Comparative analysis of the production of nitric oxide (NO) and tumor necrosis factor-α (TNF-α) from macrophages exposed to high virulent and low virulent strains of Edwardsiella tarda

Keiko Ishibe [a], Tomohiro Yamanishi [a], Yajun Wang [b], Kiyoshi Osatomi[a, b], Kenji Hara [a, b], Kinya Kanai [a, c], Kenichi Yamaguchi [a, d], Tatsuya Oda [a, d,*]

[a]Graduate School of Science and Technology, Nagasaki University, Nagasaki 852-8521, Japan

[b]Department of Marine Biochemistry, Faculty of Fisheries, Nagasaki University, Nagasaki 852-8521, Japan

[c]Labolatory of Fish pathology, Faculty of Fisheries, Nagasaki University, Nagasaki 852-8521, Japan

[d]Division of Biochemistry, Faculty of Fisheries, Nagasaki University, Nagasaki 852-8521, Japan

Keywords: *Edwardsiella tarda*; Bacterial Infection; Japanese Flounder; Fish Peritoneal Macrophages; Mouse Macrophage Cell Line; Nitric Oxide; Tumor Necrosis Factor-α

[*]Corresponding author. Tel.: +81 95 819 2831; fax: +81 95 819 2799.

E-mail address: t-oda@nagasaki-u.ac.jp (T. Oda)

Abstract

We previously reported that high virulent strain (NUF251) of *Edwardsiella tarda* has an ability to prevent the production of reactive oxygen species by macrophages, and is even capable of surviving and multiplying within Japanese flounder peritoneal macrophages, whereas the low virulent strain (NUF194) has no such ability. In this study, we found that NUF251 and NUF194 induced NO and TNF-α production from Japanese flounder peritoneal macrophages, and NUF251 caused faster induction of NO release and much higher level of TNF-α production than NUF194. In addition, similar differences between two strains in terms of the induction of NO and TNF-α production were also observed in mouse macrophage cell line RAW264.7 cells. Our results suggest that the potent ability to induce the production of NO and TNF-α from macrophages may be one of the factors responsible for the virulence of *E. tarda*.

Edwardsiella tarda, a gram negative bacterium, is one of the major bacterial pathogens in freshwater and marine fish, and natural *E. tarda* infection has been recorded predominantly in Japanese eel, Japanese flounder, channel catfish [1,2], and in many other fish species [3]. Some mammals are also known to be infected with *E. tarda* [4]. Edwardsiellosis in Japanese flounder shows suppurative inflammation and hernia as main symptoms [5], and infectious disease caused by *E. tarda* is one of the most serious problems in aquaculture industry especially to Japanese flounder. In spite of the serious threat of *E. tarda* as a fish pathogen and the increasing frequency of the disease, little is known about the exact pathogenesis of *E. tarda* infection. Dermatotoxin [6], hemolysis [7], or ability to invade epithelial cells [8] have been suggested as potential virulence factors of this bacterium. In our previous study, we found that the responses of peritoneal macrophages of Japanese flounder (*Paralichtygs olivaceus*) against high virulent strain (NUF251) and low virulent strain (NUF194) of *E. tarda* were different [9], and our results suggested that NUF251 may have a ability to prevent ROS generation by macrophages and survive inside macrophages.

Macrophages play pivotal roles in the defense mechanism against invading bacteria by producing bactericidal agents such as NO and ROS, and proinflammatory cytokines (e.g. TNF-α). NO and TNF-α are known to play not only important roles in immune defense system but also to injure host cells and tissues [10-13]. Similar to mammalian immune systems, previous studies have demonstrated that several bacterial infections in fish are also accompanied with the induction of various inflammatory mediators. For instance, *Aeromonas hydrophila* induced iNOS-mediated NO production and secretion

of TNF-α and other cytokines from kidney cells of zebrafish (*Danio rerio*) [14]. NO release was observed in carp (*Cyprinus carpio* L.) kidney leukocytes during *in vivo* bacterial infection [15]. It has been reported that production of TNF-α like factor by rainbow trout head kidney macrophages was detected by L929 cell killing assay during *in vitro* bacterial infection model [16]. However, the exact roles of inflammatory factors such as NO and TNF-α in bacterial infection in fish are unclear. Therefore, in this study, we investigated the responses of Japanese flounder peritoneal macrophages and mouse macrophage cell line RAW 264.7 cells after exposure to high and low virulent strains of *E. tarda* in terms of NO and TNF-α production.

