

Local and Systemic Up-regulation of TNF α , IL-1 β and IL-6 in Mice Intratracheally Inoculated with *Porphyromonas gingivalis*

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Abstract

Pavlica Z., A. Nemeč, A. Nemeč-Svete, D. Eržen, D.A. Crossley, M. Petelin: Local and Systemic Up-regulation of TNF α , IL-1 β and IL-6 in Mice Intratracheally Inoculated with *Porphyromonas gingivalis*. Acta Vet. Brno 2008, 77: 377-385.

The objective of the study was to find whether a single intratracheal inoculation with live *Porphyromonas gingivalis* ATCC 33277 influences local and systemic inflammatory and immune responses in mice. Twelve-week-old BALB/c mice were intratracheally inoculated with 2.9×10^9 CFU *P. gingivalis* ATCC 33277 diluted in 40 μ l sterile phosphate buffer (treated group) or with sterile PBS (control group). The animals were sacrificed 2, 6, 24, 72 and 168 h after inoculation. TNF α , IL-1 β , IL-6 and total protein concentrations were measured in the serum, lungs and kidneys. Six hours after *P. gingivalis* inoculation, TNF α concentration was significantly increased in serum ($p = 0.02$) and kidneys ($p = 0.04$), but in the lungs TNF α production was enhanced already 2 h ($p < 0.0001$) after inoculation, reaching the peak after 6 h ($p < 0.0001$). The IL-1 β concentration was also significantly increased in serum after 2 h ($p = 0.006$), remaining significantly elevated up to 3 days ($p \leq 0.0001$) after inoculation. In lungs IL-1 β levels were significantly increased 6 and 24 h ($p < 0.0001$) and in kidneys 24 h ($p < 0.0001$) and 168 h ($p = 0.01$) after inoculation. The IL-6 concentration was significantly increased in serum after 72 and 168 h ($p < 0.0001$). However, IL-6 was significantly increased in lungs after 6 h ($p < 0.0001$), remaining elevated until 72 h and in kidneys 2 and 6 h ($p < 0.0001$) after inoculation. Significantly increased total protein concentration was detected in kidneys 6 and 24 h ($p < 0.0001$) after inoculation. These results suggest that a single intratracheal inoculation with *P. gingivalis* stimulates the local and systemic inflammatory and immune response, as shown by increased tissue and serum levels of proinflammatory cytokines.

Periodontopathogenic bacteria, mice, proinflammatory cytokines, systemic effects

The human oral cavity hosts about 500 different bacterial species (Paster et al. 2001). When periodontal disease is established, periodontopathogenic bacteria such as *P. gingivalis* are numerous. Many other bacteria that can induce lung inflammation are also present in the oral cavity (Scannapieco 1994; Russell et al. 1999). Gingival fluid and saliva flow constantly rinse out bacteria from the dental plaque, so the aspiration of saliva is a possible cause of recurring lung inflammation leading to irreversible tissue damage (Scannapieco 1994; 2005). Additionally, enzymes released from diseased periodontal tissues influence bacterial colonization of the respiratory epithelium by decomposing salivary and airway mucin, which normally traps bacteria and prevents their binding to respiratory epithelium (Scannapieco and Genco 1999). Moreover, bacteria and/or their products may stimulate respiratory epithelium to produce proinflammatory cytokines leading to vasodilatation and polymorphonuclear leukocyte chemotaxis. Activated phagocytes release hydrolytic enzymes and free radicals (reactive oxygen and nitrogen species) that further damage the respiratory epithelium (Scannapieco 2005). Cytokines - tumour necrosis factor α (TNF- α), interleukins 1 (IL-1 α and β) and 6 released from inflamed periodontal tissues also stimulate

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the respiratory epithelium to produce molecules that can bind bacteria and promote their growth (Svanborg et al. 1996). TNF- α is one of the most important cytokines involved in the pathogenesis of acute and chronic inflammation. Apart from being a chemotactic agent, it also stimulates the synthesis of adhesion molecules in the lungs, leading to the formation of inflammatory cell infiltrates. TNF- α and IL-1 β also act as stimulating agents for fibroblasts and macrophages to produce chemotactic agents for phagocytes. In a murine pneumonia model, IL-6 levels are elevated resulting in antiinflammatory effects during acute inflammation due to the modulation of TNF- α levels in both the lungs and serum (Petelin et al. 2004).

