

Original Paper

The Effects of Dasatinib in Experimental Acute Respiratory Distress Syndrome Depend on Dose and Etiology

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Key Words

Dasatinib • Cytokines • Histology • Lung mechanics • Acute respiratory distress syndrome • Toll like receptor-4

Abstract

Background/Aims: Evidence suggests that tyrosine-kinase inhibitors may attenuate lung inflammation and fibrosis in experimental acute respiratory distress syndrome (ARDS). We hypothesized that dasatinib, a tyrosine-kinase inhibitor, might act differently depending on the ARDS etiology and the dose. **Methods:** C57/BL6 mice were divided to be pre-treated with dasatinib (1mg/kg or 10mg/kg) or vehicle (1% dimethyl-sulfoxide) by oral gavage. Thirty-minutes after pre-treatment, mice were subdivided into control (C) or ARDS groups. ARDS animals received Escherichia coli lipopolysaccharide intratracheally (ARDS_{Sp}) or intraperitoneally (ARDS_{Sexp}). A new dose of dasatinib or vehicle was administered at 6 and 24h. **Results:** Forty-eight hours after ARDS induction, dasatinib 1mg/kg yielded: improved lung morphofunction and reduced cells expressing toll-like receptor (TLR)-4 in lung, independent of ARDS etiology; reduced neutrophil and levels of interleukin (IL)-6, IL-10 and transforming growth factor (TGF)- β in ARDS_{Sp}. The higher dose of dasatinib caused no changes in lung mechanics, diffuse alveolar damage, neutrophil, or cells expressing TLR4, but increased IL-6, vascular endothelial growth factor (VEGF), and cells expressing Fas receptor in lung in ARDS_{Sp}. In ARDS_{Sexp}, it improved lung morphofunction, increased VEGF, and reduced cells expressing TLR4. **Conclusion:** Dasatinib may have therapeutic potential in ARDS independent of etiology, but careful dose monitoring is required.

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Introduction

Acute respiratory distress syndrome (ARDS) is a life-threatening condition induced by several clinical disorders and associated with a deregulated inflammatory response [1]. Although advances in supportive care and ventilator management for human ARDS have reduced short-term mortality [2], effective pharmacological therapies are still lacking. In this context, recent studies evaluated the role of tyrosine kinase inhibitors in experimental ARDS [3-5]. As major therapeutic agents in oncology, these pharmacological agents block the action of different classes of protein tyrosine kinases (PTKs), important molecules that regulate many intracellular signaling pathways, including the acute inflammatory response to different stimuli [6]. Dasatinib, a small-molecule tyrosine kinase inhibitor acting on both Abl- and Src-family tyrosine kinases [7], is currently used to treat chronic myelogenous leukemia and Philadelphia chromosome-positive acute lymphoblastic leukemia [8-10]. Besides its effect on malignant cells, dasatinib also inhibits cells critical for the inflammatory reaction [11] and reduces lung inflammation [12]. In addition, Src-family tyrosine kinases, the major targets of dasatinib, play critical roles in various inflammatory disease models [13, 14].

Since the pathophysiology of ARDS may differ according to the type of primary insult, resulting in the activation of different inflammatory mechanisms [15-17], we tested the hypothesis that dasatinib may act differently in experimental pulmonary (p) or extrapulmonary (exp) ARDS with similar mechanical compromise at the early phase of the lesion. The impact of different doses and possible mechanisms of action were also evaluated.

Materials and Methods

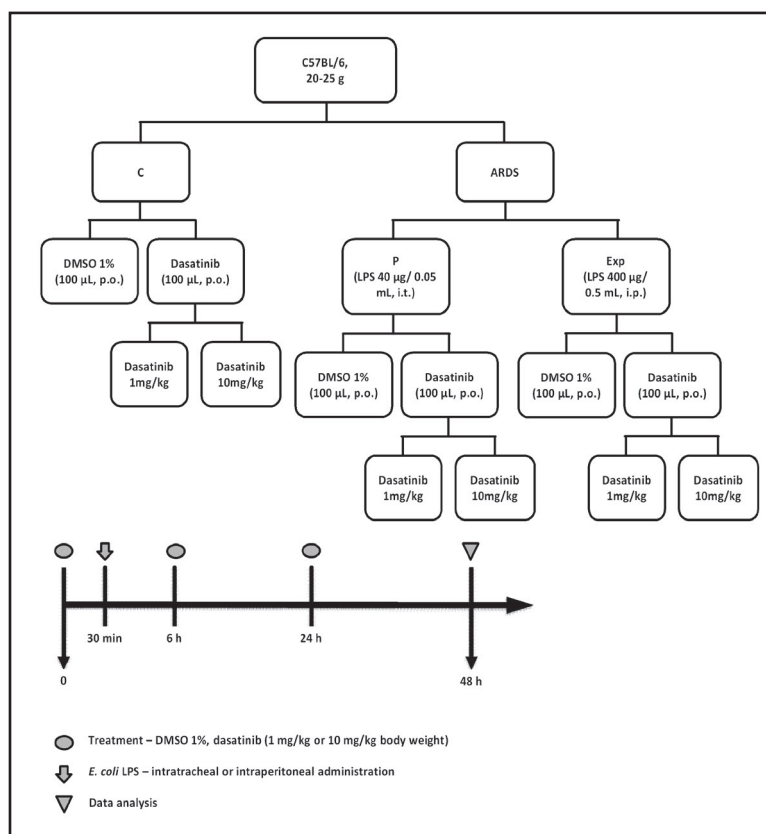
This study was approved by the Animal Welfare Committee of the Health Sciences Centre, Federal University of Rio de Janeiro (CEUA-CCS-019). All animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the U.S. National Research Council *Guide for the Care and Use of Laboratory Animals*.

Animal preparation and experimental protocol

A total of 123 C57/BL6 mice (weight 20-25 g, age 5 weeks) were used. The mice were kept under specific pathogen-free conditions, maintained without physical activities, on a 12:12 h light-dark cycle, at controlled temperature (20-22°C), and had unrestricted access to food and water. Initially, all mice were randomly divided to be pre-treated with vehicle (dimethyl sulfoxide [DMSO] 1% in saline solution, 100 µL, by oral gavage) or dasatinib, which was administered in two different doses (1 mg/kg body weight [BW] or 10 mg/kg BW, diluted in 1% DMSO, 100 µL by oral gavage). Thirty-minutes after pre-treatment with DMSO or dasatinib, animals were then randomly subdivided into a control (C) group, a pulmonary acute respiratory distress syndrome (ARDSp) group, and an extrapulmonary ARDS (ARDSexp) group. In the C groups, the animals did not undergo any surgical procedures, instillations, or injections. In the ARDS groups, mice received *Escherichia coli* O55:B5 lipopolysaccharide (LPS, Sigma Chemical Co., St. Louis, MO) either intratracheally (40 µg in 0.05 mL saline, ARDSp) or intraperitoneally (400 µg in 0.5 mL saline, ARDSexp) (Fig. 1). For intratracheal instillation, mice were anesthetized with sevoflurane, a 1-cm-long midline cervical incision was made to expose the trachea, and LPS was instilled using a bent 27G tuberculin needle. The cervical incision was closed with 5-0 silk suture and the mice returned to their cage.

