European Journal of Pharmaceutics and Biopharmaceutics 77 (2011) 384–391

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Surfactant protein A (SP-A)-tacrolimus complexes have a greater anti-inflammatory effect than either SP-A or tacrolimus alone on human macrophage-like U937 cells

Almudena López-Sánchez, Alejandra Sáenz, Cristina Casals *

Departamento de Bioquímica & Biología Molecular & CIBER Enfermedades Respiratorias, Universidad Complutense de Madrid, Madrid, Spain

ARTICLE INFO

Article history:
Available online 21 December 2010

Keywords:
Lung
Tacrolimus
Surfactant protein A
Inflammation
Macrophages
P-glycoprotein

ABSTRACT

Intratracheal administration of immunosuppressive agents to the lung is a novel treatment after lung transplantation. Nanoparticles of tacrolimus (FK506) might interact with human SP-A, which is the most abundant lipoprotein in the alveolar fluid. This study was undertaken to determine whether the formation of FK506/SP-A complexes interferes with FK506 immunosuppressive actions on stimulated human macrophage-like U937 cells. We found that SP-A was avidly bound to FK506 (K_d = 35 ± 4 nM), as determined by solid phase–binding assays and dynamic light scattering. Free FK506, at concentrations ⩽1 μM, had no effect on the inflammatory response of LPS-stimulated U937 macrophages. However, coincubation of FK506 and SP-A, at concentrations where each component alone did not affect LPS-stimulated macrophage response, significantly inhibited LPS-induced NF-κB activation and TNF-alpha secretion. Free FK506, but not FK506/SP-A, functioned as substrate for the efflux transporter P-glycoprotein. FK506 bound to SP-A was delivered to macrophages by endocytosis, since several endocytosis inhibitors blocked FK506/SP-A anti-inflammatory effects. This process depended partly on SP-A binding to its receptor, SP-R210. These results indicate that FK506/SP-A complexes have a greater anti-inflammatory effect than either FK506 or SP-A alone and suggest that SP-A strengthened FK506 anti-inflammatory activity by facilitating FK506 entrance into the cell, overcoming P-glycoprotein.

1. Introduction

Tacrolimus, also known as FK506 (Fig. 1), is a hydrophobic macrolide lactone produced by *Streptomyces tsukubaensis* [1], which acts as a powerful and clinically useful immunosuppressant through disruption of signaling events mediated by calcineurin in T lymphocytes [2,3]. In addition to its immunosuppressive effect on T cells, FK506 might act as an anti-inflammatory agent [2,4–6]. FK506 is currently available in both intravenous and oral dosage forms (commercially known as Prograf®). Clinical trials have shown tacrolimus to be more effective in lung transplantation than other oral immunosuppressants for both primary immunosuppression and rescue therapy for acute rejection recipients [7,8]. In addition, several experimental studies demonstrated that tacrolimus ameliorates ischemia–reperfusion injury after lung transplantation [5,6,9], which is the major cause of death within 30 days [7] and a risk factor for the development of bronchiolitis obliterans syndrome [10].

Nevertheless, like other immunosuppressive agents, systemically delivered tacrolimus causes substantial side effects [11]. Thus, local immunosuppression is a potential approach to increase drug levels in the graft while minimizing systemic drug levels and, therefore, systemic toxicity. Inhaled tacrolimus administered after lung transplantation could be an alternative to its systemic or oral administration, and recent studies have shown the benefits of inhaled tacrolimus for experimental lung transplantation [12], lung ischemia–reperfusion injury [6,9] and antigen-induced airway inflammation [13].

Using this route, tacrolimus would reach the alveolar fluid, where it would interact with alveolar macrophages, lymphocytes, and epithelial cells in a fluid environment characterized by the presence of pulmonary surfactant. The latter is a macromolecular complex composed of 90% lipids and 10% proteins that create a lipid-rich biological barrier that separates the alveolar gas and the

Abbreviations: FK506, tacrolimus; FKBP, FK506-binding protein; HSA, human serum albumin; LPS, bacterial lipopolysaccharide; NF-κB, nuclear factor-kappa B; MRP1, multidrug resistance associated protein; Pgp, P-glycoprotein; PMA, phorbol-12-myristate-13-acetate; SP-A, surfactant protein A; SP-R210, surfactant protein A receptor 210; TNF-α, tumor necrosis factor-α.

* Corresponding author. Departamento de Bioquímica y Biología Molecular I, Facultad de Biología, Universidad Complutense de Madrid, 28040 Madrid, Spain. Tel.: +34 913944261; fax: +34 913944672.
E-mail address: ccasalsc@bio.ucm.es (C. Casals).
URL: http://www.ucm.es/info/respira (C. Casals).

0939-6411/$ - see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2010.12.013

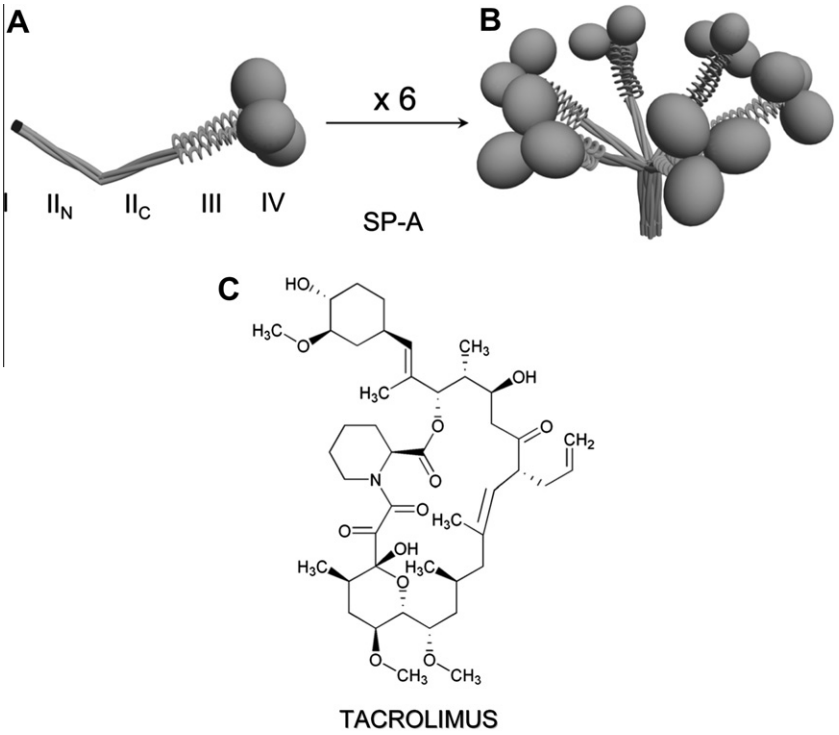

Fig. 1. Three-dimensional models of trimeric (A) and oligomeric (B) forms of SP-A, and chemical structure of FK506 (C). In A, the four structural domains of the human SP-A polypeptide chain are shown: (I) N-terminal segment involved in intermolecular disulfide bond formation; (II) collagen-like domain characterized by 23 Gly–Xaa–Yaa triplets with a sequence irregularity (kink), which divides the collagen-like domain into two parts: N-terminal (II_N) and C-terminal (II_C) portions; (III) neck region between the collagen and the globular domain; and (IV) C-terminal globular domain.

liquid that covers alveolar cell surfaces [14]. Surfactant protein A (SP-A) is the most abundant protein by mass in the alveolar fluid. SP-A is a versatile recognition protein that binds to a great variety of immune and non-immune ligands in the alveolar fluid and is principally involved in lung defense by enhancing the uptake of pathogens by phagocytes [15,16]. In addition, the binding of SP-A to receptors on immune and epithelial cells in the alveolus leads to an anti-inflammatory response, which is required to limit inflammation and avoid tissue damage [15,16]. This large oligomeric protein composed of 18 nearly identical subunits (Fig. 1) is mainly associated with surfactant lipids. Given the capability of SP-A to interact with a broad range of lipids [17], it is conceivable that SP-A binds to hydrophobic drugs such as FK506, and we found that a fluorescence derivative of tacrolimus, dansylcadaverine-FK506 (DNS-FK506), avidly binds to SP-A with an apparent equilibrium association constant of $1 \times 10^7\ M^{-1}$, which is similar to that found for FKBP/DNS-FK506 complexes ($1.5 \times 10^7\ M^{-1}$) [18].

