FKBP12 mediates necroptosis by initiating RIPK1–RIPK3–MLKL signal transduction in response to TNF receptor 1 ligation

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ABSTRACT
Necroptosis is a regulated form of necrotic cell death that is mediated by receptor-interacting serine/threonine-protein kinase 1 (RIPK1), RIPK3 and mixed-lineage kinase domain-like protein (MLKL), which mediates necrotic signal transduction induced by tumor necrosis factor (TNF). Although many target proteins for necroptosis have been identified, no report had indicated that FK506-binding protein 12 (FKBP12, also known as FKBP1A), an endogenous protein that regulates protein folding and conformation alteration, is involved in mediating necroptosis. In this study, we found that FKBP12 acts as a novel target protein in mediating necroptosis and the related systemic inflammatory response syndrome triggered by TNF. The mechanistic study discovered that FKBP12 is essential for initiating necrosome formation and RIPK1–RIPK3–MLKL signaling pathway activation in response to TNF receptor 1 ligation. In addition, FKBP12 is indispensable for RIPK1 and RIPK3 expression and subsequent spontaneous phosphorylation, which are essential processes for initial necrosome formation and necrotic signal transduction; therefore, FKBP12 may target RIPK1 and RIPK3 to mediate necroptosis in vitro and in vivo. Collectively, our data demonstrate that FKBP12 could be a potential therapeutic target for the clinical treatment of necroptosis-associated diseases.

KEY WORDS: FK506-binding protein 12, Necroptosis, Receptor-interacting serine/threonine-protein kinase 1, Receptor-interacting serine/threonine-protein kinase 3, Tumor necrosis factor

INTRODUCTION
Necroptosis is a relatively newly discovered programmed cell death process characterized by necrotic morphology, including the swelling of cellular organelles, rupture of cell membranes and, ultimately, lysis of cells (Degterev et al., 2005; Christofferson and Yuan, 2010; Pasparakis and Vandenabeele, 2015). Similar to apoptosis, necroptosis also plays a critical role in various physiological and pathological processes, such as embryonic development (Zhang et al., 2011a,b; Newton et al., 2016), tissue injury (Degterev et al., 2005; Zhang et al., 2016), inflammation (Pasparakis and Vandenabeele, 2015; Lin et al., 2016) and host defense (Mocarski et al., 2012; Nogusa et al., 2016). Necroptosis can be initiated by various necroptotic stimuli, including death receptor ligands (Micheau and Tschopp, 2003; Kim et al., 2007), Toll-like receptor (TLR)3 and TLR4 ligands (He et al., 2011; Takemura et al., 2015), virus infection sensors (Upton et al., 2012; Thapa et al., 2016; Upton and Kaiser, 2017) and interferons (Robinson et al., 2012; Thapa et al., 2013). Tumor necrosis factor (TNF), a pleiotropic proinflammatory cytokine in the family of death receptor ligands, binds TNF receptor 1 (TNFR1; also known as TNFRSF1A) on the membrane and then initiates necroptosis (Hitomi et al., 2008; Galluzzi and Kroemer, 2011). TNF-induced L929 cell necroptosis has been extensively studied, and this cell line has been identified as a well-established necrotic cellular model (Hitomi et al., 2008; Galluzzi and Kroemer, 2011).

Upon ligation with TNF, TNFR1 recruits receptor-interacting serine/threonine-protein kinase 1 (RIPK1) and several other adaptor/effecter proteins to form a TNFR1 signaling complex known as complex I, which acts as the platform to activate the nuclear factor (NF)κB and MAPK signaling pathways (Zhang et al., 2011a,b; Peltzer et al., 2016). However, in cells expressing high levels of receptor-interacting serine/threonine-protein kinase 3 (RIPK3), RIPK1 dissociates from complex I and then recruits RIPK3 to form a new complex, known as a necosome, which facilitates the phosphorylation of RIPK1 and RIPK3 (Cho et al., 2009; Li et al., 2012). The phosphorylated RIPK3 recruits and phosphorylates its substrate protein mixed-lineage kinase domain-like protein (MLKL), and then MLKL oligomerizes and translocates to the cell membrane to disrupt membrane integrity, which leads to necroptosis (Galluzzi et al., 2014; Wang et al., 2014). Therefore, RIPK1 and RIPK3 are two initiators of necroptosis, and MLKL acts as the executor of necroptosis; together, these proteins form the RIPK1–RIPK3–MLKL signaling pathway, which initiates necroptosis when triggered by TNFFR1 ligation (Sun et al., 2012; Newton, 2015).

FK506-binding protein 12 (FKBP12, also known as FKBP1A) is a 12 kDa cytosolic protein that possesses peptidyl-prolyl cis/trans isomerase (PPlase) activity (Tong and Jiang, 2015). This protein was originally identified as the binding partner of immunosuppressive drugs, such as FK506 and rapamycin, which represent two major classes of FKBP12 ligands with different structures (Harding et al., 1989). Both drugs bind FKBP12 and inhibit its PPlase activity, but the inhibition of FKBP12 does not contribute to their immunosuppressive activity (Bierer et al., 1990). Instead, binding with FKBP12 allows the drugs to subsequently interact with the mechanistic target of their action in immunosuppression. It has been suggested that the FK506–FKBP12 complex specifically interacts with calcineurin to prevent its activation (Liu et al., 1991). However, the rapamycin–FKBP12 complex targets the protein mammalian target of rapamycin (mTOR) and interferes with its kinase function, thus blocking cytokine-stimulated protein synthesis (Dumont et al., 1990). In addition,
FKBP12 also functions as a molecular chaperone promoting protein folding or catalyzes interconversion between cis/trans prolyl in its target proteins through PPIase-like activity (Siekierka et al., 1989; Lu et al., 2007). Therefore, FKBP12 is involved in diverse cellular functions, although it is not clear whether FKBP12 plays a role in programmed cell death, especially necroptosis.

In this study, we found that FKBP12 is essential for necroptosis triggered by TNFR1 ligation and that it mediates necroptotic signal transduction by initiating necrosome formation and subsequent RIPK1–RIPK3–MLKL signaling pathway activation. Moreover, FKBP12 is indispensable for RIPK1 and RIPK3 expression and spontaneous phosphorylation, the latter of which is a critical event for RIPK1 and RIPK3 in the initial formation of the necrosome and the subsequent necroptotic signal transduction. Therefore, our data demonstrate that FKBP12 is a new target protein for necroptosis induction and that it targets RIPK1 and RIPK3 to initiate necroptotic signal transduction in response to TNF stimulation. In addition, we found that FKBP12-mediated necroptosis is crucial for the high mortality associated with TNF-induced systemic inflammatory response syndrome (SIRS); therefore, FKBP12 may be a potent target for SIRS therapy, in which case FKBP12 ligands might be applied in the clinical treatment of necroptosis-associated disease, including SIRS.

