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Pulmonary Surfactant Protein A´s anti-inflammatory modulation of the IκB-α/ NF-κB signal transduction pathway

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1 INTRODUCTION

Respiration is vital to every cell in all living organisms. The degree of complexity in developing respiratory organs varies among species of different physiological states and ecological settings to meet the organisms´ specific metabolic requirements. In contrast to single cell organisms, where gas exchange occurs *via* simple diffusion, higher organisms made two major adaptations that made it possible to attain great multicellularity: they developed an efficient circulatory system that would allow transport of gases to and from the deepest tissues of the body, and an internalized respiratory system that primarily specialized in rapidly distributing obtained oxygen from the atmosphere to the circulatory system. Air-breathing vertebrates evolved an organ – the lung – that allows oxygen to move from the air into the venous blood and carbon dioxide to move out. Over the past 300 million years lungs have evolved independently in association with the diversification of the vertebrates. Among vertebrates, lungs differ considerably in structure, embryological origin and function. While the bronchoalveolar lung of mammals is a branching "tree" of tubes leading to millions of small respiratory units, termed alveoli, non-mammalian lungs are baglike with either smooth walls or large, bellows-shaped respiratory units, termed faveoli, extending from the outer wall of the lung into a central air space. The most striking lung structure is found in birds. They have developed a pair of small parabronchial lungs connected to a series of air sacs through which air is propelled in a unidirectional manner. In contrast to mammalian lungs, the lungs of fish, amphibians and reptiles always lack a bronchial tree, a diaphragm, and a separate pleuroperitoneal chamber. However, a characteristic common in all lungs is their internal, fluid-filled, gas-holding structure that inflates and deflates cyclically. But they also face potential problems related to the surface tension of the fluid. In mammalian lungs, pulmonary surfactant reduces the surface tension of this fluid lining, while surfactant present in non-mammalian lungs exhibits an antiadhesive function, lining the interface between apposed epithelial surfaces in regions of a collapsed lung.

Steady exposure of the lung to a multiplicity of microorganisms and environmental pollutants, a result of the daily inhalation of 10,000-15,000 liters of air, required strategies to recognize foreign material, and to distinguish between potentially harmful agents and most innocuous foreign material. Two interactive and protective systems evolved: the innate and the adaptive immune system. The innate (unspecific) immune system is always on guard, immediately responsive, recognizing and inactivating microbial compounds due to the recognition of

common microbial motifs. The resulting immune response is executed by innate immune cells, namely lung leukocytes and epithelial cells lining the alveolar surface and the conducting airways (Martin & Frevert, 2005). Lymphocytes, i.e. T-lymphocytes and B-lymphocytes, are specialized cells of the adaptive (specific) immune system. They respond specifically to signals from the innate immune system by producing high-affinity antibodies to very specific peptide sequences presented on antigen-presenting cells (APC). This interaction of the innate immune system with the adaptive immune system drives an efficient host defense, enabling the host to react to the myriad of microorganisms and other environmental products entering the lung every day.

1.1 Pulmonary Immunity

The lung functions as the body´s gas exchange organ. With its surface area of 50-150 square meters, the lung represents the largest surface in the body to come in contact with the outside environment (West, 5th ed., 1995). Due to this unique situation, the lung has developed various defense mechanisms to protect itself from infection. The primary contact with inhaled microorganisms, allergens, cigarette smoke and environmental pollutants, occurs in the upper respiratory tract. Large particles are trapped on the mucus layer coating the nasal epithelium and upper respiratory tract (West, 5th ed., 1995). Ciliary motion and the proper quantity of salt and water on airway surfaces assist mucus transport up to the epiglottis, where inhaled particles are swallowed (West, 5th ed., 1995, Knowles & Boucher, 2002). Smaller particles that pass the first barrier and reach the alveolar compartment are deposited on the alveolar lining layer (ALL), a thin aqueous film comprising pulmonary surfactant that lines the gas-exchanging surface of the pulmonary epithelium (McCormack & Whitsett, 2002). Since the alveoli do not have cilia, particles that are deposited on the ALL are engulfed and removed from the lung by large phagocytic cells, called macrophages.

1.1.1 Lung-specific requirements for host defense

The enormous surface area of the lung to be defended differs decisively from the skin (2.5 m^2), or from equivalent sized areas as the gut (200 m^2). Compared to the serial connection of the gut that allows sequential defenses, such as salivary amylase, gastric acid, and bile, the alveoli are exposed to the environment in parallel, each unit being self-sufficient in defense against inhaled organisms and particles. Harsh chemical environments as found in the gut, forming a physical barrier, cannot be tolerated in the delicate alveolar space. With

only two cell layers - the alveolar epithelium and the capillary endothelium - and small amounts of tissue that separate invaders from the bloodstream, the risk of pathogen dissemination is enhanced. Therefore, the objective in the alveoli is sterility rather than maintenance of a normal flora because, even moderate degrees of inflammation threatens the function of the organ. This makes the lung with its alveolar boundary the most vulnerable environmental interphase (McCormack & Whitsett, 2002). Host defense in the lung requires close interaction of constituents of both the innate immune system and the alveolar lining fluid to adequately respond to inflammation.

1.1.2 Immunocompetent cells

In order to maintain a sterile environment in the lung, immunocompetent cells are required that participate in the defense reaction to foreign material. In addition to resident alveolar macrophage, the most prominent effector cells in the lung, neutrophil granulocytes are recruited to inflammatory sites to form the first line of defense.

1.1.2.1 Alveolar macrophages

It was in the late 1800s when Ilja Metchnikoff first identified mobile ameboid cells in sea anemones and in water fleas that could engulf particulate dyes and fungal spores, respectively. Suspecting a defensive function for these cells, he inserted a rose thorn into a star fish larva and found the ameboid cells accumulating around the thorn. He termed the cells “phagocytes”, meaning “devouring cells” in Greek. He was the first to discover the importance of macrophages for distinguishing self from non-self, and responding actively to pathogens, thereby introducing modern immunology (Tauber, 1990).

The origin of alveolar macrophages (AM) and their recruitment into the lung is divers: AM either originate in the bone marrow as monoblasts, move into the blood stream as monocytes, and finally differentiate into resident macrophages in the tissue, or they are recruited by chemotactic attraction of monocytes from the lung blood pool and replicate locally in the lung. They are highly distributed throughout the body and represent the major effector cells of the innate immune system (Lohmann-Matthes et al. 1994, Monick & Hunninghake, 2002, Gordon & Taylor, 2005). Macrophages in the lung can be divided into three different types, according to their localization: 1. the alveolar macrophage; 2. the interstitial macrophage; 3. and the intravascular macrophage (Lohmann-Matthes et al., 1994).

Alveolar macrophages (AM) are the most abundant immunocompetent cells in the alveolar space, and have the most important role of all pulmonary macrophages. They are placed in close proximity of type I alveolar epithelial cells (AEC) within the alveolar surfactant film that is produced by type II alveolar lining cells, consisting of phospholipids and proteins (Lohmann-Matthes et al. 1994). Due to their unique localization at the interphase of air and lung tissue, AM are the only macrophage to be exposed to air, thus having the most prevalent contact to inhaled pathogens and particles (Lohmann-Matthes et al. 1994, Fels & Cohn, 1986). They form the first line of cellular defense by recognizing and responding to invading pathogens and are essential for maintaining a sterile environment in the pulmonary compartment (Monick & Hunninghake, 2002). AM are professional phagocytes, eliminating invading pathogens and pollutants by engulfing bacteria or particles that enter the alveolar space. The microbial clearance strongly depends on the quantity of the inoculum. Whereas a small amount of microorganisms (10^5) can be cleared by AM alone, a higher number (10^6) of microorganisms induces a modest influx of neutrophils into the alveoli (Lohmann-Matthes et al. 1994), attracted by macrophage-derived substances that include complement, arachidonic metabolites such as leukotriene B4, and chemotactic peptides such as IL-8, macrophage inflammatory peptide (MIP)-2, and related chemokines (Zhang et al., 2000). An excess of bacterial load (10^8) can only successfully be cleared by activation of T- and B-lymphocytes (Lohmann-Matthes et al., 1994). By activating the adaptive immune system, AM act as antigen-presenting cells (APC), processing and presenting microbial antigen of ingested microbes to T-lymphocytes (Fujiwara & Kobayashi, 2005). The detection and recognition of potentially pathogenic microorganisms and foreign material by AM occur through a broad range of membrane receptors expressed on the AM. Membrane receptors mediate the interaction with natural and altered-self components of the host as well as with a broad range of microorganisms. Recognition is a central function of AM that is followed by an adequate response in surface changes, uptake, signaling, altered gene expression, contributing to homeostasis, and host defense in innate and adapted immunity (Taylor et al., 2005). The uptake or phagocytosis of microbes is enhanced by opsonization. Three groups of receptors are involved in opsonization: the Fc-receptors, the complement receptors, and the lectin receptors (Lohmann-Matthes et al., 1994). Many non-opsonic receptors have been identified that mediate AM immune responses. In particular pattern recognition receptors (PRR) that recognize conserved microbial structures, so called pathogen associated molecular patterns

(PAMPs), and cytokine receptors contribute to innate immune recognition and generate pro- or anti-inflammatory responses (Janeway & Medzhitov, 2002, Taylor, 2005).

1.1.2.2 Neutrophils

Neutrophil granulocytes, or polymorphonuclear neutrophilic leukocytes (PMNs), generally referred to as neutrophils are short-lived cells that are abundant in the blood and not present in healthy tissues. They are the most abundant type of leukocytes, share characteristics with macrophages in regard to their phagocytic properties, and their key role in innate immunity. Besides phagocytosis, neutrophils release proteins and peptides in three types of granules in a process called degranulation.

Neutrophils migrate out of blood vessels to sites of infection upon macrophage-derived chemokines and cytokines where they arrive within a few hours of an inflammatory response (Janeway, 6th ed., 2005).

1.1.2.2.1 Airway antimicrobial factors

Macrophage-derived chemoattractants recruit neutrophils to sites of inflammation. Their granulae contain large amounts of antimicrobial proteins and peptides, and a number of hydrolytic enzymes whose expression is induced locally by inflammation (Rogan et al., 2006); specific or secondary granules contain lactoferrin and cathelicidin, the azurophilic or primary granules comprise secretory leucoprotease inhibitor (SLPI), α-defensins, bacteria permeability increasing protein (BPI), and tertiary granules contain e.g. cathepsin. Lysozyme is found in secretory and cytoplasmic granules but is primarily secreted by epithelial glands.

Lactoferrin is an iron-binding glycoprotein with a molecular weight of 80 kDa. It is widespread in human secretions, in particular in breast milk, tear fluid, vaginal secretions, gut-lining fluid and respiratory secretions. Lactoferrin has antimicrobial and anti-inflammatory properties.

Secretory leucoprotease inhibitor (SLPI), a 11.7 kDa non-glycosylated protein, is expressed not only by neutrophils but also by macrophages and the mucosal surface of epithelial cells. It is found in airway lavage fluid and nasal secretions, and shows anti-protease, anti-bacterial, anti-viral, and anti-inflammatory activity.

Lysozyme is a basic protein (14 kDa) known for its ability to enzymatically lyse bacterial cell walls. It is a component of phagocytic and secretory granules of neutrophils, and is produced by monocytes, macrophages and epithelial cells.

Defensins are a widely distributed family of small peptides (28-44 amino acids) that include α− and β-defensins. They have broad cytotoxic activity against bacteria, fungi, parasites, viruses and even host cells. Among the defensins only α-defensin, also known as human neutrophil peptides (HNPs), is found in azurophil granules of neutrophils.

Cathelicidin, a family of antimicrobial proteins, is found in secondary granulae of neutrophils. The only human member identified – LL-37 – is also present in certain lymphocytes, testicular tissue and airway epithelium. Active cathelicidins display antimicrobial, cytotoxic and chemotactic activity (Rogan et al., 2006, van Wetering et al., 2005).

Bactericidal Permeability-Increasing protein (BPI) is a 55 kDa protein that is stored in neutrophil primary granules. BPI is predominantly active against Gram-negative bacteria (Rogan et al., 2006).

The antimicrobial proteins and peptides present in the azurophilic and specific granules of the neutrophil are transferred to the phagolysosome where they contribute to bacterial killing. But these peptides can also be released into the extracellular space, where they not only kill microorganisms but affect other cells´ function. Though they contribute to host defense against infection and repair processes, they may also cause tissue injury and modulate immunity (van Wetering et al., 2005).

1.1.3 Phagocytosis

Professional phagocytes, such as macrophages, neutrophils and dendritic cells are highly efficient in internalizing particles. They have developed strategies to internalize microbes, particles and solutes, and to remove dead cells by mechanisms that include phagocytosis, pinocytosis and receptor-mediated endocytosis (Aderem & Underhill, 1999, Aderem, 2002). Phagocytosis ("cell eating") refers to the engulfment of large particles (> 0.5 μm), e.g. microorganisms *via* phagocytic receptors, relying on actin polymerization and the rearrangement of the plasma membrane (Liu & Shapiro, 2003; Niedergang & Chavrier, 2004). By contrast, pinocytosis ("cell drinking"), the internalization of smaller particles and solutes, and the closely related receptor-mediated endocytosis, the specific uptake of

macromolecules, viruses, and small particles are usually independent of actin polymerization but share a clathrin-based uptake mechanism (Aderem & Underhill, 1999).

Phagocytosis by macrophages is a complex process, partly due to the variety of phagocytic receptors that are able to stimulate phagocytosis and partly due to the diversity of microbes. It is required not only to initiate host defense and inflammatory cascades but also for the removal of senescent cells, for embryonic development and tissue remodeling (Aderem & Underhill, 1999, Aderem, 2002).

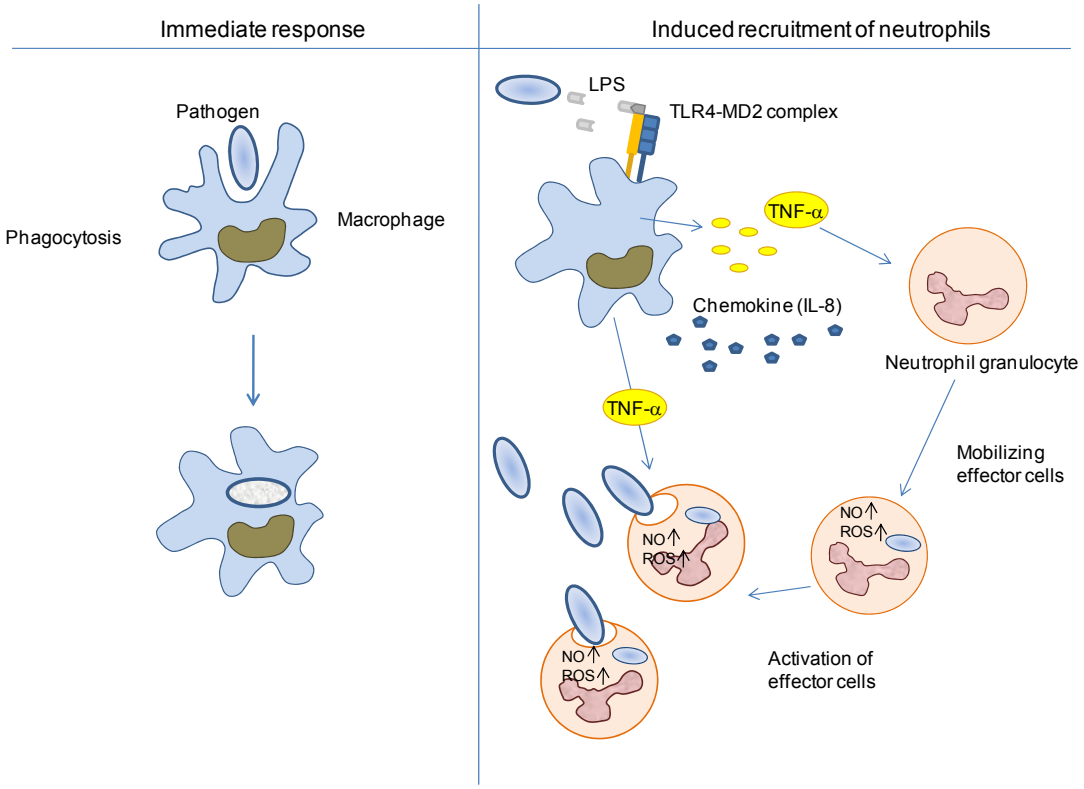


Figure 1.1: Immediate immune response vs. induced cytokine response. Invading pathogens are phagocytosed and killed by resident macrophages (left). In case of an unsuccessful clearance due to an overload of pathogens, activated macrophages release cytokines, in particular TNF-α, and chemokines that attract other effector cells, e.g. neutrophils, that help in phagocytosing and clearing pathogens (right).

1.1.3.1 Clathrin-mediated endocytosis (CME)

Phagocytosis by macrophages is essential in clearing microbial loads from the lung, however smaller particles, especially viruses, lipids and proteins, need to be removed by mechanisms involving receptor-mediated endocytosis that is clathrin dependent. Clathrin was first discovered by Barbara Pearse in 1975, who performed a biochemical analysis of coated

vesicles that attracted attention with their lattice-like external coat structure (Kirchhausen, 2000). Clathrin is the major protein found in coated vesicles. It consists of three legs radiating from a central hub resembling a spiderlike molecule that forms a trimer of 190 kDa heavy chains, each associated with a ~25 kDa light chain, termed "triskelion". The lattice-like structure formed by antiparallel interaction of the legs of triskelions centered on adjacent vertices of the lattice allows assembly of the cage that encompasses the vesicle (Kirchhausen, 2000). Clathrin assembly and disassembly proceed in several stages: 1. Initiation (nucleation or initiation of coated pit formation); 2. Coat propagation (elongation of the clathrin lattice accompanied by bilayer vesiculation and cargo recruitment); 3. Budding (completion of the lattice and pinching-off of the membrane vesicle); 4. Traffic (transport or diffusion of the coated vesicle away from the membrane); and 5. Uncoating (removal of the clathrin coat) (Kirchhausen, 2000). Clathrin coat formation is driven by adaptor proteins, such as the adaptor protein (AP)-2 complex, β-Arrestin, AP180/CALM (clathrin assembly lymphoid myeloid leukaemia), Epsin, or Eps15, and by accessory proteins, namely dynamin, amphiphysin and endophilin (Mousavi et al., 2004). Clathrin-mediated endocytosis (CME) involves either a constitutive or a signal-induced pathway (Mousavi, 2004). The constitutive pathway comprises processes that include the uptake of macromolecules and viruses from the circulation and the regulation of a number of proteins at the surface, e.g. receptors. The main function of the signal-induced pathway is to control the signaling potency of the receptor by regulating events that occur at the level of internalization, and subsequently regulate the post-endocytic fate (Mousavi, 2004).

1.1.3.2 Clathrin adaptors

The clathrin assembly machinery constitutes of a number of adaptor proteins and accessory proteins involved in clathrin-coated pit (CCP) and clathrin-coated vesicle (CCV) formation. Clathrin coat assembly at the membrane is promoted by the heterotetrameric adaptor protein (AP)-2 complex (Kirchhausen, 2000; Royle, 2006). AP-2 lies between the outer clathrin layer and inner membrane layer of the CCV, linking clathrin with the membrane (Edeling, 2006). The main function of AP-2 though is cargo recognition, followed by linking clathrin assembly to incorporation sites at the membrane (Kirchhausen, 2000). So far more than 20 clathrin adaptor proteins have been identified (Owen, 2004). Besides AP-2, the recruitment of specific cargo into CCVs involves clathrin-associated sorting proteins (CLASPs) as well as the β-arrestins (Edeling, 2006). β-arrestins were initially found to

function in the desensitization of most seven-transmembrane receptors (7TMRs). But they also act as adaptors for both AP-2 and clathrin (DeWire, 2007). They escort activated receptors to CCPs for endocytosis, and bind to other proteins involved in receptor internalization (DeWire, 2007).

1.2 Pulmonary surfactant

Pulmonary surfactant forms a thin heterogenous layer that covers the epithelial surface at the air-liquid interface (Morgenroth, 1986, Wright, 1990). Its name derived from "surface active agent", due to his best-defined function in lowering the surface tension at the air-liquid interface of the lung. The existence of a substance in the lung that reduces the surface tension in the alveoli was first predicted by the Swiss physiologist Kurt van Neergaard in 1929 when he found that the retractive force of dog and pig lungs could be removed by filling them with a gum arabic-saline solution that had comparable osmotic properties as blood serum and nearly the same surface tension, thereby eliminating all but a small fraction of "surface" free energy of the lungs. From these studies he concluded that the surface tension plays an important role in lung elasticity (Clements, 1957). A surface active agent (surfactant) was first discovered by R.E. Pattle in 1955 in attempts to treat pulmonary edema, and who found later, in 1958, that it consists of lipoprotein (Pattle, 1965; Gross, 1995). The surface tension reducing properties of surfactant were identified by John A. Clements in 1957 (Clements, 1957; Gross, 1995); the component responsible for the unusual function of surfactant though was detected later by Marshall Klaus and co-workers who analyzed the foamy substance they obtained from perfused, ventilated beef lungs. They found that this substance contained 50-70 % lipids, 74 % being phospholipids, 8 % cholesterol, 10 % triglycerides, and 8% fatty acids. But only the phospholipids displayed the unusual surface tension reducing activity (Klaus et al., 1961). The discovery by Ellen Avery and Jere Mead in 1959 that the lungs of preterm infants with neonatal respiratory distress syndrome were deficient in surfactant due to immaturity of the surfactant secretion apparatus, started a revolutionary era of surfactant treatment in premature neonates, marking the application of basic science to clinical practice in this field (Gross, 1995; Parmigiani & Solari, 2003). Since the pioneer work of van Neergaard, Pattle, Clements and Avery & Mead, numerous studies contributed to the knowledge about the composition of lung surfactant and its role in health and disease.

About 90 % of pulmonary surfactant consists of lipids, primarily phospholipids, and 8-10 % of protein. The most abundant phospholipid, phosphatidylcholine (PC), about half of which is

dipalmitoylphosphatidylcholine (DPPC), composes approximately 80 % of the phospholipid fraction. Among the remaining 8-15 % of the total phospholipid pool are phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), and phosphatidylethanolamine (PE) (Serrano & Perez-Gil, 2006; Phelps, 2001). Cholesterol, sphingomyelin, and fatty acids are included in the lipid fraction (Phelps, 2001). The protein moiety contains four specific surfactant-associated proteins: surfactant protein (SP)- A, SP-B, SP-C, and SP-D (Wright, 2005).

Surfactant lipids and all surfactant-associated proteins are synthesized and secreted by alveolar type II cells. SP-A, SP-B and SP-D are also synthesized by clara cells and submucosal cells (Wright, 2005). About 5 % of the alveolar surface area is covered by type II cells, which comprise 15 % of the peripheral lung cells. They are progenitor cells for the alveolar epithelium and responsible to restore damaged alveolar epithelium. Their major function though is to produce pulmonary surfactant (Mason, 2006). Its components are stored in densely packed concentric intracellular organelles, called lamellar bodies prior to their secretion into the alveolar space which takes place *via* fusion of the lamellar bodies with the plasma membrane (Wright et al., 1986; Wright & Dobbs, 1991; Serrano & Perez-Gil, 2006). The extrusion, the release of the lamellar body content into the alveolus is followed by the complex formation of a three-dimensional lattice-like structure called tubular myelin. Tubular myelin was thought to be a precursor form of the interfacial film, due to its efficient interfacial adsorption of surfactant preparations containing this structure (Serrano & Perez-Gil, 2006). Surprisingly, the absence of tubular myelin in SP-A knock-out mice did not alter surfactant homeostasis (Korfhagen et al., 1996). To maintain an appropriate level of functional surfactant, the surfactant pool size adjusts to alterations e.g. change in breathing frequency during exercise. The turnover of the intra-alveolar surfactant lipid pool is approximately 5-10 hours (Wright & Clements, 1987). The pathways of surfactant clearance vary: secreted surfactant can either be recycled, in which components are not degraded but reutilized by being taken up by type II cells, or it is degraded and components are reutilized to synthesize new surfactant lipids or proteins, or it is removed from the surfactant system as an intact molecule or as a degradation product such as fatty acids (Wright & Dobbs, 1991). Surfactant clearance can also occur *via* uptake by alveolar macrophages, which degrade and remove surfactant form the surfactant system (Wright & Dobbs, 1991). The mechanisms of surfactant homeostasis are not fully understood. The importance to maintain equal levels of surfactant and adapt to changes in surfactant metabolism in response to alterations in the

alveolar microenvironment is seen in patients with pulmonary diseases. The discovery of the surfactant-associated proteins further extended the knowledge of surfactant activity involving interfacial adsorption, surface tension reduction and film re-spreading by active participation in innate and adaptive host defense.

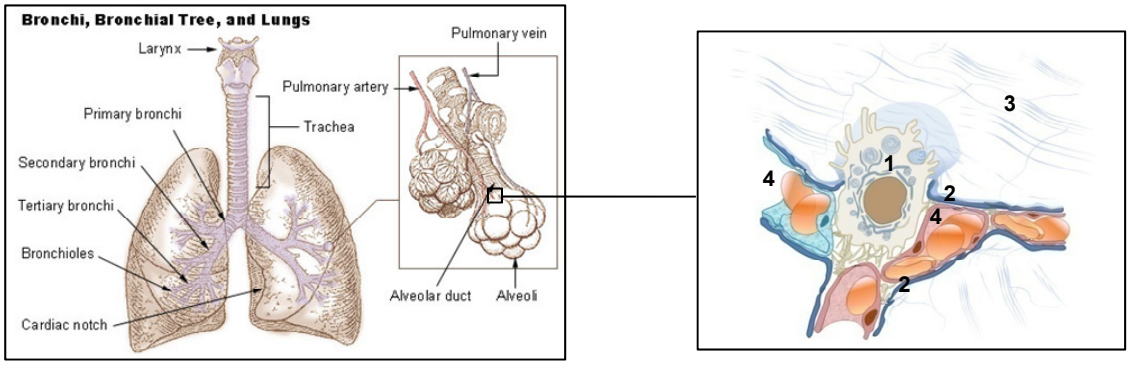


Figure 1.2: Surfactant secretion in the alveoli. Overview of the lung structure (left; source: Wikipedia Commons). Within the alveoli, the thin surfactant film is dispersed over the alveolar surface (right). Surfactant material is secreted by alveolar type II cells and forms a monomolecular film over the alveolar epithelium. Surfactant levels the unevenness between type I and type II cells. (1) alveolar type II cell; (2) alveolar type I cell; (3) surfactant film; (4) alveolar capillaries. Three-dimensional drawing, modified after Konrad Morgenroth, 1986.

1.2.1 Pulmonary surfactant proteins

Four surfactant-associated proteins have been identified so far, the hydrophobic surfactant proteins (SP)- B and SP-C, and the hydrophilic surfactant proteins SP-A and SP-D (Wright, 2005).

1.2.1.1 Hydrophobic surfactant proteins

In the late 1980s two hydrophobic peptides were detected in the organic extracts of pulmonary surfactant that were associated with the extraordinary surface-active properties of surfactant.

1.2.1.1.1 Surfactant protein (SP)-B

SP-B, formerly referred to as SPL(Phe), was first identified in 1988 as a novel pulmonary surfactant-associated protein in human surfactant and human fetal lung by Timothy Weaver and co-workers (Weaver et al., 1988). SP-B is synthesized by alveolar type II cells as a precursor of 381 amino acids that is subsequently proteolytically processed to a small polypeptide of 79 amino acids (Weaver et al., 1988; Serrano & Perez-Gil, 2006). Of all known surfactant proteins, SP-B is the only indispensable protein to initiate air breathing and it is the most active polypeptide (Serrano & Perez-Gil, 2006). SP-B promotes phospholipid adsorption from the hypophase to the air-liquid interface and facilitates the re-spreading of surfactant material during expansion achieving minimum surface tension after successive compression-expansion cycles (Serrano & Perez-Gil, 2006). Critical factors for surface activity of SP-B as well as its role in lamellar body and tubular myelin formation can be attributed to its ability to intercalate into phospholipid bilayers, to interact with surfactant lipids in particular PG, and to produce aggregation, fusion and lysis of phospholipid vesicles (Serrano & Perez-Gil, 2006). The lack of tubular myelin and immature lamellar bodies, the extracellular and intracellular form of surfactant, respectively, seen in SP-B deficient mice disrupts the routing, storage, and function of surfactant phospholipids and proteins, resulting in respiratory failure at birth (Clark et al., 1995).

1.2.1.1.2 Surfactant protein (SP)-C

The smallest surfactant protein, previously referred to as SPL(Val), is a short 34-35 amino acid lipopeptide that is proteolytically processed from a 21 kDa precursor protein (Glasser et al., 2001). SP-C is present only in pulmonary surfactant, and unlike SP-B, SP-C is

an integral membrane protein with an extremely hydrophobic transmembrane domain that spans lipid-bilayers (Beers & Fisher, 1992; Serrano & Perez-Gil, 2006). SP-C deficient mice have been generated by targeted disruption of the *sp-C* gene in mice with a Swiss black background (Glasser et al., 2001) and in mice of a congenic 129/Sv strain, showing very diverse phenotypes, with regard to the severity of lung abnormalities and susceptibility to lung injury (Ikegami et al., 2002; Glasser et al., 2003). SP-C deficiency and genetic mutations in the *sp-C* gene have been implicated in human lung diseases, e.g. pulmonary fibrosis and interstitial pneumonitis. Recent studies demonstrated that SP-C functions to limit lung inflammation, to reduce collagen accumulation, to disrupt phospholipid acyl chain packing, and plays a role in stabilization or recruitment of phospholipid films at the air-liquid interface (Ikegami et al., 2002; Glasser et al., 2001, 2003; Lawson et al., 2005).

These two small hydrophobic peptides – SP-B and SP-C – enhance the surface activity of surfactant phospholipids, promote rapid adsorption of phospholipids at the air-liquid interphase, and account for the low surface tension activity after dynamic compression. SP-B and SP-C are successfully used in surfactant replacement therapy in surfactant deficient immature lungs of preterm neonates and adult lungs with inactivated surfactant function where they restore lung function (Hawgood et al., 1996; Lewis et al., 1996; Lacaze-Masmonteil, 2007). However, their distinct contribution to surfactant function, and their role in disease is still unclear.

1.2.1.2 Hydrophilic surfactant proteins

The hydrophilic surfactant proteins SP-A and SP-D belong to the collectin family of proteins and play a key role in innate immunity.

1.2.1.2.1 Collectin structure and function

Collectins are characterized by an amino (N)-terminal collagen-like region, and a carboxy (C)-terminal lectin domain. The collagen-like region has a repeating triple helix of Gly-X-Y triplet, where X designates any amino acid and Y is often a proline or a hydroxyproline residue. Due to its disulfide-bonding pattern, the N-terminal domain maintains the molecules shape, dimension, structural stability and oligomerization. The C-terminal region has a C-type (calcium-dependent) lectin activity, also called carbohydrate-recognition domain (CRD) that enables the collectins to interact with a wide variety of pathogens resulting in pathogen opsonization and enhanced uptake by phagocytosis. The

collagen-like region is connected to the CRD *via* an α-helical coiled-coil neck region (Wright, 2005; Kishore et al., 2006). The mammalian collectin family consists currently of nine members: in addition to the two lung collectins SP-A and SP- D, the human serum collectin mannose-binding lectin (MBL), CL-L1 and CL-P1, which are expressed in the liver and vascular endothelial cells, respectively, CL-K1 and serum collectins in bovidae, namely conglutinin, collectin (CL)-43 and CL-46 have been identified (Gupta & Surolia, 2007; Wright, 2005). Structural genes that encode human SP-A (SP-A1, SP-A2 and a SP-A pseudogene), SP-D and MBL (MBL-A, and a MBL-A pseudogene) have been mapped to a cluster on chromosome 10 (Kishore et al., 2006; Wright 2005; Hawgood & Poulain, 2001).

Collectins assemble into oligomeric structures from multiple trimeric subunits, where each subunit is composed of three polypeptide chains. The degree of multimerization varies and affects the collectins´ functions (Gupta & Surolia, 2007; Wright, 2005; Kishore et al., 2006). Oligomerization begins with the trimerization of monomeric units. The coiled-coil domain aligns the collagen chains, thereby facilitating the nucleation event for the subsequent "zipper-like" folding of the collagen helix (Gupta & Surolia, 2007).

Human SP-A has a hexameric structure, and is physiologically found mainly as an octadecamer. Six structural trimeric subunits of 105 kDa each, associate and are held together *via* disulfide bonds to yield a molecule of 630 kDa, forming a bouquet-like structure that resembles MBL (Wright, 2005; Kishore et al., 2006). A structural homologue to SP-A and MBL is the first component of complement, C1q; however C1q does not contain a lectin domain, and therefore does not belong to the collectin family (Wright, 2005). SP-D forms a dodecamer in a cruciform shape. It assembles into a 520 kDa tetrameric structure with four homotrimeric subunits, 130 kDa each, linked *via* their N-terminal regions (Kishore et al., 2006). SP-D is not necessarily present as a dodecamer; it can be found as trimers, dimers and monomers in SP-D preparations, but also as a multiple of a dodecamer. Up to eight dodecamers can oligomerize, a process that might affect the recognition of and binding strength to the carbohydrate ligands on the surface of pathogens (Holmskov et al., 2003).

