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Differential nitric oxide (NO) production by macrophages from mice and guinea pigs infected with virulent and avirulent *Legionella pneumophila* serogroup 1

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SUMMARY

L-arginine-dependent reactive nitrogen intermediates have been identified as macrophage cytotoxic effector molecules against intracellular pathogens. To determine its role, *ex vivo* production of NO by peritoneal macrophages of C3H/HeN mice and Dunkin–Hartley guinea pigs infected intraperitoneally with a virulent and isogenic avirulent *Legionella pneumophila* serogroup 1 strain was compared with bacterial clearance from the lungs. While the virulent strain was cleared from mice lungs, the guinea pigs died within 96 h. *In vivo* infection with both strains resulted in the production of NO by mouse peritoneal macrophages *ex vivo*. In contrast, guinea pig macrophages did not produce detectable NO. In addition, infection by the avirulent strain led to the production of significantly more NO by mouse macrophages than the virulent parent strain, irrespective of stimulation with lipopolysaccharide (LPS) and/or interferon-gamma (IFN-γ). These results suggest that resistance to *Leg. pneumophila* infection may depend on the production of NO by host macrophages.

Keywords *Legionella pneumophila* macrophages nitric oxide virulence mice

INTRODUCTION

Legionella pneumophila, the etiologic agent of Legionnaires' disease, is a facultative intracellular Gram-negative bacterium. Upon ingestion by cells of the monocyte-macrophage series, it resists destruction and multiplies within a specialized phagosome–lysosomal vacuole [1]. The bacteria escape destruction by inhibiting both phagosome–lysosome fusion and acidification of the vacuole [2]. Virulent bacteria also inhibit the production of toxic oxygen-free radicals [3,4]. Interferon-gamma (IFN-γ) or bacterial glycoprotein-activated blood monocytes and macrophages are able to limit the growth of intracellular *Leg. pneumophila* [5,6]. Activated macrophages inhibit the growth of intracellular pathogens by oxygen-dependent killing [7], but other mechanisms such as reactive nitrogen intermediates are also involved [8–12].

NO radicals are involved in the cytostatic/cytotoxic action of macrophages against a variety of intracellular pathogens, including trophozoites of *Toxoplasma gondii* [13], *Pneumocystis carinii* [14], amastigotes of *Leishmania major* [15], *L. donavanii* [16], *Plasmodium falciparum* [17], *Cryptococcus neoformans* [9], schistosomula of *Schistosoma mansoni* [18], *Trypanosoma musculi* [19], *Tr. brucei gambiense* and *Tr. brucei brucei* [20], trophozoites of *Entamoeba histolytica* [21], *Naegleria fowleri* [22], *Listeria monocytogenes* [23], *Francisella tularensis* [24], *Mycobacterium tuberculosis* [25], *Myco. leprae* [26], and *Myco. avium* [27]. NO radicals were recently found to be involved in the killing of intracellular *Leg. pneumophila* by a murine macrophage cell line, RAW 264.7 *in vitro* [28]. NO is generated via nitric oxide synthase, which uses L-arginine as sole substrate to produce citrulline and NO, which gives rise to nitrate and nitrite [9–11].

In the present study, we investigated the role of NO radicals in resistance/susceptibility to *Leg. pneumophila* infection *in vivo* by using virulent and avirulent bacteria in two animal hosts, i.e. mice and guinea pigs. We demonstrated that macrophages from mice, which rapidly cleared the virulent bacteria from the lungs, produced significantly increased amounts of nitrite, whereas guinea pigs succumbed to the infection and their macrophages did not produce nitrite. Also, macrophages from mice infected with avirulent *Leg. pneumophila* produced higher amounts of nitrite compared with mice infected with the virulent strain, irrespective of stimulation with lipopolysaccharide (LPS) and/or IFN-γ. In addition, we also showed that *in vitro* avirulent *Leg. pneumophila* was more sensitive than the virulent strain to the cidal activity of SIN 1, a potent NO donor [29].

