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Heat stress-induced intestinal epithelial cells necroptosis via TLR3-TRIF-RIP3 pathway was dependent on p53

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ABSTRACT

Background: Heatstroke is a life-threatening disease. Present study was aimed to investigate the mechanism in heat induced intestinal epithelial cell death.
Method: Heat stress in vitro model was established on IEC cells with 42℃ for 2 h. Caspase-8 inhibitor, Caspase-3 inhibitor, RIP3 inhibitor, TLR3 agonist, poly(I:C) and p53 knockdown were used to determine the signaling pathway. Heatstroke in vivo model was established on C57BL/6 mice, with a temperature of 35.5℃±0.5℃ and a relative humidity of 60% ± 5%. The intestine necroptosis and inflammatory cytokines were measured. Pifithrin α (3 mg/kg) and p53 knockout mice were used to evaluate the role of p53.
Results: Heat stress-induced reduction of cell viability was remarkable reversed by RIP3 inhibitor. Heat stress induced upregulation of TLR3 and facilitate the formation of TRIF-RIP3 complex. The heat stress induced upregulation of RIP3 and p-RIP3 were normalized by the deletion of p53. Meanwhile, p53 knockout decreased TLR3 expression and blocked the formation of TLR3-TRIF complex. The deletion of p53 blocked the decreased cell viability and restored the activation of RIP3-MLKL signaling after heat stress, however, which were abolished by re-expression of p53 via *Tp53* OE. Increased the expression of TLR3 in the p53-deficient cells could not affect the heat stress induced necrotic cell death, which suggests that heat stress induced necroptosis via TLR3-TRIF-RIP3 signaling pathway is dependent on p53.
Conclusion: Heat stress promoted p53 phosphorylation, then upregulated TLR3 and enhanced the interaction of TRIF-RIP3, which would activate the RIP3-MLKL signaling pathway to mediate necroptosis in intestinal epithelial cells.

1. Background

Heatstroke is a life-threatening disease, characterized by core temperature over 40℃ and central nervous system dysfunction, such as coma, delirium or convulsions [1], which usually happened to those exposure to high temperatures or strenuous physical activity. Nowadays, record-breaking heatwaves were rising, leading to an increasing number of heatstroke cases. Hence, public aspects of medicine have put more attention on heatstroke and heat-related guideline or recommendation had been published. Nevertheless, the mortality could reach 26.5% and 63.2% in exertional and classic heatstroke, respectively, under intensive care [2]. This was mainly due to that these

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approaches were mostly experience-based; the key mechanism of heatstroke was still unclear. Present studies found that the pathophysiology was complex, in addition to heat, factors also including immune system dysfunction, inflammation and cytokine storm, endothelial cell injury, widespread thrombosis and bleeding, multi-organ dysfunction. Therefore, the pathophysiology of heatstroke also was called "like-sepsis reaction". Intestine owed the largest bacterial species. With its integrated intestinal mucosal barrier, balanced microenvironment and regulated immune function, the bacteria were restricted in the intestine. Under some condition, such as severe trauma, shock, infection, the injury of intestinal cell and damage of mucosal increased the permeability of intestine barrier, leading to the bacterial translocation to the systemic circulation and further inflammation. Therefore, intestine has been called the "motor" of many severe diseases. Our pervious study found that heat stress could induce intestinal epithelial injury, which was related with the systemic inflammation.

It has been reported that necroptosis could be caused by Toll-like receptor (TLR)-3 and TLR-4 agonists, tumor necrosis factor (TNF) [3]. It was modulated by receptor-interacting protein kinase (RIP) 1. Activated death receptors cause the activation of RIP1 and formation of an RIP1-RIP3-mixed lineage kinase domain-like protein (MLKL). Then, RIP3 phosphorylates MLKL, which ultimately resulting in necrosis through plasma membrane disruption and cell lysis [4]. Though the process of necroptosis was regulated, the effect of necroptosis was significantly different from apoptosis. The rupture of dead cells and release of intracellular components could lead to activation of innate immune responses and further inflammation [5]. p53, firstly found as tumor suppressor, now has been well characterized for its response to different cellular stresses, including cell cycle, apoptosis, vascular angiogenesis [6]. Our previous study found that p53 played a crucial role in heat stress-induced early apoptosis of human umbilical vein endothelial cell [7]. In addition, p53 has been found transcriptionally activated necrosis-related factor expression, which involved in the cardiomyocytes necroptosis [8]. These results suggested that p53 might involve in the heat-induced intestinal cell death, but the exact mechanism was still unclear. Present study was aimed to demonstrate the effect of p53 and signaling pathway in heat stress-induced intestine injury were explored both in vitro and in vivo.

2. Methods

2.1. Cell culture and heat stress model

Mouse-derived Intestinal Epithelioid Cells (IEC cells) were kindly given by Eusyn Medical Technology Company (Guangzhou, China). IEC cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum in a 37℃-incubator containing 5% carbon dioxide. Ethylenediaminetetraacetic acid (EDTA)-trypsin was used to passage the cells. For heat stress treatment, IEC cells were placed in an incubator at 42℃ for 2 h and then were moved to 37℃ for recovery. Cell viability and apoptosis were analyzed at 1, 3, 6 and 9 h after heat stress.

2.2. Cell viability and apoptosis measurement

IEC cells were cultured in 96-well plates and subjected to heat stress. Cell viability and apoptosis were measured by ApoTox-Glo™ Triplex Assay (Promega, G6320) according to the manufacturer's instructions. Briefly, test compounds and vehicle controls were added to appropriate wells for a final volume of 100 μl per well at the end of heat stress. Then the IEC cells were continued to culture in the incubator at 37℃ for different time. Add 20 μl of ciability reagent containing both GF-AFC Substrate and bis-AAF-R110 substrate to all wells and briefly mix by orbital shaking. Cell viability fluorescence was measured at 400_{Ex}/505_{Em} in the VICTOR Nivo microplate reader (PerkinElmer Inc.) after incubation of 30 min at 37℃. Finally, add 100 μl of caspase-Glo® 3/7 reagent to all wells, and briefly mix by orbital shaking and the cells were incubated for another 30 min at 37℃. The apoptosis was determined with the luminescence measurement.

2.3. Flow cytometry analysis

Cell death was evaluated by using the Annexin V-FITC apoptosis detection kit (Invitrogen, V13241) according to the manufacturer's instructions. Briefly, Approximately 1×10^6 IEC cells were collected after experimental treatment or not and washed in cold PBS. Cell pellets were re-suspended in binding buffer containing 1:20 Annexin V-AlexaFluor 488 and propidium iodide (PI) and incubated at room temperature for 20 min. Added 400 μl of annexin-binding buffer at the end of incubation period, mix gently, and then immediately analyzed by flow cytometry to quantify cell apoptosis and death.

