**Vibrio vulnificus** induces mTOR activation and inflammatory responses in macrophages

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**Abstract**

**Vibrio vulnificus** (V. vulnificus), a Gram-negative marine bacterium, can cause life-threatening primary septicemia, especially in patients with liver diseases. How *V. vulnificus* affects the liver and how it acts on macrophages are not well understood. In this report, we demonstrated that *V. vulnificus* infection causes a strong inflammatory response, marked expansion of liver-resident macrophages, and liver damage in mice. We demonstrated further that *V. vulnificus* activates mTOR in macrophages and inhibition of mTOR differentially regulates *V. vulnificus* induced inflammatory responses, suggesting the possibility of targeting mTOR as a strategy to modulate *V. vulnificus* induced inflammatory responses.

**Introduction**

*Vibrio vulnificus* (V. vulnificus) is a halophilic, Gram-negative and life-threatening marine bacterium [1, 2]. *V. vulnificus* infection can cause severe primary septicemia in patients with liver diseases such as viral hepatitis, advanced cirrhosis, and chronic alcoholic liver disease [3]. Despite previous studies have significantly advanced our understanding of the pathogenesis of *V. vulnificus* infection, mechanisms that regulate *V. vulnificus*-mediated responses remain unclear.

Upon infection, innate immune cells sense the pathogen and initiate cascades of events that result in the production of cytokines such as IL-1β, IL-6, and TNFα, which recruit other immune cells such as macrophages, neutrophils, and dendritic cells (DCs) to defend against pathogen invasion [4, 5]. Toll-like receptors (TLRs) or Nod-like receptors (NLRs) recognition of bacterial components activates NF-κB signaling, which is important for transcription of many cytokines [6]. In addition, activation of inflammasomes, marked by caspase-1 activation and IL-1β secretion [7–10], is an important aspect of innate response to danger signals during bacterial infection. Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that regulates both adaptive and innate immune cell development, metabolism, and function [11–16]. mTOR
forms two distinct signaling complexes, mTORC1 and mTORC2. Activated mTORC1 phosphorylates multiple downstream molecules such as S6K1 and 4E-BP1. mTORC2 phosphorylates Akt at serine 473 to regulate cell survival and nutrient uptake [17–19]. mTORC1, but not mTORC2, is sensitive to acute rapamycin treatment. Recent studies have found that mTOR and its tight regulation play important roles in innate immune responses [11, 15, 16, 20–22]. Many extracellular signals induce mTORC1 and mTORC2 activation in innate immune cells, including TLR, cytokines, and growth factors [15, 20, 23]. mTORC1 controls macrophage polarization [12, 21], is important for alveolar macrophage self-renewal [24], and regulates dendritic cell metabolism, maturation, and function [25–27]. Whether V. vulnificus can induce mTOR activation and how mTOR may regulate innate immune responses to this pathogen in macrophages is unknown.

In this report, we demonstrated that V. vulnificus could directly inflict liver damage and induce strong innate immune responses and Kupffer cell expansion. Moreover, V. vulnificus can activate mTOR in macrophages and inhibition of mTOR differentially regulates V. vulnificus-induced inflammatory responses.

**Results**

**Acute infection by V. vulnificus CGMCC1.1758 strain induced liver inflammation in C57BL/6J mice**

Patients with liver diseases are susceptible to severe V. vulnificus infection. One of the liver’s key functions is to clear blood-borne pathogens and to prevent bacteria from spreading to other organs to cause sepsis [28]. Several strains of V. vulnificus have been found to cause systemic infection in mice [29–31]. However, whether the V. vulnificus 1.1758 strain can cause liver damage has been unclear. To examine whether V. vulnificus is capable of causing liver damage, we intraperitoneally (i.p.) injected V. vulnificus strain CGMCC1.1758 into wild-type C57BL/6J mice. Mice infected with 1×10^8 CFU V. vulnificus showed higher mortality than those injected with 5×10^7 CFU (Fig 1A). V. vulnificus infection resulted in liver damage, indicated by elevated serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (Fig 1B). H&E staining showed piecemeal necrosis, focal inflammation, and portal inflammation in the liver (Fig 1C and 1D). Additionally, we detected V. vulnificus in liver homogenates (Fig 1E). These observations indicated that V. vulnificus CGMCC1.1758 is capable of inducing liver inflammatory damage in wild-type C57BL/6J mice.

