RHOE AND INFLAMMATION IN MYOCARDIAL INFARCTION

A Dissertation

by

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ABSTRACT

Cardiac inflammatory response after myocardial infarction (MI) is essential to clear necrotic myocardium for cardiac healing, while excessive and prolonged inflammation extends the infarction and promotes adverse cardiac remodeling. NF-κB is the pivot regulator upstream of inflammatory response. Overaction of NF-κB exaggerates inflammation and contributes to detrimental outcomes of MI as well as other inflammatory diseases.

Here we report RhoE as a negative inflammatory mediator by specifically inhibiting NF-κB. RhoE-deficient mice show overactivation of NF-κB in heart and develop excessive inflammation after MI. Mechanistically, RhoE interacts with p65 and p50 in cytosol and inhibits their nuclear translocation. RhoE also occupies the dimerization domain of p65 to impede the formation of p65/p50 heterodimer. Finally, we show that cardiac-specific RhoE overexpression restrains post-MI inflammation and improves cardiac function and survival. These findings identify RhoE as an important mediator of NF-κB-induced inflammation, and therefore, RhoE may serve as a potential anti-inflammatory target to achieve beneficial consequences.

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Contributors

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NOMENCLATURE

RhoE	Rho family GTPase 3
MI	Myocardial infarction
AMI	Acute myocardial infarction
CAD	Coronary artery disease
HF	Heart failure
HCM	Hypertrophic cardiomyopathy
TAC	Transverse aortic constriction
VEGF	Vascular endothelial growth factor
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IKK	IκB kinase
Ly-6G	Lymphocyte antigen 6G
F4/80	Adhesion G protein-coupled receptor E1
TNF	Tumor necrosis factor
MMPs	Matrix metalloproteinases
EMSA	Electrophoretic mobility shift assay
BiFC	Bimolecular fluorescent complementation
VN	N-terminus of fluorescent protein Venus
VC	C-terminus of fluorescent protein Venus

NTD	N-terminal domain
DD	Dimerization domain
TAD	Transactivation domain
ROCK1	Rho-associated coiled-coil-containing protein kinase 1
NLS	Nuclear localization signal

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CHAPTER I

INTRODUCTION

I.1 Myocardial Infarction (MI)

Myocardial infarction (MI), commonly known as heart attack, is a severe cardiac ischemic disease caused by coronary artery thrombotic occlusion[1]. Based on the 2016 report of American Heart Association, there are about 550,000 Americans newly suffering from myocardial infarction (defined as first hospitalized myocardial infarction) and 200,000 recurrent cases[2]. Most cases of MI occur due to coronary artery disease (CAD) and conventional risk factors of MI include cigarette smoking, high blood cholesterol, high blood pressure, diabetes and obesity.

The healing process of the infarcted heart following MI can be divided into three distinct, but overlapping phases, including the inflammatory phase, the proliferative phase, and the maturation phase[3] (Table 1).

Table 1 The healing stages after myocardial infarction.

Stages	Time from MI	Processes
Inflammatory phase	< 1 week	Clear necrotic cardiomyocytes and matrix debris
Proliferative phase	1- 3 weeks	Granulation tissue formation
Maturation phase	> 3 weeks	Scar formation

MI: myocardial infarction

During the inflammatory phase, dying cardiomyocytes-released alarmins activate innate immune signaling to induce the secretion of pro-inflammatory chemokines and cytokines[4]. Among those pro-inflammatory factors, IL-1 signaling plays a central role in recruiting leukocytes (neutrophils and macrophages) to further promote chemokines and cytokines expression[5]. The leukocytes are then recruited to the infarcted area to phagocytose dead cardiomyocytes and matrix debris. However, excessive inflammation could cause inflammatory injuries and extend the infarct area. The inflammatory phase normally lasts about one week after myocardial infarction, and apoptosis of the infiltrated neutrophils and the resolution of the acute inflammation marks the end of this phase[3]. Following the inflammatory phase, anti-inflammatory monocyte subpopulations and regulatory T cells become the predominant cell types in the infarct area to suppress inflammation. More importantly, cardiac monocytes differentiate towards anti-inflammatory subtype (M2 macrophage subtype) instead of pro-inflammatory subtype (M1 macrophage subtype), which effectively contribute to the subsequent reparative processes[6]. The inflammatory phase is most dangerous phase because most myocardial infarction death occurs in this phase.

During the proliferative phase of cardiac healing, activated myofibroblasts become the predominant cell type in the healing region[7]. These myofibroblasts are dissimilar from typical myofibroblasts in that they are phenotypically modulated fibroblasts that synthesize large amount of structural and matricellular extracellular matrix proteins instead of expressing smooth muscle cell-specific proteins[8]. Activated myoblasts migrate into the border zone of the infarct area, where they secrete the collagen and extracellular

matrix proteins to initiate the scar formation. Deposition of these structural matrix proteins into the infarct area preserves the ventricular integrity and geometry to prevent aneurysm and cardiac rupture. However, extracellular matrix deposition at remote sites from the infarct area can lead to excessive cardiac fibrosis and ventricular stiffness, which is an inevitable process that leads to adverse cardiac remodeling and constitutes the basis of the development of heart failure[8]. The proliferative phase usually ends in the first month after myocardial infarction. The maturation phase following the proliferative phase lasts more than two months to enable complete formation of a mature cross-linked scar. The infiltration of myofibroblasts into the infarct area is suppressed and the activated myofibroblasts are induced to apoptosis to end the proliferative activity in the scar. The collagenous matrix is cross-linked to form a mature scar. Thus, the lost myocardium is replaced by a collagen-based scar.

I.2 Inflammatory Response and Post-MI Inflammation

Inflammation is the biological responses of the body tissues to injurious stimuli, such as physical tissue damages, infection by pathogens, and toxins[9]. Inflammation can be classified as either acute inflammation or chronic inflammation based on the causative agent, major cells involved, primary mediators, and duration[10]. Acute inflammation occurs immediately after the tissue damages. Danger signals released from the injured tissues activate innate immune pathways to induce pro-inflammatory factors synthesis and secretion, and promote the expression of adhesion molecules on vascular endothelium. Chemokines and cytokines then recruit the leukocytes from the blood vessels into the injured tissues, which propagate and expand the inflammatory response. If the inflammation is no longer needed, the inflammatory response must be actively terminated through a process called "resolution of inflammation". If the inflammation is unresolved and prolonged, acute inflammation switches to chronic inflammation status, which is characterized by simultaneous destruction and healing of the tissue[9].

Dysregulated inflammatory response contributes to many pathological changes and human diseases. Together with immune system, inflammatory abnormality has been involved in allergic reactions, myopathies, autoimmune diseases and autoinflammatory diseases[11-13]. Moreover, inflammatory disorders have been proved to play essential roles in the pathogenesis of cancer, AIDS, atherosclerosis, hypertrophic cardiomyopathy (HCM), and ischemic cardiomyopathy[14-17].

Post-MI inflammation plays an important role in the healing and remodeling of the infarcted heart[3, 18]. During the inflammatory phase, acute inflammation is developed and large amounts of pro-inflammatory chemokines and cytokines are released, which recruit leukocyte infiltration into the infarct area. Infiltrated leukocytes, especially macrophages, actively clear the necrotic cardiomyocytes and extracellular matrix debris through phagocytosis. The inflammation then is suppressed and the healing process proceeds to the next stage. Thus, acute inflammation is a prerequisite for the repairing of the infarct myocardium. However, dysregulated post-infarction inflammation is detrimental. Overactivated inflammation leads to extra loss of myocardium and causes inflammatory injury in the inflammatory phase, resulting in extended ischemic injury and enlarged infarct size. Moreover, prolonged inflammation abates the activation of myofibroblasts and thus delays the proliferative phase. The infarct myocardium cannot be repaired timely, and the integrity and geometry of the ventricle cannot be maintained, which promotes the development of cardiac aneurysm and even ventricular rupture.