2.4. Enzyme linked immunosorbent assay (ELISA)

The protein levels of inflammatory cytokines (including TNF-α, IL-1β, IL-6, MCP-1 and CXCL1) in the medium of culture cells and in the plasma of mice were detected by using ELISA kit (Boster Biological technology, Wuhan, China) following to the manufacturer's instructions.

2.5. Animals

Pathogen-free, 8–12 weeks, male C57BL/6J mice were obtained from the Southern Medical University Animal Center (NO.: SCXK-2011–0015, Guangzhou, China). p53 knockout mice were purchased from Biocytogen Pharmaceuticals (Beijing) Co., Ltd. company (Cat: 110167, Beijing, China). Mice (male mice were used for all experiments unless otherwise indicated) were housed in ventilated cages with free access to food and water under standard laboratory conditions. All experiments were set as double-blind behavioral tests and performed in accordance with the Chinese Council on Animal Care Guidelines.

2.6. Heatstroke model

Heatstroke mice model were established according to the previously reported method [9]. Briefly, mice were randomly divided into two groups (Heat stress and Control). The mice in the heat stress group were placed in a pre-warmed incubator in which the temperature was maintained at 35.5℃ ± 0.5℃ with a relative humidity of 60% ± 5%. A rectal thermometer was used to continuously monitor the rectal temperature of mice every 30 min during the heat stress until the rectal temperature reached 42℃. The mice met the heatstroke criterion were then transferred in an incubator with an ambient temperature of 25℃ ± 0.5℃ and a humidity of 35% ± 5% for recovery. The mice in the control group were sham-heated at a temperature of 25℃ ± 0.5℃ and humidity of 35% ± 5% for a time that was comparable to that of the heat stress group. After the heat stress exposure, blood samples were rapidly collected from eyeball vein of mice and centrifuged at 3,500 rpm for 10 min and the intestine of mice was immediately isolated.

2.7. Quantitative Real-time PCR (qRT-PCR)

Total RNA was isolated from IEC cells or the intestine tissues using RNAiso Plus (Takara) according to manufacturer's instructions and was quantified with the Nanodrop 2000 (Thermo Fisher Scientific). The cDNA was synthesized by using the PrimeScript™ RT reagent Kit (Takara). Quantitative Real-Time PCR was performed in an ABI 7500 Real-Time PCR System with SYBR premix EX Taq™ (Takara). The primer sequences used for qRT-PCR are listed in Table S1. The gene expression were analyzed base on the ΔΔCt method as described before [10].

2.8. Western blot

Tissues or culture cells were homogenized in a detergent-based lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS and 1 mM PMSF) (Beyotime, Shanghai, China). The homogenate samples were centrifuged for 30 min at 16,000g at 4℃ and the supernatant was collected and then quantified with the Microplate BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of protein were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) for separation and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore). Antibodies used for western blotting were shown in supplement Table 2. All quantification was performed with AlphaEaseFC software (Alpha Innotech Corporation) and all samples were normalized to GAPDH.

2.9. Chemical compounds

The information of chemical compounds including Caspase-8 inhibitor, Caspase-3 inhibitor, p53 inhibitor Pifithrin-α, RIP3 inhibitor, TLR3 agonist, poly(I:C), recombinant mouse TNFα protein, recombinant mouse IL-1β protein and anti-TNFα antibody were shown in supplement Table 2.

2.10. Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) was performed by using Pierce™ Classic Magnetic IP/Co-IP Kit (Thermo Fisher Scientific, Cat: 88804) according to manufacturer's instructions and our describe previously [11]. Briefly, after protein extraction of IEC and intestine tissue, the protein concentrations were measured. The immune complex was prepared by combining a total 1,000 μg protein with 10 μg of IP antibody in a micro centrifuge tube for 2 h. Added the immune complex to the tube containing pre-washed magnetic beads and incubate at room temperature for 1 h with mixing. The eluate was collected by separating the beads with a magnetic stand.

2.11. Transfection of IEC cells

siRNA-*Tp53* (sc-29436), siRNA-*Ripk3* (sc-61483), siRNA-FITC (sc-36869), *Tp53* OE (sc-423509-LAC), *Ripk3* OE (sc-425224-LAC), *Tlr3* OE (sc-431258-LAC), Control lentiviral activation particles (sc-437282) and copGFP control lentiviral particles (sc-108084) were purchased from Santa Cruz Biotechnology, Inc. (USA). IEC cells were cultured in six-well plates 24 h prior to transfection until the cells grow to 40–80% confluency. Protein knockdown or overexpression in IEC cells was performed according to manufacturer's instructions, which then were following subjected to heat stress and subsequence analysis.

2.12. Histopathological analysis

Mice were deeply anesthetized with pentobarbital (80 mg/kg, i.p.) and perfused with PBS followed by 4% PFA. The intestine samples of mice were excised quickly from mice and sliced into transverse or longitudinal sections. The tissues were then embedded in paraffin blocks, and serial sections were stained with hematoxylin and eosin for microscopic evaluation.

2.13. Statistical analysis

All these data were analyzed by SPSS 20.0 software and were presented as the mean ± SEM. Two-tail Student's ***t*** tests were used to compare the difference between two groups. For multi-group comparisons, one-way ANOVA followed by the least significant difference (LSD) or Dunnett T3 test for post hoc comparisons were used to evaluate potential differences between the mean values. Two-way ANOVA was chosen when compared the differences between two groups with two factors. $p < 0.05$ was set as the significance level of these tests.

