

Heat-killed *Tsukamurella inchoensis* reduces lipopolysaccharide-induced inflammatory responses in activated murine peritoneal macrophages

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ABSTRACT: *Tsukamurella inchoensis* (*T. inchoensis*) is an aerobic species of Actinomycetales which has immunomodulatory activities when used as a suspension of killed bacilli. Here, the effects of *T. inchoensis* on lipopolysaccharide-induced inflammatory responses in mouse peritoneal macrophages have been examined. Peritoneal macrophages were harvested by lavaging with ice cold phosphate-buffered saline. Macrophages acquired from mice treated with different doses of *T. inchoensis* for seven days were cultured with 20 U/ml interferon- γ and 10 μ g/ml lipopolysaccharide for *in vivo* assays. Nitrite levels were measured by using the diazotization method based on the Griess reaction, an indirect technique to determine nitric oxide (NO) production. *T. inchoensis* inhibited lipopolysaccharide-stimulated NO production in mouse peritoneal macrophages from mice previously exposed to concentrations of 108 and 5×10^7 CFU per flask. Also, *T. inchoensis* decreased lipopolysaccharide-induced production of pro-inflammatory cytokines, including interleukin-6 and tumor necrosis factor- α . Thus, it can be concluded that *T. inchoensis* is a powerful inhibitor of lipopolysaccharide-induced NO production in activated murine macrophages, and *T. inchoensis* may be useful as a novel agent for chemoprevention in inflammatory diseases.

Keywords: Suriyan mouse; actinomycetales; nitric oxide; tumor necrosis factor- α ; TNF- α ; interleukin-6; IL-6; chemoprevention

Inflammation is a process mediated by the host's immune system to protect against a wide range of injuries (Xu et al. 2012). Dysregulated and perpetual inflammation may cause various pathophysiological conditions including hepatitis, atherosclerosis and cancer (Cousens et al. 1997). Lipopolysaccharide (LPS) and interferon (IFN)- γ play key roles in the initiation of inflammation by activating macrophages (Kopydlowski et al. 1999;

Mosser 2003). Hence, IFN- γ and LPS are often used *in vivo* and *in vitro* to induce inflammatory responses. Inflammation necessitates the activation of monocytes and macrophages, which secrete inflammatory mediators including chemokines, cytokines and nitric oxide (NO).

Nitric oxide is a small free radical which has multiple physiological and pathophysiological functions because of its broad pattern of synthesis and diverse

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mechanisms of action (Davis et al. 2001). Besides its function as a smooth muscle relaxant, NO is important in the inhibition of platelet activation, neurotransmission and the immune response (Murad 1996). Nitric oxide has both positive and negative effects; therefore, while it is useful as a modulator or messenger and for immunological self-defence, it is also potentially toxic. Nitric oxide is formed from L-arginine by the enzyme nitric oxide synthase. Since there is a causal relationship between inflammation and a large number of pathological conditions such as amyotrophic lateral sclerosis, diabetes, cancer and neurodegenerative diseases, inducible nitric oxide synthase is a molecular target of chemoprevention (Jang and Murrell 1998). Therefore, it is of great interest to identify new inhibitors of NO production.

A number of aerobic Actinomycetales species, including *Rhodococcus coprophilus*, *Gordonia bronchialis*, *T. inchonensis*, and closely related mycobacteria are able to exert subtly different adjuvant or immunomodulatory activities when injected as suspensions of killed bacilli (Tarres et al. 2012). In animal models, preparations of *T. inchonensis* have been particularly successful in preventing inflammation of the intima of arteries damaged by a balloon catheter and for use in the prevention and treatment of spontaneous type 2 diabetes mellitus (Stanford and Stanford 2012). However, the available evidence is not yet adequate to allow their use in clinical practice.

In a previous report, we evaluated the effects of *T. inchonensis* on immune responses and intestinal function in mice (Nofouzi et al. 2016). Our hypothesis was that different concentrations of *T. inchonensis* would reduce inflammatory responses in activated murine peritoneal macrophages. Therefore, the aim of our study was to evaluate the effects of this actinomycete on inflammatory responses on mice peritoneal macrophages during incubation in a CO₂ incubator for 24 h.

MATERIAL AND METHODS

Experimental animals. Male Suriyan mice (20–22 g) were obtained from the Pasteur Institute, Tehran, Iran. The experimental study was approved by the Ethics Committee of the School of Veterinary Medicine, Tabriz University. Mice (four groups, five mice per group) were randomised and domiciled five to a cage in polyester cages. The animals were maintained under standard laboratory conditions

of a temperature at $25 \pm 2^\circ\text{C}$, a photoperiod of L/D 12 : 12 h and received a standard mouse chow and water *ad libitum*.