**V. vulnificus acute infection-induced Kupffer cell and neutrophil accumulation in the liver**

To determine the infiltrating cell types in the liver after V. vulnificus infection, we performed immunofluorescence analysis of frozen liver sections of V. vulnificus–infected and control mice. As Fig 2A and 2B shows, both CD68^+ and CD11b^+ Kupffer cells and CD11b^+ Ly6G^+ neutrophils accumulated in the livers of infected mice. To further confirm Kupffer cell accumulation, we performed FACs analysis of liver mononuclear cells. As shown in Fig 2C and 2D, CD11b^+ F4/80^+ Kupffer cells were considerably increased in V. vulnificus–infected mice. Thus, acute V. vulnificus infection caused Kupffer cell accumulation in the liver.

**V. vulnificus acute infection-induced Kupffer cell proliferation and proinflammatory responses in vivo**

To determine whether Kupffer cell accumulation in the liver after V. vulnificus infection was associated with increased proliferation, we injected BrdU into mice immediately before V. vulnificus infection. Six hours after injection, Kupffer cells from V. vulnificus–infected livers...
incorporated more BrdU than those in uninfected mice, suggesting that these cells proliferated in infected mice (Fig 3A). Kupffer cells from V. vulnificus-infected mice upregulated CD71 (transferrin receptor protein 1, required for iron delivery from transferrin to cells) and CD98 (an amino acid transporter) (Fig 3B). In vitro, V. vulnificus infection of BMMΦs also upregulated CD71 and CD98 (Fig 3C), suggesting that V. vulnificus directly acted on macrophages to induce expression of these molecules. Elevated expression of CD71 and CD98 might ensure sufficient nutrients for macrophage metabolism, proliferation, and function. Together, these
observations suggest that acute *V. vulnificus* infection caused Kupffer cells to accumulate in the liver by inducing their proliferation.

To determine if *V. vulnificus* induced inflammatory responses, we measured cytokines concentrations in the serum and liver homogenates after *V. vulnificus* infection using a multiplex flow assay. In *V. vulnificus*-infected mice, multiple cytokines, such as IL-27, IL-6, IL-10, and MCP-1, obviously increased in both blood and liver (Fig 3D). Several other cytokines, such as IFN-β, IL-23, IL-12 p40, and IL-1α, increased only in the liver, while TNF-α was increased in serum, but not in the liver. The differences between liver and blood could result from differences in bacterial burdens and in local immune cell compositions in these organs/tissues. Thus, *V. vulnificus* infection triggered strong innate immune responses in vivo.

**Activation of NFκB and mTOR in macrophages by *V. vulnificus***

Transcription of many cytokines depends on NFκB after stimulating pathogen pattern recognition receptors [5, 32]. Bone marrow derived macrophages (BMMφs) infected with live 0.2 MOI *V. vulnificus* or treated with heat inactivated *V. vulnificus* (In) or *V. vulnificus* lysates for 6 hours contained increased IKKα/β and IκBα phosphorylation (Fig 4A), suggesting that *V. vulnificus* was capable of triggering IKK-NFκB activation. Low dose (0.2 MOI) *V. vulnificus*...
treatment also induced phosphorylation of mTOR, S6K1, S6, 4EBP1 and Akt. However, 2 MOI of *V. vulnificus-*treated cells did not induce phosphorylation of these proteins (Fig 4A), which was likely caused by negative feedback mechanisms triggered by over-stimulation or by death of BMMφs after 6 hours of infection. Indeed, after 3 hours treatment, 2 MOI *V. vulnificus* induced stronger S6K1 and S6 phosphorylation than 0.2 MOI *V. vulnificus* (Fig 4B). Moreover, Kupffer cells from *V. vulnificus*-infected mice also contained increased 4E-BP1 and Akt S473 phosphorylation (Fig 4C). These observations suggested that *V. vulnificus* induced both mTORC1 and mTORC2 activation in macrophages in vitro and in vivo.