3. Results

3.1. Heat stress decreased the intestinal epithelial cell viability and increased inflammatory cytokines concentration.

To investigate the damage of heat stress (HS) to the intestine, we used the mouse-derived intestinal epithelial cells (hereinafter referred to as IEC cells) to establish an in vitro heat stress model according to our previous published method, in which IEC cells were placed under 42℃ for 2 h followed by 37℃ recovery for different time (Fig. S1A). The cell viability decreased and cell death increased significantly in a time dependent form to response to heat stress, which were reached the maximum after a 6-hour or 9-hour recovery (Fig. S1B). Further investigation by flow cytometry as having double-positive staining for Annexin V and PI revealed that HS resulted in a 23.48% and 9.89% increase of necrotic cells and apoptotic cells after 6-hour recovery, respectively (Fig. S1C). Meanwhile, the pro-inflammatory cytokines (TNFα, IL-1β and IL-6) and chemokines (MCP-1 and CXCL1) were increased in the supernatant culture medium after 3-hour, 6-hour, and 9-hour recovery (Fig. S1D). Both cell death and inflammatory cytokines levels were reached the maximum after 6-hour recovery. Hence, 6-hour was used as the time point for analysis in subsequent study. The heatstroke in vivo paradigm was established with C57BL/6j mice according to our previous study [12] (Fig. 1A). Consistent with the in vitro results, the pro-inflammatory cytokines (TNFα, IL-1β and IL-6) and chemokines (MCP-1 and CXCL1) were also significantly increased in the plasma of heatstroke mice (Fig. 1B). More important, compared with the control group, those genes that involve in TNFα signaling pathway exhibited remarkable changes in their expression in response to heat stress in the intestine of mice (Fig. 1C). Moreover, immunoblotting analyses showed a significantly increased in the expression of RIP1, p-RIP1, RIP3, p-RIP3, p-Mlkl, caspase-8 and a decreased expression of p-Drp1 in the intestine of mice in heat stress group (Fig. 1D). To further investigate the effect of HS on the intestine of mice, we performed H&E staining and found that the intestine of mice in the heat stress group demonstrated profound damage manifested as villous stroma broadening, focal necrosis, cell detachment, edema and congestion (Fig. S1E). Taken together, these results demonstrate that heat stress leads to upregulation of pro-inflammatory cytokines, and one such cytokine, TNF, was sufficient to mediate cell death both in vitro and in vivo. To further explore whether TNF signaling pathway was necessary for heat stress to induce cell death, IEC cells were treated with TNFα (10 ng/mL), IL-1β (5 ng/mL) and anti-TNFα antibody (10 ng/mL) for 24 h, respectively, and then the cells were suffered to heat stress after the treatment (Fig. 1E). We found that both TNFα and IL-1β treatment decreased the IEC cells viability and increased the cell death (Fig. 1, F and G). However, blocking TNF signaling with an anti-TNFα antibody was not sufficient to reverse the heat stress-induced cell viability decrease and apoptosis increase (Fig. 1H and I). These results were further confirmed by flow cytometry analysis (Fig. S1F and G). Collectively, our data indicate that TNF signaling partially contributes to cell death after heat stress, suggesting that heat stress can induce cell death through multiple, redundant pathways.

3.2. Heat stress up-regulated the expression of TLR3, promoted TRIF-RIP3 interaction and further induced the necroptosis of intestinal epithelial cells.

Since TNF could induce both apoptotic and necroptotic cell death pathways, next we tried to find out the mode of cell death following heat stress. To differentiate between these cell deaths pathways, we treated the IEC cells with caspase-8 inhibitor (Ac-IETD-CHO, 50 μM), caspase-3 inhibitor (Ac-DEVD-CMK, 50 μM) and RIP3 inhibitor (GSK'872, 10 μM) for 12 h before heat stress (Fig. 2A). The analysis of cell viability and cell

