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Intracellular replication of *Edwardsiella tarda* in murine macrophage is dependent on the type III secretion system and induces an up-regulation of anti-apoptotic NF-κB target genes protecting the macrophage from staurosporine-induced apoptosis

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Abstract

Edwardsiella tarda **is a pathogen with a broad host range that infects both animals and humans. Resistance to phagocytic killing may be involved in the pathogenicity of this bacterium. Here we show that intracellular replication of *E. tarda* in murine macrophages is dependent on the type III secretion system and induces an anti-apoptotic effect by up-regulating anti-apoptotic NF-κB target genes. The wild-type strain replicates within the phagosomal membrane of macrophages; whereas the type III mutant does not. Microarray analysis shows the mRNA expression level of NF-κB target genes (e.g. pro-inflammatory cytokines and anti-apoptotic genes) in macrophages infected with the wild-type strain were up-regulated compared to macrophages infected with the type III mutant. Up-regulation of *Bcl2a1a, Bcl2a1b, cIAP-2,* and *TRAF1* genes induced expression of anti-apoptotic proteins to protect macrophages from apoptosis induced by staurosporine. Further, this protection was inhibited by adding kamebakaurin, an inhibitor of NF-κB activation and was confirmed using an NF-κB reporter gene assay. Up-regulation of anti-apoptotic NF-κB target genes is responsible for the anti-apoptotic activity of *E. tarda* and is**

required for intracellular replication in murine macrophages.

Keywords

Edwardsiella tarda, the type III secretion system, NF-κB, anti-apoptosis, intracellular replication, macrophage

1. Introduction

Edwardsiella tarda, a member of the family *Enterobacteriaceae*, causes infectious diseases in humans [1-5] and animals: fish [6-9], amphibians [10, 11], reptiles [12, 13], birds [14, 15], and mammals [10, 16]. *E. tarda* (=*Paracolobactrum anguillimortiferum*) was first reported as a pathogen associated with the so-called “red disease” of the Japanese eel, *Anguilla japonica* [17], and causes diseases in a wide variety of cultured seawater and fresh water fish [7, 8]. In humans, *E. tarda* is involved with sporadic cases of gastroenteritis [3, 18] and rarely causes extra-intestinal diseases where wound infections and septicemia, meningitis, cholecystitis, and osteomyelitis are described [2]. Exposure to aquatic environments or exotic animals such as reptiles and amphibians, pre-existing liver disease, conditions leading to iron overload, and dietary habits like ingesting raw fish appear to be risk factors that are associated with human *E. tarda* infections [2]. Little is known about the pathogenesis of *E. tarda* infection but some virulence factors may be involved in its pathogenicity [8] where the ability to invade epithelial cells [19] and resistance to phagocyte-mediated killing [20] are shown.

Tn*phoA* transposon tagging and proteomics methods show the sequence of the genes

involved in the type III secretion system (TTSS) of *E. tarda* [21-23]. Thirty-five ORFs code for the TTSS apparatus that are composed of chaperones, effectors, and regulators where the mutants of these genes affect adherence to and internalization within the epithelioma papillosum of carp, *Cyprinus carpio*, (EPC) cells that results in the survival and growth in fish phagocytes [23]. The TTSSs play key roles in the pathogenicity of many pathogenic bacteria [24] and may be the primary virulence mechanism of *E. tarda*.

NF-κB regulates various cellular genes involved in innate and adaptive immunity as well as genes involved in cell survival [25]. NF-κB regulates the transcription of pro-inflammatory chemokines and cytokines [26]; controls the transcription of genes involved in the immune response; and NF-κB regulates the expression of genes that confer resistance to cell death. The target genes include the caspase inhibitors c-IAP1, c-IAP2, and X-IAP, IEX-1L, a protein of unknown function, the TNF receptor-associated factors TRAF1 and TRAF2, the zinc finger protein A20, and the Bcl-2 family member A1 [27-30]. Thus, NF-κB can modulate not only the inflammatory response but also cell death.

Intracellular pathogens can regulate programmed cell apoptosis during intracellular infection. Several intracellular pathogens e.g. *Salmonella, Shigella, Yersinia, Legionella,* and *Bordetella* are reported to induce the host apoptotic response [31-35]. On the other hand,

Chlamydia [36, 37], *Porphyromonas gingivalis* [38, 39], and *Neisseria gonorrhoeae* [40] suppress the host's apoptotic response. Suppression of the host's apoptotic response is significant because the host can clear invading pathogens by initiating apoptosis where interference with the initiation of the apoptotic cascade prolongs the integrity of an intracellular niche. Thus modulation of the host's apoptotic responses promotes the intracellular survival of the invading pathogen, and contributes to the disease caused by their infection.

Chlamydia-infected HeLa cells are resistant to apoptosis and do not require NF-κB activation for *Chlamydia trachomatis* inhibition of the host epithelial cell apoptosis [41], while the anti-apoptotic activity of NF-κB is required for *Rickettsia* [42, 43] and *Helicobacter* [44]. In contrast, *Bordetella bronchiseptica* induces TTSS-dependent apoptotic effect on host cells by inactivating NF-κB in epithelial cells [35]. In this study, we show that TTSS-dependent intracellular replication of *E. tarda* in murine macrophages up-regulates NF-κB target genes including the anti-apoptotic genes and results in an anti-apoptotic effect in murine macrophages to enhance the intracellular survival of *E. tarda*. Here we use murine macrophages since the available genetic information is more abundant in the mouse than in fish, and the commercially available microarray system for the systematic analysis is

established for the mouse. Our final aim is to conduct this study to elucidate the role of TTSS involved in *E. tarda* pathogenicity in fish.

2. Results

2.1. Intracellular replication of *E. tarda* in J774 macrophages is dependent on the type III secretion system and takes place within the phagosomal membranes.

We compared the level of intracellular replication of the wild-type *E. tarda* and type III mutant, mET1229, in J774 murine macrophages at an moi of one. The number of viable cells increased for the wild-type from 3 h to 22 h after infection; whereas mET1229 decreased and where killed by 22 h (Fig.1). This suggests the type III secretion system is required for intracellular survival of *E. tarda* in murine macrophages.

J774 macrophages infected with the wild-type and type III mutant were examined at the ultra-structural level using TEM (Fig.2) and show both were observed at 0 h post-infection (Figs.2A and 2B). By 12 h postinfection, intracellular growth of the wild-type was observed

(Figs.2C-2F) and was replicating surrounded by the phagosomal membrane (Fig.2D). Larger phagosomes were formed by 12 h post-infection (Fig.2E and 2F) where many cytoplasmic components were included in the phagosome. The mechanism of formation of these large phagosomes is not clear, but may start from a small phagosome including only bacteria (Fig.2D). By 22 h post-infection, intracellular growth of the wild-type was similar to 12 h post-infection (Figs.2G and 2H), however, the total number of the wild-type had increased compared to 12 h (Fig.1). In contrast, few mutant mET1229 were observed at 12 h post-infection (Fig.1).

2.2. Intracellular replication of the wild-type in J774 macrophages induces up-regulation of the NF-κB target genes, especially the anti-apoptotic genes.

To compare the mRNA expression levels in macrophages infected with the wild-type to those infected with mET1229 at an moi of one, we carried out microarray analyses on J774 macrophages, where 30,000 mouse genes were analyzed (see Materials and Methods). At 3, 5, 12, and 22 h after infection, the total RNAs were extracted from the J774 cells infected with the wild-type or mET1229 and from the non-infected cells. The relative mRNA

expression levels in the wild-type-infected versus mET1229-infected cells varied from 11.01-fold to 0.14-fold at 3h, 93.57-fold to 0.063-fold at 5 h, 33.96-fold to 0.29-fold at 12 h, and 11.58-fold to 0.05-fold at 22 h after infection, respectively. The majority of the genes (96, 91, 99, and 94 %, respectively at each time point) exhibited little change in mRNA expression levels (between 2 and 0.5; data not shown). Among the up-regulated genes (Supplemental Table S1), there is a wide range of genes exhibiting very different and, in some cases, poorly understood cellular functions. However, up-regulation of many known NF-κB target genes was detected (Table 1). Increases was seen for several NF-κB target genes associated with inflammation. Pro-inflammatory chemokines such as MIP-2/Cxcl2 (33.96-fold increase at 12 h after infection), MIP-1α/Ccl3 (5.49-fold at 3 h), RANTES/Ccl5 (3.65-fold at 12 h), and MCP-1/Ccl2 (2.95-fold at 22 h) increased where the up-regulation of these murine macrophage chemokine expression levels is reported to be dependent on NF-κB activation [45, 46]. In addition, the cytokines such as IL-6 (17.35-fold at 12 h after infection), IFNγ (8.94-fold at 3 h), and IL-1β (3.07 at 5 h) genes are known as NF-κB target genes and were also up-regulated. The mRNA expression level of serum amyloid A3 known to be regulated by NF-κB and to be associated with inflammation was also increased (7.00-fold at 12 h) [47]. Although NF-κB dependence was not confirmed, the mRNA

expression levels of LPS-inducible genes associated with inflammation and bacterial clearance such as immune-response gene 1 (Ir1) (3.60-fold at 12 h) and the macrophage receptor MARCO (2.80-fold at 5 h) were also up-regulated [48, 49].

