that SP-A isolated

by various methods may have different or altered functions.

Another important consideration is that SP-A has been shown to bind to bacteria and to endotoxin, a lipopolysaccharide (LPS) that is a major component of the bacterial cell wall. In addition, some preparations of isolated SP-A (and other surfactant proteins) contain significant amounts of endotoxin. Since LPS can have profound effects on immune cells, it is important to measure endotoxin levels in SP-A preparations and to treat them to remove endotoxin if necessary.

In order to characterize the binding of a ligand (e.g. SP-A) to a population of cells, it is necessary to label the protein in some manner. Most studies have been carried out with radioiodinated SP-A. However, it has been shown that iodination can affect SP-A structure and function. Stuart et al. [11] reported that oxidation of SP-A (via radioiodination and exposure to H_2O_2) resulted in alterations in the sedimentation coefficient and Stokes radius values after 10 days of storage, suggesting that the SP-A had depolymerized. This breakdown was accompanied by a decrease in carbohydrate binding activity. Although the iodinated SP-A used in some studies described below was characterized with respect to retention of specific functions, the observation of Stuart and co-workers raises an important warning that highly oligomerized proteins such as SP-A (and SP-D) may be unstable when modified and that this instability may become more apparent with time.

4. Interaction of SP-A with epithelial cells

The idea that SP-A may interact with type II cells was probably primarily derived from studies of surfactant metabolism, a subject addressed in detail in the chapter by Ikegami and Jobe in this issue. Although many studies have contributed to our overall understanding of surfactant clearance, a seminal study by Hallman and co-workers [12] demonstrated that type II cells, which synthesize and secrete surfactant, are also capable of internalizing surfactant from the alveolar space. This observation raised the possibility that the type II cell may be involved in surfactant clearance. Several laboratories have attempted to identify the factors that might regulate this uptake pathway.

Subsequent studies using subfractions of surfactant isolated by differential centrifugation and enriched in specific surfactant components showed that subfractions enriched in SP-A were taken up into type II cells to a greater extent than were subfractions deficient in SP-A [13]. These studies, and others discussed in detail below, led to investigations of the possibility that SP-A is involved in regulating surfactant clearance by type II cells and to studies characterizing the binding of SP-A to type II cells.

4.1. Binding of SP-A to type II cells

Kuroki and co-workers first reported that SP-A binds to high-affinity receptors on isolated alveolar type II cells [14]. The type II cells used in this study were maintained in primary culture for approx. 20 h. Hill plots of the binding data yielded an estimated dissociation constant of 1.02 μg/ml (or 0.64 nM). This constant was calculated based on the assumption that the native molecular mass of the oligomerized form of SP-A is 1.6 MDa. Maximal binding in this study occurred at approx. 5 μg/ml. The binding was calcium-dependent and was not inhibited by treatment of type II cells with proteases including trypsin, pronase, chymotrypsin, pepsin and papain. Thus, it was suggested that the binding site was either not a protein or was a protein that was highly resistant to protease digestion. The number of cell surface binding sites was estimated to be 135 000 sites per cell. A comparison between binding to type II cells and other cells showed that macrophages and type II cells exhibited specific binding whereas adult rat lung fibroblasts and L2 cells, a cell line derived from alveolar epithelial cells, did not. The binding of SP-A to macrophages will be discussed in more detail below.

SP-A was also reported to bind with high affinity to freshly isolated type II cells [15]. Interestingly, the properties of binding to freshly isolated type II cells were both similar and different from the properties of binding to primary cultures of type II cells. For example, the number of binding sites on freshly isolated type II cells was estimated to be 40 000 sites per cell and half-maximal binding occurred at 5×10^{-10} M, assuming an oligomeric size of 650 000.

Maximal binding occurred at approx. 1 μg/ml. Using the estimated oligomeric size reported by Kuroki et al. of 1.6 MDa, then the calculated half-maximal binding occurred at approx. 2×10^{-10} M (0.2 nM), a value approx. 3-fold smaller than that reported by Kuroki et al. (The different estimates of oligomeric size of SP-A are likely due to technical differences in the methods used for size exclusion chromatography.) Surprisingly, treatment of freshly isolated cells with trypsin decreased the level of binding by approx. 50%, raising the possibility that the mechanism of binding to freshly isolated type II cells may be occurring partly via binding of SP-A to a protease-sensitive protein, whereas the binding to primarily cultures of type II cells may be via a non-protein mediated mechanism (e.g., a glycolipid, as suggested by Kuroki et al.) or via a protease-resistant protein. Binding to both freshly isolated cells and primarily cultures of cells required calcium.

An important study by Ryan et al. [16] reported that SP-A labeled with biotin binds to cultured alveolar type II cells in a saturable manner. Maximal binding was obtained at approx. 10 ng/ml. The reasons for the differences in maximal binding reported in this study and the study by Kuroki et al. and Wright et al. are not known but could be related to the vastly different methodologies used. Both Kuroki et al. and Wright et al. used radiolabeled SP-A and biochemical analysis of binding. Ryan and coworkers employed biotinylated SP-A and electron microscopic analysis. It is possible that the different labeling techniques may alter SP-A's ability to bind to type II cells. In any case, it is important to note that this study demonstrated that SP-A was internalized via a coated pit pathway by type II cells (discussed in detail below); this finding provides strong morphological support for the existence of a receptor that participates in endocytosis of SP-A.

There are important differences observed between the binding of SP-A to freshly isolated type II cells and cells that have been maintained in primary culture. Potential explanations for the differences in affinity and receptor number observed with the freshly isolated and cultured type II cells include the fact that the receptor numbers and properties may change with time in culture. In addition, some of the observed binding, especially in the freshly isolated cells, may be attributed to contaminating cells such as macrophages. Alternatively, the receptors may be differentially clustered when cells are adhered to tissue culture plastic. The possibility that culture conditions may affect binding is supported by the observation by Bates et al. [17] that binding of SP-A to isolated type II cells cultured on microporous membranes is much higher than binding to cells cultured on plastic. In addition, it is possible that receptors are damaged during the elastase treatment of the lung tissue as part of the purification procedure, and that the receptors recover during time in culture. In any case, the studies, both in vivo and in vitro, are consistent with the fact that type II cells express receptors for SP-A.

Relatively little is known about factors that regulate the expression of receptors for SP-A on type II cells. An interesting study by Chen et al. showed that treatment of type II cells with surfactant secretagogues increased receptor number [18]. In this study, the binding of ^{125}I-labeled SP-A was increased by 1.5–2-fold by exposure of isolated type II cells cultured on Transwell membranes to the surfactant secretagogues 8-bromo-cyclic AMP, phorbol 12-myristate 13-acetate, terbutaline, or ATP. It is important to note that the effect was dependent upon the substrate on which the type II cells were cultured, since cells cultured on plastic did not respond to secretagogues with increased binding of SP-A. Both baseline and secretagogue enhanced binding of SP-A to type II cells were sensitive to trypsin. These studies are consistent with the possibility that there is a link between surfactant secretion and clearance.

4.2. Receptors for SP-A on type II cells

The findings that SP-A binds to type II cells with high affinity and that the interaction has properties of receptor mediated binding have led many to attempt to identify the SP-A receptor on type II cells. Several potential SP-A receptors have been identified. The receptors will be described in this section and their potential involvement in SP-A mediated type II cell function will be addressed in the subsequent section.

Strayer and co-workers developed anti-idiotypic antibodies to identify a 30 kDa protein that is found on type II cells and ciliated cells of the conducting airways but not on alveolar macrophages [19]. This

antibody, which was directed against the SP-A binding region of anti-SP-A antibodies was used to clone distinct cDNAs from human and porcine cDNA expression libraries. These SP-A binding proteins were named SPAR, an acronym for SP-A Recognition proteins. The deduced amino acid sequences of the human and porcine SPARs were 41% similar. The proteins were reported to be similar to known cell membrane receptors including the acetylcholine, α-2C adrenergic, ryanodine, and progesterone receptors. Message was detected primarily in the lung but also in the heart, kidney and small intestine. In a subsequent study, [20] it was shown that both antibodies inhibited the binding of radiolabeled SP-A to isolated type II cells.

Using an auto-anti-idiotypic antibody approach, Stevens et al. identified an SP-A binding protein of approx. 170–200 kDa under non-reducing conditions [21]. This protein appears to be comprised of 55 kDa subunits when analyzed under reducing and denaturing conditions on SDS-PAGE gel and has been named BP55. Antibodies against BP55 were shown to bind to type II cell surface proteins and to inhibit binding of SP-A to freshly isolated type II cells. Cross-linking studies suggested that BP55 is a major type II cell surface binding protein.

Chroneos and co-workers [22] have purified an SP-A binding protein that is apparently different than the proteins identified as BP55 and SPAR. The receptor was identified on ligand blot analysis and was detected on alveolar type II cells and macrophages as well as on U937 cells. Based on its size of 210 kDa, it has been called SPR210 (surfactant protein receptor 210).