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MATERIALS AND METHODS

Reagents

PBS without Ca^{2+} and Mg^{2+}, RPMI 1640 medium without phenol red, glutamine (200 mM), and endotoxin-free fetal calf serum (FCS) were all purchased from GIBCO (Paisley, UK). Linsidomine hydrochloride (SIN 1) and N-morpholino-imino-acetonitril (SIN 1C) were kindly provided by Hoechst Labs (Paris-La Defense, France). Recombinant murine IFN-γ (2×10^7 U/mg), free of endotoxin activity, was kindly provided by Roussel-Uclaf Labs (Romainville, France). LPS from *Escherichia coli* serotype 055:B5 (LPS), N^G-monomethyl L-arginine (NMMA), N-1-naphtyl-ethylenediamine, sulfanilamide, and sodium nitrate were from Sigma Chimie (St. Quentin Fallavier, France). Ortho-phosphoric acid (H_3PO_4) was purchased from Merck Labs (Nogent-sur-Marne, France). All sterile disposable plastic materials were purchased from Becton Dickinson (Grenoble, France).

Animals

Female C3H/HeN mice (18–20 g) and male Dunkin–Hartley guinea pigs (250–300 g) were from Charles River breeders (Saint-Aubin-les-Boeuf, Cléon, France). They were housed in standard cages and given water and food *ad libitum* until use. This study was performed in accordance with prevailing regulations regarding care and use of laboratory animals in the European Communities (Journal Officiel des Communautées Européennes, 18 December 1986; L358).

Bacteria

Legionella pneumophila serogroup 1 (strain Paris CB 81-13) was isolated from the lung of a patient who died from Legionnaires' disease during a nosocomial outbreak in Paris, France. Crude lung homogenate was plated on buffered charcoal yeast extract agar supplemented with α-ketoglutarate (BCYE) and antibiotics. Plates were incubated at 35°C with 2·5% CO_2 and 95% humidity. A single colony was taken after 72 h and plated onto BCYE plates, and bacteria were grown at 35°C to mid-logarithmic phase. They were harvested in sterile distilled water and tested for viability and the absence of contaminating bacteria. The concentration of the bacteria was then adjusted to 10^9 bacteria/ml, divided into samples, and stored at –70°C until use. The virulence of this strain, passaged twice in BCYE agar, was assessed by i.p. infection of guinea pigs and intracellular multiplication in human monocyte-macrophages as previously described [6,30]. The LD_{50} for guinea pigs was 2×10^5 bacteria/ml. The avirulent strain was obtained by 76 passages of the virulent strain on BCYE over a 6-month period. A single colony from passage 76 was plated onto BCYE plates and grown at 35°C to mid-logarithmic phase. The bacteria were harvested, tested and stored as for the virulent strain. The LD_{50} for guinea pigs was $>2 \times 10^8$ bacteria/ml.

Animal infection and lung counts

We have previously shown that guinea pigs infected intraperitoneally with virulent legionellae develop severe pneumonia and peritonitis by 48 h [30]. Thus, mice and guinea pigs were infected intraperitoneally with both strains at 1×10^7 organisms/ml. They were killed 24, 48 or 72 h post-infection by ether asphyxiation and exsanguinated. The lungs were removed aseptically and contaminating blood was removed by two washes with sterile distilled water. After weighing, they were homogenized in 5–10 ml of sterile distilled water (Ultra-Turrax T25; Bioblock Scientific, Illkirch, Strasbourg, France). The homogenates were serially diluted in sterile distilled water and 100-μl aliquots were plated on BCYE–agar plates. The plates were incubated at 35°C with 2·5% CO_2 and 95% humidity. Colonies were counted after 5 days and the results expressed as viable bacteria/g of lung (wet weight).

Isolation of peritoneal exudate cells for ex vivo *production of NO*

Mice and guinea pigs were infected intraperitoneally with 1×10^7 colony-forming units (CFU) of either virulent or avirulent *Leg. pneumophila* in 1 ml of distilled water; control animals received only 1 ml of sterile distilled water. The animals were killed 48 h later and the peritoneal cavity from five mice and two guinea pigs was washed aseptically with cold PBS. The resulting peritoneal exudate cell (PEC) suspension was centrifuged at 450 ***g*** for 15 min and the cell pellet was washed twice in PBS. PEC from each group were pooled and resuspended in RPMI 1640 medium without phenol red (supplemented with 2 mM L-glutamine). The percentage of esterase-positive cells was determined by differential staining with May–Grünwald–Giemsa α-naphthyl butyrate esterase, and concentration was adjusted to contain 10^6 macrophages/ml. Cell viability was assessed by using the trypan blue dye exclusion test.