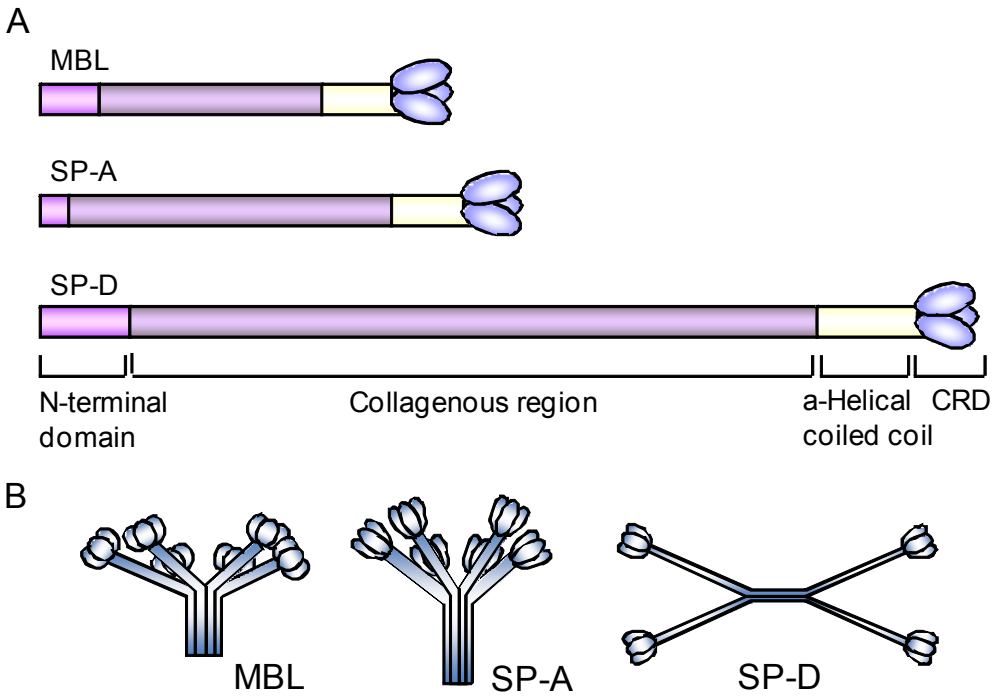


Figure 1.3: Collectin structure. (A) Collectins have a trimeric structure consisting of a collagen-like amino (N)-terminal region and C-type (calcium dependent) carbohydrate-recognition domains (CRDs). Collectins have structural subunits that are composed of trimeric polypeptide chains which assemble into oligomers. (B) SP-A and MBL form octadecamers (18-mers) consisting of six trimeric subunits. SP-D forms a dodecamer (12-mer) in a cruciform shape, consisting of four subunits. Figure modified after Wright J.R., 2005.

The C-type carbohydrate-recognition domain (CRD) was first described by Drickamer as a common element found in animal lectins by the presence of 14 invariant amino acid residues and 18 conserved residues (Drickamer, 1988). The α-helical coiled-coil region that initiates the formation of the collagenous triple-helical region also attributes to the trimerization of the heads (Holmskov et al., 2003). The collectins show selective binding to sugars such as mannose, glucose, L-fucose, N-acetyl-glucosamine (GlcNAc), and N-acetyl-mannosamine (ManNAc), whereas galactose is not bound. The CRDs of the collectins have low affinities for monosaccharides, but they show higher affinities to clustered oligosaccharides (Holmskov et al., 2003). Among mammalian collectins there are subtle differences in their sugar selectivities. The lung collectins, SP-A and SP-D, preferably bind to N-acetyl-mannosamine, L-fucose, and inositol, maltose and glucose, respectively, whereas MBL binds to higher glycans, e.g. N-acetyl-glucosamine. In addition, SP-A and SP-D bind selected lipids and proteins, e.g. lipopolysaccharide (LPS), lipoteichoic acid (LTA), phosphatidylcholine, galactosylceramide, phosphatidylinositol (PI), and glucosylceramide. (Holmskov et al., 2003;

Jack & Turner, 2003; Wright, 2005). The recognition and high-affinity binding to clustered oligosaccharides seems to be essential in their ability to distinguish self from non-self, since most carbohydrate structures in animals are terminated by sugars not recognized by collectins, such as galactose or sialic acid. The collectins´ central role in modulating innate host defense responses is due to their capacitiy to recognize and bind to pathogen-associated molecular patterns (PAMPs), and thus act as pattern recognition molecules (Holmskov et al., 2003; Wright, 2005). The interaction of the collectins with a broad range of microbial compounds is accompanied by the induction and regulation of immune cells. Collectins have been shown to opsonize pathogens, including Gram-positive and Gram-negative bacteria, viruses, fungi, allergens, and parasites, thereby preventing infection or enhancing pathogen uptake through cell surface receptors on phagocytes that are involved in microbial recognition (Holmskov, 2000; Wright 2005).

1.2.1.2.2 Surfactant protein (SP)-A

Surfactant protein A (SP-A) was the first of the four surfactant-associated proteins to be identified in 1973 (King et al., 1973). The SP-A monomer has a molecular weight of 26-36 kDa, and with a distribution of approximately 5.3 % of the total surfactant protein content, SP-A is the most abundant surfactant protein in the lung, intimately associated with lipids (Wright et al., 1987; Kishore et al., 2006). As all surfactant proteins, SP-A is primarily synthesized by alveolar type II cells where it is stored in lamellar bodies prior to secretion, the bronchiolar Clara cell and submucosal cells (Walker et al., 1986; Wright 2005), and it is metabolized by type II cells and AM (Wright & Dobbs, 1991). SP-A was first thought to play a role in surfactant homeostasis. However, a study by Tenner and colleagues demonstrated that SP-A could substitute for C1q, a structural homologue and subunit of the first component (C1) of the classical complement pathway, in modulating phagocytosis by monocytes and macrophages (Tenner et al., 1989). Later studies involving SP-A deficient mice emphasized that its main function was in modulating innate host defense responses (Crouch & Wright, 2001; Wright 2005).

Host defense functions of SP-A involve: opsonization of pathogens, functioning as an activation ligand, and regulating cell-surface-receptor expression (Wright, 2005).

SP-A´s function as an opsonin was published in 1990 by van Iwaarden and co-workers. He was the first to demonstrate that SP-A opsonizes bacteria, in this case *Staphylococcus aureus*,

and enhances phagocytosis by rat AM (van Iwaarden et al., 1990). Binding of SP-A to bacteria and viruses occurs *via* several mechanisms, e.g. *Haemophilus influenza* type A is aggregated and opsonized by SP-A through binding of the P2 outer membrane protein (McNeely & Coonrod, 1994). SP-A neutralized virus infectivity by binding to the respiratory syncytial virus (RSV) F (fusion) glycoprotein (Ghildyal et al., 1999), and *Mycoplasma pneumoniae* growth is attenuated by SP-A *via* binding to lipid components from mycoplasma membranes (Piboonpocanun et al., 2005). Several studies have shown that SP-A binds a broad range of microorganisms including viruses, Gram-positive bacteria, Gram-negative bacteria, fungi, mite extracts, and in addition, isolated rough forms of lipopolysaccharide (LPS). SP-A enhances the phagocytosis of those and of aggregated/opsonized microorganisms by immunocompetent cells, including alveolar macrophages, neutrophils, monocytes and dendritic cells (Schagat et al., 1999, 2001).

SP-A can directly activate immunocompetent cells and enhance their phagocytic activity by functioning as an activation ligand. Particles that are coated by an opsonin other than SP-A, for example by IgG, are phagocytosed by alveolar macrophages or neutrophils through direct interaction of SP-A with the phagocyte. Complement component C1q also functions as an activation ligand (Tenner et al., 1989; Lin & Wright, 2006; Wofford & Wright, 2007).

Indirect enhancement of phagocytosis by SP-A occurs through upregulation of cell-surface receptors that are involved in microbial recognition. SP-A-induced increase of cell-surface expression of scavenger receptor A, for example, enhances uptake of *Streptococcus pneumonia* (Kuronuma et al., 2004). Likewise enhances SP-A the expression of the mannose receptor by human monocyte-derived macrophages, resulting in the uptake of *Mycobacteria tuberculosis* lipoarabinomannan-coated microspheres (Beharka et al., 2002). Beharka and co-workers very recently showed that SP-A-induced activation of a Ca^{2+}/PLC/$InsP_3$ signal transduction pathway triggers an up-regulation of the mannose receptor in human monocyte-derived macrophages (Beharka et al., 2005).

Direct interaction of SP-A with macrophages can lead to pro- or anti-inflammatory mediator production. Gardai and co-workers proposed a model in which SP-A as well as SP-D are able to act in a dual manner to enhance or suppress inflammatory mediator production that was proposed to be both dependent on the cellular receptor engaged and whether SP-A, or SP-D were interacting with a host cell or with a pathogen (Gardai et al., 2003). A number of cell surface ligands and putative receptors for both collectins have been identified over the past 20

years. Receptors that have been reported to mediate SP-A's cellular functions include: C1qR (also known as CD93), calreticulin/CD91, signal-inhibitory-regulatory-protein-α (SIRP-α), SP-R210, and Toll-like-receptors (TLRs) (Wright, 2005). However, a SP-A-specific receptor has not been found so far, and the intracellular and molecular mechanisms leading to pro- or anti-inflammatory responses still remain largely unclear.

To evaluate the function of SP-A and its role in innate host defense *in vivo*, a SP-A gene-targeted mouse was generated by homologous recombination in embryonic stem cells. The SP-A-deficient mouse lacked SP-A mRNA and protein, but surprisingly, perinatal survival was unaltered compared to wild type mice, and mice grew normally in the vivarium. Lung morphology, protein levels of surfactant proteins B-D, lung tissue, alveolar phospholipid pool sizes and composition, and lung compliance was unaltered in SP-A-deficient mice compared to wild type mice, but tubular myelin figures were markedly decreased (Korfhagen et al., 1996; 1998a). When challenged with common pulmonary pathogens, e.g. group B streptococcus, a mucoid and a nonmucoid strain of *Pseudomonas aeruginosa*, respiratory syncytial virus (RSV), and *Haemophilus influenza*, mice lacking SP-A showed to be susceptible to bacterial and viral pneumonia. They displayed decreased and delayed clearance of all pathogens mentioned above, a more severe pulmonary inflammation, and an increased pulmonary infiltration of neutrophils compared to wild type mice (LeVine et al., 1997, 1998, 1999a, 1999b, 2000, 2002, Giannoni et al., 2006). SP-A-deficient mice that were infected by intranasal instillation of influenza A virus (IAV) (LeVine et al., 2002) or *Aspergillus fumigatus* (Madan et al., 2005) displayed similar outcomes. In addition, these studies found elevated T helper (Th) 1 and Th 2 cytokines, suggesting a role for SP-A in innate defense and adaptive immune response (LeVine et al., 2002; Madan et al., 2005). Administration of exogenous SP-A enhanced clearance of the pathogens and reduced lung inflammation. These *in vivo* studies demonstrated that SP-A plays an important role in innate host defense against a broad range of pulmonary pathogens.

1.2.1.2.3 Surfactant protein (SP)-D

Surfactant protein D was characterized in the late 1980s by Persson and co-workers who identified it as a pneumocyte-derived collagenous surfactant-associated protein, and changed its name from CP4 to surfactant protein D according to the nomenclature of surfactant-associated proteins (Persson et al., 1988). SP-D has a molecular weight of 43 kDa and belongs together with SP-A to the collectin family of proteins. Both lung collectins share

similar features that include recognizing, binding and clearing invading pathogens from the lung, thus contributing to innate host defense (Kingma & Whitsett, 2006). However, they have different binding preferences regarding selective lipids or proteins, and binding to the same pathogen might have different effects, for example the interaction with *Mycobacterium tuberculosis*: while SP-A enhances attachment and uptake of *M. tuberculosis* by AM, thereby promoting the organisms´ intracellular survival, SP-D reduces the uptake of *M. tuberculosis* by binding to the lipoarabinomannan moiety on the surface of the bacilli through its CRD (Gaynor et al., 1995; Pasula et al., 1997; Ferguson et al., 1999, 2002). SP-D interacts *in vitro* with bacteria, e.g. *Pseudomonas aeruginosa*, and *Klebsiella pneumonia,* fungi, e.g. *Candida albicans*, *Aspergillus fumigatus*, *Pneumocystis carinii*, respiratory viruses, e.g. influenza A virus, respiratory syncytial virus, and Rotaviruses, thereby inhibiting fungal growth and viral infectivity, and enhancing phagocytosis. The interaction of SP-D with pathogens and their agglutination is often CRD-dependent (Crouch & Wright, 2001; Haagsman et al., 2003). The functional role of SP-D has been determined in *in vivo* studies involving SP-D-deficient mice. SP-D gene-targeted mice survived and developed normally in the perinatal and postnatal periods. They display increased alveolar and tissue phosphatidylcholine pool sizes, increased numbers of large foamy AM, enlarged alveoli, and an abnormal surfactant morphology consisting of dense phospholipid membranous arrays with decreased tubular myelin. The level of surfactant protein B and SP-C mRNA and protein was unaltered, but SP-A mRNA and protein were reduced (Korfhagen et al., 1998b). SP-D-deficient mice show an overproduction of reactive oxygen species, the development of progressive pulmonary emphysema and fibrosis in association with chronic inflammation, moreover an accumulation of apoptotic and necrotic alveolar macrophages (Wert et al., 2000; Clark et al., 2002). Intratracheal administration of e.g. *Haemophilus influenza*, group B streptococcus, a nonmucoid strain of *Pseudomonas aeruginosa*, or *Aspergillus fumigatus*, resulted in increased pulmonary inflammation, deficient uptake of bacteria by AM, and an enhanced susceptibility to pulmonary hypersensitivity induced by *Aspergillus fumigatus* allergens (LeVine et al., 2000; Giannoni et al., 2006; Madan et al., 2005). The addition of exogenous SP-D reversed the observed effects, underlining the role of SP-D not only in surfactant lipid and immune homeostasis but also in innate host defense responses.

In their function as pattern recognition molecules, SP-A and SP-D participate actively in innate host defense, regulating the functions of other innate immunocompetent cells, such as alveolar macrophages, and modulating the adaptive immune response by interacting with

antigen-presenting cells and T cells, thus linking innate and adaptive immunity (Pastva et al., 2007). *In vitro* and *in vivo* investigations have contributed immensely to the knowledge we have today about the function of these two lung collectins and their unique role in lung host defense. SP-A and especially SP-D have also been localized to extrapulmonary sites, e.g. human sinus tissue, tear fluid, human lacrimal system, the gastrointestinal tract, genital organs and the skin, although their extrapulmonary functions are still unclear (Bräuer et al., 2007; Woodworth et al., 2007; Sorensen et al., 2007; Mo et al., 2007).

Genetic polymorphisms of human SP-A and SP-D have been documented and linked to pulmonary diseases, however, a human disease resulting from mutations or deletions in SP-A or SP-D has not been identified. SP-A and SP-D are considered to be biomarkers or contributors to the pathogenesis of diseases where levels of surfactant proteins are reduced, or the composition and/or function of surfactant is deranged. Allelic variations in the SP-A genes for example increase the susceptibility to RSV infection, meningococcal disease, *Aspergillus*-mediated allergies, or *Mycobacterium tuberculosis* infection, while polymorphisms of SP-D not only enhance susceptibility to infection but also influence the ability of SP-D to oligomerize. SP-A and SP-D could be used as biomarkers in some pulmonary diseases due to their changed levels found in bronchoalveolar lavage (BAL) fluid or serum of patients with acute respiratory distress syndrome (ARDS), pulmonary fibrosis, cystic fibrosis, or chronic obstructive pulmonary disease (COPD) (Pastva et al., 2007; Sorensen et al., 2007).

1.3 Bacterial lipopolysaccharide (LPS)

Bacterial lipopolysaccharides (LPS) are the major outer surface membrane components of Gram-negative bacteria that strongly stimulate the innate immune system by activating signal transduction cascades that lead to inflammatory responses. While LPS-induced responses benefit the host by sensing bacteria and mobilizing defence mechanisms, an exaggerated host response towards LPS may contribute to the development of local or systemic septic shock, multiorgan failure and death (Poltorak et al., 1998, Akira &Takeda, 2004).

1.3.1 LPS structure and function

LPS, also termed endotoxin, consist of a lipophilic membrane anchor domain – lipid A and a polysaccharide domain, containing a core region and an O-specific chain of variable length. The O-specific chain of most Gram-negative bacteria comprises of up to 50 repeating

oligosaccharide units that are formed by 2-8 monosaccharide moieties in a species- and strain-specific manner. It has been shown that the O-specific chain is dispensable for bacterial growth and survival *in vitro* but is essential *in vivo* where the O-specific chain provides protection from phagocytes. Mutants defective in or lacking the *wb** gene locus that is responsible for the synthesis of the O-specific chain, have been historically termed rough (R)-mutants, in contrast to the smooth (S)-form of wild type enterobacterial species, based on the characteristic colony morphology (Alexander & Rietschel, 2001). The core region can be distinguished between an outer core region that mainly consists of hexoses, such as D-glucose, D-galactose, D-glucosamine, N-acetylglucosamine, and N-acetylgalactosamine, and an inner core region that displays the least structural variability and contains the characteristic monosaccharide units 2-keto-3-deoxyoctulosonic acid (Kdo), often carrying additional anionic substituents, such as phosphate, diphosphate, or diphosphoethanolamine groups (Alexander & Rietschel, 2001).

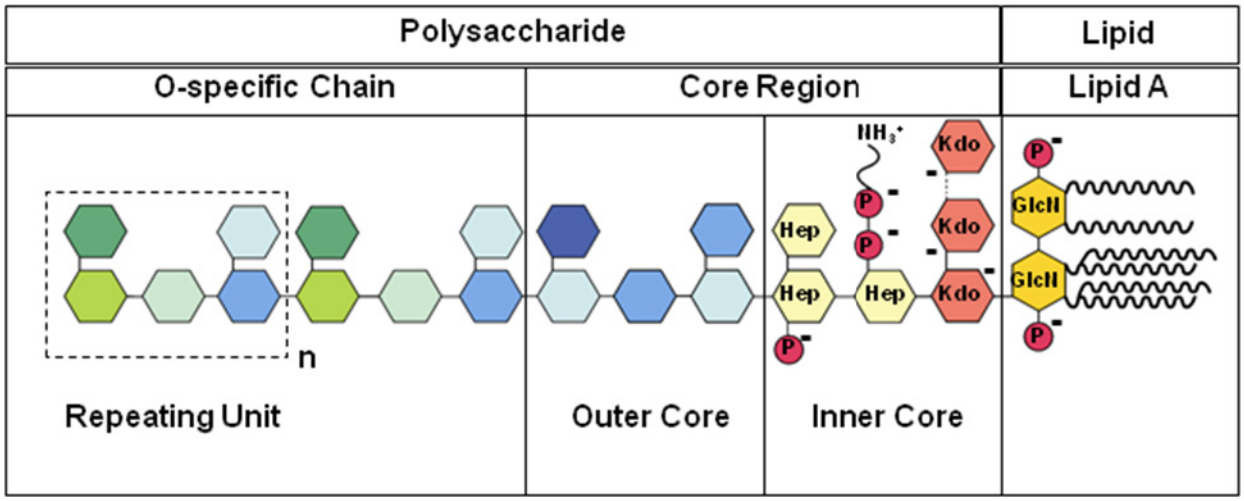


Figure 1.4: Schematic depiction of the general chemical structure of the S-type LPS from Gram-negative enterobacteria. The basic structure of LPS consist of a membrane anchoring domain – lipid A, and a polysaccharide domain, containing a core region and an O-specific chain of variable length. The core region can be distinguished between an outer and an inner core region, the latter carrying additional phosphate and ethanolamine residues. Abbreviations used: GlcN: glucosamine; Kdo: `2-keto-3-deoxyoctulosonic acid´; Hep: D-glycero-D-*manno*-heptose. Figure was kindly provided by Christian Alexander, Research Center Borstel.

The lipid A moiety of LPS is the immunostimulatory component of LPS exhibiting strong endotoxic and pyrogenic activity. It forms the outer monolayer of the outer membrane of most Gram-negative bacteria, covering almost 75 % of the total membrane surface, and it is the only part of the molecule that is recognized by immune cells of the innate immune system – on the basis of pattern recognition (Alexander & Rietschel, 2001; Raetz et al., 2007). Modifications of the lipid A moiety have been shown to modulate virulence of some Gram-negative bacteria (Raetz et al., 2007). Infection of Gram-negative bacteria initiates an immediate response by phagocytes, especially mononuclear cells that react to minute amounts (picomolar range) of bacteria-derived lipid A, subsequently activating the adaptive immune system that includes pathogen-specific T- and B-lymphocytes (Alexander & Rietschel, 2001).

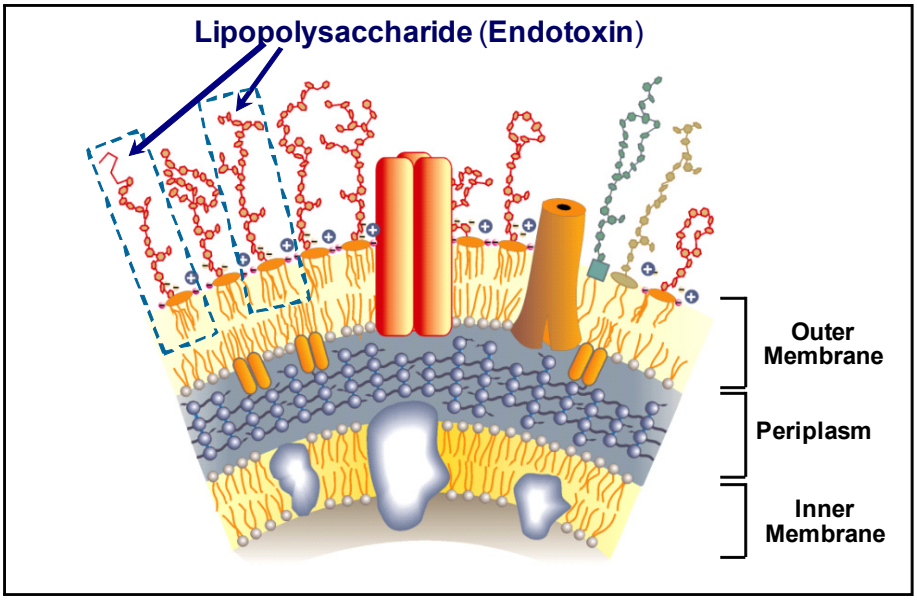


Figure 1.5: Cell wall architecture of Gram-negative enterobacteria. The cell wall of Gram-negative bacteria is characterized by two lipid bilayers, the outer and the inner (cytoplasmic) membrane, separated by the periplasm which contains the three-dimensional network of peptidoglycan (murein). Figure was kindly provided by Christian Alexander, Research Center Borstel.

1.3.2 LPS receptor complex

Membrane components of Gram-negative bacteria are identified by membrane-bound (signaling) or soluble (accessory) pattern-recognition receptors (PRR) of immunocompetent

cells, i.e. macrophages, peripheral monocytes and neutrophils. A number of those ligands are recognized by a family of mammalian transmembrane PRRs, the so called Toll-like receptors (TLRs). Initially discovered in *Drosophila melanogaster*, the fruit fly, where the Toll receptor has an essential role in fly development and immune response against fungal infection (Akira & Takeda, 2004; Miller et al., 2005), mammalian TLRs can distinguish between Gram-negative bacteria, Gram-positive bacteria, fungi and viruses, and elicit pathogen-specific transcriptional responses for an optimal immune response (Carmody & Chen, 2007). LPS is recognized by TLR4. The receptor was identified in an *in vivo* mouse model where a mutation in the TIR domain of the *tlr4* gene led to a resistance to endotoxin, yet they showed a high susceptibility to Gram-negative infection (Poltorak et al., 1998). TLRs consist of an extracellular recognition domain, containing leucine-rich repeats (LRR), a single transmembrane domain, and an intracellular Toll/IL-1 receptor homology (TIR) signaling domain (Brodsky & Medzhitov, 2007). The LPS receptor complex – TLR4-CD14 – is expressed on many cell types, including macrophages and dendritic cells (Miller et al., 2005). LPS is bound by LPS-binding protein (LBP), an accessory and acute phase protein, that circulates in the bloodstream where it recognizes and forms a high-affinity complex with the lipid A moiety of LPS. LBP then delivers LPS to CD14, forming a ternary complex. CD14 is found as a soluble form (sCD14) and a membrane bound form (mCD14) that is attached to the surface of myeloid cells *via* a glycosyl-phosphatidylinositol tail. The role of CD14 is to bind LPS and subsequently present LPS to MD2 and TLR4 (Palsson-McDermott & O´Neill, 2004; Miller et al., 2005). MD2 is a secreted glycoprotein that serves as an extracellular adaptor protein and associates with the ectodomain of TLR4. MD2 is required for LPS signal transduction as has been demonstrated in MD2-deficient mice where the lack of MD2 resulted in an unresponsiveness to LPS and an altered intracellular distribution of TLR4 (Beutler et al., 2006; Nagai et al., 2002).

1.3.3 LPS signaling pathway

LPS signaling via TLR4-MD2 leads to the rapid and coordinated activation of central intracellular pathways, including the nuclear factor-κB (NF-κB) pathway, and the MAP kinase pathway (Akira & Takeda, 2004).

The NF-κB signaling pathway plays a key role in many physiological and patho-physiological processes triggering and coordinating innate and adaptive immune responses. It encodes genes regulating inflammation, proliferation, cell adhesion, cellular-stress response,

apoptosis, tissue remodeling, and tumorigenesis. Mammalian NF-κB proteins consist of five members: Rel (c-Rel), RelA (p65), RelB, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100). They can form homo- or heterodimers which are bound to IκB (InhibitorykappaB) family proteins in unstimulated cells. All NF-κB proteins are characterized by a 300 amino acid Rel homology domain (RHD) that is located near the N-terminus and is required for dimerization. However, interaction with IκBs, and binding to DNA, can only be accomplished by Rel subfamily members, RelA, RelB and c-Rel that contain unrelated C-terminal transcriptional activation domains. In unstimulated cells, NF-κB is sequestered in the cytoplasm by IκB proteins that provide regulatory functions (Ghosh & Karin, 2002, Hayden & Ghosh, 2004; Neumann & Naumann, 2007; Perkins, 2007). Among the IκB members – IκB-α, IκB-β, Bcl-3, p105, and p100 – IκB-α and IκB-β are the most prominent and have been well characterized. The functional redundancy of both inhibitors has been investigated in knock-out mice. Because IκB-α knockout mice show increased basal NF-κB activity and die shortly after birth, an IκB-α knockout/ IκB-β knockin (AKBI) mouse was generated in which the IκB-α gene was replaced by an additional IκB-β gene (tagged to T7) that was put under the control of the *ikb-a* promoter. These mice are viable, have no apparent abnormalities, and exhibit an inducible NF-κB response similar to wild type mice (Cheng et al., 1998).

1.3.3.1 Distal/ proximal IκB-α/ NF-κB pathway

The most prominent NF-κB heterodimer p65/p50 is sequestered in the cytoplasm by a family of inhibitory proteins, among which IκB-α is the best characterized and assumed to function as the primary regulator of NF-κB in both stimulated and resting cells (Pando & Verma, 2000; Tergaonkar et al., 2005). Following LPS binding to the TLR4-MD2-CD14 complex, TIRAP (TIR-domain-containing adaptor protein) acts as a bridging adaptor between MyD88 and the TIR domain of TLR4, which allows further association of intracellular downstream signaling proteins that include IRAK1(IL-1 receptor-associated kinase), IRAK4 and TRAF6 (TNF receptor-associated factor 6) (Alexander & Rietschel, 2001; Carmody & Chen, 2007). Upon polyubiquitination of TRAF6, TAK1 (TGF-β activating kinase), TAB2 (TAK1-binding protein) and TAB3 are recruited to TRAF6. Activated TAK1 then further phosphorylates and thereby activates IKK2/β (InhibitoryκB kinase2/β), a member of the IKK complex together with IKK1/α, IKKγ/NEMO and ELKS. While NEMO is required by

upstream signals to initiate IKK activity, ELKS supposedly recruits IκB-α to the IKK complex where it is phosphorylated by IKK2/β (Ducut Sigala et al., 2004). Phosphorylation results in polyubiquitination and degradation of IκB-α at $Ser^{32/36}$ by the 26S proteasome, and thus in dissociation from NF-κB. The removal of IκB-α unmasks the nuclear localization sequence on the surface of NF-κB allowing its translocation into the nucleus where the transcription of NF-κB-dependent downstream target genes, including TNF-α (Tumor necrosis factor-α), IL-1 (Interleukin-1), and IκB-α itself occurs (Brown et al., 1993; Pando & Verma, 2000, Brown et al., 1995, Ghosh & Karin, 2002; Carmody & Chen, 2007).

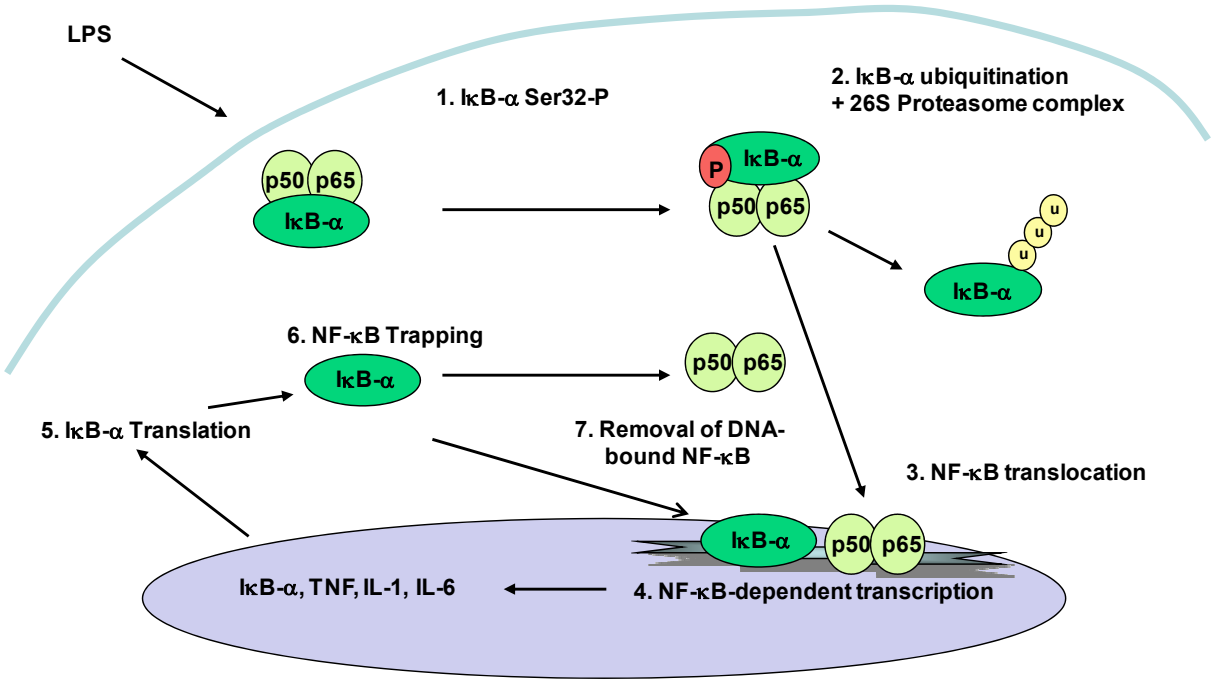


Figure 1.6: Distal/ proximal IκB-α/ NF-κB modulation. NF-κB is sequestered in the cytosol by its inhibitory protein IκB-α. Upon stimulation with e.g. LPS, IκB-α is phosphorylated at Ser^{32}, subsequently ubiquitinated and degraded by the 26S proteasome. The removal of IκB-α unmasks the nuclear localization sequence on the surface of NF-κB, allowing its translocation into the nucleus where the transcription of NF-κB-dependent genes occurs, including IκB-α. Free IκB-α within the nucleus binds to NF-κB, releases it from the DNA and leads the complex back into the cytosol where a new cycle begins.

1.4 Interaction of pulmonary SP-A with LPS

In vivo studies involving SP-A-deficient mice revealed a high susceptibility of these mice towards infection with Gram-negative bacteria. The administration of exogenous SP-A reduced pulmonary inflammation and enhanced the clearance of Gram-negative pathogens. In addition, SP-A-deficient mice intratracheally challenged with isolated LPS exhibited increased TNF-α and nitric oxide (NO) production that was restored by intratracheal administration of human SP-A (Borron et al., 2000). LPS, the major immunostimulatory factor of Gram-negative bacteria, has been shown to associate with SP-A. SP-A binds to the lipid A moiety of rough (r) LPS, but not to the smooth form of LPS (van Iwaarden et al., 1994). The first *in vitro* experiments employing the function of either SP-A alone or of SP-A on LPS-induced immune cell responses had been contradictory. Altered biological activities for SP-A and LPS individually have been observed when both agents interact (Kalina et al., 1995). Some investigators assigned pro-inflammatory properties to SP-A alone (Kremlev & Phelps, 1994; Koptides et al., 1997; Song & Phelps, 2000), others described anti-inflammatory functions, e.g. binding of SP-A to purified LPS of *Pseudomonas aeruginosa*, a respiratory pathogen, prevented TNF-α release in buffy coat cells (Hickling et al., 1998). The diverse findings may be attributed to the different cell types used, or to varying SP-A preparation protocols. A functional significance of the SP-A – R-LPS interaction was demonstrated in a study by Stamme & Wright who showed that SP-A enhances R-LPS binding and uptake by AM (Stamme & Wright, 1999). To further evaluate the mechanism involved in SP-A´s modulation of cellular responses to LPS, components of the LPS receptor complex were analyzed. SP-A inhibited the binding of LBP to Re-LPS, thereby preventing the initiation of the LBP-CD14 pathway that would lead to inflammatory responses (Stamme et al., 2002). SP-A has been shown to bind to CD14, but this interaction had no effect on the association of CD14 with rLPS (Sano et al., 1999). These findings were supported by studies employing CD14-null mice confirming that CD14 was not involved in SP-A´s inhibition of LPS pro-inflammatory cytokine production (Alcorn & Wright, 2004). Recent work suggests that SP-A reduces the catalyzing activity of LBP to transfer Re-LPS to CD14, thus modulating LPS response by altering the competence of the LBP-CD14 complex (Garcia-Verdugo et al., 2005). Besides the fact that SP-A does not bind to S-LPS, several studies have demonstrated that SP-A does modulate cellular responses towards this LPS chemotype. For example, SP-A protected lung fibroblast growth, reduced TNF-α activity and further inhibited NO and inducible nitric oxide synthase (iNOS) in AM stimulated with S-LPS (McIntosh et

al., 1996; Stamme et al., 2000). These data suggest that SP-A directly affects or modulates the immune response of the respective cell type. Interestingly, it has been shown that SP-A significantly reduced the association of recombinant soluble CD14 with S-LPS while the association with R-LPS was CD14-independent (Sano et al., 1999). In addition, work by Yamada and co-workers has demonstrated that SP-A attenuates the cell surface binding of sLPS and sLPS-induced NF-κB activation in TLR4-MD2-expressing cells (Yamada et al., 2006).