The animals recovered rapidly after surgery. The dose of LPS was selected based on previous studies demonstrating similar mechanical and morphometrical compromise in ARDSp and ARDSexp C57/BL6 mice [18-20]. At two time points after pre-treatment (6h and 24h), DMSO, dasatinib 1 mg/kg, or dasatinib 10 mg/kg were given again as appropriate per group allocation. In short, nine groups were studied: three control groups (pre-treated with DMSO, dasatinib 1 mg/kg, or dasatinib 10 mg/kg) to investigate the effects of the drug *per se*; three ARDSp groups (treated with DMSO, dasatinib 1 mg/kg, or dasatinib 10 mg/kg); and three ARDSexp groups (treated with DMSO, dasatinib 1 mg/kg, or dasatinib 10 mg/kg). Forty-eight hours after induction of ARDS, 72 animals (n = 8/group) were selected for evaluation of: a) lung mechanics and histology,

Fig. 1. Schematic flowchart of the study design and timeline. Initially, all animals were randomly allocated to be pre-treated with dimethyl sulfoxide (DMSO 1%, 100 μ L, *po*) or dasatinib (DAS, 1 mg/kg or 10 mg/kg BW, 100 μ L, *po*). After 30 min, the animals pre-treated with DMSO and dasatinib were subdivided into control (C) or acute respiratory distress syndrome (ARDS) groups. In the ARDS groups, mice received *E. coli* LPS either intratracheally (40 μ g/0.05 mL saline, ARDS_p) or intraperitoneally (400 μ g/0.5 mL saline, ARDS_{exp}), while animals in the C groups did not undergo surgical procedures, instillations, or injections. A new dose of DMSO or dasatinib was given at 6 h and then 24 h after pre-treatment. Data were collected 48 h after



ARDS induction. Eight animals per group were used for mechanical, histological, and immunohistochemical analysis, whereas five animals per group were used for BALF collection and cytokine analysis in lung tissue.

b) levels of interleukin (IL)-6, IL-10, transforming growth factor (TGF)- β , and vascular endothelial growth factor (VEGF) in lung tissue, c) number of cells expressing toll like receptor (TLR)-4 and Fas receptor in lung parenchyma, and d) liver and kidney tissue damage. In an additional group, 45 mice were subjected to the same experimental protocol and levels of IL-6, IL-10, TGF- β , and VEGF were evaluated in bronchoalveolar lavage fluid (BALF) (n = 5/group).

Lung mechanics

Forty-eight hours after pre-treatment with DMSO or dasatinib (1 mg/kg and 10 mg/kg), mice were sedated (diazepam 1 mg/kg, *ip*) and anesthetized (ketamine 67 mg/kg and xylazine 30 mg/kg, *ip*), tracheotomized, paralyzed (vecuronium bromide, 0.005 mg/kg, *iv*), and ventilated with a constant flow ventilator (Samay VR15; Universidad de la Republica, Montevideo, Uruguay) using the following parameters: respiratory frequency 100 breaths.min⁻¹, tidal volume (V_T) 0.2 mL, and fraction of inspired oxygen (FiO_2) 0.21. The anterior chest wall was surgically removed and a positive end-expiratory pressure (PEEP) of 2 cmH₂O was applied. This PEEP level was identified based on previous pilot studies. For this purpose, the chest wall was surgically removed and transpulmonary pressure was carefully measured in paralyzed mice when the chest was opened (by occluding the tracheal cannula and measuring the tracheal pressure). The mean transpulmonary pressures in C, ARDS_p and ARDS_{exp} groups presented values equal to approximately 2 cm H₂O, with no significant differences among them. Therefore, we applied a PEEP equal to 2 cm H₂O, which represented the mean transpulmonary pressure considering C and ARDS groups together.

Airflow and tracheal pressure (P_{tr}) were measured. In an open chest preparation, P_{tr} reflects transpulmonary pressure (P_l). After a 10-min ventilation period, static lung elastance (Est,L) was measured using the end-inflation occlusion method [21].

Data were analyzed using ANADAT data analysis software (RHT-InfoData, Inc., Montreal, Quebec, Canada). All experiments lasted less than 15 min.

Histology

A laparotomy was performed immediately after determination of lung mechanics and heparin (1,000 IU) was injected into the vena cava. The trachea was clamped at end-expiration (PEEP = 2 cmH₂O) and the abdominal aorta and vena cava were sectioned, yielding a massive hemorrhage that quickly killed the animals. The right lung was then removed, fixed in 4% buffered formaldehyde, and paraffin-embedded. Sections (4 μm thick) were cut and stained with hematoxylin-eosin. Photomicrographs at magnifications of x100, x200, and x400 were obtained from four non-overlapping fields of view per section using a light microscope. Diffuse alveolar damage (DAD) was quantified using a weighted scoring system by a researcher blinded to the experimental protocol [22]. Briefly, scores of 0 to 4 were used to represent the severity of septal thickening, alveolar collapse, inflammatory infiltration, and hemorrhage, with 0 standing for no effect and 4 for maximum severity. Additionally, the extent of each scored characteristic per field of view was determined on a scale of 0 to 4, with 0 standing for no visible evidence and 4 for complete involvement. Scores were calculated as the product of severity and extent of each feature, on a range of 0 to 16. The cumulative DAD score was calculated as the sum of each score characteristic, and ranged from 0 to 64.

Collagen fibers (Picrosirius-polarization method) were also quantified in the alveolar septa. Photomicrographs at x400 magnification were captured from ten non-overlapping fields of view per section with an Evolution VF Color Cooled 12-bit digital camera (Media Cybernetics, Silver Spring, MD, USA). The area occupied by fibers was determined by digital densitometric recognition, using Image-Pro Plus 6.3 Software for Windows (Media Cybernetics, Silver Spring, MD, USA) [23], and divided by the area of each studied septum, to avoid bias due to septal edema or alveolar collapse. The results were expressed as the fractional area occupied by collagen fibers in the alveolar septa. All lung histological analyses were performed in a blinded manner by an expert in lung pathology (CMT), i.e. the observer was unaware of the experimental protocol.

Kidney and liver tissue damage

The left kidney and the distal part of the right lobe of the liver were also removed after euthanasia. The tissues were fixed in 5% buffered formaldehyde, paraffin-embedded, and sections (4 μm thick) obtained. Liver sections were stained with hematoxylin-eosin, whereas kidney tissue was stained with periodic acid-Schiff reagent (PAS) to visualize the basement membrane. Ten to 15 fields per section from random tubular regions of the renal cortex and liver parenchyma were captured at a magnification of x400. Renal tubular damage was defined as tubular epithelial swelling, loss of brush border, vacuolar degeneration, and desquamation. A five-point, semi-quantitative, severity-based scoring system was used to assess each lesion parameter, graded as: 0 = normal tissue; 1 = 1–25%; 2 = 26–50%; 3 = 51–75%; and 4 = 76–100% of examined tissue.

In liver tissue, 10 fields per liver zone (central, lobular, and portal) were captured at a magnification of x400. The ratio between sinusoidal cells and total cells was computed and expressed as percentage.

