PROTECTIVE ROLE OF CORTISTATIN IN PULMONARY INFLAMMATION AND FIBROSIS

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Running Title: Cortistatin protects from experimental ALI/ARDS

Author contributions: M.B. and J.C.-S. conducted most of the experiments and analysed the data. M.C. and G.R. carried out histological processing and ELISA determinations. V.F.-P. conducted part of *in vivo* experimental models. R.B. and F.O'V. peformed the histopathological studies. M.D. conceived and designed the study, conducted *in vivo* experimental models, analysed the data and wrote the manuscript. All authors edited the manuscript.

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Word count: Introduction (557 words), Results (1953 words), Discussion (1500 words).

Acknowledgements: We thank to Dr. Luis de Lecea (Stanford University, CA, USA) for providing the CST-/- mice to establish initially the colony of animals used in this study.

Funding: This study was mainly supported by the Spanish Ministry of Science and Innovation (MICINN, grant SAF2015-67787-R). R.B. was recipient of FPI fellowship from MICIN. V.F-P. was recipient of postdoctoral fellowship from Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP (12/21767-5).

Conflict of interest: Authors declare no conflict of interest.

Ethic statement. The experiments reported in this study followed the ethical guidelines for investigations of experimental animals approved by the Animal Care and Use board and the Ethical Committee of Spanish Council of Scientific Research and performed in accordance with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Animal studies are reported in compliance with the ARRIVE guidelines and with the recommendations made by the *British Journal of Pharmacology*.

Declaration of transparency and scientific rigour. This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for Design & Analysis, Immunoblotting and

Immunochemistry and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Abstract (250 words)

Background and Purpose: Acute lung injury (ALI), acute respiratory distress syndrome (ARDS) and pulmonary fibrosis remain major causes of morbidity, mortality and healthcare burden in the critically ill patient. There is an urgent medical need for identifying factors of susceptibility and prognosis and for designing new therapeutic tools for treating these disorders. Here, we evaluate the capacity of the immunomodulatory neuropeptide cortistatin to regulate pulmonary inflammation and fibrosis *in vivo*.

Experimental Approach: ALI/ARDS and pulmonary fibrosis were induced experimentally in wild-type and cortistatin-deficient mice by pulmonary infusion of the bacterial endotoxin LPS or the chemotherapeutic drug bleomycin, and the histopathological signs, pulmonary leukocyte infiltration and cytokines and fibrotic markers were evaluated.

Key Results: Partially-deficient mice in cortistatin showed exacerbated pulmonary damage, pulmonary inflammation, alveolar oedema and fibrosis, and subsequent increased respiratory failure and mortality when challenged to LPS or bleomycin, even at low doses. Treatment with cortistatin reversed these aggravated phenotypes and protected from progression to severe ARDS and fibrosis after high-exposition to both injury agents. Moreover, cortistatin-deficient pulmonary macrophages and fibroblasts showed exaggerated *ex vivo* inflammatory and fibrotic responses. The anti-fibrotic protective effect of cortistatin was also observed in experimental scleroderma, in which lack of cortistatin predisposes to develop more severe dermal lesions and associated pulmonary fibrosis.

Conclusion and Implications: We identify to cortistatin as an endogenous break of pulmonary inflammation and fibrosis. Deficiency in cortistatin could be a marker of poor-prognosis in inflammatory/fibrotic pulmonary disorders. Cortistatin-based therapies emerge as attractive candidates to treat severe ALI/ARDS, including SARS-Cov-2-associated ARDS.

Key words: Acute lung injury, pulmonary inflammation, fibroblasts, macrophages, neuropeptide

Abbreviations:

ALI, acute lung injury

ARDS, acute respiratory distress syndrome

BALF, bronchoalveolar lavage fluid

CST, cortistatin

CTGF, connective tissue growth factor

i.d., intradermal

i.n., intranasal

IPF, idiopathic pulmonary fibrosis

MPO, myeloperoxidase

MIP2, macrophage inflammatory protein 2 (CXCL2)

SARS-CoV2, severe acute respiratory syndrome-coronavirus-2

αSMA, α-smooth muscle actin

sstr, somatostatin-receptor

Introduction

Despite major treatment efforts made over the past decades, acute lung injury (ALI) and its most severe form, acute respiratory distress syndrome (ARDS), characterized by refractory hypoxia, severe inflammation, increased vascular permeability and diffuse alveolar damage, remain major causes of morbidity and mortality in critically ill patients (Matthay et al., 2017) . ARDS can occur as a result of different clinical conditions, such as infections, pulmonary contusion and inhalation injury, that directly damage the pulmonary epithelial and endothelial cells and compromise alveolar-capillary barrier. ARDS is caused and sustained by an uncontrolled inflammatory activation characterized by massive release of cytokines and chemokines, diffuse lung oedema, inflammatory cell infiltration, and disseminated coagulation. In this sense, evidence indicates that the massive pulmonary infiltration (neutrophils and macrophages) and the subsequent inflammatory cytokine storm are closely related to secondary complications such as lung injury/ARDS, multiorgan failure and ultimately poor prognosis in the new severe acute respiratory syndrome-coronavirus-2 (SARS-CoV2) pandemic (Mehta et al., 2020; Zhou el al., 2020). Moreover, in patients who develop ARDS, the progression of ALI to pulmonary fibrosis portends a fatal outcome, with severe disruption of lung function and elevated mortality (George et al., 2020). As in other cases of pulmonary fibrosis caused by persistent infection, oxidative stress and inflammatory insults, the injury to alveolar epithelial cells activates pulmonary fibroblasts, promoting their transformation to extracellular matrix-producing myofibroblasts (Wynn, 2011). These findings highlight the urgent need to develop safe and effective therapeutic agents with capacity to limit both inflammatory and fibrotic responses in injured lung. Moreover, due to the heterogeneous progression and severity of disease in patients with ALI/ARDS, it is critical to identify factors and genes that predispose/protect to develop the most severe forms of lung injury and progressive lung fibrosis.

Cortistatin is a cyclic neuropeptide belonging to the somatostatin family that emerged as a potent immunomodulatory agent (Gonzalez-Rey and Delgado, 2008) with capacity to protect against exacerbated inflammatory and autoimmune responses in various experimental models of sepsis, rheumatoid arthritis, colitis, myocarditis and multiple sclerosis (Delgado-Maroto et al., 2017; Gonzalez-Rey et al., 2006b,c, 2007a; Souza-Moreira et al., 2013). These effects are exerted through the regulation of a plethora of inflammatory cytokines and chemokines and by deactivating macrophages and lymphocytes, pointing-out as a multitargeted and safe modulator of the cytokine storm in various tissues. However, the endogenous role of cortistatin in the modulation of immune response has been scarcely investigated, with some paradoxical effects found in cortistatin-deficient animals (Souza-Moreira et al., 2013; Qiu et al., 2020). Moreover, its role in ALI and fibrotic disorders is completely unknown, although some data point-out to a potential anti-fibrotic action of this neuropeptide. Thus, cortistatin-receptors (somatostatin-receptors sstr1-5 and ghrelin-receptor GHSR) are expressed in fibroblasts, and other sstr-agonists have been described that exert anti-fibrotic responses in various tissues, including lung (Borie et al., 2008; Egger et al., 2014; Tug et al., 2013). Therefore, in cortistatin could converge immunomodulatory and anti-fibrotic properties that synergistically might contribute to ameliorate inflammatory and fibroproliferative disorders in the lung. In this study, we will evaluate the therapeutic potential of cortistatin in two well-established experimental models of ALI and pulmonary fibrosis, as well as the immune and anti-fibrotic mechanisms involved. We will also investigate the role of cortistatin as a potential endogenous protective factor in the progression to severe ALI and pulmonary fibrosis in mice that are partially or totally deficient in this neuropeptide.

Methods

Animals and ethic statement. The experiments reported in this study followed the ethical guidelines for investigations of experimental animals approved by the Animal Care and Use board and the Ethical Committee of Spanish Council of Scientific Research and performed in accordance with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Animal studies are reported in compliance with the ARRIVE v.2.0 guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology*. Mice lacking the gene for cortistatin (CST-/-) were a generous gift of Dr. Luis de Lecea (Stanford University, La Jolla, CA, USA) and were generated in a C57BL/6 background and backcrossed with C57BL/6 mice for ten generations as previously described (Cordoba-Chacon et al., 2011). Mice heterozygous (CST+/-) for cortistatin were generated by crossing female CST-/- and male CST+/+ mice. CST+/- breeding pairs were used to generate a littermate colony of wild-type (CST+/+), heterozygous (CST+/-) and knockout (CST-/-) mice for cortistatin. Both male and female mice (20-24g body weight) were used in all experiments described in this study, and no differences were found between sexes. All animals were housed in a controlled-temperature/humidity environment (22±1°C, 60-70% relative humidity) in individual cages (10 mice per cage, with wood shaving bedding and nesting material), with a 12 h light/dark cycle (lights on at 7:00 a.m.) and fed with rodent chow (Global Diet 2018, Harlan) and tap water ad libitum. Mice were allowed to acclimatize to the experimental room for one hour before experiments. Mice were randomly assigned to the different experimental groups. Experiments were designed to make sample sizes relatively equal. However, this was not possible in some experiments due to the differential mortality rates occurring between genotypes and response to bleomycin. None of the animals were excluded from the study. Power calculations were performed using the software G*Power (www.gpower.hhu.de) to ensure that adequate group sizes were used for the studies detailed below. For *in vivo* animal models, we calculated a minimum size of five to eight mice per group in order to have a power >0.95 of detecting approximately a 30% change, assuming a standard deviation of 30% at a significance level of $p<0.05$, expecting an effect size of 1.82 for ANOVA tests. In primary cell cultures, for effect sizes between 3.1 and 4, experiments were repeated at least four times to obtain $p<0.05$ and a power >0.95.