We hypothesized that SP-A might influence FK506 bioavailability in the alveolar fluid and/or its immunosuppressive activity. Therefore, this study aimed to investigate whether the formation of FK506/SP-A complexes interferes with FK506 anti-inflammatory actions on stimulated human macrophage-like U937 cells. Macrophages are relevant cells in the alveolar fluid. Uncontrolled macrophage activation is a key initiation signal for septic insult or acute lung ischemia–reperfusion injury, since alveolar macrophage depletion attenuates disease symptoms [19,20].

2. Materials and methods

2.1. Materials

The human leukemic cell line U937 (CRL-1593.2, mycoplasma free and virus free) was supplied by the American Type Culture Collection (Manassas, VA). RPMI 1640 medium, heat-inactivated fetal bovine serum, glutamine and penicillin/streptomycin were obtained from Bio-Whittaker (Walkersville, MD). FK506 was kindly supplied by Fujisawa GmbH (Munich, Germany). ELISA kit for TNF-α immunoassays was obtained from BD PharMingen (San Diego, CA). NF-κB p50/p65 EZ-TFA Transcription Factor Assay was from Millipore (Billerica, MA). Smooth LPS from *Escherichia coli* (serotype 055:B5), phorbol 12-myristate acetate, monensin, bafilomycin A_1, nocodazole, amantadine, oligomycin B, verapamil, cyclosporin A, probenecid, and indomethacin were purchased from Sigma Aldrich (St. Louis, MO). Calcein acetoxymethyl ester (calcein-AM) was purchased from Molecular Probes (Eugene, OR). Cell proliferation reagent WST-1 was purchased from Roche Diagnostics (Barcelona, Spain). Anti-SP-R210 was kindly provided by Zissis Chroneos (Center of Biomedical Research, University of Texas Health Center, Tyler, Texas). All other reagents were of analytical grade obtained from Merck (Darmstadt, Germany).

2.2. Isolation of human SP-A

Surfactant protein A was isolated from BAL of patients with alveolar proteinosis using a sequential butanol and octylglucoside extraction [21,22]. The purity of SP-A was checked by one-dimensional SDS–Page in 12% acrylamide under reducing conditions and mass spectrometry. The oligomerization state of SP-A was assessed by electrophoresis under non-denaturing conditions, electron microscopy, and analytical ultracentrifugation as reported elsewhere [21,22]. SP-A consisted of supratrimeric oligomers of at least 18 subunits. Each subunit had an apparent molecular weight of 36,000 Da. Biotinylated SP-A was prepared as previously described for the labeling of SP-A with fluorescent Texas-Red [23]. The structure and functional activity of biotinylated SP-A was similar to that of unlabeled SP-A.

2.3. Solid-phase binding assay

To explore whether SP-A binds to immobilized FK506, solid-phase binding assay was performed with biotinylated SP-A. Wells of a 96-well polysorp microtiter plate (Nunc, Rochester, NY, USA) were coated with or without FK506 (50 μM) dissolved in methanol, overnight at room temperature, until total evaporation of the solvent. Afterwards, the non-specific binding was blocked by the addition of 5 mM Tris–HCl, 150 mM NaCl, 0.1 mM EDTA buffer, pH 7.2, containing 5% (w/v) non-fatty dried milk for 2 h. Then various concentrations of biotinylated human SP-A in 2.5% non-fatty dried milk-buffer A, with 2 mM CaCl2, were added and incubated at room temperature for 1 h. After extensive washing, streptavidin-horseradish peroxidase was added to the wells and incubations were performed for 1 h at room temperature. After a last washing step, the bound biotin-labeled SP-A was detected with *o*-phenylenediamine dihydrochloride tablets. The colorimetric reaction was stopped with 4 M sulfuric acid, and the absorbance in each well was read at 490 nm on an ELISA reader (DigiScan; Asys HiTech GmbH, Eugendorf, Austria).

2.4. Dynamic light scattering (DLS)

The hydrodynamic diameters of tacrolimus and SP-A particles as well as mixtures of these components were measured at 25 °C in a Zetasizer Nano S from Malvern Instruments (Worcestershire, UK) equipped with a 633 nm HeNe laser. Six scans were performed for each sample, and all the samples were analyzed in triplicate. The hydrodynamic diameter was calculated using the General Purpose algorithm available from the Malvern software for DLS analysis as described previously [24]. The interaction of SP-A with FK506 in solution was measured by addition of different concentrations of FK506 (from 0 to 50 μM) from a stock solution in methanol to a fixed concentration of SP-A (8 μg/ml; 12 nM), in 5 mM Tris–HCl buffer, pH 7.4, with or without 150 mM NaCl and 2 mM $CaCl_2$. The maximal final methanol concentration (<0.1%) achieved in the glass cuvettes did not affect SP-A structure as determined by circular dichroism and fluorescence spectroscopy [21,22].

2.5. Cell assays

Human monocyte-like U937 cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, and penicillin G sodium (100 units/ml)/streptomycin sulfate (100 units/ml) under a 95% air–5% CO_2 humidified atmosphere at 37 °C as reported elsewhere [21,22,25]. U937 cells were dispensed into 24-well plates at 1×10^6 cells/ml and differentiated into macrophages by incubation with 10 nM PMA for 24 h at 37 °C in a 5% CO_2 humidified atmosphere. After PMA treatment, adherent cells were washed with medium to remove PMA and non-adherent cells. Differentiated cells were further maintained in culture, as previously stated, for 24 h (recovery phase). Then cells were washed and pretreated with different concentrations of SP-A, free FK506, or combinations thereof for 5 min prior to smooth LPS (1 μg/ml) stimulation in presence of 5% heat-inactivated FBS at 37 °C. In some experiments, cells were pretreated with either 25 μg/ml of anti-SP-R210 antibody against SP-A receptor [26] or several inhibitors of endocytosis prior to 4 h LPS stimulation. The viability of differentiated U937 cells was always >90% as assessed by trypan blue exclusion. Flow cytometry analysis of PMA-treated U937 cells immunostained with antibodies to CD14 was used to assess U937 macrophage-like phenotype [27,28].

For measurement of TNF-α production, cell-free culture supernatants were collected and assayed using a human TNF-α ELISA kit. For measurements of NF-κB activation, nuclear extraction was performed and the nuclear extract assayed for NF-κB with an immunoassay kit according to the manufacturer's instructions. Results were expressed as a percentage of the level of TNF-α production or NF-κB activation by cells stimulated with LPS in the absence of FK506 and/or SP-A.

2.6. Calcein uptake assay

Differentiated macrophage-like U937 cells (10^6 cells/ml) were incubated with 0.25 μM calcein-AM for 15 min at 37 °C in the absence and presence of increasing concentrations of FK506 with or without SP-A (2.5 μg/ml). Calcein-AM is a non-fluorescent lipid soluble dye that becomes highly fluorescent through cleavage of the ester bonds by cytosolic esterases. Unlike calcein-AM, calcein is soluble and cannot penetrate plasma membrane. After washing three times with cold PBS, intracellular-retained fluorescent calcein was determined with a FLUOstar OPTIMA (BMG Labtechnologies *GmbH*, Germany) at 485 nm excitation and 530 nm emission wavelengths [29]. Results were expressed as a percentage of calcein uptake shown by differentiated U937 cells in the absence of FK506.