Image-Pro Plus 6.3 for Windows (Media Cybernetics, Silver Spring, MD, USA) was used for all analyses.

Evaluation of bronchoalveolar lavage fluid

Forty-five additional animals (n=5/group) were subjected to the same protocol described above and underwent bronchoalveolar lavage fluid (BALF) analysis. Briefly, bronchoalveolar lavage was carried out via tracheal tube with phosphate-buffered saline solution (0.5 ml) containing ethylenediamine tetraacetic acid (10 mM). BALF was centrifuged at 4°C for 10 minutes at 400×g and the cell pellet resuspended in phosphate-buffered saline for further leukocyte enumeration. The supernatant was stored at -80°C for cytokine analysis. Differential cell counts were performed in Cytospin smears stained by the May-Grünwald-Giemsa method [20]. Total leukocyte numbers were measured in a Neubauer chamber under light microscopy after diluting the samples in Türk solution (2% acetic acid).

Enzyme-linked immunosorbent assay – ELISA

IL-6, IL-10, VEGF (PeproTech, Rocky Hill, NJ, USA), and TGF-β (R&D, Minneapolis, MN, USA) levels were quantified in lung homogenate and BALF with ELISA kits, in accordance with manufacturer instructions. Lung tissue was homogenized in lysis buffer (PBS 1×, Triton X 0.01%, 1× Roche protease inhibitor cocktail [Roche Diagnostic, Mannheim, Germany]) using a glass Potter homogenizer with Teflon piston. The total amount of cytokines was quantified according to the manufacturer's protocol and normalized to the total content of protein as quantified by Bradford's reagent (Sigma-Aldrich, St Louis, MO, USA).

Immunohistochemistry

Immunohistochemical analysis for TLR4 and Fas receptor in lung tissue was done using rabbit anti-TLR4 polyclonal antibody (Abcam, catalog no. ab47093, 1:30 dilution) and rabbit anti-Fas monoclonal antibody (Santa Cruz, catalog no. SC1024, 1:200 dilution), respectively.

Paraffin-embedded tissue sections (4 μ m thick) were dewaxed and rehydrated, and heat-mediated antigen retrieval was performed with citrate buffer 10 mM (pH = 6.0) in a microwave oven for 5 minutes. Endogenous peroxidase activity was inhibited in 70% hydrogen peroxide solution in methanol. Non-specific immunoglobulin binding was blocked by 10% bovine serum albumin in phosphate-buffered saline (pH = 7.4), before primary antibody incubation. Antibodies were revealed with biotinylated secondary antibody (Nichirei-Histofine® Simple Stain Mouse Max PO®, Universal Immuno-peroxidase Polymer for mouse tissues, anti-rabbit) and detected with peroxide/DAB (Kit Dako® Liquid DAB + Substrate Chromogen System). Slides were counterstained with Harris hematoxylin (1:30).

Analysis was performed in 15-20 images of high-power fields (x400) per slide taken with an Evolution VR Cooled Color 13-bit digital camera (Media Cybernetics, Canada), manually selected under a light microscope (Nikon Eclipse 400, Nikon Instruments Tokyo, Japan). The area occupied by cells staining positively for the marker in each tissue area were then calculated and expressed as the fractional area occupied by positive cells.

Statistical analysis

Based on our experience with pharmacological therapy of endotoxin-induced ARDS in small animals [24], we expected that a sample size of six animals per group would provide appropriate power ($1-\beta=0.8$) to identify significance ($\alpha=0.05$). The normality of the data (Kolmogorov-Smirnov test with Lilliefors' correction) and the homogeneity of variances (Levene median test) were tested. Parametric data are expressed as mean \pm SD, while non-parametric data are expressed as median (interquartile range). Differences among the groups were assessed by one-way ANOVA followed by Bonferroni's test. Nonparametric data were analyzed using ANOVA on ranks followed by Dunn's post hoc test. All tests were performed using the GraphPad Prism v5.00 statistical software package (GraphPad Software, La Jolla, CA, USA). Significance was established at $p < 0.05$.

Results

Effect of dasatinib on overall survival

At 48h, the survival rate of C groups was 100% regardless of therapy and dose of dasatinib used. Treatment with DMSO led to 13% mortality in the ARDSexp group (2 of 15 animals) but no deaths in the ARDSp group. The lower dose of dasatinib resulted in 100% survival in both ARDSp and ARDSexp groups. The higher dose of dasatinib (10 mg/kg) led to 24% mortality in the ARDSp group (4 of 17 animals) but no deaths in the ARDSexp group.

Dasatinib protected lungs against morphological and functional changes observed in ARDSp and ARDSexp in a dose-dependent manner

Est,L was higher in ARDSp and ARDSexp animals treated with DMSO compared to C-DMSO (Fig. 2). The lower dose of dasatinib (1 mg/kg) led to decreased Est,L regardless of ARDS etiology. However, the higher dose of dasatinib (10 mg/kg) resulted in reduced Est,L in ARDSexp and similar Est,L in ARDSp in comparison to ARDSexp-DMSO and ARDSp-DMSO groups, respectively (Fig. 2).

All ARDS-DMSO animals exhibited alveolar collapse, septal thickening, and neutrophil infiltration. Hemorrhage was observed only in ARDSp animals. Additionally, alveolar edema was present in only two ARDSp animals. The DAD score was higher in ARDS-DMSO compared with C-DMSO (Fig. 3 and 4) animals. In both ARDSp and ARDSexp animals, dasatinib (1 mg/kg) yielded a lower DAD score compared to ARDSp-DMSO and ARDSexp-DMSO, respectively. However, the higher dose of dasatinib (10 mg/kg) produced a similar DAD score in the ARDSp group in comparison to ARDSp-DMSO, while it was effective at improving lung histology in ARDSexp animals (Fig. 3 and 4). Collagen content was more increased in ARDSp-

Fig. 2. Static lung elastance (Est,L) in experimental groups, 48h after ARDS induction. Initially, all animals were randomly allocated to be pre-treated with dimethyl sulfoxide (DMSO 1%, 100 μ L, *po*) or dasatinib (DAS, 1 mg/kg or 10 mg/kg BW, 100 μ L, *po*). After 30 min, the animals pre-treated with DMSO and dasatinib were subdivided into control (C) or acute respiratory distress syndrome (ARDS) groups. In the ARDS groups, mice received *E. coli* LPS either intratracheally (40 μ g/0.05 mL saline, ARDSp) or intraperitoneally (400 μ g/0.5 mL saline, ARDSexp), while animals in the C groups did not undergo surgical procedures, instillations, or injections. A new dose of DMSO or dasatinib was given at 6h and then 24h after pre-treatment. Values are means \pm standard error of five animals in each group. *Significantly different from respective C-DMSO group ($p < 0.05$). **Significantly different from ARDS-DMSO ($p < 0.05$). #Significantly different from ARDS-dasatinib 1 mg/kg group ($p < 0.05$).