I.3 Anti-inflammation Therapeutics in Myocardial Infarction

Based on the deleterious effects of excessive inflammation to the cardiac healing after myocardial infarction, multiple anti-inflammatory therapeutic strategies have been tested, aiming to attenuate the inflammatory injuries. These strategies can be divided into three categories: 1) enhancement of anti-inflammatory pathways; 2) inhibition of pro-inflammatory pathways; 3) shifting the balance from pro-inflammatory to reparative myeloid cell subsets[4, 19].

There are several endogenous mechanisms exist that tightly control the inflammation after myocardial infarction. The expression levels of these endogenous inflammatory inhibitors are upregulated during the processes of resolving inflammation in the inflammatory phase. Interleukin 10 (IL-10) is a pleiotropic anti-inflammatory cytokine that is primarily secreted by the infiltrated macrophages after myocardial infarction[20]. IL-10 suppresses inflammatory response by inhibiting the expression of pro-inflammatory cytokines, such as TNFα and IL-1β[21, 22]. A recent study showed that IL-10 also protects the infarcted heart from adverse cardiac remodeling by stimulating M2 subtype macrophage polarization[23]. Transforming growth factor β (TGF-β) is another pleiotropic cytokine that negatively mediates post-infarction inflammatory response. In the end stage of the inflammatory phase, the expression of TGF-β is upregulated to drive the differentiation of fibroblast to myofibroblast[24] . Inhibition of TGF-β signaling prolongs the inflammation and deteriorates the cardiac contractile function in the mouse myocardial infarction model[25].

Another anti-inflammatory strategy is the inhibition of pro-inflammatory pathways. Here, suppressions of two pro-inflammatory factors are introduced. The first one is chemokine (C-C motif) ligand 2 (CCL2). CCL2 is a chemoattractant protein that recruits monocyte/macrophage during myocardial infarction[26]. Gene therapy with an antagonistic CCL2 mutant suppresses the recruitment of macrophages into the infarct area, resulting in improved ventricular remodeling and survival after myocardial infarction[27]. The other pro-inflammatory factor is toll-like receptor 4 (TLR4), which is the specific receptor for danger-associated molecular patterns (DAMPs). TLR4-mediated recruitment of leukocytes is a critical step for the infiltration of leukocytes into the infarcted heart. Genetic ablation of TLR4 in mice showed diminished leukocyte recruitment and reduced pro-inflammatory cytokine expression after myocardial infarction[28].

The third anti-inflammatory strategy is to shift the balance of myeloid cells from pro-inflammatory subsets to reparative subsets. In the healing process of myocardial infarction, there are two subsets of monocyte existed in the infarct area: M1 subset monocytes that dominate on days 1 to 4; and M2 subset monocytes that dominate on days 5 to 10[29]. M1 subset monocytes are responsible for clearing necrotic cardiomyocytes and matrix debris by phagocytosis, and further promoting the inflammatory response by expressing pro-inflammatory factors, such as TNFα, IL-1β and MMPs. On the contrary, M2 subset monocytes contribute in resolving the inflammation by expressing IL-10 and TGF-β. M2 subset monocytes propagate the healing process and promote angiogenesis by secreting vascular endothelial growth factor (VEGF)[6, 30, 31]. Thus, shifting the monocytes from M1 subset to M2 subset may avoid excessive digestion of the infarcted myocardium

and promote the reparative process. Phosphatidylserine-presenting liposomes have shown the ability to induce M2 monocyte subset[32]. Mice treated with phosphatidylserine-presenting liposomes showed significantly decreased infarct size, increased angiogenesis, and improved cardiac functions after myocardial infarction.

Clinically, some anti-inflammatory strategies have been eventually tested in MI patients. However, most of these strategies failed to prove the same promising outcomes as in the experimental animal studies. Here, anti-inflammatory clinical trials of glucocorticoids, nonsteroidal anti-inflammatory drugs, integrins, metalloproteinases, and cytokines will be introduced.

Glucocorticoids (GCs) are a class of steroid hormones. Glucocorticoids are clinically used to suppress various allergic, inflammatory, and autoimmune disorders[33]. Glucocorticoids inhibit inflammation through three mechanisms: transcriptionally modifying the expression of inflammatory genes via glucocorticoid-responsive element; inhibiting NF-κB activation via cortisol-glucocorticoid receptor complex; activating endothelial nitric oxide synthase via membrane-associated receptors. However, clinical trials failed to show beneficial effects of glucocorticoids in patients with myocardial infarction[34]. Current clinical guidelines for AMI patients are recommend against the treatment of glucocorticoids.

Nonsteroidal anti-inflammatory drugs (NSAIDs) also broadly suppress inflammation. NSAIDs inhibit the activity of cyclooxygenase-2 (COX 2) to reduce prostanoid production from arachidonic acid[35]. Unfortunately, clinical treatment with NSAIDs in AMI patients exacerbates the outcomes of acute myocardial infarction[36].

Moreover, a recent observational study showed that the use of NSAIDs in non-AMI patients is associated with an increased risk of acute myocardial infarction[37, 38]. Collectively, NSAIDs are not recommended to be used in the treatment of myocardial infarction.

Integrins are endothelial adhesion molecules that help the activated neutrophils to migrate from the blood vessels into the infarcted myocardium. Antibodies against these endothelial adhesion molecules are proved to be beneficial in animal MI studies[39, 40], however, outcomes of the related clinical trials are conflicting. In LIMIT-AMI (limitation of myocardial infarction following thrombolysis in acute myocardial infarction) clinical trial, AMI patients treated with a monoclonal antibody against CD18 did not show diminished AMI injury[41]. Moreover, in the HALT-AMI clinical trial, the treatment with a recombinant antibody against CD11/CD18 also failed to show beneficial effects to AMI patients[42]. One possible explanation for these negative results is that the endothelial cell barrier in the heart vessels has been irreversible damaged during the myocardial infarction and thus the efficacy of the antibody is undermined to suppress the recruitment of leukocytes[22].

Metalloproteinases (MMPs) function in degrading extracellular matrix and collagen[43]. Increased activity of MMPs in the inflammatory phases contributes to cardiac aneurysm and even ventricular rupture in the infarcted ventricular wall. Overactivation of MMPs also leads to collagen-scar thinning, resulting in ventricular dilation. Several inhibitors on MMPs have been tested in the clinical trials. PG-116800, an inhibitor for MMP-2, -3, -8, -9, -13, and -14, however, failed to improve adverse ventricular

remodeling at six months after myocardial infarction in the phase II RCT PREMIER (prevention of myocardial infarction early remodeling) clinical trial[44]. On the contrary, doxycycline, another MMP inhibitor, showed beneficial effects in the phase II TIPTOP (early short-term doxycycline therapy in patients with acute myocardial infarction and left ventricular dysfunction to prevent the ominous progression to adverse remodeling) clinical trial[45]. Doxycycline significantly reduces the degree of ventricular dilation and the major adverse cardiac events at six months after myocardial infarction.

Anti-pro-inflammatory cytokines is another strategy to diminish the inflammatory injury in myocardial infarction. IL-1β, one major pro-inflammatory cytokine, activates NF-κB signaling via interaction with myeloid differentiation factor 88 and the downstream IL-1 receptor-associated kinase type 4[46]. IL-1 blockade has showed improved cardiac remodeling and cardiac function in the animal AMI models[47-49]. Clinical trials also proved the beneficial effects of IL-1 blockade. In the phase II VCU-ART (Virginia Commonwealth University acute remodeling trial) and VCU-ART2 trials, AMI patients treated with anakinra, a soluble IL-1 receptor acting to trap the circulating IL-1, tend to show improved ventricular remodeling and reduced heart failure incidence at three months after myocardial infarction[50, 51]. TNFα is another important pro-inflammatory cytokine that associates with cell death and inflammation. However, in a recent clinical trial, etanercept, a specific TNFα trapper, failed to provide beneficial effects to AMI patients[52]. Together with the disappointing results from TNFα blockers in HF patients[53], TNFα-blocking drugs are not recommended for patients with cardiovascular diseases.