The culture and maintenance of two strains of *E. tarda* (NUF251 and NUF194), the details of handling of Japanese flounder used for the preparation of peritoneal macrophages, and the culture of the macrophages were executed by the same methods as described previously [9]. To detect the production of NO, we employed Griess method that is an assay for spectrophotometrical quantification of nitrite, a stable reaction product of nitric oxide with molecular oxygen, using Griess reagent (3 mM sulfanilic acid, 30 μM N-1-naphtyl-ethlenediamine dihydrochloride, 25% glacial acetic acid). The fleshly isolated peritoneal macrophage population from Japanese flounder were cultured in 96 well-plates (2×10^4 cells/well) in RPMI 1640 medium containing 10% FBS for 24 h at 25℃, and then *E. tarda* cells (final 2×10^4 CFU/well) were added and incubated at 25℃. After the indicated periods of time, the culture medium was collected to determine nitrite concentration. As shown in Fig. 1, both strains of *E. tarda* induced NO production from Japanese flounder peritoneal macrophages in a

time-dependent manner, and NUF251 induced NO production with faster kinetics than that of NUF194. After 3 h incubation, the NO level in the culture medium reached the maximum when exposed to virulent NUF251, whereas only half of the maximum level of NO was detected in the case of low virulent strain NUF194. To ascertain whether or not Japanese flounder peritoneal macrophages can produce TNF-α into the culture medium responding to *E. tarde*, we employed sandwich enzyme-linked immunosorbent assay (ELISA) with monoclonal and polyclonal anti-mouse TNF-α antibodies (R&D Systems, Minneapolis, MN) to two different epitopes on mouse TNF-α molecule. The ELISA procedure was performed according to the manufacturer's protocol. The TNF-α concentration was estimated from a reference to a standard curve for serial twofold dilution of mouse recombinant mouse TNF-α (R&D Systems, Minneapolis, MN). As shown in Fig. 2, low but significant level of TNF-α was detected in the culture medium of Japanese flounder macrophages exposed to NUF251, whereas no significant level of TNF-α was induced by NUF194 even after 48 h incubation. Although these results suggest that NUF251 has a greater activity to induce TNF-α secretion from Japanese flounder macrophages than NUF194, it is uncertain if the exact level of fish TNF-α could be detected by our ELISA system in which anti-mouse TNF-α antibodies were used instead of the antibodies against actual Japanese flounder TNF-α. To confirm the different responses of macrophages against NUF251 and NUF194, we carried out the same analysis regarding NO and TNF-α production in mouse macrophage cell line RAW264.7.

