UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS QUÍMICAS

DEPARTAMENTO DE BIOQUÍMICA Y BIOLOGÍA MOLECULAR I

TESIS DOCTORAL

ACTIVIDAD SINÉRGICA ENTRE LA PROTEÍNA A DEL SURFACTANTE PULMONAR (SP-A) Y PÉPTIDOS ANTIMICROBIANOS EN LA DEFENSA DEL PULMÓN FRENTE A INFECCIONES

MEMORIA PARA OPTAR AL GRADO DE DOCTOR
PRESENTADA POR

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Madrid, 2015

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SYNERGISTIC ACTIVITY BETWEEN PULMONARY SURFACTANT PROTEIN A (SP-A) AND ANTIMICROBIAL PEPTIDES IN LUNG DEFENSE AGAINST INFECTION

TESIS DOCTORAL DE

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ΔH	Enthalpy of the gel-to-liquid phase transition
ANTS	1,3,6-trisulfonnic acid disodium salt (ANTS)
$\Delta T_{1/2}$	Temperature width at half-height of the DSC peak
γ	Surface tension
AAMP	Anionic antimicrobial peptide
AEC	Alveolar epithelial cell
ALI	Acute lung injury
AM	Alveolar macrophage
AMP	Antimicrobial peptide
ARDS	Acute respiratory distress syndrome
ATCC	American Type Culture Collection
BALF	Bronchoalvelar lavage fluid
BHI	Brain heart infusion
BPL	Bacterial phospholipids
β-NAD	β-Nicotinamide adenine dinucleotide hydrate.
CAMP	Cationic antimicrobial peptide
CCL	Chemokine (C-C motif) ligand 2
cDNA	Complementary deoxyribonucleic acid
COPD	Chronic obstructive pulmonary disease
CPS	Capsular polysaccharide
CRD	Carbohydrate recognition domain
CXCL8	C-X-C motif chemokines 8
DAPI	4',6-diamidino-2-phenylindole
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic acid
DPH	1,6-diphenyl-1,3,5-hexatriene

DPPC	Dipalmitoylphosphatidylcholine
DPX	p-xylene-bis-pyridinium bromide
DSC	Differential scanning calorimetry
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immune sorbent assay
FBS	Fetal bovine serum
HBD-1, -2, -3	Human beta defensin-1, -2, -3
HBSS	Hanks´s balanced salt solution
hCAP	Human cathelicidin cationic antimicrobial protein
HD	Human defensin
HDP	Host defense peptides
HNP	Human neutriohil peptides
IL	Interleukin
IPTG	Isopropyl b-d-thiogalactoside
Lα	Liquid-crystalline phase
Lβ	Gel phase
LAL	Limulus Amebocyte Lysate
LAURDAN	6-lauroyl-2-(N,N-dimethylmino)
LB	Lamellar body
LL-37	Cathelicidin
Lo	Liquid-ordered phase
LPS	Lipopolysaccharide
MBL	Mannose-binding lectine
MCP-1	Monocyte chemoattractant protein-1
MDR	Multi-drug resistant

MH-S	Murine alveolar macrophage cell line
MIP	Macrophage inflammatory protein
MLV	Multilamellar vesicle
MOI	Multiplicity of infection
MR	Mannose receptor
MVB	Multivesicular body
NET	Neutrophil extracellular trap
NF-kB	Nuclear factor-kB
NMR	Nuclear magnetic resonance
OD	Optical density
OM	Outer membrane
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PL	Phospholipid
PMB	Polymyxin B
PMBN	Polymyxin B nonapeptdie
PME	Polymyxin E or colistin
POPC	Palmitoyloleoylphosphatidylcholine
POPG	Palmitoyloleoylphosphatidylglycerol
R-LPS	Rough Lipopolysaccharide
RAW 264.7	Mouse leukaemic monocyte macrophage cell line
RDS	Respiratory distress syndrome
RNA	Ribonucleic acid

ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
S-LPS	Smooth Lipopolysaccharide
SD	Standard deviation
SDS	Sodium dodecyl-sulphate
SDS-PAGE	Sodium dodecyl-sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SIRPα	Signal inhibitory regulatory protein alpha
SP-A,-B, - B^N-C,-D	Surfactant protein A, B, B^N, C, D
SP-R210	SP-A receptor 210
TBS	Tris buffered saline
TLR	Toll-like receptor
T_m	Phase transition temperature
TM	Tubular myelin
TMB	Tetramethylbenzidine
TNF-α	Tumor necrosis factor alpha
TSA	Trypticase soy agar
TSB	Trypticase soy broth

RESUMEN

INTRODUCCIÓN

El sistema respiratorio es el encargado de suministrar oxígeno al organismo para la respiración celular. Para ser eficiente, la principal estructura donde se produce el intercambio gaseoso, el epitelio alveolar, presenta una gran superficie de contacto con la atmósfera (1). Por ello, los alveolos están continuamente expuestos a partículas inhaladas potencialmente dañinas, como alérgenos, contaminantes o patógenos. Para proteger al huésped frente a patógenos que colonizan los alveolos durante la respiración, el epitelio alveolar está equipado con una efectiva defensa inmune (2).

El sistema inmune innato en el pulmón está compuesto por un componente celular, formado por células inmunes con capacidad fagocítica, principalmente macrófagos alveolares e intersticiales; y un componente soluble, con proteínas y péptidos antimicrobianos (AMP) capaces de eliminar patógenos (3). Los AMPs, tales como β-defensinas (HBD) o el péptido derivado del extremo N-terminal de la proteína B del surfactante pulmonar (SP-B^N) (4-6), son péptidos catiónicos (HBD) o aniónicos (SP-B^N) de bajo peso molecular, evolutivamente conservados, que forman el principal sistema de defensa en la mayoría de organismos vivos (7, 8). Por otra parte, las proteínas antimicrobianas son factores solubles de mayor peso molecular, que presentan actividad bactericida y, además, son capaces de modular la respuesta inflamatoria del huésped (9, 10). Entre éstas se encuentran la lisozima, lactoferrina y las proteínas del surfactante pulmonar A y D (SP-A, SP-D) (10-12).

SP-A es la proteína más abundante en el fluido alveolar (en peso) y pertenece a la familia de colectinas, caracterizadas por la presencia de un dominio tipo lectina y un dominio tipo colágeno (11, 12). Esta familia es capaz de reconocer y facilitar la eliminación de patógenos a través del reconocimiento de patrones moleculares asociados a patógenos (13). Se ha demostrado que tanto la SP-A, como los AMPs, pueden matar directamente algunos microorganismos aumentando la permeabilidad de su membrana (13-15). La mayoría de los estudios *in vitro* con SP-A indican que se une y actúa preferentemente sobre bacterias Gram-negativas que expresan lipopolisacárido (LPS) truncado en su membrana externa (OM) (LPS rugoso), aunque también ha mostrado actividad frente a bacterias con la estructura completa del LPS (LPS liso) (16, 17). Esto podría sugerir la expresión de factores o mecanismos de resistencia a SP-A por parte de algunos patógenos pulmonares. Por otro lado, estudios *in vivo* con ratones SP-A -/- han demostrado que ratones silvestres presentan una mayor eliminación

bacteriana que ratones KO (18), incluso frente a bacterias que han sido resistentes a la acción bactericida de la SP-A *in vitro* (19, 20), indicando un papel más complejo de la SP-A *in vivo*. De este modo, nuestra hipótesis es que la SP-A actúa sinérgicamente y/o cooperativamente con diferentes AMPs presentes en el espacio alveolar para reforzar la actividad microbicida de cada factor por separado.

Esta hipótesis se basa en el hecho de que ya se han descrito previamente actividades sinérgicas en el fluido alveolar entre lisozima, lactoferrina y HBD-2 (21), así como actividades aditivas entre HBD-2 y la catelicidina LL-37 frente *E. coli* (22). El estudio de las interacciones entre la SP-A y diferentes factores solubles alveolares, y entre factores solubles y patógenos, permitiría avanzar en el conocimiento del papel de la SP-A en la inmunidad innata del pulmón. Además, ayudaría al desarrollo de nuevas terapias adyuvantes frente a infecciones por bacterias Gram-negativas, debido a la actual incidencia de bacterias multi-resistentes sin ningún antibiótico efectivo (23).

OBJETIVOS

El principal objetivo de esta tesis fue estudiar la actividad antimicrobiana de la SP-A, tanto individual como en colaboración con AMPs, en la defensa inmune innata del pulmón. Para ello, se usaron diferentes patógenos respiratorios, incluyendo *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* y *Haemophilus influenzae*, así como mutantes isogénicos de *Klebsiella* en su membrana externa, a fin de aclarar el mecanismo de resistencia bacteriana a SP-A, así como dilucidar la posible acción sinérgica de SP-A con AMPs, tanto endógenos (HBD, SP-B^{N}) como exógenos como la polimixina B (PMB) o colistina. Además, también se investigó el mecanismo cooperativo o sinérgico entre la SP-A y AMPs responsable de su actividad antimicrobiana.

Esta tesis está compuesta de 3 capítulos con los siguientes objetivos concretos:

1. Estudiar el potencial efecto protector de la SP-A frente a diferentes cepas lisas y rugosas del patógeno pulmonar *Klebsiella pneumoniae* K2; así como su habilidad para modular la actividad antimicrobiana de diversos péptidos antimicrobianos frente a varios patógenos respiratorios.
2. Determinar *in vivo* el potencial efecto terapéutico de SP-A y SP-B^{N} frente a una bacteria que es causa común de neumonía.

3. Evaluar los mecanismos mediante los cuales la SP-A y un derivado nonapeptídico de PMB (PMBN), son capaces de matar bacterias Gram-negativas sinérgicamente, cuando ni la proteína SP-A ni el péptido PMBN presentan actividad bactericida por separado.

RESULTADOS PRINCIPALES Y CONCLUSIONES

Para evaluar el papel protector de la SP-A frente a infecciones, determinamos su habilidad antimicrobiana frente al aislado clínico *Klebsiella pneumoniae* K2 (Kp wt), el cual expresa LPS liso (S-LPS) en su membrana externa (OM) y cápsula (CPS); y diferentes mutantes isogénicos de esta cepa con diferentes fenotipos de LPS rugoso, desde LPS rugoso (R-LPS) con CPS hasta LPS rugoso profundo Rc o Re sin CPS. Encontramos que la SP-A fue capaz de agregar, inhibir el crecimiento y matar mutantes descapsulados de *K. pneumoniae* con LPS rugoso profundo (Rc y Re). Por el contrario, SP-A no mostró ningún efecto antimicrobiano frente a Kp wt, mutantes rugosos descapsulados (Ra o Rb) o mutantes rugoso profundo con CPS. Además, SP-A opsonizó y aumentó la fagocitosis del mutante Rc sin CPS, y no de Kp wt, por macrófagos alveolares MH-S. Mediante experimentos de unión en fase-sólida, demostramos que la SP-A no tuvo ningún efecto frente a Kp wt, porque fue incapaz de unirse a esta bacteria. Por el contrario, SP-A sí se unió, de manera dosis-dependiente, a las cepas que fueron sensibles a ella (Rc y Re sin capsula). Estos resultados conjuntamente destacan la importancia del LPS liso y la CPS en la resistencia bacteriana a SP-A, a la vez que confirman estudios previos que indicaban la preferencia de esta proteína por bacterias que expresan LPS rugoso profundo (16, 24).

Además, evaluamos la capacidad de la SP-A para modular la actividad antimicrobiana microbicida de diferentes péptidos antimicrobianos frente a patógenos respiratorios resistentes a ella, como *K. pneumoniae* K2, *Pseudomonas aeruginosa* O1 (PAO1) and *Haemophilus influenzae* no tipable (NTHi). Para ello, se utilizaron distintos tipos de AMPs endógenos catiónicos y aniónicos, así como AMPs exógenos catiónicos. Nuestros resultados mostraron que la SP-A fue capaz de actuar sinérgicamente con concentraciones crecientes de AMPs exógenos catiónicos, como polimixina B (PMB) y colistina (PME), aumentando así la acción de dos potentes, pero citotóxicos, péptidos antibióticos de uso clínico (25, 26). Esta acción sinérgica también se vio con un derivado nonapeptídico de PMB (PMBN), que no presenta actividad bactericida, pero retiene la capacidad de unirse y alterar la estructura de la

membrana externa de bacterias Gram-negativas (27). Así, la acción sinérgica entre SP-A y PMBN derivó en una alta acción bactericida, cuando ninguno de estos factores tuvo actividad por separado.

Por otro lado, SP-A también aumentó significativamente el efecto bactericida de los AMPs catiónicos endógenos (β-defensina 2 y 3), ambos presentes en el fluido alveolar, que han sido ampliamente investigados debido a su habilidad para matar bacterias MDR (28, 29). Además, encontramos que la SP-A fue capaz de matar Kp wt en la presencia del péptido antimicrobiano aniónico, específico de pulmón, $SP\text{-}B^N$, a pH neutro. $SP\text{-}B^N$ proviene del procesamiento proteolítico de la proteína del surfactante SP-B y mata bacterias a pH ácido, pero no neutro (6). Asimismo, a través de experimentos de recuento de colonias intracelulares, encontramos que la pre-incubación de macrófagos alveolares (MH-S) y peritoneales (Raw 264.7) con SP-A y $SP\text{-}B^N$ aumentó la fagocitosis de Kp wt, comparado con células sin tratar o tratadas con SP-A o $SP\text{-}B^N$ por separado. Estos resultados juntos sugieren que la acción sinérgica de la SP-A con AMPs podría aumentar la defensa del pulmón frente a infecciones en el espacio alveolar.

Para confirmar estos resultados en condiciones fisiológicas y evaluar el posible potencial terapéutico de la SP-A en combinación con $SP\text{-}B^N$, comprobamos la actividad cooperativa entre estas proteínas *in vivo*. De este modo, 10.000 CFUs de *K. pneumoniae* K2 fueron co-administrados intratraquealmente con SP-A, $SP\text{-}B^N$ o ambos, en ratones FVB/N wt. Tras diferentes tiempos de infección, los pulmones fueron extraídos y homogeneizados para determinar la carga bacteriana por recuento de CFUs y la expresión de citoquinas por ELISA. Igualmente, se analizó el estado de los pulmones mediante histopatología, así como el numero total y el tipo de células presentes en el lavado broncoalveolar mediante tinción de centrifugados celulares. Encontramos que en los ratones tratados con $SP\text{-}A/SP\text{-}B^N$: i) la carga bacteriana pulmonar se redujo 9 veces; ii) aumentó el reclutamiento temprano de neutrófilos; y iii) mejoró la histopatología del pulmón; comparado con ratones sin tratar o ratones tratados con SP-A o $SP\text{-}B^N$ solamente. Además, se incrementó la concentración de mediadores solubles inflamatorios (TNF-α, IL-1β, -6, -17 y MIP-2) en los homogeneizados de pulmón en comparación con ratones sin tratar. De esto modo, SP-A fue capaz de cooperar con $SP\text{-}B^N$ *in vivo* para aumentar la defensa del pulmón. La acción cooperativa de $SP\text{-}A/SP\text{-}B^N$ fue consistente con: 1) una aumentada actividad microbicida de SP-A/AMP; 2) un incremento de la fagocitosis de bacterias por macrófagos alveolares inducido por SP-A/SP-

B^{N}; 3) un incremento en el reclutamiento temprano de neutrófilos en el fluido broncoalveolar inducido por SP-A/SP-B^{N}; y 3) un aumento en la respuesta inflamatoria temprana del huésped promovido por SP-A/SP-B^{N}.

Una vez que la cooperación entre SP-A y SP-B^{N} se demostró *in vivo*, evaluamos el potencial tratamiento terapéutico de SP-A/SP-B^{N}. Para ello, se infectaron ratones con *K. pneumoniae*, y tras 6h o 24h de la inoculación del patógeno, fueron tratados con SP-A más SP-B^{N}. Nuestros resultados indican que el tratamiento terapéutico de SP-A y SP-B^{N} protegió significativamente a los ratones frente a la infección por *K. pneumoniae* K2.

Finalmente, determinamos algunos de los mecanismos por los cuales SP-A mata bacterias sinérgicamente con AMPs. Para ello, estudiamos las interacciones moleculares entre SP-A y AMPs, y empleamos modelos de membranas bacterianas para investigar posibles cambios en la estructura y permeabilidad de membrana inducida por SP-A/AMPs.

Mediante experimentos de unión en fase-sólida, de dispersión de luz dinámica y cambios en la fluorescencia intrínseca de la proteína, encontramos que la SP-A es capaz de interaccionar con beta-defensina humana-3 (HBD-3), SP-B^{N} (K_d=0.39±0.3 µM), PMB (0.32 ± 0.04 µM), and PMBN (K_d =0.26 ± 0.02 µM) de manera dosis-dependiente. Además, esta interacción proteína-proteína facilita la unión de SP-A a *K. pneumoniae* K2 (con la que SP-A sola es incapaz de interaccionar).

Por otro lado, utilizamos un derivado nonapeptídico de la PMB, PMBN, sin capacidad bactericida, para estudiar el mecanismo de acción sinérgico de estas dos proteínas sobre membranas modelo de bacterias Gram-negativas. Primeramente, la acción sinérgica entre SP-A y PMBN frente a bacterias Gram-negativas (*Klebsiella pneumoniae* K2 and *Pseudomonas aeruginosa* O) se confirmó *in vitro*, donde ninguna de estas proteínas fue capaz de matar estas bacterias por separado. Dado que los modelos de membrana externa bacteriana se hacen normalmente con Re-LPS, en vez de S-LPS, también evaluamos la actividad sinérgica de SP-A y PMBN frente al mutante descapsulado isogénico de *K. pneumoniae* K2, que expresa LPS rugoso profundo y es susceptible a la acción de la SP-A. Encontramos que ni PMBN, ni SP-A a bajas concentraciones, fueron capaces de matar esta cepa de *K. pneumoniae* K2. Sin embargo, PMBN y SP-A (a bajas concentraciones de SP-A) juntas, fueron capaces de matar el mutante descapsulado de *K. pneumoniae* K2, que expresa LPS rugoso profundo.

Para identificar los mecanismos de la acción bactericida de PMBN/SP-A, medimos la capacidad de estas proteínas de insertarse en la membrana externa bacteriana mediante mediciones en la presión superficial de monocapas de Re-LPS tras la adición de las proteínas en la subfase acuosa. Encontramos que PMBN y PMBN/SP-A, pero no SP-A, fueron capaces de insertarse en estas monocapas, y que la inserción de la PMBN incrementó en presencia de SP-A. Además, mediante cambios en la fluorescencia de la sonda anfipática LAURDAN, así como por calorimetría diferencial de barrido, demostramos que la inserción de SP-A/PMBN aumentó la fluidez de membranas externas bacterianas compuestas por Re-LPS/fosfolípidos bacterianos (PL)(8/2 w/w). Estas alteraciones de membrana provocadas por las dos proteínas fueron suficiente para permeabilizar la membrana externa bacteriana, como demostramos mediante cambios en la fluorescencia del DPH (sonda hidrofóbica de membrana) y la liberación del fluoróforo hidrofílico ANTS, que fue co-encapsulado con su inactivador colisional dentro de vesículas unilamelares grandes de Re-LPS/PL (8/2 w/w). Por el contrario, la presencia de SP-A no tuvo ninguna influencia en la permeabilidad de membrana inducida por PMBN en membranas internas bacterianas compuestas por fosfolípidos bacterianos (PE/PG/CL). Analizando los resultados en conjunto, parecen indicar que la SP-A y PMBN cooperan conjuntamente para matar bacterias, porque ambas juntas son capaces de perturbar y permeabilizar la membrana externa bacteriana.

Basándonos en los resultados de la actividad sinérgica entre SP-A y PMBN, y otros AMPs, proponemos el siguiente mecanismo de acción:

i. La unión de la SP-A a AMPs permitiría el acceso de la SP-A a bacterias que expresan LPS liso en su membrana externa y/o cápsula.
ii. SP-A y AMPs inducen la perturbación de la estructura de la OM y, así, SP-A es entonces capaz de extraer moléculas de LPS de la membrana, como ha sido previamente publicado (30). Esto provoca una pérdida de LPS de la membrana, produciendo defectos transitorios en la membrana de LPS.
iii. El incremento de la fluidez de membrana y su desestabilización inducidos por SP-A/AMPs produce un incremento de la permeabilidad de la OM, que permitiría el acceso de los AMPs a la membrana interna bacteriana (IM).
iv. La perturbación de la IM bacteriana por los AMPs provocaría la muerte bacteriana y/o la translocación de los AMPs al citoplasma y su interacción con dianas intracelulares.

Basándonos en los resultados de esta tesis, concluimos que la SP-A es capaz de incrementar la actividad microbicida de AMPs catiónicos y aniónicos. De este modo, la SP-A es capaz de fortalecer la acción antimicrobiana de AMPs frente bacterias Gram-negativas. Más estudios sobre actividades sinérgicas entre factores antimicrobianos con potencial uso clínico deberían ser considerados para el desarrollo de nuevas terapias adyuvantes o alternativas para el tratamiento de infecciones respiratorias.

SUMMARY

INTRODUCTION

The respiratory system is mainly involved in providing oxygen to the organism for cellular respiration. To be efficient, the alveolar epithelium constitutes a large contact surface with the atmosphere (1). As a consequence, alveoli are constantly exposed to inhaled particles such as allergens, contaminants, and pathogens. Therefore, the alveolar epithelium is equipped with an effective immune defense in order to protect the host against pathogens reaching the alveoli during inspiration (2).

Innate immune defense in the alveolar space is composed of a cellular component, comprising immune phagocytic cells, mainly alveolar macrophages; and a soluble component, consisting of antimicrobial peptides (AMPs) and proteins able to eliminate pathogens (3). AMPs, like β-defensins (HBD) or the peptide derived from N-terminal domain of pulmonary surfactant protein B ($SP\text{-}B^N$) (4-6), are small cationic (HBD) or anionic ($SP\text{-}B^N$) conserved peptides of the innate immune response and the principal defense system for the majority of living organisms (7, 8). Alveolar antimicrobial proteins such as lysozyme, lactoferrin, SP-A, and SP-D are host soluble factors of higher molecular weight (10-12). They have direct antimicrobial activity and are able to modulate the inflammatory response of the host (9, 10).

SP-A is the most abundant protein of the alveolar fluid (by weight) and belongs to the collectin family, whose members are characterized by the presence of a lectin-like domain and a collagen-like domain. This family is able to recognize and facilitate the clearance of pathogens through interaction with pathogen-associated molecular patterns (11, 12). SP-A, like AMPs, has been proven to directly kill some microorganisms by increasing membrane permeabilization (13-15). Most *in vitro* studies of SP-A antimicrobial activity indicate that it binds to and acts preferably on Gram-negative bacteria expressing truncated LPS on their OM (rough LPS), but shows also activity against bacteria expressing the entire structure of LPS (smooth LPS) (16, 17). These findings suggest the expression in some respiratory pathogens of factors with bacterial resistance to SP-A. On the other hand, *in vivo* studies with SP-A -/- mice show much greater microbial clearance in SP-A wt than SP-A -/- mice after infection (18), even on bacteria resistant to the bactericidal activity of SP-A (19, 20); thereby suggesting more complex mechanisms of the antimicrobial activity of SP-A *in vivo*. We hypothesize that SP-A acts synergistically or cooperatively with different AMPs present in the alveolar fluid to strengthen the microbicidal activity of each factor alone.

This hypothesis is based on the facts that synergistic activity between lisozime, lactoferrin and HBD-2 has already been described in the alveolar fluid (21), and HBD-2 and cathelicidin LL-37 exhibit additive activities against *E. Coli* (22). The study of SP-A interaction with several alveolar soluble factors and between soluble factors and pathogens would further advance understanding of SP-A's role in innate immunity. It would also help to develop new adjunctive therapies against Gram-negative infections, due to the emergence of multi-drug resistant (MDR) bacteria without any effective antibiotic (23).

OBJECTIVES

The main objective of this thesis was to study both individual and cooperative antimicrobial activities of SP-A in airway host defense. To accomplish this task, several respiratory Gram-negative pathogens, including *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Haemophilus influenzae*, as well as isogenic mutants of *Klebsiella* in its OM, were used to clarify the mechanism of bacterial resistance to SP-A and the synergistic activity of SP-A with cationic and anionic AMPs, either endogenous or exogenous. In addition, the mechanism by which SP-A kills bacteria in conjunction with these factors was investigated.

This thesis is composed of three chapters with the following concrete objectives:

1. To study the potential protective effect of SP-A against different smooth and rough strains of the lung pathogen *Klebsiella pneumoniae* K2, as well as its ability to modulate the antimicrobial activity of exogenous and endogenous cationic AMPs against several respiratory pathogens.
2. To investigate the potential cooperative therapeutic action of SP-A and the lung anionic antimicrobial peptide SP-B^N against a common bacterial cause of pneumonia *in vivo*.
3. To determine the mechanism by which SP-A and PMBN, a derivative of polymyxin B, are able to kill Gram-negative bacteria synergistically, when neither of these proteins has microbicidal activity alone.

MAIN RESULTS AND CONCLUSIONS

To evaluate the protective role of SP-A against infection, we tested its antimicrobial ability against the clinical isolate *Klebsiella pneumoniae* K2 (Kp wt), which expresses smooth LPS (S-LPS) in its outer membrane (OM) and capsule (CPS), and different isogenic mutants expressing several LPS phenotypes, from rough LPS (R-LPS) with CPS to deep rough Rc- or Re-LPS without CPS. We found that SP-A was able to aggregate, inhibit the growth and kill isogenic decapsulated *Klebsiella pneumoniae* K2 mutants expressing deep rough LPS (Rc-LPS and Re-LPS). In contrast, SP-A did not have any antimicrobial effect against Kp wt, decapsulated rough mutants (Ra or Rb) or deep rough mutants expressing CPS. In addition, SP-A opsonized and enhanced phagocytosis of the Rc-LPS mutant, but not Kp wt, by alveolar macrophages. By employing solid-phase binding assay, we demonstrated that SP-A did not have any effect on Kp wt because it was unable to bind this bacterium. On the contrary, SP-A bound in a dose-dependent manner to decapsulated deep rough mutants. Together, these results highlight the importance of smooth LPS and CPS in bacterial resistance to SP-A, and confirm previous studies that indicate SP-A's preference for bacteria expressing deep rough LPS (16, 24).

Next, we evaluated whether SP-A was able to modulate the microbicidal action of different antimicrobial peptides against respiratory pathogens resistant to SP-A such as capsulated *Klebsiella pneumoniae* K2 (Kp wt), *Pseudomonas aeruginosa* O1 (PAO1), and non-typeable *Haemophilus influenzae* (NTHi). For this purpose, several kinds of cationic and anionic endogenous AMPs and cationic exogenous AMPs were used. We found that SP-A acted synergistically with increasing concentrations of exogenous cationic AMPs, such as polymyxin B (PMB) and colistin (PME), thus enhancing the action of these potent, but cytotoxic, clinical antibiotics (25, 26). This synergistic action was also found with a nonapeptide derivative from polymyxin B (PMBN) which does not have bactericidal activity but is able to bind and disturb the structure of the outer membrane of Gram-negative bacteria (27). Therefore, the synergistic action between SP-A and PMBN resulted in high bactericidal action against Gram-negative bacteria, when neither SP-A nor PMBN exhibited microbicidal activity alone.

On the other hand, SP-A significantly increased the bactericidal effect of endogenous cationic AMPs (human β-defensins 2 and 3) present in the alveolar fluid, which has been widely investigated due to their ability to kill MDR bacteria (28, 29). Furthermore, we found

that SP-A was able to kill Kp wt in the presence of the lung-specific anionic antimicrobial peptide SP-B^N at neutral pH. SP-B^N comes from the proteolytic processing of the surfactant protein SP-B and kills bacteria at acidic, but not neutral, pH (6). We found that pre-incubation of alveolar and peritoneal macrophages with SP-A and SP-B^N resulted in a dramatic uptake of Kp wt, compared with untreated cells or cells treated with SP-A or SP-B^N alone. Taken together, these results suggest that synergistic action of SP-A with AMPs in the alveolar space might enhance lung host defense against infection.

Next, we proved the cooperative action of SP-A and SP-B^N *in vivo* in physiological conditions, and evaluated the potential therapeutic treatment of SP-A in combination with SP-B^N. Therefore, 10,000 CFUs of *K. pneumoniae* K2 were intratracheally co-administered with SP-A, SP-B^N or both, in wt FVB/N mice. After several infection times, the lungs were harvested and homogenized to determine the lung bacterial burden by CFU count, and cytokine expression by ELISA. In addition, lung condition was analyzed by histopathology and the broncoalveolar lavages were used to evaluate the number and type of alveolar cells by staining of cytospin preparations. We found that in SP-A/SP-B^N-treated mice: i) lung bacterial burden was reduced 9-fold; ii) early neutrophil recruitment was enhanced; and iii) lung histopathology was ameliorated in comparison with untreated mice or mice treated with SP-A or SP-B^N alone. In addition, the concentrations of soluble inflammatory mediators (TNF-α, IL-1β, -6, -17 and MIP-2) in lung homogenates increased early in infection in comparison with untreated mice. Consequently, SP-A was able to cooperate with SP-B^N in *vivo* to enhance lung defense. This SP-A/SP-B^N's action was consistent with: 1) enhanced SP-A/SP-B^N microbicidal activity; 2) SP-A/SP-B^N-induced increase of bacterial phagocytosis by alveolar macrophages; 3) SP-A/SP-B^N-induced increase of neutrophil recruitment in the alveolar fluid; and 4) SP-A/SP-B^N-induced enhance of host early inflammatory response.

Once the cooperation between SP-A and SP-B^N *in vivo* was demonstrated, we evaluated the potential therapeutic treatment of SP-A/SP-B^N. To this end, mice were infected with *K. pneumoniae*, and after 6h or 24h of bacterial challenge, they were treated with SP-A plus SP-B^N. Our results indicate that therapeutic treatment of SP-A and SP-B^N conferred a significant protection against *K. pneumoniae* K2 infection.

Finally, we studied some of the mechanisms by which SP-A kills bacteria synergistically with AMPs. To accomplish this, we studied molecular interactions between SP-A and AMPs,

and we used bacterial model membranes to investigate possible changes in membrane structure and permeability induced by SP-A/AMP.

We found that SP-A is able to interact with human-beta-defensin-3 (HBD-3), SP-B^N (K_d=0.39±0.3 μM), PMB (0.32 ± 0.04 μM), and PMBN (K_d =0.26 ± 0.02 μM) in a dose-dependent manner as demonstrated by solid-phase binding assay, dynamic light scattering and changes in the intrinsic fluorescence of SP-A. This protein-protein interaction facilitates SP-A binding to *K. pneumoniae* K2 (which cannot be recognized by SP-A alone).

Next, we used a nonapeptide derivative from PMB (PMBN) without antimicrobial activity to study the mechanisms of synergic action of these proteins on bacterial model membranes. The synergic action of SP-A and PMBN against Gram-negative bacteria (*Klebsiella pneumoniae* K2 and *Pseudomonas aeruginosa* O) was first confirmed using an in vitro bacterial killing assay. Neither SP-A nor PMBN was able to kill these bacteria alone. Given that model membranes of the bacterial OM are usually made of Re-LPS instead of S-LPS, we also tested the synergic activity of SP-A and PMBN against the decapsulated isogenic mutant of *Klebsiella pneumoniae* K2 expressing deep rough LPS, which is susceptible to SP-A action. We found that neither PMBN nor SP-A at low concentrations was able to kill this strain of *K. pneumoniae* K2. However, PMBN and SP-A (at low SP-A concentrations) were jointly able to kill decapsulated *Klebsiella pneumoniae* K2 mutant, expressing deep rough LPS.

To identify the mechanism of PMBN/SP-A killing, we measured the capability of these proteins to insert into bacterial outer membranes by measuring the surface pressure of Re-LPS monolayers after protein injection into the aqueous subphase. We found that PMBN and SP-A/PMBN, but not SP-A, were able to insert into these monolayers, and PMBN insertion significantly increased in the presence of SP-A. SP-A/PMBN membrane insertion enhanced membrane fluidity of outer bacterial membranes composed of Re-LPS/bacterial phospholipids (PL) (8/2 w/w), as demonstrated by changes in the steady-state anisotropy of DPH (membrane hydrophobic probe), fluorescence of LAURDAN amphipathic probe, as well as by differential scanning calorimetry. These membrane disturbances induced by SP-A/PMBN, but not by SP-A or PMBN alone, resulted in membrane permeabilization, as demonstrated by changes in DPH fluorescence (membrane hydrophobic probe) and the leakage of the hydrophilic fluorophore ANTS, which was co-encapsulated with its collisional quencher DPX inside Re-LPS/PL (8/2 w/w) large unilamellar vesicles. In contrast, the presence of SP-A did not have any influence on

PMBM-induced permeability of the inner bacterial membrane composed of bacterial phospholipids (PE/PG/CL). Taken together, these results indicate that SP-A and PMBN cooperate in killing bacteria because they are jointly able to disturb and permeabilize the outer bacterial membrane.

Based on our results on the synergistic activity between SP-A and PMBN, and other AMPs, we propose the following mechanism of action:

i. SP-A binding to AMPs would allow SP-A access to bacteria that express smooth LPS in its outer membrane and/or capsule.

ii. SP-A and AMP induce perturbation of OM structure and SP-A would then be able to extract LPS molecules from the membrane as previously reported (30). This results in a loss of LPS from the membrane. As a consequence, transient defects are produced, rendering the LPS membrane leaky.

iii. SP-A/AMP-dependent increase of bacterial OM fluidity and destabilization result in increased OM permeability, which would allow AMPs access to the bacterial inner membrane (IM).

iv. AMP-induced leakage of bacterial IM would promote cell death and/or AMP translocation into the cytoplasm, and AMP interaction with intracellular targets.

Overall, based on results of this thesis, we conclude that SP-A is able to increase the microbicidal activity of cationic and anionic AMPs. Therefore, SP-A is able to strengthen the antimicrobial action of AMPs against Gram-negative bacteria. Further studies of synergistic activities between antimicrobial factors with potential therapeutic use should be considered to develop new alternative or adjuvant therapies to treat respiratory infections.

INTRODUCTION

1. RESPIRATORY SYSTEM

The main function of the respiratory system is providing continuous gas exchange between inspired air and blood in the pulmonary circulation. Survival is dependent upon this process being reliable, sustained and efficient, because cells of the organism require oxygen to generate the chemical energy necessary for the maintenance of various metabolic essential processes taking place in tissues and organs. Additionally, CO_2 generated from these processes is a metabolic waste, which has to be removed from the organism.

The respiratory system extends from mouth and nose to the alveoli and it is classically divided into upper and lower respiratory tract (Fig. 1) (31):

A. Upper respiratory tract: it consists of nose, pharynx and larynx and serves to filter airborne particles as well as to humidify and warm the inspired gases before they reach the lower respiratory zone.

B. Lower respiratory tract: it starts with the trachea and is followed by the bronchial-tree, which is progressively branched into right and left bronchi, bronchioles, alveolar sacs, and finally, into the main structure where gas exchange takes places, the alveoli.

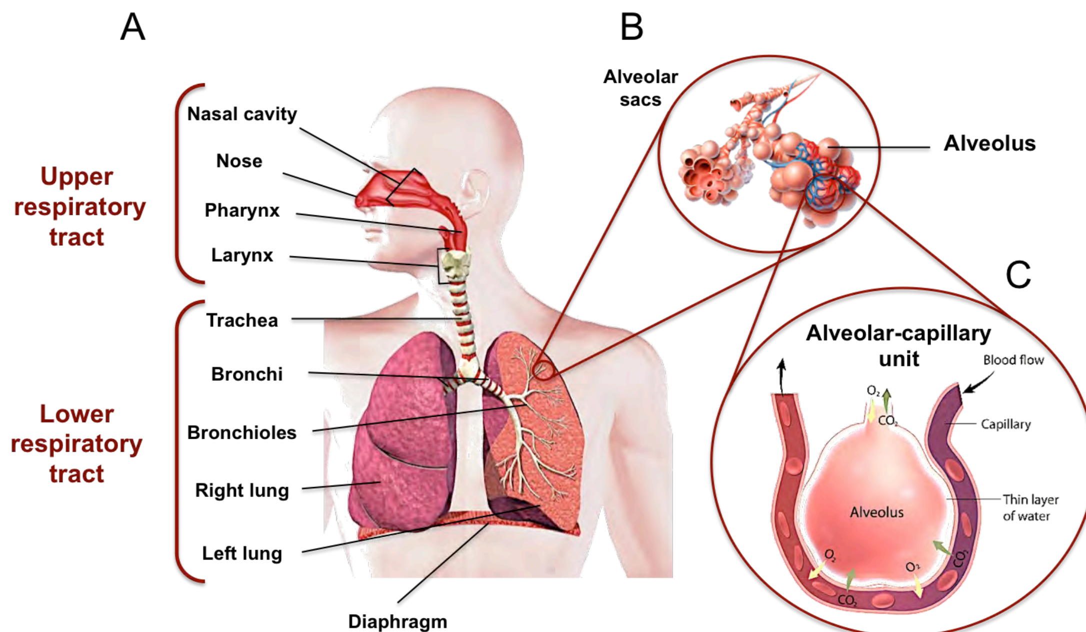

Figure 1. A) Schematic representation of the human respiratory system. B) Grouped alveoli forming two alveolar sacs. C) Schematic representation of gas exchange in the lungs.

Another classification of the respiratory tract is based most exactly on its functions and according to this, it can be divided into two zones: the conducting zone, comprising the nasal cavity, pharynx, larynx, trachea, bronchi, and bronchioles; and the respiratory zone, which is composed of the terminal and respiratory bronchioles, alveolar ducts, alveolar sacs and the alveoli, where gas exchange occurs (32).

The lung has the largest area of the body in contact with the external environment, which is necessary for gas exchange between the alveoli and pulmonary capillaries through passive diffusion. Once gas exchange occurs, oxygen enters into the blood capillaries from the alveoli and is distributed to the entire body by bloodstream. On the other hand, CO_2 is moved into the alveoli, where is removed to the atmosphere.

Consequently, the alveolar-capillary unit is the main structure involved in gas exchange (Fig. 2). It comprises three layers:

A. Capillary endothelium: composed of endothelial cells, which line the inner surface of the pulmonary capillaries and form an interface between bloodstream and alveoli. They exert critical gas exchange and metabolic functions.

B. Interstitial layer: it is made up of an intermediate connective tissue of elastic and collagen fibers where mesenchymal cells (fibroblasts) and immune system cells (lymphocytes, interstitial macrophages and mast cells) are found. It is placed between the capillary endothelium and the alveolar epithelium and support alveolar structure through the secretion of extracellular matrix proteins.

C. Alveolar epithelium: it is considered to be a physical and functional barrier, which is also involved in the clearance of environmental agents. It is found above interstitial layer and covers the entire surface of each alveolus. The alveolar epithelium (Fig. 2) is mainly composed of two types of cells, type I and type II pneumocytes or alveolar epithelial cells (1):

    - Type I pneumocytes: these cells cover 90% of the airway surface due to their large flattened phenotype and whose main function is gas exchange.

    - Type II pneumocytes: constitute the remaining 10% of the alveolar surface. They display proliferative and innate immune functions and are responsible for the synthesis and secretion of pulmonary surfactant, a lipid-protein complex that

is involved in reducing surface tension at the air/liquid interface of the alveoli. Hence, these pneumocytes exhibit a cubic morphology typical of secretory cells with microvilli on its apical area.

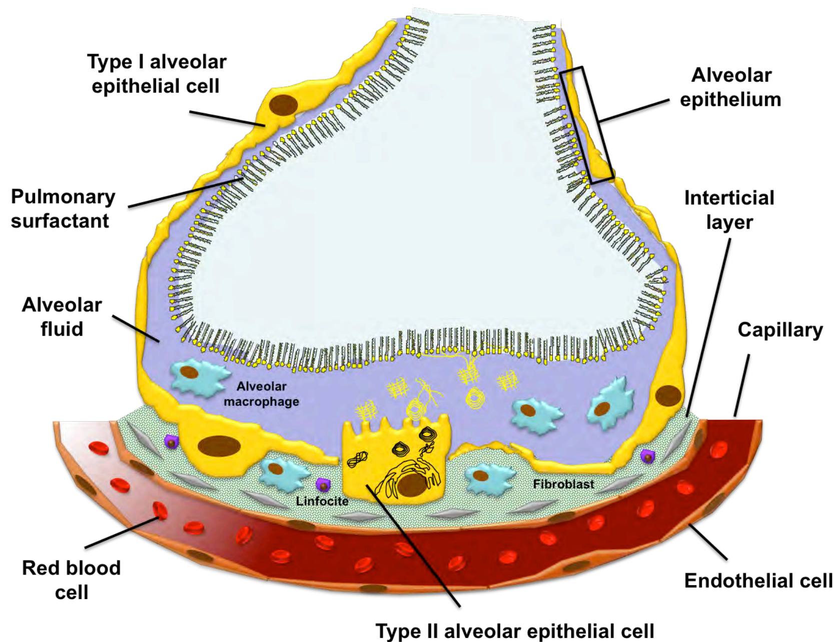

Figure 2. Schematic representation of the alveolar-capillary unit showing their three layers and their components: alveolar epithelium, interstitial layer and capillary endothelium.

Additionally, pneumocytes are covered by a thin layer of aqueous fluid, called alveolar fluid, which contains immune cells, mainly resident alveolar macrophages that coordinate host defense, as well as soluble proteins and peptides with antimicrobial and immunomodulatory activity (2). Moreover, in the alveolar fluid is also found pulmonary surfactant, which is located at the air-liquid interface allowing gas exchange while maintaining alveolar homeostasis. Thus, pulmonary surfactant keeps the structure of the alveoli against possible pressure differences and protects the body from potential pathogen invasion.

1.1. PULMONARY SURFACTANT

Pulmonary surfactant is a complex network of protein-containing extracellular membranes that overlies the alveolar epithelium and is involved in surface tension reduction and lung defense (33, 34). Since it was discovered by Clements and colleagues in the early 1960s (35),

pulmonary surfactant has been widely studied to discern its functions, composition, metabolism and importance in pulmonary diseases.

Surfactant membranes are synthesized by type II pneumocytes and form a stable monolayer at the air–liquid interface with bilayer structures attached to it (Fig. 3) (11). After its biosynthesis and prior to secretion, surfactant components are assembled into complexes stored as tightly packed membranes, called lamellar bodies. Once secreted, the lamellar bodies components transform into tubular myelin, an unusual ordered network of membranes with lattice-like structure that finally forms the surfactant film by its rapid adsorption and spreading over the air-liquid alveolar interface (Fig. 3) (33). Because of surfactant is continuously suffering compression and expansion cycles, it is constantly secreted by and recycled into the type II pneumocytes, in order to maintain constant surfactant pool size (Fig. 3) (36).

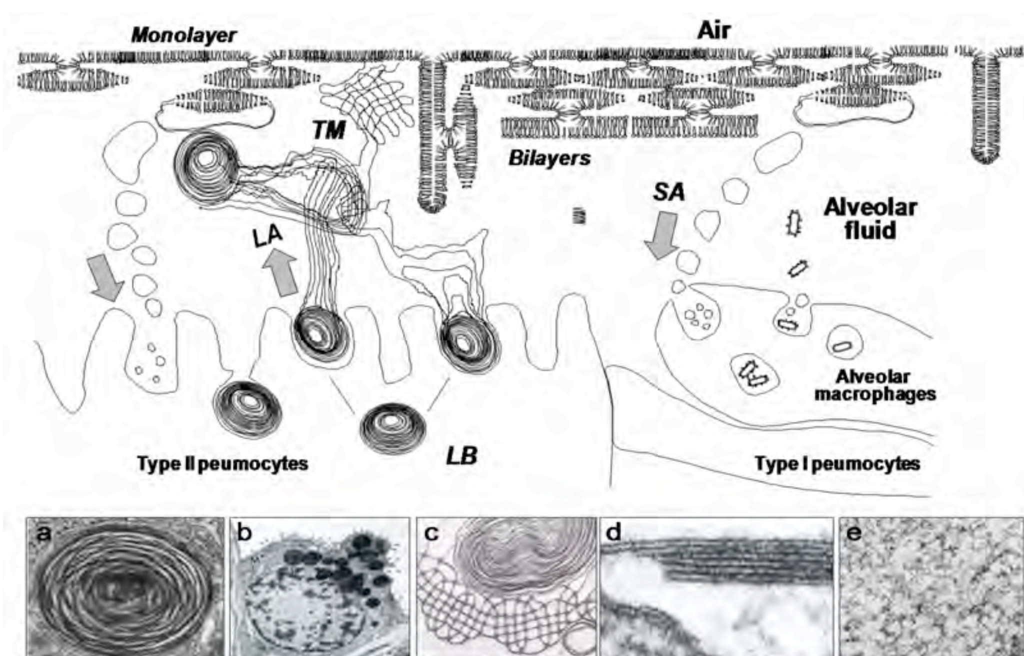

Fig. 3. Schematic representation of pulmonary surfactant secretion and recycling. After synthesis by type II pneumocytes, pulmonary surfactant is stored as tightly packed bilayer membranes called lamellar bodies (LB) (a) that are then secreted by exocytosis (b) to the alveolar fluid, where spontaneously unravel in a lattice-like structure termed tubular myelin (TM) (c) and large surfactant aggregates (LA). They have high surface activity and adsorb very rapidly to the air–liquid interface. These structures form a surface film at the alveolar air/liquid interface consisting of a phospholipid monolayer with bilayer structures attached to it (d). With surface compression and expansion cycles,

small surfactant aggregates (SA) with poor surface activity are generated, which are taken up and degraded by alveolar macrophages and type II cells for recycling. Taken from (11)

Pulmonary surfactant contains about 90 % of lipids and 10 % of proteins (by weight) (Fig. 4A). The lipid composition of mammalian extracellular surfactant is remarkably similar among diverse species (37). Lipids consist mainly of phospholipids (90-95 weight %) with a small amount of neutral lipids (5-10 weight %), mostly cholesterol, which could play a membrane structural role (11). The main lipid comprising pulmonary surfactant is dipalmitoylphosphatidylcholine, molecular species of saturated phosphatidylcholine. This lipid can be highly and densely packed at the air–water interface, becoming the main factor implicated in surface tension reduction (33).

The surfactant contains about 10% of acidic phospholipids, especially phosphatidyl glycerol and phosphatidylinositol, and also, minority fractions of sphingomyelin, phosphatidylserine or phosphatidylethanolamine (38). The presence of anionic phospholipids appears to be important in specific interactions with hydrophobic proteins, while unsaturated lipids are related to fluidizing surfactant membranes.

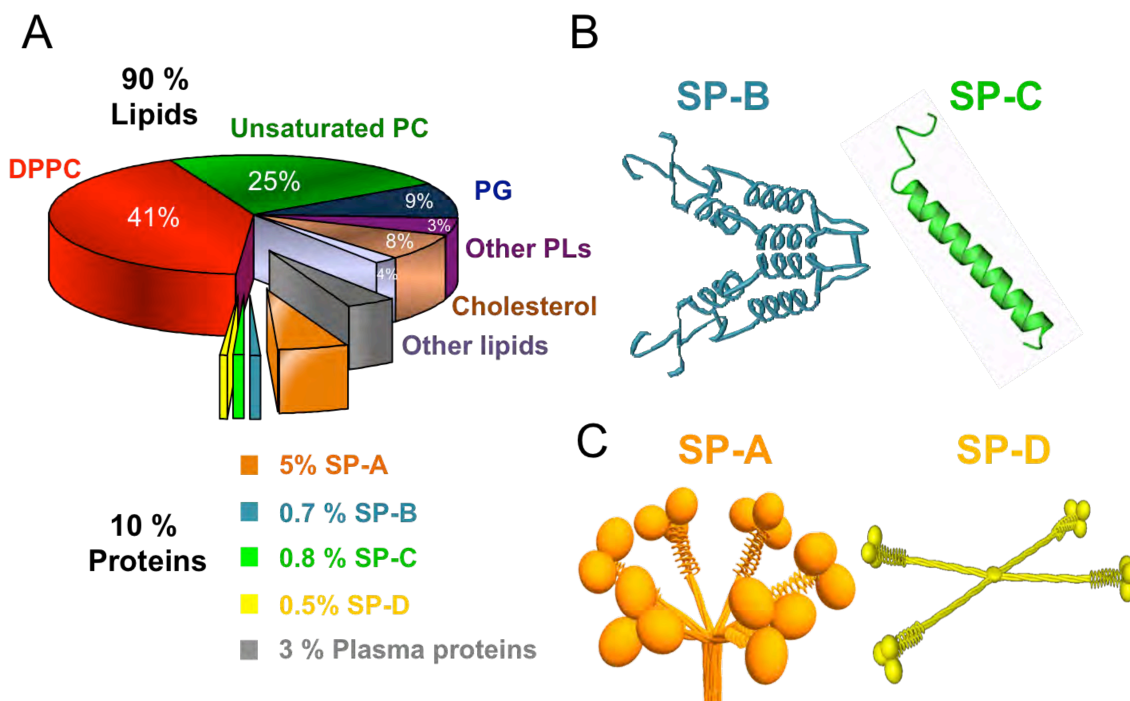

Figure 4. A) Pulmonary surfactant composition. **B)** Hydrophobic surfactant proteins. C) Hydrophilic surfactant proteins. PL: Phospholipid; DPPC: dipalmitoylphosphatidylcholine; PC: phosphatidylcholine; PG: phosphatidylglicerol; SP-: surfactant protein-.

Regarding the protein component of the pulmonary surfactant, four surfactant-associated proteins have been described: the hydrophobic lipoproteins surfactant protein B and C (SP-B and SP-C) (Fig. 4B); and the hydrophilic pulmonary collectins, surfactant protein A and D (SP-A and SP-D) (Fig. 4C). SP-B and SP-C are small and extremely hydrophobic proteins mainly involved in surface activity (39). On the other hand, SP-A and SP-D are large, hydrophilic glycoproteins that belong to the collectin mammalian super family (40). SP-A is the most abundant protein of pulmonary surfactant, comprising 50-70% of the surfactant proteins (w/w), and is mostly associated with surfactant membranes, although it is also soluble in the alveolar fluid. In contrast, SP-D is the most soluble surfactant protein, since approximately 75% of SP-D is found in the alveolar fluid, non-associated with surfactant lipids (41). Because of their ability to bind multiple ligands, they are mainly involved in host defense (12).

Pulmonary surfactant, besides permitting gas exchange, is able to maintain normal respiratory mechanics by reducing the surface tension at the air/liquid interface in the alveolar fluid; thus preventing lung collapse at the end of expiration (36). Moreover, it contributes to avoid pulmonary edema because it is able to keep alveolar fluid out from alveolar space. In addition, as pulmonary surfactant is the last structure that is found by the inspired air before entry into the body, it plays an essential role in lung defense (34).

2. DEFENSE MECHANISMS IN THE RESPIRATORY SYSTEM

To have an effective gas exchange in the lungs, respiratory system needs a large contact surface with the atmosphere. Thereby, it is continuously exposed to inhaled particles such as pathogens, allergens and contaminants. To protect the host against inhaled microbes and dangerous particles reaching the alveoli during inspiration, there are effective innate defense mechanisms (2).

2.1. PHYSICAL BARRIERS

The first defense mechanisms of the respiratory system are structural and physiochemical adaptations of the respiratory tract, which act as a mechanical barrier to block the entry of

microorganism and particles. Physical defenses are then mainly conducted by the upper airway filtering systems, the airway epithelium and the mucociliary system.

The upper airway filtering system is a mechanism by which the upper respiratory tract reduces the access of gases and particulates to the lung by physical means. Nose and nasopharinx are the structures mainly involved in this defense (42). They are remarkably efficient mechanisms that remove most airborne particles and water-soluble gases from inspired air. In addition, this anatomic mechanism is augmented by the upper airway reflexes, mostly sneezing and coughing reflexes, which clear unwanted substances from the nose, pharynx, and large airways (43).

Another physical defense is conducted by the airway epithelium, which forms a complex physicochemical barrier between the human body and the atmosphere (32). Infections occur only when pathogens can overpass these barriers. Epithelial cells are, therefore, held together by tight, adherents, and gap junctions, as well as desmosomes, in order to form an effective impermeable and mechanical barrier against the external environment (44). Consequently, in the absence of wounding or disruption, pathogens normally have to cross epithelial barriers by adhering to and colonizing these surfaces. To minimize bacterial attachment and colonization, the airway epithelia have another mechanism of defense, the mucociliary escalator, which is conducted by mucus and cilia (45).

The mucus is continuously secreted by submucosal glands and goblet cells that exist in the upper and lower airways. Therefore, epithelium is covered by an overlying mucus layer, which creates a semipermeable barrier that enables the exchange of nutrients, water, and gases while being impermeable to most pathogens (32). The airway mucus is mainly composed of mucins, which are large highly charged glycoproteins that cross-link to form the structural framework of the mucus (46). In addition, almost 200 different proteins have been identified in the mucus, such as antimicrobial substances, cytokines, and antioxidant proteins, which, along with mucins, allow this layer to also participate in the immune response of the lung (47).

Ciliated epithelial cells of the mucosal epithelia complete the mucociliary escalator defense by facilitating removal of particles and microorganism from the lung (48). The pseudostratified columnar respiratory epithelium consists of ciliated cells, non-ciliated secretory cells, and basal cells, which vary in size and ratio from the upper respiratory

epithelium to the lower respiratory epithelium (32). Therefore, ciliary movements direct overlying mucus-containing particulates and absorbed gases toward the pharynx where it is swallowed or expectorated. The efficacy of mucus flow in clearing infection is illustrated by people with defective mucus secretion or inhibition of ciliary movement, since they frequently develop lung infections caused by bacteria that colonize the epithelial surface (42).

2.2. INNATE IMMUNTY IN THE ALVEOLAR SPACE

The main protective role of the upper respiratory tract is avoiding the entrance of large inhaled particles to the respiratory zone (32). However, smaller microorganisms or allergen can escape this defense and reach the alveolar space, the last protective barrier before enter into the organism. Nevertheless, the alveoli contain a potent innate immune system, which is specialized in the non-sterile conditions of the alveolus (2).

The innate immune system in the alveolar space can be divided into the soluble component, which comprises ubiquitous and secreted antimicrobial peptides and proteins able to neutralize and eliminate microbial components and pathogens, and the cellular component, composed of resident and recruited specific cells that participate actively in host defense (3).

2.2.1 SOLUBLE/HUMORAL COMPONENT

Humoral response in the lungs is conducted by antimicrobial peptides (AMPs) and proteins present in the airways, which have an essential role in innate immunity in the lungs.

I. Antimicrobial Peptides or Host Defense Peptides

AMPs are evolutionarily conserved components of the innate immune response and the principal defense system for the majority of living organisms (49). They comprise polypeptides of fewer than 100 amino acids that are involved in defense against microorganisms, as seen by extensive research in the last decades. For example, mice lacking the endogenous beta-defensin 1 have shown increased susceptibility to *Staphylococcal* infections (50). In addition, overexpression of the human cathelicidin antimicrobial peptide LL-37 in mice protected animals against infection by *Pseudomonas aeruginosa* PAO1 (51).

