Published in final edited form as:
Pathog Dis. 2014 August ; 71(3): 357–361. doi:10.1111/2049-632X.12154.

TLR2 and TLR4 mediate the TNFα response to *Vibrio vulnificus* biotype 1

Lola V. Stamm and **Rebecca L. Drapp**
The University of North Carolina at Chapel Hill, Chapel Hill, NC

Abstract

Vibrio vulnificus (*Vv*) is a pathogenic bacterium that can cause life-threatening infections in humans. Most fatal cases are due to septic shock that results from dysregulation of cytokines, particularly TNFα, which plays a critical role in the outcome of *Vv* infection. The goal of this study was to investigate the Toll-like receptor (TLR)-mediated TNFα response to four *Vv* biotype 1 strains using mice deficient for TLR2, TLR4 and TLR2/TLR4. *Ex vivo* assays were performed with blood, splenocytes, and Kupffer cells (KC) from wild type (WT) and TLR knockout (KO) mice using formalin-inactivated *Vv* (f-*Vv*) as stimulant. All f-*Vv* biotype 1 strains elicited strong TNFα production by WT mouse blood and cells, which was TLR2- and TLR4-dependent. OxPAPC, an inhibitor of TLR2 and TLR4 signaling, effectively blunted the TLR-mediated TNFα response to f-*Vv*. Furthermore, TLR2 KO and TLR2/TLR4 KO mice were more resistant to lethal infection with *Vv* ATCC 27562 than WT mice, perhaps due to attenuation of the TNFα response. These data suggest that it may be possible to devise strategies to specifically target the harmful TLR-mediated TNFα response as an adjunct to antibiotic treatment of severe *Vv* infection.

Keywords

Vibrio vulnificus; Toll-like receptors; TLR2; TLR4; TNFα; *ex vivo* assays

Vibrio vulnificus (*Vv*) is a Gram-negative, motile bacterium that is ubiquitous in warm coastal environments (Jones & Oliver, 2009; Horseman & Surani, 2011). Infection, caused mainly by *Vv* biotype 1 strains, manifests as gastroenteritis, primary septicemia, or wound infection. Even with antibiotic treatment, mortality rates can exceed 50% for primary septicemia and 25% for wound infection (Jones & Oliver, 2009; Horseman & Surani, 2011). Fatalities are due to septic shock that results from dysregulation of cytokines, particularly TNFα, presumably owing to recognition of *Vv* agonists by Toll-like receptors (TLRs) (Espat *et al*., 1996; Shin *et al*., 2002; Powell *et al*., 2003; Toma *et al*., 2010). Previous studies showed that recognition of recombinant-produced *Vv* lipoprotein (IlpA) and flagellar filament protein (FlaB) and isolated capsular polysaccharide by TLR1/TLR2, TLR5, and TLR2, respectively, resulted in the production of inflammatory cytokines (Lee *et al*., 2006;

Correspondence: Lola V. Stamm, Infectious Diseases Program, Department of Epidemiology, Gillings School of Global Public Health, Michael Hooker Research Building, S. Columbia St., The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7435, Tel: 919-966-3809, Fax: 919-966-0584, lstamm@email.unc.edu.
Present address:
Rebecca L. Drapp, North Carolina State University, College of Veterinary Medicine, 1060 William Moore Dr., Raleigh, NC 27606

Goo *et a*l., 2007; Lee *et al*., 2010; Lee *et al*., 2011). Additionally, Stamm (2010) reported that TLR4 played a role in TNFα production by mouse blood and splenocytes following stimulation with formalin-inactivated *Vv* (f-*Vv*), and that TLR4 knockout (KO) mice, but not MyD88 KO mice, were more resistant to lethal *Vv* infection than wild-type (WT) mice. Because TLRs are potential targets for attenuating the harmful cytokine response (Boyd, 2012), the goal of this study was to investigate the TLR-mediated TNFα response to four *Vv* biotype 1strains using mice deficient for TLR2, TLR4, and TLR2/TLR4.

WT C57BL/6 and TLR2 KO mice were purchased from Jackson Laboratory (Bar Harbor, ME). TLR4 KO and TLR2/TLR4 KO mice were provided by B. Vilen and M. Heise, respectively (University of North Carolina, Chapel Hill, NC). All TLR KO mice had been backcrossed to WT C57BL/6 mice for at least eight generations. Experiments used 10–13 week old male mice. Animal procedures were approved by the UNC-CH Institutional Animal Care and Use Committee.