RAW264.7 cells were from the American Type Culture Collection (Rockville, MD),

and were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% FBS, penicillin (100 IU/mL), and streptomycin (100 μg/mL) as described previously [17]. To adherent RAW264.7 cells in 96-well plates (2 x 10^4 cells/well), *E. tarda* cells (final 2×10^4 CFU/well) were added. After the indicated periods of time, NO levels in the culture medium were determined as described above. As shown in Fig. 3A, similar to Japanese flounder macrophages, the higher level of NO production with faster kinetics from RAW264.7 cells were induced by NUF251 as compared to that induced by NUF194. The presence of an inducible pathway for NO production from L-arginine catalyzed by inducible NO synthase (iNOS) has been well documented in macrophages in response to cytokines and LPS [18,19]. Therefore, we investigated the kinetics of expression level of iNOS mRNA in RAW264.7 cells exposed to two strains of *E. tarda* by RT-PCR. The RT-PCR was performed by the following procedure. Adherent RAW264.7 cells in 12 well-plate (2×10^5 cells/well) were exposed to each strain of *E. tarda* (final 2×10^5 CFU/well). After the incubation for the indicated periods time, total RNA was isolated from the cells with Trizol reagent (Invitrogen). Total RNA (1 μg) was reverse-transcribed with an oligo dT primer in a 10-μL reaction volume using PrimeScript 1^{st} strand cDNA synthesis Kit (TaKaRa) according to the manufacturer's instructions. PCR was performed with 1 cycle of 70 sec at 95°C, 20 cycles of 55 sec at 93°C, 45 sec at 61°C, 40 sec at 72°C, and 1 cycle of 100 sec at 72°C, in a 25-μL reaction mixture containing 12.5 μL of GoTaq Green Master Mix (Promega), 0.5 μL of forward and reverse primers (1 μM each), 0.5 μL of 1^{st} strand cDNA, and 11μL of nuclease-free water. The primer sets for iNOS and β-actin were

synthesized as described previously [20,21]. Each PCR reaction (25 μL) was run on 2% agarose gels containing 0.1 μg/mL ethidium bromide. As shown in Fig. 3B, time-dependent increase in iNOS mRNA level in RAW264.7 cells was observed after exposure to NUF251, which has evidently faster kinetics than that of NUF194.

We have previously found that RAW264.7 cells are capable of producing quite high level of TNF-α in response to various stimuli [17,22]. Reflecting with this, nearly 1000 times higher levels of TNF-α were detected in the culture medium of RAW264.7 cells exposed to *E. tarda* comparing to those of Japanese flounder macrophages. This may also be partly due to the high efficiency of the ELISA system for the detection of mouse TNF-α produced by RAW264.7 cells. Consistent with the results obtained in Japanese flounder macrophages, much higher level of TNF-α was induced by NUF251 than by NUF194. These results suggest that NUF251 has a potent ability to stimulate mouse macrophages as well as fish macrophages, and induce higher levels of NO and TNF-α production. Such ability may be somehow responsible for the virulence of *E. tarda*.

NO and TNF-α generally act as defense factors toward invading bacterial pathogens, and are essential elements of antimicrobial immunity during inflammation. A number of studies have supported this conventional wisdom in immunological system of mammals. For instance, cooperation between reactive oxygen and nitrogen intermediates in killing of *Rhodococcus equi* by activated macrophages has been reported as major defense mechanism in murine model [12]. It has been reported that TNF-α plays protective roles against *Mycobaterium tuberculosis* in mice [13]. Moreover, it has been shown that membrane TNF molecules located on the cell surface

of macrophages were deeply involved in the host resistance mechanism against mycobacterial infection [23].

In addition to the defensive roles or beneficial effects in host, NO and TNF-α are also known to behave as toxic and injurious factors toward host cells and tissues when they were overproduced exceeding the critical levels. Especially during sepsis shock or acute inflammation, NO and TNF-α can be causative factors for circulatory and multiple organ failure [24,25]. Pneumolysin, a protein exotoxin produced by *Streptococcus pneumoniae*, was found to be an important pneumococcal virulence factor, and it has shown that pneumolysin exerts potent ability to induce the production of NO and various inflammatory cytokines [10]. This study also proposed that pneumolysin-induced production of large amounts of NO might be responsible for hypotension, a critical pathogenic feature of septic shock [10]. Other studies have reported that pneumococcal cell wall components are capable of inducing NO production *in vitro* [26,27]. Furthermore, two cell wall components of *Staphylococcus aureus*, lipoteichoic acid and peptidoglycan, have been reported to cause induction of NO, shock, and organ injury [28].