It seems that SP-A has developed a multitude of mechanisms to prevent LPS binding to its cognate receptor complex, or to inhibit LPS-induced pro-inflammatory cytokine production that are still largely unknown.

1.4.1 SP-A-specific IκB-α/ NF-κB modulation

LPS signals through its receptor complex TLR4-MD2-CD14, thereby activating the NF-κB-pathway leading to the production of pro-inflammatory cytokines. Many studies have shown that SP-A interferes with rough or smooth forms of LPS, thus attenuating LPS-induced inflammatory responses. How SP-A modulates the intracellular signaling cascade abrogating the classical LPS pro-inflammatory signaling pathway as described above remains elusive. Several groups have reported that SP-A inhibits NF-κB activation upon various stimuli, e.g. zymosan, peptidoglycan, NO or LPS (Sato et al., 2003; Murakami et al., 2002; Hussain et al., 2003, Wu et al., 2004). In elucidation of SP-A´s anti-inflammatory mechanism, work by Wu and co-workers shed first light on SP-A´s interference with the IκB-α/ NF-κB pathway. In that study, SP-A increased IκB-α protein expression in a dose- and time-dependent manner without enhancing IκB-α mRNA levels both basal and in the presence of LPS, or interfering with LPS-induced phosphorylation of IκB-α. Furthermore, SP-A inhibited LPS-induced NF-κB activation, independent of the LPS chemotype. In AM, SP-A employs its anti-inflammatory effects upon LPS stimulation by directly modulating the basal and LPS-coupled IκB-α turnover (Wu et al., 2004).

However, the signaling pathways controlling the SP-A-mediated attenuation of LPS-induced pro-inflammatory signals *via* IκB-α remain unknown.

1.5 Protein kinase C (PKC)

The Protein kinase C (PKC) family belongs to an extended group of serine/ threonine protein kinases, AGC (cAMP-dependent protein kinase (PKA), cGMP-dependent protein kinase (PKG), and PKC) with a broad range of tissue distribution and differential cellular localization. They are expressed in many different cell types, where they regulate a wide variety of cellular processes such as cell differentiation, cytoskeletal remodeling and gene expression in response to diverse stimuli. Based on sequence homology, their enzymatic properties and the mechanism of their regulation, individual members of the PKC family are subdivided into three classes: conventional (c) PKC, novel (n) PKC, and atypical (a) PKC (Moscat & Diaz-Meco, 2000; Tan & Parker, 2003).

1.5.1 Conventional (c) PKC and novel (n) PKC

Conventional or classical (c) PKCs comprise the α, β_I, β_{II}, and γ isotypes, whereas the β gene is alternatively spliced to produce two gene products that only differ in their extreme C-terminal ends. cPKCs are activated by phosphatidylserine (PS) and diacylglycerol (DAG) in a Ca^{2+}-dependent manner. They are also targets of the tumor-promoting phorbol ester PMA that activates these isozymes by eliminating the necessity for DAG and decreasing the Ca^{2+} concentration needed for activation (Mellor & Parker, 1998).

The novel (n) PKCs consist of the δ, ε, η, and θ isotypes. They are responsive to DAG and phorbol esters in the presence of PS, but are insensitive to Ca^{2+} (Mellor & Parker, 1998).

1.5.2 Atypical (a) PKC

The atypical (a) PKCs include the ζ and λ/ι (human PKCι and mouse PKCλ are orthologues) isotypes, based on their structural similarities. Like the nPKCs they are insensitive to Ca^{2+} but they do not bind to DAG or PMA (Mellor & Parker, 1998). Both isotypes are predominantly found in the lung (Leitges et al., 2001).

1.5.2.1 aPKC structure, activation and function

PKCζ and λ/ι consist of four functional domains and motifs: a PB1 domain in the N-terminus, a pseudosubstrate (PS) sequence, a C1 domain of a single cysteine-rich zinc-finger motif, and a kinase domain in the C-terminus. The PB1 domain recognizes specific so-called OPCA (OPR/PC/AID-atypical PKC-interaction domain) motifs of other proteins that are

found for example in PAR-6, ZIP/p62 and MEK5. The pseudosubstrate sequence blocks the substrate-binding cavity of the kinase domain. It resembles a substrate sequence with the exception of an amino acid exchange (alanine occupies the position of a serine or threonine), and is assumed to function as an autoinhibition mechanism. The C1 domain of aPKCs differs from those of cPKCs or nPKCs that contain two repeated zink-finger motifs, C1A and C1B, both of which are essential to interact with DAG and PMA. The kinase domain contains an ATP-binding region, an activation loop, a turn motif, and a hydrophobic motif.

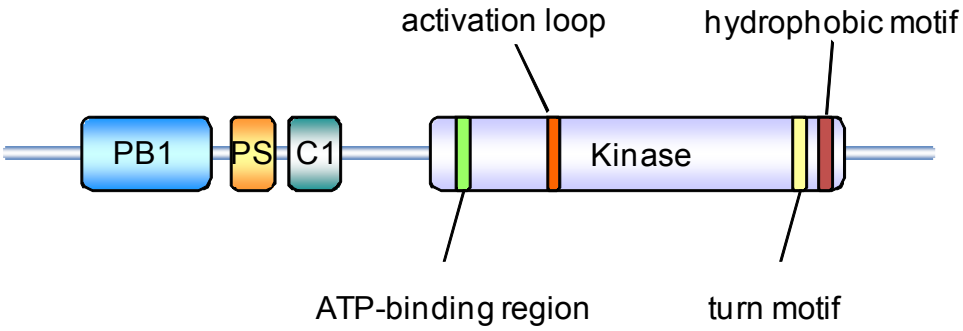


Figure 1.7: Atypical PKC structure. PKCζ and λ/ι consist of four functional domains and motifs: a PB1 domain in the N-terminus, a pseudosubstrate (PS) sequence, a C1 domain of a single cysteine-rich zinc-finger motif, and a kinase domain in the C-terminus that contains an ATP-binding region, an activation loop, a turn motif, and a hydrophobic motif.

The atypical PKCs (ζ, λ/ι) are directly or indirectly activated by phosphatidylinositol 3,4,5-triphosphate (PIP_3), phosphatidic acid, arachidonic acid, and ceramide, induced by LPS and TNF-α, but not like cPKCs or nPKCs by DAG and Ca^{2+}. This functional divergence is due to a unique N-terminal regulatory domain that lacks Ca^{2+}, phospholipids and DAG binding motifs. Atypical PKCs are activated upon release of the pseudosubstrate sequence from the substrate binding cavity, followed by the phosphorylation in the activation loop of the kinase domain. To reach the optimum catalytic output, PKCζ requires phosphorylation in the activation loop at Thr^{410}, catalyzed by phosphoinositide-dependent kinase (PDK-1) in a PI3K-dependent manner. Phosphorylation at Thr^{410} supposedly exposes the kinase domain for further phosphorylation in the turn motif at Thr^{560} that has been shown to be a key residue for activation. Activation is a prerequisite for PKCζ to function in signaling cascades that include the mitogen-activated protein kinase (MAPK) pathway and the NF-κB pathway. A role for aPKCs has been implicated in cell proliferation and in inhibiting apoptosis (Chou et al., 1998;

Moscat & Diaz-Meco, 2000; Parekh et al., 2000; Hirai & Chida, 2003; Newton, 2003; LeGood & Brindley, 2004).

Several studies have implicated a role for PKCζ in directly or indirectly interacting with components of the NF-κB pathway. PKCζ has been shown to be involved in inducing NF-κB activity by directly interacting with IKKβ, thereby activating the kinase that subsequently phosphorylates IκB-α (Lallena et al., 1999). To evaluate the role of PKCζ in NF-κB activation *in vivo*, a PKCζ knockout mouse was generated in which this kinase was inactivated by homologous recombination (Leitges et al., 2001). PKCζ-deficient mice have a fairly normal phenotype, but show alterations in secondary lymphoid organs. They exhibit impaired κB-dependent transcriptional activity as well as cytokine-induced p65 phosphorylation in embryonic fibroblasts (EFs). In whole lung extracts the lack of PKCζ results in an impairment of IKK activation upon LPS stimulation (Leitges et al., 2001). These findings suggest an essential role for PKCζ in the regulation of a stimulus-induced IKK/NF-κB signaling cascade in the lung.

1.6 Aim of this work

The pulmonary collectin, surfactant protein A, is the most abundant surfactant-associated protein in the lung. While it was first thought to function in lung homeostasis, many *in vitro* and *in vivo* studies have provided evidence that SP-A plays a central role in immune host defense by opsonizing pathogens, functioning as an activation ligand, and regulating cell-surface-receptor expression. Alveolar macrophages, the most prominent effector cells in the lung, have been shown to enhance phagocytosis of pathogens and allergens, induced by SP-A. Recently it was demonstrated that SP-A modulates alveolar macrophages´ immune responses by interfering with the IκB-α/ NF-κB pathway. SP-A enhanced IκB-α protein expression and inhibited LPS-induced NF-κB activity, independently of the LPS chemotype used, thereby employing its anti-inflammatory effects upon LPS stimulation by directly modulating the basal and LPS-coupled IκB-α turnover in alveolar macrophages. The signaling pathways though controlling the SP-A-mediated attenuation of LPS-induced pro-inflammatory signals *via* IκB-α remain largely unknown. Based on this work from our lab (Wu et al., 2004), the major and most intriguing question that evolved was: how does SP-A interfere with the IκB-α/ NF-κB pathway? In order to address this issue the following tasks were:

1. to clarify whether IκB-α is essential for SP-A´s anti-inflammatory properties in the lung, and
2. to identify which upstream/ downstream kinases are involved in SP-A-mediated IκB-α stabilization

Since many pulmonary diseases, for example ARDS, viral/ bacterial pneumonia, or bronchopulmonary dysplasia, are associated with decreased concentrations of pulmonary collectins, the risk of pulmonary infection and inflammation is increased in those patients *in vivo*. A more thorough understanding of the collectin-specific mechanisms modulating immune responses in the pulmonary compartment will potentially lead to specific therapeutic strategies that would help to improve or regain lung function.

2 MATERIALS AND METHODS

2.1 Animals

All animal experiments were approved by the Schleswig-Holstein Ministry of Environment, Nature and Forestation.

2.1.1 Rats

Male Sprague-Dawley rats, weighing 200-250 g, were obtained from Charles River (Sulzfeld, Germany) and kept under specific pathogen-free conditions.

2.1.2 Mice

2.1.2.1 IκB-α knockout/ IκB-β knockin mouse

The IκB-α knockout/ IκB-β knockin mouse was generated as described in Cheng et al., 1998, and was supplied by Prof. Dr. John F. Engelhardt, Director of the Department of Cell Biology, University of Iowa.

2.1.2.2 PKCζ knockout mouse

The PKCζ knockout mouse was generated as described in Leitges et al., 2001, and kindly provided by PD Dr. Michael Leitges, Biotechnology Centre of Oslo, University of Oslo.

2.2 Material

2.2.1 Equipment

Equipment	Description	Company
24-well plates		Nunc, Wiesbaden, Germany
96-well plates		Greiner bio-one GmbH, Frickenhausen, Germany
Accu-jet pipette controller		Brand GmbH & Co. KG, Wertheim, Germany

Balance	GX-6100	A&D Company Ltd., Ahrensburg, Germany
Balance	GX-200	A&D Company Ltd., Ahrensburg, Germany
Burner	FIREBOY eco	Integra Biosciences AG, Chur, Switzerland
Centrifuge	5810R	Eppendorf AG, Hamburg, Germany
Centrifuge	5415R	Eppendorf AG, Hamburg, Germany
Centrifuge	J2-21	Beckmann,Fullerton, CA, USA
Closures	Spectra/Por closures	Spectrum, Los Angeles, USA
Confocal laser scanning microscope	Leica TCS SP	Leica Microsystems, Bensheim, Germany
Dialysis tube	ZelluTrans	Roth, Karlsruhe, Germany
Dry Ease Mini-Gel Drying System		Invitrogen Ltd., Paisley, UK
Electrophoresis power supply	Consort E802	Consort, Thurnhout, Belgium
Gel blotting paper		Whatman, Dassel, Germany
Glass plates		Amersham Biosciences, Freiburg, Germany
Hyperfilm ECL		Amersham Biosciences, Freiburg, Germany
Imaging plate		Molecular Dynamics, Sunnyvale, USA
Incubator	UltimaII	Revco, Asheville, USA
LabTek Chamber Slides		Nunc, Wiesbaden, Germany
Light microscope	DMLS	Leica Microsystems,

		Bensheim, Germany
Nitrocellulose transfer membrane		Whatman, Dassel, Germany
Nick Columns		Amersham Biosciences, Freiburg, Germany
Parafilm		Roth, Karlsruhe, Germany
Pipettes	Rainin L2, L20, L200, L1000	Mettler-Toledo, Giessen, Germany
pH-Meter	Seven Easy	Mettler-Toledo, Giessen, Germany
Phosphor-Imager	Typhoon 8600 Variable Mode Image	Molecular Dynamics, Sunnyvale, USA
Polycarbonate centrifuge tubes		Beckman,Fullerton, CA, USA
QuixSep Dialysis		Roth, Karlsruhe, Germany
Semi-dry transfer unit	Hoefer TE77	Amersham Biosciences, Freiburg, Germany
Shaker	MiniRocker MR-1	PeqLab Biotechnology, Erlangen, Germany
Spectrophotometer	Titertek Multiskan Plus MKII	Labsystems, Finland
Standard vertical electrophoresis unit	Hoefer “SE 600 Ruby”	Amersham Biosciences, Freiburg, Germany
Stirrer	RH basic 2	IKA Janke&Kunkel, Staufen, Germany
Thermomixer	Thermomixer compact	Eppendorf AG, Hamburg, Germany
Ultra-centrifuge	L7-55	Beckmann,Fullerton, CA, USA
Ultra-centrifuge	TL-100	Beckmann,Fullerton, CA, USA
Vertical laminar airflow	BH-EN 2003	Faster, Ferrara, Italy

cabinet		
Vortexer	MS 2 Minishaker	IKA Janke&Kunkel, Staufen, Germany
Water bath	Polystat cc2	Huber, Offenburg, Germany

2.2.2 Reagents and Chemicals

Reagent	**Company**
Acrylamide/Bisacrylamide 30%	Biorad, Hercules, CA, USA
Albumin, bovine	Sigma, Munich, Germany
Ammonium persulfate (APS)	Biorad, Hercules, CA, USA
Aqua B. Braun	Melsungen, Germany
ATP	Cell signalling, Beverly, MA, USA
BCA Reagent A+B	Interchim, Montluçon Cedex, France
Boric acid	Roth, Karlsruhe, Germany
C1q	Advanced Research Technologies, Saint-Laurent, Quebec, Canada
Calcium chloride	Sigma, Munich, Germany
Dimethylsulfoxide (DMSO)	Sigma, Munich, Germany
ECL Western blotting reagents	Amersham Biosciences, Freiburg, Germany
EDTA	Roth, Karlsruhe, Germany
EGTA	Sigma, Munich, Germany
Fetal Calf Serum (FCS)	Cambrex, New Jersey, USA

Glycine	Sigma, Munich, Germany
Heparin	Sigma, Munich, Germany
ImmunoPure Plus Immobilized Protein A Agarose	Pierce, Rockford, IL, USA
Limulus amebocyte lysate assay	Cambrex, New Jersey, USA
Magnesium chloride	Sigma, Munich, Germany
Narcoren	Merial, Hallbergmoos, Germany
NF-kB GelShift nucleotides	Santa Cruz, Santa Cruz, CA, USA
Non-fat dry milk	Roth, Karlsruhe, Germany
PBS w/o $MgCl_2$ & $CaCl_2$	Gibco, Karlsruhe, Germany
Poly(dA-dT)-Poly(dA-dT)	Amersham Biosciences, Freiburg, Germany
Poly(dI-dC)-Poly(dI-dC)	Amersham Biosciences, Freiburg, Germany
Polymyxin B agarose	Sigma, Munich, Germany
Potassium chloride	Sigma, Munich, Germany
Potassium hydrogen phosphate	Sigma, Munich, Germany
Propidium iodide	Invitrogen, Eugene, OR, USA
RPMI 1640 w/o L-Glutamine	Cambrex, New Jersey, USA
Sodium chloride	Sigma, Munich, Germany
Sodium deoxycholate	Applichem, Darmstadt, Germany
Sodium dodecyl sulfate	Roth, Karlsruhe, Germany
Sodium hydroxide (4N)	Merck, Darmstadt, Germany
Sodium phosphate	Sigma, München, Germany

Sodium-ortho-vanadate	Applichem, Darmstadt, Germany
Temed	Biorad, Hercules, CA, USA
Tris	Roth, Karlsruhe, Germany
Trizma hydrochloride	Sigma, Munich, Germany
β-Glycerolphosphate	Sigma, Munich, Germany

2.2.3 Software

Adobe Photoshop 6.0	Adobe Systems Inc., Munich, Germany
GraphPadPrism 4.02	GraphPad, San Diego, USA
Irfanview 3.98	Irfan Skiljan, Wiener Neustadt, Austria
Optimas	Media Cybernetics, Silver Spring, MD, USA

2.2.4 Antibodies

2.2.4.1 Primary antibodies, unconjugated

anti-IκB-α (C-21), rabbit polyclonal	Santa Cruz	200 µg/ml
anti- rabbit IgG	Santa Cruz	200 µg/ml
anti-Actin, mouse monoclonal	Chemicon	100 µl
anti-NF-κB p65 (A), rabbit polyclonal IgG	Santa Cruz	200 µg/ml
anti-NF-κB p65 (A), mouse monoclonal	Santa Cruz	200 µg/ml
anti-PCKζ (C-20), rabbit polyclonal IgG	Santa Cruz	200 µg/ml
anti-phospho-IκB-α (Ser32/36), rabbit polyclonal	Santa Cruz	200 µg/ml
anti-phospho-PKCζ (Thr410/403), rabbit polyclonal	Cell Signaling	100 µl
anti-Phosphotyrosine, mouse monoclonal	Cell Signaling	300 µg
anti-SP-A (N-19), goat polyclonal	Santa Cruz	200 µg/ml
anti-Ub (P4D1), mouse monoclonal	Santa Cruz	200 µg/ml

2.2.4.2 Secondary antibodies, conjugated

donkey-anti-goat IgG, HRP-conjugate	Santa Cruz	100 µg/ ml
goat-anti-rabbit IgG, HRP-conjugate	Santa Cruz	100 µg/ ml
rabbit-anti-mouse IgG, Peroxidase-conjugate	Pierce	0.8 mg/ ml

Alexa Fluor 488 goat-anti-rabbit IgG (H+L)	Molecular Probes	2 mg/ml
Alexa Fluor 488 rabbit-anti-mouse IgG (H+L)	Molecular Probes	2 mg/ml
Alexa Fluor 568 goat-anti-rabbit IgG (H+L)	Molecular Probes	2 mg/ml
Alexa Fluor 568 rabbit-anti-mouse IgG (H+L)	Molecular Probes	2 mg/ml
Alexa Fluor 700 goat-anti-rabbit IgG (H+L)	Molecular Probes	2 mg/ml

2.2.5 Inhibitors

alpha-Adaptin blocking peptide	Santa Cruz, Santa Cruz, CA, USA
Apigenin	Sigma, Munich, Germany
Amantadine	Sigma, Munich, Germany
beta-Arrestin1 blocking peptide	Santa Cruz, Santa Cruz, CA, USA
Bisindolylmaleimide (Gö 6850)	Calbiochem, San Diego, CA, USA
Chelerythrine Chloride	Tocris Bioscience, Ellisville, MO, USA
Chlorpromazine	Sigma, Munich, Germany
Cytochalasin D	Sigma, Munich, Germany
Genistein	Sigma, Munich, Germany
Gö 6976	Alexis Biochemicals, Lausen, Switzerland
Phenylarsine oxide	Sigma, Munich, Germany
PKCzeta pseudosubstrate, cell permeable	Biosource, Camarillo, CA, USA
Wortmannin	Sigma, Munich, Germany

2.2.6 Protease Inhibitors

Aprotinin	Sigma, Munich, Germany
Complete Protease Inhibitor cocktail	Roche, Mannheim, Germany
Leupeptin	Sigma, Munich, Germany
Pefabloc Sc (AEBSF)	Roche, Mannheim, Germany

Pepstatin	Roche, Mannheim, Germany

2.2.7 Buffers

Blocking buffer

Dry milk, blocking grade	5 % (w/v)
Tween-20	0.1 % (v/v)

TBE (10 x)

Tris	900 mM
Boric acid	900 mM
EDTA	20 mM
	pH 8.8

TBS (10 x)

Tris	0.25 M
Sodium chloride	1.5 M
	pH 7.2

TBS-T

TBS (10 x)	1 x TBS
Tween-20	0.1 % (v/v)

TE

Tris	10 mM
EDTA	1 mM
	pH 8.0

Transfer buffer (10 x)

Glycine	1.92 M
Tris base	0.25 M

Transfer buffer (1 x)

Transfer buffer (10 x)	Transfer buffer (1x)
Methanol	20 %
ddH_2O	

FACS buffer

PBS	Gibco
Formaldehyde	1 %

2.2.7.1 SP-A purification buffer

0.5 M Tris-buffered water (TBW) (100 x)

Tris-HCl	420 mM
Tris base	81 mM
Braun H_2O	ad 200 ml
	pH 7.4, sterile filter

Octylglucopyranoside (OGP)-buffer

0.5 M TBW	5 mM
n-Octyl-β-D-glucopyranoside	20 mM
Sodium chloride	150 mM
Braun H_2O	ad 100 ml

2.2.7.2 Bronchoalveolar lavage-buffer for rats

10 x Isolation buffer

Sodium chloride	140 mM
Potassium chloride	5 mM
N-2-hydroxyethylpiperazine-*N´*-ethane sulfonic acid (HEPES)	10mM
ddH_2O	ad 500 ml
	pH 7.4

Lavage-buffer 1

Glucose	6mM
Ethyleneglycol-*bis*-(β-aminoethyl ether)-*N*,*N*´-tetraacetic acid (EGTA)	0.2mM
10 x Isolation buffer	1 x Isolation buffer
ddH_2O	ad 125 ml

Lavage-buffer 2

Glucose	6 mM
Magnesium chloride	1.3 mM
Calcium chloride	2 mM
10 x Isolation buffer	1 x Isolation buffer
ddH_2O	ad 125 ml

2.2.7.3 Bronchoalveolar lavage buffer for mice

PBS	Gibco
Ethylenediaminetetraacetic acid (EDTA)	0.2 mM

2.2.7.4 Subcellular fractionation buffer

Nuclear extraction

Basic buffer 1

Tris (pH 7.8)	10 mM

Magnesium chloride	5 mM
Potassium chloride	10 mM
EGTA (pH 7.0)	1 mM
Sucrose	0.3 M
ddH_2O	ad 500 ml

Basic buffer 2

Tris (pH 7.8)	20 mM
Magnesium chloride	5 mM
Potassium chloride	320 mM
EGTA (pH 7.0)	200 µM
Glycerol	25 %
ddH_2O	ad 100 ml

Nuclear extraction buffer 1

Calculated per sample

Basic buffer 1	400 µl
DTT	1 mM
Complete (20 x)	1.5 µl
β-glycerolphosphate	10 mM
Pefabloc	0.5 mM

Nuclear extraction buffer 2

Calculated per sample

Basic buffer 2	30 µl
DTT	1 mM
Complete (20 x)	1.5 µl
β-glycerolphosphate	10 mM
Pefabloc	0.5 mM

Membrane fractionation

Hypotonic fractionation buffer 1

Tris (pH 7.4)	10 mM
EDTA	4.5 mM
EGTA	2.5 mM
2-mercaptoethanol	2.3 mM
PMSF	1 mM
Aprotinin	10 µg/ml
Leupeptin	10 µg/ml
Pepstatin	10 µg/ml
Sodium-ortho-vanadate (Na_3VO_4)	0.1 mM

Hypotonic fractionation buffer 2

Tris (pH 7.4)	10 mM
EDTA	4.5 mM
EGTA	2.5 mM
2-mercaptoethanol	2.3 mM
PMSF	1 mM
Aprotinin	10 µg/ml
Leupeptin	10 µg/ml
Pepstatin	10 µg/ml
Sodium-ortho-vanadate (Na_3VO_4)	0.1 mM
Triton X-100	0.5 %

2.2.7.5 Immunoprecipitation buffer

RIPA-buffer

Tris-HCl (pH 8.0)	50 mM
Sodium chloride	150 mM
Nonidet P-40	1 %

2.2.7.6 Kinase assay reaction buffer

MOPS (pH 7.2)	20 mM
β-glycerolphosphate	25 mM
DTT	1 mM

EGTA	5 mM
Sodium-ortho-vanadate (Na_3VO_4)	1 mM
$MgCl_2$	75 mM
ATP	0.5 mM
[γ-^{32}P] ATP	100 µCi

Laemmli buffer

Tris (pH 6.8)	62.5 mM
SDS	2 % (w/v)
Glycerol	10 % (v/v)
Mercaptoethanol	5 % (v/v)
Bromphenol blue	0.001 % (w/v)

2.2.7.7 Electrophoretic mobility shift assay

Shift buffer (5 x)

Magnesium chloride	10 mM
DTT	5 mM
Ficoll	20 % (v/v)
Nonidet P-40	0.15 % (v/v)
ddH_2O	

Binding buffer

poly (dI/dC)	50 µg/ml
poly (dA/dT)	50 µg/ml
Potassium chloride	60 mM
ddH_2O	26.3 % (v/v)
Shift buffer (5 x)	42.9 % (v/v)

2.2.7.8 Gelelectrophoresis buffer

SDS-Polyacrylamide gelelectrophoresis

Resolving gel (12 %)

30 % Acrylamide/ Bisacrylamide (29:1)	39.1 % (v/v)
1.5 M Tris (pH 8.8)	0.37 M
SDS (10 %)	0.1 %
ddH_2O	34.9 % (v/v)
TEMED	0.05 % (v/v)
10 % Amoniumpersulfate (APS)	0.5 % (v/v)

Stacking gel (4 %)

30 % Acrylamide/ Bisacrylamide (29:1)	13.2 % (v/v)
0.5 M Tris (pH 6.8)	125 mM
SDS (10 %)	0.1 %
ddH_2O	60 % (v/v)

TEMED	0.1 % (v/v)
10 % Amoniumpersulfate (APS)	0.5 % (v/v)

10 x Gel-tank buffer

Glycine	1.92 M
Tris base	0.25
SDS	1 %

Sample buffer (5 x)

Stacking gel buffer	50 % (v/v)
Glycerol	50 % (w/v)
SDS	0.1 % (w/v)

Coomassie staining solution

Coomassie	2.4 mM
Methanol	40 % (v/v)
Pure acetic acid	10 % (v/v)
ddH_2O	50 %

Destaining buffer

Methanol	30 % (v/v)
Acetic acid	10 % (v/v)

ddH_2O	60 % (v/v)

Glycerol buffer (to dry coomassie stained gels)

Glycerol	5 % (v/v)
Ethanol	22 % (v/v)
ddH_2O	73 %

Polyacrylamide gelelectrophoresis

30 % Acrylamide/ Bisacrylamide (29:1)	17.4 % (v/v)
TBE (10 x)	0.5 x
ddH2O	77.1 % (v/v)
TEMED	0.05 % (v/v)
10 % Amoniumpersulfate (APS)	0.5 % (v/v)

2.3 Methods

2.3.1 Purification and endotoxin decontamination of SP-A

Human SP-A was purified from the bronchoalveolar lavage of patients suffering from alveolar proteinosis (AP), a disease featuring an excessive accumulation of surfactant lipoprotein in pulmonary alveoli with associated disturbance of pulmonary gas exchange. The standard therapy to remove excess alveolar surfactant material is lung lavage with saline (Presneill et al., 2006). Lung lavage fluid from AP patients used here to extract SP-A was kindly provided by Prof. K. Dalhoff from the University of Luebeck. The SP-A isolation protocol is a slightly modified version of the original protocol by Wright and co-workers (Wright et al., 1987). The bronchoalveolar lavage fluid was stored overnight at 4°C to allow sedimentation of the SP-A containing fluid. The supernatant was removed and stored at -20°C. The SP-A-containing sediment (5 ml) was slowly added *via* a syringe to a beaker that contained 250 ml butanol and stirred for 30 min. Butanol-insoluble proteins were collected by centrifugation at 11,300 rpm, 4°C, 30 min (Beckmann rotor JA-20). The supernatant was discarded and the pellet resuspended in fresh butanol. After a repeated centrifugation at 11,300 rpm, 4°C, 30 min (Beckmann rotor JA-20), the supernatant was discarded, and the resulting pellet was resuspended in 20 ml octylglucopyranoside (OGP) to extract protein. The solution was pelleted by ultra-centrifugation at 27,000 rpm, 4°C, 30 min (Beckmann rotor SW41-Ti), the pellet containing OGP-insoluble proteins was resuspended in 5 ml 5 mM Tris-buffered water (TBW), pH 7.4, and incubated with OGP at RT for 30 min. Polymyxin B agarose beads were then added to reduce endotoxin contamination and dialyzed against the same buffer for 72 h with at least six changes to remove OGP. The solution was transferred to a 15 ml conical tube centrifuged for 30 min at 2500 rpm at room temperature (Eppendorf tabletop). The supernatant was removed and transferred into an ultracentrifuge tube where insoluble proteins were removed by ultra-centrifugation (33,000 rpm, 4°C, 1 h; Beckmann rotor 70.1 Ti); the resulting supernatant contained SP-A. Purity of SP-A was assessed by SDS-PAGE and coomassie staining or by Western analysis using a SP-A antibody. SP-A preparations were tested for the presence of bacterial endotoxin using a *Limulus* amebocyte lysate (LAL) assay. Protein concentration of SP-A was assessed by bicinchoninic acid assay and ranged from 0.8 – 1.6 mg/ ml. The butanol extraction of SP-A is a well established method that does neither alter SP-A´s aggregation status nor its function (Wright et al., 1987).

2.3.2 LPS

The smooth LPS from *Salmonella friedenau* strain H909 that was extracted by the phenol/water method, purified, lyophilized, and transformed into the triethylamine salt form (Galanos & Lüderitz, 1975) was kindly provided by Prof. H. Brade, Research Center Borstel.

2.3.3 Limulus amebocyte lysate (LAL) assay

The LAL is an *in vitro* end-product endotoxin test used to detect Gram-negative endotoxin in various biological samples. The LAL is based on the observation that an infection of *Limulus polyphemus*, the horseshoe crab, with Gram-negative bacteria resulted in fatal intravascular coagulation (Bang, 1957). The clotting is a result of the interaction of endotoxin and a clottable protein in the circulating amebocytes of *Limulus* blood (Young et al., 1972). Incubation of *Limulus* amebocyte lysates with the sample to be tested will result in gelation in the presence of endotoxin, while no gelation occurs in the absence of endotoxin. All SP-A preparations used contained < 0.2 pg endotoxin/ µg SP-A. However, there are intrinsic limitations of the LAL assay, i.e. LPS associated to protein interferes with the LAL assay thereby restraining particularly the detection of lower LPS levels (Petsch & Anspach, 2000; Cooper, 1990). To exclude biologically significant endotoxin contamination of SP-A, NF-κB activation was tested in an EMSA using LPS as a positive control.

2.3.4 Bronchoalveolar lavage of rats and mice

Rats were killed by intraperitoneal injection of pentobarbital (110 mg/kg body weight) followed by exsanguination. The trachea was cannulated, and the lungs were removed and lavaged six times with 10 ml lavage buffer 1, and twice with 10 ml lavage buffer 2. Alveolar macrophages are the most prominent cell type in lung lavages (90-95 %), making AM easily accessible for *in vitro* studies. AM were collected by centrifugation at 200 x g for 10 min. The supernatant was discarded, and the pellet was resuspended in RPMI medium. Cell count and viability of the cells were carried out in a Neubauer counting chamber. The cell suspension was mixed 10:1 with erythrosin B and incubated at 4°C for four min. Erythrosin B is able to penetrate the cell wall of apoptotic cells, marking dead cells with a red colour. Cell recovery averaged 5 - 8 x 10^6 cells/ animal, and the viability of the cells averaged from 94 to 98 %. AM were plated at a density of 1 x 10^6/ ml in 24-well plates (Nunc) in the presence of 0.2 % heat-inactivated (HI)-FCS. FCS was heat-inactivated at 56°C for 30 min to inactivate complement proteins present in fetal calf serum and to inactivate undetermined growth factor inhibitors, thus providing an optimal supplement for growth media.

Selectively gene-deficient mice and respective controls were killed by intraperitoneal injection of pentobarbital (7.5 mg/ kg body weight) followed by exsanguination (either by cutting the aorta or by cardiac puncture). The trachea was cannulated; the lungs maintained within the animal and were lavaged twice with 1 ml PBS containing 0.2 mM EDTA but no Mg^{2+} and Ca^{2+}. AM were collected by centrifugation at 200 x g for 10 min. The supernatant was discarded, and the pellet was resuspended in RPMI medium. Cell recovery averaged 2-3 x 10^5 cells/ animal. The viability of the cells was determined by erythrosin B exclusion and averaged 94 to 98 %. AM were plated at a density of 2 x 10^5/ ml in 24-well plates (Nunc) in the presence of 0.2 % heat-inactivated (HI)-FCS.

