ORIGINAL ARTICLE

THE EFFECT OF INTERFERON-γ AND LIPOPOLYSACCHARIDE ON THE GROWTH OF *FRANCISELLA TULARENSIS* LVS IN MURINE MACROPHAGE-LIKE CELL LINE J774

Monika Holická, Jakub Novosad, Martina Loudová, Manuela Kudlová, Jan Krejsek

Charles University in Prague, Faculty of Medicine and University Hospital Hradec Králové, Czech Republic: Department of Clinical Immunology and Allergology

Summary: Background: *Francisella tularensis,* a causative agent of human tularemia, displaying the ability to proliferate inside the human cells. Aims: To evaluate the growth potential of *F. tularensis* LVS strain in macrophage-like cell line J774 modulated by recombinant interferon γ and *E. coli* derived lipopolysaccharide. Results: Stimulation of J774 cells either by interferon-γ or lipopolysaccharide alone, or especially in combination before infection *F. tularensis,* revealed protective effects. Higher concentrations of stimulating agents were needed to inhibit ongoing *F. tularensis* infection. Conclusions: Stimulation of J774 cell line by combination of interferon-γ with lipopolysaccharide inhibits the intracellular growth of *F. tularensis.*

Key words: *Francisella tularensis; Macrophage; Interferon-γ; Lipopolysaccharide*

Introduction

Francisella tularensis is a Gram-negative facultative intracellular bacterium and the causative agent of tularemia (12, 14), a severe and often fatal disease of human and other mammals (5). *F. tularensis* subspecies *tularensis* is highly infectious, with intradermal or inhalation routes of infections requiring only about 10 organisms to cause severe disease (4). The fact that only a few organisms at cutaneous or mucosal sites can cause overwhelming disease suggests that the organism is able to overcome innate immune clearance efficiently. Because of the practical and regulatory complications of work with fully virulent clinical isolates, most of the available studies of an early host response to *F. tularensis* have used the live vaccine strain (LVS), which is attenuated for humans but causes a fatal infection in mice (2). *F. tularensis* replicates in macrophages and macrophage-related cells (12). Growth inside macrophages is a strategy that many prokaryotic and eukaryotic pathogens use to evade the host immune responses. Growth inside macrophages not only protects microbes during the acute phase of disease but also serves as niche for the long-term survival of pathogens (6). Current opinion is that *F. tularensis* is able to escape from the phagosomes of macrophages via a mechanism that may involve degradation of phagosomal membrane (9). Santic et al. (16) found that entry of *F. tularensis* into macrophages is mediated by novel asymmetric, spacious pseudopod loops, and the nascent phagosome becomes tight fitting within seconds of formation. Biogenesis of the *Francisella*-containing phagosome (FCP) is arrested for 2–4 hrs at a unique stage within endosomal-lysosomal degradation pathway, followed by gradual bacteria escape into cytosol, where the microbes are proliferating.

Based on *in vitro* data and *in vivo* observations made in both animals and humans, one specific cytokine, interferon gamma (IFN-γ), appears to play a key role in activating of mononuclear phagocytes (15). IFN-γ is known to be the most potent activator of macrophages inducing both antimicrobicidal and antitumor mechanisms as well as up-regulating antigen processing and presentation pathways (17). LPS, bacterial lipoproteins (18) and other pathogen-associated molecular patterns (PAMPs) (17) are activating Toll-like receptors (TLR) of mononuclear phagocytes, thereby inducing proinflammatory cytokines and antimicrobial activity (18) in macrophages.

We examined the abilities of the recombinant murine lymphokine INF-γ and/or LPS derived from *E. coli* to stimulate *in vitro* antimicrobial activity of macrophage-like J774 cell line against the live vaccine strain (LVS) of *Francisella tularensis* through the abilities of activated macrophages to reduce proliferation of this bacteria. In addition, we followed dose-dependent influences of these stimulants on intracellular proliferation of *F. tularensis* and putative effect of pretreatment of J774 cells either by INF-γ or LPS alone or in combination.