Strain preparation. *T. inchonensis* was grown in Sauton's medium, harvested by centrifugation and washed in borate-buffered saline, pH 8.0. Suspensions were standardised by wet weight, re-suspended in borate-buffered saline and autoclaved at 121°C for sterilisation.

In vivo exposure to *T. inchonensis*. For the *in vivo* study, each mouse was orally administered bacterial suspension (5×10^7 , 1×10^8 and 2×10^8 CFU/mouse) consecutively for seven days by gavage. The control animals received the same amount of PBS.

Peritoneal macrophage isolation and cell culture. For the *in vitro* and *in vivo* experiments, peritoneal macrophages were harvested immediately by lavaging with ice cold sterile phosphate-buffered saline (PBS). Cells were lavaged twice and plated in RPMI 1640 (Sigma Chemical Co.) medium containing 100 U/ml penicillin/100 µg/ml streptomycin (Sigma Chemical Co.), 10% foetal bovine serum (GIBCO) and incubated for 2 h at 37°C in a 5% CO₂ humidified incubator. After 2 h of incubation, non-adherent cells were removed by gently washing with PBS and freshly prepared medium was added (Azadmehr et al. 2009). Then, the peritoneal macrophages in all groups were incubated for a further 24 h. Afterwards, the supernatants were collected and stored at -20°C for further use.

Determination of cell viability and NO production. NO production was estimated from the accumulation of nitrite (NO₂), an invariable end product of NO metabolism, in the culture medium, using the Griess reagent (0.1% naphthylendiamine and 1% sulphanilamide in 5% phosphoric acid) (Lu et al. 2011). Trypan blue was used to stain dead cells. An aliquot of the cell suspension was mixed with an equal volume of 0.4% (w/v) trypan blue in PBS and incubated for 10 min. Cells failing to exclude the dye were counted and expressed as a percentage of the total cells in the sample.

Tumor necrosis factor-α and interleukin-6 measurements. The concentrations of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were measured in peritoneal macrophage cultures using commercial ELISA kits (Ebioscience, Austria).

Statistical analysis. Data shown represent the mean \pm SEM. One-way analysis of variance (ANOVA) was carried out to determine the differences among the groups. All analyses were carried out using SPSS

software 19. Data were considered statistically significant at $P \leq 0.05$.

RESULTS

Ex vivo effect of *T. inchonensis* on NO production by mouse peritoneal macrophages

To evaluate the effect of *T. inchonensis* on NO production in mouse peritoneal macrophages stimulated by LPS plus IFN- γ , peritoneal macrophages of Suriyan mice were collected and treated with 20 U/ml IFN- γ plus 10 μ g/ml LPS for 24 h. The effects were statistically significant in all groups (Figure 1). *T. inchonensis* was not observed to have any effect on the viability of peritoneal macrophages using the trypan blue exclusion assay (cell viability was greater than 90% for all groups).

T. inchonensis inhibits TNF- α production

To investigate the suppression of TNF- α production by *T. inchonensis*, a cell-based assay was

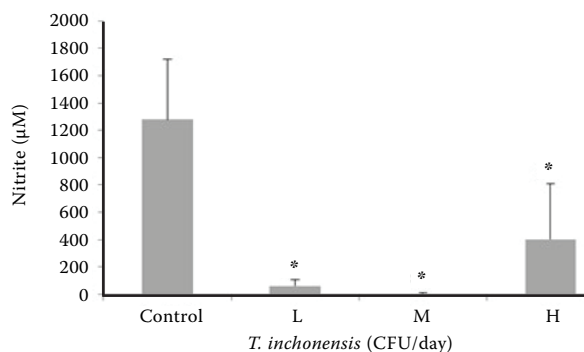


Figure 1. *Ex vivo* effect of *T. inchonensis* on lipopolysaccharide plus interferon- γ -induced NO production in mouse peritoneal macrophages (four groups, five mice per group). Peritoneal macrophages were incubated with 10 μ g/ml lipopolysaccharide plus 20 U/ml interferon- γ for 24 h. The nitrite content of culture media was analysed. Columns (vehicle) represent activated peritoneal macrophages with lipopolysaccharide plus interferon- γ . Data represent mean \pm SEM, values are from three independent experiments

H = 2×10^8 CFU/day of *T. inchonensis*; M = 1×10^8 CFU/day of *T. inchonensis*; L = 5×10^7 CFU/day

* $P < 0.05$ compared with the lipopolysaccharide plus interferon- γ -treated cells (vehicle)

performed using murine macrophages, which are known to respond to LPS and produce inflammatory cytokines such as TNF- α (Nagahira et al. 2001).

T. inchonensis suppressed LPS-induced TNF- α production in murine peritoneal macrophages as measured by ELISA (Figure 2).