An intriguing study by Momoeda and co-workers [23] suggested that neutral glycosphingolipids may serve as receptors for SP-A in the developing epithelium of the murine lung. These investigators reported that there were significant changes in the glycosphingolipid composition of murine lung in the post-natal period. SP-A was found to bind to a variety of glycolipids, consistent with previous reports [24,25]. Although the functions of these glycolipids as distinct receptors and their precise localization on the epithelium were not established, these studies are consistent with the intriguing possibility that the receptors for SP-A may be glycolipids as well as proteins.

4.3. Mechanism of binding of SP-A to isolated type II cells

Because SP-A is a multi-domain molecule it has been tempting to speculate that specific domains of SP-A may mediate specific functions. The domain structure is reviewed in the chapter by McCormack in this issue and in [26]. Briefly, the shortest domain is the N-terminal domain, which is followed by a longer collagen-like domain. This collagen-like domain is characterized by a repeating tripeptide unit typical of collagen-like proteins containing a glycine-X-Y, where X is any amino acid and Y is often a hydroxylated proline. The collagen-like domain is followed by a triple helical coiled-coil neck domain and a C-type lectin domain. The C-type lectin domain is characterized by a conserved sequence of amino acids that confer calcium-dependent carbohydrate binding (lectin) activity. This domain has been abbreviated the CRD or carbohydrate recognition domain.

Several approaches have been used to attempt to identify the domains mediating SP-A binding to type II cells. One approach has been to produce fragments of SP-A, such as the collagenase-resistant fragment or CRF. This fragment can be produced by enzymatic digestion of SP-A with collagenase. The resulting fragment is the C-terminal C-type lectin domain. This fragment retains the ability to bind carbohydrates, but seems to be unstable and to have a lower affinity for carbohydrates than does the intact protein. One limitation of this approach is that it has not been possible, thus far, to produce enzymatically a collagen-like fragment of SP-A or a fragment of the neck domain. Although others have successfully digested collagen-like proteins such as C1q with pepsin and derived a fragment of the collagen domain, nothing detectable by SDS-PAGE is recovered after digestion of SP-A with pepsin.

In order to determine whether the lectin-like domain of SP-A mediates binding, a collagenase-resistant fragment of SP-A was isolated and found to bind to a much greater extent to type II cells than to alveolar macrophages or fibroblasts [15]. Murata and co-workers [27] also showed that a collagenase-resistant fragment of SP-A competed for binding of intact SP-A and that the isolated fragment bound to type II cells in a concentration-dependent manner.

Thorkelsson and co-workers [28] used microscopic techniques to show that the lectin activity associated with SP-A is not required for, but may participate in SP-A binding to rat alveolar type II cells. In this study, it was shown that SP-A bound to horseradish peroxidase, a mannosylated protein, but that horseradish peroxidase did not alter the binding of SP-A to the type II cell surface. Thus, these data suggest that either the carbohydrate recognition domain of SP-A is not involved in binding, or that horseradish peroxidase is not an effective competitive inhibitor.

Kuroki and co-workers [29] reported that specific chemical modifications of SP-A affected its ability to bind to type II cells. For example, SP-A that was alkylated with iodoacetamide or reductively methylated did not compete with wild type SP-A for cell surface binding. In addition, neither mannose nor α-methylmannoside inhibited binding of SP-A to type II cells. Although one interpretation of this latter finding is that the lectin activity of SP-A is not involved in binding, it is also possible that the type II cell binding site is a carbohydrate that has a higher affinity for SP-A than does mannose or α-methylmannoside.

The concept that the CRD domain of SP-A is involved in type II cell binding is supported by results from a variety of methods. For example, Kuroki and co-workers have used monoclonal antibodies against SP-A to map functional domains. Two monoclonal antibodies (1D6 and 6E3) reduced the high affinity binding of SP-A to type II cells [29,30]. The monoclonal 1D6 bound to an epitope in the region of a small disulfide loop of the CRD (Cys-204 to Cys-218) [30]. Murata et al. [27] also used monoclonal anti-SP-A antibodies to help define the type II cell binding domain. One anti-SP-A monoclonal antibody (PE10) decreased the binding of SP-A to type II cells. Subsequent studies by Hiraike and co-workers [31] used the same antibody and epitope mapping to localize the region of SP-A responsible for these activities to a region in the CRD from Glu-202 to Met-207. Thus the epitope mapping studies are consistent with the CRD as the type II cell binding domain.

McCormack and co-workers employed site-directed mutagenesis to produce glycosylation deficient mutants of recombinant SP-A. They found that glycosylation of SP-A is not required for type II cell binding [32]. Interestingly, the carbohydrates of SP-A are involved in binding to viruses and bacteria [33] and mycobacterium [34,35]. These observations support the notion that different domains of SP-A may be involved in binding to type II cell and pathogens.

Site-directed mutagenesis has also been used to more precisely identify the receptor binding domain. The rationale behind this approach was to use information from the crystal structure of the homologous mannose binding protein to predict which amino acids might be involved in the binding to carbohydrates. It was shown that mutants of recombinant rat SP-A in which Glu at position 195 was changed to Gln and Arg at position 197 was changed to Asp resulted in conversion of SP-A from a mannose binding lectin to a galactose binding lectin and reduced the affinity of binding to isolated type II cells [36]. Thus, these data are also consistent with the CRD as the type II cell binding domain.

McCormack and co-workers used alanine-scanning mutagenesis to replace CRD residues Glu-195, Glu-202, Asn-214 and Asp-215 with alanine and expressed the mutant SP-A in insect cells [37]. The N214A mutant (e.g. Asn-214 replaced with Ala) bound and aggregated lipids, but these activities were reduced in the mutants E195A, E202A, and D215A. The mutant recombinant proteins did not compete effectively for binding of ^{125}I-labeled rat SP-A to type II cells. This study provides additional evidence that these residues in the CRD domain are important in SP-A interactions with type II cells.

Additional site-directed mutagenesis studies by McCormack et al. [38] investigated the role of the Cys residue at position 6 in forming intermolecular disulfide bonds and in the interactions of SP-A with type II cells and lipids. The Cys at position 6 was mutated to Ser to prevent disulfide bond formation. The Cys-6 mutant (C6S), which contained the collagen-like domain but did not form appropriate disulfide bonds, competed only weakly for receptor occupancy. This finding is consistent with the possibility that appropriate oligomerization of SP-A may be important in receptor binding and its ability to regulate type II cell function.

An additional mutant lacking the collagen-like domain (abbreviated ΔG8-P80, e.g. deletion of the peptide from the glycine at position 8 to the proline at position 80) was expressed in insect cells using the

recombinant baculovirus system [26]. The ΔG8-P80 competed only weakly for native SP-A receptor occupancy on isolated type II cells. It was concluded that the collagen-like region is required for competition with native ^{125}I-SP-A for receptor occupancy. Interestingly, it has been thought that the collagen-like domain of SP-A may be important for oligomerization of SP-A, but the ΔG8-P80 mutant remains highly oligomeric. This mutant does not bind with high affinity to alveolar type II cells.

Another approach which has yielded very interesting and sometimes surprising results is the production of chimeric molecules of SP-A with either SP-D or one of the serum C-type lectins, the mannose binding protein (MBP). This approach is very appealing because this family of proteins, known as collectins (collagen-like lectins) are highly oligomerized and oligomerization may be important in their structure and function. Therefore, the chimeric molecules may be more likely to fold and oligomerize correctly than the fragments of such complex molecules.

Using this approach, chimeras of the mannose binding protein and SP-A were produced in which the region of SP-A spanning Glu-195 to Phe-228 was replaced with the MBP-A region of Glu-185 to Ala-221 [39]. This chimera was found to bind to type II cells. Most surprisingly, a monoclonal antibody mapped to this region of SP-A bound to the chimeras, even though that region of SP-A had been re-