To examine if *V. vulnificus* could directly induce innate immune responses in macrophages, we treated BMMφs with live *V. vulnificus* or heat inactivated *V. vulnificus*, or *V. vulnificus* culture supernatant (Sup) in vitro for 6 hours. As Fig 4D shows, both live and heat-inactivated *V. vulnificus* induced IL-6, IL-10, TNF-α, and IL-1α production in these cells. In addition, live but not inactivated *V. vulnificus* induced GM-CSF, IFNβ, IL-27, and IL-1β production. Interestingly, treatment of BMMφs with rapamycin during *V. vulnificus* stimulation resulted in
Fig 4. Effects of \textit{V. vulnificus} induced mTOR activation on innate immune responses in macrophages. (A) Detection of protein phosphorylation in BMM\textsubscript{ϕ} treated with live or heat-inactivated \textit{V. vulnificus}, its lysates, or culture.
supernatant for 6 hours. (B) Detection of protein phosphorylation in BMMϕ treated with 0.2 or 2.0 MOI V. vulnificus for 3h. (C) Detection of 4E-BP1 (Thr37/46) and Akt (Ser473) phosphorylation by intracellular staining and FACS analysis in CD11b+ F4/80+ cells isolated from liver of after infection of mice with 1 × 106 CFU V. vulnificus (n = 3). Means ± SEM in the plots show MFI of 4E-BP1 (Thr37/46) and Akt (Ser473) phosphorylation. (D) Cytokine profile in the supernatants of BMMϕ after in vitro V. vulnificus infection for six hours in the presence or absence of 100 nM rapamycin. (E) Detection of protein phosphorylation in BMMϕ treated with 0.2 MOI V. vulnificus for 6h in the presence or absence of 100nM rapamycin. (F) Growth-curve of V. vulnificus in the supernatant of BMMϕs infected with 0.2 MOI V. vulnificus in the presence or absence of 100nM. Data shown represent three experiments.

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decreased expression of GM-CSF, IFNβ, IL-27, and MCP-1 without obviously affecting the production of other cytokines. In rapamycin treated BMMϕs, V. vulnificus-induced S6K1 and S6 phosphorylation was abolished and Akt phosphorylation was diminished (Fig 4E), consistent with the notion that rapamycin is able to inhibit not only mTORC1 but also mTORC2. Interestingly, V. vulnificus CFUs were lower in rapamycin treated BMMϕs compared with untreated controls (Fig 4F), which could also contribute to decreased expression of cytokines induced after treatment with rapamycin. Together, these observations suggested that V. vulnificus contain heat resistant and labile components that can induce multiple cytokine production in macrophages and that mTOR is important for V. vulnificus proliferation in BMMϕs and may selectively regulate expression of certain but not all cytokines induced by V. vulnificus.

Discussion

V. vulnificus infection causes sepsis in the host and induces inflammation via recruiting and activating immune cells such as neutrophils, monocytes, and macrophages. Extensive work has linked various virulence factors of V. vulnificus to cell death and modulation of innate immune responses to this pathogen [30, 33–37]. Patients with liver diseases such as cirrhosis are more susceptible to developing V. vulnificus infection, which can lead to sepsis, sepsis-induced organ failure, and even death [38]. Surprisingly, little is known about the liver phase of V. vulnificus infection. In this report, we showed that V. vulnificus CGMCC1.1758 was capable of inducing inflammatory responses in the liver and inflicting liver damage in C57BL/6 mice, manifested by induction of multiple inflammatory cytokines and elevated serum AST and ALT levels. These observations are consistent with a previous report that another V. vulnificus strain, M06-24/O, is capable of causing liver inflammation and damage in BALB/c mice [31].