2.7. Cytotoxicity assay

Differentiated macrophage-like U937 cells were exposed to increasing concentrations of FK506 with or without SP-A (2.5 μg/ml) for 4 h at 37 °C. Cell viability was assessed by using the WST-1 assay (Roche Biochemicals). This assay is based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenase. Absorbance of the samples was measured at 450 nm. Results were expressed as percentages of the control response of cells in the absence of FK506 or FK506/SP-A. The potential cytotoxicity of FK506 or FK506/SP-A at the concentrations assayed was also assessed as the loss of exclusion of propidium iodide (PI) after application of PI directly to the cell culture medium at 5 μg/ml final concentration as reported elsewhere [30].

2.8. Statistical analyses

Means were compared by one-way analysis of variance. We considered $p < 0.05$ as statistically significant. Data are shown as mean ± SEM. The statistical package SPSS 15.0 (Chicago, ILL) was used for the analysis.

3. Results and discussion

3.1. SP-A binding to FK506

We previously determined the binding of SP-A to dansylcadaverine-FK506 using the fluorescence and anisotropy properties of DNS-FK506 [18]. Given that derivatization of FK506 at the C22 position of the molecule with a dansyl moiety partly inhibits drug binding to the FK506-binding protein [18], we have evaluated the binding of SP-A to either immobilized FK506 or FK506 in aqueous solution. Fig. 2A shows that biotinylated SP-A bound to FK506-coated wells in a dose-dependent manner, with a $K_d = 35 \pm 4$ nM. The presence of phospholipids in the medium did not inhibit the binding of SP-A to FK506 (data not shown). Likewise, the binding of SP-A to immobilized surfactant lipids was not inhibited by the presence of FK506 in the medium (data not shown). In addition, the lipid aggregation activity of SP-A, which is of relevance in pulmonary surfactant biology, is markedly increased in the presence of FK506 [30]. Together, these results indicate that the tacrolimus-binding site of SP-A does not overlap with its lipid-binding

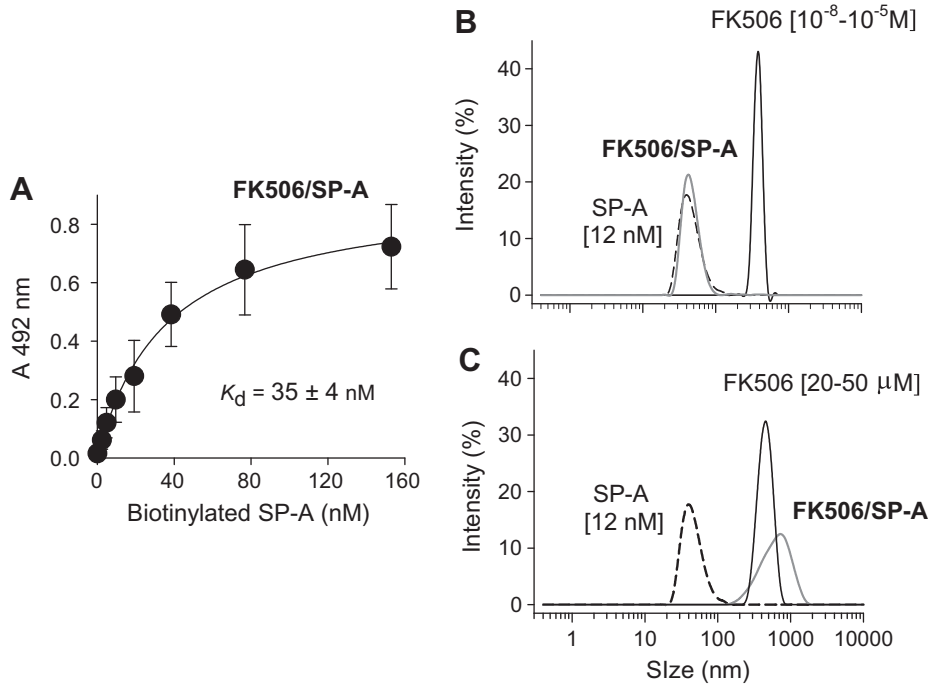

Fig. 2. SP-A binding to FK506. (A) SP-A binds to immobilized FK506 in a dose-dependent manner with a dissociation constant (K_d) of 35 ± 4 nM. Microtiter plate wells were coated with or without FK506 (50 μM). Then, biotinylated SP-A was added to the wells and the level of bound SP-A was determined with streptavidin-horseradish peroxidase. Results are means ± SD of three experiments. (B) DSL analysis of the hydrodynamic diameter of FK506 and SP-A particles in 5 mM Tris–HCl buffer, pH 7.4. The *y* axis represents relative intensity of scattered light; the *x* axis denotes the hydrodynamic diameter of particles present in the solution. Addition of different concentrations of FK506 to a solution containing a constant concentration of SP-A (12 nM) caused disappearance of the peak of FK506 particles (390 ± 20 nm) and the appearance of a SP-A/FK506 peak at 44 ± 2 nm (gray line). On the other hand, addition of higher concentrations of FK506 (ranging from 20 to 50 μM) to SP-A (12 nM) caused disappearance of the characteristics peaks of FK506 and SP-A particles and the appearance of a new SP-A/FK506 peak at 606 ± 40 nm (gray line).

site located in the C-terminal domain of the protein (Fig. 1). Moreover, no energy transfer from the tryptophan residues of SP-A (located in the C-terminal domain of the protein) to the dansyl moiety of DNS-FK506 was observed [18], and resonance energy transfer measurements suggest that the tacrolimus-binding site might be located in the neck region or in the C-terminal (II_C) portion of the collagen-like domain of SP-A [18].

The interaction of SP-A with FK506 in aqueous solution was examined by dynamic light scattering (Fig. 2B). Particle size analysis of FK506 at concentrations ranging from 0.01 to 10 μM showed a monodisperse distribution with a mean hydrodynamic diameter of 390 ± 20 nm. Particle size analysis was measured 10 min after addition of FK506 to the aqueous phase from a stock solution in methanol (FK506 particle size in methanol was 1 ± 0.5 nm). Thus, given its hydrophobic nature, tacrolimus aggregates in aqueous solution in order to reduce free energy and become a stable system. Fig. 2B also shows that SP-A particles had a hydrodynamic diameter of 42 ± 6 nm. Addition of different concentrations of FK506 (ranging from 0 to 10 μM) to a solution containing a constant concentration of SP-A (12 nM) caused disappearance of the peak of FK506 particles and the appearance of a SP-A/FK506 peak at 44 ± 2 nm (Fig. 2B). These results indicate that SP-A binds to FK506 and prevents tacrolimus self-aggregation in the aqueous medium. Similar results were found when SP-A was added to a solution containing FK506 at concentrations ⩽10 μM, indicating that the binding of SP-A to tacrolimus dissociated FK506 aggregates. Fig. 2C shows that this SP-A effect is concentration dependent, since at higher FK506 concentrations (⩾20 μM), the characteristics peaks of FK506 and SP-A particles disappear and a new SP-A/FK506 peak becomes visible with a mean hydrodynamic diameter of 606 ± 40 nm.