Vv ATCC 27562 (type strain) was purchased from Remel (Lake Charles, LA). C7184 and YJ016 were provided by J. Oliver (UNC, Charlotte, NC) and MO6-24/O by A. Wright (University of Florida, Gainesville, FL). *Vv* strains were grown in Heart Infusion (HI) broth as described previously (Stamm, 2010) and quantified with a Petroff-Hausser counting chamber. *Vv* were formalin-inactivated to prevent overgrowth during *ex vivo* assays (Stamm, 2010).

Ex vivo assays were performed with duplicate samples of heparinized mouse blood (50 μl/tube) and with splenocytes (5×10^5 cells/well) in 400μl RPMI 1640 medium without stimulant or with stimulant (i.e., 2.5×10^6 f-*Vv* or control TLR agonists) as described previously (Stamm, 2010). Splenocytes were obtained by mechanical disruption of spleens followed by lysis of red blood cells and resuspension of the washed splenocytes in RPMI 1640 medium with 5% heat-inactivated fetal bovine serum (HI-FBS). Assays with blood and splenocytes were performed three times. Liver macrophages (Kupffer cells, KC) were isolated with OptiPrep (Sigma-Aldrich, St. Louis, MO) (Froh *et al*. 2002). KC were plated at 4×10^5 cells/well, washed gently after adhering for 2h, and incubated in RPMI 1640 medium with 5% HI-FBS in 5% CO_2 at 37°C for 24h before replacement of medium with 400μl fresh medium without or with stimulant. Assays with KC were performed two times. Pam3CSK4, a synthetic mimetic of bacterial lipoprotein (InVivoGen, San Diego, CA) (50 ng) and purified *Escherichia coli* lipopolysaccharide (*Ec* LPS) (Sigma-Aldrich) (40 ng) were used as control TLR2 and TLR4 agonists, respectively. After a 24h incubation period, cell-free supernatants were collected and stored at −80°C until tested in duplicate by ELISA for TNFα (R & D Systems, Inc., Minneapolis, MN). Statistical analysis was performed with the unpaired, two-tailed *t* test for comparison of two groups or ANOVA for comparison of three or more groups followed by the Bonferroni post test or the post test for linear trend (Prism 5c, GraphPad Software, Inc., San Diego, CA). $P < 0.05$ was considered significant.

For *Vv* infection, groups of eight to ten WT, TLR2 KO, and TLR2/TLR4 KO mice were infected intraperitoneally with $5–7 \times 10^7$ *Vv* ATCC 27562 that had been grown at 33C in HI broth, washed once in phosphate buffered saline (PBS) and suspended in PBS (Stamm, 2010). WT and TLR2 KO mice were tested concurrently in three independent experiments.

TLR2/TLR4 KO mice were included in two of the experiments. Survival was monitored for 48h post infection. Mice that became irreversibly moribund based on established criteria (i.e., reduced mobility, hunched posture, poor grooming, rapid breathing) were euthanized and counted as nonsurvivors. Statistical significance of the combined data was evaluated with Fisher's exact test (Prism 5c).