Main reagents. Unless otherwise indicated, all purchased reagents used in this study were from Sigma-Aldrich (St. Louis, MO, USA). Bleomycin sulphate (with specific activity of 1.6-2.0 $U \cdot mg^{-1}$, from Sigma-Aldrich, cat#B-8616) was dissolved in saline solution (0.9% NaCl) at 1 $mg \cdot ml^{-1}$ (1.8 $U \cdot ml^{-1}$) and stored at -20ºC, and was diluted in saline at the indicated doses immediately before its injection in animals. Mouse cortistatin-29 (from Bachem, Bubendorf, Switzerland, cat#H-6444) was dissolved in ddH_2O and stored at -80ºC at a dose of 0.1 mM and was diluted in ddH_2O (used as vehicle) at the indicated dose and volume immediately before its use in the experimental models. LPS (from *E. coli* serotype 055:B5, Sigma-Aldrich, cat#L-2880) was dissolved in saline at 2 $mg \cdot ml^{-1}$ and stored at -20ºC until its use.

Antibodies. Antibodies used for flow cytometry: anti-mouse CD16/CD32 (clone 2.4G2, BD Pharmingen, San Jose, CA, USA, cat #553130), phycoerythrin (PE)-labeled anti-mouse CD45 monoclonal antibody (clone 16A, BD Pharmingen, cat#553099), allophycocyanin (APC)-labelled anti-mouse CD3 (clone 17A2, BD Pharmingen, cat#565643), APC-labelled anti-mouse CD64 (clone X54-5/7.1, eBioscience/Thermo Fisher, cat#17-0641-82) or FITC-labelled anti-mouse Ly6G antibodies (clone RB6-8C5, BD Bioscience, cat#553126). Capture and biotinylated antibodies used for sandwich ELISA: for TNFα detection (BD Pharmingen): capture antibody clone G281-2626, cat#551225; biotin-antibody clone MP6-XT3, cat#554415; for IL6 detection (BD Pharmingen): capture antibody clone MP5-20F3, cat#554398; biotin-antibody clone MP5-32C1, cat#554402; for TGFβ1 detection (BD Pharmingen): capture antibody clone A75-2,

cat#555052; biotin-antibody clone A75-3, cat#555053; for macrophage inflammatory protein-2 (MIP2/CXCL2) detection (PreproTech, London, UK): capture antibody cat#500-P130 and biotin-antibody cat#500-P130Bt; for IL1β1 detection (PreproTech): capture antibody cat#500-P51 and biotin-antibody cat#500-P51Bt. Antibodies used for immunofluorescence analysis of tissues: mouse α-smooth muscle actin (αSMA) antibody (clone 1A4, Sigma-Aldrich, cat#A5228); Alexa Fluor 568-conjugated goat anti-mouse antibody (Life Biotechnologies/Thermo Fisher, cat#A11004). Primary antibodies used for western blot analysis of pulmonary fibroblasts, all from Cell Signalling (Danver, MA, USA): rabbit anti-mouse phospho-Akt (cat#4056), mouse anti-mouse Akt (cat#2920), mouse anti-mouse phospho-p38 MAPK (cat#9216), rabbit anti-mouse p38 MAPK (cat#9212), mouse anti-mouse phospho-p42/p44 MAPK (ERK1/2, cat#9106) and rabbit anti-mouse ERK1/2 (cat#4695). Secondary antibodies used for western blot analysis of pulmonary fibroblasts, all from LI-COR Biosciences (Lincoln, NE, USA): IRDye 800CW-conjugated goat anti-mouse (cat#926-32210), IRDye 680RD-conjugated goat anti-rabbit (cat#926-68071), IRDye 680RD-conjugated goat anti-mouse (cat#926-68070) and IRDye 800CW-conjugated goat anti-rabbit (cat#926-32211). Antibodies for immunofluorescence analysis of pulmonary fibroblasts: rabbit anti-mouse Smad2/3 antibody (Cell Signalling, cat#65678); Alexa Fluor 488-conjugated donkey anti-rabbit antibody (Thermo Fisher, ca#A21206).

Induction of acute lung injury (ALI). To investigate the effect of cortistatin deficiency in the severity of ALI, CST+/+, CST+/- and CST-/- mice were infused intranasally (i.n.) with the bacterial endotoxin LPS (1 mg per kg mouse, in 20 µl volume onto the nares, approximately 20 µg LPS/mouse). To determine the therapeutic effect of cortistatin in the progression of ALI, CST+/+ mice were infused i.n. with LPS (2 mg per kg mouse, in 40 µl volume, approximately 40 µg LPS/mouse) and then treated i.p. 120 min, 24h and 48h later with vehicle or cortistatin (1 nmol/mouse, in 200 µl volume, approximately 140 µg cortistatin per kg mouse). Mice infused i.n. with saline, instead of LPS, were used as basal controls of reference. On different times after LPS or saline inhalation, animals were sacrificed by carbon dioxide affixation and bronchoalveolar lavage fluid (BALF) and lungs were isolated and processed for analysis of leukocyte infiltration, cytokine contents, vascular permeability, histopathology and myeloperoxydase (MPO) activity as described below.

Induction of experimental lung fibrosis. To investigate the effect of cortistatin deficiency in severity of lung fibrosis, bleomycin was administered intratracheally (at 1.8 U per kg mouse, in 50 µl volume) to anesthetized (i.p., ketamine 80 mg, xylazine 10 mg per kg mouse) CST+/+, CST+/- and CST-/- mice. When indicated, other doses of bleomycin (from 1.2 to 5 U per kg mouse) were assayed (see Figure 5). To investigate the therapeutic effect of cortistatin in lung fibrosis, CST+/+ mice were injected intratracheally with bleomycin (3.2 U per kg mouse, in 50 µl volume) and treated three times per week with vehicle or cortistatin via a local i.n. pathway (at 50 pmol cortistatin/mouse, in 20 µl volume) or a systemic i.p. pathway (at 1 nmol cortistatin/mouse, in 200 µl volume), starting immediately (protective acute regime) or five days (therapeutic regime) after bleomycin injection. Mice injected intratracheally with saline, instead of bleomycin, were used as basal controls of reference. Survival and body weight were daily monitored for three weeks. On different times after bleomycin injection, animals were sacrificed by carbon dioxide affixation, and BALFs and lungs were isolated and analysed for leukocyte infiltration, cytokine contents, vascular permeability, histopathological signs and fibrotic markers (collagen content and gene expression) as described below.

Induction of experimental dermal fibrosis. Skin fibrosis was induced by repetitive intradermal (i.d.) injections of bleomycin (3.2 U per kg mouse, in 100 µl volume, three times per week, during four weeks) in a localized place in the right side of saved back of CST+/+, CST+/- and CST-/- mice (under slight anaesthesia induced with 2% isoflurane). Treatment with cortistatin consisted in s.c. injections of 1 nmol cortistatin (in 100 µl volume) in a place near to bleomycin-

induced lesion, three times per week, during three weeks, starting five days after the first bleomycin administration. Animals treated with vehicle, instead of cortistatin, were used as untreated controls. Moreover, saline (in 100 µl volume) was repetitively injected i.d. in a localized point in the left side of mouse back, contralateral to bleomycin-induced lesion (used as basal control of reference). After four weeks, animals were sacrificed by carbon dioxide affixation and skin and lungs were isolated and processed for histopathological analysis and measurement of fibrotic markers as described below.