RESULTS
FKBP12 mediates necroptosis induced by TNFR1 ligation
L929 and HT22 cells are two well-established necroptotic cellular models, so we identified the role of FKBP12 in mediating necroptosis by using these two cell lines. First, we verified TNF-induced necroptosis in L929 cells by determining the morphological changes of L929 cells by performing transmission electron microscopy (TEM). As shown in Fig. 1A, the necrotic morphological changes, as induced by TNF plus Z-VAD, including the appearance of massive cytosolic vacuolation and the early loss of cell membrane integrity, were found in the dead L929 cells, confirming that L929 cells died through the necrototic pathway. Next, we detected the death of L929 cells and HT22 cells, triggered by TNF plus Z-VAD, by using the annexin V–FITC and propidium iodide double staining method; the results shown in Fig. S1 demonstrated that most of the dead cells were both annexin V–FITC- and propidium iodide double-positive (indicating necrotic cell death) and not only annexin V–FITC-single-positive (indicating apoptotic cell death), further confirming that TNF-induced cell death of L929 and HT22 cells is not occurring through apoptosis but rather through necroptosis. To analyze the exact role of FKBP12 in necroptosis, we first determined the effect of FKBP12 ligands on TNF-induced necroptosis in L929 cells. As shown in Fig. 1B, TNF-induced L929 cell death was inhibited by FKBP12 ligands, including FK506, pimecrolimus, rapamycin, everolimus, zotarolimus and ridaforolimus. Moreover, the inhibitory effect of rapamycin and FK506 on necroptosis was further confirmed by assessing cell death induced by TNF in the absence or presence of rapamycin or FK506 (Fig. 1C). To further confirm the critical role of FKBP12 in necroptosis, we determined the effect of FKBP12 knockdown on TNF-induced necroptosis. As shown in Fig. 1D, infection with FKBP12 shRNA lentivirus significantly downregulated FKBP12 in L929 cells, and FKBP12 knockdown significantly inhibited necroptosis induced by TNF in the absence or presence of Z-VAD, indicating that FKBP12 is essential for TNF-induced necroptosis. In addition, FKBP12 knockdown protected L929 cells from necroptosis induced by TNF combined with cycloheximide (CHX) or SMAC mimic (SM, such as BV-6) in the presence of Z-VAD, and even BV-6 plus Z-VAD (Fig. 1E), thus demonstrating that FKBP12 is critical for necroptosis triggered by TNFR1 ligation. Furthermore, FKBP12 knockdown also inhibited HT22 cell necroptosis induced by TNF plus Z-VAD (Fig. 1F), indicating that the critical role of FKBP12 in mediating necroptosis is not dependent on the cell type. Finally, we found that the restoration of FKBP12 protein expression in FKBP12-knockdown L929 cells led to the recovery of cell sensitivity to TNF-induced necroptosis (Fig. 1G,H), further supporting the critical role of FKBP12 in mediating necroptosis. As FKBP12 knockdown had no inhibitory effect on apoptosis induced by TNF plus cycloheximide in NIH/3T3 cells (Fig. S2), FKBP12 is specific in mediating necroptosis.

In conclusion, our results demonstrate that FKBP12 is a new target protein for necroptosis initiated by TNFR1 ligation.

FKBP12-mediated necroptosis is not associated with the mTOR signaling pathway
Previous reports have indicated that rapamycin binds to FKBP12 to form the rapamycin–FKBP12 complex, which then interacts with mTOR and suppresses its activity (Dumont et al., 1990). Because mTOR has been reported to mediate necroptosis in L929 and HT22 cells (McNamara et al., 2013; Liu et al., 2014), we next explored whether FKBP12 targets mTOR to initiate necroptosis. First, we confirmed the effect of mTOR on TNF-induced necroptosis. Similar to what was seen with rapamycin, ATP-competitive inhibitors of mTOR, including GDC0349, XL388, AZD8055 and WYE125132, inhibit TNF-induced cell death (Fig. 2A), as well as the phosphorylation of mTOR and its substrate protein 4E-BP-1 (also known as EIF4EBP1) (Fig. 2B), confirming that mTOR is critical for TNF-induced necroptosis. However, FK506, another class of FKBP12 ligand, does not suppress mTOR and its substrate protein phosphorylation (Fig. 2B), although it could inhibit TNF-induced necroptosis (Fig. 1A,B), thus demonstrating that inhibition of FKBP12 could block necroptosis in an mTOR-independent manner. Therefore, we next explored the effect of FKBP12 on mTOR signaling pathway activation during necroptosis. As shown in Fig. 2C, the phosphorylation levels of mTOR and its substrate proteins, including 4E-BP1 and p70 S6K1 (also known as RPS6KB1), increased as early as 6 h after TNF stimulation in the negative control L929 cells, indicating that TNF activated the mTOR signaling pathway during necroptosis. However, FKBP12 knockdown had no inhibitory effect on TNF-induced activation of the mTOR signaling pathway (Fig. 2C), although it significantly inhibited TNF-induced necroptosis in L929 cells (Fig. 1C,D); therefore, the mTOR signaling pathway activation does not contribute to FKBP12-mediated necroptosis in response to TNF stimulation.

Based on our results, we conclude that FKBP12 does not mediate necroptosis by activating the mTOR signaling pathway.

FKBP12 is essential for RIPK1–RIPK3–MLKL signaling pathway activation induced by TNF
The RIPK1–RIPK3–MLKL signaling pathway activation is essential for TNF-induced necroptosis (Sun et al., 2012; Newton, 2015); therefore, we determined the effect of FKBP12 on RIPK1–RIPK3–MLKL signaling pathway activation. As shown in Fig. 3A, the phosphorylation levels of RIPK1 and RIPK3 significantly increased in the negative control L929 cells but not in the FKBP12-knockdown L929 cells in response to TNF stimulation, indicating that FKBP12 is essential for RIPK1 and RIPK3 phosphorylation induced by TNF. Therefore, we next determined the effect of
FKBP12 knockdown on the phosphorylation of MLKL, the substrate protein of RIPK3; the results shown in Fig. 3B demonstrate that FKBP12 knockdown also blocked TNF-induced MLKL phosphorylation. Because phosphorylated MLKL binds to itself, via disulfide bonds, to form oligomers (Galluzzi et al., 2014; Wang et al., 2014), we next determined the effect of FKBP12 on MLKL oligomerization. As shown in Fig. 3C, there was a substantial amount of MLKL oligomers (dimers and tetramers) for the negative control cells but their levels were much lower in FKBP12-knockdown cells in response to TNF stimulation, indicating that FKBP12 knockdown also suppresses MLKL oligomerization during necroptosis. The MLKL oligomer could

