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RIP3, a kinase promoting necroptotic cell death, mediates adverse remodelling after myocardial infarction

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Aims Programmed necrosis (necroptosis) represents a newly identified mechanism of cell death combining features of both apoptosis and necrosis. Like apoptosis, necroptosis is tightly regulated by distinct signalling pathways. A key regulatory role in programmed necrosis has been attributed to interactions of the receptor-interacting protein kinases, RIP1 and RIP3. However, the specific functional role of RIP3-dependent signalling and necroptosis in the heart is unknown. The aims of this study were thus to assess the significance of necroptosis and RIP3 in the context of myocardial ischaemia.

Methods and results Immunoblots revealed strong expression of RIP3 in murine hearts, indicating potential functional significance of this protein in the myocardium. Consistent with a role in promoting necroptosis, adenoviral overexpression of RIP3 in neonatal rat cardiomyocytes and stimulation with TNF-α induced the formation of a complex of RIP1 and RIP3. Moreover, RIP3 overexpression was sufficient to induce necroptosis of cardiomyocytes. *In vivo*, cardiac expression of RIP3 was upregulated upon myocardial infarction (MI). Conversely, mice deficient for RIP3 ($RIP3^{-/-}$) showed a significantly better ejection fraction (45 ± 3.6 vs. $32 \pm 4.4\%$, $P < 0.05$) and less hypertrophy in magnetic resonance imaging studies 30 days after experimental infarction due to left anterior descending coronary artery ligation. This was accompanied by a diminished inflammatory response of infarcted hearts and decreased generation of reactive oxygen species.

Conclusion Here, we show that RIP3-dependent necroptosis modulates post-ischaemic adverse remodelling in a mouse model of MI. This novel signalling pathway may thus be an attractive target for future therapies that aim to limit the adverse consequences of myocardial ischaemia.

Keywords Programmed necrosis • Receptor interacting protein 3 • Myocardial infarction • Remodelling • Inflammation

1. Introduction

Acute myocardial infarction is one of the leading causes of death worldwide.[1] Despite considerable progress, contemporary therapy can only partially address the central problem that leads from acute ischaemia and infarction to chronic heart failure: a major loss of cardiomyocytes with subsequent remodelling and contractile dysfunction.

Apoptosis, which is mediated by death receptors like the TNF receptor[2] or fas receptor/CD95,[3] has been considered a possible target for novel therapies,[4] as this process is tightly regulated by specific signalling

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pathways and could thus potentially be inhibited. For example, TNF-α has been shown to be up-regulated during myocardial infarction (MI) and to enhance apoptosis in this clinical condition.[5] However, the overall rate of apoptotic cells in the infarcted region was <1% in this study and recent theories question a significant role of apoptosis in post-ischaemic remodelling.[6,7] Recently, a novel mechanism called 'programmed necrosis' or necroptosis has been suggested as another important mediator of cell death in the heart.[6] Similar to apoptotic cell death, this process is tightly regulated by distinct molecules, but leads to the typical morphological signs of necrosis such as defects of membrane integrity and inflammation, thus combining features of both mechanisms.[6,8–11] The signalling pathways that activate programmed necrosis are not fully understood. However, *in vitro* studies could demonstrate that TNF-α-dependent formation of a complex between receptor-interacting protein 1 (RIP1) and another kinase, receptor-interacting protein 3 (RIP3) is an essential step for inducing programmed necrosis.[8,9,12] In this process, RIP3 appears to play a key role, controlling the phosphorylation of RIP1, a necessary step in programmed necrosis.[8,12]

An important role of RIP3-mediated necroptosis has been demonstrated during viral infection of the liver and in tissue damage due to inflammatory bowel disease.[13] In the heart, inhibition of RIP1 by the small molecule necrostatin-1 leads to a reduction of infarct size, implying functional importance of necroptosis also in myocardial ischaemia.[14–16] In contrast, the functional significance of RIP3 in the heart is still unknown. Here, we demonstrate that RIP3 is expressed in the heart and RIP3 is activated during MI. Moreover, mice lacking RIP3 reveal improved cardiac performance, accompanied by a decreased inflammatory response and generation of reactive oxygen species (ROS).

2. Methods

2.1 *In vitro* experiments

For the preparation of neonatal rat ventricular cardiomyocytes (NRVCMs), 1- to 2-day-old Wistar rats were sacrificed by decapitation and hearts were immediately removed for digestion with collagenase II (Worthington) at 37°C, as previously described.[17] Cells were infected with the indicated multiplicity of infection under serum-free conditions 24 h after plating and subsequently cultured supplemented with 2% fetal calf serum for 48 h. Afterwards, cells were analysed by flow cytometric analysis, immunoblots, MTT cell viability assays, real-time PCR, immunohistology, or electron microscopy (see Supplementary material online for detailed description of applied methods).

2.2 *In vivo* studies

RIP3-deficient ($RIP3^{-/-}$) mice,[18] kindly provided by Genentech, Inc., South San Francisco, CA, USA, and littermate WT mice (C57/BL6N-background) were studied at 10–12 weeks of age. Mice were subjected to permanent left anterior descending coronary artery (LAD) ligation and subsequently assessed for left ventricular (LV) morphology and function. Furthermore, transgenic and WT mouse hearts were analysed by (immuno-)histology and electron microscopy as well as for the content of ROS. For LAD ligation as well as heart removal, mice received 0.1 mg Temgesic/kg body weight by subcutaneous injection for analgesia and were anaesthetized by inhalation of isoflurane (3–5%). For postoperative analgesia, 0.1 mg Temgesic/kg body weight was applied once daily for 5 days post-surgery. All studies were approved by the animal ethics committee of Schleswig-Holstein, Germany. Magnetic resonance imaging (MRI) experiments were performed as described elsewhere.[19] Detailed methods are available in Supplementary material online.

2.3 Statistical analysis

All results were expressed as mean ± SEM. We performed statistical analysis using one- or two-way ANOVA followed by Student–Newman–Keuls *post hoc* tests or *t*-tests as appropriate. A *P*-value of <0.05 was considered statistically significant.

3. Results

3.1 RIP3 is expressed in cardiomyocytes and co-localizes with mitochondria

In order to unravel a potential function of RIP3 in the myocardium, we first assessed its expression in the heart. While cardiac expression of RIP3 (55 kDa) was lower compared with spleen and lung, it was significantly higher than in brain and kidney (*Figure 1A* and *B*). Of note, the shifted bands above from kDa in liver and kidney might be due to post-translational modification of RIP3. RIP3 was also expressed in isolated NRVCMs (*Figure 1C*). Since it has been suggested that expression of RIP3 correlates with the functional relevance of programmed necrosis in certain cell types,[9] we hypothesized that RIP3 may also play a significant role in the heart. Adenoviral overexpression of RIP3 led to a shift in its molecular weight, indicating activation of RIP3.[20] As a negative control, this shifted band could be inhibited by RIP3-specific synthetic miRNA (*Figure 1C*). As a positive control, we stimulated L929 mouse fibroblasts that are particularly prone to programmed necrosis,[9,20] with TNF-α and Z-Val-Ala-Asp-FMK (zVAD) caspase inhibitor. After 3 h of stimulation, immunoblots from these cells revealed the shifted band at the same molecular weight as activated RIP3 in NRVCMs (*Figure 1D*), suggesting that the molecular weight shift of RIP3 in NRVCMs is indeed due to activation of RIP3. Analyses of NRVCMs by confocal laser scanning immunohistochemistry confirmed endogenous RIP3 expression in NRVCMs and showed perinuclear clustering of RIP3 (*Figure 1D*). In this regard, it has recently been reported that mitochondria reveal perinuclear clustering during programmed cell death.[21] Interestingly, co-staining of NRVCMs with a RIP3 antibody and 'Mitotracker' revealed close co-localization of RIP3 and mitochondria in these cells (*Figure 1E–G*). AdV RIP3 induced increased phosphorylation activity in NRVCMs (*Figure 2A*). In contrast, overexpression of a kinase-dead RIP3 mutant did not induce increased phosphorylation of RIP3, demonstrating that adenoviral overexpression of RIP3 promotes kinase activity of RIP3. As an additional indication of RIP3 activation, immunoblots revealed increased polyubiquitination by Lys63 (K63) residue linkage at the level of activated RIP3 (+151.9% vs. lacZ ± 24.9%, *Figure 2B* and *C*).