Histopathological analysis. For histopathologic evaluation, freshly collected lung and skin were fixed in 10% buffered formalin, embedded in paraffin and sectioned. Cross-sections (5-µm) were stained with haematoxylin/eosin (H&E), with Masson's trichrome or with Picrosirius Red using standard techniques. Images were acquired in an Axio Scope.A1 microscope (Carl Zeiss, Germany) using 5X and 10X objectives and 10X ocular and analysed with Zen 2011 Light Edition software (Carl Zeiss). All histopathological analysis and determinations were performed in a blinded manner by at least two independent researchers in whole lung sections and whole skin biopsies (0.7 cm^2), and at least three sections per mouse. ALI-induced histopathology was scored in H&E-stained lung sections determining the extent of inflammatory cell infiltration on alveolar walls, alveolar haemorrhage and alveolar septae congestion, using a semi-quantitative scale from 0 (normal and no focal inflammatory infiltrates) to 4 (severe infiltration and damage in lung structure). Bleomycin-induced pulmonary fibrosis was scored in Masson's trichrome-stained lung sections according to a semi-quantitative scale (0 to 4) evaluating alveolar thickness, damage of lung structure and fibrosis extension (Ashcroft et al., 1988): 0, normal lung or minimal fibrous thickening of alveolar or bronchial walls; 1, moderate thickening of the wall, with less than 25% of fibrotic area, but without obvious damage to lung architecture; 2, formation of fibrous bands, fibrous masses in 25-50% of lung area, and definitive damage of lung structure; 3, severe distortion of the structure and large fibrous areas (>50% of a cross-section involved); 4, total fibrous obliteration of the field. Bleomycin-induced skin fibrosis was assayed by measuring dermal thickness (distance in µm from the base membrane of epidermal layer to the hypodermal junction with subcutaneous fat, determined as the mean of three random measurements in each section) in skin cross-sections stained with Masson's trichrome and Picrosirius Red using the Fiji-ImageJ software (http://imagej.net/Fiji). Moreover, results were expressed as increase in dermal thickness comparing bleomycin-induced skin lesion and contralateral saline-injected skin in the same mouse. Density and distribution of collagen deposits and fibrotic bands and masses were analysed in Picrosirius red-stained skin samples.

BALF collection and analysis. A cannula (21G) was inserted into the trachea and ice-cold PBS (0.8 ml) was instilled twice into the lung. BALF was harvested and centrifuged (400·g, 8 min, 4°C). Cell pellets were resuspended in PBS and used to determine total cell numbers using a standard haemocytometer and to analyse the percentage of neutrophils, macrophages and T lymphocytes in BALF by flow cytometry as described below (Tager et al., 2008). Alternatively, cell populations were examined by counting at least 200 cells on Wright-Giemsa-stained BALF CytoSpin preparations. The supernatants collected from BALFs were used to determine the levels of cytokines and chemokines using sandwich ELISAs (see above for specific capture and biotinylated antibodies) following the manufacturer's recommendations, to measure total protein content using a bicinchoninic acid BCA Protein Assay Kit (Pierce/Thermo Fisher) and to determine the levels of mouse albumin using an ELISA kit (Abcam, Cambridge, UK, cat#ab207620).

Measurement of pulmonary MPO activity. Oxidative stress and neutrophil infiltration in the lung was also monitored by measuring MPO activity by using a method reported previously (Gonzalez-Rey et al., 2006a). In brief, left lung lobules were homogenized at 50 $mg \cdot ml^{-1}$ in phosphate buffer (50 mM, pH 6.0) with 0.5% hexadecyltrimethylammonium bromide. Samples

were frozen, thawed three times, and centrifuged (30,000·g, 20 min). The supernatants were diluted at 1:30 with assay buffer consisting in 50 mM phosphate buffer pH 6.0 with 167 $\mu g \cdot ml^{-1}$ o-dianisidine and 0.0005% H_2O_2, and the colorimetric reaction was measured at 450 nm between one and three minutes (ΔA_{450}) in a spectrophotometer (VersaMax Microplate Reader, from Molecular Devices, San Jose, CA, USA). MPO activity per gram of wet lung was calculated as: $13.5 x \Delta A_{450}$/lung weight. The coefficient 13.5 was empirically determined such that 1 U MPO activity is the amount of enzyme that will reduce 1 µmol H_2O_2 min^{-1}.

Pulmonary macrophage cultures. Pulmonary macrophages were enriched by plastic adherence (120 min at 37ºC, in 24-well plates) of BALFs collected from CST+/+, CST+/- or CST-/- mice 24h after LPS-induced ALI. After washing and removal of non-adherent cells, the adherent macrophages ($5x10^4$ cells) were cultured in RPMI complete medium (RPMI-1640 supplemented with 10% FBS, 100 $U \cdot ml^{-1}$ penicillin/streptomycin, 2 mM L-glutamine and 50 µM 2-mercaptoethanol, all from Gibco/Thermo Fisher) in the absence (unstimulated) or presence (stimulated) of LPS (0.5 $\mu g \cdot ml^{-1}$) and treated with or without cortistatin (10 nM). After 24h, the levels of TNFα in culture supernatants were determined by ELISA.

Measurement of pulmonary vascular permeability. Pulmonary vascular permeability was quantified by measuring albumin and protein contents in BALFs (see above) and using the Evans blue dye extravasation assay (Tager et al., 2008). Briefly, Evans blue dye (20 $mg \cdot kg^{-1}$) was injected through mouse tail vain three hours before sacrifice. At the time of killing, blood was collected into a heparinized syringe by cardiac puncture and mice were then perfused with PBS through the right ventricle to remove intravascular dye from the lungs. Lungs were dissected and homogenized and Evans blue dye was extracted by the addition of two volumes of formamide followed by incubation overnight at 60ºC. After centrifugation (5,000·g, 30 min), the absorption of Evans blue in lung supernatants and plasma was measured at 620 nm and corrected for the presence of heme pigments as follows: $A_{620} - (1.426 \times A_{740} + 0.030)$. We calculated an Evans blue index as the ratio of the amount of dye in the lungs to the plasma dye concentration. Pulmonary vascular permeability was also monitored through the ratio between lung weight measured immediately after its excision (wet weight) and lung weight after five days in an oven at 60ºC (dry weight).

Flow cytometric analysis. BALF cell pellets (10^5 cells) were incubated with anti-mouse CD16/CD32 antibody (1:100, 4°C, 10 min) to avoid non-specific binding to Fc-receptors and with 7-Aminoactinomycin D (1:100, Calbiochem/Sigma-Aldrich) to exclude dead cells. After washing in PBS/0.1% BSA, cells were surface stained with fluorophore-conjugated antibodies for CD45 and for CD3, CD64 or Ly6G (each at 4–5 $\mu g \cdot ml^{-1}$, 30 min, 4°C) and were analysed in a FACScalibur flow cytometer (BD Biosciences). Data were acquired until at least 20,000 events were collected from a live gate using forward/side scatter plots and 7-Aminoactinomycin D staining. Percentages of CD64+ macrophages, CD3+ T lymphocytes, Ly6G+ neutrophils were analysed in a gated CD45+ cell population using FlowJo v9 software, and the differential number of each cell subpopulation in BALF was calculated by multiplying this percentage by the total number of collected BALF cells.

Immunofluorescence analysis of pulmonary myofibroblasts. Formalin-fixed lung sections were incubated in 10 nM sodium citrate/0.05% Tween-20 (20 min, 100ºC) for antigen retrieval, cooled in water and then incubated twice during five min in PBS/0.025% Triton X-100. Sections were blocked with 10% goat serum/1% BSA (120 min, 20°C) and incubated with primary anti-αSMA antibody (diluted at 1:1000 in PBS/1% BSA, overnight, 4°C). After extensive washing with PBS/0.025% Triton X-100, sections were incubated with secondary Alexa Fluor 568-conjugated antibody (diluted at 1:1000 in PBS/1% BSA, 60 min, 20°C). Nuclei were DAPI-counterstained (diluted at 1:1000 in PBS, 5 min, 20ºC) and sections were mounted in Mowiol. Sections in which we omitted primary antibody were used as negative controls, showing in all

cases lack of fluorescence signal. Sections were examined in an Olympus IX81 fluorescence microscope (Olympus Life Science, Hamburg, Germany) and the images were acquired at 100X magnification (Olympus CellSens Imaging software) using the same parameters and region of interest (ROI) between samples and were quantified for the mean of fluorescence intensity using the Fiji-ImageJ software.

Measurement of collagen content in tissues. The collagen content in lungs and skin of mice was measured using the hydroxyproline assay (Reddy et al. 1996). Briefly, right lung lobes and skin biopsies (1 cm^2) were hydrolysed in 6 N HCl (approximately 100 mg tissue/ml) at 95ºC for 20h and shaking. After centrifugation (13,000·g, 15 min, 20ºC), supernatants were diluted to reach a final concentration of 4 N HCl, transferred to 96-well plates and oxidized with 1.2% chloramine-T/10% propanol in citrate-acetate buffer pH 6.5 (720 mM sodium acetate, 1% acetic acid, 200 mM citric acid, 680 mM sodium hydroxide) for 25 min at 20ºC and shacking. Ehrlich's reagent (1 vol, 15% p-dimethylaminobenzaldehyde in propanol/perchloric acid 2:1, vol:vol) was added to wells and incubated at 60ºC during one hour. Absorbance at 550 nm was measured in a spectrophotometer and extrapolated to a standard hydroxyproline curve. Collagen content was calculated by multiplying the hydroxyproline measurements by 7.40 (a coefficient according to the fact that hydroxyproline represents 13.5% of aminoacids in collagen sequence) and then expressed in µg relative to the weight of tissue.