3.2. SP-A binding to FK506 increases FK506 potency as anti-inflammatory agent

Fig. 3 shows the effect of either FK506 (Fig. 3A) or SP-A (Fig. 3B) alone on TNF-α production by macrophage-like U937 cells stimulated with smooth LPS. FK506 was a weak inhibitor of TNF-α secretion by LPS-stimulated macrophage at concentrations of FK506 lower than 10 μM. This is consistent with several studies, in which concentrations of FK506 of 1–10 μM were used to see any FK506 inhibitory effect on TNF-α secretion [31], nitric oxide production [32,33], or macrophage proliferation [34] by stimulated macrophages. This stands out against the low FK506 concentrations required to inhibit T-cell activation [1,2,30].

On the other hand, human SP-A showed a clear inhibitory effect on LPS-induced TNF-α secretion, which was dependent on the SP-A concentration. At concentrations lower than 5 μg/ml (7.5 nM) SP-A had no effect, which is consistent with previous studies [21,22,25]. SP-A or FK506 alone (without LPS) had no effect on TNF-α production by resting differentiated U937 cells for 4 h after SP-A or FK506 addition (results not shown).

We found that SP-A binding to FK506 greatly increases FK506 potency as anti-inflammatory agent. Fig. 4 shows that coincubation of FK506 and SP-A, at concentrations where each component alone did not affect LPS-stimulated macrophage response, significantly inhibited LPS-induced TNF-α secretion. Thus, FK506 concentrations as low as 0.001 μM significantly inhibited TNF-α production provided that low concentrations of SP-A (2.5 μg/ml) are present in the medium.

To find out whether this effect is SP-A-specific, we investigate whether other extracellular protein, human albumin (HSA), which like SP-A avidly binds to FK506 [18], increases FK506 potency as anti-inflammatory agent. Results in Fig. 4 show that coincubation

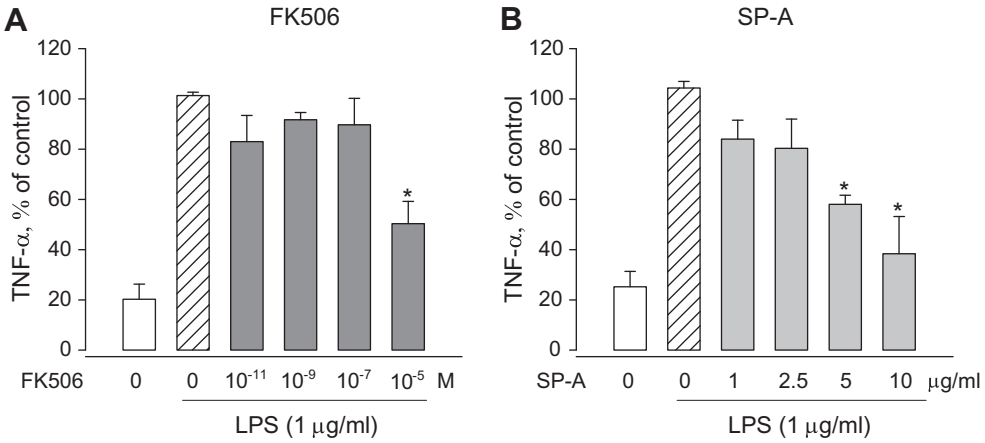

Fig. 3. Effect of FK506 (A) and SP-A (B) on TNF-α production by macrophage-like U937 cells stimulated with LPS. Differentiated U937 cells (1×10^6 cells/ml) were preincubated in the absence or presence of increasing concentrations of FK506 (A) or SP-A (B) for 5 min prior to 4-h activation with 1 μg/ml LPS in the presence of 5% FBS at 37 °C. Afterward, cell-free supernatants were collected and the levels of TNF-α were measured by ELISA. The results are presented as the means + SEM from four different cell cultures ($n = 4$). The assays from each U937 cell culture were performed in triplicate, the triplicate values were averaged, and their mean treated as a single point. Results were expressed as percentages of LPS-induced TNF-α levels. $^*p < 0.05$ compared with response elicited by LPS in the absence of either FK506 or SP-A.

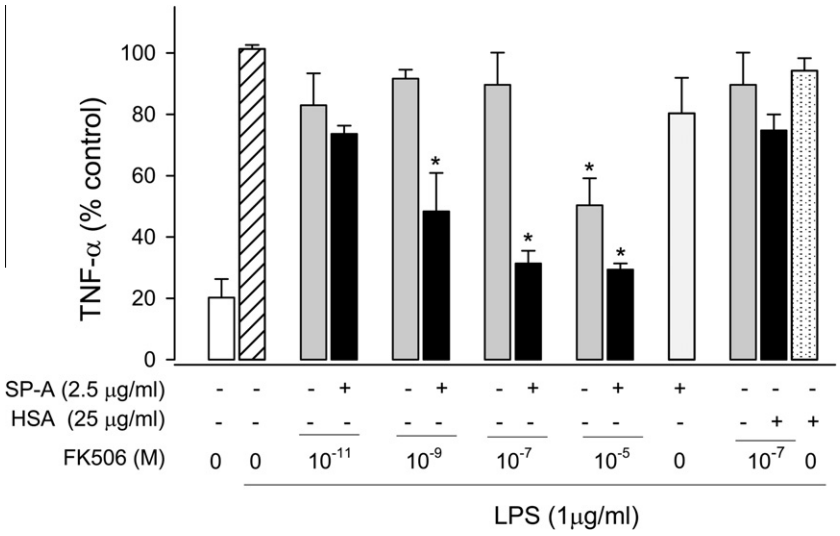

Fig. 4. FK506/SP-A complexes have greater inhibitory effect on LPS-induced TNF-α secretion than either FK506 or SP-A alone. Macrophage-like U937 cells (1×10^6 cells/ml) were preincubated in the absence or presence of increasing concentrations of FK506 with or without SP-A (2.5 μg/ml) or HSA (25 μg/ml) for 5 min prior to 4-h activation with 1 μg/ml LPS at 37 °C. Afterward, cell-free supernatants were collected and the levels of TNF-α were measured by ELISA. Data presented are from four different cell cultures ($n = 4$). The assays from each U937 cell culture were performed in triplicate, the triplicate values were averaged, and their mean treated as a single point. The results are presented as the means + SEM. Results were expressed as percentages of LPS-induced TNF-α level. $^*p < 0.05$ compared with response elicited by LPS.

of free FK506 (10^{-7} M) and excess of human albumin (25 μg/ml) did not strengthen the anti-inflammatory activity of FK506. Albumin alone had no effect on LPS-induced TNF-α secretion.

Next we examined whether the inhibitory effect of SP-A/FK506 complexes on TNF-α secretion was NF-κB dependent or independent. Fig. 5 shows that coincubation of FK506 and SP-A significantly inhibited LPS-induced NF-κB activation at concentrations where each component alone did not show any effect. Thus, SP-A/FK506 complexes seem to inhibit LPS-induced TNF-α secretion by an NF-κB-dependent manner. These results are consistent with the fact that *in vivo* intravenous or intratracheal administration of tacrolimus is protective against lung ischemia-reperfusion injury, and this protection is associated with a decrease in both NF-κB activity and proinflammatory cytokine expression [5,6]. In addition, tacrolimus was found to inhibit airway epithelial cell NF-κB activation [4].

