HLA-B27 Modulates Intracellular Growth of Salmonella Pathogenicity Island 2 Mutants and Production of Cytokines in Infected Monocytic U937 Cells

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Abstract

Background: Salmonella enterica serovar Enteritidis PT4 KS8822/88 replicates rapidly in HLA-B27-transfected human monocytic U937 cells. In this process, Salmonella pathogenicity island 2 (SPI-2) genes play a crucial role. Our previous study indicated that 118 Salmonella genes, including 8 SPI-2 genes were affected by HLA-B27 antigen during Salmonella infection of U937 cells.

Methods/Principal Findings: To further investigate Salmonella replication in HLA-B27-positive U937 monocytic cells, two SPI-2 genes, ssaS and sscA up-regulated most during Salmonella infection of HLA-B27-transfected U937 cells, were mutated by using one-step gene disruption method. Intracellular survival and replication of the mutants in the U937 cells was compared to that of the wild type strain. Surprisingly, the two mutated strains replicated significantly more than the wild type bacteria in HLA-B27-transfected cells. Secretion of tumor necrosis factor alpha (TNF-α) and interleukin 10 (IL-10) was significantly induced during the infection of HLA-B27-transfected U937 cells with the mutants. The results indicated that certain SPI-2 genes in wild type bacteria suppress Salmonella intracellular growth and production of cytokines in infected HLA-B27-transfected cells. HLA-B27-associated modulation of Salmonella SPI-2 genes and cytokine production may have importance in the persistent infection of the bacteria and the pathogenesis of reactive arthritis.

Conclusions: The study provides evidence that certain virulence factors of pathogens can reduce the intracellular growth in the host cells. We suggest that the limiting intracellular growth might be a strategy for persistence of bacteria in host cells, keeping a balance between pathogenic growth and pathogenesis.

Introduction

Reactive arthritis (ReA) is a joint inflammation that occurs following infections with certain intracellular Gram-negative bacteria, e.g. Salmonella and Yersinia [1]. The development and severity of ReA is strongly associated with the human leukocyte antigen-B27 (HLA-B27) [2,3]. However, the mechanism by which HLA-B27 confers disease susceptibility remains elusive. Nevertheless, a consistent feature of most hypotheses suggest that the interaction between the ReA-triggering bacteria and the HLA-B27-positive subjects, in whom ReA often develops, is abnormal and leads to an inefficient elimination of the causative bacteria [4,5]. Our previous studies demonstrated impaired elimination [6–8] or enhanced replication [9–11] of Salmonella Enteritidis in HLA-B27-transfected human monocytic cells, as compared with appropriate controls expressing other HLA class I antigens. However, it has been less known how HLA-B27 regulates bacterial intracellular growth in the molecular level.

Monocytes/macrophages are important cells harboring pathogens causing persistent infections or systemic disease in the body, but in order to survive and replicate the bacteria need to resist the bactericidal responses from the host cells [12]. Secretion of cytokines in host cells is closely associated with the persistence and pathogenesis of bacteria. Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine and considered one of important cytokines in host cells is closely associated with the persistence and pathogenesis of bacteria. Tumor necrosis factor alpha (TNF-α) is a pro-inflammatory cytokine and considered one of important cytokines in the development of spondyloarthropathy (SpA). Anti-TNF-α therapies have been proven to be effective in the treatment of patients suffering from SpA [13,14]. TNF-α is involved in several physiological and pathological responses of host during bacterial infection [15,16]. It is associated with host resistance phenotype, activates the bactericidal activity of macrophages [17,18], and contributes to clearance of Salmonella in animal model [19]. While TNF-α was involved in the activation of macrophages, anti-inflammatory cytokines, such as interleukin-10 (IL-10) can have antagonistic effects. IL-10 has been shown to have multiple biological activities, e.g., its major function appears to be suppression of T helper type 1 (Th1) cytokine synthesis including TNF-α and IFN-γ. It also inhibits the host defence by deactivated macrophages [20]. IL-10-treatment caused host cells...
to become permissive for growth of intracellular pathogens [21]. Activated monocytes and macrophages are the major source of IL-10 production.