Isolation and culture of primary fibroblasts. Dorsal skin biopsies (1 cm^2) and lung lobules were collected from CST+/+ and CST+/- mice (1 week-old for skin, 10 week-old for lung) and mechanically dissected in small pieces using sterile scalpels. Tissue fragments were digested in DMEM/F12 medium (Gibco) supplemented with 100 $U \cdot ml^{-1}$ penicillin/streptomycin, 2 mM L-glutamine, 140 $U \cdot l^{-1}$ Liberase (Thermolysin Low for lung samples, Dispase High for skin samples, from Roche, Basilea, Switzerland) at 37ºC with shaking. After 60 min (for lung) or 90 min (for skin), digested tissues were centrifuged (525·g, 5 min, 20ºC) and cell pellets were washed three times with complete DMEM/F12 medium (supplemented with 15% FBS, 100 $U \cdot ml^{-1}$ penicillin/streptomycin, 2 mM L-glutamine) and then cultured in complete DMEM/F12 medium in 75 cm^2-Nunc flasks (Nunc/Thermo Fisher), at 37ºC, 5% CO_2. After 3-7 days culture, medium was replaced by MEMα complete medium (MEMα supplemented with 15% FBS, 100 $U \cdot ml^{-1}$ penicillin/streptomycin, 2 mM L-glutamine, all from Gibco) and adhered fibroblasts were cultured until 80% confluence, harvested by adding Trypsin-EDTA solution (Sigma-Aldrich, cat#T4049) and were maintained at $5x10^5$ cells/flask (in 175 cm^2-Nunc flasks) at 37ºC, 5% CO_2 until their use. To evaluate gene expression by real-time qPCR, $4x10^4$ fibroblasts were cultured in 6-well-Nunc plates, cultured until 80% confluence, synchronized to G0-phase by incubation in free-FBS MEMα (overnight at 37ºC, 5% CO_2) and then cultured in complete MEMα in the absence or presence of TGFβ1 (10 $ng \cdot ml^{-1}$, PreproTech) for 24h. To evaluate Smad2/3 nuclear translocation by immunofluorescence analysis, 10^3 fibroblasts were cultured until 80% confluence in glass-coverslips which were inserted in 24-well-Nunc plates, synchronized and cultured in complete MEMα in the absence or presence of TGFβ1 (10 $ng \cdot ml^{-1}$) for 60 min. To evaluate protein expression by western blot, $5x10^5$ fibroblasts were cultured in 75 cm^2-Nunc flasks until 80% confluence, synchronized and cultured in complete MEMα in the absence or presence of TGFβ1 (10 $ng \cdot ml^{-1}$) for 24h.

Cell viability of fibroblast cultures was evaluated using Alamar-blue assay and ATP determinations. In brief, 1500 fibroblasts were seeded in 96-well plates, synchronized and cultured for different times and Alamar-Blue reagent (10% vol/vol, Sigma-Aldrich) was added during the last 4h of the culture and measured its reduction by fluorescence (excitation 550nm/emission 590nm) in a fluorescence plate reader (Tecan, Mannendorf, Switzerland). Moreover, levels of ATP were determined using CellTiter-Glo Luminescent Cell Viability Assay kit (Promega, Madison, WI, USA) following manufacturer's instructions.

Migration of fibroblasts was determined using an *in vitro* wound healing assay. In brief,10^3 fibroblasts were seeded in Culture-Inserts 2 Well in µ-Dish 35mm (Ibidi, Grafelfing, Germany)

and then cultured to confluence. After cell synchronization, the inserts were removed and complete MEMα medium was added. At different time points, wells were observed in an Olympus microscope and images were acquired at 100X magnification under phase contrast mode. The percentage of unhealed wound area was quantified using Fiji-ImageJ software and the MRI Wound Healing Tool (http://dev.mri.cnrs.fr/projects/imagej-macros/wiki/Wound_Healing_Tool).

Immunofluorescence analysis of primary fibroblasts. Lung fibroblasts were cultured in coverslips as described above and then fixed with 4% paraformaldehyde/2% glucose during 15 min at 20ºC. After extensive washing with PBS, cells were incubated with 30 mM glycine for 5 min, and permeabilized with 0.1% Triton X-100 (15 min, 20ºC). Coverslips were blocked with PBS/5% FBS/0.3% Triton X-100 (60 min, 20°C) and incubated with primary anti-Smad2/3 antibody (diluted at 1:200 in PBS/1% BSA/0.3% Triton X-100, overnight, 4°C). After extensive washing with PBS/0.025% Triton X-100, samples were incubated with secondary Alexa Fluor 488-conjugated antibody (60 min, 20°C, diluted at 1:1000 in PBS/1% BSA/0.3% Triton X-100). Nuclei were DAPI-counterstained (1:500 in PBS, 5 min, 20ºC) and were mounted in Mowiol. Samples in which we omitted the primary antibodies were used as negative controls, showing in all cases lack of fluorescence signal. Samples were examined in an Olympus IX81 fluorescence microscope and the images acquired at 400X magnification (Olympus CellSens Imaging software) using the same parameters and ROI for five independent experiments (in duplicates) and fluorescence intensity (integrated density) located specifically in nuclei was determined using the Fiji-ImageJ software. At least a mean of 200 nuclei per experiment was quantified in a blinded fashion in each experimental group.

Determination of gene expression by real-time PCR. Total RNA was isolated from lung lobes and skin samples by tissue homogenization (Ultra-Turrax T-25, from IKA, Staufen, Germany) at 13,500 rpm for 40 seconds (for lung) or 60 seconds (for skin) in TriPure reagent (Roche) and in EZNA HP total RNA Kit-Animal Tissue (Omega Bio-Tek, Norcross, GA, USA), respectively, following the manufacturers' protocols. Fibroblasts were cultured and activated as described above and then directly collected from culture plates by adding TriPure solution. Precipitated RNA (1 µg) was treated with DNase I (1 U) and then reversed transcribed using RevertAid First Strand cDNA Synthesis Kit (200 U, Thermo Fisher) and random hexamer primers (5 µM) at 42ºC for 60 min in a Mastercycler EP Gradient Thermocycler (Eppendorf, Madrid, Spain). SYBER green quantitative PCR (SensiFast Sybr No-Rox mix, from Bioline, Germany) was performed on the CFX96 Real-Time PCR system (Bio-Rad, Hercules, CA, USA) using the following conditions: 94°C for 5 minutes followed by 40 cycles at 94°C for 30 seconds, annealing at 60ºC for 30 seconds and extension at 72°C for 30 seconds. Primer sequences for cortistatin (CST), αSMA, Connective Tissue Growth Factor (CTGF) and Ribosomal Protein Lateral Stalk Subunit P0 (RPLP0) were (5' to 3'): CST-forward, GCCTTCTGACTTTCCTTGCC; CST-reverse, GAAAGCTCCCCGCTGATTGA; αSMA-forward, CAGGGAGTAATGGTTGGAAT; αSMA-reverse, TCTCAAACATAATCTGGGT; CTGF-forward, AGAACTGTGTACGGAGCGTG; CTGF-reverse, GTGCACCATCTTTGGCAGTG; RPLP0-forward, TGCACTCTCGCTTTCTGGAG; RPLP0-reverse, CTGACTTGGTTGCTTTGGCG. The expression of each gene was normalized against the expression of the housekeeping gene RPLP0 in every PCR reaction and estimating fold change expression with Delta-Delta Ct method.

Western blot analysis of fibroblast cultures. Mouse pulmonary fibroblasts were cultured and activated as described above and then lysed by incubation with lysis buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% SDS, 10 $\mu g \cdot ml^{-1}$ protease inhibitor cocktail (cat#P8465) and phosphatase inhibitor (PhosSTOP, from Roche) for 2h at 4ºC and shaking. Lysates were centrifuged (21,000·g, 15 min, 4ºC) and supernatants containing proteins extracts (10 µg) were separated on 12% SDS-

polyacrylamide gels and blotted onto polyvinylidene difluoride membranes (ImmMobilon-FL PVDF, Millipore/Thermo Fisher) using a semidry system (transfer buffer: 25 mM pH 8.3, 192 mM glycine, 20% methanol). Membranes were blocked with TBS-T buffer (10 mM Tris, 150 mM NaCl, pH 7.5, 0.1% Tween-20) and 5% BSA for 1h at 20ºC and subsequently probed overnight at 4°C with pairs of primary antibodies directed against Akt and phospho-Akt, p38 MAPK and phospho-p38 MAPK, or phospho-ERK1/2 and ERK1/2 (diluted at 1:1,000 in TBS-T/2% BSA). Immunodetection of primary antibodies was performed by incubation with secondary antibodies labeled to the near-infrared fluorophores IRDye 800CW (green dye, for phosphorylated kinases) or IRDye 680RD (red dye, for non-phosphorylated kinases) diluted at 1:20,000 in TBS-T/2% BSA/0.02% SDS for 1h at 20ºC. Images of blots were acquired in an Odyssey CLx (LI-COR Biosciences) and fluorescence intensities of specific bands corresponding to phosphorylated and non-phosphorylated (used to normalize protein expression) forms of each kinase were quantified using Fiji-ImageJ software.