Experimental protocol

Experiments were done using 96-well flat-bottomed microtitre plates (Falcon). PEC suspension of $\approx 2 \times 10^5$ macrophages in 200 μl was added to triplicate wells with 5% heat-inactivated FCS. They were then treated with medium (control), LPS (10 μg/ml), or IFN-γ (100 U/ml) in the absence or presence of 10^{-4} M NMMA, a competitive inhibitor of NO synthase, and incubated at 37°C with 5% CO_2 for 24, 48 or 72 h. NMMA was added every 24 h. The plates were centrifuged at 450 ***g*** for 10 min and cell supernatants from triplicate wells were aliquoted and kept at –70°C until nitrite determination.

Determination of nitrite

Nitrite is a stable compound produced by the reaction of NO with water and oxygen, and its accumulation in culture media reflects the amount of NO produced. Nitrite was measured by using the Griess reagent, which consists of equal volumes of 0·1% naphthyl-ethylene diamine and a mixture of 1% sulfanilamide in 2·5% H_3PO_4. Aliquots of (100 μl) cell supernatant or standards were mixed with an equal volume of Griess reagent in duplicate. After 10 min at room temperature, absorbance was recorded on a Molecular Devices spectrophotometer at 540 nm. Concentrations were determined from a standard curve constructed with sodium nitrite (1–250 μM) and expressed as nmol of nitrite per 10^6 macrophages.

Sensitivity of Leg. pneumophila *strains to NO*

The action of NO radicals on viable virulent and avirulent *Leg. pneumophila* was examined using SIN 1, a potent NO donor [29]. SIN 1 in aqueous solution donates NO radicals by 12 h. Briefly, 1×10^7 viable bacteria were suspended in sterile distilled water in 5-ml sterile Falcon screw-capped tubes with 1 mM SIN 1 in 5% glucose solution and incubated at 35°C with 2·5% CO_2 and 95% humidity for 18 h. Parallel control cultures were run as follows: (i) bacteria plus sterile distilled water; (ii) bacteria plus 1 mM SIN 1C (a precursor of SIN 1) in 5% glucose solution; (iii) bacteria plus 5% glucose solution alone; and (iv) bacteria plus 1 mM sodium nitrite in sterile distilled water. After 18 h of culture, serial dilutions of the

suspensions were plated on BCYE plates. Colonies were counted after 5 days and the results represented as the percentage of viable cells as follows:

$$\frac{\text{CFU with SIN 1, SIN 1C, 5\% glucose or 1 mM sodium nitrite}}{\text{CFU with distilled water alone}} \times 100$$

Statistical analysis

One-way analysis of variance for unequal group sizes was applied to the data using Statview software on a Macintosh Quadra 700 computer. $P \leqslant 0{\cdot}05$ was considered significant. Data were expressed as means $\pm$ 1 s.d.

RESULTS

Bacterial lung counts

Twenty-four hours post-infection, nearly all avirulent legionellae were cleared from mouse lung (Fig. 1b), whereas the virulent strain persisted for 48 h ($P < 0{\cdot}01$) and all mice survived the infection. In guinea pigs (Fig. 1a) there was a significant increase in virulent *Leg. pneumophila* CFU in the lungs 72 h post-infection ($P < 0{\cdot}001$), whereas the avirulent strain was cleared by 48 h. Guinea pigs infected with virulent *Leg. pneumophila* succumbed to infection by 96 h.

Ex vivo *nitrite production*

Significantly ($P < 0{\cdot}001$) higher amounts of nitrite were produced by macrophages obtained from mice infected by the virulent or avirulent *Leg. pneumophila* at 24 h (for only avirulent), 48 h and 72 h of culture than by macrophages from uninfected mice (Fig. 2a). Furthermore, production of nitrite by macrophages from mice infected by avirulent strain at 24, 48 and 72 h of culture was significantly higher than by macrophages from those infected by the virulent strain ($P < 0{\cdot}01$). Nitrite production by macrophages was significantly inhibited ($P < 0{\cdot}001$) by NMMA at all culture times.