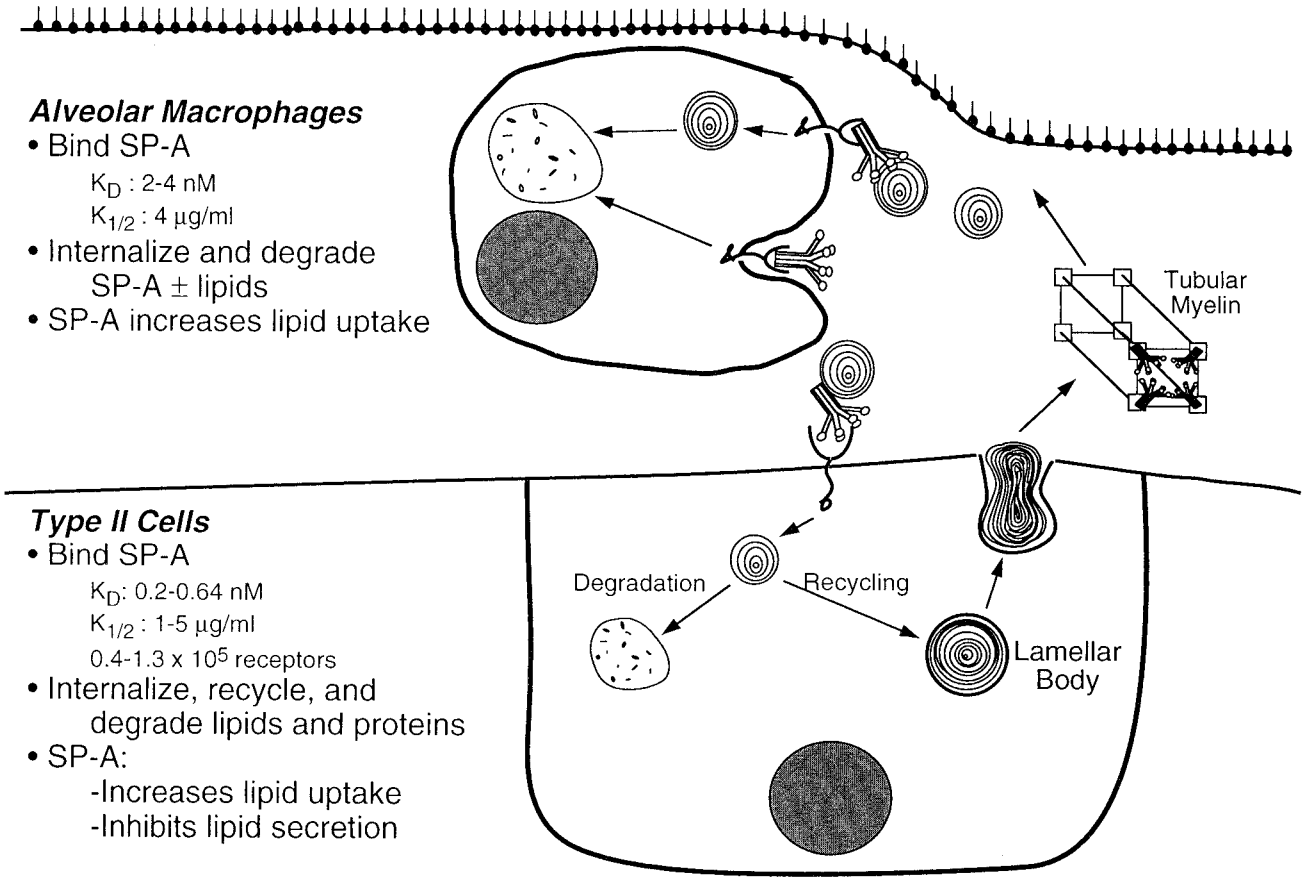

Fig. 1. Involvement of SP-A in surfactant metabolism via interactions with type II cells and alveolar macrophages. SP-A has effects on the metabolism of pulmonary surfactant by both type II epithelial cells and alveolar macrophages. Surfactant lipids are secreted by type II cells via lamellar bodies; this secretion is inhibited by SP-A. Secreted surfactant is a heterogeneous mixture with many forms, including tubular myelin and a lipid monolayer at the air-liquid interface. SP-A binds to both type II cells and alveolar macrophages with high affinity, and stimulates the uptake of lipids by both cell types. Proteins and lipids internalized by alveolar macrophages are generally degraded. Type II cells can either degrade or recycle internalized surfactant; the recycling pathway is favored in the presence of SP-A.

placed with a section of the MBP. These studies further support the concept that the conformational and oligomeric state of these complex molecules have profound effects on their function, and that data must be interpreted with this notion in mind.

The aggregate of the data considered together suggests that the CRD domain of SP-A may mediate binding to type II cells and that the N-terminal interchain disulfide bond and the collagen-like domain may contribute to appropriate folding and stability of the SP-A molecule which may be important in receptor binding.

4.4. Effects of SP-A on type II cell function

SP-A has been shown to affect a variety of type II cell functions in vitro (Fig. 1). These functions include uptake of surfactant lipid, secretion of surfactant lipid, and secretion of cytokines. Each of these effects will be discussed separately.

Wright et al. reported that SP-A stimulated lipid uptake by freshly isolated type II cells [7], and this effect was dependent on temperature and cellular metabolism. The lipids that were internalized in the presence of SP-A were degraded to a much lesser extent than were the lipids that were internalized in the absence of SP-A. The finding by Fisher and co-workers [40–42] that SP-A inhibits phospholipase A_2 raises the intriguing possibility that SP-A may regulate intracellular phospholipid metabolism by affecting degradative enzyme activity.

Bates and co-workers [17] reported that SP-A enhances the uptake of pulmonary surfactant by lung type II cells cultured on microporous membranes. A comparison of the binding of radiolabeled SP-A to cells cultured on microporous membranes and effects of SP-A on lipid uptake indicated that both binding and enhancement of lipid uptake were greater in cells cultured on microporous membranes than cells cultured on plastic.

The effect of SP-A on lipid uptake by type II cells was reported to be temperature-dependent and abolished by ATP depletion of the cells [43]. Several approaches were used to determine whether the liposomes were adhered to the cell surface or internalized, including treatment of cells with trypsin, washing the cells with EGTA, and back exchange with dioleoylphosphatidylcholine. The data were consistent with the conclusion that SP-A increases the internalization and not just the adherence of lipids to type II cells. Consistent with other studies, it was found that lipids internalized in the absence of SP-A were degraded to a lesser extent than were lipids internalized in the presence of SP-A.

Tsuzuki et al. [44] also reported that SP-A enhances uptake of phosphatidylcholine by alveolar type II cells. Internalization was demonstrated by isolating subcellular fractions of type II cells after the internalization was allowed to occur. It was reported that phosphatidylcholine incorporated in the presence of SP-A was largely intact, e.g. not degraded. Kuroki and co-workers [45] also used subcellular fractionation techniques to demonstrate that SP-A enhanced the association of liposomes with type II cell plasma membranes 4-fold. Studies with dual labeled liposomes containing dipalmitoylphosphatidylcholine and triolein suggested that lipid transfer was not enhanced by SP-A, but that the major mechanism of enhancement was via increased binding and subsequent internalization. Although SP-A increased the aggregation of liposomes, the increase in aggregation was much less than the increase in association with cells suggesting that the mechanism of increased association was not due solely to aggregation of lipids.

The cell specificity of the SP-A response was investigated by Rice and co-workers [46] who reported that SP-A enhanced the uptake of lipids in a cell specific manner; SP-A did not enhance uptake of PC by Chinese hamster ovary cells but did increase uptake by isolated type II cells.

The studies described above as well as others [30,36] have shown that SP-A enhances lipid uptake by type II cells. In contrast, Horowitz and co-workers reported that SP-A did not increase the uptake of lipids by either isolated rat type II cells maintained in primary culture or MLE-12 cells, a pulmonary adenocarcinoma cell line with alveolar cell characteristics [47]. The reasons for the apparent conflicts are not known but may be related to differences in the preparations of SP-A or the culture and isolation conditions used to obtain the cells.

SP-A has also been observed to inhibit lipid secretion in vitro. SP-A inhibited lipid secretion by primary cultures of type II cells stimulated by terbutaline, phorbol 12-myristate 13-acetate, or the ionophore A23187 [48]. Rice and co-workers also

reported that SP-A inhibited phospholipid secretion induced by phorbol ester, a β-adrenergic agonist, and a P2-purinergic agonist. Interestingly, the presence of phospholipid abrogated the SP-A inhibition [49, 50].

Studies from several laboratories using several different approaches have provided evidence that SP-A is taken up by type II cells and incorporated into lamellar bodies. For example, Ryan and co-workers used electron microscopic techniques to demonstrate that biotinylated SP-A was internalized by isolated type II cells [16]. They observed that SP-A was clustered in coated pits on the cell's surface, internalized, and eventually became associated with coated vesicles, endosomes, and multivesicular bodies. In vivo studies by Young and co-workers, using both biochemical techniques and electron microscopic autoradiography, extended these studies to the whole animal and showed that SP-A is taken up in the intact lung and incorporated into lamellar bodies [51,52]. An apparently conflicting study by Kalina and co-workers showed that gold labeled SP-A was internalized by type II cells but not incorporated into lamellar bodies [53]. Young and co-workers have reported that the method of labeling SP-A may affect its ability to be internalized (Young, personal communication).

Although the in vitro work is compelling, recent studies using mice in which the SP-A gene has been ablated by homologous recombination (SP-A (−/−) mice), show that these mice have moderately altered surfactant pool sizes, in spite of a complete lack of SP-A [54]. For example, the alveolar saturated phosphatidylcholine pools were 50% larger and the lung tissue saturated phosphatidylcholine pools were 26% larger in SP-A (−/−) mice than in wild type (SP-A (+/+)) mice. The synthesis of new PC, measured by following incorporation of radiolabeled choline and palmitate into lung tissue PC, was similar for SP-A (−/−) mice and SP-A (+/+) mice [55]. In addition, the secretion of the radiolabeled PC was similar in control and knock-out mice. Interestingly, the SP-A (−/−) mice retained more labeled PC in the lungs at 48 h, a finding consistent with observation that the pool size is increased. The authors concluded that, overall, the absence of SP-A had minimal effects on the metabolism of PC.

