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Role of Macrophages in Internalization and Early Clearance of Pseudomonas Aeruginosa from the Mouse Lung

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Abstract: This study aimed to evaluate the role of alveolar macrophages (AMs) in the internalisation and early clearance of *Pseudomonas aeruginosa* in the lung. AMs were depleted by intranasal administration of liposome-encapsulated clodronate disodium. At 24 h following the instillation of liposomes, a sublethal dose of P. aeruginosa (1 X 10^4 cfu) was inoculated intratracheally. Lung tissue was then evaluated for survival, viable bacteria and for histopathology 4 and 24 h post-infection. AM depletion of 90% did not affect the survival rate of infected mice but the clearance of *P. aeruginosa* was reduced at 24 h (P<0.05). While at 4 h AM-depleted and control mice showed similar level of PMN recruitment in the distal lung, 24 h post-infection tickening of interstitial spaces was more marked in AM-depleted mice. To evaluate the integrity of the epithelial barrier, the presence of bacteria in the spleen was assessed at 4 and 24 h. AM-depleted mice presented equal numbers of colonies as compared to control mice. At 4 h post-infection, the total and internalized numbers of bacteria were not different from the control group following a high-dose bacterial challenge (1 X 10^7 cfu). Our study show that AMs do not influence bacterial internalisation by airway epithelial cells.

Keywords: acute respiratory infection, airway epithelial cells, alveolar macrophages, Pseudomonas aeruginosa

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen that causes a broad range of acute and chronic infections, including urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, and bacteremia. Lung infections caused by *P. aeruginosa* are limited to patients who are immunocompromised, or who have defective mucociliary clearance, previous epithelial injury or foreign body placement (Williams *et al.*, 2010). Acute lung infections occur in hospitalized patients, individuals with immunodeficiency, and those with community-acquired penumonia. On the other hand, chronic or persistent lung infections is the hallmark of cystic fibrosis (CF) patients, but non-CF bronchiectasis is another cause of *P. aeruginosa* persistent lung infection.

Host defense mechanisms of the airways against bacteria are comprised of innate and acquired immune system responses and of epithelial cell barrier (Lavoie *et al.*, 2011; Williams *et al.*, 2010). Innate defenses consist mainly of the phagocytic defenses provided by the resident alveolar macrophages (AMs) and the polymorphonuclear leukocytes (PMNs) that are recruited into the lung in response to pulmonary infection. The role of AMs in defending the lung against *P. aeruginosa* and in regulating inflammatory reactions has been assessed in studies in which AM were depleted. In rodent models, AMs can be depleted to 5 to 22% of normal numbers by liposome-encapsulated clodronate disodium (L-Cl_2MDP) delivered to the pulmonary tract (Cheung *et al.*, 2000; Hashimoto *et al.*, 1996; Kooguchi *et al.*, 1998).

Epithelial respiratory cells function as part of the innate immune responses and not merely only as a physical barrier. Multiple substances with pro- and anti-inflammatory as well as antimicrobial activities are secreted by epithelial cells. Moreover, lung epithelial cells can directly interact with bacteria and therefore are involved in the colonization of the airways by *P. aeruginosa* (Allewelt *et al.*, 2000; Schroeder *et al.*, 2001). The CF transmembrane conductance regulator (CFTR) protein, expressed at

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the apical surface of airway epithelial cells, is a pattern recognition receptor (PRR) which binds to lipopolysaccharide (LPS) of *P. aeruginosa*. This binding results in lipid raft formation and interaction with caveolin-1 and 4 and allows internalisation of the bacterium (Bajmoczi *et al.*, 2009). Also, an interplay between macrophages and airway epithelial cells exist in the airways. For example, IL-1 produced by nacrophages can be sensed by the epithelium, which in turn secrete neutrophil chemokines (Mijares *et al.*, 2011). However, the effect of macrophages on other defense function of the airway epithelium towards *P. aeruginosa*, i.e. bacterial internalisation, is not known.

The main objective of this study was to further elucidate the in vivo role of AMs in early defense of the lung against *P. aeruginosa* challenge. AMs were depleted by the intranasal (i.n.) instillation of L-Cl_2MDP in mice and thereafter mice were dosed with *P. aeruginosa* by intratracheal inoculation. The effects of AM depletion on bacterial clearance in the lung and on bacterial internalization were assessed. Moreover, the spreading of bacteria in the blood circulation was studied by evaluating the presence of bacteria in the spleen.