These peptides exert potent antimicrobial activity and are rapidly mobilized to neutralize a broad range of microbes, including viruses, bacteria, fungi and eukaryotic parasites (52). In addition, AMPs are able to neutralize endotoxins and increase phagocytosis as well as

chemokines expression to aid in overcoming infection (53). In humans, the main innate AMPs of the lungs are cathelicidins and defensins, which are produced by leukocytes and epithelial cells (54). Currently, more than 2000 natural AMPs with several different sequences have been isolated from a wide range of organisms (Antimicrobial Peptide Database, http://aps.unmc.edu/AP/main.php). Due to this extensive repertory, AMPs can be classified in many different manners. They can be classified as gene coded and non-gene coded (i.e. multiple enzyme systems), as well as depending on their biological source (bacteria, plants, animal,...). Furthermore, they can be categorized according to its microbial target functions (antibactericidal, antifungical, antiviral and antiparasitic peptides) or based on molecular target (cell surface targeting peptides and intracellular targeting peptides). However, the most extended classifications are based on their secondary structures (β-sheet, α-helix, extended, and loop) and/or on their net charge (cationic and anionic peptides) (55).

II. Antimicrobial proteins

Antimicrobial proteins are host soluble factors of high molecular weight, compared to AMPs, which have antimicrobial activity against Gram-negative and positive bacteria, virus and fungi (56). Among their activities, they are able to: i) increase phagocytosis by opsonization or direct phagocytic cells stimulation; ii) kill directly microbes; iii) direct chemotaxis; and iv) modulate cytokine expression (10). Lysozyme and lactoferrin are among the most abundant and important proteins in the alveolar fluid. Both proteins are produced by neutrophils, monocytes, macrophages, and epithelial cells and can kill directly bacteria (9). Finally, as another important antimicrobial proteins are the pulmonary collectins SP-A and SP-D, which participate actively in lung host defense against infection, displaying antimicrobial and immunomodulatory activities (10).

2.2.2 CELLULAR COMPONENT

Cellular response of the lung innate immunity is coordinated by alveolar epithelial cells and phagocytic leukocytes, mostly, alveolar macrophages (AMs) and neutrophils.

I. Alveolar Epithelial Cells: besides acting as a physical barrier to block microbial infection and entry into the host, they are able to eliminate microbes by induction of immune effectors by chemokines and cytokines, as well as secretion of soluble components, which directly kill pathogens (57).

II. Alveolar Macrophages (AM): they make up approximately 95% of the leukocytes in the human airspaces and 100% of the leukocytes in the lungs of pathogen-free mice (2). AMs reside on the epithelial surface of the lung, and, in contrast to other resident macrophage populations, are in direct contact with the environment, including commensal bacteria and inhaled particles, while interacting with host-epithelial-derived factors such as surfactant components. These conditions determine their unique phenotype evolved to maintain the homeostasis and protect the lung from infection (58).

One of the main characteristics of macrophages is that they are able to promote different responses, depending on their differentiation state and microenvironment factors, in order to perform the best response to a particular inciting stimulus (59). Resident AMs are, therefore, tolerant to their continuous challenging environment, thus producing poor inflammatory responses when they are exposed to harmless antigens, and removing them by phagocytosis (60). However, if the inhaled agent is highly dangerous and exceeds the threshold response of AMs, they can initiate an inflammatory response to recruit more potent immune cells (including peripheral monocytes and neutrophils) that will help to eliminate the potential injurious agent (58).

III. Neutrophils: they are polymorphonuclear cells and the most abundant type of leukocytes in the human circulation, forming one of the main defensive cells against infection (61). In non-infectious conditions, neutrophils constitute 1-2% of the alveolar leukocytes (Fig. 5A). However, during respiratory infection, neutrophils are recruited to the lungs, reaching up to 80-90% of the immune cells in the air space (Fig. 5A). Once there, neutrophils are able to eliminate pathogens by phagocytosis or by the expression of reactive oxygen species, antimicrobial proteins and peptides (like bactericidal permeability-inducing protein and defensins), and degradative enzymes (like elastase) (62). Neutrophils also extrude neutrophil extracellular traps (NETs), which are composed of a chromatin meshwork containing antimicrobial proteins that ensnare and kill extracellular bacteria (Fig. 5B) (63). Additionally, neutrophils generate a variety of immune mediators to direct immune responses, influencing other innate and adaptive immunity cells (64).

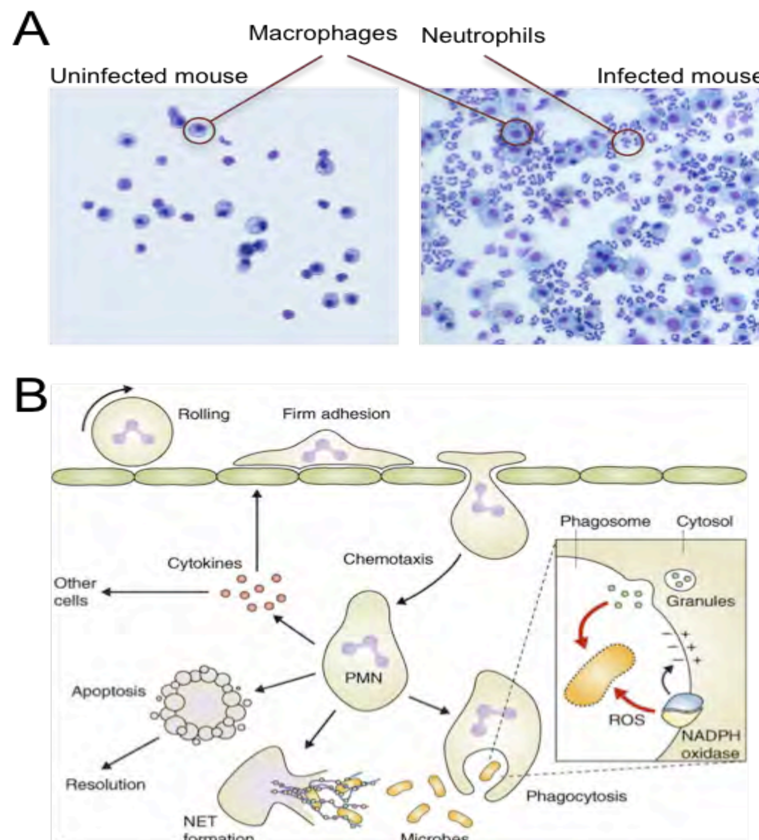

Figure 5. A) Cell types in the bronchoalveolar fluid from uninfected and infected mice. Alveolar macrophages constitute ≈95% of the leukocytes in the airspaces in non-infectious conditions. After infection, neutrophils are recruited and they can constitute up to 80-90% of the immune cells in the air space. **B)** Neutrophil activity at the site of infection. When encountering infectious particles, neutrophils (PMN) are able to fight against infection by several mechanisms, including phagocytosis and intracellular bacterial killing; secretion of neutrophil extracellular traps (NETs); and production of reactive oxygen species (ROS) and AMPs. In addition, they are able to produce cytokines, which contribute to the inflammatory reaction. Once infection is cleared, neutrophils die by apoptosis and trigger an active program to resolve inflammation. Taken from (61).

Neutrophil recruitment during early acute inflammation is critical for host defense against infection. However, an exacerbated inflammatory response resulting in neutrophil accumulation for a long time can injure the lung. The neutrophil products generated to kill microbes, such as reactive oxygen species and proteases, also kill host cells and damage host tissues (65). Therefore, host needs to subvert inflammation as soon as possible to not damage the own tissue. Thus, anti-inflammatory mediators released by the same cells that initiated the pro-inflammatory response are responsible for this subversion and coming back to non-infection conditions (66).

3. RESPIRATORY INFECTIONS

Even though the lungs have effective mechanisms of defense, several microbes overcome these mechanisms, surviving within the host and colonizing the respiratory tract. Persistence of a specific exogenous microorganism in the different parts of the respiratory system is mentioned as respiratory infection, and microorganisms involving this kind of infectious disease are known as respiratory pathogens.

Respiratory infections are normally classified as upper respiratory tract infection or lower respiratory tract infection. Upper respiratory tract infections comprise tonsillitis, pharyngitis, laryngitis, sinusitis, otitis media, and the common cold, which are frequent presenting conditions in primary care. Lower respiratory tract infections are generally more serious than upper ones. According to the European Respiratory Society, adult lower respiratory tract infections are an acute illness (present ≤ 21 days); with cough as the main symptom and ≥ 1 other respiratory tract symptom (sputum production, dyspnoea, wheeze, and/or chest discomfort/pain), and no alternative explanation (e.g. sinusitis, asthma, lung edema, or lung embolism). The two most common infections are bronchitis and pneumonia, which are an inflammatory condition of the lower respiratory tract promoted by virus and/or bacteria (67).

3.1. ACUTE LOWER RESPIRATORY TRACT INFECTION

Lower respiratory tract infections cause more disease than better-recognized threats to the public's health such as cancer, heart attacks, HIV/AIDS or malaria; as well as a greater burden than any other infectious disease (Figure 6A) (68). Consistent with the World Health Organization, ischemic heart disease, stroke, chronic obstructive pulmonary disease and lower respiratory tract infections have remained the top major cause of death during the past decade (Fig. 6B) (69). Consequently, respiratory infections are a persistent and pervasive public health problem.

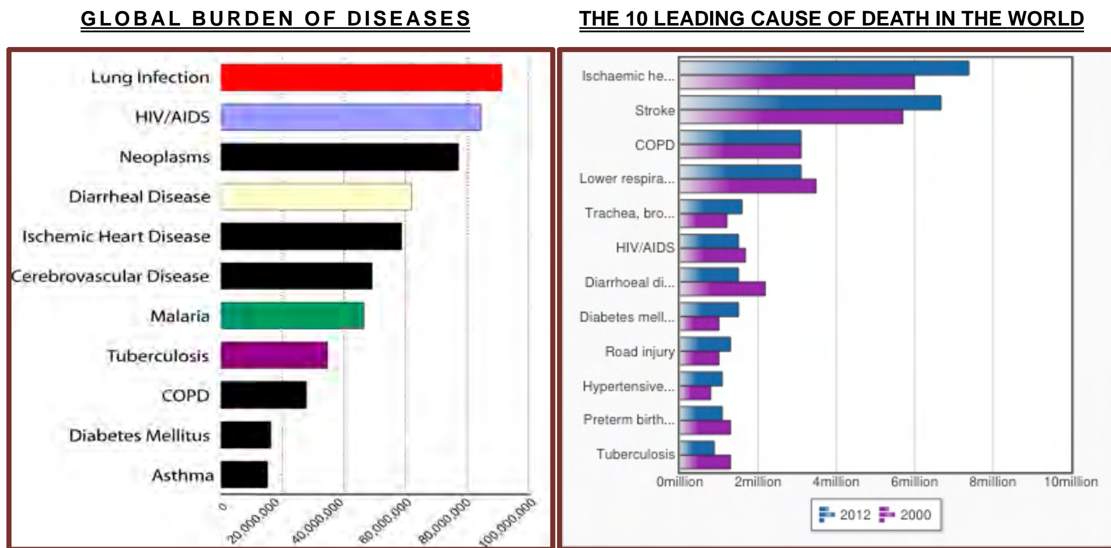

Figure 6. A) The Global Burden of Selected Diseases in 2002, as Measured by Disability-Adjusted Life Years. COPD: chronic obstructive pulmonary disease (68). **B)** The 10 leading causes of death in the world in the past decade, according to World Health Organization. Ischemic heart disease, stroke, lower respiratory tract infections and COPD have remained the top major killers. Taken from (69).

Among these infections, pneumonia remains as an important cause of morbidity and mortality (70). Pneumonia is an inflammatory condition of the lung affecting primarily alveolar sacs, which is mainly produced by bacteria or virus. It can be classified among community- or hospital-acquired infections, according to the place of acquisition. Hospital-acquired pneumonia is usually associated with invasive medical devices, mechanical ventilation, surgical procedures or immunodeficient patients, being the most common life-threatening hospital-acquired infection (71). The outcome of pneumonia is an exacerbated and persistent inflammatory condition, resulting in lung injury (72). In addition, pneumonia, especially in high-risk groups (older or inmunodeficient adults), may have complications such as bacteremia, a serious disease that occurs when infection moves into the bloodstream and quickly spread to and infect other organs.

Hospital-acquired pneumonia may be caused by a wide spectrum of bacterial pathogens, and can be polymicrobial, with organisms ranging in severity (67). Common bacterial pathogens include Gram-negative bacteria, such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Haemophilus influenzae* and *Acinetobacter* species; and Gram-positive bacteria, mainly *Staphylococcus aureus* and *Streptococcus pneumoniae* (70, 73). Among these organisms, infections caused by Gram-negative bacteria are a major challenge,

since these kinds of bacteria exert a broad spectrum of virulence factors (including adherence or invasion factors, endotoxins and exotoxins) and are highly efficient to acquire resistance to antimicrobial drugs or immune system factors (71, 74).

4. BACTERIAL VIRULENCE FACTORS

Infection is regarded as an imbalance between microbial pathogenicity factors and the host defense systems. Bacterial pathogenicity degree reflects its capacity to infect the host, and is measured by the virulence of the bacterium (75). Virulence is affected by numerous variables such as the number of infecting bacteria, host defense mechanisms, and virulence factors of the bacterium (76).

Virulence factors are, therefore, molecules and components produced by a microorganism to establish itself within a specific host and to cause disease (77). Bacteria have developed a great variety of virulence factors, which are difficult to group, because some categories can overlap (77). However, the most extended ones can be classified as following:

I. Adherence Factors: as explained in mechanical defense mechanisms, bacteria have to firstly attach to mucus and epithelial surfaces in order to cross them and invade the host. Adhesins are the main adherence factor of bacteria. They are usually hair-like appendages of the bacterium called pili or fimbriae that extend out from the bacterial surface and mediate adhesion to these surfaces (78). In addition, adhesins can be also associated with the microbial cell surface, forming the so-called nonpilus adhesins, which recognize many different elements of host-cell surfaces, such as collagens, proteoglycans, or fibrinogen (79).

II. Invasion Factors: bacteria have developed several molecular strategies to induce their invasion into target cells (intracellular invasion) or into the epithelial barriers (extracellular invasion) for colonization and/or dissemination to other host tissues (80). Extracellular invasion occurs when bacteria break down host barriers through secretion of enzymes to degrade host cell molecules, such as elastases or proteases by *P. aeruginosa*, which degrade extracellular molecules for tissue invasion (81). On the other hand, intracellular invasion include surface components and secreted proteins, which lead to rearrangements of the plasma membrane architecture in the host cell in order to induce pathogen engulfment (78). Several pathogens, for example, bind to

integrin receptors in eukaryotic cells not only to adhere but also to trigger actin cytoskeleton rearrangements leading to bacterial uptake.

III. Exotoxins: toxins can be defined as bacterial molecules that produce tissue and cell damage in host. Exotoxins are secreted proteins by the bacterium (generally enzymes) that destroy certain cellular structures (80). They are secreted into the surrounding milieu or injected into the target cell. There is a wide variety of exotoxins with several biologic effects on host cells (cytotoxins, neurotoxins or enterotoxins) and diverse sites of action (extracellular matrix, cell membrane, nucleus) (74). Exotoxin activity leads to the characteristic clinical manifestations of the infectious disease.

IV. Siderophores: the growth of bacteria in host tissue is limited not only by the host defense mechanisms but also by the presence of available iron. Iron is not usually free in solution into the host, since most of it is bound to proteins, such as hemoglobin in blood or lactoferrin in mucus or saliva (74). To overcome this, bacteria express the so-called sidephores, which are iron-binding factors that allow bacteria to compete with the host for iron (82). These factors bind to iron with a higher affinity than the iron-binding proteins of the host, thus facilitating the acquisition of this metal ion for bacterial growth (83).

V. Bacterial cell wall: as eukaryotic cells, both Gram-negative and positive bacteria are surrounded by a lipid membrane or cytoplasmic membrane, which acts as a barrier to separate essential cytoplasmic components from the environment. In addition, a cell wall is present on their outside, in which several components have been shown to be virulence and resistant bacterial factors.

Bacterial cell wall is composed of peptidoglycan, a polymer consisting of sugars and amino acids that forms a mesh-like layer (84). In case of Gram-positive bacteria, they present a thick cell wall containing many layers of peptidoglycan and lipoteichoic and teichoic acids, which are polysaccharides of glycerol phosphate or ribitol phosphate linked via phosphodiester bonds. Regarding Gram-negative bacteria, they are characteristically surrounded by other lipid membrane, which makes their cell wall substantially thicker than that of Gram-positive bacteria (Fig 7).

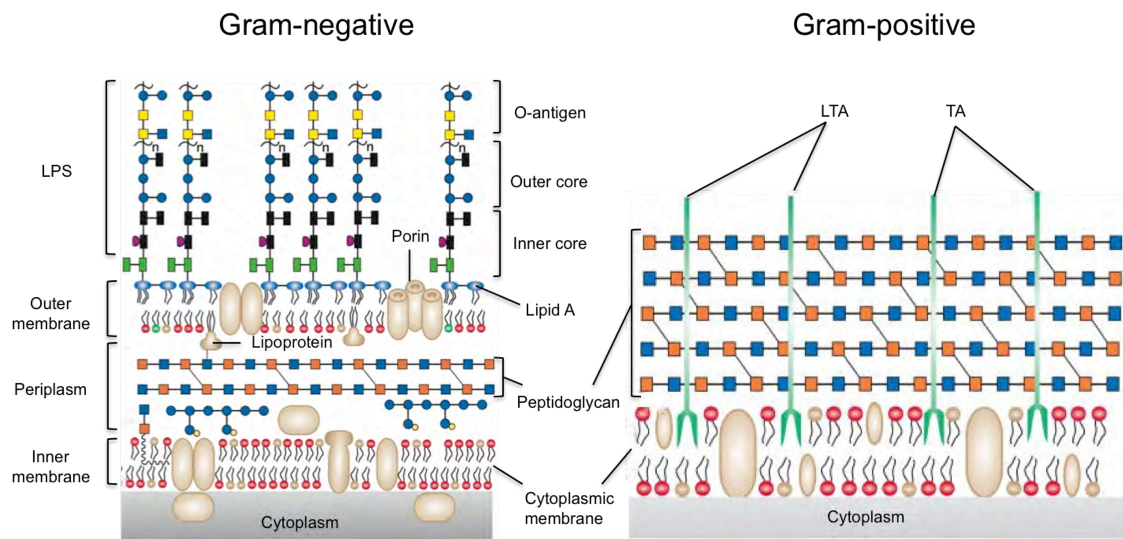

Figure 7. Schematic representation of the bacterial cell wall structure from Gram-negative and positive bacteria. LPS: lipopolysaccharide; LTA: lipoteichoic acid; TA: teichoic acid. Figure modified from (85).

Therefore, apart from the cytoplasmic membrane and peptidoglycan layers, instead of teichoic acids, Gram-negative bacteria present other lipid bilayer with polysaccharides attached to it, called the outer membrane (OM) (86). OM is an asymmetric bilayer in which the inner leaflet is composed of phospholipids and the outer leaflet comprises the bacterial lipopolysaccharide (LPS). LPS typically contains (from inside to outside) the lipid A, the oligosaccharide core, and the O-antigen, which are covalently attached to one another (Fig. 8) (87):

- Lipid A: is the hydrophobic component of the OM, which is highly conserved. Lipid A generally consists of diglucosamine phosphates attached to five or six fatty acyl chains. These hydrophobic chains anchor the LPS into the bacterial outer membrane, whereas the rest of the LPS projects from the cell surface. Thus, lipid A makes up the outer monolayer of the bacterial outer lipid bilayer.
- Oligosaccharide core: the oligosaccharide core can be divided into the outer and inner core. The inner region is usually highly conserved and is typically constructed from a few heptose residues bound to a 3-deoxy-D-manno-oct-2-ulosonic acid (KDO) residues attached to the lipid A. Inner core, beside lipid A, are commonly sprinkled with anionic groups, such as Kdo molecules, phosphate groups and, sometimes, phosphoethanolamine or pyrophosphoethanolamine substituents, which confer negative net charge to LPS. On

the other hand, the outer region is a more variable domain, which is mainly formed by hexose residues.

- O-antigen: consist of a variable number of repeating saccharide units comprising the outermost domain of the LPS molecule. It is the most variable domain of the OM and is the portion of the molecule, which usually determines serotype specificity.

There are two serotypes of LPS in nature: Smooth LPS (S-LPS), which contains the entire structure, and Rough LPS (R-LPS), which lacks the O-antigen, and has the oligosaccharide core progressively shorter, depending on the rough phenotype (Ra, Rb, Rc, Rd and Re LPS) (Fig. 8) (87). The minimal LPS required for the growth of *E. coli* consists of the lipid A and Kdo domains, i.e., expressing Re LPS. In addition to LPS, some Gram-negative bacteria usually develop a prominent polysaccharide capsular (CPS), above the LPS, composed of complex repeating subunits of anionic polysaccharides (Fig. 8) (88).

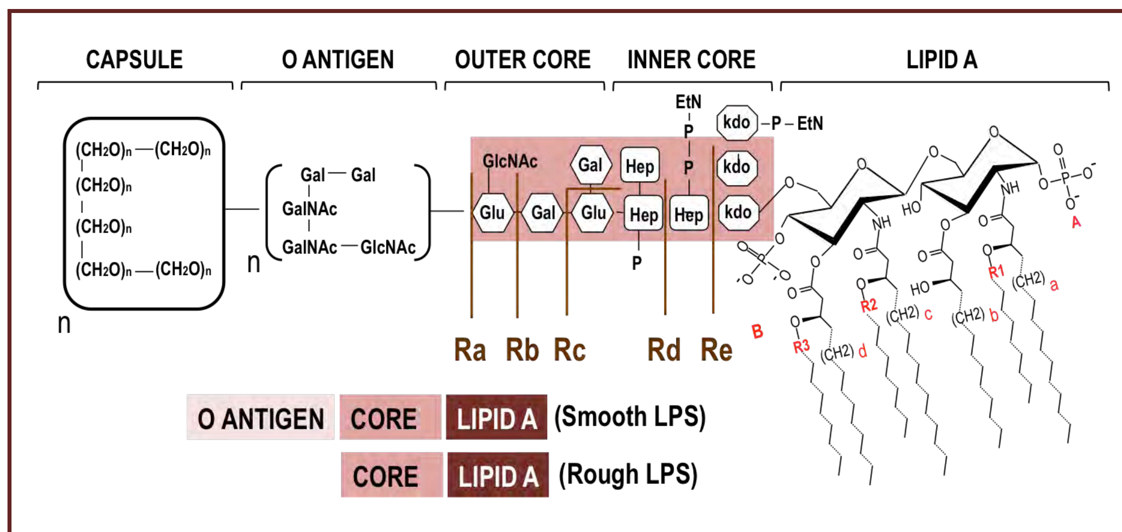

Figure 8. Chemical structure of bacterial LPS. LPS contains three different regions: i) lipid A, which contains the hydrophobic, membrane-anchoring region of LPS; ii) oligosaccharide core, comprising a variable outer core and an invariable inner core; and 3) O-antigen. Additionally, bacteria can express a capsular polysaccharide. LPS is usually named as smooth LPS when contains the entire structure or rough LPS, when lacking the O-antigen, and can be designated as Ra, Rb, Rc, Rd, and Re in order of decreasing core length. Figure modified from (89).

Besides the adhesion and invasion factors, which are usually present in the bacterial surface, other components of the bacterial cell wall are well-known virulence factors:

- Endotoxin: The LPS complex is properly and commonly referred to as endotoxin. LPS, is a potent immune response inductor, mainly because the presence of the lipid A, which is detected at picomolar levels by Toll-like Receptors (TLR) (90, 91). The lipopolysaccharide endotoxin of Gram-negative bacteria causes fever, changes in blood pressure, inflammation, septic shock, and many other toxic events. Consequently, endotoxin exerts profound biologic effects, which can be lethal for the host.

- Lipoteichoic acid: it is considered a potential virulence factor, which shares with LPS many of its pathophysiological properties. Like LPS, lipoteichoic acid strongly stimulates generation of pro-inflammatory mediators by host immune cells, and is thought to be involved in the pathophysiology of septic shock, adult respiratory distress syndrome, and toxic shock syndrome (92). In addition, lipoteichoic acid can also mediate bacterial adhesion by binding to fibronectin, membrane phospholipids or Toll-like receptors in target cells (93).

- Peptydoglican: peptidoglycans have been shown to exert cytotoxic and pyrogenic activities in mammalian hosts, and they have been implicated as mediators of septic shock (94).

- Capsular polysaccharide: capsule formation has been long recognized as a protective mechanism for bacteria within the host. *K. pneumoniae* and *P. aeruginosa* are well-recognized encapsulated strains that develop prominent capsule, which have been shown to be involved in their pathogenesis. Thus, encapsulated strains are more virulent and more resistant to phagocytosis and intracellular killing than nonencapsulated strains (95, 96). In addition, capsule can prevent killing of the bacteria by bactericidal nonimmune serum factors, one of the main soluble host defense mechanism against invading microorganisms (97).

As mentioned above, infection results from an imbalance between bacterial virulence factors and host defense mechanisms. When high virulent bacteria are able to resist host immunity and promote host infection, exogenous treatments with antimicrobials are necessary to overcome infection. Even though these treatments are very efficient to treat respiratory

infection, microorganisms have also developed mechanisms of resistance against the majority of antimicrobials.

5. ANTIMICROBIAL RESISTANCE:

Antimicrobial resistance is the capability of one microorganism to avoid the antimicrobial drug activity that was originally effective for the treatment of infections caused by it. The evolution of resistant strains is a natural phenomenon that occurs when microorganisms replicate erroneously or when resistant traits are exchanged between them. From the discovery of penicillin (1921), by Sir Alexander Fleming, to the present, conventional antibiotics have been the treatment of choice for infections.

5.1. CONVENTIONAL ANTIBIOTICS:

Antimicrobials are agents that kill microorganisms or inhibit their growth. They include antibiotics, antifungals, antiviral and antiparasitic drugs. Regarding antibiotics, they are currently defined as a chemical substance derivable from a microorganism or produced by chemical synthesis that kills or inhibits microorganisms and cures infections. Antibiotics more commonly used to treat infections can be classified, according to their structure, in: β-lactams, glycopeptides, fosfomycin, aminoglycosides, macrolides, tetracyclins, ansamycins, sulfonamides and quinolones (98, 99) In turn, they can be divided depending on their mode of action, which is based on targeting of components or functions necessaries for bacterial growth (Table 1). Accordingly, they are classified as (98, 99):

1. Cell wall synthesis inhibitors: they work by targeting penicillin binding proteins that are implicated in the synthesis of the bacterial cell wall and are present in the cytoplasmic bacterial membrane. They comprise the antibiotics β-lactams (penicillin, ampicillin, amoxicillin or carbapenems); glycopeptides (vancomycin and teicoplanin); and fosfomycin. They are all bactericidal from narrow to broad spectrum.

2. Protein synthesis inhibitors: the main target of these antibiotics is the bacterial ribosome in the cytoplasm in order to inhibit the synthesis of bacterial proteins. They include aminoglycosides (like streptomycin, kanamycin or gentamycin); macrolides

(erythromycin); tetracyclines, and ansamycins (rifampicin). These antibiotics are bactericidal or bacteriostatics (macrolides).

3. Anti-metabolites: also called folate pathway inhibitors, they act as competitive inhibitors of the enzyme dihydropteroate synthetase, an enzyme involved in folate synthesis, which is essential for the synthesis of adenine and thymine nucleobases. This group is composed of sulfonamides and co-trimoxazole, which are specially used for urinary tract infection treatment, although they present a high incidence of side effects.

4. Nucleic acid synthesis inhibitors: these antibiotics inhibit DNA synthesis by blocking bacterial topoisomerases, which are implicated in DNA supercoiling, replication, transcription and recombination. They comprise quinolones and their modern substitutes fluoroquinolones. They are broad-spectrum antibiotics that play an important role in hospital-acquired infections and others in which resistance to older antibacterial classes is suspected.

Table 1. Antibiotic classification, including their activity and common usages. LRTI: lower respiratory tract infection; URTI: upper respiratory tract infection; UTI: urinary tract infection.

<table>
<tr><th>Mechanism of action</th><th>Structure</th><th>Activity</th><th>Common usage</th><th>Examples</th></tr>
<tr><td rowspan="3">Cell wall synthesis inhibitors</td><td>β-lactams</td><td rowspan="3">Bactericidal</td><td rowspan="3">Community- and hospital-acquired infections
LRTI</td><td rowspan="3">Penicilin, Amoxicilin, Carbapenems, Vancomicin</td></tr>
<tr><td>Glicopeptides</td></tr>
<tr><td>Fosfomycin</td></tr>
<tr><td rowspan="4">Protein synthesis inhibitors</td><td>Macrolides</td><td>Bacteriostactic</td><td rowspan="4">Hospital-acquired infections
Serious LRTI</td><td rowspan="4">Streptomycin , Gentamycin, Erythromycin, Rifampicin</td></tr>
<tr><td>Aminoglycosides</td><td rowspan="3">Bactericidal</td></tr>
<tr><td>Tetracyclines</td></tr>
<tr><td>Ansamycins</td></tr>
<tr><td rowspan="2">Anti-metabolites</td><td>Sulfonamides</td><td>Bactericidal</td><td rowspan="2">UTI, LRTI and URTI
Community- and hospital-acquired infections</td><td rowspan="2">Sulfisoxazole, sulfamethizole, Sulfamethoxazole</td></tr>
<tr><td>Co-trimoxazole</td><td>Bacteriostatic</td></tr>
<tr><td rowspan="2">Nucleic acid synthesis inhibitors</td><td>Quinolones</td><td rowspan="2">Bactericidal</td><td rowspan="2">Community- and hospital-acquired infections
UTI and LRTI</td><td rowspan="2">Ciprofloxacin, Gemifloxacin, Levofloxacin, moxifloxacin</td></tr>
<tr><td>Fluoroquinolones</td></tr>
</table>

5.2 MULTIDRUG RESISTANCE

The use and misuse of antimicrobial drugs have produced the emergence of multi-drug resistant (MDR) strains (100). The repeated and successful response to emerging resistance was to discover new antibacterial drugs (100). However, in recent years this strategy has failed, with resistance accumulating faster than new antibiotics are being developed (23, 101)

OM and CPS are thought to be among the main resistance factors of bacteria. As described above, the majority of the main classes of antibiotics have to cross the cell wall to reach their intracellular targets (102). Structurally, OM appears to be an effective protective barrier to the diffusion of many hydrophobic antibiotics and host antimicrobial proteins, since lost of O-antigen and outer core in deep rough mutants confer susceptibility to a number of these antimicrobials (86, 103). Thus, pathogenic bacteria usually express a more complex OM and CPS in order to increase resistance to hydrophobic antibiotics. On the other hand, reductions in the number of anionic phosphates in lipid A are common membrane modifications of bacteria to decrease the binding and action of cationic antibiotics such as aminoglycosides or polymyxins, which disrupt bacterial cell wall by electrostatic interaction with anionic groups in the LPS (103).

Another common mechanism of resistance is the production of β-lactamases that inactivate β-lactam antibiotics by hydrolyzing the β-lactam ring (104). They include 532 distinct enzymes, where extended-spectrum β-lactamases are the most potent. Carbapenems are considered to be the agents of choice for the treatment of serious infections due to majority of extended-spectrum β-lactamases do not hydrolyze carbapenems (104). However, recent reports have found several enterobaciae bacteria, mostly *Klebsiella pneumoniae* strains, that are capable to hydrolyze them (105).

An additional mechanism of bacterial resistance is due to the presence of the so-called bacterial efflux pumps. They are active protein transporters localized in the cytoplasmic membrane, which are able to move compounds, like toxic substances or antibiotics, out of the cell (96, 106). Finally, mutational changes in enzymes targeted by antibiotics also contribute to bacterial resistance by evading the action of these selective antibiotics (107).

MDR pathogens have developed many of these resistance mechanisms against the existing antibiotics. Consequently, multidrug resistance reduces the effectiveness of

treatment, resulting in prolonged illness, higher health care expenditures, and an increase in mortality and morbidity rates (71). At this moment, the Antimicrobial Availability Task Force has identified six particularly problematic pathogens (108), including three Gram-negative organisms: *Acinetobacter baumannii*, extended-spectrum β-lactamase-producing *Enterobacteriaceae*, and *Pseudomonas aeruginosa*; two Gram-positive pathogens: methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium*; and the filamentous fungi *Aspergillus* spp (109). Among MDR Gram-positive bacteria, being MRSA the most important one (110), a handful of novel antibiotics have been recently approved for the treatment of infections caused by these organisms (111, 112). However, MDR Gram-negative bacteria are currently becoming a higher challenge, since few new antibiotics are in the drug development pipeline for their treatment (Fig. 9) (100).

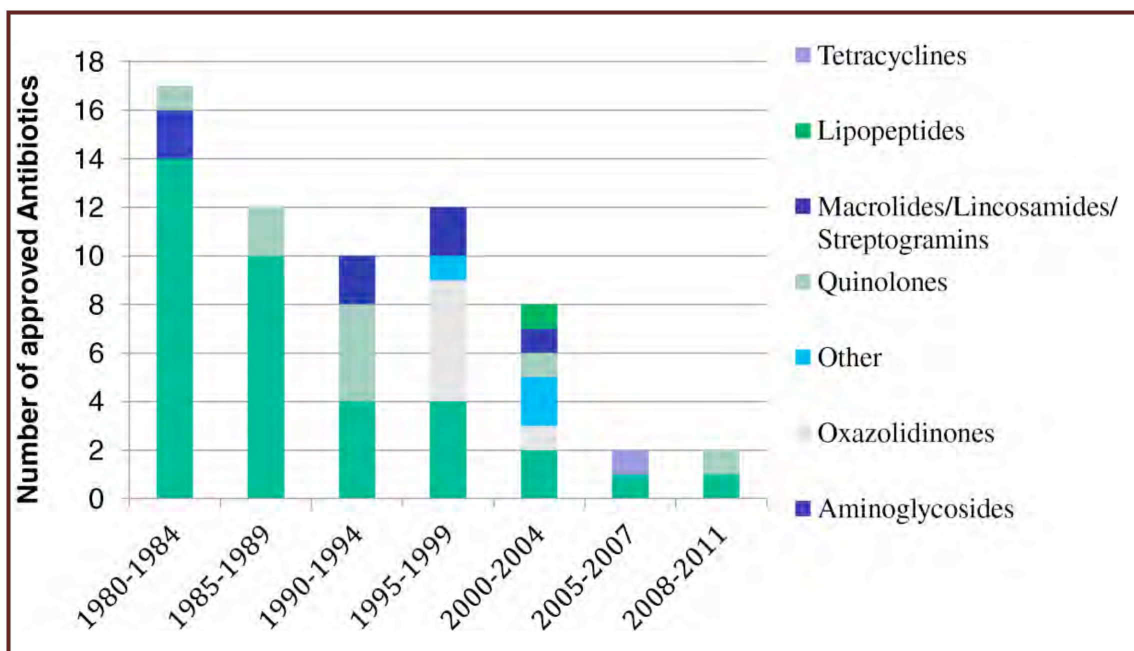

Figure 9. Number of approved antibiotics from 1980 to 2011. Since 2000, only three new classes of antibiotics have been introduced to the market for human use and one of those is limited to topical use. Taken from (100).

5.3 MULTIDRUG-RESISTANT GRAM-NEGATIVE BACTERIA

Resistance rates are increasing among several problematic Gram-negative pathogens, mainly those that usually promote serious nosocomial infections. Gram-negative bacteria are responsible for more than 30% of hospital-acquired infections, becoming hospital-acquired pneumonia one of the most difficult challenges in the treatment of infectious diseases. Additionally, in intensive care units, Gram-negative bacteria account for about 70% of these types of infections (71).

Among emergent MDR bacteria, *Enterobacteriaceae* is a large family of Gram-negative bacteria, which are able to produce hospital-acquired pneumonia, bloodstream infections and urinary tract infections (71, 113). They include pathogens such as *E. coli* and *K. pneumoniae* that usually develop large LPS molecules and can express prominent CPS on their OM. *P. aeruginosa* is another invasive opportunistic Gram-negative pathogen that is responsible for a wide range of severe nosocomial infections, especially in patients undergoing cystic fibrosis (114). Both families acquired resistance via multiple mechanisms, including production of β-lactamases and carbapenemases, upregulation of multidrug efflux pumps, complex OM, and cell wall mutations leading to a reduction in porin channels, which are proteins present in both lipid bilayers that are used by several antibiotics to cross the OM (96, 107). Additionally, *P. aeruginosa* strains produce loosely anionic CPS of alginate (115) that are able to bind themselves to form biofilms, thus providing another resistance mechanism by physical exclusion of antibiotics during infection (116). Moreover, this kind of CPS make *P. aeruginosa* intrinsically susceptible to only a limited number of antibiotics, due to its low permeability (96).

Finally, *H. influenzae* is a human-restricted respiratory pathogen, which has emerged as an important opportunistic pathogen causing infection in adults suffering obstructive lung diseases (117, 118). *H. influenzae* commonly colonizes upper respiratory tract and rarely produces severe lower respiratory infection because host immune responses can prevent its spreading to the lower respiratory tract (118). However, *H. influenzae* has developed a number of resistance mechanisms to adapt and exploit the already-impaired host immunity in patient suffering COPD, including lost or gain of resistance surface components, high number of adhesion and invasion factors or biofilm formation (118, 119). Most *Haemophilus* strains

remain susceptible to carbapenems. However, antibiotic resistance mechanisms, such as β-lactamases or bacterial efflux pumps, seem to be currently spreading in this bacterium (120).

6. NOVEL ALTERNATIVE THERAPIES TO TREAT INFECTIONS

MDR bacterial infections have emerged as one of the world's greatest health threats. Current antibiotics are becoming inefficient against several bacterial pathogens and the development of novel antibiotics, especially to treat MDR Gram-negative bacterial infections, has stagnated over the last half century. Thus, current research is focused on new alternative or adjuvants therapies to treat infections that would be able to substitute or help the classical antibiotic treatments.

Due to this emergence, components of the host immune system are being currently widely investigated as one of these alternative therapeutic strategies. Since antimicrobial peptides and proteins were discovered, extensive research has shown that these naturally occurring components are evolutionarily ancient weapons from the majority of living organisms that are highly efficient in killing microorganisms (7). Therefore, both endogenous (belonging to the human immune system) and exogenous antimicrobial peptides and proteins, as well as synthetic peptides based on these components, are becoming promising novel antimicrobial therapies (121).

6.1. SURFACTANT PROTEIN A: AIRWAY NATURAL ANTI-INFECTIVE PROTEIN

SP-A is the most abundant protein of the alveolus, by weight, representing approximately 3-5% of the total mass of pulmonary surfactant (122). It is synthesized and secreted by type II pneumocytes and nonciliated bronchial epithelial cells (Clara cells). SP-A participates actively in surfactant adsorption, recycling, and subsequent homeostasis, because of its lipid-binding ability (123). Additionally, it has been shown that together with SP-D, is a pattern-recognition molecule belonging to the pulmonary collectin family that recognizes, binds and facilitates the clearance of infectious microorganisms from the lung (18).

On the other hand, although the major site of SP-A synthesis is the lung, it has been also detected in extrapulmonary mucosal tissues as well (40). Thus, SP-A has been also found in gastric, intestinal, vaginal, skin, middle ear, Eustachian tube and renal tube epithelia;

suggesting that this collectin plays a general role in innate host defense, not only in the lungs (124).

6.1.1. STRUCTURE

SP-A belongs to the collectin family, which includes calcium-dependent carbohydrate binding proteins that are characterized by the presence of an N-terminal collagen-like region and a C-terminal lectin domain, comprising a carbohydrate recognition domain (CRD) (40). Together with SP-A and SP-D, 9 different members have been identified in the collectin family: mannose-binding lectin (MBL), conglutinin, collectin liver 1, collectin placenta 1 (CL-P1), collectin kidney 1 (CL-K1), collectin of 43 kDa (CL-43) and collectin of 46 kDa (CL-46). Apart from CL-L1 and CL-P1, all collectins are soluble and secreted proteins (125).

SP-A shows the typical structure of the collectin family, characterized by the presence of four structural domains and a supratrimeric oligomerization, which varies in several degrees depending on the collectin (Fig. 10) (40).

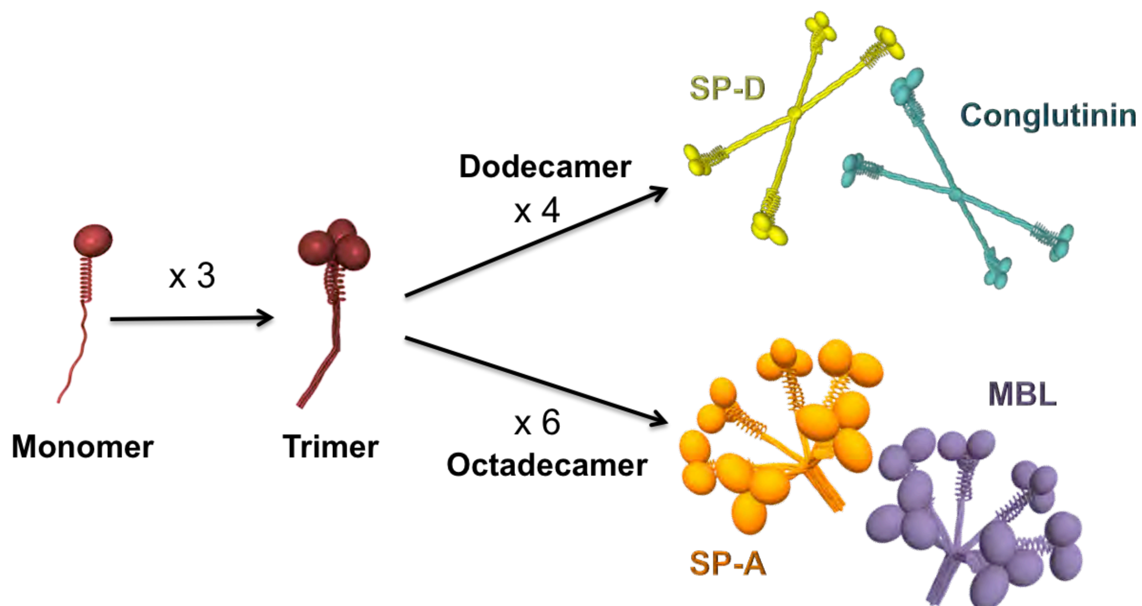

Figure 10. Collectin oligolimerization. Three monomers oligomerize to form a triple coiled-coil structure, leading to the formation of trimeric subunits. Trimeric subunits multimerize subsequently into higher oligomeric forms. MBL: mannose-binding lectin.

Each monomer of SP-A (36 kDa) is composed of (Fig. 11): an N-terminal domain (~ 8 aa), a collagen domain (~73 aa), a coiled-coil neck domain (~34 aa) and a globular or C-type lectin domain (~123 aa), comprising the carbohydrate recognition domain (CRD) (41). Once monomers are synthetized, SP-A multimerizes to form trimers, which, in turn, associate into hexamers to yield the complete protein, an octadecamer of 650 kDa and 20 nm in length, which has the appearance of a bouquet of tulips (Fig. 10) (126). It has been shown that the oligomerization of SP-A is essential for the maintenance of many of its functions (127, 128).

I. N-terminal domain:

This segment contains between 8 and 11 aminoacids, depending on species and variation in N-terminal processing. There are two cysteines residues present in this region (positions -1 and -6) that have been shown to participate in intermolecular disulfide bound formation, which stabilizes the oligomer (127, 129). The interchain disulfide bridge formed by the Cys^6 has been shown to be necessary for the interaction of the protein with surfactant lipids and type II pneumocytes (130).

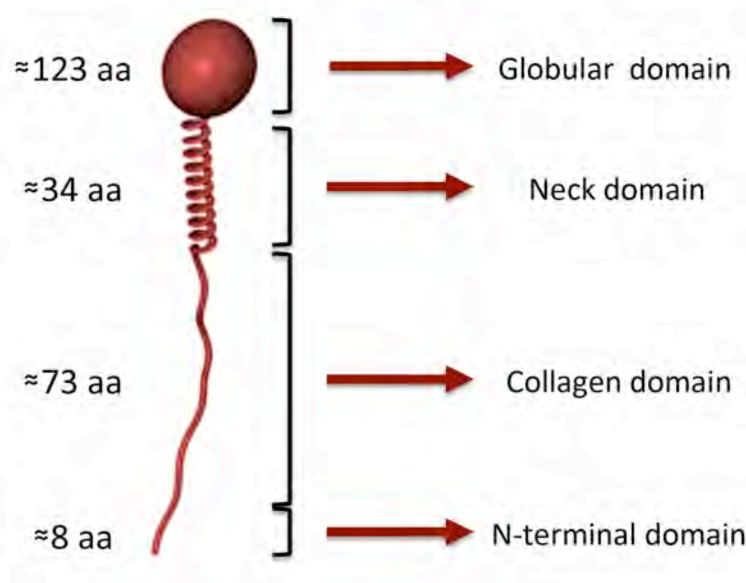

Figure 11. Structural domains of SP-A.

II. Collagen domain:

The collagen domain or collagen-like region of collectins consists of repeating motifs of Gly-X-Y, where X and Y are generally proline or hydroxyproline (40). It is usually N- or O-glycosylated. From each monomer, collagen helices are coiled between them in order to form a stable tensile domain that is relatively resistant to proteases. In SP-A, this domain has 23 Gly-X-Y repeats and is interrupted close to the middle. This interruption introduces a kink in the region that gives flexibility to the protein, allowing SP-A to fold in a bouquet-like structure (126). This domain is thought to be involved in several functions of SP-A, including protein oligomerization, chemotaxis, receptor-mediated effects, pathogen agglutination, increased phagocytosis, and binding to target ligands (41, 128, 131).

III. Neck domain:

This domain is localized between the collagen-like and globular domains and comprises a segment of 34 aa with α-helical structure. It is directly involved in the trimerization of monomers, since three neck domains associate to form a triple coiled-coil structure, maintained by hydrophobic interactions (40). These structures have been also observed in other proteins of the collectin family such as SP-D or mannose-binding lectin (40). In addition, this domain is critical for the selective binding of SP-A to various ligands, since it can guide the globular domains of SP-A trimers (126).

IV. Globular domain or carbohydrate recognition domain (CRD):

It is composed of 123 aa, which make up the C-terminal segment of the protein. This domain has 4 cysteines that form two intrachain disulfide bridges (Cys^{204}-Cys^{218} and Cys^{135}-Cys^{266}). It also has 18 highly conserved residues, which are common among C-type lectins (132). This domain resembles a globe-like structure, which is suitable for the interaction of SP-A with a range of ligands and lipid membranes (133).

In this domain there are at least two calcium-binding sites (133, 134). Near these domains are two conserved tryptophan residues at positions 191 and 213 that serve as conformational change markers of this region (135). The binding of calcium to the globular domains of SP-A causes an increase in the intrinsic fluorescence of the protein, as well as a blue-shift of the maximum of emission, indicating a conformational change that locates tryptophans in a more hydrophobic environment. This conformational change

increases the binding of SP-A to lipids, allows the binding to carbohydrates (as N-acetylmannosamine or 1-fucose) and self-aggregation of the protein (12, 135, 136).

6.1.2. FUNCTIONAL ROLES:

To date, SP-A has been shown as a multifunctional protein involved in multiple important processes of the host. Studies involving SP-A gene-deleted mice showed that SP-A is not essential for live (137). However, SP-A-/- mice displayed several complications consistent with SP-A roles *in vivo*, including lack of tubular myelin (TM) or susceptibility to lung infections (18). Therefore, *in vitro* and *in vivo* studies have shown that SP-A is implicated in homeostasis and biophysical activity of pulmonary surfactant, as well as lung innate host defense (12).

SURFACTANT-RELATED FUNCTIONS:

The ability of SP-A to bind to lipids and induce their aggregation in the presence of calcium seems to be related to its ability to modulate the biophysical properties of pulmonary surfactant.

SP-A is required to keep the tubular myelin structure, since TM is reduced in SP-A gene-deleted mice (137, 138). Thus it is responsible for maintaining a reservoir of functionally active fraction of surfactant in the alveoli (139).

SP-A increases the adsorption of phospholipids at the air-liquid interface, encouraging the formation of interfacial monolayer (140), improves the surface tension of surfactant in collaboration with SP-B (141), and favors the enrichment of the monolayer dipalmitoylphosphatidylcholine, the main factor implicated in surface tension reduction (140).

SP-A prevents surfactant membranes inactivation by serum proteins and inflammatory mediators that invade the alveolar space in many respiratory pathologies (142-144) (145). We have determined that pulmonary surfactant function can be also inactivated by factors derived from microorganisms, such as LPS and β-glucans from fungal wall (unpublished results). It has been suggested that the ability of SP-A to protect surfactant could be related to the formation of the TM, and its participation in the adsorption and maintenance of a stable interfacial monolayer (143). Recently it has been shown that SP-A might bind to different inhibitors blocking its action and maintaining the lung surfactant (144).

Additionally, SP-A participates in the regulation of pulmonary surfactant metabolic cycle, enhancing uptake and subsequently clearance of pulmonary surfactant by type II pneumocytes and alveolar macrophages for surfactant recycling (146). This function is important for the maintenance of a fully functional surfactant, because it is continuously subjected to compression and expansion cycles that generate less surface-active small aggregates, which have to be removed and replaced (139).

ROLE IN IMMUNE DEFENSE OF THE ALVEOLI:

Agglutination and Enhancement of Phagocytosis:

SP-A is able to interact through its C-terminal domain with a variety of ligands, such as allergens, LPS, and saccharides found in glycoproteins and glycolipids of bacterial, viral or fungal surfaces. This feature allows SP-A to agglutinate molecules or microorganisms (Fig. 12) enhancing mucociliary removal of inhaled dangerous particles, preventing the attachment of pathogens to cell surfaces, and/or inhibiting microbial colonization, invasion and dissemination (40).

On the other hand, SP-A is able to enhance phagocytosis of microorganisms by different mechanisms (Fig. 12) (12). The ability of SP-A to aggregate microorganisms would facilitate its uptake without an obligate interaction of SP-A with the phagocytic cell (147, 148). In addition, SP-A is capable of interacting with membrane receptors located in alveolar immune cells, such as scavenger receptor A or SP-A receptor 210 (149), resulting in enhanced phagocytosis of pathogens. SP-A can also act as an activation ligand, enhancing the phagocytosis of pathogens that have been previously coated and opsonized by an opsonin other than SP-A (e.g. IgG) (150). Finally, SP-A also stimulates phagocytosis by up-regulating the expression of immune cell-surface receptors that are involved in microbial recognition, like the macrophages cell receptors scavenger receptor A (151), complement receptor 3 (152) or mannose receptor (153), thus augmenting indirectly phagocytosis of pathogens.

Modulation of the inflammatory response:

Studies involving SP-A KO mice showed that these mice exhibited an augmented inflammatory response to a variety of stimuli, suggesting an anti-inflammatory role of SP-A (18). Thus, numerous later studies in alveolar macrophages have shown that SP-A mainly play an important anti-inflammatory role by modulating the threshold of LPS activity of these

cells towards an anti-inflammatory phenotype, via direct interaction with LPS or with immune cell receptors, mostly TLRs (Fig. 12) (154-157).

On the other hand, another studies (mainly involving nonresident alveolar macrophages) have shown that SP-A is also able to promote or subvert inflammation through regulation of the expression of numerous pro- and- anti-inflammatory mediators, suggesting that SP-A modulates the inflammatory response (Fig. 12) in a context-dependent manner (34). This modulation depends on several factors, such as: a) presence and type of pathogen or stimulus, b) type and state of activation of the responding cell, and c) period of exposure to the stimulus.

SP-A has been shown to regulate inflammatory response through the TLR family. SP-A can suppress NF-κB activation and pro-inflammatory cytokine production induced by SP-A nonligands (such as S LPS, peptidoglycan or zymosan) (156, 158), but enhances the pro-inflammatory response of TLRs to SP-A-ligands like R LPS (159). SP-A increases murine and human macrophage cytokine expression through TLR (160), but is also able to reduce indirectly cytokine expression through up-regulation of negative regulators of TLR-mediated NF-κB activation (161). SP-A differentially regulates the expression of TLR2 and TLR4 during primary human monocyte differentiation into macrophages, receptors that typically mediate pro-inflammatory responses. In contrast, SP-A diminishes TNFα secretion from these cells stimulated with TLR2 ligands (162).

Consistent with the dual role of SP-A, in an inflammatory model in the murine peritoneum, SP-A was reported to bind to the signal-inhibitory regulatory protein-α (SIRP-α) or the complex calreticulin/CD91 depending on the ligand bound to SP-A, thus promoting pro- or anti-inflammatory response (163). Additionally, SP-A was shown to inhibit the production of nitric-oxide metabolites by cells that have been pre-activated with IFN-γ and exposed to *M. pneumoniae* (164). By contrast, SP-A enhanced the production of nitric-oxide metabolites and TNF-α in response to the *Mycobacterium bovis* bacillus Calmette-Guérin (165).

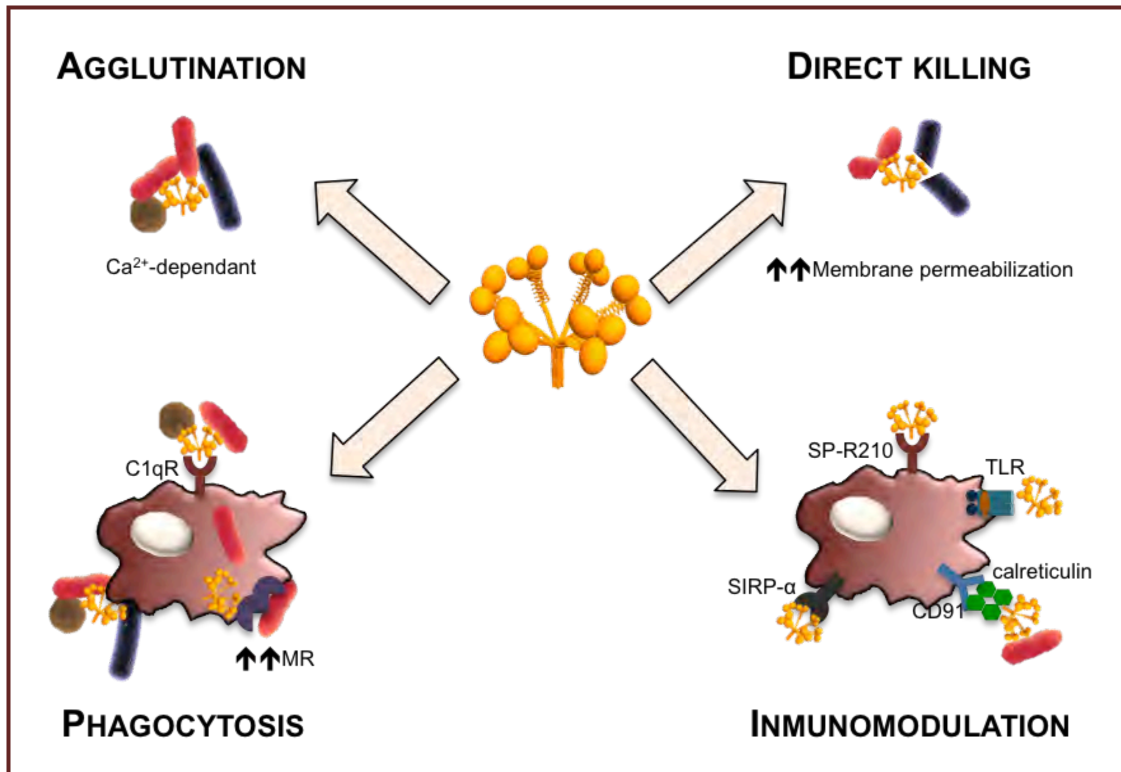

Figure 12. SP-A functions in lung innate defense against infection. SP-A displays multiple functions to add in overcome infection. Thus, SP-A is able to agglutinate and increase phagocytosis of microbes, as well as to kill directly microorganism by increasing membrane permeabilization. In addition, SP-A modulates immune system through interaction with several immune cells receptors. MR: mannose receptor; TLR: toll-like receptor; CD91: cluster of differentiation 91; SP-R210: SP-A receptor 210.