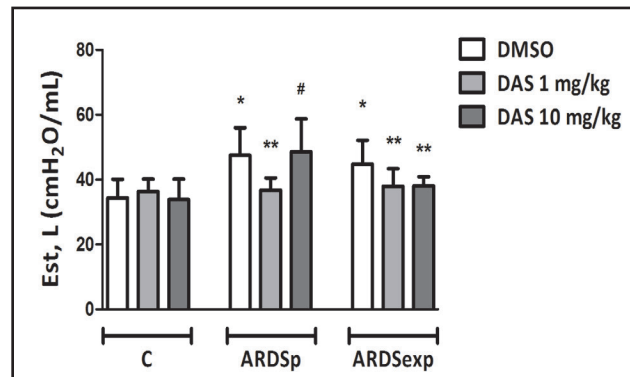
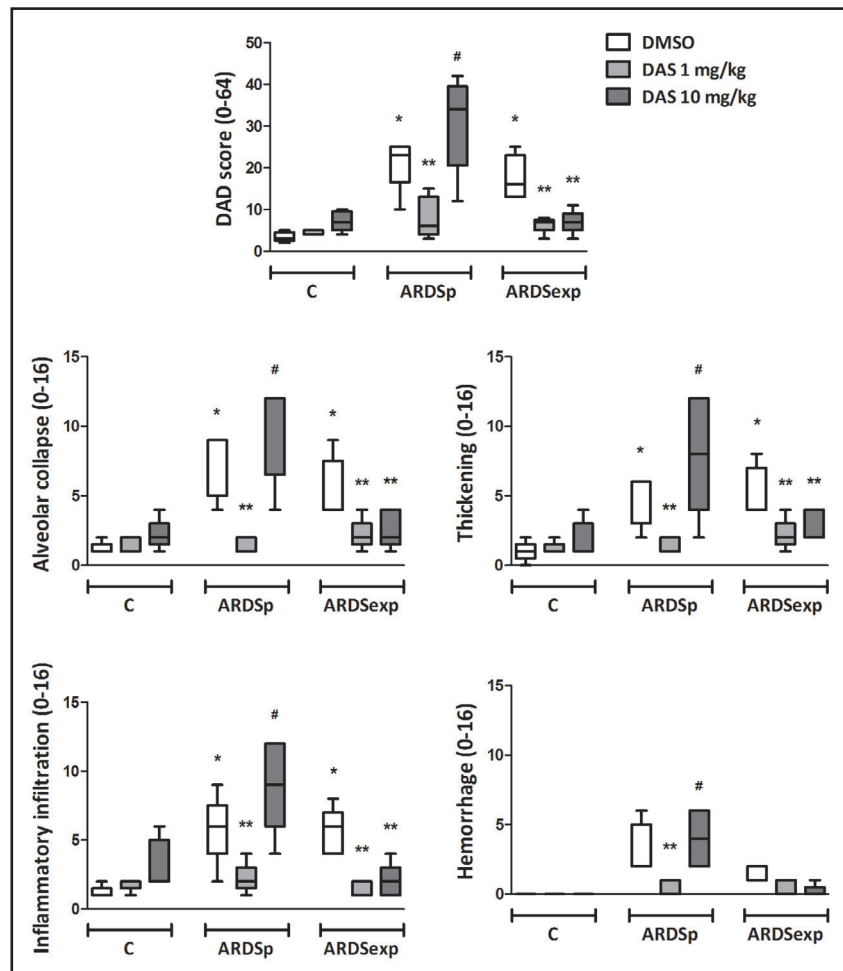


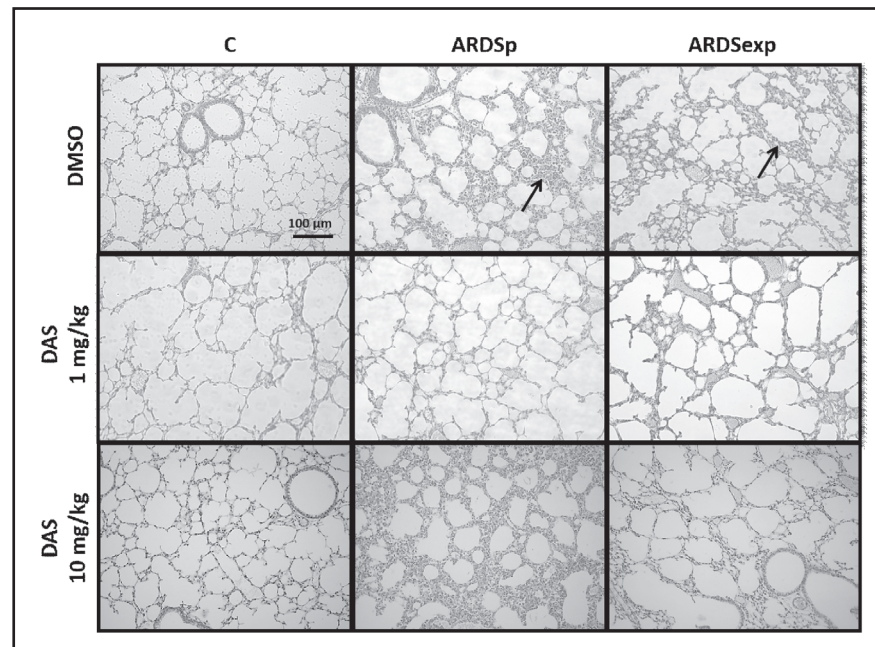
Fig. 3. Diffuse alveolar damage (DAD) score in experimental groups 48h after ARDS induction. Initially, all animals were randomly allocated to be pre-treated with dimethyl sulfoxide (DMSO 1%, 100 μ L, *po*) or dasatinib (DAS, 1 mg/kg or 10 mg/kg BW, 100 μ L, *po*). After 30 min, the animals pre-treated with DMSO and dasatinib were subdivided into control (C) or acute respiratory distress syndrome (ARDS) groups. In the ARDS groups, mice received *E. coli* LPS either intratracheally (40 μ g/0.05 mL saline, ARDSp) or intraperitoneally (400 μ g/0.5 mL saline, ARDSexp), while animals in the C groups did not un-



dergo surgical procedures, instillations, or injections. A new dose of DMSO or dasatinib was given at 6h and then 24h after pre-treatment. Values are medians (interquartile range) of eight animals in each group. A scale of 0 to 4 was used to represent the severity of septal thickening, alveolar collapse, inflammatory infiltration, and hemorrhage, with 0 standing for no effect and 4 for maximum severity. Additionally, the extent of each score characteristic per field of view was graded on a scale of 0 to 4, with 0 standing for no

appearance and 4 for complete involvement. Scores were calculated as the product of severity and extent of each feature, ranging from 0 to 16. The cumulated DAD score was calculated as the sum of single score characteristics, yielding a final score ranging from 0 to 64. *Significantly different from respective C-DMSO group ($p < 0.05$). **Significantly different from ARDS-DMSO ($p < 0.05$). *Significantly different from ARDS-DAS 1 mg/kg group ($p < 0.05$).