In addition to controlling the transcription of genes involved in the immune response, NF-κB also regulates the expression of genes that confer resistance to cell death e.g. c-IAP1, c-IAP2, X-IAP, IEX-1L, TRAF1, TRAF2, the zinc finger protein A20, and the Bcl-2 family member A1 [27-30]. As shown in Table 1, the up-regulation of anti-apoptotic NF-κB target genes including those coding for Bcl2-A1a (2.74-fold at 12 h after infection), Bcl2-A1b (2.84-fold at 12 h), cIAP-2 (5.70-fold at 5 h and 2.97-fold at 12 h), and TRAF1 (4.18-fold at 12 h) was observed. The up-regulation of these anti-apoptotic genes occurred primarily during the late stage of the infection (12 h).

2.3. Time course of transcriptional up-regulation, and classification of early and late genes.

Based on the microarray data at 3, 5, 12, and 22 h after infection, the time course of transcriptional up-regulation of J774 macrophage genes during the course of intracellular

replication of the wild-type were analyzed (Fig. 3). A set of early genes, the IFN-γ and Ccl20 genes, were induced as early as 3 h after infection and then returned to background levels. The Ccl3 gene was observed to be up-regulated after 3 h of infection where the expression remained high and then slowly decreased.

Following this early transcriptional up-regulation, induction of the genes coding for three anti-apoptotic proteins, Bcl2-A1, cIAP-2, and TRAF1, appeared at 12 h after infection and genes coding for the inflammatory proteins, Cxcl2, Ccl5, IL-6, IL-1β, serum amyloid A3, MARCO, and Ir1 appeared at 5 h to 12 h after infection and were thus classified as late genes. Among the late genes we further examined the up-regulation of four anti-apoptotic NF-κB target genes.

To confirm the increased expression of the anti-apoptotic genes coding for Bcl2-A1, cIAP-2, and TRAF1, we performed a Western-blot analysis. Fig. 4 that shows infection of J774 cells with the wild-type at an moi of one or an moi of 10 results in induction of Bcl2-A1, cIAP-2, and TRAF1 proteins at 12 h after infection, but infection with mET1229 did not. The expression level of β-actin as an internal control for the Western-blot was almost the same among all of the samples tested.

2.4. Infection of J774 macrophages with the wild-type *E. tarda* induces NF-κB activation and KA inhibits the response

On the basis of the microarray data, it was strongly suggested that intracellular replication of the wild-type in J774 macrophages induces NF-κB activation to cause up-regulation of the NF-κB target genes, especially the anti-apoptotic genes. To show NF-κB activation caused by infection with the wild-type, we performed an NF-κB reporter gene assay (Fig.5). NF-κB activity increased as the cells were treated with TNFα or LPS for 12 h without infection (positive control). Infection of J774 cells for 12 h with the wild-type at an moi of one or an moi of 10 triggered NF-κB activation; whereas mET1229 infection at an moi of one or an moi of 10 was significantly lower ($P<0.001$ for an moi of one; and $P<0.005$ for an moi of 10).

Furthermore, we determined if KA (3 μM) inhibits NF-κB activation caused by infection with the wild-type at an moi of one or an moi of 10 using the NF-κB reporter gene assay (Fig.5). NF-κB activation with the wild-type infection at an moi of one or an moi of 10 was attenuated by the addition of KA ($P<0.001$). Therefore, KA was effective in blocking activation of NF-κB caused by infection with the wild-type strain.

2.5. Infection of the J774 macrophages with the wild-type *E. tarda* protects host cells from staurosporine-induced apoptosis.

The protein kinase inhibitor, staurosporine (STS), induces apoptosis [50, 51]. STS-treated cells show many of the hallmarks of apoptosis, including cytochrome *c* release, caspase activation, and late stage DNA fragmentation [50]. Over-expression of Bcl2 and Bcl2-A1 inhibits STS-induced apoptosis [52, 53]. Up-regulation of anti-apoptotic factors, including Bcl2-A1 and c-IAP2, following infection with *Neisseria gonorrhoeae* protects host cells from STS-induced apoptosis [40]. Our microarray and Western-blot analysis shows up-regulation of the anti-apoptotic genes coding for Bcl2-A1, c-IAP2, and TRAF1 following wild-type *E. tarda* infection where the up-regulation occurred in the late stages of infection at 12 h. We used the Cell Death Detection ELISA^{PLUS} assay to quantify late-stage DNA fragmentation in J774 cells undergoing apoptosis. J774 cells were infected with the wild-type or mET1229, and then the *E. tarda* infected cells were treated with STS to determine if they exhibit resistance to STS-induced apoptosis. Before treating J774 cells with STS, we first investigated apoptosis level of J774 cells infected with the wild-type or

mET1229 at an moi of one in the complete absence of STS. As shown in Fig. 6A, J774 cells infected with the wild-type strain showed slight repression of apoptosis as compared with that infected with mET1229 (slight but significant difference between sample #2 and #3; $P<0.05$). Then, to show more defined anti-apoptotic effect of the wild-type infection, we followed the method using STS, which had been previously reported in confirmation of anti-apoptotic effect of *Chlamydia trachomatis* infection [41], that is, we also studied apoptosis level in the presence of STS. Fig. 6A shows treatment of J774 cells with STS (sample #4) increased apoptosis (~ 11-fold increase). However, the infection of STS-treated J774 cells with the wild-type at an moi of one or an moi of 10 reduced apoptosis; whereas, infection of STS-treated J774 cells with mET1229 at a moi of one or a moi of 10 did not affect STS-induced apoptosis (samples #7 and #8); and this was statistically different ($P<0.005$ for the wild-type compared to mET1229 at an moi of one; and $P<0.001$ for the wild-type compared to mET1229 at an moi of 10)].

To confirm the anti-apoptotic NF-κB dependence occurring during wild-type infection in STS-treated J774 cells, we determined if KA could inhibit the anti-apoptotic effect. Fig. 6A shows a reduction of apoptosis in STS-treated J774 cells after infection with the wild-type at an moi of one or an moi of 10 (samples #5 and #6) that was significantly attenuated by the

addition of KA (samples #10 and #11) ($P<0.05$ for #5 compared to #10; and $P<0.001$ for #6 compared to #11, respectively]. Western-blot analysis confirmed the induction of anti-apoptotic proteins Bcl2-A1, c-IAP2, and TRAF1 in the STS-treated J774 cells after wild-type infection at an moi of one or an moi of 10 that was suppressed by the addition of KA (Fig. 6B). This suggests the anti-apoptotic effect of the wild-type infection in STS-treated J774 cells is due to increased expression of NF-κB-induced anti-apoptotic proteins.

Further, we determined if the anti-apoptotic effect of the wild-type infection was associated with intracellular replication of the wild-type *E. tarda* in J774 macrophages. Fig. 7A shows the addition of KA to STS-treated J774 macrophages infected with the wild-type caused a significant decrease in the number of viable cells that replicated in J774 macrophages ($P<0.05$ at an moi of one without KA compared to with KA; and $P<0.001$ at an moi of 10 without KA compared to with KA, respectively). The induction of apoptosis by the addition of KA to STS-treated J774 macrophages infected with the wild-type may be related to the decrease in the number of viable cells for the wild type infection; whereas, the addition of KA to STS-treated J774 macrophages infected with mutant mET1229 did not have significant influence on the number of viable cells ($P>0.1$). In addition, we determined

if, in the complete absence of STS, KA has any effect on intracellular survival of the wild-type or mET1229. As shown in Fig.7B, in the complete absence of STS, KA showed significant inhibitory effect on intracellular survival of the wild-type strain ($P<0.05$ at an moi of one without KA compared to with KA; and $P<0.001$ at an moi of 10 without KA compared to with KA, respectively). This would provide evidence that NF-κB activation by the wild-type strain is somehow related to intracellular replication of this strain in J774 macrophages. On the other hand, in the complete absence of STS, KA did not exhibit significant inhibitory effect on intracellular survival of mET1229 ($P>0.1$ at an moi of one without KA compared to with KA; and $P>0.05$ at an moi of 10 without KA compared to with KA, respectively).