As tissue-resident macrophages, Kupffer cells are strategically located to clear bacteria in the liver during infection. Kupffer cells do not obviously proliferate in the steady state [39]. We have found that Kupffer cell numbers drastically increased in mice after V. vulnificus CGMCC1.1758 infection, which is at least partially caused by proliferation. Like V. vulnificus infection, systemic Listeria monocytogenes infection also results in Kupffer cell proliferation [40]. Thus, Kupffer cell expansion after bacterial infection may represent a means to increase the capacity of tissue-resident macrophages for clearance of invading pathogens. In addition to proliferation, V. vulnificus infection could increase Kupffer cells via other mechanisms. Our study provides the first evidence that V. vulnificus can cause mTOR activation. In BMMϕs, either infection with live V. vulnificus or treatment with heat-inactivated V. vulnificus or V. vulnificus lysates induces mTORC1 and mTORC2 activation reflected by 4E-BP1 and Akt S473 phosphorylation, respectively. Thus, V. vulnificus contains component(s) that can induce mTOR activation, although these components and the pattern recognition receptors that lead to mTOR activation remain to be identified. As mentioned earlier, mTOR and its tight regulation play important roles in innate immune responses [11, 15, 16, 20–22]. We have found that V. vulnificus infection
triggered strong inflammatory responses in vivo and *V. vulnificus* treatment induced production of multiple cytokines in macrophages. Among these cytokines, GM-CSF, IFN-β, IL-27, and IL-1β production but not IL-6, IL-10, TNF-α, and IL-1α production is dependent on mTOR activity. It would be interesting to further determine the underlying mechanisms by which mTOR controls production of selective cytokines during *V. vulnificus* infection.

### Materials and methods

#### Ethics statement

This study was carried out in strict accordance with the guidelines of the Zhejiang Provincial Animal Care and Use Administration Office (SYXK-ZJ-2005-0061). We performed all the animal experiments in accordance with protocols approved by the Wenzhou Medical University Animal Care and Use Committee (reference: wydw2014-0009).

#### Bacterial strains, mice and cell culture

We purchased C57BL/6J mice from Wenzhou Medical University Laboratory Animal Facility and were maintained under a SPF environment at the Central Animal Laboratory of Wenzhou Medical University. The mice were kept for 5–7 days to acclimatize after transport from the supplier. In the survival experiment, mice were observed every 30 minutes for 24 hours, and mice that survived for 24 hours were euthanized. L-929 cells were purchased from the Cell Bank of the Chinese Academy of Science in Shanghai and cultured them in RPMI1640 containing 10% heat-inactivated fetal bovine serum (Ausvin) and penicillin-streptomycin (50 IU/ml and 50 mg/ml; Beyotime). The China General Microbiological Culture Collection Center provided the *Vibrio vulnificus* CGMCC 1.1758 strain, which we grew at 37˚C in brain heart infusion broth (BHI) or on the BHI rabbit blood agar plate.

#### In vivo infection

We used i.p. injection to infect female C57BL/6J mice with *V. vulnificus*. For survival experiments, we i.p. injected mice with $5 \times 10^7$ or $1 \times 10^8$ CFU of *V. vulnificus* suspended in 200 μl PBS or with PBS alone. We euthanized and exsanguinated the mice 6 hours after infection for blood and tissue collection. The liver was fixed in 4% paraformaldehyde for histological analysis, and froze it in OCT medium (Thermo) for immunofluorescence analysis. To measure *V. vulnificus* CFU in the liver, we perfused infected mice with PBS before harvesting their livers. We cultured liver homogenate on BHI blood agar plates at 37˚C for 24 hours. We measured serum AST and ALT levels using AST and ALT kits (Njjcbio) following the manufacturer’s protocol.