2.3.5 Stimulation of alveolar macrophages

Cells were plated at 1 x 10^6/ ml in 24-well plates (Nunc) and allowed to attach for 90 min at 37°C in a 5 % CO_2 atmosphere. After a change to fresh medium the cells were treated with SP-A (20 - 40 µg/ ml) and/or LPS (10-100 ng/ ml) for indicated times at 37°C in the presence of 0.2 % HI-FCS in a 5 % CO_2 atmosphere. In separate experiments, AM were treated with a panel of kinase inhibitors to determine their possible effect on IκB-α protein expression: Gö-6976 (5 µM, 30 min), Gö-6850 (5 µM, 30 min), chelerythrine chloride (6 and 12 µM, 30 min), or atypical (a) PKC pseudosubstrate peptides (2, 5, and 10 µM, 1 h) were used to inhibit distinct subsets of conventional, novel, or atypical PKCs. Wortmannin (50 nM, 30 min) was used as phosphatidylinositol 3-kinase (PI3K) inhibitor, apigenin (30 µM, 1 h) to inhibit protein kinase CKII (formerly casein kinase II).

To determine whether SP-A uptake *via* clathrin affects its anti-inflammatory properties, AM were treated with a panel of clathrin and actin inhibitors or blocking peptides of the clathrin assembly complex: amantadine (2.5 mM, 30 min), phenylarsine oxide (2 µM, 30 min), chlorpromazine (50 mM, 1 h), or cytochalasin D (10µM, 30 min), and α-adaptin blocking peptide (10 µg, 10 min), β-arrestin blocking peptide (10 µg, 10 min). Because clathrin and actin inhibitors or blocking peptides detach AM from their wells, these experiments were performed in pre-coated microfuge tubes at 37°C in a thermomixer with gentle shaking.

Experiments employing the inhibitors or blocking peptides mentioned above were performed under four different conditions: i) in the absence of SP-A and LPS (basal), ii) in the presence of SP-A (40 µg/ ml, 1 h) (constitutive), iii) in the presence of LPS (10 ng/ ml, 30 min or

100 ng/ ml, 1 h) (induced), and iv) in the presence of SP-A (40 µg/ ml, 1 h) prior to LPS (10 ng/ ml, 30 min or 100 ng/ ml, 1 h) (modulated).

2.3.6 Isolation of peripheral blood mononuclear cells (PBMC)

MNC from wild type or AKBI mice were isolated by density gradient using Ficoll-Histopaque-1083 (Sigma). Heparinized blood was mixed with an equal amount of Hanks´ balanced salt solution and centrifuged at 420 x g. The interphase layer containing the MNC fraction was collected, washed twice with Hanks´ balanced salt solution and once in serum-free RPMI. Cells were plated at a density of 1x 10^6/ ml in 24-well plates. The isolation of PBMC from heparinized blood was performed in the lab of PD Dr. Andra Schromm.

2.3.7 Nuclear extraction

After treatment, the supernatant was discarded and the cells were scraped off with a rubber policeman in 500 µl cold PBS and spun at 4500 x g, 5 min, 4°C. The resulting pellet was resuspended in 400 µl nuclear extraction buffer 1 and incubated on ice for 15 min. 25 µl NP-40 was added, and the suspension was vortexed for 10 seconds. Thereafter samples were centrifuged at 4500 x g, 5 min, 4°C. The supernatant was taken to represent the cytosolic fraction. The pellet was resuspended in 30 µl nuclear extraction buffer 2 and incubated on ice for 15 min, followed by centrifugation at 16100 x g, 10 min, 4°C. The supernatant containing the nuclear fraction was transferred to a new vial.

2.3.8 Bicinchoninic acid (BCA) assay

Cytosolic, nuclear and membrane fractions were assayed for protein content by the bicinchoninic acid (BC) reagent (Uptima) according to the manufacturers´protocol.

Smith and co-workers supplied the Biuret assay with bicinchoninic acid combining both methods to achieve a sensitive and colorimetric protein detection system (Smith et al., 1985). Like the Lowry assay, the BCA assay is based on the reduction of Cu^{2+} to Cu^{+} by peptide bounds of proteins. Bicinchoninic acid chelates Cu^{+} ions with very high specificity and forms a water soluble purple-coloured complex. Samples are mixed 1:50 with a freshly prepared bicinchoninic acid/ copper sulphate solution, and incubated at 37°C for 30 min, after which absorbance of the final Cu^{+} complex is measured at 562 nm. Bovine serum albumin (2 mg/ml) was used as protein standard, ranging from 20 µg/ ml to 2 mg/ ml. The measured protein concentration, being equivalent to the absorbance, was referred to the standard curve and analyzed with GraphPad Prism.

2.3.9 Western blotting

The name Western blotting derived from the inventor of the Southern blot, E. Southern, who developed a method to separate DNA fragments and their subsequent hybridisation (Southern, 1971). Following blotting techniques were named accordingly Northern blotting (RNA), or Western blotting (Protein). Western blotting (or immunoblotting) is a technique to transfer protein of previously separated samples onto a membrane (often a nitrocellulose membrane) with subsequent immuno-detection. Samples were separated according to their molecular size by sodium-dodecyl-sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The SDS-PAGE technique under reducing condition with its discontinuous buffer system used here was based on the original method described by U.K. Laemmli (Laemmli, 1970). The SDS-PAGE contains two types of gels: a large-pore stacking gel (4 %, pH 6.8) and a small-pore resolving gel (12 %, pH 8.8). Samples were mixed with 5x sample buffer, DTT and 2 µl bromphenol blue, heated for 5 min at 95°C, and subsequently separated on 12 % SDS-PAGE. Western analysis was performed on cytosolic and nuclear extracts, membrane fractions, and immunoprecipitated samples. Blotting paper, nitrocellulose membrane, and gel were bathed in transfer buffer and stacked onto the blotting apparatus in the order: blotting paper-membrane-gel-blotting paper. The transfer was performed at 100 V, 200 mA, 100 W for 1 h. Afterwards, membranes were blocked in 5 % milk powder (blotting grade)/ TBS-T for 1 h and were further incubated with anti-IκB-α, anti-aPKC, anti-PKCThr $^{410/403}$, anti-NF-κB p65 (all rabbit polyclonal), or β-actin (mouse monoclonal) at a 1:700, 1:200, 1:700, 1:500 and 1:1000 dilution, respectively for 2 h (room temperature) or overnight (4°C). The linearity of the Abs was determined by performing a dilution series (1-80 µg) after which the optimized protein concentration (30-40 µg) was applied on the gel. After incubation, membranes were washed three times with TBS-T for 5 min, followed by the addition of the secondary Abs for 1 h at room temperature: Goat anti-rabbit IgG-HRP conjugate or rabbit anti-mouse IgG-peroxidase conjugate. The membranes were washed five times with TBS for 5 min before immunoreactive proteins were visualized using the ECL Western blotting detection system (Amersham).

2.3.10 Immunoprecipitation

An antigen-antibody reaction in solution often leads to a loss of solubility of the antigen-antibody complexes, subsequently resulting in precipitation. It´s a method used to enrich a specific protein to some degree of purity, or – in the case of co-immunoprecipitation

(or pull-down) – to determine protein-protein interactions, or to identify members of protein complexes in cell extracts. Immunoprecipitated samples were used in Western analysis and to determine kinase activity. Rat AM (2 x 10^6/ well) were plated in 24-well plates and allowed to adhere for 90 min at 37°C in a 5 % CO_2 atmosphere. After a change to fresh medium containing 0.2 % HI-FCS, cells were stimulated for the indicated times with SP-A, LPS, or, for some experiments, with C1q that shares structural and functional homology with SP-A. After culture, the supernatant was discarded, the cells washed once with cold PBS, and then lysed on ice for 30 min in 500 µl RIPA buffer. The lysates were spun at 9,300 x g for 15 min, then the supernatants were precleared by adding protein A-agarose (20 µl) and incubated for 45 min at 4°C, followed by centrifugation at 9,300 x g for 10 min. The precleared supernatant was centrifuged once more at 100,000 x g at 4°C for 1 h. The supernatant was transferred to a new vial and incubated with anti-aPKC Ab, or anti-NF-κB p65 Ab for 2 h or overnight at 4°C. The immune complexes were incubated with 20 µl of protein A-agarose for 2 h at 4°C with gentle rotation, collected by centrifugation at 9,300 x g for 5 min at 4°C and washed three times with 500 µl cold RIPA buffer by centrifugation at 200 x g for 1 min. The immunoprecipitated complexes were carefully resuspended in 50 µl 5x sample buffer and released by boiling, followed by centrifugation at 9,300 x g for 5 min. The supernatant was transferred to a new vial and mixed with 10 µl bromphenol blue and 15 µl 0.5M DTT.

2.3.11 *In vitro* immunocomplex kinase assay

Kinases have many regulatory functions and play key roles in e.g. signal transduction pathways. The immunocomplex kinase assay is able to determine kinase activity by immunoprecipitating the target kinase adding the kinases´ substrate, a phosphate source, and an adequate buffer. The kinase phosphorylates its substrate by using radioactive phosphorus (^{32}P) incorporated into ATP as a phosphate source. When a substrate is phosphorylated, the radioactivity is transferred to the substrate, and – depending on the amount of incorporated ^{32}P – is indicative for the kinase´s activity. Immunoprecipitated aPKC was incubated in 100 µl kinase assay reaction buffer and 10 µl myelin basic protein (MBP) as substrate at 37°C for 30 min with gentle shaking. The reaction was stopped by adding 50 µl LAEMMLI buffer. Immunocomplexes were released from agarose beads by boiling and centrifugation (16,100 x g, 1 min). The supernatant was transferred to a new vial, and 50 µl of the sample was subjected to 12% SDS-PAGE (180 V, 110 mA, 3 h). The gel was analyzed with a PhosphorImager (Molecular Dynamics) to quantitate band intensities.

2.3.12 Membrane fractionation assay

Rat AM were treated with SP-A (40 µg/ml) for various amounts of time. Incubation was stopped with ice-cold PBS, and the cells were resuspended in 300 µl of hypotonic fractionation buffer 1, incubated for 20 min at 4°C while rotating, and sonicated for 1 min. Total cell lysates were then centrifuged at 100,000 x g for 30 min at 4°C to separate cytosolic from particulate fractions. The cytosolic fraction was transferred to a new vial; the resulting pellet was extracted in 150 µl of hypotonic fractionation buffer 2, and centrifuged at 16,000 x g for 20 min at 4°C. The resulting supernatant was taken to represent the membrane fraction. Equal amounts of membrane protein for each sample (30-50 µg) were separated by SDS-PAGE (12%) and blotted with anti-aPKC Ab.

2.3.13 DNA labeling

NF-κB oligonucleotides (50 ng) were incubated with 10 x kinase buffer (Roche), [γ-^{32}P] ATP (8 pmol), and 10 U of T4 polynucleotide kinase (Roche) at 37°C for 1 h. The reaction was stopped with 50 µl TE buffer, and applied to a NICK-column (Amersham), followed by the addition of 400 µl TE buffer. The first flow-through was discarded and another 400 µl TE buffer applied to the column. This time the flow-through containing the end-labeled NF-κB oligonucleotides was collected, its cpm (counts per minute) measured and stored at 4°C. The cpm measured ranged from 40,000 to 80,000.

2.3.14 Electrophoretic mobility shift assay (EMSA)

An electrophoretic mobility shift assay (EMSA) is used to study protein-DNA or protein-RNA interactions, and to determine whether a protein or a protein mixture is capable of binding to a given DNA or RNA sequence. The activity of NF-κB in the nuclear extracts was determined by a standard electrophoretic mobility shift assay (EMSA). NF-κB oligonucleotides were end-labeled with [γ-^{32}P] ATP using T4 kinase as described above. Two µg of crude nuclear extract was incubated for 15 min in shift buffer, followed by the incubation of 20 min in binding buffer containing 50 µg of poly (dI/ dC)/ml with 7.5 fmol of the ^{32}P-labeled oligonucleotides encoding the consensus NF-κB site 5'-AGCTCAGAGGGGACTTTCCGAGAGAGC-3'. Samples were separated by electrophoresis on 5% polyacrylamid gels for 2 h at 180 V, after which gels were analyzed with a PhosphorImager.

2.3.15 Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay is a widely-used immunological technique based on the specific antigen-antibody interaction. The amount of a specific antigen in solution is quantitatively determined by a chromogenic conversion that can be achieved either by an enzyme-linked antigen (competitive ELISA), or an additional antibody that is coupled to an enzyme and detects the same antigen (sandwich ELISA). The chromogenic conversion is achieved by addition of the enzyme´s substrate. TNF-α was determined in pooled cell-free supernatants of stimulated cells by sandwich DuoSet ELISA (R&D Systems, Wiesbaden, Germany) using goat anti-mouse TNF-α Ab and biotinylated goat anti-mouse TNF-α Ab. The TNF-α ELISA was performed in the lab of PD Dr. Andra Schromm according to the manufacturer's protocol.

2.3.16 Confocal microscopy

The confocal microscopy is an optical imaging technique that uses a spatial pinhole to eliminate out-of-focus light or flare in specimens that are thicker than the focal plane, thus improving image quality considerably compared to conventional fluorescence microscopy. In addition, as at one time only one point is illuminated three-dimensional images can be reconstructed by scanning the specimen over parallel scanning lines. Cellular and subcellular structures can be detected by fluorescence-labeled antibodies. Rat AM were plated at a density of 1 x 10^5 cells on 8-well Lab Tek II chamber slides (Nunc). Cells were allowed to attach for 90 min in the presence of 0.2% HI-FCS. After incubation with the indicated stimuli, cells were fixed with 500 µl ice-cold (-20°C) methanol for 2 min, washed three times with PBS, and permeabilized with 500 µl 0.25% Triton X-100 for 8 min. After repeating washings the cells were blocked with 10% bovine serum albumine (BSA)/ PBS for 30 min, and incubated with isoform specifc anti- aPKC Ab, anti-PKCζ Ab, anti-aPKCThr $^{410/403}$, anti-p65 Ab, anti-PKCι Ab, or FITC-labeled SP-A (40 µg/ml), at a 1:250, 1:200, and a 1:50 dilution, respectively. Goat anti-rabbit IgG conjugated to Alexa Fluor 488 or 568, or Rabbit-anti-mouse IgG conjugated to Alexa Fluor 488 or 568 served as secondary Abs. Cell nuclei were counterstained with propidium iodide (PI) or TOTO-3 iodide (Molecular Probes). Samples were analyzed using a Leica TCS SP confocal laser scanning microscope (Leica Microsystems, Bensheim, Germany). Images were acquired with the Leica TCSNT software and assembled using Adobe Photoshop 6.0.

2.3.17 SP-A labeling

Purified SP-A was labeled with fluorescein-5-isothiocyanate isomer I (FITC), using a FluoReporter FITC protein labeling kit (Invitrogen) with a modified manufacturer´s protocol. SP-A was incubated with sodium bicarbonate buffer, pH 9 (100 mM) and FITC for one hour at room temperature. The reaction was stopped by adding hydroxylamine hydrochloride, pH 8.5 (300 mM) and dialyzed over night in a QuixSep Micro Dialyzer (Roth, Karlsruhe, Germany) against 1x TBW to remove unbound FITC.

2.3.18 Coating of microfuge tubes

Experiments employing clathrin or actin inhibitors were performed in pre-coated microfuge tubes due to the detaching effect of the inhibitors on the AM. Autoclaved microfuge tubes were filled with 1 % BSA/ PBS and incubated overnight at 4°C. BSA/ PBS was removed and centrifuged at 16,300 x g for 1 min. Tubes were washed twice with PBS by centrifugation at 16,300 x g for 1 min. After the last washing, the supernatant was removed and the tubes again centrifuged in order to remove all fluids from the tubes. The pre-coated microfuge tubes were stored at 4°C.

2.3.19 Fluorescence-activated cell sorting (FACS) analysis

Cell sorter in general can isolate and sort single cells. The fluorescence-activated cell sorting is a specialized form of flow cytometry as it provides sorting of single cells due to their specific light scattering and fluorescent characteristics. It is a fast, objective and quantitative technique to record fluorescent signals from individual cells, as well as isolate cells of particular interest. Freshly isolated rat AM were suspended in RPMI medium to a final concentration of 1 x 10^6 cells/ 500 µl, divided into BSA-pre-coated 1.5 ml microfuge tubes, and incubated in the presence of 0.2 % HI-FCS and in the absence or presence of clathrin and β-actin inhibitors prior to the addition of FITC-labeled SP-A with gentle rotation at 37°C. After stimulation cells were centrifuged at 200 *g* for 5 min, at 4°C. The supernatant was discarded, and the cells were washed with PBS without $CaCl_2$ and $MgCl_2$ by centrifugation (200 *g*, 5 min, 4°C). Cells were then transferred to a new pre-coated vial and washed twice more with PBS. The pellet was resuspended in 100 µl FACS buffer (PBS containing 1 % formaldehyde) and transferred to a FACS tube. To exclude dead cells, 1 ml propidium iodide (1 µg/ ml) was added and immediately centrifuged at 290 *g*, 10 min, 4°C. The supernatant was discarded and the pellet resuspended in 100 µl FACS-buffer. Samples

(20,000 cells/ treatment) were analyzed for relative fluorescence per cell at 514 nm after excitation at 488 nm.

2.3.20 Statistics

Data were analyzed by paired or unpaired student´s *t*-test, one-way analysis of variance (ANOVA) with a post-hoc Newman-Keuls test or Dunnett´s post-test, and two-way ANOVA, as indicated in the figure legends. Values were considered significant when $p < 0.05$. Data were presented as mean ± standard error (SEM).

3 RESULTS

3.1 PART I: SP-A-induced activation of atypical protein kinase C ζ in IκB-α-dependent anti-inflammatory immune regulation

3.1.1 Purification and activity of isolated SP-A

To investigate the modulatory effects of SP-A on the IκB-α/ NF-κB pathway, SP-A was purified from bronchoalveolar lavage fluid of patients suffering from alveolar proteinosis (AP) as described in the "Materials and Methods" section (page 50). The puritiy and protein concentration of isolated SP-A was assessed by SDS-PAGE with subsequent coomassie staining and BCA assay, respectively. SP-A preparations were further tested for the presence of bacterial endotoxin using a *Limulus* amebocyte lysate (LAL) assay. All SP-A preparations used contained < 0.2 pg endotoxin / µg SP-A.

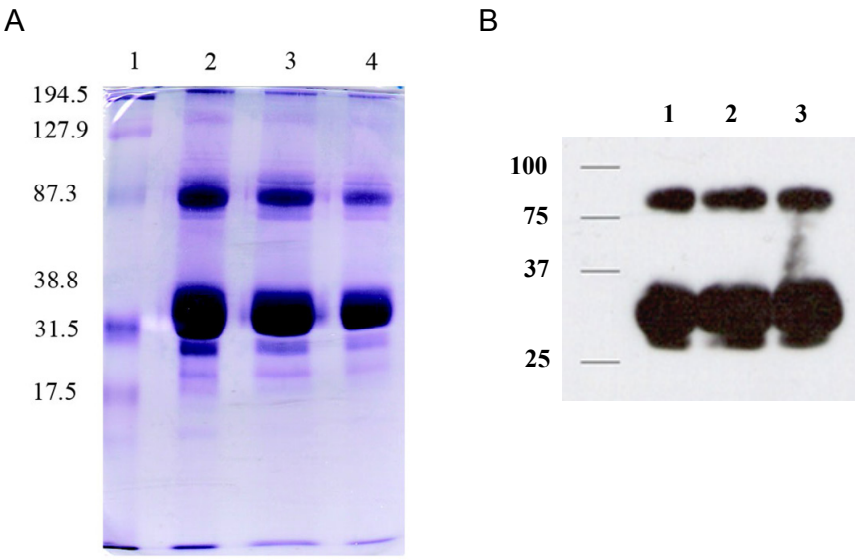


Figure 3.4: Coomassie-stained SDS-PAGE and Western blot after SP-A purification. (A) Coomassie-stained SDS-PAGE: lane 1: protein standard; lane 2: SP-A control (40 µg/ ml); lane 3: purified SP-A (40 µg/ ml); lane 4; purified SP-A (20 µg/ ml). SP-A exhibits multiple bands under denaturing conditions. (B) Western blot analysis using an anti-SP-A Ab: lane 1: SP-A control (20 µg/ ml); lane 2: FITC-labeled SP-A (20 µg/ ml); lane 3: purified SP-A (20 µg/ ml).

It has been shown that SP-A has no NF-κB activation potency but inhibits LPS-induced NF-κB activity and increases IκB-α protein expression alone and in the presence of either

rough (r) or smooth (s) LPS (Wu et al., 2004). Therefore and to exclude undetected endotoxin contamination by the LAL assay, due to SP-A-LPS interaction (Cooper, 1990), the activity of isolated SP-A was tested in an electrophoretic mobility shift assay (EMSA) and in Western blot analysis prior to its use in the following experiments.

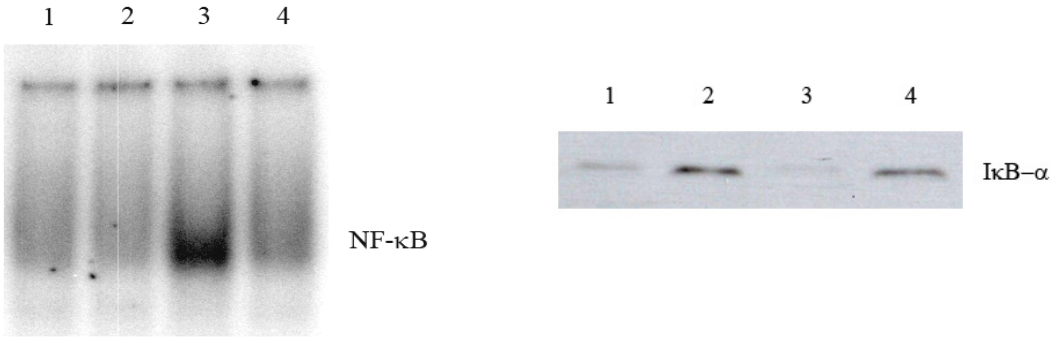


Figure 3.2: EMSA and Western analysis of purified SP-A. Freshly isolated AM were left untreated (lane 1) or were treated with SP-A (lane 2: 40 µg/ ml, 1 h), sLPS (lane 3: 100 ng/ ml, 1 h) or both (lane 4). SP-A inhibits LPS-induced NF-κB activity as shown in a NF-κB activation assay (left panel), and increases IκB-α protein expression in the presence of LPS (right panel).

Freshly isolated alveolar macrophages (AM) were left untreated or were treated with SP-A (lane 2: 40 µg/ ml, 1 h), sLPS (lane 3: 100 ng/ ml, 1 h), or with SP-A prior to the addition of LPS (lane 4). SP-A alone does not induce NF-κB activation, instead it further reduces basal NF-κB activation levels (lane 2, left panel). SP-A inhibits LPS-induced NF-κB activity (lane 4, left panel), confirming previous findings on SP-A´s anti-inflammatory modulation of LPS-induced NF-κB activity. IκB-α protein expression on the other hand is increased in the presence of SP-A (lane 2, right panel) compared to resting cells (lane 1, right panel). Furthermore, SP-A enhances IκB-α protein expression in the presence of LPS, matching the anti-inflammatory properties of SP-A regarding its inhibitory effect on LPS-induced NF-κB activation. After confirming SP-A´s anti-inflammatory activity, it was used in the following experiments.

3.1.2 SP-A fails to inhibit LPS-induced TNF-α release by IκB-α knockout/ IκB-β knockin (AKBI) cells

To confirm previous findings on the role of IκB-α in the anti-inflammatory activity of SP-A, IκB-α-deficient mice were obtained and primary immune cells from those mice were isolated. While IκB-α-deficient mice are postnatal lethal (Beg et al., 1995), an IκB-α knockout/IκB-β knockin (AKBI) mice was generated, in which the integrated IκB-β gene is under the control of the IκB-α promoter, and at the same time a null mutation is introduced into the IκB-α gene (Cheng et al., 1998). AKBI mice have a normal phenotype, and NF-κB induction by PMA or TNF-α in AKBI mouse fibroblasts and thymocytes is identical compared to wt cells (Cheng et al., 1998). Fan and co-workers showed that AKBI mice reveal a functional redundancy of IκB-α and IκB-β upon LPS challenge *in vivo* supporting the findings of Cheng et al. (Fan et al., 2004).

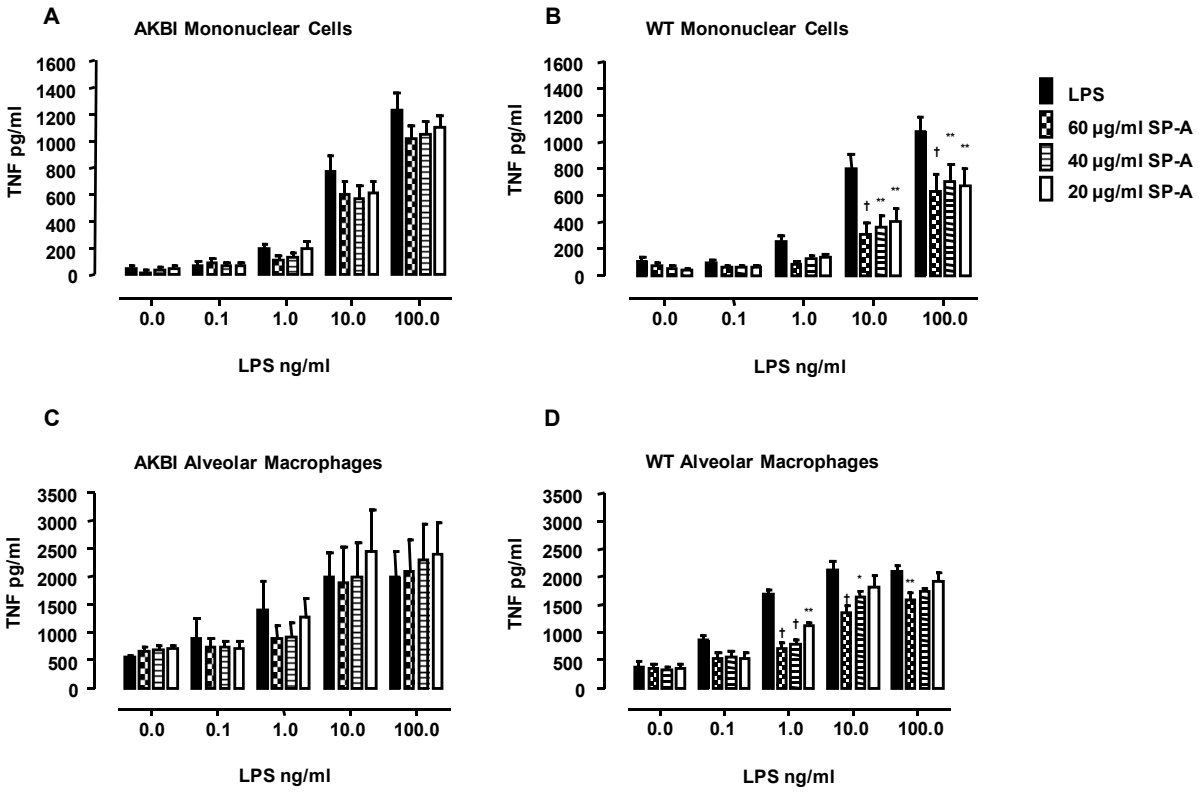


Figure 3.3: SP-A fails to inhibit LPS-induced activation of IκB-α knockout/IκB-β knockin (AKBI) cells. Pooled PBMC or alveolar macrophages (AM) from either three to four AKBI (A, C) or three wt mice (B, D) were left untreated or treated with 20-60 µg/ml SP-A (37°C, 1 h), and were then exposed either to media, or to 0.1-100 ng/ml LPS (37°C, 1 h). Cell-free supernatants were harvested after 4 h for the determination of TNF-α by ELISA. The data shown are mean ± SE of three

independent experiments. Statistical analysis was performed using a two-way ANOVA with a Bonferroni post-test. * $p < 0.05$, ** $p < 0.01$, † $p < 0.001$ (versus LPS-induced TNF-α release in the absence of SP-A).

AM isolated by BAL, and peripheral blood mononuclear cells (PBMC) obtained by cardiac puncture from either three or four AKBI and respective wild type (wt) mice, were left untreated or were treated with various amounts of SP-A (20-60 µg/ ml, 1 h) prior to the addition of LPS (0.1-100 ng/ ml, 1 h). Cells were harvested after four hours of incubation, and the supernatant was used for the determination of TNF-α release by ELISA. PBMC (Figure 3.3A) and AM (Figure 3.3C) from AKBI mice released similar amounts of TNF-α in response to the various amounts of LPS (0.1-100 ng/ ml) compared to wt cells (Figure 3.3B and D) confirming previous findings by Fan and colleagues. Pretreatment of the cells with different concentrations of SP-A did not alter the levels of TNF-α release. Wild type PBMC and AM released comparable amounts of LPS-induced TNF-α in a dose-dependent manner. In both cell types SP-A (20-60 µg/ ml) significantly (*$p < 0.05$ to †$p < 0.001$) and in a concentration-dependent manner inhibited LPS-induced TNF-α release (Figure 3.3B and D), whereas SP-A failed to inhibit LPS-induced TNF-α release by PBMC (Figure 3.3A) or AM (Figure 3.3C) from AKBI mice. These data confirm that SP-A-mediated inhibition of LPS-induced TNF-α production in PBMC and AM critically depends on the presence of IκB-α, and that IκB-β could not compensate for the lack of IκB-α under these conditions.

3.1.3 SP-A fails to inhibit LPS-induced p65 nuclear translocation in AKBI alveolar macrophages

LPS-induced TNF-α release is preceded by p65 nuclear translocation and NF-κB activation (Ghosh & Karin, 2002). To determine whether TNF-α release is associated with an increase in p65 nuclear translocation, the effect of SP-A on LPS-induced p65 localization in AKBI and wt AM was investigated by confocal microscopy. AM of AKBI and wt mice were isolated by BAL, seeded into 8-well Chamber slides at a density of 1×10^5, and allowed to adhere at 37°C in the presence of 0.2 % HI-FCS in a 5 % CO_2 atmosphere. Cells were left untreated (Figure 3.4 a, e) or treated with SP-A (40 µg/ ml, 1 h; Figure 3.4 b, f), LPS (100 ng/ ml, 1 h; Figure 3.4 c, g) or pretreated with SP-A prior to the addition of LPS (Figure 3.4 d, h). After stimulation the cells were further treated as described in “Materials and Methods”. An Ab against NF-κB p65 was detected by goat-anti-rabbit IgG conjugated to Alexa Fluor 488.

Cell nuclei were counterstained with propidium iodide (PI). In wt AM reasonable amounts of p65 were present in resting cells and in cells treated with SP-A (Figure 3.4A a, b). Interestingly, treatment of AM with SP-A resulted in an accumulation of p65 around the plasma membrane. Wild type AM stimulated with LPS exhibited an increased p65 nuclear as well as cytosolic accumulation (Figure 3.4A c) that was almost completely prevented (60 ± 5 %) by pretreatment of the cells with SP-A (Figure 3.4A d, 4B). In contrast, LPS-induced p65 nuclear accumulation in AKBI AM (Figure 3.4A g) could not be inhibited by SP-A (Figure 3.4A h, 3.4B). As expected, comparable amounts of nuclear p65 are seen under resting or SP-A conditions in wt and AKBI AM. Together the data suggest that SP-A-mediated inhibition of LPS-induced p65 nuclear translocation critically depends on the presence of IκB-α. Nuclear p65 was statistically analyzed from an average of 120 counted cells per independent experiment by measuring the pixel densitometry of NF-κB p65 in the nucleus (Figure 3.4B).