MATERIALS AND METHODS

Bacterial strain and growth conditions. The non-mucoid laboratory strain *P. aeruginosa* PAO1 was grown on 5% sheep blood Columbia agar plates (Heipha, Heidelberg, Germany) and then cultured in Trypticase Soy Broth (TSB) (Difco, Becton Dickinson, Maryland, U.S.A.) overnight at 37°C and the culture adjusted to an optical density at 600 nm (OD_{600}) of 0.05 with TSB. Bacteria were grown for 2 h to reach the log-phase and then diluted to the desired concentration for inoculation.

Animals. Specific pathogen-free C57Bl/6 male mice of 8-10 weeks and weighing 24 to 26 g were obtained from Charles River (Calco, Italy). All mice were housed under SPF conditions in the animal care facility at the H.S. Raffaele Scientific Institute, Milano, Italy. The animal procedures were reviewed and approved by the H.S. Raffaele Animal Care and Use Committee, Milano, Italy.

Preparation of L-PBS and L-Cl_2MDP. Liposomes were prepared as previosuly described (Van Rooijen & Sanders, 1994). Briefly, 86 mg of egg phosphatidylcholine (Sigma-Aldrich, Milan, Italy) were dissolved in 10 ml of chloroform in a 500-ml round-bottom flask. The chloroform was evaporated by rotation under vacuum, and a thin phospholipid film was formed around the flask. At room temperature, the lipid was dispersed in 10 ml of phosphate-buffered saline (L-PBS) or 0.6 M Cl_2MDP (2.5 g in 10 ml of PBS). Cl_2MDP was generously provided by Boehringer Mannheim Roche. The suspension was kept at room temperature for 2 h under nitrogen gas and then sonicated for 3 min in a water bath sonicator and kept overnight at 4°C. L-PBS and L-Cl_2MDP were centrifuged and washed three times with sterile PBS at 10,000 X g for 15 min at 4°C to remove free Cl_2MDP. The final pellet was resuspended in 4 ml of sterile PBS and used immediately or stored at 4°C under nitrogen gas. The liposomes were used within 7 days of preparation.

Intranasal (i.n.) instillation of liposomes. Mice were anesthetized by an intraperitoneal injection of a solution of 2.5% Avertin (2,2,2-tribromoethanol and tert.-amyl alcohol) (Sigma-Aldrich) in 0.9% NaCl and administered at a volume of 0.015 ml/g body weight. They were inoculated with a 2-ml aliquots of L-PBS or L-Cl_2MDP repeatedly via the external nares for a total volume of 50 ml. The entire procedure required 15 to 20 min per mouse. To assess the efficiency of L-Cl_2MBP in depleting AMs, BALs were performed on mice before and after i.n. challenge with liposomes, as previously described (Trotta *et al.*, 2012). L-PBS and L-Cl_2MDP were delivered 24 h prior to bacterial infections.

Intratracheal administration of *P. aeruginosa*. Mice were anesthetized by an intraperitoneal injection of Avertin, as described before, and were placed in dorsal recumbency. The trachea was directly visualized by a ventral midline incision, exposed and intubated with a sterile, flexible 22-g cannula (Becton Dickinson, Heidelberg, Germany) attached to a 1 ml syringe. A 50 µl inoculum of a bacterial suspension was implanted via the cannula into the lung and all lobes were inoculated. After inoculation, the incisions were closed by sutures. The mice were maintained under specific pathogen-free conditions in sterile cages which were put into a ventilated isolator (Charles River). After 4 and 24 h, mice were euthanised. For quantitative bacteriology, mouse lungs and spleens were excised aseptically and homogenised in 1 ml PBS using the DIAX 900 homogenizer (Heidolph GmbH, Schwabach, Germany). One-hundred µl of the homogenates and 10-fold serial dilutions were spotted onto TSB plates and cfu determined after 24 h growth at 37°C. For histopathology, lungs were removed *en bloc*, rinsed in PBS, fixed in 4% paraformaldehyde in PBS, pH 7.4, at 4°C for 24 h, and processed for obtaining cryosections. Longitudinal sections of 7 µm were taken with a cryostate at regular intervals in the proximal, medial and distal region. Sections were stained with Harrys.