For this study, *ex vivo* assays were used to investigate the role of TLR2 and TLR4 in the TNFα response of mice to four *Vv* biotype 1 strains that were isolated previously from septicemic patients (Rouche *et al*., 2005; Chatzidaki-Livanis *et al*., 2006; Thiaville *et al*., 2011). Aliquots of blood from WT, TLR2 KO, TLR4 KO, and TLR2/TLR4 KO mice were incubated in medium without stimulant or in medium with f-*Vv* or control TLR agonists. A high level of TNFα was detected in supernatants from WT blood stimulated with each of the f-*Vv* strains ($P < 0.01$) (Fig. 1A) or with the control TLR agonists ($P < 0.01$) (Supplemental, Fig. 1a) compared with supernatants from WT blood without stimulant in which TNFα was undetectable (i.e., below the 31 pg ml^{-1} detection limit) (data not shown). Compared with WT supernatants, the TLR2 KO and TLR4 KO supernatants contained less TNFα after stimulation with each of the f-*Vv* strains ($P < 0.01$) (Fig. 1A) or with the control TLR agonists, PAM3CSK4 and *Ec* LPS, respectively ($P < 0.01$) (Supplemental, Fig. 1a). The TNFα level of supernatants from TLR4 KO blood stimulated with the f-*Vv* strains was higher than that of supernatants from comparably stimulated TLR2 KO blood ($P < 0.01$). Interestingly, variability was observed in the TNFα level among supernatants from WT blood stimulated with the four f-*Vv* strains ($P < 0.01$) or among supernatants from TLR2 KO or TLR4 KO blood stimulated with these strains ($P < 0.01$) suggesting that the expression and/or structure of *Vv* TLR2 (i.e., capsule, lipoproteins) and TLR4 (LPS) agonists may differ among the strains. The TNFα level of supernatants from TLR2/TLR4 KO blood stimulated with the f-*Vv* strains was typically low or undetectable compared to that of supernatants from comparably stimulated WT, TLR2 KO or TLR4 KO blood ($P < 0.01$) (Fig. 1A). These results indicated that virtually all of the TNFα response of WT blood to the f-*Vv* strains was mediated by TLR2 and TLR4 signaling. Support for these findings was obtained when WT blood was incubated with f-*Vv* ATCC 27562 in the presence of OxPAPC (InVivoGen). OxPAPC is a mixture of oxidized phospholipids, which blocks TLR2 and TLR4 signaling by competing with accessory proteins (i.e., CD14, LBP, and MD2) that interact with TLR2 and TLR4 agonists (i.e., capsule, lipoproteins, LPS) (Erridge *et al*., 2008; Zughaier, 2011). Consistent with the TLR KO data, the TNFα response of WT blood to f-*Vv* ($P < 0.01$) and to the control TLR agonists ($P < 0.01$) was strongly inhibited by the addition of OxPAPC (Fig. 1B).

To evaluate further the role of TLR2 and TLR4 in the TNFα response to the f-*Vv* strains, *ex vivo* assays were performed with WT and TLR KO splenocytes. Results were similar to those observed with blood. A high level of TNFα was detected in supernatants of WT splenocytes stimulated with the four f-*Vv* strains ($P < 0.01$) (Fig. 1C) or with the control TLR agonists ($P < 0.01$) (Supplemental, Fig. 1b) compared to supernatants from WT splenocytes without stimulant in which TNFα was undetectable (data not shown). Supernatants from TLR2 KO and TLR4 KO splenocytes stimulated with the f-*Vv* strains ($P < 0.01$) or with the control TLR agonists ($P < 0.01$) contained less TNFα than those from corresponding WT

splenocytes stimulated with the f-*Vv* strains (Fig. 1C) or with the control TLR agonists (Supplemental Fig. 1b). Comparison of the TNFα level of supernatants from TLR4 KO and TLR2 KO splenocytes stimulated with the f-*Vv* strains showed that the TNFα level was different for MO6-24/O and C7184 (P <0.01), but not for ATCC 27562 or YJ016 (P >0.54). Variability was observed in the TNFα level among supernatants from WT splenocytes stimulated with the four f-*Vv* strains (P <0.03) or among supernatants from TLR2 KO or TLR4 KO splenocytes stimulated with these strains (P <0.01). The TNFα level of supernatants from TLR2/TLR4 KO splenocytes stimulated with the f-*Vv* strains was very low or undetectable compared with that of supernatants from comparably stimulated WT, TLR2 KO or TLR4 KO splenocytes (P <0.01) (Fig. 1C).