3. Discussion

Intracellular pathogens modulate host cell apoptosis during intracellular infection to facilitate the survival of the bacteria in host cells. Macrophage apoptotic pathways are influenced by a variety of factors, including cytokines, NO, and NF-κB [54]. With several

intracellular pathogens e.g. *Salmonella, Shigella, Yersinia,* and *Legionella,* the cell death of the host macrophage through apoptosis is reported to be important in the dissemination of the pathogens [31-34]. In contrast, several bacterial pathogens including *Chlamydia*, *Porphyromonas gingivalis*, *Neisseria gonorrhoeae*, *Rickettsia,* and *Helicobacter* are reported to suppress the host apoptotic response and thereby prolong the integrity of an intracellular niche that promotes the intracellular survival of the invading pathogens. We show *E. tarda* elicits an anti-apoptotic effect on murine macrophages through the up-regulation of the NF-κB target anti-apoptotic genes that promotes the intracellular survival of the pathogen. With *Chlamydia*, the suppression of apoptosis allows this intracellular bacterium to maintain an environment necessary for replication that prevents the uptake and clearance of infected cells by neighboring phagocytes [37]; whereas, alveolar macrophage apoptosis is reported to contribute to pneumococcal clearance in pulmonary infection [55]. As apoptosis marks unwanted cells to direct recognition for engulfment and degradation by phagocytes [56], blocking apoptosis might be an important strategy for *E. tarda* to survive in phagocytes. However, it is still unclear if this protection against apoptosis in pahgocytes is relevant to *E. tarda* pathogenesis. When mET1229 was intramuscularly injected into the zebrafish attenuation in virulence was observed (unpublished results). Relationship between

anti-apoptotic mechanism of *E. tarda* in host phagocytes and virulence of *E. tarda* to host has to be examined in future study using susceptible host such as zebrafish.

The regulation of apoptosis is a highly complicated process involving the coordinated activity and interaction of multiple factors. Primary sites of control include the regulation of cytochrome *c* localization and the activity of a group of cysteine proteases termed caspases. The localization of cytochrome *c* and the activation of caspases are often controlled by the activities of the Bcl-2 family; that is, apoptosis can be either favored or inhibited by the gene products of the Bcl-2 family. One of these family members, Bcl2-A1, is a rapidly inducible gene product that was initially characterized in murine macrophages [57] and functions to prevent apoptosis [58]. Bcl2-A1 is shown to bind to the pro-apoptotic Bcl-2 family member, Bid, that transmits apoptotic signals to the mitochondria [59, 60] and blocks its collaboration with pro-apoptotic Bax or Bak in the plane of the mitochondrial membrane [61]. This binding of Bcl2-A1 to Bid prevents the release of cytochrome *c* from mitochondria that initiates the apoptotic cascade. STS was used here as a potent inducer of apoptosis that triggers apoptosis in various types of cells. The induction of apoptosis by STS is mediated by the mitochondrial pathway [62, 63]. Apoptosis induction by STS targets mitochondrial Bax making mitochondrial outer membranes permeable releasing cytochrome *c*. This is

followed by caspase activation and the development of the apoptotic morphology. It is unclear how mitochondrial opening is developed during apoptosis, but Bcl2-A1 may directly take part and regulate this stage [64]. Recently, the bacillus of Calmette-Guerin (BCG) was shown to induce Bcl2-A1 expression protecting murine macrophages from NO-induced apoptosis in both wild-type peritoneal exudate macrophages (PEM) and J774 cells but not in *Bcl2-A1-a*$^{-/-}$ PEM. This indicates a requirement for *Bcl2-A1-a* induction in BCG-mediated survival of inflammatory macrophages [65]. Further reports show all of three genes encoding the closely related iso-forms of Bcl2-A1 (from the *Bcl2-A1-a, Bcl2-A1-b, and Bcl2–A1-d* genes) were expressed in J774 cells but the *Bcl2-A1-b* iso-form showed the greatest increase upon treatment with IFN-γ (11-fold), IFN–γ + BCG (38-fold), and BCG (5-fold) while the *A1-a* and *A1-d* iso-forms were increased to a lesser degree [65]. In our microarray data, significant up-regulation of the *Bcl2-A1-a* and *Bcl2-A1-b* genes was detected at 12 h and 22 h after infection in J774 cells using the wild-type *E. tarda* (Table 1 and Fig. 3). Further, transient expression of murine Bcl-2-A1-a in COS-7 cells is reported to cause resistance to STS-induced apoptosis, although the precise mechanism of the resistance of Bcl-2-A1-a to STS-induced apoptosis in COS-7 cells is unknown [53]. These reports and our findings suggest an association of Bcl2-A1 with the regulation of STS-induced apoptosis in J774

cells.

In addition to the up-regulation of Bcl-2-A1, the up-regulation of cIAP-2 was also detected in J774 cells infected with the wild-type. cIAP-2 belongs to the inhibitor of apoptosis protein (IAP) family and is involved in suppressing the host cell death response downstream of cytochrome *c* release [66]. Over-expression of cIAP-2 is reported to suppress apoptosis induced by a variety of stimuli including STS [66]. Therefore, the up-regulation of both Bcl-2-A1 and cIAP-2 may be important for *E. tarda* to prevent STS-induced apoptosis i.e. Bcl-2-A1 may inhibit the induction of the apoptosis cascade by STS before cytochrome *c* release and cIAP-2 may interfere with the downstream cytochrome *c* release.

Members of the tumor necrosis factor receptor superfamily (TNFR) can induce a wide spectrum of cellular responses, including apoptosis, cell proliferation, and differentiation. These functions are primarily mediated by a family of intracellular TNFR-binding proteins, the TNFR-associated factors (TRAFs) [67]. Six TRAF family proteins (TRAF1 to TRAF6) are described in humans and mice where TRAF1 is a unique member of the TRAF family because of its molecular architecture. TRAF1 protein lacks the conserved N-terminal RING domain found in the TRAF family proteins and fails to induce NF-κB or to activate stress kinases when over-expressed in cells. TRAF1 is reported to be transcriptionally up-regulated

by NF-κB together with TRAF2, cIAP-1, and cIAP-2 [28]. In our microarray data, significant up-regulation of the *TRAF1* gene along with the *cIAP-2* gene was observed. This up-regulation is thought to be a consequence of NF-κB activation caused by the intracellular replication of *E. tarda*, but the role of the increased expression of TRAF1 on the anti-apoptotic effect of *E. tarda* against STS-induced apoptosis is unclear and further investigation will be required.

At 12 and 22 h post-infection, intracellular growth of the wild-type was observed using TEM where the wild-type was replicating in J774 macrophages surrounded by phagosomal membranes (Figs.2C-2H). Replication of the wild-type was occurring in many large phagosomes that had cytoplasmic components (Fig.2E-2H). Additionally, the microarray analysis shows intracellular replication of the wild-type *E. tarda* in J774 cells results in an increase in the NF-κB target genes. How are these many NF-κB target genes up-regulated? There is little experimental evidence for the recognition of bacteria in host intracellular compartments in animal cells. However, in mammalian systems the presence of surveillance mechanisms that sense and respond to bacteria in the cytosol are shown [68], although the host factors that are associated with such recognition are still poorly understood. Nucleotide-binding oligomerization domain (NOD) proteins, a growing family of cytosolic

proteins are implicated in the innate recognition of bacteria and the induction of inflammatory responses through NF-κB activation [69]. Two members, NOD1 and NOD2, are shown to recognize bacterial lipopolysaccharide (LPS) and/or peptidoglycan [69]. The muramyl dipeptide (MDP) derived from peptidoglycan is an essential structure of bacteria and is recognized by NOD2 [70, 71]. Signaling through NOD1 and NOD2 is mediated using the kinase RICK (RIP-like interacting CLARP kinase) interacting with NOD1 and NOD2 through the homophilic CARD (caspase recruitment domain)-CARD interactions. RICK mediates the activation of NF-κB using stimulation of the IKK and promotes NF-κB-dependent gene transcription [69]. How are LPS and/or MDP delivered to the cytosol? Phagocytic cells contain intracellular hydrolases which digest bacterial peptidoglycan that release the muropeptides. Muropeptides derived from peptidoglycan from most species of bacteria consist of GlcNAc-MurNAc that is linked with short peptides that possess the conserved dipeptide present in MDP [69]. These LPS and/or MDP-like molecules derived from the phagocytosed bacteria may be recognized by NOD1 and/or NOD2 [69]. However, it remains unclear how LPS and/or muropeptides enter the cytosol from phagosomes across phagosome membranes. With *E. tarda,* LPS and/or muropeptides may not have to enter the cytosol across phagosome membranes to be recognized by NOD

proteins because the replication of *E. tarda* within large phagosomes included cytoplasmic components. We propose NOD proteins present in these cytoplasmic components contained in the large phagosomes may detect LPS and/or the MDP-like molecule and results in NF-κB activation. However, further investigations using NOD protein-deficient murine macrophages will be required.