On the other hand, SP-A has also shown chemoattractive properties. SP-A enhances chemotaxis of alveolar macrophages (166) and inflammatory alveolar neutrophils (167) whereas it has the opposite effect on peripheral neutrophils (167). SP-A enhances chemotaxis through the stimulation of neutrophil chemotactic factors release (168). In addition, exogenous administration of SP-A in mechanically ventilated preterm lambs, which are characterized by defects in both immune and innate host defenses, stimulates the recruitment of neutrophils in the lungs (169).

Although the majority of SP-A functions are involved in innate immune response, numerous studies have shown that SP-A is also implicated in adaptive immunity in the lungs (170). Thus, SP-A is able to modulate several functions of dendritic cells and T cells, both involved in adaptive response (171). SP-A has been shown to prevent hypersensitivity in allergic response (124) and inhibit IgE binding to *Aspergillus fumigatus*, thus blocking

histamine release from human basophils (172). In addition, SP-A inhibits mast cell degranulation *in vitro* and *in vivo*, decreasing susceptibility to allergic antigens (172, 173).

C. Direct Microbicidal Activity

One of the main characteristics of SP-A as an antimicrobial protein is its ability to directly kill microorganisms (Fig. 12) (13). This activity derived from findings showing that SP-A KO mice were more susceptible than wt mice to lung infection by Gram-negative and positive bacteria like *Group B Streptococcus* (174), *P. aureginosa* (20), *H. influenzae* (175) or *K. pneumoniae* (176), as well as fungal or viral pathogens like *Histoplasma capsulatum* (177) or respiratory syncytial virus (178). In addition, exogenous administration of SP-A was able to restore bacterial clearance in mice lungs (174, 178).

Consequently, direct killing of SP-A has been widely investigated since purified SP-A was reported to inhibit the growth of Gram-negative bacteria or fungi (16, 177). Furthermore, the microbicidal role of SP-A *ex vivo* was demonstrated in studies involving macrophage-free bronchoalveolar lavages from SP-A -/- mice, which showed lower bactericidal activity against *E. coli* K-12 than those lavages from SP-A +/+ mice (14).

SP-A performs this activity mainly because its capability to bind to various ligands existing on the surface of microbes, such as LPS on Gram-negative bacteria, glycoprotein on fungi, lipoarabinomannan on mycobacteria, phospholipids on mycoplasma, and glycoproteins on virus surface. Subsequently, even though little is known about the SP-A mechanisms of microbicidal activity, SP-A has been shown to kill bacteria and fungi, to inhibit the growth of mycoplasma and mycobacteria as well as to neutralize viral infection (13).

I. Virus

Direct activity of SP-A on virus can be referred to as viral neutralization or inhibition of hemagglutination activity. In the case of influenza A virus, hemagglutinins allows the recognition of host cell and facilitates the entry of the viral genome into the target cells (179). SP-A can interfere with this process through interaction between sialic acid residues on its CRD and (presumably) the sialic acid receptor present on the hemagglutinins (180). Additionally, SP-A can also interact with herpes simplex virus type 1 (181), respiratory syncytial virus (182) and human immunodeficiency virus (183) in order to neutralize their

infectivity, as seen by reduction in the number of host cells expressing viral nucleoprotein after infection.

II. Fungi

The interaction of SP-A with fungi seems to be directed by two mechanisms: binding to structural polysaccharides consisting of repetitions of the same oligosaccharide elements, and interaction with glycosylated proteins express by fungi on their surface (40). SP-A has been reported to inhibit the growth of *Histoplasma capsulatum* by altering the cell membrane, as seen by proteins leak from the microorganism and enhanced access to the impermeant alkaline phosphatase substrate ELF97 after exposure to SP-A (177).

III. Bacteria

SP-A microbicidal activity has been mainly investigated in Gram-negative bacteria. SP-A directly inhibits the growth of Gram-negative bacteria such as *E. coli, Bordetella pertussis*, *Legionella pneumophila* and *P. aeruginosa* by increasing bacterial membrane permeability (16, 19, 24, 184). In general, rough bacterial mutants containing truncated LPS species were more readily permeabilized by SP-A than smooth strains (16, 24, 185). It has been suggested that bacterial permeabilization and killing require interaction between SP-A and LPS, most likely through the protein binding to the proximal core and/or lipid A moieties of LPS (17). This hypothesis is supported by the finding that deletion of the terminal sugar of *B. pertussis* and *B. bronchiseptica*, the causative agent of whooping cough, renders the organisms susceptible to aggregation and permeabilization by SP-A (24).

On the other hand, although there are no reports about direct killing of SP-A in Gram-positive bacteria, it has been shown that SP-A is able to inhibit the growth of other bacterial groups, as mycoplasma or mycobacteria, which are well-known opportunistic pathogens (186, 187). SP-A binds *Mycoplasma pneumonia* or *Mycobacterium avium* through lipids expressed on its OM and decreases metabolic activity and bacterial DNA replication, thus attenuating its growth (188, 189).

6.1.3. EFFECT OF SP-A ON GRAM-NEGATIVE BACTERIA MODEL MEMBRANES

SP-A has been reported to kill Gram-negative bacteria by increasing membrane permeability. However, the mechanisms by which SP-A increases the permeability of the bacterial membrane *in vivo* are still poorly understood. Nevertheless, there are various reports comprising model bacterial membranes that help to understand, at least, some of the molecular mechanisms involved in SP-A bacterial killing.

The CRD domain of SP-A is able to recognize and bind various components of the bacterial surface. Thus, SP-A has been shown to bind to rough LPS (159), di-mannose repeating units of some capsular polysaccharides (147), phospholipids such as phosphatidylcholine (which can be found on diverse bacteria) (123), and membrane proteins such as P2 outer membrane protein of *H. Influenzae* (148).

Bacterial membrane is responsible for mediating many essential functions in microbial pathogens, such as selective permeability and maintenance of gradients; energy obtaining by electron transport and oxidative phosphorylation; synthesis and cross-linking of peptidoglycan or another biopolymers; motility; and processing or display of adhesins or other key virulence determinant (190). Consequently, membrane disruption by SP-A might interfere with some of these essential bacterial functions, thus resulting on bacterial death.

Using Re-LPS model membranes, it has been shown that SP-A may disrupt the bacterial membranes by extracting Re-LPS molecules thereof. This would make more fluid the bacterial membranes and create packing defects that facilitate the interaction of SP-A with its main ligand on Gram-negative bacteria, the lipid A. This would cause the membrane permeabilization, which would allow the entry or exit of small molecules, such as water, through the bilayer of LPS (30). This ability of SP-A to disrupt bacterial membranes is dependent on its binding to the LPS, since this does not occur in vesicles containing 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (17).

Even though a mounting body of evidence seems to support that membrane permeabilization alone appears insufficient to cause cell death (191), this effect contributes, al least, to mechanisms by which SP-A exerts their antimicrobial activity. Therefore, additional or complementary mechanisms could be involved in the mechanism of action of SP-A, such as targeting of intracellular components like some AMPs (191).

6.1.4. SP-A BACTERIAL RESISTANCE

Despite SP-A is specialized to target specific and essential molecular patterns expressed by pathogens; microorganisms inevitably develop diverse strategies for its own defense.

The main constitutive resistance strategy of Gram-negative bacteria against SP-A appears to be the expression of more complex LPS. Numerous studies have shown the binding preference of SP-A by deep rough LPS. SP-A binds to LPS from either the Re-mutant of *Salmonella minnesota* or the J5-mutant of *E. coli*; but not to *E. coli* O111 LPS (159, 192). SP-A interacts with Rb LPS mutant from *E. coli* strain LCD 25 (131). Consistent with that, SP-A has been reported to kill *E.coli* strains K12 (14) or J5 (16), both expressing deep rough LPS as well as rough mutants of *B. pertussis* (24) or *P. aeruginosa* O1 (19, 185, 193). In contrast, SP-A did not increase permeability of *E. coli* O111, which expresses smooth LPS, wild-type strains of *B. pertussis* or *P. aeruginosa*, or liposomes containing palmitoyloleoylphosphatidylethanolamine and smooth LPS (17).

Besides LPS, resistance against SP-A may be due to other components of the bacterial cell wall, such as the capsular polysaccharide, pili, and flagellum. SP-A binds to specific strains of *K. pneumoniae* expressing the sequence mannose-α-2/3-mannose on its CPS, unlike *Klebsiella* strains expressing CPS lacking this sequence (147). These *Klebsiella* strains have been also found to be resistant to other lectins (194, 195). In fact, it has been proposed that the degree of virulence conferred by a particular CPS might be connected to its mannose content, since the lack of this oligosaccharide could avoid direct killing and phagocytosis by lectins and macrophages (196). This is consistent with the observed predominance of *Klebsiella* expressing capsular serotype K2 infections, one of the most virulent of *Klebsiella* (97). On the other hand, flagellum and type IV pilus expressed by *P. aeruginosa* have been reported to confer resistance to SP-A in this bacterium. Thus, *P. aeruginosa* mutants in these components were preferentially cleared by SP-A +/+ mice, but survived in SP-A KO mice (185, 193). In addition, SP-A was able to increase membrane permeability and/or aggregate these mutants, unlike wt strain.

There is another set of mechanisms that make bacteria resistant to the microbicidal action of SP-A, like the secretion of exoproteases. Incubation of SP-A with several clinical isolates of *P. aeruginosa* organisms resulted in a concentration- and temperature-dependent degradation of SP-A, consistent with the presence of a *Pseudomonas* elastase B (197). This

was also tested *in vivo* where isogenic mutant strains of *P. aeruginosa* nonexpressing this protease were attenuated in virulence when compared to the wild-type strain during lung infection in SP-A +/+ mice (197). Additionally, a secreted serine protease of *Pseudomonas*, called protease IV, was also found to degrade SP-A from cell-free bronchoalveolar lavage fluid, thus inhibiting SP-A-mediated bacterial aggregation and macrophages uptake (198).

6.2. ANTIMICROBIAL PEPTIDES

As mentioned in this thesis, antimicrobial peptides (AMPs) are among the main components of the innate immune response and the principal defense system for the majority of living organisms (49). Currently, more than 2000 natural AMPs with several different sequences have been isolated from a wide range of organisms (Antimicrobial Peptide Database, http:// aps.unmc.edu/AP/main.php), of which only 6% are anionic. Therefore, almost all research has been developed with cationic antimicrobial peptides (CAMPs). However, anionic antimicrobial peptides (AAMPs) are receiving more attention, since they have been shown as another integral and important part of the innate immune system.

Exogenous and endogenous AMPs are mostly amphipathic molecules, with a high to moderate content of cationic or anionic residues, and hydrophobic amino acids (199). These characteristics allow them to kill a broad-spectrum of microorganisms as well as to display immunostimulatory and anti-inflammatory properties (8).

Based on peptide secondary structure, AMPs can be classified into (200): α- helical, β-sheet, extended and loop-structured peptides (Fig. 13).

- α-helical peptides: are unstructured or disordered peptides that fold into amphipathic α-helices when get in contact with a target membrane. Magainins**,** which are expressed on the skin of frogs (201), or human cathelicidins (202), are one of the most representative CAMPs of this group. Dermcidin is one of the most studied human AAMPs that also adopts a α–helix structure upon contact with lipid bilayer surface (203).

- β-sheet peptides: contain between 2 to 4 disulfide bridges that stabilize β-sheet structures and adopt cyclic-like conformations that are thought to be essential for antibacterial activity. Defensins are among the main components of this group in

mammals (52). Another example is HE2C, which is an anionic β-defensin-like peptide expressed in human testis (204).

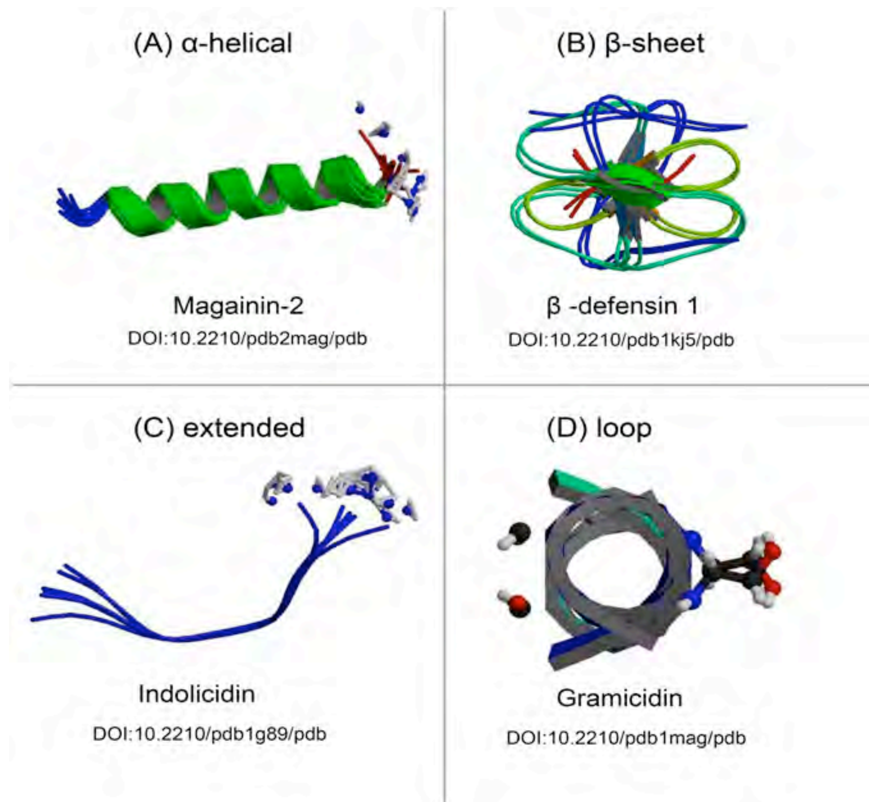

Figure. 13. Protein models of the four classes of antimicrobial peptides according to their secondary structure. Taken from (121).

- Linear extended peptides: less common that the above mentioned, these peptides adopt an unconventional linear structure, without α- or β-structures. They are characterized by the overexpression of one or more amino acids, like histidine residues on histatins (205), tryptophan residues on indolicidins (206), or proline and arginine residues on the proline-arginine (PR)-rich antibacterial peptide (207).

- Loop-structured peptides: this peptides assembly into loops due to the presence of a single disulfide bond and/or cyclization of the peptide chain. An example is bactenein found in cattle and sheep (208). Another examples are gramicidin S and polymyxins, which are cationic decapeptides that adopt cyclic structures in their peptide chains (209).

6.2.1. MAIN FEATURES FOR ANTIMICROBIAL ACTIVITY

AMPs share common features, which are responsible for their selective toxicity for the microbial target, but not for the host. They are mainly characterized by forming amphipathic structures, with cationic net charge at physiological pH (although as mentioned before, there is a small group of them that are anionic) and a moderate hydrophobicity, with up to 50% hydrophobic amino acids (199). These features allow AMPs to exert its activity against bacteria and not the host. Alternatively, AMPs toxicity to potentially vulnerable host tissue may be limited by localization and/or highly regulated expression of AMPs (191).

I. Charge:

Almost all antimicrobial peptides are cationic. Subsequently, they display a net positive charge, ranging from +2, like human α-defensin 1 (HNP-1), to +9, like human β-defensin 3 (HBD-3) (210). These characteristics are responsible for the initial binding of CAMPs to bacterial membrane through electrostatic interactions. In bacteria, outer phospholipid bilayer is heavily populated with lipids negatively charged, like phosphatidil-glicerol, -serine and cardiolipin. In contrast, the outer one of the animal cells is composed principally of lipids with no net charge (zwitteronic), and most of the lipids with negatively charged headgroups are facing the cytoplasm, into the inner leaflet. The elevated presence of acidic phospholipids on bacterial membrane, together with LPS and/or teichuronic acids (negatively charged), permit mutual electroaffinity between AMP and bacterial membrane, and confers selective antimicrobial targeting relative to host tissues (191).

In the case of anionic antimicrobial peptides, they contain aspartic acid residues as the main responsible for their negative charge, which generally ranges from -1 to -7 (211, 212). It still not clear how these anionic antimicrobial peptides are able to interact with bacterial surfaces, but it seems that they use metal ions to form cationic salt bridges with negatively charged components of microbial membranes, thereby facilitating interaction with their target organisms (212).

II. Amphipathicity:

Amphipathicity refers to the existence and polarization of hydrophobic and hydrophilic domains within a molecule. In proteins and peptides, it can be achieved by several conformations. For α-helical peptides, amphipathicity is often accomplished by a periodicity of

polar and apolar residues of about three to four, which can be achieved when the peptides are in contact with the target membrane. β-sheet peptides often show spatial separation of polar and hydrophobic residues due to the presence of a variable number of β-strands, which are frequently antiparallel (213).

As AMPs, biomembranes are also amphipathic. Cellular membranes are composed of a phospholipids bilayer, where hydrophobic acyl chains of phospholipids are placed into the membrane, whereas its hydrophilic headpolars are exposed to the outsides of the membrane, in contact with water. Consequently, amphiphatic conformation of AMPs seems to be the optimal structure for interaction with and insertion into bacterial membranes (191). Hydrophilic residues would interact with the polar headgroups of the phospholipids, while hydrophobic residues could be inserted into the membrane and interact with the acyl chains. In addition, the amphipathic nature of AMPs may enable them to form transmembrane channels and/or to cross the membrane for intracellular targeting (15).

III. Hydrophobicity:

Hydrophobicity is defined as the percentage of hydrophobic residues within a peptide. For most AMPs it is approximately 50%, and the hydrophobic residues more common are alanine, leucine, phenylalanine or tryptophan (199). This characteristic enables water-soluble antimicrobial peptides to partition into the membrane lipid bilayer and is required for effective membrane permeabilization (191). An increase of peptide hydrophobicity is associated with an increase in antimicrobial activity. However, increasing levels of hydrophobicity above a threshold have been strongly correlated with mammalian cell toxicity and loss of antimicrobial specificity (214).

6.2.2. MECHANISMS OF ACTION IN BACTERIAL KILLING

Several techniques like circular dichroism, solid-state NMR spectroscopy or microscopic analyses as well as studies involving model bacterial membranes have permitted to discern the mechanisms of action of AMPs (191). These findings have shown that AMPs exert different effects on microbial cells with diverse target sites or mechanisms of activity. However, regardless of the specific mechanism, the antimicrobial activity of AMPs has three commons steps that, in some cases, have been shown to be independent. These steps are: I)

peptide attachment; II) peptide insertion and membrane permeabilization; and III) cell death (15).

I. Peptide attachment: correlation between cationicity and membrane binding ability (215), as well as strong conservation of positive charge in CAMPs, seems to indicate that the initial binding of CAMPs with bacterial membrane may be initially conducted by electrostatic interactions (15, 216). In the OM of Gram-negative bacteria, CAMPs promote displacement of divalent cationic ions (Ca^{2+}), which are bound to CPS and LPS molecules maintaining the OM closely packed (217). Such displacement is likely to be energetically favorable given that the binding affinity of a typical antimicrobial peptide for LPS is ≈3 orders of magnitude greater than that of divalent cations (191). In Gram-positive bacteria, its envelope is enriched in negatively charged teichoic and teichuronic acids, which are responsible for peptide interaction.

AAMP Attachment is less studied. Many AAMPs, like dermcidin in human sweat, show higher activity at acidic pH and high salt conditions, which seem to function similar to catonic metal ions, enhancing the binding between the peptide and the bacterial membrane (203). On the other hand, AAMPs have been also speculated to interact with membranes via the use of receptors to cross the bacterial membrane and target intracellular components (212). However, the mechanisms underlying the antimicrobial effect of AAMPs are still unclear and remain to be further studied.

II. Peptide insertion and Membrane permeabilization: once CAMPs cross bacterial cell wall, they gain access to the membrane (outer and inner membrane in Gram-negative bacteria), which is permeabilized by peptide insertion. It has been suggested that peptide insertion into the membrane is sequential and related to peptide concentration (218). Peptides begin to bind parallel to the membrane until they arise a threshold concentration at which peptide molecules orientate perpendicularly and insert into the bilayer, forming transmembrane pores to permeabilize the bacterial membrane [210].

Several models of insertion have been proposed, according to experimental findings with the techniques previously mentioned (Fig. 14):

a. Barrel-stave model (219): proposed for alamethicin. Peptides aggregate in the bilayer surface until they reach a determined concentration where their helices form

a bundle in the membrane with a central lumen. Then, the hydrophobic peptide regions align with the lipid core region of the bilayer and the hydrophilic peptide regions form the interior region of the pore.

b. Toroidal-pore model (220): proposed for magainins, protegin and melittin. The attached peptides aggregate and induce the lipid monolayers to bend continuously till peptides connect the two leaflets of the membrane, forming a curved pore.

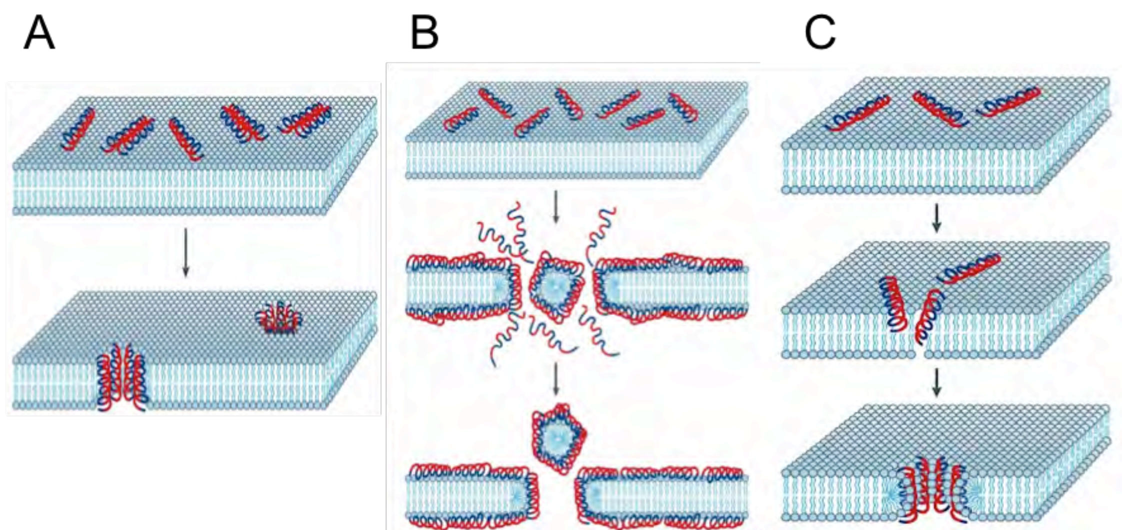

Figure 14. Proposed models of the killing induced by AMPs. A) Barrel-stave model. B) Carpet model. C) Toroidal model. Hydrophilic regions of the peptides are shown coloured red and hydrophobic regions of the peptide are shown coloured blue. Taken from (15).

c. Carpet model (221): peptides disrupt the membrane by orienting parallel to the surface of the lipid bilayer (forming an extensive layer or carpet) that, at high peptide concentrations, disrupt the bilayer in a detergent-like manner, eventually forming micelles.

III. Cell death: in early studies, AMPs permeabilization was thought as the primary mechanism of killing, due to impairment of essential functions of the membranes as well as by leakage of ions and metabolites (191). However, there is increasing evidence that indicates that AMPs have intracellular targets. Buforin II, a proline α-helical peptide, penetrates the bacterial outer membrane without permeabilizing it, and

accumulates into the cytoplasm, exerting its cytotoxic activity (222). CAMPs rich in arginine residues are able to translocate across both the cellular and nuclear membranes, where they interact with DNA, RNA and/or proteins to inhibit synthesis pathways (223). There is no general mechanism of intracellular killing. Once AMPs translocate into the cytoplasm, they are able to inhibit cell-wall synthesis, enzymatic activity or DNA, RNA and protein synthesis and/or to activate autolysin (191).

6.2.3. IMMUNOMODULATION:

Several AMPs have been found to modulate both innate and adaptive immune systems and have been recently renamed (or grouped) as host defense peptides (HDPs) (8). Among this function, HDPs exert numerous roles including direct and indirect modulation of pro- and anti-inflammatory responses; increase of extracellular and intracellular bacterial killing; promotion of polarized dendritic cell maturation and macrophage differentiation; and modulation of wound repair, apoptosis and pyroptosis (53).

HDPs can reduce endotoxin-induced inflammation, because are able to bind to free LPS reducing its endotoxicity (224). HDPs decrease the expression of pro-inflammatory mediators, such as TNF-α, in human primary cells in response to various host and endogenous molecules, while upregulating anti-inflammatory cytokines and pathways (225). HDPs also displayed anti-inflammatory properties *in vivo* in both Gram-negative and Gram-positive bacterial infection models (226). On the other hand, they promote chemotaxis indirectly by stimulating the expression of well-known chemokines (227, 228), like CXCL8/IL8 and CCL2/MCP1, and directly by acting as chemokines themselves to recruit a variety of immune cells (229).

HDPs have been proposed to link the innate and adaptive immune responses by inducing the differentiation of dendritic cells and macrophages (230, 231). Additionally, HDPs have been shown to induce autophagy to increase intracellular pathogen clearance (232) and promote or inhibit apoptosis, depending on cell type (233) as well as induce pyroptosis (234), thus augmenting pathogen clearance and/or decreasing an excessive inflammation (8).

7. EXOGENOUS CATIONIC ANTIMICROBIAL PEPTIDES: POLYMYXINS AND THEIR NOVEL DERIVATIVES

Polymyxins are nonribosomal cyclic lipodecapeptides that are strongly cationic, due to the presence of five free amino groups (235). They are synthetized by different species of *Bacillus polymyxia* and are able to kill Gram-negative bacteria. They were discovered in 1947 and were subsequently used in intravenous therapy. However, due to their high toxicity for humans and the discovery of better-tolerated drugs, they were largely abandoned in the 1960s (236). Nevertheless, polymyxins have the ability to kill the current MDR bacteria. Thus, they have been reinstated as the last-line therapy for severe MDR infections (26, 237).

7.1. POLYMYXIN B AND COLISTIN:

Polymyxins comprise a group of five chemically different compounds (polymyxins A–E), but the most studied and the only available for clinical use are polymyxin B (PMB) and E, also named colistin (5). They have typical features of CAMPs, including: high cationic character (+5), provided by 5 α,γ-diaminobutyric acid residues; and hydrophobicity and amphiphaticity, provided by the presence of phenylalanine and leucine residues and a fatty acid tail. PMB and colistin share a common primary sequence, but differ at position 6, which is occupied by D-Phe in PMB and D-Leu in colistin. All amino acid residues are of the L-configuration, except for these D-amino acids at position 6 (Fig. 15) (238).

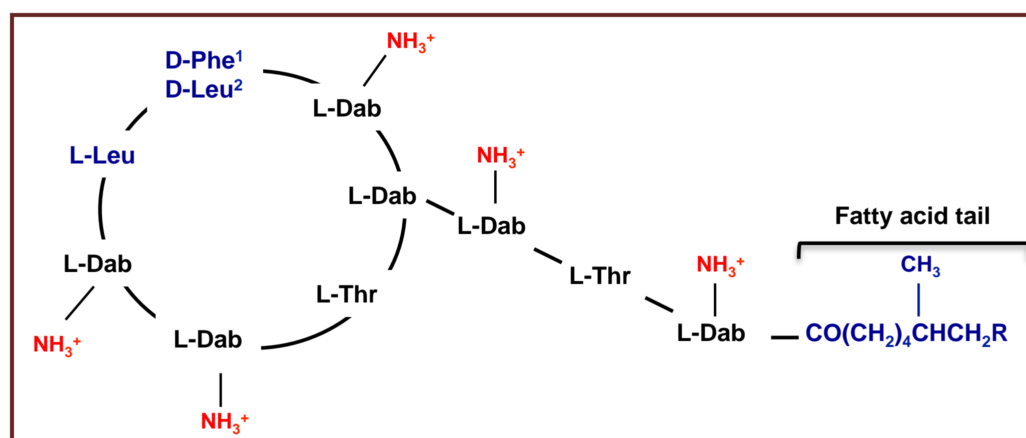

Figure 15. Polymyxin structure. Polymyxins are pentacationic lipodecapeptides composed of a heptapeptidic ring and a fatty acid tail. They comprise PMB and colistin, which only differ at position 6 that is occupied by D-Phe in PMB ([1]) and D-Leu in colistin ([2]). Hydrophobic regions are shown colored in blue and cationic regions are shown colored in red. Dab: diaminobutyric acid.

There are several proposed model for the mechanism of action of polymyxins, but the most accepted, consistent with available and extensive experimental data in the literature, is the mechanism of "self-promoted uptake" (217). Polymyxins firstly bind to and accumulate onto the OM though electrostactic interactions between the positive charges of the diaminobutyric acid residues and the negative charges of LPS molecules, thus promoting access to the outer lipid bilayer. This initial electrostatic interaction temporarily stabilizes the complex and brings the N-terminal fatty acyl chain of the polymyxin molecule into proximity with the lipid A of the outer lipid bilayer. Thereby, polymyxins bind to the lipid A monolayer through hydrophobic and electrostatic interactions, inserting, subsequently, the lipophilic face (fatty acyl chain and the Phe and Leu hydrophobic domain) into the hydrophobic core of the bacterial outer membrane. This appears to promote a loose packing of the adjacent lipid A fatty acyl chains, causing OM disruption (238). Afterward, the polymyxin molecule inserts and disrupts the physical integrity of the inner cytoplasmic membrane (inner membrane of Gram-negative bacteria) by forming channels that lead to the leakage of cytoplasmic molecules and likely cell death (239). Additionally, as many other AMPs, it is though that polymyxins may target intracellular components of bacteria, which also would contribute to bacterial death.

Polymyxins exert neurotoxicity and, mainly nephrotoxicity, which is higher in patients with abnormal renal function, correlates with the age and varies in the recent literature from 10% to 30%, reaching up to 45% or 55% in some case (236, 240-242). The nephrotoxicity of polymyxins is principally associated with acute tubular necrosis and increased serum creatinine concentrations (236). The mechanisms of polymyxin-induced nephrotoxicity are not clear, but seem to be related to kidney tubular cell apoptosis, since PMB- and colistin-induced tubular apoptosis have been reported (243). In addition, toxicity of polymyxins might be linked to that of aminoglycosides, another group of cationic antibiotics that produce damage renal proximal tubuli by interaction with the brush-border membrane of the proximal tubular cells, which are rich on acidic phospholipids (244). Moreover, oxidative stress and reactive oxygen species have been suggested to play a key role in their nephrotoxicity (244, 245).

7.2. POLYMYXIN DERIVATIVES:

Numerous polymyxin derivatives have been developed, in order to reduce the polymyxins-associated toxicity for patients or to obtain more potent and efficient antimicrobials (246). Thus, derivatives lacking the fatty acid tail or two strategic positions of the five cationic charges of polymyxins differ from polymyxins in their renal handling and affinity to kidney brush-border membrane, and are in preclinical studies (27).

7.2.1. DES-FATTY ACYL DERIVATIVES

7.2.1.1. POLYMYXIN B NONAPEPTIDE

Polymyxin B nonapeptide (PMBN) is a derivate from PMB that lacks the fatty acid tail and the N-terminal amino acyl residue Dab (Fig. 16). Its net charge is therefore + 4, instead of +5 of PMB. PMBN is less bactericidal or not bactericidal at all, but retains a significant OM-perturbing action (247). It has been shown to only produce a mild release of periplasmic proteins, unlike PMB, which is able to cause a high efficiently periplasmic and cytoplasmic protein release (248). However, PMBN is still able to bind to LPS with high affinity, which is likely retained by proper three-dimensional conformation and cationicity (249).

Although PMBN is an extremely poor antimicrobial compound, it has been shown to reduce at low concentrations the MICs of many hydrophobic antibiotics for several pathogens (250-252), which is consistent with its LPS binding affinity (249). Intraperitoneal treatment of PMBN + erythromycin was reported to protect mice infected with *K. pneumoniae* or *P. Aeruginosa*, whereas PMBN or erythromycin alone were inactive (253). PMBN was also able to sensitize various Gram-negative pathogens to the direct bactericidal effect of human serum (254, 255) and neutrophils (256). Therefore, PMBN appears to have an effective synergistic ability with other antimicrobials by disturbing the bacterial OM, which could establish a novel therapeutic direction (250, 253).

PMB	MHA/MOA	-Dab^{+}	-Thr	-Dab^{+}	-cy[Dab	-Dab^{+}	-DPhe	-Leu	-Dab^{+}	-Dab^{+}	-Thr]
Colistin	MHA/MOA	-Dab^{+}	-Thr	-Dab^{+}	-cy[Dab	-Dab^{+}	-DLeu	-Leu	-Dab^{+}	-Dab^{+}	-Thr]
PMBN	-	-	-Thr	-Dab^{+}	-cy[Dab	-Dab^{+}	-DPhe	-Leu	-Dab^{+}	-Dab^{+}	-Thr]
[Ser^{3}]-PMB (3-10) (Des-Fatty acyl derivative)	-	-	-	$^{+}$Ser	-cy[Dab	-Dab^{+}	-DPhe	-Leu	-Dab^{+}	-Dab^{+}	-Thr]
[Ser^{2}-Dap^{3}]-PMB (2-10) (Des-Fatty acyl derivative)	-	-	$^{+}$Ser	Dap	-cy[Dab	-Dab^{+}	-DPhe	-Leu	-Dab^{+}	-Dab^{+}	-Thr]
[Ser^{1}]-PMB (Des-Fatty acyl derivative)	-	$^{+}$Ser	-Thr	-Dab^{+}	-cy[Dab	-Dab^{+}	-DPhe	-Leu	-Dab^{+}	-Dab^{+}	-Thr]
NAB7061	OA	-	-Thr	-Abu	-cy[Dab	-Dab^{+}	-DPhe	-Leu	-Dab^{+}	-Dab^{+}	-Thr]
NAD741	Ac	-	-Thr	-DSer	-cy[Dab	-Dab^{+}	-DPhe	-Leu	-Dab^{+}	-Dab^{+}	-Thr]
NAD739	OA	-	-Thr	-DSer	-cy[Dab	-Dab^{+}	-DPhe	-Leu	-Dab^{+}	-Dab^{+}	-Thr]

Figure 16. Primary structures of polymyxin B (PMB), colistin and some of their derivatives. Boxed parts indicate locations where the compounds are not identical. MHA/MOA, mixture of methyl octanoyl and methyl heptanoyl (fatty acid tail); Dab: diaminobutyryl; Abu, aminobutyryl; Dap, diaminopimelyl; OA: octanoyl; Ac, acetyl; cy: cyclic portion indicated with brackets. The positive charge of the free α- and γ-amino group is also shown. Modified from (27).

Regarding toxicity, PMBN was 15-fold less toxic than polymyxin in an acute-toxicity assay in mice, 25-fold less active in releasing histamine from rat mast cells, and 150-fold less active in causing neuromuscular blockade (246). However, PMBN still suffers from being nephrotoxic and current research is trying to develop novel derivative less toxic (257).

Additionally, PMBN has been extensively used for studying the mechanism by which polymyxins and CAMPs kill bacteria, showing that three-dimensional conformation of cyclic ring of polymyxins seem to be more important than their chirality or LPS binding for their antimicrobial activity (249, 258).

7.2.1.2. DES-FATTY ACYL-PMB ANALOGS

Recently, more des-fatty acyl derivatives have been developed, which comprise the peptide ring portion of PMB and N-terminal moieties (linear peptide portion) that were changed to basic or hydrophilic amino acids (Fig. 16) (27). These synthetic peptides have smaller amino acid side chains and shortened length of the linear peptide. Unlike PMBN, they ha an antimicrobial activity against *P. aeruginosa* equivalent to that of PMB (259). In

addition, their binding activity to *E. coli* and *P. Aeruginosa* was comparable with PMB and they have lower acute toxicity after intravenous administration in mice (259, 260).

7.2.2. NAB COMPOUNDS

These derivatives have their cyclic peptide portion identical to that of PMB, but their linear peptide portions are uncharged, thus containing the only three positive charges of the peptide ring portion, whereas PMB and colistin contain five (Fig. 16) (27). NAB7061 and NAB741 have linear portions consisting of uncharged threonyl-D -serinyl and acetyl-Thr-D-Ser, respectively (261, 262). NAB739 differs from NAB741 by having an octanoyl group at the N-terminus, instead of acetyl (261).

Both NAB7061 and NAB741 lack potent direct antibacterial activity, but, like PMBN, are effective sensitizers by increasing the OM permeability to hydrophobic and large hydrophilic antibiotics in Gram-negative bacteria, (262, 263). In addition, combinatorial treatment of NAB7061 and erythromycin was effective in an experimental *E. coli* peritoneal infection, unlike the administration of these components individually (264). Both components induced less necrosis on renal proximal tubular cells than PMB (257), and showed higher renal clearance and less renal accumulation than (262, 265).

NAB739 exerts bactericidal activity similar or several-fold higher than PMB against *Enterobacteriaceae* or *A. baumannii* and *P. aeruginosa*, respectively (263, 266), and has been effective in treating mice i.p. infected with *E. coli* (264). In addition, it has shown combinatorial activity with antibiotics, like rifampicin (261). NAB739 was 26-fold less toxic than PMB and 7.5-fold less toxic than colistin to human kidney proximal tubular cells (267). Consequently, NAB739 is undergoing preclinical studies, although more toxicity and *in vivo* studies are necessaries.

8. HUMAN CATIONIC ANTIMICROBIAL PEPTIDES

8.1. DEFENSINS:

Defensins are small, multifunctional CAMPs, which typically contain 18–45 amino acid residues and six conserved cysteines that form three intramolecular disulfide bonds that stabilize a large β-sheet structure (Fig. 17) (268). They comprise an evolutionarily conserved family of CAMPs divided into three subfamilies: α-defensins, only expressed in mammals; β-

defensins, expressed in vertebrates; and θ-defensin, only found in certain non-human primates (269). α- and β-defensins subfamilies are expressed in humans and differ in the length of peptide segments between the six cysteines and the pairing of these cysteines that are connected by disulphide bonds (270).

- Human α-defensins: are composed of 29–35 amino acids and the cysteine arrangement is C1–C6, C2–C4 and C3–C5 (271). Four members, named human neutrophil peptides (HNPs) 1–4, have been primarily found in the azurophilic granules of neutrophils, but also in monocites, lymphocytes and natural killer cells. The other two members, human defensins (HD)-5 and -6, are located in secretory granules of intestinal Paneth's cells and in female genital epithelial cells (269). HNPs comprise 30–50% of the total protein of azurophilic organelles, and are expressed constitutionally by microbial signals, developmental signals or pro-inflammatory cytokines (29). When stimulated, both neutrophils and Paneth cells degranulate, releasing α-defensins into the local environment to fight against infection (8).

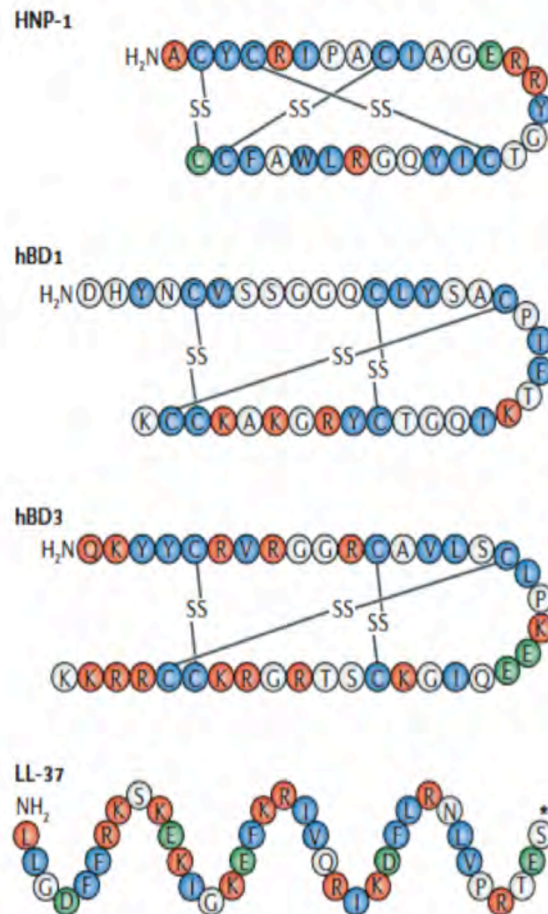

Figure 17. Structures of representative defensin and cathelicidin peptides. Residues with a net positive charge are in red, those with a negative charge are in green, and hydrophobic residues are in blue. N termini are indicated with NH2. The star at the end of LL-37 indicates that the C terminus is amidated. HNP-1, human neutrophil peptide 1; hBD, human β-defensin; SS, disulphide bonds. Taken from 77.

- Human β-defensins (HBDs): HBDs are 36–42 amino acids in length and have their 3 disulfide bridges arranged in a C1–C5, C2–C4, C3–C6 pattern (272). More than 28 HBDs have been identified, but the most studied are HBD-1, -2 and -3. HBD-1 is

expressed in respiratory epithelial cells, urogenital tract and reproductive tract; HBD-2 is expressed in skin, trachea and lung; and HBD-3 is expressed predominantly in the skin but is also in the respiratory trachea (273). HBD-1 is constitutively expressed and HBD-2 and -3 have been also found at very low basal amounts upon noninducible conditions. In addition, HBDs are induced by the presence of microorganisms or through pro-inflammatory cytokines such as TNF-α and IL-1β (274). HBD-3 represents the most potent CAMP of HBDs and has a broad-spectrum antimicrobial activity, including MDR bacteria. In addition, unlike the other HBDs, HBD-3 retains bactericidal ability upon physiological concentrations of sodium chloride or in the presence of various divalent cations (275, 276).

As AMPs, defensins exert microbicidal activity against Gram-negative and positive bacteria, fungi and virus as well as immunomodulatory functions into the host (272, 274).

8.2. CATHELICIDINS

These peptides have a strong cationicity (+ 6 at pH 7.4) and a high content of basic and hydrophobic amino acids (277). They are known as cathelicidins, due to their main structural feature, consisting on a high-level sequence identity in the 5′ region, named the N-terminal cathelin domain, because it is also found in cathelin, a cysteine protease inhibitor. The antimicrobial properties of cathelicidins are found in their highly variable C-terminal domain (278).

In humans, there is only one cathelicidin precursor, hCAP-18, which is processed by serine protease 3 to generate a 37 amino acid peptide called LL-37 (Fig. 17) (279). The structure of LL-37 in aqueous solution is relatively disordered, but adopts a typical α-helical assembly of CAMPs upon contact with the bacterial wall (280). LL-37 is expressed in immune cells, such as neutrophils, monocytes, dendritic cells, and macrophage, as well as in mast and epithelial cells. Its expression can be constitutive or induced by the presence of inflammatory mediators and microbial structures (278).

LL- 37 has been shown to display a broad range of immune functions both *in vitro* and *in vivo*. It is able to directly kill different microorganisms, including *P. aeruginosa*, *K. pneumoniae*, and *S aureus* (281-283). LL-37 has anti-fungal (284) and antiviral activity (285).

Moreover, LL-37 displays immunomodulatory activities, including both direct and indirect neutrophils, monocytes and T cell recruitment, and regulation of angiogenesis and cell growth (286).

Consequently, cathelicidins, together with defensins, have become a promising therapeutic agent to treat infectious and inflammatory diseases (280).

9. HUMAN ANIONIC ANTIMICROBIAL PEPTIDE: SP-B^N

In humans, AAMPs were firstly discovered in bronchoalveolar lavage fluid and airway epithelial cells. These peptides occur in millimolar concentrations, are antimicrobial against both Gram-positive and Gram-negative organisms and were shown to require zinc as a cofactor for antimicrobial activity (287). Later, a variety of AAMPs have been found in plasma and cells of human blood, which are mainly encrypted within the primary structures of proteins. Dermcidin is one of the most studied human AAMPs, which is proteolytically processed to give rise to 14 dermcidin-derived peptides, from which the anionic peptides DCD-1L and DCD-1 show antimicrobial activity (288). Unlike most of AMP, DCD-1L maintains antimicrobial activity over a broad pH range and at high salt concentration (289). This is consistent with others AAMPs, as the recently discovered human AAMPs surfactant protein B^N (SP-B^N), a soluble segment of the surfactant proprotein SP-B found in human airspace, which shares some features with dermcidin and has antimicrobial activity only at low pH (6).

SP-B^N is an 80 amino acid, anionic, saposin-like peptide, which comes from the proteolytic processing of the NH_2-terminal propeptide of SP-B. SP-B^N is secreted into the airspace with surfactant and exerts robust antimicrobial activity against Gram-positive and -negative bacteria, but only at acidic pH (6). Processing of SP-B occurs during the surfactant secretory pathway, in which is glycosylated in the endoplasmic reticulum, transported to the Golgi apparatus and thence to multivesicular bodies and is finally stored in lamellar bodies (290). SP-B is firstly translated into a larger precursor protein in which the mature SP-B peptide (residues 201–279) is flanked by an N-terminal prepropeptide of 200 amino acids and a C-terminal peptide of 102 amino acids; both of them containing saposin-like domains (291). Cleavage of both terminal propeptides occurs in the lumen of the multivesicular body, prior to or during their fusion with the lamellar body, and it is thought to be performed by proteases

such as napsin A and cathepsin H (292). Finally, the saposin-like domain of the N-terminal propeptide is also cleaved to obtain the AAMP SP-B^N. Moreover, the C-terminal domain encodes another saposin-like domain with unknown functions (6).

SP-B^N has been shown not to partition with surfactant lipids and to be secreted into the alveolar fluid, since it was recovered in the mouse bronchoalveolar lavage fluid and in nonciliated bronchiolar cells, alveolar type II epithelial cells, and alveolar macrophages (6). Overexpression of SP-B^N in targeted mice protected mice against infection with *P. aeruginosa* and *S. aureus*. *In vitro*, SP-B^N localized to lysosomes in phagocytic cells and was able to increase bacterial phagocytosis and killing by alveolar macrophages. In addition, SP-B^N directly killed *K. pneumoniae* and *S. aureus*, but only at acidic pH, which, together with lysosomal localization, was consistent with a potential role in intracellular killing by alveolar macrophages (6).

The mechanism of bactericidal action of SP-B^N is still unknown. SP-B^N shares striking structural and functional similarities with amoebapores, other member of the saposin-like proteins family. Amoebapores are a family of three cytolytic peptides (amoebapore A, B and C) from *Entamoeba histolytica* that kill ingested bacteria by the amoeba in acidic phagolysosomes (293). They are highly hydrophobic and amphipathic and permeabilize bacterial membranes in a pH- and oligomerization-dependent manner by forming a stable transmembrane pore that disrupts the cytoplasmic membrane (294). Both peptides are predicted to have very similar secondary structures and three-dimensional structure according with the so-called “saposin-fold”. Thus, by nuclear magnetic resonance, amoebapore A was found to contain five helices connected by three disulfide bonds; whereas, SP-B^N has been predicted to contain four amphipathic helices and three disulfide bridges arising from six invariant cysteine residues. This saposin-folding confers them resistance to denaturation and proteolysis, and promotes protein stability and likely antimicrobial activity in an acidic environment. Moreover, SP-B^N has been found to form a coiled coil structure at acidic pH, which could be implicated in its pH-dependent antimicrobial activity (295, 296).

Although still unknown, it is possible that these features allow SP-B^N to depolarize bacterial membrane via formation of pores, like amoebapores (6). Additionally, since it is secreted into the alveolar fluid together with another antimicrobials factors, whether SP-B^N could kill bacteria *in vivo* at neutral pH also remains to be determined.

10. SYNERGISTIC AND ADDITIVE ACTIVITY OF HOST DEFENSE FACTORS

The innate immune system of higher organism is usually composed of different antimicrobial peptides and proteins, which are virtually expressed at a particular site, like skin or alveolar space in humans (10). The presence of multiple antimicrobial factors might provide redundancy in bacterial killing and increase the spectrum of antimicrobial activity to strengthen host defense (22). Consequently, most of these factors might act cooperatively in their microbicidal activity (273). Antimicrobial cooperation occurs when two antimicrobial factors work in conjunction to reduce the individual concentration of each molecule required to kill a microorganism. Whether the combined activity is greater than the sum of the individual activities, it is synergistic activity. If it is similar to the sum of both, the combination is additive.

Both activities have been observed in AMPs of different organisms, not only between them, but also with antimicrobial proteins. Frog peptides, including members of the dermaseptin family and magainin, have shown synergistic activity (297, 298). Two peptides found in rabbit neutrophil granules, also act synergistically in killing extracellular pathogens (299). Peptides representing each of the structural classes found in mammals, protegrin-1 (β-hairpin), LL-37 (α-helical), bactenecin (loop), and indolicidin (extended), demonstrated combinatorial activity against representative human pathogens (300). In addition, HBD-2 and LL-37 had additive activity against *E. coli*, and lactoferrin and lysozyme acted synergistically in combination with HBD-2 or in triple combinations with the proteinases inhibitor secreted by leukocytes (22).

On the other hand, AMPs, both exogenous and endogenous, have been studied to synergize with antibiotics. Polymyxins showed synergistic and additive activity when combined with lipophilic and amphiphilic antibiotics, such as rifampin, macrolide antibiotics, fusidic acid and novobiocin (26). Additionally, AMPs like peptide p18, defensins, and cathelicidins have been found to act in conjunction with commonly used antibiotics against several microorganisms (301).

11. THERAPEUTIC POTENTIAL OF HOST DEFENSE FACTORS

As mentioned throughout the introduction, numerous studies involving SP-A have indicated the essential role of SP-A in innate immune defense of the host. In addition to its antimicrobial and immunomodulatory activities found *in vitro* and *in vivo*, the role of SP-A in the pathobiology of several pulmonary diseases has been demonstrated. Varying serum and bronchoalveolar lavage fluid levels of SP-A as well as polymorphisms in genes encoding SP-A have been associated with a range of infectious and non-infectious diseases (which may undergo infection), including idiopathic pulmonary fibrosis (302), cystic fibrosis (303, 304), pulmonary alveolar proteinosis (305), hypersensitivity pneumonitis (306), bacterial pneumonia (307), acute lung injury/acute respiratory distress syndrome (308, 309) or interstitial lung disease (310). Moreover, lower respiratory tract pathogens have been shown to impair SP-A function to potentially leave the host vulnerable to develop severe infection (311). Consequently, administration of exogenous SP-A might be useful for both delaying microbial invasion and increasing host defense mechanism to overcome infection.

Usage of naturally occurring antimicrobial factors as future therapeutics is being widely investigated, not only with SP-A. AMPs are currently great promising alternatives to treat respiratory infections (312). Together with SP-A, they are implicated in host defense, including microbial clearance, immunomodulation and neutralization of endotoxins (53). In addition, are unaffected by classical antibiotic resistance mechanisms and are less likely to produce new bacterial resistance (121). Most importantly, one of the main desirable characteristics is its ability to kill MDR bacteria, (313).

With multi-drug resistance accumulation without new effective antibiotics, many companies are trying to introduce the antimicrobial peptide products into the market (313, 314). AMPs have been or are in various stages of development, ranging from early preclinical studies to phase III clinical studies. Pexiganan (analog of maganin 2) was the first AMP developed commercially, which arrived into phase III clinical trials, but failed to be accepted for clinical use, because it was not more effective that other current antibiotics (315). Since then, AMPs have been in clinical phases for more than 10 years. However none has been yet approved for clinical use for several reasons, including low oral bioavailability, weak activity *in vivo*, nonspecific cytotoxicity, apparent susceptibility to proteolysis, and their propensity to be rapidly metabolized (314). Additionally, they are affected by a very expensive synthesis

compared to antibiotics, since production of one gram of an AMP can cost up to 400US$, whereas for a conventional antibiotic this price can be under 1US$ (313).

Polymyxins are one of the most promising AMPs that are currently used clinically (5). Both polymyxins B and colistin are used for topical treatments of infection by *P. aureginosa* and *A. baumannii* without side effects and with low bacterial resistance (313). Although these AMPs are too toxic to be used systemically as anti-bactericidal or anti-endotoxic reagents; both colistin and polymyxin B are increasingly being used as salvage therapy in critically-ill patients with MDR Gram-negative infections (26, 316).

Due to the appearance of these MDR bacteria, functional recombinant proteins of SP-A (containing full-length SP-A or some of its domains) as well as synthetics analogs of polymyxins or AMPs are being currently developed to reduce their toxicity and/or improve their clinical activity for the treatment of several infections, including respiratory infections (13, 246). Additionally, some of these factors, as well as their analogs, have shown synergistic activity with both antibiotics and other antimicrobial factors of the host (301, 317). This feature is particularly interesting, since combinatorial use between these molecules and/or with antibiotics could reduce clinical doses of both, thus decreasing associated toxicity and/or increasing effectiveness of antibiotics against MDR bacteria.

OBJECTIVES

Gas exchange between oxygen and CO_2 is essential for the maintenance of metabolic processes in the organism, and requires a large contact surface with the atmosphere. The alveolar epithelium is continuously exposed to inhaled particles such as pathogens, allergens and contaminants. To protect the host against microorganisms reaching the alveoli during inspiration, there are some effective defense mechanisms.

Beside a cellular component, the lung innate immune system is composed of a soluble component, consisting of proteins and antimicrobial peptides (AMPs) able to kill, directly or indirectly, microorganisms as well as modulate the inflammatory response of the host (3, 8). SP-A is a large protein present in the lung with immune defense functions (12, 18). SP-A belongs to the collectin family, and it is the most abundant protein of the alveolar fluid by weight. SP-A acts as an opsonin and enhances phagocytosis of several pathogens including *S. aureus* and *P. aeruginosa* (149, 197). SP-A is able to modulate the alveolar immune cell response and also exert direct bactericidal activity against some Gram-positive and Gram-negative bacteria, virus and fungi *in vitro* and *in vivo* (12, 14). It has been reported that SP-A enhances lysosomal delivery of GFP-transfected *E. coli* in alveolar macrophages (318), suggesting that SP-A could impair pathogens' evasion of lysosomal fate. SP-A has also been shown to have a protective role in *K. pneumoniae* infections since SP-A knock-out mice show a higher mortality index upon *K. pneumoniae* challenge (176). Finally, it has been reported that pathogen elimination is very deficient in SP-A -/- mice (18), whereas SP-A +/+ mice showed a greater microbial clearance, even on bacteria resistant to SP-A killing *in vitro* (19, 20).

Since the alveolar fluid contains other soluble factors able to recognize and eliminate pathogens, like AMPs, it is possible that synergistic or cooperative action between several airway antimicrobial factors might strengthen the microbicidal activity of each factor alone. Therefore, our hypothesis is that SP-A could act in collaboration with AMPs of the alveolar space to increase its antimicrobial activity in the alveoli. This would contribute to enhance the clearance of respiratory pathogens. In this regard, synergies between lysozyme, lactoferrin and human-beta defensin-2 (HBD-2) have been described (21), while cathelicidin LL37 and HBD-2 have demonstrated additive action against *E. coli* (22).

This is particularly important given the emergence of antibiotic bacterial resistance and spread of MDR-bacteria to current antimicrobials. Thus, it has increased urgency to explore

alternative means of combating pathogenic assault (23, 109). A better understanding of the biological and molecular basis of SP-A-pathogen and SP-A-AMPs interactions opens stimulating perspectives on future alternative or adjuvant treatments.

Therefore, the main objective of this thesis was **to study both individual and cooperative antimicrobial activities of SP-A in airway host defense**. To accomplish this task, several respiratory Gram-negative pathogens were used in order to clarify the mechanism of bacterial resistance to SP-A, as well as to elucidate the potential synergistic activity of SP-A with AMPs. Additionally, the mechanism by which SP-A kills bacteria in conjunction with these factors was investigated.