Excessive amount of TNF-α secretion has also been suggested to lead to lethal damage to the host during the inflammation. It has been well documented that LPS from Gram-negative bacteria stimulates monocytic cells to induce TNF and other proinflammatory cytokines that mediate multi organ failure and lethality in experimental animal models of endotoxin shock [11]. In addition, TNF has been identified as a common mediator of hepatocellular apoptosis and liver injury in

experimental mouse models [29]. In the case of acute hepatotoxicity caused by *Pseudomonas aeruginosa* exotoxin A in mice, it has been shown that neutralization of TNF by anti-TNF antibody prevented liver injury [30]. These findings evoke the notion that inflammatory mediators such as NO and TNF-α produced by macrophages in response to invading bacteria can cause serious detrimental effects on the host apart from the beneficial effects. Therefore, we would like to propose that the potent ability of virulent *E. tarda* (NUF251) to induce NO and TNF-α production from macrophages is one of the important virulence factors. Although the exact mechanism is still unclear, highly lethal pathogenesis of NUF251 in fish can be attributed to the cooperation between the ability of preventing ROS generation by macrophages and surviving inside macrophages which may enable the bacteria to cause septic shock, and the ability of induction of large amounts of NO and TNF-α from macrophages which may contribute to serious host inflammatory damage. Further studies are required to gain insight into the action mode of NUF251 on macrophages and the underlying mechanism or responding factors.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research from Nagasaki University, Japan, and by grants from Nagasaki Prefecture collaboration of Regional Entities for Advancement of Technological Excellence, Japan Science and Technology Agency, and from the Ministry of Education, Science, Sports and Culture, Japan.

Reference

[1] Kusuda R, Salati F. Major bacterial diseases affecting mariculture in Japan. Ann Rev Fish Dis 1993; 3: 69-85.

[2] Darwish A, Plumb JA, Newton JC. Histopathology and pathogenesis of experimental infection with *Edwardsiella tarda* in Channel Catfish. J Aquat Anim Health 2000; 12: 255-266.

[3] Thune RL, Stanley LA, Cooper RK. Pathogenesis of gram-negative bacterial infections in warm water fish. Annu Rev Fish Dis 1993; 3: 37-68.

[4] Janda JM, Abbott SL. Infections associated with the genus *Edwardsiella*: the role of *Edwardsiella tarda* in human disease. Clin Infect Dis 1993; 17: 742-748.

[5] T. Miyazaki, N. Kaige. Comparative histopathology of edwardsielosis in fishes. Fish Pathol 1985; 20, 219-227

[6] Ullah MA, Arai T. Pathological activities of the naturally occurring strains of Edwardsiella tarda. Fish Pathol 1983; 18: 65-70.

[7] Hirono I, Tange N, Aoki T. Iron-regulated haemolysin gene from *Edwardsiella tarda*. Mol Microbiol 1997; 24: 851-856.

[8] Ling SHM, Wang XH, Xie L, Lim TM, Leung KY. Use of green fluorescent protein (GFP) to track the invasion pathways of *Edwardsiella tarda* in *in vivo* and *in vitro* fish models. Microbiology 2000; 146: 7-19.

[9] Ishibe K, Osatomi K, Hara K, Kanai K, Yamaguchi K, Oda T. Comparation of the responses of peritoneal macrophages from Japanese flounder (*Paralichthys olivaceus*) against high virulent and low vilurent strains of *Edwardsiella tarda.* Fish

Shellfish Immunol. 2008; 24(2): 243-51

[10] Braun JS, Novak R, Gao G, Murray PJ, Shenep JL. Pneumolysin, a protein toxin of Streptococcus pneumoniae, induces nitric oxide production from macrophages. Infect Immun. 1999; 67 (8): 3750-6.

[11] Freudenberg MA, Galanos C. Tumor necrosis factor alpha mediates lethal activity of killed gram-negative and gram-positive bacteria in D-galactosamine-treated mice. Infect Immun. 1991; 59(6): 2110-5.

[12] Darrah PA, Hondalus MK, Chen Q, Ischiropoulos H, Mosser DM. Cooperation between reactive oxygen and nitrogen intermediates in killing of *Rhodococcus equi* by activated macrophages. Infect Immun. 2000; 68 (6): 3587-93.

