

Title:

The Role of Death Receptor Signaling Pathways in Mouse Sertoli Cell Avoidance of Apoptosis During LPS- and IL-18-induced Inflammatory Conditions

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Abstract

Lipopolysaccharide (LPS) triggers infectious acute inflammation, and interleukin (IL)-18 is an inflammasome-mediated cytokine. We previously demonstrated that endogenous IL-18 induces testicular germ cell apoptosis during acute inflammation when plasma IL-18 levels are high. Additionally, high-dose recombinant IL-18 (rIL-18) induced Leydig cell apoptosis. The blood-testis barrier formed by Sertoli cells protects testicular germ cells from both exogenous and endogenous harmful substances. However, the impact of LPS and IL-18 on Sertoli cells remained unclear. We stimulated TM4 cells, a mouse Sertoli cell line, with LPS (200 or 1000 ng/mL) or rIL-18 (0.1–100 ng/mL) at levels that induced Leydig cell apoptosis in our previous study and assessed caspase 3 cleavage and the mRNA expression of inflammatory cytokines and markers of apoptotic pathways (*Tnfr1*, *Fasl*, *Fas*, *Fadd*) after stimulation. *Il6* mRNA was increased by LPS stimulation. *Tnfa* mRNA was increased by 200 ng/mL LPS but not 1000 ng/mL LPS. *Fas* was increased, but *Fasl* was decreased, by LPS. LPS had little influence on *Tnfr1* or *Fadd* mRNA expression and did not induce apoptosis. *Il18* mRNA was not increased, and *Il18r1* was significantly decreased following LPS treatment. Treatment with rIL-18 increased *Il18r1* mRNA and induced inflammation, but decreased *Tnfr1* and had little influence on apoptosis, as indicated by *Tnfa*, *Fasl*, *Fas*, *Fadd* and cleaved caspase 3. These results suggested that Sertoli cells do not easily undergo apoptosis despite strong inflammatory stimuli. Additionally, Sertoli cells may resist inflammation and play a larger role in protecting testicular homeostasis than other component cells of the testis.

Keywords

apoptosis, inflammation, interleukin-18, Sertoli cell, testicular homeostasis

47

48 **Abbreviations**

49 DMEM, Dulbecco's modified eagle medium; Fadd, Fas associated death domain protein;

50 FasL, Fas ligand; IL, interleukin; IL-18r1, interleukin-18 receptor 1; LPS,

51 lipopolysaccharide; PVDF, polyvinylidene difluoride; rIL-18, recombinant IL-18; Tfre,

52 transferrin receptor; Tnfr, tumor necrosis factor receptor.

Introduction

In 2017, an estimated 48.9 million people worldwide suffered from sepsis, a life-threatening complication of severe infection (Rudd et al., 2020). Post-intensive-care syndrome is defined as new or worsening impairments in physical, cognitive, or mental health status arising after a critical illness and persisting beyond acute care hospitalization (Needham et al., 2012). Recently, increases in the incidence of sepsis and number of septic shock survivors have promoted investigation into the quality of life after sepsis (Genga and Russell, 2017). Approximately 20% of adult male patients suffering sepsis are between the peak reproductive ages of 18 to 45 years (Beale et al., 2009). Several physical impairments resulting from post-intensive-care syndrome have been reported, such as pulmonary and neuromuscular problems (Johanna Josepha et al., 2022; Martín-Vicente et al., 2021), and a few reports have described reproductive dysfunction and altered gonad physiology after a critical illness (Sengupta et al., 2022; Omolaoye et al., 2022; Gacci et al., 2021). In patients with severe acute respiratory syndrome, for example as a result of SARS-coronavirus-2/COVID-19 infection, oxidative stress causes many cytokines to be overproduced, leading to a systemic cytokine storm and systemic inflammation. Germ cell apoptosis, impaired spermatogenesis, and sperm DNA fragmentation are consequences of this inflammatory response (Sengupta et al., 2022;

Omolaoye et al., 2022). Oligo-crypto-azoospermia has been reported in 25% of male patients who have recovered from COVID-19 (Gacci et al., 2021).

Interleukin (IL)-18 is a known inflammasome-mediated proinflammatory cytokine, and IL-18 levels are increased in mouse testes by inflammatory stimulation, such as lipopolysaccharide (LPS) administration (Abu Elhija et al., 2008c; Abu Elhija et al., 2008b). In addition, IL-18 is an important cytokine to maintain testicular homeostasis. IL-18 is produced by germ cells, testicular somatic cells (Leydig cells and Sertoli cells), and resident macrophages and may regulate testicular function via autocrine/paracrine signaling under physiologic conditions (Abu Elhija et al., 2008a; Abu Elhija et al., 2008d; Komsky et al., 2012; Strand et al., 2005). We previously studied the effect of endogenous IL-18 on mouse testicular germ cells apoptosis caused by *E.coli* LPS-induced endotoxemia in mice (Inoue et al., 2015). During the acute phase of inflammation, endogenous IL-18 induced germ cell apoptosis; whereas, IL-18 suppressed germ cell apoptosis during recovery from inflammation. We also demonstrated that LPS and high IL-18 levels induce Leydig cell apoptosis *in vitro* (Inoue et al., 2020). However, the impact of LPS and IL-18 on Sertoli cells remained unclear.

The primary function of Sertoli cells is to maintain and control spermatogenesis (Yamamuro et al., 2021) and to protect testicular germ cells from harmful substances by

constituting the blood-testis barrier (Cheng and Mruk, 2012). Therefore, any damage to Sertoli cells could severely impact spermatogenesis (Murphy and Richburg, 2015). When considering the quality of life of patients with post-intensive-care syndrome, maintaining the function of cells that support spermatogenesis is very important to avoid male infertility. Therefore, we mimicked infectious inflammatory stimulation using LPS or IL-18 and investigated whether these stimuli induced Sertoli cell apoptosis, which may affect spermatogenesis and male fertility.

Materials and methods

Cell culture, LPS and rIL-18 stimulation, and cell viability

A Sertoli cell line, TM4, was purchased from the American Type Culture Collection (Manassas, VA, USA), and cells that had undergone 4 passages were used for our experiments. The TM4 cells were cultured in Dulbecco's modified eagle medium (DMEM)/F12 medium (American Type Culture Collection) supplemented with penicillin (50 U/mL), streptomycin (50 µg/mL; MP Biomedicals, Illkirch, France), 5% horse serum (American Type Culture Collection), and 2.5% fetal calf serum (American Type Culture Collection). The cell lines were maintained in a humidified atmosphere composed of 95% air and 5% CO₂ at 37°C. Prior to LPS treatment, TM4 cells were cultured on 6-well

microplates (Collagen I Cellware, Corning, Corning, NY, USA) with 1×10^5 cells and 2 mL DMEM/F12 per well for 30 hours. The cells were then incubated with 200 ng/mL LPS or 1000 ng/mL LPS (*E.coli* O55:B5, #L2880, Sigma–Aldrich, St. Louis, MO, USA) dissolved in DEME/F12. LPS is an endotoxin found in the cell wall of gram-negative bacteria and induces sepsis (Gabarin et al., 2021). The LPS-stimulated cells were collected 0, 1, 6, 12, 18, and 24 hours after treatment. TM4 cells were also cultured with recombinant IL-18 (rIL-18; mouse recombinant IL-18 without BSA; Medical & Biological Laboratories Co., Ltd., Aichi, Japan) at increasing concentrations (0.1, 1, 10, and 100 ng/mL), and collected 18 hours after treatment. The LPS and rIL-18 concentrations used in this study were based on our previous study (Inoue et al., 2020). We intentionally used the same lot of LPS in this study as in our previous study (Inoue et al., 2020). Control cells were incubated with medium only. Cell viability was determined with trypan blue staining (Thermo Fisher Scientific, Waltham, MA, USA).