Fig. 4. Representative photomicrographs (light microscopy) of lung parenchyma of C-DMSO, C-DAS 1 mg/kg, C-DAS 10 mg/kg, ARDSp-DMSO, ARDSp-DAS 1 mg/kg, ARDSp-DAS 10 mg/kg, ARDSexp-DMSO, ARDSexp-DAS 1 mg/kg, and ARDSexp-DAS 10 mg/kg animals 48h after pre-treatment. Arrows show alveolar collapse. Note higher alveolar collapse in



the ARDSp-DMSO, ARDSexp-DMSO, and ARDSp-DAS 10 mg/kg groups. Photomicrographs were taken at an original magnification of x200 from slides stained with hematoxylin-eosin. Bars = 100 µm.

Table 1. Collagen fiber content. Values are median (interquartile range) of collagen fibers (n=6 animals/group). C: control, ARDS: acute respiratory distress syndrome, p: pulmonary, exp: extrapulmonary, DMSO: dimethyl sulfoxide, DAS: dasatinib. *Significantly different from C-DMSO ($p < 0.05$). **Significantly different from ARDS-DMSO ($p < 0.05$)

	C			ARDS					
	DMSO	DAS 1 mg/kg	DAS 10 mg/kg	DMSO	DAS 1 mg/kg	DAS 10 mg/kg	DMSO	DAS 1 mg/kg	DAS 10 mg/kg
Collagen (%)	4.0 (3.7-4.0)	4.0 (3.2-4.8)	2.9 (2.3-4.0)	6.7 (4.7-11.5)*	3.8 (1.5-6.6)**	4.7 (2.3-6.9)**	5.1 (3.9-8.9)	4.2 (3.3-4.9)	3.1 (2.5-7.7)

DMSO compared to C-DMSO, and dasatinib therapy reduced it regardless of dose (Table 1). In ARDSexp, the amount of collagen fibers was similar in all groups.

Histological analysis of kidney and liver tissue revealed no difference between C and ARDS groups or between DMSO and dasatinib treatment (Table 2).

Dasatinib modulated the recruitment of inflammatory cells and the cytokine profile in lung tissue and BALF

To support our data of inflammatory infiltration included in the DAD score, we also evaluated the number of total cells, mononuclear cells, and neutrophils in BALF. The number of total and mononuclear cells, as well as of neutrophils, was higher in both ARDS groups treated with DMSO compared to C-DMSO. In ARDSp, dasatinib at 1 mg/kg led to a lower number of total cells and neutrophils compared to DMSO, whereas dasatinib at 10 mg/kg

Table 2. Kidney and liver damage. Kidney: Values are median (interquartile range) of kidney damage score (n=5 animals/group). Liver: Values are mean \pm SD of the percentage of sinusoidal cells in liver zones (portal, central, and lobular). C: control, ARDS: acute respiratory distress syndrome, p: pulmonary, exp: extrapulmonary, DMS

	C			ARDS					
	DMSO	DAS 1 mg/kg	DAS 10 mg/kg	DMSO	p		exp		
	DMSO	DAS 1 mg/kg	DAS 10 mg/kg	DMSO	DAS 1 mg/kg	DAS 10 mg/kg	DMSO	DAS 1 mg/kg	DAS 10 mg/kg
Kidney									
Swelling	0.5 (0-1)	1 (1-1)	0 (0-0.25)	1 (0-1)	0.5 (0-1)	1 (0-1)	1 (0-1)	1 (0.75-1)	1 (1-1)
Loss of brush border	1 (1-1.25)	1 (1-1)	1 (1-1)	1 (1-1)	1 (1-1.25)	1 (1-2)	1 (1-2)	0.5 (0-1)	1 (0-1)
Vacuolar degeneration	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0.25)	0 (0-1)
Desquamation	1 (0.75-1)	1 (0.75-1)	0.5 (0-1)	1 (1-1)	0.5 (0-1)	0 (0-1)	0 (0-1)	0 (0-0.25)	1 (0-1)
Liver									
Central	27.6 \pm 4.6	28.8 \pm 5.8	29 \pm 6.7	33.2 \pm 7.6	30.6 \pm 4.5	32.6 \pm 5.7	37.1 \pm 5	27.4 \pm 4.8	26 \pm 6.3
Lobular	34.4 \pm 6.4	35.4 \pm 5.2	34 \pm 5.1	33.1 \pm 7.4	34 \pm 5.8	31.4 \pm 7.2	41.3 \pm 6	30.3 \pm 7.1	31.2 \pm 7.4
Portal	25.2 \pm 9.7	31 \pm 4.2	37.3 \pm 8.4	28.5 \pm 6.9	32.3 \pm 3.8	36.2 \pm 4.9	34.2 \pm 4.1	28.5 \pm 3.8	29.8 \pm 4.3

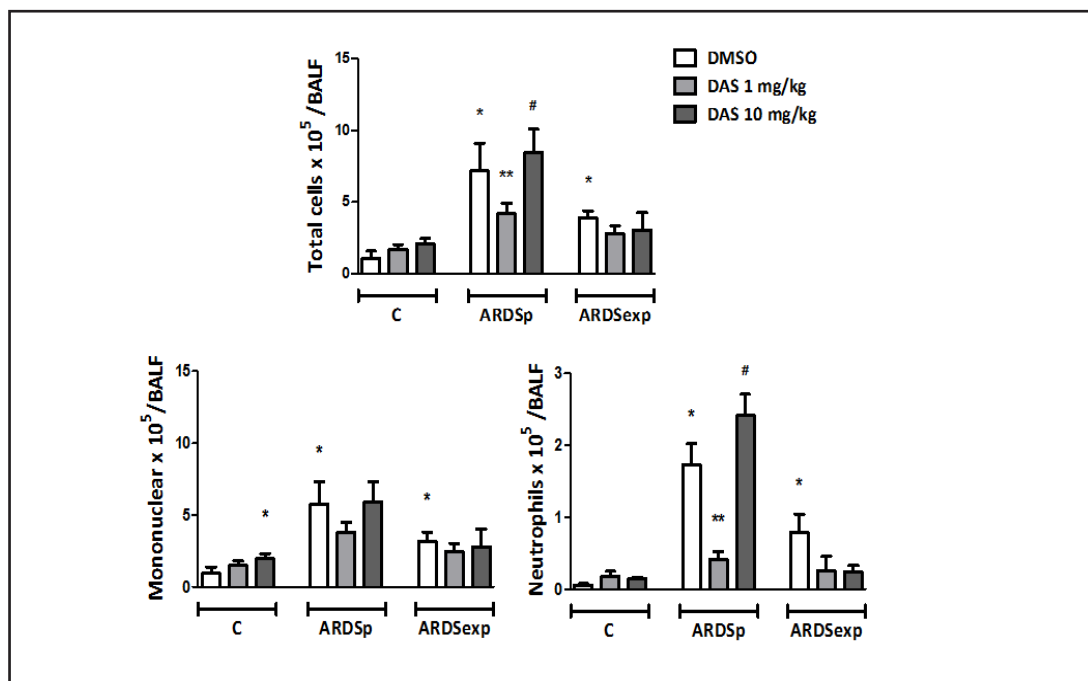


Fig. 5. Number of total cells, mononuclear cells, and neutrophils in bronchoalveolar lavage fluid (BALF) 48h after ARDS induction. Initially, all animals were randomly allocated to be pre-treated with dimethyl sulfoxide (DMSO 1%, 100 μ L, *po*) or dasatinib (DAS, 1 mg/kg or 10 mg/kg BW, 100 μ L, *po*). After 30 min, the animals pre-treated with DMSO and dasatinib were subdivided into control (C) or acute respiratory distress syndrome (ARDS) groups. In the ARDS groups, mice received *E. coli* LPS either intratracheally (40 μ g/0.05 mL saline, ARDSp) or intraperitoneally (400 μ g/0.5 mL saline, ARDSexp), while animals in the C groups did not undergo surgical procedures, instillations, or injections. A new dose of DMSO or dasatinib was given at 6h and then 24h after pre-treatment. Values are means \pm standard error of five animals in each group. *Significantly different from respective C-DMSO group ($p < 0.05$). **Significantly different from ARDS-DMSO ($p < 0.05$). #Significantly different from ARDS-dasatinib 1 mg/kg group ($p < 0.05$).

did not modify the number of total cells and neutrophils, which was similar to that of DMSO animals (Fig. 5).

