Surfactant protein A prevents IFN-γ/IFN-γR interaction and attenuates classical activation of human alveolar macrophages (1)

Carlos M. Minutti*†#, Belén García-Fojeda*†#, Alejandra Sáenz*†, Mateo de las Casas-Engel‡, Raquel Guillamat-Prats†¶, Alba de Lorenzo*, Anna Serrano-Mollar†¶, Ángel L. Corbí‡, and Cristina Casals*†§

*Departamento de Bioquímica y Biología Molecular I; Universidad Complutense de Madrid; 28040 Madrid, Spain.

†Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBERES); Instituto de Salud Carlos III, 28029 Madrid, Spain.

‡Centro de Investigaciones Biológicas; Consejo Superior de Investigaciones Científicas, 28040 Madrid, Spain.

¶Departamento de Patología Experimental; Instituto de Investigaciones Biomédicas de Barcelona; Consejo Superior de Investigaciones Científicas; 08036 Barcelona, Spain.

Running title: **SP-A binding to IFN-γ**

§Corresponding Author:

Cristina Casals, Ph.D.

Department of Biochemistry and Molecular Biology I; Faculty of Biology; Complutense University of Madrid; 28040 Madrid, Spain
Tel.: 34-913944261- Cell Phone: 34-670492968; Fax: 34-913944672
E-mail: ccasalsc@ucm.es

\# *These authors contributed equally*.

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ABSTRACT[2]

Lung surfactant protein A (SP-A) plays an important function in modulating inflammation in the lung. However, the exact role of SP-A and the mechanism by which SP-A affects IFN-γ-induced activation of alveolar macrophages remains unknown. To address these questions, we studied the effect of human SP-A on rat and human alveolar macrophages stimulated with IFN-γ, LPS, and combinations thereof, and measured the induction of pro-inflammatory mediators, as well as SP-A´s ability to bind to IFN-γ or IFN-γR1. We found that SP-A inhibited [IFN-γ+LPS]-induced TNF-α, iNOS, and CXCL10 production by rat alveolar macrophages. When rat macrophages were stimulated with LPS and IFN-γ separately, SP-A inhibited both LPS-induced signaling and IFN-γ-elicited STAT1 phosphorylation. SP-A also decreased TNF-α and CXCL10 secretion by *ex vivo* cultured human alveolar macrophages and M-CSF-derived macrophages stimulated by either LPS, IFN-γ, or both. Hence, SP-A inhibited up-regulation of IFN-γ-inducible genes (*CXCL10, RARRES3,* and *ETV7*) as well as STAT1 phosphorylation in human M-CSF-derived macrophages. In addition, we found that SP-A bound to human IFN-γ (K_D =11 ± 0.5 nM) in a Ca^{2+}-dependent manner and prevented IFN-γ interaction with IFN-γR1 on human alveolar macrophages. We conclude that SP-A inhibition of [IFN-γ+LPS]-stimulation is due to SP-A attenuation of both inflammatory agents and that the binding of SP-A to IFN-γ abrogates IFN-γ effects on human macrophages, suppressing their classical activation and subsequent inflammatory response.

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[2] **Abbreviations: aMϕs:** Alveolar macrophages; **CXCL10**: C-X-C motif chemokine 10; **ETV7:** Transcription factor ETV7; **IFN-γR1:** Interferon gamma receptor 1 or CD119; **Mϕ(M-CSF)**: human M-CSF-derived macrophages; **sIFN-γR1:** soluble fraction of interferon gamma receptor 1; **SP-A:** Surfactant Protein A; **RARRES3:** Retinoic acid receptor responder 3.

INTRODUCTION

Pulmonary surfactant is a lipoprotein complex that lines the alveolar surface. Its main function is to reduce alveolar surface tension (1); however, it also functions as a modulator of immune responses. The two principal surfactant components involved in innate immunity in the alveolus are surfactant proteins A (SP-A) and D (SP-D) (2).

SP-A is an oligomeric extracellular protein that is found mainly in the alveolar fluid, associated with surfactant extracellular membranes that line the alveolar epithelium and with alveolar cells. SP-A recognizes pathogen-associated molecular patterns on some microorganisms, resulting in aggregation, opsonization, or permeabilization of microorganisms and facilitation of microbial clearance (2,3). Moreover, SP-A is also able to bind to membrane receptors present in macrophages, epithelial cells, and lymphocytes, modifying their response to different stimuli (2). It has been reported that SP-A-deficient mice show decreased microbe clearance from the alveolar space and increased tissue markers of inflammation (4). These findings make SP-A's protective role in alveolar immune defense evident.

The major effector cells of innate immunity in the alveolus are the alveolar macrophages (aMφs) that constitute a unique class of professional phagocytes (5). Macrophages change their phenotype reversibly in response to stimuli. This process is called macrophage activation, which varies from classical activation to alternative activation (6,7). The term ''classical activation" refers to macrophages stimulated with IFN-γ (host factor) and pathogen products (e.g., TLR agonists such as LPS) (6,7). IFN-γ is the main cytokine associated with classical activation of macrophages. It is mainly produced by T_H1 and natural killer cells and exerts its effects through interactions with its IFN-γ receptor complex, composed of the IFNγR1 and IFNγR2 chains, whose cytoplasmic tails are associated with JAK1 and JAK2 kinases respectively. IFN-γ binds to IFNγR1 with high affinity and activates crosslinking of two molecules of each IFNγR1 and IFNγR2 chain, which results in activation of tyrosine kinases JAK1 and JAK2 and phosphorylation of STAT1. Activated P-STAT1 translocates to the nucleus, where it mediates the transcription of IFN-γ-induced genes such as CXCL10 or IRF transcription factors, among others (7,8). On the other hand, the transmembrane TLR4 serves as the primary mediator of LPS signaling, which leads to activation of NF-κB, MAPK, AP-1, IRFs, and early growth response family members, many of which participate in the IFN-γ response (9). LPS-stimulated macrophages produce pro-inflammatory molecules such as TNF-α, IL-1β, and CXCL10, among others (7). IFN-γ can 'prime' macrophages to give an enhanced response to TLR ligands, such as LPS (10,11). Synergy between IFN-γ and LPS occurs at multiple levels, ranging from signal recognition to convergence of signals at the promoters of target genes (11). Thus the presence of both IFN-γ and TLR ligands induces resting macrophages to rapidly acquire a set of effector functions (production of inflammatory cytokines, chemokines, and reactive oxygen species) that contribute to microbial clearance.