Materials and methods

In vitro infection of J774 cells

Conditions of infection were selected to ensure the multiplicity of infection (MOI) (i.e., ratio of bacterial cells to

J774 cells) to be 200. J774 cells derived from female BALB/c mouse (ECACC, No: 85011428) were cultured for 48 hrs in a complete culture medium without gentamicin. *Francisella tularensis* LVS strain (ATCC 29684, American Type Culture Collection, Manassas, Va.) was cultured on McLeod agar plates for 48 hrs at 37 °C in atmosphere containing 5% CO_2. J774 cells were exposed to LVS strain for 2 hrs at 37 °C in culture medium without gentamicin. Infected cells were than cultivated in culture medium supplemented with gentamicin for 1 hrs at 37 °C in atmosphere containing 5% CO_2 and then washed three times with culture medium without gentamicin. Infected macrophages were incubated in 6-well cultivation platelets ($2x10^6$ cells per 4 ml of medium Dulbecco's MEM with Glutamax-1 with 10% BSA, Gibco™). This was the time point 0.

Proliferation of Francisella tularensis, viability and number of cells

To determine the number of intracellular bacteria, cells were washed once in PBS and suspended in 1 ml of PBS 100-µl portions of each sample, serially diluted in PBS, were cultured on McLeod agar plates. The number of cells and viability were calculated using trypan blue exclusion test (0.5% TB in saline). Samples were collected each 3 hrs during 12 hrs of cultivation of cells and than after 24 hrs.

Stimulation of macrophage-like J774 cells

Recombinant murine interferon γ (mouse recombinant, expressed in *E. coli*, SIGMA, Saint Louis, Missouri, USA), in concentrations 100 I.U. per 1ml or 1000 I.U. per 1ml of medium, and/or bacterial lipopolysaccharide derived from *E. coli* (from *E. coli* serotype O55:B5, SIGMA, Saint Louis, Missouri, USA), in concentration 10 ng per 1 ml of medium or 50 ng per 1ml of medium, was used for stimulation immediately after infection (at the time 0) or 3 hrs before infection.

Statistical analysis

Results expressed as mean ± S.D. were derived from three independent experiments. Analysis of variance followed by Scheffe's evaluation of contrasts was exploited to reveal significant differences.

Results

Infection of J774 macrophages by F. tularensis with subsequent stimulation by INF-γ or/and LPS

In the first experiment, macrophage-like J774 cells were treated with 100 or 1000 IU/ml of INF-γ and/or 10 or 50 ng/ml of LPS that was added into infected cell cultures at the time 0. The growth of *Francisella tularensis* LVS was monitored in the course of 12 hrs, each 3 hrs and at the time 24 and 48 hrs (event. 72 hrs) thereafter.

Concentrations 1000 IU/ml of INF-γ (Fig. 1) effectively suppressed ($p<0.01$) growth of *Francisella tularensis* LVS at the time 48 hrs and 72 hrs (data not shown) of cultivation of infected cells. The bacterial counts were decreased by approximately 2 log10. This effect was not observed in the case of concentration 100 IU/ml of INF-γ. Furthermore, the proliferation of *F. tularensis* in J774 cell treated by 1000 IU/ml INF-γ was significantly lower ($p<0.01$) compared to cell cultures stimulated by 100 IU/ml INF-γ at the 48 hrs of cultivation. Treatment of macrophages with LPS (Fig. 2) displayed only a little effect on the growth of *F. tularensis* LVS with the only exception at the time 48 hrs, when concentration 50 ng/ml of LPS slightly suppressed proliferation of *F. tularensis* LVS ($p<0.05$). This effect was not observed at 72 hrs of cultivation of cells (data not shown).