How can these studies with SP-A knock-out mice be reconciled with the in vitro work? It is, of course, possible that the in vitro work is artifactual. It is also possible, however, that compensatory mechanisms may function in the absence of SP-A and result in adjustments that obscure evidence of a large direct effect of SP-A on lipid metabolism. This possibility seems reasonable in light of the studies that have shown that many animals in which genes have been ablated have no obvious phenotype, possibly due to redundant compensatory mechanisms [56–58]. In addition, it seems possible that when the SP-A deficient animals are stressed, e.g. by exercise, an acute inflammation, lung injury or infection, the regulatory role of SP-A may become more evident. Further investigations to elucidate the role of SP-A in regulating surfactant metabolism are important.

The same approaches that have been used to map type II cell binding domains have been employed to determine which domain is involved in SP-A regulation of type II cell function. For example, Kuroki et al. [29,30] observed that two monoclonal antibodies (1D6 and 7E3) blocked ability of SP-A to inhibit phospholipid secretion. The monoclonal antibody 6E3, which binds the neck region of SP-A, blocked lipid uptake into type II cells but not lipid aggregation or lipid binding. Another monoclonal antibody, 1D6 which binds the CRD, blocked the interaction of SP-A with phospholipid and the ability of SP-A to increase lipid uptake [30]. Thus, these studies support the concept that the CRD may be involved in regulating type II cell function.

SP-A in which Glu-195 was changed to Gln-195 and Arg-197 was changed to Asp-197 by site-directed mutagenesis had a decreased ability to inhibit phospholipid secretion [36]. A recombinant SP-A lacking the collagen-like domain was a potent inhibitor of surfactant secretion, in spite of the fact that it did not compete with wild type SP-A for receptor occupancy. This deletion mutant also enhanced lipid uptake by type II cells [26]. In contrast, the SP-A mutant lacking Cys-6 only weakly inhibited surfactant secretion. Neither C6S nor ΔG8-P80 mutants enhanced lipid association with type II cells [38]. The mutants in which alanine-scanning mutagenesis was used to replace Glu-195, Glu-202, Asn-214 and Asp-215 with alanine were much less effective at regulating surfactant secretion and uptake by type II cells [37]. The mutants E195Q and R197D were no longer

able to enhance lipid uptake by type II cells. These data are consistent with the possibility that SP-A interactions with type II cells and regulation of type II cell function is complex.

The oligomeric state of the SP-A molecule also has effects on its function. For example, Hattori and co-workers reported that oligomerized SP-A isolated from lavage of patients with alveolar proteinosis had a reduced ability to inhibit phospholipid secretion and had a lower affinity for binding to type II cells [59].

Several studies have also attempted to identify which receptor is involved in SP-A mediated effects on lipid metabolism. An auto-anti-idiotypic antibody against BP55, a type II cell surface protein that binds SP-A, inhibited the SP-A mediated enhancement of lipid uptake, and it was suggested that BP55 may be the receptor responsible for the SP-A mediated enhancement of lipid uptake [43]. Interestingly, this antibody did not decrease the ability of SP-A to inhibit phospholipid secretion, suggesting that different receptors may mediate these functions of SP-A.

Two antibodies against the 30 kDa SPAR protein were shown to modulate the SP-A mediated inhibition of phospholipid secretion by SP-A [20]. Both antibodies inhibited SP-A's effect on secretion of phospholipid stimulated by any of several surfactant secretagogues, including ATP, dibutyryl cAMP, terbutaline, and ionomycin. Taken together these studies suggest that this receptor may be involved the SP-A mediated regulation of phospholipid secretion.

The 210 kDa receptor identified by Chroneos and co-workers may also be involved in regulating the effects of SP-A on phospholipid secretion [22]. Anti-receptor polyclonal antibodies inhibited the binding of SP-A to both type II cells and macrophages and reduced the SP-A mediated inhibition of phospholipid secretion in vitro. The anti-receptor antibodies also blocked SP-A mediated macrophage effects (discussed in detail below). Thus, it is possible that more than one receptor (the 30 kDa protein described by Strayer and the 210 kDa protein described by Chroneos and co-workers) may both participate in SP-A mediated inhibition of phospholipid secretion. The possibility that these antibodies may react with different subunits of the same receptor has not been addressed.

5. Interaction of SP-A with phagocytes

SP-A has been shown to bind to a number of phagocytic cells, with different binding characteristics and downstream effects of that binding. In examining the role of SP-A in pulmonary host defense, the interactions of SP-A with alveolar macrophages and peripheral blood monocytes have been well studied; several studies have also looked at the interaction of SP-A with other phagocytic cells, such as neutrophils, bone marrow-derived macrophages, monocyte-derived macrophages, and various monocytic cell culture lines. While there is some controversy about the specific receptors involved in these processes, and more controversy about the specific nature of SP-A stimulation of phagocytes, the vast majority of studies clearly delineate a role for SP-A in the regulation of phagocyte activity in the lung.

5.1. Binding of SP-A to phagocytic cells

Not surprisingly, the initial characterizations of SP-A interaction with phagocytic cells looked at binding to whole cells, in attempts to define the cell types to which SP-A binds as well as the nature of that binding. Initial binding studies focused mainly on SP-A binding to type II cells, but also included alveolar macrophages for purpose of comparison. In 1988, Kuroki and colleagues were the first to demonstrate that SP-A binds with high affinity to rat alveolar macrophages using ^{125}I-labeled rat SP-A [14]. Shortly thereafter, Wright et al. confirmed these results in another study of type II cells, showing not only that SP-A bound in a specific manner to alveolar macrophages, but that this binding was calcium-dependent and that collagen was less effective in competing for this binding than for binding to type II cells [15].

Later studies focused specifically on SP-A binding to phagocytic cells, and began to define the mechanism of SP-A binding to these cells. In three related studies, Schlepper-Schäfer and colleagues examined the binding of recombinant human SP-A-coated gold particles to human peripheral blood monocytes and monocyte-derived macrophages [60] and of both SP-A-coated gold and unbound SP-A to alveolar macrophages [61,62] by electron microscopy. In the first of these studies, Wintergerst et al. found that

both monocytes and monocyte-derived macrophages bind and internalize SP-A-gold particles, and that macrophage uptake of SP-A-gold was 2.4-fold higher than that of monocytes. Additionally, they found that the uptake of SP-A-gold particles occurred through coated pits, and that this uptake was inhibited in both cell types by mannosyl-BSA, but not galactosyl-BSA [61].

These results were confirmed by the later studies on alveolar macrophages; alveolar macrophages bound and internalized more SP-A-gold particles in less time, and mannosyl-BSA showed slightly less inhibition of SP-A-gold binding and uptake than in the previous study (59% inhibition with alveolar macrophages vs. 73% inhibition with monocyte-derived macrophages) [61]. Additionally, it was shown that the interaction between SP-A-gold particles and alveolar macrophages was specific (inhibited by an excess of uncoupled SP-A) and calcium-dependent (inhibited in the presence of 10 mM EDTA). In a third study, unbound SP-A was incubated with alveolar macrophages, which were then incubated with anti-SP-A antiserum and protein A-coupled gold particles [62]. The results of this study correlated nicely with the previous two; SP-A binding to alveolar macrophages was found to be calcium-dependent, and inhibitable to some extent by mannosyl-BSA (to a maximum inhibition of 36%, lower than in either of the previous studies), the collagenase-resistant fragment of SP-A (maximum inhibition of 44%) and the collagen-like fragment of C1q (maximum inhibition of 19%), indicating the possibility of two mechanisms for SP-A binding to phagocytes, via both the collagenous and carbohydrate binding domains.

Other investigators used radioiodinated rat SP-A to characterize SP-A binding to rat alveolar macrophages [63]. This was the first study to quantitate the binding characteristics of SP-A to alveolar macrophages; in it, it was shown that SP-A binding to alveolar macrophages is specific as well as time-, temperature-, and concentration-dependent, and, assuming that the SP-A oligomer has a molecular mass of 1.6 MDa, has an apparent K_d of between 2 and 4 nM [14]. Additionally, the concentration of SP-A at which binding was half-maximal was measured to be 4 μg/ml. In an apparent contradiction of the results of Manz-Keinke et al. which implicated the carbohydrate binding domain of SP-A in cell binding, Pison and colleagues found that large molar excesses of both collagen and the SP-A structural homologue complement component 1q (C1q), while less effective than unlabeled SP-A, could inhibit the binding of labeled SP-A to cells, and that heating SP-A to 50°C decreases cell binding by 58% [63]. This heat treatment corresponds to the thermal denaturation temperature of the collagen-like triple helices of SP-A [64–66]; these findings taken together were used to support the hypothesis that the collagenous domain of SP-A is at least partially responsible for the interaction of SP-A with alveolar macrophages.