SP-A has been reported to inhibit LPS-induced signaling (p-IκBα, p-ERK, p-p38, and p-Akt) in human monocyte-derived macrophages (12) and to increase expression of the negative regulators of LPS-induced signaling in murine (13) and human monocyte-derived macrophages (14). SP-A also inhibited the production of pro-inflammatory mediators by human (15) and rat (16,17) aMϕs, human monocyte-derived macrophages (18), and human macrophage-like U937 cells (16, 19-21) stimulated with LPS and other TLR ligands. Moreover, SP-A has been reported to modulate aMϕs responses to IFN-γ in the absence or presence of LPS or pathogens, but contradictory observations have also been made. For example, Stamme et al. (22) have shown that SP-A enhances production of NO and iNOS in rat aMϕs stimulated with IFN-γ or IFN-γ plus LPS. In contrast, other studies indicate that SP-A suppresses NO production by murine aMϕs stimulated with IFN-γ– and IFN-γ plus *M. avium* (23) or IFN-γ plus *M. tuberculosis* (24).

To understand the modulatory effects of SP-A on the response of aMϕs to IFN-γ and IFN-γ+LPS stimuli [Mϕ(LPS/IFN-γ)], we studied the effect of SP-A on IFN-γ-stimulated human and rat alveolar macrophages and human M-CSF-derived macrophages (Mϕ(M-CSF)) in the absence or presence of LPS. Our data show that SP-A reduces IFN-γ-triggered inflammation in rat and human aMϕs and human Mϕ(M-CSF). SP-A inhibition of LPS/IFN-γ-induced macrophage stimulation is due to SP-A attenuation of both inflammatory agents. Our findings show, for the first time, that SP-A binds to IFN-γ preventing IFN-γ interaction with IFN-γR1 on the cell surface. We conclude that this could be one of the mechanisms by which SP-A attenuates IFN-γ effects.

MATERIALS AND METHODS

Isolation, purification and characterization of human SP-A

Surfactant protein A was isolated from bronchoalveolar lavage of patients with alveolar proteinosis using the sequential butanol and octylglucoside extraction (25,26). Endotoxin content of isolated human SP-A was about 300 pg endotoxin/mg SP-A as determined by Limulus amebocyte lysate assay (Lonza, Basel, Switzerland). The purity of SP-A was checked by one-dimensional SDS-PAGE in 12 % acrylamide under reducing conditions and mass spectrometry. SP-A consisted of supratrimeric oligomers of at least 18 subunits (MW, 650 KDa). The oligomerization state of SP-A was assessed by electrophoresis under nondenaturing conditions (25,26), electron microscopy (26), and analytical ultracentrifugation as reported elsewhere (25). Each subunit had an apparent molecular weight of 36 kDa. Biotinylated SP-A was prepared using the Mini-biotin-XX protein labeling kit (Invitrogen, Carlsbad, CA) as previously described (27). The structure and functional activity of biotinylated SP-A was similar to that of unlabeled SP-A.

Animal handling and human lung tissue procurement

Rat aMϕs were obtained from Sprague Dawley male rat lungs. Rats (approximately 350 g) were anesthetized with Ketamine (Merial, Duluth, Georgia) (80 mg/kg) and Xylazine (Bayer, Leverkusen, Germany) (10 mg/kg). The cardiopulmonary block was extracted to perform bronchoalveolar lavages with PBS, 0.2 mM EDTA. All animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals (28) and Spanish guidelines for experimental animals.

As a source of human lung tissue we used multiple organ donors. The review board and the ethics committee of the Hospital Clinic of Barcelona, as well as the Spanish and Catalan Transplant Organizations, approved this study, which was conducted in accordance with the guidelines of the World Medical Association's Declaration of Helsinki. Donors with recent history of tobacco smoking, obesity or any radiological pulmonary infiltrate were excluded from this study. Immediately after obtaining the lungs, we performed a bronchoalveolar lavage at 4°C using 1 liter of 0.9 % NaCl to isolate human aMϕs.

Isolation and culture of primary alveolar macrophages

Bronchoalveolar cells were separated from lavage fluid by centrifugation (250 x g, 7 min). The sedimented cells were washed twice with PBS and the cell pellet resuspended in RPMI 1640 medium (10 % heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, supplemented with glutamine 2 mM) (Lonza). Human and rat aMϕs were purified by adherence for 90 min at 37°C under a 95% air-5 % CO_2 atmosphere in 150-cm^2 culture flasks as previously reported (29,30). Adherent cells were 94.0 ± 1.1 % viable (trypan blue exclusion test). To evaluate the purity of the isolated human macrophages, cells were cytospun in a CytoSpin 3 Cytocentrifuge (Shandon Scientific Ltd, Waltham, Massachusetts) and the cytospin preparations were stained by Diff-Quick kit (Diagnostics Grifols S. A., Barcelona, Spain) following the manufacturer's protocol. Four fields of each sample were counted. Adherent cells were found to be composed of 94.8 ± 0.8 % of aMϕs. On the other hand, flow cytometry analysis of rat macrophages immunostained with anti-CD11c (AbD Serotec, Kidlington, UK) confirmed the purity of rat aMϕ preparations.

Incubation Conditions

Adherent cells were gently scraped, plated in 96-well plastic dishes (7.5 x 10^4 cells per well) in 0.2 ml of RPMI with 5 % FBS and precultured overnight. Cells were incubated for another 24 h in the presence or absence of smooth LPS (*Escherichia coli* 055:B5, 1 ng/ml) (Sigma, St. Louis, Missouri), either rat or human recombinant IFN-γ (Calbiochem, Darmstadt, Germany) (0.05-10 ng/ml), human SP-A (5, 12.5, 25 and 50 µg/ml), and combinations thereof. Higher doses of both LPS (10-100 ng/ml) and IFN-γ (100 ng/ml) were also assayed. At the SP-A concentrations used the effect of SP-A was greater at lower doses of LPS and/or IFN-γ than at higher doses. Cell viability was higher than 97 % under assay conditions. Macrophage cultures were plated in triplicate wells, and each series of experiments was repeated at least three times.