Thus, SP-A/FK506 complexes, but not SP-A or FK506 alone at low concentrations, inhibited LPS-induced NF-κB activation. SP-A itself mediates modulation of the IκB-α/NF-κB pathway, but SP-A concentrations required for this immunomodulatory effect are $\geqslant$20 μg/ml [35]. We hypothesized that FK506 is involved in the inhibition of LPS-induced NF-κB activation, while SP-A might facilitate FK506 entrance into the cell. In the cytosol, FK506 is bound to its cytosolic immunophilin: FKBP [2]. This drug-immunophilin complex targets calcineurin and inhibits the Ca^{2+}/calmodulin-dependent phosphatase activity of calcineurin. The inhibition of calcineurin in turn reduces the expression of a number of nuclear transcription factors, including NF-κB. Calcineurin inactivates IκB, an inhibitor of NF-κB, therefore increasing the levels of active NF-κB in the nucleus, which leads to increased production of mRNA for proinflammatory cytokines [36–38]. The ability of calcineurin to participate in the induction of NF-κB-dependent promoters is not T-cell specific and has been described in macrophage-like U937 [37]. In addition, a recent study demonstrates that FK506-induced inactivation of calcineurin in macrophages or calcineurin deletion can induce a form of LPS tolerance and protect the host from LPS toxicity *in vivo* [39].

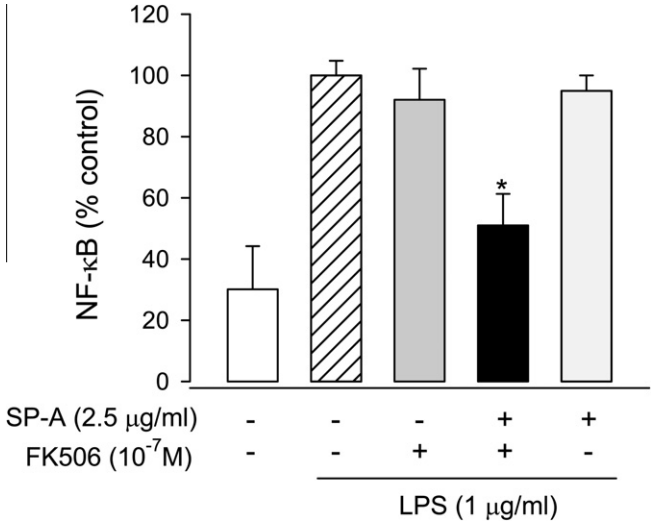

Fig. 5. Coincubation of FK506 and SP-A significantly inhibits LPS-induced NF-κB activation at concentrations where each component alone does not show any effect. Macrophage-like U937 cells (1×10^6 cells/ml) were preincubated in the absence or presence of free FK506 (10^{-7} M), SP-A (2.5 μg/ml), or FK506 (10^{-7} M) + SP-A (2.5 μg/ml) for 5 min prior to 1-h activation with 1 μg/ml LPS at 37 °C. Then nuclear extracts were obtained and the levels of the p50 subunit of NF-κB were measured. The results are presented as the means + SEM from four different cell cultures ($n = 4$). Results were expressed as a percentage of LPS-induced NF-κB activation. $^*p < 0.05$ compared with response elicited by LPS.

3.3. SP-A binding to FK506 prevents FK506 efflux by P-glycoprotein

FK506 partitions into membranes, and no receptors have been demonstrated for FK506 in cellular membranes. Thus, FK506 enters the cell by passive diffusion and it is recognized by FKBP at the cytosolic side of plasma membrane. However, in some cells, a drug efflux pump P-glycoprotein (Pgp) actively transports FK506 out of target cells, thereby reducing its efficacy [40]. We hypothesized that SP-A/FK506 complexes might facilitate FK506 entrance into macrophages bypassing Pgp.

Pgp is widely expressed in many biological barriers in the body, including the lung epithelial alveolar barrier [41]. Pgp and MRP1 were also found in normal bronchial and bronchiolar epithelial layers, as well as in seromucinous glands and alveolar macrophages, where they prevent the accumulation of toxic substances [42]. To ascertain if Pgp and/or MRP1 were expressed in macrophage-like U937 cells, we performed a functional test for the presence of both efflux pump activities based on the calcein-AM assay [29]. Calcein-AM passes through the plasma membrane easily due to its hydrophobicity. However, it is actively extruded by Pgp or MRP1 before its intracellular esterase-dependent conversion to free calcein, which is soluble and highly fluorescent. Intracellularly trapped calcein does not bind to cellular components and its fluorescence is insensitive to changes in pH, Ca^{2+}, or Mg^{2+}. Pgp and MRP1 activities can be evaluated in the presence of specific inhibitors of either Pgp or MRP1. When Pgp and MRP1 pumps are active, the uptake of calcein-CM is low. However, when these pumps are inhibited, the uptake of calcein-AM increases and consequently the fluorescence of intracelullarly retained calcein [43].

Fig. 6A shows that calcein-AM uptake greatly increased in the presence of specific Pgp inhibitors (the calcium channel blocker verapamil and the immunosuppressive drug cyclosporin A) but not in the presence of MRP1-specific inhibitors (indomethacin and probenecid). These results indicate that macrophage-like U937 cells express Pgp but not MRP1. These results are consistent with those that indicate that Pgp expression is part of the macrophage differentiation process [43,44]. On the other hand, Fig. 6B shows that FK506 is a substrate of Pgp but not FK506/SP-A complexes. Free FK506 showed a clear inhibitory effect on the calcein-AM efflux mediated by Pgp. As a result, an FK506 concentration-dependent increase in intracelullarly trapped calcein was observed. These results indicate that in the absence of SP-A, FK506 must be actively transported out of cells, thereby reducing its intracellular concentration and immunosuppressive effects. The binding of FK506 to SP-A facilitates FK506 entrance into the cell by a mechanism that overcomes Pgp.

3.4. SP-A facilitates FK506 entrance into the cell by endocytosis

Recent studies show that SP-A binds very rapidly to the macrophage surface and that SP-A uptake by these cells involves a clathrin-dependent endocytic pathway [45,46]. Thus, if FK506/SP-A complexes are taken up by macrophages through endocytosis, then their inhibitory effect on TNF-α release should be totally or partly reverted by endocytosis inhibitors. Fig. 7 shows that the inhibitory effect of 10^{-7} M FK506 plus 2.5 μg/ml SP-A on LPS-induced TNF-α secretion was abolished by the presence of several endocytosis inhibitors: amantadine, which is a clathrin-dependent endocytosis inhibitor; monensin, an ionophore that prevents endosomal acidification and maturation; bafilomycin A and oligomicin B, inhibitors of the

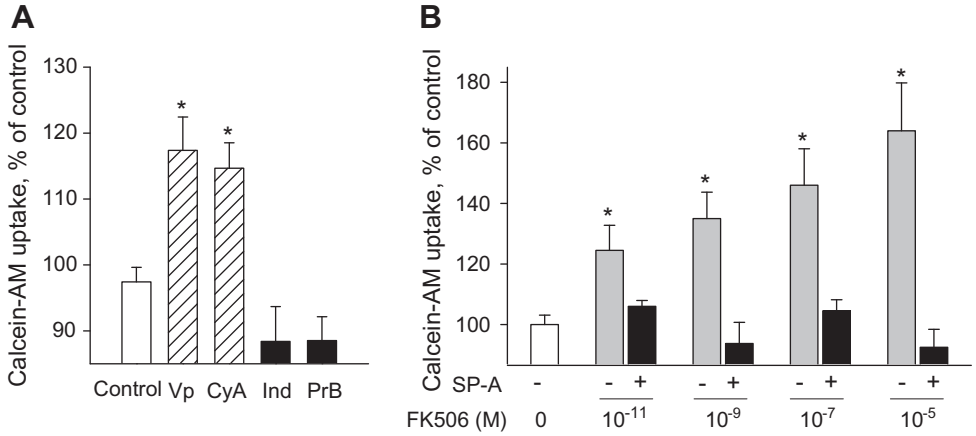

Fig. 6. Calcein-AM uptake assay for Pgp activity (A) Macrophage-like U937 cells were incubated with 0.25 μM calcein-AM for 15 min at 37 °C in the absence and presence of different inhibitors of either Pgp (25 μM verapamil and 2 μM cyclosporin A) or MRP1 (0.2 mM indomethacin and 10 mM probenecid). (B) Cells were incubated with 0.25 μM calcein-AM for 15 min at 37 °C in the absence and presence of increasing concentrations of FK506 with or without SP-A (2.5 μg/ml). Calcein-AM uptake was measured as calcein-specific fluorescence (λ_{ex} = 488 nm and λ_m = 530 nm). Results were expressed as percentage of calcein-AM uptake shown by differentiated U937 cells in the absence of modulators. The results are presented as the means + SEM from four different cell cultures ($n = 4$) ($^*p < 0.05$).