[13] Flynn JL, Goldstein MM, Chan J, Triebold KJ, Pfeffer K, Lowenstein CJ, Schreiber R, Mak TW, Bloom BR. Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice. Immunity. 1995; 2(6): 561-72.

[14] Rodríguez I, Novoa B, Figueras A. Immune response of zebrafish (Danio rerio) against a newly isolated bacterial pathogen Aeromonas hydrophila. Fish Shellfish Immunol. 2008; 25(3): 239-49.

[15] Pietsch C, Vogt R, Neumann N, Kloas W Production of nitric oxide by carp (Cyprinus carpio L.) kidney leukocytes is regulated by cyclic 3',5'-adenosine monophosphate.Comp Biochem Physiol A Mol Integr Physiol. 2008; 150(1):58-65.

[16] Qin QW, Ototake M, Noguchi K, Soma G-I, Yokomizo Y, Nakanishi T. Tumor necrosis factor alpha (TNF α)-like factor produced by macrophages in rainbow

trout, *Oncorhynchus mykiss.* Fish Shellfish Immunol. 2001; 11(3):245-56.

[17] Yamanishi T, Yamamoto Y, Hatakeyama T, Yamaguchi K, Oda T.CEL-I, an invertebrate N-acetylgalactosamine-specific C-type lectin, induces TNF-alpha and G-CSF production by mouse macrophage cell line RAW264.7 cells. J Biochem. 2007; 142(5): 587-95.

[18] Sethi, G. and Sodhi, A. In vitro activation of murine peritoneal macrophages by ultraviolet B radiation: up regulation of CD18, production of NO, proinflammatory cytokines and signal transduction pathway. Mol Immunol 2004; 40: 1315-1323.

[19] Eason, S. and Martin, W. Involvement of tyrosine kinase and protein kinase C in the induction of nitric oxide synthase by LPS and IFN g in J774 macrophages. Arch Int. Pharmacodyn 1995; 330: 225-236.

[20] Imanishi N, Andoh T, Sakai S, Satoh M, Katada Y, Ueda K, Terasawa K, Ochiai H. Induction of inducible nitric oxide (NO) synthase mRNA and NO production in macrophages infected with influenza A/PR/8 virus and stimulated with its ether-split product. Microbiol Immunol 2005; 49(1): 41-8.

[21] Chang, S. H., Mun, S. H., Ko, N. Y., Lee, J. H., Jun, M. H., Seo, J. Y., Kim, Y. M., Choi, W. S., and Her, E. () The synergistic effect of phytohemagglutinin and interferon-γ on the expression of tumor necrosis factor-α from RAW264.7 cells. Immunol Letters 2005; 98:137-143.

[22] Yamamoto Y, Kurachi M, Yamaguchi K, Oda T.Induction of multiple cytokine secretion from RAW264.7 cells by alginate oligosaccharides. Biosci Biotechnol Biochem 2007 Jan;71 (1):238-41.

[23] Dambuza I, Allie N, Fick L, Johnston N, Fremond C, Mitchell J, Quesniaux VF, Ryffel B, Jacobs M.Efficacy of membrane TNF mediated host resistance is dependent on mycobacterial virulence. Tuberculosis (Edinb) 2008 May; 88 (3): 221-34.

[24] Thiemermann, C. Nitric oxide and septic shock. Gen Pharmacol 1997; **29**: 159–166.

[25] Waage, A. Tumour necrosis factor and septic shock. Lancet. 1998; **351**:603.

[26] Freyer, D; Weih, M; Weber, J R; Bürger, W; Scholz, P; Manz, R; Ziegenhorn, A; Angestwurm, K; Dirnagl, U. Pneumococcal cell wall components induce nitric oxide synthase and TNF-alpha in astroglial-enriched cultures. Glia 1996; 16: 1-6.

[27] Orman, K L; Shenep, J L; English, B K. Pneumococci stimulate the production of the inducible nitric oxide synthase and nitric oxide by murine macrophages. J Infect Dis 1998; 178: 1649–1657.

