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Heat killed *Dietzia maris* reduces lipopolysaccharide-induced inflammatory responses in murine adherent peritoneal cells

K. Nofouzi[1*], B. Ghahvechi-Dizaji[2], G.H. Zarrini[2], B. Baradaran[3], G.H. Hamidian[4], R. Safaralizadeh[2], G. McIntyre[5], P. Ripley[6], S. Mousavi[7]

[1]Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran

[2]Department of Animal Biology, Faculty of Natural Science, University of Tabriz, Tabriz, Iran

[3] Immunology Research Center, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

[4]Department of Basic Sciences, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran

[5] Centre for Infectious Diseases and International Health, Windeyer Institute of Medical Sciences, University College London, 46 Cleveland Street, London, United Kingdom

[6] BioEos Ltd., Mill House, Claygate, Marden, Near Tonbridge, Kent, UK

[7]Department of Food Hygiene and Aquatic Animals, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran

ABSTRACT:

Dietzia maris (*D. maris*) is a gram-positive, aerobic, mycolic acid-containing actinomycete without mycelium. Actinomycetes such as *Tsukamurella inchonensis* reduce lipopolysaccharide- induced inflammatory responses in activated murine peritoneal macrophages. Here, the effects of *D. maris* on LPS-induced inflammatory responses were examined in mouse adherent peritoneal cells. *D. maris* was grown, harvested, and washed. Suspensions were standardized by wet weight, re-suspended in borate-buffered saline, and autoclaved. For *in vivo* study, each mouse was orally administered by bacterial suspension (5×10^7, 1×10^8 and 2×10^8 CFU/Mouse) consecutively for seven days. Control animals received the same amount of phosphate-buffered saline (PBS). Adherent peritoneal cells were harvested for *in vitro* experiments. Cells were lavaged and plated in RPMI 1640 medium, stimulated with LPS (100 ng/ml), and incubated for 2 h. Afterward, non-adherent cells were removed followed by adding freshly prepared medium. Supernatants (50 µl) were collected, centrifuged, mixed with Griess reagent, and the absorbance was measured at 560 nm. *D .maris* inhibited LPS-stimulated nitric oxide (NO) production in murine macrophages at concentrations of 5×10^7, 1×10^8 and 2×10^8 CFU/Mouse. Also, *D. maris* decreased LPS-induced production of pro-inflammatory cytokines of interleukin (IL)-6 at all doses. By contrast, tumor necrosis factor (TNF)-α was not effected by *D. maris* treatment of mice. Our results indicate that *D. maris* is a potent inhibitor of LPS-induced NO production. *D. maris* may be useful as a novel agent for the chemoprevention of inflammatory disease.

***Keywords*:** Adherent peritoneal cells; Anti-inflammation; *Dietzia maris*; IL-6; Lipopolysaccharide

Corresponding Author:
K. Nofouzi, Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran
E-mail address: nofouzi@tabrizu.ac.ir

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INTRODUCTION

Inflammation is a process mediated by the host's microcirculatory system to protect against a wide range of injuries (Xu et al., 2012). Inflammation is the result of complex interactions between immune and inflammatory cells, their mediators, as well as regulators, and is part of the innate immune response. There are several differences between inflammation in health and disease. One of the most important differences is that in a disease state, inflammatory cells and pathways continue to perpetuate inflammatory cycles regardless of the tissue structure; on the contrary, during inflammation in a state of health, the response is highly self-limited to a specific stimulus working with a "pro-gramme" to minimize tissue damage (Gallo et al., 2017). Nitric oxide (NO) is a short-lived biomolecule that mediates many biological functions, including host defense, vasoregulation, platelet aggregation, and neurotransmission (Azadmehr et al., 2009). In addition to its physiological functions, NO is also implicated in the pathology of many inflammatory diseases, including arthritis, myocarditis, colitis, and nephritis, and a large number of pathological conditions such as amyotrophic lateral sclerosis (ALS), cancer, diabetes, and neurodegenerative disease (Davis et al., 2001). Therefore, inhibition of NO production has become a therapeutic target of treatment for inflammatory disease.