Bacterial antigens and toxins may enter the circulation during inflammation and can therefore stimulate systemic immune responses leading to various immune-mediated diseases including glomerulonephritis (Lah et al. 1993; Renvert et al. 1996). In dogs *P. gingivalis* is reported to bind to endothelial cells of glomerular capillaries causing immune-mediated glomerulonephritis (DeBowes et al. 1996).

P. gingivalis has a vast array of potential virulence factors and it can modulate host immune processes, its lipopolysaccharide (LPS) alone, which is released from the bacterial cell in membrane vesicles, is capable of modulating the cytokine network (Lamont and Jenkinson 1998). Intratracheal inoculation with live *P. gingivalis* is known to cause mild pneumonia with increased local cytokine production in BALB/c mice (Kimizuka et al. 2003) and systemic cytokine modulation occurs after a single intratracheal exposure of mice to sonicated *P. gingivalis* (Petelin et al. 2004). Therefore, the aim of the study was to find whether a single intratracheal inoculation with live *P. gingivalis* ATCC 33277 also influences local and systemic inflammatory and immune responses in mice.

Materials and Methods

Animals

One hundred and ten male specific pathogen free (SPF) BALB/c mice, 12 weeks of age, were obtained from the Centre for Animal Genomics, Veterinary Faculty, University of Ljubljana. All animals received a standard laboratory diet (Teklad Global 16% Protein Rodent Diet, Harlan Teklad) and water *ad libitum*. All animal protocols were approved by the Veterinary Administration of the Republic of Slovenia No. 323-02-187/2004/2.

P. gingivalis culture

Challenge suspensions of *P. gingivalis* strain ATCC 33277 were supplied by the Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana. *P. gingivalis* was maintained frozen in a sterile vial containing porous beads that serve as carriers to support microorganisms (Microbank; Pro-lab Diagnostics) and kept at -70 °C. Briefly, bacteria were grown on Brucella Blood Agar (BBL, Oxoid) supplemented with 5% sheep blood, hemin (5 μ g/ml) (Sigma) and vitamin K1 (1 μ g/ml) (Sigma) and kept in anaerobic conditions (GENbox anaer; bioMerieux) for 10 days at 35 °C. The cell concentration in the inoculum was adjusted to approximately 2.9×10^9 CFU in 40 μ l of PBS.

Experimental design

Mice were anaesthetized with an intramuscular (i.m.) injection of 0.1 ml of anaesthetic solution that was prepared as follows: 0.5 ml ketamine (100 mg/ml; Bioketan, Vetoquinol Biowet) + 0.25 ml xylazine (20 mg/ml;

Table 1. Experimental groups and the number of animals included in each experimental group

Experimental group	Procedure	Number of mice
1	Treated group: <i>P. gingivalis</i> (2.9×10^9 CFU diluted in 40 μ l sterile PBS, intratracheally) inoculation at the start. Sacrificed at: 2 h, 6 h, 24 h, 72 h, 168 h	50 (10 per time interval)
2	Control group: 40 μ l sterile PBS intratracheally at the start. Sacrificed at: 2 h, 6 h, 24 h, 72 h, 168 h	50 (10 per time interval)
3	Untreated control group: immediate sacrifice	10

Rompun, Bayer) + 0.1 ml acepromazine (10 mg/ml; PromAce, Fort Dodge) + 0.15 ml water for injection (Aqua ad iniectionabilia; B. Braun Melsungen AG). The stock mixture was further diluted 1 : 1 with water for injection prior to use. Anaesthetised mice were inoculated with 2.9×10^9 CFU live *P. gingivalis* ATCC 33277 (50 animals) diluted in 40 μ l sterile phosphate buffer (PBS, pH = 7.4; Gibco, Invitrogen) using a plastic cannula to place the inoculum into the trachea (treated group). Fifty mice were given only 40 μ l of sterile PBS (control group). These two groups were kept separately. Mice were sacrificed at intervals: 2, 6, 24, 72 and 168 h after inoculation, at every time interval 10 mice from each group were euthanized by cervical dislocation once a surgical plane of general anaesthesia was achieved (typically within 3 min of injection of 0.5 ml of anaesthetic mixture).