Collectively, the major anti-inflammatory therapeutics have been introduced as above. Most of the clinical trials failed to prove the similar beneficial effects of anti-inflammatory therapeutics in the animal studies. The disappointing results of these translational studies may be high due to the following reasons: 1) experimental animals significantly differ from humans; 2) surgical ligation of the coronary artery to induce myocardial infarction in animals is largely different from the process of atherothrombosis-induced coronary artery narrowing in human; 3) the variance of post-AMI inflammatory status is more complex in human than in the experimental animals. Therefore, more studies need to be performed to clearly understand the complexity of the inflammation during myocardial infarction. Also, novel mechanisms are required to provide new potential therapeutic targets to properly suppress the post-infarction cardiac inflammation.

I.4 NF-κB Signaling

I.4.1 Structure of the NF-κB family

Transcription factor NF-κB controls the expression of many genes involved in a broad range of biological processes[54, 55]. This part will introduce the members and their structure of the NF-κB family, the regulation of NF-κB signaling, and the clinical significance of dysregulated NF-κB signaling.

NF-κB was first discovered as a DNA binding protein complex in the nuclei of activated B lymphocytes about thirty years ago[56]. Further studies revealed that the NF-κB complex is composed of 5 different subunits that contain a conserved amino sequence in the amino-terminal end termed Rel homology region (RHR) (Fig. 1). The Rel homology region contains two function domains, the amino-terminal domain (NTD) that is responsible for DNA binding activity; and the dimerization domain (DD) that mediates the homo- and hetero-dimerization of the NF-κB subunits. Based on their structure and functional domains, these 5 subunits are further divided into two classes: class I includes p50 and p52; class II includes p65, c-Rel and RelB. The carboxy-terminus of class I proteins contains glycine-rich regions and has transrepression activity, while class II proteins has a transactivation domain (TAD) in their carboxy-terminal end.

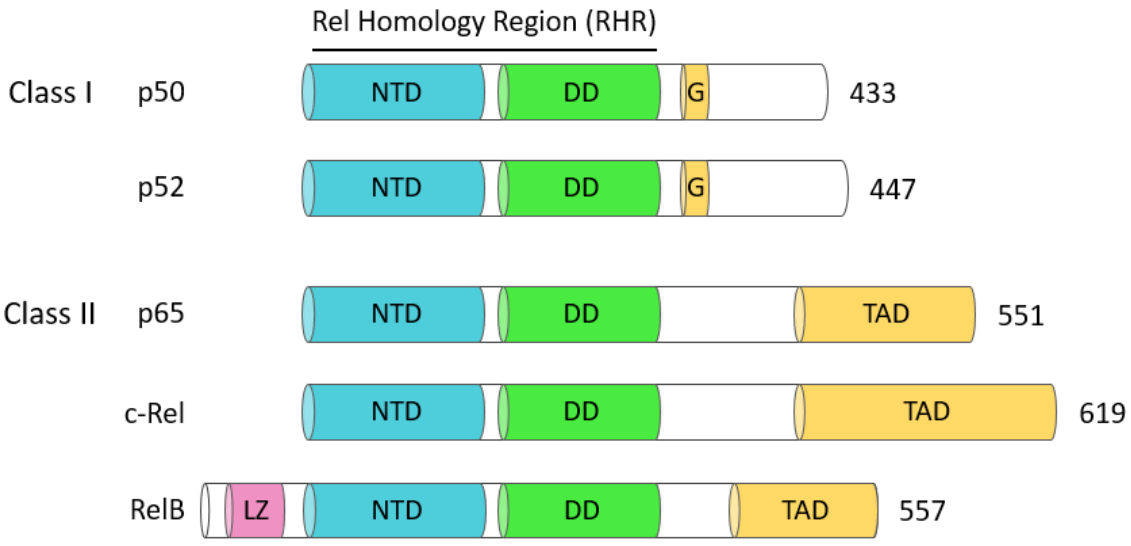

Figure 1. Schematic structure of the NF-κB family subunits.

There are five subunits in the human NF-κB family. Each subunit contains the Rel homology region (RHR) in the amino-terminal end. The Rel homology region contains two functional domains: amino-terminal domain (NTD) and dimerization domain (DD). Two of the subunits, p50 and p52, contain a glycine-rich region in the carboxy-terminal end, which has transrepression activity. The other three subunits, p65, c-Rel and RelB, contain a transactivation domain (TAD) in the carboxy-terminal end, which has transactivation activity. RelB also contains a predicted leucine zipper motif (LZ) in its amino-terminal end, which is essential to RelB transcriptional activity.

I.4.2 NF-κB dimerization

NF-κB subunits form homo- or hetero-dimer to exert their functions in DNA binding and transcriptional activation. In theory, the 5 NF-κB subunits should form 15 unique homo- and hetero-dimers. However, only 12 of them have been identified in vivo. The three unidentified dimers are RelB/RelB, RelB/c-Rel, and p52/c-Rel[57]. The dimerization is conducted by the dimerization domain of the subunits. Two NF-κB subunits interact with each other to form the dimerization surface. Of these, p65 and p50 creates the most stable NF-κB dimer interface. The positions of Phe-213 and Asn-200 in p65 are occupied by Tyr-269 and Asp-256 in p50 to form a highly stable hydrogen bond. Thus, p65/p50 is the most stable and abundant NF-κB dimer in vivo.

Studies on the X-ray structures of the NF-κB:DNA complex have revealed the interaction between the NF-κB dimer and DNA containing κB sequence. The NF-κB dimers form a butterfly-like structure through the dimerization domain, and use both the amino-terminal and dimerization domain to encircle the DNA containing κB and activate the its transcription.

I.4.3 Regulation of NF-κB signaling

There are two distinct NF-κB activation pathways, the canonical pathway and the alternative pathway (Fig. 2). The primary NF-κB complex in the canonical pathway is the p65/p50 complex, which is sequestered in the cytosol by the bound inhibitor of NF-κB (IκBα). Meanwhile, RelB/p100 is the major NF-κB complex in the alternative pathway. These two NF-κB pathways are activated by two distinct sets of stimuli, and regulates different biological processes. The canonical pathway is activated by antigens, LPS or pro-inflammatory cytokines, such as TNFα and IL-1β, which are recognized by antigen receptor, Toll-like receptors, or cytokine receptors. The stimuli are further transmitted to the IκB kinase (IKK) complex and the major component IKKβ is activated and phosphorylated. Then the phosphorylated IKKβ induces the ubiquitin-proteasome-mediated degradation of the inhibitor of NF-κB (IκBα) through phosphorylating IκBα. The active p65/p50 heterodimer then translocates into the nucleus to activate its target genes, including genes involved in inflammation, survival, and proliferation.

The alternative pathway is stimulated by lymphotoxin, B-cell activating factor (BAFF), CD40, OX40, or CD 27. The corresponding receptors then activate the NF-κB inducing kinase (NIK), which further induces the activation of the IKKα. Active IKKα then phosphorylates p100 at Ser-866 and Ser-870 sites. The phosphorylated p100 is processed to p52 via the ubiquitin-proteasome-mediated degradation of its carboxy-terminus. The RelB/p52 heterodimer then translocates into the nucleus to regulate its target genes, including chemokines and genes involved in lymphoid organogenesis.