Current evidence indicates that macrophages play an essential role in the pathogenesis of the inflammatory responses by their ability to produce cytokines such as IL-6 and TNF-α. TNF-α is a monocyte-macrophage derived cytokine that acts as an essential mediator in the defense mechanism of the host in response to bacterial colonization or invasion and causes immunopathologic disorders when secreted in excess (Delgado et al., 1999). The toxicity caused by Gram-negative bacteria has been ascribed to LPS (Lipopolysaccharides), an outer membrane component of bacteria. LPS represents one of the most potent inducers of TNF-α and at high concentrations causes tissue injury, fever, disseminated vascular coagulation, and septic shock, often resulting in death (Tracey and Cerami, 1993).

Interleukin-6 (IL-6) is a cytokine with a broad range of biological activities (Remick et al., 2005). IL-6 functions as an essential and sensitive indicator of inflammation within the body. IL-6 is a key player in chronic inflammation, and IL-6 levels are elevated in inflammatory diseases in humans. Expression of IL-6 is increased at the site of inflammation and blockade of IL-6, and IL-6 signaling is effective in the prevention and treatment in models of inflammatory diseases.

Dietzia maris (*D. maris*) is a Gram- positive, aerobic, mycolic acid-containing actinomycete without aerial mycelium (Pidoux et al., 2001). In a previous report, it was found that *Tsukamurella inchonensis* (*T. inchonensis*), an aerobic species of Actinomycetales, could reduce inflammatory responses in murine peritoneal macrophages (Nofouzi et al. 2017). Therefore, our study aimed to assess the effects of another actinomycete, *D. maris* on mice adherent peritoneal cells.

MATERIALS AND METHODS

Animals

This experimental study was approved (FVM. REC. 1395.59) by the Ethics Committee of the School of Veterinary Medicine, Tabriz University. Male albino laboratory mice, *Mus musculus* Linn. (20-22 g; 7-8 weeks old) were obtained from the Pasteur Institute (Tehran, Iran). Mice (four groups, five mice per group) were randomized and housed in polyester cages. Because study did not aim to examine sex-associated differences, only male mice were used here to minimize the stress associated with intracage fighting. The animals were maintained under standard laboratory conditions at 25 ± 2 ºC and a photoperiod of L/D 12: 12 h, and received a standard mouse chow and water *ad libitum*. Animal care and handling throughout the experimental procedures was in accordance to Iran National Committee for Ethics in Biomedical Research legislations and Presidential Decree 56/2013, in compliance with the Directive 2010/63/EU on the protection of animal used for scientific purposes.

Strain preparation

D. maris was grown in Sauton's medium, harvested by centrifugation, and washed in borate-buffered saline (pH 8.0). Suspensions were standardized by wet weight, re-suspended in borate-buffered saline, and autoclaved at 121 ºC for sterilization.

In vivo exposure to *D. maris*

For the *in vivo* study, after successful completion of a two-week quarantine period, each mouse received orally 200 µl of administered bacterial suspension (5×10^7, 1×10^8 and 2×10^8 cells/mouse) by gavage consecutively for 7 days (Fig. 1). An expert experimenter in oral gavage of mice administered all treatments

using a straight, 20-gauge stainless steel bulb tipped gavage needle once daily between 09:00 and 10:00 am. The needle was never forced down the esophagus and all animals were monitored for at least 15 min after oral gavage. The control animals received the same amount of phosphate-buffered saline (PBS). After 7 days of treatment, the mice were anesthetized with isoflurane (1.5-2.5%) to harvest peritoneal macrophages.