Data and statistical analysis. All experiments are randomized and blinded. All data are expressed as mean±SD, unless when specified (i.e. Figure 6). To control for unwanted sources of variation between individual experiments, data obtained from qPCR and western blot analysis of fibroblast cultures (Figure 6B,6C) were normalized to unstimulated CST+/+ fibroblasts. The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). In accordance with journal policy, statistical analysis was performed only when a minimum of $n = 5$ independent samples was acquired. We analysed data for statistical differences between groups using the unpaired Student's t-test or the non-parametric Mann-Whitney U-test and, if appropriate, by Kruskal-Wallis analysis of variance test. Survival curves were analysed by the Kaplan-Meier log-rank test. All analyses were performed using GraphPad Prism v5.0 software (La Jolla, CA, USA). We considered P-values < 0.05 (two-tailed) as significant.

Results

Protective role of cortistatin in bacterial endotoxin-induced ALI. We first investigated the role played by cortistatin in pulmonary inflammation by using a well-characterized experimental model of ALI that is induced in mice by i.n. injection of the bacterial endotoxin LPS, which mirrors important aspects of human ARDS, and is widely used to assay novel therapeutic agents (Tsushima et al., 2009). Pulmonary administration of LPS (2 $mg \cdot kg^{-1}$) caused a rapid influx of circulating inflammatory cells, mainly of neutrophils, into the alveolar spaces (Figure 1A), which was accompanied by excessive levels of inflammatory cytokines and chemokines (Figure 1B) and resulted in enhanced permeability of pulmonary capillaries, alveolar protein leakage and interstitial oedema (Figure 1C). Histopathological analysis of H&E-stained lung sections confirmed that pulmonary infusion of LPS resulted in marked damage in lung structure, including substantial inflammatory cell infiltration, abundant alveolar exudation and disseminated haemorrhages, indicating the occurrence of ALI (Figure 1D). We observed that the systemic administration of cortistatin ameliorated severity of ALI, as denoted by a significant reduction in leukocyte infiltration, lung inflammation, interstitial and alveolar oedema and the histopathological signs (Figure 1A-D). Moreover, macrophages isolated from BALFs of mice with LPS-induced ALI that were treated with cortistatin produced less inflammatory factors than those isolated from untreated mice (Figure 1E). In this sense, *ex vivo* treatment with cortistatin significantly reduced the production of inflammatory cytokines by macrophages isolated from BALF of mice with ALI (Figure 1F). These data suggest that cortistatin impairs the acute inflammatory cascade induced by exposition to bacterial endotoxins in the lung and avoid subsequent disruption of epithelial integrity.

In order to investigate the capacity of endogenous cortistatin to regulate pulmonary inflammation, we induced ALI in mice that partially (CST+/-) or fully (CST-/-) lack cortistatin

gene. Infusion of LPS at low-dose (1 mg·kg^{-1}) caused exacerbated development of ALI in mice with a total, and even with a partial, deficiency in cortistatin (Figure 2). In comparison to wild-type mice (CST+/+), we observed excessive leukocyte infiltration (Figure 2A), severe histopathological signs of pulmonary inflammation, oedema and damage (Figure 2B) and enhanced presence of inflammatory mediators (Figure 2C) and protein leakage (Figure 2D) in BALFs of CST+/- and CST-/- mice. Moreover, pulmonary macrophages isolated from CST+/- and CST-/- mice with ALI significantly produced more inflammatory TNFα than macrophages isolated from BALF of CST+/+ mice (Figure 2E). Importantly, exogenous administration of cortistatin to CST+/- mice reversed the severe ALI phenotype that we observed in these animals (Figure 3). Moreover, *in vitro* treatment with cortistatin impaired the enhanced TNFα production by BALF macrophages isolated from CST+/- and CST-/- mice with ALI (Figure 2E). These findings indicate that a partial deficiency of cortistatin predisposes to develop exacerbated acute pulmonary inflammatory responses and severe lung damage after exposition to bacterial endotoxins.

Deficiency in cortistatin exacerbates pulmonary fibrosis in bleomycin-challenged mice. We further investigated the role played by cortistatin in a well-characterized model of pulmonary fibrosis induced by intratracheal injection of bleomycin, which shares significant similarities with human IPF, and has been widely used for studying pulmonary fibrogenesis and evaluating the effect of therapeutic antifibrotic strategies (Kolb et al., 2020). In this model, as occurred with exposition to infection, toxins or radiation, the antineoplastic drug bleomycin causes alveolar epithelial injury that induces the release of profibrotic cytokines/growth factors (i.e., TGFβ1, TNFα and CTGF), which activate lung fibroblasts and their subsequent transformation into αSMA-expressing myofibroblasts that are responsible of the excessive extracellular matrix protein deposition that characterizes the fibrotic lung. We firstly found that mice lacking cortistatin showed significantly earlier and higher mortality after bleomycin challenge, relative to wild-type animals (Figure 4). Significant was the fact that bleomycin at doses as low as 1.2-1.8 U·kg^{-1}, that did not compromise survival of CST+/+ mice, increased the mortality rate above 50% in CST+/- and CST-/- mice. In order to investigate the clinical markers and mechanisms involved in this susceptibility to bleomycin, we further used a dose of 1.8 U·kg^{-1}. Exposure of CST+/- and CST-/- mice to this dose of bleomycin resulted in a significant loss of body weight (up to 20%) and death rates ranging from 75% to 80% (Figure 5A). Histopathological examination of Masson's trichrome-stained pulmonary sections showed that, whereas lungs of bleomycin-challenged CST+/+ mice had moderate thickening of the alveolar walls, less than 25% of fibrotic area and no obvious damage in lung architecture, mice that are partially or fully deficient in cortistatin had lungs with large fibrous areas (above 60%), in many cases with total fibrous obliteration of the field, showing severe distortion of pulmonary structure (Figure 5B). We observed this increased fibrosis score in cortistatin-deficient mice as early as seven days after bleomycin instillation, coinciding with the drop in survival (Figure 5A and 5B). Moreover, the exacerbated bleomycin-induced lung fibrosis observed in CST+/- and CST-/- mice was accompanied by an excessive early leukocyte infiltration (mainly composed by neutrophils and macrophages) and increased levels of inflammatory mediators (Figure 5C). Moreover, deficiency in cortistatin increased pulmonary vascular leak after bleomycin-induced injury, as indicated by elevated total protein content in BALFs and Evans blue extravasation in lungs of CST+/- and CST-/- mice in comparison to CST+/+ mice (Figure 5D). Finally, we observed that bleomycin challenge resulted in marked enhancement of fibrotic markers and fibrogenic mediators in mice lacking cortistatin. Thus, collagen contents, TGFβ1 levels and gene expression of CTGF were significantly elevated in lungs and BALFs collected from CST+/- and CST-/- mice 7-10 days after bleomycin instillation (Figures 5C and 5E). Moreover, immunofluorescence analysis of lung sections showed that deficiency in cortistatin significantly increased the presence of αSMA-expressing myofibroblasts in peribronchiolar and parenchymal

areas of dense fibrotic remodelling at three weeks after bleomycin challenge (Figure 5E). All together, these findings indicate that cortistatin is a key regulator of pathological pulmonary fibrosis induced by tissue-damage agents, and that these effects are probably exerted both by indirectly limiting pulmonary inflammation and by directly impairing fibrogenic responses.

Cortistatin-deficient fibroblasts show increased fibrogenic responses. To investigate whether endogenous cortistatin directly regulates fibrosis, independently of its immunoregulatory effects, we evaluated the fibrogenic responses of primary pulmonary fibroblasts isolated from wild-type and cortistatin-deficient mice. Because *in vivo* experiments demonstrated that partially-deficient and totally-deficient mice for cortistatin showed similar exacerbated pulmonary fibrosis in response to bleomycin, and individuals with partial deficiency in this neuropeptide will be more frequent within human population than those fully-deficient, we focused *in vitro* experiments on CST+/- fibroblasts. First, we confirmed that mouse lungs and pulmonary fibroblasts expressed cortistatin (Figure 6A). Interestingly, activation of pulmonary fibroblasts with TGFβ1 almost completely abrogated cortistatin gene expression, and bleomycin challenge moderately reduced its expression in lungs (Figure 6A). CST+/- pulmonary fibroblasts expressed significantly higher levels of the profibrogenic factor CTGF and the myofibroflastic marker αSMA than CST+/+ fibroblasts (Figure 6B). Interestingly, deficiency in cortistatin generated a fibroblast that, in basal conditions, expressed CTGF levels comparable to those expressed by TGFβ1-activated CST+/+ fibroblasts (Figure 6B), suggesting the existence of an overactivated state in cortistatin-deficient fibroblasts. The fibrogenic phenotype found in CST+/- pulmonary fibroblasts correlated with the hyperactivation of various intracellular factors that are critically involved in profibrogenic signalling (Wynn, 2011; Wynn and Ramalingam, 2013). Thus, the activation and subsequent nuclear translocation of Smad2/3 was significantly increased in CST+/- fibroblasts (Figure 6C, Figure S1A). Moreover, the basal levels of activated phosphorylated forms of various protein kinases, including Akt, p38 MAPK and ERK1/2, were markedly increased in cortistatin-deficient fibroblasts, showing similar or even higher activation levels than those showed by TGFβ1-activated CST+/+ fibroblasts (Figure 6C, Figure S1B). Furthermore, in comparison to wild-type fibroblasts, cortistatin-deficient pulmonary fibroblasts showed accelerated and increased migratory responses in a wound healing assay (Figure 6D). However, lack of cortistatin did affect neither fibroblast growth nor viability (Figure S2). Therefore, these findings indicate that cortistatin could act as an endogenous break of activation, migration and differentiation of fibroblasts.