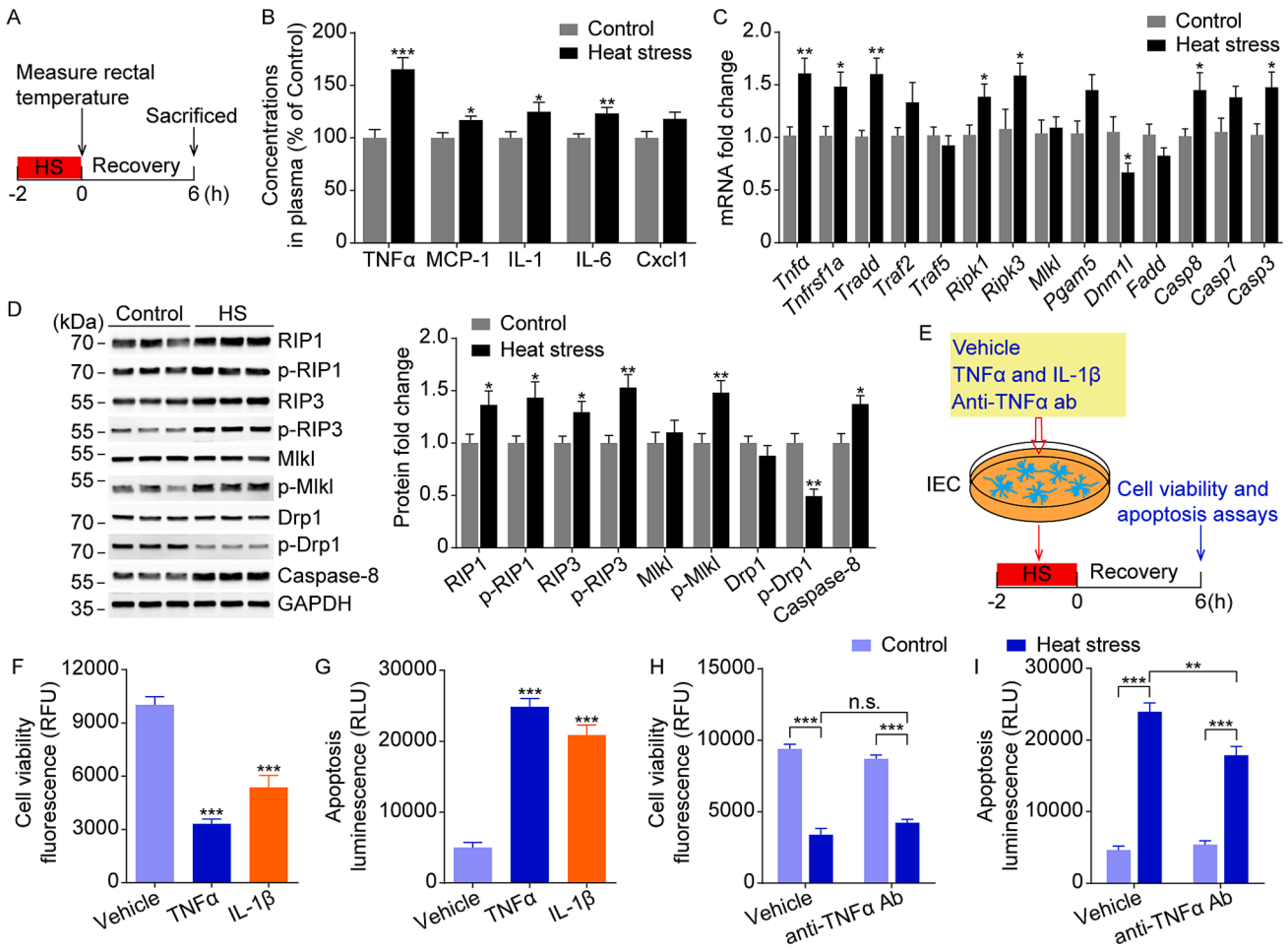

Fig. 1. Injury and cell death were increased in the intestine of mice following heat stress. A. Schematic of the in vivo experimental design. B. Heat stress induced upregulation of the levels of inflammatory cytokines in plasma of heatstroke mice (N = 6 for each group). C. The genes involved in TNF signaling pathway exhibited remarkable changes in their expression in response to heat stress in the intestine of mice (N = 6 for each group). D. The expression of RIP1, p-RIP1, RIP3, p-RIP3, Mlkl, p-Mlkl, Drp1, p-Drp1 and caspase-8 in the intestine of mice were measured by western blot after heat stress (N = 6 for each group). E. Schematic of the in vitro experimental design for TNFα, IL-1β and TNFα antibody treatment on IEC cells. F. Analysis results showed that both TNFα (10 ng/mL) and IL-1β (5 ng/mL) treatment for 24 h decreased the cell viability and increased the cell death. H and I. Heat stress-induced reduction of cell viability (H) and increased cell death (I) were normalized by the treatment of TNFα antibody (10 ng/mL) for 24 h. Data are presented as the mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001.

death by flow cytometry revealed that the treatment of caspase-8 or caspase-3 inhibitor could only partially reverse the heat stress-induced reduction of cell viability and cell death, while treatment of RIP3 inhibitor could almost abolished the effect of heat stress on IEC cells (Fig. S2A and B). In addition, immunoblotting analysis showed that inhibition of caspase-8 or caspase-3 had no effect on the phosphorylation of Mlkl after heat stress; however, the inhibition of RIP3 nearly blocked the heat stress-induced activation of Mlkl (Fig. 2B). Our results indicate that heat stress leads to necroptotic cell death in IEC cells.

We next sought to define whether the activation of RIP3 was necessary for heat stress induced necrotic cell death. First, the IEC cells were treated with *Ripk3* OE to upregulate the expression of RIP3 or treated with siRNA-*Ripk3* to knock down the expression of RIP3 before they were suffered to heat stress (Fig. 2A). We found that upregulation of RIP3 in IEC cells made them more sensitive to heat stress; by contrary, downregulation of RIP3 nearly blocked the heat stress-induced reduction of cell viability (Fig. S2C and D) and restored the activation of Mlkl induced by heat stress (Fig. 2C). These results showed that heat stress induced necroptotic cell death was dependent on the activation of RIP3.

TLR3, one member of the toll-like receptor family, is another pathways that can lead to the induction of necroptosis [13]. To evaluate whether TLR3 will trigger the TRIF-RIP3 signal to mediates the induction of necroptosis during heat stress, we first used the poly(I:C) (25 μM), a TLR3 agonist, to treat the IEC cells (Fig. S2C). We found that activation of TLR3 by poly (I:C) treatment could not only induce cell viability decrease to lead to cell deaths (Fig. S2F and G), but also increase the phosphorylation of RIP3 and Mlkl (Fig. 2D). Furthermore, previous study had already elucidated that TLR3 drive RIP3 activation directly via the adapter protein TIR domain-containing adapter-inducing interferon-β (TRIF), which acts to phosphorylate and activate Mlkl to induce necrotic cell death [13]. Our results showed that treatment of poly(I:C) facilitated the formation of TRIF-RIP3 complex (Fig. 2E). These results demonstrate that activation of TLR3 will trigger TRIF-RIP3 signal activate to further induce necroptotic cell death. To further determine whether heat stress induced necroptosis by activating TLR3-TRIF-RIP3 signal, we examined expression of TRL3 and TRIF in the intestine of mice after heat stress. Immunoblotting analysis showed that heat stress increased both of these two protein expression in the intestine of heatstroke mice (Fig. 2F). What is more important, heat stress also facilitated the formation of TRIF-RIP3 complex in the intestine (Fig. 2G). Taken together, our data show that upregulation of TLR3 to trigger TRIF-RIP3 signal activation underlies induction of necroptosis by heat stress.