3.2 Overexpression of RIP3 induces the formation of RIP1/RIP3 complex and necrosis of cardiomyocytes

Cell culture experiments in human cell lines showed that association of RIP1 and RIP3 in response to TNF-α stimulation represents the crucial initial step in programmed necrosis.[8,12] To test whether TNF-dependent formation of RIP1/RIP3 complexes is also present in cardiomyocytes, we infected NRVCMs with an adenoviral vector encoding for rat RIP3 (NCBI Reference Sequence: NM_139342.1) or GFP (green fluorescent protein) as control. Forty-eight hours after infection, NRVCMs were pretreated with a 50 μM zVAD-fmk caspase inhibitor, since caspases have been shown to inhibit RIP3–RIP1 complex formation *in vitro*,[12] and stimulated with 100 ng/mL of recombinant TNF-α for different

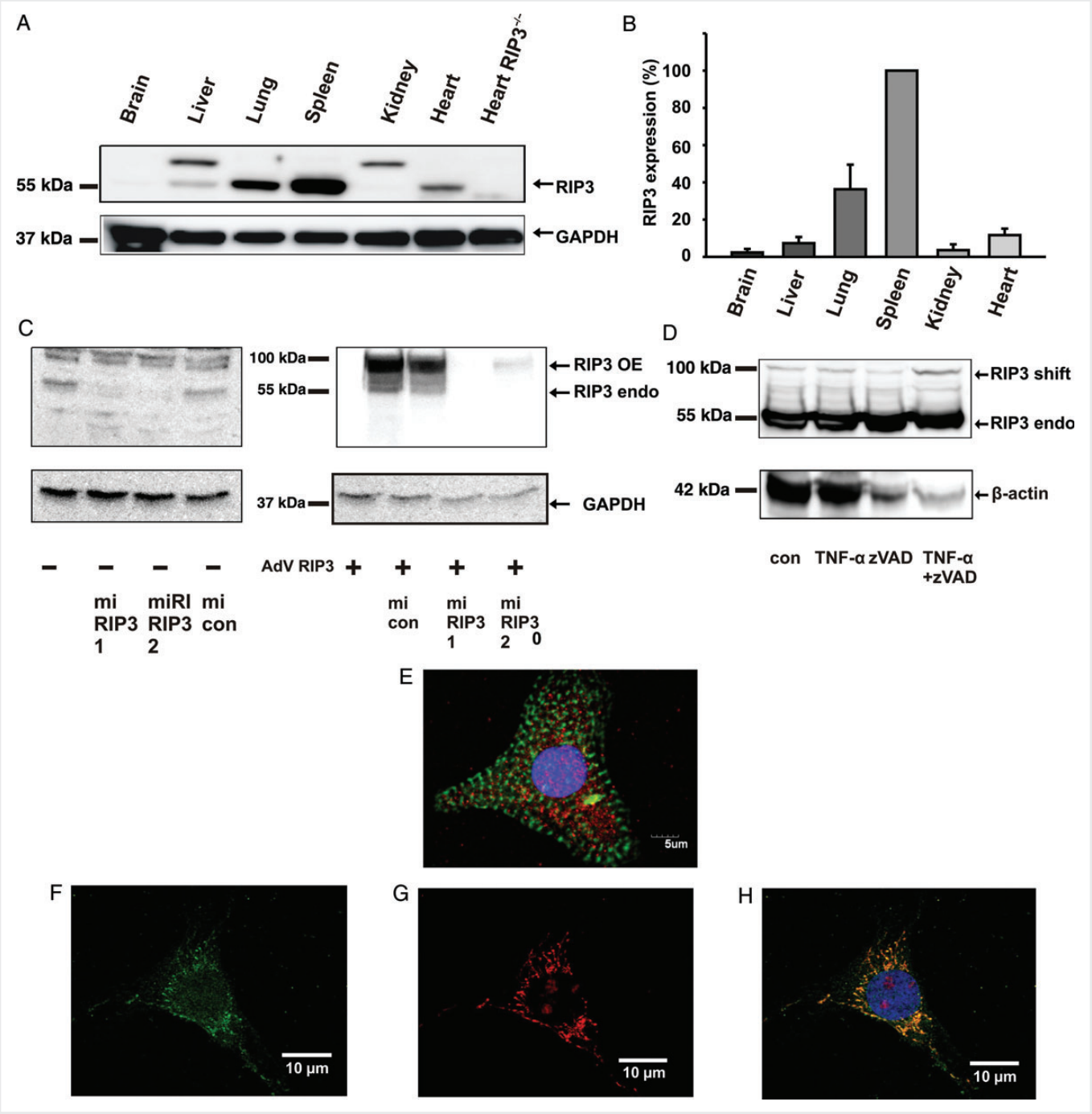

Figure 1 RIP3 is expressed in cardiomyocytes. (*A*) Immunoblot revealing RIP3 expression in the mouse heart. GAPDH was used as loading control. RIP3 expression in the heart is lower compared with lung and spleen, higher than in brain and kidney ($n = 3$, *B*). (*C*) Immunoblot showing that RIP3 is expressed in NRVCMs. Adenoviral overexpression of RIP3 induced a shift of RIP3 in molecular weight (RIP3 OE). Both endogenous and weight-shifted RIP3 expression could be inhibited by two miRNA viruses: miRIP3-1 and miRIP3-2, but not by overexpression with miRNA control virus (micon), demonstrating the specificity of the shifted RIP3-bar. (*D*) As a positive control, stimulation of L929 mouse fibroblast with TNF-α and zVAD induced a shift in the molecular weight of RIP3. (*E*) Confocal laser immunocytology: co-staining of RIP3 (stained red) and α-actinin in an NRVCM. In NRVCMs, RIP3 showed a perinuclear clustering (*F*, stained green), similar to mitochondria (*G*, stained red). (*H*) Co-staining of mitochondria and RIP3. $n = 3$ independent experiments. Con: unstimulated control; AdV RIP3: RIP3 adenovirus.

time intervals. Immunoprecipitation with a RIP1 antibody followed by immunoblotting with a RIP3 antibody revealed rapid formation of RIP1/RIP3 complexes as early as 30 min after TNF stimulation with a maximum at 60 min (*Figure 3A*). As a sign of increased turnover of RIP1/RIP3 complexes in RIP3 overexpressing NRVCMs, RIP1 content was significantly decreased in these cells ($-87.6 \pm 2.7\%$, $P < 0.001$, $n = 3$; *Figure 3B* and *C*). Next, we assessed the consequences of RIP3 activation on cardiomyocyte survival. Of note, adenoviral overexpression