Quantitative real-time RT-PCR

Total RNAs were extracted from the TM4 cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reverse transcription was performed using iScript cDNA synthesis kits (Bio-Rad Laboratories, Hercules, CA, USA) according to the

manufacturer's instructions. The expression of the mRNAs for IL-6, IL-18, IL-18
 receptor 1 (IL-18r1), tumor necrosis factor (Tnf)- α , tumor necrosis factor receptor (Tnfr)
 1, Fas, Fas ligand (Fasl), and Fas associated death domain protein (Fadd) were detected
 using quantitative real-time reverse transcription PCR (LightCycle480, Roche
 Diagnostics K.K. Tokyo, Japan) with SYBR green real-time PCR master mix (Toyobo,
 Osaka, Japan). The expression of 13 housekeeping genes in TM4 cells was analyzed using
 a Mouse Housekeeping Gene Primer Set (Takara Bio Inc., Shiga, Japan). Transferrin
 receptor (*Tfrc*) was determined to be the most stable housekeeping gene by BestKeeper
 (Pfaffl et al., 2004). The relative expression of each gene was calculated using the ddCt
 method after normalization to *Tfrc* mRNA levels. Table 1 shows the primer sequences
 used for PCR. A commercially available primer set was used to detect *Il18r1* (#VMPS-
 3068, Biomol GmbH, Hamburg, Germany).

Western blotting

Rabbit polyclonal antibodies against cleaved caspase-3 (cleaved at Asp175,
 #9661) and caspase-3 control cell extracts (#9663, extracts from untreated Jurkat cells
 [negative control] or Jurkat cells treated with cytochrome c [positive control]) were
 purchased from Cell Signaling Technology (Danvers, MA, USA). Rabbit polyclonal

antibodies against transferrin receptor (ab84036) were purchased from Abcam (Cambridge, UK). Peroxidase-conjugated anti-rabbit IgG antibodies were purchased from Jackson ImmunoResearch Inc. (West Grove, PA, USA).

Protein was extracted from TM4 cells with PRO-PREP solution (iNtRON Biotechnology Inc, Seoul, Korea) according to the manufacturer's instruction. Equal concentrations of each pooled sample were subjected to SDS-polyacrylamide gel electrophoresis on Mini-PROTEIN TGX Stain-Free Gels (Bio-Rad Laboratories) and transferred onto polyvinylidene difluoride (PVDF) membranes (Trans-Blot Turbo Transfer Pack, Bio-Rad Laboratories) using a Trans-Blot Turbo Transfer System (Bio-Rad Laboratories). The membranes were blocked with Bullet Blocking One for Western Blotting (Nacalai tesque, Kyoto, Japan) and incubated with primary antibodies diluted in Can Get Signal immunoreaction enhancer solution 1 (Toyobo) overnight at 4°C and then incubated with secondary antibodies diluted in Can Get Signal solution 2 (Toyobo) for 1 hour at room temperature. Blots were developed using the ECL-plus Western Blotting Detection System (GE Healthcare) and exposed on an ImageQuant LAS 4010 system (GE Healthcare). Transferrin receptor was used as the internal control. Western blotting was performed in quadruplicate using pooled samples ($n = 3$ per group).

Statistical analysis

All data are presented as the mean \pm standard deviation. Student's t-test or Welch's t-test was used to compare average differences between 2 groups. Responses to LPS and rIL-18 were compared between each treatment group and the vehicle (control) group. A probability level of $p < 0.05$ was considered statistically significant.

Results

Sertoli cells reduced FasL expression to avoid apoptosis of LPS-mediated death receptor signaling

To investigate the tolerance of Sertoli cells to inflammation, we treated a Sertoli cell line, TM4 cells, with LPS and examined apoptosis as indicated by cleaved caspase-3 protein expression. The amount of cleaved caspase-3 in TM4 cells did not increase after treatment with either 200 ng/mL or 1000 ng/mL LPS for up to 24 hours (Fig. 1A). TM4 cell viability did not change after treatment with either 200 ng/mL or 1000 ng/mL LPS for up to 24 hours (Table 2).

To examine whether the death-receptor-mediated apoptotic pathway was activated by LPS in the TM4 cells, we examined the expression of mRNAs encoding important regulators of death-receptor-mediated apoptosis (Tnfr1, Fas, FasL, and Fadd).

Fas mRNA was significantly increased after 1 hour treatment with either 200 or 1000 ng/mL LPS and remained elevated after 24 hours of treatment with LPS (Fig. 1B). The *Fasl* mRNA was significantly decreased after 6 hours or 24 hours of LPS stimulation with wide variability between the sample replicates at other timepoints examined (Fig. 1C). LPS had little influence on the *Tnfr1* mRNA expression (Fig. 1D). *Fadd* mRNA levels were significantly increased 1 hour after 200 ng/mL LPS treatment and decreased to baseline within 6 hours after administration of LPS (Fig. 1E), but *Fadd* mRNA levels remained elevated 24 hours after treatment with 1000 ng/mL LPS treatment (Fig. 1E). These results suggested that, even though apoptosis was not detected, the death-receptor-mediated apoptotic pathway was activated.