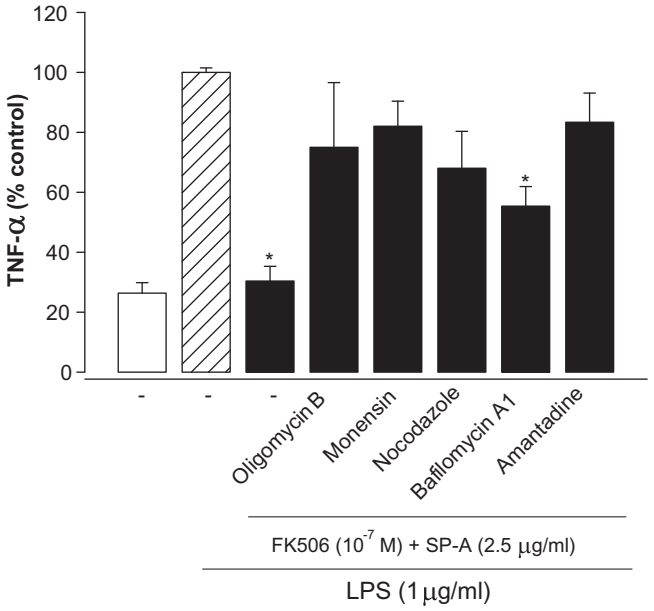

Fig. 7. Endocytosis inhibitors block FK506/SP-A inhibitory effect on TNF-α secretion by LPS-stimulated macrophages. Differentiated macrophage-like U937 cells (1×10^6 cells/ml) were pretreated with different endocytosis inhibitors (25 μM monensin, 0.2 μM bafilomycin A_1, 10 μM nocodazole, 2.5 mM amantadine, and 10 μg/ml oligomycin B) for 10 min prior to 4 h incubation with 1 μg/ml LPS in the presence or absence of 10^{-7} M FK506 plus 2.5 μg/ml SP-A. Afterward, cell-free supernatants were collected and the levels of TNF-α were measured by ELISA. In the absence of FK506/SP-A, these inhibitors had no effect on LPS-induced TNF-α secretion at the concentrations assayed (data not shown). Results were expressed as a percentage of LPS-induced TNF-α level. The results are presented as the means + SEM from four different cell cultures ($n = 4$) ($^*p < 0.05$).

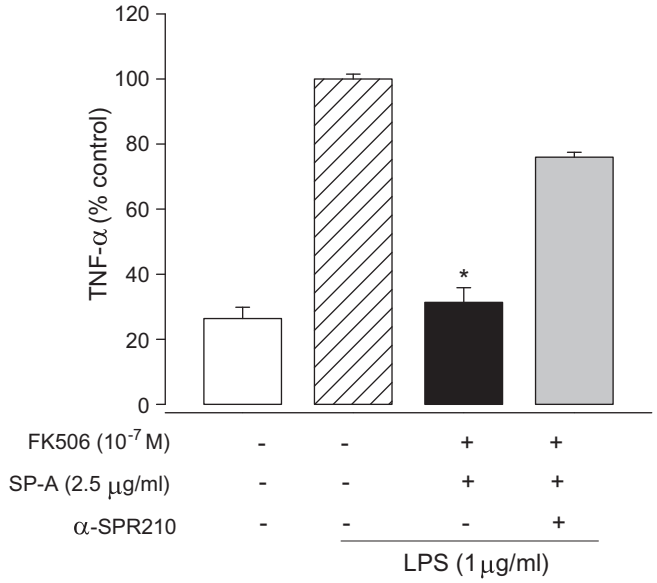

Fig. 8. The inhibitory effect of FK506/SP-A on TNF-α secretion is partially blocked in the presence of an antibody against SP-A receptor (SP-R210). Differentiated U937 cells (1×10^6 cells/ml) were preincubated for 30 min in the absence or presence of FK506 + SP-A, with or without 25 μg/ml anti-SP-R210 antibody. After 4-h incubation with 1 μg/ml LPS, cell-free supernatants were collected and the levels of TNF-α were measured by ELISA. Results were expressed as a percentage of LPS-induced TNF-α level. The results are presented as the means + SEM from four different cell cultures ($n = 4$) ($^*p < 0.05$).

vacuolar proton pumps; and nocodazole, a microtubule-disrupting agent [47]. Some of these endocytosis inhibitors have been demonstrated to prevent SP-A internalization by macrophages [46] and also by type II alveolar epithelial cells, which are involved in the synthesis, secretion, and reuptake of SP-A [48]. Our results indicate that FK506/SP-A complexes are taken up by the cells through endocytosis. This mechanism of entry into the cell prevents FK506 binding to Pgp and FK506 transport out of the cell.

Although the interaction of SP-A (and FK506/SP-A complexes) with macrophages seems to be a receptor-mediated process, the receptors mediating this process are not clear, although SP-A binding to several receptors has been described [15]. It has been demonstrated that the binding of SP-A to its endocytosis receptor(s) takes place by direct protein–protein interaction but not by the carbohydrate recognition domain located at the C-terminal domain (Fig. 1) [45]. SP-R210 is an SP-A-specific receptor sited in the plasma membrane of different cell types, such as U937, alveolar macrophages, or type II cells [49], and it has been recently identified as unconventional myosin 18A [26]. Furthermore, SP-A binds to SP-R210 via its collagen domain but not via its C-terminal domain [50]. If this receptor is involved in the endocytosis of FK506/SP-A complexes, an anti-SP-R210 antibody would hamper SP-A binding to SP-R210 and therefore FK506/SP-A complex internalization. Fig. 8 shows that the inhibitory effect of 10^{-7} M FK506 plus 2.5 μg/ml SP-A on LPS-induced TNF-α secretion was reduced by the presence of anti-SP-R210 in the medium, indicating that this SP-A receptor is implicated in the uptake of FK506/SP-A complexes.

4. Conclusions

Available oral or parenteral formulations of tacrolimus are clinically hampered due to dose-related efficacy and toxicity. Respiratory delivery of FK506 nanoparticles is a new and very promising approach for lung transplantation. However, it is not known whether tacrolimus interacts with proteins present in the alveolar environment, which can reduce its drug potency.

In this study, we have analyzed the interaction of tacrolimus with SP-A, the most abundant protein in the alveolar fluid. We have demonstrated that SP-A binds to FK506 with high affinity and prevents tacrolimus self-aggregation in the aqueous medium. The formation of SP-A/tacrolimus complexes increases tacrolimus potency as an anti-inflammatory agent on macrophages, since SP-A facilitates FK506 entrance into macrophages by endocytosis. This mechanism of entry into the cell prevents FK506 binding to Pgp and FK506 transport out of the cell. One of the major limitations of FK506 and other immunosuppressant and antimitotic drugs is that they are substrates of the Pgp efflux pump [40]. Thus, they are actively excluded from cells that express Pgp such as lung epithelial cells and alveolar macrophages [41,42]. Our finding that SP-A, by binding to FK506, increases drug potency in a macrophage-like U937 cell line that expresses Pgp is significant.

