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## TNF- $\alpha$ response in macrophages depends on clinical *Legionella pneumophila* isolates genotypes

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### ABSTRACT

Legionnaires' Disease (LD) is a severe pneumonia mainly caused in Europe by *Legionella pneumophila* serogroup 1 (Lp1). Sequence-based typing methods reveal that some sequence types (ST) are overrepresented in clinical samples such as ST1 and ST47, suggesting that some strains are more fit for infection than others. In the present study, a collection of 108 Lp1 clinical isolates were used to evaluate the strain-dependent immune responses from human macrophages. Clinical Lp1 isolates induced differential TNF $\alpha$  secretion from macrophages. ST1 isolates induced a significantly higher TNF- $\alpha$  secretion than non-ST1, whereas ST47 isolates induced a significantly lower TNF- $\alpha$  secretion than non-ST47 isolates. ST1 isolates induced a significantly higher cell death than ST47 isolates evaluated by lactate dehydrogenase activity (cytotoxicity) and caspase-3 activity (apoptosis). Treatment of macrophages with anti-TNF- $\alpha$  antibodies significantly reduced the cell death in macrophages infected with ST1 or ST47 strains. The TNF- $\alpha$  secretion was neither explained by a differential bacterial replication nor by the number or type (bystander or infected) of TNF- $\alpha$  producing cells following infection but by a differential response from macrophages. The Paris ST1 reference strain elicited a significantly higher *TNF- $\alpha$*  gene transcription and a higher induction of NF- $\kappa$ B signaling pathway than the Lorraine ST47 reference strain.

Clinical Lp1 isolates induce a diverse immune response and cell death, which could be related to the genotype. The two predominant sequence-types ST1 and ST47 trigger opposite inflammatory response that could be related to the host susceptibility.

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## Introduction


*Legionella pneumophila* (Lp) is a Gram-negative bacterium that replicates within eukaryotic microorganisms in aquatic ecosystems [1,2]. It can be an accidental human pathogen if contaminated droplets from colonized man-made water systems are inhaled. In the lungs, after phagocytosis by alveolar macrophages, Lp rapidly injects around 300 effectors via a Dot/Icm type IV secretion system (T4SS) in order to avoid destruction, hijack the host's defenses, and create its replicative vacuole [1,2]. The resulting infection is a severe pneumonia named Legionnaires' disease (LD). The main risk factors for LD include age >50 years, smoking, or immunosuppression [3]. In particular, the incidence of LD in patients suffering from chronic inflammatory diseases and receiving therapies against the pro-inflammatory cytokine Tumor Necrosis Factor alpha (TNF- $\alpha$ ) is 13-fold

higher than the rest of the population [4]. Moreover, LD can be fatal in around 8% of cases, and up to 30% in patients admitted to an intensive care unit [5,6]. A previous study reported that both a high inflammatory status and corticosteroid therapy were associated with fatal LD outcome [7]. LD cases are mainly due to the serogroup 1 (Lp1) which account for up to 80% in Europe and USA [8,9]. The latter can be further subdivided into Sequence Type (ST; based on DNA sequencing) [10]. On circa 3000 STs described so far, 5 main STs (ST1, ST23, ST37, ST47, and ST62) are the most prevalent in Europe, accounting for approximately half of cases [9,11]. This suggests that ST isolates are not equally fit to create or maintain a successful infection in human lungs.

The immune response following Lp phagocytosis has been extensively studied and recently reviewed [12,13]. Notably, alveolar macrophages can detect Lp,

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 Supplemental data for this article can be accessed [here](#).

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both extracellularly and intracellularly, and trigger secretion of cytokines such as TNF- $\alpha$ . This pro-inflammatory cytokine is recognized by TNF receptors (TNFR1 & TNFR2), which could subsequently activate the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) signaling pathway, leading to transcriptional activation of pro-survival and pro-inflammatory genes including *TNF- $\alpha$*  itself creating an auto-inducing loop [14]. TNFR1 additionally possesses a death-domain that can trigger cell death via apoptosis [14]. TNF- $\alpha$  is then essential for the host's defenses, by recruiting other immune cells and/or inducing cell death as a way to restrict intracellular pathogens, and is therefore often targeted by microbes [15]. The efficiency of TNF- $\alpha$  against Lp replication has additionally been demonstrated *in vitro* in different models [16–20]. However, these studies were conducted using laboratory model strains. The strain-dependent response is less-well explored, although the epidemiological data indicate that some STs are clinically predominant [9,11], which could be related to specific bacterial determinants, inadequate immune response from the host, or a combination of both. We therefore used the large bacterial collection associated with clinical cases previously described [7] to evaluate the ability of Lp1 clinical strains to induce differential immune responses in a macrophage cell line model, in particular TNF- $\alpha$  secretion and cell death due to the essential role of this cytokine during LD.

## Materials and methods

### Bacterial strains

We used a collection composed of 108 Lp1 isolates (Table S1) corresponding to LD cases previously described [7]. The collection encompasses 40 different STs, including the main STs isolated in France (ST1, ST20, ST23, ST40, ST47, ST62, ST146, ST259, ST701). The three most prevalent STs (ST1, ST23, and ST47) account for 39.8% of isolates in the collection, which is consistent with reported LD in France [21]. Whole-genome sequences were deposited in the European Nucleotide Archive under the accession no. PRJEB15241 [22]. The Paris ST1 (CIP107629) and Lorraine ST47 (CIP 108729) strains were used as reference strains.

### Bacterial and cell cultures

All Lp1 strains were cultured either in Buffered Charcoal Yeast Extract (BCYE; solid) or in ACES Yeast Extract (AYE; liquid), both supplemented with iron and L-cysteine and incubated at 37°C. The human monocyte U937 cell line (ATCC® CRL-1593.2;

Manassas, VA, USA) and the human monocyte THP1-XBlue cells (InvivoGen; San Diego, CA, USA) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) at 37°C and 5% CO<sub>2</sub> in a humidified atmosphere. Cells were differentiated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Saint Louis, MO, USA) for 48 h before use.

### Macrophages infection

Lp1 strains were grown from frozen stocks for 3 days at 37°C on BCYE dishes before being freshly patched for 24 h. Bacteria were suspended in liquid medium at an optical density adjusted to 0.05 and the growth was followed at  $\lambda = 600$  nm on a TECAN Infinite M200 Pro (Mannedorf, Switzerland) multiplate reader until the transition between the exponential and the stationary growth phases. Except for cytometry, bacteria were then diluted in cell culture medium in order to reach a multiplicity of infections (MOI) of 1 for one hour at 37°C to induce a stress before infecting macrophages differentiated with PMA. Plates were centrifuged for 5 min at 500 g to favor interactions and incubated for 1 h at 37°C. The wells were then washed with medium in order to remove unincorporated bacteria. For cell secretions, supernatants were harvested at several time points post-infection and stored at -80°C until measurement. For some experiments, the macrophages were treated with anti-TNF- $\alpha$  antibody (20  $\mu$ g/mL, clone MAB1; eBioscience, Thermo Fisher Scientific, Waltham, MA, USA) or with the corresponding isotype control (20  $\mu$ g/mL, clone P3.6.2.8.1; eBioscience) for 24 h. For replication, macrophages were scraped and pooled with supernatants in order to collect external and intracellular bacteria; the number of colony-forming units (CFU) was determined by serial dilutions of the supernatants plated on BCYE petri dishes at the designated post-infection time points.