Conversely, combination of INF-γ and LPS (Fig. 3) was remarkable effective. This effect was observed after 24 hrs of cultivation of macrophages. Proliferation of *F. tularensis* LVS was suppressed by both combinations of stimulants (100 IU/ml of INF-γ + 10 ng/ml of LPS and 1000 IU/ml of INF-γ + 50 ng/ml of LPS) ($p<0.01$) in comparison with cells infected by *F. tularensis* only. At the time of 48 hrs, the combination of higher doses suppressed growth of bacteria more effectively compared to combination of lower doses of stimulants ($p<0.01$). At the time 48 hrs of cultivation of infected cells the number of *F. tularensis* decreased approximately 4 log10 in comparison with infected cells only.

No direct effect of stimulants on the viability and number of J774 cells in the entire course of cultivations of cells was observed (data not shown). However, higher doses of INF-γ only or in combination with higher doses of LPS reduced the number of cells at 24 hrs and 48 hrs of cultivation of macrophage-like J774 cell line.

Stimulation of J774 macrophages by INF-γ or/and LPS with subsequent infection by F. tularensis

In these experiments, macrophage-like J774 cells were treated 3 hrs before infection with 100 or 1000 IU/ml of rINF-γ and/or 10 or 50 ng/ml of LPS. Macrophage-like J774 cells were washed after stimulation and infected with *F. tularensis* LVS. The ability of INF-γ and LPS to activate macrophages and inhibit the replication of *F. tularensis* LVS was monitored at the same time as stimulation of infected cells.

The number of bacteria at 48 hrs of cultivation of cells in the presence 1000 IU/ml of INF-γ (Fig. 4) was decreased in comparison with control (only infected cells) ($p<0.05$). At that time the bacterial number decreased approximately 2 log10.

Treatment of macrophages with LPS alone (Fig. 5) had no effect on the growth of *F. tularensis*, but this effect was monitored through 24 hrs of cultivation only.

The effect of combinations of 100 IU/ml of INF-γ with 10 ng/ml of LPS and 1000 IU/ml of INF-γ with 50 ng/ml of LPS (Fig. 6) on the growth of *F. tularensis* LVS were signi-

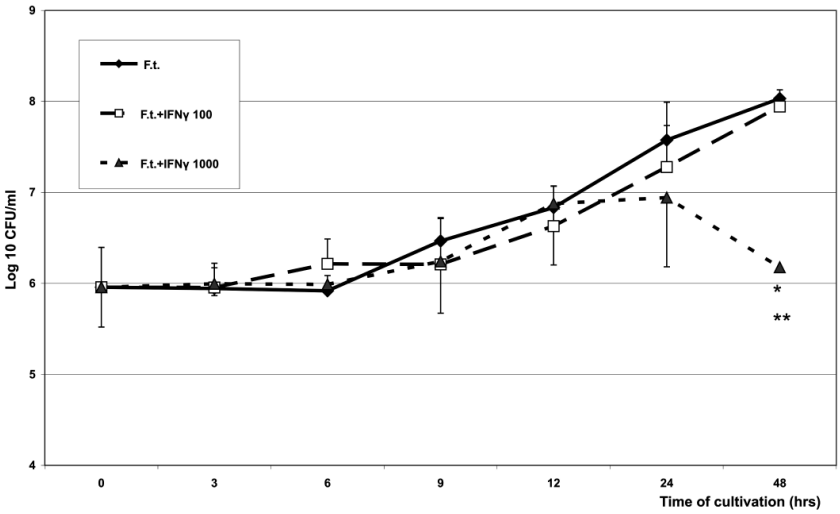

Fig. 1: Proliferation of *F. tularensis* LVS in macrophage-like cell line J774. The infection of cells by *F. tularensis* LVS with subsequent stimulation by 100 IU/ml of IFNγ or 1000 IU/ml of IFNγ. *p<0.01 compared with infection by *F. tularensis* only, **p<0.01 compared with infection by *F. tularensis* with consequential stimulation by 100 IU/ml of IFNγ.