Fig. 1. See next page for legend.
Fig. 1. FKBP12 mediates TNF-induced necroptosis. (A) Necrotic ultrastructural changes of L929 cells induced by TNF plus Z-VAD. Cells were treated with TNF (100 ng/ml) plus Z-VAD (20 μM) for 12 h, and the ultrastructural changes of cell death was analyzed by TEM. Representative results from three independent experiments are shown. (B) FKBP12 ligands protect L929 cells from death induced by TNF. Cells were pre-treated with FKBP12 ligands, including FK506, pimecrolimus, rapamycin, everolimus, zotarolimus and ridaforolimus at 10 μM, and then treated with TNF (100 ng/ml) for 48 h. The cell survival rate was measured through the Trypan Blue exclusion method. Representative measurements of at least three independent experiments are shown. The cell survival values represent the mean±s.d. of three separate experiments (n=3), **P<0.01 (one-way ANOVA) compared with cells in the group treated with TNF in the presence of DMSO (as these FKBP12 ligands were dissolved in DMSO, DMSO was used as the negative control). The sample size (n=3) and the number of repeated experiments (three or more) were also used in all the Trypan Blue exclusion experiments shown in this figure. (C) FK506 and rapamycin inhibit TNF-induced necroptosis in L929 cells. Cells were pre-treated with DMSO (negative control), FK506 or rapamycin for 1 h, and then treated with or without TNF for 48 h. The proportion of cells that had undergone cell death was determined by measuring the proportion of propidium iodide positive-stained cells. Three independent experiments were performed, and representative results are shown. **P<0.01 (one-way ANOVA) compared with cells in the group treated with TNF in the presence of DMSO. (D) FKBP12 knockdown blocks TNF-triggered necroptosis. L929 cells were infected with the FKBP12 shRNA or the negative control shRNA lentivirus and then treated with TNF in the absence or presence of Z-VAD for an additional 48 h. Western blotting was performed to detect the knockdown efficiency of FKBP12. Actin was used as a loading control. The proportion of propidium iodide-positive cells was measured via flow cytometry and used as the cell death value (%). The cell death values are shown as the mean±s.d., and they were analyzed by a two-tailed t-test. **P<0.01 (one-way ANOVA). The cell death values (%) are shown as the mean±s.d. of three separate experiments (n=3) for C and D. (E) FKBP12 knockdown inhibits L929 cell necroptosis induced by TNFR1 ligation. FKBP12-knockdown and negative control L929 cells were treated with TNF plus Z-VAD in the presence of cycloheximide (T+CHX+Z) or SMAC mimetic (T+SM+Z), or with SMAC mimetic plus Z-VAD (SM+Z) for 24 h, and cell survival was determined through microscopy (200× magnification). More than three fields in each group were observed, and representative images are shown. Cell survival was also quantified by using the Trypan Blue exclusion method. The cell survival values are shown as the mean±s.d. **P<0.01 (two-tailed t-test). (F) FKBP12 knockdown inhibits HT22 cell necroptosis induced by TNF. HT22 cells were infected with FKBP12 shRNA or Con shRNA lentivirus for 48 h, and then treated with or without TNF for another 48 h. The cell survival rate was measured by the Trypan Blue exclusion method, and FKBP12 knockdown efficiency was evaluated by western blotting. The percentage of cell survival is shown as the mean±s.d. **P<0.01 (two-tailed t-test). (G) Restoration of FKBP12 expression recovers the sensitivity of L929 cells to TNF-induced cytotoxicity. L929 cells were infected with the indicated lentiviruses and then treated with or without TNF plus Z-VAD for 24 h. Western blotting was performed to evaluate the expression levels of FKBP12 and HA (from control plasmid, pCDH-HA-control, or plasmid for expression of HA-tagged FKBP12, pCDH-HA-FKBP12), and actin was used as a loading control. Cell survival was determined by microscopy (200× magnification). More than three fields in each group were observed, and representative images are shown. Cell survival was also determined by means of a Trypan Blue exclusion assay. The cell survival values are shown as the mean±s.d. **P<0.01 (two-tailed t-test). All the western blot analyses were repeated independently three or more times, with representative images shown. Statistical analysis of the relative density of protein bands is shown in Fig. S3.

In addition, we confirmed the interaction between MLKL protein molecules by performing a Duolink proximity ligation assay (PLA), which can detect the interaction between different proteins or the same proteins. As shown in Fig. 3D, the green spots, which represent the interaction of MLKL proteins, significantly increased in the negative control L929 cells but not the FKBP12-knockdown L929 cells after TNF plus Z-VAD treatment, suggesting that FKBP12 knockdown suppresses MLKL dimerization. Moreover, the PLA signals representing the protein interaction were also analyzed by using the Image Tool software, and the results demonstrated that the normalized PLA signals increased in the negative control L929 cells but not in the FKBP12-knockdown L929 cells treated with TNF plus Z-VAD, further confirming that FKBP12 knockdown blocks the TNF-induced MLKL homo-interaction. Finally, oligomerized MLKL has been reported to translocate to the membrane and disrupt the membrane integrity (Galluzzi et al., 2014; Wang et al., 2014); therefore, we determined the effect of FKBP12 knockdown on MLKL membrane translocation. The results shown in Fig. 3E demonstrate that the MLKL oligomer translocates to the membrane in response to TNF plus Z-VAD stimulation but that the FKBP12 knockdown inhibits MLKL translocation.

Collectively, our data demonstrate that FKBP12 is essential for RIPK1–RIPK3–MLKL signaling pathway activation during necroptosis induced by TNFR1 ligation.

FKBP12 is required for necrosome formation and the RIPK1 or RIPK3 oligimerization induced by TNF

Necrosome formation has been reported to occur upstream of the RIPK1–RIPK3–MLKL signaling pathway and is essential for the activation of this pathway during necroptosis (Cho et al., 2009; Li et al., 2012); therefore, we next analyzed the effect of FKBP12 knockdown on necrosome formation. As shown in Fig. 4A, the interaction between RIPK1 and RIPK3 significantly increased in the negative control cells but not in the FKBP12-knockdown cells in response to TNF plus Z-VAD stimulation, suggesting that FKBP12 knockdown inhibits necrosome formation. In addition, the interaction between RIPK1 and RIPK3 was also measured through Duolink PLAs, and the PLA signals representing the RIPK1 and RIPK3 interaction were significantly enhanced in the negative control cells but not in the FKBP12-knockdown cells after TNF plus Z-VAD treatment (Fig. 4B), further confirming the critical role of FKBP12 in necroptosome formation.