Ten mice were euthanized at the beginning of the study to determine basal levels of cytokines in serum and supernatant of organs (Table 1).

Preparation of specimens

Blood was collected into serum tubes immediately after sacrifice by cardiac puncture and allowed to clot for 20 min prior to being centrifuged for 10 min at 1200 g at 4 °C. The serum was then frozen and stored at -70 °C until further examination. Lungs and kidneys were harvested from sacrificed animals, weighed and homogenised with 400 μ l PBS. The homogenate was centrifuged for 15 min at 20000 g at 4 °C. The supernatant was then stored frozen at -70 °C until further testing.

Cytokine and total protein levels in supernatant and serum

The levels of TNF- α , IL-1 β and IL-6 were determined in the serum and supernatant obtained from the lung and kidney homogenates using commercial ELISA test kits (R&D Systems, Minneapolis) according to the manufacturer's guidelines. The Bradford's method (Bradford 1976) was used to assay the level of proteins in the supernatants and serum.

Statistical analysis

Analysis of variance was used to evaluate the difference between average levels of measured indicators of inflammation in different experimental groups according to the time period of the test. When the model was statistically significant, least squared difference test was used as a multiple comparison test to determine the significant differences between subgroup means in an analysis of variance setting. Values of $p < 0.05$ were considered significant. The analysis was performed with the use of commercial statistical software SAS 9.00 (PROC ANOVA) (SAS Institute Inc, Cary, NC).

Results

Clinical course of the experiment

As demonstrated by histological examination in our preliminary study (Pavlica et al. 2006), single intratracheal inoculation with 2.9×10^9 CFU *P. gingivalis* in 40 μ l PBS induces moderate pneumonia in mice, which is not life-threatening. During the present

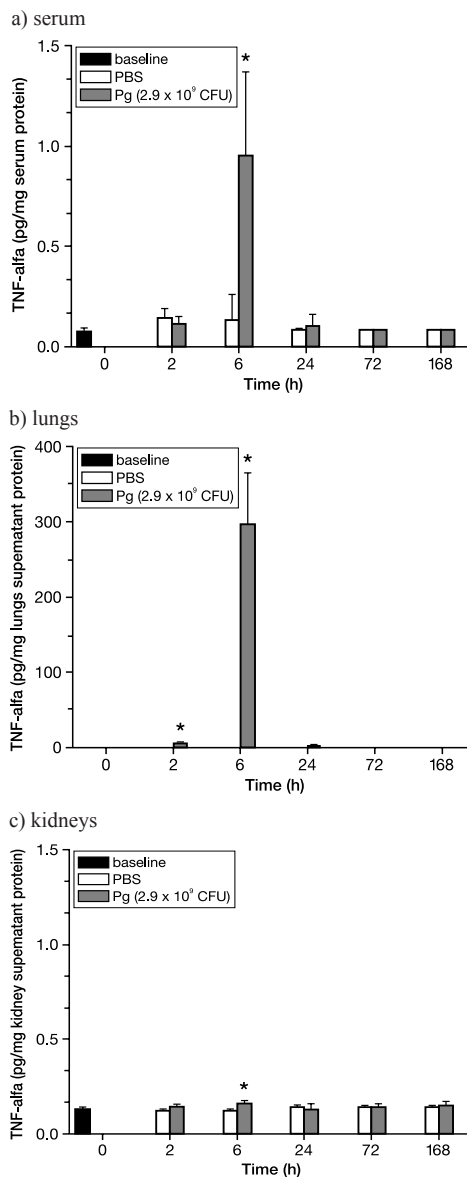


Fig. 1. TNF- α levels in serum (a), lungs (b) and kidneys (c) at different time intervals after inoculation.

* Significant difference ($p = 0.02$ (a), $p < 0.0001$ (b), $p = 0.04$ (c)) between PBS-treated and *P. gingivalis*-inoculated mice.