The virulence genes in Salmonella responsible for infection and pathogenicity are mainly clustered in distinct 40 kb chromosomal region called Salmonella pathogenicity islands (SPI), which have been acquired by horizontal gene transfer [22,23]. SPI-1 and SPI-2 are known as pathogenicity islands, which encode their own type III secretion systems (T3SS) to export bacterial effector proteins into the host [24,25]. The SPI-1 secretion system mediates bacterial invasion into host intestinal epithelial cells [26] while the SPI-2 is required for Salmonella replication inside host cells and systemic infections in mice [27]. The SPI-2 genes were specifically induced inside host cells selected by fluorescence-activated cell sorting [28]. Signals specific to the intracellular environment are sensed and modulated by a two-component regulatory system ssaA/B encoded in the SPI-2 region, leading to induction of SPI-2 gene expression [29]. Further study revealed that the expression of ssaA/B itself is regulated by OmpR-EnvZ, another two-component system with global regulatory function located outside the SPI-2 region [30]. Moreover, the SPI-2 region contains large blocks of horizontally acquired genes which has a higher A+T content than other parts of Salmonella DNA [24,31]. The AT-rich DNA is bound by the histone-like nucleoid structuring protein (H-NS), leading to the silencing of the binding genes [32]. The H-NS-promoted silencing might protect Salmonella from the detrimental effect of over-expressing of SPI-2 virulence genes at inappropriate times [33].

Our recent study indicated that the expression of HLA-B27 in monocytic cells influenced global Salmonella gene response during intracellular infection [11]. Expression of Salmonella genes was significantly changed during infection of HLA-B27-transfected cells compared to the infection of HLA-A2-transfected cells. SPI-2 genes were the most up-regulated. To learn more about the interplay between the SPI-2 genes and HLA-B27 antigen, two of the SPI-2 genes were mutated and intracellular growth of the mutants was investigated during the course of infection. In addition, the production of cytokines TNF-α and IL-10 was also determined in Salmonella infected cells.

Results

Cell surface expression of HLA-B27 and HLA-A2 molecules

The expression of transfected HLA-B27 and HLA-A2 was confirmed by flow cytometry always in new batches of the cells as before [10,11]. The level of expression of the transfected molecules in U937 cells was similar to that of HLA-B51, one of the MHC class I molecules endogenously expressed by U937 cells. In addition, the surface expression levels of the transfected molecules corresponded to the levels of those molecules endogenously expressed on peripheral blood monocytes [6].

Construction of Salmonella SPI-2 mutants

To further study the intracellular growth of Salmonella in HLA-B27-positive U937 monocytic cells, two SPI-2 genes ssaS and sscA, significantly increased their expression in HLA-B27-transfected cells, were disrupted. Within the internal region of the gene ssaS in wild type S. Enteritidis PT4 KS8822/88, 33 nucleotides were replaced with the kanamycin gene cassette, and within the internal region of the gene sscA, total 72 nucleotides were replaced with the kanamycin gene cassette. Candidate mutant colonies were selected on LB plates with antibiotic kanamycin because the wild type bacteria failed to grow on these plates. Further confirmation was performed by PCR using the flanking primers (Table 1), external to the site of mutation in disrupted genes. The genomic DNA isolated from the wild type and mutant strains respectively were used as templates, and the PCR products with expected sizes were produced (Fig. 1). This indicates that the homologous recombination occurred in the mutated strains and the expected mutants were obtained.

Growth of mutant and wild type strains in LB broth

To investigate whether the mutations on the SPI2 genes affected the growth of the bacteria in vitro in LB medium, the mutants and wild type strain were grown as routinely in LB broth overnight. The bacteria cultures were then transferred into fresh LB broth to get a logarithmic growth phase of bacteria for each of them. The bacteria cultures were further diluted and put on LB plates, supplemented with 30 µg/mL of kanamycin for mutant strains to grow. The number of bacteria was calculated, reported as CFU, and the results were shown in Fig. 2. The results suggested that these mutants and wild type strains had a similar growth rate in LB broth.