Necrosome formation has been reported to enable the recruitment of free RIPK3 in necrosomes to form RIPK3 dimers or oligomers, which promotes RIPK3 intramolecular autophosphorylation (Wu et al., 2014). In addition, phosphorylated RIPK3 facilitates accumulation of reactive oxygen species (ROS), which then promotes RIPK1 oligomerization through crosslinking in the form of oxidized disulfide bonds (Zhang et al., 2017). Because RIPK3 autophosphorylation results from an intramolecular reaction in the oligomers that enable RIPK1 to bind RIPK3 to form a necrosome, RIPK3-dependent ROS are involved in a positive-feedback circuit on necrosome formation and subsequent necroptotic signal transduction (Zhang et al., 2017). Therefore, RIPK1 and RIPK3 oligomerization is essential for their phosphorylation and their ability to form necrosomes during necroptosis; therefore, we next determined the effect of FKBP12 on RIPK1 and RIPK3 oligomerization. As shown in Fig. 4C, there was a substantial amount of RIPK1 oligomer in negative control cells but its level was much lower in FKBP12-knockdown cells treated with TNF plus Z-VAD, indicating that FKBP12 knockdown suppressed RIPK1 oligomerization. Consistent with a previous study (Zhang et al., 2017), RIPK1 oligomers were detected only in nonreduced protein samples, confirming that RIPK1 forms oligomers through ROS-induced crosslinking of oxidized disulfide bonds. In
addition, the Duolink PLA signal for RIPK1 homodimerization in the negative control cells was significantly higher than that in FKBP12-knockdown cells after TNF plus Z-VAD stimulation (Fig. 4D), further confirming the essential role of FKBP12 in mediating RIPK1 oligomerization. Similar to RIPK1, RIPK3 also undergoes a significant amount of dimerization or oligomerization in the negative control cells treated with TNF and Z-VAD, whereas FKBP12 knockdown decreased RIPK3 homo-interaction (Fig. 4E). Moreover, the Duolink PLA signals representing RIPK3 homodimerization were significantly higher in the negative control cells than the FKBP12-knockdown cells (Fig. 4F), further supporting a critical role of FKBP12 in mediating RIPK3 oligomerization.

Based on our results, FKBP12 is essential for necrosome formation and RIPK1 and RIPK3 oligomerization.

**FKBP12 is essential for RIPK1 and RIPK3 expression and spontaneous phosphorylation**

To further explore the mechanism of FKBP12 in mediating necroptosis, we next determined the effect of FKBP12 knockdown on the expression of some proteins involved in complex I formation, which is upstream of necrosome formation during TNF-induced signal transduction. As shown in Fig. 5A, FKBP12 knockdown had no inhibitory effect on the expression of some proteins involved in complex I, including TNFR1, TRADD, FADD, TRAF2, cIAP1 (also known as BIRC2), CYLD and the long isoform of c-FLIP (FLIP/L, encoded by CFLAR), indicating that FKBP12 may not mediate necroptosis by targeting complex I. RIPK1 and RIPK3 expression and spontaneous phosphorylation in the early signal transduction stage are also critical for necroson formation and RIPK1–RIPK3–MLKL signaling pathway activation (Degterev et al., 2008; Christofferson et al., 2014; Zhang et al., 2017). Therefore, we next determined the effect of FKBP12 on the expression and spontaneous phosphorylation of RIPK1, RIPK3 and MLKL. As shown in Fig. 5B, C, the mRNA and protein levels of RIPK1 and RIPK3 decreased significantly in FKBP12-knockdown cells compared with those in the negative control cells. Moreover, RIPK1 and RIPK3 expression was also increased by the persistent ectopic expression of FKBP12 in L929 cells or transient overexpression of FKBP12 in BHK21 cells (Fig. 5D,E). Therefore, FKBP12 is essential for RIPK1 and RIPK3 expression. Consistent with the alteration of RIPK1 and RIPK3 expression, the spontaneous phosphorylation of RIPK1 and RIPK3 decreased in FKBP12-knockdown cells (Fig. 5B) but increased in FKBP12 overexpression cells (Fig. 5D), demonstrating that FKBP12 is also critical for controlling RIPK1 and RIPK3 spontaneous phosphorylation by regulating their expression. However, the MLKL protein level showed no significant changes in response to FKBP12 knockdown or overexpression (Fig. 5B,D), indicating that MLKL is not the target protein for FKBP12 in mediating necroptosis.

Fig. 2. mTOR signaling pathway is not involved in FKBP12-mediated necroptosis. (A) ATP-competitive inhibitors of mTOR block TNF-induced necroptosis in L929 cells. Cells were treated with or without TNF in the absence or presence of ATP-competitive inhibitors of mTOR, including GDC0349, XL388, AZD8055 and WYE125132, and cell death was quantified by measuring the proportion of propidium iodide positive-stained cells using flow cytometry. The cell death values (%) are shown as the mean±s.d. (n=3). **P<0.01 (one-way ANOVA test), compared with cells in the group treated with TNF. (B) mTOR signaling pathway activation was suppressed by ATP-competitive inhibitors of mTOR and by rapamycin, but not FK506. L929 cells were treated with ATP-competitive inhibitors of mTOR, rapamycin and FK506 for 6 h, and western blotting was used to detect the phosphorylation level of mTOR (p-mTOR) and its substrate protein 4E-BP-1 (p-4E-BP-1). Actin was used as a loading control. (C) FKBP12 knockdown does not suppress TNF-induced activation of the mTOR signaling pathway. FKBP12 knockdown and the negative control L929 cells were treated with TNF for the indicated time points, and western blotting was used to evaluate FKBP12 knockdown efficiency and the phosphorylation level of mTOR and its substrate protein, including 4E-BP-1 and p70 S6K1 (p-p70 S6K). Actin was used as a loading control. All the western blot analyses were repeated independently three or more times, with representative images shown. Statistical analysis of the relative density of protein bands is shown in Fig. S4.
Collectively, FKBP12 is essential for RIPK1 and RIPK3 expression and spontaneous phosphorylation, which may contribute to FKBP12-mediated necroptotic signal transduction.

**FKBP12 is essential for TNF-induced systemic inflammatory response syndrome**

Reports have indicated that TNF-induced necroptosis is essential for the TNF-induced systemic inflammatory response syndrome (SIRS) (Duprez et al., 2011; Linkermann et al., 2012); therefore, we verified the in vivo function of FKBP12 on necroptosis in mouse TNF-induced SIRS model. Because deletion of the Fkbp12 gene in mice is embryonically lethal, we downregulated FKBP12 in mice by injecting mice with purified FKBP12 shRNA lentivirus via the tail vein. As shown in Fig. 6A,B, the level of FKBP12 mRNA and protein in FKBP12-knockdown mice caecum was significantly lower than that in the negative control mice (injected with the same amount of purified Con shRNA lentivirus) or mock-treated mice (which received an injection without lentivirus, denoted ‘blank’), indicating that FKBP12 knockdown in mice is efficient. Consistent with previous reports (Chen et al., 2015), dramatic decreases in temperature were observed
over time after TNF injection in the negative control mice, whereas FKBP12 knockdown significantly reversed the decrease in body temperature induced by TNF (Fig. 6C). Moreover, compared with the negative control mice, FKBP12-knockdown mice survived longer and at high rates (Fig. 6D). Therefore, FKBP12-knockdown mice were protected from severe hypothermia and death induced by TNF.
administration. In addition, we analyzed the effect of FKBP12 on necroptosis-mediated tissue damage of the caecum and liver in TNF-induced SIRS. As shown in Fig. 6E, at 6 h after TNF injection, the negative control mice showed focal necrosis in the caecum, but the necrosis was much milder in the FKBP12-knockdown mice. Moreover, analysis of caecum lysates showed that TNF-induced phosphorylation of RIPK1 and RIPK3 in the negative control mice was significantly higher than that in FKBP12-knockdown mice (Fig. 6B). Therefore, our data demonstrate that FKBP12 is essential for TNF-induced necroptotic signal transduction and subsequent