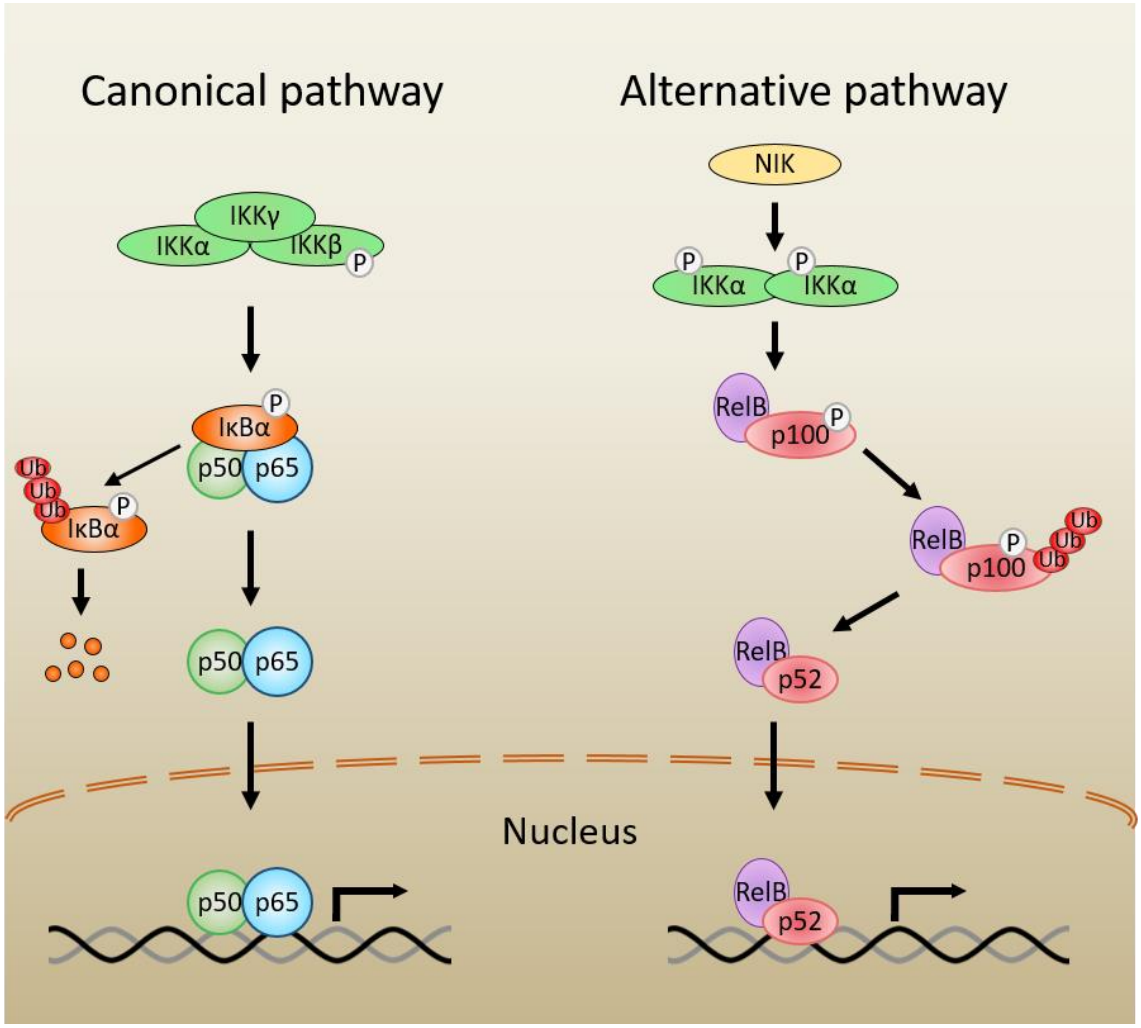

Figure 2. The canonical and alternative NF-κB pathways.

Canonical pathway is primarily stimulated by pro-inflammatory stimuli, such as LPS, TNFα and IL-1β. The activated IKK complex phosphorylates IκBα and induces IκBα degradation. The free p65/p50 heterodimer then translocates into the nucleus to regulate genes related to inflammation, proliferation, and survival. The alternative pathway is closely involved in the lymphoid organogenesis. Lymphotoxin and B-cell activating factor activate NIK, which phosphorylates and activated IKKα. Activated IKKα then phosphorylates p100, leading the procession of p100 to p52. The RelB/p52 heterodimer then translocates into the nucleus to regulate its target genes.

I.5 RhoE and Its Functions in the Heart

I.5.1 The atypical small GTPase RhoE

RhoE belongs to a member of the Rho family of GTPases. RhoE is primarily located in the cytosol, and translocates to the cell membrane when farnesylated and dephosphorylated[58-60]. The tissue expression of RhoE is universal while the levels could be varied[61]. RhoE has a unique characteristic in that it lacks GTPase activity and stays in the GTP-bound state. Therefore, RhoE is not regulated by GTP/GDP cycling, but instead by the expression level and by protein modifications such as prenylation and phosphorylation[62, 63].

The initial function of RhoE is linked to cell actin cytoskeleton dynamics, cell migration and apoptosis through the inhibition of its effector, Rho kinase 1 (ROCK1). Recent studies have revealed much diversified functions of RhoE in regulation of other biological processes, including calcium homeostasis, angiogenesis, cell cycling, tumorigenesis, and cancer metastasis[61, 63-70].

I.5.2 Study of RhoE in the heart

Compared to RhoE's roles in tumorigenesis, the functions of RhoE in the heart has been less studied. Our lab have revealed a diversified functions of RhoE in the cardiac physiology, including cardiomyocyte apoptosis, cardiac angiogenesis, and cardiac calcium handling. We found that RhoE expression level is downregulated in the heart of patients with end-stage heart failure[67].

RhoE is essential to suppress ROCK1-mediated cardiomyocyte apoptosis, and RhoE deficiency in mouse led to increased apoptotic cardiomyopathy, resulting in higher tendency of heart failure after pressure overload by transverse aortic constriction (TAC). RhoE also plays an important role in regulating the cardiac angiogenesis in response to pressure overload[65]. RhoE is required to stabilize the hypoxia-inducible factor 1α (HIF-1α), which is the primary transcription factor of vascular endothelial growth factor (VEGF). RhoE deficiency in mouse led to decreased the cardiac VEGF expression and impaired angiogenesis.

Finally, RhoE is also involved in mediating the cardiac calcium homeostasis[64]. RhoE-null mouse died at the embryonic stage with fetal arrhythmias. Mechanistically, depletion of RhoE triggers severe calcium leakage. RhoE deficiency impairs lysosomal targeting and degradation of the β2-adrenergic receptor, leading to increased β2-adrenergic receptor level and hyperactivated protein kinase A (PKA) signaling. PKA further destabilizes the ryanodine receptor type 2 channels and prompted the calcium leakage.

CHAPTER II

MATERIALS AND METHODS

II.1 Animals

The Institutional Animal Care and Use Committee (IACUC) of Texas A&M University Health Science Center Institute of Biosciences and Technology approved the experiments with animals. The following mouse lines were used in the study: Wild-type (WT) mouse; RhoE floxed control ($RhoE^{flox/+}$) mouse; cardiomyocyte-specific RhoE hyploinsufficient ($RhoE^{flox/+}$;MHC-Cre) mouse; cardiomyocyte-specific RhoE overexpression transgenic (RhoE-TG) mouse.

II.2 Mouse MI Model

Male mice at the age of 12 weeks were used in the MI surgery. Acute myocardial infarction was induced as described in the earlier studies as described in our and other earlier studies[71]. In brief, mice were fully anesthetized with 2% isoflurane gas and oxygen. Then mice were subject to endotracheal intubation and mechanically ventilated with a rodent respirator. Left thoracotomy was performed to open the chest cavity. The heart was then exposed and the left anterior descending (LAD) coronary artery was permanently ligated with a 6-0 silk suture at the site of about 2 mm lower than the tip of the left auricle. Successful LAD ligation was confirmed by appearance of a paler color in the anterior wall of the left ventricle. The chest was then closed and post-operative care was given. The mice were carefully monitored twice each day after the surgery.

II.3 Reagents

The following key chemicals were used in the study (Table 2).

Table 2 Key chemicals used in the experiments.

Reagent name	Company	Catalog number
γ^{-32}P,ATP	PerkinElmer	BLU002H250UC
Poly(dI-dC)	Sigma-Aldrich	P4929
Recombinant murine TNFα	PeproTech	315-01A
His-p65 recombinant protein	Adipogen Life Sciences	AG-40T-0020-C002
His-p50 recombinant protein	Adipogen Life Sciences	AG-40T-0021-C002
On-targetplus non-targeting siRNAs	GE Dharmacon	D-001810-10
On-targetplus mouse RhoE siRNA	GE Dharmacon	L-064484-01

II.4 Antibodies

The following antibodies were used in the experiments (Table 3).