### Enzyme Linked Immunosorbent Assay (ELISA), cytotoxicity assay, and caspase-3 activity assay

Amounts of secreted TNF- $\alpha$  were measured in supernatants using Ready-Set-Go ELISA kits (ThermoFisher). Levels of released lactate dehydrogenase (LDH) by membrane-damaged cells were enzymatically measured using the Cytotox 96 non-radioactive cytotoxicity assay and values were expressed as percentages compared to a maximum LDH release (Promega, Fitchburg, WI, USA). Caspase-3 activity in cells was measured using the Apo-ONE Homogeneous Caspase-3/7 Assay and values

were expressed as relative fluorescence units (RFU; Promega). These assays were performed following manufacturer's recommendations.

### **Intracellular TNF- $\alpha$ staining**

To measure TNF- $\alpha$  production, infections were carried out at MOI 5 for 1 h and followed by Brefeldin A incubation (10  $\mu$ g/mL, eBioscience) for an additional 3 or 7 h. Cells were washed twice with Phosphate-Buffered Saline (PBS; Gibco) and fixed using 4% paraformaldehyde (Sigma-Aldrich) for 20 minutes. Macrophages were permeabilized with PBS containing 0.1% Triton X-100 and 1% FBS for 15 min and stained with PhycoErythrin anti-human TNF- $\alpha$  Antibody (clone MAB11; BioLegend, San Diego, CA, USA), rabbit anti-*Legionella pneumophila* (homemade) and goat anti-rabbit pacific orange (Invitrogen, Thermo Fisher Scientific) for 60 min. An Attune acoustic focusing cytometer (Life Technologies, Thermo Fisher Scientific) was used for flow cytometry, and data were analyzed using FlowJo software (version 10, Ashland, OR, USA).

### **Quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis**

At 4 and 16 h post-infection, RNA from U937 cells were extracted using TriZol reagent as recommended by the manufacturer (Life Technologies, Thermo Fisher Scientific). RNA was purified using the Quick-RNA kit (ZymoResearch, Irvine, CA, USA) following the manufacturer's recommendations. Residual DNA was removed by treatment with Turbo DNA-free kit (Ambion, Thermo Fisher Scientific), and cDNA synthesized using the qScript Flex cDNA Synthesis kit (Quanta biosciences, Gaithersburg, MD, USA) by reverse transcription of 0.5 mg of total RNA. The expression of target genes was analyzed by quantitative PCR using a LightCycler Fast Start SYBR Green kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. Relative quantification of target cDNA was determined by calculating the difference in cross-threshold (CT) values after normalization to hypoxanthine phosphoribosyltransferase 1 (HPRT1) cDNA signals ( $\Delta\Delta$ CT method), taking the 4 h non-infected control as a reference. The primers used were as follows: HPRT1 forward primer 5'-ctccgttatggcgacc-3' and reverse primer 5'-caccctttccaatcctcag-3'; TNF- $\alpha$  forward primer 5'-ctgctgcactttggagtgat-3' and reverse primer 5'-agatgatctgactgctggg-3'; interleukin (IL)-1 $\alpha$  forward primer 5'-actgcccaagatgaagacca-3' and reverse primer 5'-ccgtgagttcccagaagaa-3'; IL-1 $\beta$  forward primer 5'-gaagctgatggcctaaca-3' and reverse primer 5'-aagccctgtctgtagtg-3'.

### **Quantification of NF- $\kappa$ B-induced SEAP activity**

THP1-Blue™ NF- $\kappa$ B cells (InvivoGen) are THP-1 cells stably transfected with a secreted embryonic alkaline phosphatase (SEAP) reporter gene under the control of a promoter inducible by the NF- $\kappa$ B transcription factor family. Upon stimulation, NF- $\kappa$ B is activated and subsequently the secretion of SEAP is promoted. Levels of SEAP in supernatants collected 4, 8, and 24 h post-infection were enzymatically detected with Quanti-Blue medium (InvivoGen) at  $\lambda = 650$  nm using a multiplate reader.

### **Statistical analysis**

The mean  $\pm$  standard deviation (SD) of values are presented, and differences investigated using paired and unpaired Student's t-test or one-way ANOVA and post-hoc comparison by Tukey's test. The correlation between variables was evaluated by Pearson's R test. Probability values less than 0.05 were considered significant. Data were analyzed and graphics generated with the Prism program (version 5.0, GraphPad Software, Inc., San Diego, CA, USA).

## **Results**

### **Lp1 clinical isolates induce differential TNF- $\alpha$ secretion from macrophages**

We first determined whether Lp1 clinical isolates might differentially induce TNF- $\alpha$  secretion from macrophages. The U937 cell line was used as a standardized model of human macrophages and infected at a multiplicity of infections (MOI) of 1 for 24 h with 108 Lp1 isolates. All clinical strains induced TNF- $\alpha$  secretion from human macrophages, but this was highly variable (Figure S1). Interestingly, some overlap of a phylogenetic tree constructed from the whole-genome sequencing data of the isolates with their corresponding TNF- $\alpha$  secretion suggests an association between the TNF- $\alpha$  secretion and certain genotypes (Figure 1, Table S1). Because ST1, ST23, and ST47 are predominant in the collection (39.8%) and clinically relevant STs, we primarily focused our analysis on these. ST1 strains induced significantly more TNF- $\alpha$  secretion from U937 cells compared to non-ST1 infected cells (Figure 2a). There was no significant difference in TNF- $\alpha$  secretion between ST23 and non-ST23 infected cells (Figure 2b), whereas ST47 isolates induced significantly less TNF- $\alpha$  secretion compared to non-ST47 infected cells (Figure 2c). U937 cells infected by ST1 strains produced a higher TNF- $\alpha$  response than the ST23 and the ST47 isolates, whereas ST47 induced a lower TNF- $\alpha$  response than the ST1 and ST23 isolates (Figure 2d). Interestingly, in the 108 Lp1 collection used,

ST1 isolates were significantly more frequent in immunosuppressed patients (cancer/malignancy and corticosteroid therapy) compared to other STs, and ST47 isolates were significantly less frequently isolated from patients suffering of cancer/malignancy compared to ST1 isolates (Table 1). For the rest of the study, we focused our investigations on the comparison of the two ST groups with opposite phenotype, namely ST1 and ST47.