Generation of human M-CSF-derived macrophages

Human PBMC were isolated from buffy coats from normal donors over a lymphoprep gradient (Nycomed Pharma, Oslo, Norway), according to standard procedures. Monocytes were purified from PBMC by magnetic cell sorting using CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes (95 % $CD14^+$ cells) were cultured at 0.5 x 10^6 cells/ml for 7 days in RPMI 1640 supplemented with 10 % FBS at 37°C in a humidified atmosphere with 5 % CO^2, and containing M-CSF (ImmunoTools, Friesoythe, Germany) (10 ng/ml) to generate M-CSF-derived macrophages as previously described (31). Cytokines were added every 2 days. Cells were treated with human recombinant IFN-γ (1 ng/ml), human SP-A (50 μg/ml), and combinations thereof on the 8th day. Cultures were plated in triplicate wells, and each series of experiments was repeated at least three times.

Cytokine determinations

Secreted cytokines were quantified in supernatants of treated human and rat aMϕs using specific ELISA kits following the supplier's instructions. Rat and human TNF-α and human CXCL10 ELISA kits were purchased from BD Biosciences (San Diego, CA) and rat CXCL10 from Peprotech (Rocky Hill, NJ). In brief, antibodies were coated on a 96-well Nunc-Immuno Plate MaxiSorp Surface (Thermo Scientific, Waltham, Massachusetts) in 0.1 M sodium carbonate, pH 9.5, overnight. After blocking with PBS, 10% FBS, and extensive washing, samples and standards were incubated for two hours at room temperature. Cytokines were detected with biotinylated detection antibodies and streptavidin-horseradish peroxidase. The colorimetric reaction was developed with tetramethylbenzidine (BD Biosciences) and was stopped with 4 M sulfuric acid (Sigma), and the absorbance at 450 nm was read on an ELISA reader (DigiScan; Asys HiTech GmbH, Eugendorf, Austria).

Western blot analysis

Cells were lysed with three freezing-thawing cycles in a buffer containing: 10 mM HEPES, pH 7.9, 15 mM MgCl, 10 mM KCl, 0.5 mM EDTA, 0.2 % Triton X-100, 1 mM benzamidine, 200 μg/ml aprotinin, 200 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (Sigma). When phosphorylated proteins were analyzed, phosphatase inhibitors were added to the buffer: 20 mM β-glycerophosphate, 10 mM NaF, 10 mM sodium pyrophosphate, and 2 mM orthovanadate (Sigma). Samples were resolved by SDS-PAGE in reducing conditions and transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, California). After blocking with 2.5 % skim milk, membranes were washed in PBS, 0.1% tween, and incubated with anti-iNOS, GAPDH, P-ERK, total ERK, P-STAT1, and total STAT1 (Cell Signaling, Danvers, Massachusetts). Membranes were incubated with horseradish-peroxidase-labeled anti-rabbit IgG (Sigma), then washed and exposed to ECL reagents (Merk Millipore, Darmstadt, Germany). Immunoreactive bands were densitometered (Quantity One Software, BioRad) and then normalized to GAPDH for iNOS quantification and to the respective total protein for quantification of phosphorylated proteins.

Quantitative real-time RT-PCR

Total RNA was extracted using an RNeasy kit from Quiagen (Venlo, Netherlands), retrotranscribed and amplified in triplicates with the reverse transcription system kit (Applied Biosystems, Waltham, Massachusetts) (32). Oligonucleotides for selected genes were designed according to the Universal Probe Library system (Roche Diagnostics, Rotkreuz, Switzerland) for quantitative real-time PCR (qRT-PCR). The analyses for selected genes were made by lightcycler 480 (Roche). For quantification of selected genes, the next oligonucleotides were used: 5´-aagcagttagcaaggaaaggtc-3´ and 5´-gacatatactccatgtagggaagtga-3´ for *CXCL10*, 5´-gacccagggggtctgttc-3´ and 5´-aaggagcagctgatacacgtaa-3´ for *ETV7*, 5´-ctcctcttggcttcgagatg-3´ and 5´-aaggcggaaaatctcaatca-3´ for *RARRES3*, and 5´-agccacatcgctcagacac-3´ and 5´-gccaatacgaccaaatcc-3´ for *GAPDH*. Levels of mRNA were quantitated using ΔΔCt method (33) and were then normalized to maximal expression levels obtained in the presence of IFN-γ.

Solid-phase binding assays

Solid-phase binding assays were performed as previously described (27) with minor modifications. Either rat IFN-γ, human IFN-γ, human sIFN-γR1 (R&D, Minneapolis, MN), or human serum albumin (HSA) (Sigma) (1 µg per well) was coated on a 96-well maxisorp microtiter plate in 0.1 mM sodium bicarbonate buffer, pH 9.5, overnight at 4°C. The wells were washed three times with buffer A (5 mM Tris-HCl, pH 7.4, containing 150 mM NaCl) with 0.1 mM EDTA. Wells were blocked with buffer A with 0.1 mM EDTA containing 5 % skim milk for 2h. After the plate was washed, biotinylated SP-A, in concentrations ranging from 0 to 470 nM (0 to 333 μg/ml), was added to the wells in buffer A in the presence or absence of 2 mM $CaCl_2$. Incubations were performed for 1 h at RT. After extensive washing, streptavidin-horseradish peroxidase (Sigma) was added to the wells. To assay the inhibition of the IFN-γ/sIFN-γR1 interaction by SP-A the mixture of IFN-γ (0.1 μg/ml) and SP-A (in concentrations ranging from 0 to 160 μg/ml) was added to the sIFN-γR1-coated wells in buffer A, with or without 2 mM $CaCl_2$, and incubated at RT for 1 h. The binding of IFN-γ to sIFN-γR1 was detected using a polyclonal anti-human IFN-γ (Abcam, Cambridge, UK) and horseradish peroxidase-conjugated anti-rabbit antibody. The binding of either biotin-labeled SP-A or anti-human IFN-γ was detected with tetramethylbenzidine. The colorimetric reaction was stopped with 4 M sulfuric acid, and the absorbance was read at 490 nm on an ELISA reader.

