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In vivo effect of adhesion inhibitor heparin on *Legionella pneumophila* pathogenesis in a murine pneumonia model

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Franco Dante Menozzi has died since this study was conducted.

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Abstract Objective: To examine the effect of intratracheal heparin instillation on *Legionella pneumophila*-related acute lung injury (ALI) and systemic dissemination. **Design:** Prospective, controlled experimental study. **Setting:** University research laboratory. **Interventions:** A/J mice received 5 µg of sulfated heparin intratracheally co-instilled with 10⁶ or 10⁸ colony-forming units (CFU) of a virulent isolate of *L. pneumophila*. **Measurements and results:** ALI was assessed in control groups (PBS and PBS-heparin) and on days 1, 2 and 3 post-infection, in terms of the lung wet-to-dry (W/D) weight ratios and of lung endothelial permeability to radio-labeled albumin (Perm-I¹²⁵). Lung bacterial loads were measured and systemic spread was assessed by blood and target organ culture. The alveolar inflammatory response was eval-

uated by measuring the cytokine levels (TNF-α, IFN-γ, IL-6 and IL-12p70) in bronchoalveolar lavage fluids (BALF). Co-instilled heparin improved mouse survival after the 10⁸ CFU challenge ($p < 0.01$). On day 2, heparin co-instillation significantly reduced the W/D ratio and Perm-I¹²⁵ ($p < 0.01$ and $p < 0.001$ respectively), improved lung bacterial clearance ($p < 0.001$), prevented systemic dissemination (blood, liver, spleen, kidneys and brain cultures, all $p < 0.05$) and significantly increased IFN-γ and IL-12p70 levels in BALF ($p < 0.05$). **Conclusions:** Heparin co-instillation during intratracheal *L. pneumophila* challenge has a protective effect on the alveolar-capillary barrier and prevents bacterial dissemination. These results tend to confirm the competitive inhibition by heparin of *L. pneumophila* attachment to lung epithelium in vivo, and point to the possible involvement of a heparan-sulfate adhesin in *L. pneumophila* binding to pneumocytes.

Keywords Acute lung injury · Heparin · Pneumonia · *Legionella pneumophila* · Legionnaires' disease

Introduction

Legionella pneumophila, a Gram-negative facultative intracellular pathogen, is the causative agent of legionellosis. *L. pneumophila* causes legionnaires' disease, a severe form of pneumonia, and Pontiac fever, a milder illness. An estimated 8,000 to 18,000 people contract legionnaires' disease in the USA every year [1]. When reaching lung alveoli after inhalation in aerosols, *L. pneumophila* attaches to and is internalized by alveolar macrophages and epithelial cells [2–5]. *L. pneumophila* then replicates within host cells in a parasitic relationship. The hallmarks of human bacterial pneumonia are increased alveolar permeability with edema and gradual impairment of gas exchanges due to alveolar–capillary barrier injury; these changes are collectively referred to as acute lung injury (ALI) [6, 7]. ALI can be studied in murine models by measuring functional parameters such as lung extravascular water, alveolar – capillary membrane permeability and the lung inflammatory response [8, 9]. Cytokine production during *L. pneumophila* infection has been characterized as a T1 polarization [10–14].

In the susceptible hosts, pathogens adhere to target tissues in order to escape mechanical clearance and to establish an infectious focus from which dissemination may occur. A number of saccharides can inhibit *L. pneumophila* attachment to the human respiratory cell line A549 [15, 16]. Polymeric sulfated saccharide heparin is among the most efficient competitive inhibitors of *L. pneumophila* attachment to A549 cells [15], suggesting that bacteria bind to sulfated glycosaminoglycans borne by trans-membrane proteoglycans (PGs) at the surface of host alveolar pneumocytes. This attachment involves a specialized microbial surface-exposed adhesin [17]. Adhesion to and subsequent invasion of pneumocytes could be an important pathway of ALI development and bacterial dissemination in *L. pneumophila* pneumonia and could thus represent an attractive therapeutic target. To test the hypothesis that pneumocyte adhesion is central to the pulmonary pathogenicity of *L. pneumophila*, we examined whether inhibition of bacterial adhesion to lung epithelia through the use of a known competitive inhibitor, sulfated heparin, would reduce *L. pneumophila*-related ALI, prevent extrapulmonary bacterial dissemination and modulate host cytokine responses.