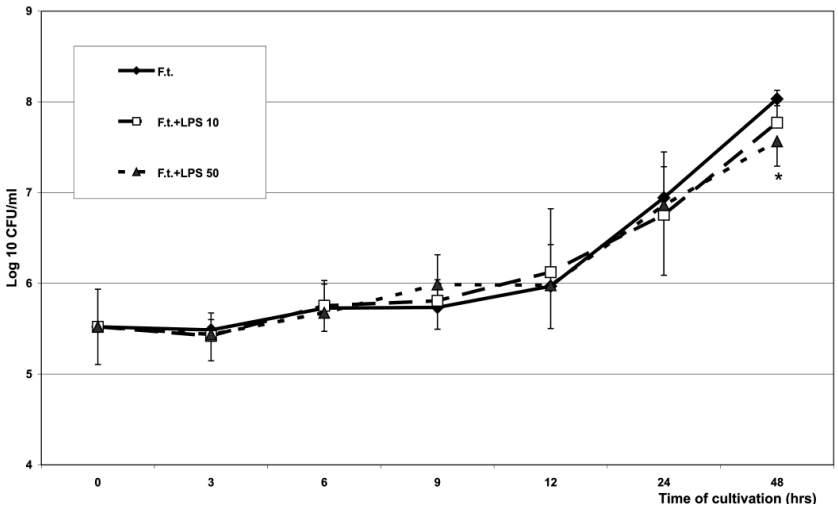

Fig. 2: Proliferation of *F. tularensis* LVS in macrophage-like cell line J774. The infected cells were subsequently stimulated by 10 ng/ml of LPS or 50 ng/ml of LPS. *p<0.05 compared with infection by *F. tularensis* only.

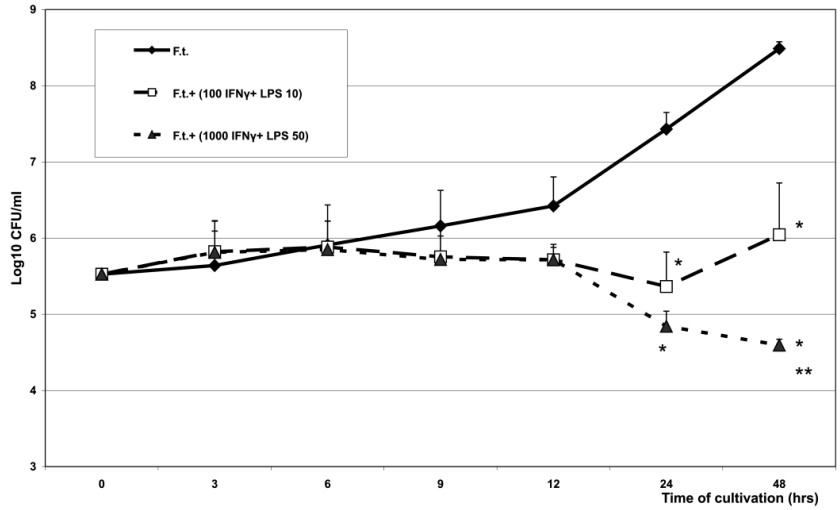

Fig. 3: Proliferation of *F. tularensis* LVS in macrophage-like cell line J774. The infection of cells by *F. tularensis* with subsequent stimulation by combination of 100 IU/ml of IFNγ with 10 ng/ml of LPS or 1000 IU/ml of IFNγ with 50 ng/ml of LPS. *p<0.01 compared with infection by *F. tularensis* only, **p<0.01 compared with infection by *F. tularensis* with consequential stimulation by 100 IU/ml of IFNγ with 10 ng/ml of LPS.

Legends: Results expressed as mean ± S.D. were derived from three independent experiments.