The NF-κB reporter gene assay shows *E. tarda* infection triggers NF-κB activation in J774 cells where KA completely inhibits this activation (Fig.5). In the NF-κB reporter gene assay, we used LPS as a positive control for NF-κB activation. LPS induces NF-κB activation in J774 cells (Fig.5). Previous reports show LPS treatment of J774 cells results in NF-κB activation due to the increase in the nuclear level of NF-κB p50 subunit as well as the degradation of IκBα [72-74]. If LPS derived from phagocytized *E. tarda* is important for NF-κB activation induced by *E. tarda* infection, the p50 subunit of NF-κB would be primarily involved in this activation. Further, NF-κB activation in J774 cells by infection with the wild-type was completely inhibited by the addition of KA (Fig.5). Because KA is shown to preferentially affect the p50-mediated DNA-binding activity of NF-κB rather than RelA and has no effect on either LPS-induced degradation of IκBα or nuclear translocation of NF-κB [75], the complete inhibition of NF-κB activation by KA would suggest the

involvement of p50 subunit in NF-κB activation in J774 cells by infection with the wild-type *E. tarda*.

Here we used murine macrophages to examine the anti-apoptotic mechanism caused by *E. tarda* infection. We used the murine macrophage model rather than fish macrophages because the available genetic and biological information is more abundant in the mouse than in fish, and the microarray system is established for the mouse. It is reported the TTSS mutants have decreased survival and growth in fish phagocytes [23] where our results using murine macrophages agree. Therefore, the TTSS-dependence of *E. tarda* virulence against mammalian macrophages and fish phagocytes is now established. Our aim is to clarify the anti-apoptotic mechanism of *E. tarda* in fish phagocytes to study the mechanism of the virulence of *E. tarda* in fish as they are the major susceptible natural host.

4. Experimental/materials and methods

4.1. Bacterial strains, cell culture, and media.

E. tarda FK1051 was isolated from a diseased Japanese flounder, *Paralichthys olivaceus*,

and was used as the wild-type strain. The type III secretion system (TTSS)-deficient mutant, mET1229, was constructed as follows: we focused on the *E. tarda esaV* gene [23] reported to be a *Yersinia enterocolitica yscV* homolog. Disruption of the *yscV* homologs in several pathogens results in decreased pathogenicity [76-79]. A knockout of the *E. tarda esaV* gene was performed using the *in vitro* transposon insertion and marker exchange methods. The *Tn5-Km* gene was inserted into the *esaV* ORF using an EZ::TN <KAN-2> Insertion Kit (Epicentre Technologies) according to the manufacturer's instructions. The mutant *esaV* gene containing a single *Tn5-Km* insertion was excised using *Not*I digestion and the *Not*I fragment was cloned into the pSacBCos vector, originally constructed by replacing the *Nco*I fragment of SuperCos1 (Stratagene) with the *sacB* gene [80]. The resultant plasmid was introduced into the FK1051 strain by electroportation. Plasmid curing and marker exchange were performed by growing kanamycin-resistant transformants on trypticase soy agar (TSA, Nissui) plates containing kanamycin and 5 % sucrose. After showing the *Tn5-Km* insertion within the *esaV* ORF by PCR, one of the resultant kanamycin-resistant, sucrose-resistant, and ampicillin-sensitive colonies was used for further studies and named mET1229. When mET1229 was intramuscularly injected into the zebrafish, *Danio rerio,* attenuation in virulence was observed (unpublished results). FK1051 and mET1229 were grown in

trypticase soy broth (TSB, Nissui) at 30℃.

J774 cells, a murine macrophage-like cell line derived from BALB/c mice, were obtained from American Type Culture Collection. It was maintained in RPMI1640 (Sigma) supplemented with 10 % FCS.

4.2. Infection of J774 cells with *E. tarda* strains.

A 24-well tissue culture plate was inoculated with 7.5 x 10^4 J774 cells/well and incubated overnight at 37℃ under 5 % CO_2. The cells were infected with the wild-type and mET1229 strains at a multiplicity of infection (moi) of one for 30 min; then pre-warmed tissue culture medium containing 200 μg/ml gentamicin was added. After 1.5-h incubation, the medium was removed, washed twice with PBS, and new tissue culture medium without gentamicin was added. The J774 cells were incubated for 0, 3, 5, 12, or 22 h at 37℃ under 5 % CO_2. At the end of each time period, the cells were washed twice with PBS and lysed with 1 % Triton X-100 and inoculated on TSA, incubated, and counted (for CFU). To purify the total RNA used for the microarray analysis, at each time period, ISOGEN (Nippon gene) was added to the cells and total RNA was isolated following the manufacturer's instructions.

The protein expression level at the end of the12-h incubation was determined by washing the cells twice with PBS and Western-blot analysis was performed as described below.

4.3. Microarray analysis.

Total RNA was isolated from three independent experiments using 6-well tissue culture plate at 3, 5, 12, or 22 h after infection as described above. For microarray analysis, the total RNAs isolated from three independent experiments at each time period were used for antisense amino allyl RNA (aRNA) synthesis and for hybridization to the AceGene Mouse Oligo Chip 30K (Hitachi), on which 30,000 mouse genes were printed, according to the manufacturer's instructions. RNA amplification and labeling were performed using an Amino Allyl MessageAmp^{TM} aRNA Kit (Ambion) following the manufacturer's protocol. Double-stranded cDNAs were synthesized from one microgram of total RNAs using T7 oligo(dT) primer. Using these double-stranded cDNAs as templates, aRNA was then generated with 5-(3-aminoallyl)-UTP (aaUTP). After purification of the aRNA, dye coupling reaction between the amino allyl modified UTP residues on the aRNA and amine reactive dyes [Cy3- and Cy5-monoreactive dyes (Amersham)] was carried out. The Cy3- and Cy5-labeled aRNAs were purified using the microbio-spin column P30 (BioRad) and were

fragmented. The AceGene Mouse Oligo Chips were then hybridized with the fragmented Cy3- and Cy5-labeled aRNAs at 42℃ for 17 h. After hybridization, the oligo chips were washed with 2xSSC (standard saline citrate; 0.3 M NaCl, 0.3 M Na-citrate) and 0.1 % sodium dodecyl sulfate for 5 min at 30℃, rinsed with 2xSSC and 1xSSC, and finally dried. The fluorescent signal was detected using a microarray scanner (ScanArray Lite, PerkinElmer).

The fluorescent intensities on a scanne image were quantified, corrected for backgroud noise, and normalized with the software DNASIS Array (Hitachi Software Engineering) as described previously [81, 82]. In brief, data either from control spots or from spots containing high intensities of artificial signals were removed. The signal intensity of each spot was then normalized to equalize total signal intensity. The normalized signal intensity of each spot was plotted on a scatter plot with the Cy3 fluorescence on the *x*-axis and Cy5 fluorescence on the *y*-axis (data not shown). The ratio of Cy5 fluorescence (gene expression in J774 cells infected with the wild-type strain) to Cy3 fluorescence (gene expression in J774 cells infected with the mET1229 or gene expression in the non-infected J774 cells) was calculated and genes with an outstanding Cy5 : Cy3 ratio (Cy5-wild : Cy3-mET1229 ratio) more than 2.0 or less than 0.5 were listed in Supplemental Tables S1 and S2, respectively.

From among the up-regulated genes listed in Supplemental Table S1, wellknown NF-κB target genes were selected and listed in Table 1.

4.4. Transmission electron microscopy (TEM).

A six-well tissue culture plate was inoculated with 3.0 x 10^5 J774 cells/well and incubated overnight at 37℃ under 5 % CO_2. Each well was then infected with either the wild-type or mET1229 at an moi of one. At 0, 12, or 22 h after infection, the cells were washed twice with PBS, fixed with 2.5 % glutaraldehyde in 0.05 M cacodylate buffer (pH7.4) for > 2 h on ice, and embedded in 812. Ultrathin sections were counterstained with uranyl acetate and lead citrate.