An experimental A/J mouse model of *L. pneumophila* lung infection was set up for this study. *L. pneumophila*-induced ALI was studied in mice that received a single intratracheal dose of heparin by measuring the lung wet-to-dry lung tissue weight ratio and by assessing alveolar–capillary barrier permeability. Bacterial clearance was assessed by measuring the lung bacterial load over time. Extrapulmonary dissemination was assessed by testing blood and target organs (liver, spleen, kidneys and brain) for *L. pneumophila*. The cytokine response was assessed

by measuring IFN- γ , TNF- α , and IL-6, -10, and -12p70 in bronchoalveolar lavage fluid.

Materials and methods

Animals

Female pathogen-free 8- to 9-week-old A/J mice (Harlan UK Laboratory, Oxford, UK) were used for all experiments. They were housed in the Lille University Animal Care Facility and provided with food and water ad libitum. All the experiments were approved by the Lille Institutional Animal Care and Use Committee.

Bacteria and culture conditions

The clinical strain *L. pneumophila* serogroup 1 strain Lens wild type was kindly provided by the French National Reference Center for *Legionella*. This epidemic strain, which was recently sequenced, caused the largest outbreak of community-acquired legionnaires' disease in France (2003–2004), with a high case fatality rate (21%) [18, 19]. It was grown on buffered charcoal-yeast extract (BCYE) agar with L-cysteine (bioMerieux, Marcy l'Etoile, France) for 48 h. For mouse challenge, colonies were resuspended in sterile phosphate buffered saline (PBS) at 2×10^7 colony-forming units (CFU)/ml or 2×10^9 CFU/ml according to the group.

Preparation of a heparin instillate

Lipopolysaccharide-free sulfated heparin sodium salt with an average molecular weight of 6,000 Da extracted from porcine intestinal mucosa (Sigma-Aldrich, USA) was added to PBS and to PBS bacterial suspension at a concentration of 100 $\mu\text{g}/\text{ml}$ corresponding to 5 μg of heparin per mouse, 30 min prior to instillation.

Mouse challenge

A/J mice were infected by intratracheal (i.t.) inoculation, in dorsal recumbence, after sevoflurane anesthesia (Servo-rane™, Abbott, UK). A 24-G animal feeding needle was used to instill 50 μl of bacterial suspension (10^6 or 10^8 bacteria per mouse). Negative control mice received 50 μl of sterile PBS or PBS-heparin in the same conditions.

Bacterial cultures

Immediately after the instillation (T0) and on days 1, 2 and 3, groups of mice were exsanguinated by intracardiac

puncture. The lungs, liver, spleen, kidneys and brain were aseptically removed. The organs were homogenized in sterile containers with 1 ml of sterile PBS, serially diluted, and cultured on *Legionella* BCYE agar plates for 3 days at 37°C. The results were expressed in CFU/ml.

Bacteria were also quantified 5 h post-infection. Mice were killed humanely, and bronchoalveolar lavage (BAL) was performed by cannulating the trachea with a 22-G catheter and 1.5 ml of sterile PBS. The BAL samples were filtered (5 µm pore size) to eliminate resident phagocytes. They were then serially diluted and cultured on *Legionella* BCYE agar plates for 3 days at 37°C.