Dynamic light scattering

The hydrodynamic diameters of human and rat IFN-γ, human sIFN-γR1, and human SP-A, as well as mixtures of these components, were determined at 25°C in a Zetasizer Nano S (Malvern Instruments, Malvern, UK) equipped with a 633-nm HeNe laser as previously reported (3, 27). Six scans were performed for each sample, and all of the samples were analyzed in triplicate. The interaction of SP-A with IFN-γ in solution was measured by the addition of different SP-A concentrations (from 0 to 150 nM; 0-100 μg/ml) to 322 nM (7.7 μg/ml) IFN-γ in buffer A in the absence and presence of 175 μM or 2.5 mM $CaCl_2$.

Binding of [^{125}I]-IFN-γ to IFN-γR1 at the cell surface of human alveolar macrophages and inhibition by SP-A

Recombinant human IFN-γ was labeled with [^{125}I]-Bolton Hunter reagent (PerkinElmer, Waltham, Massachusetts) as described previously (34). In brief, 10 μg of human IFN-γ in 10 μl of 0.1 M sodium borate buffer, pH 8.5, was added to 0.7 mCi of [^{125}I]-Bolton Hunter reagent, and the reaction mixture was agitated for 15 minutes at RT. The reaction was stopped by the addition of 0.5 ml of 0.2 M glycine in 0.1 M borate buffer, pH 8.5 for 5 minutes at RT. Free-iodine was separated from the labeled protein using disposable PD-10 desalting columns (GE Healthcare, Waukesha, WI) according to manufacturer's instructions. The concentration of the labeled IFN-γ was determined by sandwich-type ELISA by using a polyclonal anti-human IFN-γ, a horseradish peroxidase-conjugated anti-rabbit antibody and tetramethylbenzidine for color detection.

Human aMϕs were plated in 96-well plates (1 x 10^5 cells per well) in 0.2 ml of RPMI containing 5% FBS and primed overnight with LPS (1 ng/ml) (37°C, under 5 % CO_2 atmosphere). Control wells with no cells were coated with the same medium supplemented with 5% HSA. Subsequently, the cells were washed with PBS. Wells were blocked with PBS plus 0.5% HSA for 30 minutes at 4ºC. After the plate was washed with PBS, [^{125}I]-IFN-γ (10 ng/ml) and SP-A (in concentrations ranging from 0 to 100 µg/ml) were added to the cells in RPMI either with 2.5 mM $CaCl_2$ or with 5 mM EDTA. Incubations were performed for 2 h at 4ºC. After extensive washing, the cells were lysed with 10% SDS, and radioactivity was counted with a Perkin Elmer Wallac Wizard 1470-020 Gamma Counter. To confirm that the detected [^{125}I]-IFN-γ bound specifically to its receptor at the cellular surface, we used a mouse mAb (BD Biosciences) (50 µg/ml), which recognizes the region of human IFN-γR1 that binds to IFN-γ, to inhibit IFN-γ interaction with its receptor (35). An irrelevant, isotype-matched mouse IgG1 mAb (AbD Serotec) was used as a control for these experiments.

Statistics

Data are presented as means ± SEM. Differences in means between groups were evaluated by one-way ANOVA followed by the Bonferroni multiple-comparison test. An α level $\leq$ 5% ($P \leq 0.05$) was considered significant.

RESULTS

SP-A inhibits LPS and IFN-γ effects on rat alveolar macrophages

To determine the effects of SP-A on the classical activation of aMϕs, primary aMϕs isolated form rat lungs were activated with LPS and/or IFN-γ in the absence and presence of SP-A. Figures 1A and 1B show that LPS induced significant TNF-α secretion and iNOS expression in rat aMϕs, and that the induction of both factors was even greater when macrophages were simultaneously incubated with LPS plus IFN-γ. This indicates that IFN-γ treatment results in efficient priming/amplification of TLR4-induced cellular responses. However, IFN-γ alone did not induce TNF-α secretion and iNOS expression in rat aMϕs, which is contrary to previous results in RAW 264.7 peritoneal macrophages cell line (36), where IFN-γ up-regulates TNF-α and iNOS production.

When different concentrations of SP-A were tested (5, 12.5, and 25 µg/ml), we found that SP-A significantly inhibited, in a dose-dependent manner, TNF-α secretion (Figure 1A) and iNOS production (Figure 1B) by rat aMϕs stimulated with either LPS alone or LPS plus IFN-γ. The effect of SP-A on LPS-stimulated rat aMϕs is consistent with previous results (15,16,19,20). In line with previous findings (12), SP-A also significantly inhibited LPS-induced phosphorylation of ERK1/2 (T202/Y204) in a dose-dependent manner (Figure 2A and B), whereas ERK activation was not significantly induced in the presence of either SP-A alone or IFN-γ (Figure 2A and B).

SP-A attenuates IFN-γ effects on rat alveolar macrophages

IFN-γ signaling is mediated by the cytosolic factor STAT1 that is activated during IFN-γ-dependent JAK-STAT activation (11). STAT1 is phosphorylated at two sites (tyrosine 701 and serine 727) following IFN-γ exposure (11). To determine whether the inhibitory effect of SP-A on aMϕs stimulated with LPS plus IFN-γ was also due to SP-A attenuation of IFN-γ, we measured the effect of SP-A on IFN-γ-induced STAT1 Y701 phosphorylation and CXCL10 secretion by rat aMϕs (Figure 3).