[28] Kengatharan, K M; De Kimpe, S; Robson, C; Foster, S J; Thiemermann, C. Mechanism of gram-positive shock: identification of peptidoglycan and lipoteichoic acid moieties essential in the induction of nitric oxide synthase, shock, and multiple organ failure. J Exp Med 1998; 188: 305-315.

[29] Leist, M., F. Gantner, I. Bohlinger, G. Tiegs, P. G. Germann, A. Wendel. Tumor necrosis factor-induced hepatocyte apoptosis precedes liver failure in experimental murine shock models. Am J Pathol 1995; 146:1220.

[30] Schümann J, Angermüller S, Bang R, Lohoff M, Tiegs G. Acute hepatotoxity of *pseudomonas aeruginosa* Exotoxin A in Mice Depends on T cell and TNF. J

Immunol 1998 Nov 15; 161(10):5745-54

Figure legends

Fig. 1. Time-course analysis of NO production from Japanese flounder peritoneal macrophages after exposure to high virulent strain NUF251 (●) and low virulent strain NUF194 (○) of *E. tarda*. To the adherent macrophages in 96-well plates (2 x 10^4 cells/well) in RPMI 1640 medium containing 10% FBS, each bacterial cell suspension was added (final 2 x 10^4 CFU/well). After the indicated periods of time at 25℃, the culture supernatant was withdrawn from each well, and subjected to the determination of NO level as described in the text. Each point represents an average of triplicate measurements. Each bar represents standard deviation. Asterisks indicate significant differences between two strains ($^*P<0.05$).

Fig. 2. Time-course analysis of TNF-α production from Japanese flounder peritoneal macrophages after exposure to high virulent strain NUF251 (●) and low virulent strain NUF194 (○) of *E. tarda*. To the adherent macrophages in 96-well plates (2 x 10^4 cells/well) in RPMI 1640 medium containing 10% FBS, each bacterial cell suspension was added (final 2×10^4 CFU/well). After the indicated periods of time at 25℃, the culture supernatant was withdrawn from each well, and subjected to the determination of TNF-α level as described in the text. Each point represents an average of triplicate measurements. Each bar represents standard deviation. Asterisks indicate significant differences between two strains ($^*P<0.05$).

Fig. 3. Time course analysis of NO production (a) and expression of iNOS mRNA (b) in

RAW264.7 cells after exposure to high virulent strain NUF251 and low virulent strain NUF194 of *E. tarda*. (a) To the adherent cells in 96-well plates (2 x 10^4 cells/well) in RPMI 1640 medium containing 10% FBS, bacterial cell suspension of NUF251 (●) or NUF194 (○) was added (final 2×10^4 CFU/well). After the indicated periods of time at 25℃, the culture supernatant was withdrawn from each well, and subjected to the determination of NO level as described in the text. Each point represents an average of triplicate measurements. Each bar represents standard deviation. Asterisks indicate significant differences between two strains ($^*P<0.05$). (b) Adherent cells (2 x10^5 cells/ well in 12-well plates) were incubated with strain NUF251 or NUF194 (final 2×10^5 CFU/well) for the indicated periods of time, and then the cells were subjected to the analysis of iNOS mRNA levels by RT-PCR as described in the text.

Fig. 4. Time-course analysis of TNF-α production from RAW264.7 cells after exposure to high virulent strain NUF251 (●) and low virulent strain NUF194 (○) of *E. tarda*. To the adherent cells in 96-well plates (2 x 10^4 cells/well) in RPMI 1640 medium containing 10% FBS, each bacterial cell suspension was added (final 2×10^4 CFU/well). After the indicated periods of time at 25℃, the culture supernatant was withdrawn from each well, and subjected to the determination of TNF-α level as described in the text. Each point represents an average of triplicate measurements. Each bar represents standard deviation. Asterisks indicate significant differences between two strains ($^*P<0.05$).