Evaluation of alveolar–capillary barrier injury

Albumin flux across the endothelial barrier was measured with a method initially described for use in a rat model [20]. Briefly, 0.5 ml of ¹²⁵I-labeled bovine serum albumin (1 µCi) (HAS; CIS Biointernational, Gif-sur-Yvette, France) was injected intraperitoneally 2 h before intraperitoneal injection of pentobarbital sodium (Sanofi, Libourne, France), followed by sternotomy, exsanguination and lung removal. Radioactivity and the hemoglobin (Hb) concentration were measured in blood. The lungs were weighed and radioactivity was counted prior to homogenization and centrifugation (Polytron, PT 1600E; Fischer Bioblock Scientific, Switzerland). The Hb content of the supernatant was also measured. Blood and lung-homogenate samples were then desiccated to calculate extravascular lung water. The wet-to-dry weight ratio (W/D) of each pair of lungs was determined from the difference between the wet weight and the dry weight (measured after 7 days in a 37°C incubator) [8]. A permeability index was used to express the permeability of the alveolar–capillary membrane [20].

Cytokine levels in BAL fluid

At T0 and on days 1 and 2 post-inoculation, groups of mice were killed humanely and their lungs were lavaged with two 0.8-ml aliquots of sterile PBS. BAL fluid was filtered and immediately frozen at –80°C after collection. It was then concentrated down to 50 µl by ultrafiltration on a Millipore Ultrafree-0.5 filter device (Biomax-5 membrane). BAL cytokines were quantified with BD™ Cytometric Bead Array (CBA) technology (Becton-Dickinson), which employs a series of particles with discrete fluorescence intensities to simultaneously detect multiple soluble analytes in a small sample volume. The BD™ CBA Mouse Inflammation Kit was used as recommended by the supplier to quantitatively measure gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6) and -12p70 (IL-12p70) levels in the different samples. Briefly, each concentrated

BAL sample was incubated for 2 h at room temperature in the presence of mixed antigen-coupled beads (50 ml) and Phycoerythrin Detection Reagent (50 ml), followed by two washes. Bead fluorescence was then measured using the BD FACSArray™ Bioanalyzer. The median relative fluorescence intensities were converted into cytokine concentrations by using nine-point calibration curves created by serially diluting cytokine standards.

Experimental groups

The experimental groups were: the controls instilled with sterile PBS or PBS containing 100 µg/ml of heparin ($n = 5$ per group) and the infected groups challenged i.t. with *L. pneumophila* strain Lens with or without 100 µg/ml of co-instilled heparin ($n = 8$ per group). Groups of animals were killed humanely at T0 and on days 1, 2 and 3. All the experiments were conducted in duplicate, except for bacterial load assay in blood and peripheral target organs.

Statistical analysis

Mortality rates were compared between groups by using the log-rank test with Kaplan–Meier analysis. Bacterial loads in blood and organs were compared with the χ^2 test (with Yates correction if necessary) and BAL fluid culture results with the Mann–Whitney U test. Quantitative parameters were compared using two-way analysis of variance (ANOVA). Post hoc comparisons used the Bonferroni's test (Version 4.00, Graph Pad Prism, USA). Data are expressed as means \pm standard error of the mean (SEM). *P* values below 0.05 were considered as significant.

Results

Effect of co-instilled heparin on mouse survival

A set of in vitro work demonstrated that heparin inhibits *L. pneumophila* adhesion to human bronchial and alveolar cell lines [15, 16]. This effect was observed in a concentration-dependent manner with a maximum efficacy at 100 µg of heparin in the medium culture (data not shown). For this reason, this dose only was tested in the study, which was not designed to be an in vivo dose-response study.

Survival was recorded for 7 days after i.t. challenge with non-lethal and lethal doses of *L. pneumophila* (10^6 and 10^8 CFU respectively) (Fig. 1). The survival rate on day 7 was 100% with the non-lethal challenge, regardless of heparin co-instillation. With the lethal challenge, the survival rate on day 7 was 0% without heparin co-instillation and 30% with heparin co-instillation