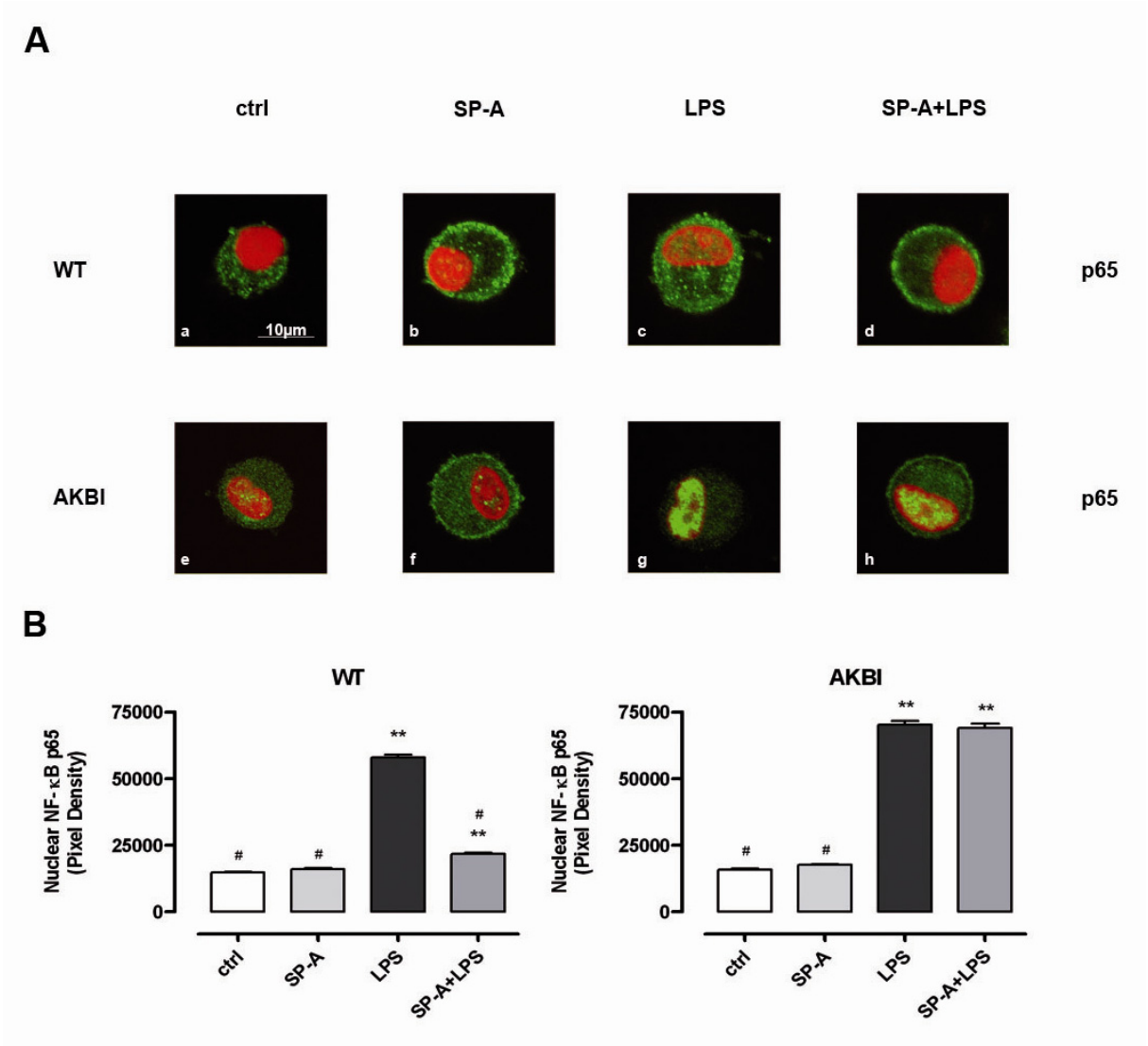


Figure 3.4: SP-A fails to inhibit LPS-induced p65 nuclear translocation in AKBI AM. (**A**) AM from wild type (a-d) or AKBI (e-h) mice were left untreated or treated with 40 µg/ml SP-A (37°C, 1 h), exposed to media, or to 100 ng/ml LPS (1 h), and then analyzed by confocal microscopy. An Ab against NF-κB p65 was detected by goat-anti-rabbit IgG Ab conjugated to Alexa Fluor 488 (green), cell nuclei were counterstained with propidium iodide (PI; red). Overlays of single stainings are shown. Images are representative of three independent experiments. (B) Pixel densitometry of nuclear NF-κB p65 was quantified and statistically analyzed with one-way ANOVA and Newman-Keuls post-hoc test. ** $p < 0.001$ (versus control), $^{\#}p < 0.001$ (versus LPS).

3.1.4 An atypical PKC is involved in IκB-α stabilization

In order to elucidate the underlying molecular mechanisms of SP-A-mediated immune protection, the focus was laid on identifying upstream regulators involved in the stabilization of IκB-α by SP-A. Since members of the PKC family have been shown to be involved in

IκB-α turnover (Bren et al., 2000) and SP-A immune functions (Schagat et al., 1999), a panel of PKC inhibitors was used with different specificity to test the role of various PKC in SP-A-mediated IκB-α stabilization in primary rat AM. AM were isolated by BAL and experiments were performed under four different conditions: i) distinct inhibitors alone (basal, Figure 3.5A), ii) inhibitors plus SP-A (40 µg/ ml) (constitutive, Figure 3.5B), iii) inhibitors plus LPS (10 ng/ ml) (induced, Figure 3.5C), and iv) inhibitors plus SP-A (40 µg/ ml) followed by the addition of LPS (10 ng/ ml) (modulated, Figure 3.5D). The inhibitors used included: Gö-6976, that specifically inhibits conventional (c) PKC isoforms (α, β_I, β_{II}, γ) which are diacylglycerol (DAG)-sensitive and Ca^{2+}-responsive; Gö-6850 (Bisindolylmaleimide), which inhibits the cPKCs α, and γ as well as the DAG-sensitive but Ca^{2+}-insensitive novel (n) PKC isoforms δ and ε; chelerythrine chloride (CC) an inhibitor of Ca^{2+}- and DAG-dependent and -independent PKCs, including the DAG-insensitive atypical (a) isoforms ζ and λ/ι. Since both SP-A and LPS have been shown to activate PI3K in human macrophages (Beharka et al., 2005) and in THP-1 cells (Guha & Mackman, 2002), respectively, the PI3 kinase inhibitor wortmannin was utilized. Apigenin, a selective CKII inhibitor was employed due to the fact that in mouse embryonic fibroblasts CKII has been shown to be critically involved in the basal turnover of IκB-α (Pando & Verma, 2000). After stimulation nuclear and cytosolic fractions were extracted as described in "Materials and Methods". The protein content of the cytosolic fractions was determined by BC assay, and the samples were further subjected to SDS-PAGE. IκB-α protein expression was determined by Western analysis.

Neither of the inhibitors used had a significant effect on IκB-α protein expression under basal conditions (Figure 3.5A). In line with previous data (Wu et al., 2004), SP-A significantly increased IκB-α protein expression by 210 ± 44 % ($p < 0.05$) under constitutive conditions (Figure 3.5B). The effect of SP-A on IκB-α stabilization was still significant (149 ± 12 % of control) in the presence of Gö-6976 and Gö6850, whereas pretreatment with CC, wortmannin and apigenin abrogated SP-A´s stabilizing effect on IκB-α protein expression (Figure 3.5B). IκB-α protein expression in the presence of LPS was significantly reduced by pretreatment of AM with wortmannin (Figure 3.5C), supporting a previous report by Guha & Mackman (Guha & Mackman, 2002). SP-A significantly enhanced IκB-α protein expression in the presence of LPS by 137 ± 10 % ($p < 0.05$) (Figure 3.5D). Whereas treatment of AM with

Gö-6976 and Gö-6850 did not inhibit the SP-A-mediated stabilization of IκB-α (Figure 3.5B) it inhibited SP-A's effect in the presence of LPS (Figure 3.5D). In sharp contrast, CC treatment of the cells abolished the stabilizing effect of SP-A on IκB-α both constitutively and in the presence of LPS suggesting that aPKCs might be involved in SP-A´s effects (Figure 3.5B and D). Wortmannin abrogated SP-A's effect on IκB-α stabilization both constitutively and in the presence of LPS (Figure 3.5B and D). Apigenin, a selective CKII inhibitor, also inhibited SP-A-mediated IκB-α stabilization constitutively and in the presence of LPS (Figure 3.5B and D).

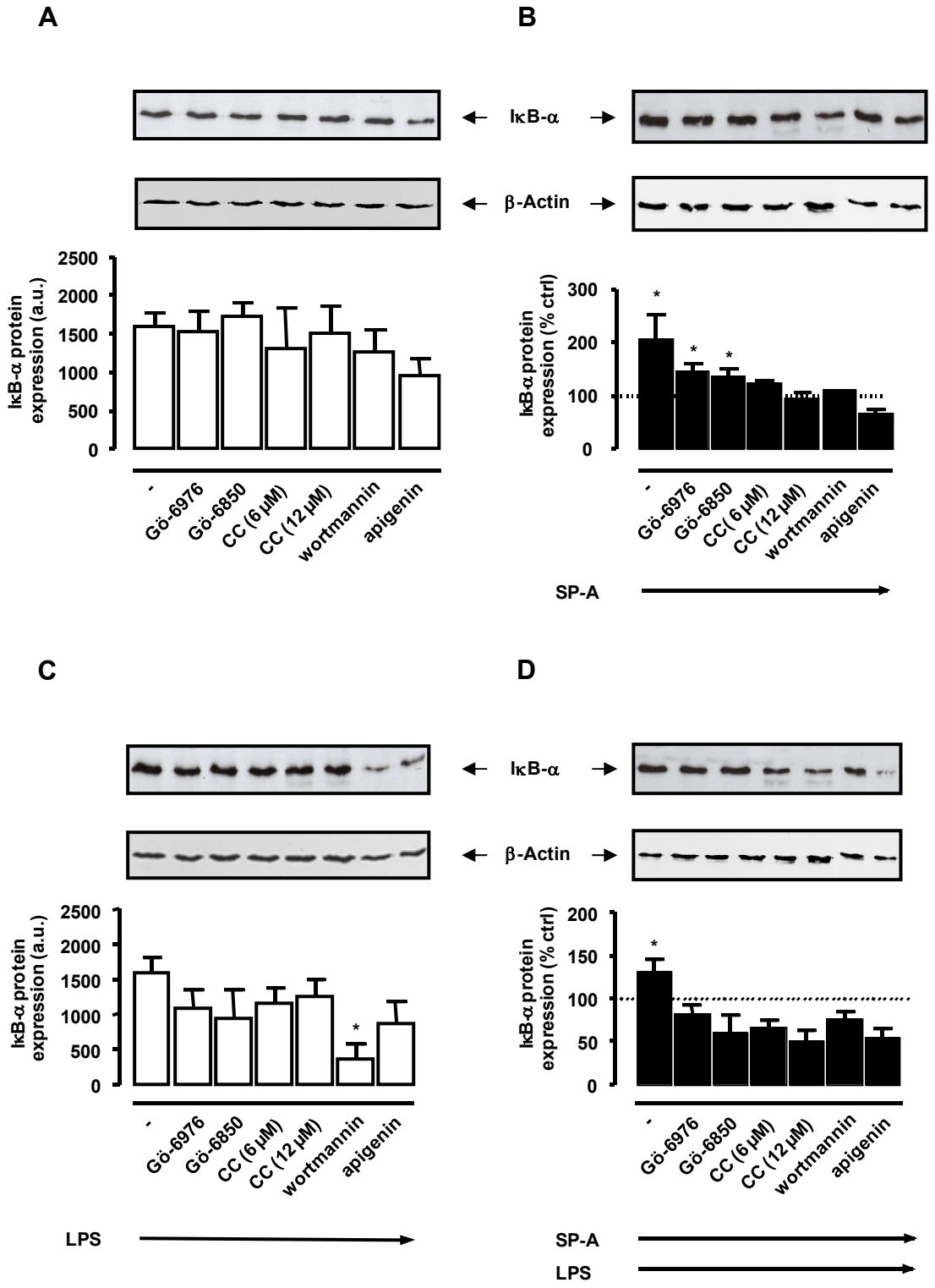


Figure 3.5: An atypical PKC is involved in IκB-α stabilization. (**A-D**) Representative cytosolic IκB-α and β-actin Western blots. (**A**) Primary rat AM were left untreated or treated with 5 µM Gö-6976, 5 nM Gö-6850, 6 and 12 µM chelerythrine chloride (CC), 50 nM wortmannin (37°C, 30 min), or 30 µM apigenin (37°C, 1 h). Equal amounts (30-40 µg of

protein) of the cytosolic fractions were subjected to SDS-PAGE and immunoblotted for IκB-α or β-actin (A-D). Analysis of band intensities was performed using Optimas software (Media Cybernetics, Silver Spring, MD). Data are expressed as arbitrary units (a.u.) (mean ± SE) from four to five independent experiments. (**B**) AM were left untreated or preincubated (37°C, 30 or 60 min) with the inhibitors as in (**A**) and were then exposed to 40 μg/ ml SP-A (1 h). Data are expressed as percentage of IκB-α protein expression in the absence of SP-A (dotted line, 100%) (mean ± SE) from four to five independent experiments. (**C**) AM were left untreated or treated with the inhibitors as in (**A**) and were then exposed to 10 ng/ ml LPS (30 min). Data are expressed as a.u. (mean ± SE) from four to five independent experiments. (**D**) AM were left untreated or treated with SP-A as in (**B**) and were then exposed to 10 ng/ml LPS (30 min). Data are expressed as percentage of IκB-α protein expression in the absence of SP-A (dotted line, 100%) (mean ± SE) from four to five independent experiments. Data in Figure 3.5A and C were analyzed by one-way ANOVA followed by Newman-Keuls post hoc-test; data in Figure 3.5B and D were analyzed by a paired Student's *t*-test when expressed as percentage of the control response. * $p < 0.05$ (versus IκB-α expression in the absence of SP-A).

Since among aPKCs, PKCζ has been shown to modulate IκB-α turnover (Bren et al., 2000) and IKK/ NF-κB activation in both nuclear and whole lung extracts (Leitges et al., 2001), the role of aPKC isoforms in SP-A's immunomodulation of AM was investigated in detail. An isoform-specific cell-permeable inhibitory myristoylated peptide derived from the pseudosubstrate (ps) motif of aPKCs was used to silence the activity of aPKCs, as the aPKC pseudosubstrate mimics the substrate and maintains aPKC in its nonactive form. Of note, the ps peptides are not specific for either isoform, since the ps sequences (SIYRRGARRWRKL) are identical in both isoforms PKCζ and PKCλ/ι (Standaert et al., 1997).

3.1.5 Atypical PKC inhibition suppresses SP-A-mediated IκB-α stabilization

AM were isolated by BAL and experiments were performed under four different conditions: i) aPKCps alone (basal, Figure 3.6A), ii) aPKCps plus SP-A (40 μg/ ml) (constitutive, Figure 3.6B), iii) aPKCps plus LPS (10 ng/ ml) (induced, Figure 3.6C), and iv) aPKCps plus SP-A (40 μg/ ml) followed by the addition of LPS (10 ng/ ml) (modulated, Figure 3.6D). After treatment, nuclear and cytosolic fractions were extracted as described in "Materials and Methods". The protein content of the cytosolic fractions was determined by BC assay, and the samples were further subjected to SDS-PAGE. IκB-α protein expression was determined by Western analysis. Treatment of the cells with aPKCps (2-10 μM) did not significantly affect IκB-α protein level under basal (Figure 3.6A) or LPS (Figure 3.6C) conditions. Compared to basal conditions, SP-A significantly increased IκB-α protein expression both constitutively (Figure 3.6B) and in the presence of LPS (Figure 3.6D). After

stimulation nuclear and cytosolic fractions were extracted as described in "Materials and Methods". The protein content of the cytosolic fractions was determined by BC assay, and the samples were further subjected to SDS-PAGE. IκB-α protein expression was determined by Western analysis. Treatment of the cells with aPKCps at 2 μM, 5 μM and 10 μM decreased basal IκB-α protein levels in a concentration-dependent manner (Figure 3.6A). Pretreatment of AM with aPKCps at 2 μM had no significant effect on SP-A-mediated IκB-α stabilization. However, pretreatment with 10 μM resulted in a significant inhibition of IκB-α by SP-A (Figure 3.6B). aPKCps treatment of AM prior to the addition of LPS did not alter IκB-α protein expression at any concentration (Figure 3.6C). Interestingly, in the presence of LPS, aPKCps pretreatment at any concentration resulted in a significant inhibition ($p < 0.02$) of IκB-α protein levels by SP-A (Figure 3.6D) when compared to the corresponding aPKCps concentration in the absence of SP-A. Taken together, these results suggest that an aPKC isoform is involved in SP-A-mediated IκB-α stabilization both constitutively and in the presence of LPS.

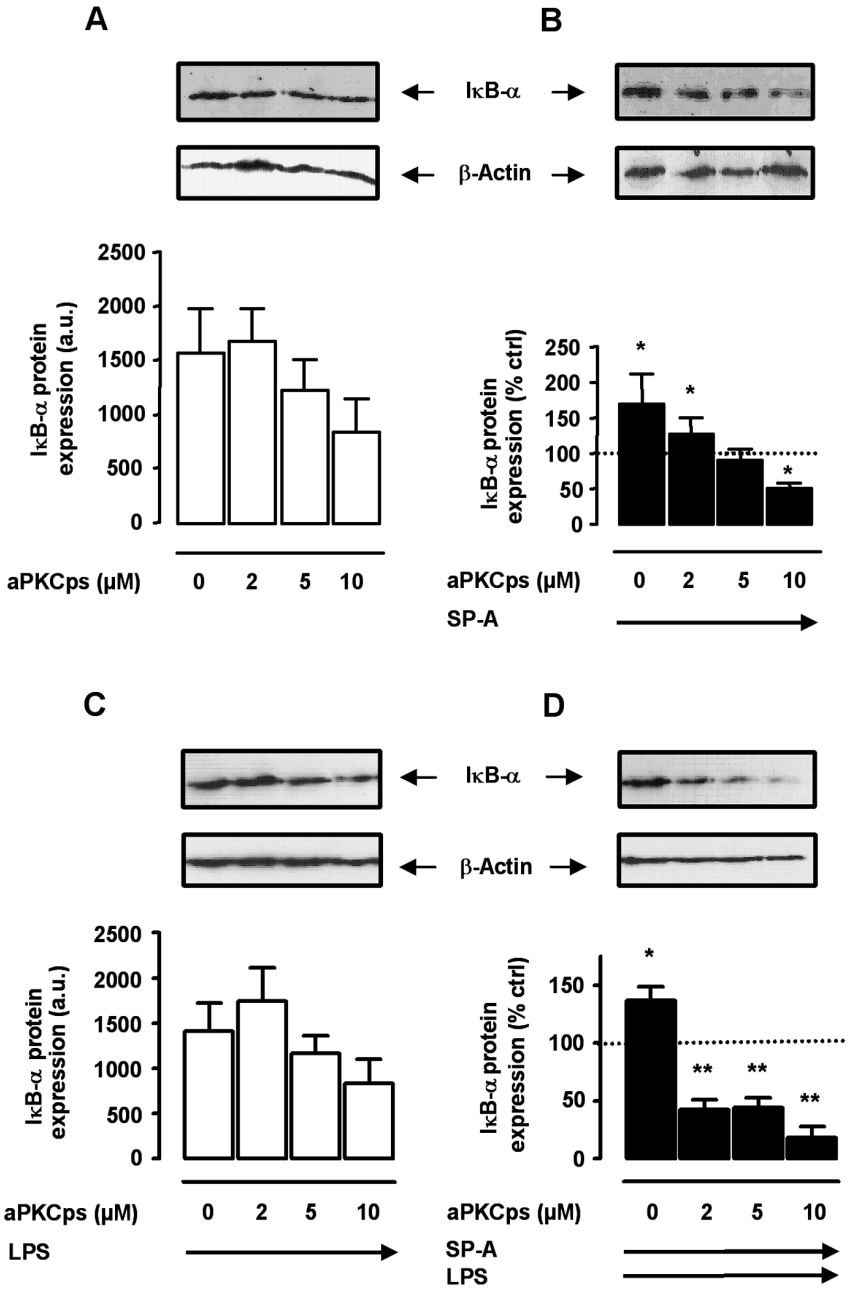


Figure 3.6: Atypical PKC inhibition suppresses SP-A-mediated IκB-α stabilization. (**A-D**) Representative cytosolic IκB-α and β-actin Western blots. (**A**) Primary rat AM were left untreated or treated with 2-10 μM atypical PKC pseudosubstrate (ps) (37°C, 1 h) (control). Cytosolic cell fractions were immunoblotted for IκB-α or β-actin. Densitometric results are in arbitrary units (a.u.) (mean ± SE) from five independent experiments. (**B**) AM were left untreated or treated as in (**A**) and were then exposed to 40 μg/ ml SP-A (1 h). Densitometric results are expressed as percentage of IκB-α protein expression in the absence of SP-A (control, dotted line) (mean ± SE) from five independent experiments. (**C**) AM were left untreated or treated as in (**A**) and were then exposed to 10 ng/ ml LPS (30 min). Densitometric results are in arbitrary units

(a.u.) (mean ± SE) from five independent experiments. (**D**) AM were left untreated or treated with SP-A as in (**B**) and were then exposed to 10 ng/ ml LPS (30 min). Data are expressed as percentage of IκB-α protein expression in the absence of SP-A (control, dotted line) (mean ± SE) from five independent experiments. Data in Figure 3.6A and C were analyzed by one-way ANOVA followed by Newman-Keuls post-hoc test; data in Figure 3.6B and D were analyzed by a paired Student's *t*-test when expressed as percentage of the control response. * $p < 0.05$, ** $p < 0.02$ (versus control).

3.1.6 SP-A stimulates aPKCThr$^{410/403}$ phosphorylation and kinase activity

To investigate whether SP-A can stimulate aPKC activation, the phosphorylation status of aPKC was determined. To activate aPKC, phosphorylation of the activation loop consensus threonine residue Thr^{410} by PI3K-dependent PDK1 is substantial (LeGood et al., 1998). AM were treated with SP-A (20 µg/ ml) for different time points. Cells were then lysed and aPKC – or ctrl IgG – was immunoprecipitated from whole cell lysates. Anti-rabbit IgG was utilized as a control to show specificity for aPKC. Samples from each time point were subjected to SDS-PAGE. The presence of aPKCThr$^{410/403}$ was detected by Western analysis with the corresponding Ab, whereas an anti-aPKC Ab was used to evaluate aPKC loads. Treatment of AM with SP-A significantly increased aPKCThr$^{410/403}$ phosphorylation after 1-10 min (Figure 3.7A) of incubation and then declined to baseline (Figure 3.7B). The specificity for aPKC was shown by the absence of aPKCThr$^{410/403}$ when ctrl IgG had been immunoprecipitated from whole cell lysates.

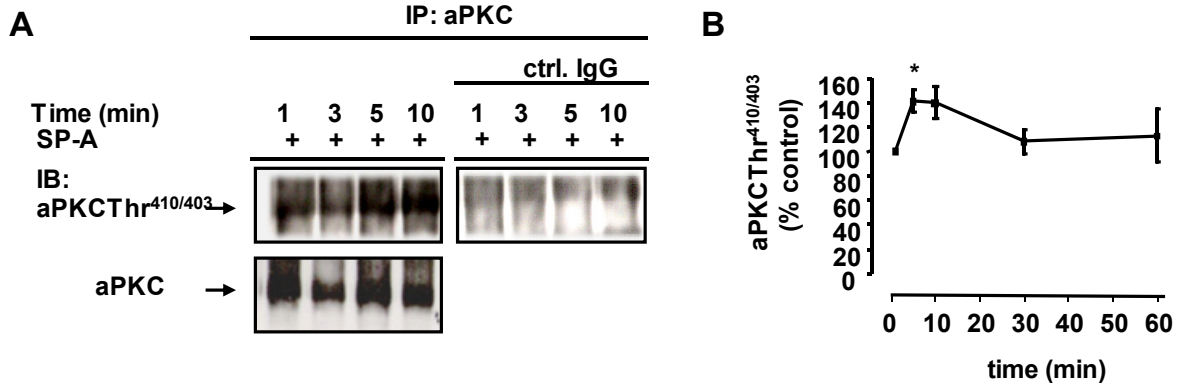


Figure 3.7: SP-A stimulates aPKCThr$^{410/403}$ phosphorylation. (**A**) AM were incubated with 20 µg/ ml SP-A at 37°C for 1, 3, 5, or 10 min. Atypical PKC was immunoprecipitated from whole cell lysates. The presence of aPKCThr$^{410/403}$ was detected by immunoblotting with the corresponding Ab. Anti-aPKC Ab was used to evaluate the aPKC loads. Anti-rabbit IgG Ab was used as a control to show specificity for aPKC. Blots are representative of three independent experiments. (**B**) AM were treated with 20 µg/ ml SP-A for 0, 5, 10, 30, or 60 min. Atypical PKC was immunoprecipitated from whole cell lysates. The presence of aPKCThr$^{410/403}$ was detected by immunoblotting with the corresponding Ab. Densitometric results (mean ± SE) are given as percentage of the zero time point (control) from four independent experiments.

Phosphorylation at Thr^{410} in the activation loop of aPKC precedes the kinases´ activation. It was then investigated whether the observed SP-A-mediated increase in aPKC$Thr^{410/403}$ phosphorylation correlates with an increased kinase activity. AM were left untreated for different time points, or were treated with SP-A (20 µg/ ml) for the same time points. Cells were then lysed and aPKC was immunoprecipitated from whole cell lysates and an *in vitro* immunocomplex kinase assay was performed as described in "Materials and Methods". In separate experiments complement component C1q (20 µg, 1 h) was used as a control due to its structural homology to SP-A. Untreated cells did not exhibit aPKC kinase activity, while treatment with SP-A – but not C1q (Figure 3.8B) – stimulates aPKC kinase activity in a time-dependent manner, reaching a maximum at 1 h ($p < 0.05$) (Figure 3.8A and B).

To show specificity of SP-A-induced aPKC activity, AM were left untreated, were treated with SP-A (40 µg/ ml, 1 h), LPS (100 ng/ ml, 1 h), or were pretreated with aPKCps prior to the addition of SP-A (Figure 3.8C). Cells were then lysed and aPKC was immunoprecipitated from whole cell lysates, followed by an *in vitro* immunocomplex kinase assay. SP-A-induced aPKC activity (Figure 3.8C, lane 2) was comparable to that induced by LPS (Figure 3.8C, lane 3), used as a positive control (Herrera-Velit, 1997). Pre-incubation of AM with aPKCps peptides (Figure 3.8C, lane 4) abolished the aPKC activity in the presence of SP-A. Together, the results demonstrate that SP-A induces aPKC activation by phosphorylating aPKC in the activation loop at Thr^{410}. The data further support the idea that SP-A mediates IκB-α stabilization in primary AM *via* activation of aPKC.

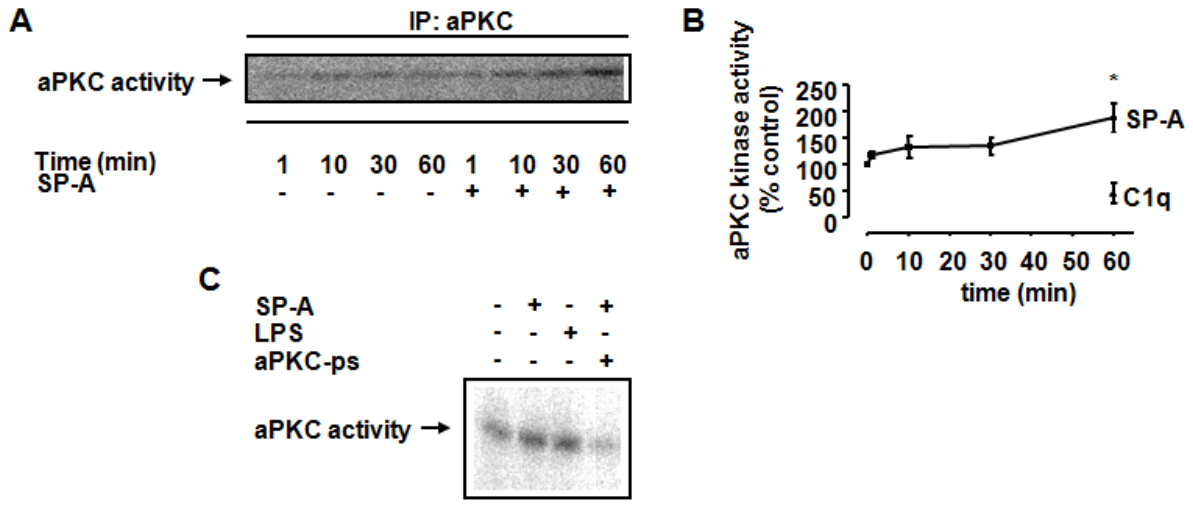
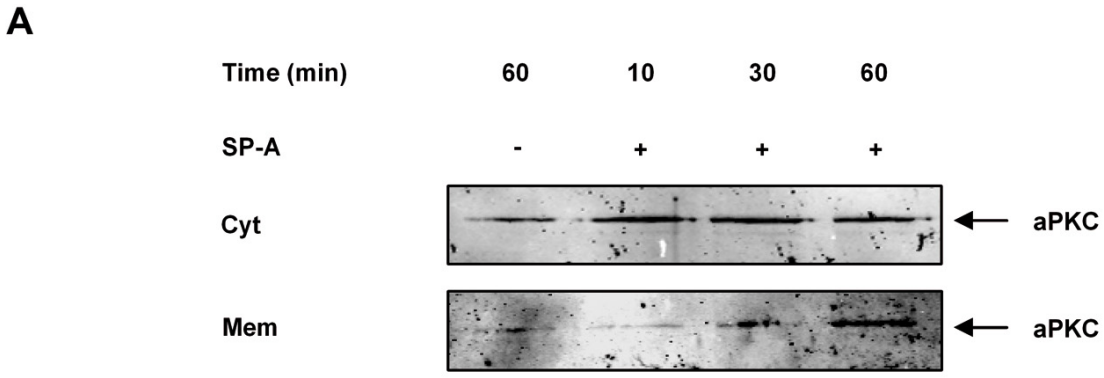


Figure 3.8: SP-A induces kinase activity. (**A**) AM were left untreated or treated with 20 µg/ ml SP-A for the times indicated. Atypical PKC was immunoprecipitated from cell lysates and subjected to an *in vitro* kinase assay using MBP as a substrate. Atypical PKC kinase activity was determined as described in "Materials and Methods". The phosphorylated proteins were run on a 12 % SDS-PAGE gel. (**B**) Densitometrical analysis of four independent experiments. The SP-A structural homologue C1q (20 µg, 1 h) was included as control protein. Data are expressed as percentage of aPKC activity at the corresponding time point (control). (**C**) AM were left untreated (lane 1), or treated with 40 µg/ ml SP-A (lane 2), 100 ng/ ml LPS (lane 3), or pretreated with 10 µM aPKCps peptides prior to the addition of 40 µg/ ml SP-A (lane 4). Cells were subjected to an in *vitro* aPKC kinase assay. The gel is representative of three independent experiments. Data were analyzed by a one-way ANOVA with a Dunnett's post-hoc test, or with paired Student's *t*-test. * $p < 0.05$ (versus control).

Even though phosphorylation at Thr^{410} facilitates kinase activity, it is not sufficient to provide full activity. A rapid autophosphorylation is necessary for a concomitant translocation of PKCζ (Chou et al., 1998; Parekh et al., 2000). Therefore, the effect of SP-A on aPKC translocation was examined by two approaches, i.e. membrane fractionation and confocal microscopy.

3.1.7 SP-A favors the accumulation of PKCζ in the plasma membrane

Freshly isolated AM were left untreated or were incubated with SP-A for 10 min, 30 min, or 60 min. Treatment of the cells was followed by subcellular fractionation using a membrane fractionation assay as described in "Materials and Methods", to obtain cytosolic and membrane extracts. The protein content of both fractions was determined by BC assay, and the samples were further subjected to SDS-PAGE. Atypical PKC protein expression was determined by Western analysis. SP-A increased cytosolic aPKC levels compared to resting cells, and SP-A further stimulated aPKC membrane translocation in a time-dependent manner, reaching a maximum at 1 h (Figure 3.9A).



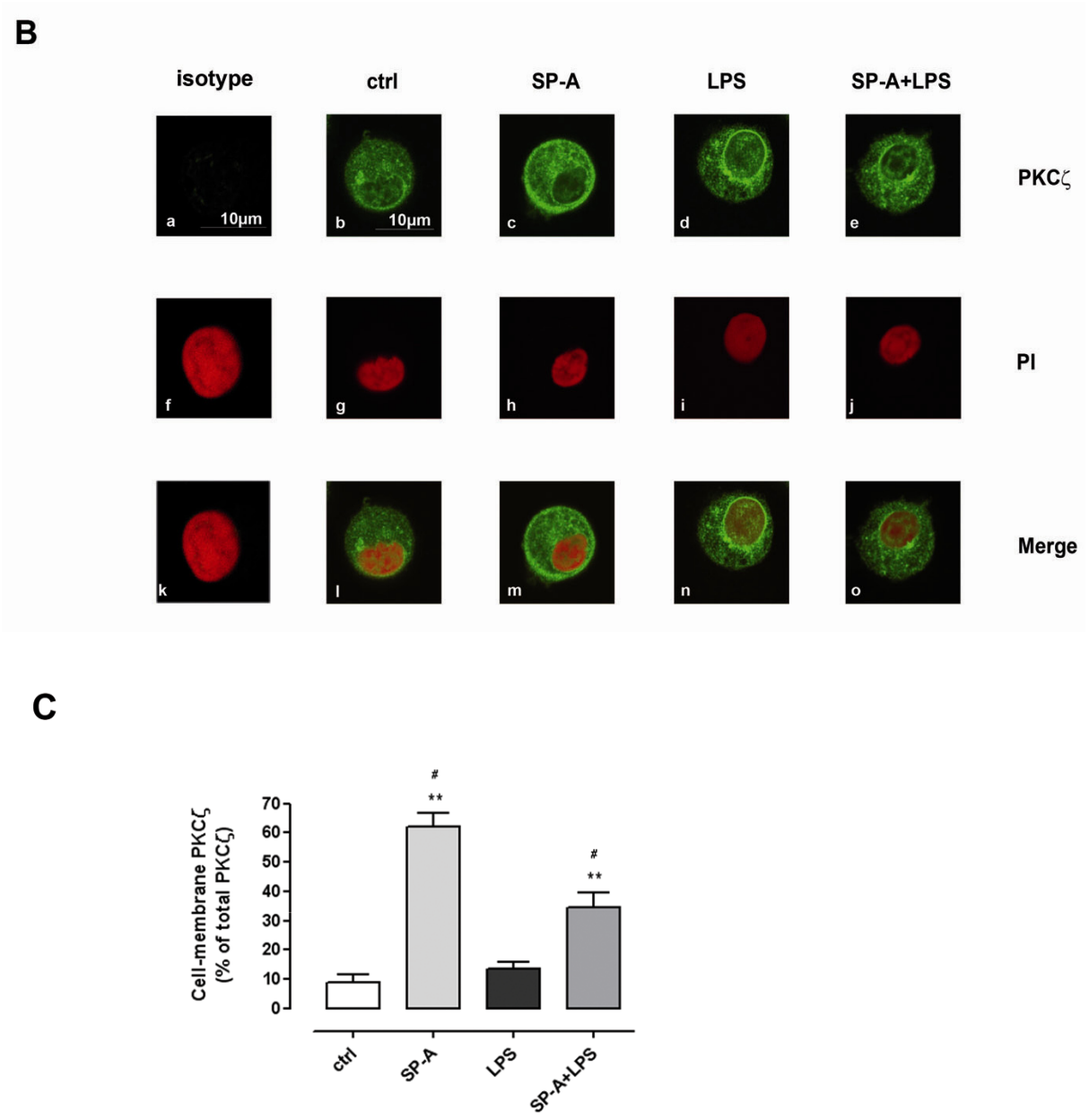


Figure 3.9: SP-A favors PKCζ plasma membrane translocation. (**A**) AM were left untreated or treated with 40 µg/ ml SP-A for the times indicated. Cell membrane isolation was performed as described in "Materials and Methods". Cytosolic and membrane fractions were subjected to SDS-PAGE and immunoblotted for aPKC. Data shown are representative of three experiments. (**B**) AM adhered to Chamber slides at 1 x 10^5 cells/ well and were left untreated and then exposed to media or treated with 40 µg/ml SP-A (1 h), and were then further either exposed to 100 ng/ ml LPS (1 h) , or treated with 100 ng/ ml LPS (1 h) alone. Slides were blocked and incubated with an isotype-specific Ab (control IgG; a), or a specific Ab against PKCζ (b-e) that was detected by goat-anti-rabbit IgG Ab conjugated to Alexa Fluor 488. (f-j) Cell nuclei were counterstained with propidium iodide (PI). Cells were visualized by confocal microscopy. (k-o) Overlay of single stainings. Images shown are representative of three independent experiments with similar results. (**C**) Pixel Density of cell-membrane PKCζ was quantified from total PKCζ and statistically analyzed by one-way ANOVA and Newman-Keuls post-hoc test. ** $p < 0.001$ (versus control), $^{\#}p < 0.001$ (versus LPS).