In a study designed to look at the distribution of cell surface receptors for SP-A by comparing the recovery of cells with SP-A-coated magnetic beads, Oosting and Wright found that preincubation of alveolar macrophages with SP-A significantly inhibited their binding to SP-A-coated beads [67]. This study demonstrated that recovery of alveolar macrophages with SP-A-coated beads was greater than that of any other cell type. The other cells tested, in decreasing order of recovery, were peritoneal macrophages, neutrophils, alveolar type II cells, COS cells, and, in contrast to the results of Malhotra et al. [68], monocytic U937 cells [67].

Studies of the interaction between SP-A and other phagocytic cells are not as comprehensive as those with alveolar macrophages. In the previously described study by Ohmer-Schröck et al., no binding of fluorescently labeled SP-A (FITC-SP-A) was detected to peritoneal macrophages or liver macrophages (Kupffer cells), although results of this study were not quantified [62]. Chroneos and colleagues found specific, saturable, high-affinity ($K_d = 2.2$ nM) binding of SP-A to bone marrow-derived macrophages, which was calcium-dependent, and not inhibited by either mannan or C1q [22]. In a quantified study using FITC-SP-A, Geertsma et al. examined the binding of SP-A to peripheral blood monocytes, granulocytes, and lymphocytes, and found that while FITC-SP-A binding to monocytes was dramatically higher than to either of the other cell types tested, this binding was neither saturable (at concentrations as high as 50 μg SP-A/ml) nor blocked by excess unlabeled SP-A; preincubation of cells with a 200-fold molar excess of unlabeled SP-A failed to significantly reduce binding of FITC-SP-A to monocytes

[69]. Interestingly, preincubation of monocytes with C1q at molar ratios as low as 5:1 significantly reduced FITC-SP-A binding to them, and preincubation with a 20-fold molar excess of C1q completely eliminated FITC-SP-A binding to the cells [69].

5.2. Cell surface receptors for SP-A on phagocytes

The studies summarized above served to support the previously developed hypothesis that receptors for C1q are responsible for SP-A binding to monocytic cells. In a prior set of studies by Malhotra et al., a 115 kDa receptor for C1q (C1qR) was identified on the monocytic cell line U937 and postulated to be responsible for SP-A binding to those cells [68,70,71]. The first studies examined the ability of C1q, SP-A, MBP, conglutinin, and collagen to inhibit binding of ^{125}I-labeled C1qR to C1q immobilized on microtiter plates. In two separate studies, it was shown that conglutinin, C1q, MBP, and SP-A all inhibit binding of C1qR to immobilized C1q [70,71]. This inhibition was shown for C1qR:inhibitor molar ratios of approx. 1:2800, 1:1400 [70] and 1:140 [71]. In a third study, Malhotra and colleagues demonstrated specific, saturable, salt-dependent, high-affinity ($K_d = 0.7$ nM) binding of SP-A to U937 cells [68]. They found this binding to be competed by both C1q and C1qR, supporting their hypothesis that SP-A binds to C1qR on the surface of U937 cells.

In addition to C1qR, two other proteins have been identified that bind SP-A in different fractions of whole lung homogenate. These proteins are SPR210 and cellular myosin. Only SPR210 has been localized to the cell surface, and its cellular distribution is not limited to phagocytic cells [22]. Michelis et al. reported in 1994 that SP-A binds myosin, and that this binding is concentration-dependent [72]. While this study postulated that this interaction could have functional importance in lung injury, further studies led to the possibility that the coiled-coil motif was important in SP-A binding (Michelis, unpublished results). Chroneos and colleagues identified a 210 kDa cell-surface protein which binds SP-A, SPR210 [22]. Purified on a column made by binding SP-A to maltose-coupled sepharose beads, SPR210 likely binds the collagen-like domain of SP-A; it was further purified from U937 cell membranes, and found on bone marrow-derived macrophages and type II cells [22].

5.3. Mechanism of SP-A interaction with phagocytes

Many groups of investigators have used binding and receptor studies to draw conclusions about the mechanism of SP-A-phagocyte interaction. These experiments have been complicated and often difficult to interpret; as a result, evidence exists to support several different hypotheses about the domain or domains of SP-A involved in binding to phagocytic cells.

The most common technique used to examine SP-A binding is a competition assay in which the ability of various proteins to compete for SP-A binding to cells or to stimulate function is assessed. Those seeking to examine binding to the C1q receptor have used unlabeled C1q and iodinated SP-A to provide evidence for their claims that SP-A binds [68] or does not [22] bind to cells via the C1q-R. In examining the role of the collagenous domain of SP-A in its interactions with cells, types IV and V collagen have also been used to block SP-A binding to cells [15,63].

Thus far in the literature, competition assays have proven inconclusive. Assays using collagen as a competitor for SP-A have yielded conflicting results. Extreme excesses of collagen (an almost 300:1 molar ratio) have been shown to block the binding of labeled SP-A to alveolar macrophages by 30% [15]. Other studies, however, seem to conflict with this data. In one report, collagen was shown to be less effective than C1q in competing for SP-A binding and only marginally more effective than BSA except at molar ratios approaching and exceeding 1000:1, and then only to about 40% less than control levels [63]. While C1q has been more effective in competition assays looking at binding to U937 cells [68], and alveolar macrophages [63], another study found that the addition of unlabeled C1q increased SP-A binding to rat bone marrow-derived macrophages by over 100% [22].

Oosting and Wright, in their study of cell recovery assay using SP-A-coupled magnetic beads, addressed one potential explanation for the conflicting results in these studies: the direct interaction of SP-A with other proteins in solution. In this study, they exam-

ined the binding of ^{125}I-labeled SP-A to proteins immobilized on microtiter plates, and found that SP-A binds to itself, SP-D, and C1q, a previously unreported result which complicates coincubation competition experiments with either whole cells or immobilized proteins [67]. In addition, this study characterized the binding of cells to SP-A-coupled beads after preincubation or coincubation of cells with several potential competitors for cell binding and in the presence of carbohydrates and carbohydrate-bound BSA. Specifically, the study found that coincubation of cells with SP-A-coated beads and SP-A, SP-D, C1q, mannosyl-BSA, or heat-treated or deglycosylated SP-A significantly reduced alveolar macrophage recovery. Interestingly, it was also found that only preincubation of the cells with SP-A (untreated, deglycosylated, and heat-treated SP-A all were effective) reduced alveolar macrophage recovery; pre- or coincubation with collagenase-treated SP-A, mannose binding protein, type V collagen, mannan, or fucoidin did not [67]. These data, taken together, support the hypothesis that SP-A binding to alveolar macrophages is at least partially due to the collagen-like domain of SP-A, and that it is independent of SP-D, C1q, and MBP binding.

Several studies support a model of SP-A-phagocyte interaction via the other domains of SP-A. Some studies have argued that the interaction of SP-A with cells involves the carbohydrate binding domains of SP-A. These studies have shown that SP-A binding to monocyte-derived macrophages can be inhibited by the addition of mannosyl-BSA [60,61]. In another study, it was shown that the collagenase-resistant fragment of SP-A binds non-specifically to alveolar macrophages [15]. Furthermore, the binding of SP-A to cells has been shown by many groups to be calcium-dependent [15,67] suggesting that the structure of the collagenase-resistant portion of SP-A is vital to cell binding. It is possible, however, that a calcium-dependent conformational change, independent of the calcium-dependent lectin activity of SP-A, is important for SP-A binding to cells.

5.4. Functional consequences of SP-A binding to phagocytes

In order to examine further the mechanism of SP-A binding to phagocytes, it became necessary to define the functional consequences of that binding. Almost all recent studies seeking to characterize the interaction of SP-A with various cell types have relied on functional assays to draw their conclusions. A variety of techniques, from competition to receptor clustering assays, have been used to examine both the domains of SP-A responsible for cell binding and the cell surface proteins responsible for functional interactions.

Alveolar macrophages are the predominant cell type within the alveolus, and are responsible for the first line of pulmonary host defense [73]. Therefore, most studies that sought to characterize functional interactions with phagocytes have focused on alveolar macrophages. Because alveolar macrophages do not divide in culture, they must be taken directly from lung lavage; this isolation method has caused some investigators to look elsewhere for the targets of functional studies: peripheral blood monocytes, monocyte-derived macrophages, and bone marrow-derived macrophages are the most common other cells used in these assays, with limited success. Some recent research has also been done on neutrophils, the second most common cell type in lung lavage, and an important part of the phagocytic response to some infections.

Functional studies involving SP-A and phagocytes can be divided into several categories. The large majority of these studies have focused on the relationship between SP-A and the uptake and/or phagocytosis of pathogens, foreign particles, and surfactant lipids; other studies have looked at intracellular signaling pathways stimulated by SP-A, the stimulation of cellular responses such as actin polymerization and chemotaxis, and the regulation of cytokine and reactive species production and secretion. The interaction of these processes forms the basis of a model for SP-A involvement in pulmonary host defense (Fig. 2).