Statistical analysis. Statistical significance was analysed using the StatView program (Abacus Concepts Inc., Berkeley, CA) on a Power Macintosh iBook. ANOVA tests, with post hoc Fisher PLSD or Scheffe F-test, or Mann-Whitney tests were run to determine P values. A value of P<0.05 was

considered statistically significant

RESULTS AND DISCUSSION

This study was undertaken to investigate the role of AMs in the early clearance and bacterial internalization of *P. aeruginosa* in an acute model of pulmonary infection. Preliminary studies from our laboratory showed that *P. aeruginosa* PAO-1 strain proliferated in the C57Bl/6 murine lung for at least 24-72 h and then it was cleared from the airways (Conese *et al.*, 2008). *P. aeruginosa* internalization by lung cells has been shown to peak 4 h post-infection (Schroeder *et al.*, 2001). Based on these data, bacterial internalization and clearance and blood spreading were evaluated at 4 and 24 h post-infection, respectively.

Depletion of AM by i.n. instillation of L-Cl_2MDP results in reduced bacterial clearance but not in systemic bacterial spreading

To deplete AMs, we instilled intranasally L-Cl_2MDP (Cheung *et al.*, 2000). Fig. 1 shows that 24 h after i.n. instillation of 50 l of L-PBS or L-Cl_2MDP, the number of AMs recovered from L-Cl_2MDP-treated mice was significantly lower than that recovered from untreated and L-PBS-treated mice.

The number of bacteria in the lungs after intratracheal instillation of *P. aeruginosa* PAO-1 (1 X 10^4 cfu) in AM-depleted mice and control mice is shown in Fig. 2A. There were significantly more bacteria in the lungs of AM-depleted mice than in those of control mice at 24 h. No difference in bacterial number in control mice and AM-depleted mice at 4 h was observed.

To evaluate the integrity of the epithelial barrier in AM-depleted mice, the presence of bacteria in spleen was assessed following 1 X 10^4 cfu administration to the lung. The number of colonies found in spleens of control mice at 24 h did not differ as compared at 4 h post-infection (Figure 2B). AM-depleted mice presented equal number of colonies as compared to control mice (Figure 2B).

Histopathological findings are shown in Figure 3. In control mice without *P. aeruginosa* instillation, there was a normal distribution of AMs (Fig. 3a); these were absent in the AMs-depleted mice without *P. aeruginosa* instillation (Fig. 3b). Four hours after the bacterial instillation, PMNs were infiltrated into airspace in AM-depleted mice, as well as in control mice (Fig. 3c and d). Twenty-four hours after the bacterial instillation, thickening of interstitial spaces was more marked in AM-depleted mice (Fig. 3f).

These results confirm in part those obtained by Kooguchi *et al.* (1998). These Authors observed in CD-1 mice that eight hours after the bacterial instillation, few PMNs were infiltrated into airspaces in AM-depleted mice, in contrast to control mice which showed significant predominance of PMNs in the airspaces. At 48 h, they observed destruction of alveolar structure and thickening of interstitial spaces more marked in AM-depleted mice. Differences at early times (4-8 h) could be due to the mouse strain used or to the delivery method of clodronate-containing liposomes and the extent of AMs depletion. However, in the present study, about 92% of AMs could be depleted by i.n. instillation of L-Cl_2MDP, similarly to what obtained by Kooguchi *et al.* (1998) with aerosol inhalation.

Depletion of AMs doe not influence bacterial Internalisation

Bacterial internalization by lung cells after the intratracheal instillation of PAO-1 (1 X 10^7 cfu) was evaluated at 4 h. Total and internalized cfu were similar in both groups (Fig. 4). Although the amount of internalized cfu/total cfu was lower in L-Cl_2MDP-treated mice than in L-PBS-treated mice, this was not statistically significant (L-PBS: 0.12 ± 0.01% of total cfu; L-Cl_2MDP: 0.06 ± 0.03% of total cfu).