T. inchonensis inhibits IL-6 production

IL-6 production by LPS-challenged macrophages was significantly inhibited by *T. inchonensis* (Figure 3).

DISCUSSION

In this study, we used an *ex vivo* strategy to determine the effect of oral administration of *T. inchonensis* on cytokine production by cultured leukocytes. The main finding of our study is that prior exposure to certain actinomycete bacteria alters IL-6, TNF- α cytokine and nitric oxide production by peritoneal cells.

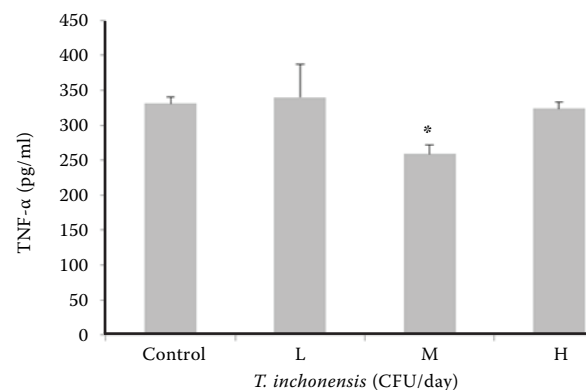


Figure 2. Tumor necrosis factor- α (TNF- α) levels in peritoneal cell (1.5×10^5 cells/ml) cultures from mice fed three doses of *T. inchonensis*. Peritoneal cells were obtained 10 days after administration of *T. inchonensis* as described in the Material and Methods and cultured in the presence of inducing agents (lipopolysaccharide, 1 μ g/ml). Culture supernatants were collected after two days and assayed for tumor necrosis factor- α by ELISA. Data represent mean \pm SEM of triplicate cultures ($n = 5$)

H = 2×10^8 CFU/day of *T. inchonensis*; L = 5×10^7 CFU/day of *T. inchonensis*; M = 1×10^8 CFU/day of *T. inchonensis*

*Significant differences with respect to the control group ($P \leq 0.05$)

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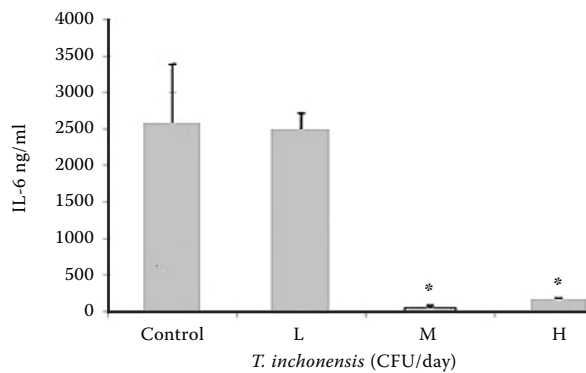


Figure 3. Interleukin-6 (IL-6) levels in peritoneal cell (1.5×10^5 cells/ml) cultures from mice fed three doses of *T. inchoensis*. Peritoneal cells were obtained 10 days after administration of *T. inchoensis* as described in the Material and Methods and cultured in the presence of inducing agents (lipopolysaccharide, 1 μ g/ml). Culture supernatants were collected after two days and assayed for interleukin-6 by ELISA. Data represent mean \pm SEM of triplicate cultures ($n = 5$)

H = 2×10^8 CFU/day of *T. inchoensis*; M = 1×10^8 CFU/day of *T. inchoensis*; L = 5×10^7 CFU/day of *T. inchoensis*

*Significant differences with respect to the control group ($P \leq 0.05$)

This study examined the effect of two different mitogenic signals on peritoneal cells. One signal was provided by LPS from *Pseudomonas aeruginosa*, which is a macrophage activator (Moore et al. 2000) at the concentration (10 μ g/ml) employed here. Moreover, IFN- γ , which primes macrophages for activation but does not in itself activate macrophages (Mosser 2003), was used. These two compounds can affect both macrophages and T cells (Tewari et al. 2007). The peritoneal cavity is often used as a concentrated source of mature murine macrophages. Peritoneal lavage yields $2\text{--}8 \times 10^6$ cells of which 20 to 40% are macrophages; however, other cell types are also present (Tejada-Simon et al. 1999). Thus, other cells besides macrophages may contribute to the findings observed in peritoneal cell cultures.

The results indicated that heat-killed *T. inchoensis* enhanced the suppression of nitric oxide production in two treatment groups. Similar results have been observed with lactobacilli (Tejada-Simon et al. 1999), but no anti-inflammatory effect of *T. inchoensis* has previously been described. Consequently, in the present study, the effect of heat-killed *T. inchoensis* on NO production in murine macrophages *ex vivo* at doses of 5×10^7 , 1×10^8 and 2×10^8 CFU/mouse was investigated.