Treatment with cortistatin ameliorates bleomycin-induced pulmonary inflammation and fibrosis. Our previous results indicate that cortistatin has a critical role in the regulation of pulmonary inflammation and fibrosis and that administration of cortistatin is a potential strategy for the prevention and treatment of lung injury-induced fibrosis. The systemic injection of cortistatin at the early stage significantly prevented the profound body weight loss, high mortality and severe pulmonary fibrosis that were induced by the administration of high-dose bleomycin (Figure 7A). These protective effects correlated with inhibition of pulmonary inflammation, injury and vascular leak (Figure 7A). Importantly, cortistatin also therapeutically attenuated bleomycin-induced mortality and the severity of pulmonary fibrosis at the later stages following bleomycin instillation (Figure 7B). Indeed, treatment of animals with cortistatin beginning five days after bleomycin challenge, once that pulmonary inflammation was fully established, significantly improved fibrosis score, reduced collagen deposition and decreased the presence of αSMA-expressing myofibroblasts in lung (Figure 7B). We found similar therapeutic efficiencies using both systemic (i.p.) and local (i.n., at five-fold lower doses) routes of administration (Figure 7B). Noteworthy from a therapeutic point of view is the fact that cortistatin treatment was able to reverse the susceptibility to suffer severe pulmonary fibrosis in mice that had a partial deficiency

in cortistatin after their exposition to low-doses of bleomycin (Figure 8). These results suggest that cortistatin-based therapies could impair pulmonary inflammation and attenuate the established pulmonary fibrosis.

Protective role of cortistatin in an experimental model of scleroderma. Finally, we investigated whether the protective effect showed by cortistatin in pulmonary fibrosis could be extended to other fibrotic pathologies and organs, such as skin fibrosis. As observed in pulmonary fibroblasts, profibrotic stimulation decreased the expression of cortistatin in primary mouse skin fibroblasts (Figure 9A), suggesting that skin is a potential target-tissue for cortistatin regulation. We evaluated skin fibrosis using an experimental mouse model of scleroderma induced by repetitive i.d. injections of bleomycin during four weeks, which mirrors many histopathological signs found in human systemic sclerosis (Yamamoto, 2010; Tsujino and Sheppard, 2016). As expected, bleomycin administration to CST+/+ mice generated skin lesions showing a twofold increase in dermal thickness (Masson's trichrome-staining) and dense accumulation of extracellular matrix (picrosirius red-staining), relative to naïve or saline-injected animals (Figure 9B). Bleomycin-challenged skin from CST+/- and CST-/- mice showed significantly thicker dermal lesions and denser fibrotic bands than that showed by the dermis of CST+/+ mice (Figure 9B). Moreover, skin lesions of cortistatin-deficient mice showed wide areas of epidermal hypertrophy and marked accumulation of inflammatory cells in the deeper dermal layers and perivascular spaces, that are significantly less frequents in wild-type skin lesions (Figure S3). The exacerbated histopathological signs of fibrosis observed in the dermis of cortistatin-deficient mice correlated with increased collagen contents and CTGF gene expression (Figure 9B). Whereas the regime of bleomycin challenge used in this study did not affect survival of CST+/+ mice, we observed a mortality rate of 20% and 25% in CST+/- and CST-/- mice, respectively, at three or four weeks after the i.d. bleomycin injection. Necropsy revealed the occurrence of oedematous/haemorrhagic lungs, pointing out to pulmonary failure as a probable cause of death in these animals. Because this model of dermal fibrosis may also course with slight signs of pulmonary fibrosis (Yamamoto, 2010), and a percentage of patients with scleroderma suffer pulmonary complications that are related to chronic fibrosis (Wei et al., 2011), we analysed histopathological signs of fibrosis in the lung collected from mice with dermal fibrosis. In these animals, we observed that the deficiency in cortistatin predisposed to develop severe pulmonary fibrosis, in contrast to wild-type mice that course with mild lung fibrosis (Figure 9C). Interestingly, local perilesional treatment with cortistatin of fibrotic skin almost abrogated the increase in dermal thickness and other histopathological signs and significantly reduced lung fibrosis that is associated to scleroderma (Figure 9D). These findings demonstrated that cortistatin also acts as a critical regulator of skin fibrosis, and its presence may avoid the appearance or progression of pulmonary fibrosis-associated complications.

Discussion

Inflammation and wound-healing are two physiological processes aimed at restoring normal tissue structure and function after an insult or injury. However, they can be more damaging than the insult itself if uncontrolled, excessive or prolonged. In the lung, a dysregulated inflammation causes excessive leukocyte accumulation and increased permeability of endothelial and alveolar epithelial barriers. A wound-healing response that has gone out of control after lung injury causes pulmonary fibrosis, which is characterized by progressive loss of alveolar structure, disruption of the epithelial-endothelial barrier, activation of fibroblasts and their differentiation to myofibroblasts, excessive deposition of extracellular matrix and tissue remodelling. Far of restoring host pulmonary homeostasis, aberrant inflammatory and fibrotic responses, in some cases being part of the same cascade, contribute to the pathogenesis of severe lung disorders, such as ALI/ARDS and IPF. A precise balance of inflammatory/fibrogenic versus immunomodulatory/antifibrotic factors must exist to tune adequately these responses, and the identification of factors that limit or reverse both processes is critical for understanding the pathophysiology and identifying new therapeutic targets for these disorders. In this study, by using two well-characterized experimental models of ARDS and pulmonary fibrosis, we point-out to cortistatin as an endogenous protective factor. We found that a deficiency in cortistatin predisposes for developing exacerbated inflammatory and fibrotic responses in injured lungs after exposition to bacterial endotoxins or chemotherapeutic drugs, even at low doses, and to subsequently suffer more severe disease progression and increased mortality. Moreover, our data show that a treatment with cortistatin is able to mitigate these pathological processes.

We envision the involvement of various non-excluding and complementary cellular and molecular mechanisms that could explain the protective effect of cortistatin in pulmonary inflammation and fibrosis (Figure 10). Firstly, previous reports demonstrated the anti-inflammatory activity of cortistatin on macrophages, and described its protective effect in murine models of sepsis and endotoxemia, mainly acting by regulating a wide panel of inflammatory mediators (Gonzalez-Rey et al., 2006b,c). Here, we confirmed in the lung that, cortistatin downregulates the production of various inflammatory cytokines and chemokines by activated BALF macrophages, and importantly, we found that infiltrating pulmonary macrophages that are deficient in cortistatin produced excessive levels of cytokines that are responsible of the pathophysiology of ALI/ARDS. This effect could be exerted in an autocrine/paracrine manner, since macrophages express both cortistatin and its receptors (Dalm et al., 2003; Gonzalez-Rey et al., 2006b,c; Markovics et al., 2012), and both sstr and ghrelin-receptors are involved in the anti-inflammatory activity of cortistatin (Gonzalez-Rey and Delgado, 2007). Moreover, its action on macrophages could orchestrate the infiltration of other leukocytes (i.e., neutrophils) involved in pulmonary inflammation and fibrosis. Furthermore, the immunomodulatory activity of cortistatin on lymphocytes is widely recognized, and beside a potent suppressive effect on Th1 cell-mediated inflammatory responses, we previously found that a major mechanism involved in the generation of immune tolerance by cortistatin is the induction of regulatory T cells (Gonzalez-Rey et al., 2007b), a T-cell subpopulation that is involved in amelioration of ALI/ARDS and pulmonary fibrosis (D'Alessio et al., 2009). These findings open the possibility that, by regulating the inflammatory/immune response in the lung, cortistatin could impair an initial step in the profibrotic cascade, and thus mitigate subsequent progression to pulmonary fibrosis. Although the immunomodulatory action of cortistatin could contribute to its anti-fibrotic role, evidence suggests a direct and additional action of cortistatin in fibrogenic effectors in the lung. We found that pulmonary fibroblasts isolated from cortistatin-deficient mice showed overactivated TGFβ1-signaling pathways, including Smad2/3, Akt and MAPKs (p38 and ERK1/2), that drive the expression of genes (collagen, CTGF, αSMA) that drive pathological fibrosis (Wynn, 2011). Again, this effect could be mediated in a paracrine fashion, since fibroblasts express both cortistatin and its receptors (this study, Borie et al., 2008; Egger et al., 2014; Tug et al., 2013). In agreement, various studies reported the effect of cortistatin in MAPKs

and Akt in other cell types (Duran-Prado et al., 2013; Morell et al., 2014), and other sstr-agonists regulate the activation of fibroblasts (Borie et al., 2008; Wang et al., 2013). Interestingly, we observed a negative correlation between expression of cortistatin and the activation of pulmonary fibroblasts, confirming that cortistatin acts as an endogenous break that needs to be released for allowing fibroblast activation. Finally, the fact that treatment with cortistatin efficiently reduced fibrotic responses in bleomycin-challenged mice when initiated once that the inflammatory response was fully established in the lung also supports the capacity of cortistatin to directly limit the fibrogenic response.