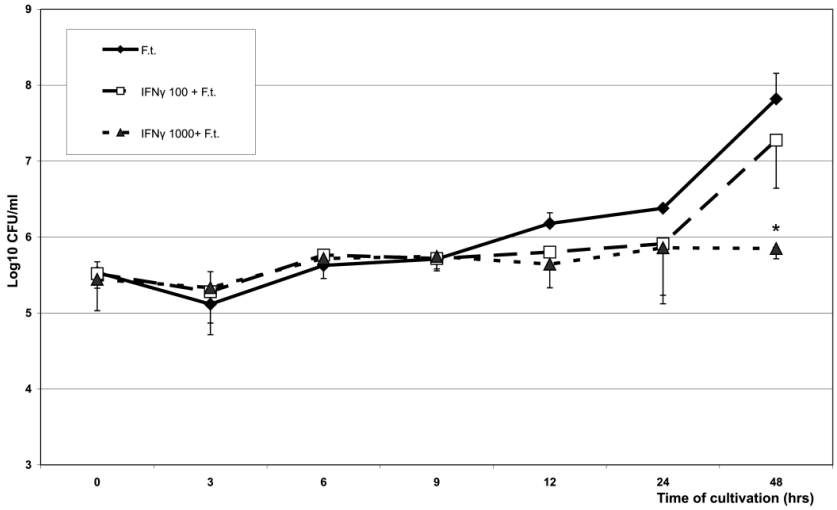

Fig. 4: Proliferation of *F. tularensis* LVS in macrophage-like cell line J774. The cells were stimulated by 100 IU/ml of IFNγ or 1000 IU/ml of IFNγ with subsequential infection by *F. tularensis.* *p<0.05 compared with infection by *F. tularensis* only.

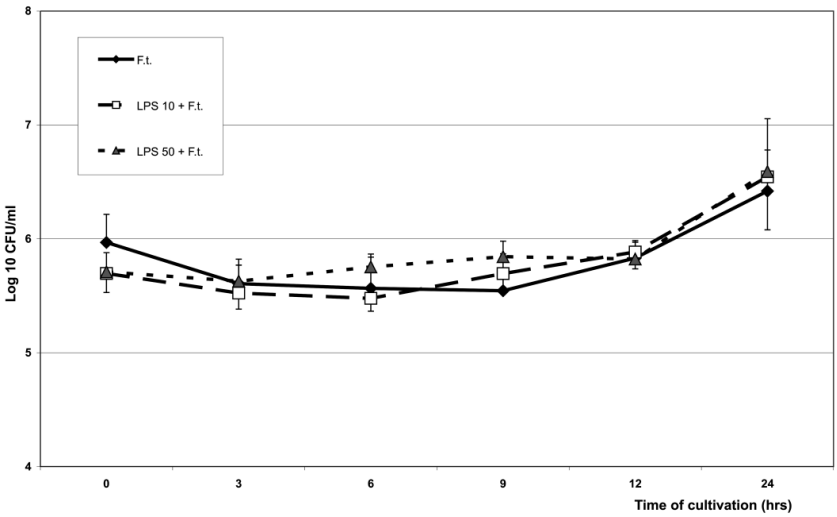

Fig. 5: Proliferation of *F. tularensis* LVS in macrophage-like cell line J774. The cells were stimulated by 10 ng/ml of LPS or 50 ng/ml of LPS with subsequent infection by *F. tularensis*.