Because KC are a major source of TNFα when activated by TLR agonists of blood and gut pathogens (Bilzer *et al*., 2006; Wu *et al*., 2009), *ex vivo* assays were also performed with WT and TLR KO KC. A high level of TNFα was detected in supernatants from WT KC stimulated with the four f-*Vv* strains (P <0.01) (Fig. 1D) or with the control TLR agonists (P <0.01) (Supplemental, Fig. 1c) compared to supernatants from WT KC without stimulant in which TNFα was undetectable (data not shown). As observed for blood and splenocytes, supernatants from TLR2 KO and TLR4 KO KC (P <0.01) stimulated with the f-*Vv* strains or with the control TLR agonists (P <0.01) contained less TNFα than supernatants from corresponding WT KC stimulated with the f-*Vv* strains (Fig. 1D) or with the control TLR agonists (Supplemental Fig. 1c). The TNFα level of supernatants from TLR2/TLR4 KO KC stimulated with the f-*Vv* strains was very low or undetectable compared with that of supernatants from WT, TLR2 KO or TLR4 KO KC (P <0.01) (Fig. 1D). Although variability was not observed in the TNFα response among supernatants from WT KC (P =0.23) or among supernatants from TLR4 KO KC (P > 0.24) stimulated with the four f-*Vv* strains, variability was observed in the TNFα response among supernatants from TLR2 KO KC (P <0.01) stimulated with these strains (Fig. 1D). In contrast to the TNFα level of supernatants from TLR4 KO blood stimulated with the f-*Vv* strains, which was consistently higher than that from comparably stimulated TLR2 KO blood (P <0.01), the TNFα level of supernatants from TLR4 KO KC stimulated with the f-*Vv* strains was consistently lower than that from comparably stimulated TLR2 KO KC (P <0.01). These results suggest that the TNFα response of KC to f-*Vv* was largely mediated by TLR4. Information concerning mouse KC TLR expression is limited. However, because KC are a more homogenous cell population than blood, variation in TLR expression levels may account for the different trends observed in the TLR2- and TLR4-mediated TNFα response of KC and blood.

Stamm (2010) reported previously that TLR4- or TNFα-deficiency protected against lethal infection with *Vv* ATCC 27562 in a mouse sepsis model. Because our ex vivo assays showed that production of TNFα following stimulation with f-*Vv* was dependent upon TLR2 and TLR4, we examined the effect of TLR2 KO and TLR2/TLR4 KO on the susceptibility of mice to lethal infection with *Vv* ATCC 27562. There was no difference in survival between the TLR2 KO (88.5% survival) and TLR2/TLR4 KO mice (78.9% survival) (P =0.43). However, these TLR KO mice were more resistant than WT mice (31% survival) to lethal infection, indicating that a deficiency of TLR2 or TLR2/TLR4 (P <0.01) is protective.

In summary, *ex vivo* assays were used to examine the TLR-mediated TNFα response of mouse blood, splenocytes, and KC to four *Vv* biotype 1 strains. All f-*Vv* strains elicited strong TNFα production by WT mouse blood and cells, which was TLR2- and TLR4-dependent. Furthermore, TLR2 KO and TLR2/TLR4 KO mice were more resistant to lethal infection with *Vv* than WT mice, perhaps due to attenuation of the TNFα response. These data suggest that it may be possible to devise strategies to selectively target the harmful TLR-mediated TNFα response. Spiller *et al*. (2008) showed that blockade of TLR2 and TLR4 signaling with monoclonal antibodies, at the initiation of antibiotic therapy, is feasible for treatment of experimental Gram-negative bacterial infections. Thus, we propose that OxPAPC, which inhibited the TLR2- and TLR4-mediated TNFα response to f-*Vv*, warrants investigation in the mouse model of *Vv* sepsis as an adjunct to antibiotic therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was support by NIH grant RO3 AI082129 to LVS. We thank J. Oliver and A. Wright for providing *Vv* strains, B. Vilen and M. Heise for providing TLR4 and TLR2/TLR4 breeder mice, B. Bradford, V. Soldatow, and J. Card for suggestions for KC isolation, and P. Stewart for advice on statistical analysis.

References

Bilzer M, Roggel F, Gerbes AL. Role of Kupffer cells in host defense and liver disease. Liver Int. 2006; 26:1175–1186. [PubMed: 17105582]

Boyd JH. Toll-like receptors and opportunities for new sepsis therapeutics. Curr Infect Dis Rep. 2012; 14:455–461. [PubMed: 22805995]

Chatzidaki-Livanis M, Hubbard MA, Gordon K, Harwood VJ, Wright AC. Genetic distinctions among clinical and environmental strains of *Vibrio vulnificus*. Appl Environ Microbiol. 2006; 72:6136–6141. [PubMed: 16957239]

Erridge C, Kennedy S, Spickett CM, Webb DJ. Oxidized phospholipid inhibition of toll-like receptor (TLR) signaling is restricted to TLR2 and TLR4: roles for CD14, LPS-binding protein, and MD2 as targets for specificity of inhibition. J Biol Chem. 2008; 283:24748–24759. [PubMed: 18559343]