Table 3 Antibodies used in the experiments.

Reagent name	Company	Catalog number	Application
RhoE	Cell Signaling	3664	WB
p65	Cell Signaling	8242	WB, IP
p50	Cell Signaling	3035	WB, IP
IκBα	Cell Signaling	4814	WB
Phospho-IκBα (Ser32)	Cell Signaling	2859	WB
Phospho-p65 (Ser536)	Cell Signaling	3033	WB
GAPDH	Cell Signaling	5174	WB
Lamin B1	Cell Signaling	13435	WB
myc	Cell Signaling	2278	WB, IP
HA	Cell Signaling	3724	WB, IP
GST	Cell Signaling	2622	WB, IP
GFP	Cell Signaling	2956	WB, IP
flag	Sigma-Aldrich	F7425	WB, IP
Anti-rabbit IgG, HRP-linked	Cell Signaling	7074	WB
Anti-mouse IgG, HRP-linked	Cell Signaling	7076	WB
Mouse anti-rabbit IgG (conformation specific)	Cell Signaling	5127	WB, IP
Rabbit IgG isotype control	Abcam	ab27478	IP
ly-6G	Abcam	ab25377	WB, IHC
F4-80	Abcam	ab6640	WB, IHC

WB: western blotting, IP: immunoprecipitation, IHC: immunohistochemistry staining.

II.5 Mammalian Expression Constructs

NF-κB-dependent firefly reporter plasmid and EF1α promoter-dependent Renilla reporter plasmid were kindly provided by Dr. Xin Lin (University of Texas MD Anderson Cancer Center, USA). Human RhoE cDNA was subcloned into pCMV-myc-N vector (Clontech, 635689) to generate myc-RhoE expression construct. Human p65 cDNA was subcloned into p3xFLAG-CMV (Sigma Aldrich, E4026) to generate flag-p65 expression construct. Human p65 cDNA was subcloned into pEGFP-C3 (Clontech) to generate GFP-p65 expression construct. The truncated mutants of GFP-p65 were generated using Q5 site-directed mutagenesis kit (New England Biolabs, E0554S). Human p50 cDNA was subcloned into pCMV-HA-N (Clontech, 635690) to generate HA-p50 expression construct. The truncated mutants of HA-p50 were generated using Q5 site-directed mutagenesis kit (New England Biolabs, E0554S). All expression constructs used in the experiments are summarized in Table 4.

Table 4 Mammalian expression constructs used in the experiments.

Construct	Application
NF-κB-dependent firefly reporter	Luciferase assay
EF1α promoter-dependent Renilla reporter	Luciferase assay
myc-RhoE	Luciferase assay; EMSA; WB; Co-IP
GFP-p65	Co-IP
GFP-p65 (1-286 aa)	Co-IP
GFP-p65 (286-551 aa)	Co-IP
GFP-p65 (1-186 aa)	Co-IP
GFP-p65 (187-286 aa)	Co-IP
flag-p65	Co-IP
HA-p50	Co-IP
HA-p50 (1-245 aa)	Co-IP
HA-p50 (24-433 aa)	Co-IP
VN	BiFC assay
VC	BiFC assay
VN-RhoE	BiFC assay
VC-p65	BiFC assay
VC-p50	BiFC assay
VC-p65 (1-286 aa)	BiFC assay
VC-p65 (286-551 aa)	BiFC assay
VC-p65 (1-186 aa)	BiFC assay
VC-p65 (187-286 aa)	BiFC assay

EMSA: electrophoretic mobility shift assay, WB: western blotting,
Co-IP: Co-immunoprecipitation, BiFC: bimolecular fluorescence complementation.

II.6 Cell Culture and Transfection

Mouse C2C12 myoblast cells were obtained from ATCC (CRL-1772) and cultured in DMEM medium supplemented with 20% fetal bovine serum. Human embryonic kidney HEK293T cells were obtained from ATCC (CRL-11268) and cultured in DMEM medium supplemented with 10% fetal bovine serum. The adult mouse cardiomyocytes were isolated from adult mouse heart using the isolation kit (Cellutron Life Technologies, ac-7031). The isolated cardiomyocytes were cultured in AW medium (Cellutron Life Technologies, m-8034).

The transfections in HEK293T cells were performed using lipofectamine 2000 transfection reagent (Thermofisher, 11668027). The transfections in C2C12 cells were performed using the Neon electroporation system (Thermofisher, MPK10025). siRNA was transfected into cells using Lipofectamine RNAiMAX transfection reagent (ThrmoFisher, 13778150)

II.7 Dual-luciferase Assay

Dual-luciferase assay was performed according to our earlier study[65]. Briefly, C2C12 cells were seeded in triplicates in a 12-well plate overnight to reach 70% confluency. The cells in each well were transfected with 50 ng of NF-κB-dependent firefly reporter construct and 5 ng of Renilla reporter construct together with either 10 pmol of control siRNA or 10 pmol of RhoE-specific siRNA. 16 hours after the transfection, TNFα was added with a final concentration of 40 ng/ml and the cells were harvested 4 hours later. Dual-luciferase

activities were measured using the dual-luciferase reporter assay system (Promega, E1910). The Renilla luciferase activity was used for the normalization purpose.

II.8 Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts of the C2C12 cells were prepared by NE-PER nuclear and cytoplasmic extraction reagents (ThermoFisher, 78833). 10 μg nuclear extracts and 2 μl of 1 μM ^{32}P-labeled annealed κB probes (5'- AGTTGAGGGGACTTTCCCAGGC-3' and 5'-GCCTGGGAAAGTCCCCTCAACT-3') were incubated in the binding buffer (50 mM HEPES, 100 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM EGTA, 1.25 mM DTT, 0.2 μg/ul poly-dI-dC) at room temperature for 15 minutes. The samples were separated by a non-denaturing polyacrylamide gel, and the gel was then dried at 80°C for 2 hours followed by the X-ray film detection.

II.9 GST Pull-down Assay

Human RhoE cDNA was subcloned into pGEX-6P-1 (GE Healthcare) to generate GST-RhoE construct. GST and GST-RhoE recombinant proteins were expressed in E. coli and extracted by glutathione sepharose (GE Healthcare). The proteins were purified by high-performance liquid chromatography. GST or GST-RhoE protein was conjugated to glutathione beads in 1ml of NP-40 lysis buffer followed by the addition of His-p65 or His-p50 protein. After 3-hr incubation at 4°C, the proteins were eluted for the immunoblotting analysis.

II.10 Immunoprecipitation and Immunoblotting

For immunoprecipitation, cells were lysed in NP-40 lysis buffer containing 1.0 mM PMSF and protease inhibitor cocktail (Roche, 11873580001). The cell lysates were incubated with protein G-Sepharose beads (GE Healthcare, 17061801) together with either IgG or the specific antibodies at 4°C overnight. The beads were washed with lysis buffer for 4 times and boiled with 2 x SDS loading buffer (Santa Cruz Biotechnology, sc-24945). The samples were separated in 10% Bis-Tris protein gel (ThermoFisher) and transferred to the nitrocellulose membrane. The membranes were then incubated with specific primary antibodies, following by incubation with the secondary antibody. We used the confirmation specific second antibody that only recognized the primary antibody but not heavy or light chains of the antibody used for immunoprecipitation.

II.11 Bimolecular Fluorescence Complementation (BiFC) Assay

The BiFC assay was performed with a modified protocol according to the earlier study[72]. Venus is an improved version of yellow fluorescent protein (YFP). RhoE was subcloned into the N-terminus of Venus expression construct; p65 and p50 was cloned into the C-terminus of Venus individually. C2C12 cells were transfected with different pairs of BiFC constructs as indicated in the experiments. 24 hours after the transfection, cells were fixed in 4% paraformaldehyde. The fluorescent signals were taken by Nikon A1 confocal laser microscope.