5.5. Non-phagocytic cellular responses to SP-A

Binding studies to whole cells are consistent with the existence of at least one cell surface receptor for SP-A; functional data support this, and, in fact, a tenable hypothesis is that numerous SP-A binding receptors exist with different functions. The first con-

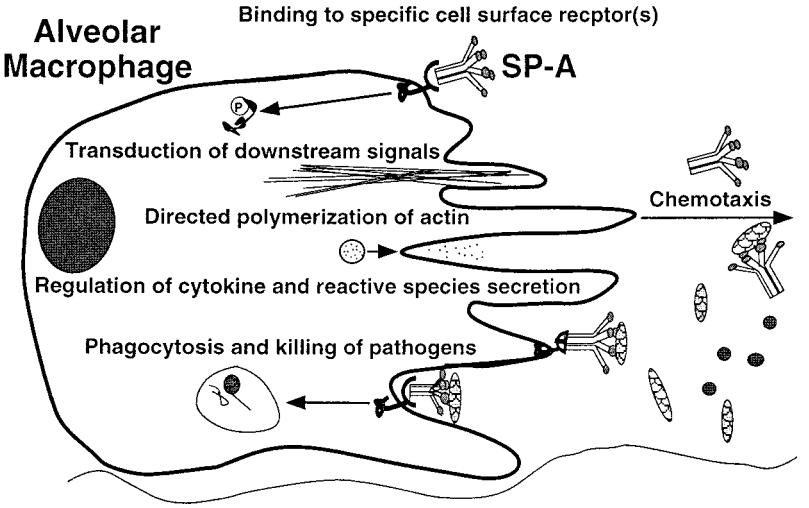

Fig. 2. Model for SP-A involvement in pulmonary host defense. The interaction of SP-A with alveolar macrophages leads to the regulation of many of the host defense properties of the macrophage. SP-A binding to cell surface receptors triggers the transduction of specific intracellular signalling cascades, including the tyrosine phosphorylation of proteins (shown), the release of intracellular calcium, and the production of inositol triphosphate, an active signaling molecule. These signals have many effects on cellular processes. SP-A has been shown to stimulate the directed polymerization of actin microfilaments, consistent with its role as a macrophage chemoattractant; macrophages migrate towards higher concentrations of SP-A in vitro. SP-A has also been shown to have various effects on the expression of proteins such as iNOS, and the subsequent production of reactive species of nitrogen. SP-A also regulates the secretion of reactive oxygen species and cytokines such as TNF-α, IL-1β, and IL-6 by alveolar macrophages. Finally, SP-A binds to pathogens and stimulates their phagocytosis by alveolar macrophages; phagocytosis often leads to the inactivation and/or killing of potentially harmful organisms.

sequence of receptor binding is the transmission of intracellular signals; certain signaling pathways are common motifs, and various groups have examined the signaling pathways activated by SP-A in phagocytes.

SP-A increases intracellular calcium and inositol triphosphate (IP3) concentrations in alveolar macrophages. In two separately reported studies by Ohmer-Schröck and colleagues, it was reported that SP-A stimulates an increase in intracellular calcium concentration, as measured by the increase in fluorescence of fura-2, a calcium indicating dye [62]. In their initial study, they found that the free cytosolic calcium concentration in adherent alveolar macrophages increased immediately after the addition of SP-A to a final concentration of 60 μg SP-A/ml, and that the increase reached a plateau more than 2 min after stimulation [62]. These findings were corroborated by the later study, which also found that this stimulation is concentration-dependent in that increased SP-A concentrations lead to a higher percentage of responding cells, and that the calcium response correlates with an increase in the generation of IP3, an active intracellular signaling molecule [74]. This study also reported that the increase in calcium was necessary for SP-A stimulated phagocytosis, a finding that correlates with findings on IgG-mediated phagocytosis [75].

Another common signaling pathway in phagocytes is the activation of tyrosine kinases, which regulate protein activity by phosphorylating tyrosine residues on them. In a study presented in an abstract, we have shown that SP-A stimulates protein tyrosine phosphorylation in alveolar macrophages, but not in peripheral blood monocytes or peritoneal macrophages [76]. In a related study, it was demonstrated that both tyrosine phosphorylation and actin polymerization are also necessary for SP-A-stimulated phagocytosis [77].

Receptor binding and subsequent downstream signals are also necessary for the directed rearrangement of the actin cytoskeleton, a process tightly controlled in motile phagocytic cells (reviewed in [78]). Studies characterizing actin polymerization and motility in alveolar macrophages have revealed not only that SP-A stimulates alveolar macrophage chemo-

taxis in a concentration-dependent manner [79], but that it also stimulates directional actin polymerization in alveolar macrophages [80]. Comparisons with peripheral blood monocytes have revealed that SP-A stimulates neither function in them although SP-A does stimulate the migration of peritoneal macrophages [79].

The study which established that SP-A stimulates alveolar macrophage chemotaxis also examined the mechanism of this stimulation. Wright and Youmans [79] found that SP-A stimulation of alveolar macrophage chemotaxis is blocked by the addition of a 20-fold excess of collagen, and that this stimulation is significantly decreased after heat treatment of the SP-A at 50°C, a temperature which denatures the collagen-like triple helices of SP-A. These findings support the hypothesis that SP-A stimulates chemotaxis through a cell interaction involving its collagen-like domain, although interactions involving other domains were not ruled out; this hypothesis was further supported by studies which showed that the collagen-resistant fragment of SP-A does not stimulate chemotaxis by itself [79].

SP-A also affects the production of several important mediators of inflammation and immune defense, including cytokines such as TNF-α and colony stimulating factors (CSFs) as well as reactive species of oxygen and nitrogen. The precise balance of these cell products is essential in the lung as elsewhere; cytokine release controls the inflammatory response and the proliferation and recruitment of other immune cells, and reactive species can lead to tissue damage if uncontrolled. The precise contribution of SP-A to this balance is the matter of some controversy in the field, as various groups have reported different results using SP-A purified by different methods.

The effect of SP-A on cytokine release by alveolar macrophages has been studied by several groups. Blau and colleagues found that SP-A stimulates the production of CSFs by both type II cells and alveolar macrophages [81]. While the bioassay used did not differentiate between the several different types of CSFs, the morphological characteristics of colonies formed in medium conditioned by SP-A-stimulated alveolar macrophages were similar to those grown in medium with IL-3, or conditioned with LPS- or IL-1-stimulated alveolar macrophages. Significantly, SP-A did not stimulate CSF production in peritoneal macrophages [81].

The role of SP-A in regulating lung inflammation is unclear. SP-A has been reported both to stimulate production of a host of inflammatory cytokines, including TNF-α, and to inhibit LPS-stimulated TNF-α production. In studies by Kremlev and colleagues, SP-A stimulated release of TNF-α and interleukins 1β and 6 (IL-1β and IL-6) by peripheral blood mononuclear cells, alveolar macrophages, splenocytes, [82] and monocytic cell line THP1, as well as the cell surface expression of proinflammatory cell surface markers CD14, CD54, and CD11b on THP-1 cells [83]. Investigators in this group have also reported that SP-A activates the transcription factor NF-κB in THP-1 cells; gel-shift assays were used to demonstrate a stimulation of activity of this factor, an important regulator of proinflammatory protein expression. Contrary to these studies are those done by McIntosh et al., who found that SP-A reduced TNF-α activity in medium conditioned by LPS-stimulated alveolar macrophages, and that this reduction was dose-dependent, serum-independent, and reversible [84].

The major difference between these studies is the method used for the purification of SP-A. Kremlev and colleagues use an isoelectric focusing method after solubilization of alveolar proteinosis (AP) proteins in 3 M urea, 1% 2-mercaptoethanol, and 0.1% NP-40 [10,82,83]; the results obtained by McIntosh et al. have been reproduced with SP-A purified by either butanol extraction from AP lavage [84] or a gentler, non-butanol-based EGTA extraction method [9] (unpublished results). Recent in vivo studies using knock-out mice may be the key to resolving this controversy; the first reports on this subject support the role of SP-A in attenuating lung inflammation [85].

SP-A has been reported by various groups to have effects on the production of reactive oxygen and nitrogen metabolites by phagocytic cells. These products are important mediators of host defense; they are important in the killing of ingested and extracellular pathogens, and they participate in both intracellular and intercellular signaling pathways. While there are significant differences among the findings of these studies, some of them appear to be explainable.

In two different studies, it has been demonstrated that SP-A stimulates the release of reactive oxygen metabolites from alveolar macrophages [86,87]; two studies have found that SP-A decreases PMA-stimulated superoxide production in canine neutrophils and alveolar macrophages [88] and rat alveolar macrophages [89]. In the first study, van Iwaarden and colleagues reported that human AP-derived SP-A induces a lucigenin-dependent chemiluminescence response in rat alveolar macrophages; this response, indicative of the production of oxygen radicals, was shown to be concentration-dependent at concentrations up to 3 μg SP-A/ml and independent of the response generated by *Staphylococcus aureus*. Furthermore, this stimulation was specific to alveolar macrophages, and not seen in rat peritoneal macrophages, rat or human neutrophils, or human peripheral blood monocytes [86], a finding consistent with that of Goodman and Tenner, who demonstrated that AP SP-A coated onto plates did not stimulate reactive oxygen production in neutrophils, although C1q and fragments of the C1q collagen-like domain did [90], by a mechanism later attributed to binding via CD18 [91].