Espat NJ, Auffenberg T, Abouhamze A, Baumhofer J, Moldawer LL, Howard RJ. A role for tumor necrosis factor-alpha in the increased mortality associated with *Vibrio vulnificus* infection in the presence of hepatic dysfunction. Ann Surg. 1996; 223:428–433. [PubMed: 8633922]

Froh M, Konno A, Thurman RG. Isolation of liver Kupffer cells. Curr Protoc Toxicol. 2002; 14:4.1–4.12. [PubMed: 23045093]

Goo SY, Han YS, Kim WH, Lee KH, Park SJ. *Vibrio vulnificus* IlpA-induced cytokine production is mediated by Toll-like receptor 2. J Biol Chem. 2007; 282:27647–27658. [PubMed: 17640874]

Horseman MA, Surani S. A comprehensive review of *Vibrio vulnificus*: an important cause of severe sepsis and skin and soft-tissue infection. Int J Infect Dis. 2011; 15:e157–166. [PubMed: 21177133]

Jones MK, Oliver JD. *Vibrio vulnificus*: Disease and pathogenesis. Infect Immun. 2009; 77:1723–1733. [PubMed: 19255188]

Lee BC, Kim MS, Choi SH, Kim TS. Involvement of capsular polysaccharide via a TLR2/NF-κ B pathway in *Vibrio vulnificus*-induced IL-8 secretion of human intestinal epithelial cells. Int J Mol Med. 2010; 25:581–591. [PubMed: 20198307]

Lee NY, Lee HY, Lee KH, Han SH, Park SJ. *Vibrio vulnificus* IlpA induces MAPK-mediated cytokine production via TLR1/2 activation in THP-1 cells, a human monocytic line. Mol Immunol. 2011; 49:143–154. [PubMed: 21903273]

Lee SE, Kim SY, Jeong BC, et al. A bacterial flagellin, *Vibrio vulnificus* FlaB, has a strong mucosal adjuvant activity to induce protective immunity. Infect Immun. 2006; 74:694–702. [PubMed: 16369026]

Powell JL, Strauss KA, Wiley C, Zhan M, Morris JG Jr. Inflammatory cytokine response to *Vibrio vulnificus* elicited by peripheral blood mononuclear cells from chronic alcohol users is associated with biomarkers of cellular oxidative stress. Infect Immun. 2003; 71:4212–4216. [PubMed: 12819121]

Rosche TM, Yano Y, Oliver JD. A rapid and simple PCR analysis indicates there are two subgroups of *Vibrio vulnificus* which correlate with clinical or environmental isolation. Microbiol Immunol. 2005; 49:381–389. [PubMed: 15840964]

Shin SH, Shin DH, Ryu PY, Chung SS, Ru JH. Proinflammatory cytokine profile in *Vibrio vulnificus* septicemic patients' sera. FEMS Immunol Med Microbiol. 2002; 33:133–138. [PubMed: 12052568]

Spiller S, Elson G, Ferstl R, Dreher S, Mueller T, Freudenberg M, Daubeuf B, Wagner H, Kirschning CJ. TLR4-induced IFN-γ production increases TLR2 sensitivity and drives Gram-negative sepsis in mice. J Exp Med. 2008; 205:1747–1754. [PubMed: 18644971]

Stamm LV. Role of TLR4 in the host response to *Vibrio vulnificus*, an emerging pathogen. FEMS Immunol Med Microbiol. 2010; 58:336–343. [PubMed: 20059544]

Thiaville PC, Bourdage K, Wright AC, Farrell-Evans M, Garvan CW, Gulig PA. Genotype is correlated with but does not predict virulence of *Vibrio vulnificus* biotype 1 in subcutaneously inoculated iron dextran-treated mice. Infect Immun. 2011; 79:1194–1207. [PubMed: 21199909]

Toma C, Higa N, Koizumi Y, et al. Pathogenic *Vibrio* activate NLRP3 inflammasome via cytotoxins and TLR/nucleotide-binding oligomerization domain-mediated NF-κ B signaling. J Immunol. 2010; 184:5287–5297. [PubMed: 20348425]

Wu J, Meng Z, Jiang M, et al. Toll-like receptor-induced innate immune responses in non-parenchymal liver cells are cell type-specific. Immunol. 2009; 129:363–374.