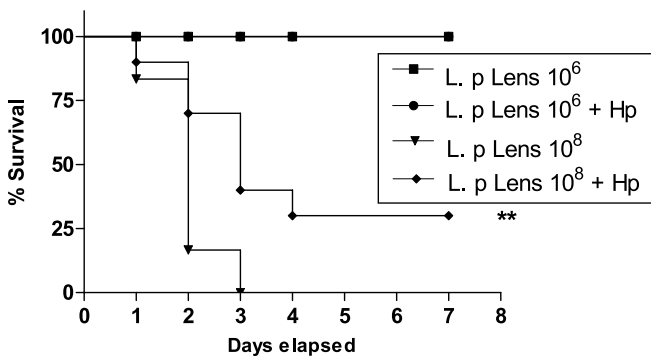


Fig. 1 Effect of co-instilled heparin on mouse survival during 7 days after i.t. instillation of a non-lethal challenge (10^6 CFU/mouse) and a lethal challenge (10^8 CFU/mouse) of *L. pneumophila*. $N = 10$ mice per group. $**p < 0.01$ vs. *L.p* Lens 10^8 . The data were confirmed in a replicate experiment

($p < 0.01$). Thus, co-instilled heparin improved the survival after *L. pneumophila* challenge.

Effect of co-instilled heparin on lung and BAL fluid bacterial loads

As previously described with *L. pneumophila* strain Philadelphia [10], the number of bacteria in the lungs increased exponentially until day 2 and started to decline on day 3 (Fig. 2a). In heparin co-instilled mice, the lung bacterial load tended to be lower than in *L. pneumophila*-instilled mice on day 1 and the difference reached statistical significance on day 2 ($p < 0.001$). Lung counts started to decline on day 3 in both groups. Thus, co-instilled heparin increased *L. pneumophila* clearance from lungs.

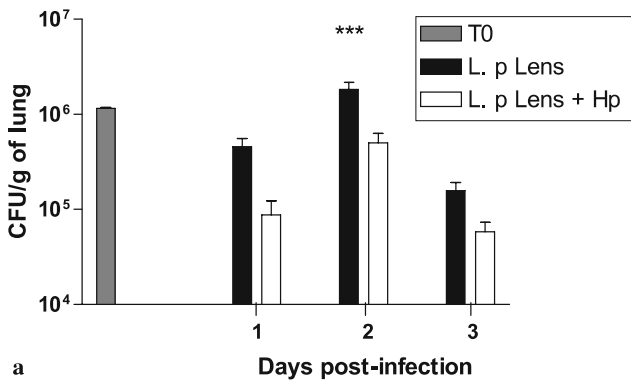


Fig. 2 Effect of co-instilled heparin on lung and BAL fluid bacterial loads in *L. pneumophila* pneumonia. **a** The number of colony-forming units (CFU) in lungs at T0 and days 1, 2 and 3. **b** CFU were counted on BCYE plates in filtered BAL fluids collected 5 h

post-challenge. The data are means \pm standard error (SE) (indicated by error bars). $N = 5$ mice in control groups, $n = 8$ mice in infected groups. $***p < 0.001$ vs. *L.p* Lens+Hp, $**p < 0.01$ vs. *L.p* Lens. The data were confirmed in a replicate experiment

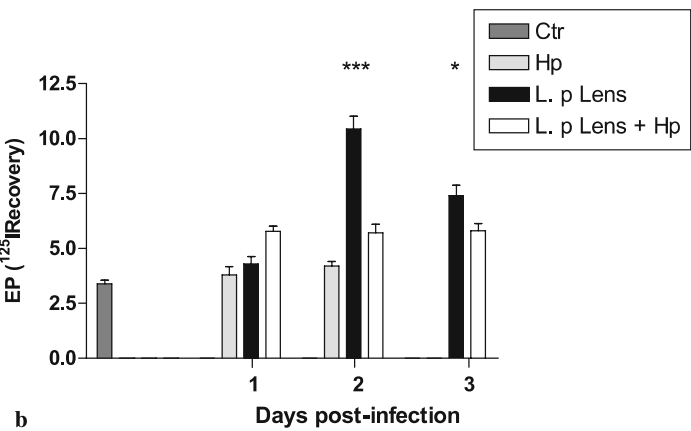
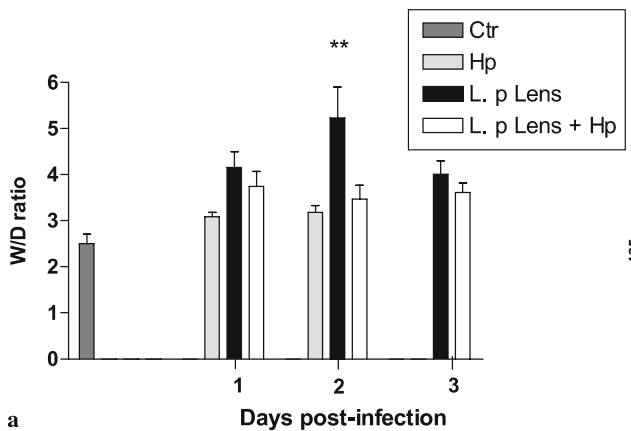