II.12 RNA Isolation and RT-PCR

Isolation of total RNA from mouse heart was performed using TRIzol reagent (Life Technologies, 15596018). cDNAs were synthesized from 1 μg total RNA with the qScript One-Step RT-qPCR kit (Quanta Biosciences, 19780). Quantitative RT-PCR was performed using SYBR Green PCR Master Mix (Life Technologies, 4309155) in the StepOnePlus Real-Time PCR System according to the manufacturer's instructions. The following primers were used in the qRT-PCR assay (Table 5).

Table 5 Primers used in the qRT-PCR assay.

Gene	Species	Primer sequence	Target location
IL-1β	Mouse	Forward: 5′-TGTGAAATGCCACCTTTTGA-3′	Exon 2
		Reverse: 5′-TGTCCTCATCCTGGAAGGTC-3′	Exon 4
TNFα	Mouse	Forward: 5′-CCACCACGCTCTTCTGTCTA-3′	Exon 1
		Reverse: 5′-GGTTGTCTTTGAGATCCATGC-3′	Exon 4
MMP2	Mouse	Forward: 5′-CAGGGAATGAGTACTGGGTCTATT-3′	Exon 11
		Reverse: 5′-ACTCCAGTTAAAGGCAGCATCTAC-3′	Exon 11
MMP9	Mouse	Forward: 5′-CATGCACTGGGCTTAGATCA-3′	Exon 8
		Reverse: 5′-TGGGACACATAGTGGGAGGT-3′	Exon 9
GAPDH	Mouse	Forward: 5′-GGTGAAGGTCGGTGTGAACGGATTT-3′	Exon 2
		Reverse: 5′-GCAGAAGGGGCGGAGATGATGA-3′	Exon 4

II.13 Immunohistochemistry (IHC) Staining

Paraffin sections of the whole heart were used for detecting Ly-6G and F4/80 expression. The tissue sections were subjected to antigen retrieval, and then incubated with primary antibodies overnight at 4°C, followed by incubation with the HRP-conjugated secondary antibody. The pictures were taken by Leica DM2000 histology microscope.

II.14 2, 3, 5-Triphenyltetrazolium Chloride (TTC) Staining

Three days after myocardial infarction, the mouse heart was isolated and cut in 1.0 mm slices. The slices were then incubated with 1% w/v TTC at 37°C for 15 minutes, followed by fixation in 10% formalin for 20 minutes.

II.15 Statistical Analysis

In the two group comparisons, unpaired, 2-tailed student's *t*-test was used. All values are presented as means $\pm$ SD; *n* refers to the sample size. A value of $P < 0.05$ was considered statistically significant.

CHAPTER III

RESULTS

III.1 RhoE Is a Negative Regulator of Cardiac Inflammation

III.1.1 RhoE deletion upregulates pro-inflammatory factors in the heart

We previously defined the essential roles of RhoE in the cardiac physiology[64, 65, 67]. RhoE is downregulated in human failing heart, and RhoE closely participates in the regulation of cardiac ventricular remodeling, calcium handling and cardiac angiogenesis. To further explore the cardiac function of RhoE, gene expression profiles of wild-type and RhoE knockout mouse E10.5 hearts were analyzed. We observed a significant upregulation of a large set of pro-inflammatory factors, including cytokines/chemokines and their modulators, members of tumor necrosis factor (TNF) superfamily, interferons, immunoreceptors, matrix metalloproteinases (MMPs), etc. (Fig. 3). Since immune cells have not been developed at mouse embryonic day 10.5[73], RhoE deletion-induced elevation of pro-inflammatory factors in the heart is unexpected and intriguing.

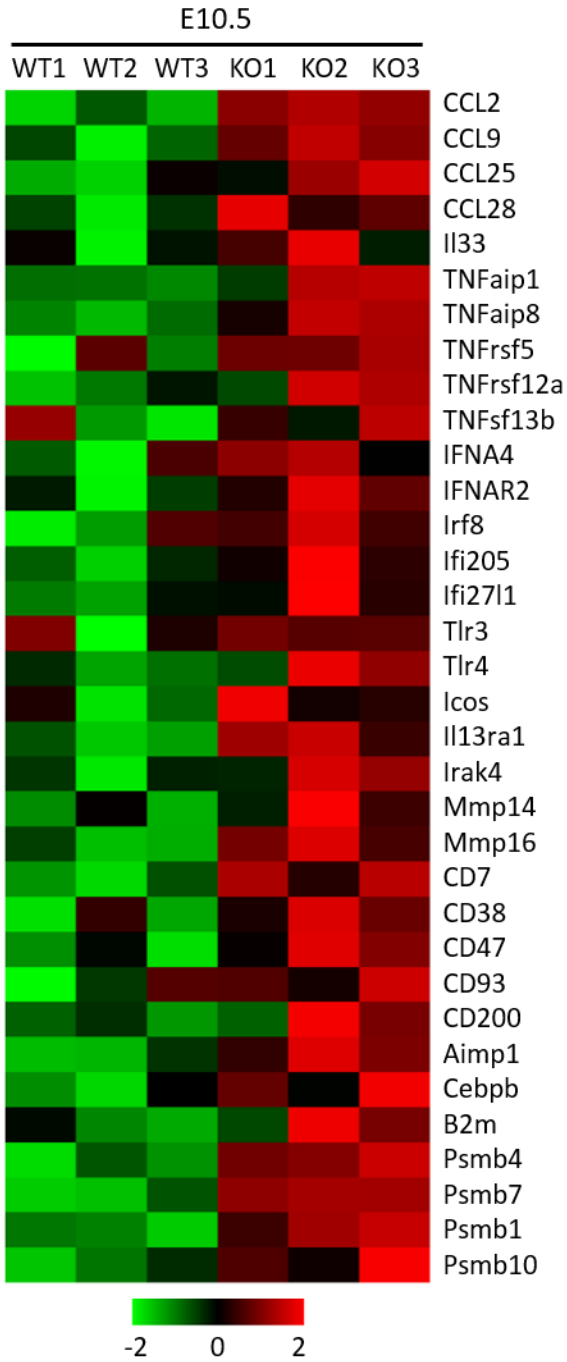

Figure 3. RhoE deletion upregulates pro-inflammatory factors in embryonic mouse heart.

Heat map represents the expression profile of proinflammatory genes in wild-type (WT) and RhoE knockout (KO) embryonic mouse E10.5 hearts.

III.1.2 RhoE deficiency promotes post-MI inflammation

To study the correlation between RhoE and the inflammatory response in the heart, we generated RhoE-floxed mouse (Fig. 4A). RhoE-floxed mouse was crossed with αMHC-Cre transgenic mouse to generate cardiac-specific RhoE haploinsufficient ($RhoE^{flox/+}$;MHC-Cre) mouse. The RhoE deficiency in the heart was examined by western blot and deceased RhoE expression was observed (Fig. 4B). The $RhoE^{flox/+}$;MHC-Cre mice were viable and did not show any obvious physical differences compared with the floxed control ($RhoE^{flox/+}$) mice. We used $RhoE^{flox/+}$;MHC-Cre and $RhoE^{flox/+}$ mice for the following studies.