Uptake of wild type and mutant Salmonella strains by transfected U937 cells

HLA-B27- or HLA-A2-transfected U937 cells were infected with the mutants and wild type bacteria, and uptake of the bacterial strains by U937 cells was measured at 1 hour after infection. The number of living bacteria per cell was counted after

Table 1. Primers used in the gene mutagenesis in this study.

| Genes | Primers | Sequences |
|-------|---------|-----------|
| ssaS  | ssaS-Km-F | 5'-CAATTTGTAACCACTTTATGACTTTCCTTTATCCGTTAGCGGTATGGTGAGAACCCGCTTGTGTCCA-3' |
|       | ssaS-Km-R | 5'-ATGGAATATTTAATATCACTGAACTGCGTGTGATCTTGCACCGGCTTGTGTCCA-3' |
|       | ssaS-F    | 5'-GACGAATTTTGAACGCAA-3' |
|       | ssaS-R    | 5'-AACCATGCTTCCCAATTGCT-3' |
| sscA  | sscA-Km-F | 5'-TATATATGATGGCTGCTATGGTCTAGTCTGGTCATGGAAGATGTGCACCAGGCTTAAAAGCCACGGTGTCGCTGAA-3' |
|       | sscA-Km-R | 5'-GCGCTTGGATGTAATGCAGACGTATACAACACACGGAGATAGACCTCCGTGGCCACGGCTAGGCTGCTGAA-3' |
|       | sscA-F    | 5'-GCGTATGTTGAGTGAGA-3' |
|       | sscA-R    | 5'-CATCTTTCTGACAGATGTC-3' |

*: 80-nucleotide (nt)-long primers including 60 nt homology extensions complementary to the targeted regions of the genes and 20 nt priming sequences (underlined) for the synthesis of the kanamycin resistance cassette gene from E. coli MC4100 yebW::Km.

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Enhanced intracellular replication of mutants in U937 cells

The cells infected with wild type and mutant Salmonella strains were incubated for indicated time points and the numbers of intracellular bacteria were determined. To our surprise, intracellular replication of the two mutant strains in U937 cells was increased compared to wild type strain (Fig. 4A and B). Intracellular replication of both mutants was significantly enhanced in HLA-B27 cells compared to in HLA-A2 cells (Fig. 4C and D). Among the two mutants, the number of the sscA::Km mutant bacteria was higher than that of the ssaS::Km mutant, and the highest amount of bacteria was detected at 3 days after infection. The increased number of mutated bacteria in infected HLA-B27-transfected U937 cells must be due to enhanced replication since no difference was seen in uptake of mutants and wild type bacteria (Fig. 3). The results suggest that the two SPI-2 genes in some way suppressed intracellular growth in wild type Salmonella. It seems that sscA had more negative effect on Salmonella intracellular growth than ssaS did. The two mutants had a small growth advantage when grown in HLA-A2 cells compared to the wild type bacteria. This growth advantage in HLA-A2 cells was much less than in HLA-B27 cells. These results indicate that HLA-B27 significantly modulates Salmonella intracellular replication.

Increased production of TNF-α in HLA-B27-transfected cells induced by the mutants

To investigate increased replication of bacteria in HLA-B27 cells, secretion of TNF-α in culture supernatants was detected using ELISA method. The results indicate that TNF-α was rapidly induced in Salmonella-infected U937 cells starting at 1 hour after infection, and maximal production was reached at 8 h after infection with the two mutants (Fig. 5A and B). Compared to HLA-A2 cells, the two mutants caused significantly increased production of TNF-α in HLA-B27 cells (Fig. 5C and D). Moreover, the concentration of TNF-α induced by ssaS::Km mutants in HLA-B27 positive cells was at least 2-fold higher than that by the wild type strain, while the mutant sscA::Km caused only slightly increased amount of TNF-α. Taken together, these findings suggest that the production of TNF-α is related to the presence of HLA-B27 antigen and Salmonella intracellular growth in infected HLA-B27 cells.

Increased production of IL-10 in HLA-B27-transfected cells by the mutants

IL-10 was detectable at 4 hours after infection and maximal production occurred at 24 h after infection. The mutants produced more IL-10 in both cell lines than the wild type bacteria (Fig. 6A and B). Mutant ssaS::Km-infected HLA-B27 cells produced over 2-fold amount of IL-10 compared with the wild-type–infected cells, whereas mutant sscA::Km-infected HLA-B27 cells showed a moderate increase in IL-10 production. In addition, the mutants caused more production of IL-10 in HLA-B27 cells.
than in HLA-A2 cells (Fig. 6C and D), indicating that secretion of IL-10 was also affected by HLA-B27 during *Salmonella* infection.