Fig. 5. FKBP12 is essential for RIPK1 and RIPK3 expression. (A, B) The effect of FKBP12 knockdown on the expression of proteins involved in complex I and the necrosome. FKBP12 knockdown and the negative control L929 cells were lysed to analyze the level of proteins involved in complex I (A) and necrosome (B). t, antibody against total protein; p, antibody against phosphorylated form. Actin was used as a loading control. (C) FKBP12 knockdown suppresses RIPK1 and RIPK3 transcription. mRNA was extracted from FKBP12 knockdown and the negative control L929 cells, reverse transcribed into cDNA, and then used to measure the levels of RIPK1, RIPK3 and FKBP12 mRNA via qPCR. The experiments were performed at least three times, and representative results are shown. The relative expression values are shown as the means±s.d. of three separate experiments (n=3). **P<0.01 (two-tailed t-test). (D) FKBP12 ectopic expression (HA-tagged FKBP12 from pCDH-HA-FKBP12) promotes RIPK1 and RIPK3 expression and phosphorylation. L929 cells were infected with negative control lentivirus or lentivirus encoding FKBP12 with an HA tag and then lysed to detect the protein levels of FKBP12, HA, RIPK1 and RIPK3, and the phosphorylation levels of RIPK1 and RIPK3. Actin was used as a loading control. (E) Transient ectopic expression of FKBP12 in BHK21 cells (baby hamster kidney fibroblast cells) increases RIPK1 and RIPK3 expression. Cells were transfected with the negative plasmids (pCMV3-Myc-vector) or plasmids containing the Myc-taggedFKBP12 cDNA (pCMV3-Myc-FKBP12) sequence for 48 h and then lysed to determine the protein levels of FKBP12, Myc, RIPK1 and RIPK3. Actin was used as a loading control. All the western blot analyses were repeated independently three or more times, with representative images shown. Statistical analysis of the relative density of protein bands is shown in Fig. S7.
necroptosis in vivo. We also assessed liver damage in mice with TNF-induced SIRS, and there was no evident focal necrosis in the liver for either the negative controls or in FKBP12-knockdown mice (Fig. 6F) after TNF injection; therefore, damage to the caecum, but not to the liver, may contribute to the pathology of TNF-induced SIRS in our study.

To further analyze the in vivo functions of FKBP12 in mediating necroptosis, we evaluated the effect of FKBP12 ligands
on TNF-induced SIRS. As shown in Fig. 7A,C, mice pretreated with FKBP12 ligands, such as FK506 and rapamycin, were significantly protected from hypothermia induced by TNF injection. Moreover, mice pretreated with FK506 or rapamycin survived longer and at higher rates (Fig. 7B,D). Therefore, these results further confirm the essential role of FKBP12 in mediating necroptosis in vivo.

Fig. 7. FKBP12 ligands protect against TNF-induced SIRS. FK506 (A,B) and rapamycin (C,D) inhibit mouse hypothermia and death in the TNF-induced SIRS model. Mice (n=10) were pretreated with FK506 (12 mg/kg body weight per day, intragastrically once a day for 7 days), Rapamycin (5 mg/kg body weight, intraperitoneally) or an equal amount of vehicle for 1 h and then injected with mouse TNF (100 μg/kg body weight). Mice body temperature and survival rates were measured at the indicated time points. The representative results of three independent experiments are shown. The mouse body temperature values are shown as the mean±s.d. of ten mice (n=10). **P<0.01 (two-tailed t-test). (E) Model for FKBP12 in mediating necroptosis triggered by TNFR1 ligation. FKBP12 is essential for RIPK1 and RIPK3 expression, which is critical for their subsequent spontaneous phosphorylation. In addition to the crucial role of RIPK1 and RIPK3 spontaneous phosphorylation in necrosome formation and the subsequent necroptotic signal transduction, FKBP12 mediates necroptosis through initiating RIPK1–RIPK3–MLKL signaling pathway activation in response to TNFR1 ligation.
Collectively, FKBP12 is essential for the necroptosis-mediated mortality of TNF-induced SIRS; therefore, FKBP12 is an important target for necroptosis in vivo.

**DISCUSSION**

In this study, FKBP12 was identified as a novel target in mediating necroptosis induced by TNFR1 ligation, which targets RIPK1 and RIPK3 to initiate necroosome formation, RIPK1–RIPK3–MLKL signaling pathway activation and subsequent necroptosis. Reports have indicated that necrototic signal transduction is mediated by sequential events of RIPK1–RIPK1 homo-interaction, RIPK1–RIPK3 hetero-interaction and RIPK3–RIPK3 homo-interaction (Wu et al., 2014). Moreover, the hetero-interaction between RIPK1 and RIPK3 enables RIPK3 to recruit other free RIPK3 to the necroosome to form RIPK3 homodimers or oligomers, which facilitates RIPK3 autophosphorylation via intramolecular reactions (Wu et al., 2014). Furthermore, phosphorylated RIPK3 not only recruits and phosphorylates its substrate protein MLKL but also phosphorylates RIPK1 in the necroosome (Cho et al., 2009; Galluzzi et al., 2014; Wang et al., 2014). Moreover, it has been reported that phosphorylated RIPK3 also promotes ROS accumulation (Zhang et al., 2009; Schenk and Fulda, 2015), which oxidizes the disulfide bonds between cysteine residues in the RIPK1 protein peptide to form crosslinks between RIPK1 molecules, thereby leading to RIPK1 oligomerization and subsequent autophosphorylation (Zhang et al., 2017). Because RIPK1 autophosphorylation enables RIPK1 to recruit RIPK3 to form the functional necrosome, RIPK3-dependent ROS function in a positive feedback circuit that ensures the effective induction of necroptosis (Zhang et al., 2017). Therefore, these homodimers and heterodimers between RIPK1 and RIPK3 promote their own phosphorylation, which then promotes MLKL activation and subsequent necroptosis. In this study, we found that FKBP12 is essential for ROS-dependent RIPK1–RIPK3 homo-interactions, necroosome formation and RIPK3 oligomerization, and, hence, that FKBP12 is required to mediate necroptosis via the initiation of RIPK1–RIPK3–MLKL sequential phosphorylation. Therefore, the initiation of RIPK1–RIPK3–MLKL signaling pathway activation is the potential mechanism through which FKBP12 mediates necroptosis.