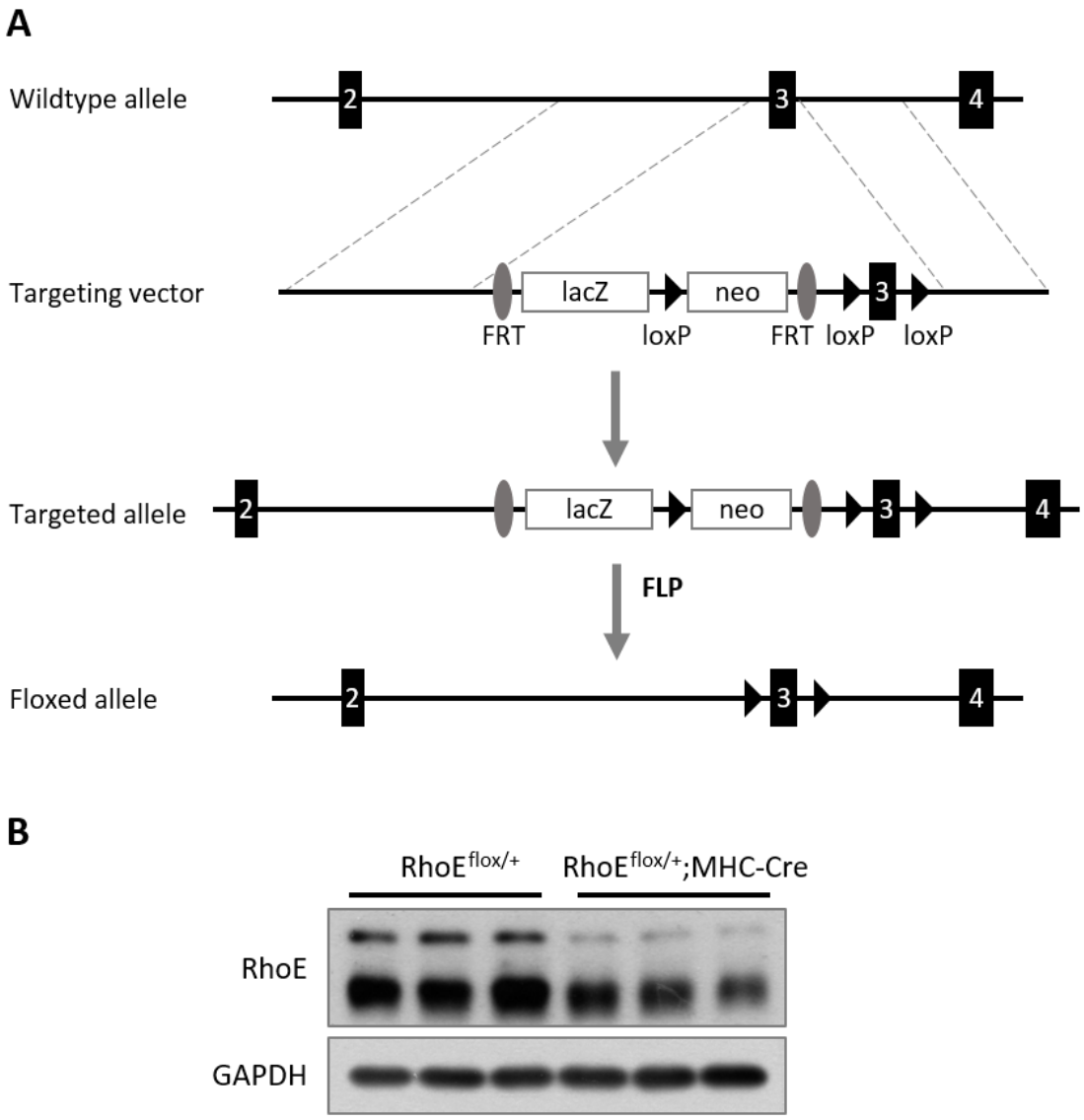

Figure 4. Generation and validation of cardiac-specific RhoE haploinsufficient mouse.

(A) Schematic diagrams of the targeting strategy for generating mouse with floxed RhoE allele.

(B) Immunoblot to validate RhoE deficiency in the $RhoE^{flox/+}$;MHC-Cre mouse heart.

Acute myocardial infarction surgery was performed in $RhoE^{flox/+}$;MHC-Cre and $RhoE^{flox/+}$ mice to induce cardiac acute inflammatory response. The cardiac inflammation was then analyzed three days after MI. We observed a significant increase of macrophage and neutrophil infiltration in the $RhoE^{flox/+}$;MHC-Cre mouse hearts compared to the $RhoE^{flox/+}$ mouse hearts (Fig. 5, A and B).

Besides the infiltration of inflammatory cells, the production of pro-inflammatory cytokines, chemokines and MMPs also represent the severity of inflammatory response. We found that RhoE deficiency also promoted the production of pro-inflammatory cytokines (IL-1β and TNFα) and MMPs (Fig. 6), indicating excessive inflammation occurred in the RhoE-deficient mouse heart after acute myocardial infarction.

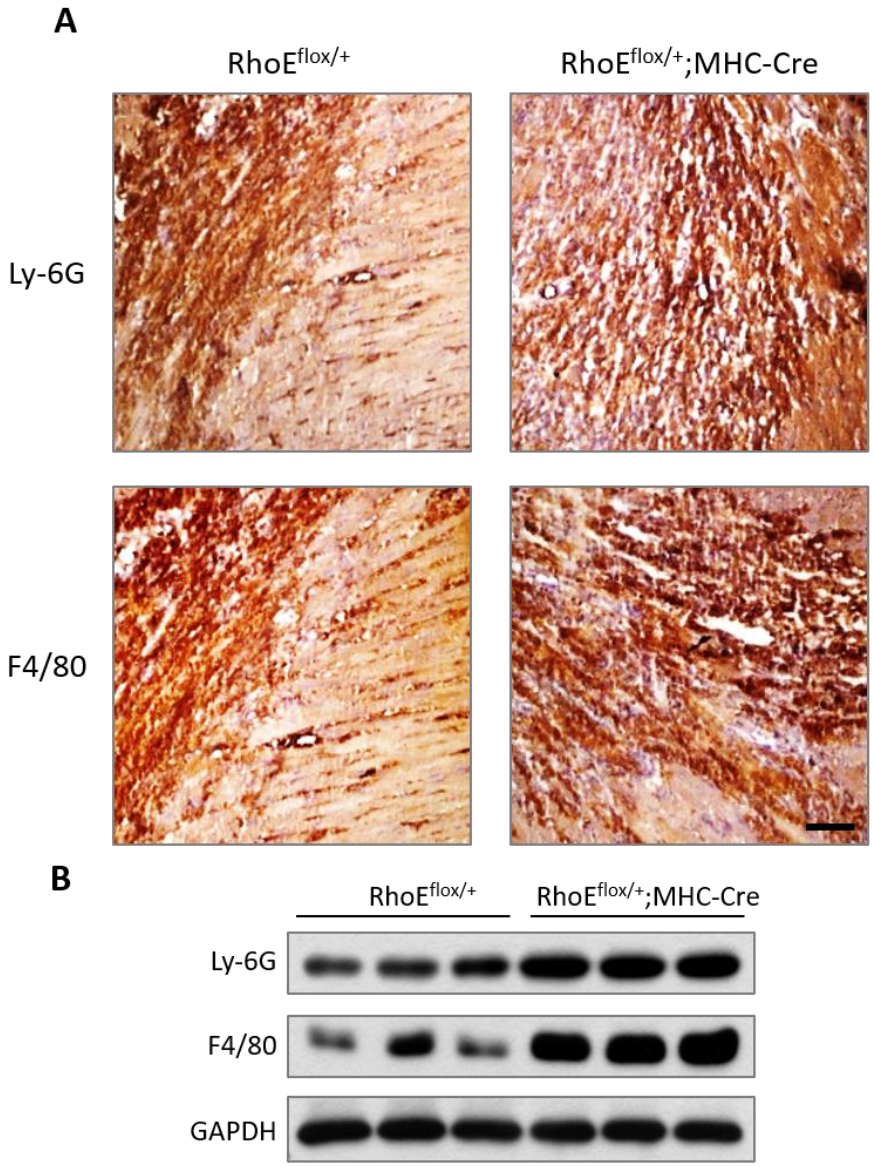

Figure 5. RhoE deficiency promotes post-MI inflammation.

(A) RhoE$^{flox/+}$ and RhoE$^{flox/+}$;MHC-Cre mouse hearts on day 3 after MI were conducted for the Immunohistochemistry staining for the neutrophil marker (Ly-6G) and the macrophage marker (F4/80). Scale bar: 0.2 mm.

(B) RhoE$^{flox/+}$ and RhoE$^{flox/+}$;MHC-Cre mouse hearts on day 3 after AMI were conducted for the immunoblotting of Ly-6G and F4/80.