Our findings have multiple clinical implications from both therapeutic and diagnostic points of view. First, we demonstrated that cortistatin-based therapies emerge as attractive alternatives for treatment of pulmonary disorders that course with hyperactivated inflammatory and fibrotic responses, such as ALI/ARDS and IPF. Despite the enormous progression made in the identification of the pathogenic mechanisms involved in the initiation and progression of these diseases, they are an enduring problem in respiratory and critical medicine that remains therapeutically unsolved, since they remain as major causes of morbidity and mortality world-wide (Matthay et al., 2017). This urgent need has acquired global dimensions lately during the pandemic covid-19 with the association of severe ARDS and pulmonary fibrosis with poor prognosis in SARS-Cov2-infected patients (Mehta et al., 2020). Due to the redundancy and complexity of the cytokine and fibrogenic network, the multitargeted action of cortistatin as an immunomodulatory agent on a plethora of mediators of the cytokine storm offers obvious advantages versus other therapies based on neutralization of a single molecule (i.e., monoclonal antibodies). Moreover, cortistatin-based therapies could limit the start of late-onset pulmonary fibrosis in patients with ARDS or covid-19 in post-infection stages. It is important mentioning that cortistatin has a favourable safety profile in humans and demonstrated clinical efficiency in patients with Cushing's disease (Giordano et al., 2007). Furthermore, the interest of the pharmaceutical companies in developing cortistatin-based analogues with improved half-life in serum has increased lately, and a recent report demonstrated their efficiency in inflammatory conditions (Rol et al., 2021). We have recently assisted to an example of repositioning of a therapy based in another immunomodulatory and anti-fibrotic neuropeptide, vasoactive intestinal peptide or Aviptadil (Chorny & Delgado, 2008; Prasse et al., 2010), for the treatment of ARDS in patients with severe covid-19 (Scavone et al., 2020; clinical trial: NCT04311697).

Second, the fact that a simple partial deficiency in cortistatin could predispose for developing exacerbated inflammatory and fibrotic responses could be used to anticipate the diagnostic of more severe forms of pulmonary disorders. In this sense, this study opens the possibility of initiating further clinical research that corroborates plasma cortistatin level as a biomarker of protection or prognosis in patients with ARDS or IPF. If this is the case, our results also suggest that a treatment based in cortistatin injection would easily correct the deficiency and improve disease progression. Although the endogenous and environmental factors that could influence in the body levels of cortistatin are mostly unknown, one could anticipate that these levels will change along our life under different circumstances and scenarios, and thus, our susceptibility to suffer certain disorders. In any case, a percentage of the human population is heterozygous for cortistatin from birth, since cortistatin gene is located at chromosome 1p36.22, and monosomy of 1p36 is the most common subtelomeric terminal deletion syndrome (Jordan et al., 2015). Beside a spectrum of neurologic and sensorial defects, these individuals develop cardiac fibrosis and cardiomyopathy, although no studies describe increased incidence of pulmonary disorders, despite their short life expectancy.

Beside the potential to treat pulmonary disorders as IPF, our data in a model of scleroderma point-out to the possibility to extend the use of cortistatin for the treatment of progressive fibrosis affecting other tissues. Noteworthy is that cortistatin-deficient mice not only developed exacerbated skin lesions, but they also showed severe pulmonary fibrosis and associated

mortality in response to dermal bleomycin exposition. It is intriguing to speculate with the possibility that a deficiency in cortistatin could contribute to the susceptibility to suffer pulmonary fibrosis-related complications that occur in a percentage of patients with skin scleroderma (Wei et al., 2011), in which factors involved in this differential susceptibility are mostly unknown.

Finally, bleomycin is a chemotherapeutic drug used for treating many types of cancer. Unfortunately, a proportion of bleomycin-treated patients develop severe side-effects that are associated to appearance of pulmonary and skin fibrosis that mostly obligate to treatment withdrawal. Understanding that deficiency in a factor like cortistatin could predispose to such adverse effects could help to select the correct chemotherapy or try to correct them by cortistatin-based treatments.

In summary, this study provides new insights into the function of cortistatin in pulmonary inflammation and demonstrates a novel role of this neuropeptide in fibrosis, which could be extrapolated beyond lung tissue. Overall, we demonstrate that deficiency in cortistatin could be considered a potential biomarker of susceptibility to suffer severe forms of pulmonary disorders including ARDS, IFP or pneumonia, and that therapies based in the use of cortistatin or its stable analogues emerge as attractive alternatives for treating diseases that are still unresolved from a clinical point of view.

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Figure legends

Figure 1. Treatment with cortistatin ameliorates ALI. A. Mice with LPS-induced ALI were treated i.p. with vehicle or cortistatin (LPS+CST) as described in the scheme. Animals treated i.n. with saline were used as basal controls. The number of total cells and leukocyte subpopulations in BALF were determined at the indicated times. MPO activity in lung extracts was determined to confirm the presence of neutrophils. n=8 mice/group. **B.** Cytokine contents in BALF isolated 12h after LPS-induced ALI (n=6 mice/group). **C.** Lung oedema was determined by measuring protein and albumin contents in BALFs and wet/dry weight ratios of lungs isolated 48h after LPS-induced ALI (n=7 mice/group). **D.** Histopathological scores were determined in H&E-stained sections of lungs collected 48h after LPS-induced ALI (n=8 mice/group, scale bar: 100 µm). **E.** Spontaneous production of inflammatory cytokines by macrophages isolated from BALFs recovered 48h after LPS-induced ALI (n=5 mice/group, each by duplicated cell cultures). **F.** Cytokine production by BALF macrophages collected two days after LPS-induced ALI and restimulated *ex vivo* with medium or LPS in the absence or presence of CST (n=5 mice/group, each by duplicated cell cultures). Results are the mean±SD with dots representing individual values of biologically independent animals. *p<0.05 vs. LPS+vehicle.

Figure 2. Deficiency in cortistatin exacerbates severity of ALI and pulmonary inflammation. ALI was induced in wild-type (CST+/+), partially-deficient (CST+/-) or totally-deficient (CST-/-) mice for cortistatin by i.n. instillation of LPS (1 $mg \cdot kg^{-1}$). **A.** The number of total cells in BALFs and MPO activity in lung extracts were determined in samples isolated 24h after LPS inhalation. n=10 mice/group. **B.** Histopathological scores were determined in H&E-stained lung sections isolated 24h after LPS-induced ALI (n=7 mice/group, scale bar: 200 µm). **C.** Cytokine contents in BALF collected 12h after LPS-induced ALI (n=5 mice/group). **D.** Lung oedema was assayed by measuring the protein contents in BALFs collected 48h after LPS-induced ALI (n=7-10 mice/group). **E.** Production of inflammatory TNFα by macrophages isolated from BALFs collected from CST+/+, CST+/- or CST-/- mice 24h after LPS-induced ALI and *ex vivo* restimulated with medium or LPS in the absence (none) or presence of cortistatin (n=5 mice/group, each by duplicated cell cultures). Results are the mean±SD with dots representing individual values of biologically independent animals. *p<0.05 vs. CST+/+ mice. #p<0.05 vs. untreated macrophages in panel E.

Figure 3. Administration of cortistatin reverses the exacerbated ALI phenotype showed by cortistatin-deficient mice. ALI was induced in partially-deficient mice for cortistatin (CST+/-) by i.n. injection of LPS and then treated i.p. with vehicle or cortistatin. Histopathological scores were determined in H&E-stained lung sections (n=6 mice/group, scale bar: 200 µm), and leukocyte infiltration, protein and cytokine contents were determined in BALFs isolated 24h after LPS-induced ALI (n=7 mice/group). Dashed grey horizontal lines correspond to values obtained from LPS-infused CST+/+ mice. Results are the mean±SD with dots representing individual values of biologically independent animals. *p<0.05 vs. vehicle-treated CST+/- mice.

Figure 4. Deficiency in cortistatin significantly increases the mortality in experimental lung fibrosis. Pulmonary fibrosis was induced by intratracheal injection of different doses of bleomycin in wild-type (CST+/+), partially-deficient (CST+/-) or totally-deficient (CST-/-) mice for cortistatin and survival was daily monitored. Each panel includes the number of mice that were used per group. *p<0.05 vs. CST+/+ mice.