4.5. NF-κB reporter gene assay.

Plasmid p55IgκLuc carrying the three NF-κB DNA-binding sites that drive the expression of the luciferase gene [83] was a gift from Dr. Suzuki's laboratory. The pRL-CMV vector carrying CMV-driven renilla-luciferase reporter gene was used as an internal control reporter and was purchased from Promega. J774 cells (2 X 10^5) were grown in 6-well plates for 24 h were co-transfected with 1580 ng of the reporter plasmid p55IgκLuc

and 340 ng of the control plasmid pRL-CMV using FuGENE HD Transfection Reagent (Roche) followed by incubation for 18 h. The transfected cells were infected with the wild-type *E. tarda* and mET1229 at an moi of one or an moi of 10 for 30 min before pre-warmed tissue culture medium containing 200 μg/ml gentamicin was added. After 1.5-h incubation, the medium was removed, washed twice with PBS, and tissue culture medium without gentamicin was added. The J774 cells were incubated for 12 h at 37℃ under 5 % CO_2. At 12 h after infection, cells were washed with PBS and lysed in 500 μl of cell lysis buffer. Luciferase activity in the cell lysate was assayed using the Dual-Luciferase Reporter Assay System (Promega). Relative NF-κB-luciferase activity was defined as the mean ± S.D. x 10^{-3} (n = 3) of the activity ratio of luciferase compared to renilla-luciferase. Statistical analysis was performed using a two-tailed *t* test. TNFα and LPS were purchased from PeproTech EC and Sigma, respectively.

To determine if kamebakaurin (KA) inhibits NF-κB activation caused by infection with the wild-type, the infected cells were treated with KA to inhibit NF-κB activity. The NF-κB inhibitor, KA, was purchased from Calbiochem. KA inhibits NF-κB by targeting the DNA-binding activity of p50 and blocks the expression of anti-apoptotic NF-κB target genes [75]. KA (3 μM) was used to pre-treat the cells for 2 h before infection with *E. tarda* strains,

and KA (3 μM) was also added to all tissue culture medium used after infection. Additionally, KA (3 μM) did not affect growth of *E. tarda* in culture media.

4.6. Induction of host cell apoptosis using staurosporine (STS) and the effect of NF-κB inhibition.

A 24-well tissue culture plate was inoculated with J774 (7.5 x 10^4 cells/well) and incubated overnight at 37℃ under 5 % CO_2. Then they were infected with the wild-type or mET1229 strains at an moi of one or 10 for 30 min; and pre-warmed tissue culture medium containing gentamicin was added at a final concentration of 200 μg/ml. After 1.5-h incubation, the medium was removed, washed twice with PBS, and tissue culture medium without gentamicin containing 0.125 μM STS (Wako) or mock buffer without STS (Dimethyl Sulfoxide) was added. The J774 cells were incubated for 12 h at 37℃ under 5 % CO_2. At the end of the time period, the cells were washed twice with PBS, and used for the apoptosis assay, Western-blot analysis or bacterial count.

The effect of NF-κB inhibition on STS-treated or STS-untreated J774 cells infected with the wild-type or mET1229 was determined by using KA. KA at 3 μM was used to pre-treat the cells for 2 h before infection with *E. tarda* strains, then KA (3 μM) was also added to all

tissue culture medium after infection.

4.7. Apoptosis assay.

Late-stage DNA fragmentation was quantitatively evaluated using the Cell Death Detection ELISA^{PLUS} (Roche Molecular Biochemicals) according to the manufacturer's instructions. The enrichment of mono- and oligonucleosomes released into the cytoplasm was calculated as the ratio of the absorbance in the sample cells compared to the absorbance of the control cells. The enrichment factor was used as a parameter of apoptosis and is shown on the y axis as the mean ± S.D. of triplicate experiments. Statistical analysis was performed using a two-tailed *t* test. An enrichment factor of one represents background or spontaneous apoptosis [84].

4.8. Western-blot analysis.

The cells washed with PBS (described above) were lysed with lysis buffer (10 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, and 0.2 % Triton X-100). Samples were electrophoresed using a 10 % SDS-polyacrylamide gel followed by blotting onto a PVDF membrane (Immobilon-P, Millipore). After incubating in blocking solution (2 % BSA,

0.05 % sodium azide in TBS), the membrane was incubated with anti-Bcl2-A1, anti-cIAP-2, anti-TRAF1 or anti-β-actin antibodies. After washing with TBS-0.05 % Tween-20, bound proteins were detected with anti-rabbit antibody or anti-mouse IgG antibody. Anti-Bcl2-A1, anti-cIAP-2, and anti-TRAF1 rabbit antibodies were purchased from Santa Cruz, and anti-β-actin mouse antibody was purchased from Chemicon.

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Figure captions

Fig. 1. Intracellular growth of the wild-type *E. tarda* and mET1229 in J774 macrophages.

Mono-layers of J774 macrophages were infected with 7.5 x 10^4 bacteria, incubated for the indicated time, treated with gentamicin (200 μg/ml) for 1.5 h, and then lysed with 1 % Triton X-100. The number of viable bacteria are shown as the mean ± S.D. of three samples.

Fig. 2. Representative electron micrographs of *E. tarda*-infected J774 macrophages.

Representative electron micrographs of wild-type-infected cells at 0 h post-infection (A), 12 h post-infection (C-F), and 22 h post-infection (G and H). A representative electron micrograph of mET1229-infected cells at 0 h post-infection is shown (B). The phagosomal membranes are indicated by the white arrows. The wild-type *E. tarda* replicating within the large phagosomes is shown with a thick black arrow (F). Abbreviations: Edt, the wild-type; Mut, mET1229.

Fig. 3. Time-course analysis of gene expression.

J774 cells were infected for 3, 5, 12, or 22 h with the wild-type *E. tarda* or mET1229. After total RNA extraction, cRNA synthesis was performed. The resulting cRNA was hybridized to the microarray chips (AceGene Mouse Oligo Chip 30K, Hitachi). The *vertical axis* represents the fold-increase in gene expression between the wild-type-infected *versus* mET1229-infected cells.

Fig. 4. Detection of anti-apoptotic proteins induced by infection with the wild-type *E. tarda*.

J774 cells were infected for 12 h with the wild-type *E. tarda* or mET1229 at an moi of one or an moi of 10. Increased expression of the genes coding for Bcl2-A1, cIAP-2, and TRAF1 was detected using Western-blot analysis. The expression of β-actin was used as an internal control for Western-blot.

Fig. 5. NF-κB activation by infection of the J774 macrophages with the wild-type *E. tarda* and effect of KA on the response.

Relative NF-κB-luciferase activity in J774 cells after infection with the wild-type (gray bars) and mET1229 (black bars) or without infection (control culture, open bar) was determined in

the presence or absence of KA (3 μM) as described in Materials and Methods. TNFα and LPS were added as positive controls without infection (hatched bars).

Fig. 6. J774 macrophages are protected from staurosporine (STS)-induced apoptosis by infection with the wild-type and this protection is prevented by treatment with an NF-κB inhibitor.

Panel A. Quantification of nucleosomal DNA fragmentation using the Cell Death Detection ELISA^{PLUS} assay. J774 cells infected with the wild-type *E. tarda* or mET1229 at an moi of one or an moi of 10 were treated with mock buffer (samples #5-8) or the NF-κB inhibitor. kamebakaurin (KA), (samples #10-13) followed by apoptosis induction with STS (samples #4-13) or mock buffer (samples #2 and #3) for 12 h. Treatment of J774 cells with only STS (sample #4) was a positive control for apoptosis induction and "no treatment" represents the background level whose enrichment factor was one (sample #1). The rate of apoptosis is reflected by the enrichment of nucleosomes in the cytoplasm, as shown on the *y* axis (n=3, mean ± S.D.). The differences between the wild-type infection and mET1229 infection at a moi of 1 (samples #5 and #7) and at a moi of 10 (samples#6 and #8) were found to be significant at $P<0.005$ and $P<0.001$, respectively. The differences between KA-untreated

wild-type infection and KA-treated wild-type infection at a moi of 1 (samples #5 and #10) and at a moi of 10 (samples#6 and #11) were found to be significant at $P<0.05$ and $P<0.001$, respectively. Abbreviations: W1 and W10, wild-type infection at an moi of 1 and 10, respectively; M1 and M10, mET1229 infection at an moi of 1 and 10, respectively. *Panel B*, Induction of anti-apoptotic proteins by the wild-type infection of STS-treated J774 cells is suppressed by the addition of KA. Western-blot analysis was performed as described under "Materials and Methods". Abbreviations: STS, staurosporine; KA, kamebakaurin.

Fig. 7. Intracellular growth of the wild-type within STS-treated or STS-untreated J774 macrophages is attenuated by treatment with the NF-κB inhibitor.