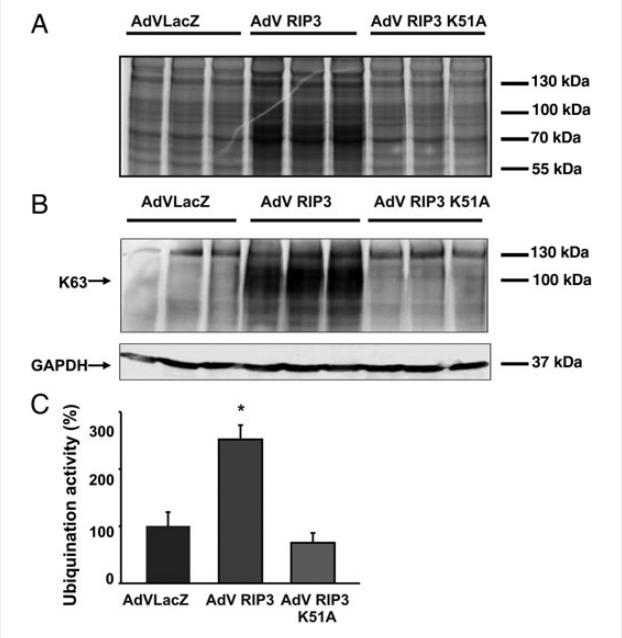

Figure 2 Increased phosphorylation and ubiquitination activity of RIP3 in neonatal rat cardiomyocytes. (*A*) Semi-quantitative phosphogel staining showing increased phosphorylating activity in cells overexpressing RIP3. Overexpression of a kinase-dead RIP3 mutant (AdRIP3 A51 K) led to no increase of phosphorylation activity ($n = 3$). (*B*) Immunoblot revealing polyubiquitin chains formed by Lys63 (K63) residue linkage at the level of activated RIP3 in NRVCMs that were infected with a RIP3 adenovirus vs. control cells that were infected with a LacZ virus (AdVLacZ), demonstrating increased ubiqitination activity of activated RIP3 ($+151.9\%$ vs. lacZ $\pm 24.9\%, n = 3, C$). NRVCMs overexpressing a kinase-dead RIP3 mutant (AdV RIP3 K51A)) served as negative control, and immunoblot against GAPDH served as loading control. $^{*}P < 0.01$.

of RIP3 significantly increased the amount of propidium iodide (PI)-positive cells (68.9 ± 1.2 vs. $26.8 \pm 1.3\%$ in unstimulated control cells, $P < 0.01$, *Figure 3D* and *E*), as measured by FACS analysis. Notably, overexpression of a kinase-inactive RIP3 mutant (RIP3 K51A) did not alter the proportion of PI-positive cells, demonstrating that kinase activity of RIP3 is required for the induction of cell death of NRVCMs (see Supplementary material online, *Figure S1*). Combined 7AAD/annexinV staining revealed no difference in apoptotic cell rates between both groups (data not shown). Simultaneous TNF-α stimulation and caspase inhibition by zVAD-FMK slightly decreased the RIP3-dependent cell death rate (*Figure 3E*). However, neither TNF-α stimulation nor caspase inhibition alone affected RIP3-dependent cell death rate (see Supplementary material online, *Figure S2*), confirming that RIP3-induced cell death is not executed via the 'canonical' apoptotic caspase-dependent pathway. In line with the FACS analyses, adenoviral overexpression of RIP3 markedly reduced survival of NRVCMs ($-57 \pm 0.83\%$ vs. control, $P < 0.01$, *Figure 3F*), as measured by another cell viability assay (MTT assay) and significantly increased cell lysis, as measured by augmented troponin T release into the cell culture media (2143 ± 337 pg/mL vs. 801 ± 89 pg/mL, $P < 0.01$, *Figure 3G*). In contrast, adenoviral miRNA-based knockdown of RIP3 did not alter the basal cell death rate of cultured cardiomyocytes (data not shown). This effect might favour the concept that RIP3-dependent effects are confined to stress conditions and are dispensable under basic conditions. Of note, miRNA-based knockdown of RIP1 (see Supplementary material online, *Figure S2C*) only weakly inhibited RIP3-dependent cell death (see Supplementary material online, *Figure S2D*), indicating that RIP1 activity is not required for RIP3-mediated cell death of cardiomyocytes. In conclusion, we demonstrate that RIP3 is sufficient for induction of programmed necrosis in cardiomyocytes.

3.3 RIP3 expression is induced in myocardial infarction

To probe for a potential pathophysiological role of RIP3 *in vivo*, we examined activation of RIP3-dependent signalling in a mouse model of MI. Interestingly, 24 h after permanent ligation of the LAD, significantly up-regulated RIP3 protein levels were detected in ischaemic mouse hearts ($+70.69 \pm 14.36\%, P < 0.05$, *Figure 4A* and *B*), consistent with activation of RIP3-dependent signalling pathways in response to myocardial ischaemia. Histological analyses revealed up-regulation of RIP3 especially in the peri-infarct zone of the LV (*Figure 3E*), implying functional relevance of RIP3 in myocardial infarction.

3.4 RIP3 mediates inflammation and ROS generation after myocardial infarction

To further examine the functional relevance of RIP3 in MI, we subjected $RIP3^{-/-}$ mice[18] to permanent LAD ligation ($n = 22$). This intervention did not result in differences with regard to the survival rate compared with WT controls (see Supplementary material online, *Figure S3A*). Next, we evaluated the effects of RIP3 deficiency on biochemical, histological, and ultrastructural levels. Haematoxylin–eosin stainings of WT and $RIP3^{-/-}$ hearts after MI are shown in *Figure 5A*. To examine the role of apoptosis in RIP3-dependent remodelling, we measured caspase 3 cleavage in WT and $RIP3^{-/-}$ hearts 24 h after LAD ligation. By immunohistology and western blot, we did not detect alterations of caspase 3 cleavage in both $RIP3^{-/-}$ hearts and WT controls (*Figure 5B–D*). Furthermore, stainings for Cd31 revealed a comparable decrease of endothelial cells in the infarcted areas in both genotypes (*Figure 5E*). Likewise, electron microscopy of both WT and $RIP3^{-/-}$ hearts revealed no obvious morphological differences between both groups (*Figure 5F*). In contrast, 4 days after infarction we observed a marked attenuation of inflammatory cell invasion into infarcted regions in RIP3-deficient hearts (*Figure 5B*). Inflammation is a major consequence of myocardial ischaemia[19] and —in contrast to apoptotic cell death—a pivotal concomitant effect of programmed necrosis *in vivo*.[9,12] We therefore systematically assessed whether the degree of inflammation was decreased in $RIP3^{-/-}$ hearts after LAD ligation. Indeed, CD3 staining and quantification of T-cell invasion revealed a significantly lower number of inflammatory cells in $RIP3^{-/-}$ hearts (*Figure 6A* and *B*). The decreased inflammatory response of $RIP3^{-/-}$ hearts was accompanied by an unaltered systemic level of leucocytes and T cells in $RIP3^{-/-}$ mice compared with controls. Furthermore, we could not detect any morphological alterations of livers, lungs, kidneys, and spleens of RIP3 and WT mice 4 days after LAD ligation (see Supplementary material online, *Figure S4*). In addition to inflammation, generation of ROS has been presumed to be another key mediator of RIP3-induced organ damage.[6,10] We therefore examined $RIP3^{-/-}$ hearts and WT controls for ROS content 24 h after LAD ligation. $RIP3^{-/-}$ hearts revealed significantly decreased ROS generation vs. WT hearts (2.31 ± 0.58 vs. 5.32 ± 1.18, $P < 0.05$, *Figure 6C*). In a reverse approach, we also assessed cultivated NRVCMs overexpressing RIP3 for ROS contents by FACS analysis.