LPS decreased IL-18r1 mRNA expression but did not increase IL-18 mRNA expression

To confirm that an inflammatory response was initiated by LPS stimulation in the TM4 cells, we measured the expression of inflammatory cytokine mRNAs after LPS treatment. *Il6* mRNA expression in LPS-stimulated cells (both 200 ng/mL and 1000 ng/mL) was markedly increased 1 hour after treatment and remained significantly higher than in the control cells for 24 hours after treatment (Fig. 2A). *Tnfa* mRNA expression was significantly increased 1 hour after 200 ng/mL LPS treatment ($p < 0.01$) and

decreased to baseline within 18 hours after LPS administration (Fig. 2B). In contrast, *Tnfa* mRNA expression did not increase after administration of 1000 ng/mL LPS and was significantly decreased 12 and 18 hours after treatment as compared with controls (Fig. 2B). The expression of *Il18* mRNA was not increased following treatment with either concentration of LPS, but *Il18* mRNA levels increased after 24 hours in the control cells but not the LPS-treated cells (Fig. 2C). The expression of *Il18r1* mRNA was decreased by LPS in a concentration-dependent manner (Fig. 2D). Remarkably, 1000 ng/mL LPS significantly decreased *Il18r1* expression at all time points. These results suggest that LPS induces inflammation in Sertoli cells but does not induce IL-18 expression. The observed decrease in the mRNA for the IL-18 receptor may indicate that LPS inhibited the homeostatic IL-18/IL-18R signaling cascade.

rIL-18 stimulation did not induce Sertoli cell apoptosis

To further investigate the role of IL-18 in Sertoli cell apoptosis, we examined caspase cleavage and upregulation of the death-receptor-mediated apoptotic pathway in TM4 cells treated with rIL-18 for 18 hours. *Il18r1* mRNA expression was increased by stimulation with 1, 10, and 100 ng/mL rIL-18 (Fig. 3A), and rIL-18 also increased *Il6* mRNA expression (Fig. 3B). *Tnfa* mRNA expression was not significantly changed

following treatment with rIL-18 (Fig. 3C) and *Tnfr1* mRNA expression decreased (Fig. 3D). High-dose rIL-18 (100 ng/mL) increased *Fasl* mRNA expression (Fig. 3E) and did not significantly change *Fas* or *Fadd* mRNA expression (Fig. 3F, G). Treatment with rIL-18 did not induce TM4 cell apoptosis, as assessed by western blot for cleaved caspase-3 (Fig. 3H). This analysis of death-receptor signaling demonstrated that IL-18 did not increase apoptotic signaling in TM4 cells. Moreover, TM4 cell viability did not change after treatment with rIL-18 for 18 hours (Table 2).

Discussion

This study demonstrated that LPS induced an inflammatory response in TM4 Sertoli cells, potentially increasing their sensitivity to apoptotic signals. However, LPS treatment did not stimulate the production of apoptosis-inducing ligands in the TM4 Sertoli cells, and as a result, apoptosis was not induced in response to LPS treatment. Additionally, our study showed that rIL-18 did not induce apoptosis in Sertoli cells. These results contrast with our previous study, which demonstrated that Leydig cell apoptosis was induced by LPS and IL-18 administered at the same concentrations tested in the Sertoli cells via Fas/FasL/caspase-8-dependent and TNF/TNFR1/caspase-8-dependent pathways (Inoue et al., 2020). This study suggests that Sertoli cells may have some

tolerance to apoptosis in response to infectious inflammatory stimulation (tested using LPS) and inflammasome-mediated cytokine stimulation (tested using IL-18). Our results, when coupled with our previous work (Inoue et al., 2020), suggest that different somatic cell types in the testes have different sensitivities during acute inflammation.

In this *in vitro* study, Sertoli cell apoptosis was not induced by LPS despite activation of an inflammatory response and increased *Fas* mRNA expression. LPS also did not affect TM4 cell viability. This was somewhat surprising because LPS-induced systemic inflammation impaired testicular function in a mouse model (Wang et al., 2019). Additionally, Liu and colleagues (2016) found that LPS (50 μ M) decreased mouse Sertoli cell viability and induced apoptosis *in vitro*. The discrepancy between their results and ours may be due to differences in the LPS concentrations administered. Consistent with this explanation, Wang and colleagues (2015) showed that FasL expression in boar Sertoli cells was dependent on LPS concentration: a low LPS concentration (0.01 μ g/mL) decreased FasL expression, but a high LPS concentration (10.0 μ g/mL) increased FasL expression as compared with controls. We could not convert the concentration of 50 μ M LPS used by Liu and colleagues (2016) to ng/mL because the molecular weight of the LPS used in their study was not detailed in their publication. The molecular weight of LPS has been reported as 1-3 $\times 10^6$ (Okuda et al., 2016), so we estimate that 50 μ M was

greater than the 10 µg/mL LPS shown to induced FasL expression (Wang et al, 2015). FasL is an important factor to induce apoptosis. In our study, LPS treatment did not increase *FasL* mRNA expression in TM4 Sertoli cells despite of enhancement of *FasL* expression; therefore, apoptosis was not initiated.

The TNF-TNFR1 pathway also induces apoptosis, but increased *Tnfr1* expression was only synchronized with increased *Tnfa* expression under one of the conditions assessed in this study— both were increased relative to controls 1 hour after treatment with 200 ng/mL LPS. This was a peak time point for *Tnfa* mRNA expression. *Tnfa* mRNA expression was also increased significantly, as compared with controls, 6 and 12 hours after treatment with 200 ng/mL LPS, but this increase did not influence *Tnfr1* expression. Surprisingly, high-dose LPS (1000 ng/mL) significantly decreased *Tnfa* mRNA expression, as compared with controls, and did not influence *Tnfr1* expression. TNF-α/TNFR1 and TNF-α/TNFR2 signaling regulate adherens junctions (germ cell-Sertoli cell, Sertoli cell-Sertoli cell) and tight junctions (Sertoli cell-Sertoli cell; blood-testis barrier) in the testes via the p38 MAPK and JNK pathways (Ni et al., 2019). Therefore, these results suggest that Sertoli cells may possess mechanisms, such as decreasing *FasL* to avoid death-receptor-mediated apoptosis and not altering *Tnfr1* expression, to protect spermatogenesis by maintaining the stability of adherens junctions

and tight junctions during acute inflammatory conditions.

In the testes, IL-18 levels increase during endotoxin-induced acute systemic inflammation (Abu Elhija et al., 2008c). However, *Il18* mRNA was not increased in TM4 Sertoli cells after LPS stimulation in our study. These results suggest that Sertoli cells do not actively synthesize IL-18 despite induction of the inflammatory response during acute inflammatory conditions. Accordingly, neither Sertoli cells nor Leydig cells (Inoue et al., 2020) are likely the main cell type in the testes that produces IL-18 in response to inflammation. IL-18 regulates testicular function, development, and spermatogenesis under physiologic conditions (Abu Elhija et al., 2008a; Abu Elhija et al., 2008d; Komsky et al., 2012; Strand et al., 2005). Our previous study demonstrated that endogenous IL-18 suppresses testicular germ cell apoptosis during the recovery phase after acute inflammation when plasma IL-18 levels return to baseline (Inoue et al., 2015). Hence, the physiological concentration of IL-18 and IL-18/IL-18R signaling are crucial for testicular homeostasis. In this study, LPS decreased *Il18r1* expression in TM4 Sertoli cells. Accordingly, we postulate that LPS-induced inflammation inhibits IL-18/IL-18R1 signaling, which is linked with homeostasis of the testicular environment including the function of the Sertoli cells.

In this study, rIL-18 treatment led to an increase in *Il18r1* mRNA expression.