Fig. 3 Effect of co-instilled heparin on *L. pneumophila*-related alveolar–capillary barrier injury at days 1, 2 and 3 post-infection. **a** evaluation of the wet-to-dry lung weight ratios (W/D). **b** evaluation of endothelial permeability (EP) of the alveolar–capillary barrier to 125 I-labeled bovine serum albumin. The data

are means \pm SE (indicated by error bars). $N = 5$ mice in control groups, $n = 8$ mice in infected groups. $**p < 0.01$ vs. *L.p* Lens+Hp, $***p < 0.001$ vs. *L.p* Lens+Hp, $*p < 0.05$ vs. *L.p* Lens+Hp. The data were confirmed in a replicate experiment

To examine whether heparin blocked *L. pneumophila* primary adhesion to airway epithelial cells, filtered acellular BAL fluid was obtained 5 h after instillation and cultured. Bacterial counts were significantly higher in co-instilled heparin mice than in *L. pneumophila*-instilled mice ($p < 0.05$) (Fig. 2b).

Effect of co-instilled heparin on *L. pneumophila*-related alveolar–capillary barrier injury

A dynamic time-course analysis of the extra-vascular lung water and the protein flux across endothelial barrier was performed from days 1 to 3 post-challenge with *L. pneumophila*. Pharmacokinetics of heparin instilled into the lungs in a liquid form are not well known, but those of heparin inhaled into the lungs have been published [21, 22]. Therefore, we deduced that the residual amount of 5 μg of heparin in the lungs past 48 h was likely to be negligible and we did not pursue the experi-

ments in the control heparin-instilled mice past this time-point.

In *L. pneumophila*-instilled groups, the lung W/D weight ratio doubled on day 2 post-challenge and started to decline on day 3 (Fig. 3a). Heparin did not affect this ratio in uninfected mice. In infected mice, however, co-instilled heparin significantly attenuated the increase on day 2 ($p < 0.01$).

Endothelial permeability measured after IP injection of ^{125}I -labeled albumin increased threefold in the first 2 days in *L. pneumophila*-instilled mice and started to decline on day 3 (Fig. 3b). Consistently with former W/D results, co-instilled heparin did not affect endothelial permeability in uninfected mice. In infected mice, however, co-instilled heparin significantly attenuated the increase in endothelial permeability on days 2 and 3 ($p < 0.001$ and $p < 0.05$ respectively).

Thus, alveolar–capillary barrier injury was roughly halved on day 2 when heparin was co-instilled with *L. pneumophila*, and no rebound effect was noted.