Zughaier SM. *Neisseria meningitidis* capsular polysaccharides induce inflammatory responses via TLR2 and TLR4. J Leukoc Biol. 2011; 89:469–480. [PubMed: 21191086]

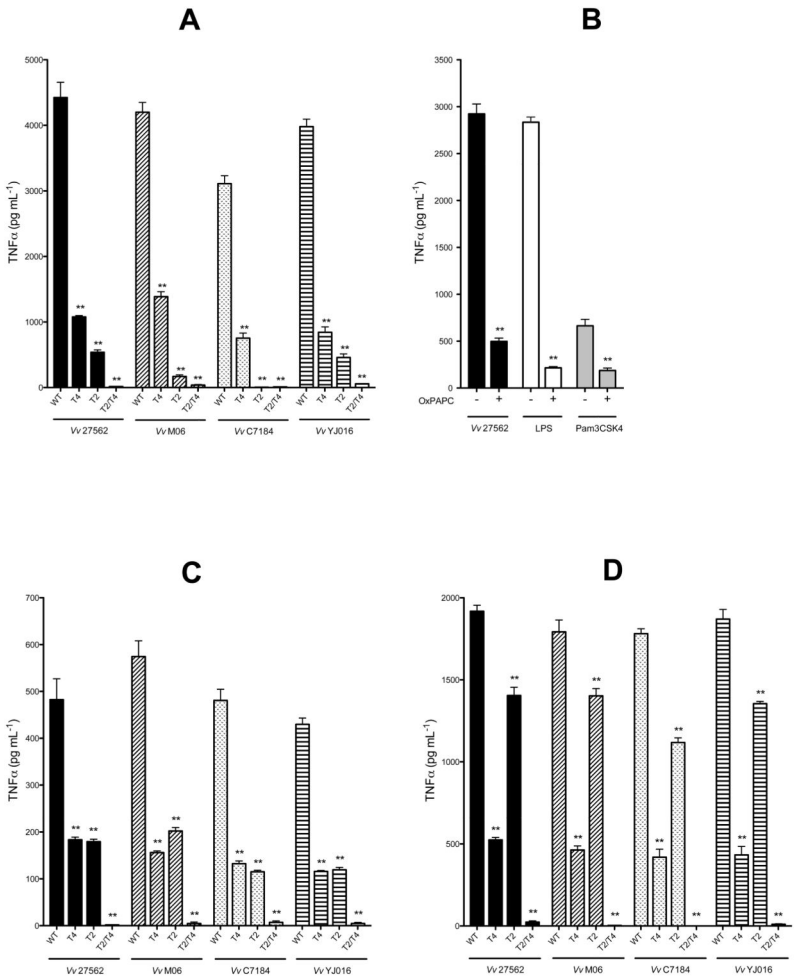

Fig. 1.

TNFα response of mouse blood and cells to stimulation with formalin-inactivated *Vibrio vulnificus* (f-*Vv*) biotype 1 strains is mediated by TLR2 and TLR4. *Ex vivo* assays were performed with blood or isolated cells pooled from two mice per each genotype. Duplicate samples of wild-type (WT), TLR4 (T4) knockout (KO), TLR2 (T2) KO, and TLR2/TLR4 (T2/T4) KO blood (A), splenocytes (C), or Kupffer cells (KC) (D) were incubated in duplicate without stimulant or with 2.5×10^6 f-*Vv* strains (ATCC 27562, MO6/24-O, C7184, and YJ016) for 24h. Duplicate samples of WT blood (B) were incubated in duplicate with 2.5×10^6 f-*Vv* ATCC 27562, *Ec* LPS (40 ng) or PAM3CSK4 (50 ng) with (+) or without (−) OxPAPC (15 μg) for 24h. Duplicate supernatants were collected, pooled, and tested in duplicate for TNFα by ELISA. Values are the mean (± SEM). Results are representative of three independent experiments (A and C) or two independent experiments (B and D). The TNFα level of supernatants from all WT and TLR KO samples incubated without stimulant was below the assay detection limit (31 pg ml^{-1}) and is not shown. ** Different compared with WT samples with the same stimulus (P <0.01) (A, C, D). ** Different compared with WT samples without OxPAPC (P <0.01) (B).