We found that SP-A dose-dependently inhibited IFN-γ–stimulated CXCL10 secretion (Figure 3A) and STAT1 Y701 phosphorylation (Figure 3B, C) by rat aMϕs. LPS alone had no effect on STAT1 Y701 phosphorylation after 30 minutes of LPS-stimulation (Figure 3B, D). This was expected since S727, but not Y701, is phosphorylated by p38 MAPK activated by TLR agonists (11). However, LPS alone induced CXCL10 secretion (137 ± 15 pg/ml) by rat aMϕs, and simultaneous stimulation with LPS plus IFN-γ induced higher CXCL10 secretion than with IFN-γ alone (305 ± 12 pg/ml and 172 ± 12 pg/ml, respectively) (Figure 3A). The CXCL10 promoter contains response elements for STAT1, NF-kB, and AP-1 (11), which would explain why aMϕs produce CXCL10 after stimulation with IFN-γ and/or LPS. Secretion of CXCL10 induced by LPS, IFN-γ, or LPS plus IFN-γ was also inhibited by SP-A in a dose-dependent manner (Figure 3A), consistent with the results obtained with TNF-α and iNOS described above. Together, these data demonstrate that SP-A inhibition of [IFN-γ+LPS]-stimulation is due to SP-A attenuation of both inflammatory agents.

SP-A inhibits LPS and IFN-γ effects on *ex vivo* cultured human alveolar macrophages

Given the above set of data on rat aMϕs, we sought to extend our findings to the case of primary human aMϕs. Figure 4A shows that SP-A inhibited TNF-α secretion by human aMϕs stimulated with LPS alone or LPS plus IFN-γ in a dose-dependent manner. Similarly to the observations using rat aMϕs presented above, IFN-γ itself was not capable of significantly stimulating TNF-α secretion. However, IFN-γ increased the levels of LPS-induced TNF-α secretion by human aMϕs, as previously reported (29), and this further increase was significantly impaired by SP-A. Therefore, similar to the case of rat aMϕs, SP-A is capable of limiting the production of pro-inflammatory cytokines by human aMϕs exposed to the classical activation stimuli LPS+IFN-γ.

Moreover, as shown in Figure 4B, SP-A dose-dependently inhibited IFN-γ-induced CXCL10 secretion by *ex vivo* cultured human aMϕs. Simultaneous stimulation with IFN-γ and LPS induced higher CXCL10 secretion than with IFN-γ alone (367 ± 36 pg/ml and 268 ± 16 pg/ml, respectively), and SP-A also inhibited [LPS+ IFN-γ]-induced CXCL10 secretion (Figure 4B). On the other hand, iNOS protein did not increase in response to

LPS or LPS plus IFN-γ in human aMϕs (Supplementary Figure 1). This was expected since little to no iNOS and NO have been detected in human macrophages obtained from normal donors (15, 37), mainly due to epigenetic silencing of NOS2 (38).

SP-A inhibits IFN-γ effects on human monocyte-derived macrophages Mϕ(M-CSF)

We then used human monocyte M-CSF-derived macrophages [human Mϕ(M-CSF)]. Alveolar macrophages show phenotypical features of anti-inflammatory macrophages (39) and M-CSF prime monocytes to differentiate into anti-inflammatory macrophages (40). As in the case of *ex vivo* cultured human aMϕs, SP-A inhibited TNF-α and CXCL10 secretion by Mϕ(M-CSF) stimulated by LPS, IFN-γ, and LPS+IFN-γ (Supplementary Figures 2A and B). Human Mϕ(M-CSF) stimulated with IFN-γ and/or LPS led to higher CXCL10 secretion (21.7 ± 1.5 ng/ml, 17.4 ± 2.9 ng/ml, and 13.1 ± 1.7 ng/ml in response to LPS+IFN-γ, IFN-γ, and LPS, respectively) than *ex vivo* cultured human aMϕs (367 ± 36 pg/ml, 268 ± 16 pg/ml, and 164 ± 7 in response to LPS+IFN-γ, IFN-γ, and LPS, respectively).

CXCL10 is one of the paradigmatic genes induced by IFN-γ (11). To find out whether SP-A affected other IFN-γ-regulated genes, we evaluated the effect of SP-A on the IFN-γ–dependent gene expression in human Mϕ(M-CSF). To that end, human Mϕ(M-CSF) were stimulated with IFN-γ in the presence and absence of SP-A, and IFN-γ-induced genes were analyzed by quantitative real-time RT-PCR. Figure 4C shows that IFN-γ up-regulated the expression of *CXCL10* (fold change 65 ± 6), *RARRES3* (fold change 34 ± 13) identified as a retinoic acid responder gene (41), and *ETV7* (fold change 177 ± 43) (also known as the human ETS family gene TEL2/ETV7, which promotes proliferation and has a role in oncogenesis (42)). Importantly, SP-A inhibited the IFN-γ-induced expression of *CXCL10*, *RARRES3,* and *ETV7* genes by 49 %, 85 %, and 70 %, respectively (Figure 4C). Moreover, we found that SP-A inhibited IFN-γ–stimulated STAT1 Y701 phosphorylation in human Mϕ(M-CSF) (Figure 4D). Together, these findings demonstrate that SP-A also suppressed IFN-γ effects in the absence and presence of LPS on human Mϕ(M-CSF).

SP-A binds to IFN-γ and prevents IFN-γ binding to its receptor on human alveolar macrophages

To determine the mechanism by which SP-A diminishes IFN-γ effects on rat and human aMϕs as well as human Mϕ(M-CSF), we first studied the potential interaction between SP-A and IFN-γ in a solid phase binding assay. Figure 5A shows that biotinylated SP-A bound to human IFN-γ-coated wells in a dose- and Ca^{2+}-dependent manner, with K_D =11 ± 0.5 nM. Biotinylated SP-A bound neither to IFN-γ-coated wells in the absence of Ca^{2+}, nor to wells coated with human serum albumin, nor to wells containing buffer alone, regardless of the presence of Ca^{2+}. Biotinylated SP-A also bound to rat IFN-γ in a dose- and Ca^{2+}-dependent manner with K_D = 28 ± 4 nM (data not shown). Figure 5B shows that the percentage of biotinylated SP-A binding to human IFN-γ-coated wells decreased by addition of unmodified SP-A, indicating that binding of biotinylated SP-A to IFN-γ-coated wells was not due to the biotin moiety.