Data show that in the absence of AM, bacterial internalisation occurred, likely by the other predominant resident cell type in the airways, i.e. airway epithelial cells. Overall, these results indicate that internalisation of bacteria by airway epithelial cells is negligible at early times of infection. AM depletion compromises the capacity of C57Bl/6 mice to clear *P. aeruginosa* from the lung at 24 h but it has no effect on the function of airway epithelial cells as nonprofessional phagocytes. This is confirmed by the lack of increased number of colonies found in the spleen in AM-depleted mice as compared to control mice. AM depletion was noted by others to profoundly affect pulmonary host defenses against *P. aeruginosa* and other respiratory pathogens (Broug-Holub *et al.*, 1997; Cheung *et al.*, 2000; Hickman-Davis *et al.*, 1997; Kooguchi *et al.*, 1998). However, to our knowledge this is the first report on the influence of AM depletion on the early response of epithelial cell capability of internalisation of *P. aeruginosa* planktonic bacteria. Our data clearly indicate that bacterial internalisation does not play a pivotal role in the immune surveillance at early times of infection, while AMs are necessary to clear *P. aeruginosa* at later times, likely due to their capacity to recruit other phagocytic cells (PMNs) rather than to their intrinsic phagocytic function (Cheung *et al.*, 2000). Indeed, some studies indicate that macrophages can make a small but measurable contribution to early bacterial clearance (Kannan *et al.*, 2009; Kooguchi *et al.*, 1998; Manicone *et al.*, 2009), while PMNs play a primary and unambiguous role in *P. aeruginosa* clearance during acute pulmonary infection (Koh *et al.*, 2009; Tsai *et al.*, 2000).

In summary, this study indicates that lung macrophages do not have an influence on the uptake of *P. aeruginosa* planktonic bacteria by other respiratory cell types, i.e. airway epithelial cells, while it suggests a pivotal role of AMs in recruiting PMNs for appropriate clearance of this opportunistic pathogen from the lung.

ACKNOWLEDGEMENTS
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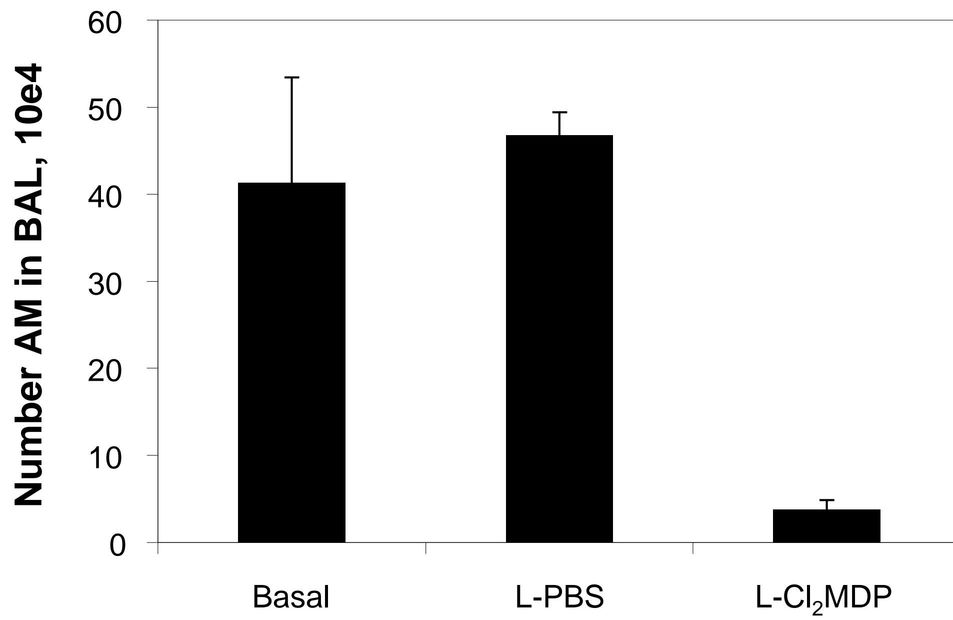
FIGURE LEGENDS

Fig. 1. Depletion of AM in vivo by intranasal instillation of Cl_2MDP. Fifty microliters of L-PBS or L-Cl_2MDP was delivered to mice by intranasal instillation; 24 h after the instillation of liposomes, the number of AM in the BAL fluid was determined from hemocytometer counts and cytocentrifuged preparations. The number of AM in BAL fluid from untreated, L-PBS-treated and L-Cl_2MDP-treatmed mice (n= 10/group) is shown. Data are expressed as the means ± SEM of three experiments. *$P<0.05$.

Fig. 2. Effect of AM depletion on bacterial clearance and systemic spreading. **A**. Mice were treated with L-PBS or L-Cl_2MDP (n=15/group) and then challenged with *P. aeruginosa* PAO-1 (1 X 10^4 cfu). At 4h and 24 h, bacterial counts in lung homogenates were determined. *$P<0.05$. **B**. Mice were treated with L-PBS or L-Cl_2MDP (n=15/group) and then challenged with *P. aeruginosa* PAO-1 (1 X 10^4 cfu). At 4h and 24 h, bacterial counts in spleen homogenates were determined. Bacterial counts are represented as the percentage of the cfu counted in the lung. In A and B, data are expressed as the means ± SEM of three experiments.