This thesis is composed of **three chapters** with the following concrete objectives:

1. To study the potential protective effect of SP-A against different smooth and rough strains of the lung pathogen *Klebsiella pneumoniae* K2, as well as its ability to modulate the antimicrobial activity of cationic AMPs, both exogenous and endogenous, against several respiratory pathogens, including *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Haemophilus influenzae*.
2. To determine the potential cooperative therapeutic action of SP-A and the anionic peptide SP-B^N against a common bacterial cause of pneumonia *in vivo*.
3. To evaluate the mechanisms by which SP-A and a nonapeptide derived from polymyxin B (PMBN) kill bacteria synergistically.

MATERIALS AND METHODS

1. Materials

1.1. Surfactant Protein A (SP-A)

Human surfactant protein A was isolated from bronchoalveolar lavage fluid (BALF) of patients with alveolar proteinosis using a sequential butanol and octylglucoside extraction similar to (319). Aliquots from BALF (1.5 mL) were re-suspended in 25 mM Tris, 150 mM NaCl buffer (25 mL) and subsequently added to 1-butanol, HPLC grade (Scharlab) (1:30, w/v) according to the amount of total protein in the aliquot determined by lowry protein assay (320). After 30 min of stirring at RT, surfactant/butanol mixture was centrifuged at 5000 g for 30 min at 15ºC to deposit the butanol-insoluble protein. Pellet was dried under nitrogen and washed twice with 5 mM Tris, 150 mM NaCl, 20 mM octyl-/3-D-glucopyranoside (Sigma) pH 7.4. The obtained pellet was suspended in 5mM Tris pH 7.4 and dialyzed against the same buffer for 24h at 4ºC to remove detergent. After that, solution was centrifuged at 100,000 g, 4ºC, for 30 min. Supernatant, containing soluble protein SP-A, was finally stored in small aliquots at -20ºC after evaluating the amount of protein by lowry protein assay (320). The endotoxin level of SP-A was measured by the toxinsensor™ chromogenic limulus amebocyte lysate endotoxin assay kit according to the manufacturer's instructions (GenScript, USA). Endotoxin levels were less than 0.15 EU/ml.

Isolated human SP-A was characterized by several experimental approaches:

- **Polyacrylamide Gel Electrophoresis (PAGE)**

The purity of SP-A was checked by 1-dimensional SDS-PAGE with 12% acrylamide/bisacrylamide under reducing conditions, and its oligomerization state was assessed by 1-dimensional PAGE in 4-12% acrylamide under non-denaturing conditions (131). After electrophoresis in these conditions, SP-A was detected by either coomassie blue or silver staining methods (321, 322). Under reducing conditions, SP-A migrated as monomer (≈36 kDa) and nonreducible dimer (≈ 72 kDa) (Fig. 1), as previously described for this protein (323). Under non-denaturing conditions, SP-A migrated as higher oligomers, mainly octadecamers (≈650 kDa), as previously characterized (324). Thus, SP-A consisted of supratrimeric oligomers of at least 18 subunits. Each subunit had an apparent molecular weight of 36,000 Da.

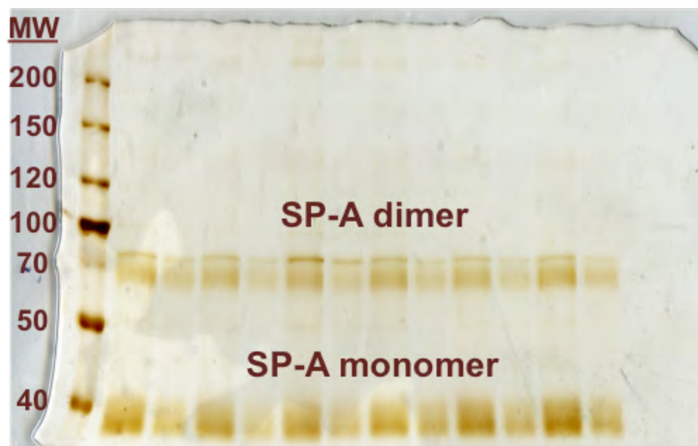

Figure 1. Silver stain of human SP-A analyzed by 1-dimensional SDS-PAGE in 12% acrylamide under reducing conditions. Upper bands correspond to SP-A dimer (≈ 72 kDa); and lower bands correspond to SP-A monomers (≈36 kDa) after protein reduction.

- **Intrinsic Fluorescence Spectroscopy**

Fluorescence measurements of isolated SP-A were carried out in 5 x 5 quartz cuvettes using an SLM-Aminco AB-2 spectrofluorimeter equipped with a thermostated cuvette holder (±0.1 °C; Thermo Fisher Scientific, Waltham, MA, USA). Fluorescence emission spectra of SP-A (10 µg/mL) were measured at 25 °C in 5 mM Tris buffer, pH 7.4. Blanks and protein were excited at 295 nm. Emission spectra were recorded from 305 to 400 nm, and the slit-widths were 4 nm for the excitation and emission beams. The emission spectrum of SP-A is dominated by the contribution of two conserved tryptophan residues located at positions 192 and 213 of the globular COOH-terminal domain (135). The emission spectrum of human isolated SP-A had a maximum at 337 nm (Fig. 2A), as previously described (135).

- **Dynamic Light Scattering (DLS)**

DLS, sometimes referred to as Quasi-Elastic Light Scattering, is a technique that allows to measure the size distribution of molecules and particles dispersed or dissolved in a liquid (325). For this, a laser is focused on a small region of the sample. A certain proportion of the incident light is scattered and detected at an angle of 90 °, regarding to the direction of the incident beam. The Brownian motion of the scattering particles causes fluctuations in light

scattering intensity. The velocity of the Brownian motion of the particles can be calculated and related to their hydrodynamic size (326).

The hydrodynamic diameters of SP-A (Fig. 2B) in 5mM Tris buffer, pH 7, were measured at 25°C in a Zetasizer Nano S (Malvern Instruments, Malvern, UK) equipped with a 633-nm HeNe laser as explain later in section 5.1.2-DLS. The hydrodynamic size of isolated human SP-A was around 30-40 nm, as seen previously seen (144).

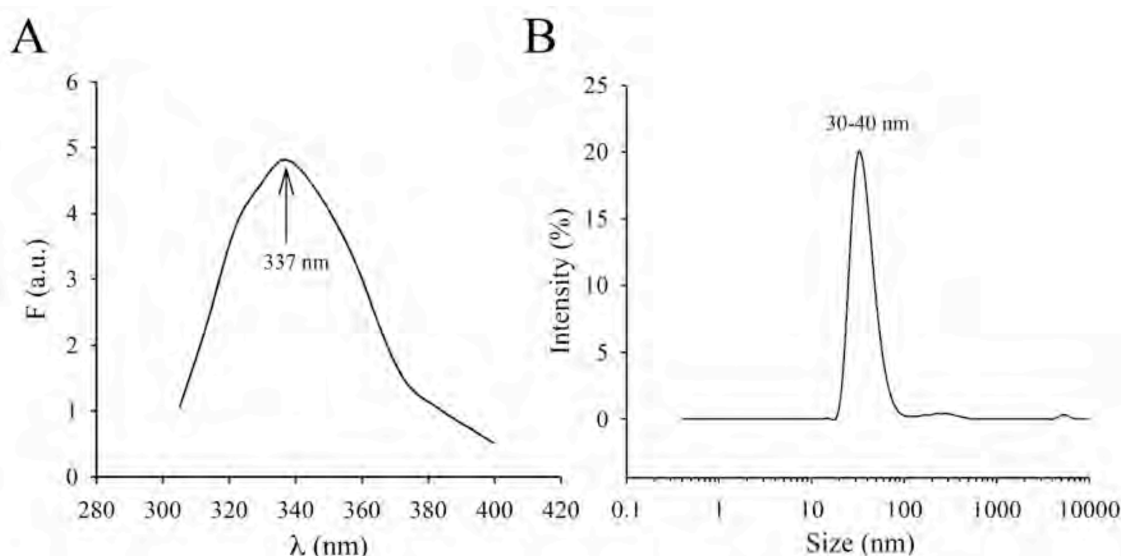

Figure 2. Representative graphs of emission spectra **(A)** and hydrodynamic size **(B)** of isolated human SP-A. SP-A shows a maximum at 337 nm when excited at 295 nm, and its hydrodynamic size is usually around 30-40 nm in 5 mM Tris buffer, pH 7.

- **Self-Aggregation Assay**

Self-aggregation of SP-A is one of its main intrinsic abilities, which it is principally Ca^{2+}-dependent, but also occurs at high-ionic strength or mildly acidic pH (136). Ca^{2+}-dependent self-association of SP-A (Fig. 3) was performed at 37 °C by measuring the change in protein absorbance at 360 nm in a DU-800 spectrophotometer (Beckman Coulter, Fullerton, USA) as previously described (327). Sample and the reference cuvettes were first filled with 0.4 ml of 5 mM Tris buffer, pH 7.4. After 10 min equilibration at 37 ºC, SP-A (20 μg) was added to the sample cuvette and the change in absorbance at 360 nm was monitored at 1-min intervals over 10 min. Next, Ca^{2+} (5 mM, final concentration) was added to both the sample and the reference cuvette and the change in absorbance was monitored again. Self-association of SP-

A was finally reversed by adding EDTA (10 mM, final concentration).

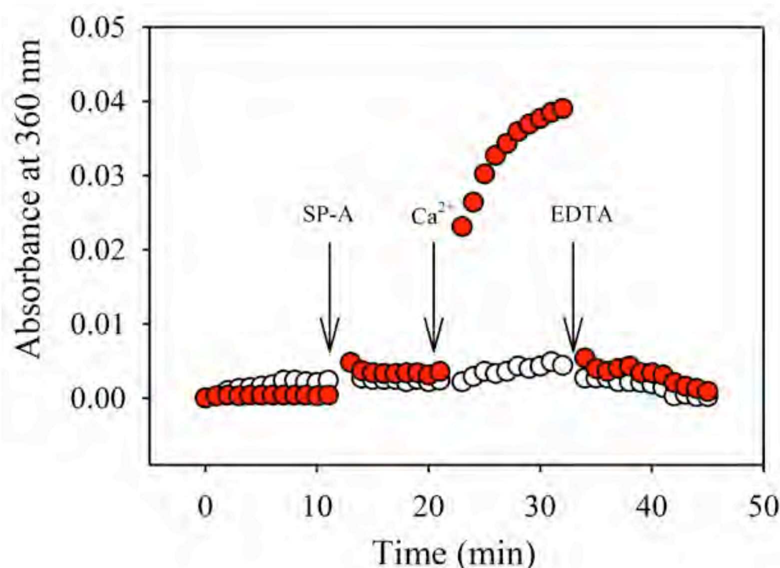

Figure 3. Representative graph of self-aggregation of SP-A in the presence of Ca^{2+}. Absorbance of 20 mg SP-A (red circle) in 0.4 ml of 5 mM Tris buffer, pH 7.4, or buffer alone (white circle) was measured at 360 nm before and after addition of Ca^{2+} (5 mM). Self-aggregation was reverted by addition of EDTA (10 mM).

- **Ability to aggregate bacteria expressing deep rough LPS.**

SP-A has been shown to aggregate bacteria, preferably expressing deep rough LPS (13). Thus, to analyze the biological activity of human isolated SP-A, we performed bacterial aggregation assays of SP-A against *Escherichia coli* J5, a rough mutant of *E. coli* 0111:B4, which express deep rough LPS (328) and has been reported to be bound and aggregated by SP-A (192, 329).

Bacteria were grown at 37ºC in 5 mL of Luria-Bertani (LB) medium and harvested (500 g for 10 min) on exponential-phase. The pellet was washed twice with 10 mM phosphate buffer saline (PBS) pH 7.2 and re-suspended in Hank's balanced salt solution (HBSS) buffer (0.137 M NaCl, 5,4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 4.2 mM $NaHCO_3$). Optical density (OD) of this suspension was measured in a spectrophotometer DU-800 (Beckman Coulter, Fullerton, USA) at 700 nm and adjusted to a final OD_{700} = 1. This OD was previously established to be equivalent to a bacterial concentration of 10^9 colony forming units (CFU)/mL by plating serial dilutions on agar plates of bacteria growth in the same conditions, and counting the number of CFU after ON

incubation at 37°C. SP-A 25 μg/mL was, then, added to 150 μL of this solution and incubated for 30 min at 37 ºC with 30 seconds of gentle shaking each 5 min. The aggregation was determined by monitoring the changes in absorbance at 700 nm during 2h at 37ºC without shaking (Fig. 4A). The results are shown as percentage of absorbance versus absorbance at time 0 (100%). Furthermore, *E. coli* J5 transformed with the plasmid pZsGreen expressing GFP was used for visualization of bacterial aggregation by fluorescence microscopy (Leica TCS SP2 Confocal System) (Fig. 4B).

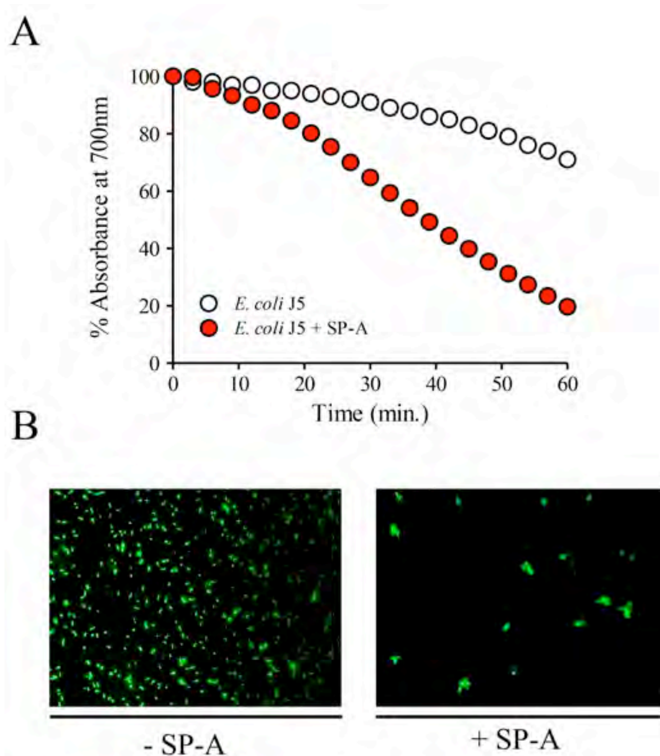

Figure 4. A) Representative graph of ability of SP-A (25 μg/mL) to aggregate *E. coli* J5. Aggregation was determined by monitoring the changes in absorbance at 700 nm during 1h at 37ºC without shaking. **B)** Representative images of *E. coli* J5 expressing GFP aggregated by SP-A.

1.2. Antimicrobial Peptides

- Polymyxin B (PMB), Polymyxin E or colistin (PME) and polymyxin B nonapeptide (PMBN) were obtained from Sigma Aldrich (St. Louis, MO, USA).

- Recombinant human β-defensins (HBDs) HBD-1, HBD-2 and HBD-3 were obtained from PrepoTech (Rocky Hill, NJ, USA).

- NH_2-terminal propeptide of surfactant protein B (SP-B^N) was obtained in the Prof. Timothy E. Weaver laboratory (Cincinnati Children's Hospital Medical Centre, OH, USA) during a predoctoral stay, as following. SP-B^N cDNA (encoding residues 61–146; Fig. 5A) was generated from mouse type II cell RNA by RT-PCR using upstream primer 5'-GGG AAT TCC ATA TGC ATG CAG GAG CTA ATG ACC TG-3' and downstream primer 5'-CCG CTC GAG CTG CCC ACG TGG GCA CAG GCC-3'; restriction sites for NdeI and XhoI were encoded in the upstream and downstream primers, respectively.

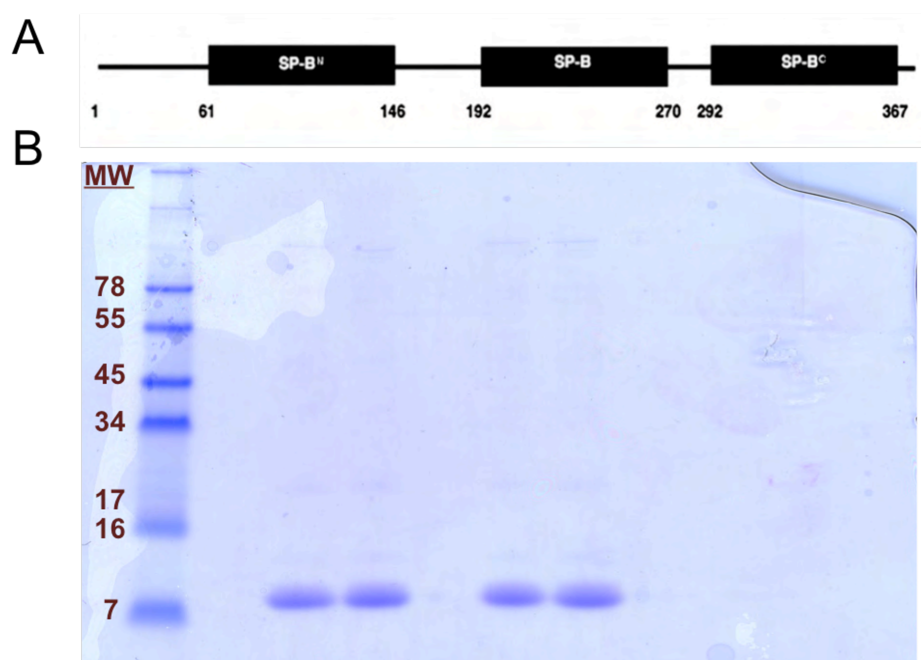

Figure 5. A) Schematic representation of preproSP-B protein. The boxes indicate the predicted location of three saposin-like domains in mouse preproSP-B: N-terminal peptide (SP-B^N): residues 61–146; mature peptide (SP-B): residues 192–270; and C-terminal peptide (SP-B^C): residues 292–367. Taken from (6). **B)** Recombinant SP-B^N on 10-20% Tricine-SDS PAGE gradient gel stained with comassie blue.

The amplified 258-bp fragment was cloned into the NdeI/XhoI sites of PET21a vector (Novagen, Madison, WI). SP-B^N was expressed in *Escherichia coli* BL21 (DE3). Transformed bacteria were grown in LB medium supplemented with 50 μg/ml carbenicillin to an OD_{600} of 0.6; protein expression was induced by the addition of 0.1 mM

isopropyl b-d-thiogalactoside (IPTG) for 3 h at 37° C. Ten to 20% tricine–SDS-PAGE of bacterial lysates expressing SP-B^N detected a band, Mr = 9 kDa, following IPTG induction (Fig. 5B). The broth was centrifuged, and the isolated bacterial pellet was lysed by sonication in 20 mM Tris buffer, pH 7.4, 4°C. Inclusion bodies were recovered by centrifugation, washed in Tris buffer, and solubilized in 20 mM Tris, 6 M urea, 50 mM DTT buffer, pH 7.4. Denatured, solubilized inclusion body protein was diluted (1:10) in 20 mM Tris, 6 M urea, 0.5 M NaCl, 3 mM reduced glutathione, 0.3 mM oxidized glutathione, pH 7.4, and dialyzed three times against 10 volumes of the same buffer in which the urea concentration was reduced to 2 M followed by dialysis against 10 volumes of 20 mM Tris, 0.5M NaCl (nickel-nitrilotriacetic acid [Ni-NTA] binding buffer), pH 7.9. After centrifugation, the supernatant was applied to a Ni-NTA agarose column (Novagen). The column was washed and eluted according to the manufacturer's protocol. Eluted protein was dialyzed against sodium phosphate buffer, pH 7.0, and stored in aliquots at -80°C. The endotoxin level of SP-A was measured by the toxinsensor™ chromogenic limulus amebocyte lysate endotoxin assay kit according to the manufacturer's instructions (GenScript, USA). Endotoxin levels were less than 0.12 EU/ml.

1.3.Biotynilation Assay

SP-A and SP-B^N were biotynilated by the FluoReporter® Mini-biotin-XX Protein Labeling Kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). This kit contains watersoluble biotin-XX sulfosuccinimidyl ester, which readily reacts with protein amines to yield a biotin moiety covalently attached via two aminohexanoic chains ("XX"). This 14-atom spacer has been shown to enhance the ability of the biotin moiety to bind to avidin's relatively deep binding sites. Proteins (1 mg/ml) in 100 mM sodium bicarbonate buffer, pH 8, were incubated with biotin (biotin/protein molar ratio of 3-8:1) for 90 min in darkness at room temperature. To remove unreacted fluorescent reagent, the mixture was exhaustively dialyzed against 5 mM Tris-HCl, pH 7.4. After that, protein amount was determined by lowry protein assay. The structure and functional activity of biotinylated proteins were similar to that of unlabeled SP-A and SP-B^N.

1.4.LPS and Lipids

Rough LPS from *Salmonella minnesota* (serotype Re 595) were purchased from Sigma (St. Louis, MO, USA). Total *E. coli* phospholipid extract was purchased from Avanti Polar Lipids (Birmingham, AL, USA). The organic solvents (methanol and chloroform) used to dissolve lipids were HPLC grade (Scharlau, Barcelona, Spain).

1.5.Fluorescent Probes

The fluorences probes Rhodamine Phalloidin and 4',6-diamidino-2-phenylindole (DAPI) were obtained from Invitrogen (Waltham, MA, USA). The fluorescent probes propidium iodide, 1,6-diphenyl-1,3,5-hexatriene (DPH), 6-lauroyl-2-(N,N-dimethylmino) naphthalene (LAURDAN), 8-aminonaphthalene-1,3,6-trisulfonic acid disodium salt (ANTS) and p-xylene-bis-pyridinium bromide (DPX) were obtained from Molecular Probes Inc. (Eugene, OR, USA).

All other reagents were obtained from Sigma Aldrich (St. Louis, MO, USA) or where indicated.

2. Mice, Bacteria and Cell lines

2.1.Bacteria

Klebsiella pneumoniae 52145 (serotype K2:O1) is a clinical isolate that has been described previously (330) (Fig. 6) and was kindly provided by Dr. José A. Bengoechea (Fundación Caubet-Cimera, Centro Internacional de Medicina Respiratoria Avanzada, Mallorca, Spain) as well as used during a predoctoral stay in his laboratory. The chemical structure of its CPS has been reported (331). To obtain *K. pneumoniae* mutant strains with defects in LPS core, chromosomal in-frame nonpolar waa deletions were generated by Dr. JA Bengoechea laboratory as described in (95, 332). The isogenic mutants 52145-Δ*wabK* (Kp Rc+CPS), which express Rc-LPS and CPS; 52145-Δwca_{K2}-Δ*wabK* (Kp Rc-CPS), which express Rc-LPS and does not express CPS; and 52145-Δ*wabC* (Kp Re-CPS), which express

Re-LPS and does not express CPS have also been described previously (95, 331). All *Klebsiella strains* were grown in LB medium at 37 ºC.

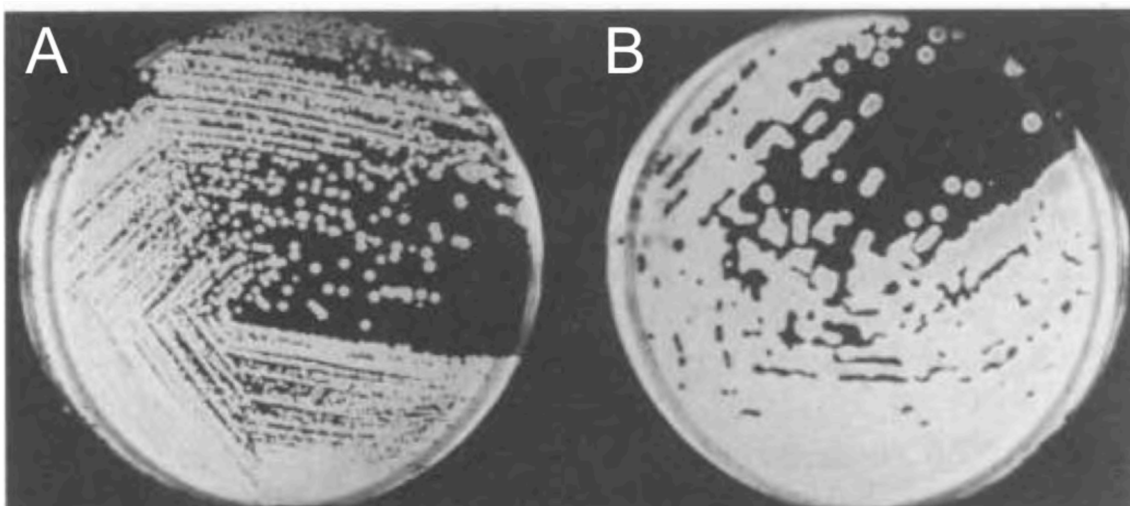

Figure 6. Aspects of colonies from a non-mucoid strain of *K. pneumoniae* (KP110) **(A)** and the mucoid strain of our strain of study, *K. pneumoniae* (52145) **(B)**. Taken from (330).

Pseudomonas aeruginosa O1 (PAO1) wild type is a clinical isolate that has been described previously (333) and was kindly provided by Dr. José A. Bengoechea as well as used during a predoctoral stay in its laboratory. This bacterium was also grown in LB at 37 ºC.

Non-typable *Haemophilus influenzae* (NTHi) strain 375 is an otitis media isolate (334), and was kindly provided by Dra. Junkal Garmendia (Instituto de Agrobiotecnología, Navarra, Spain) as well as used during a predoctoral stay in her laboratory. NTHi was grown at 37 °C and 5% CO_2 on chocolate agar plates (BioMérieux, Marcy l'Etoile, France) or brain heart infusion (BHI) broth supplemented with 10 µg/mL haemin and 10 µg/mL β-Nicotinamide adenine dinucleotide hydrate (β-NAD).

2.1.1. Bacteria expressing Green Fluorescent Protein

To obtain fluorescents bacteria, bacteria were made competent using the $CaCl_2$ method (335), and subsequently transformed with the plasmid pZsGreen (Clontech Laboratories, CA, USA), which encodes a variant of wild-type *Zoanthus* sp. green fluorescent protein (GFP). These variant is flanked by the lac operon and is expressed upon induction of IPTG. In

addition, this vector encodes carbenicilin resistance gene, to select transformed bacteria. Aliquot of 100 μl of competent bacteria were transformed with 2 ng of plasmid pZsGreen. Transformed bacteria were plated on LB-agar with 100 μg/ml of carbenicillin to select bacteria expressing GFP. The next day, plates were observed in a STATION Kodak 4000MM IMAGE filter equipment using λ_{ex} 465 nm and λ_{em} 535 nm and colonies with greater fluorescence intensity were selected.

2.2. Cell Cultures

MH-S (mouse alveolar macrophages) and Raw 264.7 (mouse peritoneal macrophages) cell lines was purchased from American Type Culture Collection (Rockville, MD) (Fig. 7). Both were grown at 37ºC and 5% CO^2 in RPMI 1640 medium supplemented with 2mM glutamine, 100 units/mL penicillin, 100 units/mL streptomycin and 10% fetal bovine serum (FBS).

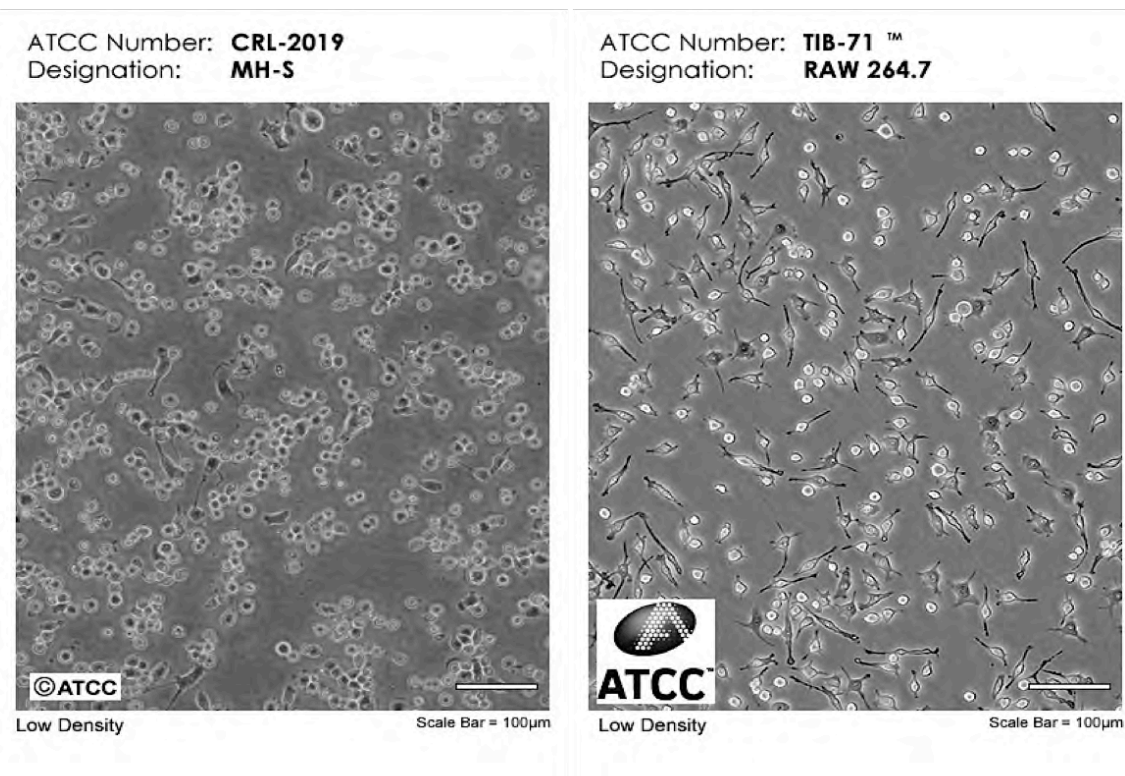

Figure 7. Optic micrograph showing MH-S and Raw 264.7 cells morphology.

2.3.Mice

Five to six weeks old males FVB/N were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) for *in vivo* experimental approaches. All mice were housed in a pathogen-free barrier facility and were handled according to the Institutional Animal Care and Use Committee guidelines at Cincinnati Children's Hospital Medical Center.

3. Antimicrobial Activity *in vitro* Assays

3.1.Bacterial Aggregation

Bacteria were grown at 37ºC in 5 mL of LB medium, harvested (500 g for 10 min) on exponential-phase and the pellet was washed twice with 10 mM phosphate buffer saline (PBS) pH 7.2, re-suspended in Hank's balanced salt solution (HBSS) buffer (0.137 M NaCl, 5,4 mM KCl, 0.25 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 4.2 mM $NaHCO_3$), and adjusted to a final concentration of 10^9 CFU/mL (OD_{700} = 1). SP-A 25 µg/mL was added to 150 µL of this solution and it was incubated for 30 min at 37 ºC with 30 seconds of gentle shaking each 5 min. The aggregation was determined by monitoring the changes in absorbance at 700 nm during 2h at 37ºC without shaking, in a spectrophotometer DU-800 (Beckman Coulter, Fullerton, USA). The results are shown as percentage of absorbance versus absorbance at time 0 (100%).

K. pneumoniae 52145 and mutant strains expressing GFP were used to visualize bacterial aggregation by fluorescence microscopy (Leica TCS SP2 Confocal System).

In addition, a suspension of 1 mL from *K. pneumoniae* 52145 and mutant strains re-suspended in HBSS buffer and adjusted to a final concentration of 10^9 CFU/mL were incubated in culture tubes with SP-A 25 µg/mL in the same conditions above mentioned, for direct visualization of bacterial aggregation by turbidity and bacterial pellet formation.

3.2.Inhibition of Bacterial Growth

10 µL bacteria on stationary-phase were diluted 1/200 in 200 µL LB in the absence or presence of 25 µg/mL SP-A to perform the experiment at the beginning of the growing-phase. Tubes were shaken at 250 rpm and 37ºC for up to 6 hours. Bacterial density was monitored by

measuring light scattering at 400 nm in an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA) at 1-hour intervals.

3.3. Bacterial Killing

To evaluate the microbicidal activity of SP-A, AMPs or both, colony count on plate assay was used. Bacteria were grown in corresponding medium (BHI for *H. influenzae* and LB for the other ones) at 37ºC with continuous shaking to exponential phase. The bacteria were harvested from broth by centrifugation at 500 g for 10 min, and the pellet was washed twice with 10 mM PBS (pH 7.2), re-suspended in PBS and adjusted to a final concentration of 5 x 10^5 CFU/mL. Aliquots of this suspension (5 µL) were mixed with SP-A (100 µg/mL) alone in 5 mM Tris (pH 7.2), 150 mM NaCl and 2 mM $CaCl_2$, or with AMPs (different concentrations) in the absence or presence of 100 µg/mL of SP-A in 10 mM PBS (pH 7.2), 1% TSB and 100 mM NaCl for 1h at 37ºC. In the case of SP-B^N, bacteria were incubated with SP-A (100 µg/mL), SP-B^N (10 µg/mL) or both in 20 mM phosphate buffer, pH 7.0, or 2 mM sodium acetate buffer, pH 5 for 1 h at 37 °C, in the absence and presence of 150 mM NaCl with or without 2 mM Ca^{2+}. In all cases the final volume of incubation was adjusted to 30 µL. After incubation, bacteria were plated on agar plates and incubated for 18 h at 37ºC. Viable bacteria were estimated by colony count and results are expressed as survival percentages with respect to bacteria not exposed to antibacterial agents.

3.4. Microscopic examination of bactericidal activity of SP-A

Bacterial membrane integrity and direct bactericidal activity of SP-A against *K. pneumoniae* and mutants were also assessed by staining bacteria with the membrane-impermeant fluorescent probe, propidium iodide. *K. pneumoniae* 52145 and deep rough mutant strains expressing GFP were harvested from broth by centrifugation at 500 g for 10 min, and the pellet was washed twice with 10 mM PBS (pH 7.2), re-suspended in 5 mM Tris (pH 7.2), 150 mM NaCl and 2 mM $CaCl_2$ and adjusted to a final concentration of 10^8 CFU/mL. Aliquots of 100 µL were incubated with or without 100 µg/mL of SP-A for 1 h at 37°C. PMB (5 µg/mL) was used as positive control of killing. After treatment, samples were stained with propidium iodide at 30 µM for 15 min in the dark. Bacteria were pelleted, and re-suspended in the same buffer to one-tenth the original volume and mounted on a glass slide.

Living (green color) and dead/dying bacteria (red color) were visualized by fluorescence microscopy (Leica TCS SP2 Confocal System).

3.5.Phagocytosis of bacteria by macrophages

To determine the ability of SP-A to enhance phagocytosis of bacteria by itself, MH-S cells were seeded in 24-well tissue culture plates at a density of $5x10^5$ cells per well 15 h before the experiment. Macrophages were infected with *Klebsiella pneumoniae* 52145 wt or Rc LPS nonexpressing CPS (multiplicity of infection = 50) in HBSS and bacterial phagocytosis assay in the presence of 100 µg/ml SP-A was performed with three different approaches: a) Bacteria were pre-incubated 2h with SP-A before infection, b) Macrophages were pre-incubated with SP-A 2h before infection and, c) macrophages were infected with a suspension of bacteria and SP-A.

To examine the synergistic activity of SP-A and AMPs to increase phagocytosis, MH-S or Raw 264.7 cells were pre-incubated 4h with or without SP-A, $SP\text{-}B^N$ or with both proteins, before infection with *Klebsiella pneumoniae* 52145 ($5 X 10^7$ CFU).

To synchronize infection in both cases, plates were centrifuged at 200 g for 5 min. Plates were incubated at 37°C in a humidified 5% CO_2 atmosphere. After 1h of infection, monolayer cells were washed 3 times with PBS and incubated with RPMI containing 10% FCS, 10 mM Hepes, gentamicin (300 µg/mL) and polymyxin B (15 mg/mL) for 90 min. to kill extracellular bacteria. Cells were extensible washed again to remove gentamicin and polymyxin, and lysed with 0.5% saponin re-suspended in PBS. The intracellular bacteria were serial diluted for CFU enumeration on agar plates.

Immunofluorescence of phagocytosis was performed as previously described (336). MH-S cells were seeded on 12 mm circular coverslips in 24-well tissue culture plates. Infections were carried out as described before with *K. pneumoniae* 52145 and decapsulated deep rough mutant strain expressing GFP. After 1h, cells were washed three times with PBS, and fixed with 3.7% paraformaldehyde in PBS pH 7.4. The actin cytoskeleton was stained with Rhodamine-Phalloidin diluted 1:100 and DNA was stained with 1 µg/ml 4',6-diamidino-2-phenylindole. Stains were carried out in 10% horse serum, 0.1% saponin in PBS for 15 min. Coverslips were washed twice in PBS containing 0.1% saponin, once in PBS and once in

H_2O, mounted on Mowiol 4-88 containing 2.5% (w/v) 1,4-diazobicyclo-[2.2.2]-octane (DABCO) and analyzed with a Leica TCS SP2 Confocal System.

4. Antimicrobial Activity *in vivo* Assays

4.1. Intratraqueal Administration

For *in vivo* experimental approaches, all bacterial infection and treatments were delivered via intratracheal (Fig. 8). Briefly, isoflurane was administered to mice until adequate anesthesia was achieved. Anesthetized mice were hanged vertically by their two upper incisors on a horizontally suspended string on a plastic supportive table. This position was maintained during the entire administration procedure. The tongue was extended with 2 pipette tips and held in place with one of them. With the mouth open, 50µl of bacteria and/or proteins were injected into the pharynx while covering the nose with the thumb. After 2-3 deep mouth inhalations the thumb was removed, and mice were taken down and allowed to recover from anesthesia. This procedure is highly effective to deliver proteins and cells to the lungs and avoids tracheal incision and cannulation.

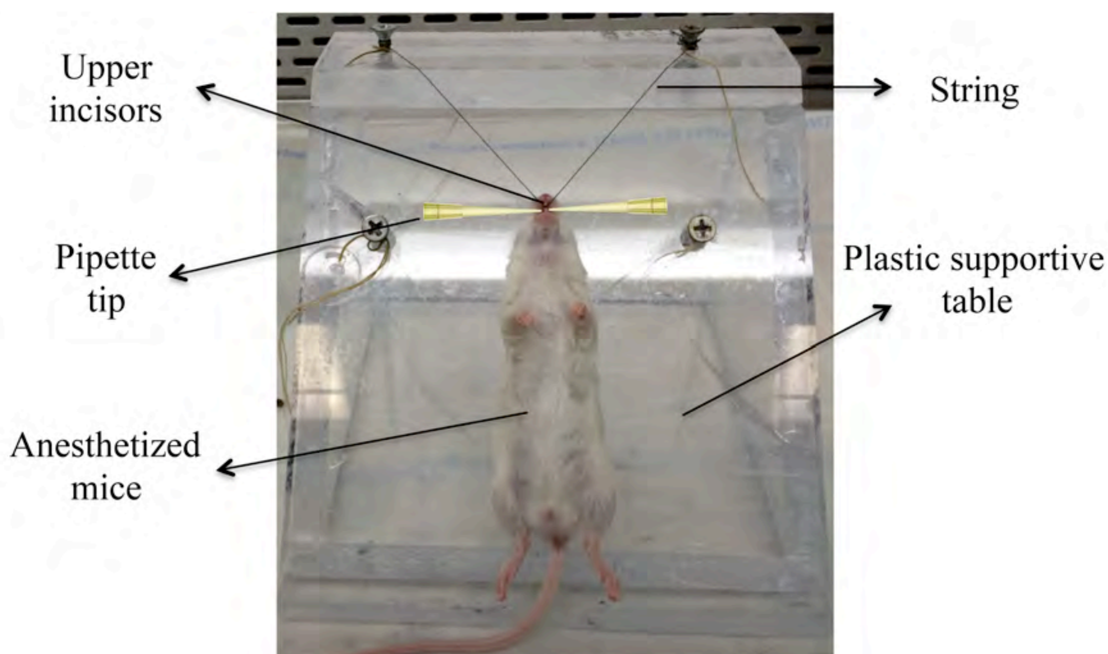

Figure 8. Illustrative image of intratracheal administration procedure. Mice are anesthetized with isoflurane and hung vertically by their upper incisors. 2 pipette tips are used to extend the tongue and open the mouth of the animal. While tongue is kept extended with one pipette tip, bacteria and/or proteins are then injected into the pharynx while covering the nose with the thumb.

4.2.Bacterial Killing *in vivo*

Preliminary experiments identified a dose of intratracheal *K. pneumoniae* K2 that caused some inflammation with minimal mortality in wt FVB/N mice. Ten thousand CFUs of *K. pneumoniae* were then administered intratracheally as mentioned above. This bacterium is heavily encapsulated and particularly virulent in mice. To minimize variability in virulence, all bacteria were selected from aliquots of the same passage stored at -70°C in 16% glycerol/trypticase soy broth (TSB), and prepared as previously described (337). For each experiment, a trypticase soy agar (TSA) (Difco, Detroit, MI) plate was streaked with an inoculum from the glycerol stock and incubated overnight at 37°C. A single colony was inoculated into 1 mL of TSB, incubated at 37°C with vigorous shaking for 10 hours, transferred to 50 ml of LB medium and grown to mid-logarithmic phase at 37°C with vigorous shaking. The bacteria were harvested from the broth by centrifugation at 200g for 10 min, washed, and re-suspended in sterile PBS. The number of viable bacteria was determined by plating serial dilutions of the stock on TSA plates and counting the number of CFU after overnight incubation at 37°C. The bacterial suspension was stored at 4°C and used within 48 hours. For each experiment, the dose was confirmed by plating an aliquot from the bacterial suspension that was injected intratracheally.

To examine the potential synergistic protection of SP-A and SP-B^N *in vivo*, a combination of *K. pneumoniae* K2 and 100 µg SP-A, 20 µg SP-B^N or both proteins re-suspended in 50 µL of 5 mM Tris and 150 mM NaCl were administered intratracheally to mice. For therapeutic application of SP-A/SP-B^N, mice infected with *Klebsiella pneumoniae* K2 were treated with both proteins in combination 6h or 24h after the bacterial challenge. Mice were exsanguinated after a lethal dose of sodium pentobarbital i.p at 24 h or 48 h post-infection, respectively. In both applications, after infection time, left lungs were harvested, weighed and homogenized in 1 mL sterile PBS. Dilutions of lung homogenates were plated on LB agar plates and the number of CFUs determined after an overnight incubation at 37°C. Each experimental group included 6 mice and data were expressed as CFU ± SEM/mouse.

4.3.Bronchoalveolar lavage cell count

Right lungs of mice infected and treated as mentioned in bacterial killing *in vivo* experiments were used to obtain bronchoalveolar lavage fluids (BALFs) as described

previously (337). The lungs were lavaged three times with 1-mL aliquots of PBS, BALF was centrifuged at 200 g for 10 min to precipitate BALF cells, and the cell pellet was re-suspended in 1 mL of PBS. A 50-mL aliquot was stained with an equal volume of 0.4% trypan blue (Life Technologies) for total cell count on a hemocytometer. Differential cell counts were made on cytospin preparations stained with the May-Grünwald Giemsa stain (Scientific Products, McGraw Park, IL).

4.4. Lung Histopathology

Mice were exsanguinated after a lethal dose of sodium pentobarbital i.p. and their lungs inflation-fixed with 4% paraformaldehyde 6 or 24 h following intratracheal instillation of 5 mM Tris and 150 mM NaCl or *K. pneumoniae* with or without SP-A+SP-B^N, as mentioned above. Serial 5-µm paraffin lung sections from each lobe were loaded onto polylysine-coated slides (Fisher, Atlanta, GA) and stained with hematoxylin and eosin (Sigma).

4.5. Cytokine levels in lung homogenates

At 6 or 24h after treatment, levels of cytokines associated with severe lung injury (mouse interleukin [IL]-1β, IL-6, IL-17a, tumor necrosis factor-α [TNF-α], and macrophage inflammatory protein (MIP)-2) were assessed in lung homogenates by enzyme-linked immunosorbent assay (ELISA) using MilliplexTM Multiplex kits (Millipore, Billerica, MA) according to manufacturer's protocol. Briefly, cytokine levels presents in the lung were measured by an indirect ELISA where the secondary antibodies were conjugated with horseradish peroxidase. The quantity of cytokines bound to the inmunosorbent was assayed by the addition of streptavidin-RPE. Samples were read using luminex technology on the Bio-PlexTM (Bio-Rad, Hercules, CA). Concentrations were calculated from standard curves using recombinant proteins and expressed in pg/mL. All samples were assayed in duplicate. The limits of sensitivity as described by the manufacturer are <3.2, <3.2, <0.64, <3.2 and <16 pg/mL, respectively.

5. Experimental Approaches for Mechanism of Action of SP-A and AMPs

5.1.Binding Assays

5.1.1. SP-A binding to Bacteria

To explore the ability of SP-A alone to bind to *Klebsiella pneumoniae* 52145 and mutant strains, or to *Klebsiella pneumoniae* 52145 in the presence of AMPs, binding assays were performed as previously described (338). Microcentrifuge tubes were filled with PBS containing 10% fetal bovine serum (FBS) for 24 h at 4°C to block nonspecific binding of SP-A to plastic. Next day, bacteria were grown at 37ºC in 5 mL of LB, harvested (500 g for 10 min) on log-phase and re-suspended to a concentration of 10^7 CFU/mL in 5mM Tris buffer pH 7.4 containing 150 mM NaCl and 2 mM Ca^{2+}. Bacteria were then incubated on blocked microcentrifuge tubes with several concentrations of biotinylated SP-A in the absence or presence of PMB (5 µg/mL), PMBN (5 µg/mL), SP-B^N (10 µg/mL) or human serum albumin (HSA) (5 or 10 µg/mL) at room temperature (RT) for 30 min. with gentle shaking. Suspensions were then centrifuged (500 g for 10 min) to pellet bacteria, washed twice with PBS and re-suspended in 200 µL of carbonate buffer 0.1 M pH 9.5. Bacteria-associated SP-A was then measured by solid-phase binding as described below.

The concentration of biotinylated SP-A bound to bacteria was determined by solid-phase binding assay. Samples were applied to a 96 well-plate maxisorp (Nunc, Rochester, NY, USA) and allowed to bind 1 h at 37°C. Controls in absence of bacteria were performed to estimate the nonspecific binding of SP-A to plastic. The plate were blocked 1 h at 37ºC with 5mM Tris containing 10 % FBS. After extensive washing with PBS, streptavidin-horseradish peroxidase (1:2000) was added to the wells and incubated for 1h at RT. The bound biotin-labeled SP-A was detected by adding *3,3',5,5'-tetramethylbenzidine liquid substrate* (TMB; Sigma). The colorimetric reaction was stopped with 4 M sulfuric acid, and the absorbance in each well was read at 450 nm on an ELISA reader (DigiScan; Asys HiTech GmbH, Eugendorf, Austria). A standard curve was generated with 156 ng/mL to 1.2 µg/mL of SP-A in 0.1 M buffer carbonate pH 9.5. Results obtained were expressed as nanograms of SP-A per concentration of bacteria.

5.1.2. SP-A binding to AMPs

Intrinsic Fluorescence experiments. Fluorescence measurements were carried out in 5 x 5 quartz cuvettes using an SLM-Aminco AB-2 spectrofluorimeter equipped with a thermostated cuvette holder (±0.1 °C; Spectronic, Waltham, MA, USA). Fluorescence emission spectra of SP-A (10 µg/mL) in the absence or presence of increasing concentrations of AMPs were measured at 25 °C in 5 mM Tris-HCl buffer (pH 7.4), 150 mM NaCl. Blanks and protein were excited at 295 nm. Emission spectra were recorded from 305 to 400 nm, and the slit-widths were 4 nm for the excitation and emission beams.

The apparent dissociation constants (K_d) at 25 ºC for the complex SP-A/AMP were obtained by analyzing the change in the fluorescence of SP-A at 337 nm upon addition of increasing amounts of AMPs. The data from equilibrium binding titrations of SP-A were analyzed by nonlinear least-squares fitting to equation [1]:

$$\Delta F = \Delta F_{max} \cdot \frac{[AMP]^n}{K_d + [AMP]^n} \qquad [1]$$

, where ΔF is the change in fluorescence intensity at 337 nm relative to the intensity of free SP-A; ΔF_{max} is the change in fluorescence intensity at saturating AMP concentrations; K_d is the apparent equilibrium dissociation constant; *[AMP] is* the molar concentration of free AMP; and n is the Hill coefficient.

Dynamic light scattering (DLS). DLS, as we have seen before, allows measuring the size distribution of molecules dissolved in a liquid by measuring the proportion of the incident light that is scattered. The Brownian motion of the scattering particles causes fluctuations in light scattering intensity. The velocity of the Brownian motion of the particles can be calculated and related to their hydrodynamic size (326).

The hydrodynamic diameters of AMPs and SP-A, as well as mixtures of these components, were measured at 25°C in a Zetasizer Nano S (Malvern Instruments, Malvern, UK) equipped with a 633-nm HeNe laser. Six scans were performed for each sample, and all of the samples were analyzed in triplicate. The hydrodynamic diameter was calculated using

the general purpose algorithm available from the Malvern software for dynamic light scattering analysis, which correlates the diffusion coefficient to the hydrodynamic diameter through the Stokes-Einstein equation [2]:

$$d_H = \frac{K_\beta T}{3\pi\eta D} \quad [2]$$

, where K_β is the Boltzmann constant, T is the temperature, η is the viscosity, and D is the translational diffusion coefficient. The multiple narrow modes algorithm was also used to verify the results obtained by the general purpose method. The interaction of SP-A with AMP in solution was measured by the addition of different AMP concentrations to 15 nM SP-A in 5 mM Tris buffer (pH 7.4) in the absence or presence of 150 mM NaCl.

Solid-phase binding assay. Solid-phase binding assay was also performed with biotinylated SP-B^N to explore whether SP-B^N binds to immobilized SP-A. Wells of a 96-well maxisorp microtiter plate (Nunc, Rochester, NY, USA) were coated with either SP-A (25µg) or human serum albumin (25 µg) in 0.1 mM sodium bicarbonate buffer, pH 9.5, overnight at 4°C. Wells were washed (5 mM Tris HCl, pH 7.4, containing 150 mM NaCl, 2 mM $CaCl_2$ and 0.1% Tween 20) and blocked with washing buffer containing 2.5% nonfatty dried milk for 2 h. Following washing, biotinylated SP-B^N in concentrations ranging 0 to 12 µM was added to the wells in the same buffer. Incubations were performed for 1 h at room temperature. To detect the bound biotin-labeled SP-B^N, streptavidin-horseradish peroxidase was added to the wells and absorbance was read in the same conditions mentioned in 5.1.1. Results obtained were expressed as OD_{450nm} of biotinylated SP-B^N.

5.2.Experiments on Model Bacterial Membranes

5.2.1. Bacterial Model Membrane preparation

Total bacterial phospholipid extract (BPL) and Re-LPS were used to prepare bacterial model membranes. Bacterial stock solutions of PL in chloroform/methanol (3:1, v:v) and Re LPS in chloroform/methanol/water (17:7:1, v:v:v) were prepared and stored at -20ºC. The sample solution was prepared by mixed stocks solutions of the BPL and Re-LPS, to achieve the desired Re-LPS/PL ratio. The organic solvent was then evaporated to dryness under a

stream of nitrogen, and traces of solvent were subsequently removed by evacuation under reduced pressure overnight. Multilamellar vesicles of Re-LPS/BPL (8/2, w/w) were prepared by hydrating the dry lipid film in a saline buffer and allowing them to swell for 1h at a temperature above the transition temperature (Tm). After vortexing, the resulting multilamellar vesicles (MLVs) were used for different assays. Unilamellar vesicles were used for spectroscopic measurements to reduce light scattering artifacts. To obtain them, MLVs were sonicated during 4 min at 390 w/cm (burst of 0.6 s, with 0.4 s between bursts) in a UP 200S sonifier with a 2mm microtip.

5.2.2. Monolayer experiments

Monolayer experiments were performed at 25°C using a thermostated Langmuir-Blodgett trough (102M micro Film Balance; NIMA Technologies, Coventry, UK) equipped with an injection port and magnetically stirred. The trough is equipped with two symmetrical movable barriers controlled by an electronic device. The subphase buffer employed was 5 mM Tris buffer (pH 7.4), 150 mM NaCl containing 0.175 mM $CaCl_2$. A concentrated solution (1 mg/mL) of Re LPS dissolved in chloroform/methanol 3/1 (v/v) was used for monolayer experiments. For penetration studies, monolayers were formed by spreading different volumes of a concentrated solution of Re LPS at the air-water interface to give the indicated initial surface pressures (from 0 to 40 mN/m). After organic solvent evaporation, the monolayer was allowed to stabilize for a few minutes before 100 μL of PMBN (1 μg/mL), SP-A (1.25 μg/mL), or the mixture PMBN+SP-A was injected into the subphase without disturbing the lipid monolayer. Then, protein-induced changes in the monolayer surface pressure at constant surface area were measured.

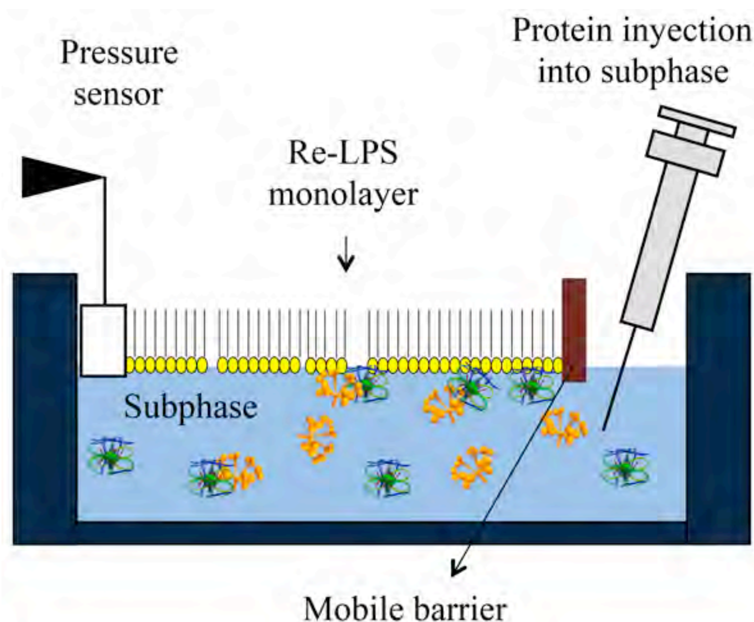

Figure 9. Schematic representation of a Langmuir balance. A monolayer of Re-LPS is deposited at the air-liquid interface at a given initial pressure. SP-A, PMBN or both are injected into the subphase while going by monitoring changes in surface pressure induced by the insertion of these molecules in the Re-LPS monolayer.

5.2.3. Fluorescence Experiments

Fluorescence experiments were carried out using an SLM-Aminco AB-2 spectrofluorimeter equipped with Glam prism polarizers and a thermostated cuvette holder (±0.1ºC) (Thermo Spectronic, Waltham, MA, USA). We have used 5X5 mm path-length quartz cuvettes.

5.2.3.1. DPH fluorescence measurements.

The required amounts of Re-LPS/BPL (8/2 w/w) were mixed with DPH at a probe/lipid molar ratio of 1:200 (final lipid concentration of 1 mg/mL) as previously described (339). DPH concentration was determined spectrophotometrically by absorbance at 350 nm, using a molar extinction coefficient in methanol of 88000 $M^{-1}cm^{-1}$. MLVs of Re-LPS/BPL (8/2, w/w) were prepared in 5 mM Tris buffer (pH 7.4), 150 mM NaCl. To reduce the size of these particles, MLVs were sonicated as described before to form LUVs. Exposure to light was minimized throughout the preparation of Re LPS/PL aqueous dispersions doped with DPH.