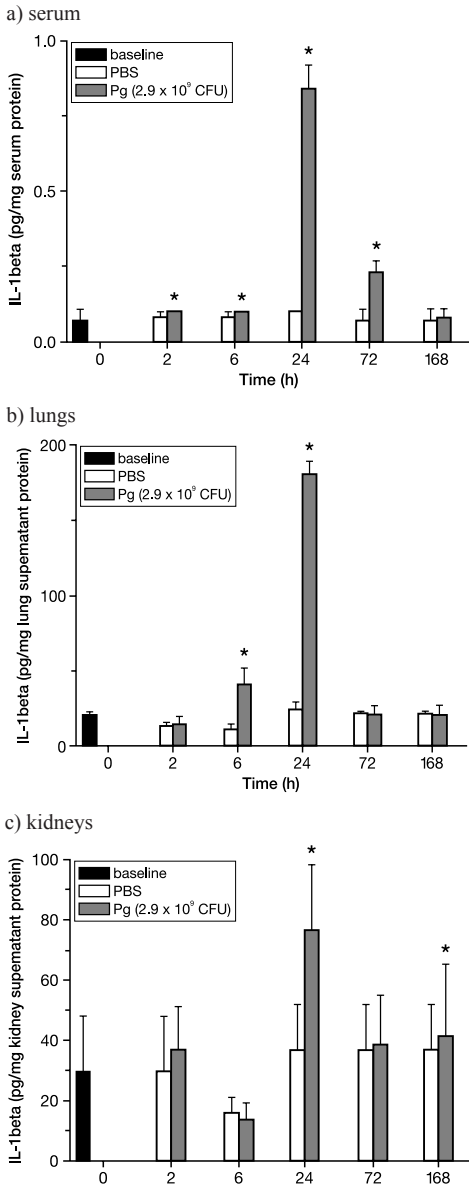


Fig. 2. IL-1 β levels in serum (a), lungs (b) and kidneys (c) at different time intervals after inoculation.

* Significant difference ($p \leq 0.006$ (a), $p < 0.0001$ (b), $p \leq 0.01$ (c)) between PBS-treated and *P. gingivalis*-inoculated mice.

The weight of harvested kidneys was significantly higher ($p < 0.05$) at all time intervals in the treated group compared to the weight of kidneys obtained from the control group of animals (Fig. 4b).

experiment, mice showed clinical signs of illness with polydipsia and inappetence from 6 to 72 h after inoculation, however, at the end of the trial they were clinically healthy.

Significantly higher levels of TNF- α were detected in the serum of the treated group compared with control mice at 6 h after inoculation ($p = 0.02$) (Fig. 1a) and in lungs at both 2 h ($p < 0.0001$) and 6 h ($p < 0.0001$) after inoculation (Fig. 1b). TNF- α was significantly higher in kidneys 6 h after inoculation ($p = 0.04$) (Fig. 1c).

In addition, IL-1 β levels were already significantly higher in the serum 2 h after inoculation ($p = 0.006$), remaining significantly elevated 6 ($p = 0.0001$), 24 ($p < 0.0001$) and 72 ($p < 0.0001$) h after inoculation (Fig. 2a) in the treated group compared with the control group of mice.

In the supernatant obtained from the lung tissue of the animals from the treated group, IL-1 β levels were significantly higher 6 h after inoculation ($p < 0.0001$) and remained elevated 24 h after inoculation ($p < 0.0001$) (Fig. 2b).

In the supernatant of the kidneys the IL-1 β levels were significantly higher in the treated group 24 h ($p < 0.0001$) and 168 h ($p = 0.01$) after inoculation (Fig. 2c).

IL-6 was elevated in the serum of treated animals 72 and 168 h after inoculation ($p < 0.0001$) (Fig. 3a).

In lungs the IL-6 levels were raised 6 h after inoculation ($p < 0.0001$) and remained elevated until 72 h after inoculation (Fig. 3b), while in the kidneys the IL-6 levels increased within 2 h after inoculation ($p < 0.0001$) and remained significantly higher until 6 h after inoculation (Fig. 3c).