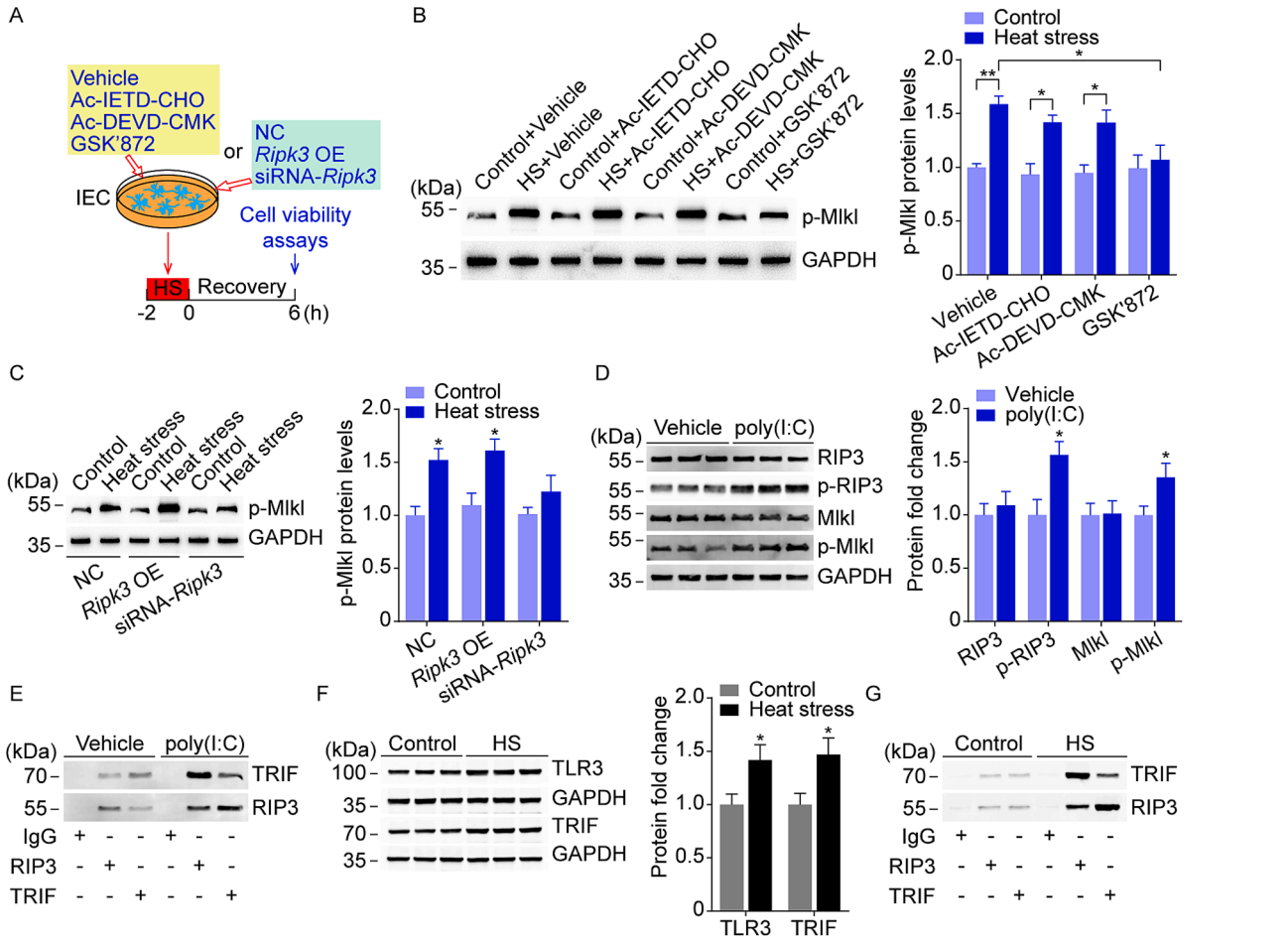

Fig. 2. Heat stress induced necroptotic cell death by activating TLR3-TRIF-RIP3 signal. A. Schematic of the in vitro experimental design. IEC cells were treated with caspase-8 inhibitor Ac-IETD-CHO (50 μM), caspase-3 inhibitor Ac-DEVD-CMK (50 μM) and RIP3 inhibitor GSK'872 (10 μM) for 12 h or were treated with *Ripk3* OE and siRNA-*Ripk3* to upregulate or knock down the expression of RIP3 before they were subjected to heat stress. B. Effect of caspase-8, caspase-3 and RIP3 inhibitor on the phosphorylation of Mlkl after heat stress. C. Immunoblotting analysis showed that heat stress-induced activation of Mlkl was blocked by downregulation of RIP3, in contrast, which were not affected by upregulation of RIP3. D. The expression of RIP3, p-RIP3, Mlkl and p-Mlkl increased in IEC cells after the treatment of poly (I:C). E. The treatment of poly (I:C) facilitated the formation of TRIF-RIP3 complex. F and G. Immunoblotting results showed that heat stress induced upregulation of TLR3 and TRIF in the intestine of heatstroke mice (F) and promoted the formation of TRIF-RIP3 complex (G) (N = 6 for each group). Data are presented as the mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001.

3.3. p53 was required for heat stress-induced necroptosis in the intestine of mice.

p53 has been proved to involve in necroptosis in tumor cells [14]. To determine whether p53 may have function in the regulation of TLR3-TRIF-RIP3 signal to mediate heat stress-induced necroptosis, we first explored the function of the p53 pathway in IEC cells in response to heat stress. Immunoblotting analysis showed that p53 phosphorylation increased significantly after heat stress (Fig. S3A). qRT-PCR analysis revealed that the canonical p53 targets *Cdkn1a* (p21) and *Gadd45a* were significantly upregulated in heat stress group (Fig. S3B). To determine whether p53 is needed to mediate necrotic cell death in response to heat stress, we knocked down p53 in IEC cells by using siRNA-*Tp53* and evaluated the effect of heat stress on the cells after p53 knockdown (Fig. 3A). Analysis results showed that p53 knockdown could markedly reversed the reduction of cell viability induced by heat stress (Fig. S3C and D). Furthermore, immunoblotting analysis revealed that downregulation of p53 also normalized the heat stress-induced increased phosphorylation of RIP3 and inflammatory cytokines concentrations (Fig. 3, B and C). To further investigate the role of p53, we inhibited p53 activation by a daily intraperitoneal administration of Pifithrin-α (3 mg/kg body weight) in C57BL/6J mice for three days before they were subjected to heat stress (Fig. 3D). In line with in vitro results, our immunoblotting analysis and ELISA results showed that both heat stress-induced RIP3 activation in the intestine and upregulation of inflammatory cytokines in plasma of mice were rescued by the inhibition of p53 activation (Fig. 3, E and F). Histopathological analysis further demonstrated that the treatment Pifithrin-α could prevented the intestine of mice from HS-induced damage. Compared with the control group, intestine from HS mice showed significant focal necrosis, cell detachment and neutrophil infiltration, while the intestine from Pifithrin-α treatment mice showed intact structure and no apparent neutrophil infiltration (Fig. S3E). Taken together, these results indicate that p53 is required for heat stress-induced necroptosis in the intestine.

Our data had demonstrated that p53 play a role in mediating necrotic cell death following heat stress, but whether p53 contributes to heat stress activates TLR3-TRIF-RIP3 signal to lead to necroptosis is still unclear. Actually, our results revealed that heat stress increased TLR3 expression both in the intestine of mice (Fig. 2F) and IEC cells (Fig. S3D). However, downregulation of p53 would diminish the expression of TLR3 in IEC cells, which blocked the formation of TRIF-RIP3 complex. In contrast, upregulation of p53 would raise the expression of TLR3 and promote the formation of TRIF-RIP3 complex (Fig. S3, D and E). Collectively, these data indicate that heat stress activated p53 to