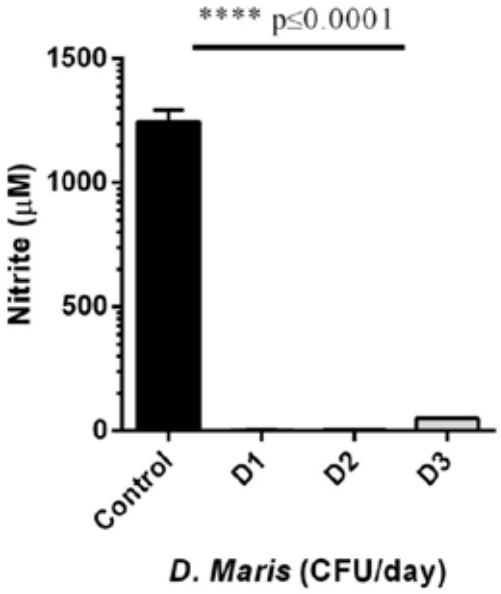

Figure 1. Inhibition of LPS-induced NO production by ***D. maris*** in murine macrophages. Murine macrophages were treated with LPS (100 ng/ml) in PBS (control), 5×10^7 of ***D. maris*** (D1), 1×10^8 of ***D. maris*** (D2) and 2×10^8 of ***D. maris*** (D3) for two h. The cell culture supernatant was collected and used to determine NO levels. Data are expressed as the mean ± SEM of triplicate experiments. Statistical analysis was performed using one-way ANOVA andTurkey's multiple-comparison post hoc tests.

Adherent peritoneal cells isolation, cell culture, and determination of NO production

For *in vitro* experiments, adherent peritoneal cells were harvested immediately by lavaging with ice-cold sterile PBS. Cells were lavaged twice and plated in RPMI 1640 medium (Sigma Chemical Co.) containing 100 U/mL penicillin/100 µg/mL streptomycin (Sigma Chemical Co.), 10% Fetal Bovine Serum (GIBCO), stimulated with LPS (100 ng/ml), and incubated in a 5% CO_2 humidified incubator at 37 ˚C for 2 h. Afterward, non-adherent cells were removed by gently washing with PBS, followed by adding a freshly prepared medium (Nofouzi et al., 2017). Supernatants (50 µl) were collected, centrifuged at 600 × g for 5 min, and mixed with 100 µl of Griess reagent [1% sulfanilamide in 5% phosphoric acid and 0.1% N-(1-naphthyl) ethylenediamide dihydrocholide in distilled water]. Absorbance was measured at 530 nm using a Wallac 1420 ARVO Sx (Perkin Elmer, Inc., Waltham, MA).

Cytokine protein array

Peritoneal cells were harvested by lavage of the corresponding site with ice-cold PBS. The lavage was performed in a single round with 60 ml of PBS. The cell extract was concentrated by centrifugation (600×g, 10 min, 4 °C), and the pellet was resuspended and incubated with ACK lysis buffer at room temperature for 3 min. The resulting cell extract was then pelleted by centrifugation, and resuspended in RPMI 1640 medium (Sigma Chemical Co.) containing 100 U/mL penicillin/100 µg/mL streptomycin (Sigma Chemical Co.), 10% Fetal Bovine Serum (GIBCO), stimulated with LPS (100 ng/ml), and incubated in a 5% CO_2 humidified incubator at 37 ˚C for 2 h. The cells were then plated at a density of 1×10^6 live cells (trypan blue exclusion) per well in 12-well, flat-bottom polystyrene plates, and cultured at 37 ˚C and 5% CO2. Non-adherent cells were discarded twice: the first time at 30 min after incubation with a medium containing 2% FBS, and the second time after overnight incubation with a medium containing 10% FBS. Subsequently, the adhered cells were examined in the medium containing 10% FBS. A 1-h resting period was allowed between the second medium change and the stimulation with LPS to generate supernatants. Macrophages (5×10^5 cells) on 24 well plates were preincubated for 24 h and then treated with LPS (100 ng/ml) for 2 h. IL-6 and TNF-α in the culture medium were screened using commercial ELISA kits (Ebioscience, Austria).