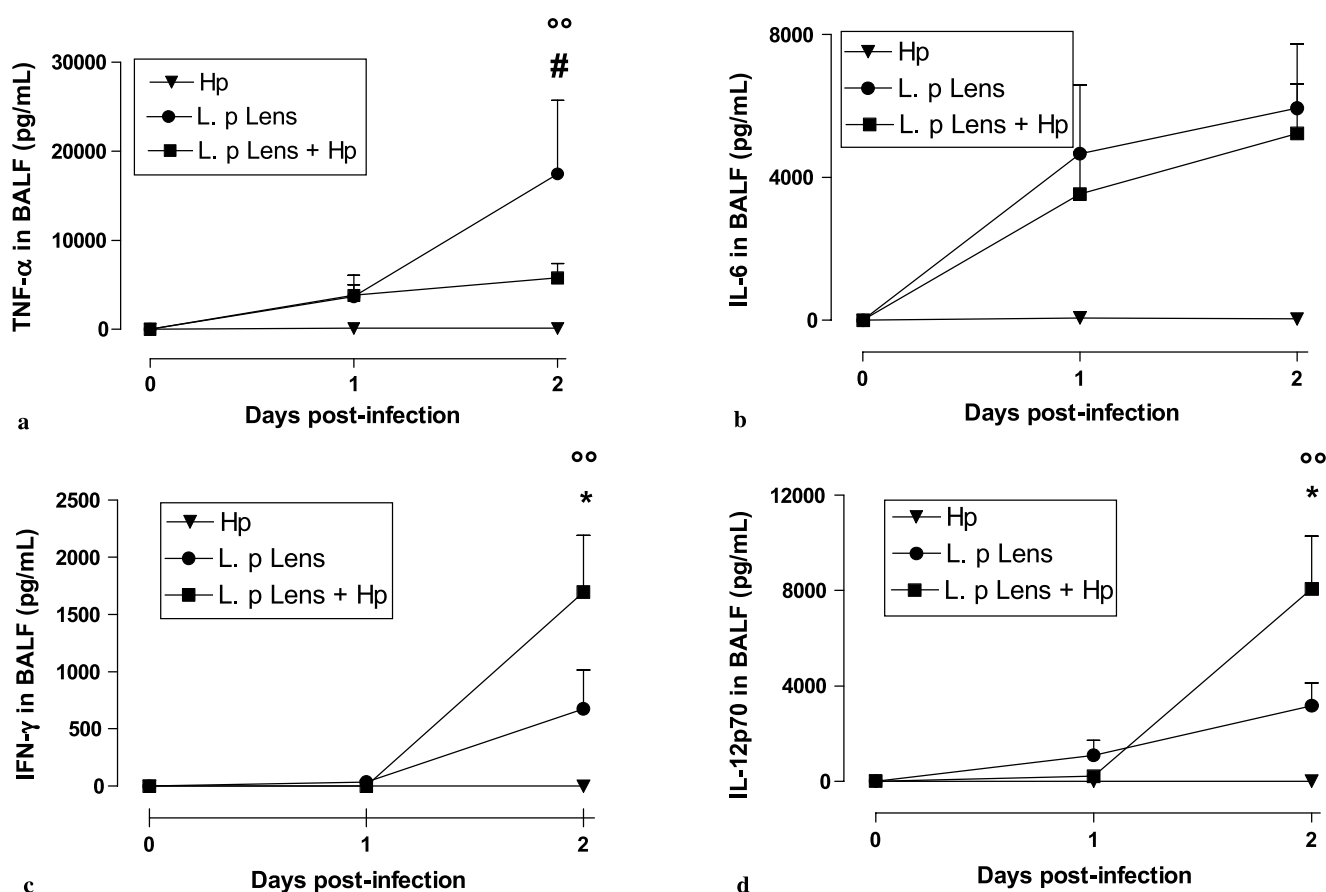


Fig. 4 Effect of co-instilled heparin on cytokine concentrations (pg/ml) in bronchoalveolar lavage fluid (BALF) on days 1 and 2 of *L. pneumophila* challenge. **a** TNF- α levels; **b** IL-6 levels; **c** IFN- γ levels; **d** IL-12p70 levels. The data are means \pm SE (indicated

by error bars). $N=5$ mice in control groups and $n=8$ mice in infected groups. # $p < 0.05$ vs. L. p Lens+Hp, * $p < 0.05$ vs. L. p Lens, °° $p < 0.01$ vs. Hp. The data were confirmed in a replicate experiment

Table 1 Effect of co-instilled heparin on blood and target organ bacterial loads. CFU were detected on BCYE plates at days 1 and 2 post-challenge. The percentages of positive samples (%) are shown. $p < 0.05$ between L.p Lens and L.p Lens+HP