In a second approach using confocal microscopy, AM were seeded into 8-well Chamber slides at a density of 1 x 10^5, and allowed to adhere at 37°C in the presence of 0.2 % HI-FCS in a 5 % CO_2 atmosphere. Cells were left untreated (Figure 3.9B b) or treated with SP-A (40 µg/ ml, 1 h; Figure 3.9B, c), LPS (100 ng/ ml, 1 h; Figure 3.9B, d) or pretreated with SP-A prior to the addition of LPS (Figure 3.9B, e). After stimulation the cells were further treated as described in "Materials and Methods". An isoform-specific Ab against PKCζ was detected by goat-anti-rabbit IgG conjugated to Alexa Fluor 488. Cell nuclei were counterstained with propidium iodide (PI). An isotype specific Ab (ctrl IgG; Figure 3.9B, a) was used to show specificity for the detection of PKCζ. The confocal images confirmed that SP-A, compared to basal conditions, favors a translocation of PKCζ to the plasma membrane in primary AM, as detected by the isoform-specific Ab (Figure 3.9B, l and m). In contrast, LPS stimulation of AM induced a translocation of PKCζ (Figure 3.9B, n) towards the nucleus. Pretreatment of the cells with SP-A, however, largely reduced LPS-mediated nuclear translocation of PKCζ (Figure 3.9B, o). Membrane-bound PKCζ was statistically analyzed from an average of 100 counted cells per independent experiment by subtracting cell-membrane PKCζ from total PKCζ. SP-A significantly induced a translocation of PKCζ to the membrane, even in the presence of LPS (Figure 3.9C).

Both experimental approaches indicate that SP-A not only induces aPKC phosphorylation and kinase activity but also promotes the concomitant translocation of PKCζ to the membrane.

3.1.8 SP-A modulates p65 localization and p65/PKCζ co-immunoprecipitation

Stimulus-induced PKCζ associates with and phosphorylates *trans*activating p65 (Leitges et al., 2001; Duran et al., 2003). We next asked if SP-A-favored PKCζ membrane localization is associated with the distribution of p65. Therefore, AM were seeded into 8-well Chamber slides at a density of 1 x 10^5, and allowed to adhere at 37°C in the presence of 0.2 % HI-FCS in a 5 % CO_2 atmosphere. Cells were left untreated (Figure 3.10A, b) or treated with SP-A (40 µg/ ml, 1 h; Figure 3.10A, c), LPS (100 ng/ ml, 1 h; Figure 3.10A, d) or pretreated with SP-A prior to the addition of LPS (Figure 3.10A, e). An Ab against NF-κB p65 was detected by goat-anti-rabbit IgG conjugated to Alexa Fluor 488. Cell nuclei were counterstained with propidium iodide (PI). An isotype specific Ab (ctrl IgG; Figure 3.10A, a) was used to show specificity for the detection of NF-κB p65. After stimulation the cells were further treated as described in "Materials and Methods". We confirmed our initial observation

in wt mouse AM (Figure 3.4A) in rat AM (Figure 3.10A) and indeed revealed that p65 distribution strongly parallels that of PKCζ (Figure 3.9B) under both SP-A or LPS conditions (Figure 3.9B, 3.10A, b, c; 3.10B). SP-A significantly induced a p65 translocation towards the cell membrane alone and in the presence of LPS. In addition, as in wt mouse AM, pretreatment of the cells with SP-A almost abolished LPS-induced p65 nuclear translocation and induced an accumulation of p65 at the plasma membrane (Figure 3.10A, e, 3.10B). Membrane-bound NF-κB p65 was statistically analyzed from an average of 100 counted cells per independent experiment by subtracting cell-membrane NF-κB p65 from total NF-κB p65 (Figure 3.10B).

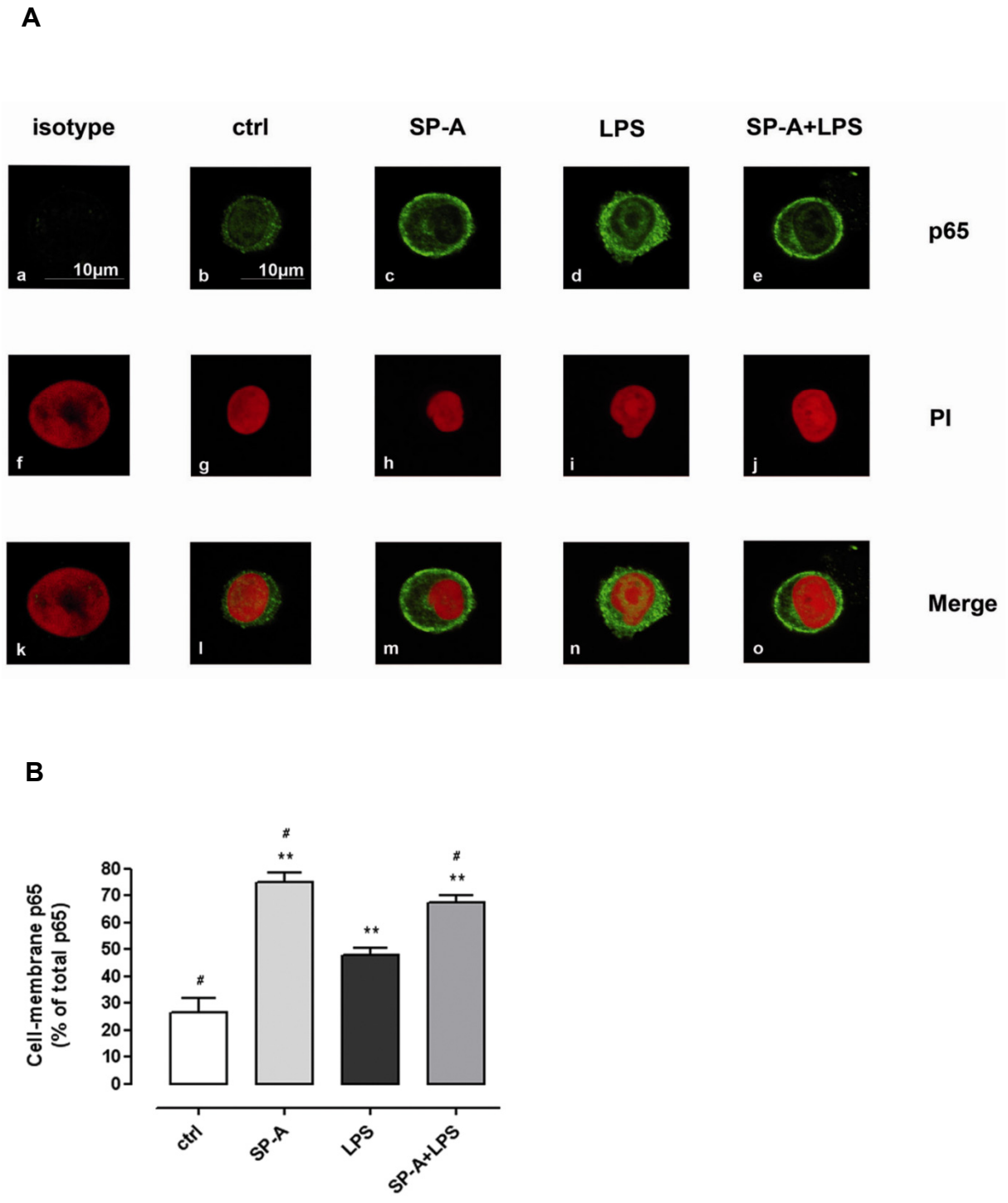


Figure 3.10: SP-A favors p65 plasma membrane translocation. (**A**)AM were left untreated or treated with 40 µg/ml SP-A (1 h), and then exposed to media, or to 100 ng/ml LPS (1 h) and further treated as described for (3.9B). (a-e) Cells were incubated with an isotype-specific Ab (control IgG; a), or an Ab against NF-κB p65 (b-e) that was detected by goat-anti-rabbit IgG Ab conjugated to Alexa Fluor 488. (f-j) Cell nuclei were counterstained with propidium iodide (PI), (k-o) overlay of single stainings. Images shown are representative of three independent experiments. (**B**) Pixel density of cell-membrane

NF-κB p65 was quantified from total NF-κB p65 and statistically analyzed by one-way ANOVA and Newman-Keuls post-hoc test. ** $p < 0.001$ (versus control), $^{\#}p < 0.001$ (versus LPS).

Since SP-A, alone or in the presence of LPS, favored both PKCζ and p65 membrane localization, the effect of SP-A on PKCζ-p65 interaction was examined next. AM were treated with different concentrations of SP-A (40 µg/ ml and 60 µg/ ml). Cells were then lysed and p65 – or ctrl IgG – was immunoprecipitated from whole cell lysates. Anti-rabbit IgG was utilized as a control to show specificity for p65, no Ab served as a negative control. Samples from each time point were subjected to SDS-PAGE. The presence of aPKC was detected by Western analysis with the corresponding Ab, whereas an anti-p65Ab was used to evaluate p65 loads.

SP-A, significantly and in a concentration-dependent manner, increased aPKC-p65 co-immunoprecipitation under constitutive conditions (Figure 3.11A, B). Since the SP-A-enhanced interaction obviously has no NF-κB *trans*activating potency, the hypothesis was that this effect of SP-A directly or indirectly prevented p65 nuclear translocation.

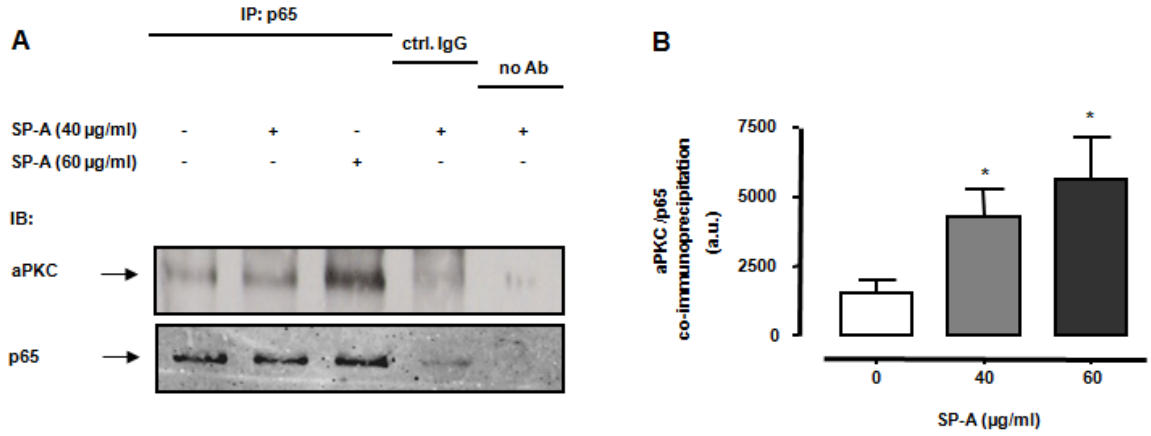


Figure 3.11: SP-A enhances aPKC/p65 co-immunoprecipitation under resting conditions. (**A**) AM were left untreated or treated with 40 µg/ ml or 60 µg/ ml SP-A (1 h). NF-κB p65 was immunoprecipitated from whole cell lysates as described in "Materials and Methods". Anti-rabbit IgG or no Ab was used as a control. Atypical PKC was detected by immunoblotting with the corresponding Ab. Anti-p65 Abs were used to evaluate p65 loads. (**B**) Densitometric results of three independent experiments. Data are expressed as arbitrary units (a. u.) (mean ± SE). Data were analyzed by one-way ANOVA with Dunnett´s post test. * $p < 0.05$ (versus untreated).

3.1.9 SP-A does not inhibit IκB-α ubiquitination

PKCζ has been shown to influence proteasome-mediated protein degradation (Scott et al., 2002, Hernandez-Pigeon et al., 2005). IκB-α turnover is regulated mainly by phosphorylation, ubiquitination and subsequent degradation by the 26S proteasome. It has been shown previously that SP-A neither induces IκB-α phosphorylation at $Ser^{32/36}$ nor enhances IκB-α mRNA levels both basal and in the presence of LPS (Wu et al., 2004). Since phosphorylation precedes ubiquitination the effect of SP-A on IκB-α ubiquitination was investigated. AM were left untreated or were treated with SP-A (40 μg/ ml), the proteasome inhibitor PSI (15 μM), aPKC pseudosubstrate peptides (5 μM), or combinations of the three agents. Cells were then lysed and IκB-α was immunoprecipitated from whole cell lysates. Samples were subjected to SDS-PAGE. The presence of ubiquitin was detected by Western analysis with the corresponding Ab; an anti-IκB-α Ab was used to evaluate IκB-α loads. Compared to unstimulated cells, treatment with PSI and aPKCps alone increased ubiquitin-conjugated IκB-α which was further enhanced when both inhibitors were used in combination. Interestingly, SP-A alone and in combination with PSI and/or aPKCps elevated ubiquitin-conjugated IκB-α, suggesting that SP-A does neither interfere with nor inhibit the ubiquitin-dependent pathway.

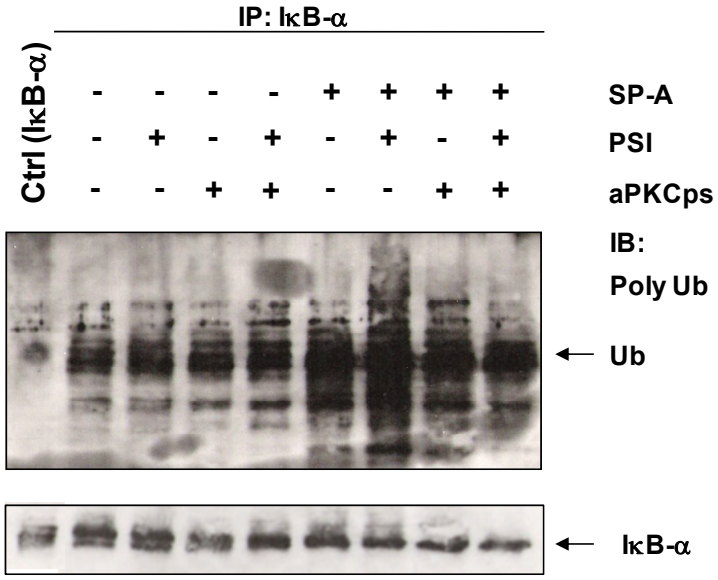


Figure 3.12: SP-A does not interfere with the ubiquitin-dependent degradation pathway. AM were left untreated or treated with 40 µg/ml (1 h), 5 µM aPKCps (1 h), 15 µM PSI (30 min). IκB-α was immunoprecipitated from whole cell lysates as described in "Materials and Methods". Ubiquitin was detected by immunoblotting with the corresponding Ab. Anti-IκB-α Abs were used to evaluate IκB-α loads.

3.1.10 PKCζ is essential for SP-A-mediated IκB-α stabilization and inhibition of LPS-induced NF-κB activity

In order to establish the selective role of PKCζ in mediating the SP-A effect described, mice deficient for the PKCζ isoform were used (Leitges et al., 2001). Whereas PKCλ/ι$^{-/-}$ mice are embryonic lethal (Soloff et al., 2004), PKCζ$^{-/-}$ mice are grossly normal but exhibit an impairment of IKK activation in whole lung extracts after LPS challenge (Leitges et al., 2001).

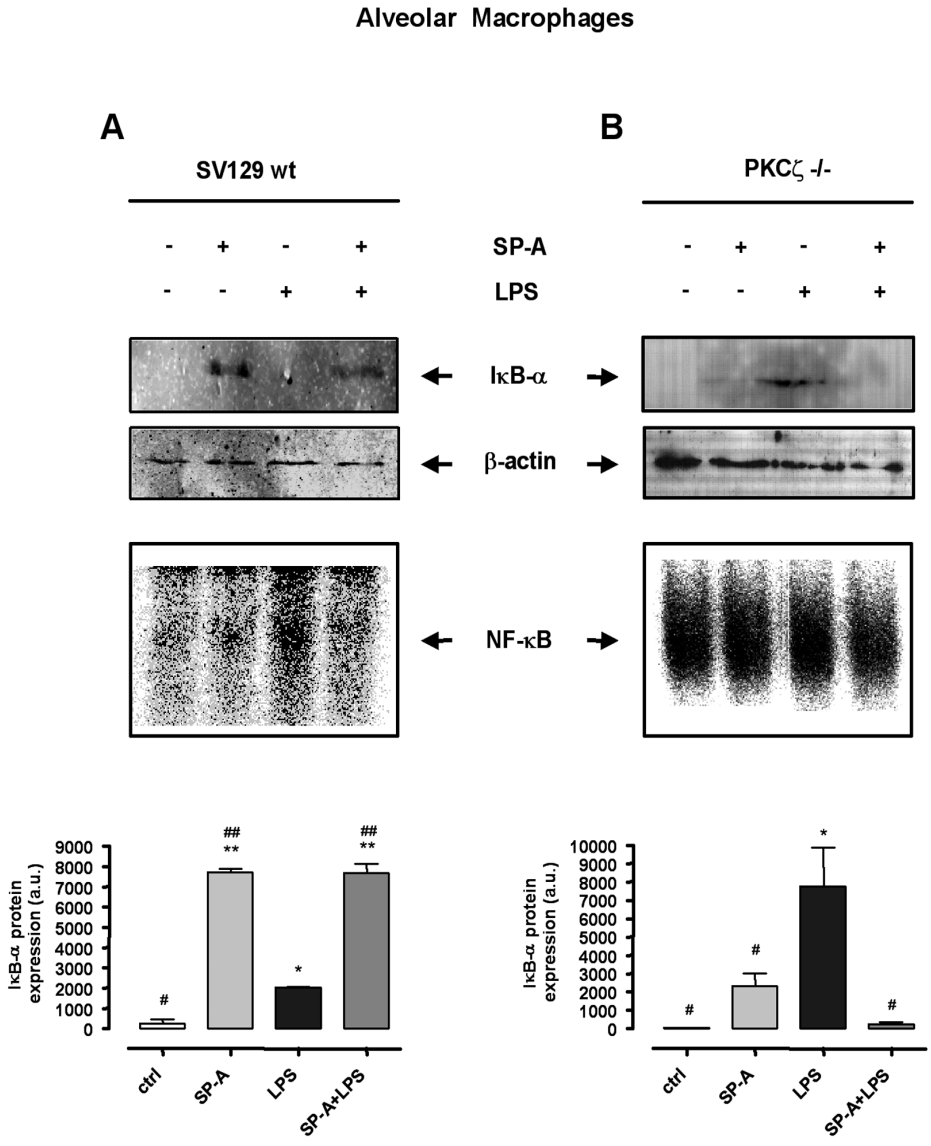


Figure 3.13: PKCζ is vital for SP-A-mediated IκB-α stabilization and inhibition of LPS-induced NF-κB activity. (A-B) Pooled AM from either four wild-type (A) or four PKCζ-deficient mice (B) were left untreated or treated with

40 µg/ ml SP-A (1 h), and then exposed either to media or to 100 ng/ ml LPS (1 h). Cytosolic cell fractions were immunoblotted for IκB-α (upper panel). Nuclear extracts of the cells were analyzed by EMSA for NF-κB DNA binding activity (mid panel). Data shown are representative of three independent experiments. Densitometric results are expressed as arbitrary units (a.u.). Data were analyzed by one-way ANOVA with a post-hoc Newman-Keuls test. * $p < 0,01$, ** $p < 0,001$ (versus control); $^{\#}p < 0.01$, $^{\#\#}p < 0.001$ (versus LPS).

In freshly isolated AM from PKCζ$^{-/-}$mice, however, NF-κB activation was apparent both basal and LPS-induced. SP-A failed to inhibit basal and only slightly reduced LPS-induced NF-κB DNA binding compared to wt AM (Figure 3.13A and B, lower panel). Compared to wt AM, SP-A-mediated IκB-α stabilization was almost abolished in PKCζ$^{-/-}$AM (Figure 3.13A and B, upper panel). Surprisingly, in the presence of LPS, IκB-α was enhanced in PKCζ$^{-/-}$ AM compared to wt AM and this effect was abolished when cells had been pretreated with SP-A (Figure 3.13B, upper panel). Taken together these data indicate that PKCζ, constitutively and in the presence of LPS, is indispensible for SP-A-mediated anti-inflammatory signaling in AM.

3.1.11 PKCι is expressed and differentially distributed within AM upon diverse stimuli

It has been shown that in lung homogenates of PKCζ-deficient mice the protein level of PKCλ/ι was unaltered (Leitges et al., 2001). Since the aPKC pseudosubstrate peptides and the aPKC Ab work well for both atypical PKC isozymes, a specific PKCι Ab (kindly provided by M. Leitges) was used to elucidate whether aPKCι is expressed in AM by confocal microscopy. AM were seeded into 8-well Chamber slides at a density of 1 x 10^5, and allowed to adhere at 37°C in the presence of 0.2 % HI-FCS in a 5 % CO_2 atmosphere. Cells were left untreated (Figure 3.14A, a) or treated with SP-A (40 µg/ ml, 1 h; Figure 3.14A, b), LPS (100 ng/ ml, 1 h; Figure 3.14A, c) or pretreated with SP-A prior to the addition of LPS (Figure 3.14A, d). A specific Ab against PKCι was detected by goat-anti-rabbit IgG conjugated to Alexa Fluor 488. Cell nuclei were counterstained with propidium iodide (PI). After stimulation, the cells were further treated as described in "Materials and Methods". PKCι distribution strongly parallels that of NF-κB p65 under all conditions (Figure 3.10A) and of PKCζ under both SP-A or LPS conditions (Figure 3.9B). SP-A significantly induced a PKCι translocation towards the cell membrane alone and in the presence of LPS. Another similarity to the p65 distribution was that pretreatment of the cells with SP-A almost

abolished LPS-induced PKCι nuclear translocation and induced an accumulation of PKCι at the plasma membrane (Figure 3.14d).

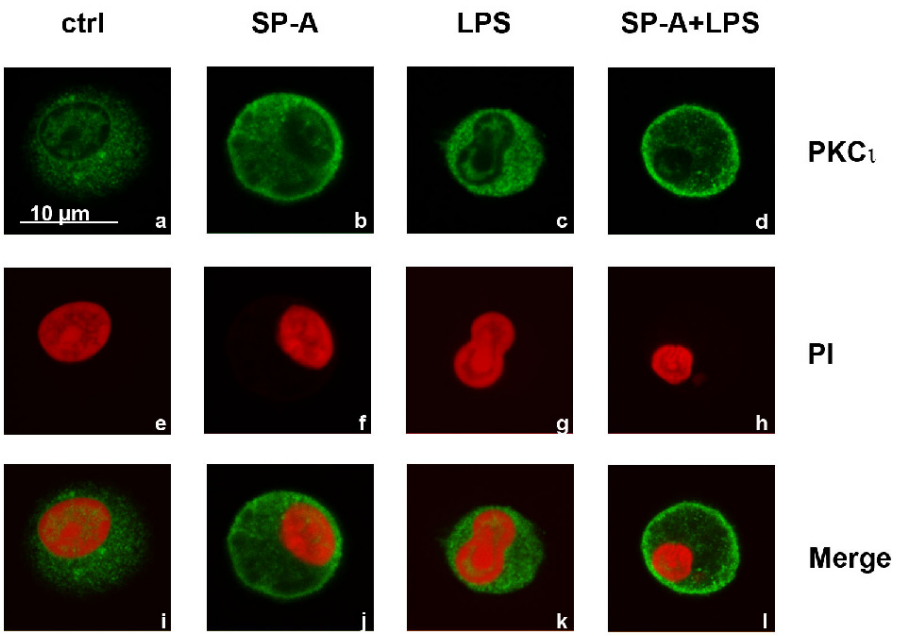


Figure 3.14: Distribution of PKCι in AM. AM adhered to Chamber slides at 1 x 10^5 cells/ well and were left untreated and then exposed to media or treated with 40 µg/ml SP-A (1 h), and were then further either exposed to 100 ng/ ml LPS (1 h) , or treated with 100 ng/ ml LPS (1 h) alone. Slides were blocked and incubated with a specific Ab against PKCι (a-d) that was detected by goat-anti-rabbit IgG Ab conjugated to Alexa Fluor 488. (e-h) Cell nuclei were counterstained with propidium iodide (PI). Cells were visualized by confocal microscopy. (i-l) Overlay of single stainings. Images shown are representative of two independent experiments with similar results.

3.2 PART II: Role of clathrin-mediated endocytosis (CME) of SP-A by alveolar macrophages in anti-inflammatory signaling

3.2.1 SP-A-mediated IκB-α stabilization in AM does require calcium but is not inhibitable by mannose

SP-A modulates anti-inflammatory responses of alveolar macrophages (AM) by stabilizing the main inhibitor of NF-κB, IκB-α. Previous studies showed that SP-A binding and uptake by AM is calcium-dependent, but does not involve the carbohydrate recognition domain (CRD) of SP-A. It was further demonstrated that SP-A is cleared by AM in a clathrin-dependent manner, trafficking through the endolysosomal pathway (Jain et al., 2005; Crowther et al., 2005). The hypothesis was that SP-A uptake by AM is a prerequisite for SP-A-mediated IκB-α stabilization and thus for its anti-inflammatory effects. To confirm the previously reported dependence of Ca^{2+} in SP-A binding and uptake by AM, SP-A was labeled with FITC. Freshly isolated AM were either left untreated, were treated with FITC-labeled SP-A (40 µg/ ml), EDTA (10-50 mM), or were treated with EDTA prior to the addition of FITC-SP-A. To further exclude that the CRD is involved, AM were treated with mannose (1 %) which is preferably bound by the CRD of SP-A, or with mannose prior to the addition of FITC-SP-A. Cell-association (i.e. binding and/ or uptake) of FITC-SP-A was analyzed by FACS as described in "Materials and Methods". Cell-associated FITC-SP-A was inhibited when cells had been pretreated with EDTA but not with mannose, confirming that cell-association is dependent on Ca^{2+} but does not involve the CRD.

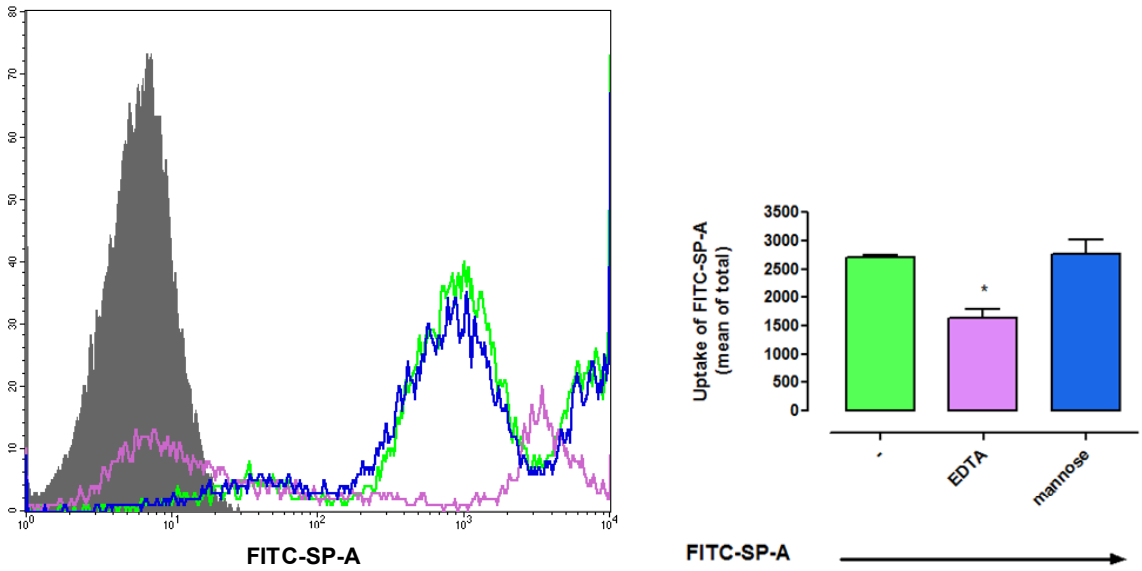


Figure 3.15: Cell-association of FITC-SP-A is reduced by EDTA. AM were left untreated (grey area, left panel), or treated with 40 µg/ ml FITC-labeled SP-A (1 h), or were treated with 10 mM EDTA (20 min) and 1 % mannose (20 min) prior to the addition of FITC-SP-A. FACS analysis was performed and cell-associated FITC-SP-A was determined (right panel). Data shown are representatives of two independent experiments. * $p < 0.05$

It was then investigated whether the prevention of SP-A´s cell-association affects SP-A-mediated IκB-α stabilization. AM were either left untreated, or treated as before with SP-A (40 µg/ ml), EDTA (10-50 mM), EDTA prior to the addition of SP-A, mannose (1 %), or mannose prior to treatment with SP-A. After stimulation, nuclear and cytosolic fractions were extracted as described in "Materials and Methods". The protein content of the cytosolic and nuclear fractions was determined by BC assay, and the samples were further subjected to SDS-PAGE. IκB-α protein expression was determined by Western analysis, NF-κB DNA binding activity was analyzed by EMSA.

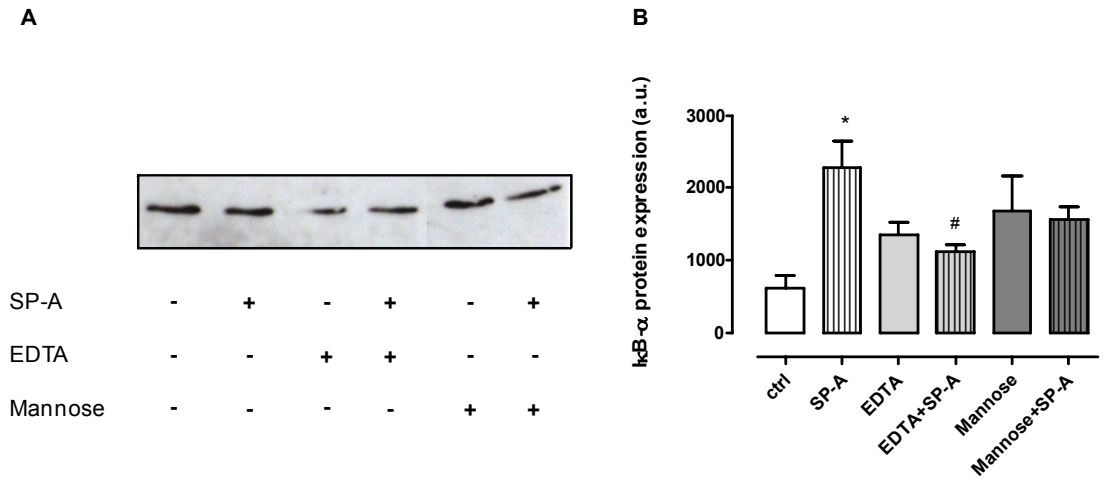


Figure 3.16: SP-A-mediated IκB-α stabilization is dependent on Ca^{2+}. (A) Representative Western blot. AM were left untreated, were treated with SP-A (40 µg/ ml), EDTA (50 mM), Mannose (1 %), or were pretreated with EDTA or Mannose prior to the addition of SP-A. Data shown are representative of three independent experiments. Cytosolic cell fractions were immunoblotted for IκB-α. Densitometric results are expressed as arbitrary units (a.u.). (B) Statistical analysis was performed by one-way ANOVA with a post-hoc Newman-Keuls test. * $p < 0.01$ (versus ctrl); # $p < 0.05$ (versus SP-A).

SP-A-mediated IκB-α stabilization was significantly ($p < 0.05$) inhibited by pretreatment of AM with EDTA (Figure 3.16B), indicating that Ca^{2+} was involved in the enhancement of IκB-α protein expression by SP-A (Figure 3.16A). Treatment of AM with 1% mannose, which is bound preferably by the CRD of SP-A, did not have any effect on SP-A-mediated IκB-α protein expression (Figure 3.16A, B). Together the results indicate that Ca^{2+} but not the CRD is involved in SP-A´s cell-association with AM as well as SP-A-mediated IκB-α stabilization.

3.2.2 SP-A-mediated IκB-α stabilization is clathrin-dependent

The initial hypothesis was that SP-A uptake by AM is a prerequisite for its anti-inflammatory effects. It has been shown that SP-A is cleared by AM in a clathrin-dependent manner (Jain et al., 2005; Crowther et al., 2005). Therefore clathrin inhibitors were used to prevent SP-A uptake and to investigate the effect on SP-A-mediated IκB-α stabilization. Amantadine (Aman), a specific clathrin inhibitor that prevents budding of clathrin-coated vesicles (CCV), phenylarsine oxide (PAO) that prevents formation of CCVs and works upstream of amantadine, Chlorpromazine (Chlpr), which inhibits the assembly of the clathrin adaptor protein AP2 on clathrin-coated pits (CCP), and also cytochalasin D (CytoD), which

induces actin depolymerization have been shown to significantly decrease SP-A uptake and degradation by AM (Jain et al., 2005; Baritussio et al., 2000).