Figure 2b shows the effect of LPS (10 μg/ml) on nitrite production by macrophages. LPS increased the production of nitrite by macrophages from both infected and uninfected mice. Macrophages from mice infected with both strains produced significantly more nitrite relative to macrophages from control mice ($P < 0{\cdot}01$). While no significant differences were observed in the production of nitrite by the two strains at 24 h, macrophages from mice infected with the avirulent strain produced significantly more nitrite at 48 and 72 h of culture than macrophages from mice infected by the virulent strain ($P < 0{\cdot}05$). After 24 h of culture, nitrite production by macrophages from infected and uninfected mice was constant for the remaining 48 h.

Figure 2c shows the effect of recombinant murine IFN-γ (100 U/ml) on nitrite production by macrophages. Like LPS, IFN-γ also increased the production of nitrite by macrophages from both infected and uninfected mice. IFN-γ resulted in a significant increase ($P < 0{\cdot}01$) in nitrite production by macrophages from mice infected with virulent and avirulent *Leg. pneumophila* relative to macrophages from uninfected mice, at 24 h of culture. At later times (48–72 h) values were similar in uninfected mice and mice infected with the virulent strain, whereas macrophages from mice infected with the avirulent strain produced significantly ($P < 0{\cdot}01$) more nitrite than the other two groups. Like LPS, IFN-γ also induced constant nitrite production during the next 48–72 h of culture from infected and uninfected mice.

Figure 2d shows the effect of IFN-γ plus LPS on nitrite production by macrophages. Like LPS, the association of LPS with IFN-γ also increased the production of nitrite by macrophages from both infected and uninfected mice. However, in the presence of LPS and IFN-γ, macrophages from uninfected mice produced increased amounts of nitrite compared with LPS alone ($P < 0{\cdot}05$). Macrophages from mice infected with both strains produced significantly more nitrite relative to macrophages from control mice ($P < 0{\cdot}01$). The kinetics of nitrite production by macrophages from infected mice was similar to that with LPS alone.

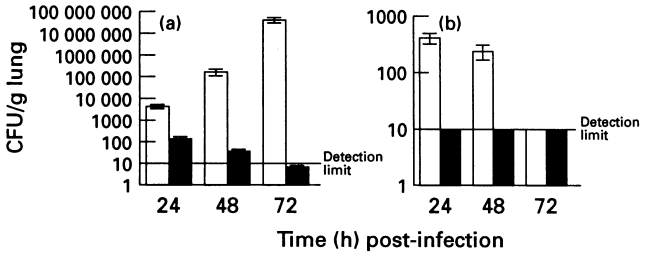

Fig. 1. Bacterial clearance from the lungs of guinea pigs (a) and mice (b) 24, 48 and 72 h after infection with virulent (□) and avirulent (■) *Legionella pneumophila*. Each column represents the mean $\pm$ s.d. for 10 guinea pigs and 20–25 mice in three and five different experiments, respectively. Mean values in guinea pigs at 72 h (virulent) are for the four surviving animals. CFU, Colony-forming units.

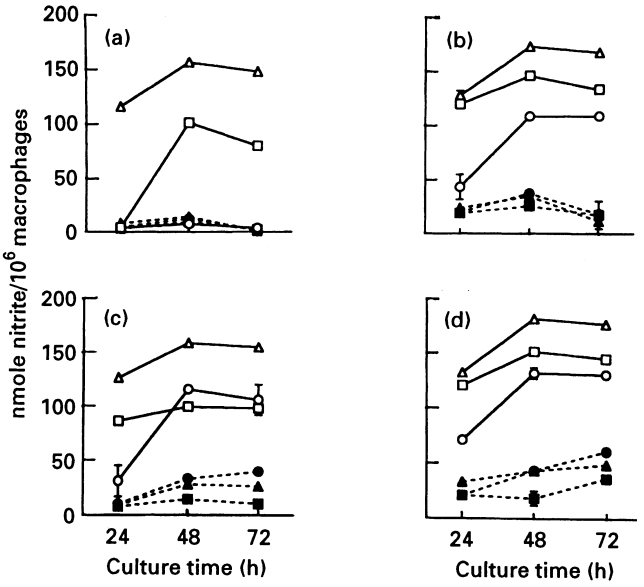

Fig. 2. *Ex vivo* production of nitrite by peritoneal macrophages obtained from uninfected control mice (injected with distilled water; C, ○) and mice infected with the virulent (V, □) and avirulent (Av, △) *Legionella pneumophila* strains 48 h post-infection. (a) Cells alone. (b) Effect of lipopolysaccharide (LPS; 10 μg/ml). (c) Effect of recombinant murine IFN-γ (100 U/ml). (d) Effect of LPS (10 μg/ml) and IFN-γ (100 U/ml). Nitrite production was determined after 24, 48 and 72 h of culture as described in Materials and Methods. The effect of 10^{-4} M N^G-monomethyl L-arginine (NMMA) on *ex vivo* production of nitrite by peritoneal macrophages is shown for uninfected (C, ●), and *Leg. pneumophila*-infected (V, ■, and Av, ▲) mice, respectively. Each point represents the mean $\pm$ s.d. of triplicate cultures in four experiments.