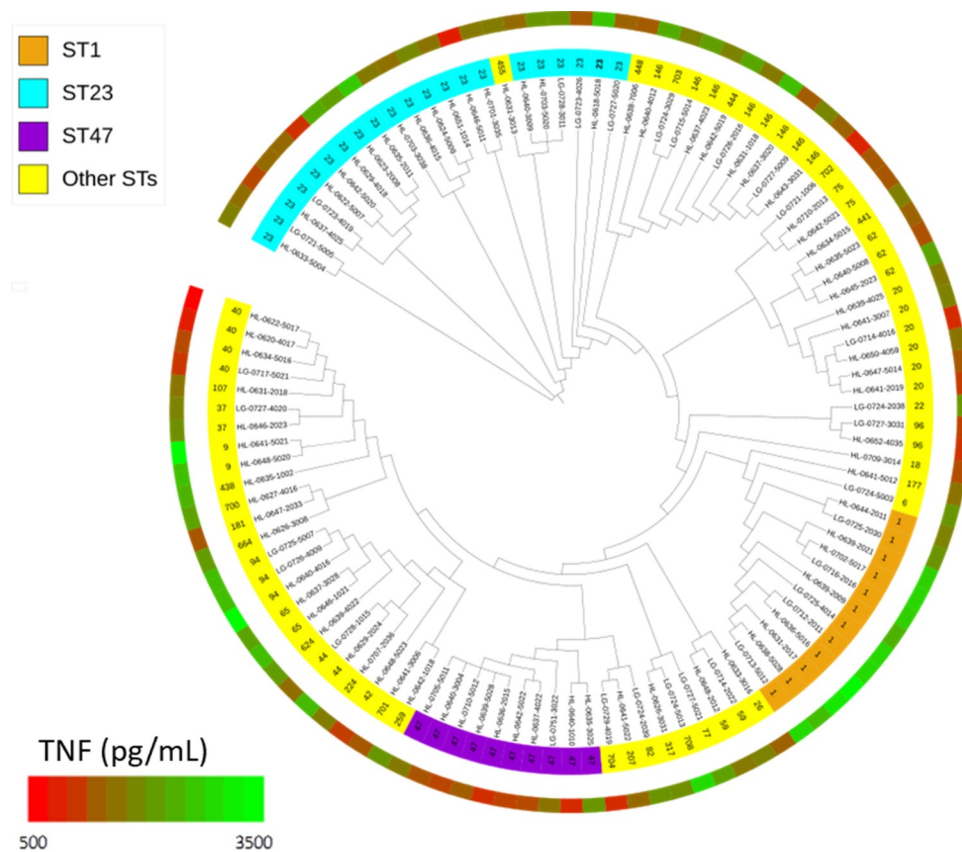
### **TNF- $\alpha$ secretion in Lp1 infected macrophages correlates with host cell death**

U937 macrophages were challenged with Lp1 ST1 and ST47 clinical isolates and lactate dehydrogenase (LDH) and caspase-3 activities were measured to evaluate cytotoxic and apoptotic activities, respectively. For these experiments, the control group was reduced to the main STs (Table S1). ST1 strains induced significantly higher LDH and caspase-3 activities from U937 cells than non-ST1 infected cells (Figure 3a, Figure 3b). ST47 isolates induced a significantly lower LDH and caspase-3 activities than non-ST47 infected cells (Figure 3c, d). ST1 isolates induced significantly higher LDH and caspase-3 activities from

macrophages than ST47 isolates (Figure 3e, f). A positive correlation was found between TNF- $\alpha$  secretion and LDH activity, as well as between TNF- $\alpha$  secretion and caspase-3 activity (Figure 4a, b). The addition of anti-TNF- $\alpha$  antibodies reduced slightly but significantly the LDH activity, whereas caspase-3 activity was highly and significantly reduced in U937 cells infected with ST1 or ST47 strains (Figure 4c, d). These results indicate that the Lp1-induced cell death, in particular apoptosis, is TNF- $\alpha$  dependent.

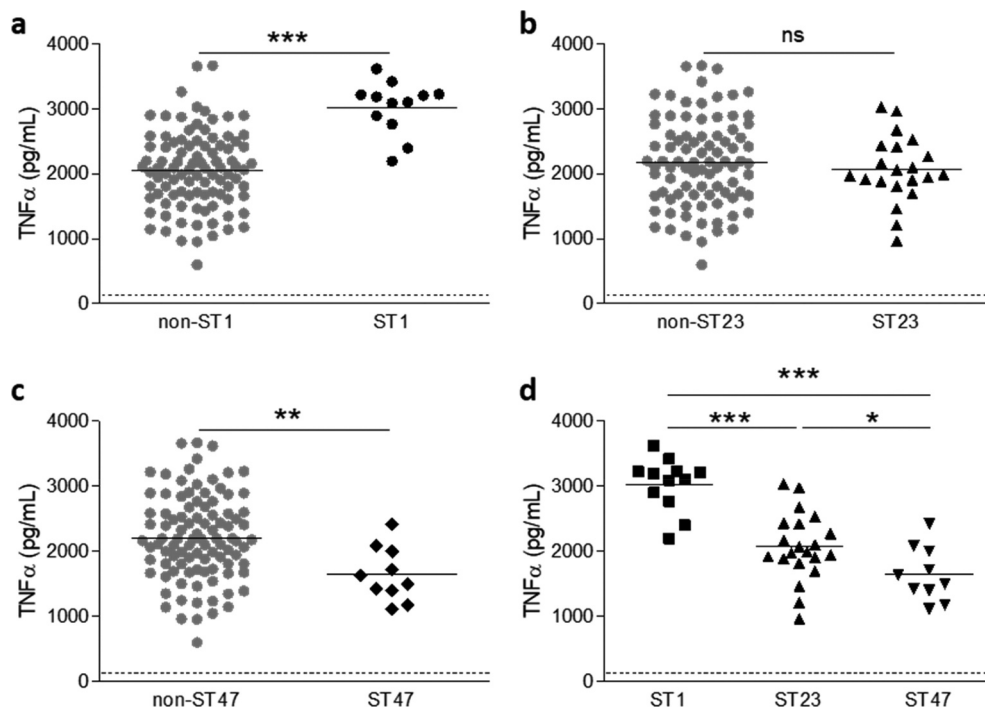
*The differential TNF- $\alpha$  secretion between ST1 and ST47 isolates is independent of the bystander macrophages response and the bacterial replication*

A recent study [23] has indicated that, during Lp infection in mice, uninfected (*i.e.* bystander) cells are the primary producers of TNF- $\alpha$ , favoring the TNF- $\alpha$ -induced cell death of infected cells. We therefore hypothesized that the difference in TNF- $\alpha$  secretion and cell death between ST1 and ST47 isolates could be associated to a differential TNF- $\alpha$  production from bystander cells. To identify TNF- $\alpha$ -producing macrophages and Lp1-infected macrophages we established a double-staining technique, allowing the simultaneous detection of those cells at single-cell



**Figure 1.** Whole genome-based phylogenetic tree of 108 Lp1 clinical isolates. The interior ring corresponds to clinical isolates STs and the exterior ring corresponds to a heatmap representing TNF- $\alpha$  secretion levels by infected U937 cells.





**Figure 2.** TNF- $\alpha$  secretion from human U937 macrophages infected by Lp1 clinical strains. U937 cells were infected (MOI 1) with 108 Lp1 clinical isolates, and the levels of TNF- $\alpha$  in culture supernatants were determined by ELISA at 24 h post-infection. Scattergram of secreted TNF- $\alpha$  concentrations by U937 cells upon infection with (a) non-ST1 ( $n = 96$ ) and ST1 ( $n = 12$ ) strains, (b) non-ST23 ( $n = 87$ ) and ST23 ( $n = 21$ ) strains, (c) non-ST47 ( $n = 98$ ) and ST47 ( $n = 10$ ) strains, and (d) ST1 ( $n = 12$ ), ST23 ( $n = 21$ ) and ST47 ( $n = 10$ ) strains. Data points show mean of triplicate. The lines represent the mean value for each group and the dashed lines represent the mean value in non-infected cells. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; ns, non-significant. Data are representative of two independent experiments.