Panel A. J774 cells infected with the wild-type *E. tarda* or mET1229 at an moi of 1 or an moi of 10 were treated with mock buffer (samples #1, 3, 5, and 7) or the NF-κB inhibitor, kamebakaurin (KA), (samples #2, 4, 6, and 8) followed by apoptosis induction with STS (samples #1-8) for 12 h. Viable cells were counted as described under "Materials and Methods". The difference between KA-untreated wild-type infection and KA-treated wild-type infection at an moi of one (samples #1 and #2) and at an moi of 10 (samples#3 and #4) were found to be significant at $P<0.05$ and $P<0.001$, respectively; whereas, the

differences between KA-untreated mET1229 infection and KA-treated mET1229 infection at an moi of one (samples #5 and #6) and at an moi of 10 (samples#7 and #8) were found to be insignificant, $P>0.1$, respectively. Abbreviations: STS, staurosporine; KA, kamebakaurin. *Panel B.* J774 cells infected with the wild-type *E. tarda* or mET1229 at an moi of 1 or an moi of 10 were treated with mock buffer (samples #1, 3, 5, and 7) or the NF-κB inhibitor, kamebakaurin (KA), (samples #2, 4, 6, and 8) followed by treatment with mock buffer without STS (samples #1-8) for 12 h. Viable cells were counted as described under "Materials and Methods". The difference between KA-untreated wild-type infection and KA-treated wild-type infection at an moi of one (samples #1 and #2) and at an moi of 10 (samples#3 and #4) were found to be significant at $P<0.05$ and $P<0.001$, respectively; whereas, the differences between KA-untreated mET1229 infection and KA-treated mET1229 infection at an moi of one (samples #5 and #6) and at an moi of 10 (samples#7 and #8) were found to be insignificant, $P>0.1$ and $P>0.05$, respectively.

Supplemental Material

Supplemental Table S1. Up-regulated genes which show the highest relative increase in mRNA expression at 3, 5, 12 and 22 h after infection of J774 cells with the wild-type and Type III mutant of *E. tarda*.

Supplemental Table S2. Down-regulated genes which show the lowest relative increase in mRNA expression at 3, 5, 12 and 22 h after infection of J774 cells with the wild-type and Type III mutant of *E. tarda*.

Table 1. Up-regulated NF-κB target genes which showed high relative increase in mRNA expression after infection of J774 cells with the wild-type and Type III mutant of *E. tarda*

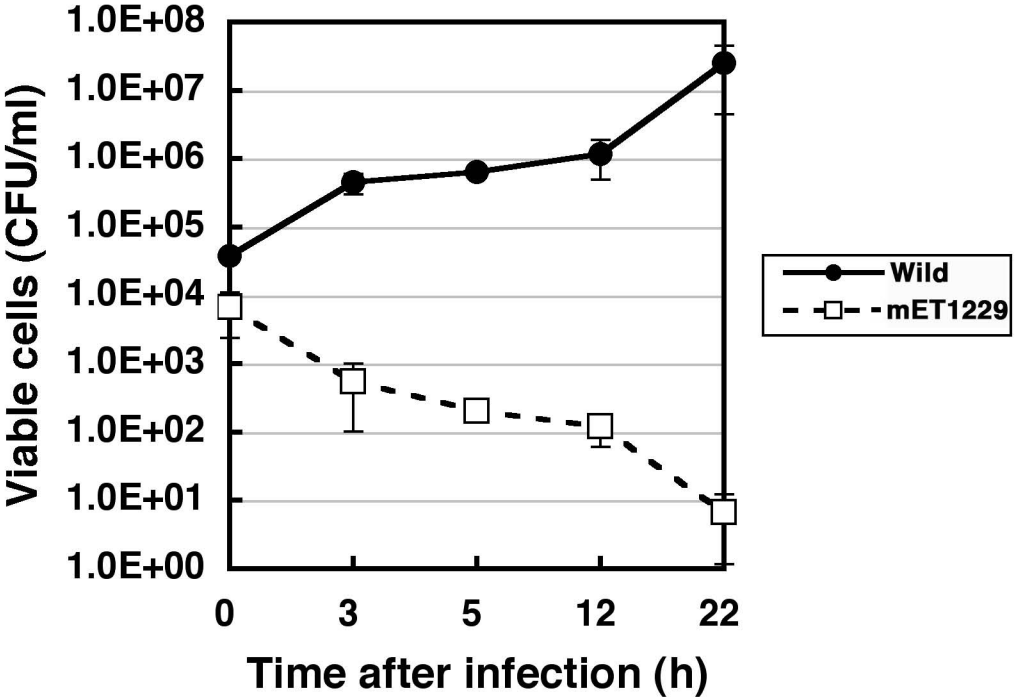
Time after infection[a]	Description[b]	Accession Number	Fold increase (wild/mutant)	(wild/NI[c])	(mutant/NI)[d]
3h	interferonγ	M28621	8.94	58.10	6.50
	chemokine (C-C motif) ligand 3 (Ccl3); macrophage inflammatory protein-1α (MIP	NM_011337	5.49	29.77	5.43
	chemokine (C-C motif) ligand 20 (Ccl20); macrophage inflammatoryprotein-3α (MI	NM_016960	3.50	3.16	0.90
5h	**baculoviral IAP repeat-containing 3, apoptosis inhibitor 2 (cIAP-2)**	**NM_007465**	**5.70**	**6.83**	**1.20**
	interleukin 1β	NM_008361	3.07	6.82	2.22
	macrophage receptor with collagenous structure (MARCO)	NM_010766	2.80	19.28	6.88
12h	chemokine (C-X-C motif) ligand 2 (Cxcl2); macrophage inflammatory protein-2 (MI	NM_009140	33.96	8.60	0.25
	interleukin 6	NM_031168	17.35	63.66	3.67
	serum amyloid A3	NM_011315	7.00	12.25	1.75
	TNF receptor-associated factor 1 (TRAF1)	**NM_009421**	**4.18**	**6.88**	**1.64**
	chemokine (C-C motif) ligand 5 (Ccl5); RANTES	NM_013653	3.65	10.63	2.91
	immune-responsive gene 1	L38281	3.60	3.29	0.91
	baculoviral IAP repeat-containing 3, apoptosis inhibitor 2 (cIAP-2)	**NM_007465**	**2.97**	**4.37**	**1.47**
	B-cell leukemia/lymphoma 2 related protein A1b (Bcl2A1b)	**NM_007534**	**2.84**	**4.66**	**1.64**
	B-cell leukemia/lymphoma 2 related protein A1a (Bcl2A1a)	**NM_009742**	**2.74**	**3.35**	**1.22**
22h	chemokine (C-C motif) ligand 2 (Ccl2); monocyte chemoattractant protein-1 (MCP	NM_011333	2.95	3.37	1.14

[a]For each time point after infection, results are shown in order of decreasing fold increase of (wild/mutant) ratio.
For the 5 h time point, the up-regulated NF-κB target genes that are already included in the 3 h time point are not shown.
For the 12 h time point, the up-regulated NF-κB target genes that are already included in the 3 or 5 h time point are not shown except for cIAP-2.
For the 22 h time point, the up-regulated NF-κB target genes that are already included in the 3, 5, or 12 h time point are not shown.
Exceptionally, the up-regulation of the gene encoding cIAP-2 is shown at both 5 and 12 h time points.

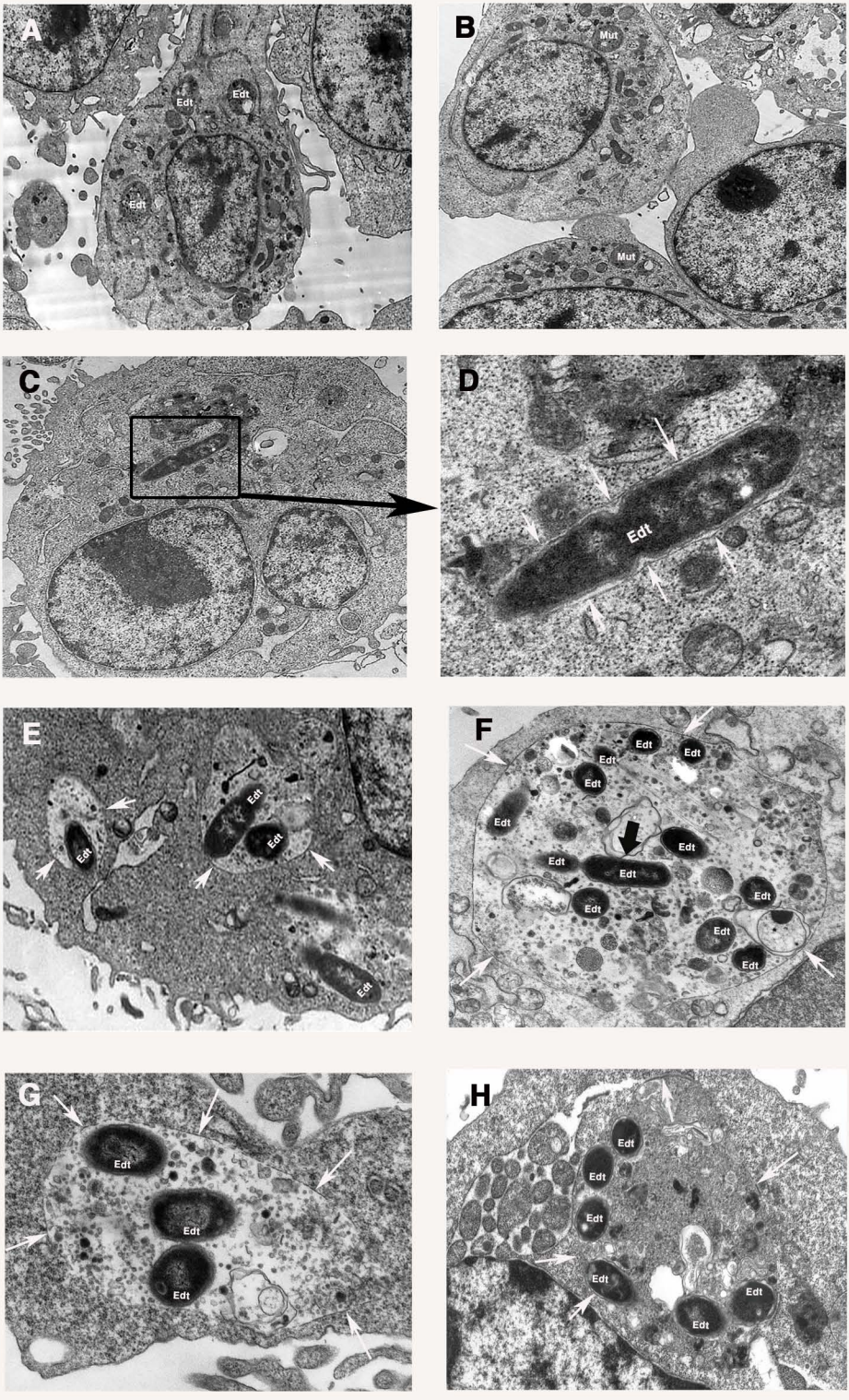
[b]Genes shown in boldface are anti-apoptotic NF-κB target genes.