Because IL-18 is induced by macrophages, we postulate that IL-18/IL-18R1 signaling can be kept at a desired homeostatic level even in the presence of inflammation. *Il6* levels indicated that rIL-18 induced inflammation in the TM4 Sertoli cells; however, *Tnfr1* expression was reduced in the presence of rIL-18, which likely circumvented apoptosis. LPS treatment also increased *Il6* levels in the cells, but apoptosis was likely avoided by decreasing *FasI* expression. The result of avoiding apoptosis was same, but the mechanisms of circumventing apoptosis appear different between LPS-initiated and IL-18-initiated events in TM4 Sertoli cells.

The rIL-18 doses used in this study were based on our previous study, in which we demonstrated plasma IL-18 levels of ~50 ng/ml after administration of LPS to C57BL/6J mice (Inoue et al., 2015). Our findings suggest that Sertoli cells have a tolerance to the acute inflammatory conditions previously shown to cause germ cell (Inoue et al., 2015) and Leydig cell apoptosis (Inoue et al., 2020). Gurunathan and colleagues (2019) reported that TM3 Leydig cells were more sensitive to the cytotoxic effects of graphene oxide than TM4 Sertoli cells. Accordingly, our studies suggest differences in sensitivity to inflammatory stimuli and harmful substances between Leydig cells and Sertoli cells. Moreover, IL-18 stimulation decreased *Tnfr1* expression. These results suggest that IL-18 has beneficial effects, both at physiological concentrations and

at pharmacological concentrations that mimic inflammation, that allow the Sertoli cells to escape apoptosis, and that Sertoli cells differ from germ cells and Leydig cells in this respect.

This study has some limitations. First, the reaction of Sertoli cells to LPS and FasL co-stimulation could not be evaluated. Second, Sertoli cell-immune cell interactions, such as Sertoli cells and T cells, which produce soluble FasL, or Sertoli cells and macrophages, which produce pro-inflammatory cytokines, could not be evaluated. Accordingly, future studies are planned to investigate Sertoli cell-immune cell interactions under inflammatory condition using co-culture systems. Third, the protein mediators of these effects were not studied directly, and the response of Sertoli cells was not examined in vivo; these will be the focus of future studies. Additionally, other functional changes in Sertoli cells, such as alterations in tight junction integrity, were not examined in this study. Previous studies have reported that proinflammatory cytokines, including IL-18, induced by exogenous harmful substances, such as LPS, directly affect tight junction structure, independent of apoptotic signaling pathways (Feng et al., 2022; Li et al., 2012; Capaldo and Nusrat, 2009; Bruewer et al., 2003). These are important areas for future investigation.

In conclusion, this study demonstrated that Sertoli cells seem to be less sensitive

to LPS and IL-18 toxicity than Leydig cells or germ cells, and apoptosis was not induced in response to either stimulus. This was correlated with decreased *Fasl* and *Tnfr1* expression, suggesting a mechanistic explanation for the observed resistance to apoptosis. This study, when coupled with our previous work (Inoue et al., 2020), revealed differences between testicular somatic cells—Sertoli and Leydig cells—in their sensitivity and apoptotic response to harmful substances, such as LPS and high-levels of IL-18.

Declaration of interest

The authors declare that there are no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Abu Elhija, M., Lunenfeld, E., Eldar-Geva, T., Huleihel, M., 2008a. Over-expression of IL-18, ICE and IL-18 R in testicular tissue from sexually immature as compared to mature mice. *Eur. Cytokine. Netw.* 19 (1), 15-24.
- Abu Elhija, M., Lunenfeld, E., Eldar-Geva, T., Huleihel, M., 2008b. Lipopolysaccharide increased the expression levels of IL-18, ICE and IL-18 R in murine Leydig cells. *Am. J. Reprod. Immunol.* 60 (2), 151-159.
- Abu Elhija, M., Lunenfeld, E., Huleihel, M., 2008c. LPS increases the expression levels of IL-18, ICE and IL-18 R in mouse testes. *Am. J. Reprod. Immunol.* 60 (4), 361-371.
- Abu Elhija, M., Lunenfeld, E., Persky, L., Huleihel, M., 2008d. Constitutive expression of IL-18 binding protein in murine testicular tissues and cells. *Eur. Cytokine. Netw.* 19 (1), 25-29.
- Beale, R., Reinhart, K., Brunkhorst, F.M., Dobb, G., Levy, M., Martin, G., Martin, C., Ramsey, G., Silva, E., Vallet, B., Vincent, J.L., Janes, J.M., Sarwat, S., Williams, M.D.; PROGRESS Advisory Board., 2009. Promoting Global Research Excellence in Severe Sepsis (PROGRESS): lessons from an international sepsis registry. *Infection.* 37 (3), 222-232.
- Bruewer, M., Luegering, A., Kucharzik, T., Parkos, C.A., Madara, J.L., Hopkins, A.M.,

- 358 Nusrat, A., 2003. Proinflammatory cytokines disrupt epithelial barrier function by
359 apoptosis-independent mechanisms. *J. Immunol.* 171 (11), 6164-6172.
- 360 Capaldo, C.T., Nusrat, A., 2009. Cytokine regulation of tight junctions. *Biochim. Biophys.*
361 *Acta.* 1788 (4), 864-871.
- 362 Cheng, C.Y., Mruk, D.D., 2012. The blood-testis barrier and its implications for male
363 contraception. *Pharmacol. Rev.* 64 (1), 16-64.
- 364 Feng, R., Adeniran, S.O., Huang, F., Li, Y., Ma, M., Zheng, P., Zhang, G., 2022. The
365 ameliorative effect of melatonin on LPS-induced Sertoli cells inflammatory and tight
366 junctions damage via suppression of the TLR4/MyD88/NF- κ B signaling pathway in
367 newborn calf. *Theriogenology.* 179, 103-116.
- 368 Gabarin, R.S., Li, M., Zimmer, P.A., Marshall, J.C., Li, Y., Zhang, H., 2021. Intracellular
369 and Extracellular Lipopolysaccharide Signaling in Sepsis: Avenues for Novel
370 Therapeutic Strategies. *J. Innate. Immun.* 13 (6), 323-332.
- 371 Gacci, M., Coppi, M., Baldi, E., Sebastianelli, A., Zaccaro, C., Morselli, S., Pecoraro, A.,
372 Manera, A., Nicoletti, R., Liaci, A., Bisegna, C., Gemma, L., Giancane, S., Pollini, S.,
373 Antonelli, A., Lagi, F., Marchiani, S., Dabizzi, S., Degl'Innocenti, S., Annunziato, F.,
374 Maggi, M., Vignozzi, L., Bartoloni, A., Rossolini, G.M., Serni, S., 2021. Semen
375 impairment and occurrence of SARS-CoV-2 virus in semen after recovery from