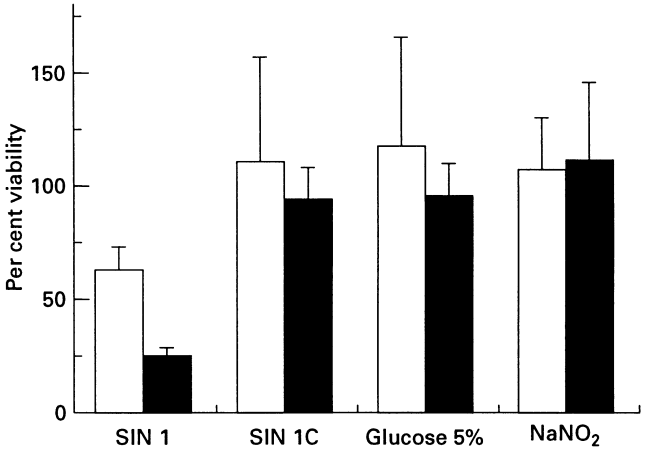

Fig. 3. *In vitro* susceptibility of virulent (□) and avirulent (■) *Legionella pneumophila* to a nitric oxide donor, SIN 1. Bacteria were incubated with 1 mM SIN 1 and with 5% glucose, 1 mM SIN 1C (a precursor of SIN 1), and 1 mM sodium nitrite ($NaNO_2$) as negative controls. After 18 h of incubation at 35°C, viable bacteria were determined as described in Materials and Methods. Each column represents mean ± s.d. of duplicate cultures in four experiments.

Nitrite production by macrophages stimulated with LPS and/or IFN-γ was inhibited by NMMA at all culture times ($P < 0{\cdot}001$) (Fig. 2b–d).

Ex vivo *production of nitrite by peritoneal macrophages from guinea pigs infected with virulent and avirulent* Leg. pneumophila
As shown in Fig.1, intense bacterial multiplication was found in guinea pig lungs 48 h post-infection with the virulent strain, while counts of the avirulent strain were restricted. We therefore chose this time point to measure *ex vivo* nitrite production by macrophages from infected or uninfected guinea pigs. No nitrite production by macrophages stimulated with or without LPS from uninfected guinea pigs and those infected with the virulent or avirulent strain was detected at any time of culture (24, 48 and 72 h) (data not shown).

The effect of IFN-γ alone and in combination with LPS could not be tested in our study for the following reasons: (i) guinea pig IFN-γ is not available commercially; and (ii) the effect of IFN-γ being species-specific, murine IFN could not be used.

Sensitivity of Leg. pneumophila *strains to NO* in vitro
Virulent and avirulent *Leg. pneumophila* were both sensitive to the action of SIN 1 (Fig. 3). However, the avirulent strain was significantly more susceptible to killing by NO than the virulent strain ($P < 0{\cdot}01$). The viability of the virulent and avirulent *Leg. pneumophila* strains was not affected by 1 mM sodium nitrite, 5% glucose, or 1 mM SIN 1C.

DISCUSSION

To our knowledge, this is the first *ex vivo* study of the role of NO in anti-legionella activity in mice infected with virulent and avirulent *Leg. pneumophila* strains. Macrophages from mice infected with both virulent and avirulent *Leg. pneumophila* produced significantly more nitrite than uninfected mice. The inhibition of nitrite production by NMMA, a competitive inhibitor of L-arginine [10,11], showed that this production was due to induction of NO synthase activity. In addition, macrophages from mice infected with the avirulent strain produced higher amounts of nitrite *ex vivo* than those infected with the virulent strain.