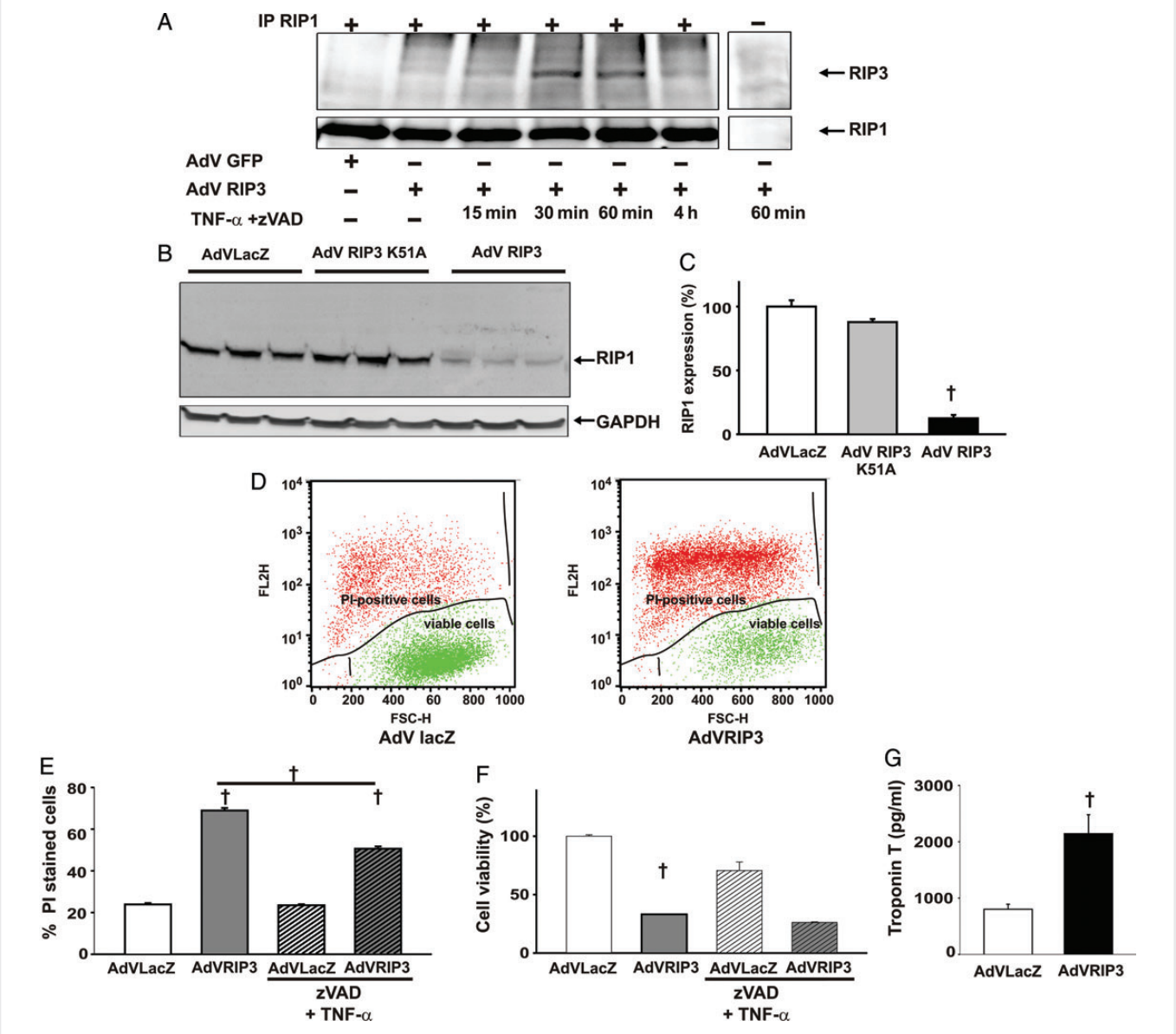

Figure 3 RIP3 induces programmed necrosis of cardiomyocytes. (*A*) Adenoviral overexpression of RIP3 in NRVCMs stimulates the formation of a RIP1/RIP3 complex, as shown by immunoprecipitation after stimulation with TNF-α and pre-incubation with the pan-caspase inhibitor zVAD-fmk. (*B*) Immunoblot revealing that adenoviral overexpression of RIP3 leads to a significant decline in RIP1 expression ($-87.6 \pm 2.7\%$, $P < 0.001$, $n = 3$, *C*). (*D*) PI staining and FACS analysis show significantly increased cell death of NRVCMs overexpressing RIP3. zVAD-fmk and TNF-α stimulation did not significantly alter the proportion of PI-positive cells (*E*), $n = 4$ independent experiments. (*F*) Treatment of NRVCMs with zVAD-fmk and TNF-α did not significantly alter cell viability, whereas overexpression of RIP3 in NRVCMs markedly decreased cardiomyocyte survival ($n = 4$). (*G*) Overexpression of RIP3 in NRVCMs lead to a significant increase of cell lysis, as measured by troponin T release into culture media ($n = 4$). Con: unstimulated control; AdV GFP, AdV LacZ: control adenovirus; AdV RIP3: RIP3 adenovirus; AdV RIP3 K51A: RIP3 virus expressing a kinase-inactive RIP3 mutant; PI: propidium iodide; FL2H: fluorescence signal (high); FSC-H: forward scatter; $^{*}P < 0.05$; $^{\dagger}P < 0.01$; $^{\ddagger}P < 0.001$.

Interestingly, these cells showed an increased ROS content ($+25 \pm 3.6\%$ vs. LacZ-expressing cells, *Figure 6D*), emphasizing the functional role of ROS generation as a potential primary effector of RIP3 in the heart. Taken together, ROS generation and inflammation could both function as mediators of RIP3-dependent myocardial damage due to ischaemia. To further assess the role of altered cardiac energetics in RIP3-dependent remodelling, we used ^{31}P magnetic resonance spectroscopy. We found a higher PCr/ATP ratio of RIP$^{-/-}$ mouse hearts vs. WT controls; however, the difference did not reach statistical significance (see Supplementary material online, *Figure S5*). Thus, likely additional mechanisms are also relevant in RIP3-dependent cardiac remodelling.