Weight of the lungs and kidneys and total protein amount in kidneys

The weight of harvested lungs was significantly higher 6 h after inoculation in the treated group ($p = 0.03$) comparing to the weight of lungs obtained from control mice and remained higher until the end of the experiment (Fig. 4a).

The total protein content in kidneys was significantly higher 6 h ($p < 0.0001$) and 24 h ($p < 0.0001$) after inoculation in the treated group compared to the values obtained from controls (Fig. 4c).

Discussion

A single intratracheal inoculation of mice with 2.9×10^9 CFU live *P. gingivalis* ATCC 33277 stimulated local and systemic inflammatory and immune responses, the mice showing clinical signs of illness with polydipsia and inappetence from 6 to 72 h after inoculation, and complete clinical recovery until the end of the trial.

Inoculation with lipopolysaccharide (LPS) of *E. coli* is reported to raise TNF- α levels in the bronchoalveolar fluid but not in the serum of rats (Nelson et al. 1989). The same authors proposed that TNF- α , which is produced locally in the lungs, does not enter systemic circulation. On the other hand, it is reported that intratracheal inoculation with live *E. coli* stimulates TNF- α levels in the lung lavage and in the serum of rats (Karzai et al. 2003), which is consistent with the findings of our study, as in the mice inoculated with live *P. gingivalis* TNF- α levels were significantly elevated 6 h after inoculation in the lungs and serum. However, Petelin et al. (2004) reported for mice that TNF- α was elevated in lungs already 2 h after inoculation with killed *P. gingivalis*. In our study, TNF- α levels were only slightly, but significantly elevated in lungs 2 h after inoculation, increasing up to 6 h, when increased TNF- α production was also detected in the kidneys. We therefore presume that host alveolar macrophages, neutrophils and monocytes required some time to recognize the live bacteria and start increasing the cytokine release, similarly as suggested in the case when mice were intratracheally infected with live or killed *Legionella pneumophila* (Kikuchi et al. 2004). It is reported that only free LPS molecules are toxic for the host; free LPS occurs through vesicle formation, during cell division or after destruction of the bacterial cell wall of dead bacteria (Wolf et al. 2005).

According to our results, IL-1 β of the tested cytokines seemed to be the most involved cytokine in the local and systemic inflammatory and immune responses, yet its levels were

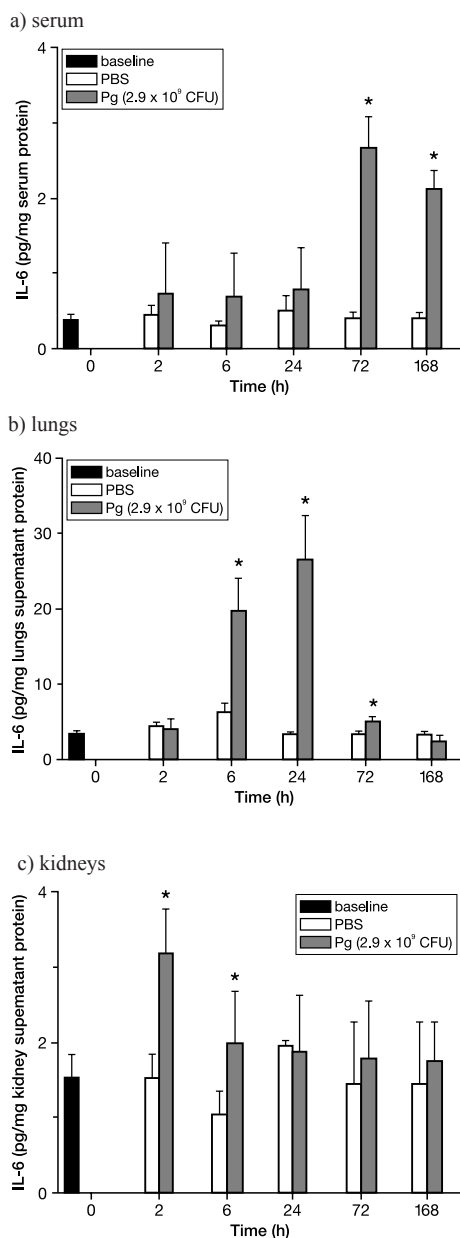


Fig. 3. IL-6 levels in serum (a), lungs (b) and kidneys (c) at different time intervals after inoculation.