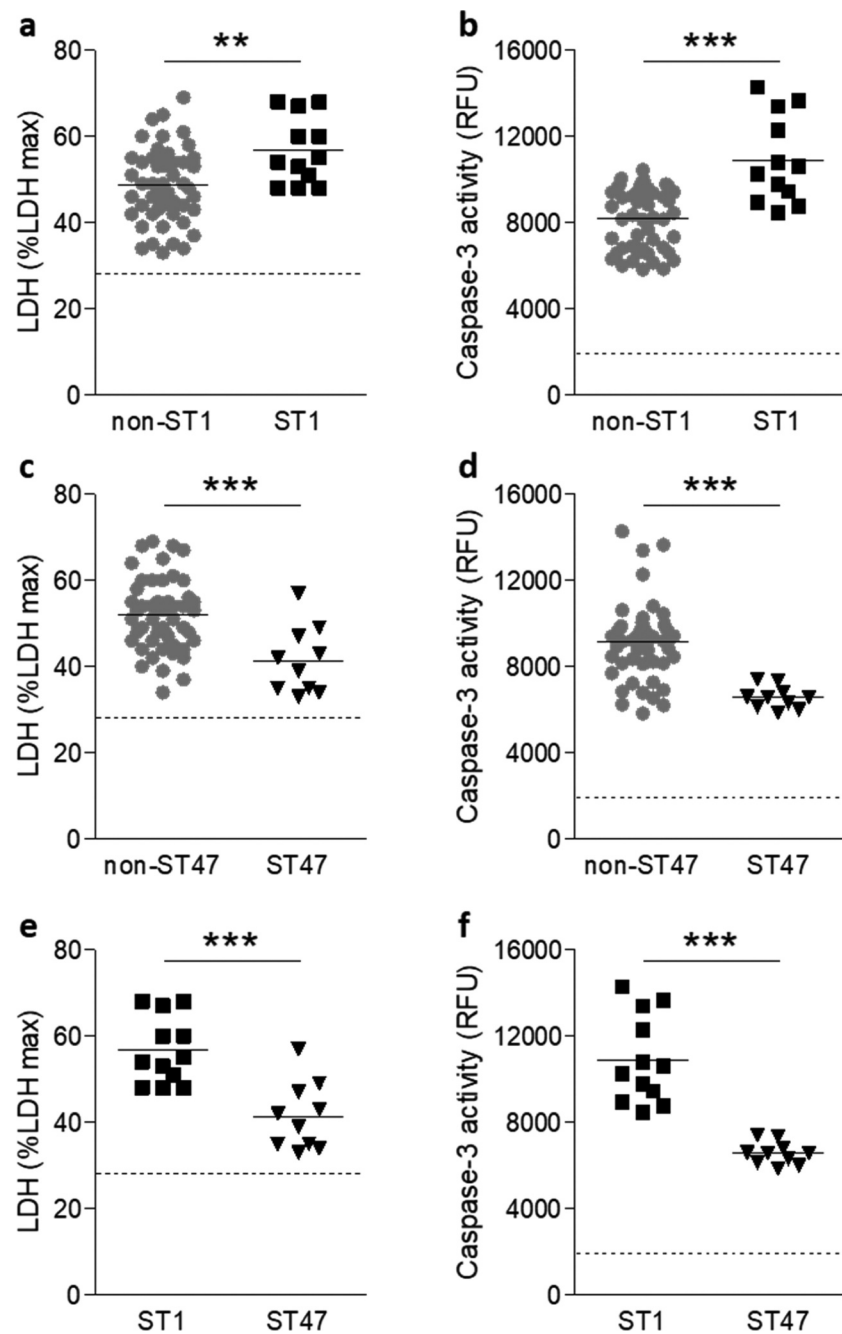
**Table 1.** Epidemiological characteristics of all 108 cases of Legionnaires' disease.

|  | ST1              | ST47             | Other STs      | All cases        |
|--|------------------|------------------|----------------|------------------|
| <b>No. of subjects</b>                         | 12               | 10               | 86             | 108              |
| <b>Sex</b>                                     |                  |                  |                |                  |
| Female   | 5 (41.7)         | 5 (50.0)         | 19 (22.0)      | 29 (26.9)        |
| Male   | 7 (58.3)         | 5 (50.0)         | 67 (88.0)      | 79 (73.1)        |
| <b>Age, years</b>                              | 67.6 [48.5–89.9] | 57.0 [28.4–87.6] | 56.1 [21–92.8] | 56.8 [21.1–92.8] |
| <b>Death<sup>a</sup></b>                       | 5 (41.7)         | 4 (40.0)         | 14 (19.3)      | 23 (24.8)        |
| <b>Risk factors<sup>d</sup></b>                |                  |                  |                |                  |
| Tobacco smoking                                | 4 (33.3)         | 6 (60.0)         | 50 (58.1)      | 60 (64.8)        |
| Diabetes mellitus                              | 1 (8.3)          | 1 (10.0)         | 17 (19.7)      | 18 (19.4)        |
| Cancer/malignancy <sup>b,c</sup>               | 5 (41.7)         | 0                | 7 (8.1)        | 12 (12.9)        |
| Corticosteroid therapy <sup>c</sup>            | 6 (50.0)         | 2 (20.0)         | 8 (9.3)        | 16 (17.3)        |
| Other immunosuppressive medication             | 1 (8.3)          | 1 (10.0)         | 6 (6.9)        | 8 (8.6)          |
| At least one risk factor                       | 10 (83.3)        | 8 (80.0)         | 70 (81.4)      | 88 (95.0)        |
| <b>Intensive care unit admission</b>           | 9 (75.0)         | 5 (50.0)         | 49 (56.9)      | 63 (68.0)        |
| <b>Hospital acquired infection<sup>c</sup></b> | 4 (33.3)         | 0                | 3 (4.8)        | 7 (7.6)          |

Data are expressed as number (%) of cases or as median [range]. ST, sequence type. <sup>a</sup>Not mutually exclusive; <sup>a</sup> $p < 0.05$ , ST1 versus other STs; <sup>b</sup> $p < 0.05$ , ST1 versus ST47; <sup>c</sup> $p < 0.001$ , ST1 versus other STs.  $p$  values calculated with Fisher's exact test or Student's  $t$  test.

level by flow cytometry (Figure 5a) at 4 and 8 h post-infection. The results indicated that, regardless of the STs, TNF- $\alpha$  is produced more significantly by bystander cells at 4 h and produced similarly by bystander and infected cells at 8 h post-infection (Figure 5b). Although the proportion of TNF- $\alpha$  producing cells (bystander as well as infected) was somewhat higher

after infection with ST1 compared to ST47, the differences were not statistically significant at both time points (Figure 5b). We then investigated whether the isolates from the ST1 and ST47 groups replicated equally and, according to the number of colony-forming units, the two groups displayed a similar replication pattern (Figure 6). The differential TNF-