**Discussion**

The present study demonstrates that intracellular replication of the two SPI-2 mutants of *S. Enteritidis* was remarkably increased compared to the replication of wild-type bacteria in HLA-B27 positive human U937 monocytic cells. The results suggest that intracellular growth of wild type bacteria was suppressed in HLA-B27 cells. The observations provide the evidence for the new concept that certain pathogens limit their intracellular growth to keep the balance between the growth and persistence. The two *Salmonella* SPI-2 mutants were significantly increased mainly in HLA-B27 cells compared to in HLA-A2 cells (Fig. 4), suggesting that HLA-B27 antigen plays a key role. Previously, several different theories were proposed to explain the role of HLA-B27 in the pathogenesis of ReA [4,34]. Our earlier studies showed evidence that HLA-B27 influences the intracellular persistence of arthritis-triggering *Salmonella* within monocytes [6,10]. These results support the hypothesis of ineffective elimination of microbes in the pathogenesis of ReA [35]. ReA-triggering bacteria may persist for an abnormally long time in HLA-B27-positive patients with ReA [36]. Nevertheless, the interplay between genetic predisposition of HLA-B27 and *Salmonella* genes could lead to the explanation on the increased intracellular growth and long persistence of the pathogens in the HLA-B27 positive patients with ReA.

HLA-B27 probably modulates intracellular environment where bacteria reside which then affects bacterial gene expression. Our previous investigation revealed that 118 *S. Enteritidis* PT4 KS8822/88 genes were up- or down-regulated by HLA-B27 antigen during infection of U937 cells. Among regulated genes SPI-2 genes were up-regulated most [11]. The virulence genes clustered in SPI-2 are necessary for *Salmonella* intracellular growth [37]. The present study demonstrates that the two SPI-2 mutants remarkably increased intracellular growth when compared with that of the wild type *Salmonella* in HLA-B27-transfected macrophages. This highly enhanced growth of mutants was caused by HLA-B27 since only slight difference in the growth rate of the bacteria in HLA-A2 positive cells (Fig. 2) was observed. This gene “function-gain mutation” has been found also in other bacteria. Zahrt and Deretic reported that the growth of the gene $R^{0981}::Km$ mutant was significantly higher than the growth of

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**Figure 4. Intracellular growth of wild type and mutant strains in U937 cells.** The growth of wild type, the ssaS::Km and sscA::Km mutants were compared in HLA-B27-transfected (A) or HLA-A2-transfected U937 cells (B). The comparison for the mutant ssaS::Km growth in HLA-B27 and HLA-A2 cells (C) and for the mutant sscA::Km growth in HLA-B27 and HLA-A2 cells (D) was shown. Values represented the mean and standard deviation of at least three independent experiments with duplicate samples. *, ** and *** indicate $P<0.05$, $P<0.01$ and $P<0.001$, respectively, when the mutant-infected cells compared to the wild type (WT)-infected cells (A and B) or the mutant-infected HLA-B27 cells to HLA-A2 cells (C and D). Data were compared using Student’s paired 2-tailed t-test.

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wild-type *Mycobacterium tuberculosis* H37Rv after infection of murine macrophage-like J774 cells and bone marrow-derived macrophage cells, but the mutants were attenuated for virulence [38]. These results were against the gene “function-loss mutation” concept. This phenomenon might reflect the balance between disease-causing bacteria and their persistence during bacterial pathogenesis [39].

TNF-α is considered an important cytokine in the development of arthritis [16]. Anti-TNF-α therapies have been effective in the treatment of SpA, indicating that TNF-α plays a major role in the pathogenesis of SpA [13,14]. The central role of TNF-α was further supported by genetic study that TNFRp55-deficient mice were highly susceptible to *Salmonella* infection [18]. The present study demonstrated that SPI-2 mutants induced higher production of TNF-α than wild-type bacteria in infected HLA-B27-transfected cells, suggesting that the production of TNF-α may be associated with the growth of *Salmonella* in HLA-B27-transfected U937 cells. On the other hand, both the amount of bacteria and production of TNF-α were obviously enhanced by HLA-B27, indicating that HLA-B27 has a regulatory role in the *Salmonella* replication and secretion of certain cytokines intracellularly. Gamma interferon (IFN-γ) is a major activator of macrophages and the role of IFN-γ in natural resistance to bacteria in mice and host cells has been documented [40]. TNF-α and IFN-γ appear to cooperate in the activation of macrophages [41]. However, no IFN-γ was detectable from either HLA-B27-transfected cells or HLA-A2-transfected cells (data not shown).