* Significant difference ($p < 0.0001$ (a), $p < 0.0001$ (b), $p < 0.0001$ (c) between PBS-treated and *P. gingivalis*-inoculated mice.

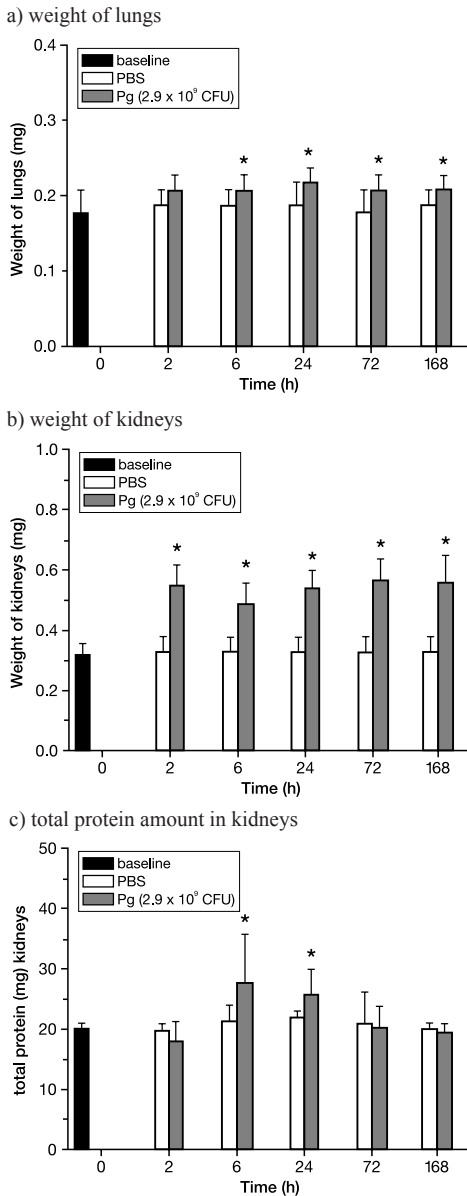


Fig. 4. Weight of the lungs (a), weight of the kidneys (b) and total protein amount in the kidneys (c) at different time intervals after inoculation.

* Significant difference ($p = 0.03$ (a), $p < 0.05$ (b), $p < 0.0001$ (c)) between PBS-treated and *P. gingivalis*-inoculated mice.

elevated at different times after inoculation. That could also be a specific feature of BALB/c mice as they lack the IL-1 β receptor inhibitor (Van den Berg 2001). Besides, IL-1 β acts synergistically with TNF- α (Van den Berg 2001).

Bacteria and their lipopolysaccharides react with antibodies produced, forming immune complexes (Cook and Sullivan 1991). These complexes accumulate in glomeruli during glomerular filtration (MacDougall et al. 1986; Ortiz et al. 1991) where they act as chemoattractants to polymorphonuclear leucocytes, macrophages and platelets, which produce cytokines, leukotriens, growth factors, platelet activating factor and reactive nitrogen species (Baylis 1987; Sharma et al. 2000; Khlgatian et al. 2002). Formation of cytotoxic iron-nitrosyl-sulphur complexes may occur, inhibiting Fe-dependent enzymes and damaging the DNA of the cells in kidneys (Cook and Sullivan 1991). Cytokines may lead to coagulation abnormalities and induce metabolic changes in endothelial cells (Nieto 1998; Franek et al. 2005). Similarly, cytokines may also influence metabolism in glomerular mesangial cells (Sedor et al. 1993). The results of our study show increased TNF- α levels in kidneys 6 h after inoculation with 2.9×10^9 CFU *P. gingivalis*, which is indicative of an inflammatory and immune response in distant tissues. At the same time, the weight of kidneys and total protein amount were also higher in mice inoculated with 2.9×10^9 CFU *P. gingivalis* than in non-inoculated mice (Figs 4b, 4c).