- 376 COVID-19. Hum. Reprod. 36 (6), 1520-1529.
- 377 Genga, K.R., Russell, J.A., 2017. Update of Sepsis in the Intensive Care Unit. J. Innate.
- 378 Immun. 9 (5), 441-455.
- 379 Gurunathan, S., Kang, M.H., Jeyaraj, M., Kim, J.H., 2019. Differential Cytotoxicity of
- 380 Different Sizes of Graphene Oxide Nanoparticles in Leydig (TM3) and Sertoli (TM4)
- 381 Cells. Nanomaterials (Basel). 9 (2), 139.
- 382 Inoue, T., Aoyama-Ishikawa, M., Kamoshida, S., Nishino, S., Sasano, M., Oka, N.,
- 383 Yamashita, H., Kai, M., Nakao, A., Kotani, J., Usami, M., 2015. Endogenous
- 384 interleukin 18 regulates testicular germ cell apoptosis during endotoxemia.
- 385 Reproduction. 150 (2), 105-114.
- 386 Inoue, T., Aoyama-Ishikawa, M., Uemura, M., Yamashita, H., Koga, Y., Terashima, M.,
- 387 Usami, M., Kotani, J., Hirata, J., 2020. Interleukin-18 levels and mouse Leydig cell
- 388 apoptosis during lipopolysaccharide-induced acute inflammatory conditions. J.
- 389 Reprod. Immunol. 141, 103167.
- 390 Johanna Josepha Op't Hoog, S.A., Eskes, A.M., Johanna van Mersbergen-de Bruin, M.P.,
- 391 Pelgrim, T., van der Hoeven, H., Vermeulen, H., Maria Vloet, L.C., 2022. The effects
- 392 of intensive care unit-initiated transitional care interventions on elements of post-
- 393 intensive care syndrome: A systematic review and meta-analysis. Aust. Crit. Care. 35

- 394 (3), 309-320.
- 395 Komsky, A., Huleihel, M., Ganaiem, M., Kasterstein, E., Komorovsky, D., Bern, O.,
 396 Raziell, A., Friedler, S., Ron-El, R., Strassburger, D., 2012. Presence of IL-18 in
 397 testicular tissue of fertile and infertile men. *Andrologia*. 44 (1), 1-8.
- 398 Li, X., Akhtar, S., Choudhry, M.A., 2012. Alteration in intestine tight junction protein
 399 phosphorylation and apoptosis is associated with increase in IL-18 levels following
 400 alcohol intoxication and burn injury. *Biochim. Biophys. Acta*. 1822 (2), 196-203.
- 401 Liu, X.R., Wang, Y.Y., Dan, X.G., Kumar, A., Ye, T.Z., Yu, Y.Y., Yang, L.G., 2016. Anti-
 402 inflammatory potential of β -cryptoxanthin against LPS-induced inflammation in
 403 mouse Sertoli cells. *Reprod. Toxicol.* 60, 148-155.
- 404 Martín-Vicente, P., López-Martínez, C., Lopez-Alonso, I., López-Aguilar, J., Albaiceta,
 405 G.M., Amado-Rodríguez, L., 2021. Molecular mechanisms of postintensive care
 406 syndrome. *Intensive Care. Med. Exp.* 9 (1), 58.
- 407 Murphy, C.J., Richburg, J.H., 2015. Implications of Sertoli cell induced germ cell
 408 apoptosis to testicular pathology. *Spermatogenesis*. 4 (2), e979110.
- 409 Needham, D.M., Davidson, J., Cohen, H., Hopkins, R.O., Weinert, C., Wunsch, H.,
 410 Zawistowski, C., Bemis-Dougherty, A., Berney, S.C., Bienvenu, O.J., Brady, S.L.,
 411 Brodsky, M.B., Denehy, L., Elliott, D., Flatley, C., Harabin, A.L., Jones, C., Louis, D.,

- 412 Meltzer, W., Muldoon, S.R., Palmer, J.B., Perme, C., Robinson, M., Schmidt, D.M.,
413 Scruth, E., Spill, G.R., Storey, C.P., Render, M., Votto, J., Harvey, M.A., 2012.
414 Improving long-term outcomes after discharge from intensive care unit: report from a
415 stakeholders' conference. *Crit. Care. Med.* 40 (2), 502-509.
- 416 Ni, F.D., Hao, S.L., Yang, W.X., 2019. Multiple signaling pathways in Sertoli cells: recent
417 findings in spermatogenesis. *Cell Death Dis.* 10 (8), 541.
- 418 Okuda, S., Sherman, D.J., Silhavy, T.J, Ruiz, N., Kahne, D., 2016. Lipopolysaccharide
419 transport and assembly at the outer membrane: the PEZ model. *Nat Rev Microbiol.* 14
420 (6), 337-345.
- 421 Omolaoye, T.S., Jalaaliddine, N., Cardona Maya, W.D., du Plessis, S.S., 2022.
422 Mechanisms of SARS-CoV-2 and Male Infertility: Could Connexin and Pannexin
423 Play a Role? *Front. Physiol.* 13, 866675.
- 424 Pfaffl, M.W., Tichopad, A., Prgomet, C., Neuvians, T.P., 2004. Determination of stable
425 housekeeping genes, differentially regulated target genes and sample integrity:
426 BestKeeper--Excel-based tool using pair-wise correlations. *Biotechnol. Lett.* 26 (6),
427 509-515.
- 428 Rudd, K.E., Johnson, S.C., Agesa, K.M., Shackelford, K.A., Tsoi, D., Kievlan, D.R.,
429 Colombara, D.V., Ikuta, K.S., Kissoon, N., Finfer, S., Fleischmann-Struzek, C.,

- 430 Machado, F.R., Reinhart, K.K., Rowan, K., Seymour, C.W., Watson, R.S., West, T.E.,
 431 Marinho, F., Hay, S.I., Lozano, R., Lopez, A.D., Angus, D.C., Murray, C.J.L., Naghavi,
 432 M., 2020. Global, regional, and national sepsis incidence and mortality, 1990-2017:
 433 analysis for the Global Burden of Disease Study. *Lancet*. 395 (10219), 200-211.
- 434 Sengupta, P., Dutta, S., Roychoudhury, S., D'Souza, U.J.A., Govindasamy, K.,
 435 Kolesarova, A., 2022. COVID-19, Oxidative Stress and Male Reproduction: Possible
 436 Role of Antioxidants. *Antioxidants (Basel)*. 11 (3), 548.
- 437 Strand, M.L., Wahlgren, A., Svechnikov, K., Zetterstrom, C., Setchell, B.P., Soder, O.,
 438 2005. Interleukin-18 is expressed in rat testis and may promote germ cell growth.
 439 *Mol. Cell. Endocrinol.* 240 (1-2), 64-73.
- 440 Wang, F., Liu, W., Jiang, Q., Gong, M., Chen, R., Wu, H., Han, R., Chen, Y., Han, D.,
 441 2019. Lipopolysaccharide-induced testicular dysfunction and epididymitis in mice: a
 442 critical role of tumor necrosis factor alpha. *Biol. Reprod.* 100 (3), 849-861.
- 443 Wang, Y., Zhang, J.J., Yang, W.R., Luo, H.Y., Zhang, J.H., Wang, X.Z., 2015.
 444 Lipopolysaccharide-induced expression of FAS ligand in cultured immature boar
 445 sertoli cells through the regulation of pro-inflammatory cytokines and miR-187. *Mol.*
 446 *Reprod. Dev.* 82 (11), 880-891.
- 447 Yamamuro, T., Nakamura, S., Yamano, Y., Endo, T., Yanagawa, K., Tokumura, A.,