Statistical analysis

Data are shown as means ± SD (standard deviation) from five mice. Comparisons between groups were made using one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison post hoc test (SPSS software for windows release 21.0; SPSS Inc., Chicago, USA). Differences were considered statistically significant at $p < 0.05$. In order to monitor the risks for decision error in statistical inference from a minimum of five mice per group, the statistical power of the study sample size was analyzed by G Power software (Version 3.1., Heirich Heine University, Düsseldorf, Germany) to detect an efficient group size at a 80% power (1-β err prob = 0.8), 95% confidence (α = 0.05), and an anticipated effect size

d>1. The actual power of statistical analysis of this study was 0.82.

RESULTS

Effects of *D. maris* on LPS-induced NO production in mouse macrophages

As shown in Figure 2, *D. maris* significantly inhibited LPS-induced NO production ($p < 0.001$) in all groups. *D. maris* 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).

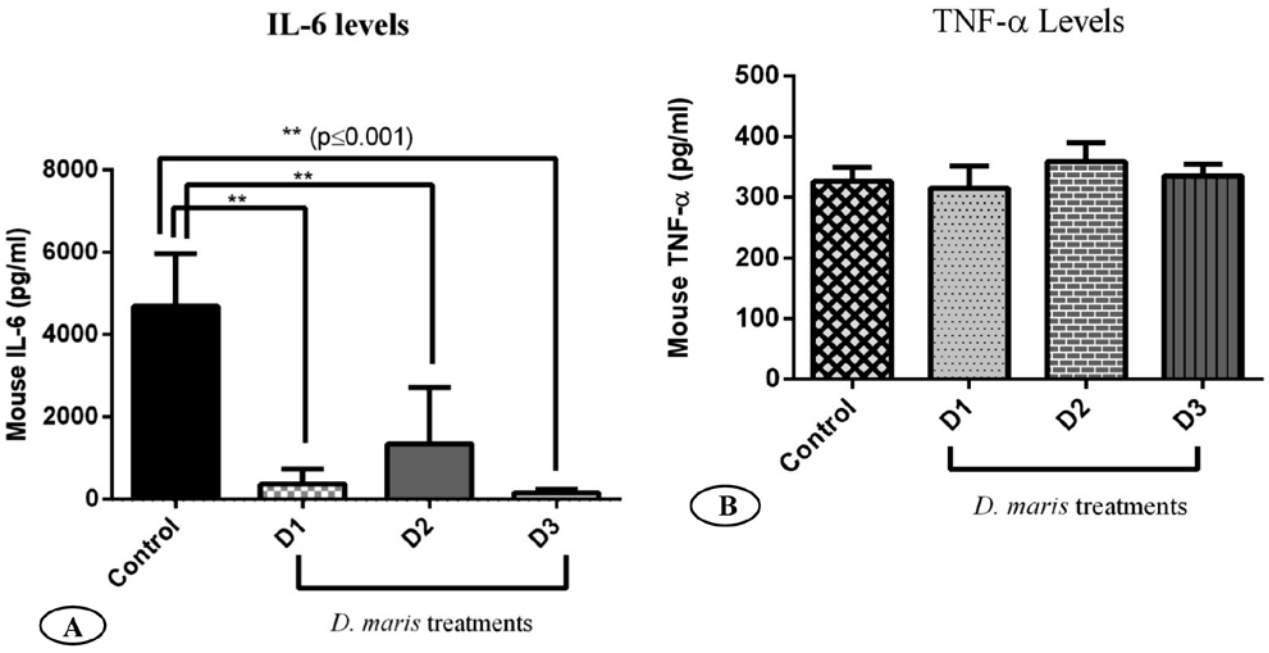

Figure 2. Levels of IL-6 and TNF-α produced by peritoneal macrophages from mice orally treated with *D. maris*. A) IL-6 level significantly different from PBS (control) group. B) *D. maris*did not alter TNF-α level. Murine macrophages were treated with LPS (100 ng/ml) in PBS (control), 5×10^7 of *D. maris* (D1), 1×10^8 of *D. maris* (D2) and 2×10^8 of *D. maris* (D3) for 2 h. Vertical bars represent the mean of measurements of five mice per group, with error bars denoting the standard error of mean.Statistical analysis was performed using one-way ANOVA and Turkey's multiple-comparison post hoc tests.