3.5 RIP3 mediates adverse remodelling after myocardial infarction

To examine whether RIP3 impairs cardiac remodelling after MI, we assessed cardiac morphology and function of RIP3$^{-/-}$ mice and WT controls under basal conditions, 24 h, and 30 days post-MI by cardiac

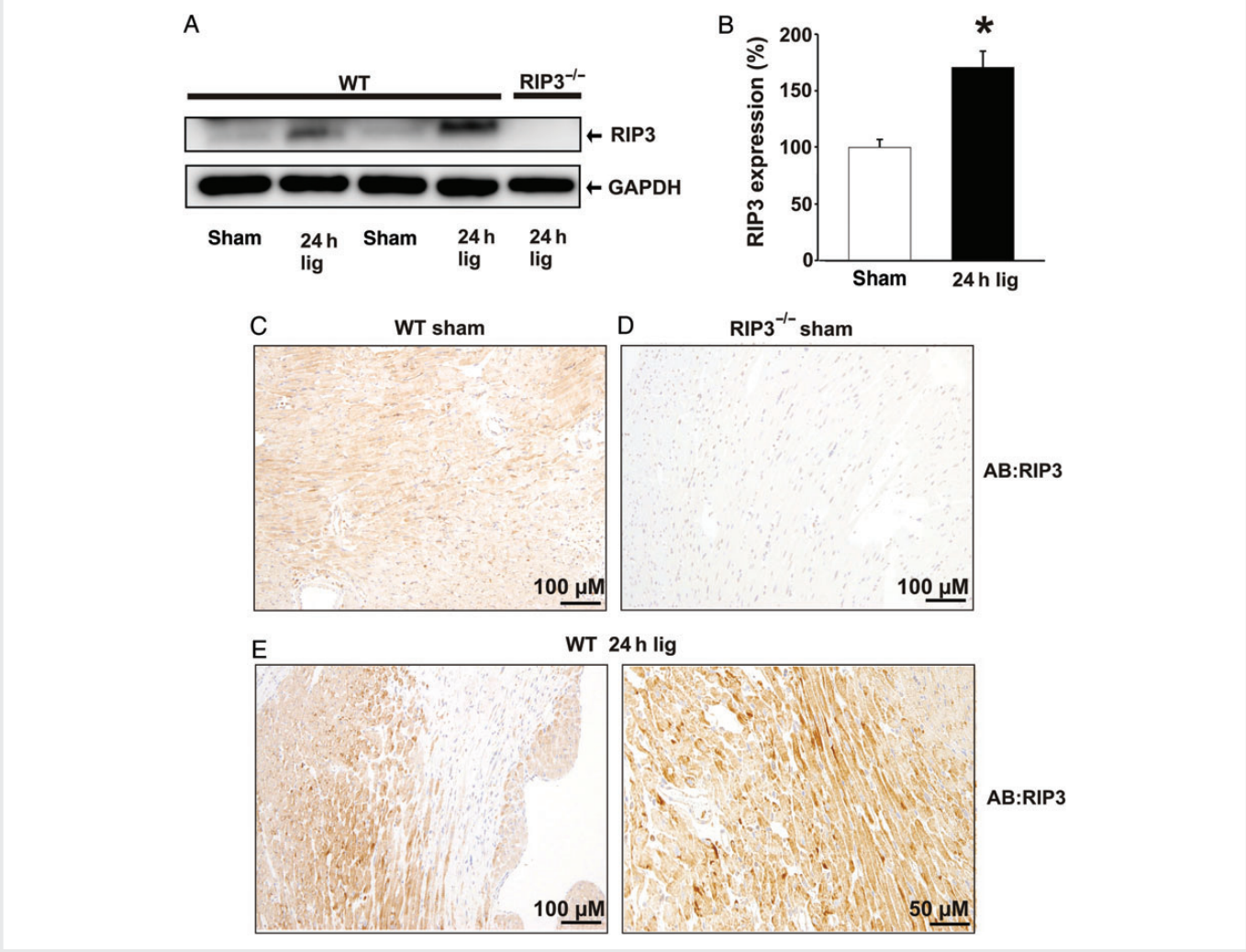

Figure 4 RIP3 is up-regulated in ischaemic hearts. (*A* and *B*) Immunoblot demonstrating significant up-regulation of RIP3 24 h after permanent ligation of the LAD in mouse hearts ($n = 4$). (*C*) Representative immunohistological staining of RIP3 in a sham-operated WT mouse heart. (*D*) As a negative control, RIP3$^{-/-}$ mouse hearts were not stained by the RIP3 antibody. (*E*) 24 h after LAD ligation, WT hearts revealed increased staining of RIP3 in the peri-infarct area ($n = 4$).

MRI (*Figure 7* and see Supplementary material online, *Movies*). Basic morphological and functional parameters were comparable between both groups (*Table 1*). Noteworthy, gadolinium-enhanced MRI scans of the left ventricle 24 h after LAD ligation and histology-based quantification of fibrotic areas 30 days after LAD ligation showed no significant differences in infarct size between both groups (*Figure 7B* and *D*, and see Supplementary material online, *Figure S6*).

However, 30 days after LAD ligation, RIP3$^{-/-}$ mice demonstrated a significantly better ejection fraction than WT controls ($45 \pm 3.6\%$, $n = 11$ vs. $32 \pm 4.4\%$, $n = 10$, $P < 0.05$; *Figure 7F*). Consistently, RIP3$^{-/-}$ hearts showed less hypertrophy than WT controls, as demonstrated by a decreased LV/body weight ratio (3.50 ± 0.13 mg/g, $n = 11$ vs. 4.20 ± 0.21 mg/g, $n = 10$, $P < 0.05$; *Figure 7G*). Moreover, real-time PCR experiments revealed lower levels of B-type natriuretic peptide (BNP) expression in RIP3-deficient hearts (*Figure 7E*) and serum troponin T levels were significantly lower in RIP3-deficient mice (see Supplementary material online, *Figure S3B*), also consistent with improved myocardial remodelling of these mice post-infarction. Taken together, these data indicate that RIP3 does not alter short-term infarct size, but promotes long-term adverse post-infarct remodelling *in vivo*.

4. Discussion

Programmed necrosis mediated by RIP3 has recently been identified as a novel mechanism of cell death with major functional importance in several organs, including liver,[12] pancreas,[9] and bowel.[13] We here show that RIP3 is also expressed in the heart, forms a complex with RIP1 in cardiomyocytes, and can be activated upon TNF stimulation. Nevertheless, cardiomyocytes appear to be less prone to necroptosis compared with, e.g. Jurkat cells,[12] since caspase inhibition and TNF-α stimulation alone were not sufficient to induce significant cell death. In contrast, forced overexpression of RIP3 is a potent stimulus for necroptosis of NRVCMs, in line with the hypothesis that protective effects may act upstream of RIP3. For example, inhibition of the RIP1/RIP3 complex by caspase 8 has recently been suggested as one possible mechanism protecting cells from necroptosis.[22,23] The need of RIP3 overexpression