A decrease in SP-A in the alveolar space is a condition associated with lung dysfunction such as acute lung injury and ischemia–reperfusion injury after lung transplantation [15,51]. Thus, in the face of acute inflammation, addition of recombinant human SP-A to the tacrolimus dispersion for nebulization might improve the anti-inflammatory and immunosuppressive pulmonary therapy.

Acknowledgements

This work was supported by the Ministerio de Ciencia e Innovación (SAF2009-07810), and Instituto de Salud Carlos III (CIBERES-CB06/06/0002). We thank Dr. Zissis Chroneos (Center of Biomedical Research, University of Texas Health Center, Tyler, Texas) for providing polyclonal antibody against surfactant protein A receptor 210 (SP-R210). We also thank Fujisawa GmbH/Astellas Pharma for providing FK506.

References

[1] T. Kino, H. Hatanaka, M. Hashimoto, M. Nishiyama, T. Goto, M. Okuhara, M. Kohsaka, H. Aoki, H. Imanaka, FK-506, a novel immunosuppressant isolated from a Streptomyces. I. Fermentation, isolation, and physico-chemical and biological characteristics, J. Antibiot. (Tokyo) 40 (1987) 1249–1255.

[2] J. Clardy, The chemistry of signal transduction, Proc. Natl. Acad. Sci. USA 92 (1995) 56–61.

[3] S. Feske, H. Okamura, P.G. Hogan, A. Rao, Ca2+/calcineurin signalling in cells of the immune system, Biochem. Biophys. Res. Commun. 311 (2003) 1117–1132.

[4] T. Deuse, F. Blankenberg, M. Haddad, H. Reichenspurner, N. Phillips, R.C. Robbins, S. Schrepfer, Mechanisms behind local immunosuppression using inhaled tacrolimus in preclinical models of lung transplantation, Am. J. Respir. Cell Mol. Biol. 43 (2010) 403–412.

[5] B. Krishnadasan, B. Naidu, M. Rosengart, A.L. Farr, A. Barnes, E.D. Verrier, M.S. Mulligan, Decreased lung ischemia-reperfusion injury in rats after preoperative administration of cyclosporine and tacrolimus, J. Thorac. Cardiovasc. Surg. 123 (2002) 756–767.

[6] S.M. Woolley, A.S. Farivar, B.V. Naidu, M. Rosengart, R. Thomas, C. Fraga, M.S. Mulligan, Endotracheal calcineurin inhibition ameliorates injury in an experimental model of lung ischemia-reperfusion, J. Thorac. Cardiovasc. Surg. 127 (2004) 376–384.

[7] J.D. Christie, L.B. Edwards, P. Aurora, F. Dobbels, R. Kirk, A.O. Rahmel, J. Stehlik, D.O. Taylor, A.Y. Kucheryavaya, M.I. Hertz, The registry of the international society for heart and lung transplantation: twenty-sixth official adult lung and heart-lung transplantation report-2009, J. Heart Lung Transplant. 28 (2009) 1031–1049.

[8] Y. Fan, Y.B. Xiao, Y.G. Weng, Tacrolimus versus cyclosporine for adult lung transplant recipients: a meta-analysis, Transplant Proc. 41 (2009) 1821–1824.

[9] A.S. McCourtie, H.E. Merry, P.S. Wolf, E. FitzSullivan, J.C. Keech, A.S. Farivar, M.S. Mulligan, Synergistic protection in lung ischemia-reperfusion injury with calcineurin and thrombin inhibition, Ann. Thorac. Surg. 89 (2010) 1766–1771.

[10] S.M. Fiser, C.G. Tribble, S.M. Long, A.K. Kaza, J.A. Kern, D.R. Jones, M.K. Robbins, I.L. Kron, Ischemia-reperfusion injury after lung transplantation increases risk of late bronchiolitis obliterans syndrome, Ann. Thorac. Surg. 73 (2002) 1041–1047. discussion 1047–1048.

[11] S.M. Bhorade, E. Stern, Immunosuppression for lung transplantation, Proc. Am. Thorac. Soc. 6 (2009) 47–53.

[12] A. Ingu, K. Komatsu, S. Ichimiya, N. Sato, Y. Hirayama, M. Morikawa, T. Abe, Effects of inhaled FK 506 on the suppression of acute rejection after lung transplantation: use of a rat orthotopic lung transplantation model, J. Heart Lung Transplant. 24 (2005) 538–543.

[13] Y. Morishita, Y. Hirayama, K. Miyayasu, K. Tabata, A. Kawamura, Y. Ohkubo, S. Mutoh, FK506 aerosol locally inhibits antigen-induced airway inflammation in Guinea pigs, Int. Arch. Allergy Immunol. 136 (2005) 372–378.

[14] J. Goerke, Pulmonary surfactant: functions and molecular composition, Biochim. Biophys. Acta 1408 (1998) 79–89.

[15] J.R. Wright, Immunoregulatory functions of surfactant proteins, Nat. Rev. Immunol. 5 (2005) 58–68.

[16] C. Casals, I. Garcia-Verdugo, Molecular and functional properties of surfactant protein A, in: K. Nag (Ed.), Lung Surfactant Function and Disorder, Boca Ratón, Florida, 2005, pp. 59–86.

[17] C. Casals, Role of surfactant protein A (SP-A)/lipid interactions for SP-A functions in the lung, Pediatr. Pathol. Mol. Med. 20 (2001) 249–268.

[18] O. Canadas, A. Saenz, G. Orellana, C. Casals, Equilibrium studies of a fluorescent tacrolimus binding to surfactant protein A, Anal. Biochem. 340 (2005) 57–65.

[19] M. Zhao, L.G. Fernandez, A. Doctor, A.K. Sharma, A. Zarbock, C.G. Tribble, I.L. Kron, V.E. Laubach, Alveolar macrophage activation is a key initiation signal for acute lung ischemia-reperfusion injury, Am. J. Physiol. Lung Cell Mol. Physiol. 291 (2006) L1018–L1026.

[20] E.L. Martin, T.A. Sheikh, K.J. Leco, J.F. Lewis, R.A. Veldhuizen, Contribution of alveolar macrophages to the response of the TIMP-3 null lung during a septic insult, Am. J. Physiol. Lung Cell Mol. Physiol. 293 (2007) L779–L789.

[21] F. Sanchez-Barbero, J. Strassner, R. Garcia-Canero, W. Steinhilber, C. Casals, Role of the degree of oligomerization in the structure and function of human surfactant protein A, J. Biol. Chem. 280 (2005) 7659–7670.

[22] F. Sanchez-Barbero, G. Rivas, W. Steinhilber, C. Casals, Structural and functional differences among human surfactant proteins SP-A1, SP-A2 and co-expressed SP-A1/SP-A2: role of supratrimeric oligomerization, Biochem. J. 406 (2007) 479–489.

[23] O. Canadas, I. Garcia-Verdugo, K.M. Keough, C. Casals, SP-A permeabilizes lipopolysaccharide membranes by forming protein aggregates that extract lipids from the membrane, Biophys. J. 95 (2008) 3287–3294.

[24] A. Saenz, A. Lopez-Sanchez, J. Mojica-Lazaro, L. Martinez-Caro, N. Nin, L.A. Bagatolli, C. Casals, Fluidizing effects of C-reactive protein on lung surfactant membranes: protective role of surfactant protein A, FASEB J. 24 (2010) 3662–3673.