The IL-6 level in lung tissue was higher in ARDSp-DMSO compared to C-DMSO animals. No statistically significant differences were observed in IL-10, TGF- β , or VEGF levels among

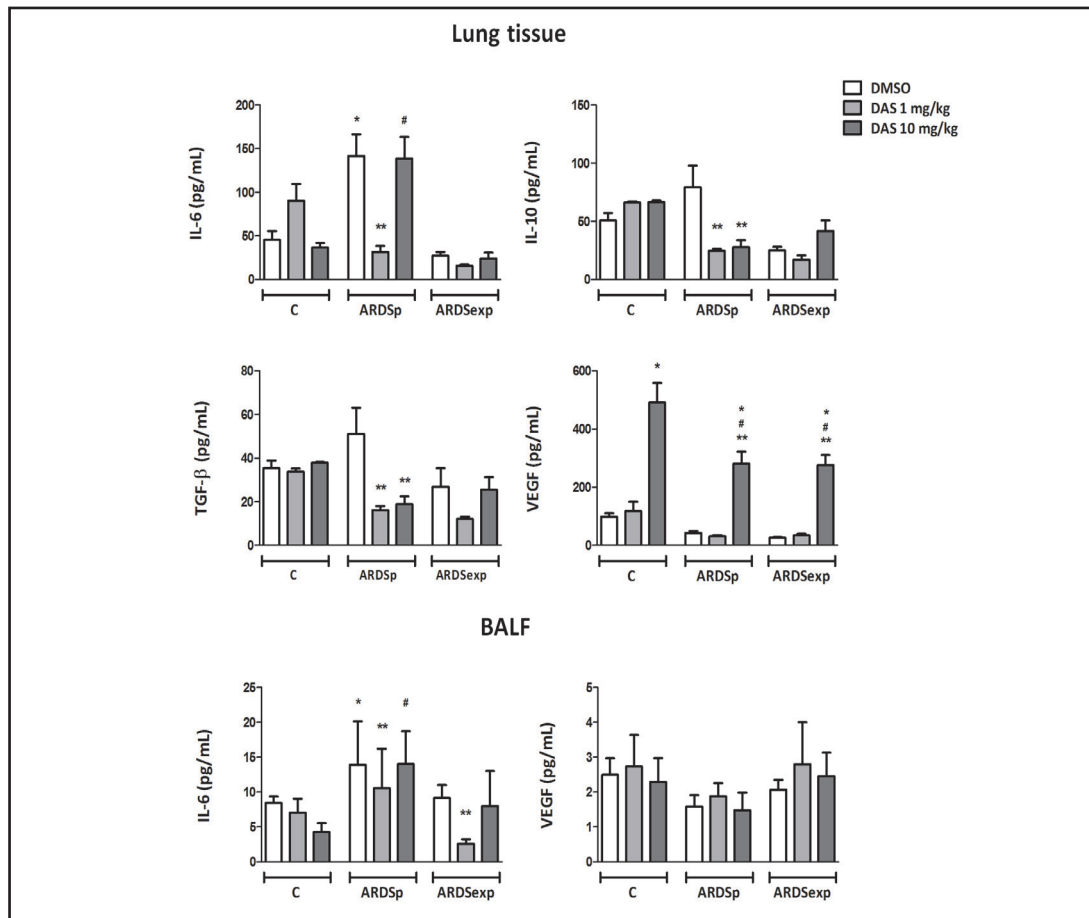


Fig. 6. Enzyme-linked immunosorbent assay (ELISA) of IL-6, IL-10, TGF- β , and VEGF expressions in lung tissue and IL-6 and VEGF in BALF (pg/mL) 48h after ARDS induction. Initially, all animals were randomly allocated to be pre-treated with dimethyl sulfoxide (DMSO 1%, 100 μ L, *po*) or dasatinib (DAS, 1 mg/kg or 10 mg/kg BW, 100 μ L, *po*). After 30 min, the animals pre-treated with DMSO and dasatinib were subdivided into control (C) or acute respiratory distress syndrome (ARDS) groups. In the ARDS groups, mice received *E. coli* LPS either intratracheally (40 μ g/0.05 mL saline, ARDSp) or intraperitoneally (400 μ g/0.5 mL saline, ARDSexp), while animals in the C groups did not undergo surgical procedures, instillations, or injections. A new dose of DMSO or dasatinib was given at 6h and then 24h after pre-treatment. Values are means \pm standard error of five animals in each group. *Significantly different from respective C-DMSO group ($p < 0.05$). **Significantly different from ARDS-DMSO ($p < 0.05$). #Significantly different from ARDS-dasatinib 1 mg/kg group ($p < 0.05$).

the C-DMSO, ARDSp-DMSO, and ARDSexp-DMSO groups. ARDSp animals treated with the lower dose of dasatinib (1 mg/kg) displayed reduced levels of IL-6, IL-10, and TGF- β in ARDSp compared to ARDSp animals treated with DMSO (Fig. 6). In ARDSexp mice treated with dasatinib 1 mg/kg, no significant changes in IL-6, IL-10, and TGF- β were observed between groups. ARDSp animals treated with the higher dose of dasatinib (10 mg/kg) exhibited decreased levels of IL-10 and TGF- β , but no significant changes in IL-6 compared to ARDSp mice treated with DMSO. The higher dose of dasatinib resulted in increased VEGF levels in C, ARDSp, and ARDSexp groups.

On analysis of BALF, only ARDSp-DMSO animals presented a higher IL-6 level compared to the C-DMSO group. VEGF was not altered among groups. Treatment with dasatinib 1 mg/kg resulted in a lower level of IL-6 in ARDSp and ARDSexp compared to ARDSp-DMSO and ARDSexp-DMSO groups, respectively (Fig. 6). The higher dose of dasatinib (10 mg/kg) was associated with similar levels of IL-6 in ARDSp compared to ARDSp-DMSO animals.