Contrasting this study is another with a similar finding. Weissbach et al. [87] reported several years later that only SP-A bound to a surface stimulates superoxide production by alveolar macrophages, measured in a similar fashion to van Iwaarden and co-workers. In this study, they found that this stimulation was concentration-dependent up to a coating concentration of 80 μg SP-A/ml, and that no enhancement of oxygen radical production was seen in either alveolar macrophages stimulated with soluble canine or rat SP-A or in peripheral blood monocytes adhered to SP-A-coated surfaces. The authors of this study postulated that the differences between this study and that of van Iwaarden et al. could be explained if SP-A purified from alveolar proteinosis patients was aggregated in such a fashion as to mimic binding to a surface.

Supporting this claim are two studies which demonstrated that soluble SP-A not only does not stimulate reactive oxygen production, but decreases the production of reactive oxygen species in PMA-stimulated cells. In the earlier of these studies, Weber and colleagues demonstrated that preincubation of either canine alveolar macrophages or blood-derived neutrophils with SP-A for 30 min significantly decreases their respiratory burst stimulated with PMA, and that SP-A has no effect on reactive oxygen production in unstimulated cells [88]. Interestingly, collagenase-treated SP-A had an opposite effect on neutrophils in this study, increasing oxygen radical production; this finding would have been more significant had a wide variety of test proteins, including such things as porcine lactate dehydrogenase, canine alkaline phosphatase and bovine serum albumin, not had similar effects [88]. In the only study to use a different system of superoxide measurement, Katsura et al. found that rat SP-A alone had no effect on rat alveolar macrophage superoxide production, as measured by ferricytochrome c reduction, and that preincubation of cells for at least 2 h with SP-A concentrations between 1 and 10 μg/ml decreases their oxidative response to PMA; human AP SP-A also had a significant inhibitory effect on the PMA-stimulated response in this study [89].

Nitric oxide (NO) is a reactive species of nitrogen which also has a role in host defense properties of phagocytic cells. Increases in alveolar macrophage NO production are generally caused by induction of nitric oxide synthase (iNOS) synthesis; these increases have been obtained by many inflammatory stimuli. Blau and colleagues have reported that SP-A induces expression of iNOS, with a corresponding increase in NO production [92]. This study found that although less effective than IFN-γ or LPS in stimulating nitrite release, both rat and human AP-derived SP-A stimulated NO production in a concentration- and time-dependent manner. In addition, this group found that peritoneal macrophages and type II cells were not stimulated by SP-A to produce NO, although both cell types were stimulated by LPS and IFN-γ.

5.6. SP-A stimulation of phagocytosis and uptake

The interaction of SP-A with phagocytic cells has been looked at most thoroughly as a stimulus for endocytic and/or phagocytic uptake of various particles, from endogenous surfactant lipids and proteins to inhaled pollen grains [93] and pathogens. This work has established a clear role for SP-A in regulating both the clearance and metabolism of sur-

factant and in stimulating non-inflammatory host defense pathways in the lung.

Initial characterizations of the functional interactions of SP-A with phagocytes examined the uptake and processing of surfactant lipids and SP-A by alveolar macrophages, both in vivo and in vitro (Fig. 1). Early studies showed that alveolar macrophages had immunoreactive surfactant proteins in their secondary lysosomes, both in the presence and absence of surfactant lipids [6]. Using exogenous surfactant with fluorescein-labeled protein components, Stern and colleagues confirmed the finding that alveolar macrophages take up surfactant proteins, also postulating that this uptake occurred independently of lipid catabolism [94].

Later studies looked specifically at the uptake of SP-A by alveolar macrophages and the effect of SP-A on their uptake and metabolism of surfactant lipids. An early study of DPPC uptake by alveolar macrophages showed that SP-A (then called SP 26-36) stimulated the uptake of ^{14}C-DPPC-containing liposomes by alveolar macrophages to levels almost 20-fold greater than without it; in this study, SP-A had no significant effect on lipid uptake by fibroblasts [7]. Later studies found that this stimulation is dose-, time-, and temperature-dependent, and that neither C1q nor SP-D enhanced DPPC uptake by macrophages [95]. The degradation of internalized lipids, however, was not changed in the presence of SP-A, a finding consistent with earlier studies by Miles et al. which examined the degradation of lipids from whole surfactant preparations and ones from which the major hydrophilic proteins had been extracted [96].

The study by Wright and Youmans also demonstrated for the first time that alveolar macrophages degrade SP-A in a time- and temperature-dependent manner, and that this uptake is independent of the presence of surfactant lipids [95]. These findings were confirmed in a later study by Bates and Fisher, who found that rat macrophages degraded SP-A in a time- and concentration-dependent manner, and that they degraded human and rat SP-A much more than bovine SP-A [97]. This degradation was confirmed to be intracellular and independent of surfactant lipids [97]. A follow-up to this study demonstrated that activation of macrophages (both alveolar and tissue) by overnight culture on tissue culture plastic in medium containing 10% fetal calf serum significantly enhanced the ability of the cells to degrade SP-A; overnight culture had no effect on cell association of SP-A, nor did it affect the degradation of phospholipids [98].

Many studies have looked at the effect of SP-A on the binding and phagocytosis of foreign particles, including a wide variety of pathogenic organisms. Initial studies compared the effects of SP-A to the known properties of C1q, a serum homologue involved in the initiation of the complement cascade and the activation of phagocyte response to IgG. In these studies, it was demonstrated that SP-A enhances FcR- and CR1-mediated phagocytosis by peripheral blood monocytes and monocyte-derived macrophages in a manner similar, though not identical, to stimulation by C1q [99]. In this study, cells were adhered to wells coated with C1q, SP-A, or, as a control, transferrin (see Fig. 3). Monocytes adhered to SP-A or C1q phagocytosed significantly more IgG- or C4b-opsonized sheep erythrocytes than did the control cells [99].

Van Iwaarden and colleagues examined the effect of SP-A on the binding of serum-opsonized *S. aureus* (SAE) to alveolar macrophages [86]. In an assay that measured the association of ^{3}H-labeled SAE with alveolar macrophages, it was demonstrated that preincubation of the macrophages with either SP-A or surfactant significantly enhances the association of serum-opsonized SAE with the cells. The results seen with SP-A were dose-dependent, and not seen when SAE were not first preopsonized with serum or when bacteria (and not cells) were preincubated with SP-A [86]. These results did not support the findings of several earlier studies, which showed that the addition of alveolar lining material significantly enhanced the inactivation of *S. aureus* with alveolar macrophages [100], and that human lung lavage enhanced the association of *S. aureus* with alveolar macrophages [101].

Many subsequent studies tested whether SP-A could act as an opsonin for phagocytosis (Fig. 3A). In studies using a variety of methods, most of which did not distinguish bound from internalized pathogens, SP-A was shown to enhance the phagocytosis of herpes simplex 1 with rat alveolar macrophages [102], and the association of logarithmically growing *Escherichia coli*, both log- and stationary-phase *Pseu-*

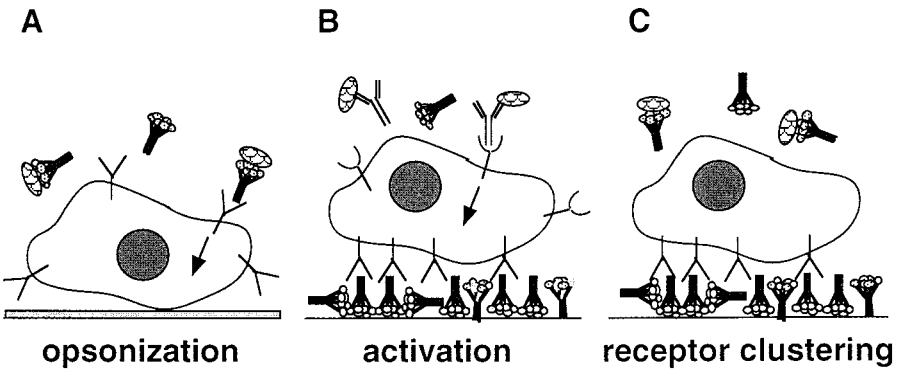

Fig. 3. Effect of various methods of presenting SP-A to cells on phagocyte function. SP-A presented to phagocytes in different states can lead to the stimulation or inhibition of various macrophage functions. (A) SP-A bound to bacteria or viruses can serve as an opsonin, directly mediating the interaction between pathogens and cells, and stimulating the phagocytosis of the pathogens. (B) Cells adhered to an SP-A-coated surface are activated to produce reactive oxygen species, as well as to phagocytose particles opsonized with molecules such as IgG, which mediates the interaction of particles with cells via the Fc receptor. (C) Cells adhered to an SP-A-coated surface also lack a response to soluble SP-A, presumably because SP-A binding cell surface receptors are clustered on the basal surface of the cell and are therefore unavailable to interact with SP-A in solution. These cells do not respond to SP-A presented as an opsonin for bacterial phagocytosis.

domonas aeruginosa, and log-phase *S. aureus* with rat alveolar macrophages [103], *S. aureus* but not pneumococci with both rabbit alveolar macrophages and human monocyte-derived macrophages [104], type A but not type B *Haemophilus influenzae* with rabbit alveolar macrophages [105], *S. aureus* with human peripheral blood monocytes [69], rough but not smooth *E. coli* with alveolar macrophages [106], and *Pneumocystis carinii* with rat alveolar macrophages [107]. While these studies created some controversy about the nature of SP-A-pathogen interactions, and the precise types of pathogens which are opsonized by SP-A, all of them demonstrated the ability of SP-A alone to directly stimulate the association of pathogens with phagocytic cells.