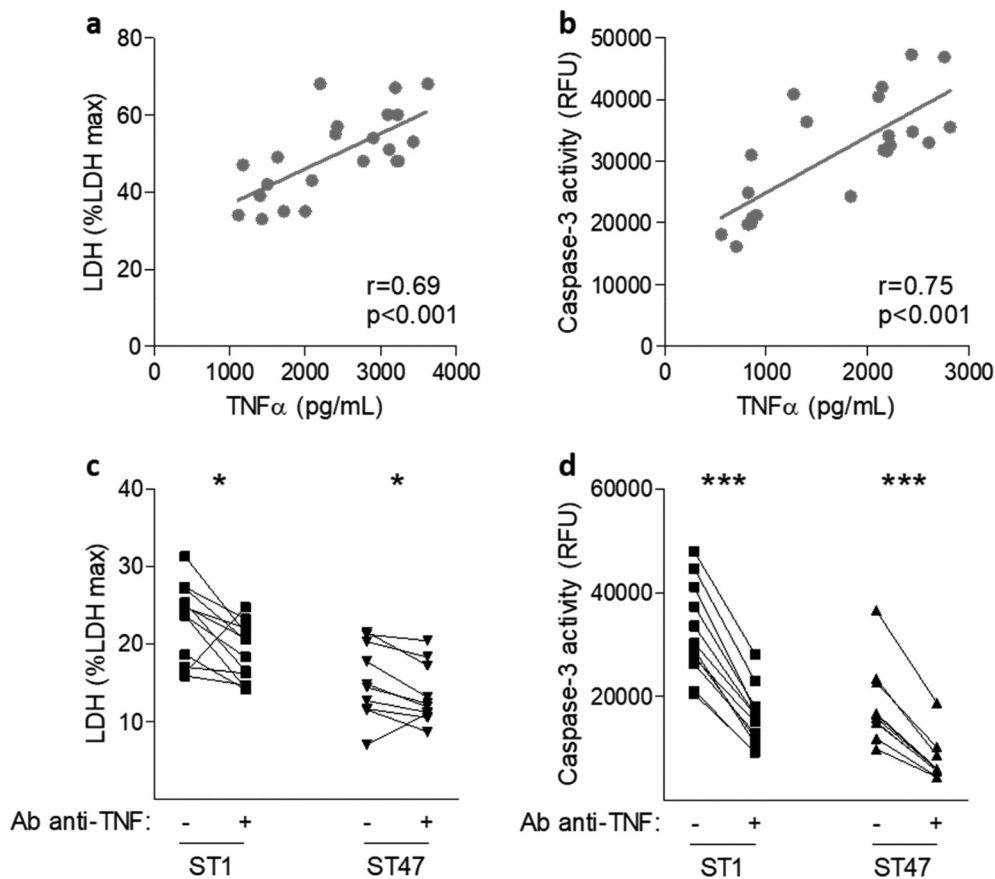


**Figure 3.** LDH and caspase-3 activities from macrophages infected with ST1 and ST47 clinical strains. U937 cells were infected (MOI 1) with 61 Lp1 clinical isolates, belonging to the main STs (*i.e.* ST1, ST20, ST23, ST40, ST47, and ST146), and the activities of LDH and caspase-3 were determined in culture supernatants at 24 h post-infection. Scattergram of LDH (**a**, **c**, **e**) and caspase-3 activities (**b**, **d**, **f**) in U937 cells upon infection with (a, b) non-ST1 ( $n = 49$ ) and ST1 ( $n = 12$ ) strains, (c, d) non-ST47 ( $n = 51$ ) and ST47 ( $n = 10$ ) strains, and (e, f) ST1 ( $n = 12$ ) and ST47 ( $n = 10$ ) strains. Data points show mean of triplicate. The lines represent the mean value for each group and the dashed lines represent the mean of LDH or caspase-3 activities in non-infected cells. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . Data are representative of at least three independent experiments.

$\alpha$  secretion between ST1 and ST47 groups is therefore neither explained by a different number of TNF- $\alpha$ -producing cells, nor by a different number of replicative bacteria.

#### ***Lp1-dependent TNF- $\alpha$ secretion is due to increased TNF- $\alpha$ transcription***

As it is difficult to investigate the molecular mechanisms behind the differential TNF- $\alpha$  secretion using



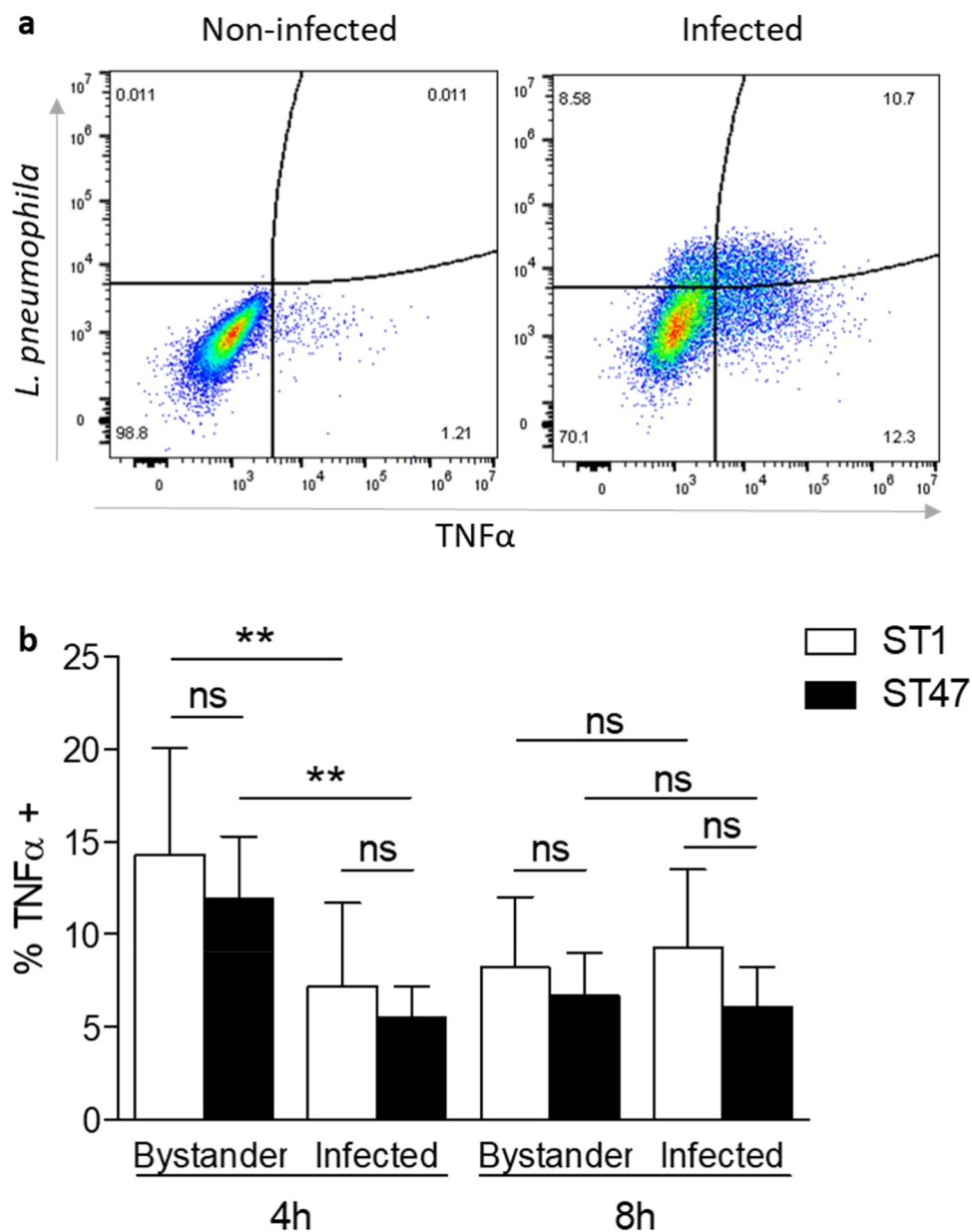
**Figure 4.** Involvement of TNF- $\alpha$  in LDH and caspase-3 activities from macrophages infected with ST1 and ST47 clinical strains. U937 cells were infected (MOI 1) with ST1 ( $n = 12$ ) or ST47 ( $n = 10$ ) clinical isolates and treated with anti-TNF- $\alpha$  antibody. The levels of TNF- $\alpha$  secretion and LDH and caspase-3 activities were determined in culture supernatants at 24 h post-infection. Correlation between TNF- $\alpha$  levels and LDH activity (a) and between TNF- $\alpha$  levels and caspase-3 activity (b) in U937 cells infected with ST1 or ST47 clinical isolates. Graph showing the LDH (c) and caspase-3 activities (d) in U937 cells upon infection with ST1 and ST47 strains treated with anti-TNF- $\alpha$  antibody (+) or the corresponding isotype control (-). Data points show mean of triplicate. \* $p < 0.05$ ; \*\*\* $p < 0.001$ , treated versus non-treated cells.  $r$ , Pearson's correlation coefficient. Data are representative of at least three independent experiments.