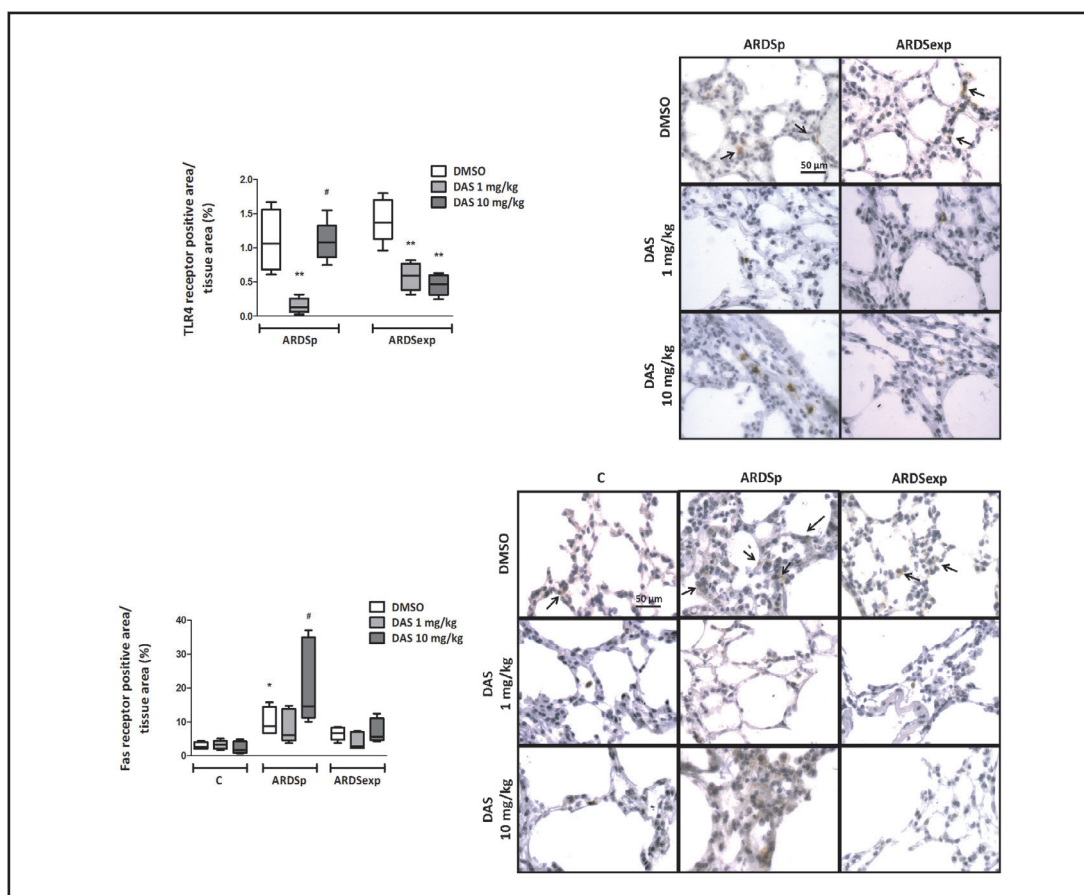


Fig. 7. Immunohistochemistry of apoptosis receptor Fas and TLR4 in lung tissue 48h after ARDS induction. Initially, all animals were randomly allocated to be pre-treated with dimethyl sulfoxide (DMSO 1%, 100 μ L, *po*) or dasatinib (DAS, 1 mg/kg or 10 mg/kg BW, 100 μ L, *po*). After 30 min, the animals pre-treated with DMSO and dasatinib were subdivided into control (C) or acute respiratory distress syndrome (ARDS) groups. In the ARDS groups, mice received *E. coli* LPS either intratracheally (40 μ g/0.05 mL saline, ARDSp) or intraperitoneally (400 μ g/0.5 mL saline, ARDSexp), while animals in the C groups did not undergo surgical procedures, instillations, or injections. A new dose of DMSO or dasatinib was given at 6h and then 24h after pre-treatment. Values are means \pm standard error of five animals in each group. *Significantly different from respective C-DMSO group ($p < 0.05$). **Significantly different from ARDS-DMSO ($p < 0.05$). #Significantly different from ARDS-DAS 1 mg/kg group ($p < 0.05$).

The number of cells expressing Fas receptor and TLR4 in lung tissue was influenced by dasatinib treatment

A similar number of positive cells for TLR4 expression was found in the ARDSp-DMSO and ARDSexp-DMSO groups (Fig. 7). The C groups were not represented in the graph because the sensitivity of the technique used to identify the positive cells to TLR4 was insufficient for use in control lung tissue. The lower dose of dasatinib (1 mg/kg) led to a lower number of cells expressing TLR4 in both ARDS groups (Fig. 7), whereas the higher dose of dasatinib (10 mg/kg) induced a similar number of positive cells in ARDSp compared to ARDSp-DMSO and a decreased number of cells expressing TLR4 in ARDSexp compared to ARDSexp-DMSO (Fig. 7).

A higher number of positive cells expressing the Fas receptor was observed only in ARDSp-DMSO compared to C-DMSO animals. The lower dose of dasatinib (1 mg/kg) did not change the number of cells expressing the Fas receptor in either ARDS group, while the higher dose of dasatinib (10 mg/kg) led to an increased number of Fas-positive cells in ARDSp compared to ARDSp-dasatinib 1 mg/kg (Fig. 7).

Discussion

In the present study, dasatinib at the lower dose tested (1 mg/kg) led to: 1) reductions in Est,L, diffuse alveolar damage, levels of IL-6 in BALF, and number of cells expressing TLR4, regardless of ARDS etiology; 2) reduced neutrophil infiltration and lower levels of IL-6, IL-10, TGF- β in lung tissue in ARDSp. Conversely, the higher dose of dasatinib (10 mg/kg) was associated with: 1) similar Est,L, diffuse alveolar damage, neutrophil infiltration, levels of IL-6 (BALF and lung tissue), increased VEGF in lung tissue, number of cells expressing TLR4, and higher number of cells expressing the Fas receptor in ARDSp animals compared to animals treated with DMSO; 2) improved lung morphofunction, increased VEGF, and lower number TLR4-positive cells in ARDSexp. Therefore, the beneficial effects of dasatinib in ARDS depend on its dose and on the etiology of the syndrome.

Many drugs currently used for targeted therapy of cancer are tyrosine kinase inhibitors, which target enzymes that deregulate expression and activity of tumor genes. The small-molecule inhibitor imatinib can specifically inactivate the Bcr-Abl tyrosine kinase, whose normal mechanism of auto-inhibition is disrupted in chronic myelogenous leukemia [25]. Nilotinib and dasatinib were developed for use in imatinib-resistant patients, and both are equivalent as a treatment for chronic myeloid leukemia in terms of cytogenetic and major molecular response [26]. Recent studies reported the ability of dasatinib to modulate the innate immune response in a model of endotoxemia [12, 27]. Mice pre-treated with dasatinib which received LPS intraperitoneally had reduced serum levels of tumor necrosis factor (TNF)- α and increased IL-10; however, secretion of IL-6 and accumulation of neutrophils in the lung was not affected [10]. In rats pre-treated with nilotinib for 1 week prior to exposure to aerosolized LPS, nilotinib caused downregulation of inflammatory cytokines and inducible nitric oxide synthase (iNOS) levels in the lung [28]. Post-treatment with the selective Src-tyrosine kinase inhibitor 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP1) after intratracheal instillation of LPS led to reductions in total protein content in BALF, neutrophil recruitment, nuclear factor (NF)- κ B activation, and levels of TNF- α [29]. Treatment with imatinib or nilotinib in LPS-instilled mice during recovery from neutropenia attenuated pulmonary edema, histological changes, and concentrations of TNF- α , IL-1 β , IL-6, and myeloperoxidase in BALF [30].