448 Matsumura, T., Kobayashi, K., Mori, H., Enokidani, Y., Yoshida, G., Imoto, H.,
449 Kawabata, T., Hamasaki, M., Kuma, A., Kuribayashi, S., Takezawa, K., Okada, Y.,
450 Ozawa, M., Fukuhara, S., Shinohara, T., Ikawa, M., Yoshimori, T., 2021. Rubicon
451 prevents autophagic degradation of GATA4 to promote Sertoli cell function. PLoS.
452 Genet. 17 (8), e1009688.
453

Figure Legends

Figure 1.

Death-receptor-mediated apoptosis in the TM4 Sertoli cells after LPS stimulation. (A) Expression of cleaved caspase-3 (Cl. Casp-3) in TM4 cells. Representative blots are shown. Relative expressions of (B) *Fas*, (C) *FasL*, (D) *Tnfr1*, and (E) *Fadd* mRNAs 0, 1, 6, 12, 18, and 24 hours after LPS stimulation. All data are presented as the mean \pm standard deviation. Closed circle, 200 ng/mL LPS; Open circle, 1000 ng/mL LPS; square, control (vehicle). $n = 3$ per group. *, $p < 0.05$, **, $p < 0.01$, significant difference between 200 ng/mL LPS and control. †, $p < 0.05$, ††, $p < 0.01$, significant difference between 1000 ng/mL LPS and control. NP; no protein, NC; negative control, PC; positive control.

Figure 2.

Inflammatory cytokine levels in the TM4 Sertoli cells after LPS stimulation. Relative expression of (A) *Il6*, (B) *Tnfa*, (C) *Il18*, (D) *Il18r1* mRNAs in TM4 cells 0, 1, 6, 12, 18, and 24 hours after LPS stimulation. All data are presented as the mean \pm standard deviation. Closed circle, 200 ng/mL LPS; Open circle, 1000 ng/mL LPS; square, control (vehicle). $n = 3$ per group. *, $p < 0.05$, **, $p < 0.01$, significant difference between 200 ng/mL LPS and control. †, $p < 0.05$, ††, $p < 0.01$, significant difference between 1000

ng/mL LPS and control.

Figure 3.

Death-receptor-mediated apoptosis in the TM4 Sertoli cells 18 hours after treatment with recombinant IL-18 (rIL-18). Relative mRNA expression levels for (A) *Il18r1*, (B) *Il6*, (C) *Tnfa*, (D) *Tnfr1*, (E) *Fasl*, (F) *Fas*, and (G) *Fadd*. (H) Cleaved caspase-3 (Cl. Casp-3) protein levels in TM4 cells after rIL-18 treatment. Representative blots are shown. All data are presented as the mean \pm standard deviation. $n = 3$ per group. *, $p < 0.05$, **, $p < 0.01$, significant difference between rIL-18 and vehicle (control). NP; no protein, NC; negative control, PC; positive control.

Table 1. Quantitative PCR primer sequences and annealing temperatures

Gene	Primer sequence (5' to 3')		Annealing temperature
	Forward	Reverse	°C
<i>Tnf</i>	AGAAGAGGCACTCCCCCAAAA	CCGAAGTTCAGTAGACAGAAGAGCG	63
<i>Tnfr1</i>	AGAACCAGTTCCAACGTACC	TCTGAGTCTCCTTACAGGGGAT	57
<i>Fas</i>	GAACCTCCAGTCGAAACCA	GCTGTGTCTTGGATGCTGTCA	62
<i>Fasl</i>	TCAGTCTTGCAACAACCAGCC	GATTGAATACTGCCCCCAGGT	62
<i>Fadd</i>	CAGGTGGCATTGACATTGTG	ACCGAGGCGTTCTTCTTCTCA	62
<i>Il6</i>	TGGGAAATCGTGGAAATGAGA	CAAGTGCATCATCGTTGTTCATAC	63
<i>Il18</i>	GCCATGTCAGAAGACTCTTGCGTC	GTACAGTGAAGTCGGCCAAAGTTGTC	66

Fadd, Fas-associated death domain protein; Fasl, fas ligand; Il, interleukin; tnfr, tumor necrosis factor alpha; tnfr, tumor necrosis factor receptor.

Table 2. Viability of TM4 Sertoli cell after LPS or IL-18 stimulation

	Length of LPS Stimulation					
	0 h	1 h	6 h	12 h	18 h	24 h
Cell viability in control TM4 cells (%)	99.5 ± 0.9	100.0 ± 0.0	100.0 ± 0.0	99.4 ± 1.1	99.1 ± 1.0	100.0 ± 0.0
Cell viability with 200 ng/mL LPS (%)	99.2 ± 0.7	99.6 ± 0.7	100.0 ± 0.0	98.6 ± 0.2	99.2 ± 0.7	100.0 ± 0.0
Cell viability with 1000 ng/mL LPS (%)	100.0 ± 0.0	99.6 ± 0.6	99.7 ± 0.5	100.0 ± 0.0	99.6 ± 0.7	99.8 ± 0.4
	Recombinant IL-18 concentration					
	0 ng/mL	0.1 ng/mL	1.0 ng/mL	10 ng/mL	100 ng/mL	
Cell viability (%)	99.8 ± 0.4	100.0 ± 0.0	99.7 ± 0.3	99.5 ± 0.4	99.8 ± 0.4	

Data are presented as mean ± standard deviation.

IL, interleukin; LPS, lipopolysaccharide.