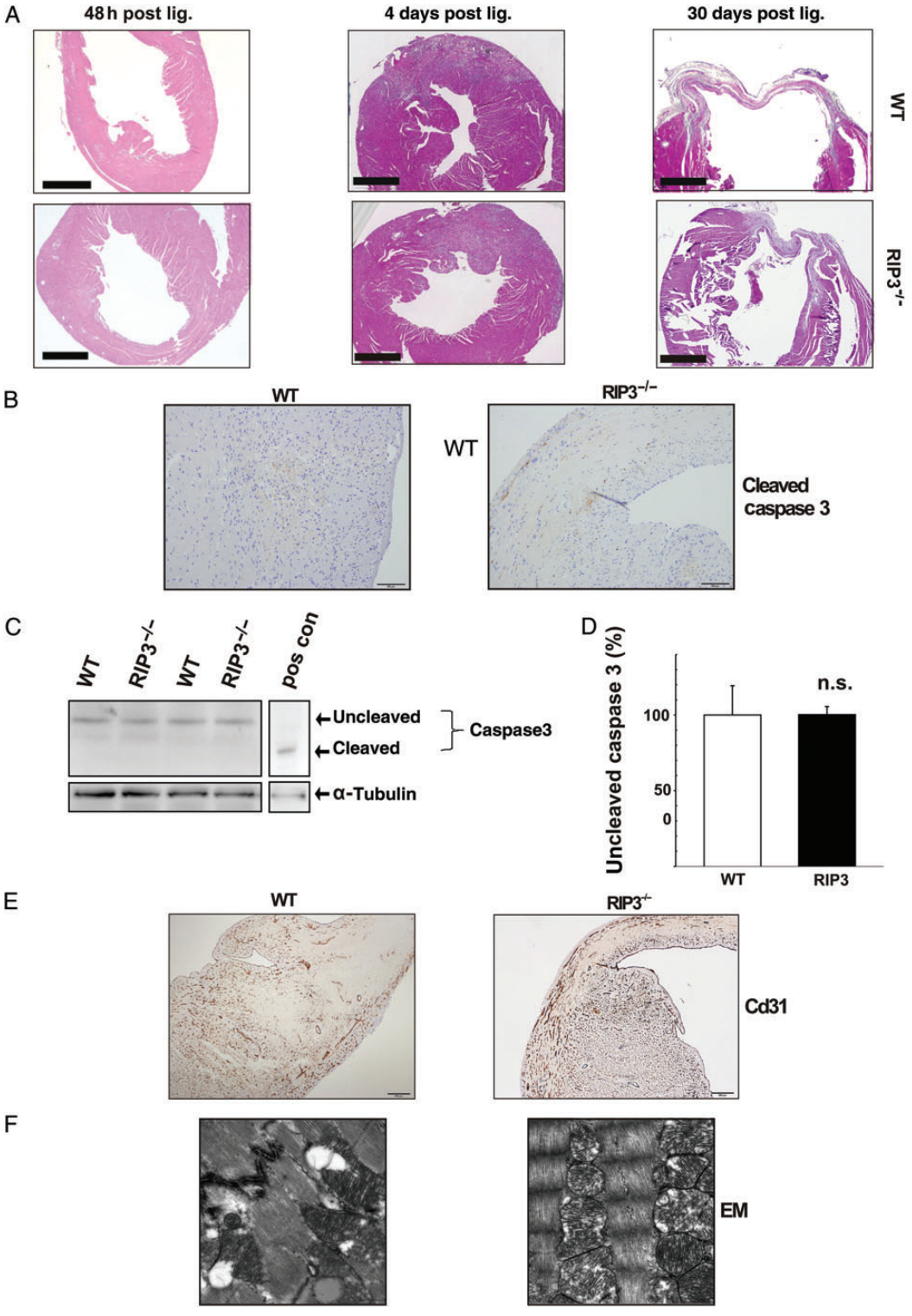

Figure 5 No altered apoptosis rate in infarcted RIP3$^{-/-}$ hearts. (*A*) Representative haematoxylin–eosin stainings of WT and RIP3$^{-/-}$ hearts 48 h, 4 days, and 30 days after LAD ligation. Scale bars denote 1000 μM. (*B*) Immunohistological stainings of both WT and RIP3$^{-/-}$ mouse hearts revealed discretely scattered cells that were positive for cleaved caspase 3. (*C*) Immunoblot of WT and RIP3$^{-/-}$ protein extracts 24 h post LAD ligation showing no significant difference in caspase 3 cleavage in both groups. Liver extract from LPS-treated TAK1 knockout mice 15 was used as positive control (pos con) for caspase 3 cleavage. The content of uncleaved caspase 3 was unaltered between both groups ($n = 4$) (*D*). (*E*) As a marker of angiogenesis, both WT and RIP3$^{-/-}$ hearts showed an attenuated staining for Cd31 in infarcted areas. (*F*) Representative electron microscopy imaging of border zones of infarcted areas of WT and RIP3$^{-/-}$ mouse hearts 24 h after LAD ligation, revealing amorphous matrix densities. n.s.: no statistically significant difference.

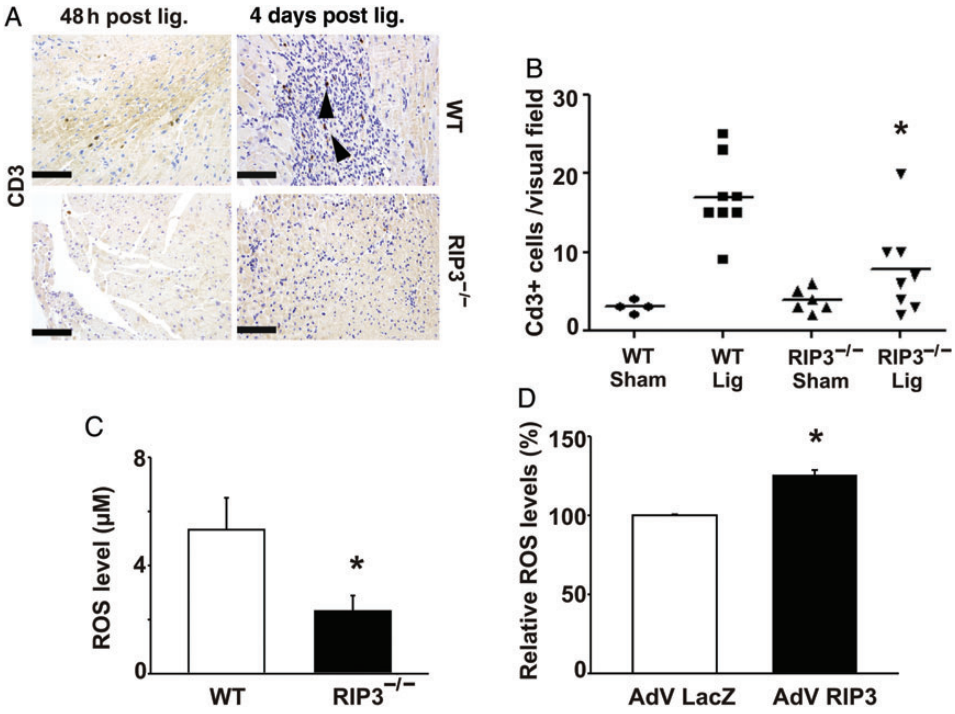

Figure 6 RIP3 mediates inflammation and generation of ROS in myocardial infarction. (*A*) RIP3$^{-/-}$ hearts revealed a significantly decreased invasion of CD3-positive cells 4 days after LAD ligation vs. WT controls. (*B*) Scale bars denote 100 μM. (*C*) RIP3$^{-/-}$ hearts revealed significantly decreased levels of ROS vs. WT controls 24 h after LAD ligation ($n = 8$). (*D*) In line with this ROS-generating effect of RIP3 *in vivo*, neonatal rat cardiomyocytes overexpressing RIP3 showed a moderately increased ROS production compared with NRVCMs expressing a control virus (LacZ, $n = 4$). LV: left ventricular weight; Lig.: LAD ligation. *$P < 0.05$.