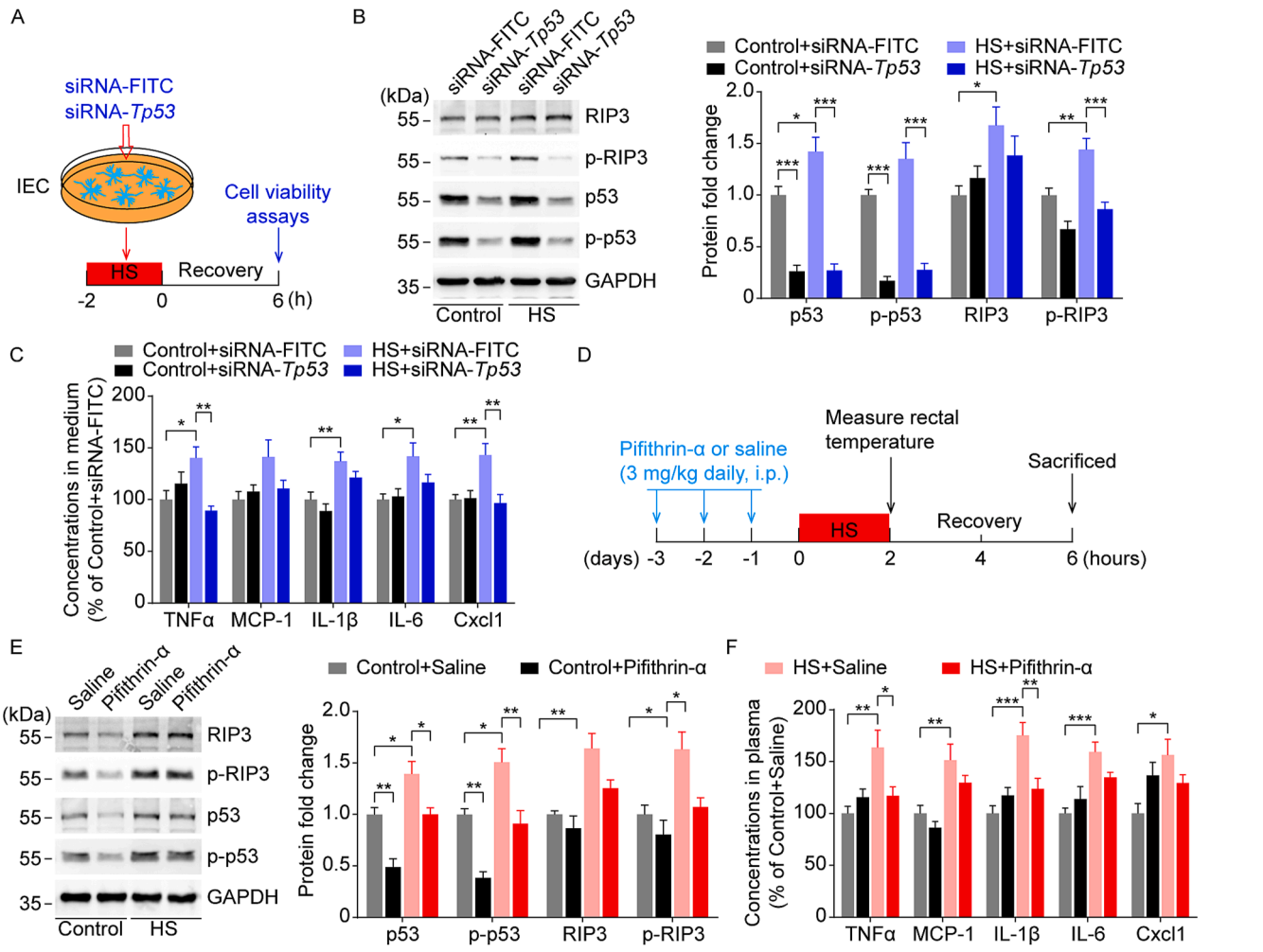

Fig. 3. p53 was required for heat stress-induced necroptosis in the intestine of mice. A. Schematic of the in vitro experimental design for downregulation of p53 in IEC cells before they were subjected to heat stress. B. Effects of the treatment of siRNA-*Tp53* on the expression of p53, p-p53, RIP3 and p-RIP3 in IEC cells following heat stress. C. Heat stress-induced upregulation of inflammatory cytokines in IEC cells were restored by knockdown of p53. D. Schematic of the in vivo experimental design for the treatment of Pifithrin-α. In brief, mice were received a daily intraperitoneal (i.p.) injection with Pifithrin-α (3 mg/kg body weight) for three days before they were subjected to heat stress. E and F. Effects of the treatment of Pifithrin-α on the expression of p53, p-p53, RIP3 and p-RIP3 in the intestine (E) and the levels of inflammatory cytokines in plasma of heatstroke mice following heat stress (F) (N = 6 for each group). Data are presented as the mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001.

regulate the function of TLR3, which then triggered the TRIF-RIP3 signal activation to lead to necrotic cell death in the intestine.

3.4. *Heat stress-induced intestinal epithelial cells necroptosis via TLR3-TRIF3-RIP3 pathway was dependent on p53 in vivo.*

To further characterize the role of p53 in mediating the TLR3-TRIF-RIP3 signal to induce necroptosis during heat stress, we generated a transgenic mouse model in which *Tp53* (encode p53) was deleted (hereinafter referred to as $Tp53^{-/-}$). Our genomic PCR genotyping and immunoblotting results showed that, compared with levels in the intestine of wildtype littermates ($Tp53^{+/+}$), the levels of p53 were completely knocked out in the intestine of $Tp53^{-/-}$mice (Fig. S4, A and B). No differences were observed in the body weight between $p53^{-/-}$ and $p53^{+/+}$ littermate mice (Fig. S4C). Next, we further investigated whether *Tp53* deletion could blocked heat stress-induced necrotic cell death. We subjected $Tp53^{-/-}$ and $Tp53^{+/+}$ mice to the heat stress paradigm (Fig. 4A). The levels of inflammatory cytokines in plasma of $Tp53^{+/+}$ mice exhibited progressive heat stress-induced reductions, which were normalized by the deletion of *Tp53* in $Tp53^{-/-}$mice (Fig. 4B). Histopathological analysis revealed that the HS-induced damage on the intestine of mice was also blocked in $Tp53^{-/-}$ mice (Fig. S4D). Meanwhile, the heat stress-induced activation of RIP3 and Mlkl in the intestine of mice was significantly reversed by the deletion of *Tp53* (Fig. 4C). We did not observe any significant differences in TRIF expression between the $Tp53^{-/-}$ and $Tp53^{+/+}$ mice; However, deletion of *Tp53* markedly decreased the expression of TLR3 in the intestine (Fig. 4D).