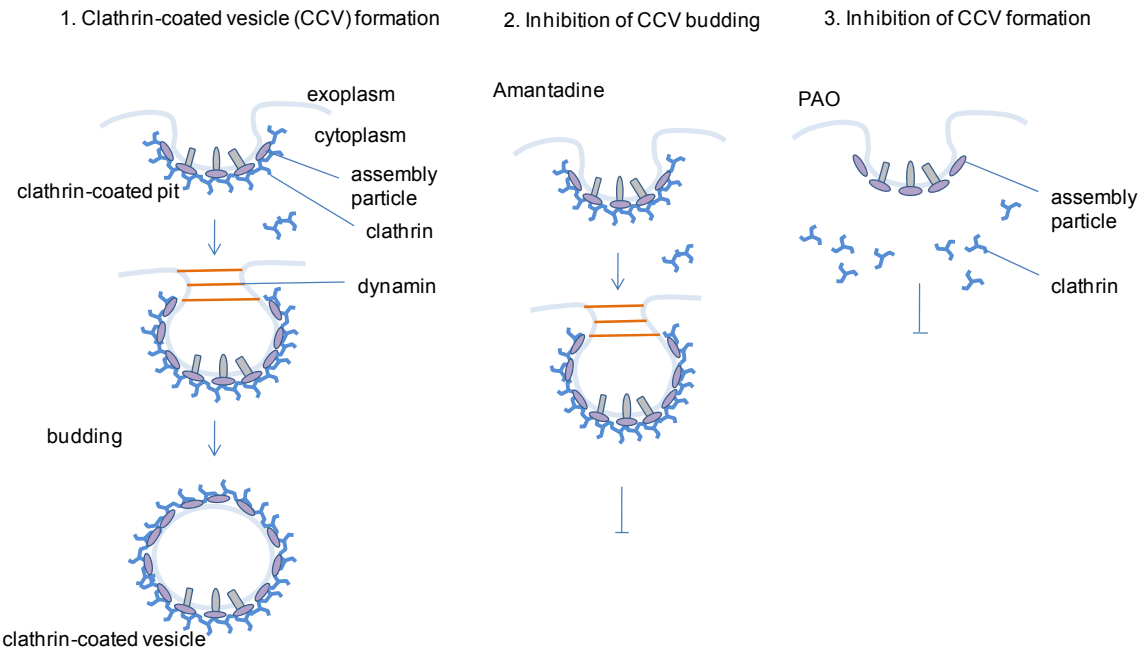


Figure 3.17: Schematic depiction on the interference of clathrin inhibitors with clathrin-coated vesicle formation. 1. Clathrin-coated vesicle (CCV) formation under normal circumstances: clathrin-coated pit formation is followed by coat propagation and budding, releasing a complete vesicle. **2.** Amantadine inhibits the budding of forming vesicles from the membrane. **3.** Phenylarsine oxide (PAO) prevents CCV formation.

Experiments employing the clathrin inhibitors as well as CytoD were performed in BSA-pre-coated microfuge tubes because of their detaching effect on adhered AM. Freshly isolated AM were left untreated or were treated with SP-A (40 µg/ ml, 1 h), Aman (2.5 mM, 30 min), PAO (2 µM, 30 min), Chlpr (50 µM, 1 h), or CytoD (10 µM, 30 min), or were pretreated with the inhibitors prior to the addition of SP-A. After stimulation, nuclear and cytosolic fractions were extracted as described in “Materials and Methods”. The protein content of the cytosolic and nuclear fractions was determined by BC assay, and the samples were further subjected to SDS-PAGE or Polyacrylamide gel electrophoresis. Cytosolic IκB-α protein expression was determined by Western analysis, NF-κB activity was analyzed by EMSA.

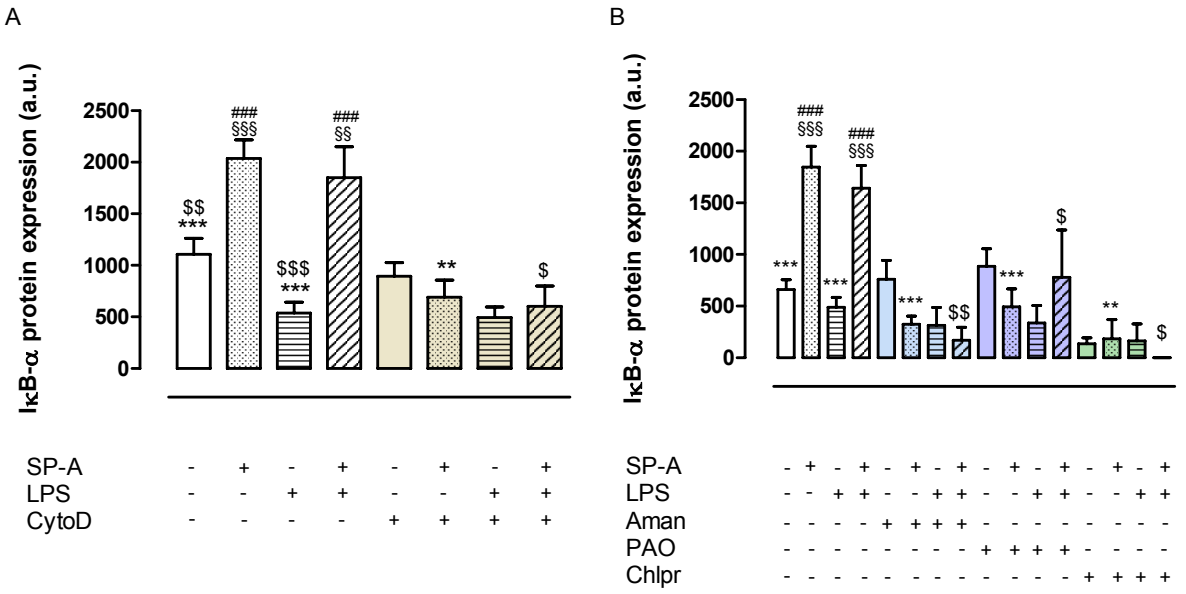


Figure 3.18: SP-A-mediated IκB-α stabilization is clathrin-dependent. AM were left untreated, were treated (A) with SP-A (40 µg/ ml), LPS (100 ng/ ml), and CytoD (10 µM), or (B) with SP-A (40 µg/ ml), LPS (100 ng/ ml), Aman (2.5 mM), PAO (2 µM), Chlpr (50 µM), or were pretreated with the inhibitors prior to the addition of SP-A. Data shown are representative of three to five independent experiments. Cytosolic cell fractions were immunoblotted for IκB-α. Densitometric results are expressed as arbitrary units (a.u.). Data were analyzed by one-way ANOVA with a post-hoc Newman-Keuls test. ** $p < 0.01$; *** $p < 0.001$ (versus SP-A), §§ $p < 0.01$; §§§ $p < 0.001$ (versus control); $^{###}$ $p < 0.001$ (versus LPS), $^{\$}$ $p < 0.05$; $^{\$\$}$ $p < 0.01$; $^{\$\$\$}$ $p < 0.001$ (versus SP-A+LPS).

Pretreatment of AM with Aman, PAO, Chlpr, or CytoD significantly inhibited SP-A's enhancing effect on IκB-α protein as well as SP-A-mediated inhibition of LPS-induced IκB-α degradation, suggesting that clathrin-mediated internalization of SP-A by AM as well as an intact actin cytoskeleton is indispensible for SP-A's anti-inflammatory effects.

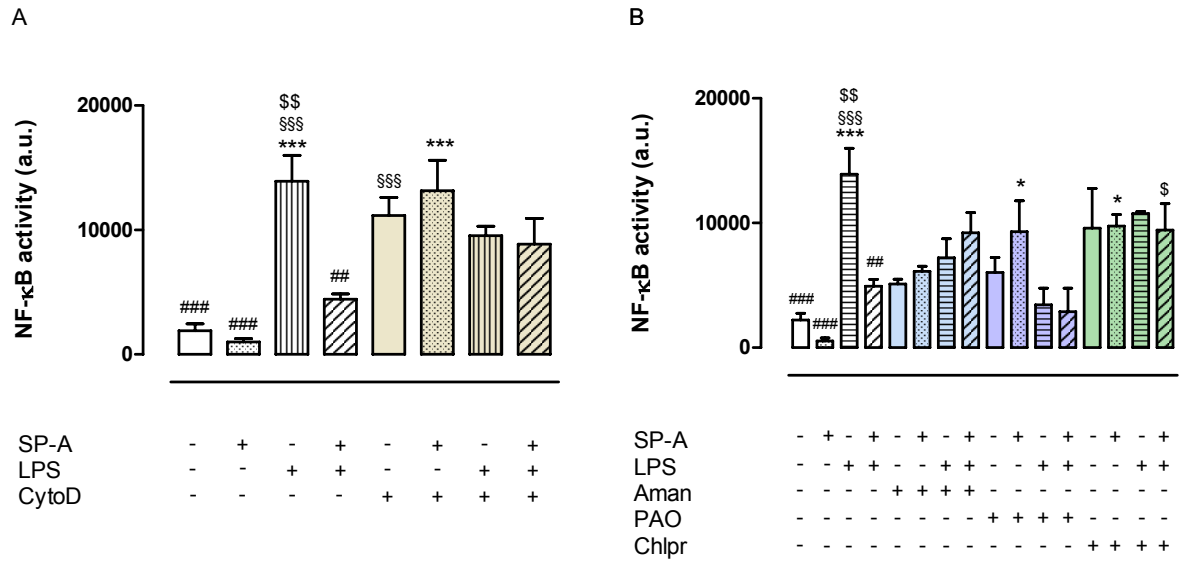


Figure 3.19: SP-A-mediated inhibition of NF-κB acivity is clathrin-dependent. AM were left untreated, were treated (A) with SP-A (40 µg/ ml), LPS (100 ng/ ml), and CytoD (10 µM), or (B) with SP-A (40 µg/ ml), LPS (100 ng/ ml), Aman (2.5 mM), PAO (2 µM), Chlpr (50 µM), or were pretreated with the inhibitors prior to the addition of SP-A. Data shown are representative of three to five independent experiments. Nuclear extracts of the cells were analyzed by EMSA for NF-κB DNA binding activity. Densitometric results are expressed as arbitrary units (a.u.). Data were analyzed by one-way ANOVA with a post-hoc Newman-Keuls test. * $p < 0.05$; *** $p < 0.001$ (versus SP-A); §§§ $p < 0.001$ (versus control); $^{\#\#}$ $p < 0.01$; $^{\#\#\#}$ $p < 0.001$ (versus LPS), $^{\$}$ $p < 0.05$; $^{\$\$}$ $p < 0.01$(versus SP-A+LPS).

Pretreatment with CytoD (Figure 3.19 A) resulted in an enhanced NF-κB activity in resting cells which was further increased in the presence of SP-A. Inhibition of clathrin with Aman, PAO, and chlorpromazine slightly enhanced NF-κB activity in resting cells (Figure 3.19 B). While treatment with Aman prior to the addition of SP-A only slightly increased NF-κB activity, pretreatment with PAO and chlorpromazine significantly enhanced NF-κB binding activity in the presence of SP-A. In addition, pretreatment with chlorpromazine abrogated SP-A´s inhibitory effect on LPS-induced NF-κB activation. These results suggest that the inhibition of the actin cytoskeleton and CME (i.e. inhibition with PAO and chlorpromazine) abrogates SP-A-mediated inhibition of NF-κB activity under constitutive conditions and in the presence of LPS (i.e. by pretreatment with chlorpromazine), corresponding with the data obtained from Western analyses.

3.2.3 Cell-association of FITC-SP-A by AM is significantly reduced by clathrin inhibitors

In order to evaluate the direct effect of clathrin inhibitors on SP-A´s uptake, SP-A was labeled with FITC, and AM were treated with FITC-SP-A (40 µg/ ml, 1 h), or with Aman (2,5 mM, 30 min) and PAO (2 µM, 30 min) prior to the addition of FITC-SP-A. FACS analysis was performed as described in "Materials and Methods". This way only newly bound or endocytosed FITC-SP-A by AM is detected by FACS, and the inhibition of clathrin-mediated endocytosis can be directly compared to FITC-SP-A alone. Cell-associated FITC-SP-A was significantly (* $p < 0.05$) inhibited in the presence of Aman and PAO.

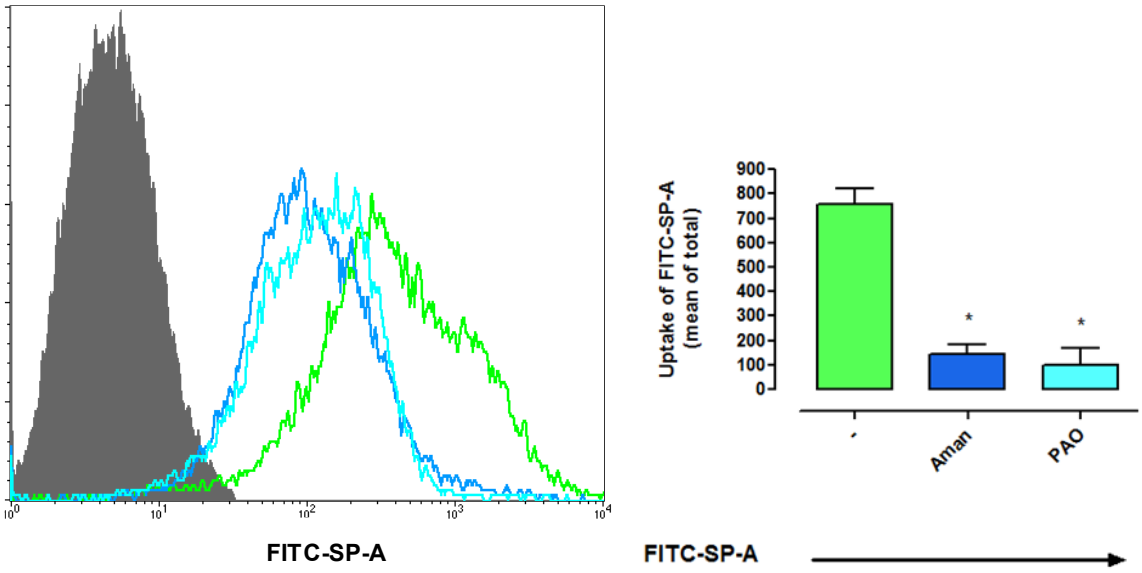


Figure 3.20: Cell-association of FITC-SP-A is reduced by clathrin inhibitors. AM were left untreated (grey area, left panel), or treated with 40 µg/ ml FITC-labeled SP-A (1 h), or were treated with 2,5 mM Aman (30 min) and 2 µM PAO (30 min) prior to the addition of FITC-SP-A. FACS analysis was performed and cell-association of FITC-SP-A was determined (right panel). Data shown are representatives of two independent experiments. * $p < 0.05$

In another approach using confocal microscopy the effect of the clathrin inhibitors amantadine and PAO was visualized. AM were seeded into 8-well Chamber slides at a density of 1 x 10^5, and allowed to adhere at 37°C in the presence of 0.2 % HI-FCS in a 5 % CO_2 atmosphere. Cells were left untreated or treated with FITC-labeled SP-A (40 µg/ ml, 1 h), or pretreated with Aman (2.5 mM, 30 min) or PAO (2 µM, 30 min) prior to the addition of

FITC-SP-A (Figure 3.21). In a control condition, cells were treated with FITC alone. After stimulation, the cells were further treated as described in "Materials and Methods". The cytoskeleton was stained to show cell boundaries. An Ab against β-actin was detected by rabbit-anti-mouse IgG conjugated to Alexa Fluor 568. FITC was detected at 514 nm after excitation at 488 nm.

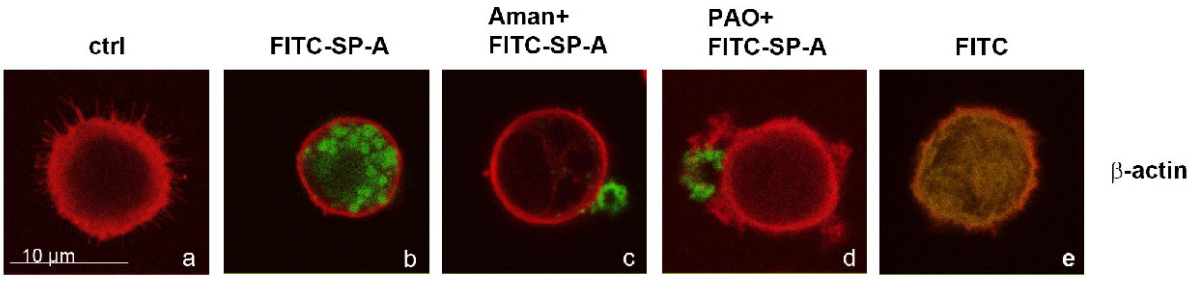


Figure 3.21: Uptake of FITC-SP-A is reduced by clathrin inhibitors. AM adhered to Chamber slides at 1 x 10^5 cells/ well and were left untreated (a) and then exposed to media or treated with FITC alone (1 h; e), 40 µg/ml FITC-SP-A (1 h; b), or were treated with 2.5 mM Aman (30 min; c) or 2 µM PAO (30 min; d) prior to the addition of FITC-SP-A. Cells were blocked and incubated with an Ab against β-actin that was detected by rabbit-anti-mouse IgG Ab conjugated to Alexa Fluor 568. Cells were visualized by confocal microscopy. Overlay of single stainings. Images shown are representative of two independent experiments with similar results.

FITC-SP-A is endocytosed by AM (Figure 3.21 b), however pretreatment with amantadine (Figure 3.21 c) or PAO (Figure 3.21 d) prevented endocytosis of FITC-SP-A by AM.

3.2.4 Both β-arrestin and α-adaptin are involved in SP-A-mediated IκB-α stabilization

In the search for clathrin adaptors involved in SP-A-mediated anti-inflammation the most prominent clathrin associated adaptors AP2 and β-arrestin were targeted. The α-subunit of the AP2 complex binds to PtdIns(4,5)P_2 and recruits AP2 to the plasma membrane where clathrin is connected to AP2 *via* β-arrestins (Owen, 2004). The arrestins, in particular β-arrestin 2, have been shown to bind directly to IκB-α, thereby preventing IκB-α degradation by the proteasome and inhibiting NF-κB activation (Gao et al., 2004). After inhibiting these two clathrin adaptors with their respective blocking peptides (b.p.), the SP-A-mediated IκB-α stabilization was investigated by Western analysis and NF-κB activation assay (EMSA). AM were either left untreated, or treated with SP-A (40 µg/ ml), LPS (100 ng/ ml), or treated with SP-A prior to the stimulation with LPS. Cells were further

pretreated with the blocking peptides prior to the addition of SP-A, LPS or both. After stimulation nuclear and cytosolic fractions were extracted as described in "Materials and Methods", and their protein content was determined by BC assay.

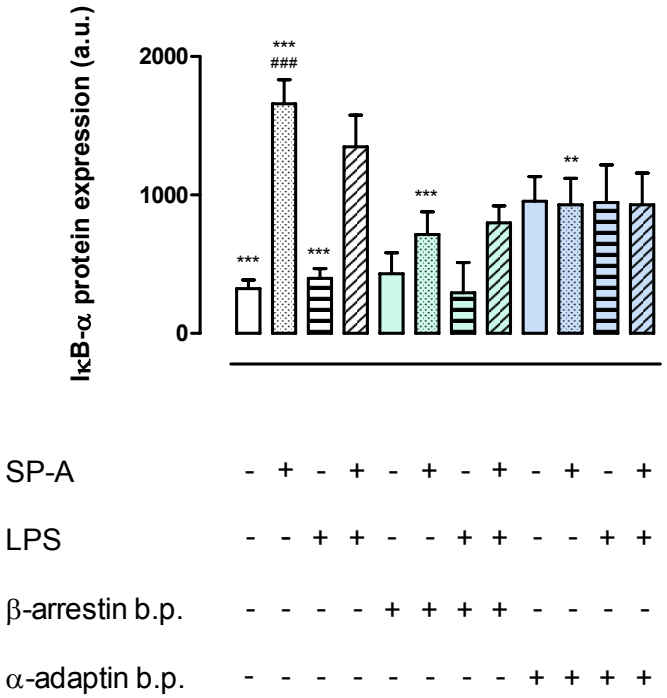


Figure 3.22: The clathrin adaptors β-arrestin and α-adaptin are involved in SP-A-mediated IκB-α stabilization. AM were left untreated or were treated with 40 µg/ ml SP-A (1 h), 100 ng/ ml LPS (1 h), or both, 10 µg/ ml β-arrestin b.p. (10 min), 10 µg/ ml α-adaptin b.p. (10 min), or cells were pretreated with the blocking peptides and were further exposed to SP-A. Cytosolic fractions were subjected to SDS-PAGE and Western analysis was performed. Data are representative of three to five independent experiments. $^{###}$ $p < 0.001$ (versus control), ** $p < 0.01$, *** $p < 0.001$ (versus SP-A).

SP-A-mediated IκB-α stabilization was significantly ($p < 0.001$ and $p < 0.01$, respectively) decreased after pretreatement of the cells with both β-arrestin b.p. and α-adaptin b.p. (Figure 3.22), suggesting that both β-arrestin and α-adaptin are involved in SP-A-mediated IκB-α stabilization.

It was then investigated whether the decrease in IκB-α protein expression in the presence of the inhibitors and SP-A correlates with an increase in NF-κB DNA binding activity under the same conditions. Nuclear extracts were subjected to PAGE and analyzed by EMSA for NF-κB activity.

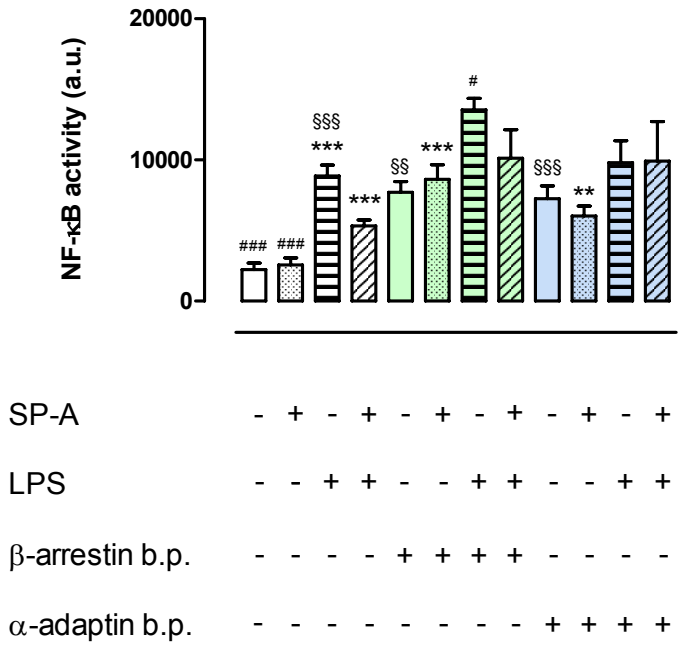


Figure 3.23: The inhibition of the clathrin adaptors β-arrestin and α-adaptin increases NF-κB activity. AM were left untreated or were treated with 40 µg/ ml SP-A (1 h), 100 ng/ ml LPS (1 h), or both, 10 µg/ ml β-arrestin blocking peptide (b.p.) (10 min), 10 µg/ ml α-adaptin b.p. (10 min), or cells were pretreated with the blocking peptides and were further exposed to SP-A. Nuclear extracts were analyzed by EMSA for NF-κB DNA binding activity. Data are representative of three to five independent experiments. §§ $p < 0.01$, §§§ $p < 0.001$ (versus control); $^{\#}$ $p < 0.05$, $^{\#\#\#}$ $p < 0.001$ (versus LPS); * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$ (versus SP-A).

The inhibition of β-arrestin and α-adaptin alone with the corresponding blocking peptide already significantly ($p < 0.001$) increased basal NF-κB activity. In the presence of SP-A, NF-κB activity was significantly enhanced when either clathrin adaptor was blocked (Figure 3.23). The previously reported SP-A-mediated inhibition of LPS-induced NF-κB activity was abrogated when cells had been pretreated with the β-arrestin and α-adaptin blocking peptides. LPS-induced NF-κB activity was further enhanced in the presence of the β-arrestin blocking peptide. Witherow and co-workers previously demonstrated that the suppression of β-arrestin in HeLa cells lead to an increase in NF-κB activity upon stimulation with TNF-α. They further found that β-arrestin 1 and 2 attenuate NF-κB activation (Witherow et al., 2004). In support of this notion, the inhibition of β-arrestin resulted in an increase in NF-κB activity in AM upon LPS stimulation, as well as in the presence of SP-A.

Together these results suggest that clathrin, as well as both clathrin adaptors β-arrestin and α-adaptin, are important components in SP-A endocytosis as well as in SP-A signaling.

3.2.5 SP-A-induced aPKC kinase activity is abrogated by clathrin inhibition

PKCζ kinase activity has been previously shown to be critically involved in SP-A-mediated anti-inflammation (Moulakakis et al., 2007). To investigate further effects involved in the inhibition of SP-A uptake, SP-A-induced aPKC kinase activity was investigated in the presence of the clathrin inhibitor amantadine. AM were left untreated, were treated with SP-A (40 µg/ ml, 1 h), LPS (100 ng/ ml, 1 h), and Aman (2.5 mM, 30 min), or were pretreated with Aman prior to the addition of SP-A or LPS (Figure 24). Cells were then lysed and aPKC was immunoprecipitated from whole cell lysates, followed by an *in vitro* immunocomplex kinase assay. Phosphorylated proteins were subjected to SDS-PAGE and analyzed as described in "Materials and Methods".

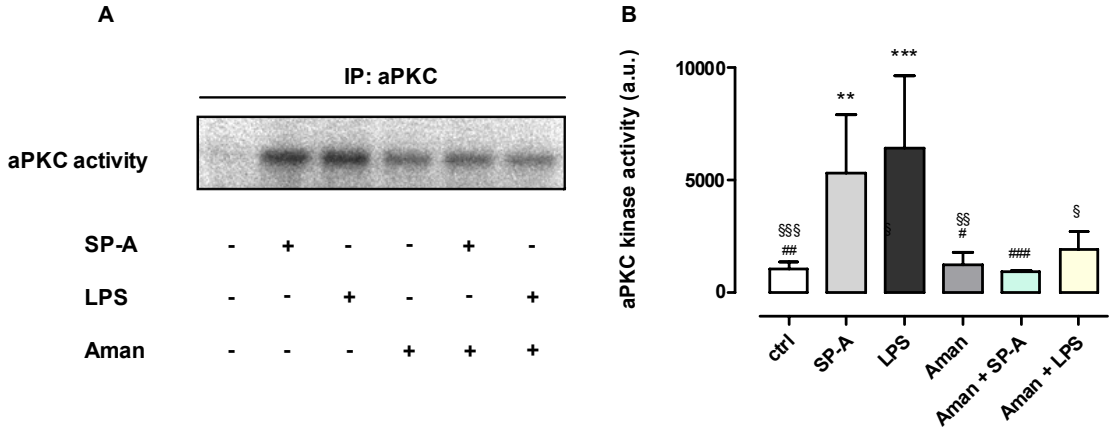


Figure 3.24: SP-A-induced aPKC kinase activity is abrogated by clathrin inhibition. (A) Representative Kinase Assay. AM were left untreated, or treated with 40 µg/ ml SP-A, 100 ng/ ml LPS, and 2.5 mM Aman, or pretreated with Aman prior to the addition SP-A or LPS. Cells were subjected to an in *vitro* aPKC kinase assay. Atypical PKC was immunoprecipitated from cell lysates and subjected to an *in vitro* kinase assay using MBP as a substrate. Atypical PKC kinase activity was determined as described in "Materials and Methods". The phosphorylated proteins were run on a 12 % SDS-PAGE gel. **(B)** Statistical analysis of three independent experiments that were analyzed by an unpaired Student´s *t*-test. ** $p < 0.01$, *** $p < 0.001$ (versus control); $^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$, $^{\#\#\#}$ $p < 0.001$ (versus SP-A); § $p < 0.05$, §§ $p < 0.01$, §§§ $p < 0.001$ (versus LPS).

Compared to control conditions, aPKC activity is significantly enhanced in the presence of SP-A and LPS, as has been shown previously. Pretreatment of AM with the clathrin inhibitor

Aman alone did not alter control conditions. However, pretreatment with Aman prior to the addition of either SP-A or LPS, resulted in an abrogation of SP-A- or LPS-induced aPKC activity. These results strongly suggest that the uptake of both SP-A and LPS is a prerequisite for SP-A- and LPS-induced aPKC kinase activation.

4 DISCUSSION

In the recent past, more and more reports emerged, demonstrating an important role for the pulmonary collectin SP-A in anti-inflammatory immunomodulation of innate immune responses in the lung (Wright, 2005). Within the scope of this study the direct modulation of SP-A on the IκB-α/ NF-κB signal transduction pathway was investigated. In a "proof-of-principle" experiment using AKBI mice, IκB-α was found to be essential for SP-A´s anti-inflammatory immunomodulation. Further investigations to determine potential upstream kinases involved in SP-A signaling revealed that the activity of the atypical PKCζ was critically involved in SP-A´s anti-inflammatory macrophage activation *via* stabilization of IκB-α. In addition, SP-A was found to induce PKCζ phosphorylation, kinase activity and translocation. *Ex vivo* experiments utilizing PKCζ-deficient mice supported the data obtained from *in vitro* investigation and implicated a possible role for PKCζ as a positive or negative regulator in the IκB-α/NF-κB signaling pathway in a stimulus-specific manner in the lung.

In a second approach to determine SP-A´s anti-inflammatory immunomodulatory functions, clathrin-mediated endocytosis (CME) and components of the clathrin assembly complex were inhibited, and SP-A signaling *via* the IκB-α/ NF-κB pathway was examined. The inhibition of CME and related components decreased SP-A-mediated IκB-α stabilization and abrogated SP-A´s inhibitory effect on LPS-induced NF-κB activity. It further abrogated SP-A-induced PKCζ activity.

4.1 PART I: Identification of atypical PKCζ in SP-A-mediated and IκB-α-dependent anti-inflammatory immune regulation

The pivotal role of SP-A in anti-inflammatory immunomodulation of innate immune responses of the lung to LPS has been repeatedly demonstrated in recent years (McCormack and Whitsett, 2002; Wright, 2005). Pulmonary inflammation induced by molecular components of Gram-positive and Gram-negative bacteria, in particular the local or systemic LPS release from Gram-negative bacteria is still a major cause of life-threatening diseases (Piantadosi & Schwartz, 2004). While LPS recognition benefits the host by sensing bacteria and initiating defense mechanisms, an overactive response to LPS would harm the host and contribute to the development of systemic or septic shock syndrome. To prevent autotoxic mediator release by AM, the major effector cell of the pulmonary innate immune system,

especially in response to LPS, negative signaling cascades have been studied intensively in recent years. Of particular interest in this context was the modulation of the NF-κB activation threshold by SP-A. Inhibition of LPS-induced NF-κB activity by SP-A has been suggested to occur *via* direct interaction or interference of SP-A with components of the LPS receptor complex, including LBP, CD14, TLR-4, and MD2 that would prevent LPS from binding to its receptor. SP-A-mediated inhibition of LPS-induced NF-κB activity has also been reported to occur independently of LPS-specific signal transduction pathways (Sano et al., 1999; Gardai et al., 2003; Garcia-Verdugo et al., 2005; Alcorn & Wright, 2004; Wu et al., 2004; Sano et al., 2000; Stamme et al., 2002). Intracellulary, the pivotal role of IκB-α in NF-κB regulation has recently been highlighted by Tergaonkar and co-workers demonstrating that IκB-α (as well as β and ε) is essential for both preventing NF-κB DNA binding and NF-κB-dependent gene expression in the basal state of cell activation. It was further shown that IκB-α increases the responsiveness of p65/IκB complexes to diverse stimuli (Tergaonkar et al., 2005). Previous work from our labsuggested that SP-A inhibits LPS-induced NF-κB activation by post-transcriptionally slowing the basal turnover of IκB-α in primary AM (Wu et al., 2004). However, the *in vivo* proof and the intracellular signaling pathways involved have not yet been defined.

SP-A was isolated and purified from bronchoalveolar lavage fluid of patients suffering from alveolar proteinosis, a disease characterized by an excessive accumulation of surfactant lipoprotein in the pulmonary alveoli with associated disturbance of pulmonary gas exchange (Presneill et al., 2006). The purity of newly isolated SP-A was verified by coomassie-stained SDS-PAGE and Western analysis. The existence of bacterial endotoxin was tested by a *Limulus* amebocyte lysate (LAL) assay for all SP-A preparations used. However, there are intrinsic limitations of the LAL assay. LPS has the capability to interact with other substrates including proteins, thereby masking endotoxin molecules and escaping removal procedures. This interaction interferes with the LAL assay by preventing the detection of particularly lower LPS levels (Petsch & Anspach, 2000). To exclude hidden endotoxin molecules that could not be detected by the LAL assay, each SP-A preparation was tested in a NF-κB activation assay, using LPS as a positive control. SP-A did not induce NF-κB DNA binding activity and inhibited LPS-induced NF-κB activation. Western analysis showed that SP-A increased basal IκB-α protein expression even in the presence of LPS. Together the results

confirm SP-A´s anti-inflammatory immunomodulatory functions constitutively as well as in the presence of LPS, and could therefore be used in the following experiments.