to detect the RIP1/RIP3 complex in NRVCMs may be due to a faster turnover of complexes, e.g. due to rapid RIP1 cleavage[12] in NRVCMs compared with Jurkat cells. Consistently, we demonstrated that RIP3 overexpression leads to a significant decrease of RIP1 protein content in NRVCMs. Another explanation might be based on low RIP3 expression in the heart under basal conditions, which hampers detection of the RIP1/RIP3 complexes in NRVCMs. Up-regulation of RIP3 under stress conditions (*Figure 4A*) implies that a functional relevance of RIP3 and programmed necrosis in the heart is limited to stress situations like myocardial ischaemia. In line with this notion, RIP3$^{-/-}$ mice show normal life span and fertility[18] as well as normal cardiac function under basal conditions. Moreover, miRNA-based knockdown of RIP3 in NRVCMs did not alter viability of NRVCMs compared with LacZ-infected control cells (data not shown) and knockdown of RIP3 did have no effect on TNF-α-stimulated NRVCMs (see Supplementary material online, *Figure S2*). In this context, complex formation of RIP1 and RIP3 and subsequent induction of programmed necrosis due to RIP3 overexpression may mimic stress situations, consistent with the occurrence of programmed necrosis in cardiomyocytes.

The complex formation of RIP1 and RIP3 as early as 30 min after TNF-α stimulation of NRVCMs as well as up-regulation of RIP3 24 h after myocardial ischaemia imply that RIP3-dependent effects are executed in an early response to exogenous stress. Recent work by Oerlemans *et al.*[15] demonstrates a reduction of infarct size due to chemical inhibition of RIP1 by Nec-1 24 h after induction of ischaemia. Consistently, we could demonstrate the 'canonical' pathway of TNF-induced complex formation of RIP1 and overexpressed RIP3 in NRVCMs. However, overexpression of RIP3 alone was sufficient to induce programmed necrosis of NRVCMs without a need for additional stimulation by TNF-α (*Figure 3*). In this regard, cardiomyocytes differ from other cells, e.g. L929 cells. In these cells, RIP3 overexpression requires additional TNF-α stimulation to induce programmed necrosis.[24] Two explanations for this interesting result are possible:

first, RIP3 overexpressed in NRVCMs is constitutively active, 'bypassing' the upstream TNF-related activation pathway. This explanation is supported by the fact that knockdown of RIP1 did not significantly inhibit RIP3-dependent cell death of cardiomyocytes either (see Supplementary material online, *Figure S2C*), demonstrating a RIP1-independent effect of RIP3 in cardiomyocytes.

Secondly, in the heart, additional yet unknown activation pathways of RIP3 might exist apart from TNF-α. In this regard, recent studies demonstrated that several different receptors and signalling pathways upstream from RIP3 may activate necroptosis.[25] The specific activation pathway of RIP3 seems to be cell type-dependent and also depending on the specific activating stress stimulus.[25] In respect to our model of myocardial ischaemia, this explanation is supported by the fact that we did not find significant differences in the regulation of TNF-α-related genes between WT and RIP$^{-/-}$ mouse hearts (see Supplementary material online, *Figure S7*). Moreover, treatment of WT and RIP3$^{-/-}$ hearts with the TNF-α inhibitor, etanercept, did not effectively rescue WT mice after LAD ligation (data not shown). Thus, most likely other upstream pathways apart from TNF mediate RIP3 activation in myocardial ischaemia.

In contrast to these early effects of RIP1 inhibition in the model of Oerlemans *et al.*, infarct size of RIP3$^{-/-}$ mice is not altered in our model. At first sight, it is therefore surprising that RIP3 nevertheless

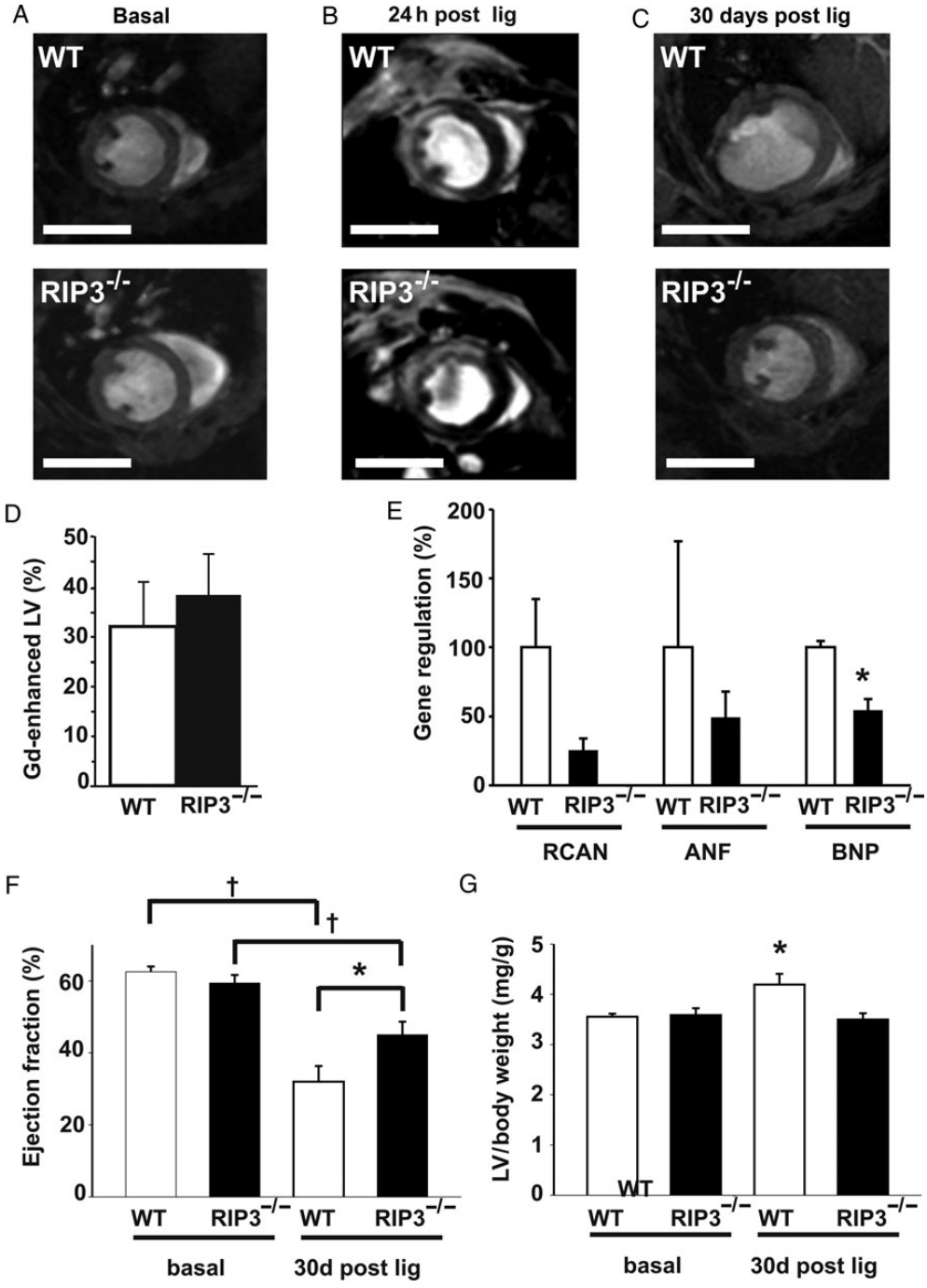

Figure 7 RIP3 mediates adverse left ventricular remodelling in response to myocardial ischaemia. (*A*) Cardiac MRI from WT and RIP3$^{-/-}$ mice under basal conditions. (*B*) Gadolinium-enhanced MRI short-axis views of WT and RIP3$^{-/-}$ hearts 24 h after LAD ligation, showing that infarct size is not altered between both groups, as quantified in (*D*) ($n = 4$). (*C*) MRI short-axis views 30 days after LAD ligation, scale bars: 5 mm. Thirty days after LAD ligation, RIP3$^{-/-}$ hearts revealed a significant down-regulation of BNP vs. WT controls, a significantly better ejection fraction (EF) (*E*) and less hypertrophy (*F*) than WT controls ($n = 12$, $^{*}P < 0.05$, $^{\dagger}P < 0.01$).