To determine whether the deletion of *Tp53* rescued heat stress-induced cell death, we subjected the $Tp53^{-/-}$ mice derived IEC cells (hereinafter referred to as IEC $Tp53^{-/-}$) to heat stress paradigm (Fig. S4D). We found that heat stress induced decreased of cell viability and led to cell death in the $Tp53^{+/+}$ mice, by contrary, the deletion of p53 restored the heat stress-induced reduction of cell viability, which prevented the cells from death (Fig. S4E). Finally, to further understand the function of p53 in heat stress-induced necroptosis, we carried out experiments by restored the expression of p53 or upregulated the expression of TLR3 in IEC $Tp53^{-/-}$ cells before they were suffered to heat stress (Fig. 4E). Similarly, heat stress-induced necrotic cell death was blocked in IEC $Tp53^{-/-}$ cells, which were re-induced by restoration of p53 expression; however, there was no effect on heat stress-induced induction of necroptosis by upregulation of TLR3 when p53 was deficiency (Fig. 4, F and G). Collectively, these data indicate that p53 plays a

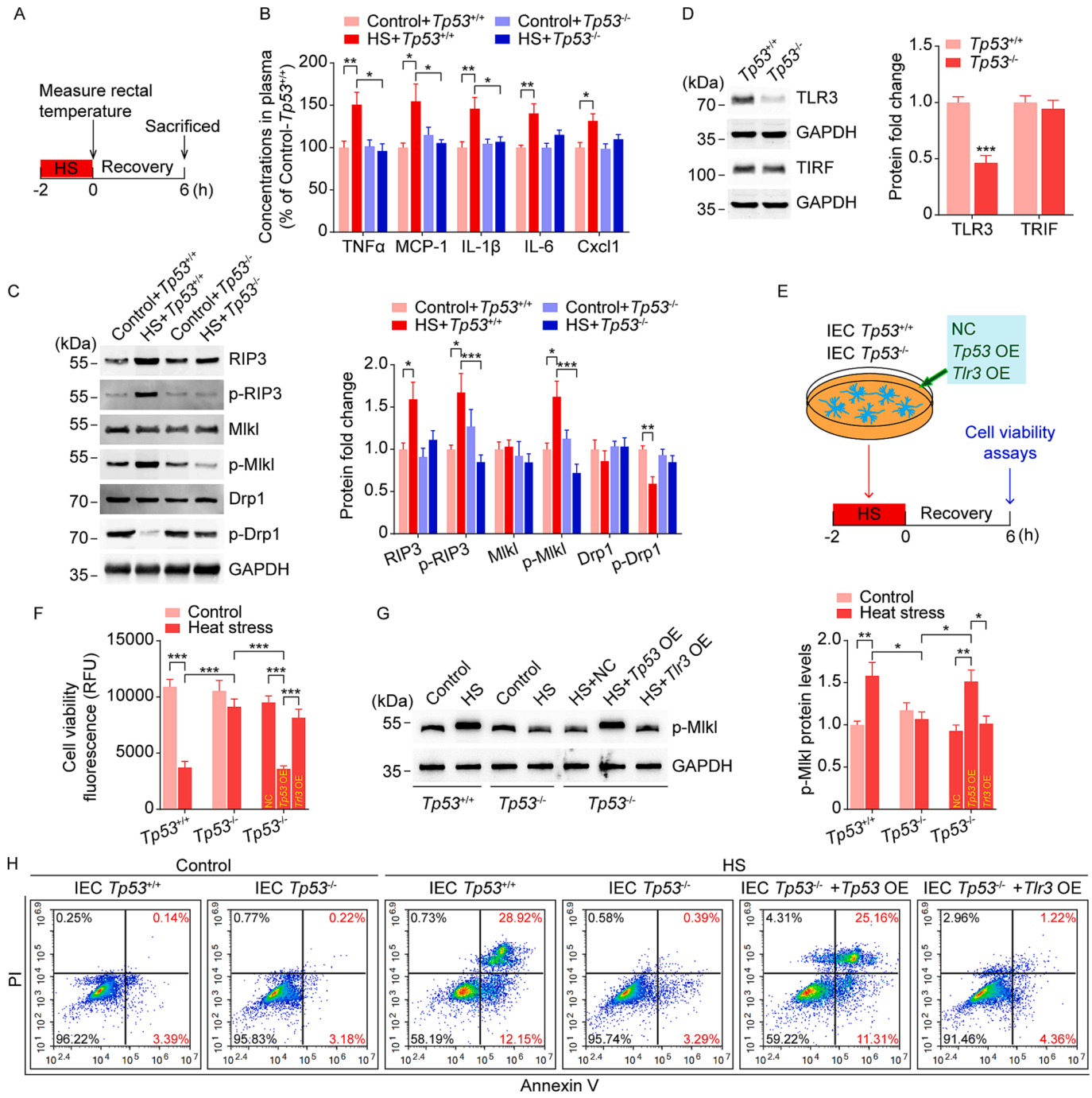

Fig. 4. Heat stress-induced intestinal epithelial cells necroptosis via TLR3-TRIF3-RIP3 pathway was dependent on p53. *Tp53*$^{-/-}$ mice. B. Effect of p53 deletion on the levels of inflammatory cytokine in the plasma of mice (N = 6 for each group). C. Heat stress-induced upregulation of RIP3, p-RIP3, p-Mlkl and reduction of p-Drp1 were normalized by p53 deletion (N = 6 for each group). D. Effect of p53 deletion on the expression of TLR3 and TRIF in the intestine of mice (N = 6 for each group). E. Schematic of the in vitro experimental design. IEC *Tp53*$^{-/-}$ cells were treated with *Tp53* OE to re-express p53 or with *Tlr3* OE to upregulate the expression of TLR3 before they were subjected to heat stress. F. Effect of re-expression of p53 and upregulation of TLR3 on IEC *Tp53*$^{-/-}$ cells to response to heat stress. G. Immunoblotting analysis showed that heat stress-induced activation of Mlkl was blocked by the deletion of p53, which was activated again by re-expression of p53. H. Flow cytometry with Annexin V-FITC/PI staining was used to evaluate the induction of HS-induced cell death. Data are presented as the mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001.

critical role in regulating TLR3 function to further mediate TRIF-RIP3 signal transduction to trigger necroptosis following heat stress.

4. Discussion

Intestine was sensitive to heat stress. Pervious study showed that, when the core temperature reached 40°C, marked epithelial necrosis has been observed, manifested as lesions exhibiting epithelial necrosis and villi desquamation. As the core temperature increased to 42C, the damage to the villi increased [12]. It has been found intestinal cells could appear apoptosis, necrosis, ferroptosis and autophagy [11,12,15,16]. In addition, heat stress also down-regulated the tight junction proteins. Both the cell death and decreased tight junction proteins lead to the increased permeability of intestine. As the intestine

contains a large bacteria and endotoxins, intestinal injury, and the increased permeability-induced bacterial translocation and endotoxemia have been implicated in the pathophysiological process of heatstroke. Microarray analysis of gene expression profiles of rat small intestine in response to heat stress showed that differentially expressed genes were mainly related to stress, immune regulation, and metabolism processes [17]. By RNA sequencing and targeted transcriptome analyses, Koch *et al* found that heat stress induced activated immune- and phagocytosis-related pathways with LPS and cytokines in bovine intestine [18]. The penetration of toxic and bacterial compounds may trigger a modulated immune repertoire and induced an antioxidative defense mechanism. In general, intestine was not only the first damaged organ, but also the 'motor' for further systemic inflammation and multi-organ dysfunction. In present study, our results showed that heat stress could induce intestinal epithelial cell necroptosis, suggesting that targeted necroptosis could help maintain the intestine barrier and prevent further systemic inflammation of heatstroke.