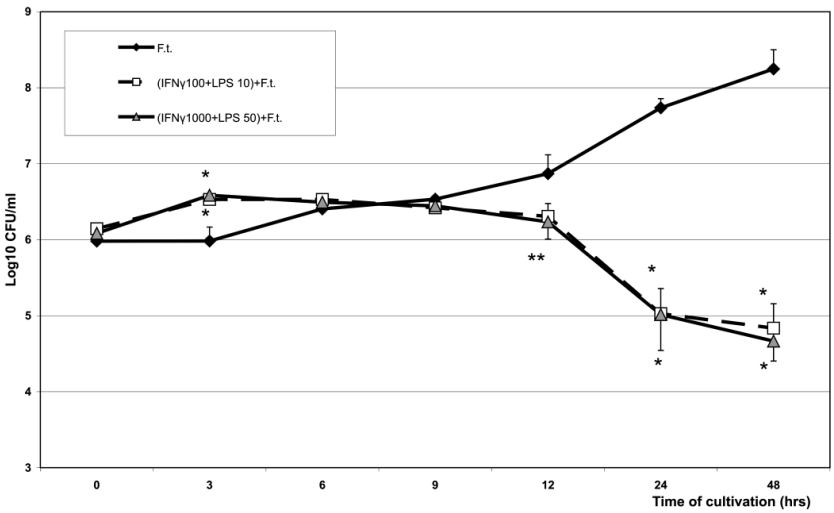

Fig. 6: Proliferation of *F. tularensis* LVS in macrophage-like cell line J774. The cells were stimulated by combination of 100 IU/ml of IFNγ with 10 ng/ml of LPS or 1000 IU/ml of IFNγ with 50 ng/ml of LPS with subsequent infection by *F. tularensis*. *p<0.01 compared with infection by *F. tularensis* only, **p<0.05 pretreatment by combination of 1000 IU/ml of IFNγ with 50 ng/ml of LPS compared with infection by *F. tularensis* only.

Legends: Results expressed as mean ± S.D. were derived from three independent experiments.

ficant. The accumulation of bacteria inside the cells was observed after 3 hrs of cultivation of macrophages. This effect was observed in the case of both lower and higher combination of stimulants ($p<0.01$). This effect was not dose dependent. Down regulation of growth of *F. tularensis* LVS was observed after 12 hrs of cultivation of cells. Only higher combination of stimulants suppressed proliferation of bacteria in comparison with the only infected control ($p<0.05$). The strong effect on growth of *F. tularensis* was observed after 24 hrs of cultivation of macrophages, when bacterial number decreased by 3 log10. This effect was not dose dependent.

We found a negative influence of combination of stimulants on the viability of macrophage cells (data not shown). Cell viability was decreased at the time 9 hrs of cultivation; 100 IU/ml of INF-γ + 10 ng/ml of LPS ($p<0.01$) or 1000 IU/ml of INF-γ + 50 ng/ml of LPS ($p<0.01$), compared with infection by *F. tularensis* only. On the other hand, cell viability was increased at 48 hrs of cultivation; 100 IU/ml of INF-γ + 10 ng/ml of LPS ($p<0.01$), or 1000 IU/ml of INF-γ + 50 ng/ml of LPS ($p<0.05$), compared with infection by *F. tularensis* only.

Discussion

Macrophage is an important effector cell for both cellular and humoral immunity. Pathogens have complex strategies for intracellular survival in these cells. These strategies are as diverse as the microorganisms themselves (8). Little is know about the means *F. tularensis* bacteria survive within host cells, since, unlike many other facultative intracellular bacteria, it produces no toxins and no secretion systems (13). Lai et al. have shown that LVS strain is well adapted to the intracellular environment of macrophages. Recently, it has been shown that *F. tularensis* is capable to escape effectively from phagosomes of macrophages (9, 16). The ability of *F. tularensis* to survive and multiply in various cell types is well recognized (1, 3, 5, 8, 10). In agreement with results of previous studies, we found a rapid multiplication of *F. tularensis* in untreated cultures of J774 line after 12 hrs of infection with comparison to the works by Lai et al. (13) and Hrstka et al. (11). However, compared to Lai et al. who revealed 2 log10 increase in the member of viable *F. tularensis* bacteria in the 12 to 24 hrs of cultivation we found only 1 log10 increase. However, our results are apparently in the concordance as MOI used by us was the half only. Similar results were also obtained in the case of resident peritoneal macrophages (8). INF-γ is particularly important to host defence, exspecially early in the response to primary infection, perhaps through their ability to activate macrophages for more efficient killing of *F. tularensis* (1, 2, 8). Similarly, LPS activates Toll-like receptors of mononuclear phagocytes, thereby inducing proinflammatory cytokines and antimicrobial activity (18). We only found the positive effect of INF-γ on the reduction of *F. tularensis* if infected cells were exposed to the higher concentration of INF-γ for 48 hrs of cultivation irrespective to the stimulants used.