In addition, the interaction of SP-A with human and rat IFN-γ was examined in solution by dynamic light scattering. Figure 5C (left panel) shows that human IFN-γ alone displays a major peak, which corresponds to particles with a hydrodynamic diameter of 10.5 ± 0.5 nm. The hydrodynamic diameter of human IFN-γ determined in these experiments approaches the predicted diameter of 7 ± 1 nm, which does not take into account water molecules enclosing the protein in solution (43). In the case of human SP-A, two identifiable peaks were recognized for SP-A alone, one corresponding to SP-A particles with a hydrodynamic diameter of 38 ± 5 nm and another minor peak corresponding to SP-A aggregates with a hydrodynamic diameter of 1000 nm (data not shown). Self-aggregation of SP-A occurs in a Ca^{2+}- and NaCl-dependent manner (44). To reduce SP-A self-aggregation, experiments were performed in the presence of 175 μM $CaCl_2$.

Figure 5C (central panel) shows that the addition of increasing concentrations of SP-A (ranging from 0 to 120 nM; 0-80 μg/ml) to an IFN-γ solution (322 nM) containing calcium caused an SP-A concentration-dependent decrease of the IFN-γ peak. At an IFN-γ/SP-A molar ratio of approximately 3:1, only one peak (58 nm) was observed with a hydrodynamic diameter higher than those of SP-A and IFN-γ alone. This new peak likely consists of SP-A/IFN-γ complexes. This peak did not appear in the absence of Ca^{2+}

(Figure 5C, right panel). Similar results were obtained with rat IFN-γ (data not shown). Therefore, this set of experiments demonstrates that SP-A and IFN-γ interact in solution in a Ca^{2+}-dependent manner.

To clarify whether SP-A has a direct effect on IFN-γ-induced cell response by interacting not only with IFN-γ but also with its cellular receptor, the extracellular domain of IFN-γR1 (sIFN-γR1) was coated onto microtiter plate wells. Biotinylated SP-A was then added to the wells, and the level of bound SP-A was determined with streptavidin-horseradish peroxidase. No detectable binding of SP-A to sIFN-γR1 was observed (Figure 6A). In addition, no detectable SP-A/sIFN-γR1 interaction was observed in solution when examined by dynamic light scattering (Figure 6B).

To determine whether SP-A interferes with the binding of IFN-γ to its receptor, IFN-γ binding to coated sIFN-γR1 was determined by ELISA using a polyclonal anti-human IFN-γ and in the presence and absence of SP-A (Figure 6C). We found that SP-A interfered with the binding of IFN-γ to IFN-γR1 in the presence but not the absence of Ca^{2+}. The inhibition caused by SP-A was saturable and dose- and Ca^{2+}-dependent, with a ~80 % inhibition at concentrations equal to or higher than 20 μg/ml (30 nM). As expected, the binding of IFN-γ to its receptor IFN-γR1 was not affected by Ca^{2+} (data not shown).

We next examined whether SP-A could effectively inhibit the binding of IFN-γ to IFN-γR1 on the cell surface. To achieve this, we analyzed the binding of [^{125}I]-IFN-γ to IFN-γR1 on human aMϕs. To confirm that the detected [^{125}I]-IFN-γ bound specifically to its receptor at the cellular surface, we used a monoclonal antibody (mouse anti-human CD119) that recognizes the region of IFN-γR1 that binds to IFN-γ to block [^{125}I]-IFN-γ binding to its receptor. Figure 6D shows that, in the absence of SP-A, [^{125}I]-IFN-γ bound to human aMϕs and that this binding was specifically abrogated by the blocking antibody. Conversely, an isotype-matched mouse IgG1 control did not inhibit [^{125}I]-IFN-γ binding to human aMϕs (data not shown). However, addition of SP-A clearly inhibited the [^{125}I]-IFN-γ binding to IFN-γR1 in a dose-dependent manner in the presence but not absence of calcium. Similar results were found on rat aMϕs. Therefore, our results indicate that SP-A impairs IFN-γ recognition by IFN-γR1 on the cell surface. This could be at least one of the mechanisms by which SP-A suppresses IFN-γ effects.

DISCUSSION

Innate immune defense in the alveolar space is characterized by a delicate balance between an effective inflammatory response and the maintenance of tissue integrity. Alveolar macrophages play a major role in this equilibrium by producing and releasing a variety of biologically active products in response to stimuli such as LPS and IFN-γ (5,39). Macrophage activation and the initiation of inflammation involve a complex balancing act between activating and repressing signals. The results of the present study show that SP-A has an anti-inflammatory effect on rat and human aMϕs and human Mϕ(M-CSF), counteracting the stimulation exerted by IFN-γ or LPS+IFN-γ.

In this study we have shown that SP-A, used at concentrations within the ranges found in healthy individuals (15-105 μg/mL) (45), inhibited the production of pro-inflammatory molecules such as TNF-α and CXCL10 by rat and human aMϕs and human Mϕ(M-CSF) stimulated with IFN-γ, LPS, and LPS+IFN-γ. SP-A also inhibited iNOS production by rat aMϕs stimulated with LPS and LPS+IFN-γ. iNOS production by stimulated human aMϕ was not detected, confirming previous results that indicate that human aMϕ and blood-monocyte-derived macrophages obtained from normal donors and stimulated *in vitro* generally do not express iNOS (15, 37, 38). SP-A also inhibited IFN-γ-induced STAT1 phosphorylation in rat aMϕs and human Mϕ(M-CSF) and inhibited up-regulation of IFN-γ-inducible genes (*CLCX10*, *RARRES3,* and *ETV7*) by human Mϕ(M-CSF). In addition, we have shown for the first time that human SP-A bound to IFN-γ with high affinity (K_D = 11 ± 0.5 nM for human IFN-γ and K_D = 28 ± 4nM for rat IFN-γ) and prevented IFN-γ interaction with its receptor IFN-γR1 on human aMϕs. These data disclose a novel mechanism by which SP-A controls inflammation in the alveolus.

Our observation that SP-A reduces IFN-γ-triggered inflammation in rat and human aMϕs and human Mϕ(M-CSF) is in agreement with the previous studies that showed that SP-A suppresses NO production by murine alveolar Mϕs stimulated with IFN-γ– and IFN-γ plus M. avium (23) or IFN-γ plus M. tuberculosis (24). However, our results appear to contradict the data from Stamme and colleagues (22) using rat aMϕs. We do not currently have a clear explanation for these contrasting results, although there might be some differences in the experimental design. For instance, starting cell number, media and supplements, different types of plastic, and other conditions such as whether macrophages are rested prior to activation might have substantial effects on activation status. In addition, the source and concentration of cytokines is also important.