numerous isolates, we decided to use ST1 and ST47 reference strains, namely the Paris and Lorraine strains, respectively. We first explored the TNF secretion, LDH and caspase-3 activities, as well as the intracellular growth rate for both reference strains (Figure S2, and Figure S3). We confirmed that for the parameters described above that the Paris and Lorraine reference strains are representative of ST1 and ST47 clinical isolates, respectively. We then explored the mRNA expression of TNF- $\alpha$  in U937 cells challenged with both reference strains. Compared to non-infected cells (controls), the level of TNF- $\alpha$  mRNA transcription was significantly higher in macrophages infected with Paris reference strain at both 4 and 16 h post-infection and with macrophages infected with Lorraine reference strain at 16 h post-infection. TNF- $\alpha$  expression was significantly upregulated in cells infected with the Paris reference strain compared to

the ones infected with the Lorraine reference strain at both 4 and 16 h post-infection (Figure 7a). These findings are consistent with the temporal differences in TNF- $\alpha$  release observed (Figure 7b). Together, these findings suggest that the differential TNF- $\alpha$  secretion from infected macrophages is mediated by early events.

#### ***Lp1-dependent TNF- $\alpha$ secretion is associated with NF- $\kappa$ B signaling pathway***

It is already known that Lp infection activates the NF- $\kappa$ B signaling pathway, which is involved in proinflammatory genes transcription [13]. To investigate whether the NF- $\kappa$ B signaling pathway was differentially activated by ST1 and ST47 isolates, we therefore challenged the reporter THP1-XBlue cells with Paris or Lorraine reference strains. At 4, 8 and 24 h post-infection, the NF- $\kappa$ B-induced SEAP



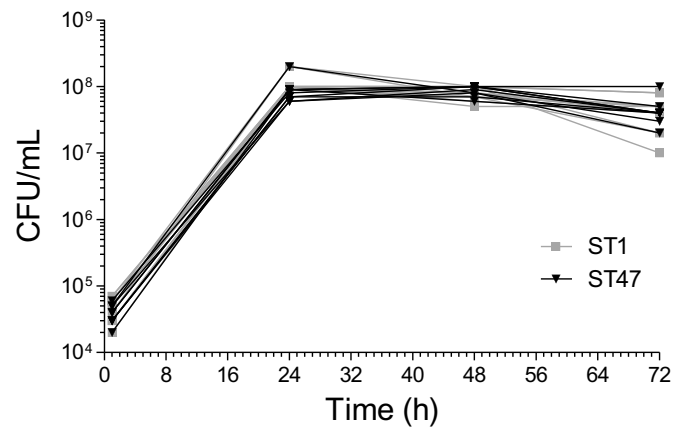
**Figure 5.** TNF- $\alpha$  is produced by both infected and bystander macrophages. U937 cells were infected at MOI 5 for 4 or 8 h with ST1 ( $n = 8$ ) or ST47 ( $n = 10$ ) and sorted by flow cytometry. (a) Representative plots from non-infected cells (negative control) and cells infected by a ST47 clinical strain at 8 h post-infection. (b) Relative contribution of bystander (uninfected) and infected cells to TNF- $\alpha$  production in macrophages challenged with Lp1 clinical strains. Values are means  $\pm$  SD. \*\* $p < 0.01$ ; ns, non-significant.

activity was significantly increased in cells infected with Paris reference strain compared to cells infected with Lorraine reference strain (Figure 8). This differential activation was correlated with the TNF- $\alpha$  secretion (Figure S4), and similar to the results obtained with U937 cells at 24 h (Figure S2a). These data suggest that the differential TNF- $\alpha$  gene transcription and secretion from macrophages infected by ST1 compared to ST47 strains are mediated by a differential activation of the transcription factor NF- $\kappa$ B.

## Discussion

The present study found that all Lp1 clinical strains can induce TNF- $\alpha$  secretion from human macrophages, but this secretion is highly variable and appears dependent on the genotype. Notably, isolates from ST1 induced a higher TNF- $\alpha$  secretion than most isolates from other ST of the collection while ST47 induced a lower secretion. This result was neither explained by a differential bacterial replication nor by the number of TNF- $\alpha$





**Figure 6.** Intracellular replication of ST1 and ST47 clinical strains in human macrophages. U937 cells were infected (MOI 1) with ST1 (n = 11) or ST47 (n = 9) strains and the number of colony-forming units (CFU) was determined at 1, 24, 48 and 72 h post-infection. Data are representative of two independent experiments performed in triplicate and expressed as mean values.