Figure 5. Deficiency in cortistatin increases the severity in an experimental model of lung fibrotic disease. Pulmonary fibrosis was induced by intratracheal injection of bleomycin (1.8 $U \cdot kg^{-1}$) in wild-type (CST+/+), partially-deficient (CST+/-) or totally-deficient (CST-/-) mice for cortistatin. **A.** Body weight loss (n=10 mice/group) and survival (n=28 CST+/+, n=42 CST+/-, n=40 CST-/-) were daily monitored. **B.** Pulmonary fibrosis scores were determined at the indicated times after bleomycin injection in Masson's trichrome-stained lung sections (n=8 mice/group, unless 6 mice/group at day 14, scale bar: 200 µm). **C.** The number of total cells, macrophages, neutrophils and T lymphocytes in BALFs (n=8 mice/group), MPO activity in lung extracts (n=6 mice/group) and BALF cytokine contents (n=6 mice/group) were determined at the indicated times. **D.** Lung oedema was determined by measuring protein contents in BALFs (n=8 mice/group) and Evans-blue extravasation index in lungs (n=5 mice/group) isolated at the indicated times. **E.** Markers of lung fibrosis were determined at the indicated times by measuring collagen contents and TGFβ1 levels in lung protein extracts (n=6 mice/group), CTGF mRNA expression in lungs (n=12 mice/group) and αSMA-positive immunofluorescence in lung sections (n=5-6 mice/group, scale bar: 100 µm). Dashed horizontal lines correspond to values obtained from naïve mice (n=6). Results are the mean±SD with dots representing individual values of biologically independent animals. *p<0.05 vs. CST+/+ mice.

Figure 6. Cortistatin-deficient fibroblasts show exacerbated profibrotic responses. A. Gene expression of cortistatin by unstimulated and TGFβ1-activated mouse primary lung fibroblasts (n=3 cultures) and by lungs isolated from naïve or bleomycin-treated mice (n=5 mice/group). **B-D.** Primary lung fibroblasts were isolated from wild-type (CST+/+) and partially-deficient (CST+/-) mice for cortistatin were cultured in the absence or presence of TGFβ1 (10 $ng \cdot ml^{-1}$) stimulation. **B.** αSMA and CTGF gene expression was determined after 24h culture (13 unstimulated cultures, 9 stimulated cultures). **C.** Nuclear translocation of activated Smad2/3 was determined by immunofluorescence analysis and the levels of activated phosphorylated Akt, p38 MAPK and ERK1/2 were analysed by western blot after 1h of culture (n=4-5 independent experiments, in triplicates). See online Figure S1 for representative immunofluorescence images and western blots. Data in panels B and C are relative to unstimulated CST+/+ fibroblasts. **D.** Fibroblast migration activity was measured at different time points using an *in vitro* wound healing assay (n=6 independent cultures). Results are the mean±SEM with dots representing individual values of independent experiments. *p<0.05.

Figure 7. Treatment with cortistatin reduces mortality and disease severity of experimental lung fibrosis. A and B. Mice with bleomycin-induced severe pulmonary fibrosis were treated with vehicle or with cortistatin following an early protective regime (**A**) or a delayed therapeutic regime (**B**) as indicated in the two schemes. Mice injected intratracheally with saline instead bleomycin were used as basal controls (n=6). Mortality, body weight loss (8 mice/group) and histopathological signs of lung fibrosis (8 mice/group in **A**, and 7-13 mice/group in **B**) were determined at the indicated time points. Pulmonary leukocyte infiltration, levels of inflammatory cytokines and oedema (protein levels) were assayed in BALFs (6 mice/group). Fibrogenic markers including the content of collagen (10 mice/group) and the presence of αSMA-positive myofibroblasts (expressed as fluorescence mean, 7 mice/group) were determined in lungs isolated at the indicated time points. Scale bars: 150 µm. Results are the mean±SD with dots representing individual values of biologically independent animals. *p<0.05 vs. Bleo+vehicle-treated mice.

Figure 8. Exogenous administration of cortistatin reversed the exacerbated fibrogenic phenotype observed in cortistatin-deficient mice. Lung fibrosis was induced in partially-deficient mice (CST+/-) for cortistatin and treated with vehicle or cortistatin as indicated in the scheme. Bleomycin-challenged CST+/+ mice were used as controls of reference. Mortality, histopathological signs of fibrosis (5-11 mice/group) and the presence of αSMA-positive myofibroblasts (5 mice/group) were determined in lungs isolated at the indicated time points. Scare bars: 100 µm. Results are the mean±SD with dots representing individual values of biologically independent animals. *p<0.05.

Figure 9. Cortistatin regulates dermal and pulmonary fibrosis in an experimental model of scleroderma. A. Gene expression of cortistatin by unstimulated and TGFβ1-activated mouse primary skin fibroblasts (n=3 cultures) and by skin biopsies isolated from naïve or bleomycin-treated mice (n=6 mice/group). **B.** Skin fibrosis was induced in wild-type (CST+/+), partially-deficient (CST+/-) and totally-deficient (CST-/-) mice for cortistatin by repetitive i.d. local injections of bleomycin as indicated in the scheme. The increase in dermal thickness and fibrosis was determined in Masson's trichrome- and Picrosirius red-stained sections of skin samples isolated on day 28 (n=7-10 mice/group, representative images are shown, scale bar: 100 µm). CTGF gene expression (5-10 mice/group) and collagen content (5 mice/group) in skin samples were quantified on day 28. Horizontal dashed lines represent values obtained from skin samples i.d. injected with saline instead bleomycin (n=8 mice). **C.** Analysis of fibrogenic markers in lungs isolated from CST+/+, CST+/- and CST-/- mice with bleomycin-induced skin scleroderma (representative images of Masson's trichrome-stained sections are shown, scale bar: 100 µm). The presence of αSMA-positive myofibroblasts in lung sections was analysed by immunofluorescence. n=8-10 mice/group. **D.** Mice with bleomycin-induced dermal fibrosis were treated locally with cortistatin as indicated in the scheme. The increase in dermal thickness (10 mice/group) relative to saline-injected skin (dashed line) and lung fibrosis scores (5 mice/group) were determined in tissue samples isolated at day 28 (representative images of Masson's trichrome- and Picrosirius red-stained sections are shown, scale bar: 100 µm). Results are the mean±SD with dots representing individual values of biologically independent animals. *p<0.05 vs. CST+/+ mice (panels B and C) or vehicle-treated mice (panel D).

Figure 10. Scheme illustrating the cellular and molecular mechanisms involved in the protective effect of cortistatin on pulmonary inflammation and fibrosis. Panel A: Structure of a healthy alveolus showing a cleared alveolar space and intact and thin epithelial-endothelial barrier and surfactant layer that allow normal gas exchange. **Panel B:** Bacterial infection (i.e., LPS), pneumonia or tissue damage by chemicals (i.e., bleomycin) may cause ALI, which is characterized by neutrophil recruitment to the lung, with both alveolar (by resident macrophages) and systemic release of inflammatory cytokines (TNFα, IL1, IL6) and chemokines (MIP2). Exaggerated alveolar inflammation and oxidative stress induce apoptosis/necrosis of epithelial and endothelial cells and damage the alveolar-capillary barrier, leading to the development of pulmonary oedema and hypoxemia. Moreover, the release of inflammatory cytokines and growth factors (TNFα, TGFβ1) activates pulmonary fibroblasts and induces secretion/deposition of extracellular matrix components (i.e., collagen, fibronectin), generating an interstitial fibrotic scar that contributes to the impairment of gas exchange. **Panel C:** The subsequent course of ARDS is aggravated in the absence of cortistatin. In cortistatin-deficient mice, even after the exposition to low injury-inducing agents, the exacerbated and persistent pulmonary inflammation and the progression to intra-alveolar fibrosis/scarring by hyperactivated fibroblasts and αSMA^{+}-myofibroblasts, avoid the reabsorption of alveolar oedema fluid and repair of the injured alveolar epithelium, do not allow recovery from respiratory failure and in some cases causes the death. **Panel D:** Cortistatin, which is produced

endogenously in the lung by alveolar macrophages (Mϕ) and fibroblasts or provided exogenously from other tissues (i.e., infiltrating inflammatory cells, pulmonary circulation) or through cortistatin-based treatments, is able to: 1) deactivate alveolar and infiltrating macrophages and reduce production of inflammatory cytokines and chemokines that damage alveolar structure and activate/attract neutrophils; 2) impair the production by macrophages of cytokines/growth factors that signal for fibrogenic responses in fibroblasts; 3) reduce the activation of fibroblasts and their differentiation to myofibroblasts by acting through its receptors (CST-R) as a break of intracellular signal factors (Smad2/3, Akt and MAPKs) that are critical for gene expression of profibrogenic mediators (see in square-box). Interestingly, fibrogenic activation impairs the secretion of cortistatin by fibroblasts, thus releasing this endogenous break.