[c]Non-infected cells.

[d]Fold increase of (mutant/NI) ratio was calculated by dividing (wild/NI) ratio by (wild/mutant) ratio.



Okuda et al. Fig. 1



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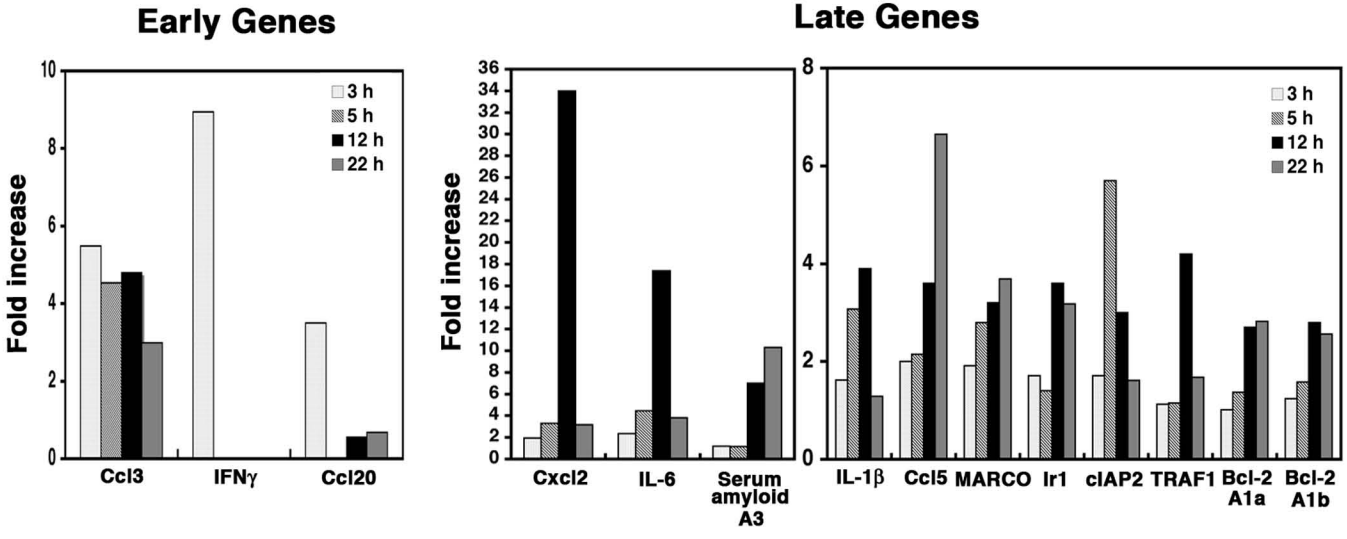

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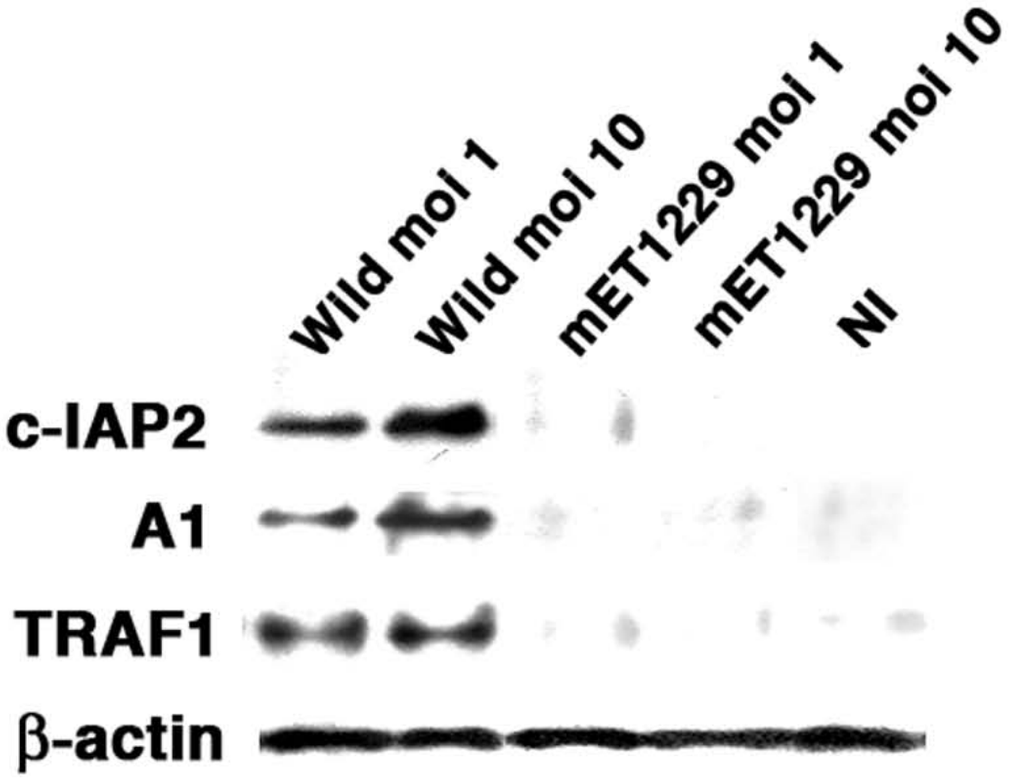

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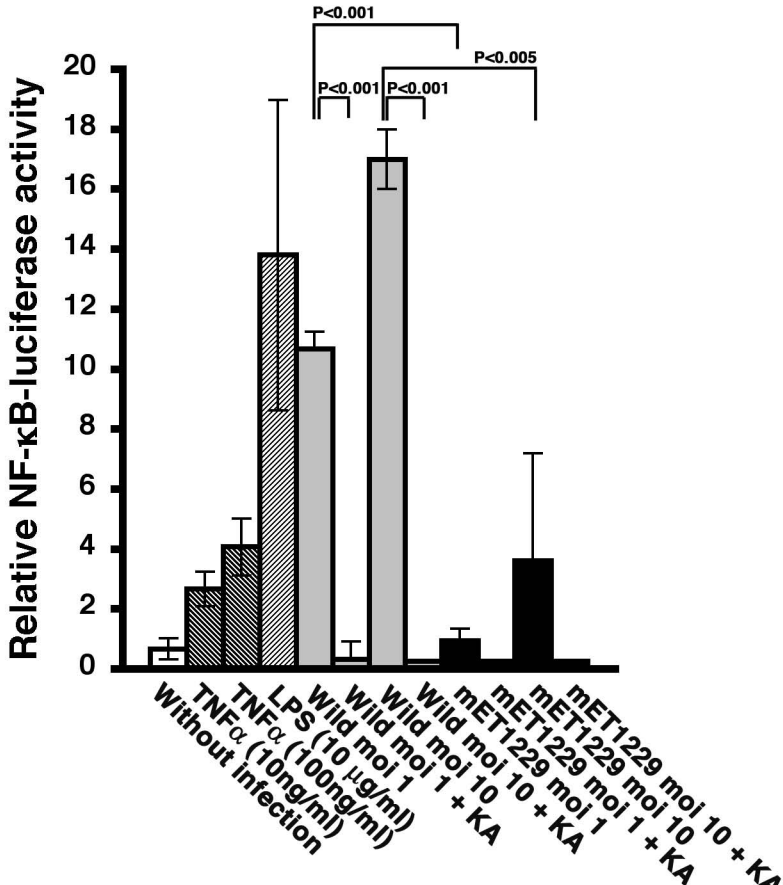