mediates long-term cardiac remodelling as well as contractile function. Yet, a significant difference between programmed necrosis and apoptosis is the strong inflammatory response driven by necrosis.[12] Consistently, we could demonstrate that the post-ischaemic inflammatory response of RIP3$^{-/-}$ mice is markedly decreased compared with WT mice. Thus, apoptotic cell death was unlikely to account for the differences between both groups. Moreover, we could not detect any evident change in apoptotic cell death between both groups, as measured by caspase 3 cleavage. Since a role of inflammation in post-infarction remodelling has been demonstrated before,[26] reduced inflammation may be one possible mechanism for the observed protective effects of RIP3 ablation on adverse remodelling in our study. This mechanism seems to be heart-specific, as the total count and proportion of T cells in the blood of RIP3$^{-/-}$ mice was unaltered compared with WT mice.

Table 1 Morphological and functional parameters of RIP3$^{-/-}$ mice hearts and WT controls under basal conditions and 30 days post LAD ligation, assessed by MRI

	WT		RIP3$^{-/-}$	
	Basal	30 days post LAD ligation	Basal	30 days post LAD ligation
Weight (g)	23.6 ± 0.5	27.7 ± 0.4	24.9 ± 0.6	26.8 ± 0.5
Heart rate (/min)	487 ± 15.1	562 ± 14.6	495 ± 22.0	531 ± 15.1
EDV (μL)	62 ± 2.3	121 ± 19.9	71 ± 4.8	83 ± 8.6
ESV (μL)	23 ± 1.6	89 ± 20.4	30 ± 3.9	48 ± 8.7
Stroke volume (μL)	39 ± 1.3	32 ± 1.8	42 ± 1.6	35 ± 1.4
Ejection fraction (%)	63 ± 1.6	32 ± 4.4	59 ± 2.3	45 ± 3.6*
Cardiac output (mL/min)	19 ± 0.6	18 ± 1.3	21 ± 1.3	19 ± 0.6
LV weight (mg)	89 ± 4.0	116 ± 6.0	84 ± 2.3	94 ± 4.4*
LV/body weight (mg/g)	3.6 ± 0.1	4.2 ± 0.2	3.6 ± 0.1	3.5 ± 0.1*

WT, wild type; EDV, end-diastolic left ventricular volume; ESV, end-systolic left ventricular volume; LV, left ventricular.
*$P < 0.05$ vs. WT.

However, since our results are based on the constitutive knockout of RIP3, we cannot definitely rule out that RIP3 deficiency of leucocytes may have a cardiomyocyte-independent effect in this regard.

In addition to a decreased inflammatory response, we could demonstrate decreased generation of ROS in RIP3$^{-/-}$ mice after LAD ligation (*Figure 6C*). Generation of ROS has been considered a crucial mechanism of RIP3-induced cell damage.[8,10] In our model of permanent myocardial ischaemia, ROS might be a key mediator that 'translates' short-term effects of RIP3 signalling into long-term adverse ischaemic remodelling.[27] Oxidative stress has been demonstrated to activate prohypertrophic signalling pathways like mitogen activated protein kinases[28] and to cause a slippage of myofibrils and LV dilation,[29] ultimately leading to decreased ventricular contractility. Thus, inhibition of ROS generation is likely to be causative for preserved contractility in the RIP3 knockout mouse. Of note, oxidative stress also induces inflammatory pathways in the heart;[29] thus, the relative contribution of these two mechanisms to RIP-mediated LV remodelling cannot be considered independently. Mitochondria have been recognized to be a source of excessive ROS generation in MI.[28] The co-localization of RIP3 and mitochondria implies that mitochondrial ROS generation is crucial in RIP3-dependent myocardial remodelling. In line with this notion, several recent publications have identified the mitochondrial proteins such as mixed lineage kinase domain-like protein (MLKL) and phosphoglycerate mutase family member 5 (PGAM5) as downstream effectors of RIP3.[20,30–33] These results have even led to the consideration of the RIP1/RIP3 complex being a 'mitochondrial attack complex',[20,30] with dissipation of the mitochondrial transmembrane potential ($\Delta\psi_M$) ultimately leading to fragmentation of mitochondria under specific stress conditions[32] Indeed, we could detect a trend towards improved energetics of RIP3$^{-/-}$ mouse hearts after LAD ligation *in vivo*. Moreover, the co-localization of RIP3 and mitochondria in cardiomyocytes is consistent with the functional importance of mitochondria in RIP3-mediated cardiac damage. However, RIP3 overexpression in cardiomyocytes did neither cause fragmentation of mitochondria nor significant alteration of the mitochondrial transmembrane potential ($\Delta\psi_M$) compared with control cells (data not shown). As possible explanations for this interesting finding, ROS might be generated by other sources, like endosomes,[34] peroxisomes,[35] or infiltrating immune cells. In line with this assumption, a novel report by Tait *et al.*[36] generally challenges the functional role of mitochondria in programmed necrosis, demonstrating that in some cells, mitochondria are not necessarily required in this process. It thus appears possible that RIP3 may affect regulatory functions on metabolism apart from mitochondrial damage, especially in an energy-dependent organ like the heart.

In conclusion, we here demonstrate that RIP3 exerts negative effects on post-ischaemic cardiac remodelling. In addition to the data of Oerlemans *et al.* on RIP1 in ischaemia–reperfusion injury, these results imply a fundamental role of pathways mediating programmed necrosis in cardiac ischaemia. Because late cardiac remodelling with subsequent post-ischaemic heart failure is still a major clinical problem, these signalling pathways might represent attractive targets for future therapeutic interventions.

5. Limitations of this study

The *in vivo* results presented in this study are based on a constitutive knockout of RIP3. Although we have not observed any morphological differences between transgenic and WT control mice in peripheral blood cells as well as other organs, we cannot definitely rule out that RIP3 deficiency of other cell types may have contributed to the observed effects in the heart. Moreover, our study does not provide a quantitative comparison of apoptotic vs. necroptotic cell death in the course of myocardial ischaemia. Thus, we cannot definitively conclude that necroptosis is the dominant form of cell death in post-ischaemic myocardial damage. Nevertheless, we feel that the alterations of myocardial remodelling in the RIP3 knockout mouse strongly argue for an important role of the necroptotic RIP3 signalling pathway in post-MI remodelling. Further studies are needed to finally compare the relative contribution of each form of cell death in this and other disease models.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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