In addition to TNF-induced phosphorylation of RIPK1 and RIPK3, the spontaneous phosphorylation of RIPK1 and RIPK3 in the early signaling stage before TNF treatment is also indispensable for necroptosis induction because suppression of RIPK1 and RIPK3 spontaneous phosphorylation through kinase-dead mutation blocks necroosome formation and necroptosis (Vandenabeele et al., 2010; Kaiser et al., 2014; Liu et al., 2017). In this study, we found that FKBP12 knockout significantly inhibited RIPK1 and RIPK3 expression and spontaneous phosphorylation. Therefore, FKBP12 may mediate necroptotic signal transduction and the subsequent necroptosis triggered by TNFR1 ligation by facilitating RIPK1 and RIPK3 spontaneous phosphorylation. Moreover, TNF-induced phosphorylation of RIPK1 is mediated by ROS-dependent RIPK1 oligomerization (Zhang et al., 2017), although the associated mechanism involved in mediating RIPK1 spontaneous phosphorylation in the early signaling stage without ROS accumulation remains unclear. Because RIPK1 can form homo-interactions via the RHIM domain and is highly expressed in a necrototic cellular model (Li et al., 2012; Orozco et al., 2014; Wu et al., 2014), it is possible that RIPK1 forms a dimer or oligomer through the RHIM domain before TNF treatment. Therefore, RIPK1 spontaneous phosphorylation may be mediated by RHIM domain-dependent RIPK1 oligomerization. Because a high level of RIPK1 protein guarantees that RIPK1 will have sufficient opportunities for oligomerization and subsequent spontaneous phosphorylation, the downregulation of RIPK1 would decrease its spontaneous phosphorylation. Therefore, based on the critical role of FKBP12 on RIPK1 expression, it is reasonable that FKBP12 promotes RIPK1 spontaneous phosphorylation by facilitating RIPK1 expression. In addition, our speculated mechanism for FKBP12 in mediating RIPK1 spontaneous phosphorylation might also explain the role for FKBP12 in promoting RIPK3 spontaneous phosphorylation. Collectively, FKBP12 may mediate necroosome formation and subsequent RIPK1–RIPK3–MLKL signaling pathway activation in response to TNFR1 ligation by promoting RIPK1 and RIPK3 expression and subsequent spontaneous phosphorylation.

Reports have indicated that TNF-induced SIRS is sensitized by Z-VAD but inhibited by RIPK1 kinase suppression or Ripk3 gene ablation; therefore, necroptosis contributes to the mortality of TNF-induced SIRS (Duprez et al., 2011; Chen et al., 2015). Here, by using both genetic and drug-based approaches, we revealed a decisive role for FKBP12 in determining the mortality of TNF-induced SIRS, confirming the in vivo function of FKBP12 in mediating necroptosis. Consistent with previous reports, we found that TNF-induced tissue injury occurred in the caecum of the negative control mice, whereas FKBP12 knockout significantly attenuated caecum damage and suppressed RIPK1 and RIPK3 phosphorylation in caecum lysates after TNF injection; therefore, FKBP12-mediated necroptosis in the caecum contributes to the pathology of TNF-induced SIRS. In addition, no significant focal necrosis was detected in the liver tissue of the negative control and FKBP12-knockdown mice after TNF administration. These findings challenge an earlier view that liver damage, but not intestinal damage, contributes to TNF-induced mortality because liver damage results from necroptosis, but intestinal damage is caused by apoptosis (Duprez et al., 2011). Reports have indicated that liver damage is usually induced by strong necrototic signals, including a high dose of TNF or TNF plus Z-VAD, and milder necrototic stimuli cannot cause prominent liver damage, although it is enough to trigger caecum injury and mortality. In addition, the caecum has been identified as the most sensitive organ to TNF-induced tissue injury (Tracey et al., 1986; Chen et al., 2015). Therefore, the apparent discrepancy may be explained by the difference in necrototic stimuli types and strengths used in the SIRS model.

In conclusion, FKBP12 was identified as a new target protein for necroptosis in this study, which initiates necroptosis induced by TNFR1 ligation by promoting necroosome formation and subsequent RIPK1–RIPK3–MLKL signaling pathway activation. Moreover, FKBP12 is essential for RIPK1 and RIPK3 expression and spontaneous phosphorylation, which are critical for the initial necroosome formation; therefore, FKBP12 might target RIPK1 and RIPK3 to initiate necroptotic signal transduction in response to TNFR1 ligation (Fig. 7E). In addition, based on the protective effect of FKBP12 genetic knockdown and kinase inhibition on the mortality of TNF-induced SIRS, FKBP12 was identified as a potential therapeutic target for the treatment of life-threatening conditions of SIRS, and its ligands could be a therapeutic option used in the clinical treatment of necroptosis-associated disease.

**MATERIALS AND METHODS**

**Cells and reagents**

L929 fibrosarcoma cells and HT22 cells were purchased from the Cell Culture Center, Beijing Institute of Basic Medical Science of the Chinese Academy of Medical Science (Beijing, China). 293TN cells were obtained from System Biosciences (SBI, Mountain View, CA). The cells...
were cultured in Dulbecco’s modified Eagle medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS; Kangyuan Biology, China). Necrostatin-1 (50 μM), Z-VAD-FMK (20 μM), FK506 (10 μM), rapamycin (10 μM), pimecrolimus (10 μM), everolimus (10 μM), zotarolimus (10 μM), ridaforolimus (10 μM) and SMAC mimetics (BV-6, 2 μM) were purchased from MedChem Express (Beijing, China). The inhibitors of mTOR, including GDC0349 (10 μM), XL388 (10 μM), AZD8055 (10 μM) and WYE125132 (2 μM), were purchased from TargetMol (Beijing, China). TNF (100 ng/ml) was obtained from R&D Systems Inc. Minneapolis, MN; ridaforolimus (10 μM), pimecrolimus (10 μM), rapamycin (10 μM), Z-VAD-FMK (20 μM), Smac mimetics (BV-6, 2 μM), were purchased from Sino Biological (Beijing, China) and directly transfected into BHK21 cells using the Chemifect transfection reagents (Fengrui Biology), and the protein A/G Plus Agarose (Santa Cruz Biotechnology) at 4°C for 2 h for pre-clearing. The pre-cleared cell lysate was subsequently incubated with the appropriate primary antibody overnight at 4°C. Immunoprecipitation was completed by adding protein A/G Plus Agarose (Santa Cruz Biotechnology) to the samples, incubating the samples for 2 h at 4°C, and washing the protein A/G Plus Agarose three times with lysis buffer. Finally, the immunoprecipitants were denatured by the addition of Laemmli sample buffer (Bio-Rad) and boiling for 10 min at 100°C before being subjected to western blot analysis as described above.

**Cell death analysis**

Cell death was assessed by microscopy (at a magnification of 200×) based on the presence of specific morphological changes. Three fields in each group were observed, and representative images are shown. Cell death was also quantified by means of flow cytometry by measuring the ratio of propidium iodide-positive cells to the total number of cells. Briefly, the cells were collected by trypsinization and stained with propidium iodide. Then, cell death was assessed by flow cytometry and CellQuest software (both FACS Calibur, BD). More than 10,000 cells were analyzed for each measurement. In addition, a Trypan Blue exclusion method was also used to determine cell death and survival. In brief, cells were collected by trypsinization, stained with Trypan Blue, loaded on a hemocytometer and then examined immediately under a microscope at low magnification. The number of blue-stained and nonstained cells was counted, and cell viability was calculated using a specific formula.