Alveolar macrophages reside in a tissue compartment that is constantly exposed to contaminated air. Thus alveolar macrophage activation is tightly controlled through several cell-cell and soluble mediator interactions to limit unwanted inflammatory responses (39). SP-A is one of the soluble factors that contribute to create an anti-inflammatory state in the lungs through various mechanisms. First, SP-A blocks the binding of TLR ligands to their receptors by direct SP-A interaction with TLR4 (21), TLR2 (16), the TLR co-receptor MD2 (21), and CD14 (19,20). Second, SP-A modifies macrophage response to TLR ligands by modulating signaling cascades. For example, SP-A increases the expression of negative regulators of TLR-signaling, such as IRAK-M (14) and β-arrestin 2 (13), thereby inhibiting LPS-induced stimulation of macrophages. Moreover, SP-A promotes PKCζ activation and IκBα stabilization through mechanisms that require SP-A endocytosis by macrophages (17). Internalized SP-A also inhibits IκBα, ERK, p38, and Akt phosphorylation by macrophages stimulated with TLR2 and TLR-4 ligands (12). Third, SP-A reduces the production of reactive oxygen intermediates by inhibiting NADPH oxidase activity in human monocyte-derived macrophages activated by PMA or serum-opsonized zymosan (18). Here we showed that the binding of SP-A to IFN-γ, which suppressed IFN-γ interaction with its receptor IFN-γR at the cell surface, is another mechanism by which SP-A limits inflammation and maintains a tolerant lung environment in the steady state. However, following an infection, a harsh IFN-γ induction would potentially override SP-A capability to block IFN-γ, leading to a desirable inflammatory response to fight against infection. The fact that SP-A is induced in response to IFN-γ (46) suggests that SP-A may also be implicated in the regulation of detrimental inflammation at the resolution phase after infection.

IFN-γ is essential for anti-mycobacterial immunity, and disorders of IFN-γ production confer predisposition to mycobacterial disease in humans (47). However, high levels of secreted IFN-γ may be harmful. It has been shown that IFN-γ and IFN-γ-induced CXCL10 are directly involved in the exacerbation of different lung inflammatory diseases (acute lung injury and bronchiolitis) in murine experimental models and/or humans (48,49). Moreover, IFN-γ causes emphysema and alterations in pulmonary protease/antiprotease balance when expressed in pulmonary tissues (50). Furthermore, the administration of neutralizing antibodies against IFN-γ or CXCL10 has been shown to attenuate lung injury and/or improve mice survival rate (51,52).

The alveolar fluid from normal lungs contains high concentrations of SP-A that probably minimize the biological effects of low concentrations of endotoxins that enter the alveolus and IFN-γ. In patients with acute lung injury in which proinflammatory cytokines and neutrophils accumulate in the air spaces, the concentration of SP-A significantly decreases (53,54). The observation that SP-A restores lung tissue integrity in response to sterile inflammation (55) supports the hypothesis that SP-A may be important in modulating inflammation and epithelial integrity in the lung in response to acute injury.

In summary, we have shown that human SP-A inhibits IFN-γ, LPS, and LPS+IFN-γ effects on rat and human aMϕs and human Mϕ(M-CSF) and that SP-A binds to IFN-γ with high affinity, inhibiting IFN-γ recognition by its receptor on the cell surface. These data unravel a previously unknown mechanism by which SP-A/IFN-γ interaction plays a significant role in tipping the balance of inflammation to protect the alveolar epithelium.

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FIGURE LEGENDS

Figure 1. Inhibitory effect of SP-A on TNF-α and iNOS production by rat alveolar macrophages stimulated with IFN-γ and/or LPS. Purified rat aMϕs were cultured in the presence or absence of IFN-γ (10 ng/ml), LPS (1 ng/ml), SP-A (5, 12.5, 25 µg/ml), and combinations thereof. We measured TNF-α secretion by ELISA (A) and iNOS production by Western blot (B) after 24 hours of IFN-γ and/or LPS-stimulation. The results are expressed as percent of LPS plus IFN-γ-stimulated TNF-α and iNOS production in the absence of SP-A. The mean value of LPS plus IFN-γ-induced TNF-α secretion in the absence of SP-A was 6.8 ± 0.5 ng/ml (100%). The data shown are means ± SEM of three different aMϕs cultures. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ when compared with the response elicited by LPS in the absence of SP-A. $^{\circ\circ\circ}P < 0.001$ when compared with the response elicited by LPS+ IFN-γ without SP-A.

Figure 2. Inhibitory effect of SP-A on LPS induced ERK phosphorylation by rat alveolar macrophages. Purified rat aMϕs were cultured in the presence or absence of LPS (1 ng/ml), IFN-γ (10 ng/ml), SP-A (5, 12.5, 25 µg/ml), and combinations thereof. We measured ERK phosphorylation by Western blot after 30 minutes of LPS-stimulation in the presence or absence of SP-A or IFN-γ. In A, the results are presented as means (± SEM) from three different aMϕs cultures, and expressed as percentages of LPS-induced ERK phosphorylation. $^{\#\#}P < 0.01$ when compared with the response elicited by LPS in the absence of SP-A. In B, representative Western blot images of ERK phosphorylation in rat aMϕs exposed to either LPS and/or SP-A (upper panel) or LPS and/or IFN-γ (lower panel) are shown.