#### Bacterial treatment of BMMϕs in vitro and Western blot

We isolated bone marrow cells and cultured them with 10% L-929 cell culture medium as described previously [15]. We plated BMMϕs in 35 mm dishes and cultured them for 12 hours. We then added live or heat inactivated *V. vulnificus* at the indicated multiplicity of infection (MOI) or *V. vulnificus* lysates to the cells. We collected supernatants at the indicated times for cytokine quantification. We then subjected the infected cells to lystate preparation and Western blot analysis by following a previous protocol [41]. Anti-phosho-mTOR, anti-phosho-S6K1, anti-phosho-S6, anti-phosho-Akt, anti-phosho-4EBP1, anti-phosho-IKKα/β, anti-phosho-IκBα, and anti-β-actin antibodies all came from Cell Signaling Technology.
Measurement of cytokines by multiplex flow assay

We measured multi-cytokines by using a LEGENDplex multi-analyte flow assay kit (13-plex) for mouse inflammation panel (Biolegend). We assessed the cytokine concentrations in sera, liver homogenates, and supernatants of cultured cells according to the manufacturer’s instructions. We collected data using a BD FACSaria II and analyzed them with LEGENDplex data analysis software (Biolegend).

BrdU incorporation

We intraperitoneally injected the mice with 1 mg BrdU (BD Bioscience) in 200 μl PBS, then with 1×10⁸ CFU of V. vulnificus two hours later. We harvested the liver from each infected mouse six hours after infection, isolating liver monocytes by following the previous protocol [42]. Briefly, livers were mashed in IMDM with 10% FBS. The upper cell suspension was filtered by nylon mesh and spun at 4˚C, 2000 rpm for 5 min. The cell pellet was resuspended in 12 ml 35% Percoll (GE Healthcare), and carefully underlaid with an equal volume of 75% Percoll, and centrifuged at 1000 x g for 20 min at room temperature without break. Cells at the interface were collected, washed and resuspended in IMDM (10% FBS) for further staining. We stained the cell surface with CD11b and F4/80 (Biolegend) and used a BrdU Flow Kit (BD Bioscience) for BrdU intracellular staining, following the manufacturer’s protocol.

Flow cytometry

We performed cell surface staining with fluorescence-conjugated antibodies in 2% FBS–PBS. We obtained fluorochrome-conjugated anti-CD11b, anti-F4/80, anti-CD71, and anti-CD98 from Biolegend. We identified cell death using a violet Live/Dead kit (Invitrogen). We performed intracellular staining for Alexa Fluor 647-conjugated p-Akt (Ser473) and p-4EBP1 (Thr37/46) (Cell Signaling Technology) using BD Biosciences Cytofix/Cytoperm and Perm/Wash solutions. We collected data using a BD FACSaria II and analyzed them with FlowJo (Treestar).

Histological and immunofluorescence analysis

For histology analysis, we imbedded the fixed livers in paraffin, then cut thin sections, and following standard procedures, stained them with hematoxylin and eosin. We quantified the liver histological score of inflammation according to Ishak inflammation score [43, 44]. For immunofluorescence analysis, we cut frozen sections into 8μM slices in a cryostat microtome (Thermo) at -20˚C and permeablized and blocked them. We probed the tissues with appropriate, fluorescently labelled antibodies, including APC-conjugated anti-CD11b, PE-conjugated anti-CD68, and PE-conjugated anti-Ly6G (Biolegend). Finally, we dried the sections and observed them with a Nikon A1 confocal microscope.

Statistical analysis

We analyzed statistical significance using the Student t test and determined survival difference by log-rank survival analysis, performing all statistics using Graphpad Prism 5.0 software. P values are defined as follows: P < 0.05, P < 0.01, and P < 0.001.

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