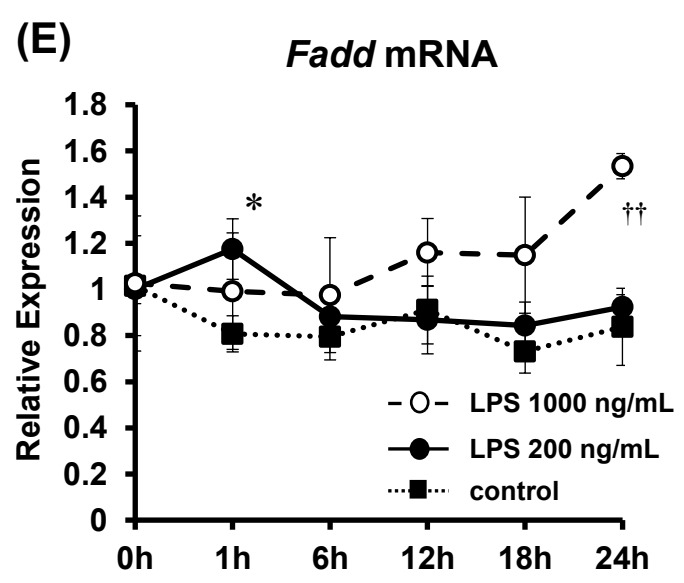
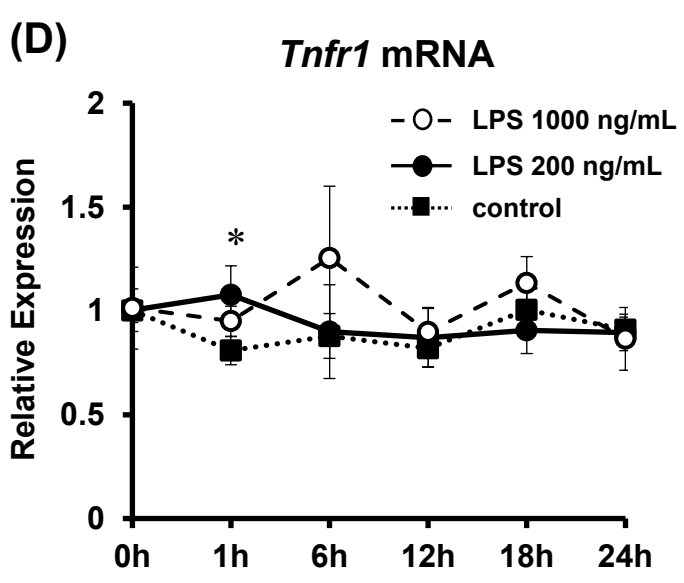
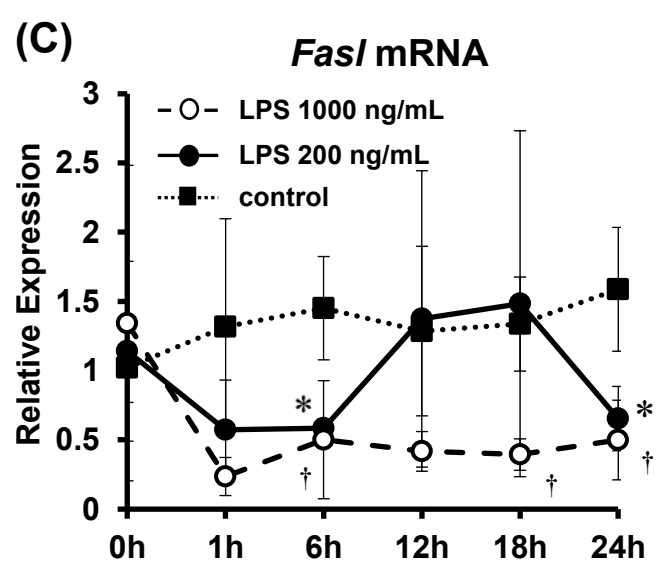
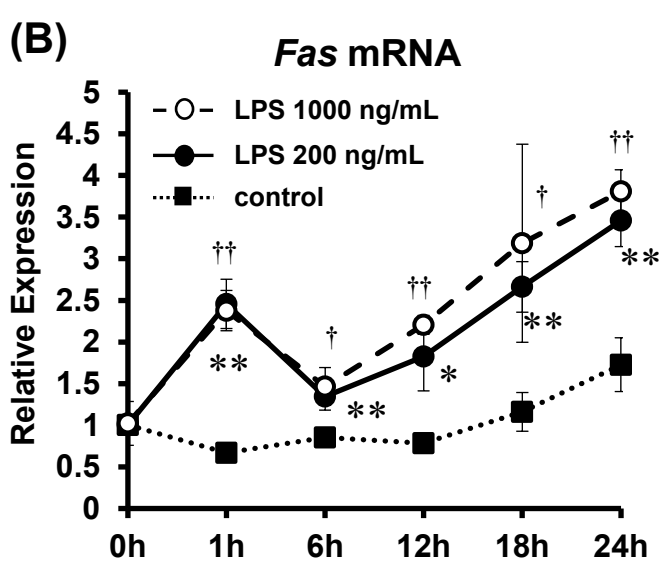
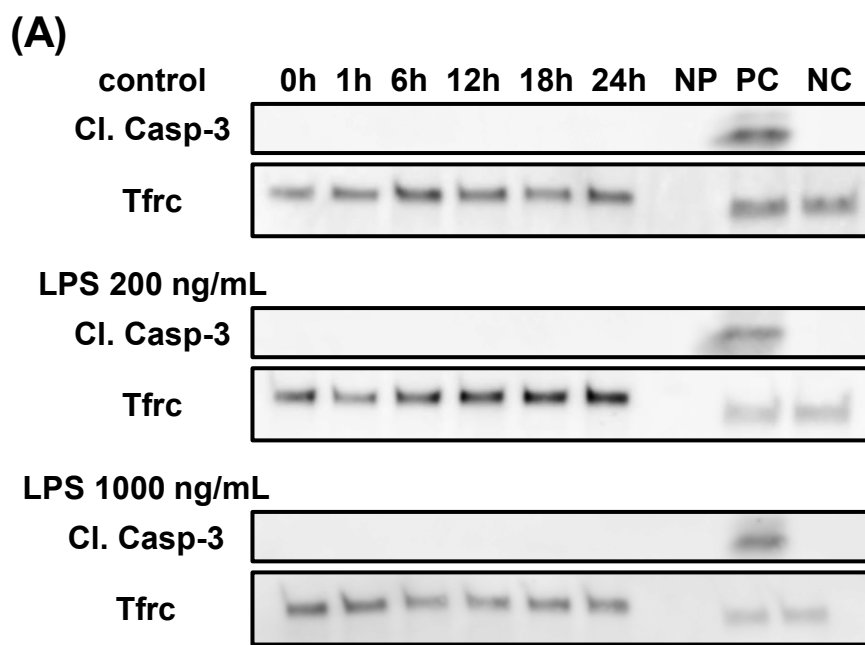