Two more recent studies have used methods that effectively distinguish between intracellular and extracellular bacteria, in order to separate cell associated bacteria from internalized ones; both of these studies have confirmed the ability of SP-A to directly stimulate the phagocytosis of pathogens by macrophages. In the first of these studies, trypan blue dye was used to quench the fluorescence of extracellular bacteria, using a method developed by Newman et al. [108] to study the phagocytosis of yeasts by alveolar macrophages. In this study, it was demonstrated that SP-A stimulates the phagocytosis of *H. influenzae*, Group A *Streptococcus*, and *S. pneumoniae* by alveolar macrophages; this stimulation was shown to be concentration-dependent, and antibodies to SP-A blocked the stimulation of phagocytosis by bronchoalveolar lavage fluid [109]. The later study used electron microscopy to measure phagocytosis, and showed that SP-A enhances the uptake of *Bacillus Calmette-Guérin* (BCG) by bone marrow-derived macrophages [110].

The stimulation of phagocytosis by SP-A is generally seen as an enhancement of pulmonary host defense. In two studies, another perspective on this interaction has emerged. Two different studies have shown that SP-A enhances the association of *Mycobacterium tuberculosis* (MTB) with alveolar macrophages [111] and monocyte-derived macrophages [35]; binding of MTB to cells is an important first step in the tuberculosis infection, as MTB lives and divides intracellularly. Downing and colleagues examined factors in the lavage of patients with human immunodeficiency virus (HIV) infections which increased cell association of MTB; SP-A was identified as the factor which enhanced MTB association 3-fold over control groups, and was postulated to be an important contributor to the development of tuberculosis during the progression of HIV infection [111]. In the study by Gaynor and colleagues, SP-A increased the association of MTB with monocyte-derived macrophages; this association was confirmed

to lead to increased phagocytosis by electron microscopy, and was inhibited by both mannan and antibodies to the anti-mannose receptor [35].

Several studies of SP-A-mediated phagocytosis have examined the cell surface receptors likely to be involved in this stimulation. Receptor clustering studies have led to some controversy about the exact receptors involved in the stimulation of phagocytosis by SP-A; adherence of cells to a ligand-coated surface has been shown in some systems to cluster receptors for that ligand on the basal surface of the cells, making them unavailable to interact with soluble or particle-bound ligand (Fig. 3) [112]. In the study by Geertsma and colleagues [69], peripheral blood monocytes were adhered to surfaces coated with a high concentration of C1q; this coating eliminated the SP-A mediated enhancement of *S. aureus* association with cells. These results were interpreted as supporting the hypothesis that C1q receptors mediate the stimulation of phagocytes by SP-A.

In a later study, however, we demonstrated that while monocytes adhered to C1q-coated surfaces lose responsiveness to SP-A, alveolar macrophages retain their responsiveness to SP-A on these surfaces; additionally, it was shown that SP-A does not stimulate the phagocytosis of *H. influenzae* in alveolar macrophages adhered to SP-A-coated surfaces, and that adherence to C1q-coated surfaces significantly inhibits baseline phagocytosis of bacteria in both cell types [109]. Recently, Weikert and colleagues have shown that antibodies to SPR210, an SP-A binding protein expressed on many cell types, block SP-A enhancement of BCG uptake by bone marrow-derived macrophages; this is the first direct demonstration of the involvement of a specific protein in SP-A stimulation of a phagocyte [110]. This study also showed that the macrophage mannose receptor was not responsible for this stimulation, as studies on MTB had postulated.

Further studies are needed to determine the specific receptor(s) on alveolar macrophages necessary for SP-A interaction and stimulation. The nature of the SP-A – soluble, pathogen-bound, surface-bound and/or lipid-bound – may determine the precise set of processes stimulated. Additionally, the cell types with which future studies are conducted may prove essential; differences in cellular responsiveness to SP-A have been shown among phagocytes (see Table 1). The cellular distribution of receptors known to bind SP-A does not match the cellular specificity of stimulation, the strong possibility exists that several receptors are responsible for the various interactions of SP-A with phagocytic cells.

Table 1
Effects of SP-A on the host defense functions of mononuclear phagocytes

Function	Alveolar macrophages	Peripheral blood monocytes or monocytic cell lines
SP-A binding	Specific Saturable High-affinity Bind and internalize SP-A-gold SPR-210	Non-saturable Bind and internalize SP-A-gold C1q-R (U937) SPR-210 (U937)
Stimulates the phagocytosis or association of non-opsonized organisms	+	+
Activation ligand for phagocytosis via FcR and CR1	n.d.	+
Stimulates chemotaxis	+	−
Stimulates actin polymerization	+	−
Increases intracellular calcium and IP3	+	n.d.
Stimulates protein tyrosine phosphorylation	+	−
Affects the production of:	TNF-α IL-1β Reactive O_2 species Nitric oxide	IL-1β IL-6
Increases the expression of:	iNOS	CD14, CD54, CD11b (THP1)

+, functions stimulated by SP-A; −, functions not affected by SP-A; n.d., not done.

6. Future perspectives

In summary, numerous studies employing a wide variety of techniques have provided evidence that SP-A can interact with and affect the functions of alveolar epithelial and immune cells. These studies are all consistent with the possibility that both type II cells and immune cells express specific receptors for SP-A. Although several SP-A binding proteins on type II cells and macrophages have been identified, little is known about the putative receptors and how they may transduce SP-A mediated to the cells. Important future areas will be the cloning and expression of these receptors and elucidation of the mechanism by which they interact with SP-A and regulate cell function.

In vitro studies show that SP-A appears to be involved in regulating surfactant metabolism (Fig. 1). These studies have been the basis for a model in which SP-A regulates surfactant pool size. In this model, when surfactant is in excess in the extracellular space, SP-A enhances lipid uptake by either type II cells, where it can be recycled or degraded and reused to synthesize new surfactant, or by macrophages, which actively degrade both lipid and SP-A. Further secretion is inhibited by the interaction of SP-A with the type II cells. Little is known about the mechanisms by which SP-A exerts these effects, although recent studies have identified potential receptors and SP-A binding domains involved in this process. Although these in vitro studies provide compelling evidenced that SP-A is involved in regulating surfactant metabolism, mice in which SP-A has been deleted by homologous recombination were found to have relatively small perturbations to their extracellular surfactant pool sizes. One possible explanation for the observation that the pool size perturbations were smaller than might have been expected is that alternative compensatory mechanisms may regulate surfactant pool size in the absence of SP-A. These compensatory mechanisms could possibly be overwhelmed in scenarios in which the animals are stressed by exercise, inflammation, or infection.

The involvement of SP-A in regulating immune cell function is supported by both in vitro studies and in vivo studies. In general, the in vitro studies have shown that SP-A enhances the effects of immune cells such as phagocytosis and bacterial killing as well as chemotaxis. There are some conflicting reports in the literature about the role of SP-A in regulating cytokine and free radical production. Possible explanations for these conflicts include different methods of preparation of SP-A and the possibility that the 'purified' SP-A may be contaminated with endotoxin and immunoglobulins among other factors. Alternatively, specific immune cells or cell lines may have unique responses to SP-A. In vivo studies using mice in which the SP-A gene has been ablated support the idea that SP-A functions in host defense; these mice are more susceptible to pulmonary infections than are wild type mice. Although the studies with the knock-out mice have provided confirmation for the in vitro studies, there are still many unanswered questions about the role of SP-A in host defense. For example, the relative roles of different immune cells, such as macrophages, monocytes, neutrophils and T-cells, are not known. The possible interaction of SP-A with other immune proteins such as SP-D and those of the adaptive immune system has not been thoroughly investigated.

Another important unanswered question is what regulates the relative contribution of SP-A to regulating type II cell or immune cell function. SP-A as isolated from lung lavage is largely bound to lipid. Therefore, it seems important to elucidate the effects of lipids on SP-A mediated functions. However, since SP-A is also synthesized by the airway Clara cell, it seems likely that SP-A may be secreted without lipid from this cell type and then become lipid associated during the lavage procedure. The effects of these potentially different forms of SP-A have not been investigated. Finally, little is known about the functions of SP-A in the injured or infected lung and the factors that regulate the expression of SP-A under such conditions.

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