	Blood			Liver			Kidneys			Spleen			Brain		
	Positive culture(s)/n	%	P	Positive culture(s)/n	%	P	Positive culture(s)/n	%	P	Positive culture(s)/n	%	P	Positive culture(s)/n	%	P
Day 1															
L. p Lens	6/20	30		10/20	50		5/18	28		6/18	33		5/18	28	
L. p Lens + HP	3/20	15	0.25	3/10	30	0.29	3/10	30	0.9	4/10	40	0.72	0/10	0	0.04
Day 2															
L. p Lens	10/20	50		13/15	87		12/15	80		12/17	71		7/15	47	
L. p Lens + HP	1/25	4	0.0003	2/10	20	0.0008	1/10	10	0.0006	3/10	30	0.04	0/10	0	0.01

Effect of co-instilled heparin on extrapulmonary dissemination of *L. pneumophila*

In *L. pneumophila*-instilled mice, bacterial translocation from the lungs was detectable on day 1 (Table 1). On day 2, *L. pneumophila* culture was positive in the liver, kidneys and spleen (87%, 80% and 71% respectively) than in blood and brain (about 50% for both). Heparin co-instillation roughly halved the rates of positive blood cultures and prevented brain infection on day 1. On day 2, in heparin co-instilled mice, blood, liver, kidney spleen and brain culture positivity rates were 4%, 20%, 10%, 30% and 0% respectively, representing a drastic *L. pneumophila* growth restriction in this group (all $p < 0.05$ versus *L. pneumophila*-instilled group).

Effect of co-instilled heparin on lung proinflammatory cytokine levels

On day 2, TNF- α production was significantly higher in *L. pneumophila*-instilled group than in control heparin and heparin co-instilled groups ($p < 0.01$ and $p < 0.05$ respectively) whereas no difference in IL-6 levels was noted (Fig. 4a, b). IFN- γ and IL-12p70 levels increased significantly more from day 1 to 2 in co-instilled heparin groups than in heparin control and *L. pneumophila*-instilled groups (for both cytokines $p < 0.01$ and $p < 0.05$ respectively) (Fig. 4c, d).

Thus, co-instilled heparin led to a dissociated pattern of inflammation from day 1 to 2, with a minor increase in the acute-phase cytokine TNF- α and a major increase in the Th1 cytokines IFN- γ and IL-12p70.

Discussion

In this study, a single intratracheal dose of sulfated heparin co-instilled with a virulent isolate of *L. pneumophila* in A/J mouse lungs lowered the mortality rate, protected the alveolar-capillary barrier, prevented systemic bacterial dissemination and stimulated Th1 cytokine production.

To characterize the anti-adhesion effect of heparin, we chose a bacterial inoculum of 10^6 cells per mouse, which mimics fairly mild legionnaires' disease [10]. A single dose of heparin was instilled along with *L. pneumophila* as uptake of this pathogen by host cells occurs within minutes after entry into the lung alveoli [23].

A preliminary in vitro study showed differences in the ability of some *L. pneumophila* mutants to attach to macrophages and respiratory epithelial cells, indicating that some of the mechanisms of attachment are different [5]. The molecular mechanisms underlying *L. pneumophila* invasion of respiratory epithelial cells, which constitute more than 95% of the surface area of the alveolar surface area, are poorly known, including the