**Electron microscopy**

Cells were fixed with 2.5% glutaraldehyde for at least 30 min, treated with 1.5% osmium tetroxide, dehydrated with acetone, and embedded in Durcupan resin. Small pieces were cut (thick section, 0.5–1 μm) from the epoxy cast making note of the side with the cells. Thin sections were poststained with lead citrate and examined using a TECNAI 10 electron microscope (Philips, Holland) at 80 kV.

**Western blotting**

For the western blotting experiments, the cells were lysed in Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) and the protein concentration in the lysate was quantified with a BCA protein assay kit (Pierce, Rockford, IL). Total protein (60 μg) was loaded in each lane, and then the proteins were separated by SDS-PAGE and electrically transferred to a polyvinylidene difluoride (PVDF) membrane (Sigma-Aldrich). After being blocked with 5% skim milk, the membrane was blotted with the appropriate primary antibodies for 12–16 h at 4°C and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (Zhongshan Biotechnology, Beijing, China) for 1–2 h at room temperature. The proteins were detected using the Tanon™ High-sig ECL Western Blot Substrate (Tanon Science & Technology, Shanghai, China), and digital images were obtained using a gel-imaging system (Tanon 5200, Shanghai, China). The following antibodies were used for the experiments: anti-FADD (1:1000, 610399), anti-FKBPI2 (1:1000, 610808) and anti-RIPK1 (1:3000, 610458) (BD Transduction Laboratories, San Jose, CA); anti-RIPK3 (1:3000, 2283, ProSci, San Diego, CA); anti-MLKL (1:1000, 21066-1-AP, 66675-1-lg, Proteintech, Rosemont, IL); anti-TRIF (1:2000, MAB425, R&D Systems Inc. Minneapolis, MN); anti-phospho-RIPK3 (1:1000, ab195117), anti-MLKL (1:1000, ab127868), anti-phospho-MLKL (1:1000, ab196436) (Abcam, Cambridge, MA); anti-RIPK3 (1:1000, sc-374639) (Santa Cruz Biotechnology, CA); anti-eIF1 (1:1000, 4952), anti-RIPK1 (1:3000, 3493), anti-RIPK3 (1:3000, 15828), anti-CYLD (1:1000, 8462), anti-Myc-tag (1:2000, 2276), anti-HA tag (1:2000, 3724), anti-Flag tag (1:2000, 14793), anti-FLIP (1:1000, 56343), anti-TRAF2 (1:1000, 4724), anti-α-actin (1:1000, 3010), anti-phospho-mTOR (1:1000, 5536), anti-phospho-4E-BP1 (1:1000, 2855), anti-phospho-p70 S6K (1:1000, 9206), anti-phospho-RIPK1 (1:1500, 31122), anti-phospho-RIPK3 (1:1000, 57220) (Cell Signaling Technology (CST), Beverly, MA); anti-TRADD (1:1000, ABP52634), anti-β-tubulin (1:1500, ABM40030) and anti-GAPDH (1:1500, ABP52783) (Abbkine, Redlands, CA); anti-β-actin (1:3000, A5441) (Sigma-Aldrich). All western blot assays were performed three or more times, and the representative results are shown.

**Immunoprecipitation**

For the immunoprecipitation experiments, the cells were lysed in NP40 buffer containing 1% NP40, 50 mM Tris-HCl pH 8.0, 150 mM NaCl and complete protease inhibitor (Topsunirse, Beijing, China) at 4°C for 30 min. The cell lysates were subsequently centrifuged at 10,000 g for 10 min to remove cellular debris, and the protein concentration in the lysate was quantified with a BCA protein assay kit (Pierce). Approximately 500 μl of total protein in the lysate was incubated with an isotype IgG control antibody (2 μg/sample, Zhongshan Biotechnology) and protein A/G Plus Agarose (Santa Cruz Biotechnology) at 4°C for 2 h for pre-clearing. The pre-cleared cell lysate was subsequently incubated with the appropriate primary antibody overnight at 4°C. Immunoprecipitation was completed by adding protein A/G Plus Agarose (Santa Cruz Biotechnology) to the samples, incubating the samples for 2 h at 4°C, and washing the protein A/G Plus Agarose three times with lysis buffer. Finally, the immunoprecipitants were denatured by the addition of Laemmli sample buffer (Bio-Rad) and boiling for 10 min at 100°C before being subjected to western blot analysis as described above.

**Gene repression experiment**

For the gene repression experiment, the genes were downregulated via lentivirus transfection with specific shRNAs. The lentiviral vector pLKO.1-TRC was used to construct the shRNA vectors. The DNA fragments encoding shRNAs targeting specific genes or a nonspecific gene (Con shRNA) were synthesized by Geneiz (Beijing, China) and inserted into the Age I and EcoR I site of the pLKO.1-TRC vector and verified by DNA sequencing. The constructed lenti-shRNA vectors were subsequently cotransfected into 293TN cells (SBI) with the indicated second-generation packaging systems (psPAX2, #12260, and pMD2.G, #12259, vectors; Addgene) using Chemifect transfection reagents (Fengrui Biotechnology, Beijing, China). The lentivirus-containing supernatant was harvested after 48–72 h of transfection and filtered through a 0.45 μm filter. Transduction was performed in the presence of 10 μg/ml polybrene for 48–72 h, and gene knockdown efficiency was verified by western blotting. DNA sequences targeting the following specific genes were inserted into the lenti-shRNA vectors: mouse FKBP12, 5'-TGCTTTGAAGATGGAAAGAAT-3' and negative control, 5'-TTCCTCCGACGTGCACGT-3'.

**Ecotropic expression of FKBP12**

FKBP12 cDNA with HA tag-encoding sequence was inserted into the lentivirus vector pCDH-CMV-MCS-EF1-Puro (SBI) and verified by DNA sequencing. The newly constructed vector was co-transfected into 293TN cells (SBI) with the indicated second-generation packaging systems (psPAX2 and pMD2.G) using the Chemifect transfection reagent (Fengrui biotechnology). The lentivirus-containing supernatant was harvested after 48–72 h of transfection and filtered through a 0.45 μm filter. Transduction was performed in the presence of 10 μg/ml polybrene for 48–72 h, and the level of FKBP12 protein was verified by western blotting. In addition, the pCMV3-Myc-FKBPI2 plasmid was purchased from Sino Biological (Beijing, China) and directly transfected into BHK21 cells using the Chemifect transfection reagents (Fengrui Biotechnology), and the protein levels of FKBP12 and HA tag were determined by western blotting.