NO synthase activity in macrophages can be induced by various stimulants (particularly LPS and IFN-γ) and play a major role in macrophage activity against several bacterial pathogens, as discussed earlier. In our study, LPS and IFN-γ increased nitrite production by macrophages from mice infected with both the virulent and avirulent strain. However, we consistently found higher nitrite production by macrophages from mice infected with the isogenic avirulent strain than with the virulent strain. Thus, the differences in nitrite production by macrophages from mice infected by the two strains could be linked to their virulence. Virulent and avirulent *Leg. pneumophila* are recognized differently by specific MoAbs [30]. One such antibody is directed against an antigenic determinant of *Leg. pneumophila* LPS [31], suggesting that the differences in LPS [32], a major component of the bacterial cell wall, could explain the differences in NO synthase activity in macrophages.

In addition, our data suggest a direct role of NO-mediated cidal activity against *Leg. pneumophila*, since SIN 1, a generator of NO [29], had direct cidal activity against both the virulent and avirulent *Leg. pneumophila* strains *in vitro* (Fig. 3). A similar direct bactericidal action of another NO generator, sodium nitroprusside, has been shown with *Leg. pneumophila* by Summersgill *et al.* [28]. We found that the avirulent strain was significantly more susceptible to SIN 1 than the virulent strain (25% *versus* 72% survival among the avirulent and virulent strains, respectively).

The differences in clearance of avirulent and virulent *Leg. pneumophila* from the lungs of infected mice could be related to: (i) the intense production of NO by macrophages; and (ii) the fact that the isogenic avirulent strain was more susceptible than the parent virulent strain to NO *in vitro*.

The parallel study in guinea pigs showed a lack of detectable NO production and intense multiplication of the virulent strain in the lungs (Fig. 1a). These results suggest a link between the two phenomena, but other mechanisms of defence cannot be ruled out [33]. Thus the effective clearance of avirulent bacteria from the lungs of guinea pigs was mainly, if not entirely, due to NO-independent mechanisms, since no NO activity could be detected either *ex vivo* or *in vitro*.

Several mechanisms have been proposed to explain the bactericidal/bacteriostatic activity of phagocytes against *Leg. pneumophila*: (i) the production of oxygen-derived species by activation of NADPH-oxidase [34]; (ii) the inhibition of intracellular multiplication of *Leg. pneumophila* by IFN-γ-activated macrophages due to a limited availability of intracellular iron, which occurs as a consequence of IFN-γ-induced coordinate down-regulation of transferrin receptor expression and intracellular ferritin concentrations [5,33,35]. NO production by activated murine macrophages lends further support to the above-mentioned mechanisms: Summersgill *et al.* [28] have shown that IFN-γ induced the production of nitrite in a murine macrophage cell line, RAW 264.7, which was directly responsible for killing *Leg. pneumophila.* These authors have also shown that this bactericidal activity correlates with the depletion of intracellular iron in this cell line. Nitric oxide radicals generated by IFN-γ-activated macrophages react with iron–sulphur prosthetic groups of the enzymes necessary for cellular functions such as (i) mitochondrial respiration, which may limit the ability of host cells to produce ATP [10,11]; (ii) DNA replication requiring ribonucleotide reductase [36]; and (iii) the citric acid cycle [12,37], and these radicals are released

extracellularly as iron–nitrosyl–sulphur complexes. This results in a net decrease in intracellular iron in the macrophages, and has been shown to be responsible for NO-cidal activities against intracellular pathogens [10,11,28,38,39].

In conclusion, our results suggest that NO production by macrophages plays a major role in defences against *Leg. pneumophila* in resistant hosts such as mice, and that the intensity of NO production induced by the virulent and avirulent strain could be related to intrapulmonary growth. The lack of NO production in animals susceptible (guinea pigs) to virulent *Leg. pneumophila* could partially explain the intense multiplication in the lungs leading to death. In humans, although inducible NO synthase activity by macrophages is still controversial [14,27,40], the lack of this defence mechanism may account for susceptibility to Legionnaires' disease due to virulent *Leg. pneumophila* serogroup 1. The report by Summersgill *et al.* [28], that in the human cell line HL-60 *Leg. pneumophila* growth was only contained, and that neither bactericidal activity nor NO production was induced, is in keeping with this hypothesis. However, our data must be interpreted with caution, since other defence mechanisms were indirectly evidenced in this study by the elimination of avirulent legionellae from guinea pig lungs in the absence of detectable inducible NO synthase activity.

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