nature of the host receptor involved in a non-complement-mediated uptake. Many microorganisms express adhesins that bind sulfated glycosaminoglycans (GAGs) borne by proteoglycans (PGs) at the surface of host epithelial cells. Bacterial interaction with PGs facilitates epithelial cell invasion by *Neisseria gonorrhoeae* and systemic dissemination of *Mycobacterium tuberculosis* [24, 25]. *L. pneumophila* shares many traits with *M. tuberculosis*, such as initial pulmonary infection, spread to other tissues, and the use of professional alveolar phagocytes as a major site of replication. Interactions with non-phagocytic cells may also have an important role in the pathogenesis of legionnaires' disease. *M. tuberculosis* complex strains produce at their surface a protein named heparin-binding hemagglutinin adhesin (HBHA) [26]. Binding of *M. tuberculosis* to epithelial cells, but not to macrophages, can be inhibited by anti-HBHA antibodies and by competition with heparin, which is a highly acidic mucopolysaccharide with sulfaminic bridges, suggesting that HBHA-mediated adherence is specific for non-phagocytic cells [26]. In vitro, heparin inhibited the adhesion of *L. pneumophila* to human bronchial and alveolar cell lines by 93% and 60% respectively [15, 16]. These percentages suggest that more than half of the epithelial cells receptors involved in *L. pneumophila* attachment are heparan sulfate molecules. The main consequence of the anti-adhesion effect of sulfated heparin may be the trapping of "free" heparin-coated forms of *L. pneumophila* unable to adhere to airway epithelial cells in the alveolar spaces. Two findings favor this hypothesis: first, filtered BAL fluids cultures collected 5 h post-infection contained a larger number of cultivable bacteria when heparin was co-instilled. The 5-h time point was too early for *L. pneumophila* replication to have occurred in alveolar macrophages. Second, the amount of *L. pneumophila* released in the lungs during the first 48 h was lower in heparin co-instilled mice than in *L. pneumophila*-instilled mice. This could explain the limited alveolar–capillary barrier damages in the presence of heparin, as shown by a 50% decrease in extravascular lung water and in endothelial permeability to radiolabeled albumin. Heparin inhibition of *L. pneumophila* epithelial adhesion might also explain the lower systemic spread measured by blood and organ culture. Finally, the larger amounts of IFN- γ and IL-12p70 in BAL fluid on day 2 may be related to the increased numbers of non-adherent "free" forms of bacteria in the alveolar spaces of heparin-treated animals. Indeed, IFN- γ and IL-12p70 are two Th1 cytokines involved in the host response to pathogens, and particularly in restriction of bacterial growth and clearance

from infected sites [27–29]. The conjunction of all these factors would tend to limit *L. pneumophila* pathogenicity and dissemination, ultimately leading to improved animal survival.

Although there are no studies in the specific context of *L. pneumophila* pneumonia, ALI leads to systemic and local lung imbalances in coagulant factors, resulting in fibrin deposition [30]. In addition, levels of natural anticoagulants such as activated protein C (APC), antithrombin (AT) and tissue factor pathway inhibitor (TFPI) decline during sepsis, owing both to decreased production and to enhanced breakdown [31]. As heparin is not inert with respect to coagulation, and possibly inflammation too, the effect of heparin observed in this study at the very beginning of the infectious process could also be due in part to these effects. However, heparin effects on both coagulation and inflammation are controversial. In human healthy volunteers, inhaled heparin reduced the level of inflammatory mediators such as histamine and increased TFPI release in blood [32]. These data were not confirmed in a smoke inhalation-induced lung injury model, which could reduce neither lung dysfunction nor leukocyte accumulation [33]. In fact, exogenous heparin exhibits paradoxical in vitro functioning, since it is an acceleratory cofactor for anticoagulant AT but can also induce its inactivation in pathologic conditions reinforcing procoagulant activity [34, 35]. Thus, the in vivo ability of heparin to block fibrin deposition in the lung and thereby to improve lung function is uncertain [36]. This is why attempts to prevent fibrin deposition in human pneumosepsis have evolved toward natural anticoagulant systemic supply, such as APC [37, 38].

In the setting of *L. pneumophila*-related ALI, a strategy capable of preventing primary bacterial attachment to airway epithelial cells might limit the pulmonary pathogenicity of this microorganism. The protective effect of heparin in vivo indirectly supports the evidence of a bacterial mechanism of attachment operating through the binding to pneumocyte surface GAGs. Attempts are underway to identify *L. pneumophila* GAG-binding adhesin(s). This would provide the possibility of developing new specific anti-adhesive therapy that might be a potential alternative to conventional antimicrobial chemotherapy.

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