NO is a short-lived free radical, synthesized from arginine and with very high reactivity. It possesses a variety of physiological activities involved in the regulation of blood vessel dilation and the immune response, and it functions as a neurotransmitter (Moncada and Erusalimsky 2002; Azadmehr et al. 2009). Inducible nitric oxide synthase, which is expressed in many cells including macrophages, is induced by proinflammatory cytokines and LPS (Xu et al. 2012). However, excess production of NO is associated with several diseases such as atherosclerosis, septic shock and chronic inflammatory diseases (Azadmehr et al. 2009; Luiking et al. 2010). In the current study, heat-killed *T. inchoensis* significantly inhibited NO production in mouse peritoneal resident macrophages activated by LPS plus IFN- γ in a concentration-dependent manner. These results show that *T. inchoensis* is a strong inhibitor of NO production in activated macrophages. As NO is an important mediator of inflammation, increased NO production has been implicated in inflammatory disease and autoimmunity. Therefore, it was expected that *T. inchoensis* should have beneficial anti-inflammatory effects. The suppressive effects that were observed were not attributable to cytotoxicity.

Treatment with *T. inchoensis* altered the production of IL-6. Interestingly, IL-6 was downregulated by *T. inchoensis*, although it has been reported that the pro-inflammatory cytokine IL-6 is induced by LPS (Riedemann et al. 2003; Beurel and Joep 2009). Interleukin-6 is a 212-amino acid polypeptide known to have a variety of biological effects (Riedemann et al. 2003). Elevated IL-6 levels in both humans and animals are associated with increased mortality (Vyas et al. 2005). Interleukin-6 is considered to be one of the major markers of lethal sepsis (Bozza et al. 2007), for example, as demonstrated in studies using IL-6 knockout mice (Remick et al. 2005). However, it does not appear to be a viable target for treatment, because anti-IL-6 strategies were unsuccessful in short-term mortality studies (Vyas et al. 2005). IL-6 is considered to be a proteolysis-inducing factor in muscle. It is implicated in cancer cachexia where muscle atrophy is evident (Tsujioka et al. 1996) and induces skeletal muscle protein breakdown (Goodman 1994). IL-6 also induces hepatocyte proliferation; this process is mediated through STAT3 (Ren et al. 2002). On the other hand, increased brain IL-6 has been associated with acute cognitive impairments (Beurel and Joep 2009)

and likely contributes to the long-term cognitive and neuroanatomical sequelae of sepsis, such as behavioural deficits and neuronal loss (Semmler et al. 2007). The reports of Riedemann et al. (2003) and Gennari and Alexander (1995) indicate that limiting the biological activity of IL-6 improves survival.

In the present study, we have shown that *T. inchoensis* inhibited LPS-induced TNF- α in a dose-dependent manner. This inhibition did not have effect on macrophage viability. The slight increase in TNF- α levels seen in the low dose group is likely due to non-specific effects or macrophage activation, which has been shown to occur in isolated peritoneal macrophages (Howard et al. 2009). Dexamethasone impedes the production of TNF by endotoxin-stimulated macrophages, but pretreatment of macrophages with dexamethasone prior to endotoxin stimulation was essential for effective inhibition (Morrison 1987). Tumor necrosis factor- α , one of the most important pro-inflammatory cytokines, is secreted mainly from activated monocyte/macrophages in response to diverse extracellular stimuli, and plays a major role in inflammatory diseases such as septic shock and rheumatoid arthritis (Nagahira et al. 2001). Tumor necrosis factor is intrinsically pyrogenic, similar to IL-1. It also has the ability to induce IL-1 with its consequent spectrum of potent biological activities (Dinarello et al. 1986). Lipopolysaccharide is known to be a strong stimulator of TNF- α production (Ohnishi et al. 1994). Lipopolysaccharide-stimulated TNF- α production in monocyte/macrophages is positively regulated at the transcriptional and translational levels (Beutler 1990). Interferon- γ has been shown to augment TNF biosynthesis (Beutler 1990), apparently acting to up-regulate both the transcriptional and post-transcriptional responses to LPS. Current evidence indicates that macrophages play an important role in the pathogenesis of inflammatory responses through their ability to produce proinflammatory cytokines (Delgado et al. 1999). Proinflammatory cytokines, which include TNF- α and IL-6, are generated in tissues infected by microbial pathogens as well as in tissues subjected to generalised trauma such as ischaemia/reperfusion injury. Production of inflammatory mediators serves a necessary function by facilitating wound healing, partly by recruiting various immune cell populations.

In conclusion, this preliminary study showed that oral administration of heat-killed *T. inchoensis*

has advantageous effects in the mouse, affecting parameters such as nitric oxide, TNF- α and IL-6 levels. Further investigations are needed to fully understand the interaction between this organism and different animals at the molecular level.

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