Fig. 1

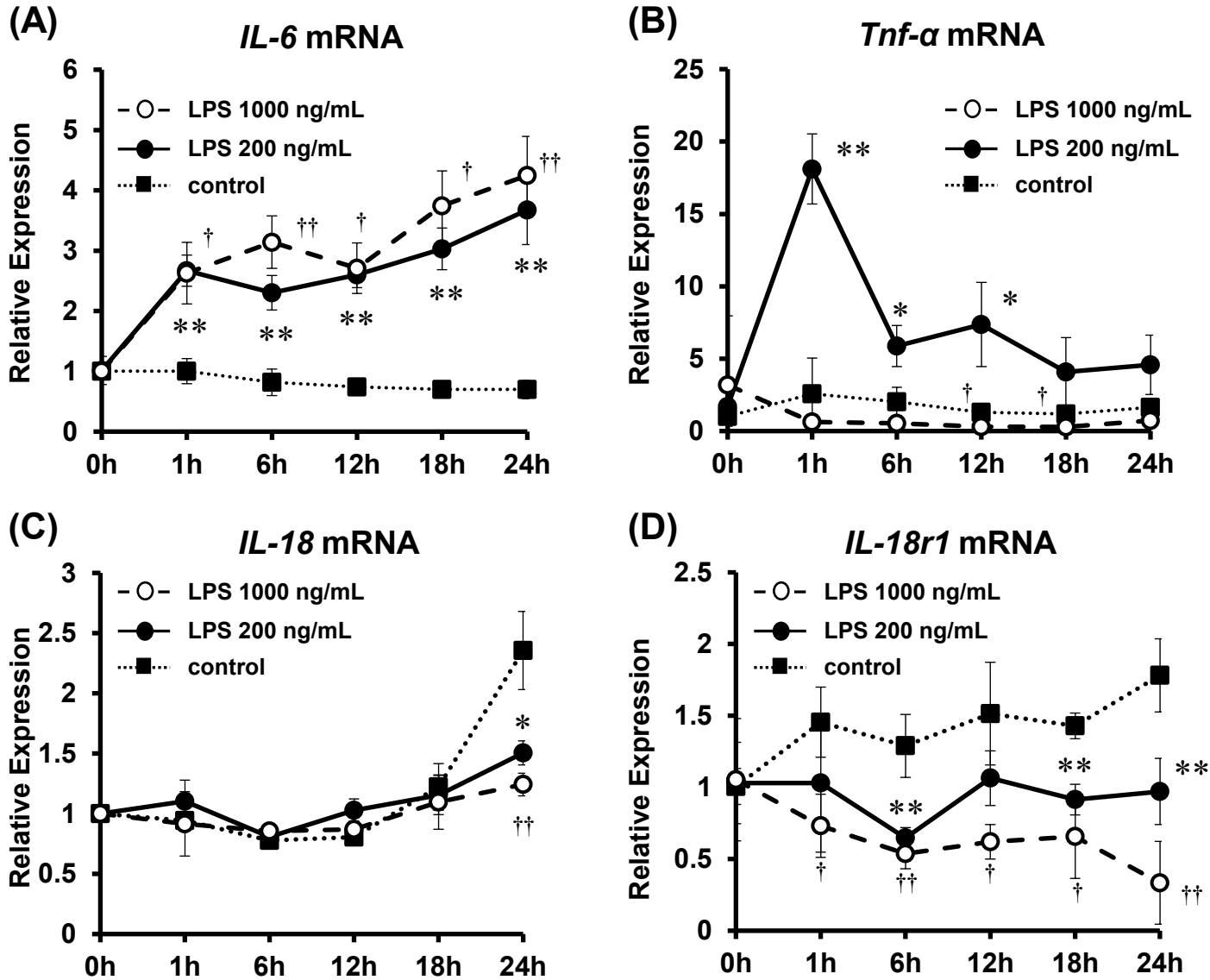


Fig. 2

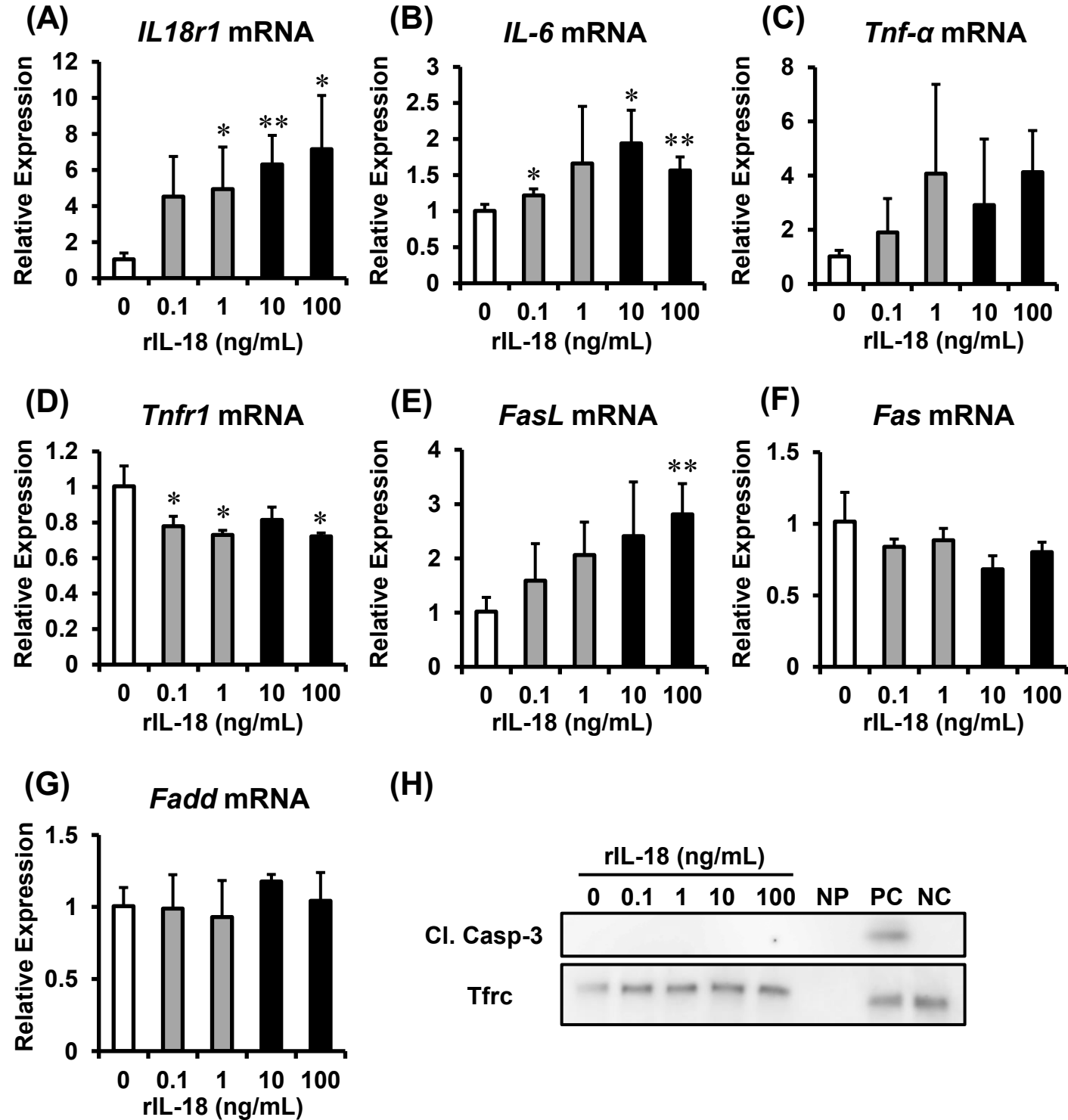


Fig. 3