To study the effect of PMBN, SP-A or both on bacterial model membrane, measurements of fluorescence intensity and fluorescence emission anisotropy of DPH were done.

Fluorescence emission anisotropy. For each sample, fluorescence emission intensity data in parallel and perpendicular orientations with respect to the exciting beam were collected ten times each and then averaged. Background intensities in DPH-free samples due to the vesicles and proteins were substracted from each recording of fluorescence intensity. Excitation and emission wavelengths were set at 360 and 430 nm, respectively. Anisotropy, r, was calculated as [3]:

$$r = \frac{I_{II} - GI_{\perp}}{I_{II} - 2GI_{\perp}} \qquad [3]$$

,where I_{II} and $I_{\perp}$ are the parallel and perpendicular polarized intensities measured with the vertically polarized excitation light and G is the monochromator grating correction factor.

DPH fluorescence intensity measurements. The emission spectra were recorded with the emission polarizer set at the magic angle (m= 54.7°) relative to the vertically polarized excitation beam. This was done to reduce contributions from scattering due to Re-LPS/BPL dispersions and to avoid intensity artifacts due to molecular rotation during the lifetime of the excited state (340). Moreover, background intensities in probe-free samples were subtracted from each re-cording of fluorescence intensity.

5.2.3.2. LAURDAN GP Function

Re-LPS/BPL (8/2, w/w) liposomes were mixed with the amphiphilic fluorescent probe LAURDAN at a probe/phospholipid molar ratio of 1:100. LUVs were prepared in 5 mM Tris buffer (pH 7.4), 150 mM NaCl and then steady-state fluorescence emission spectra of LAURDAN were measured at 25°C in the absence and presence of different PMBN and/or SP-A concentrations. The LAURDAN emission maximum is near 440 nm in the lipid gel phase but is red-shifted to 490 nm in the liquid-crystalline phase (341). To quantify these emission spectrum changes, the excitation generalized polarization (GP_{ex}) of LAURDAN was calculated according to Parasassi et al. (341), using Eq. [4]:

$$GP = \frac{I_B - I_R}{I_B + I_R} \quad [4]$$

,where I_B and I_R are the intensities at the blue (440 nm) and red (490 nm) edges of the emission spectrum (λ_{ex} = 364 nm).

5.2.4. Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) is a technique, which measures the thermodynamic properties of temperature-induced phase transitions. A sample and an appropriate reference material (usually the same buffer in which the sample has been prepared) are simultaneously heated in different cells at the same predetermined rate. If the sample undergoes a thermally induced phase transition, absorption or release of heat takes place (depending on whether the process is endothermic or exothermic). The instrumental control system has to supply more (or less) heat to the sample cell to maintain its temperature equal to that of the reference cell.

The recorded parameter in a DSC scan is the excess heat capacity (C_p) of a sample with respect to the reference as a function of temperature. Endothermic transitions are represented as positive peaks. Three thermodynamic parameters can be directly determined from the thermogram (Fig. 10). Firstly, the phase transition temperature, T_m, corresponds to the maximum C_p of the peak. For symmetric curves, T_m represents the temperature at which the transition is half complete. Secondly, the relative cooperativity of the process is related to the sharpness of the peak, which is commonly expressed as the temperature width at half-height of the peak, $\Delta T_{1/2}$. And finally, the enthalpy of the transition (ΔH) can be calculated by integrating the area below the thermogram (342, 343).

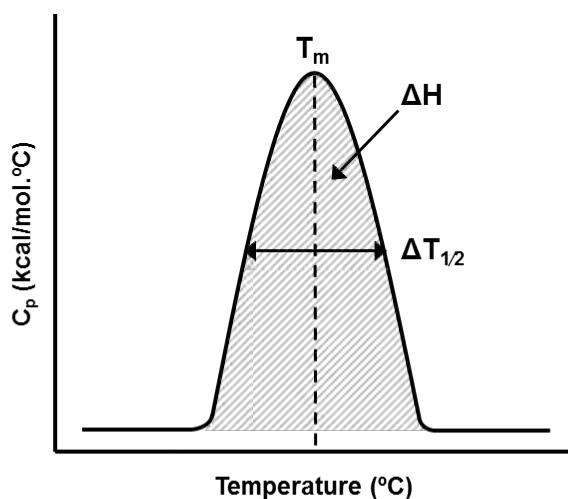

Figure 10. DSC thermogram for a two-state endothermic process. T_m is the phase transition temperature, $\Delta T_{1/2}$ is the temperature width at half-height of the peak, and ΔH is the enthalpy of the thermodynamic event.

Biological membranes undergo a temperature-induced phase transition from a relatively ordered crystalline-like state (gel phase (L_β)) to a relatively disordered fluid-like state (liquid-crystalline phase (L_α)). The properties of the gel-to-liquid phase transition of different lipid mixtures can be therefore studied by DSC.

Calorimetric measurements were performed as previously reported (144, 339) in a Microcal VP differential scanning calorimeter (Microcal Inc., Northampton, MA, USA) at a heating rate of 0.5 ºC/min. Multilamellar vesicles of Re-LPS/PL (8/2, w/w) (0.5mM) prepared in 5 mM Tris buffer (pH 7.4), 150 mM NaCl, were loaded in the sample cell in the absence and presence of different amounts of PMBN (1.25 µg/mL to 95 µg/mL) or/and 1.25 µg/mL SP-A. Buffer alone was loaded in the reference cell of the microcalorimeter. Four calorimetric scans were collected from each sample between 10ºC and 60ºC. The standard Microcal Origin software was used for data acquisition and analysis. The excess heat capacity functions were obtained after subtraction of the buffer-buffer baseline.

5.2.5. Analysis of liposomal permeability

An assay was developed to assess the effect of SP-A, PMBN and the mixture SP-A/PMBN on the permeability of model bacterial membranes. The fluorophore ANTS and its collisional quencher DPX were co-encapsulated into Re-LPS/BPL (8/2, w/w) vesicles. Re-

LPS/BPL (8/2, w/w) dry lipid film was rehydrated in a buffer containing the probes to form MLVs (5 mM Tris buffer (pH 7.4), 150 mM NaCl, 12.5 mM ANTS, 45 mM DPX). Then, sample was subjected to 5 freeze-thaw cycles (liquid N_2) and passed through an extruder to obtain LUVs (100 nm pore membrane). Free probes were removed in a sephadex G50 chromatography. Then, 300 µl of vesicles were loaded in 5x5 mm path-length quartz cuvettes to measure fluorescent intensity as a function of time using excitation and emission wavelengths of 353 nm and 520 nm, respectively. These experiments were carried out using an SLM-Aminco AB-2 spectrofluorimeter equipped with Glam prism polarizers and a thermostated cuvette holder (±0.1ºC) (Thermo Spectronic, Waltham, MA, USA).

After leakage of aqueous contents by addition of a potential lytic peptide (SP-A, PMBN or both), the probes will dilute into surrounding medium, and ANTS fluorescence will increase because quenching by DPX will be diminished. Triton X-100 was used as positive control. We have also obtained the emission spectra of the samples at the beginning and at the end of the experiments to measure the percentage of leakage according to this equation [5]:

$$\%L = \frac{(Fp - Fo)}{(F100 - Fo)} X\ 100 \qquad [5]$$

,where F_0 is the fluorescence at 520 nm of the sample before adding any protein, F_p is the fluorescence after the addition of proteins, and F_{100} is the fluorescence obtained after the addition of triton X-100 (total permeabilization).

6. Statistical analysis

Means were analyzed by Student´s t-test for unpaired samples or analysis of variances following by Bonferroni test when appropriate. p value <0.05 was considered statistically significant. Analyses were performed using Prism 5 program for MAC OS X (GraphPad Software, Inc. La Jolla, CA).

CHAPTER 1

Pulmonary Surfactant Protein A enhances exogenous and endogenous antimicrobial peptide activity against lung pathogens

1. ABSTRACT

Pulmonary surfactant protein-A (SP-A) is a large protein secreted to the alveolar fluid that has been proven to have macrophage-independent antimicrobial activity *in vivo*. In this study, we explore the effect of SP-A on aggregation, growth, and viability of several pathogenic Gram-negative bacteria as well as SP-A's possible synergistic action with several antimicrobial peptides (AMPs). Results indicate that SP-A was able to aggregate, inhibit the growth of, and kill isogenic decapsulated *Klebsiella pneumoniae* (serotype K2:O1) mutants expressing deep rough lipopolysaccharide on their outer membranes. Conversely, SP-A did not bind or have any effect against capsulated or decapsulated *K. pneumoniae* K2:O1 expressing smooth lipopolysaccharide or capsulated deep rough *K. pneumoniae* mutants. However, we found that SP-A acted synergistically with exogenous cationic AMPs (polymyxin B and colistin) as well as with endogenous cationic AMPs (human β-defensins 2 and 3) present in the alveolar fluid. SP-A enhanced AMP microbicidal activity on different pulmonary pathogen strains (*K. pneumoniae* K2, *P. aeruginosa* O1, and nontypeable *Haemophilus influenzae*), which are resistant to SP-A. In addition, we found that AMPs facilitate the binding of SP-A to bacteria that did not interact with SP-A alone through a protein-protein interaction mechanism. These results indicate that the synergic action of SP-A with AMPs might be important in lung defense against infection.

2. INTRODUCTION

The alveolar epithelium in the organism is continuously exposed to inhaled particles such as, allergens, contaminants and pathogens. To protect the host against pathogens reaching the alveoli during inspiration, there must be some effective defense mechanisms. *Klebsiella pneumoniae* is a common Gram-negative bacterium that infects the respiratory tract of immune compromised patients (344). One of the main virulence factors of this kind of bacteria is the lipopolysaccharide (LPS) present in its outer membrane (OM), which is composed by a highly conserved and amphipathic fraction called lipid A, an oligosaccharide core and a variable domain of repeated units of polysaccharides called O-antigen (345). The LPS core domain is usually divided into inner and outer core and it is frequently named as smooth LPS (S-LPS), when it contains all the structure mentioned; or rough LPS (R-LPS), when it lacks the O-antigen, but keeps the lipid A and the oligosaccharide core which could

be progressively shorter, depending on the rough phenotype (Ra-, Rb-, Rc-, Rd- and Re-LPS) (346).

K. pneumoniae expresses S-LPS and two major core structures in their OM (type 1 and 2), which differ only in the outer-core region (347). In addition, *Klebsiella* usually develops a prominent capsule polysaccharide (CPS) composed of complex repeating subunits of acid polysaccharides. The CPS, consisting of four to six sugars and, very often, uronic acids (as negatively charged components), can be classified into 77 serological types, being K1 and K2 the most virulent serotypes (344). LPS and CPS structure of *K. pneumoniae* have been shown to be involved in their pathogenicity and resistance to antimicrobial factors in the alveolar fluid (348, 349).

The innate immune system in the alveolar space is composed, besides a cell component, by multiple soluble factors able to recognize and eliminate pathogens, through direct bactericidal action, or indirectly by increasing their phagocytosis by phagocytic cells present in the alveolar space (350). Among these factors, there are antimicrobial peptides (AMPs) like cathelicidins or defensins, and proteins like C-reactive protein, lactoferrin, lysozyme, galectins, complement proteins and pulmonary surfactant collectins (SP-A and SP-D), which are all secreted by the alveolar epithelium, alveolar macrophages and/or neutrophils (12). Several evidences show that host lung defense against bacterial infections is partly due to the presence of these antimicrobial factors in the airways (12, 350).

Surfactant protein-A (SP-A) belongs to the collectin family and is the most abundant protein of pulmonary surfactant (by weight) (12). It is able to modulate and facilitate alveolar immune cells response, to opsonize microbes and also exerts direct bactericidal activity against some Gram-positive and -negative bacteria, virus and fungi (351). Wu and co-workers (2003) have shown that SP-A decreases the growth of Gram-negative bacteria by increasing membrane permeability (16). Moreover, it has been reported that SP-A -/- mice are more susceptible than WT mice to lung infections (352) and that macrophages-free bronchoalveolar lavages from SP-A +/+ mice showed a bactericidal effect greater than lavages from SP-A -/- mice, suggesting a direct microbicidal role of SP-A *in vivo* (353). In this point, the antimicrobial ability of SP-A is well established. However, multiple bacterial resistance factors to SP-A have been reported, being bacterial strain and the LPS and CPS types the most important ones (354). In general, Gram-negative bacteria susceptible to SP-A

have a truncated (deep R-LPS) or abbreviated OM like *Escherichia coli* J5 or K12 (17, 353, 355). However, the highly virulent respiratory pathogens like *K. pneumoniae* K2 or *P. aeruginosa* O1, which express more complex OM, have been shown to be resistant to SP-A-mediated membrane permeabilization or SP-A binding (356, 357). Due to the wide variety of Gram-negative strains and serotypes, the real bacterial killing spectrum of SP-A needs more clarification.

In addition to SP-A, the immune system in the alveolar space is also composed by AMPs, which are small cationic or anionic peptides involved in the innate and adaptive immune systems that show antimicrobial activity against bacteria, virus and fungi (350). AMPs can be endogenous (part of the innate immune system) and exogenous, which are administered for the treatment of infections (358). Among the endogenous cationic AMPs (CAMPs) present in the alveolar fluid are the human beta-defensins (HBDs) (273). Several HBDs have been described, but the most studied are HBD-1, -2 and -3. In the alveolar space, HBD-1 is constitutively expressed, while HBD-2 and HBD-3 are expressed by cytokine induction or the presence of pathogens. These beta defensins show a higher antimicrobial activity than HBD-1 (29). On the other hand, the exogenous CAMPs Polymyxin B and colistin (PMB and PME, respectively) are polypeptide antibiotics capable of killing Gram-negative bacteria. They became available for clinical use in the 1960s, but they were replaced by antibiotics considered less toxic (359). However, due to the ability of polymyxins and naturally occurring defensins to kill multidrug-resistant (MDR) Gram-negative pathogens, they have gained considerable attention and clinical interest (26, 121).

Given the rising phenomenon of microbial resistance among important pathogens (360), several studies have suggested the importance of cooperative actions between different soluble factors with microbicidal activity *in vitro* and *in vivo* (22, 361). Consequently, it is possible that SP-A may act synergistically or cooperatively with different AMPs in the alveolar space to strengthen the microbicidal activity of each factor alone. The study of these interactions would allow the development of new therapies against MDR Gram-negative bacteria.

Therefore, in this work, we proposed to evaluate the ability of SP-A to enhance or block the microbicidal activity of several CAMPs (PMB, PME, and HBDs) against three respiratory pathogens, as well as to determine some of the mechanism by which these proteins are able to

act synergistically. For this purpose, we firstly characterized the individual antimicrobial activity of SP-A, using a clinic strain of *K. pneumoniae* and several isogenic mutants expressing truncated OM.

3. EXPERIMENTAL DESIGN

K. pneumoniae K2 is a common and high virulent Gram-negative bacterium that infects the respiratory tract of immune compromised patients. To study the potential protective effect of SP-A against this bacterium and/or its mechanism of resistance to SP-A, chromosomal in-frame nonpolar waa deletions were generated in order to obtain *K. pneumoniae* mutant strains with defects in LPS core. Thus, we used the next smooth and rough strains (Fig. 1): i) the clinic strain *K. pneumoniae* 52145, which express S LPS and CPS (Kp wt); ii) the isogenic mutant 52145-Δ*wabK* (Kp Rc+CPS), which express Rc LPS and CPS; iii) the isogenic mutant 52145-Δwca_{K2}-Δ*wabK* (Kp Rc-CPS), which does not express CPS and express Rc-LPS; and the isogenic mutant 52145-Δ*waaC* (Kp Re-CPS), which does not express CPS and express Re-LPS.

To determine the antimicrobial action of SP-A against these bacteria (Fig 1), a single colony of bacteria was inoculated into 10 ml of LB, incubated at 37°C with vigorous shaking O.N., transferred to 5 ml of LB medium and grown to mid-logarithmic phase at 37°C. Bacteria were, then, harvested (500 g for 10 min), the pellet was washed twice with PBS, pH 7.2, re-suspended in corresponding buffer, and incubated with SP-A to determine its ability to:

1. Aggregate bacteria: evaluated by changes in bacterial suspension absorption at 700nm, by microscopic examination of bacteria expressing GFP and by direct visualization of bacterial pellet formation.
2. Kill bacteria: determined by CFU counts and staining of bacteria expressing GFP with the membrane-impermeant fluorescent probe, propidium iodide following fluorescence microscopy.
3. Enhance phagocytosis of bacteria: assessed by intracellular CFU count on the cell line of mouse alveolar macrophages MH-S.
4. Bind to bacteria: estimated by solid-phase binding assays of bacteria incubated with biotinylated SP-A.

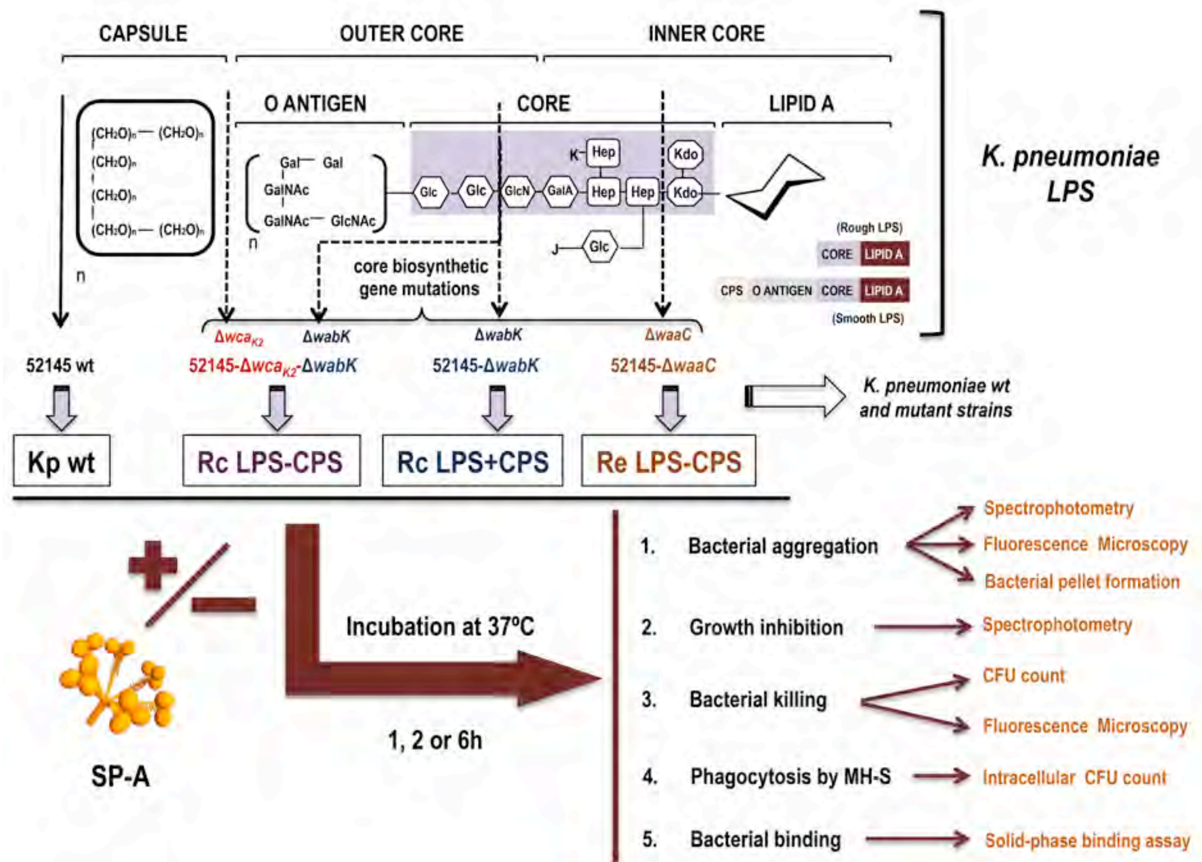

Fig. 1. Experimental design used to determine the individual antimicrobial action of SP-A. *K. pneumoniae* 52145 (Kp wt) OM structure is based on a published study (331). Dashed lines denote the truncation level for the different core biosynthetic gene mutations, which were used to obtain the several rough strains for this study.

To elucidate the potential synergistic activity of SP-A with AMPs (Fig. 2), both exogenous (polymyxin B and E) and endogenous CAMPs (human beta defensin-1, -2 and -3) were used. SP-A in the absence and presence of CAMPs was incubated with the respiratory pathogens *K. pneumoniae* (Kp wt), *P. aeruginosa* O1 (PAO1) and non typable *H. influenzae* (NTHi) and bacterial killing assays were assayed by CFU count. In addition, the ability of SP-A to bind to *K. pneumoniae* in the presence of AMPs was investigated by solid-phase binding assay. Finally, the potential molecular interactions between SP-A and CAMPs were determined using dynamic light scattering (DLS) assays and changes in the intrinsic fluorescence of SP-A (Fig. 2).

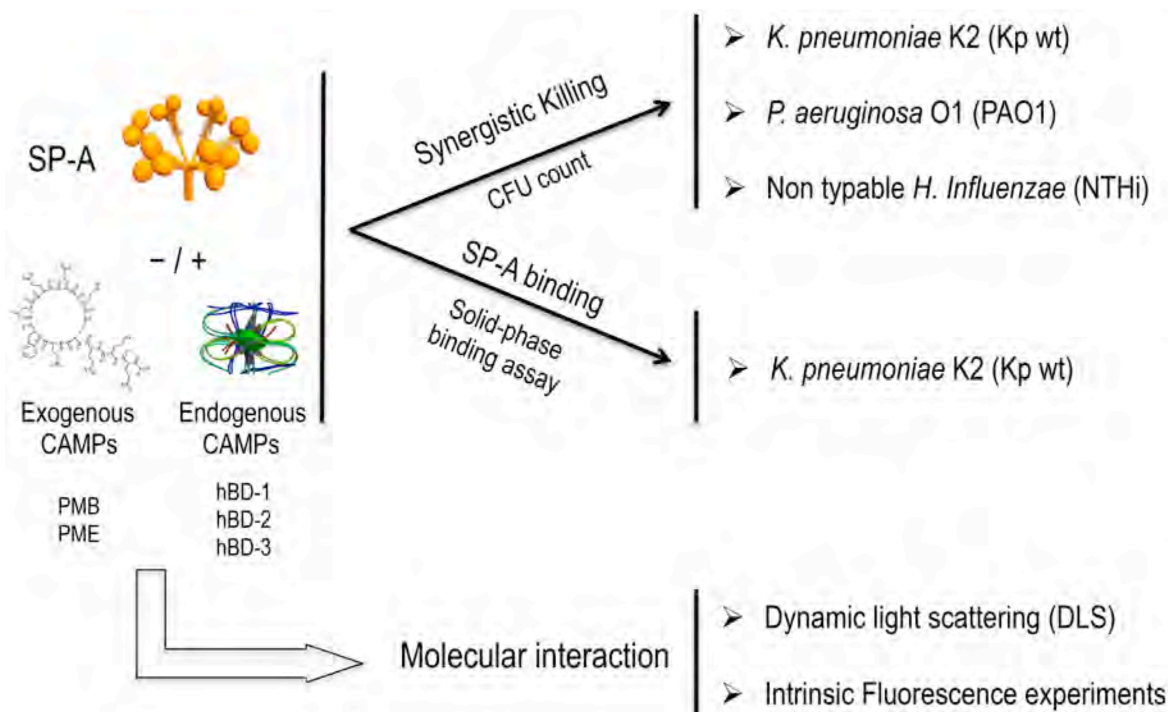

Fig. 2. Experimental design used to determine the synergistic action of SP-A with cationic antimicrobial peptides. CAMPs: cationic antimicrobial peptide; PMB: polymyxin B: PME: polymyxin E; hBD: human beta-defensin.

4. RESULTS

4.1. SP-A antimicrobial effect on *K. pneumoniae* 52145.

Since *K. pneumoniae* is a common lung pathogen and an important cause of nosocomial infections (344), we investigated the potential antimicrobial activity of SP-A against this microorganism, determining its ability to: a) aggregate bacteria, b) inhibit bacterial growth, c) cause bacterial death, and d) increase phagocytosis by alveolar macrophages. Several studies have shown that SP-A interacts with bacteria expressing preferably rough LPS (17, 131, 362). Thus, we have used different isogenic mutants of *K. pneumoniae* expressing different LPS phenotypes, from smooth LPS with CPS to deep rough Re-LPS without CPS, in order to examine its antimicrobial activity.

Bacterial aggregation assays *in vitro* were performed with Kp wt, Kp Rc+CPS, Kp Rc-CPS and Kp Re-CPS. The spectrophotometry results showed that SP-A, in the presence of

calcium, was able to aggregate Rc and Re strains nonexpressing CPS over 2h incubation at 37ºC (Fig. 3A and B). In contrast, there was no significant difference in the monitoring of the Kp wt and Kp Rc+CPS strains in the presence or absence of SP-A (Fig. 3A and B).

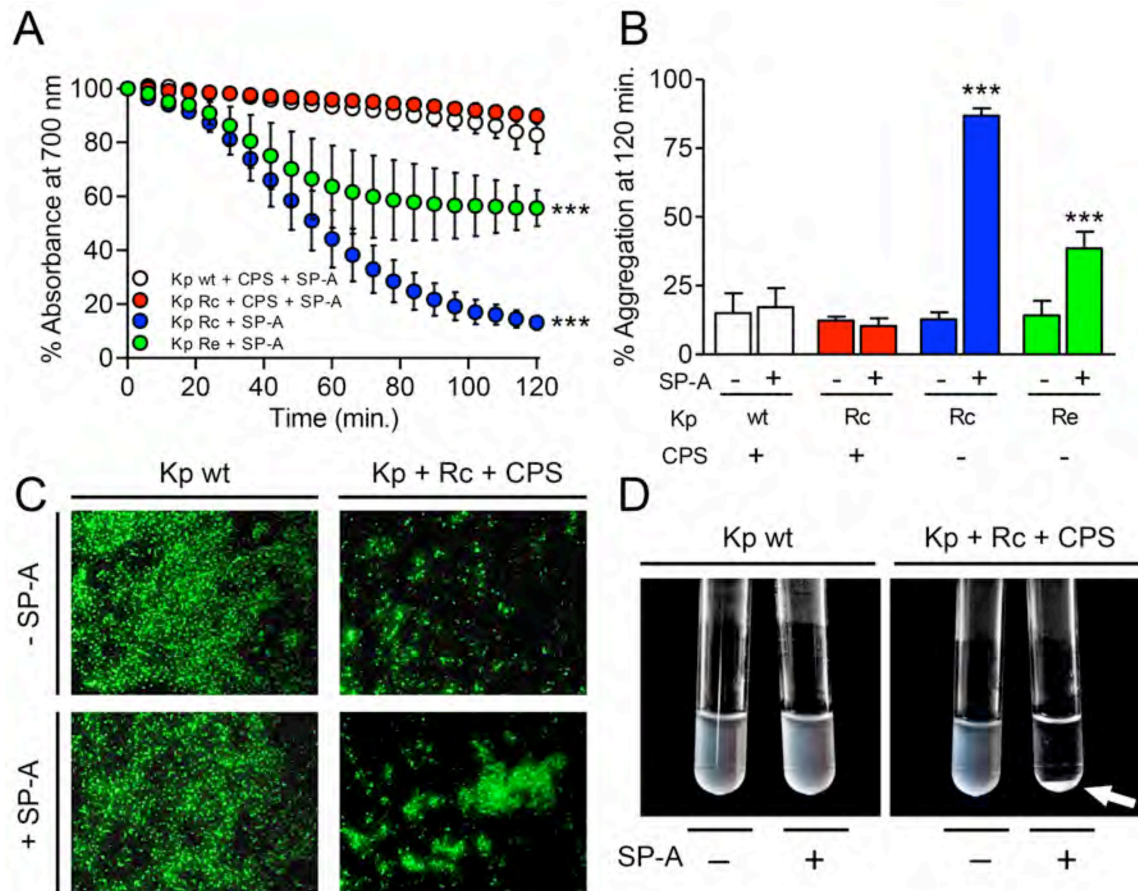

Figure 3. SP-A aggregates deep rough decapsulated *K. pneumoniae*. Kp wt, Kp Rc+CPS, Kp Rc-CPS and Kp Re-CPS were incubated in the absence or presence of SP-A 25 µg/mL Hank's balanced salt solution (HBSS) buffer. **A)** Aggregation was determined by monitoring the changes in absorbance at 700 nm during 2h at 37ºC without shaking. **B)** Percentage of aggregation at 120 min. is shown as absorbance at that time versus time 0. Absorbances of strains incubated in the absence of SP-A are around 100% (data not shown). Data represent mean ± SD (n=3, each one in duplicate) and significant differences between control and treatments are indicated by asterisks, where *** $p < 0.001$ vs control. **C)** SP-A aggregation was visualized by fluorescence microscopy using Kp wt and Kp Rc-CPS expressing GFP. **D)** SP-A bacterial aggregation of Kp Rc-CPS but not Kp wt was also visualized by turbidity and bacterial pellet formation (white arrow) after 2h of incubation with SP-A (25 µg/mL).

The SP-A ability to aggregate deep rough strains of *K. pneumoniae* lacking CPS but not smooth capsulated strains was also observed by fluorescence microscopy using bacteria expressing GFP (Fig. 3C). Similarly, it is shown in culture tubes containing Kp wt and Kp Rc-CPS, untreated or treated with SP-A, where deep rough strain aggregation is confirmed in form of bacterial pellet and culture transparency. In contrast, for the wt strain, no bacterial pellet was detected and culture turbidity remained after 2h of incubation with SP-A, which indicates that the protein does no induce the aggregation of Kp wt (Fig. 3D).

Some studies have shown that SP-A inhibits the growth of several Gram-negative strains (355). Therefore, we also investigated whether SP-A has this ability against the strains used in this study. Our results showed that SP-A produced a significant decrease in the growth of the Re-CPS strain, starting at 4 hours of incubation, as seen by optical density decline of the strain at 400 nm, in the presence of SP-A. However, SP-A had no effect on the growth of Kp wt and Kp Rc+CPS (Fig. 4A and 4B).

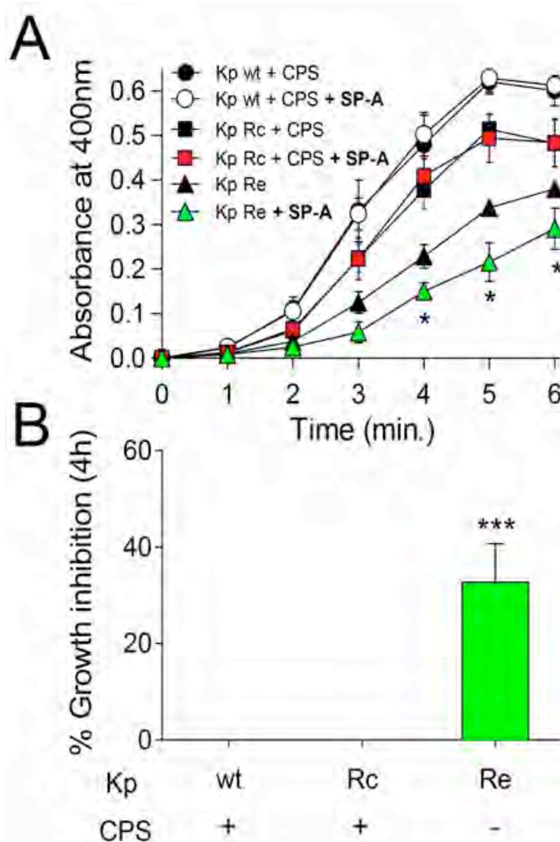

Figure 4. SP-A inhibits the growth of *K. pneumoniae* 52145 expressing deep rough LPS and non-expressing CPS. 200 µl of a suspension of bacteria Kp wt, Kp Rc+CPS or Kp Re-CPS in the beginning of the growing-phase were incubated 6h at 37ºC and 250 rpm in the absence or presence of 25 µg/ml of SP-A. **A)** Growth inhibition was monitored by measuring the absorbance at 400 nm each hour. **B)** Also is shown the percentage of inhibition by plotting the percentage of decrease in absorbance at 4 hours from treated samples compared to untreated control at the same time. Bars represent mean ± SD (n=3, each one in duplicate) and significant differences between control and treatments are indicated by asterisks, where * $p < 0.05$.

Next, we tested the direct killing of SP-A by colony-forming units (CFU) count on LB-agar plates. These experiments demonstrated that deep rough decapsulated strains of *K. pneumoniae* showed susceptibility to SP-A by a 2-fold decrease in the CFU count of the treated samples.

However, the absence of a decrease in the number of CFUs for Kp wt and Kp Rc+CPS strains indicates the resistance of these strains to SP-A (Fig. 5A).

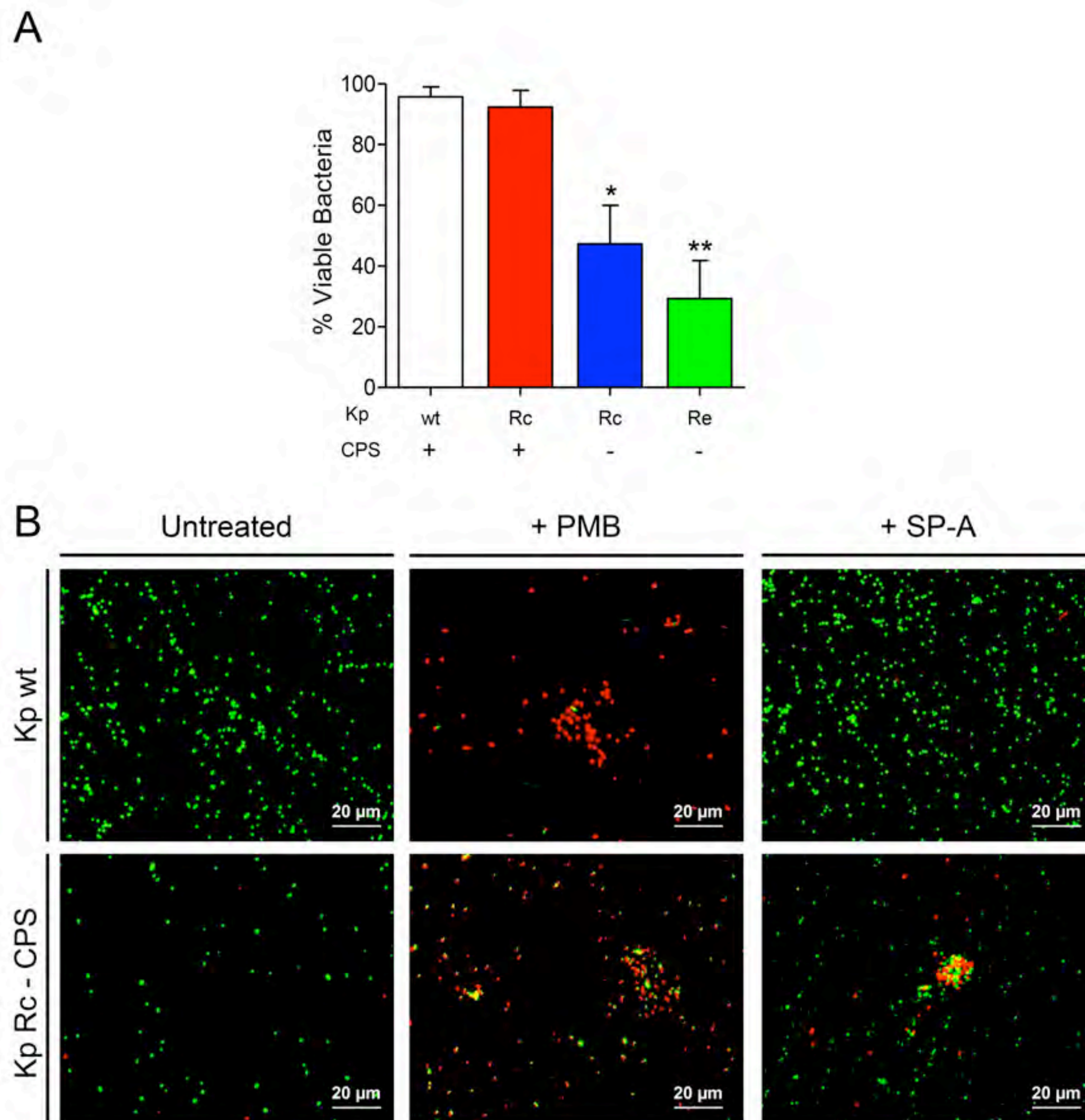

Figure 5. SP-A directly kills decapsulated strains *K. pneumoniae* Rc and Re expressing deep rough LPS. A) Bacteria (10^5 CFUs/mL) were incubated 1h at 37ºC in the absence or presence of SP-A 100 µg/mL 5 mM Tris (pH 7.2), 150 mM NaCl, 2 mM $CaCl_2$ buffer and immediately plated on LB agar for CFU count after 18h of incubation at 37ºC. The survival of bacteria in the presence of SP-A is shown as a percentage of the number of colonies of the same strain not exposed to agent. Bars represent mean ± SD (n=3, each one in duplicate) and significant differences in survival between control and treatments are indicated by asterisks, where **p < 0.01 and *p <0.05. B) *K. pneumoniae* wt and Rc-CPS both expressing GFP (to visualize living bacteria in green) were incubated with or without 100 µg/mL SP-A or 5µg/mL of PMB, as positive control of killing, for 1h at 37ºC. After that, both strains were stained with propidium iodide (stains dead/dying bacteria in red) following by fluorescence microscopy to assess viability (original magnification 50X).

The killing of deep rough decapsulated strains of *K. pneumoniae* by SP-A was also determined using the membrane-impermeant DNA-specific dye propidium iodide. Following incubation with SP-A, Kp Rc-CPS strain formed aggregates and most of bacteria were stained with propidium iodide (Fig. 5B). This indicates cell death, which is in agreement with the 60% of killing determined by the CFU count assay (Fig. 3A). In contrast, Kp wt neither aggregated nor was stained with propidium iodide in the presence of SP-A. Both strains were stained with propidium iodide after treatment with 5µg/mL of PMB as positive control of cell death.

Finally, we explored the ability of SP-A to enhance the phagocytosis of Kp wt as well as its isogenic mutant Kp Rc-CPS by mouse alveolar macrophages (MH-S) using an intracellular CFU count assay. In the absence of SP-A, after 1h of incubation, the uptake of Kp Rc-CPS bacteria by MH-S cells was greater than that of the wt bacteria, as previously seen by others (363) (Fig. 6A). Bacterial phagocytosis assays in the presence of SP-A were performed with three different approaches: a) Bacteria were pre-incubated 2h with SP-A before infection, b) macrophages were pre-incubated with SP-A 2h before infection, and c) macrophages were infected with a suspension of bacteria and SP-A. In the last two approaches, the presence of 100 µg/mL of SP-A did not increase the uptake of any of these bacteria by alveolar macrophages after 1h of infection (data not shown). However, when bacteria were pre-incubated with SP-A 2h before infection, SP-A was able to increase phagocytosis of the isogenic mutant Kp Rc-CPS, but not of the Kp wt strain (Fig. 6A). This is in agreement with the microscopic observation of intracellular bacteria expressing GFP (green) on alveolar macrophages, in which cytoplasm was stained with rhodamine-phalloidin (red) and nucleus with DAPI (blue). At the same conditions for intracellular CFUs count, higher numbers of intracellular Kp Rc-CPS compared to Kp wt were observed by confocal microscopy (Fig. 6B). In addition, these data suggest that enhanced phagocytosis of the mutant strain was due to the opsonic effects of SP-A, and not to a direct stimulatory effect of SP-A on macrophages. This is consistent with the ability of SP-A to aggregate Kp Rc+CPS (Fig. 3).

Taken together, our results suggest that SP-A has no antimicrobial effect on *K. pneumoniae* 52145 wild type strain and this could be due to the structure of its OM, since deep rough strains nonexpressing CPS are susceptible to SP-A. As *K. pneumoniae* is one of the lung pathogens that are isolated more frequently in patients with ARDS (196), it is conceivable that this bacterium has developed mechanisms to evade the bactericidal activity

of different soluble factors present in the alveolar space. One of these mechanisms could be to avoid being recognized and bound by SP-A present in the alveolar space. Hence, we evaluated the ability of SP-A to bind to Kp wt.

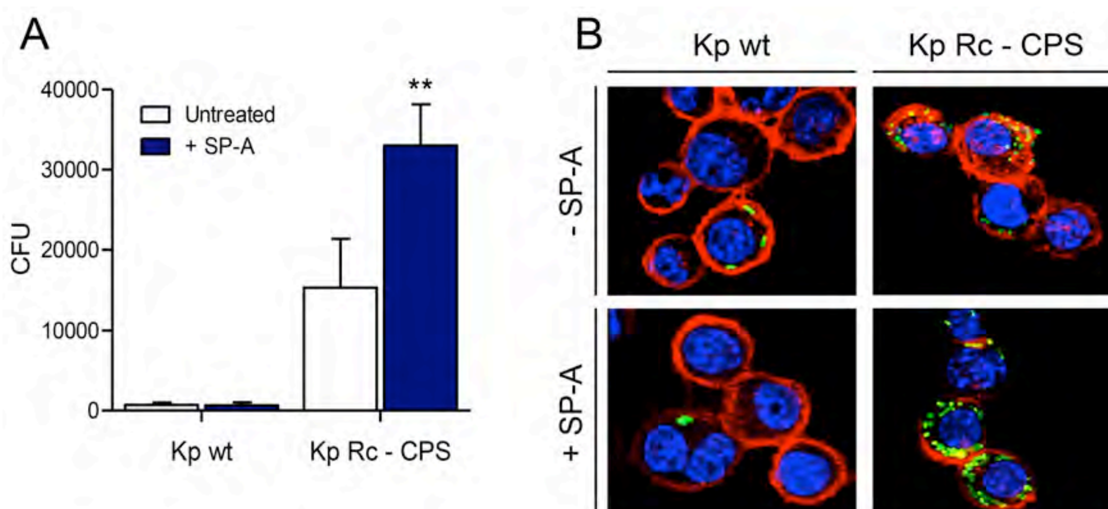

Figure 6. SP-A increases alveolar macrophages phagocytosis of decapsulated *K. pneumoniae* 52145 expressing Rc-LPS. A) Macrophages were infected 1h with *Klebsiella pneumoniae* 52145 wt or Rc-LPS nonexpressing CPS (multiplicity of infection = 50) pre-incubated with or without 100 µg/mL SP-A during 2h before infection, and treated with gentamicin (300 µg/mL) and PMB (15 µg/mL) during 90 min. to kill extracellular bacteria. Cells were lysed with 0.5% Saponin re-suspended in PBS and intracellular bacteria were serial diluted for CFU enumeration on agar plates. Bars represent mean ± SD (n=3) and significant differences between control and treatments are indicated by asterisks, where ** = $p < 0.01$. B) Immunofluorescence confocal microscopy of mouse alveolar macrophages infected with Kp wt and Rc-CPS both expressing GFP. Actin cytoskeleton was stained with Rhodamine-Phalloidin (red) and DNA was stained with DAPI (blue) to visualize cells with intracellular bacteria expressing GFP (green). Images are representative of three independent experiments.

4.2. SP-A Binding to *K. pneumoniae* 52145 strains.

The ability of SP-A to bind to the different *K. pneumoniae* strains used in this study was assessed by a solid-phase binding assay. *K. pneumoniae* and isogenic mutants were incubated 30 min. with increasing concentrations of biotinylated SP-A (0 to 1.25 µg/mL), and the amount of bacteria-associated SP-A was measured. As our previous results suggested, SP-A showed greater binding ability to decapsulated deep rough strains (Kp Rc-CPS and Re-CPS) and this binding was in a dose-dependent manner (Fig. 7). The fact that SP-A was unable to

recognize and bind to the wt strain would explain why this protein does not present microbicidial activity against it.

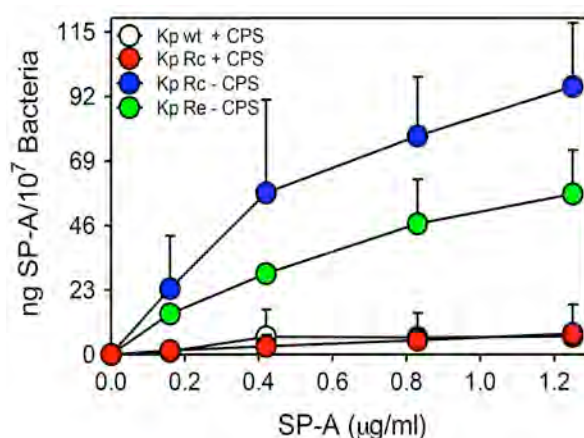

Figure 7. SP-A binds to decapsulated *K. pneumoniae* 52145 expressing deep rough LPS. *K. pneumoniae* strains (10^7 CFU) were re-suspended in 5 mM Tris pH 7.4 with 150mM NaCl and 2 mM Ca^{2+} and incubated with biotinylated SP-A (0 to 1.25 μg/mL) at room temperature for 30 minutes. Total *Klebsiella*-associated SP-A was measured by solid-phase binding and expressed as total nanograms of SP-A/10^7 bacteria. Bars represent mean ± SD (n=3, each one in duplicate).

So far, our results have shown that SP-A lacks of a direct antimicrobial effect against *K. pneumoniae* 52145 wt. However, *in vivo* studies have demonstrated that SP-A has antimicrobial activity against *in vitro* SP-A-resistant strains (20, 364). Since the SP-A is not alone in the alveolar fluid, the second objective of this study was to investigate whether SP-A was able to collaborate with antimicrobial peptides, both endogenous as exogenous modulating its microbicidal activity against various Gram-negative respiratory pathogens.

4.3. Synergistic action of SP-A with exogenous cationic antimicrobial peptides.

To evaluate whether SP-A is able to modulate the antimicrobial activity of exogenous cationic antibiotics with potential clinical use, we used PMB and E and three lung pathogens resistant to SP-A: *K. pneumoniae* 52145 wt (Kp wt), *P. aeruginosa* O1 (PAO1) and non-typeable *H. influenzae* (NTHi).

We performed survival bacterial assays by CFU count with different concentrations of PMB or PME in the presence or absence of SP-A. As it is known, PMB or PME alone showed

bactericidal effect in a dose-dependent fashion, being observed a decrease in bacterial survival according to the PMB or PME concentrations used (Fig. 8). SP-A alone (up to 100 µg/mL) had no effect in these bacteria; however, when bacteria were incubated with 100 µg/mL of SP-A and increasing concentrations of PMB (Fig. 8, upper graphs) and PME (Fig. 8, lower graphs) we obtained a higher bacterial killing for all pathogens studied. By contrast, when similar experiments were performed with lower concentrations of SP-A, up to 5 µg/mL, any synergistic effect was observed (data not shown). Thereby, we proved that SP-A significantly enhanced the polymyxin action, and it could increase the effect of these antibiotics in the alveolar space.

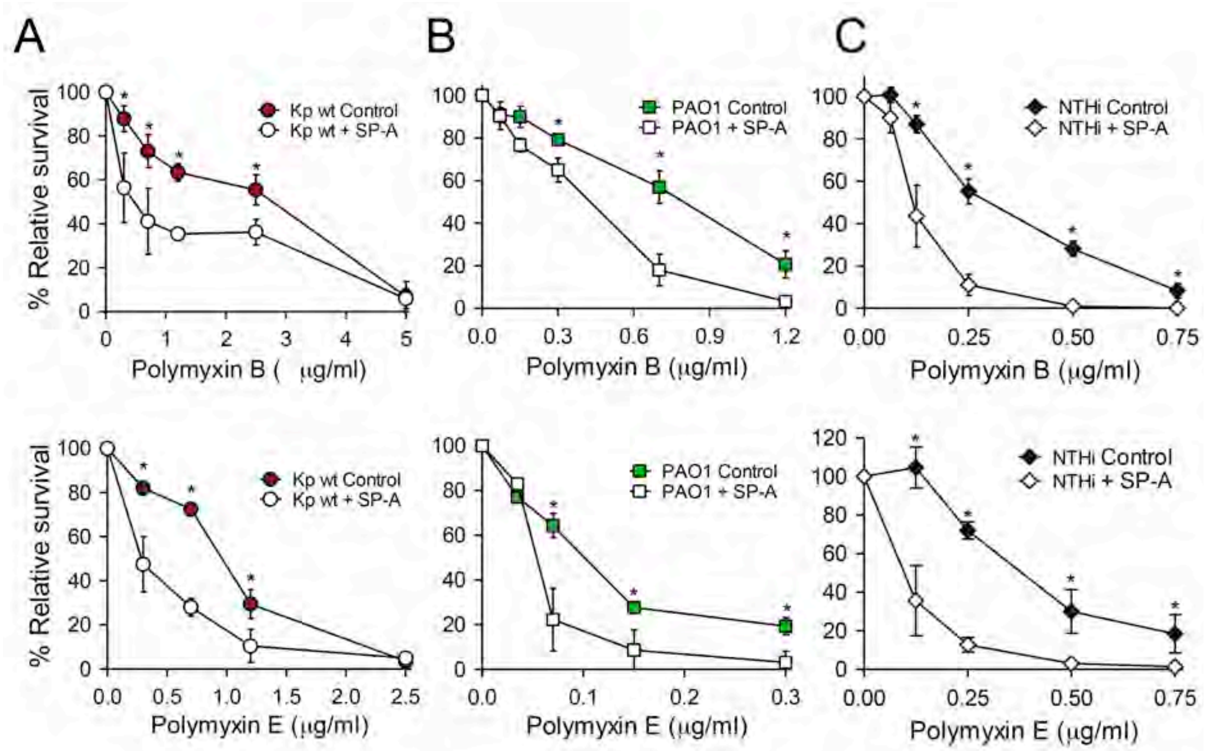

Figure 8. SP-A acts synergistically with exogenous CAMPs to kill Gram-negative bacteria. 10^5 CFUs/mL of *K. pneumoniae* 52145 wt **(A)**, *P. aeruginosa* O1 **(B)** or *non-typeable H. influenza* **(C)** were incubated with different concentrations (0.07 µg/mL to 5 µg/mL) of PMB (upper line) and PME (lower line) in 10 mM PBS (pH 7.2), 1% TSB and 100 mM NaCl buffer in the absence (colored symbol) or presence (white symbol) of 100 µg/mL SP-A for 1h at 37ºC and immediately plated on LB agar for CFU count after 18h of incubation at 37ºC. The results are shown as % relative survival (percentage of live colony counts compared to untreated control). Data represent mean ± SD (n=4, each one in duplicate) and significant differences in survival between the two treatments are indicated by asterisk, where * = $p < 0.05$.

Since SP-A can act synergistically with CAMPs that do not belong to lung defense mechanisms, such as PME and PMB, we examined whether SP-A shows this effect on humans AMPs present in the alveolar fluid.

4.4. Synergistic action of SP-A with endogenous cationic antimicrobial peptides.

To clarify the SP-A role in the innate immune system of the alveolar space and given that most of the antimicrobial factors present in the alveolar fluid could act cooperatively (273), we used human β-defensins (HBDs) to evaluate the potential synergistic action of SP-A with endogenous CAMPs.

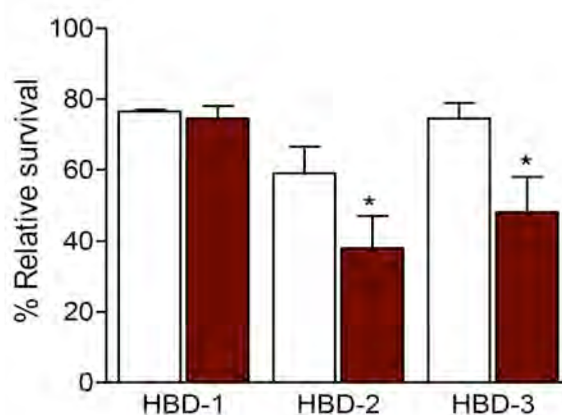

Figure 9. SP-A acts synergistically with the endogenous cationic AMPs human β-defensin 2 and 3 to kill *K. pneumoniae* 52145 wt. 10^5 CFUs/mL of *K. pneumoniae* 52145 re-suspended in in 10 mM PBS (pH 7.2), 1% TSB and 100 mM NaCl buffer were mixed with sublethal concentrations of HBDs (2.5 µg/mL HBD-1, 0.75 µg/ml HBD-2 and 1.5 µg/ml HBD-3) in the absence (white bars) or presence (red bars) of 100 µg/mL SP-A, incubated for 1h at 37 ºC and immediately plated on LB agar for CFUs count after 18h of incubation at 37 ºC. Results are shown as % relative survival (percentage of live colony counts compared to untreated control). Bars represent mean ± SD (n=3, each one in duplicate) and significant different in survival between the two treatments are indicated by asterisks, where * = $p < 0.05$.

To verify whether SP-A is able to enhance the bacterial killing of HBDs, we used sublethal concentrations of defensins (that produced a 20-30% decreased survival of the bacteria). To establish these concentrations, mortality curves were performed for all HBDs against *K. pneumoniae* 52145 (data not shown). The results obtained indicated that 100 µg/mL SP-A did not increase or block the bactericidal effect of HBD-1, while it significantly increased the bactericidal effect of HBD-2 and HBD-3 (Fig. 9). Therefore, the two inducible

variants with greater bactericidal effect of HBDs showed a synergistic effect with SP-A, similar to that found with the exogenous bactericidal peptides PMB and PME (Fig. 9).

4.5. SP-A Binding to *K. pneumoniae* 52145 in the presence of AMPs.

To explore the reasons why the presence of SP-A increases the bactericidal activity of these AMPs we performed solid-phase binding assays of SP-A to Kp wt in the presence or absence of AMPs. *K. pneumoniae* was incubated 30 min. with increasing concentrations of SP-A (0 to 1.25 µg/mL), with or without PMB (5 µg/mL) or HSA (5 µg/mL), and the amount of bacteria-associated SP-A was measured. As shown above (Fig. 7), SP-A alone did not bind to *K. pneumoniae* wt, not even in the presence of HSA (Fig.10). However, SP-A was able to bind to *K. pneumoniae* in the presence of PMB in a dose-dependent manner (Fig. 10). Therefore, these results suggest that AMPs facilitates the binding of SP-A to bacteria.

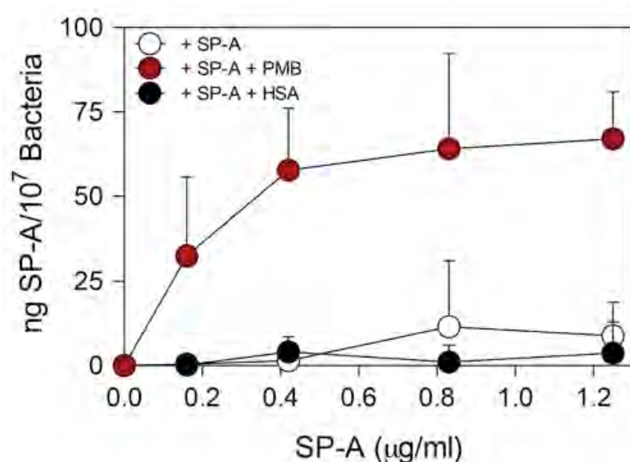

Figure 10. SP-A is able to bind *K. pneumoniae* 52145 wt in the presence of polymyxin B. Increasing concentration of SP-A were incubated with 10^7 CFU of *K. pneumoniae* 52145 wt in the absence (white symbol) or presence of PMB 5 µg/mL (black symbol) or HSA 5 µg/mL (red symbol) at room temperature for 30 minutes. Total *Klebsiella*-associated SP-A was measured by solid-phase binding and expressed as total nanograms of SP-A/10^7 bacteria. Bars represent mean ± SD (n=3, each one in duplicate).