This work confirms that IκB-α is critically involved in SP-A´s anti-inflammatory effects. In IκB-α knockout/ IκB-β knockin (AKBI) mice, an integrated T7-tagged IκB-β gene is expressed under the promoter and regulatory sequence of the IκB-α gene and at the same time a null mutation is introduced into the IκB-α gene (Cheng et al., 1998). AKBI mice exhibit no apparent abnormalities and an induced NF-κB response is comparable to that o wild-type animals, revealing a functional redundancy of IκB-α and IκB-β upon LPS challenge *in vivo* (Fan et al., 2004). In line with this, the data demonstrated that LPS-induced TNF-α release by AKBI cells was similar to that by wt cells confirming the described *in vivo* redundancy of IκB-α and IκB-β in response to LPS (Fan et al., 2004). However, whereas SP-A significantly inhibited LPS-induced TNF-α release (Figure 3.3B and D) and p65 nuclear translocation in wt cells (Figure 3.4E, d), it failed to inhibit TNF-α production (Figure 3.3A and C) as well as p65 nuclear accumulation in AKBI cells (Figure 3.4E, h). These data strongly suggest, that IκB-β could not compensate the lack of IκB-α in SP-A-mediated inhibition of LPS-induced cell activation.

The present study further identifies atypical PKCζ as an essential upstream mediator of IκB-α/ NF-κB regulation by SP-A in primary cells. In PKCζ$^{-/-}$ AM, SP-A-mediated IκB-α stabilization was almost completely abrogated (Figure 3.13B), strongly supporting the experiments employing the inhibitory atypical PKC pseudosubstrate peptides in rat AM (Figure 3.6A, B). The combined data confirm that the PKCζ isoform is critically involved in SP-A-mediated IκB-α stabilization and suggest that the inhibition or the lack of PKCζ potentiates the rate of IκB-α turnover in resting AM.

In contrast to the well characterized IκB-α turnover under stimulus-induced conditions, where IκB-α is transcriptionally regulated by NF-κB in a tight autoregulatory loop that is sensitive to and rapidly induced by NF-κB activating stimuli, little is known about the mechanisms that regulate the basal turnover of IκB-α. Work by Pando & Verma demonstrated in mouse embryonic fibroblasts that an efficient basal turnover of IκB-α requires the carboxy-terminal CKII phosphorylation at Ser^{293} whereas IKK phosphorylation at Ser^{32} and Ser^{36} plays no part under basal conditions (Pando and Verma, 2000) pointing out the complex regulatory

mechanisms involved in maintaining a sensitive NF-κB pathway. Even though IκB-α is not a direct substrate of PKCζ (Janosch et al., 1996), PKCζ-associated CKII preferentially phosphorylates Ser^{293} compared to non-associated CKII in transfected NIH3T3 cells, thereby potentially accelerating the basal turnover of IκB-α (Bren et al., 2000). In the present study however, the inhibition or the lack of PKCζ expedited IκB-α turnover under basal conditions in AM. In addition, apigenin, a selective CKII inhibitor, abolished the SP-A-mediated stabilization of IκB-α, suggesting that in AM both PKCζ and CKII activity contribute to this process.

One central route controlling IκB-α turnover is the proteasome-mediated degradation of ubiquitinated IκB-α. PKCζ has been shown to influence proteasome-mediated protein degradation in diverse ways: by either increasing protein degradation, or by stabilizing the protein and thus inhibiting degradation by the proteasome (Scott et al., 2002, Hernandez-Pigeon et al., 2005). However, when the effect of SP-A on IκB-α ubiquitination in AM was investigated, SP-A enhanced the presence of ubiquitin-conjugated IκB-α (and other proteins) alone and in combination with a proteasome inhibitor and/or atypical PKC pseudosubstrate peptides. These results indicate that SP-A-activated PKCζ does not stabilize IκB-α by inhibiting its ubiquitination and subsequent degradation *via* the ubiquitin-dependent proteasomal pathway (Figure 3.12).

Since LPS- or cytokine-induced PKCζ activity was previously shown to induce NF-κB activation by distinct mechanisms – first through its activation of IKK (Diaz-Meco et al., 1999; Lallena et al., 1999) and secondly by phosphorylation of p65 – the finding that this PKC isozyme is involved in SP-A-mediated anti-inflammation was surprising (Duran et al., 2003; Anrather et al., 1999). Besides PKCζ, kinases implicated in p65 phosphorylation include CK II, protein kinase A, IKK2, Akt, p38, p42, and p44 (Chen and Greene, 2004). However, among these kinases, only PKCζ has been shown to directly interact with p65 (Leitges et al., 2001) and phosphorylates p65 at Ser^{311} in TNF-α stimulated mouse fibroblasts (Duran et al., 2003). In the present study, SP-A enhanced the interaction of PKCζ with p65 in primary AM under constitutive conditions in a concentration-dependent manner (Figure 3.11). Since SP-A alone has no NF-κB *trans*activating potency it was assumed that SP-A, directly or indirectly, prevents p65 nuclear translocation. Confocal microscopy and cell fractionation analysis of p65 localization in rat AM revealed a translocation of p65 to the plasma membrane

and a substantial inhibition of LPS-induced p65 nuclear translocation by SP-A. Based on the combined results a model was designed in which SP-A-mediated PKCζ activation stabilizes IκB-α by preventing p65 nuclear translocation in primary AM.

Previous work by Leitges and co-workers showed a reduced NF-κB DNA binding activity in nuclear lung extracts of PKCζ$^{-/-}$ mice treated with LPS or IL-1 intraperitoneally. In addition they found that in whole lung extracts the lack of PKCζ resulted in an impairment of IKK activation upon LPS stimulation suggesting a substantial role of PKCζ in the control of stimulus-induced IKK/ NF-κB signaling cascade in the lung (Leitges et al., 2001). In the present study, IκB-α expression in response to LPS was decreased in wt AM (Figure 3.13A), as expected. Surprisingly, LPS enhanced IκB-α in PKCζ$^{-/-}$ AM (Figure 3.13B). A possible explanation might include that the lack of PKCζ impairs the LPS-induced IKK activation and the subsequent IκB-α degradation as previously suggested (Leitges et al., 2001). On the other hand, the IκB-α increase observed in PKCζ$^{-/-}$ AM could result from *de novo* transcription and might be referred to a compensatory increase of PKCλ/ι. However, Western blot analysis of lung extracts from wt and PKCζ-deficient mice for PKCζ and PKCλ/ι protein expression with specific antibodies revealed that the level of PKCλ/ι is not affected by the loss of PKCζ (Martin et al., 2005), rendering the latter explanation unlikely. The LPS-mediated IκB-α increase in PKCζ$^{-/-}$ AM was abolished by pretreatment of the cells with SP-A, proposing that SP-A not only fails to stabilize IκB-α but provides a signal that triggers IκB-α degradation, presumably by activating IKK in a PKCζ-independent way. Of note, in PKCζ$^{-/-}$ AM, SP-A failed to inhibit basal and LPS-induced NF-κB activation compared to wt cells, suggesting that the down-modulation of constitutive and LPS-induced NF-κB activity by SP-A is dependent on PKCζ (Figure 3.13). PKCλ/ι has been shown to be expressed in AM and distributed within the cell in a strikingly similar pattern to the distribution of NF-κB p65 or PKCζ. Although PKCλ/ι was shown not to be increased in lung extracts, the distribution pattern suggests a compensatory effect to some extent (Figure 3.14). The role of PKCλ/ι as an oncogene in tumorigenesis does not affect the NF-κB pathway but instead activates the Rac1 → Pak → MEK1,2 → ERK1,2 signaling pathway required for transformed growth (Regala et al., 2005). This would explain why PKCλ/ι did not compensate for the lack of PKCζ that resulted in the strong NF-κB activity seen in PKCζ-deficient mice.

The mechanisms underlying PKCζ activation are not completely understood and display substantial differences for different cell types, stimuli, and incubation conditions (Hirai & Chida, 2003). PKCζ can be activated *in vitro* by phosphatidylinositol-3,4,5-triphosphat (PIP_3) (Herrera-Velit et al., 1997; Nakanishi et al., 1993), phosphatidic acid (Limatola et al., 1994), ceramide and arachidonic acid (Muller et al., 1995; Procyk et al., 2000) and/or by direct interaction with binding proteins (Jaken & Parker, 2000). To activate PKCζ, phosphorylation of the activation loop consensus threonine residue Thr^{410} by PI3K-induced phosphoinositide-dependent kinase (PDK) 1 is substantial (Le Good et al., 1998). Besides different activation mechanisms, distinct intracellular localizations may have a major impact on cell-specific PKCζ function (Tan & Parker, 2003). It was demonstrated that SP-A enhanced aPKC$Thr^{410/403}$ phosphorylation (Figure 3.7), aPKC kinase activity (Figure 3.8), and translocation (Figure 3.9) in primary rat AM. In line with previous work (Jaken & Parker, 2000), LPS treatment of AM was found to favor a nuclear accumulation of PKCζ, in contrast to the cell membrane accumulation of the kinase in the presence of SP-A. However, pretreatment of the cells with SP-A largely prevented LPS-induced subcellular localization of the kinase (Figure 3.9). The biological significance though remains to be established. Interestingly, recent data suggest that LPS-induced and PI3K-dependent PKCζ activation can be modified by anti-inflammatory mediators. As shown in RAW 264.7 cells, LPS-induced PKCζ activation is potentiated by the lipid mediator 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 resulting in an inhibition of IKK and NF-κB activity (Castrillo et al., 2003).

Figure 4.1 depicts a model implicating SP-A-induced PKCζ as a p65 interacting kinase that reduces macrophages inflammatory responsiveness by promoting IκB-α stabilization under resting conditions, subsequently leading to the inhibition of LPS-induced NF-κB activation. However, the mechanisms of PKCζ activation as well as the PKCζ effectors operative in IκB-α-dependent anti-inflammatory modulation by SP-A in resting AM were not identified in the present study, and will be the focus of future efforts.

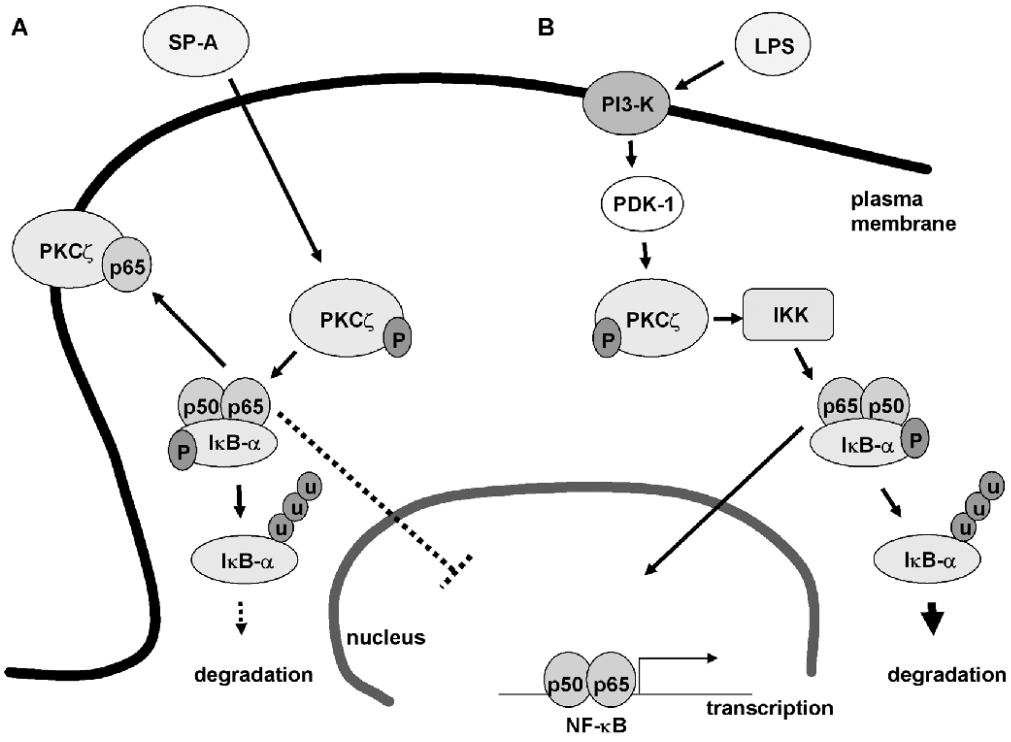


Figure 4.1: A model implicating PKCζ in SP-A-mediated IκB-α stabilization, leading to inhibited NF-κB-induced cell activation. (**A**) In resting alveolar macrophages, SP-A-activated PKCζ is implicated as a p65 interacting kinase that reduces macrophage inflammatory responsiveness by promoting IκB-α stabilization *via* retarded degradation (broken arrow), subsequently altering the threshold for NF-κB activation. (**B**) Classical LPS-induced NF-κB activation pathway. LPS-induced PKCζ leads to IκB-α phosphorylation, ubiquitination and degradation (bold arrow) *via* IKK, allowing p65 nuclear translocation and NF-κB activation.

In summary, the evidence presented here indicates that anti-inflammatory macrophage activation *via* IκB-α by SP-A critically involves PKCζ activity. In addition, the data imply a possible role of PKCζ as a positive or negative regulator in IκB-α/ NF-κB signaling pathways in a stimulus-specific manner in the lung. As uncontrolled NF-κB activation may well be deleterious, tonic activation of PKCζ by SP-A may serve as a physiological brake on induction of TNF-α in AM localized to sites of pulmonary inflammation.

4.2 PART II: Role of CME in SP-A´s anti-inflammatory signaling

The pulmonary collectins are critical components of the immune host defense of the lung, mediating inflammatory responses of innate and adaptive immune cells to protect the host from lung damage or impaired gas exchange (Pastva et al. 2007). The anti-inflammatory effects of SP-A have been thoroughly investigated over the past several years. It has been previously shown that SP-A modulated the IκB-α/ NF-κB pathway by stabilizing IκB-α thereby preventing NF-κB activation (Wu et al., 2004). Recently, atypical PKCζ was identified as an upstream kinase and critical factor involved in SP-A-mediated IκB-α stabilization (Moulakakis et al., 2007).

Most recently, it was investigated how SP-A uptake by AM influences SP-A-mediated IκB-α stabilization constitutively and in the presence of LPS, and thus affects SP-A anti-inflammation.

Endocytosis has been considered as a means to terminate signals and prevent autotoxic mediator release by the effector cell. Recent data suggest that endosomes have functional importance by providing compartment-specific molecular interactions leading to the assembly of unique signaling complexes (Miaczynska et al., 2004). It has been reported that SP-A uptake by AM is clathrin-dependent (Jain et al., 2005), and further that SP-A travels along the endolysosomal pathway (Crowther et al., 2005). We confirmed that SP-A cell-association was Ca^{2+}-dependent but CRD-independent (Figure 3.15). In the current study clathrin was inhibited with amantadine, phenylarsine oxide and chlorpromazine prior to the addition of SP-A to evaluate whether SP-A uptake was connected to SP-A-mediated IκB-α stabilization. Western analysis and NF-κB activation assays revealed that IκB-α protein expression was significantly decreased in the presence of all the inhibitors used while NF-κB activity was significantly enhanced after pretreatment with CytoD, PAO and chlorpromazine. Pretreatment with amantadine increased NF-κB DNA binding activity, however a statistically significant effect could not be achieved due to the large SEM. SP-A-mediated inhibition of LPS-induced NF-κB activity is abrogated after treatment with amantadine and PAO, though the effect is statistically not significant due to the large deviation. Actin-depolarization achieved by pretreatment of the cells with cytochalasin D had similar effects, indicating that deformation/invagination for CCP formation is actin-dependent, and a requirement for endocytosis. Compared to constitutive conditions where FITC-labeled SP-A is endocytosed by AM, confocal microscopy revealed that FITC-SP-A remains on the outer membrane of

AM in the presence of the clathrin inhibitors amantadine and PAO. Together the data imply that indeed SP-A uptake *via* clathrin is a prerequisite for SP-A-modulated IκB-α stabilization and anti-inflammation and SP-A-mediated inhibition of (LPS-induced) NF-κB activity.

The clathrin assembly machinery constitutes of a number of adaptor proteins and accessory proteins involved in clathrin-coated pit (CCP) and clathrin-coated vesicle (CCV) formation. Clathrin-coat formation was thought to be initiated by the adaptor protein (AP) 2-complex, however recent data suggest that AP2 might not be a prerequisite for the formation of functionally active CCPs at the plasma membrane, but that its major function lies in cargo recruitment (Conner & Schmid, 2003). β-arrestin on the other hand is an adaptor for both, clathrin and AP2. Both clathrin adaptors differ by means of cargo recognition. While α-adaptin is a cargo-motif adaptor, β-arrestin is a cargo-specific adaptor (Owen, 2004)

The α-subunit of AP2 binds to PtdIns(4,5)P_2, bridging clathrin and the membrane. An α-adaptin specific blocking peptide was used to inhibit AP2 binding to the membrane, thus preventing a stable lattice formation *via* AP2. Pretreatment of the cells with the α-adaptin blocking peptide resulted in an enhanced NF-κB activation, and a decrease of SP-A-mediated IκB-α stabilization, as determined by electrophoretic mobility shift assays (EMSA) and Western analysis, respectively. Interestingly, the inhibition of α-adaptin induced NF-κB activity already in resting cells that was further increased by the addition of SP-A and/or LPS, suggesting that AP2 plays an important role not only by participating in SP-A endocytosis but also in maintaining a steady availability of clathrin close to the membrane in resting cells.

β-arrestin adaptor proteins play important roles in G protein-coupled receptor internalization where they have been shown to bind various signaling molecules, e.g. Src family kinases (McPherson et al., 2001).

It has been demonstrated that β-arrestin inhibits NF-κB activity through direct interaction with IκB-α (Witherow et al., 2004). In support of this notion, the inhibition of β-arrestin with a specific blocking peptide resulted in a significantly reduced SP-A-mediated IκB-α stabilization, while NF-κB activity was enhanced in cells pretreated with the blocking peptide prior to the addition of SP-A and/or LPS. The inhibitory effect of SP-A on NF-κB activation could not be re-established in the presence of all the inhibitors tested, strongly supporting the hypothesis that SP-A uptake is a prerequisite for its anti-inflammatory actions both constitutively and in the presence of LPS. These results further suggest that besides clathrin,

both α-adaptin and β-arrestin play an active role in SP-A uptake and SP-A-modulated anti-inflammation.

The consequences of CME inhibition not only affected SP-A-mediated IκB-α stabilization and NF-κB inhibition, but also prevented SP-A-induced PKCζ kinase activity. The atypical PKCζ which has been shown previously to be essential in SP-A-mediated IκB-α stabilization and anti-inflammation (Moulakakis et al., 2007), could not be activated by either SP-A or LPS in the presence of amantadine, demonstrating that CME is strongly involved in SP-A signaling.

The initial observation made by Bergeron and colleagues that activated epidermal growth factor receptors (EGFRs) and their downstream signaling factors such as Shc, Grb2 ans mSos were not found on the plasma membrane but on early endosomes, suggest that EGFR signaling persists in the endosomal compartment (DiGuglielmo et al., 1994). Emerging evidence demonstrating that other activated receptors were found in endocytic organelles, and that the impairment of CME reduced the activity of downstream signaling events, lead to the "signaling endosome" hypothesis (Miaczynska et al., 2004, von Zastrow & Sorkin, 2007). Husebye and co-workers demonstrated earlier that the inhibition of the clathrin-dependent endocytic pathway involved in the LPS/Toll-like receptor (TLR) 4 signaling cascade resulted in an enhanced LPS signaling, and thus in an increased NF-κB activation (Husebye, 2006). The severity of an exaggerated host response towards LPS may result in the development of local or systemic septic shock, multiorgan failure and death (Poltorak, 1998, Akira&Takeda, 2004). While LPS uptake by CME terminates LPS signaling in response of the host to the invader, it is hypothesized that SP-A has to enter the cell *via* endocytosis which is clathrin- and actin-dependent to be able to start its signaling cascade, and thus maintain an anti-inflammatory environment in the lung.

The current findings demonstrate the importance of SP-A´s modulation of the IκB-α/ NF-κB pathway in the pulmonary compartment to maintain an anti-inflammatory environment. CME and the atypical PKCζ have been identified as key components involved in SP-A´s immunomodulation. As uncontrolled NF-κB activation may well be deleterious, SP-A uptake and its subsequent tonic activation of PKCζ may serve as a physiological brake on induction of pro-inflammatory cytokine release in AM localized to sites of pulmonary inflammation. Further studies on the role of SP-A´s immunoregulatory mechanisms unique to the lung will

potentially solve the SP-A-signaling pathway and lead to specific therapeutic strategies in inflammatory and infectious human lung diseases.

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6 ABBREVIATIONS

µCi	micro-Curie
7TMRs	seven-transmembrane receptors
Ab	Antibody
AEC	Alveolar epithelial cells
AKBI	IκB-α knockout/ IκB-β knockin
ALL	Alveolar lining layer
AM	Alveolar macrophage
Aman	Amantadine
ANOVA	Analysis of variance
AP	Adaptor protein
APC	Antigen presenting cell
aPKC	atypical PKC
aPKCps	atypical PKC pseudosubstrate
ARDS	Acute respiratory distress syndrome
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BPI	bacteria permeability increasing protein
C1q	Complement component C1q
Ca^{2+}	Calcium
CC	Chelerythrine chloride
CCP	Clathrin-coated pits
CCV	Clathrin-coated vesicles
CLASPs	Clathrin-associated sorting proteins
CME	Clathrin-mediated endocytosis
COPD	Chronic obstructive pulmonary disease
cPKC	conventional PKC
CRD	Carbohydrate-recognition domain
CytoD	Cytochalasin D
DAG	Diacylglycerol
DPPC	Dipalmitoylphosphatidylcholine
DTT	Dithiothreitol
EDTA	Ethylendiaminetetraacetic acid
EGTA	Ethyleneglycol-bis-(2-aminoethyl ether)-N,N,N´,N´-tetraacetic acid
EMSA	Electrophoretic mobility shift assay
FACS	Fluorescence-activated cell sorting
GlcNAc	N-acetyl-glucosamine
HEPES	*N*-2-hydroxyethylpiperazine-*N´*-ethane sulfonic acid
HI-FCS	Heat inactivated-fetal calf serum
HRP	Horse raddish peroxidase
Ig	Immunglobulin

IKK	Inhibitory kappa B kinase
IL	Interleukin
IRAK1	IL-1 receptor-associated kinase
IκB-α	Inhibitory kappa B-alpha
IκB-β	Inhibitory kappa B-beta
KCl	Potassium chloride
LBP	LPS-binding protein
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
ManNAc	N-acetyl-mannosamine
MAPK	Mitogen-activated protein kinase
MBL	Mannose binding lectin
MBP	Myelin basic protein
$MgCl_2$	Magnesium chloride
MIP	Macrophage inflammatory protein
mRNA	messenger ribonucleic acid
MyD88	Myeloid differentiation factor 88
Na_3VO_4	Sodium-ortho-vanadate
NF-κB	Nuclear factor-kappa B
NOS	Nitric oxide synthase
nPKC	novel PKC
PAMP	Pathogen-associated molecular pattern
PAO	Phenylarsine oxide
PBMC	Peripheral blood mononuclear cells
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Propidium iodide
PI3K	Phosphatidylinositol-3-kinase
PIP_3	Phosphatidylinositol-triphosphate
PKC	Protein kinase C
PKCζ	Protein kinase C zeta
PRR	Pattern-recognition receptor
PS	Phosphatidylserine
PtdIns	Phosphatidylinositol
SDS-PAGE	Sodium-dodecyl-sulfate-Polyacrlyamide gelelectrophoresis
Ser	Serine
SLPI	secretory leucoprotease inhibitor
SP-A	Surfactant protein A
SP-B	Surfactant protein B
SP-C	Surfactant protein C
SP-D	Surfactant protein D
TAB2	TAK1-binding protein
TAK1	TGF-β activating kinase

Thr	Threonine
TIR	Toll/IL-1 receptor homology
TIRAP	TIR-domain-containing adaptor protein
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
TRAF6	TNF receptor-associated factor 6

SUMMARY

Emerging evidence demonstrates a pivotal role for the pulmonary collectin surfactant protein (SP)-A in anti-inflammatory modulation of lung immunity. Mice lacking SP-A are highly susceptible to bacterial and viral pneumonia displaying decreased and delayed clearance of pathogens. Administration of exogenous SP-A enhanced clearance of the pathogens and reduced lung inflammation. In its function as a pattern recognition molecule, SP-A participates actively in innate host defense by regulating the functions of other innate immunocompetent cells, such as alveolar macrophages, e.g. SP-A enhances the phagocytosis by immunocompetent cells, acts as an activation ligand, or regulates the expression of cell-surface receptors. However, the mechanisms underlying SP-A-mediated macrophage activation and inhibition of LPS-induced NF-κB activation leading to anti-inflammation *in vivo* and *in vitro* are not completely understood.

In order to elucidate the role of IκB-α in SP-A-mediated anti-inflammation, *ex vivo* experiments were performed employing IκB-α knockout/ IκB-β knockin (AKBI) mice. Although alveolar macrophages (AM) and mononuclear cells of AKBI mice released similar amounts of TNF-α in response to LPS compared to wild type cells, IκB-α was found to be essential for SP-A-mediated inhibition of LPS-induced TNF-α release and p65 nuclear translocation. Further investigations to determine potential upstream kinases involved in SP-A signaling revealed that the activity of the atypical PKCζ was critically involved in SP-A´s anti-inflammatory macrophage activation *via* stabilization of IκB-α. SP-A was found to transiently trigger aPKCThr$^{410/403}$ phosphorylation, aPKC kinase activity and translocation in primary rat AM. *Ex vivo* experiments utilizing PKCζ-deficient mice supported the data obtained *in vitro.* Co-immunoprecipitation experiments revealed that SP-A induces aPKC/p65 binding under constitutive conditions.

In order to investigate the mechanisms underlying SP-A-mediated macrophage activation, and thus modulating the intracellular signaling pathway in those cells, it was examined whether endocytosis of SP-A by AM is a prerequisite for this modulation. Clathrin-mediated endocytosis (CME) is a key uptake mechanism of SP-A by AM and type II cells and is generally considered as a means to terminate signaling. In this context it was examined whether CME inhibition would alter the SP-A-modulated IκB-α/ NF-κB pathway. The inhibition of clathrin significantly impaired SP-A-mediated IκB-α stabilization and inhibition of LPS-induced NF-κB activation. It further abrogated SP-A-induced PKCζ activity. When the most prominent adaptor proteins responsible for bridging clathrin and the plasma

membrane – α-adaptin and β-arrestin – were inhibited, the same results were observed. Obviously, CME of SP-A does not terminate signaling but rather contributes to and promotes SP-A-mediated anti-inflammatory signaling *via* the IκB-α/ NF-κB pathway.

In this study the direct modulation of SP-A on the IκB-α/ NF-κB signal transduction pathway was investigated. In the course of this task the atypical protein kinase Cζ (zeta) was identified as a pivotal upstream kinase involved in SP-A´s anti-inflammatory macrophage activation *via* IκB-α, thus attributing a novel, stimulus-specific signaling function to PKCζ in SP-A-modulated pulmonary immune response. In addition, the uptake of SP-A in a clathrin-dependent manner has proved to be essential in SP-A´s immunomodulation, providing novel insights into the requirements for SP-A anti-inflammatory signaling and modulation of the IκB-α/NF-κB pathway.

ZUSAMMENFASSUNG

Seit einigen Jahren häufen sich Forschungsergebnisse, die darauf hinweisen, dass das pulmonale Collectin Surfactant Protein (SP)-A essentiell an der anti-inflammatorischen Modulation der angeborenen Lungenimmunität beteiligt ist. SP-A-defiziente Mäuse zeigen eine gesteigerte Anfälligkeit gegenüber pulmonalen Infektionen bakterieller und viraler Herkunft, einhergehend mit einer reduzierten und verzögerten „clearance“ der Pathogenen. Die erhöhte Entzündungsreaktion in diesen Mäusen konnte durch eine exogene SP-A Substitution korrigiert werden. In seiner Funktion als „pattern recognition molecule“ stimuliert SP-A die Phagozytose und Zytokinproduktion immunkompetenter Zellen, z.B. Makrophagen. Desweiteren kann SP-A als Aktivierungsligand fungieren oder die Expression von Oberflächen-Rezeptoren regulieren. Allerdings blieben die *in vivo* und *in vitro* ermittelten anti-inflammatorischen Mechanismen, die zur SP-A-vermittelten Makrophagen-Aktivierung und Inhibition der LPS-induzierten NF-κB Aktivierung führen, bislang unverstanden.

Im Rahmen dieser Arbeit wurde die direkte Modulation von SP-A auf den IκB-α/ NF-κB Signaltransduktionsweg untersucht. Um die Rolle von IκB-α in der SP-A-vermittelten anti-Entzündungsreaktion aufzuklären, wurden *ex vivo* Experimente an IκB-α knockout/ IκB-β knockin (AKBI) Mäusen durchgeführt. Obgleich die TNF-α Freisetzung nach LPS-Stimulation in Alveolarmakrophagen (AM) und mononuklearen Zellen von AKBI-Mäusen vergleichbar mit Wildtyp-Zellen war, konnte festgestellt werden, dass für die SP-A-vermittelte Hemmung von LPS-induzierter TNF-α Freisetzung und NF-κB p65 nuklearer Translokation IκB-α unerlässlich ist. Die Suche nach potentiellen upstream Kinasen, die frühzeitig in diesen Signaltransduktionsweg eingreifen und an der SP-A-vermittelten anti-inflammatorischen Reaktion beteiligt sind, ergab, dass die Aktivität der atypischen (a) PKCζ essentiell an der anti-inflammatorischen Makrophagen Aktivierung, über die Stabilisierung von IκB-α, durch SP-A beteiligt ist. Es zeigte sich, dass SP-A eine transiente Steigerung der Phosphorylierung von aPKCThr$^{410/403}$, der Kinase-Aktivität und der Translokation von aPKC in primären AM der Ratte induziert. *Ex vivo* Experimente mit Zellen PKCζ-defizienter Mäuse bestätigten die zuvor *in vitro* erlangten Ergebnisse. Co-Immunpräzipitations-Experimente belegten, dass SP-A die Bindung von aPKC/ p65 unter konstitutiven Bedingungen steigert.

Weitere Untersuchungen, die zur Aufklärung der Mechanismen führen sollten, über die SP-A Makrophagen aktiviert und den Signaltransduktionsweg in diesen Zellen moduliert, gingen

von der Annahme aus, dass die Aufnahme/ Endozytose von SP-A durch AM eine Voraussetzung dieser Modulation ist. Die Clathrin-vermittelte Endozytose (CME) ist der Hauptaufnahme-Mechanismus von SP-A durch AM und alveolare Typ II Zellen. Generell wird die CME als ein Mechanismus betrachtet, der Signale, nach Aufnahme des entsprechenden Liganden, beendet. In diesem Zusammenhang wurde untersucht, ob die Inhibition von Clathrin den SP-A-modulierten IκB-α/ NF-κB Signaltransduktionsweg verändert. Die Hemmung von Clathrin führte zu einer signifikanten Reduzierung der SP-A-vermittelten IκB-α Stabilisierung und Inhibition der LPS-induzierten NF-κB Aktivierung. Desweiteren verhinderte die Inhibition von Clathrin die Aktivierung von PKCζ durch SP-A. Ähnliche Ergebnisse wurden erzielt, als die wichtigsten Clathrin-Adapter-Proteine – α-Adaptin und β-Arrestin – blockiert wurden, die dafür verantwortlich sind, dass Clathrin mit der Plasmamembran verknüpft wird. Offensichtlich führt die Clathrin-vermittelte Aufnahme von SP-A nicht zu einer Beendigung des Signals, sondern trägt vielmehr zu der SP-A-vermittelten anti-inflammatorischen Signalgebung über den IκB-α/ NF-κB Signaltransduktionsweg bei.

Im Verlauf dieser Arbeit konnte die atypische Protein Kinase Cζ als essentielle Kinase identifiziert werden, die an der SP-A-vermittelten anti-inflammatorischen Makrophagen-Aktivierung über die Stabilisierung von IκB-α beteiligt ist. Damit erhält PKCζ eine neuartige, Stimulus-spezifische Funktion in der SP-A-modulierten pulmonalen Immunantwort. Darüber hinaus konnte gezeigt werden, dass die Clathrin-abhängige Aufnahme von SP-A eine zentrale Bedeutung in der SP-A-vermittelten Immunmodulation hat. Diese Ergebnisse geben einen neuen Einblick darüber, welche Voraussetzungen gegeben sein müssen, damit SP-A eine anti-inflammatorische Modulation des IκB-α/ NF-κB Signaltransduktionsweges einleiten kann.

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PUBLICATIONS

Published manuscripts

1. Lee SJ, **Moulakakis C**, Koning SM, Hausner W, Thomm M and Boos W. TrmB, a sugar sensing regulator of ABC transporter genes in *Pyrococcus furiosus* exhibits dual promoter specificity and is controlled by different inducers. *Mol Microbiol*. 57(6):1797-807, 2005.
2. **Moulakakis C,** Adam S, Seitzer U, Schromm AB, Leitges M and Stamme C. Surfactant Protein A Activation of Atypical Protein Kinase C {zeta} in I{kappa}B-{alpha}-Dependent Anti-Inflammatory Immune Regulation. *J Immunol* 179: 4480-4491, 2007.
3. **Moulakakis C** and Stamme C. Role of clathrin-mediated endocytosis of surfactant protein A by alveolar macrophages in anti-inflammatory signaling. *Manuscript in preparation.*

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1. **Moulakakis C,** Adam S and Stamme C. Effects of Surfactant Protein A on proximal IκB-α/ NF-κB signaltransduction pathways. *European Respiratory Journal* 26: Supplement 49: 3561, 2005
2. **Moulakakis C,** Adam S, Seitzer U, Leitges M and Stamme C. Pivotal role of atypical Protein Kinase Cζ in Surfactant Protein A anti-inflammatory signaling. *Proceedings of the American Thoracic Society* 3: Abstract Issue: A191, 2006.

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