IL-10, one of inhibitory cytokines, generally deactivates macrophages and permits enhanced bacterial intracellular growth during infection [21,42]. This study shows that SPI-2 mutants induced more IL-10 than did the wild type, correlating to increased number of mutant bacteria in HLA-B27 cells. The SPI-2 mutants caused a moderate increase of IL-10 in HLA-A2 cells, but not so much as in HLA-B27 cells (Fig. 6), suggesting that HLA-B27 regulated IL-10 secretion. We showed earlier that HLA-B27-transfected cells produced more IL-10 during the first three days.

**Figure 5. TNF-α production in culture supernatants of U937 cells infected with wild type and mutant strains.** Culture supernatants were collected at the indicated time points after infection, the production of TNF-α induced by the wild type, ssaS::Km and sscA::Km mutants were determined in HLA-B27-transfected U937 cells (A) and in HLA-A2-transfected U937 cells (B). The comparison of TNF-α production induced by the mutant ssaS::Km in HLA-B27 and HLA-A2 cells (C) and by the mutant sscA::Km in HLA-B27 and HLA-A2 cells (D) was shown. The results represented means ± standard deviation from at least three independent experiments. *, ** and *** indicate \( P<0.05, \) <0.01 and <0.001, respectively, when the mutant-infected cells compared to the wild type (WT)-infected cells (A and B) or the mutant-infected HLA-B27 cells to HLA-A2 cells (C and D). Data were compared using Student’s paired 2-tailed t-test.

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postinfection than HLA-A2-transfected cells which was correlated with increased bacterial survival in the HLA-B27 cells [8]. However, the administration of neutralizing mAb against IL-10 did not notably affect the clearance of the bacteria. This may be explained by the perhaps incomplete elimination of endogenous IL-10 due to the addition of anti-IL-10 being not enough [8].

HLA-B27 modulates interaction between host and Salmonella during infection [43]. This interaction involves the exchange of biochemical signals, called “cross-talk” [44]. As a result of this talk, Salmonella translocates a number of effector proteins into host cells via type III secretion systems to elicit host cell’s signaling pathway, leading to a variety of host response, including induction of cytokines [45]. Uchiya et al [21] showed SPI-2 gene spIC (ssaB) to be involved in the protein kinase A (PKA) signaling pathway that induces IL-10 expression in Salmonella-infected macrophages. This played an important role in intracellular growth of Salmonella. In this study, we showed that SPI-2 genes suppress secretion of IL-10 and bacterial intracellular replication, and the extent of suppression is augmented by HLA-B27. Collectively, these observations indicate that SPI-2 genes trigger host cell signaling transduction pathways to moderate production of cytokines and affect intracellular growth.

In conclusion, we demonstrated enhanced intracellular growth of two SPI-2 mutants of Salmonella within HLA-B27-transfected U937 cells. The observations suggest that SPI-2 genes in wild-type Salmonella somehow suppress intracellular growth and secretion of cytokines TNF-α and IL-10. This study provides evidence that wild-type Salmonella limits its intracellular growth in the HLA-B27-transfected cells to keep the balance between its growth and pathogenesis. However, further experiments will be still required to determine how Salmonella SPI-2 genes affect intracellular signalling pathways and modify Salmonella replication in HLA-B27 positive cells.

**Materials and Methods**

**Cell lines and transfection**

The human monocytic cell line U937 was obtained from American Type Culture Collection (Rockville, USA). The U937 cell line expresses the HLA class I alleles A3, A26, B18, B31, Cw1, and Cw3 [46]. Co-transfection of the cells with the vectors harbouring HLA-B*2705 or HLA-A2 genomic DNA with the plasmid pSV2neo (to confer resistance to genetin [G-418]) was carried out by electroporation. The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS; PAA laboratories, Linz, Austria), 1.8 mM L-glutamine and 50 μg/ml gentamicin (both from Biological Industries, Kibbutz Beit Herennek, Israel) at 37°C in a humidified