[25] I. Garcia-Verdugo, F. Sanchez-Barbero, K. Soldau, P.S. Tobias, C. Casals, Interaction of SP-A (surfactant protein A) with bacterial rough lipopolysaccharide (Re-LPS), and effects of SP-A on the binding of Re-LPS to CD14 and LPS-binding protein, Biochem. J. 391 (2005) 115–124.

[26] C.H. Yang, J. Szeliga, J. Jordan, S. Faske, Z. Sever-Chroneos, B. Dorsett, R.E. Christian, R.E. Settlage, J. Shabanowitz, D.F. Hunt, J.A. Whitsett, Z.C. Chroneos, Identification of the surfactant protein A receptor 210 as the unconventional myosin 18A, J. Biol. Chem. 280 (2005) 34447–34457.

[27] C. Casals, J. Arias-Diaz, F. Valino, A. Saenz, C. Garcia, J.L. Balibrea, E. Vara, Surfactant strengthens the inhibitory effect of C-reactive protein on human lung macrophage cytokine release, Am. J. Physiol. Lung Cell Mol. Physiol. 284 (2003) L466–L472.

[28] Z.L. Huang, M.L. Failla, P.G. Reeves, Differentiation of human U937 promonocytic cells is impaired by moderate copper deficiency, Exp. Biol. Med. 226 (2001) 222–228.

[29] L. Homolya, M. Holló, M. Müller, E.B. Mechetner, B. Sarkadi, A new method for a quantitative assessment of P-glycoprotein-related multidrug resistance in tumour cells, Br. J. Cancer 73 (1996) 849–855.

[30] O. Canadas, R. Guerrero, R. Garcia-Canero, G. Orellana, M. Menendez, C. Casals, Characterization of liposomal tacrolimus in lung surfactant-like phospholipids and evaluation of its immunosuppressive activity, Biochemistry 43 (2004) 9926–9938.

[31] I.M. Conboy, D. Manoli, V. Mhaiskar, P.P. Jones, Calcineurin and vacuolar-type H+ATPase modulate macrophage effector functions, Proc. Natl. Acad. Sci. USA 96 (1999) 6324–6329.

[32] M. Hamalainen, A. Lahti, E. Moilanen, Calcineurin inhibitors, cyclosporin A and tacrolimus inhibit expression of inducible nitric oxide synthase in colon epithelial and macrophage cell lines, Eur. J. Pharmacol. 448 (2002) 239–244.

[33] D.J. Dusting, K. Akita, H. Hickey, M. Smith, V. Gurevich, Cyclosporin A and tacrolimus (FK506) suppress expression of inducible nitric oxide synthase in vitro by different mechanisms, Br. J. Pharmacol. 128 (1999) 337–344.

[34] M. Comalada, A.F. Valledor, E. Sanchez-Tilló, I. Umbert, J. Xaus, A. Celada, Macrophage colony-stimulating factor-dependent macrophage proliferation is mediated through a calcineurin-independent but immunophilin-dependent mechanism that mediates the activation of external regulated kinases, Eur. J. Immunol. 33 (2003) 3091–3100.

[35] C. Moulakakis, S. Adam, U. Seitzer, A.B. Schromm, M. Leitges, C. Stamme, Surfactant protein A activation of atypical protein kinase Cξ in IκB-α-dependent anti-inflammatory immune regulation, J. Immunol. 179 (2007) 4480–4491.

[36] B. Frantz, E.C. Nordby, G. Bren, N. Steffan, C.V. Paya, R.L. Kincaid, M.J. Tocci, S.J. O'Keefe, E.A. O'Neill, Calcineurin acts in synergy with PMA to inactivate I kappa B/MAD3, an inhibitor of NF-kappa B, EMBO J. 13 (1994) 861–870.

[37] N.M. Steffan, G.D. Bren, B. Frantz, M.J. Tocci, E.A. O'Neill, C.V. Paya, Regulation of IkB alpha phosphorylation by PKC- and Ca(2+)-dependent signal transduction pathways, J. Immunol. 155 (1995) 4685–4691.

[38] K. Hughes, A. Antonsson, T. Grundstrom, Calmodulin dependence of NfkappaB activation, FEBS Lett. 44 (1998) 132–136.

[39] C. Jennings, B. Kusler, P.P. Jones, Calcineurin inactivation leads to decreased responsiveness to LPS in macrophages and dendritic cells and protects against LPS-induced toxicity in vivo, Innate Immunol. 15 (2009) 109–120.

[40] T. Saeki, K. Ueda, Y. Tanigawara, R. Hori, T. Komano, Human P-glycoprotein transports cyclosporin A and FK506, J. Biol. Chem. 268 (1993) 6077–6080.

[41] L. Campbell, A.N. Abulrob, L.E. Kandalaft, S. Plummer, A.J. Hollins, A. Gibbs, M. Gumbleton, Constitutive expression of p-glycoprotein in normal lung alveolar epithelium and functionality in primary alveolar epithelial cultures, J. Pharmacol. Exp. Ther. 304 (2003) 441–452.

[42] G.L. Scheffer, A.C. Pijnenborg, E.F. Smit, M. Müller, D.S. Postma, W. Timens, P. van der Valk, E.G. de Vries, R.J. Scheper, Multidrug resistance related molecules in human and murine lung, J. Clin. Pathol. 55 (2002) 332–339.

[43] O. Legrand, G. Simonin, J.Y. Perrot, R. Zittoun, J.P. Marie, Pgp and MRP activities using calcein-AM are prognostic factors in adult acute myeloid leukemia patients, Blood 91 (1998) 4480–4488.

[44] N.J. Combates, P.O. Kwon, R.W. Rzepka, D. Cohen, Involvement of the transcription factor NF-IL6 in phorbol ester induction of P-glycoprotein in U937 cells, Cell Growth Differ. 8 (1997) 213–219.

[45] J.E. Crowther, L.S. Schlesinger, Endocytic pathway for surfactant protein A in human macrophages: binding, clathrin-mediated uptake, and trafficking through the endolysosomal pathway, Am. J. Physiol. Lung Cell Mol. Physiol. 290 (2006) L334–L342.

[46] C. Moulakakis, C. Stamme, Role of clathrin-mediated endocytosis of surfactant protein A by alveolar macrophages in intracellular signaling, Am. J. Physiol. Lung Cell Mol. Physiol. 296 (2009) L430–L441.

[47] A.I. Ivanov, Pharmacological inhibition of endocytic pathways: is it specific enough to be useful?, Methods Mol Biol. 440 (2008) 15–33.

[48] S.R. Bates, C. Dodia, J.Q. Tao, A.B. Fisher, Surfactant protein-A plays an important role in lung surfactant clearance: evidence using the surfactant protein-A gene-targeted mouse, Am. J. Physiol. Lung Cell Mol. Physiol. 294 (2008) L325–L333.

[49] Z.C. Chroneos, R. Abdolrasulnia, J.A. Whitsett, W.R. Rice, V.L. Shepherd, Purification of a cell-surface receptor for surfactant protein A, J. Biol. Chem. 271 (1996) 16375–16383.

[50] P. Borron, F.X. McCormack, B.M. Elhalwagi, Z.C. Chroneos, J.F. Lewis, S. Zhu, J.R. Wright, V.L. Shepherd, F. Possmayer, K. Inchley, L.J. Fraher, Surfactant protein A inhibits T cell proliferation via its collagen-like tail and a 210-kDa receptor, Am. J. Physiol. 275 (1998) L679–L686.

[51] C. Casals, V. Varela, M.L. Ruano, F. Valino, J. Perez-Gil, N. Torre, E. Jorge, F. Tendillo, J.L. Castillo-Olivares, Increase of C-reactive protein and decrease of surfactant protein A in surfactant after lung transplantation, Am. J. Respir. Crit. Care Med. 157 (1998) 43–49.