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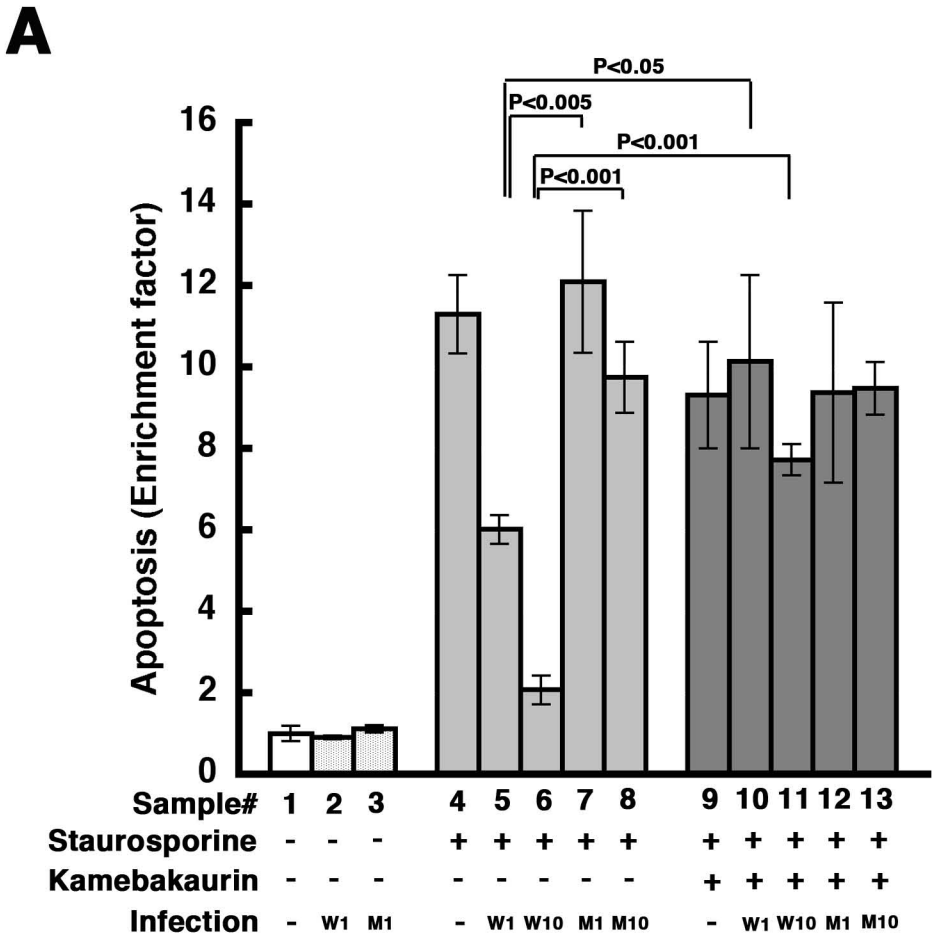
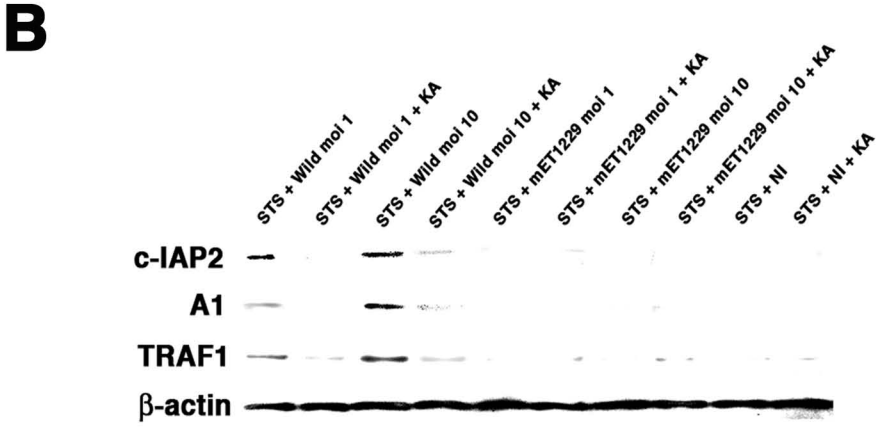
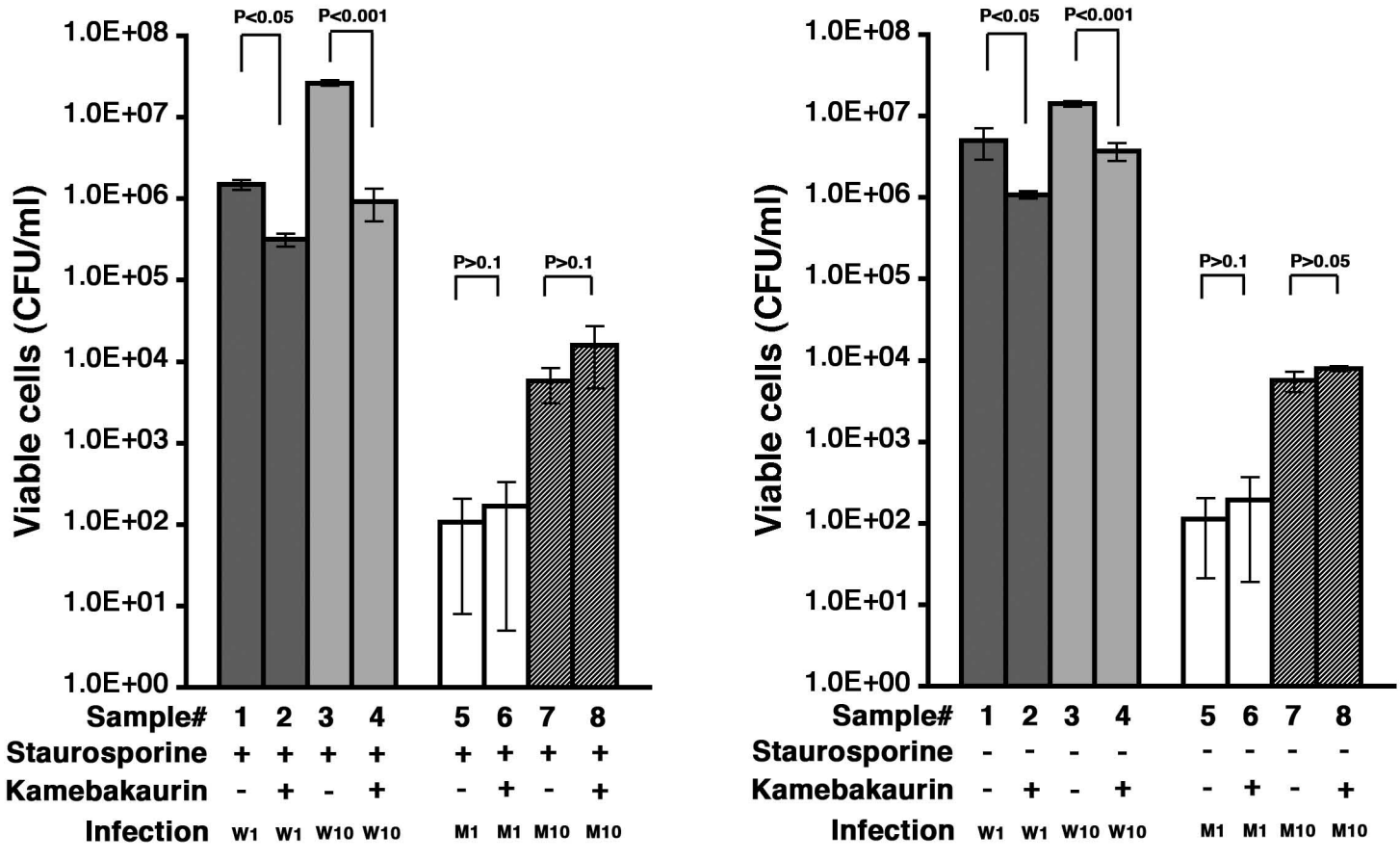




Okuda *et al.* Fig.6



Okuda *et al*. Fig.7