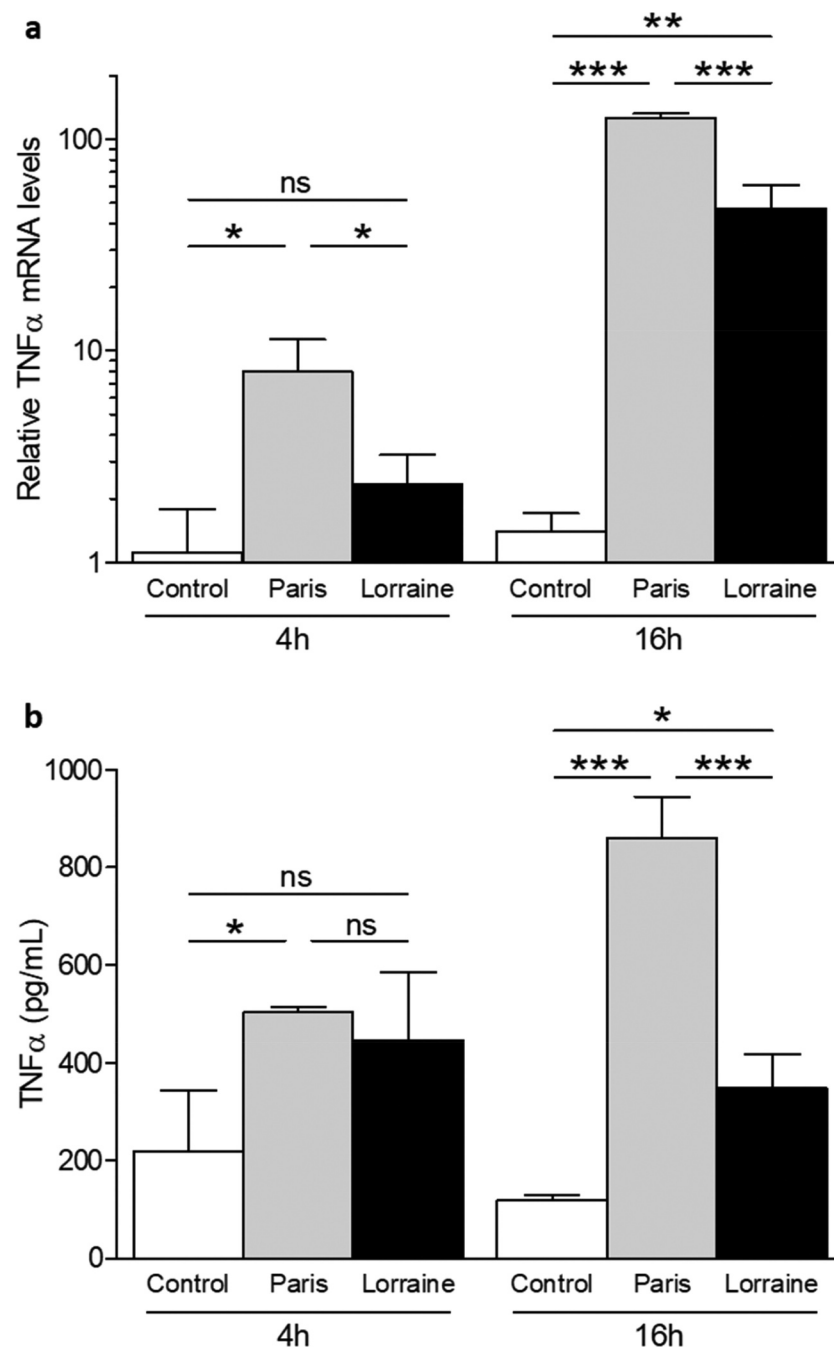
producing cells following infection, but rather by a differential response of macrophages according to the STs. ST1 isolates induced a higher NF- $\kappa$ B signaling pathway, followed by higher *TNF- $\alpha$*  gene transcription than did ST47 isolates. The higher *TNF- $\alpha$*  secretion observed for ST1 isolates consequently induced a higher cell death, in particular apoptosis, than ST47 isolates. Overall, the results presented herein suggest that ST1 isolates trigger a more robust immune response from human macrophages than do ST47 isolates.

The role of *TNF- $\alpha$*  against *Legionella* infections has been well established by clinical [4,24] as well as *in vivo* [25–27] and *in vitro* studies [16–20]. Notably, it has been reported that *TNF- $\alpha$*  counteracts intracellular *Lp* replication through lysosomal acidification and activity of unknown caspases [27]. However, the full mechanism by which *TNF- $\alpha$*  contributes to *Lp* growth restriction has yet to be elucidated. In the present study, even though ST1 isolates induced a *TNF- $\alpha$*  secretion roughly twice higher than did ST47 isolates, no impact on bacterial replication between the two STs was found. Lowering the MOI delays the plateau but still did not reveal any difference between the two ST (Figure S3) indicating that the growth curves are similar for both STs. Moreover, previous studies also reported that the cytokine levels produced in response to *Legionella* infections are not necessarily correlated with bacterial replication [28,29]. Taken together, the differences in terms of *TNF- $\alpha$*  secretion observed between ST1 and ST47 isolates may therefore reflect a true difference in the activating potential of these clinical isolates.

We then sought to determine whether this differential *TNF- $\alpha$*  secretion could be explained by the number, or type, of *TNF- $\alpha$* -producing cells. The latter is of

potential importance as it has been reported that, in mice, *Legionella*-infected macrophages produced *IL-1 $\alpha$*  that alerted uninfected (bystander) cells of an ongoing infection. Bystander cells in turn secreted *TNF- $\alpha$* , becoming the primary producers of *TNF- $\alpha$* . This signaling cascade could represent a way to bypass multiple blockade due to *Lp* infection, including the *TNF- $\alpha$* -induced cell death [23]. Using a different technical approach, it was found herein that human U937 bystander cells were the primary producers of *TNF- $\alpha$*  at an early stage of *Lp* infection, similarly to the description in mice. However, *TNF- $\alpha$* -positive cells were equally distributed between infected and bystander cells later in the bacterial life cycle. Additionally, there was no significant difference between the proportion of *TNF- $\alpha$* -positive cells obtained with ST1 compared with ST47 isolates. Therefore, although a differential regulation at the single-cell level is not excluded, the higher *TNF- $\alpha$*  secretion observed with ST1 isolates cannot be explained by a higher number of *TNF- $\alpha$* -producing cells, or by the type (bystander or infected) of cells.

The mRNA profiles were more informative. The Paris ST1 reference strain markedly induced *TNF- $\alpha$* , *IL-1 $\alpha$* , and *IL1 $\beta$*  mRNA expression (the latter two – *data not shown*) as early as 4 h post-infection compared to the Lorraine ST47 reference strain. These findings support the concept that the differential responses of macrophages toward ST1 and ST47 isolates are mediated by early events in cell activation. The transcription of these genes is notably controlled by NF- $\kappa$ B signaling pathway, which could be modulated by intracellular pathogens, including *Legionella* [13,15,29], and it was found herein that the Paris ST1 reference strain induced a more robust NF- $\kappa$ B