4.6. Interaction of SP-A with AMPs.

Once proved that AMPs allow the binding of SP-A to bacteria with which SP-A alone cannot interact, we studied the potential interaction between SP-A and different AMPs in solution by measuring changes in the intrinsic fluorescence or in the hydrodynamic diameter of SP-A. Dynamic light scattering (DLS) studies showed that PMB alone has a unique peak (independent of the concentration), which corresponds to particles with a hydrodynamic diameter of 20 ± 3 nm (data not shown). For SP-A, two identifiable peaks were recognized for SP-A alone (15 nM), one corresponding to SP-A particles with a hydrodynamic diameter of

34 ± 5 nm (Fig. 11A, black line) and another corresponding to SP-A aggregates with a hydrodynamic diameter of 1000 nm (data not shown), according with previous data of our group (365). The addition of increasing concentrations of PMB (ranging from 0 to 75 μM) to a SP-A solution caused a PMB-concentration-dependent increase of the small size SP-A peak, up to a PMB molar concentration of 15 μM (Fig. 11A). This new peak (916 ± 22 nm for the highest amount of PMB added), is higher than the one determined for SP-A or PMB alone (at the same concentrations) and suggests the formation of a SP-A/PMB complex.

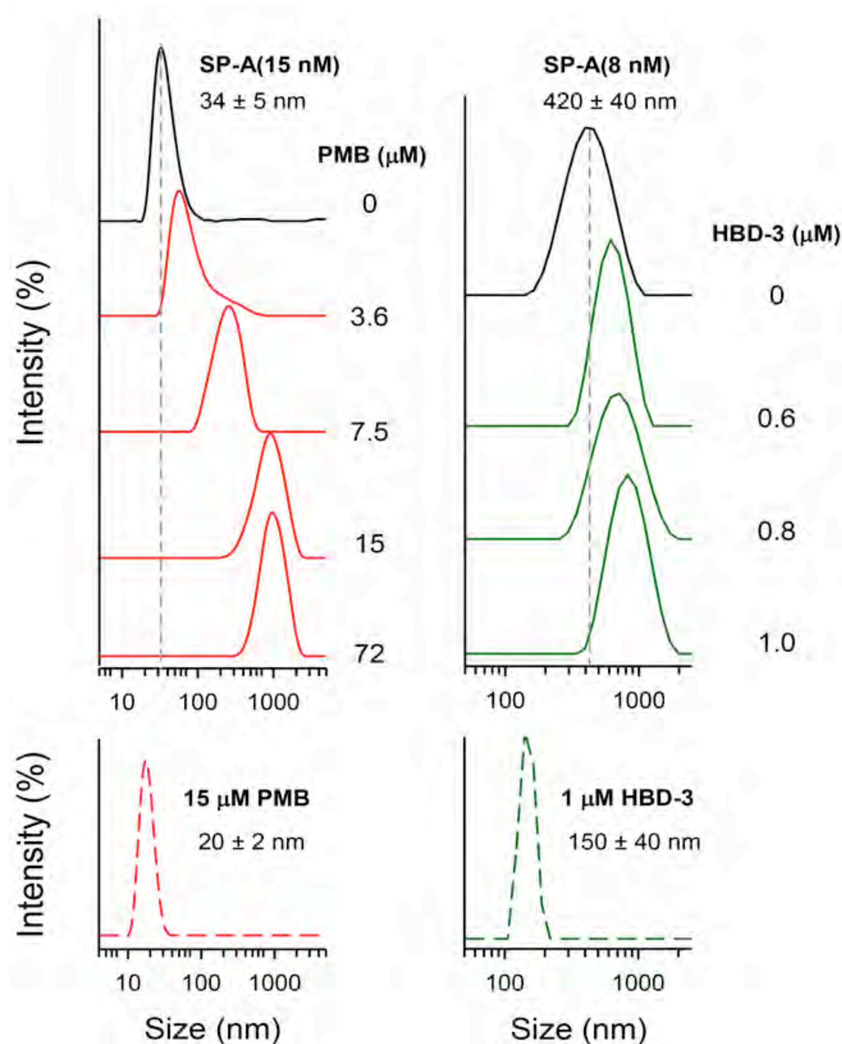

Figure 11. SP-A interacts with polymyxin B and HBD-3. DSL analysis of the hydrodynamic diameter of SP-A (34 ± 5 nm) in the absence or presence of increasing concentration of PMB (left graphs), in 5 mM Tris buffer (pH 7.4), or HBD-3 (right graphs), in 5mM Tris buffer (pH 7.4) containing 150 mM NaCl. The y axis represents the relative intensity of the scattered light; the x axis denotes the hydrodynamic diameter of the particles present in the solution.

We also evaluated the possible binding between SP-A and AMPs by measuring changes in the intrinsic fluorescence emission spectrum of SP-A upon the addition of increasing concentrations of AMPs. In the absence of AMPs, the emission spectrum of SP-A is dominated by the contribution of two conserved tryptophan residues located at positions 192 and 213 of the globular COOH-terminal domain (135), which are sensitive markers of conformational changes in this region (324). The emission spectrum of SP-A had a maximum at 338 nm, indicative of Trp residues partially exposed to solvent (Inset Fig. 11B) (366). The addition of increasing concentrations of PMB (0 to 150 μM) produced a concentration-dependent decrease in the fluorescence intensity of SP-A without changes in its maximum wavelength (Inset Fig. 11B). This could indicate that PMB bound to the globular domain of SP-A or that conformational changes in SP-A (that exposed Trp residues to solvent) occurred due to the interaction with PMB. Figure 11 also shows the intensity data from titration of 15 nM SP-A to PMB. Following the changes in fluorescence intensity as a function of PMB concentration, we obtained a estimated dissociation constant for the SP-A/PMB complex of K_d = 0.32 ± 0.04 μM. In addition, the Hill coefficient value was greater than 1, indicating a positive cooperative binding.

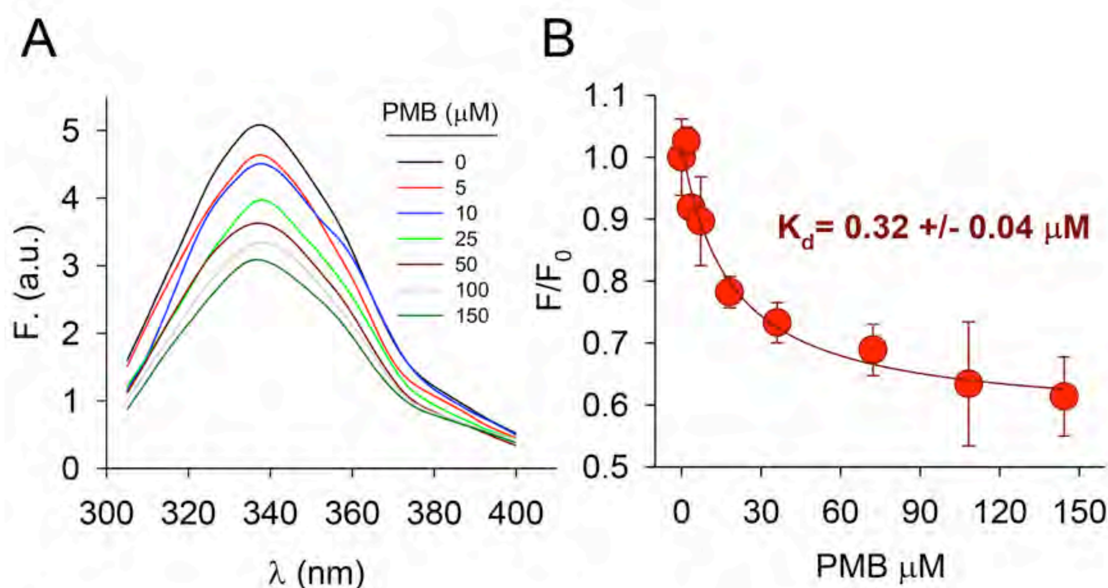

Fig. 12. SP-A interacts with polymyxin B. A) Representative graph of the fluorescence emission spectra of SP-A (15 nM, black line) measured with or without increasing concentrations of PMB (0 to 150 μM, colored lines) at 25 °C in 5 mM Tris buffer (pH 7.4) containing 150 mM NaCl. **B)** Results are also expressed as F/F_0 in each PMB concentration at maximum emission of SP-A, where F is the observed intensity of SP-A and F_0 is the observed intensity of SP-A+PMB. Results are means ± SD of 4 experiments.

Binding experiments using DLS and changes in the intrinsic fluorescence of SP-A were also carried out to evaluate the binding of this protein to another AMPs, such as HBD3, estimating a binding constant which was similar to that described by Saito and coworkers (367) (data not shown).

5. DISCUSSION

In this study, SP-A neither bound nor showed bactericidal activity against the clinic isolate *K. pneumoniae* 52145 wild type, which expresses both smooth LPS and CPS on its outer membrane, not even against the deep rough *K. pneumoniae* mutants expressing CPS. Although SP-A exerts antimicrobial activity in several microorganisms (351), several studies on SP-A antimicrobial ability *in vitro* against Gram-negative bacteria indicate that it binds to and acts on strains that expresses rough LPS, especially deep rough phenotype Rc or Re (17, 131, 355). This is consistent with our data, where SP-A was able to aggregate, kill and enhance phagocytosis by opsonization of Kp Rc-CPS and Re-CPS (Fig. 3-6), indicating that truncated LPS from inner core and the absence of CPS on *K.pneumoniae* 52145 made bacteria susceptible to SP-A.

Multiple reports have shown the SP-A-resistance of different bacterial strains expressing S-LPS (356, 357, 368). LPS is generally thought to be a protective wall with two potential barriers, the hydrophobic barrier provided by the hydrocarbon chain region (inner core) and the hydrophilic part provided by the oligosaccharide core (outer core), which is densely packed (369). Since the surface polysaccharides molecular network is lower in rough strains, S-LPS could prevent or decrease the SP-A binding to its main ligand in Gram-negative bacteria, the lipid A (370), protecting bacteria against SP-A action. In fact, we found that SP-A only had activity against decapsulated *K. pneumoniae* mutants expressing deep rough LPS, because it was able to bind these mutants (Fig. 7).

With regards to the role of CPS in bacterial resistance, it has been shown that binding and action of SP-A against some *K. pneumoniae* strains depends on the sugar composition of the CPS (344). It is known that, among saccharides, SP-A binds preferably mannose and fucose residues and only binds to *K. pneumoniae* strains expressing manose-α2/3-manose sequence in their capsule (capsular serotype K21) which is not present in *K. pneumoniae* 52145 CPS (capsular serotype K2) used in this study (344, 356). This also might explains why SP-A

neither bound nor showed antimicrobial activity against this clinical strain and remarks the importance of CPS to confer bacterial resistance against SP-A.

Taken together, these results highlight the importance of LPS and CPS in the bacterial resistance against SP-A, and confirm previous studies that indicate the SP-A preference to deep rough LPS bacteria(16). Finally, these findings provide new insights into the structural basis of SP-A/LPS interactions to the findings already reported (24).

Although we have not seen bactericidal effect of SP-A against this clinical strain of *K. pneumoniae*, it has been reported that this protein has a protective role in *K. pneumoniae* infections since SP-A knock-out mice show a higher mortality index upon *K. pneumoniae* challenge (364). Furthermore, SP-A knockout mice are much more susceptible to bacterial infections and macrophages-free broncho-alveolar lavages from SP-A +/+ mice shown a greater bactericidal effect than lavages from SP-A -/- mice, suggesting a microbicidal role of SP-A *in vivo*, even on bacteria expressing CPS and smooth LPS and resistant to SP-A *in vitro* (20, 353). Thus, taken these findings together, they suggest a complex microbicidal role of SP-A in the innate immune system of the alveolar space.

In this work, we show for the first time that SP-A, besides the bactericidal activity displayed against some pathogens, has another important role in the innate immune system, cooperating with AMPs present in the alveolar space to kill Gram-negative lung pathogens resistant to SP-A. Thus, we have shown that SP-A is able to act synergistically with antimicrobial peptides present in the alveolar fluid (such as HBD-2 and HBD-3). In this regard, it was observed an increase in the microbicidal activity of HBD-2 and -3 for *K pneumoniae* K2 strain against which SP-A showed no effect (Fig. 9). Among the innate immune system in the alveolar space, several reports have suggested the importance of cooperative actions between different soluble factors with microbicidal activity. It has been found that lactoferrin and lysozyme act synergistically in combination with HBD-2 (21), while HBD-2 and LL-37 have demonstrated an additive action against *E. coli* (22). Likewise, it has also found a synergistic activity in triple combinations of lysozyme, lactoferrin, and the proteinases inhibitor secreted by leukocytes (SLPI) (22). In addition, SP-A and SP-D have been shown to have additive neutralizing activity with human cathelicidin LL-37 against influenza A viruses susceptible to SP-A (371).

Moreover, we have shown that SP-A can also increase the bactericidal effect of two potent, but cytotoxic, bactericidal exogenous peptides, PMB and PME, against three different bacterial strains which SP-A did not have bactericidal effect, like *K. pneumoniae* K2, *P. aeruginosa* O1 or *non-typable H. influenzae* (Fig. 8). Since PMB and colistin are antibiotics of last resort against MDR microorganisms because of their high toxicity for the patient, and that this toxicity has been suggested to be dose dependent (372), their combinatorial use with SP-A would allow the use of a lower concentration of polymyxins, reducing their toxicity without decreasing their bactericidal effect. Therefore, the synergistic action found in this study, should be considered for the development of new therapies, though combinatorial use of SP-A and AMPs as therapeutic agents.

The mechanism of action by which SP-A enhances the antimicrobial activity of these AMPs is still unknown and must be deeply investigated. A possible explanation could be that the presence of these AMPs facilitates the binding of the protein to SP-A resistant pathogens (with which SP-A alone cannot interact), since solid-phase binding assays showed that SP-A was able to bind *K. pneumoniae* K2 wt when PMB was added in the solution (Fig. 10). The ability of AMPs to facilitate the binding of SP-A to these bacteria could be through a protein-protein interaction mechanism. Using dynamic light scattering and changes in the fluorescence of SP-A, we have demonstrated that SP-A interacts with PMB (Fig 11) and HBD-3 (data not shown) in a dose-dependent manner.

SP-A ability to bind to antimicrobial peptides have been previously described. Saito et al determined that SP-A binds- to HBD3 with a binding constant of 17.3 µM, which was very similar to what we have observed, and it is able to block the cytotoxicity of hBD-3 without inhibiting its antimicrobial activity (at lethal concentrations) (367). In addition, we showed that SP-A increased its microbicidal activity (at sub-lethal concentration) (Figure 7). This suggests a complex interaction between both factors that would improve the activity of the innate immune system in the lung defense against pathogens. Currently, we are further examining the direct mechanisms of action by which these proteins kill synergistically bacteria. Perhaps, one of them is the previous bacterial OM disruption, since we have seen that SP-A killed or helped to kill bacteria when OM was perturbed by two different ways: mutational alteration of the LPS structure; and the use of drugs able to bind and perturb bacterial OM. In addition, AMPs might act as SP-A carriers, through direct interaction, in order to allow SP-A binding to its bacterial target, thus permitting its bactericidal activity.

In summary, we have found that SP-A has antimicrobial activity against pathogenic Gram-negative bacteria, when bacterial OM was mutated up to deep rough LPS without CPS. Moreover, in smooth LPS bacteria expressing CPS (as clinical strains of *K. pneumoniae* K2), which are capable to avoid the bactericidal activity of SP-A, SP-A is able to act synergistically with cationic AMPs, through a protein-protein interaction mechanism, enhancing the innate immune system in lung defense against Gram-negative lung pathogens. However, more antimicrobial *in vitro* experiments would be necessary to gain insight into the interaction mode of SP-A with AMPs, and *in vivo* studies that could confirm this finding at physiological conditions. On the other hand, biophysical and biochemical experiments to investigate mechanisms by which these factors kill bacteria would be necessary to better understand their role in the innate immune system. The study of synergistic activity between SP-A and AMPs may provide new insights to the development of new efficient therapeutic treatments against MDR pathogens.

CHAPTER 2

Natural anti-infective pulmonary proteins: In vivo cooperative action of surfactant protein SP-A and the lung antimicrobial peptide SP-B^N

1. ABSTRACT

The anionic antimicrobial peptide SP-B^N, derived from the N-terminal saposin-like domain of the SP-B proprotein, and SP-A are lung anti-infective proteins since SP-A-deficient mice are more susceptible than WT mice to lung infections, and bacterial killing is enhanced in transgenic mice overexpressing SP-B^N. However, SP-B^N showed *in vitro* microbicidal activity only at middle acidic pH, and several microorganisms have been proven resistant to SP-A. Thus, we hypothesize that SP-B^N and SP-A might act cooperatively in the alveolar fluid to strengthen each other's microbicidal activity. Our results indicate that both proteins acted synergistically *in vitro* against SP-A- and SP-B^N-resistant capsulated *Klebsiella pneumoniae* (serotype K2) at neutral pH. SP-A and SP-B^N were able to interact in solution (K_d = 0.39±0.02 μM), which enabled SP-A binding to bacteria with which SP-A or SP-B^N alone could not interact. *In vivo*, we found that treatment of *Klebsiella pneumoniae*-infected mice with SP-A and SP-B^N conferred more protection against *K. pneumoniae* infection than each protein individually. Relative to untreated infected mice, SP-A/SP-B^N-treated infected mice showed significant reduction of bacterial burden, early neutrophil recruitment enhancement, and ameliorated lung histopathology. In addition, the concentrations of inflammatory mediators in lung homogenates increased early in infection in contrast with the weak inflammatory response of untreated *K. pneumoniae*-infected mice. Finally, we found that therapeutic treatment of SP-A and SP-B^N after 6h or 24h of bacterial challenge conferred significant protection against *K. pneumoniae* infection. These studies show novel anti-infective pathways that could drive development of new therapies for pulmonary infections.

2. INTRODUCTION

Lung infection is a frequent cause of morbidity and mortality especially in cystic fibrosis and other chronic lung diseases. Multidrug-resistant (MDR) bacteria strains are widespread and the pipeline for new antibiotics is insufficient to match the health burden posed by lung infection. Thus, the effectiveness of current therapeutic strategy against pneumonia is threatened (360).

Klebsiella pneumoniae is a frequent cause of nosocomial infections and may be responsible for as much as 20% of respiratory infections in the neonatal intensive care unit (373). The emergence of MDR strains of *K. pneumoniae* has significantly complicated the management and treatment of infections involving this organism and it is contributing to

increased bacterial drug resistance, toxic effects and health care costs (373). In this regards, innate host defense proteins secreted into the airway are less likely to induce bacterial resistance that regular exogenous antibiotics, mostly since outer membrane (OM) is the main target of these proteins. Moreover, they have other desirable features as new classes of antibiotics, including a broad spectrum activity, neutralization of endotoxins, activity *in vivo* and possible synergy with antibiotics (374). Consequently, a variety of host defense proteins are gaining considerable attention and clinical interest, because of their importance in maintaining lung homeostasis in ambient conditions and their potential to kill MDR microorganisms (375). Among them, surfactant protein- (SP) A and the saposin-like domain derived from NH_2-terminal propeptide of surfactant protein B (SP-B^N) have been shown as potent lung innate immune molecules (6, 12).

SP-A is a pulmonary protein member of the collectin family, which plays a key role in innate immunity through different activities. Directly interacts with and agglutinates various microbes and enhances phagocytosis by opsonization or direct phagocytes stimulation (376). It is able to regulate inflammatory mediator production depending upon the pathogen, the responding cell, and the cytokine environment (377). In addition, SP-A directly inhibits the growth of and kills some microorganisms by membrane permeabilization (362). *In vivo*, deficiency in SP-A in gene-targeted mice cause no apparent pathological changes but results in increased susceptibility to lung infections (352). Moreover, macrophage-free bronchoalveolar lavages from SP-A +/+ mice showed greater bactericidal activity against bacteria than those lavages from SP-A -/- mice (353). On the other hand, we recently discovered a new anionic AMP, SP-B^N, derived from the proteolytic processing of the NH_2-terminal segment of the proprotein SP-B, which possess strong antimicrobial activity (6). Overexpression of SP-B^N in the distal airway epithelium protects mice against infection with *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* (6). *In vitro*, SP-B^N indirectly promotes the uptake of bacteria by macrophage cell lines and directly kills bacteria at acidic pH, consistent with a lysosomal antimicrobial function (6). Despite the importance that these proteins have in lung defense against pathogens, there are limitations to consider: i) several studies have described the existence of SP-A-resistant microorganisms, including *K. pneumoniae* (chapter 1, (354, 378) and ii) SP-B^N is secreted into the alveolar fluid, but is not able to kill microorganism at physiological pH (6).

We have previously shown that SP-A is able to act synergistically with AMPs against several respiratory pathogens resistant to SP-A (chapter 1). In addition, several reports have suggested the importance of cooperative interaction between antimicrobial factors of the alveolar space in order to strengthen the microbicidal defense of the lungs (22, 371, 379); even though, little is known about the *in vivo* interaction between these factors in innate host defense. Hence, SP-A and SP-B^N may interact in the airways to aid in killing bacteria, even on resistant ones.

We confirmed the bactericidal activity of both, SP-A and SP-B^N, *in vitro* and assessed the potential therapeutic benefit of these proteins in combination to enhance host defense against a common cause of pneumonia, *Klebsiella pneumoniae*. Harnessing the host's natural antimicrobial proteins might provide an adjunct to the current therapy for pneumonia.

3. EXPERIMENTAL DESIGN

For this work, both *in vitro* and *in vivo* potential synergistic activity of SP-A and SP-B^N was evaluated. For *in vitro* experiments (Fig. 1), we firstly evaluated their direct microbicidal ability as well as their ability to enhance phagocytosis of capsulated *K. pneumoniae* K2. Killing was evaluated by CFU count on bacteria incubated with SP-A (100 µg/mL), SP-B^N (10 µg/mL) or both in 20 mM phosphate buffer, pH 7.0, or 2 mM sodium acetate buffer, pH 5, both buffers containing or not 150 mM NaCl with or without 2 mM Ca^{2+}. For phagocytosis assays, alveolar and peritoneal mouse macrophages (MH-S or Raw 264.7 cells) were infected with a suspension of bacteria and SP-A and/or SP-B^N and uptake of bacteria were enumerated by CFU count of intracellular bacteria after macrophages lysis. On the other hand, the ability of SP-B^N to bind to *K. pneumoniae* at acidic and neutral pH as well as the ability of SP-A to bind to this bacterium in the presence of SP-B^N was investigated by solid-phase binding assay. Finally, as determined in chapter 1 for CAMPs, the potential molecular interactions between SP-A and SP-B^N were determined using DLS assay and changes in the intrinsic fluorescence of SP-A.

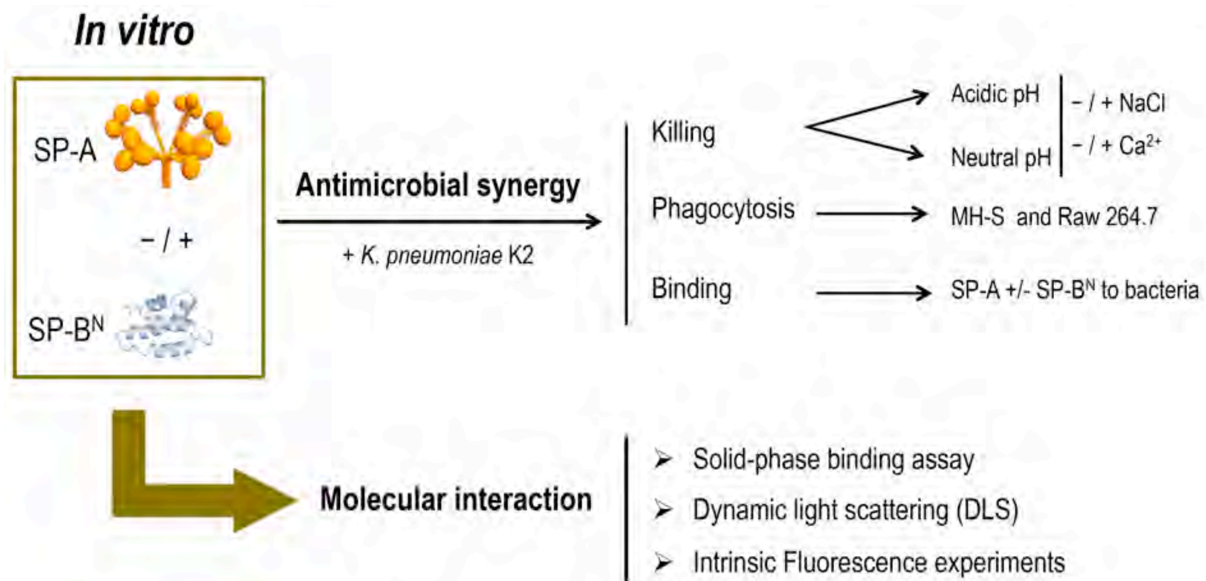

Fig. 1. *In vitro* experimental design used in chapter 2.

For *in vivo* experiments (Fig. 2), 10,000 CFUs of *K. pneumoniae* K2 re-suspended in 100 µL of 5 mM Tris/150 mM NaCl were intra-tracheally co-administered with SP-A, SP-B^N or both, in wild type FV/BN mice. Then, after 6, 24 or 72h of infection, on one hand, the left lobe of the lungs was harvested and homogenized in 1 mL of sterile PBS to assay lung bacterial burden by CFU count or to determine cytokines expression by ELISA. On the other hand, the right lobes were used to obtain the bronco-alveolar lavage (BAL). Total cell counts from BAL fluid were counted in a hemocytometer, and then, a suspension of cells was cytocentrifuged to analyze the types of cells by staining of cytospin preparations. In some experiments, lungs were inflation-fixed with 4% paraformaldehyde 6 or 24 h following intra-tracheal instillation of bacteria and proteins, and lung sections from each lobe were loaded onto polylysine-coated slides and stained with hematoxylin and eosin for analysis of lung histopathology. For therapeutic application of SP-A/SP-B^N, mice infected with 10,000 CFUs of *K. pneumoniae* K2 were treated with both proteins in combination 6 or 24h after the bacterial challenge and sacrificed at 24 or 48 h post-infection, respectively. Then, lung bacterial burden and total cell numbers and type of cells were analyzed as mentioned above.

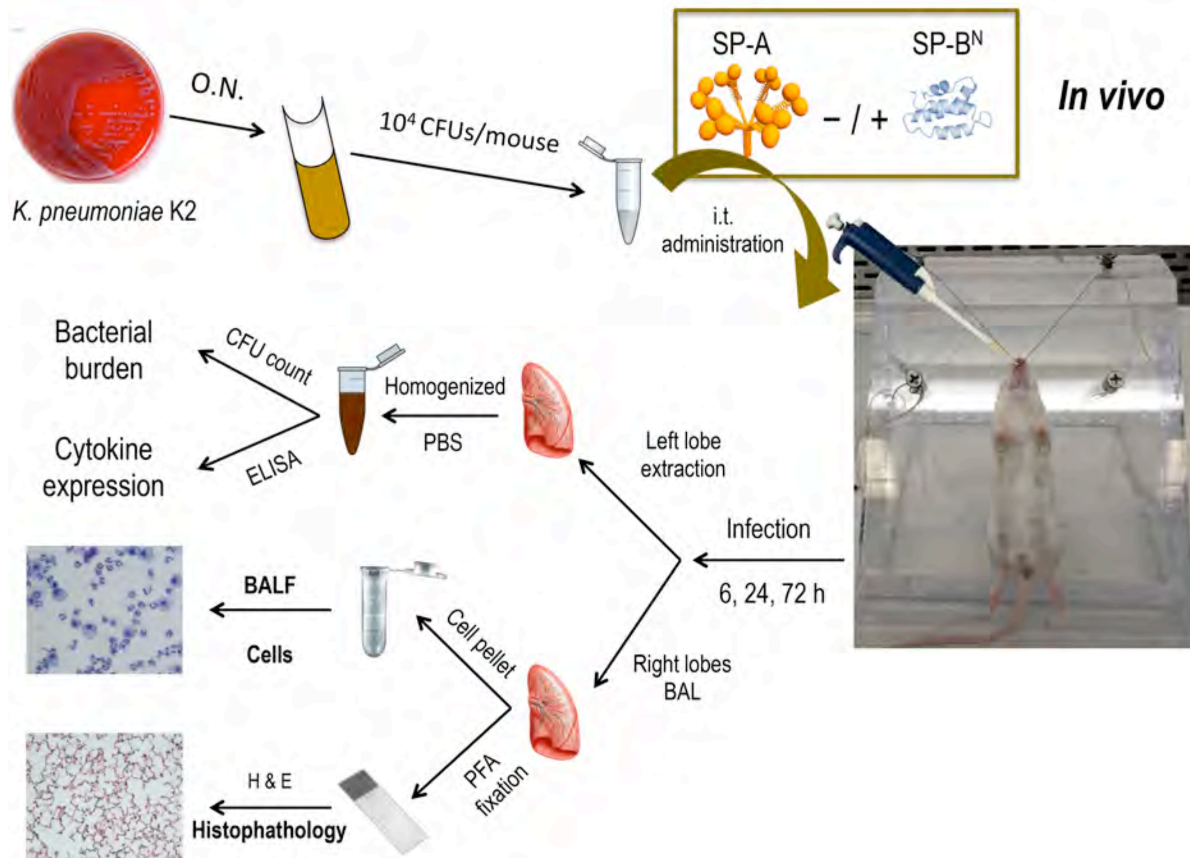

Fig. 2. *In vivo* experimental design used in chapter 2. CFU: colony-forming units; PFA: paraformaldehyde; BALF: bronchoalveolar lavage fluid; H&E: Hematoxylin & eosin

4. RESULTS

4.1. SP-A and SP-B^N are able to act synergistically against *Klebsiella pneumoniae* K2 at neutral pH *in vitro*

To assess potential synergistic interaction between SP-B^N and SP-A in bacterial killing, *in vitro* killing assays were performed using the common and highly virulent pathogen *K. pneumoniae* (serotype K2). Bacteria were incubated with SP-A and/or SP-B^N at pH 7 (Fig. 2A) or pH 5.5 (not shown). As previously reported by us, SP-B^N and SP-A was unable to kill *Klebsiella pneumoniae* K2 at neutral pH (chapter 1, (6)). However, we found that in combination, both proteins effectively killed *K. pneumoniae* at neutral pH, where neither of

these proteins had bactericidal activity individually. Addition of a buffer containing 150 mM NaCl enhanced SP-B^N/SP-A mediated killing independently of the presence of calcium (Fig. 3A), indicating that the presence of ionic strength in the medium is important but not essential for bactericidal activity. Given that SP-B^N is able to kill bacteria at acidic pH (6), we also performed the experiment under these conditions. However, at this pH, the bactericidal action of SP-B^N was not increased by the presence of SP-A, with or without NaCl or Calcium (data not shown).

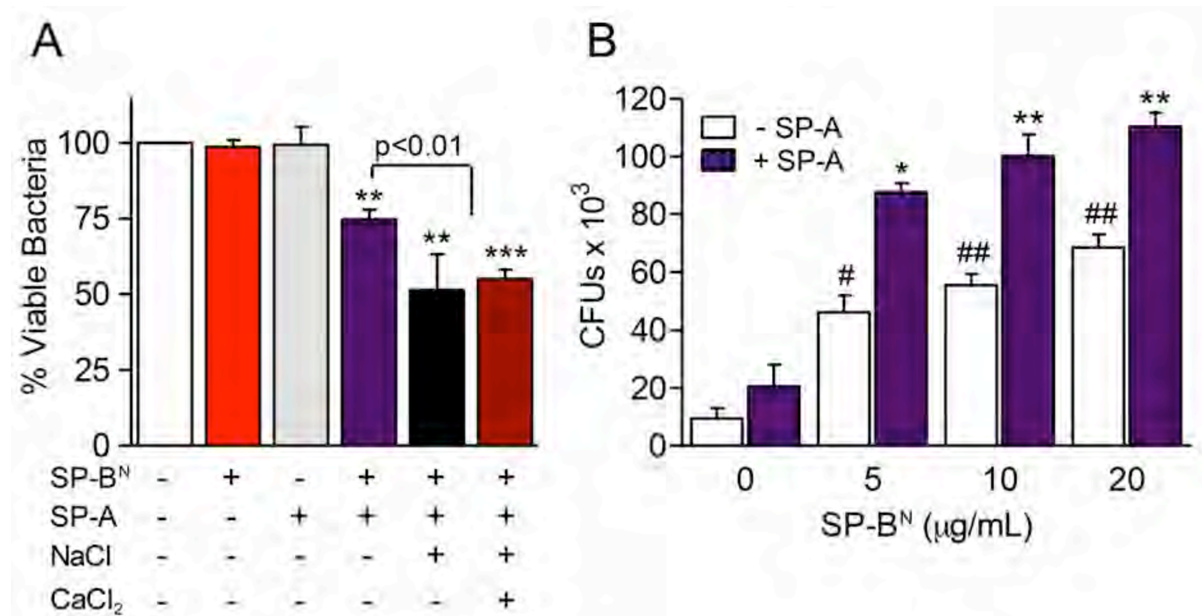

Figure 3. SP-A and SP-B^N are able to kill synergistically *K. pneumoniae* K2 at neutral pH and enhance phagocytosis by macrophages. A) Bacteria (10^5 CFUs/mL) were incubated 1h at 37ºC with SP-B^N (10 µg/mL), SP-A (100 µg/mL) or both in 20mM phosphate buffer, pH 7, containing or not 150 mM NaCl and/or 2mM $CaCl_2$. The number of viable bacteria was assessed by CFU count and results expressed as mean ± SD (n=4, each one in duplicate). **B)** RAW264.7 cells were pre-incubated with SP-B^N (0 to 20 µg/mL) with or without SP-A (100 µg/mL) for 4 h. Cells were washed twice with PBS, and infected 1 h with *K. pneumoniae* (5 X 10^7 CFU). Bacterial uptake was assessed by quantitative culture of cell lysates. Results are means ± SD (n=4, each one in duplicate). ***$p < 0.001$ and **$p < 0.01$ vs samples treated with proteins individually; ### $p < 0.001$ and ## $p < 0.01$ vs untreated samples.

4.2. SP-A acts together with SP-B^N to enhance bacterial uptake *in vitro*

Individually, SP-A and SP-B^N enhance phagocytosis of several respiratory pathogens (6, 380). We tested the potential cooperative action between these proteins to enhance uptake of *K. pneumoniae* by the mouse macrophages cell lines MH-S and Raw 264.7. Pre-incubation of bacteria with both proteins prior to infection or infection of macrophages with a suspension of bacteria and SP-A and/or SP-B^N did not increase phagocytosis after 1 h of infection (data not shown), suggesting that both proteins did not directly promote the uptake of this bacteria. In contrast, pre-incubation of MH-S (data not shown) or RAW264.7 cells (Fig. 3B) with SP-A (100 μg/mL) in combination with increasing amount of SP-B^N (5 to 20 μg/mL) for 4 h prior to the addition of bacteria resulted in a dramatic increase in intracellular bacteria, compared with untreated cells or cells treated with SP-A or SP-B^N alone (Fig. 3B). Therefore, these data suggest that SP-A and SP-B^N act in conjunction to indirectly increase phagocytosis of *Klebsiella*.

4.3. SP-A is able to bind *K. pneumoniae* in the presence of SP-B^N

As we have seen in Chapter 1, SP-A is only capable of recognizing and binding to Kp wt in the presence of other AMP (chapter 1, Fig. 10). Thus, we proposed to study if SP-B^N could also facilitate the binding of SP-A to bacteria. To this end, we firstly analyzed the ability of SP-B^N to bind to this bacterium at both pH 7 and 5.5. Bacteria were incubated with biotinylated SP-B^N (0 to 2.5 μg/mL) and *Klebsiella*-associated SP-B^N was assessed by solid-phase binding assay. Fig 4A shows that SP-B^N was able to bind to *K. pneumoniae* at acidic pH, but not at neutral pH, which is in accordance with its ability to kill bacteria only at acidic pH (6). On the other hand, binding of biotinylated SP-A (0 to 1.25 μg/mL) to bacteria in the presence or absence of SP-B^N (10 μg/mL) or HSA (10 μg/mL) was also evaluated. As previously shown (chapter 1, (356)), SP-A alone was unable to bind to *K. pneumoniae* K2, even in the presence of HSA (Fig. 4B). However, SP-A bound avidly to *Klebsiella* in a dose-dependent manner when SP-B^N was added to the solution (Fig. 4B), indicating that SP-B^N facilitates binding of SP-A to bacteria even at neutral pH.

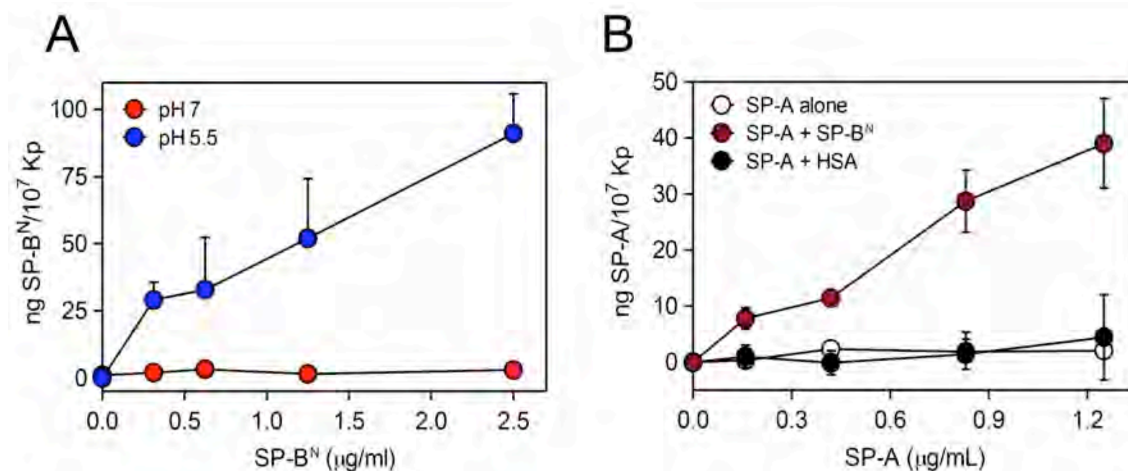

Figure 4. SP-A binds to *K. pneumoniae* K2 in the presence of SP-B^N. A) Biotinylated SP-B^N were incubated at RT for 30 minutes with *K. pneumoniae* K2 (10^7 CFU) in 20mM phosphate buffer, pH 7 (red symbol), or 5mM sodium acetate buffer, pH 5.5 (blue symbol), both containing 150 mM NaCl and 2mM $CaCl_2$. Total *Klebsiella*-associated SP-B^N was measured by solid-phase binding and expressed as total nanograms of SP-B^N per 10^7 bacteria. **B)** Increasing concentration of SP-A were incubated alone (white symbol) or in the presence of SP-B^N (10 µg/mL) (red symbol) or HSA (10 µg/mL) (black symbol) in the same conditions above mentioned. Total *Klebsiella*-associated SP-A was measured by solid-phase binding and expressed as total nanograms of SP-A/10^7 bacteria. Results are means ± SD of 4 experiments, each one in duplicate.

4.4. SP-A and SP-B^N interact directly

We next determined the mechanism by which the combination of SP-A and SP-B^N are able to bind and kill *K. pneumoniae*. First, we studied the potential interaction between SP-B^N and immobilized SP-A. Solid-phase binding assay showed that SP-B^N interacted with immobilized SP-A (38 nM) in a dose-dependent (0 to 11 µM) and saturable (K_d = 0.42 ± 0.08 µM) manner (Fig. 5A, red circles). SP-B^N did not bind to wells coated with HSA (Fig. 5A, black circles) or wells containing buffer alone.

Interaction of SP-A and SP-B^N in solution was next examined by monitoring changes in the intrinsic fluorescence of SP-A. The fluorescence emission spectrum of SP-A in the absence of SP-B^N showed a maximum at 338 nm, indicating that Trp residues (positions 191 and 213 of the globular domains) are partially exposed to solvent (381). Addition of increasing concentrations of SP-B^N (0 to 5.5 µM) significantly decreased in amplitude of the emission spectrum of SP-A, without changes in its maximum wavelength, in a dose-dependent manner (Fig 5B). These data suggest that the interaction of SP-B^N with SP-A takes

place in tryptophans region or that the binding produces conformational changes in SP-A resulting in the quenching of the two SP-A tryptophans (324).

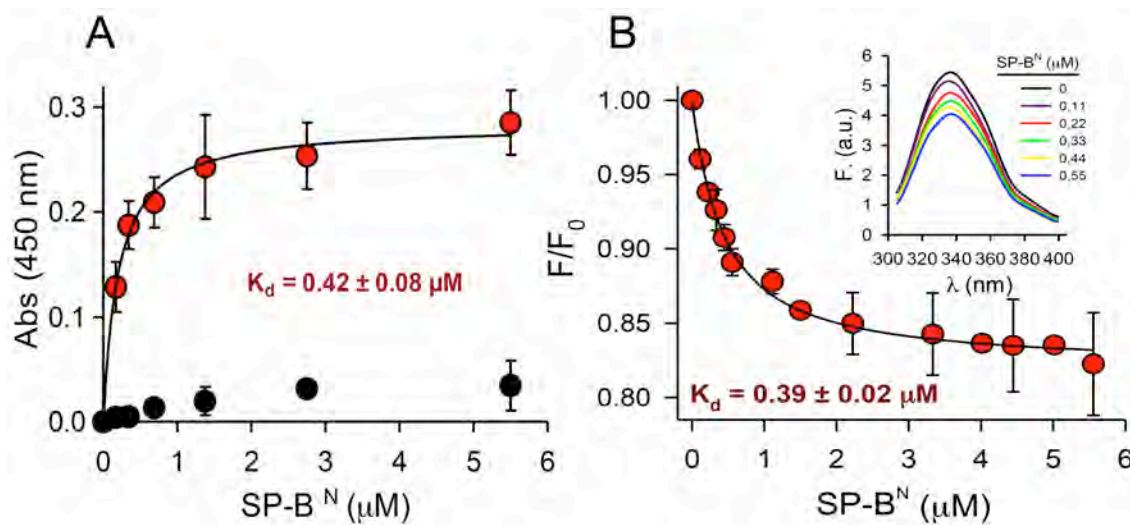

Figure 5. SP-A and SP-B^N interact in a dose-dependent manner. A) 25μg of SP-A (red symbol) or HSA (black symbol) was coated onto microtiter plate wells and biotinylated SP-B^N (0 to 11 μM) was then added to the wells. The level of bound SP-B^N was determined with streptavidin-horseradish peroxidase. Results are means ± SD of 4 experiments. **B)** Fluorescence emission spectra of SP-A (15 nM) was measured with or without increasing concentrations of SP-B^N (0 to 5.55 μM) at 25 °C in 5 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl. Results are expressed as F/F_0 in each SP-B^N concentration at maximum emission of SP-A, where F is the observed intensity of SP-A and F_0 is the observed intensity of SP-A with SP-B^N. Results are means ± SD of 4 experiments. In addition, inset graph shows a representative graph of fluorescence emission spectra of SP-A in the absence (black line) and the presence of increasing concentrations of SP-B^N (colored lines).

Using the Hill equation according to (chapter 1), an estimated K_d value for the SP-A/SP-B^N complex was obtained by measuring the changes in the fluorescence intensity of SP-A at 338 nm upon the addition of increasing concentrations of SP-B^N. This dissociation constant (K_d = 0.39 ± 0.02 μM) was quite similar to that one calculated by solid-phase binding assay.

4.5. Killing of *K. pneumoniae* in mice treated with SP-A and SP-B^N

To determine an appropriate bacterial dose for *in vivo* studies, 10^3 to 10^5 CFUs of *K. pneumoniae* K2 (6 mice/group) in wild-type FVB/N mice were inoculated intratracheally. Administration of *Klebsiella* was well tolerated, and all animals survived the 72-h study period at the 10^4-CFUs dose. Alterations in activity or physical appearance of the animals

were not detected until 72 h post-infection. *K. pneumoniae* K2 was co-administrated with SP-A, SP-B^N or both to wt FVB/N mice and bacterial burden was assessed. At 6 hours post-infection, lung bacterial burden was decreased 2.5-fold in SP-A- or SP-B^N-treated mice (p=0.02) and 6.6-fold in SP-A+SP-B^N-treated mice, p=0.001 (Fig. 6). Bacterial burden was also significantly decreased at 24 h when mice were treated with SP-A (2-fold), SP-B^N (4-fold) or SP-A+ SP-B^N (9-fold) (Fig. 6), indicating that these proteins are more effective when they are co-administrated. Although SP-A and SP-B^N treatment slowed down bacterial growth, total bacterial burden increased from 6 to 24 hours post-infection and by 72 hours post-infection. There was no difference among the groups at that last time post-infection. These data suggest that exogenous SP-A and SP-B^N are consumed after 24 hours and that retreatment and/or higher doses will be required to control infection.

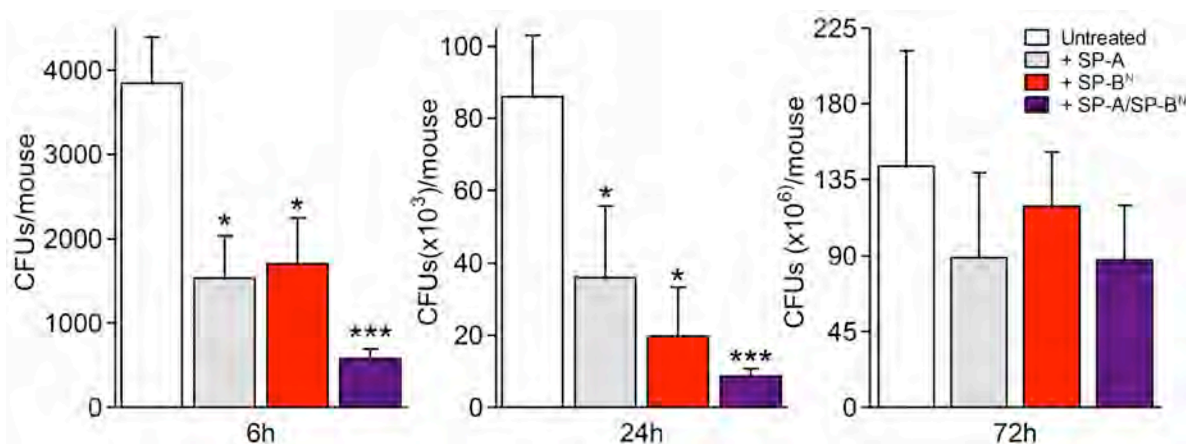

Figure 6. SP-A and SP-B^N treatment enhances bacterial clearance following intratracheal instillation of *K. pneumoniae* K2. *K. pneumoniae* K2 (10^4 CFUs) was co-administrated with SP-A (100 μg), SP-B^N (20 μg) or SP-A (100 μg) + SP-B^N (20 μg) in wt FVB/N mice and lungs were harvested, weighed, and homogenized at indicated time points. The numbers of viable bacteria were assessed by colony counting and expressed as CFUs/Mouse (n=6 mice/group/time point). Results are means ± SEM (n=6); ***$p < 0.001$ and *$p < 0.05$ vs untreated mice.

4.6. Inflammation in SP-A and SP-B^N-treated mice after *K. pneumoniae* infection

A mild increase in the total cells in BALF of untreated mice was observed 6 h and 24 h after infection with *K. pneumoniae*, compared to uninfected mice (Fig. 4A). Total cell numbers in BALF from SP-A- or SP-B^N-treated groups were significantly increased

compared to untreated animals. Combined SP-A/SP-B^N treatment further enhanced cell recruitment at 6 and 24 h post-infection (Fig. 4A). Histologic examination of lung sections showed mild alveolar infiltration in lungs of infected mice at 6h post-infection, which was higher in mice infected and treated with SP-A+SP-B^N (Fig. 4B). By 24h, alveolar infiltration increased in both groups of infected mice (Fig 4B), and areas of lobular pneumonia were detected, especially in untreated mice (Fig 4B, black arrows).

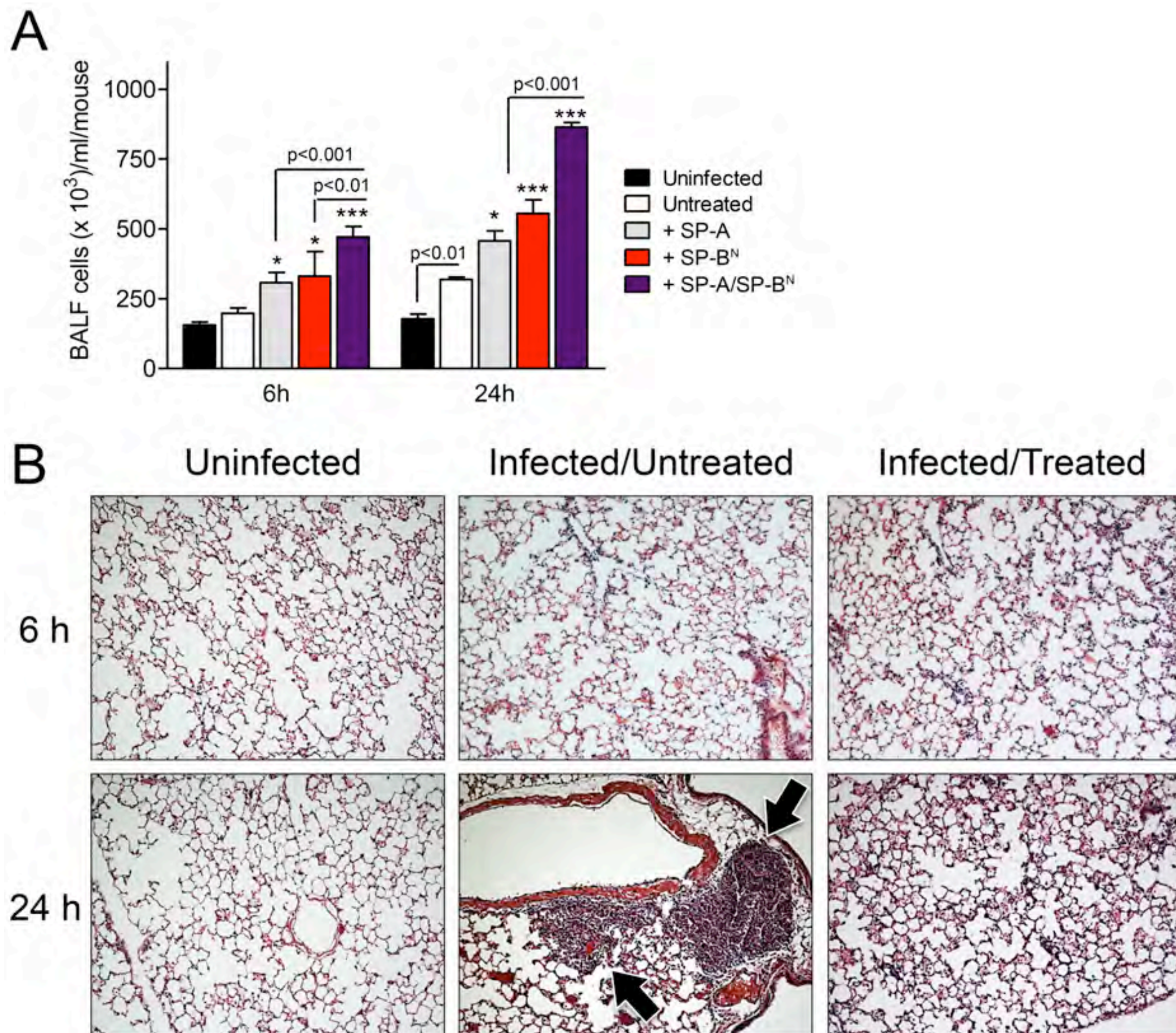

Figure 7. SP-A and SP-B^N treatment increase total cells in BALF and ameliorate lung histopathology of mice infected with *Klebsiella pneumoniae*. A) wt FVB/N mice were instilled with buffer or infected with 10^4 CFUs of *K. pneumoniae* K2 co-administrated with SP-A (100 µg), SP-B^N (20 µg) or SP-A (100 µg)+SP-B^N (20 µg), and sacrificed at 6h or 24h to assess total cell count. Six mice were evaluated for each group at each time point. Results are means ± SEM (n=6), ***p < 0.001 and *p < 0.05 vs untreated mice. **B)** Lung sections from uninfected mice or mice instilled with *K. pneumoniae* (10^4 CFUs) with or without SP-A(100µg)+SP-B^N(20µg) were stained with hematoxylin and eosin after 6 h (upper line) or 24 h (lower line) of administration. Representative lung histology from each group is shown. Arrows indicate lobular pneumonia.

Analyses of cell types in BALF from uninfected mice revealed that nearly all cells were alveolar macrophages while practically there were no neutrophils (Fig. 8). In infected mice, both treated and untreated ones, we could observe an increase in the recruitment of neutrophils, both at 6h and 24h post-infection (Fig. 8A and B). Neutrophil recruitment was significantly greater in mice treated with SP-A/SP-B^N compared to mice treated with SP-A or SP-B^N (Fig. 8A and B). In contrast, administration of both molecules in uninfected mice only promoted a minimal inflammation (data not shown).

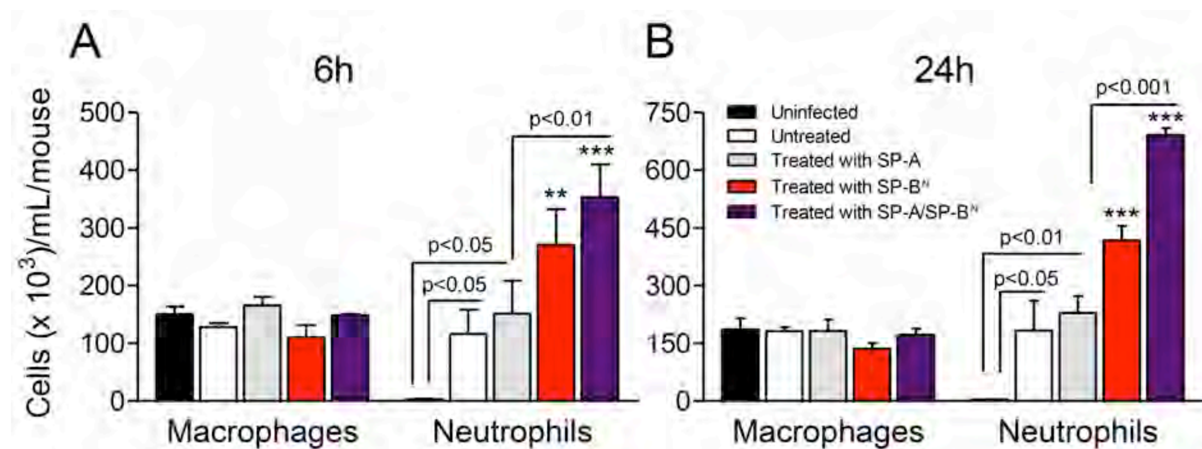

Figure 8. SP-A and SP-B^N treatment enhances early neutrophils recruitment in infected mice. wt FVB/N mice were instilled with buffer or infected with 10^4 CFUs of *K. pneumoniae* K2 co-administrated with SP-A (100 µg), SP-B^N (20 µg) or SP-A (100 µg) + SP-B^N (20 µg). Mice were sacrificed at 6h **(A)** or 24h **(B)** and total macrophages and neutrophils count in bronchoalveolar lavage fluid (BALF) were assessed. Six mice were evaluated for each group at each time point. Results are means ± SEM (n=6). ***$p < 0.001$ and **$p < 0.01$ vs untreated mice.