Fig.1

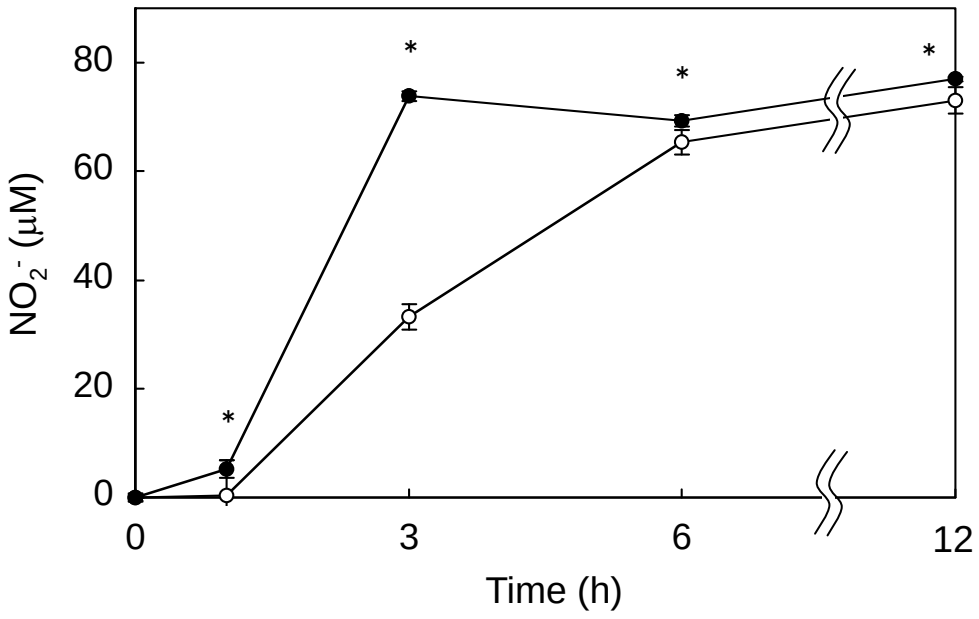


Fig .2

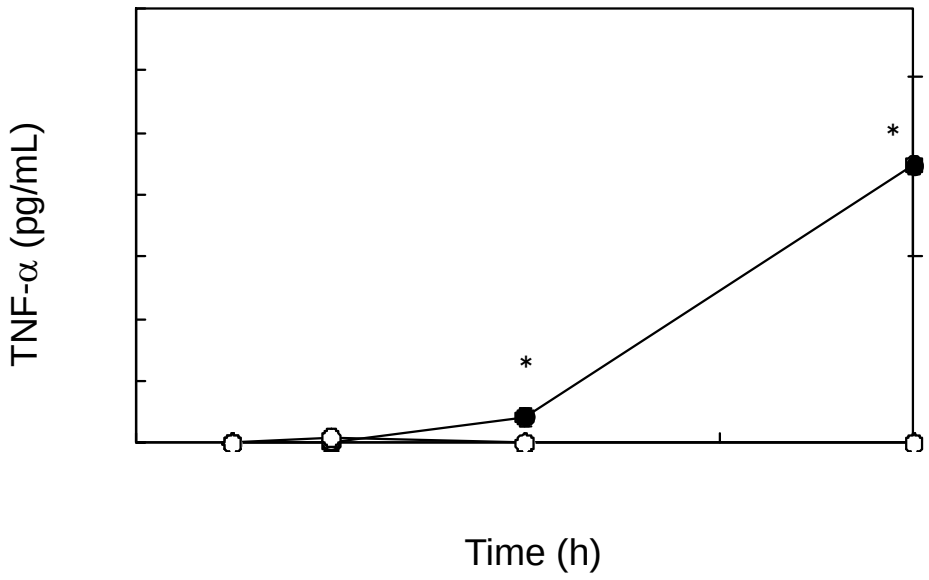
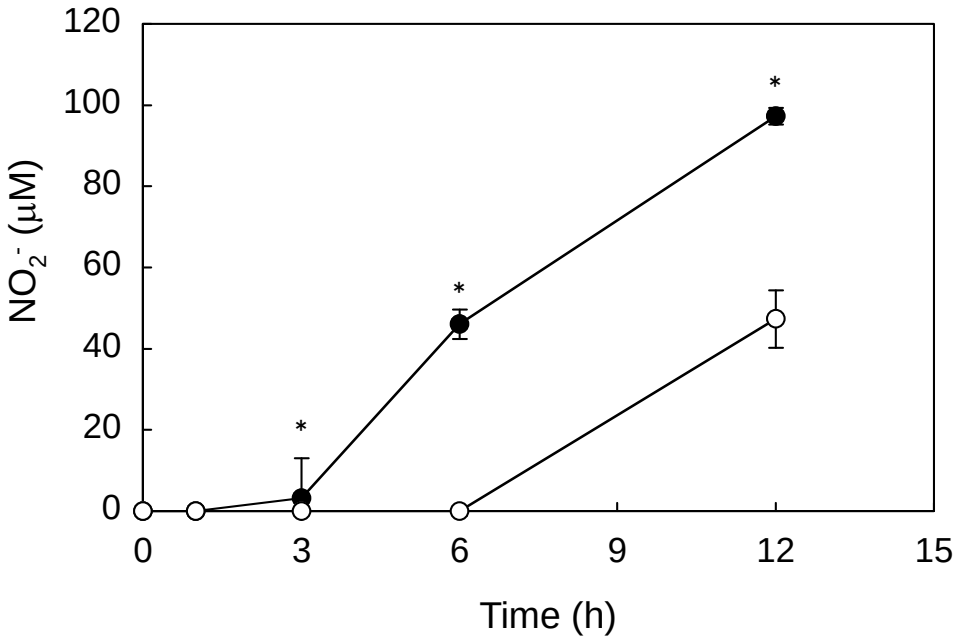