Figure 3. Inhibitory effect of SP-A on IFN-γ-induced STAT1 Y701 phosphorylation and CXCL10 secretion by rat alveolar macrophages. Purified rat aMϕs were cultured in the presence or absence of IFN-γ (1-10 ng/ml), LPS (1 ng/ml), SP-A (5, 12.5, 25 µg/ml), and combinations thereof. (A) CXCL10 secretion was measured after 24 hours IFN-γ (1 ng/ml) and/or LPS-stimulation by ELISA. The results are presented as means (± SEM) from three different cell cultures and expressed as percentages of LPS plus IFN-γ-induced CXCL10 secretion. The mean value of LPS plus IFN-γ-induced CXCL10 secretion

by rat aMϕs in the absence of SP-A was 305 ± 12 pg/ml (100 %). (B) STAT1 Y701 phosphorylation was measured after 30 minutes stimulation with IFN-γ (10 ng/ml), LPS (1 ng/ml), or both, by Western blot. The data shown are means ± SEM of three different aMϕs cultures and were expressed as percentages of IFN-γ-induced STAT1 phosphorylation. (C, D) Representative Western blot images of STAT1 phosphorylation in rat aMϕs exposed to either IFN-γ (10 ng/ml) and/or SP-A (C) or IFN-γ (10 ng/ml) and/or LPS (1 ng/ml) (D) are shown. $^{\#}P < 0.05$ when compared with LPS stimulation without SP-A. $**P < 0.01$, $***P < 0.001$ when compared with the response elicited by IFN-γ alone in the absence of SP-A. $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$ when compared with the response elicited by LPS+IFN-γ without SP-A.

Figure 4. Inhibitory effect of SP-A on IFN-γ and/or LPS-challenged human macrophages. In A and B, human aMϕ were cultured in the presence or absence of IFN-γ (1 ng/ml), LPS (1 ng/ml), SP-A (12.5, 25, 50 μg/ml), and combinations thereof. We measured TNF-α (A) and CXCL10 (B) secretion after 24 h IFN-γ and/or LPS-stimulation. In (C) Human Mϕ(M-CSF) were cultured in the presence or absence of IFN-γ (1 ng/ml), SP-A (50 μg/ml), and combinations thereof. We measured *CXCL10*, *RARRES3,* and *ETV7* induction by RT-qPCR after 24 hours IFN-γ-stimulation. In (D) Human Mϕ(M-CSF) were cultured in the presence or absence of IFN-γ (0.05 ng/ml), SP-A (50 μg/ml), and combinations thereof. STAT1 Y701 phosphorylation was evaluated by Western blot after 30 minutes of stimulation. In (A) and (B) the results are expressed as percent of LPS plus IFN-γ-stimulated secretion of TNF-α (A) or CXCL10 (B) in the absence of SP-A. The mean values of LPS plus IFN-γ-induced TNF-α and CXCL10 secretion in the absence of SP-A were 875 ± 134 pg/ml and 367 ± 36 pg/ml, respectively (100 %). In (C) results were expressed as percentages of IFN-γ-induced gene in the absence of SP-A. In (D) results were expressed as percentages of IFN-γ-induced STAT1 phosphorylation. The data shown are means ± SEM of three different Mϕ cultures. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ when compared with the response elicited by LPS without SP-A. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ when compared with the response elicited by IFN-γ in the absence of SP-A. $^{\circ\circ\circ}P < 0.001$ when compared with the response elicited by LPS+IFN-γ in the absence of SP-A.

Figure 5. SP-A binds to IFN-γ. (**A**) Either human IFN-γ or human serum albumin (HSA) (1 μg) was coated onto microtiter plate wells. Biotinylated SP-A (0-470 nM; 0-333 μg/ml) was then added to the wells in the presence or absence of 2.5 mM Ca^{2+}, and levels of bound SP-A were determined with streptavidin-horseradish peroxidase. (**B**) Competition by unlabeled SP-A of biotinylated SP-A binding to human IFN-γ**.** Unlabeled SP-A was added simultaneously with biotinylated SP-A (100 nM; 80 μg/ml) to human IFN-γ-coated wells (1 μg) in presence of 2.5 mM Ca^{2}. In A and B, the data shown are means ± SEM of three different experiments. The assays from each SP-A concentration were performed with six independent determinations. (**C**) DLS analysis of the hydrodynamic diameter of human IFN-γ (10.5 ± 0.5 nm) and SP-A (38 ± 5 nm). 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. The analysis of the hydrodynamic diameter of particles after the addition of increasing concentrations of SP-A (0-120 nM) (0-80 μg/ml) to a solution containing a constant concentration of IFN-γ (322 nM) (7.7 μg/ml) is shown in the presence or absence of calcium. In C, one representative experiment of three is shown.

Figure 6. SP-A prevents IFN-γ interaction with IFN-γ receptor 1 (IFN-γR1/CD119). (A) sIFN-γR1 (1 μg) was coated onto microtiter wells. Coated sIFN-γR1 was detected using rabbit polyclonal anti-human IFN-γR1. Either biotinylated (B-SP-A) or unlabeled SP-A (160 μg/ml) was incubated for 1 h in the presence of 2.5 mM Ca^{2+}. SP-A binding to coated sIFN-γR1 was determined with streptavidin-horseradish peroxidase. **(B)** DLS analysis of the hydrodynamic diameter of particles after the addition of increasing concentrations of SP-A (0-80 μg/ml) to a solution containing a constant concentration of sIFN-γR1 (5 μg/ml) (**C**) sIFN-γR1 (1 μg) was coated onto microtiter wells. IFN-γ (100 ng/ml) and different concentrations of SP-A (0-160 μg/ml) in the presence or absence of 2.5 mM Ca^{2+} were incubated for 1 h. The binding of IFN-γ to sIFN-γR1-coated wells was detected using a polyclonal anti-human IFN-γ and horseradish peroxidase-conjugated anti-rabbit antibody. $^{**}P < 0.01$, $^{***}P < 0.001$, when compared with IFN-γ binding without SP-A (defined as 100 %.) (**D**) LPS primed human aMϕs were blocked and incubated with $[^{125}I]$-IFN-γ (10 ng/ml) and SP-A (at concentrations ranging from 0

to 100 µg/ml) with either 2.5 mM $CaCl_2$ or 2.5 mM EDTA. Incubations were performed for 2 h at 4 ºC. Cells were lysed with 10 % SDS and radioactivity was measured. Human aMϕs incubated with anti-IFN-γR1 antibody were used as a positive control to prevent interaction between IFN-γ and its receptor. The gamma counts of IFN-γ binding without SP-A were defined as 100 %. The data are means ± SEM of three different experiments (with different human aMϕ cultures in D). The assays from each macrophage preparation were performed with triplicate determinations. $^{**}P < 0.01$, $^{***}P < 0.001$, when compared with IFN-γ binding to its receptor in the absence of SP-A.