**Duolink proximity ligation assay**

The formation of the RIPK1–RIPK3 heterodimer and the RIPK1 or RIPK3 homodimers was determined by performing a Duolink in situ proximity ligation assay (PLA) kit (Sigma-Aldrich) according to the manufacturer’s instructions. Cells were planted on glass-bottom cell culture dishes, treated with TNF at the indicated time points, and then washed twice with PBS. Cells were fixed with 4% paraformaldehyde in PBS for 30 min and permeabilized by the addition of 0.2% Triton X-100 for 15 min at room temperature. The cells were then incubated with Duolink blocking solution in a preheated humidity chamber for 30 min at 37°C followed by incubation with primary antibodies, all used at 1:80, including mouse anti-RIPK1 (BD, 610458) and rabbit anti-RIPK3 (CST, 15828) (for detecting the
RIPK1–RIPK3 heterodimer), mouse anti-RIPK1 (BD, 610458) and rabbit anti-RIPK1 (CST, 3493) (for detecting RIPK1 homodimer), or mouse anti-RIPK3 (Santa Cruz Biotechnology, sc-374639) and rabbit anti-RIPK3 CST, 15828) (for detecting RIPK3 homodimer) antibodies, overnight at 4°C. After washing twice in Duolink washing buffer A for 5 min, the cells were incubated with secondary antibodies conjugated to oligonucleotides (PLA probes anti-rabbit plus and anti-mouse minus) for 1 h in a preheated humidity chamber at 37°C. Unbound PLA probes were removed by washing twice in Duolink washing buffer A for 5 min, and then the Duolink ligation solution was applied to the slides for 30 min in a preheated humidity chamber at 37°C followed by washing in Duolink washing buffer A twice for 2 min. The Duolink amplification-polymerase solution was applied to the slides in a dark preheated humidity chamber for 100 min at 37°C. The slides were then washed twice in 1× Duolink washing buffer B for 10 min. The cells were then mounted using Duolink in situ mounting medium with DAPI, and observed with an LSM 510 confocal microscope (Carl Zeiss). PLA signals were recognized as green fluorescent spots and analyzed using the Duolink Image Tool software (Sigma-Aldrich); results were normalized for each cell by calculating the average level of green fluorescent spots in each cell in the image. Independent experiments were performed at least three times, and the representative results are shown.

**Real-time quantitative PCR**

Total RNA was isolated from FKBP12 shRNA and Con shRNA L929 cells using the Total RNA Kit II (#R6834-01) purchased from OMEGA Bio-Tek (Norcross, GA, USA) and reverse transcribed into cDNA with random hexamers using the PrimeScript RT Reagent Kit (TAKARA, Dalian, China) according to the manufacturer’s instructions. Real-time quantitative PCR (qPCR) was carried out using a CFX36 Touch™ Real-Time PCR System (Bio-Rad), and the amplifications were performed using the KOD SYBR® qPCR Mix (#QKD-201, TOYOBO, Beijing, China). Reactions were performed in triplicate in three independent experiments. Expression data were normalized to the geometric mean of the values obtained from the β-actin housekeeping gene keeping the variation in expression levels and were analyzed using the 2−ΔΔCT method. The primer sequences used in the experiments are the following: mouse RIPK1, forward, 5′-GAAGACAGACCTTACAGACACGG-3′ and reverse, 5′-CAGTAGCTTCACCACTCGAC-3′; mouse RIPK3, forward, 5′-CAGTGGGACCTCTGTCCG-3′ and reverse, 5′-CAAGCTGTGTAGGTAGCACATC-3′; mouse FKBP12, forward, 5′-AGACCATCTCCTCGGAGAGC-3′ and reverse, 5′-GTGGCTCCATAGGATGCTG-3′; and mouse actin, forward, 5′-CATCCTTGGCTGAAGTCC-3′ and reverse, 5′-ATCATTTTCAAGACCCTACAAC-3′.

**TFN-induced SIRS mouse model**

C57BL/6 mice were purchased from Vital River Laboratories (Beijing, China). The care and use of laboratory animals were in strict adherence with the guidelines from ‘Laboratory animal Guideline for ethical review of animal welfare (GB/T 35892-2018), General Administration of Quality Supervision, Inspection and Quarantine of the People’s Republic of China’. Six-to-eight-week-old female mice (average weight of ~20 g) were injected with 100 μg/kg TNF (Genscript, Nanjing, China) diluted in endotoxin-free PBS via the tail vein. Mice in the negative control group were injected with PBS. The body temperature of the mice was determined every 2 h for the first 24 h via an animal thermometer (Haichuang Gaoke Technology, Beijing, China). Mice that were moribund or had a body temperature below 23°C were killed.

**FKBP12-knockdown mouse model**

FKBP12 shRNA lentivirus and the negative control Con shRNA lentivirus in the cell culture medium were concentrated to 10^11 plaque-forming units (pfu) through ultrafiltration with the Macrosep Advance centrifugal device (PALL, Beijing, China). The C57BL/6 mice were injected with the concentrated lentivirus (100 μl/mouse) three times at the interval of 3 days via the tail vein. At 2 weeks after the first injection, the mice were killed to isolate various tissues. Proteins were extracted from isolated mouse colon tissues by using the Tissue or Cell Total Protein Extraction Kit (#CS10003, Sangon Biotech, Shanghai, China) according to the manufacturer’s instructions. Briefly, 10–50 mg colon tissues were weighed and cut into small pieces in the 1.5 ml micro-centrifuge tubes, and then 0.2 ml tissue lysis buffer (containing protease inhibitors) were added into the tube. The tissues were homogenized by using a mini pestle-homogenizer, and the supernatant was transferred to a new 1.5 ml tube after centrifugation. The amount of extracted proteins in the supernatant were quantified by using the Bradford method and then western blotting was used to determine the FKBP12 protein level.

**Histopathology**

Tissues were immediately collected from euthanized mice and fixed in 10% neutral buffered formalin for 48 h. The fixed tissues were dehydrated in ethanol, cleared in xylene, and embedded in paraffin blocks. Sections (of 5 μm) were cut and mounted on adhesion microscope slides and then stained with hematoxylin and eosin (H&E, #G1120, Solarbio Biotechnology, Beijing, China) for analyses. Three H&E-stained tissue sections were detected for each caecum or liver tissue sample, and representative images were captured using identical settings on a Leica DM2500 optical microscope.

**Statistical analysis**

GraphPad prism 7 software was used to analyze the data and construct graphs. Statistical significance was analyzed by performing a two-tailed Student’s t-test or one-way ANOVA test and defined as *P*<0.01. All experiments were repeated at least three times, and the data are expressed as the mean±s.d. from representative experiments. The normality of the data used in the ANOVA was checked via the Shapiro–Wilk test using SPSS software; the results indicated that no significant differences were found among data in the same group (*P*>0.05). Therefore, the data used in the ANOVA test were normally distributed. In addition, the equality of the data used in the ANOVA test was checked with the Levene’s test for equality of variance (Homogeneity-of-Variance) using SPSS software. The results indicated that significant differences were not observed in the data and the data could be used for the ANOVA test (*P*>0.05).

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

Methodology: J.Y., G.C.; Software: G.C.; Validation: G.C.; Formal analysis: J.F.; Investigation: Z.W.; Data curation: Z.W.; Writing - original draft: G.C.; Writing - review & editing: G.C.; Supervision: J.F.; Project administration: J.Y., G.C.; Funding acquisition: G.C.

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**Supplementary information**

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