Moreover, neutrophil recruitment was strongly associated with elevated levels of TNF-α, IL-1β, IL-6, IL-17α, and MIP-2 (Fig. 9). The cytokine response was only detected in animals treated with SP-A/SP-B^N and was transient, returning to baseline 24h after treatment.

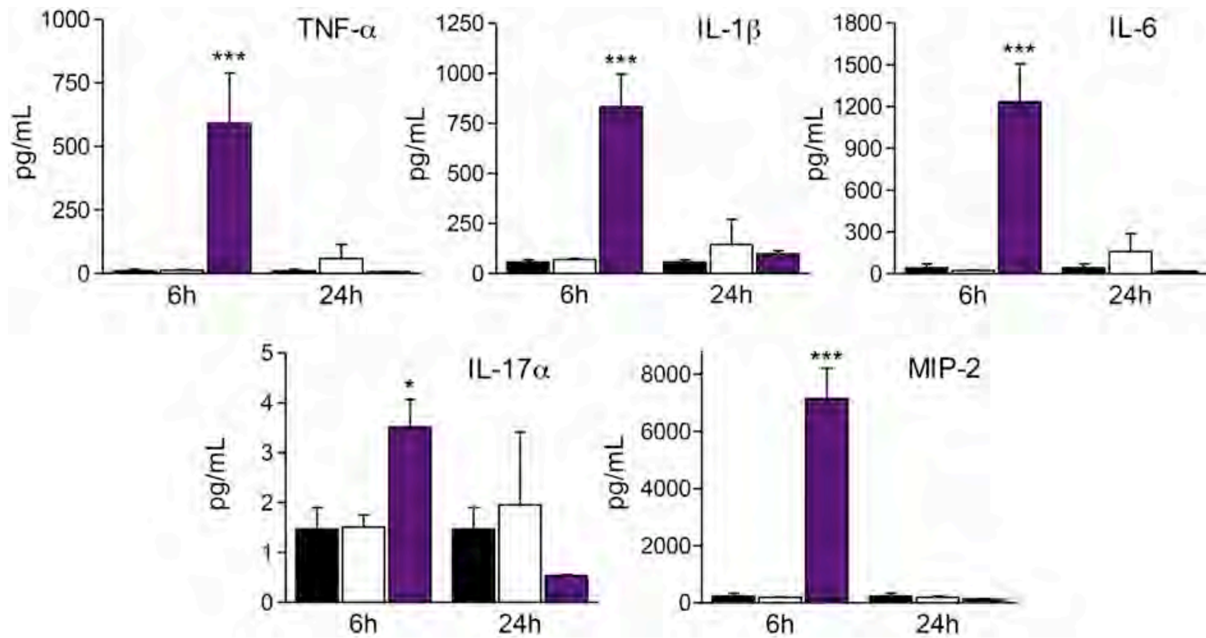

Figure 9. Levels of cytokines in lung homogenates after intratracheal co-instillation of *K. pneumoniae* K2 and SP-A/SP-B^N. wt FVB/N mice were instilled with 5 mM Tris, 150 mM NaCl buffer (*black bars*) or 10^4 CFUs of *K. pneumoniae* K2 without (*white bars)* or with (*purple bars*) 100 µg of SP-A + 20 µg of SP-B^N. Lungs were harvested and homogenized at 6h and 24 hours after infection and concentrations of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, IL-17α and macrophage inflammatory protein-2 (MIP-2) were quantitated by ELISA (n = 5 mice for each group). Results are means ± SEM, ***$p < 0.001$ and *$p < 0.05$ vs untreated mice.

4.7. Therapeutic effect of SP-A and SP-B^N in mice with an established infection

Once we have verified that both proteins are able to act in conjunction *in vivo*, we asked if they could be used as treatment once infection was established. To assess the therapeutic potential of SP-A/SP-B^N treatment, wt FVB/N mice were first infected with *K. pneumoniae* and subsequently treated with exogenous proteins 6 or 24h post-infection. Lung bacterial burden and BALF cells were analyzed 24h post-infection for animals treated at 6h, and 48h post-infection for animals treated at 24h.

SP-A/SP-B^N treatment reduced bacterial burden when administered 6h or 24h post-infection (Fig. 10A). Reduced bacterial burden was associated with increased cell infiltration (Fig. 10B) with neutrophils being the predominant cell type (Fig. 10B and 10C). Therefore, therapeutic treatment of SP-A and SP-B^N was also protective against *K. pneumoniae* infections.

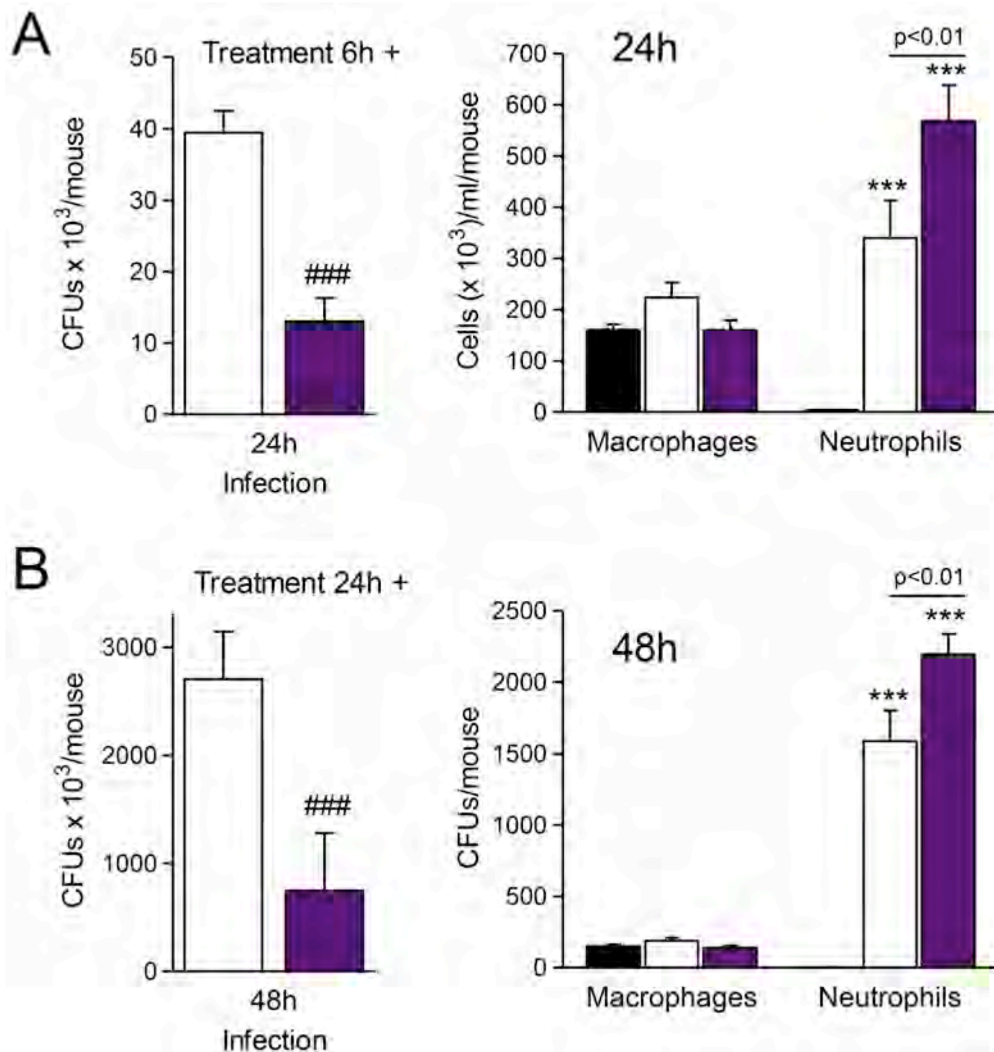

Figure 10. Therapeutic application of SP-A/SP-B^N increased bacterial clearance and neutrophils recruitment in mice with an established *K. pneumoniae* K2 infection. wt FVB/N mice were instilled with PBS (*black bars*) or 10^4 CFUs of *K. pneumoniae* K2 and instilled with Tris 5mM NaCl 150mM pH 7 (*white bars*) or SP-A (100 µg) + SP-B^N (20 µg) (*purple bars*) 6h **(A)** or 24h **(B)** after the bacterial challenge. Mice were then sacrificed at 24h or 48 hours post-infection, respectively, to assess lung bacterial burden in lungs (left graphs) and macrophages and neutrophils count in BALF (right graphs). Six mice were evaluated for each group at each time point. Results are means ± SEM (n=6). ***p< 0.001 vs uninfected mice. ###p< 0.001 vs untreated mice.

5. DISCUSSION

Due to the emergence of multi-resistant bacteria and the ineffectiveness of current treatments (360), research is focusing on naturally occurring lung proteins that may provide novel adjunctive therapies. SP-A and SP-B^N have been shown to have *in vivo* anti-infective role individually. Overexpression of SP-B^N in targeted mice is associated with a significant protective effect and survival following infection (6) and SP-A -/- mice are more susceptible to lung infection (352). In the current study, we demonstrated *in vivo* cooperative action between SP-A and SP-B^N against MDR-*K. pneumoniae* infection, which corroborates previous data showing synergistic *in vitro* activity between antimicrobial soluble factors of the alveolar space (22, 371).

SP-A and SP-B^N have shown several *in vitro* antimicrobial activities. Both proteins alone enhance bacterial uptake and killing by macrophages (151, 152). Microbicidal role of SP-A has been demonstrated by *in vitro* (16) and *in vivo* (14) studies showing bactericidal activity in Gram-negative bacteria. However, several SP-A-*in vivo*-susceptible microorganisms (20, 176) have been shown resistant to SP-A *in vitro* (16, 19) (Chapter 1), and we reported that SP-A acts synergistically with the cationic antimicrobial peptides human beta-defensin 2 and 3 to aid in killing *K. pneumoniae* K2 (Chapter 1), suggesting that SP-A might require collaboration with AMP in the alveolar space. On the other hand, the anionic saposin-like peptide SP-B^N kill bacteria only at acidic pH, but it is has been shown to be secreted into the alveolar fluid (6), where could cooperate with another antimicrobials factors, like SP-A. In the current study, we found that *in vivo* protection of SP-A/SP-B^N treatment against *Klebsiella pneumoniae* K2 was consistent with a combinatorial antimicrobial activity between these proteins. Therefore, we found that SP-A and SP-B^N further enhanced macrophages-mediated phagocytosis of *Klebsiella pneumoniae* in combination and were able to kill bacteria synergistically *in vitro* at neutral pH, whereas neither of these proteins had bactericidal activity individually. In this sense, several reports have found synergistic and additive interactions between antimicrobial factors of the alveolar fluid, including synergistic combination between lactoferrin and lysozyme against *E. coli* (22) and additive antiviral activity of SP-A with human cathelicidin LL-37 (371).

This collaboration in bacterial killing seems to be in connection with SP-A ability to bind *Klebsiella*, since only occurs in the presence of SP-B^N and probably through the interaction of SP-A with SP-B^N (K_d=0.39±0.3 μM). Ability of collectins, such as SP-A and SP-D, to bind

several ligands, including proteins, has been widely studied (365, 367). They showed several effects, from additive or antagonist (361, 382) to anti-cytotoxicity ones (367), which suggest complex interactions between alveolar soluble factors. Thus, in our case, protein-protein interaction showed synergistic activity and appears to be essential for this activity. The mechanisms by which SP-A and SP-B^{N} produce bacterial death synergistically remain unknown. It has been reported that SP-A is able to permeabilize bacterial outer membrane (14, 30) and suggested that SP-B^{N} might kill bacteria via pore formation, given the striking similarities with the anionic AMPs amoebapores, including saposin-like folding and pH- and oligomerization-dependent bactericidal activity (6). Therefore, it is possible that both proteins could enable each other to perturb bacterial OM through the interaction between them, which permit binding to bacterial LPS. However, additional experiments are necessary to confirm this presupposition.

The ability of SP-A and SP-B^{N} to enhance clearance of *K. pneumoniae in vivo* appears to be related to their capability to modulate host inflammatory response, since SP-A and SP-B^{N} were able to enhance early immune response to *Klebsiella*. SP-A and SP-B^{N} enhanced the expression of several pro-inflammatory cytokines, including IL-17, which is widely implicated in host response against infection by *K. pneumoniae* (383); as well as the expression of an important chemokine implicating in neutrophils recruitment, MIP-2 (384). Consequently, SP-A and SP-B^{N} promoted an increase in early neutrophil recruitment in BALF during infection with *Klebsiella*, which was greater in combination. In contrast, instillation of SP-A and SP-B^{N} in uninfected mice only promoted a minimal inflammation (data not shown).

Although data supporting the role of SP-B^{N} in inflammation are sparse, anionic AMPs have been shown to induce cytokine and chemokine production. Dermcidin is an antimicrobial peptide secreted into sweat, which shares some features with SP-B^{N}, such as anionicity, antimicrobial activity at acidic pH and constitutive expression (385). Dermcidin-derived peptides have been shown to activate normal human keratinocytes by inducing pro-inflammatory and chemoattractive mediators (386). In addition, AMPs have been reported to promote chemotaxis (8), both indirectly by stimulating the expression of well-known chemokines, like CXCL8/IL8 and CCL2/MCP1(227); and directly by acting as chemokines themselves to recruit a variety of immune cells (229). On the other hand, even though the main role of SP-A in inflammation seems to be anti-inflammatory (18), it has been also

shown to increase inflammation and modulate host innate defense in a context-dependent manner (34). SP-A stimulates production of pro-inflammatory cytokines during the early phase of lung injury (387). SP-A enhances chemotaxis of inflammatory BAL neutrophils toward chemokines such as MIP-2 (167) or the stimulation of neutrophil chemotactic factors release (168). In addition, influx of neutrophils into bronchoalveolar lavage fluid and pro-inflammatory cytokine expression was enhanced after exogenous administration of SP-A in mechanically ventilated preterm lambs (169). Thus, according to our results, SP-A and SP-B^N could collaborate *in vivo* to immunomodulate host immune response during infection.

The collaboration between both factors in the alveolar space might also depend on the bacterial pathogen or toxin that is attacking the host. Evidence indicated that *Klebsiella* infections are characterized by lacking an early inflammatory response, because *K. pneumoniae* antagonizes the activation of NF-kB in order to subvert the host inflammatory response in its own benefit (388, 389). In addition, several reports have demonstrated that activation of an inflammatory response is essential to clear *Klebsiella* infections (383, 390-392). In our case, SP-A and SP-B^N seem to counteract the anti-inflammatory response induced by this bacterium, since mice treated with both proteins showed an increase in early inflammation and neutrophils recruitment. In fact, studies involving mice infected with *K. pneumoniae* have found that the marked reduction of bacterial loads in response to the AMP a-defensin-1 is attributed to the increased accumulation of leukocytes at the site of infection (393). In addition, it is possible that the lower bacterial burden found in the lungs contributed to this increase in pro-inflammatory response, due to the presence of fewer bacteria to suppress the cytokine response. In any case, our data suggest that SP-A/SP-B^N treatment might enhance endogenous protective mechanisms to aid in the clearance of *K. pneumoniae.*

In summary, we found that two surfactant components, SP-A and SP-B^N, act in conjunction to protect the lungs against *Klebsiella pneumoniae* infection. These findings are consistent with an SP-A+SP-B^N-mediated increase in: 1) microbicidal activity; 2) bacterial phagocytosis; 3) neutrophil recruitment in BALF and 4) host early inflammatory response. Given the alarming increase in MDR Gram-negative bacteria in the face of a paucity of new antibiotics (376), we have shown that naturally occurring lung proteins may provide a novel model of adjunctive therapy for Gram-negative infections.

CHAPTER 3

Mechanisms of the Synergic Antimicrobial Action of Lung Surfactant Protein A and Polymyxin B Nonapeptide

1. ABSTRACT

Surfactant protein A (SP-A), the most abundant protein of the alveolar fluid, has been proven to directly kill some microorganisms; however, several bacteria are resistant to SP-A. In this study we investigate whether a nontoxic nonapeptide derived from polymyxin B (PMBN) cooperates with SP-A in killing Gram-negative bacteria and the mechanism by which both molecules exert their action. We found that, although PMBN and SP-A did not exhibit antimicrobial activity against *Klebsiella pneumoniae* K2:O1 and *Pseudomonas aeruginosa* PAO1, the combined action of both proteins resulted in efficient bacterial killing. PMBN was able to interact with SP-A (K_d =0.26 ± 0.02 μM), which allowed SP-A to bind to bacteria with which SP-A alone could not interact. To identify the mechanism of bacterial killing mediated by PMBN/SP-A, we measured the capability of these proteins to insert into bacterial outer membranes. We found that PMBN and PMBN/SP-A, but not SP-A, were able to insert into LPS monolayers, and PMBN insertion significantly increased in the presence of SP-A. Membrane disturbances induced by SP-A/PMBN, but not by SP-A or PMBN alone, resulted in membrane permeabilization of vesicles composed of bacterial outer membrane lipids. In contrast, the presence of SP-A did not have any influence on PMBN-induced permeability of bacterial inner membranes. Taken together, these results indicate that SP-A and PMBN cooperate in killing bacteria because they are jointly able to disturb and permeabilize the outer membrane of Gram-negative bacteria.

2. INTRODUTION

Polymyxins are pentacationic lipopeptides isolated from *Bacillus polymyxa* (394) that are well-known clinically used antibiotics (242, 317). Among them, polymyxin B (PMB) and polymyxin E, also called colistin, have been used in clinical practice since 1950s. Polymyxins are highly bactericidal against Gram-negative organisms, which are increasingly used as the last-line therapeutic options for treatment of infections caused by multidrug-resistant (MDR) Gram-negative bacteria, particularly *Pseudomonas aeruginosa, Acinetobacter baumannii*, and *Klebsiella pneumoniae* (26, 395).

To understand the mechanisms of antibacterial activity of the polymyxins and their resistance, it is crucial to have knowledge of their chemical structures (217, 247). Polymyxins are cyclic lipopeptides that can be characterized by the general structure illustrated in figure 1.

Their decapeptide sequence contains an intramolecular cyclic heptapeptide loop between the amino group of the side chain of the diaminobutyric acid (Dab) residue at position 4 and the carboxyl group of the C-terminal threonine residue at position 10. Polymyxins also have several other distinguishing structural features, including: five nonproteogenic Dab residues, which makes them polycationic at pH 7.4; hydrophobic residues at positions 6 and 7; and an N-terminal fatty acyl group (238).

Like many others antimicrobial peptides, the mixture of lipophilic and hydrophilic groups makes polymyxins amphipathic, a chemophysical property that is essential for their antibacterial activity (191, 238). Polymyxins are membrane surface-active proteins, which bind to lipid A, an important polymyxin-binding target in the outer membrane of Gram-negative species (238). The mechanism of action of polymyxins is based on self-promoted uptake system and it was explain in detail by Robert E Hancock (217) and Martti Vaara (247). It purports that the amphipathic nature of polymyxins is crucial to enable uptake of the polymyxin molecule across the outer membrane barrier (216), also permitting the passage of a variety of molecules, including hydrophobic compounds and small antimicrobial proteins.

Polymyxins are considered drugs of last resort because of their high neuro- and nephrotoxicity (236, 267, 396). In an attempt to counter this, a derivative from PMB, called polymyxin B nonapeptide (PMBN), was synthesized (246, 247). This derivative is characterized by the absence of the fatty acid tail and the N-terminal amino acyl residue Dab (Fig. 1). PMBN thereby lost the bactericidal activity of polymyxin B and is noticeably far less toxic (397-399). PMBN does, however, still possess endotoxin-binding activity via interacting with the anionic groups of LPS (400), although the deacylated derivative is also less active in its anti-endotoxin activity than that of its parent compound (401). By such interaction at the surface of viable Gram-negative bacteria, this nonapeptide facilitates the insertion of the membrane attack complex of the complement system into the bacterial membrane. In addition, the outer membrane-disorganizing effect of PMBN was described to enhance the permeability of the bacterial membrane for hydrophobic antibiotics (250, 255).

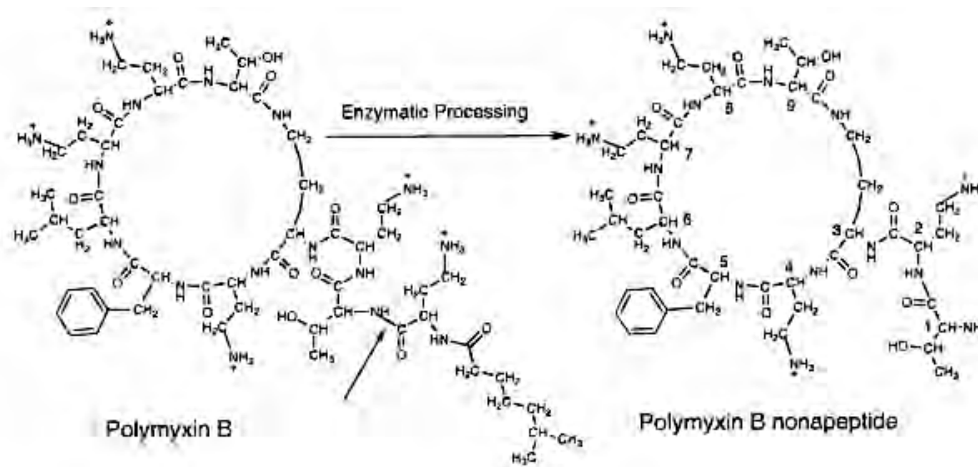

Fig. 1. Generation of polymyxin B nonapeptide. PMBN is obtained by proteolytic removal of the fatty acid tail of PMB. Taken from (249).

Surfactant protein A (SP-A) is a pulmonary collectin, which has been widely studied partly because of its antimicrobial properties in the lungs (12). Therefore, SP-A is able to aggregate, inhibit the grown and kill some microorganisms among Gram negative and positive bacteria, virus and fungi (18). We have recently shown that one function of the surfactant protein A (SP-A) in the alveolar innate immune system is its ability to act synergistically with antimicrobial peptides (AMPs), facilitating the clearance of various lung pathogens, even if they are resistant to the direct antimicrobial activity of SP-A (chapter 1 and 2). To this extent, we have seen that SP-A acted synergistically with cationic AMPs, both exogenous (PMB and colistin) and endogenous (HBD-2 and -3) presents in the alveolar fluid, leading to high bactericidal action against *Klebsiella pneumonia* K2 (which is resistant to SP-A). In addition, is important to notice, that there is also a synergistic action between SP-A and SP-B^N, which do not exhibit bactericidal activity alone at neutral pH. This synergistic action is possible because AMPs facilitate the binding of SP-A to bacteria (with which SP-A alone cannot interact), probably through a protein-protein interaction mechanism (chapter 1 and 2).

Despite having lost their bactericidal capacity, PMBN maintains the ability to bind to bacterial outer membranes by interaction with LPS (400) and it is able to act collaboratively with complement proteins and other hydrophobic antibiotics (250), suggesting that PMBN could act in collaboration with SP-A. Moreover, we have shown that SP-A interacts with PMB increasing its antimicrobial activity, most likely through a of protein-protein interaction mechanism. Therefore, in this work, using a variety of biophysical techniques, we propose to

study the effect that these proteins together have on bacterial model membranes in order to determine the mechanism by which SP-A kills synergistically bacteria with polymyxins. For this purpose, we firstly evaluate whether the derivative from polymyxin PMBN could act in collaboration with SP-A to cause bacterial death, although none of them possesses bactericidal capacity separately.

3. EXPERIMENTAL DESIGN

For this work, we have used the exogenous CAMP polymyxin B nonapeptide, to investigate the mechanism by which SP-A and AMPs act synergistically. As mentioned before, PMBN is a derivative from polymyxin B, which lack of the acid fatty tail and is unable to kill bacteria but retains its ability to bind and disrupt bacterial OM. Then, the integrity and properties of bacterial model membranes of Re-LPS/BPL after incubation with SP-A and PMBN was assessed. First, we evaluated the potential synergistic killing of SP-A and PMBN against *K. pneumoniae* K2 and *P. aeruginosa* O1 by CFU count. In addition, to mimic condition of biophysical experiments, its synergy was also determined on *K. pneumoniae* K2 mutant expressing Re-LPS (Fig. 2).

For the study of mechanisms of action, as used for the other AMPs, we firstly determined the ability of SP-A to bind to *K. pneumoniae* K2 in the presence of PMBN by solid-phase binding assay as well as the potential molecular interactions between SP-A and PMBN by DLS and changes in the intrinsic fluorescence of SP-A (Fig. 2). Next, we tested the ability of PMBN together with SP-A to insert into monolayers of Re-LPS by measuring increases in the surface pressure of the monolayer after addition of PMBN, SP-A or both.

To obtain further information about mechanism of SP-A- and PMBN-promoted bacterial membrane disruption, we employed liposomes containing Re-LPS and bacterial phospholipid extract (BPL) composed of PE/PC/PG. Liposomes were prepared by mixed stocks solutions of the BPL and Re-LPS to achieve the desired Re-LPS/BPL ratio (80/20, w/w). Dry lipid films were hydrated in a saline buffer and allowing them to swell for 1h at a temperature above the transition temperature (Tm). After vortexing, the resulting multilamellar vesicles (MLVs) were used for measuring the thermotropic behavior of Re-LPS/PL vesicles in the absence or presence of both proteins by differential scanning calorimetry (DSC). Unilamellar vesicles, obtained by sonication of MLVs, were used for spectroscopic measurements to

reduce light scattering artifacts. To determine membrane fluidity of bacterial model membranes in the absence and presence of SP-A and PMBN, the hydrophobic membrane fluorescent probes DPH as well as the amphiphilic fluorescent dye LAURDAN were used (Fig. 2). Changes in their fluorescence emission are indicative of membrane perturbation. Finally, the fluorophore ANTS and its collisional quencher DPX were co-encapsulated into Re-LPS/BPL or BPL vesicles to assess the ability of SP-A, PMBN or both to permeabilize bacterial model membranes by measuring the increase of the ANTS fluorescence after leakage of the aqueous contents by addition of a potential lytic peptide (Fig. 2)

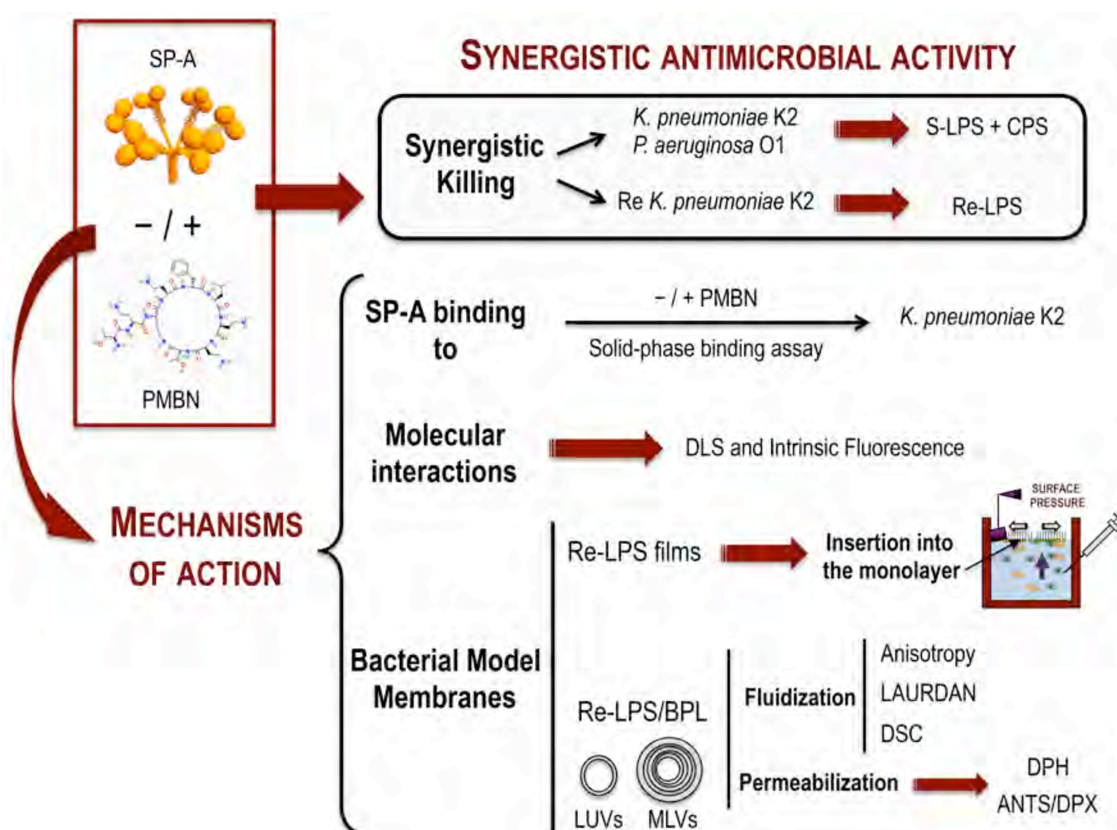

Fig. 2. Experimental design used in chapter 3. Fluorescent probes = DPH, LAURDAN, ANTX/DPX. DLS: dynamic light scattering; DSC: differential scanning calorimetry. BPL: phospholipid extract; LUVs: large unilamellar vesicle; MLVs: multilamellar vesicle.

4. RESULTS

4.1. Synergistic activity of SP-A and PMBN

Previous studies from our group showed that SP-A had no bactericidal activity against *Klebsiella pneumoniae* K2, but nevertheless was able to act synergistically with polymyxin B and colistin increasing their antimicrobial activity (chapter 1). In addition, it was also capable of acting in collaboration with SP-B^{N}, an anionic antimicrobial peptide present in the alveolar fluid, against pulmonary pathogens resistant to SP-A, reducing the bacterial burden and increasing the early inflammatory response (chapter 2). Consequently, for this work, we firstly confirmed synergistic interaction between SP-A and PMBN against two Gram-negative bacteria, *K. pneumoniae* (serotype K2) and *Pseudomonas aeruginosa* O1 using an *in vitro* bacterial killing assay. As previously reported, neither SP-A nor PMBN possessed antimicrobial activity against *Klebsiella or Pseudomonas* (chapter 1,(247)). In contrast, we found that in combination, SP-A and PMBN effectively killed these two Gram-negative bacteria (Fig. 3A).

Due to the difficulty of working with smooth LPS *in vitro*, to make bacterial model membranes we have used a rough LPS. Therefore, we tested the synergistic activity of SP-A and PMBN using an isogenic mutant of *K. pneumoniae* expressing deep rough LPS in its bacterial outer membrane. We have previously described that deep rough decapsulated strains of *K. pneumoniae* showed susceptibility to SP-A (chapter 1), which agrees with several studies showing that SP-A preferably interacts with bacteria expressing rough LPS (16, 17, 131). To see the synergistic effect of both proteins, without any of them taking effect separately, it was necessary to decrease the concentration of SP-A. Thus, in figure 3B we can see that SP-A at 25 μg/ml did not have bactericidal effect on deep rough decapsulated strains of *K. pneumoniae*. Neither PMBN showed bactericidal effect by itself on this strain. However, PMBN and SP-A together demonstrated antimicrobial effect on this *K. pneumoniae* strain.

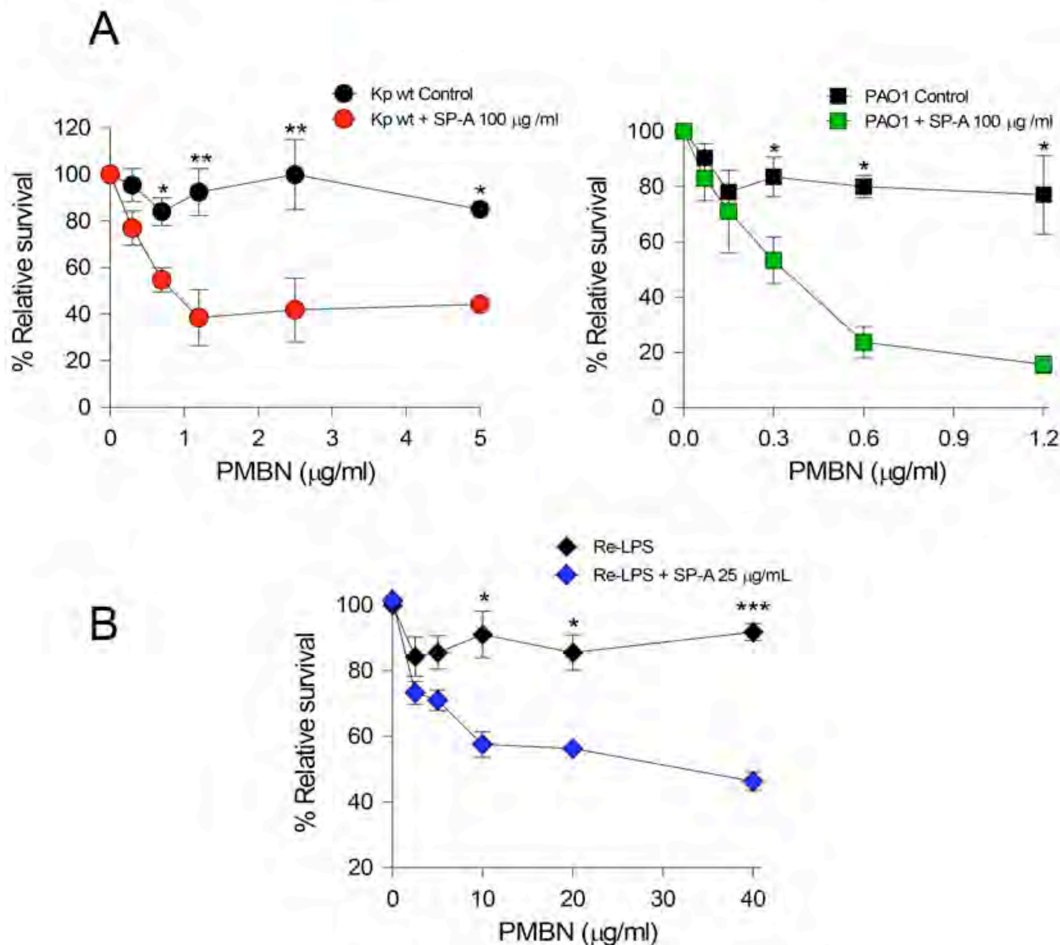

Figure 3. SP-A and PMBN are able to kill synergistically Gram-negative bacteria expressing S LPS or Re LPS. A) 10^5 CFUs/ml of *K. pneumoniae* K2 (left graph) or *P. aeruginosa* PAO1 (right graph) were incubated with increasing concentrations of PMBN in the absence (black symbol) or presence (colored symbol) of 100 µg/mL of SP-A in 10 mM PBS (pH 7.2), 1% TSB and 100 mM NaCl for 1h at 37ºC and immediately plated on LB agar for CFUs count after 18h of incubation at 37ºC. **B)** 10^5 CFUs/ml of *K. pneumoniae* K2 expressing Re-LPS were mixed with several concentrations of PMBN in the absence (black symbol) or presence (blue symbol) of 25 µg/ml SP-A in the same conditions above mentioned. Results are shown as % relative survival (percentage of live colony counts compared to untreated control). Bars represent mean ± SD (n=4, each one in duplicate), *p < 0.05, **p < 0.01 ***p <0.001 vs control.

4.2. PMBN facilitates binding of SP-A to bacteria through a protein-protein interaction mechanism

To gain insight into the mechanism by which SP-A and PMBN were able to act synergistically against *K. pneumoniae*, whereas neither of these proteins had bactericidal

activity individually, we tested the ability of these proteins to bind to bacteria surface. It has been described that PBMN lacks antimicrobial activity, but retains its ability to bind to LPS (247). In this work, we have performed solid-phase binding assays to examine the ability of SP-A to bind *K. pneumoniae* wild type in the presence of PMBN. Bacteria were incubated 30 min. with increasing concentrations of biotinylated SP-A (0 to 1.25 µg/mL) in the absence and presence of PMBN (1 µg/mL) or HSA (1 µg/mL) and then bacteria-associated SP-A was measured. As we previously reported (chapter 1), SP-A alone did not bind *K. pneumoniae* wt, even in the presence of HSA. However, SP-A was able to bind *Klebsiella* surface in the presence of PMBN in a dose-dependent manner (Fig. 4).

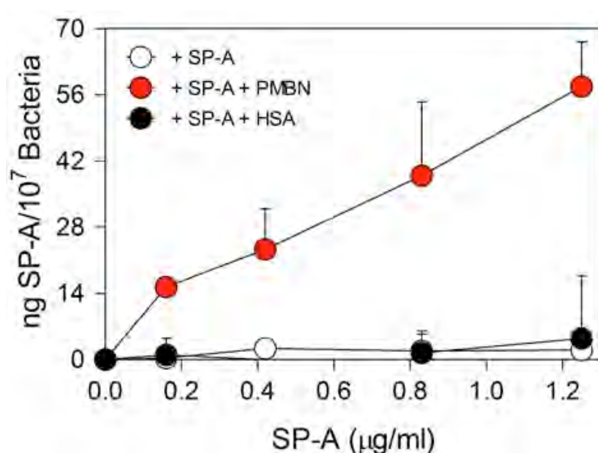

Figure 4. SP-A interacts with *K. pneumoniae* K2 only in the presence of PMBN. Increasing concentration of SP-A were incubated with 10^7 CFU of Kp wt in the absence (white symbol) or presence of PMBN 5 µg/ml (red symbol) or HSA 5 µg/ml (black symbol) at room temperature for 30 minutes. Total *Klebsiella*-associated SP-A was measured by solid-phase binding and expressed as total nanograms of SP-A/10^7 bacteria. Error bars represent standard deviations from the mean of three experiments, each one in duplicate.

To try to understand how PMBN facilitates the binding of SP-A to bacteria, we evaluated potential interaction between both proteins in solution by monitoring changes in the intrinsic fluorescence or in the hydrodynamic diameter of SP-A.

Emission spectra for SP-A were recorded from 300 to 400 nm after excitation at 295 nm. It was characterized by a maximum at 338 nm (Fig. 5A), due to the contribution of two conserved tryptophan residues located at positions 191 and 213 of the globular COOH-terminal domain (135). Addition of increasing concentrations of PMBN (0 to 200 µM) resulted in a concentration-dependent decrease in fluorescence intensity (Fig. 5A and 5B) of SP-A, indicating that there is an interaction between these proteins. Following the changes in fluorescence intensity as a function of PMBN concentration we could obtain a dissociation

constant for the SP-A/PMBN complex of K_d =0.26 ± 0.02 μM. The dissociation constant obtained is similar to that obtained for the SP-A/PMB complex (chapter 1), indicating that the binding between the SP-A and PMB does not take place through the fatty acid tail. In addition, the Hill coefficient value was greater than 1, indicating a positive cooperative binding.

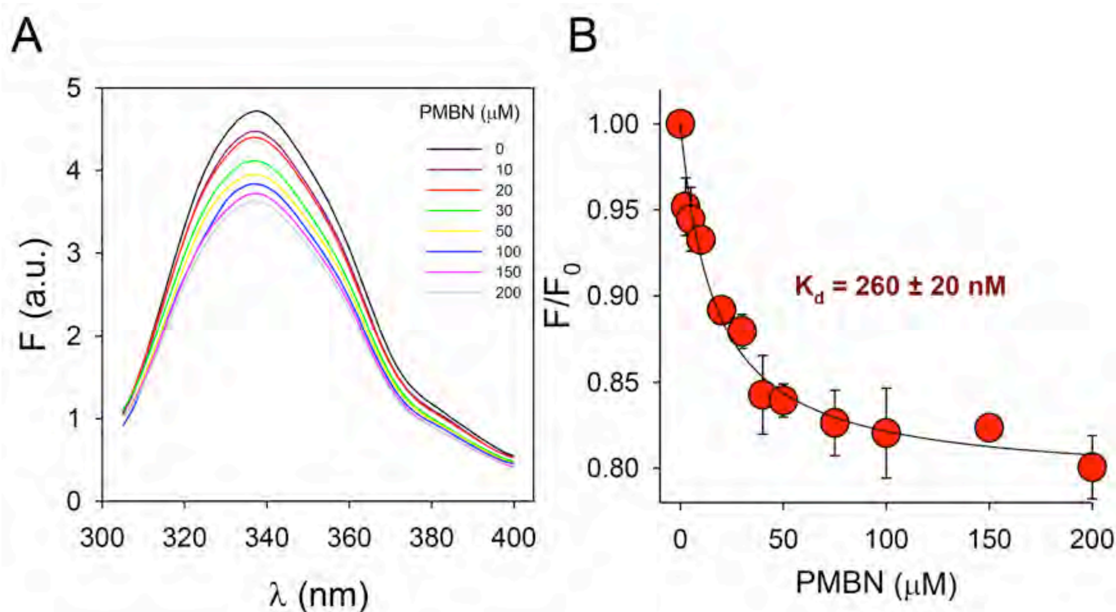

Figure 5. SP-A interacts with polymyxin B nonapeptide. A) Representative graph of the fluorescence emission spectra of SP-A (15 nM, black line) measured with or without increasing concentrations of PMBN (0 to 200 μM, colored lines) at 25 °C in 5 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl. **B)** Results are also expressed as F/F_0 in each PMBN concentration at maximum emission of SP-A, where F is the observed intensity of SP-A and F_0 is the observed intensity of SP-A+PMB. Results are means ± SD of 4 experiments.

Interaction of SP-A and PMBN in solution was also determined by dynamic light scattering. These experiments were carried out with the same buffer of *in vitro* killing assay, even though self-aggregation of SP-A occurs in the presence of calcium and/or NaCl (136). SP-A alone showed two peaks, the major one corresponds to SP-A aggregates with a hydrodynamic diameter of 421.25 ± 37.54 nm (Fig. 6A) and another corresponding to higher SP-A aggregates with a hydrodynamic diameter of >1000 nm (data not shown). In contrast, PMBN showed a unique peak of 441.26 ± 57.63 nm (Fig. 6A).

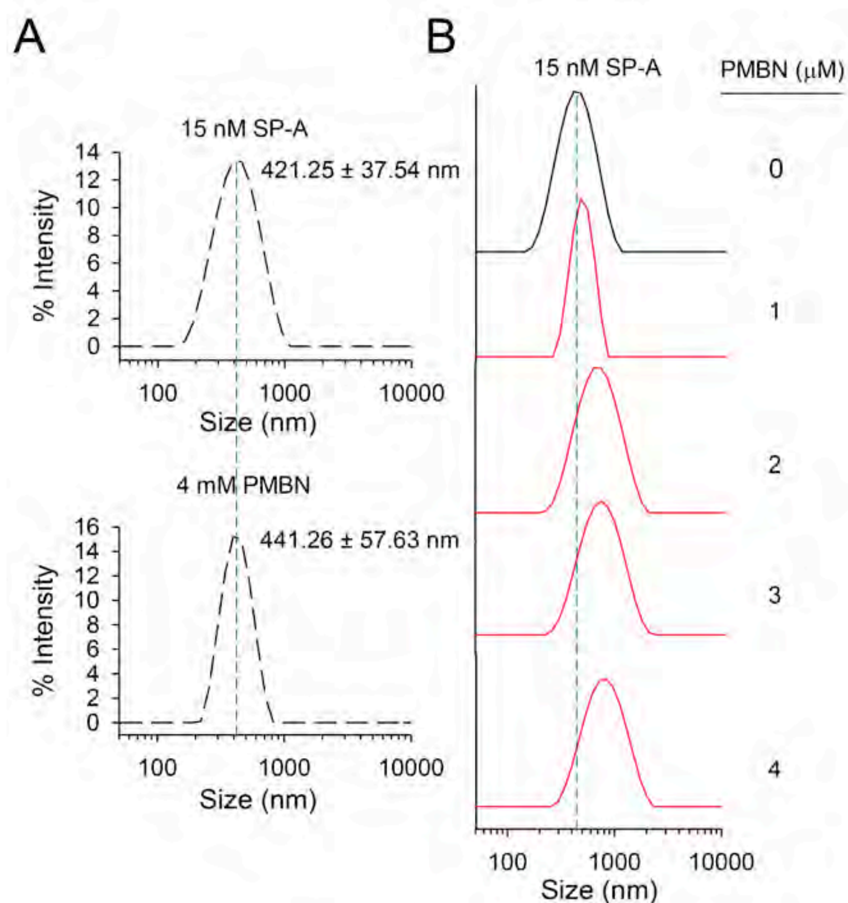

Figure 6. SP-A interacts with polymyxin B nonapeptide. Binding of SP-A and PMBN was also examined by DLS in 5 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl. **A)** DSL analysis of the hydrodynamic diameter of SP-A (15 nM) and PMBN (4 μM). **B)** DSL analysis of the hydrodynamic diameter of SP-A (15 nM) in the absence (black line) or presence (colored lines) of increasing concentration of PMBN (0-4μM). The y axis represents the relative intensity of the scattered light; the x axis denotes the hydrodynamic diameter of the particles present in the solution.

It is known that PMB and PMBN have a different structure in solution that coupled with bacterial membrane. The solution structure of LPS-bound PMB was elucidated as an envelope-like fold of the peptide ring and a β-turn type II for the free peptide (238, 402). This structure of the free peptide could favor the formation of aggregates in solution, which would explain the size we get from DLS measurements. Figure 6B shows that the addition of increasing concentrations of PMBN (ranging from 0 to 4 μM) to a SP-A solution (15 nM) caused a PMBN concentration-dependent increase of the SP-A peak. This new peak, could exhibit a hydrodynamic diameter higher than that observed for SP-A or PMBN alone, depending on the PMBN concentration. These results are consistent with a cooperative

binding. Its important to notice that in the presence of SP-A, PMBN aggregates become dissociated and they hide their hydrophobic residues through its binding to the SP-A. Consequenlty, changes observed in SP-A size in the DLS would be due to the formation of different SP-A/PMBN complexes.

Together, these data suggest that PMBN permits binding of SP-A to bacteria, probably through a protein-protein interaction mechanism, similarly to what happened with polymyxin B (chapter 1).

4.3. SP-A increase PMBN insertion into Re LPS monolayers

Studies involving lipid monolayers at the air/water interface, which mimic biological membranes, have been widely used for the study of protein-membrane interactions (403). The penetration of proteins into monolayers of Re-LPS gives information that can be significant to understand the mechanism of action of these proteins on the OM of Gram-negative bacteria. We tested the ability of SP-A and PMBN to insert into Re-LPS films by measuring the increase in the lipid monolayer surface pressure at a given initial pressure. An increase in pressure (π) is indicative that protein is able to insert into the monolayer, resulting in a more tightly packed film. For these experiments, SP-A, PMBN or both were injected into the aqueous sub-phase of Re-LPS monolayers at several initial surface pressures (π_i). Pressure increase was measured until equilibrium adsorption pressure (π_e) (steady-state pressure after the addition of the proteins) is reached. By plotting the surface pressure increase induced by the insertion as a function of initial surface pressure ($\Delta\pi = \pi_e - \pi_i$), the maximum insertion pressure (MIP) for each protein can be obtained by extrapolation of the regression line to a surface pressure increase equal to zero. This pressure indicates the maximum π_i above which no more protein molecules are able to penetrate into a membrane and increase surface pressure.

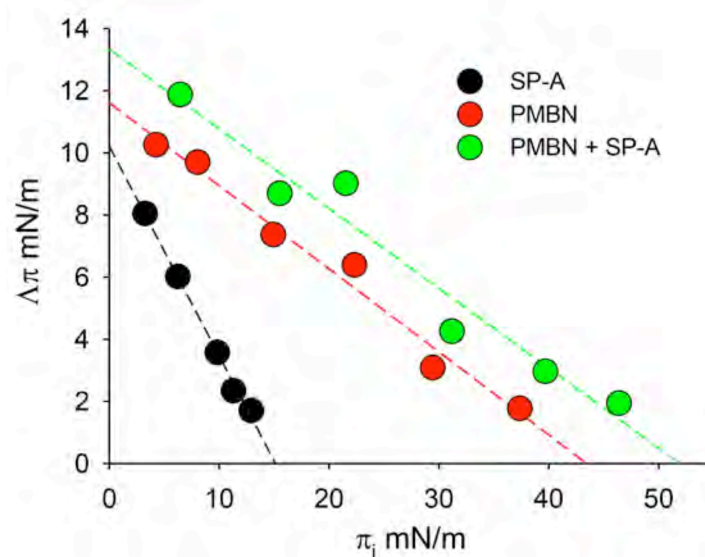

Figure 7. SP-A increases PMBN insertion into Re LPS monolayers. Re LPS monolayers were formed by spreading 10 µL of a concentrated solution of Re LPS (1 mg/mL) at the air-water interface of the subphase buffer (5 mM Tris buffer, pH 7.4, 150 mM NaCl and 0.175 mM CaCl2) to give the indicated initial surface pressures (0 to 50 mN/m). PMBN (1 µg/mL), SP-A (1.25 µg/mL), or the mixture PMBN+SP-A was injected into the subphase and protein-induced changes in the monolayer surface pressure at constant surface area were measured using a thermostated Langmuir-Blodgett trough. Straight line was obtained by linear regression.

Considering that the estimated lateral pressure for a biological membrane is between 30-35 mN/m (404, 405), we can see in figure 7 that SP-A was not able to be inserted into a bacterial membrane where it reaches a MIP about 15 mN/m. Clausell et al. (239) described that PMB and different polymyxin B analogues are able to bind LPS, thus inserting between the molecules and expanding the lipid film. These experiments were performed at an initial pressure of 20 mN/m, which is lower than the estimated lateral pressure for biological membranes. We have extended these measurements for the PMBN, determining that it had a MIP value of 42 mN/m, indicating that PMBN would be able to insert into the bacterial outer membrane. When both proteins are together, the reaching MIP value is greater (51mN/m) indicating that SP-A is able to increase PMBN insertion into bacterial model membranes

4.4. PMBN and SP-A fluidize bacterial model membranes

DPH fluorescence

Given that PMBN inserts into bacterial model membranes, we investigated whether PMBN influences membrane fluidity using the highly hydrophobic fluorescent dye DPH. This probe is very sensitive to polarity and fluidity of the membrane. Its fluorescence decreases rapidly in the presence of water, indicating changes in membrane properties. Decrease in DPH steady-state anisotropy can be due by two reasons: i) a reduction of the lipid molecular order surrounding DPH and a consequent hastening in DPH rotational diffusion, which indicate fluidization; or ii) changes in DPH fluorescence lifetime and hence, changes in DPH steady-state fluorescence intensity, indicating membrane perturbation and water entry (406). Figure 8A shows that addition of increasing concentrations of PMBN to Re-LPS/BPL (8/2, w/w) unilamellar vesicles resulted in a significant decrease in the steady-state emission anisotropy of DPH incorporated in bacterial model membranes.

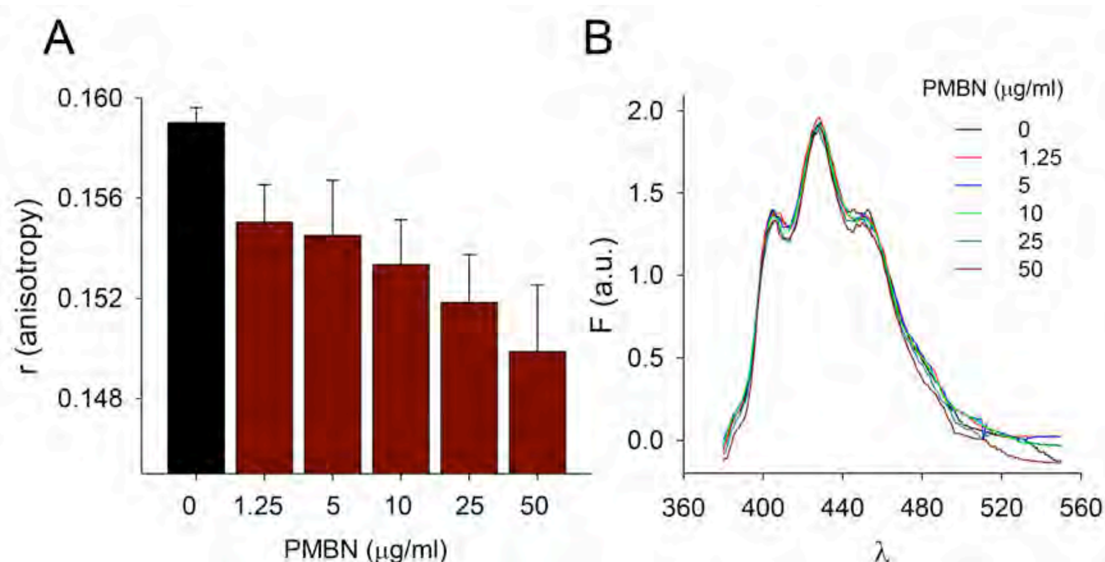

Figure 8. PMBN and SP-A increase membrane fluidity. A) Effect of increasing PMBN concentrations on steady-state anisotropy of DPH embedded in Re-LPS/BPL vesicles (1 mg/mL). Values are expressed as means ± SD of 3 experiments. **B)** Representative graph of the effect of different amounts of PMBN on the fluorescence emission spectra of DPH in Re-LPS/BPL (1 mg/mL) vesicles upon excitation at 340 nm.

To find out the reason by which PMBN decreases DPH steady-state anisotropy in Re-LPS/BPL (8/2, w/w) vesicles, we determined the effect of different amounts of PMBN on the fluorescence emission spectra of DPH in Re LPS/BPL (8/2, w/w) vesicles upon excitation at 340 nm. The lack of changes in the fluorescence emission of DPH with increasing amounts of PMBN (Fig. 8B), allows us to infer that PMBN decreases the lipid order of these bacterial model membranes.

LAURDAN fluorescence

To further examine whether PMBN affects membrane properties near the bilayer surface, we used the amphiphilic fluorescent dye LAURDAN. This probe is localized near the polar head groups of lipids and displays high sensitivity to the polarity of its environment, this is, its fluorescence emission intensity and maximum emission wavelength depends on the number and/or mobility of water molecules present at the membrane interface (341, 407). When the local environment of LAURDAN is a membrane, its emission depends strongly on the packing of the lipid chains. At temperatures below the phase transition (gel state), the emission maximum is near 440 nm. At temperatures above the phase transition (liquid crystalline state), the emission maximum is red-shifted to 490 nm (341).

The steady-state fluorescence parameter known as the LAURDAN generalized polarization (GP) quantitatively relates LAURDAN spectral changes by taking into account the relatively fluorescence intensities of the blue and red edge regions of the emission and excitation spectra, respectively (341).

Figure 9A shows that the emission spectrum of LAURDAN in Re LPS/PL vesicles (8/2, w/w) in the absence of PMBN showed a maximum centered at 440 nm, denoting that these membranes contain a high percentage of ordered phase. Addition of increasing PMBN concentrations caused a decrease in the fluorescence emission intensity at 440 nm and a red shift of the spectra. Figure 9B shows that the LAURDAN GP values decreased with increasing PMBN concentrations. This clearly indicates that PMBN is fluidizing these bacterial model membranes.

Additionally, pre-incubation of 50 μg/ml of PMBN with different SP-A concentrations before addition to Re LPS/PL vesicles (8/2, w/w) resulted in a higher decrease of LAURDAN GP values with respect to those obtained with PMBN alone (Fig. 9C). This indicates that the

fluidizing activity of PMBN is enhanced in the presence of SP-A, which is consistent with DPH data.