Traditionally, apoptosis was thought to be the only programmed cell death pathway. With the development of research, several programmed cell death modes have been found. Necroptosis was a recently identified regulated necrosis with passive and active proinflammatory functions [19]. Apoptosis was a kind of silent cell death, while necroptosis act as "whistle blowers", which lead to the release of cytokines and other proinflammatory signals into the cellular surroundings [20,21]. In present study, we found heat stress could induce intestinal epithelial cell necroptosis. Heat stress significantly increased the expression levels of RIP1, RIP3, phosphorylated RIP1, RIP3 and MLKL, with the increased pro-inflammatory cytokines in mice plasma and culture medium supernatant. Thus, the necroptosis was correlated with the inflammatory response. These results indicating that targeting necroptosis could be beneficial to the clinical treatment. So far, developed RIP3 inhibitors includes GSK'840, GSK'843 and GSK'872 [13]. Ponatinib and pazopanib, which were found could inhibit RIP1 and RIP3, has been approved by Food and Drug Administration (FDA) as chemotherapeutics in clinical treatment [22]. However, the applications of these tyrosine kinase inhibitors were limited due to their cardiotoxicity. The development of relevant application drugs for necroptosis has broad prospects.

Therefore, targeting necroptosis could help alleviate the severe inflammatory response under heatstroke condition at least by two patterns: inhibiting the necroptosis induced inflammatory response and preventing the bacterial or endotoxin translocation by maintaining intestine barrier [23]. Therefore, it was crucial to clarify the key factor in heat stress induced necroptosis. Hence, we explore the role of p53 in heat stress induced necroptosis of intestinal epithelial cells. The process of necroptosis could be p53 dependent or p53 independent. Previous studies have showed that p53 could mediate necroptosis via interactions with CypD and Drp1, which correlated with mitochondrial permeabilization pore opening [24]. Besides, Lin *et al* also found that GTP-induced necroptosis was modulated by the p53-independent pathway [25]. Our results show that heat stress-induced intestinal epithelial cells necroptosis was dependent on p53. These results showed that p53 could be the therapeutic target for heatstroke.

Necroptosis could be activated by many stimuli, such as activation of death receptors, stimulation of Toll-like receptors, activation of the T-cell receptor and anti-cancer drugs. Activation of TLR3 could induce necroptosis in a direct and indirect way [13,26,27]. TLR3 usually activated by double-stranded (ds)RNA under infection condition. Notably, in present study, compared with p53 knockout cells, TLR3 overexpression cells with p53 knockout has no effect on cell viability and apoptosis luminescence. This suggested that TLR3 mediated necroptosis was dependent on p53 under heat stress. Taura et al found that p53 could regulate the transcription of TLR3 and further regulate the function on TLR3 in human epithelial cell lines [28]. The reduced binding of p53 to the TLR3 promoter, resulting in reduced TLR3 gene transcription and inhibiting the TLR3 mediated pro-inflammatory cytokines and chemokines release [29]. Together, our results indicated that p53 played key role in heat stress induced TLR3-TRIF-RIP3 activation and necroptosis in intestine epithelial cells.

5. Conclusion

Our results indicate that p53 contributes to heat stress induction of necrotic cell death. We found that heat stress promoted the activation of p53 to regulate TLR3 function, which further enhanced the TRIF-RIP3 signal transduction to trigger the activation of RIP3-MLKL signal to mediate necroptosis in intestinal epithelial cells. In addition, the heat stress-induced necroptosis in intestine promoted the development of intestinal inflammation, which in turn aggravates the intestine injury (Fig. 5).

Declarations.

Ethics approval and consent to participate.

The experimental protocols were approved by the Institutional Animal Care and Use Committee at The Southern Medical University and General Hospital of Southern Theater Command of PLA.

Availability of data and materials.

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication.

All authors reviewed the manuscript and approved the publication.

Authors' contributions.

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. JZ, ZG and LS were responsible for study concept and design. JZ, LL, XQ, ZZ and ZG were responsible for experiment measurement. JZ, DT, ZG and LS were responsible for drafting the manuscript.

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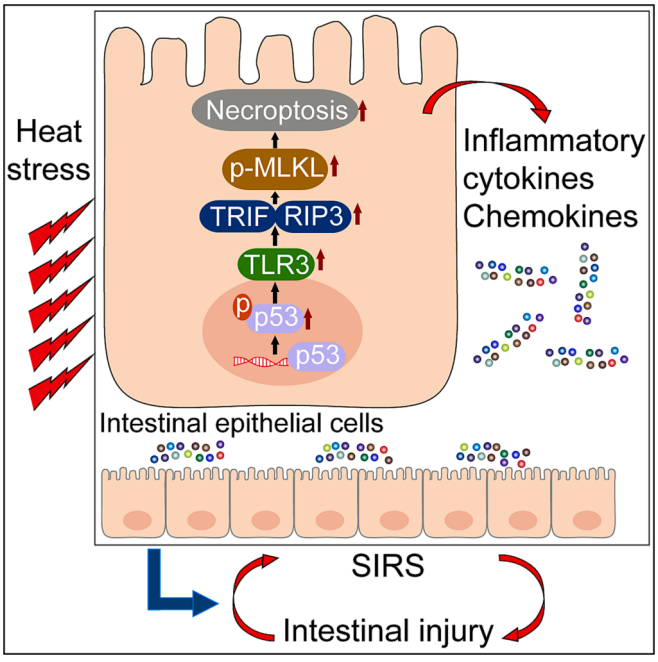

Fig. 5. Schematic diagram of p53 meditated in heat-induced intestinal epithelial cells. Heat stress promoted the phosphorylation of p53, which further upregulated TLR3 and enhanced the interaction of TRIF-RIP3, leading to the activation of RIP3-MLKL mediated necroptosis in intestinal epithelial cells. The necroptosis of intestinal epithelial cells amplificated the inflammatory of intestine. In turn, inflammation further aggravate the intestine injury.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

Not available.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2023.110574.

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