The effect of the stimulation of J774 cells by LPS was very limited, if any. Telepnev et al. (18) published that *F. tularensis* is capable in macrophage-like cell line J774 of suppressing the capability of the cells to respond to LPS or bacterial lipopeptide. It could be the reason for the fact that J774 cells stimulated by LPS are not capable to supports the growth of *F. tularensis*. Rather different results were obtained if J774 cells were either pretreated or treated with the combination of INF-γ with LPS. This combination significantly suppressed the proliferation of *F. tularensis* in both pretreated and treated cells. If we stimulated the cells after infection by combination of stimulatory agents, a dose dependent effect was evident. Growth suppression of *F. tularensis* was prolonged up to 48 hrs of cultivation only if higher concentrations of stimulatory agents were used. On the other hand, the *F. tularensis* growth at 3 hrs after infection was significantly stimulated by the pretreatment by INF-γ with LPS irrespective concentrations used compared to untreated cell only. At that time J774 cells have already been exposed to the stimulation for 9 hrs. One plausible explanation of this fact could be better internalisation of *F. tularensis* by pretreated cells. This phenomenon was not identified at the any other points of observation. In contrast, we found decreased counts of *F. tularensis* at those intervals. Similar decreases were achieved if infected J774 cells were subsequently stimulated. The significant decrease in the number of *F. tularensis* was apparent after stimulation by 1000 IU/ml of INF-γ and 50 ng/ml of LPS before the start of infection already after 12 hrs of cultivation compared with the cells treated after infection. The multiplication of *F. tularensis* in pretreated J774 cells was significantly diminished after 24 hrs of cultivation being similar in both concentrations of INF-γ with LPS.

The most important limitation of our study is the fact that *E. coli* LPS was used to stimulate J774 cells. There are substantial differencies in the capacity of LPSs isolated from different bacterial species or even from various strains of the same species to stimulate host response. This has been recently addressed by Duenas et al. (7) who found much lower potency of *F. tularensis* LPS to stimulate human monocytes via TLR4 compared to *E. coli* LPS.

To summarize, it is apparent from our results that INF-γ alone has a protective effect regarding *F. tularensis* infection of macrophage-like J774 cells. This effect was even more pronounced if cells were treated by the combination of INF-γ with LPS. If cells were stimulated before infection by *F. tularensis* LVS, lower concentrations of INF-γ (tenfold) and LPS (fivefold), respectively, were sufficient to diminish the proliferation of *F. tularensis* LVS. If the infection of J774 macrophages by *F. tularensis* has already been in progress, higher concentrations of INF-γ and LPS were necessary to suppress the growth of *F. tularensis* in macrophage-like J774 cell line.

Acknowledgments

The presented study was fully supported by Ministry of Education, Youth and Sport, Czech Republic, grant

No. LN00A033. We thank Mrs. Hana Kotlandová for her assistance.