Although IL-6 is considered to enter the blood stream immediately after the stimulation of cells with LPS (Wong and Clark 1988; Molloy et al. 1993) or live bacteria (Karzai et al. 2003), the peak of IL-6 in serum occurred as late as 72 and 168 h after inoculation. IL-6 is supposed to have inhibitory effects on TNF- α levels; local application of IL-6 to rats inoculated concurrently with LPS has been reported to inhibit TNF- α production (Ulich et al. 1991). This can also be assumed from the results of our study, where the systemic drop in TNF- α level was simultaneous with an IL-6 increase. Besides, Xing et al. (1998) reported that IL-6 has anti-inflammatory effects. However, local time-dependent production of IL-6 was

different from that seen in the serum. The levels of IL-6 started to increase already 6 h after inoculation and remained elevated up to 72 h in the lungs. It is therefore possible that IL-6 locally acts synergistically with TNF- α and IL-1 β .

In kidneys, IL-6 levels were already increased within 2 h after inoculation, which is in accordance with Wong and Clark's (Wong and Clark 1988) hypothesis of rapid IL-6 production and release after LPS stimulation.

IL-6 is mainly produced by fibroblasts, monocytes and macrophages when these cells are exposed to gram negative bacteria. This cytokine acts by binding to specific receptor, leading, together with TNF- α and IL-1 β , to synthesis and release of acute phase proteins from the liver (Molloy et al. 1993; Ebersole et al. 2002).

Therefore, periodontal disease, which is a chronic infection with periodontopathogenic bacteria (i.e. gram negative *P. gingivalis*) might also lead to systemic inflammatory and immune responses causing damage in distant tissues as demonstrated for lungs and kidneys in the present study.

Lokální a systémová reakce TNF α , IL-1 β a IL-6 u myši, kterým byla intratracheálně inokulována *Porphyromonas gingivalis*

Z infikovaných plic zvířat a lidí s periodontitidou byla izolována *Porphyromonas gingivalis*. Bakteriémie a toxémie spojená s touto infekcí může vést k imunitním a zánětlivým reakcím ve vzdálených tkáních. Dvanáctitýdenním BALB/c myšim bylo intratracheálně aplikováno 40 μ l sterilního fosfátového pufru s $2,9 \times 10^9$ CFU *P. gingivalis* ATCC 33277 (experimentální skupina), nebo sterilní PBS (kontrolní skupina). Zvířata byla utracena 2, 6, 24, 72 a 168 hodin po aplikaci. V séru, plicích a ledvinách byly stanovovány koncentrace TNF α , IL-1 β , IL-6 a celkového proteinu. Šest hodin po aplikaci byla významně zvýšená koncentrace TNF α v séru ($p = 0,02$) a ledvinách ($p = 0,04$). V plicích byla produkce TNF α zvýšená již 2 h po aplikaci ($p < 0,0001$), přičemž nejvyšších hodnot bylo dosaženo 6 h po aplikaci ($p < 0,0001$). Koncentrace IL-1 β byla po 2 hodinách v séru rovněž významně zvýšená ($p = 0,006$) a zůstala významně zvýšená 3 dny ($p \leq 0,0001$). V plicích byla koncentrace IL-1 β významně zvýšená po 6 a 24 hodinách ($p < 0,0001$) a v ledvinách po 24 ($p < 0,0001$) a 168 hodinách ($p = 0,01$) od aplikace. Koncentrace IL-6 v séru byla významně zvýšená po 72 a 168 hodinách ($p < 0,0001$). Nicméně jeho koncentrace v plicích byla zvýšená již po 6 hodinách ($p < 0,0001$) a zůstala zvýšena do 72 hodin, a v ledvinách po 2. a 6. hodině ($p < 0,0001$). V ledvinách byla hladina celkového proteinu významně zvýšená po 6. a 24. hodině od aplikace ($p < 0,0001$). Tyto výsledky ukazují, že jednorázová intratracheální inokulace *P. gingivalis* stimuluje lokální a systémovou imunitní a zánětlivou reakci, jak dokládají zvýšené koncentrace prozánětlivých cytokinů v séru a tkáních.

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