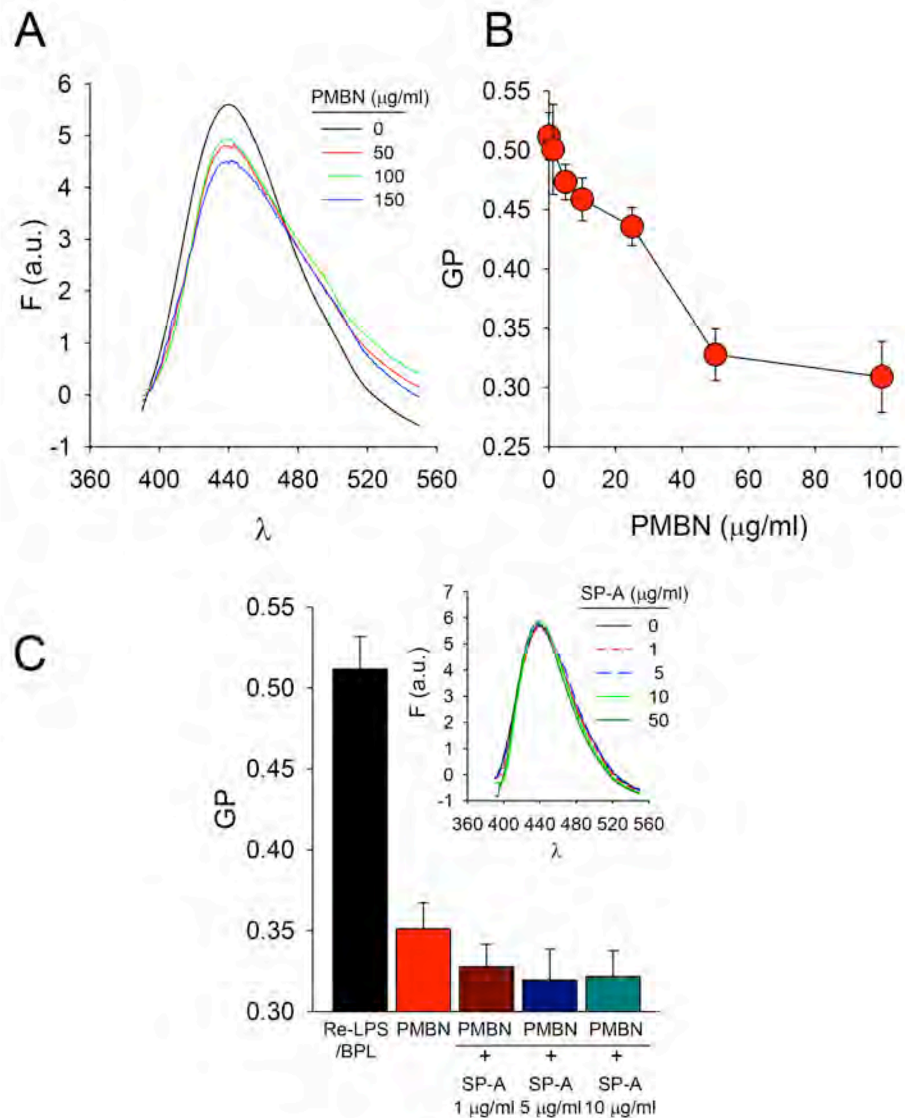

Figure 9. LAURDAN fluorescence to ascertain PMBN- and SP-A-fluidizing effects on Re LPS/PLliposomes. A) Representative graph of the effect of increasing PMBN concentration on the fluorescence emission spectrum of LAURDAN embedded in Re LPS/PL vesicles at 25 ºC. B) Effect of increasing PMBN concentrations on the GP values of LAURDAN. C) GP values of LAURDAN embedded in Re LPS/PL vesicles after addition of PMBN (50 μg/mL) in the absence or presence of increasing concentrations of SP-A (1, 5 and 10 μg/mL). Inset graph shows the representative graph of the effect of increasing SP-A concentrations on the fluorescence emission spectrum of LAURDAN.

DSC measurements

For a more detailed analysis of the thermotropic properties, DSC experiments were performed with Re LPS/PL (8/2, w/w) in the absence and presence of PMBN at different concentrations. The thermal transition of Re LPS/PL (8/2, w/w) was characterized by gel to fluid main transition temperature (T_m) of 22.54 ± 0.43 ºC and a transition enthalpy of 2.81 ± 0.98 kcal/mol (Fig. 10A). Addition of increasing amount of PMBN (1.25 µg/mL to 96 µg/mL) caused a decrease in the main transition temperature of these membranes until it is completely abolished at 96 µg/mL (Fig. 10A and Table 1). This is probably indicating a destabilization of the gel phase. Additionally, at a concentration of 1.25 µg/mL of PMBN, a shoulder in the endotherm was observed. Brandenburg et al. have observed something similar with Re LPS in contact with polymyxin B, and have suggested a demixing of the phase due the formation of endotoxin-peptide clusters (408).

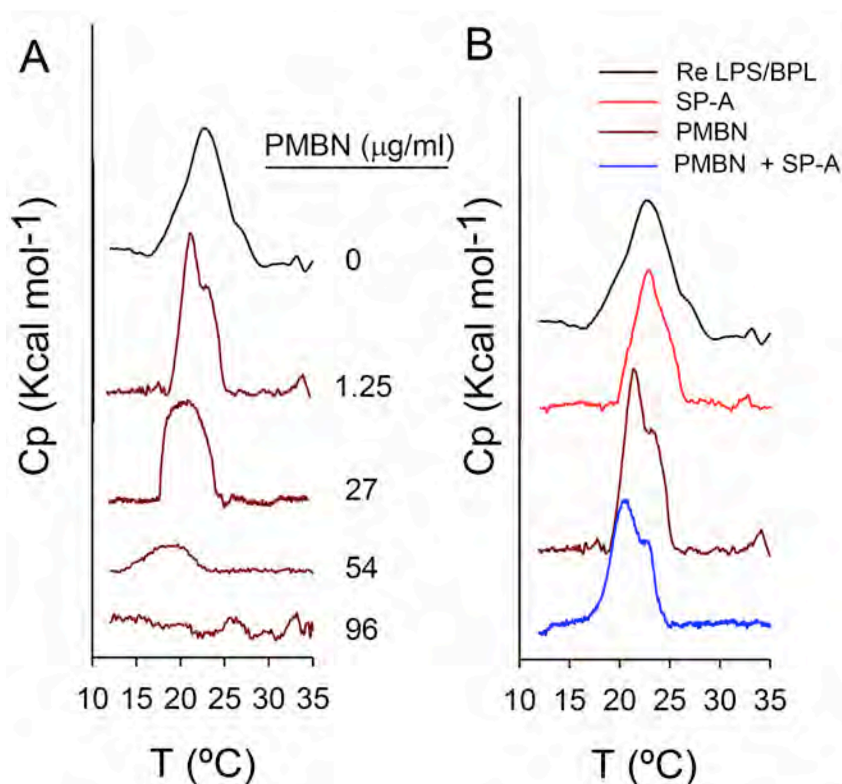

Figure 10. PMBN decreases the main transition temperature of Re LPS/PL multilamellar vesicles. A) Effect of PMBN on DSC heating scans of Re LPS/BPL liposomes (8/2, w/w). B) Multilamellar vesicles were also studied in the presence of 1 µg/mL SP-A alone (red line),1.25 µg/mL PMBN (dark red line) or both (blue line). Calorimetric scans were performed at a rate of 0.5°C/min. Three consecutive scans were recorded for each sample. One representative experiment of 3 is shown.

In contrast, when SP-A was added to Re-LPS/BPL (8/2, w/w), no SP-A-induced effects on the T_m were observed (Fig. 10B). This might be because at this concentration of SP-A (1 µg/ml) there are electrostatic repulsions between negative charges in the SP-A surface and the negatively charged Re LPS molecules, which would prevent the interaction of the protein with Re LPS (30). Finally, in figure 10C we can see that the effect of PMBN over Re LPS/PL (8/2, w/w) membranes was higher in the presence of SP-A. This thermal phase transition was also characterized by a complex double-peak due to electrostatic interactions between the positively charged PMBN and the anionic LPS, but it is more shifted to lower temperatures, indicating that PMBN/SP-A fluidized bacterial model membranes more than PMBN alone.

Table 1: Thermodynamic data of MLV (0.5mM) composed of Re LPS/PL (8/2, w/w) in the absence or presence of increasing concentrations of PMBN (1.25, 27, 54 and 96 µg/mL).

	ΔH (Kcal/mol °C)	T_m	$T_{1/2}$
Re-LPS/PL (8/2,w/w)	2.8 ± 1.0	22.5 ± 0,4	3.8 ± 0,5
+ 1.25 µg/ml PMBN	3.6 ± 0.4	21.3 ± 0.1	3.8 ± 0.4
+ 27 µg/ml PMBN	3.9 ± 0.8	20.5 ± 1,4	6.3 ± 1.6
+ 54 µg/ml PMBN	1.2 ± 0.2	18.3 ± 0,7	6.3 ± 0.9
+ 96 µg/ml PMBN	No phase transition observed		

Data are means ± SD of 3 experiments.

4.5. Ability of SP-A and PMBN to destabilize and permeabilize inner and outer bacterial model membranes

DPH fluorescence

As mentioned before, DPH fluorescence is very sensitive to the polarity of the membrane environment. This probe has a low quantum yield and a very short lifetime when exposed to water. Thus, its fluorescence is sensitive to the amount of water that penetrates into the lipid bilayer. Thus, we found that addition of increasing concentrations of SP-A causes a decrease in the fluorescence intensity of DPH membranes inserted in Re-LPS/BPL (8/2, w/w) (Fig. 11A), due to water penetration in the Re-LPS bilayer as a consequence of SP-A-induced Re

LPS membrane destabilization (30). As seen in Figure 9B, this destabilization of bacterial model membrane is greater in the presence of PMBN, which alone does not decrease DPH fluorescence (Fig. 11B).

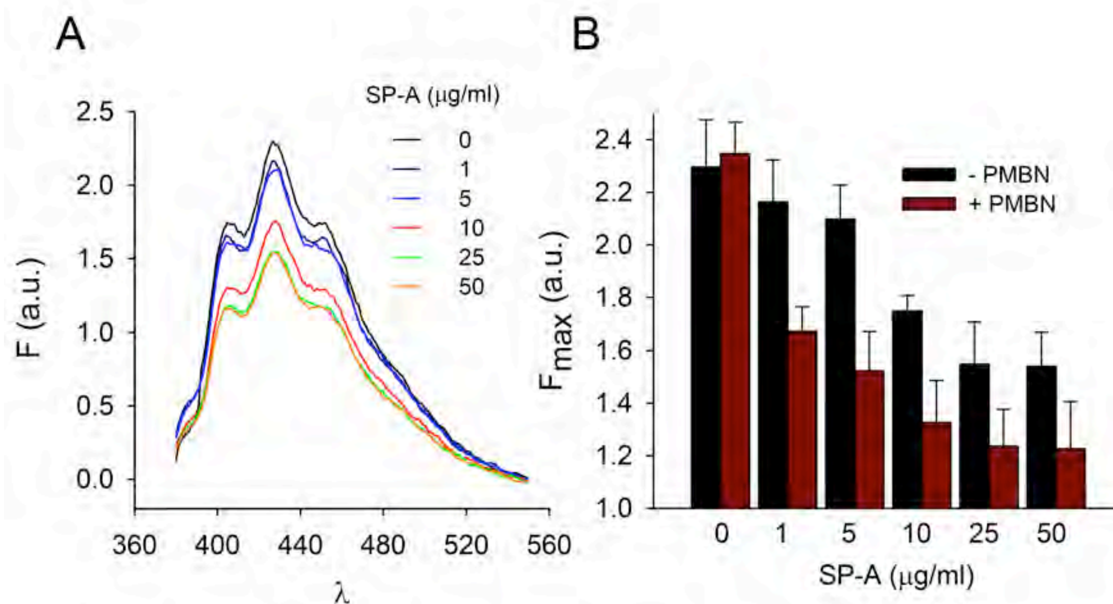

Fig. 11. A) Representative graph of the effect of different amounts of SP-A on the fluorescence emission spectra of DPH in Re-LPS/BPL (1 mg/mL) vesicles upon excitation at 340 nm. **B)** Effect of increasing concentrations of SP-A on fluorescence intensity of DPH embedded in Re-LPS/BPL vesicles (1 mg/mL) in the absence and presence of PMBN (50 μg/mL). Values are expressed as means ± SD of 3 experiments.

Leakage of ANTS/DPX probes

We have seen how the two proteins are capable of insert into bacterial model membranes and destabilize them when they are together. The next step is to see if the destabilization arising in the membrane is sufficient to produce bacterial permeabilization and death.

Experiments were performed to characterize the direct permeabilizing effects of SP-A and/or PMBN on liposomal membranes composed of bacterial phospholipids (BPL) in the presence and absence of Re-LPS. Unilamellar vesicles were loaded with a set of self-quenching probes (ANTS and DPX), which fluorescence as they diffuse apart. Experiments were performed with vesicles composed of BPL (PE/PG/CL) or Re-LPS/PL (8/2, w/w) as

models of inner and outer bacterial membrane, respectively. Triton-X100 was used as positive control of membrane permeabilization.

We have seen that SP-A was able to destabilize bacterial model membranes, probably because favored the penetration of water molecules in Re-LPS membranes, disturbing Re-LPS acyl chain packing by taking up Re-LPS molecules from the membrane (30). Although, this membrane perturbation is insufficient to cause membrane permeabilization (neither inner nor outer membrane), as we have seen by leakage experiments (Fig. 12 and 13).

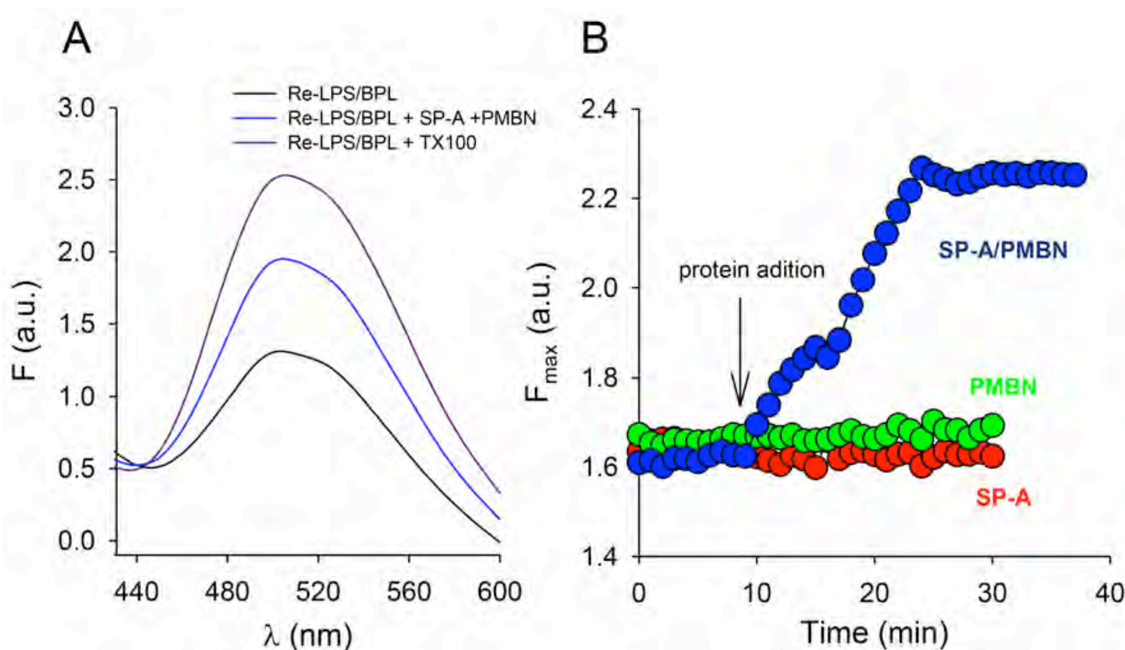

Figure 11. SP-A and PMBN synergistically induce membrane permeability of liposome mimicking bacterial OM. A) Representative graph of the fluorescence emission spectrum of ANTS after the exposure of Re-LPS/BPL vesicles (mimicking the OM of bacteria) to SP-A (25 µg/mL)+ PMBN (50 µg/mL) or TX100 (positive control). Experiments were performed in the presence of 0.150 mM $CaCl_2$. B) Changes in ANTS fluorescence during time. The arrow marks the moment when SP-A, PMBN or SP-A/PMBN were added.

The fact that the SP-A was unable to permeabilize the inner membrane coincides with the data obtained by Kuzmenko et al (17) where they found that incubation of 100 µg/ml of SP-A with liposomes composed of POPE alone resulted in no changes from the background level of fluorescence. However, Kuzmenko and coworkers found SP-A was able to permeabilize

bacterial model membranes containing Re LPS in a calcium dependent way (17). Probably, the differences observed between our results were due to the calcium concentration that we have used in our experiments was very low (0.150 mM), in order to not interfere in the activity of PMBN, which is proposed to compete with LPS-bound calcium ions, to bind to and destabilize LPS adjacent molecules (396).

Furthermore, while PMBN (50 μg/ml) was unable to permeabilize the bacterial outer membrane composed of Re-LPS/BPL (8/2, w/w) (Fig. 11), it was able to permeabilize vesicles mimicking inner membrane of Gram-negative bacteria (non-containing LPS), indicating that LPS confers protection to PMBN activity. According to equation 5, we could estimate that incubation with PMBN resulted in a 44.6 % of leakage, relative to triton-X100 (100%) (Fig. 12).

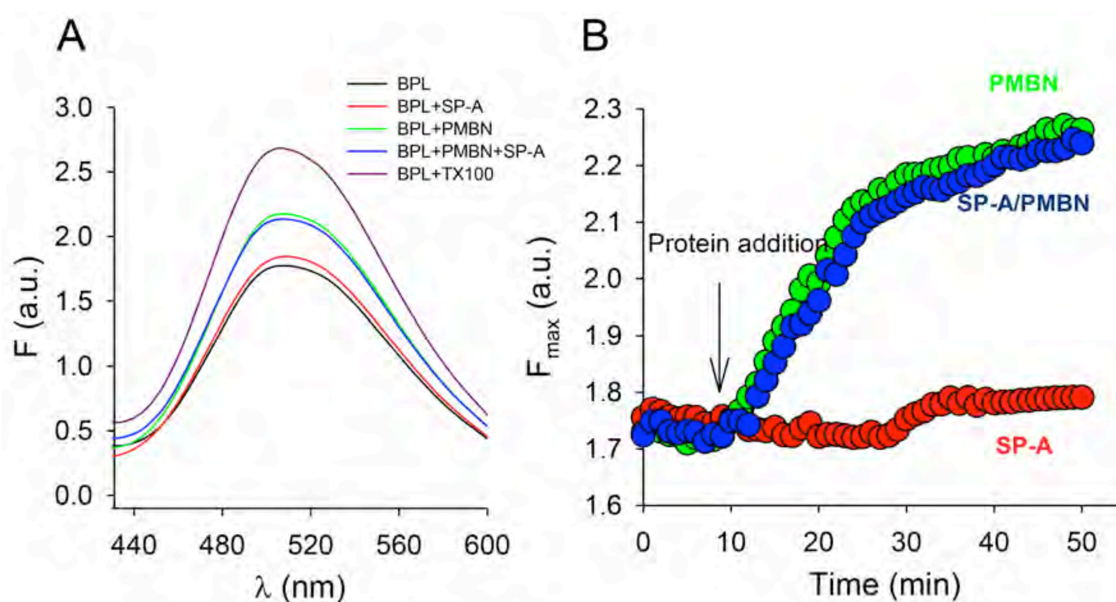

Figure 12. PMBN increase membrane permeability of liposome mimicking bacterial inner membrane. A) Representative graph of the fluorescence emission spectrum of ANTS after the exposure of BPL vesicles (mimicking the inner membrane of bacteria) to SP-A (25 μg/mL)+ PMBN (50 μg/mL) or TX100 (positive control). Experiments were performed in the presence of 0.150 mM $CaCl_2$. **B)** Changes in ANTS fluorescence during time. The arrow mark the moment where SP-A, PMBN or SP-A/PMBN were added.

As we see in figure 11 the combined action of the two proteins (SP-A and PMBN) is required to produce bacterial outer membrane permeabilization (Re LPS/PL [8.2, w/w]), obtaining a 51.5 % of leakage. Furthermore, the combined action of both proteins did not improve the PMBN permeabilizing capacity on bacterial inner membrane (Fig. 12).

5. DISCUSSION

The global emergence of multidrug-resistant bacteria is an important clinical problem that is increasingly limiting the use of currently known antibiotics. This is related to the mechanism of action of traditional antibiotics, with five main specific targets: cell-wall synthesis, DNA gyrase, metabolic enzymes, RNA polymerase and protein synthesis (409). Bacteria can develop genetic resistance toward these mechanisms of action. The actual mechanism of action for PMB and its derivate is not yet fully understood but has been proposed to involved the formation of molecular contacts between the inner and outer membranes of the bacteria and the induction of lipid exchange, thus resulting in loss of the compositional specificity of the membranes and osmotic instability (410, 411). It is extremely difficult for the bacteria to generate genetic resistance to such a physical mechanism of action based on ionic and hydrophobic interactions. In fact, resistance to polymyxins is rare (412, 413), a reason that explains the renewed interest in its clinical use (26, 395, 414). Although PMB is bactericidal to MDR Gram-negative bacteria and able to neutralize the toxic effects of released LPS, its therapeutic applications are very limited because of its relative high toxicity (415, 416). Because most of the toxic activity of PMB resides at the N-terminal fatty amino acid 6-methylheptanoic/octanoic-Dab, the removal of this segment by proteolytic cleavage, generated a nontoxic peptide named polymyxin B nonapeptide (399).

PMBN is extremely poor antimicrobial compound, but it is still capable, like PMB, of binding to LPS (400). The binding of PMBN to LPS is therefore independent of the lipophilic acid tail of PMB and may be mediated by two processes: electrostatic interactions between the positives charges of the PMBN and the negative charges of the phosphates of LPS, and hydrophobic contacts of the D-Phe-Leu with the hydrophobic core of the lipid A moiety of LPS (238, 239, 402).

Once PMBN is bound to its target, LPS, is able to disturb the bacterial membrane. It is commonly referred that the PMBN has “sensitizing activity”, thus rendering Gram-negative

bacteria susceptible to various hydrophobic antibiotics (246, 250, 417) against which the intact outer membrane is an effective barrier. A clear example of this was described by Ofek and coworkers who showed that PMBN was able to protect mice challenged with *Klebsiella pneumoniae* in combination with erythromycin (253). This ability for disturbing bacterial outer membrane points at a novel therapeutic direction.

In this work we have studied the mechanism by which PMBN alters the bacterial outer membrane allowing it to act synergistically with other antimicrobial agents like SP-A. Although neither SP-A nor PMBN possess bactericidal activity against *Pseudomonas aeruginosa* or *Klebsiella pneumoniae,* in this chapter we show that they are able to act synergistically to produce bacterial death (Fig. 2) and we move into its mechanism of action.

PMBN binds to LPS and is capable of insert at least partially into the bacterial outer membrane (Fig. 7). However, these processes are insufficient to permeabilize bacterial model membranes (Fig. 11), but might be sufficient to sensitize them. PMBN interaction with the membrane would give rise to the formation of clusters of PMBN/LPS as seen by calorimetry (Fig. 10). This would lead to destabilization of the gel phase becoming the bacterial membranes more fluid, which corroborates the data obtained by fluorescence anisotropy of DPH and GP of laurdan (Fig. 8A and 9A and B). It must take into account that every biological membrane has a certain fundamental biophysical characteristics for the performance of its functions (11, 144). Thus, changes in the lateral structure or fluidity of the membrane may be sufficient to sensitize it and turn it weaker to other antimicrobial agents.

Besides being able to sensitize membranes against other agents, PMBN has another very important role, facilitating the binding of SP-A to bacteria. As we have seen in chapter 1 of this thesis, SP-A was unable to bind to *Klebsiella pneumanie* wild type. However, SP-A is capable of binding to bacteria in the presence of PMBN (Fig. 4), possibly through protein-protein interaction mechanism (Fig. 5 and 6). Binding of SP-A to PMBN appears to be the first important step of their synergistic activity. Interaction of collectins, like SP-A and SP-D, with a variety of host defense molecules has been reported (13). SP-A is able to bind to beta-defensins and polymyxins (chapter 1;(367)) while SP-D, has been reported to interact with alpha-defensins and LL-31 (361, 382). Now we have described that SP-A binds to PMBN, with a K_d which is very similar to that obtained for the SP-A/PMB complex (Chapter 1), showing that the fatty acid tail of PMB is not involved in its binding to the SP-A. SP-A-bound

PMBN would retain its ability to bind LPS, thus allowing SP-A access and binding to inner targets in OM, as lipid A, the main target of SP-A in Gram negative bacteria (192). Thus, SP-A could overcome one of the most important factors involved in resistance to SP-A, Smooth LPS (16, 418) (chapter 1), in order to add in outer membrane permeabilization.

Once SP-A comes in contact with the bacterial membrane, it has been reported to favor the penetration of water molecules in Re LPS membranes and to cause a sharp decrease of the overall transition enthalpy of these membranes (30). As we have seen, SP-A is unable to insert into Re-LPS membranes, probably because it is a large oligomeric protein (Fig. 7). Thereby, the observed changes must be due to protein-induced perturbation of the lipid packing, as we have demonstrated by the decrease in the fluorescence intensity of DPH induced by SP-A on bacterial outer model membranes. SP-A has been shown to extract LPS molecules of the membrane and to form supramolecular lattice-like protein-LPS aggregates in the bacterial membrane surface (30) that could explain the changes observed in bacterial membranes, and might be the initial step of its outer membrane disturbance, which was higher in the presence of PMBN. Despite this, SP-A alone was also unable to permeabilize bacterial model membranes (Fig. 11). However, SP-A was able to act in collaboration with PMBN, enhancing even more the instability of the membrane caused by this peptide. SP-A increased peptide insertion into the membrane, probably favored by the extraction of LPS (Fig. 7) and the increase in fluidity caused by PMBN (Fig. 8-10), thus leading to the permeabilization of the bacterial OM (Fig. 11).

Once the OM has been permeabilized, PMBN could translocate into the periplasmic space and from there, as seen above, permeabilizes the inner membrane (Fig. 12). Finally, PMBN would promote cell death by disruption of this membrane or/and translocation into the cytoplasm and interaction with intracellular targets. In addition, given that microbial cell membranes are responsible for multiple and essential functions (191), it is also likely that SP-A add in killing bacteria, not only by increasing PMBN access through OM, but also by collaborating in OM destabilization, resulting in irreversible disruption in these vital functions.

In this chapter, we have tried to approach into the mechanism by which SP-A and PMBN are capable of producing the death of Gram-negative bacteria, when separated neither of them can. We conclude that the binding between the two proteins may facilitates the interaction of

SP-A with the bacterial membrane. Once on membrane surface, both proteins increase its insertion, at least partially, leading to changes in the biophysical properties of the membranes, thus promoting membrane permeabilization and eventually bacterial death. There are still many unknowns in this process. We do not know if SP-A or PMBN cause permeabilization after the other factor weakened the bacterial OM or it is a combination of the membrane alterations produced by both proteins. Probably it would be necessary make more experiments about this. In any case, the fact that there is synergy with SP-A, causes that PMBN become relevant within the therapeutic field and could be used instead of PMB as a drug of last resort in the fight against MDR organisms such as *Klebsiella pneumoniae*.

GENERAL DISCUSSION

The purpose of this doctoral thesis was to further investigate the antimicrobial role of SP-A in airway host defense. The main finding of this thesis is that SP-A is able to protect host against infection by synergistic actions in the alveolar space, thus strengthening the antimicrobial potential of the lungs. Additionally, we elucidated some of the factors of bacterial resistance to SP-A as well as the mechanism by which SP-A act cooperatively with AMPs to kill bacteria. Therefore, our findings provide new insights to better understanding the complex role of this multifunctional protein during infection.

SP-A is a pulmonary collectin with an essential role in host defense against infection. Bacterial agglutination and increase of phagocytosis are among the main roles of SP-A in innate host defense, as seen by extent *in vitro* evidences (12). Multiple reports have been published in which SP-A is able to bind and enhance phagocytosis of Gram-negative bacteria, including: 1) *Escherichia coli* J5 and K12 (19, 329), deep rough mutant strains; 2) mucoid and flagellated strains of *P. aeruginosa* (419), but not others isolates of *P. aeruginosa* (420, 421); 3) *Klebsiella pneumoniae* strains expressing capsular serotype K21, but not K2 (147); 4) and type a but not type b *Haemophilus influenzae* (148, 420). In addition, SP-A also enhances macrophages uptake of Gram-positive bacteria, such as *Staphylococcus aureus* (149, 422), *Streptococcus pneumoniae* (151) or Group A and B *Streptococcus* (420). The potential *in vivo* role of SP-A in innate defense, supported by *in vitro* findings, was proved once SP-A gene-targeted mice were successfully generated (137, 174). These mice were then infected with several of these respiratory pathogens showing more susceptibility than wt mice to these bacteria (18).

After initial findings as opsonin, SP-A was then reported to directly kill Gram-negative bacteria *in vitro* (16) and *ex vivo* (14) by increase membrane permeabilization. SP-A showed preference by bacteria expressing rough LPS phenotype, including *E. coli* K-12 and J5, beside other rough strains of *E. coli* (16). SP-A was found to kill, aggregate and enhance phagocytosis only rough mutants of *Bordetella pertussis* and *Bordetella bronchiseptica*, unlike the wild-type strains, which was resistant to these activities (24). Comparative signature-tagged mutagenesis on *Pseudomonas* showed that mutants with reduced LPS and low outer membrane integrity were susceptible to SP-A membrane permeabilization, unlike the other ones (19). In the present thesis, we confirmed these previous studies in *Klebsiella pneumoniae*, a common Gram-negative bacterium that infects the respiratory tract of immune compromised patients, which only was susceptible to SP-A when was mutated to deep rough

LPS nonexpressing CPS. Thus, SP-A was only able to aggregate, kill and enhance phagocytosis of decapsulated *Klebsiella* mutants expressing deep rough Rc and Re LPS, indicating that truncated LPS up to inner core on *K. pneumoniae* made bacteria susceptible to SP-A.

On the other hand, reports of bacterial killing activity of SP-A on bacteria with S-LPS phenotype are sparse. SP-A exhibited antimicrobial activity against two *Enterobacter aerogenes* species and two *Klebsiella pneumoniae* clinical isolates (with unknown serotype), all of them expressing smooth LPS (16). SP-A bound to and inhibited the growth of *Legionella pneumophila*, a respiratory pathogen that express S LPS and can replicate within human alveolar macrophages (184). In our case, SP-A did not have any activity against *Klebsiella* wt, which express S LPS and CPS. Accordingly, there are several reports of bacteria expressing S LPS that have been resistant to SP-A, including the mentioned *Bordetella* species, *Pseudomonas aeruginosa* (which also express exoproteases that are able to degrade SP-A) (19, 197) and *E. coli* O111 (16, 329). On the other hand, there are no reports of SP-A killing on several bacteria in which SP-A has been shown to interact and/or to enhance phagocytosis or clearance *in vivo*, such as *K. pneumoniae*, *H. influenzae*, *S. aureus* or *Group B Streptococcus*; suggesting that opsonization by SP-A may be not always linked to its direct bacterial action.

A number of microbial surface components have been shown to be ligands for SP-A. For instance, SP-A binds to P2 outer membrane protein, found on particular *H. influenza* species (148), and flagellin, the major structural protein of the flagella of *P. aeruginosa* (421), thus enhancing phagocytosis of these pathogens. However, flagellum of *Pseudomonas* has been also reported to confer clearance resistance *in vivo* to SP-A, since mutants lacking flagella were attenuated in SP-A+/+ mice, but survived in SP-A KO mice (185). On the other hand, several studies involving LPS isolated from smooth and rough bacteria revealed that the main target ligand of SP-A in Gram-negative bacteria appears to be in the LPS membrane (13). SP-A only bound to deep rough LPS, mainly through the lipid A, which is anchored in the outer leaflet of the outer lipid bilayer (131, 159, 192). In contrast, SP-A did not associate with smooth forms of LPS. This is consistent with our studies, since SP-A only bound to deep rough *Klebsiella* mutants, in which it had antimicrobial activity. In addition, SP-A has been shown to interact only with specific capsular polysaccharide of some *K. pneumoniae* serotypes expressing manose-α2/3-manose sequence (serotype K21), unlike our bacteria of

study, which express capsular serotype K2 and lacks of this sequence (147). In fact, it is though that *Klebsiella* serotypes, which do not express this sequence, are more virulent because they are able to avoid the antimicrobial activity of lectins (like SP-A) and macrophages, since they are not recognized by these immune components (196).

Even though SP-A is unable to act against *Klebsiella pneumoniae* K2, SP-A KO mice have been shown to be more susceptible to this bacterium (176). In addition, these mice also exhibited delayed microbial clearance after intratracheal inoculation of *P. aeruginosa* (20), bacterium resistant to SP-A *in vitro* (19, 185, 197). One explanation of these disparate results is likely because SP-A have been shown to exert a multiple roles during infection, such as increase of phagocytosis or immunomodulation. Thus, deficiency on SP-A could decrease some of these functions. It is also possible that respiratory pathogens display OM heterogeneity during infection (smooth or rough LPS, with or without CPS) depending on its needs to infect the host (423-425), which could make bacteria susceptible to SP-A bactericidal activity *in vivo*. On the other hand, clinical isolate used in several studies could have molecular adaptations that increase their resistance to antimicrobial activity of SP-A, such as expression of a more complex OM or proteases. In this thesis, we approached another possibility, regarding to a potential role of SP-A to cooperate with another antimicrobial factors of the alveolar space, in order to aid in killing bacteria to overcome infection.

The microbicidal role of SP-A *in vivo,* where it shares location with another antimicrobial factors and surfactant phospholipids (to which SP-A is intimately associated), has been suggested by studies involving macrophages-free bronchoalveolar lavages from SP-A +/+ mice, which showed a greater bactericidal effect than lavages from SP-A -/- mice (14). In addition, since several bacterial strains showed resistance to SP-A *in vitro*, bacterial killing activity of SP-A *in vivo* was proposed to require the combinatorial interactions with other antimicrobial defenses in the airspace (16). In this sense, several reports have suggested the importance of cooperative interaction between antimicrobial factors of the alveolar space in order to strengthen the microbicidal activity of each factor alone. It has been found that AMPs representing each of the structural classes found in mammals demonstrated combinatorial activity against representative human pathogens (300). Lactoferrin and lysozyme acted synergistically in combination, while HBD-2 and human cathelicidin LL-37 demonstrated an additive action against *E. coli* (22). Likewise, it has also found synergistic activity in triple combinations of lysozyme, lactoferrin, and the proteinases inhibitor secreted by leukocytes

(22). In addition, SP-A and SP-D has been shown to have additive neutralizing activity with LL-37 against influenza A viruses susceptible to SP-A (382).

Therefore, in the present thesis, we found that SP-A was able to act synergistically with endogenous components of the innate system, including the cationic AMPs β-defensin 2 and 3 as well as the recently discovered anionic AMPs SP-B^N. In the latter case, SP-A and SP-B^N showed combinatorial bactericidal activity at neutral pH, whereas none of them had antimicrobial activity individually; and enhanced macrophages-mediated phagocytosis. In addition, we found that SP-A+SP-B^N treatment in wt mice was more protective against *K. pneumoniae* K2 infection than administration of proteins individually. Thus, our findings indicate that SP-A is able to enhance host innate defense against infection, even on bacteria capable of escape from SP-A antimicrobial activity.

The ability of SP-A, together with AMPs, to enhance clearance of *K. pneumoniae in vivo* seems to be also in connection with their capability to modulate host inflammatory response, since SP-A and SP-B^N were able to enhance early immune response to *Klebsiella*. SP-A and SP-B^N enhanced the expression of several pro-inflammatory cytokines, which are widely implicated in host response to infection (TNF-α, IL-1β, -6) (72), and specially, to *K. pneumoniae* one (IL-17) (383); as well as the expression of an important chemokine implicating in neutrophils recruitment, MIP-2 (384). Consequently, SP-A and SP-B^N promoted an increase in early neutrophil recruitment in bronchoalveolar lavage fluid during infection with *Klebsiella*, which was greater in combination. In contrast, instillation of SP-A and SP-B^N in uninfected mice only promoted a minimal inflammation (data not shown).

Although data supporting the role of SP-B^N in inflammation are sparse, anionic AMPs have been shown to induce cytokine and chemokine production. Dermcidin is an antimicrobial peptide secreted into sweat, which shares some features with SP-B^N, such as anionicity, antimicrobial activity at acidic pH and constitutive expression (385). Dermcidin-derived peptides have been shown to activate normal human keratinocytes by inducing pro-inflammatory and chemoattractive mediators (386). In addition, AMPs have been reported to promote chemotaxis (8), both indirectly by stimulating the expression of well-known chemokines, like CXCL8/IL8 and CCL2/MCP1(227); and directly by acting as chemokines themselves to recruit a variety of immune cells (229). On the other hand, even though the main role of SP-A in inflammation appears to be anti-inflammatory (18), it has been also

shown to increase inflammation (13). SP-A stimulates production of pro-inflammatory cytokines during the early phase of lung injury (387). SP-A enhances chemotaxis of inflammatory BAL neutrophils toward chemokines such as MIP-2 (167) or the stimulation of neutrophil chemotactic factors release (168). In addition, influx of neutrophils into bronchoalveolar lavage fluid and pro-inflammatory cytokine expression was enhanced after exogenous administration of SP-A in mechanically ventilated preterm lambs (169). The majority of studies involving the pro-inflammatory role of SP-A have been performed with nonresident alveolar macrophages, unlike studies showing its anti-inflammatory role, which might indicate that SP-A exert several roles on inflammation depending of the target cell.

The collaboration between antimicrobial factors in the alveolar space might also depend on the bacterial pathogen or toxin that is attacking the host. Evidence indicated that *Klebsiella* infections are characterized by lacking an early inflammatory response, because *K. pneumoniae* antagonizes the activation of NF-kB in order to subvert the host inflammatory response in its own benefit (388, 389). In addition, several reports have demonstrated that activation of an inflammatory response is essential to clear *Klebsiella* infections (383, 390, 391). In our case, SP-A and SP-B^N seem to counteract the anti-inflammatory response induced by this bacterium, since mice treated with both proteins showed an increase in early inflammation and neutrophils recruitment. In fact, studies involving mice infected with *K. pneumoniae* have found that the marked reduction of bacterial loads in response to the AMP α-defensin-1 is attributed to the increased accumulation of leukocytes at the site of infection (393). Therefore, our data suggest that SP-A/SP-B^N treatment might enhance endogenous protective mechanisms to aid in the clearance of *Klebsiella.* Thus, SP-A and AMPs could collaborate *in vivo* to immunomodulate host immune response during infection.

On the other hands, to the recently appearance of multi-drug resistant (MDR) Gram-negative bacteria without new effective antibiotic non-toxic (100), we also found that SP-A is able to act synergistically with the exogenous cationic AMPs polymyxins against three respiratory pathogens. Thus, SP-A enhanced the bactericidal effect of two potent, but cytotoxic, exogenous antibiotic peptides, PMB and colistin against *K. pneumoniae*, *P. aeruginosa* and non-typable *Haemophilus influenzae*. Both polymyxins have been shown to enhance OM permeability to lipophilic and amphiphilic antibiotics like rifampin, macrolide antibiotics, fusidic acid and novobiocin, and kill MDR bacteria very efficiently (26). For this reason, and due to their associated toxicity for the patient, they are currently re-used as the

last resort against infections by MDR microorganisms in clinic (396). Since this toxicity has been suggested to be dose dependent (236), the combinatorial use with SP-A would allow the use of a lower concentration of polymyxins, reducing their toxicity without decreasing their bactericidal effect. Additionally, SP-A showed the same combinatorial effect with polymyxin B nonapeptide (PMBN), a derivative from polymyxin B, which lacks of antimicrobial activity but retains the polymyxin ability to bind bacterial outer membrane (OM) (246). As PMBN by itself has a lower toxicity than PMB and colistin, the use of SP-A in conjunction with PMBN could be very effective to treat infection by MDR bacteria.

The mechanism of action by which SP-A enhances the antimicrobial activity of these AMPs is still unknown. In the present thesis, the major finding regarding this part is that SP-A and AMPs might kill bacteria synergistically because SP-A/AMPs are able to promote changes on bacterial OM structure and permeability through a protein-protein interaction mechanism. Consistent with that, we elucidated some steps of the antimicrobial synergistic activity of these proteins.

Through several experimental approached, we found that the first step of their combinatorial activity seems to be the interaction between both factors, which allow SP-A binding to bacteria. Thus, SP-A was able to bind to human beta-defensin-3 (HBD-3), SP-B^N (K_d=0.39±0.3 μM), PMB (0.32 ± 0.04 μM), and PMBN (K_d =0.26 ± 0.02 μM) in a dose-dependent manner, thus increasing its interaction with *K. pneumoniae* K2, a bacterium that is not recognized by SP-A (147). Interaction of collectins, like SP-A and SP-D, with a variety of host defense molecules has been reported (13). SP-A, unlike SP-D, bound to β-defensins (367). In addition, SP-D, but not SP-A, has been reported to interact with α-defensins and LL-31 (361, 382). They showed several effects, from additive or antagonist (361, 382) to anti-cytotoxicity ones (367), suggesting complex interactions between these factors. In our case, SP-A and AMPs binding had a synergistic effect and seems to be essential for this activity. Taken together, we found that AMPs facilitated the binding of SP-A to *Klebsiella*, probably, through a protein-protein interaction mechanism.

The antimicrobial activity of cationic AMPs (CAMPs) has been shown to required three commons and independent steps, including peptide attachment; peptide insertion and membrane permeabilization; and cell death (15). The most accepted mechanism of peptide attachment to bacterial membrane is the initial electrostatic interactions between cationic

residues of the peptides and negatively charged structures of the bacterial surface, in order to gain access to the outer lipid bilayer, in Gram-negative bacteria. Thus, CAMPs are able to promote displacement of divalent cationic ions (Ca^{2+}), which maintain CPS and LPS molecules of the OM closely packed. These displacements then promote disorganization of the bacterial LPS (191, 216). It is possible that the LPS-disorganizing and -binding ability of CAMPs allow SP-A access and binding to inner targets in OM, as lipid A, the main target of SP-A in Gram negative bacteria (192). Thus, SP-A could overcome one of the most important factors involved in resistant to SP-A, smooth LPS (16, 19, 24), in order to add in OM permeabilization. In the case of synergistic activity with $SP\text{-}B^N$, not only SP-A but also $SP\text{-}B^N$ was unable to bind to *Klebsiella* at neutral pH separately. In contrast, SP-A was able to enhance its interaction in the presence of $SP\text{-}B^N$, which only bind to *Klebsiella* and has activity at acidic pH. Several anionic AMPs have been shown to use metal ions to form cationic salt bridges with negatively charged components of microbial membranes (212). In addition, $SP\text{-}B^N$ and amaebopores have been proposed to permebealize bacterial membranes in a pH- and oligomerization-dependent manner (6, 294, 426). Thus, it is possible that both proteins could enable each other to bind to bacterial OM through conformational changes promoted by protein interactions. However, additional experiments are necessary to confirm this presupposition.

The next step of the combinatorial action between SP-A and AMPs appears to be in accordance with their ability to insert into the OM. In this thesis, monolayers of Re-LPS were used to examine the mechanism by which SP-A and PMBN kills bacteria synergistically. Model membrane systems mimicking the interfacial environment of bacterial membrane have been extensively used to investigate how proteins interact with membranes (404). Thus, we found that PMBN binds to LPS and is capable of partially insert into the bacterial outer membrane, corroborating reports about PMBN insertion ability on LPS (239). In contrast, SP-A did not insert into the monolayers, likely because it is a very large protein and it has been suggested to form supramolecular lattice-like protein-LPS aggregates in the bacterial membrane surface (30). However, the presence of SP-A significantly increased insertion of PMBN into monolayers of Re-LPS, which may indicate the initial step of their combinatorial killing activity.

Once AMPs insert into the membrane of the target bacteria, they have shown to disrupt the bacterial membrane by different mechanisms, from transmembrane pore formation to

disruption in a detergent-like manner (7, 15). Polymyxins, once they cross LPS and arise to the outer lipid bilayer, bind to lipid A and insert into the bilayer through their hydrophobic part (fatty acyl tail and Phe and Leu hydrophobic domain), thus promoting disruption of the highly packed lipid A fatty acyl chains of the OM (238). In addition, it has been also proposed that PMB promote the formation of molecular contacts between the inner and outer membranes of the bacteria and the induction of lipid exchange, thus resulting in loss of the compositional specificity of the membranes and osmotic instability (410, 411). In the case of PMBN, the hydrophobic segment together with peptide accumulation into the OM, as well as stereospecificity is probably the cause of the OM perturbation (249, 258). However, deficiency of a higher amphiphaticity and hydrophobicity (due to the lost of the fatty acyl tail) is responsible for its absence of bacterial killing (247). We found that PMBN interaction with the membrane would give rise to the formation of clusters of PMBN/LPS, as we have seen by calorimetry. This would lead to destabilization of the gel phase becoming the bacterial membranes more fluid, which corroborates the data obtained by fluorescence anisotropy of DPH and GP of laurdan. It must take into account that every biological membrane has a certain fundamental biophysical characteristics for the performance of its functions (11, 144). Thus, changes in the lateral structure or fluidity of the membrane may be sufficient to sensitize it and turn it weaker to other antimicrobial agents.

Regarding SP-A, it is reported that is able to extract LPS molecules from bacterial OM to perturb it (30), thus likely rendering membrane leaky and creating defects to permit small molecules, such as water, penetrate through the bilayer LPS. This is in accordance with the SP-A-induced perturbation of bacterial outer model membranes that we have seen by the decrease in DPH florescence intensity by SP-A. In addition, the presence of PMBN increases this SP-A-promoted destabilization, corroborating the collaboration of both proteins in membrane disturbance. Despite this individual action of SP-A, it was unable to permeabilize bacterial model membranes. However, SP-A acted in collaboration with PMBN, enhancing even more the instability of the membrane caused by this peptide. SP-A increased peptide insertion into the membrane, probably favored by the extraction of LPS and the increase in fluidity caused by PMBN, thus leading to the permeabilization of the bacterial OM. The presence of SP-A/PMBN complex increased membrane perturbation and permeabilization of bacterial outer membranes, as demonstrated by changes in DPH fluorescence and leakage of the encapsulated probes ANTS and DPX. In contrast, the presence of SP-A did not have any

influence on PMBN-induced permeability of the inner bacterial membrane composed of bacterial phospholipids (PE/PG/CL). Taken together, these results indicate that SP-A and PMBN cooperate in killing bacteria because they are jointly able to disturb and permeabilize the outer bacterial membrane.

In this thesis, through several experimental approaches (differential calorimetry scanning, mobility of membrane probes and leakage of coated probe) on liposomes containing Re-LPS/bacterial phospholipids, mimicking the bacterial OM, we proved that SP-A and PMBN were able to promote changes on outer bacterial membrane structure and permeability, which eventually might produce bacterial death. Thus, we demonstrated that SP-A/AMPs-mediated disturbance and permeabilization might be the last step of the synergistic activity of SP-A with AMPs.

Since SP-A was shown to be an essential part of the host immune defense against infection, numerous efforts have been conducted in order to better understanding its functions in host immunity and its therapeutic potential. Therefore, the results presented in this thesis provide new insights, both *in vitro* and *in vivo*, about the role of SP-A as antimicrobial protein. More importantly, we have shown a new potential role of SP-A for futures clinical applications, since it is able to enhance the bactericidal activity of two potent but cytotoxic exogenous AMPs, currently used to treat MDR infections (396). In addition, in the face of the promising role of endogenous AMPs to treat respiratory infections (53, 121), we found that SP-A could be a very interesting partner of these AMPs as adjuvant therapy to overcome infections. Finally, we elucidated the mechanism by which SP-A act in conjunction with one AMP in order to optimize future treatment of these antimicrobials factor. Thus, we proposed the next mechanism of action of the synergistic activity between SP-A and AMPs:

i) AMPs interact with SP-A permitting SP-A access to bacteria that express smooth LPS and/or capsule on its bacterial outer membrane (OM); ii) SP-A is now available to bind to LPS and extract LPS molecules to perturb OM; iii) SP-A/AMP-dependent increase of bacterial OM fluidity and destabilization increase OM permeabilization, which would enhance AMPs access to the inner membrane of Gram negative bacteria; iv) finally, AMPs promote cell death by disruption of this membrane or/and translocation into the cytoplasm and interaction with intracellular targets.

To conclude, given the alarming increase in MDR Gram-negative bacteria and the paucity of new effective antibiotics, a better understanding of the synergistic activity between naturally occurring antimicrobial factors, especially concerning physiological conditions *in vivo*, may provide new insights to the development of new efficient adjunctive therapies against infection by MDR bacteria.

CONCLUSIONES

El trabajo presentado en esta tesis doctoral revela nuevos detalles sobre el papel de la SP-A en la defensa del pulmón frente a la infección por bacterias Gram negativas. Además, proporciona datos de interés para el desarrollo de nuevas terapias alternativas o adyuvantes para el tratamiento de infecciones respiratorias por patógenos multiresistentes (MDR). Tomando todos los resultados en consideración, concluimos que:

1. La SP-A sólo presenta actividad antimicrobiana frente *Klebsiella pneumoniae* K2, cuando carece de capsula y su membrana externa presenta LPS rugoso profundo. Estos resultados destacan la importancia del LPS completo (con antígeno O) y la capsula en la resistencia bacteriana a SP-A.

2. La SP-A incrementa la capacidad bactericida de péptidos antibióticos con potencial uso clínico, tales como polimixina B (PMB) y colistina. Además, SP-A actúa de forma sinérgica sobre *Klebsiella pneumoniae* K2 con un derivado nonapeptídico de PMB (PMBN) que no tiene efecto bactericida pero mantiene su capacidad de unión membrana externa bacteriana.

3. La SP-A también es capaz de actuar sinérgicamente con péptidos antimicrobianos (AMPs) endógenos tanto catiónicos (HBD-2 y -3), como aniónicos ($SP\text{-}B^{N}$), frente a patógenos respiratorios Gram-negativos resistentes a SP-A. De este modo, la acción sinérgica entre SP-A y AMPs podría ser importante en la defensa del pulmón frente a infecciones por bacterias Gram negativas.

4. El efecto cooperativo entre la SP-A y AMPs endógenos se ha demostrado también *in vivo*. La SP-A junto con $SP\text{-}B^{N}$ fue capaz de disminuir la infección provocada por el patógeno *K. pneumoniae* K2. La acción cooperativa de $SP\text{-}A/SP\text{-}B^{N}$ fue consistente con: 1) una aumentada actividad microbicida directa de $SP\text{-}A/SP\text{-}B^{N}$; 2) un aumento de fagocitosis de *Klebsiella pneumoniae* K2 por macrófagos alveolares inducido por $SP\text{-}A/SP\text{-}B^{N}$; 3) un incremento en el reclutamiento temprano de neutrófilos en el fluido bronco-alveolar inducido por $SP\text{-}A/SP\text{-}B^{N}$; y 4) una aumentada respuesta inflamatoria temprana del huésped. Además, SP-A y $SP\text{-}B^{N}$ fueron capaces de actuar sinérgicamente *in vivo* una vez que la infección por *K. pneumoniae* K2 estaba establecida, demostrando así su utilidad terapéutica.

5. La SP-A interacciona con PMB, PMBN, HBDs-2 y -3, y $SP-B^N$ con una K_d en el rango de 0.2 a 17 μM, lo que facilita la unión de SP-A a bacterias capsuladas o con LPS liso (con las que no puede interaccionar) a través de un mecanismo de interacción proteína-proteína.

6. Utilizando membranas modelo de la membrana externa de bacterias Gram-negativas hemos estudiado el mecanismo de acción sinérgico de SP-A y PMBN. El complejo SP-A/PMBN es capaz de unirse e insertarse en la membrana externa bacteriana, pero no la SP-A, provocando cambios en las propiedades físicas de las membranas y promoviendo, de este modo, la permeabilización de la membrana y, eventualmente, la muerte de la bacteria.

La acción sinérgica entre la SP-A y AMPs demostrada en esta tesis puede ser la base para un nuevo modelo de terapia adyuvante en infecciones pulmonares. El uso de factores naturales del pulmón como agentes terapéuticos debería considerarse para aumentar la eliminación directa de patógenos invasivos, así como incrementar mecanismos de protección endógenos del huésped para combatir infecciones. Además, puesto que PMB y colistina son antibióticos de ultimo recurso frente a microorganismos multi-resistentes a antibióticos, y teniendo en cuenta su alta toxicidad para el paciente, su uso en combinación con SP-A permitiría disminuir la dosis de polimixinas, reduciendo así su toxicidad sin disminuir su efecto bactericida. En este caso, el uso de SP-A con PMBN podría resultar más efectivo, puesto que PMBN por sí mismo presenta una menor toxicidad que PMB o colistina, y nuestros datos indican una eficiente acción microbicida de PMBN en combinación con SP-A.

CONCLUSIONS

The research presented in this doctoral thesis reveals new insights about the role of SP-A in airway host defense. In addition, it provides interesting evidences concerning the development of new alternative or adjuvant treatments against respiratory infections by multi-drug resistant pathogens. Taking all data into consideration, we conclude that:

1. SP-A has antimicrobial activity against *Klebsiella pneumoniae* K2 only when the bacterium lacks the capsule (CPS) and its outer membrane (OM) expresses deep rough LPS. These results highlight the importance of smooth LPS and CPS in the bacterial resistance against SP-A.

2. SP-A strengthens the microbicidal effect of antibiotics with potential clinical use, such as polymyxin B (PMB) and colistin. In addition, SP-A acts synergistically with a nonapeptide derivative of PMB (PMBN), which is not bactericidal, but retains its ability to bind to Gram-negative bacteria.

3. SP-A is able to act synergistically with endogenous cationic AMPs (HBD-2 and -3) as well as anionic AMPs ($SP\text{-}B^N$) against Gram-negative respiratory pathogens resistant to SP-A. Therefore, synergic action of SP-A with AMPs might be important in lung defense against infection.

4. *In vivo*, SP-A was also able to collaborate with $SP\text{-}B^N$ to decrease bacterial infection by the respiratory pathogen *Klebsiella pneumoniae* K2. Cooperative action between these proteins was consistent with: 1) an enhanced SP-A/AMP microbicidal activity; 2) an indirect increase of phagocytosis by alveolar macrophages; 3) an increase in early neutrophils recruitment in the alveolar fluid from infected SP-A/AMP-treated mice; and 4) an enhanced early host inflammatory response.

5. SP-A is only able to kill Kp in the presence of cationic or anionic AMPs, because AMPs facilitate the binding of SP-A to bacteria (with which SP-A alone cannot interact) through a protein-protein interaction mechanism.

6. Using model bacterial membranes of Gram-negative bacteria, we found that SP-A/PMBN, but not SP-A alone, is able to insert into the bacterial outer membrane,

promoting changes on outer membrane structure and permeability, and eventually, bacterial death.

The synergistic action found in this study between SP-A and AMPs may provide a novel model of adjunctive therapy for pulmonary infections. Combinatorial use of naturally occurring lung factors as therapeutic agents should be considered to enhance killing of invasive pathogens as well as to increase endogenous protective mechanisms of the host to overcome infection. Moreover, since PMB and colistin are antibiotics of last resort against MDR microorganisms, the combinatorial use with SP-A would allow the use of a lower concentration of polymyxins, reducing their toxicity without decreasing their bactericidal effect. In this case, the use of SP-A in conjunction with PMBN could be more effective, since both together showed efficient bacterial killing, and PMBN by itself has much lower toxicity than polymyxins.

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