References

1. Anthony LS, Ghadirian E, Nestel FP, Kongshavn PA. The requirement for gamma interferon of mice to experimental tularemia. Microbial Pathog 1989;7:421–8.
2. Barker JH, Weiss J, Apicella M, Nauseef WM. Basis for the failure of *Francisella tularensis* lipopolysaccharide to prime human polymorphonuclear leukocytes. Infect Immunity 2006;74:3277–84.
3. Bosio CM, Elkins KL. Susceptibility to secondary *Francisella tularensis* live vaccine strain infection in B-cell-deficient mice is associated with neutrophilia but not with defects in specific T-cell-mediated immunity. Infect Immun 2001; 69:194–203.
4. Carlson Jr PE, Carrol JA, O'Dee DM, Nau GJ. Modulation of virulence factors in *Francisella tularensis* determines human macrophage response. Microbial Pathogen 2007;42:204–14.
5. Chen W, KuoLee R, Shen H, Busa M, Conlan JW. Toll-like receptor 4 (TLR4) plays a relatively minor role in murine defense against primary intradermal infection with *Francisella tularensis* LVS. Immunol Lett 2005;97:151–4.
6. Cowley SC, Myltseva SV, Nano FE. Phase variation in *Francisella tularensis* affecting intracellular growth, lipopolysaccharide antigenicity and nitric oxide production. Mol Microbiol 1996;20:867–74.
7. Duenas AI, Aceves M, Orduna A, Diaz R, Sanchez Crespo M, Garcia-Rodriguez C. *Francisella tularensis* LPS induces the production of cytokines in human monocytes and signals via Toll-like receptor 4 with much lower potency than *E. coli* LPS. Int Immunol 2006;18(5):785–95.
8. Fortier AH, Polsinelli T, Green SJ, Nacy CA. Activation of macrophages for destruction of *Francisella tularensis*: Identification of cytokines, effector cells, and effector molecules. Infect Immun 1992;60:817–25.
9. Golovliov I, Baranov V, Krocova Z, Kovarova H, Sjöstedt A. An attenuated strain of the facultative intracellular bacterium *Francisella tularensis* can escape the phagosome of monocytic cells. Infect Immun 2003;71(10):5940–50.
10. Golovliov I, Sandström G, Ericsson M, Sjöstedt A, Tärnvik A. Cytokine expression in the liver during the early phase of murine tularemia. Infect Immun 1995;63:534–8.
11. Hrstka R, Stulik J, Vojtesek B. The role of MAPK signalling pathway during *Francisella tularensis* LVS infection-induced apoptosis in murine macrophages. Microbes Infect 2005;7(4):619–25.
12. Kieffer TL, Cowley S, Nano FE, Elkins KL. *Francisella novicida* LPS has immunobiological activity in mice than *F. tularensis* LPS, and contributes to *F. novicida* murine pathogenesis. Microbes Infect 2003;5(5):397–403.
13. Lai XH, Golovliov I, Sjöstedt A. *Francisellla tularensis* induced cytopathogenicity and apoptosis in murine macrophages via a mechanism that requires intracellular bacterial multiplication. Infect Immun 2001;69:4691–4.
14. Li H, Nookala S, Bina XR, Bina JE, Re F. Innate immune response to *Francisella tularensis* is mediated by TLR2 and caspase-1 activation. J Leukoc Biol 2006;80: 766–73.
15. Murray HW. Gamma interferon, cytokine-induced macrophage activation, and antimicrobial host defence. Diag Microbiol Infect Dis 1990;13:411–21.
16. Santic M, Molmeret M, Klose KE, Kwaik YA. *Francisella tularensis* travels, a novel, twisted road within macrophages. Trends Microbiol 2006;14:37–44.
17. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-γ: an overview of signals, mechanisms and functions. J Leuk Biol 2004;75:163–89.
18. Telepnev M, Golovliov I, Grundström T, Tärnvik A, Sjöstedt A. *Francisella tularensis* inhibits Toll-like receptor-mediated activation of intracellular signalling and secretion of TNF-α and IL-1 from murine macrophages. Cell Microbiol 2003; 5(1):41–51.

Received: 31/07/2009.
Accepted: 25/09/2009.

Corresponding author:

Prof. RNDr. Jan Krejsek, CSc., Charles University in Prague, University Hospital Hradec Králové, Department of Clinical Immunology and Allergology, Sokolská 581, 500 05 Hradec Králové, Czech Republic; e-mail: krejsek@fnhk.cz