ARDS may be caused by a direct (pulmonary) insult to alveolar epithelium and/or by an indirect (extrapulmonary) lesion, which primarily affects the endothelium and results from an acute systemic inflammatory response [17, 31]. Some studies have reported that the response to pharmacological therapies might differ depending on the primary insult [17, 24, 32]. The experimental models of pulmonary and extrapulmonary ARDS used herein were induced by intratracheal or intraperitoneal injection of *Escherichia coli* LPS, respectively, and resulted in similar degrees of lung mechanical changes at days 1 [18-20] and 2 [19]. In the present study, ARDSp and ARDSexp animals treated with DMSO exhibited increases in Est,L and DAD score, as well as neutrophil infiltration, compared to the C-DMSO group.

Thus, as dasatinib has a broad spectrum of action and its putative effects on ARDS need to be clarified, we opted to test this drug rather than other tyrosine-kinase inhibitors. To the best of our knowledge, this is the first study to investigate the effects of dasatinib in models of pulmonary and extrapulmonary ARDS.

We choose to administer dasatinib in DMSO by oral gavage because it is soluble in organic solvents, such as DMSO. As DMSO presents low toxicity at low concentrations (2-4%) [33], we dissolved dasatinib in DMSO 1% in saline solution to minimize adverse effects. Mice were treated with dasatinib or DMSO 30 minutes before the administration of LPS to allow systemic distribution and maximum serum concentrations of dasatinib for the duration of the experiment [34]. In addition, we tested two different doses of dasatinib (1 mg/kg or 10 mg/kg) in order to evaluate optimal dose and toxicity in our models of LPS-induced lung injury.

The lower dose of dasatinib (1 mg/kg) produced improvement in lung mechanics, histological damage, and neutrophil levels in BALF in both ARDS groups as compared with

vehicle (DMSO) only. In a previous study from our group, we demonstrated, twenty-four hours after LPS administration, cytoplasmic degeneration of type II epithelial cell and alveolar-capillary membrane injury mainly in ARDSp [15], which may result in reduced surfactant release. Additionally, there was an increase in neutrophil infiltration in alveolar space, associated with increased levels of inflammatory mediators. The combination of this morphological change in type II epithelial cell with lung inflammation may lead to surfactant dysfunction and alveolar collapse, as it was observed in DAD score.

In addition, in the ARDSp group, we observed reduced expression of TGF- β in lung and IL-6 in lung tissue and BALF, which contributed to lower Est,L. Consistent with the modulation of TGF- β in the ARDSp group and the reported effect of platelet-derived growth factor receptor (PDGFR) in fibrotic disease [35], we found lower collagen content with both doses of dasatinib (1 mg/kg and 10 mg/kg). We also observed a smaller number of TLR4-positive cells in both ARDS groups. Activation of the innate immune response by binding of microbial products or cell injury-associated endogenous molecules to pattern recognition receptors, such as the TLRs on the epithelium and alveolar macrophages, is recognized as a key event for lung inflammation. TLR4, one of the best-characterized pattern recognition receptors of the innate immune system, recognizes not only LPS of Gram-negative bacteria but also endogenous ligands to mediate ARDS of many etiologies, as demonstrated in experimental studies [36, 37]. Once TLR4 binds with its ligands, it activates NF- κ B through a MyD88-dependent pathway that ultimately stimulates the expression of pro-inflammatory cytokines, leading to the pathological changes of ARDS [38]. The lower number of positive cells could be explained by a reduced number of inflammatory cells, as demonstrated in the DAD score. Indeed, dasatinib is known to impair neutrophil migration and adhesion *in vitro* by completely blocking integrin- and Fc-receptor-mediated neutrophil functions [11]. On the other hand, the 10 mg/kg dose of dasatinib did not affect lung morphofunction, number of neutrophils in BALF, cytokine levels, or number of TLR4-positive cells in ARDSp; these parameters remained similar to those of ARDSp-DMSO animals.

Another important mechanism related to the pathophysiology of ARDS is apoptosis. Patients with ARDS have been found to present higher concentrations of Fas receptor and Fas ligand in pulmonary edema fluid [39]. We also evaluated the number of cells expressing the Fas receptor in lung tissue. A higher number of cells expressing the Fas receptor was observed only in the ARDSp-DMSO compared to the C-DMSO group. Additionally, the number of cells expressing the Fas receptor was higher in ARDSp-dasatinib (10 mg/kg) than ARDSp-dasatinib (1 mg/kg) animals, which may be attributed to the deleterious effects of high-dose dasatinib, enhancing recruitment of inflammatory cells and boosting activation of the TLR4 pathway and production of pro-inflammatory mediators, such as IL-6, thus increasing apoptosis. In contrast, in ARDSexp, neither the lower nor the higher dose of dasatinib changed the number of cells expressing Fas receptor. This may be associated with the improvement in lung mechanics and reduction in lung histological damage observed.

Although we did not find a significant difference in mortality between the experimental groups, the higher mortality observed with the higher dose of dasatinib (10 mg/kg) reflected its toxicity in the ARDSp group. Conversely, in the ARDSexp group, both doses were beneficial. This could be explained by the presence of a direct (pulmonary) and an indirect (extrapulmonary) primary insult, with more systemic repercussions in the ARDSexp group. However, we did not observe histological changes in liver and kidney tissues that could suggest greater systemic impairment in the experimental groups.

Limitations

The current study has several limitations: 1) we used models of ARDS induced by *E. coli* LPS administered intratracheally or intraperitoneally to reproduce pulmonary and extrapulmonary pathways of lung insult; thus, our results cannot be extrapolated to other ARDS models or directly to the clinical setting; 2) the first dose of dasatinib was administered before the induction of lung damage due to the pharmacokinetics of the drug, thus hindering immediate clinical application. However, in cancer patients the price to pay

for increasing treatment intensity and duration with the goal to improving survival is an increasing susceptibility to infection and the risk of developing ARDS. Since dasatinib is an oral medication used for treating cancer, its use may reduce the risk of ARDS; 3) we analyzed all data 48h after the pre-treatment, and, consequently, we have no information about earlier or later effects; and 4) DMSO may be associated with toxic effects, but due to the low concentration used in this study, we believe it did not affect any parameters of interest.

Conclusions

The lower dose of dasatinib (1 mg/kg) tested in this study was effective at preventing lung morphofunction impairment independent of the etiology of ARDS; however, the modulation of the inflammatory process differed according to the etiology of ARDS and the dose administered. All these data expand our knowledge regarding the lung protective effects of dasatinib in ARDS and are clinically valuable, especially given its use and relatively favourable safety profile of these agents. However, careful dose monitoring is required.

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Disclosures Statement

The authors have not disclosed any potential conflicts of interest.

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