Fig.3

(a)


120
100
80
60
40
20
0
NO_2^- (μM)
0
3
6
9
12
15
Time (h)
*
*
*

(b)

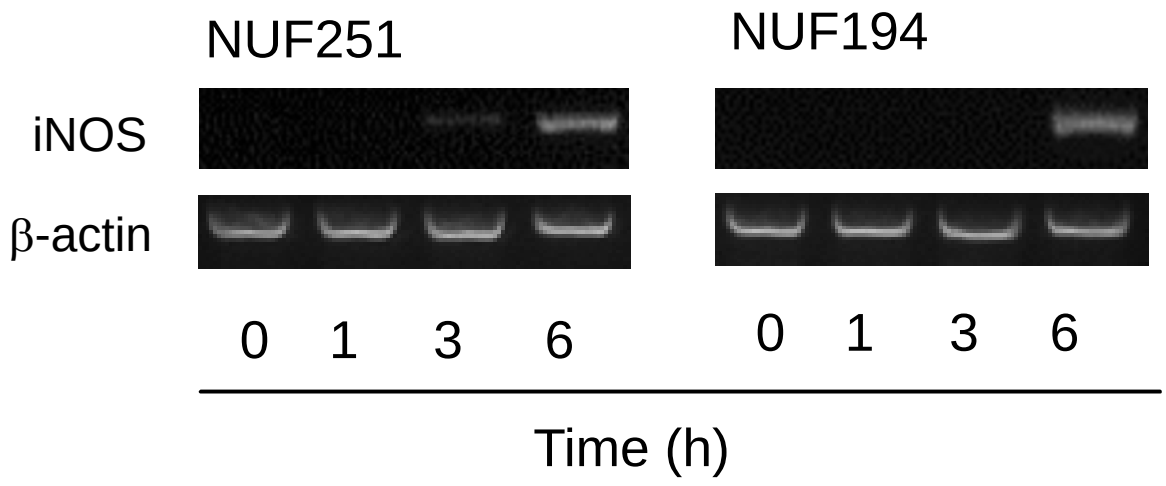

NUF251
NUF194
iNOS
β-actin
0 1 3 6
0 1 3 6
Time (h)

Fig .4