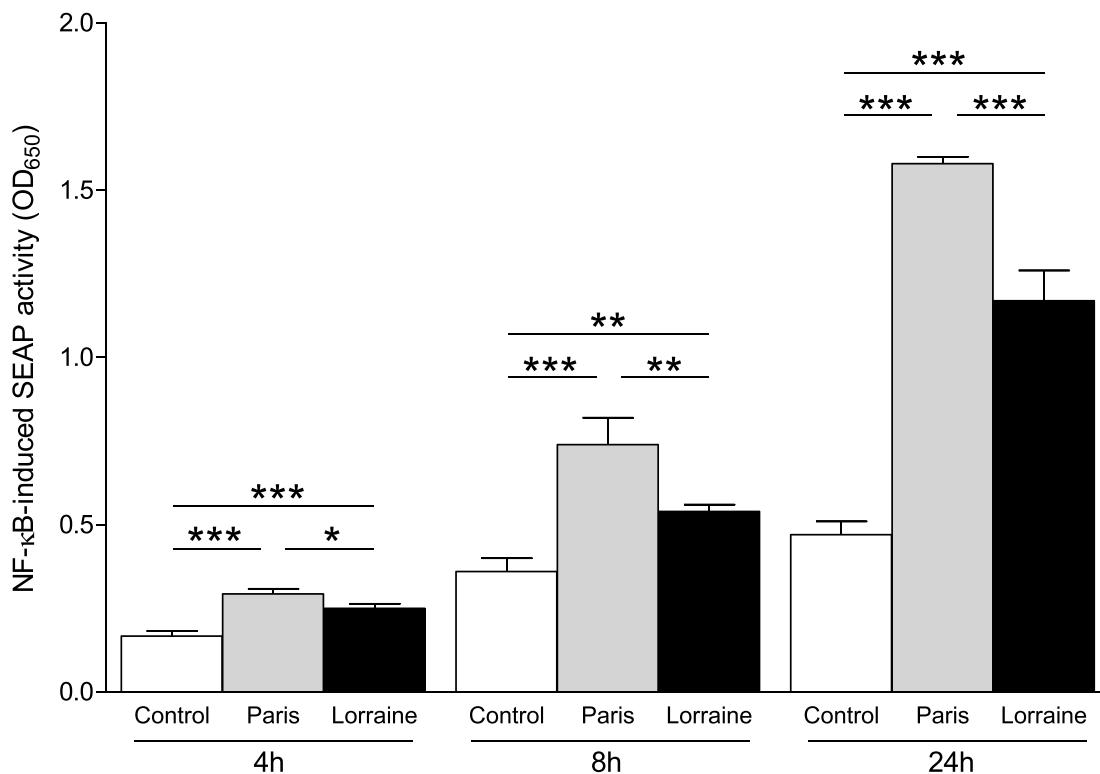


**Figure 7.** TNF- $\alpha$  expression and secretion from macrophages infected with the ST1 Paris and ST47 Lorraine reference strains. U937 cells were infected (MOI 1) with the Paris or Lorraine reference strains, and the levels of *TNF- $\alpha$*  mRNA (a) and TNF- $\alpha$  in culture supernatants (b) were determined at 4 h and 16 h post-infection. Values are means  $\pm$  SD and the white bars (control) represent the levels obtained in non-infected cells. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; ns, non-significant. Data are representative of at least three independent experiments performed in triplicate.

pathway activation compared to the Lorraine ST47 reference strain. These data collectively suggest that, although it is not possible to discriminate the contribution of infected from bystander cells at the single-cell level, ST1 isolates induced a stronger NF- $\kappa$ B pathway activation in human macrophages than ST47

isolates, followed by a higher transcriptional activation and a more elevated TNF- $\alpha$  secretion.

Another major role of TNF- $\alpha$  is the induction of cell death via its binding to TNFR1, which possesses a death domain leading cells to undergo apoptosis [14]. For immune cells, the induction of cell death



**Figure 8.** NF- $\kappa$ B-induced SEAP activity from macrophages infected with the ST1 Paris and ST47 Lorraine reference strains. THP1-Blue™ NF- $\kappa$ B cells are macrophages expressing a secreted embryonic alkaline phosphatase (SEAP) reporter gene driven by the NF- $\kappa$ B promoter. Cells were infected (MOI 1) with the Paris or Lorraine reference strains, and SEAP activity (reflecting NF- $\kappa$ B activation) was determined in culture supernatants at 4, 8 and 24 h post-infection. Values are means  $\pm$  SD. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . Data are representative of at least two independent experiments performed at least in triplicate.

represents the ultimate way to restrict intracellular infections. To counteract the premature cell death that would be detrimental to their life cycle, intracellular bacteria have developed strategies to inhibit cell death and/or to promote cell survival [15]. Lp actively delays apoptosis until 18 h post-infection in U937 macrophages to complete its life cycle, before activating caspase-3-dependent apoptosis to facilitate egress [30,31]. It was found herein that ST1 isolates induced cell death, in particular caspase-3-dependent apoptosis, more efficiently than ST47 isolates. The strong correlation between cell death and TNF- $\alpha$  levels in infected macrophages was further experimentally confirmed using antibodies against TNF- $\alpha$  that dramatically reduced apoptosis in both STs. Taken together, the data indicate that the activation of caspase-3-dependent apoptosis in macrophages infected by Lp1 requires the action of TNF- $\alpha$  produced by macrophages themselves during the infection.

One major virulence factor of *Legionella* is the Dot/Icm T4SS. This allows the injection in the host cytosol around 300 effectors to hijack the host defense mechanisms [1], and mutants defective for functional Dot/Icm T4SS are unable to create their replicative vacuole and

are rapidly destroyed by macrophages. We observed that a  $\Delta dotA$  mutant in the genetic background Paris ST1 reference strain induced a similar mean TNF- $\alpha$  secretion to the one of the non-infected cells (*data not shown*), suggesting that Dot/Icm T4SS play an essential role in the TNF- $\alpha$  response. Although not investigated in the present study, several effectors have been described to interfere with the pathways reported herein, such as SdhA and SidF that contribute to the prevention of host cell death [32]. Conversely, VipD, Ceg18, Lem12, LegS2, and Lpg0716 induce caspase-3 activation and apoptosis [33]. In addition, LegK1 and LnaB activate NF- $\kappa$ B signaling pathway, which controls the transcription of pro-inflammatory but also pro-survival genes [34,35], and, more recently, it was reported that LegC4 increases TNF- $\alpha$  secretion from murine bone-marrow-derived macrophages and contributes to Lp clearance [36]. Beside the Dot/Icm T4SS, it was established in mice that TNF- $\alpha$  is produced in response to Lp in a flagellin-independent manner [37] but other pathogen-associated molecular patterns (PAMPs) like lipopolysaccharide, lipoproteins, or dsDNA could be involved [2,13]. Taken together, these effectors and PAMPs constitute interesting

candidates, and future studies should evaluate if they are differentially regulated or constituted between ST1 and ST47 isolates.

Although the underlying mechanisms are only partially elucidated, it is demonstrated herein for the first time that ST1 and ST47 isolates differ in triggering immune response and cell death from human macrophages. This is particularly interesting as these STs infect people with opposite immune status. Whereas ST1 isolates preferentially cause disease in immunocompromised patients treated with corticosteroid therapies, ST47 isolates more often afflict immunocompetent patients [21,38]. The risk of developing LD is increased for patients under corticosteroid or other immunosuppressive therapies, especially for those treated with anti-TNF- $\alpha$  therapies [4,24]. Therefore, and with the necessary caution required to extrapolate data from patients and experimental results, it can be hypothesized that ST1 isolates are hindered by an effective immunological response, particularly the one relying on TNF- $\alpha$ , explaining their success to create infection in people defective for this pathway. Conversely, ST47 isolates trigger less TNF- $\alpha$  secretion and TNF- $\alpha$ -dependent cell death from our standardized macrophages model than most of STs of the collection, including ST1. From the results presented herein, we speculate that ST47 isolates induce a moderate inflammatory response, which could limit the macrophage ability to alert other immune cells and to trigger apoptosis. These results could contribute to the understanding of the ST47 isolates strategies to establish a successful infection in an immunocompetent system context. The validation of these data in patients combined with the recent development of PCR specifically designed to identify ST1 and ST47 from patients' respiratory samples [39,40] pave the way to a personalized follow-up of patients during LD.

In conclusion, the present study found that clinical Lp1 isolates induce, *in vitro*, a diverse immune response and cell death, which are associated with genotype. These results suggest that specific bacterial determinants are involved in inadequate inflammatory responses during LD. These data also contribute to a better understanding of host susceptibility in LD involving ST1 and ST47 isolates.

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No potential conflict of interest was reported by the author(s).



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## Data availability statement

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

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