Fig. 3. Histologic assessment (Harrys staining) of mouse lung tissue upon AM depletion. Mice were exposed to L-PBS (a, c, and e) or L-Cl_2MDP (b, d, and f). (a and b) Tissues from mice without *P. aeruginosa* instillation. Arrows indicate AMs. (c and d) Tissues from mice 4 h after *P. aeruginosa* instillation. (e and f). Tissues from mice 24 h after *P. aeruginosa* instillation. Original magnification, X20.

Fig. 4. AM depletion does not influence bacterial internalisation. Mice were treated with L-PBS or L-Cl_2MDP (n=10/group) and then challenged with PAO-1 (1 X 10^7 cfu). At 4 h, single cell suspensions were obtained from lungs and number of internalized bacteria were determined by the gentamicin exclusion assay. Data are shown as cfu/g lung tissue and expressed as the means ± SEM of three experiments.


Number AM in BAL, 10e4
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50
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30
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0
Basal
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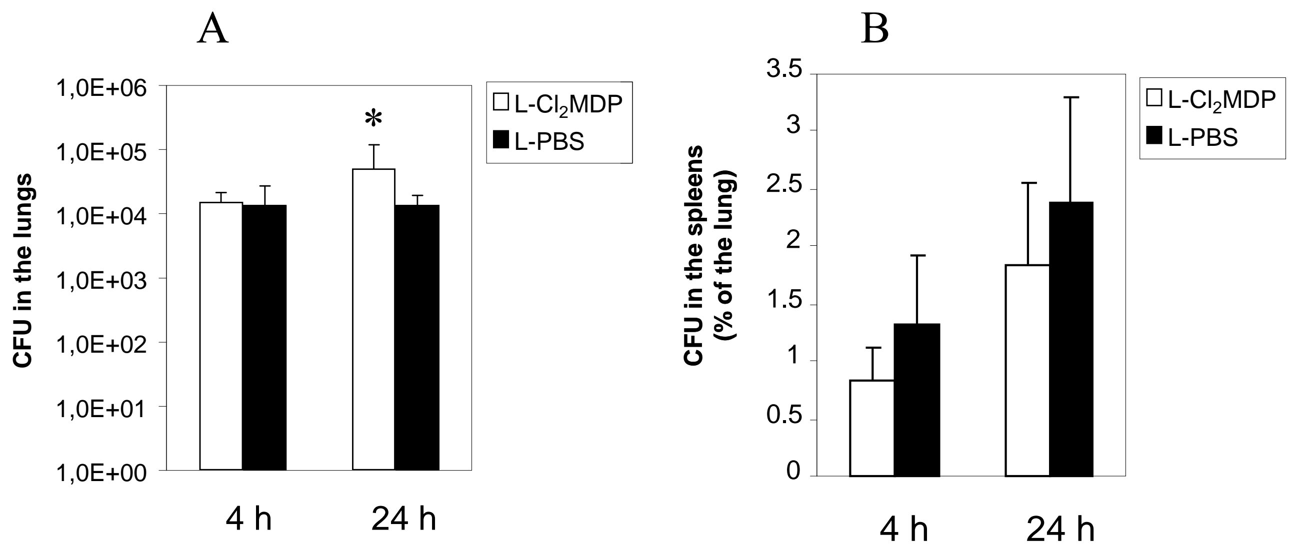
A
CFU in the lungs
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1,0E+05
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24 h
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B
CFU in the spleens
(% of the lung)
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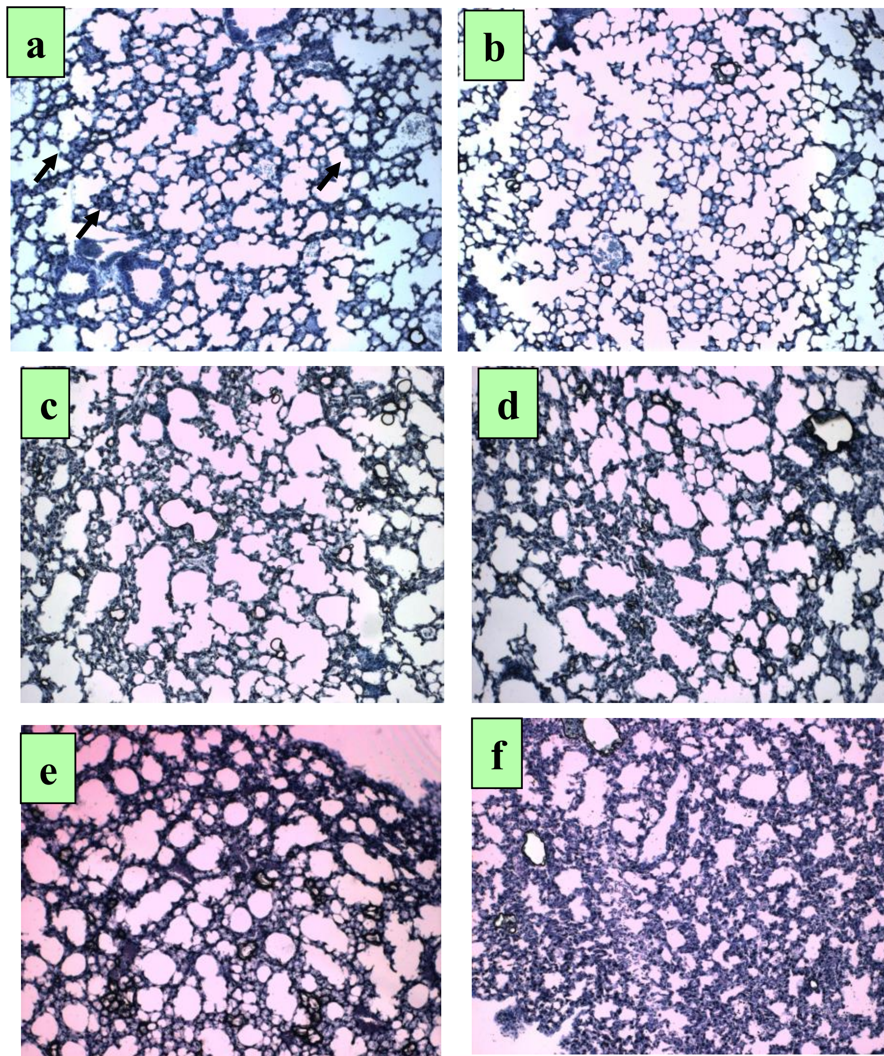
3
2.5
2
1.5
1
0.5
0
4 h
24 h
L-Cl_2MDP
L-PBS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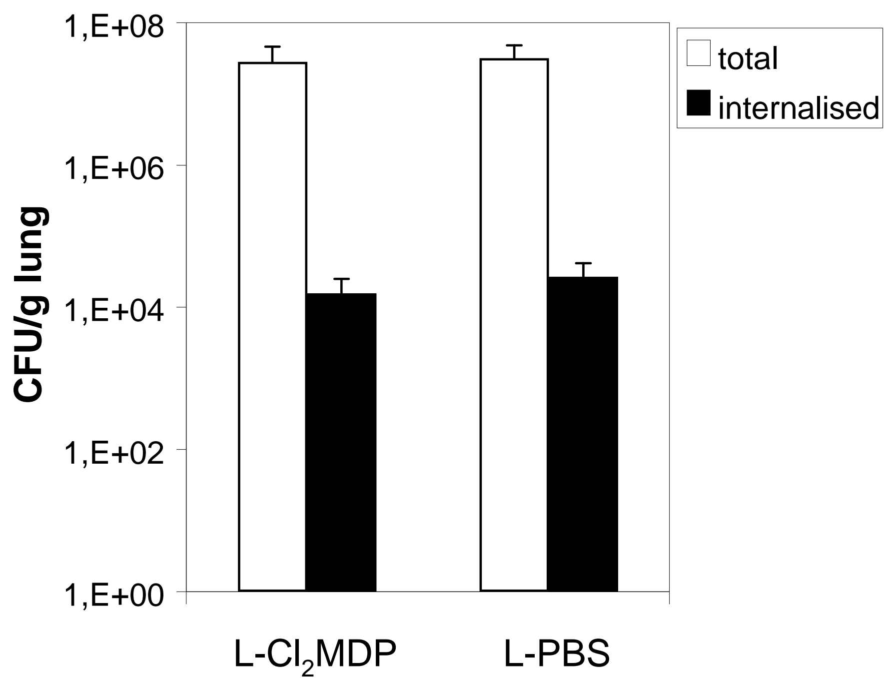
CFU/g lung
1,E+08
1,E+06
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internalised
L-Cl2MDP
L-PBS