![Figure 6. IL-10 production in culture supernatants of U937 cells infected with wild type and mutants. Culture supernatants were collected at the indicated time points after infection, the production of IL-10 induced by the wild type, ssaS::Km and sscA::Km were determined in HLA-B27-transfected U937 cells (A) and in HLA-A2-transfected U937 cells (B). The comparison of IL-10 production induced by the mutant ssaS::Km in HLA-B27 and HLA-A2 cells (C) and by the mutant sscA::Km in HLA-B27 and HLA-A2 cells (D) was shown. The results represented means ± standard deviation from at least three independent experiments. *, ** and *** indicate P<0.05, <0.01 and <0.001, respectively, when the mutant-infected cells compared to the wild type (WT)-infected cells (A and B) or the mutant-infected HLA-B27 cells to HLA-A2 cells (C and D). Data were compared using Student’s paired 2-tailed t-test.

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The Effect of HLA-B27 on SPI-2 Mutants

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atmosphere of 5% CO₂/95% air incubator. The expression of transfected HLA-B27 or HLA-A2 molecules on the surface of the U937 cells was examined by FACSscan flow cytometry (BD Immunocytometry Systems, San Jose, CA) each time when the new batch of cells was thawed for use. Cells were stained with fluorescein isothiocyanate-conjugated anti-human HLA-B27 monoclonal antibody (mAb) (clone FD705-9E11; One Lambda, Canoga Park, CA) and anti-HLA-A2 monoclonal antibody (mAb) (Clone BB7.2; One Lambda Inc., Canoga Park, CA) as described previously [6].

Bacterial strains, plasmids and culture conditions

The wild type Salmonella Enteritidis strain was originally isolated from a stool of a patient with Salmonella-triggered ReA [6], typed as phage type 4 (PT4) and named as Salmonella Enteritidis PT4 KS8822/88 [11]. The mutants from Salmonella wild type were made as described below. Escherichia coli carrying temperature-sensitive plasmid (pKOBEGA) or MC4100 ybeW:Km were gifts from Dr. Ghigo. The Salmonella and E. coli strains were routinely grown in Luria-Bertani (LB) broth or on LB agar plates at 37°C or as indicated. Media were supplemented with ampicillin (100 µg/ml) and kanamycin (30 µg/ml) (both from Sigma) as required.

Infection of cells with S. Enteritidis PT4 KS8822/88

The U937 cells were diluted to a concentration of 1.0 × 10⁶ cells/ml and seeded in tissue culture flasks (75-cm²) or 24-well plates (Greiner, Germany). For differentiation to adherent macrophages, the cells were incubated with 10 ng/ml phorbol myristate acetate (PMA; Sigma-Aldrich, St Louis, MO) for 24 hours. Meanwhile, Salmonella strains were grown overnight in LB broth to the logarithmic phase of growth. Then Salmonella bacteria were used to infect the PMA-stimulated U937 cells at a ratio of 50:1 (bacteria:cell) without antibiotics for 1 h at 37°C [11]. Then the infected cells were washed three times to remove non-adherent bacteria and overlaid with fresh 1640 medium containing gentamycin (50 µg/ml) and kanamycin (30 µg/ml) (both from Sigma) as indicated. Media were supplemented with ampicillin (100 µg/ml) and kanamycin (30 µg/ml) (both from Sigma) as required.

Measurement of cytokines in cell-culture supernatants of infected cells

The cytokines were measured using enzyme-linked immunosorbent assay (ELISA). Supernatants were collected from 24-well plates at the defined time points: 1, 4, 8, 24, 72 h, 3 or 7 d after infection. The assay was performed in 96-well plates (Nunc) with capture anti-human antibodies. The antibody pairs: mAb1 and mAbm11 for measurement of TNF-α; and JESS9D7 and JESS3-12G8 for IL-10 were purchased from Pharmingen (San Diego, California). The absorbances were measured with a Victor™ Multilabel Counter (Wallac Oy, Turku, Finland) at a wavelength of 405 nm. Concentrations were calculated from a standard curve generated from each plate.

Statistical analysis

Differences in cytokine levels in cell cultures as well as intracellular growth of wild type and mutant strains were analysed using unpaired two-tailed Student’s t test. P values of 0.05 or less were considered significant.

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Author Contributions

Conceived and designed the experiments: SCG QSH KG. Performed the experiments: SCG. Analyzed the data: SCG QSH KG. Contributed reagents/materials/analysis tools: KG. Wrote the paper: SCG QSH KG.

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