Effects of *D. maris* on IL-6 production

Since macrophage-derived cytokines are critical in a variety of inflammatory processes, the impact of *D. maris* was evaluated on the output of two cytokines by peritoneal macrophages using an ELISA technique (Fig. 3 and 4). The creation of IL-6 was suppressed by *D. maris* (by 5×10^7, 1×10^8 and 2×10^8 cells/mouse *vs*. LPS-treatment) as shown in Figure 3.

Effects of *D. maris* on TNF-α production

To investigate the suppression of TNF-α production by *D. maris*, a cell-based assay was performed using murine macrophages, which are known to respond to LPS and produce inflammatory cytokines such as TNF-α. *D. maris* could not suppress LPS-induced TNF-α production in murine peritoneal macrophages as measured by ELISA (Fig. 4).

DISCUSSION

D. maris has been isolated from soil, and the skin and intestinal tract of carp (Koerner et al., 2009). Several species of aerobic Actinomycetales, including *Gordonia bronchialis (G. bronchialis*), *Rhodococcus coprophilus (R. coprophilous*), and *T. inchonensis*, are capable of subtly different adjuvant or immunomodulatory activities when injected as suspensions of killed bacilli (Tarres et al., 2012). In animal models, it was found that preparation of this species was especially fortunate in preventing the inflammation of the intima of arteries damaged with a balloon catheter (Stanford and Stanford, 2012), and for the prevention and medical care of spontaneous type 2 diabetes mellitus (Tarres et al., 2012). Previously, we demonstrated the stimulatory effects of *T. inchonensis* on immune functions and enhancing immune barri-

er function in the intestines of mice (Nofouzi et al., 2016). A previous study reported even anti–inflammatory and immunomodulatory impacts of this species (Nofouzi et al., 2017). In the present study, therefore, the effects of *D. maris* at doses of 5×10^7 cell, 1×10^8 cell, and 2×10^8 cell *D. maris*/mouse were examined on NO production and some cytokines in mice. As our previous study, five mice were used per group because our ethics committee did not approve the use of extra mice in the present study. However, our work is confirmed by previous studies that used three to five mice per group (Azadmehr et al., 2009; Kalischuk et al., 2009; Gasting et al., 2010; Nofouzi et al., 2016). The main finding of our study is that prior exposure to certain actinomycete bacteria, alters IL-6, and nitric oxide production by peritoneal cells or not?

The spectra of products observed in our study with peritoneal cells suggest that macrophages are a crucial target for *D. maris* immunomodulatory activity. The bacteria capable of secreting IL-6 and NO could exert a wide scope of effects including stimulatory or inhibitory impacts. The potential to alter macrophage task is significant for several reasons. Macrophages constitute the other significant cell population of the immune system with phagocytosis being a primary task. They originate from bone marrow, and, after migration and maturation, settle in the tissue as mature macrophages (Tejada-Simon et al., 1999). These can be triggered by a variety of stimulants, and their main functions involve phagocytosis of foreign particles, antigen presentation, and making cytokines (IL-6, TNF-α, IL-1, IL-12) or reactive oxygen mediators, such as NO, which recruit other inflammatory cells. Thus, macrophages can participate in both humoral and cell-mediated immune responses.

Lipopolysaccharide has been exposed as the main initiating agent in Gram-negative sepsis due to the activation of inflammatory cells, particularly the mononuclear phagocyte, which releases a series of additional mediators including TNF-α, IL-1, and IL-6 (Revelli et al., 1999). Additionally, LPS stimulates iNOS gene expression and NO making. An earlier work demonstrated that *Helicobacter pylori* attenuated LPS-induced nitric oxide production by murine macrophages (Lu et al., 2011). The repressive effect was not due to cytotoxicity. As NO is an evaluative mediator in inflammation, increased NO production has been implicated in inflammatory and autoimmune diseases Although, NO has very high reactivity and a variety of physiological activities involved in the regulation of blood vessel dilation and immune response, and functions as a neurotransmitter (Azadmehr et al., 2009). If the inflammation becomes extreme or chronic, however, healthy host cells may also be damaged and killed by NO, resulting in inflammatory pathologies (Xu et al., 2012). The results of our study indicated that heat-killed *D. maris* enhanced the suppression of NO production in the two treatment groups. Similar results were observed with lactobacilli (Tejada-Simon et al., 1999) and *T. inchonensis* (Nofouzi et al., 2017).

In addition to the association of IL-6 in the generation of B and T cell-mediated responses, it should be recalled that this cytokine is primarily involved in the elicitation of the acute phase response to injury (Snick 1990). The IL-6 output by LPS-stimulated macrophages at all doses was significantly inhibited by *D. maris*. The same may be true for benzimidazole and *T. inchonensis* induced the suppression of IL-6 output (Revelli et al., 1999; Nofouzi et al, 2017). In contrast, Wang et al. demonstrated that *Ganoderma lucidum* enhanced LPS-induced IL-6 in mice macrophages (Wang et al., 1997).

There is plenty of confirmation that TNF-α is an essential mediator of shock and organ failure complicating Gram-negative sepsis (Delgado et al., 1999). TNF is combined and secreted very quickly by macrophages in response to endotoxin and may be detected in the circulation within one hour in experimental animals given endotoxin. Following synthesis, TNF is widely delivered in tissues and is then rapidly degraded (Morrison, 1987). TNF-α modulates the immune response by triggering the production of several other regulatory cytokines (Shin et al., 2006). The effect of *D. maris* on TNF-α release was inconclusive as clarifications should be made as to why NO is inhibited whereas TNF appears intact. Maybe the kinetics are different for testing both compound productions, for which more time point evaluations are necessary. Reis et al. (2008) found that proteasome inhibitors are potent inhibitors of NO production by LPS-stimulated macrophages, with TNF-α being inhibited to a lesser extent. *D. maris* likely causes depletions in inflammatory mediators by blocking proteasomal activity, rather than antagonizing one cytokine at a time. Our data are supported by those of Ahmadi-Renani and McCruden (1997) who found no change in TNF-α release with alpha dihydrotestestrone. Morrison and Silverstein (2000) reported that treatment of mice with killed *S. aureus* and *E. coli* did not affect TNF- α levels. Ishida-Fujii et al. (2007) described that peritoneal macrophages from rats fed

with *Lactobacillus casei* and intraperitoneally infected with *E.coli* could improve phagocytic activity and produced higher quantities of TNF- α. Neumann et al. (2009) showed that *L. delbrueckii* UFV-H2b20 stimulated the production of TNF-α in mouse *in vitro* . In our previous work on *T. inchonensis*, however, the bacteria decreased lipopolysaccharide-induced production of TNF- α level (Nofouzi et al., 2017).

CONCLUSION

According to our observations, *D. maris* acts as a NO inhibitor in adherent peritoneal cells. Also, this species attenuated LPS-induced IL-6 production. *D. maris*, therefore, demonstrates potential as a potent inhibitor of responses induced by inflammatory stimuli. Additional works will, however, be required before full potential therapeutic benefits of *D. maris* can be wholly understood and evaluated.

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CONFLICT OF INTEREST STATEMENT

None declared by the authors.

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