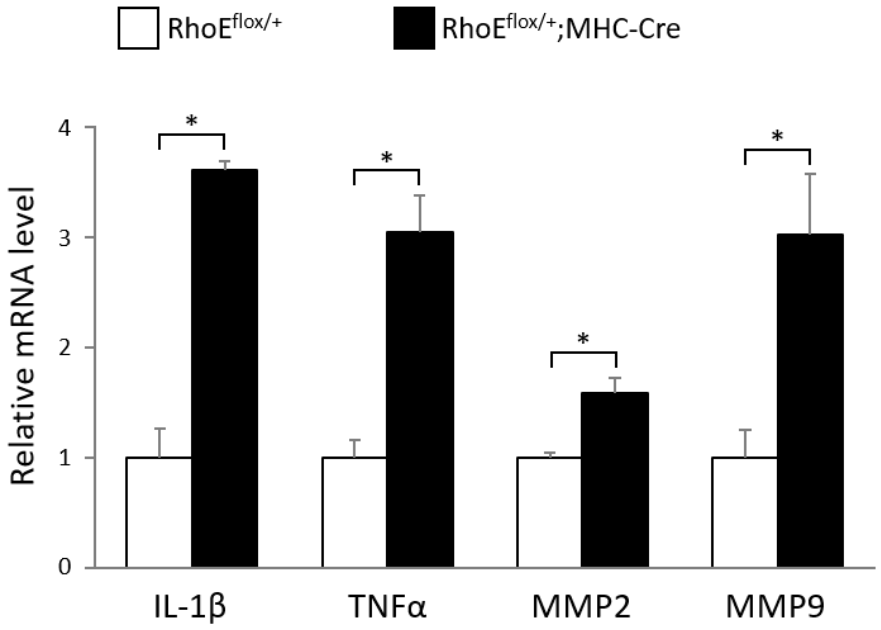

Figure 6. RhoE deficiency promotes post-MI expression of pro-inflammatory cytokines and MMPs.

$RhoE^{flox/+}$ and $RhoE^{flox/+}$;MHC-Cre mouse hearts on day 3 after MI were analyzed by qRT-PCR for the expression levels of IL-1β, TNFα, MMP2 and MMP9 relative to GAPDH. *: $P < 0.05$.

Consistent with the excessive cardiac inflammation after acute myocardial infarction, enlarged infarct size (Fig. 7A), more deteriorated cardiac function (Fig. 7B), and higher mortality rate (Fig. 8) were exhibited in RhoE deficient mice.

Collectively, these data suggest that RhoE is required in the heart to restrain post-MI inflammation, and RhoE deficiency promotes post-MI inflammation, leading to more severe myocardial infarction injuries.

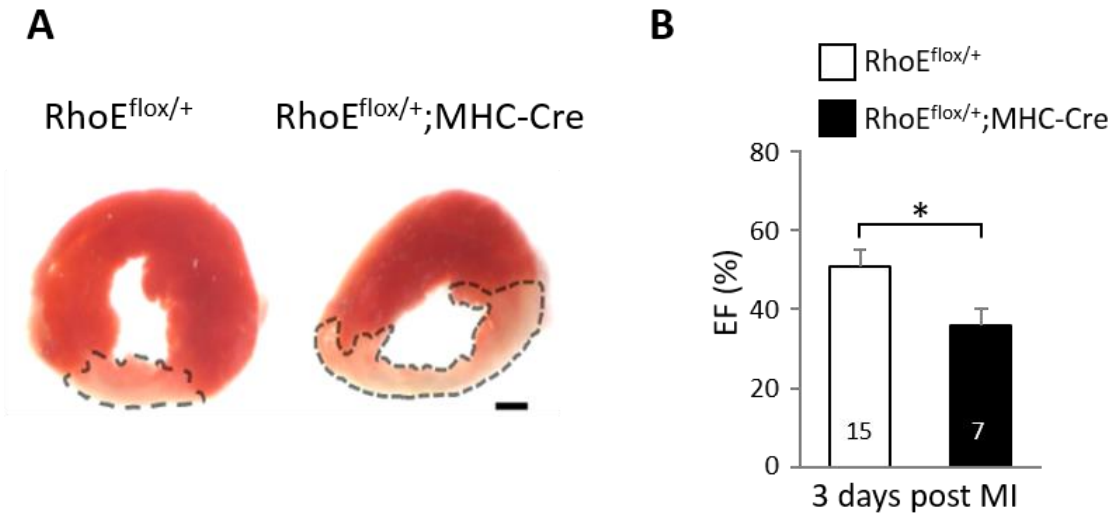

Figure 7. RhoE deficiency exaggerates AMI-induced cardiac injuries.

(A) Triphenyltetrazolium chloride (TTC) staining for the infarct size. The infarct areas were highlighted by the dashed lines. Scale bar: 1.0 mm.

(B) Ejection fraction assayed by echocardiography. *: $P < 0.05$. n: number of the mice used for the assessment.

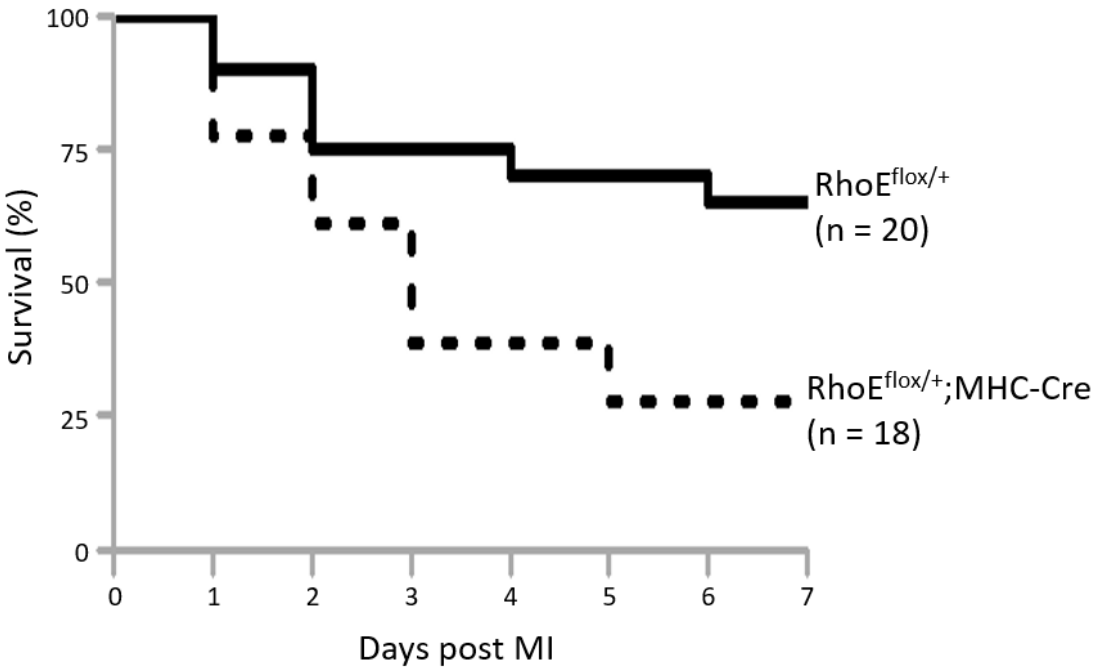

Figure 8. RhoE deficiency induces higher mortality in the first week after acute myocardial infarction.

Kaplan-Meier survival curves of the RhoE$^{flox/+}$ and RhoE$^{flox/+}$;MHC-Cre mouse in the first week post MI. n: number of the mice used for the assessment.

III.1.3 Acute myocardial infarction induces RhoE expression

Interestingly, we also observed that acute myocardial infarction induced the expression of RhoE in wild-type mouse heart during the first week post myocardial infarction (Fig. 9, A and B). RhoE protein level was upregulated during the first week after acute myocardial infarction with the peak at day 3, indicating the tight and active participation of RhoE in resolving post-MI inflammation.

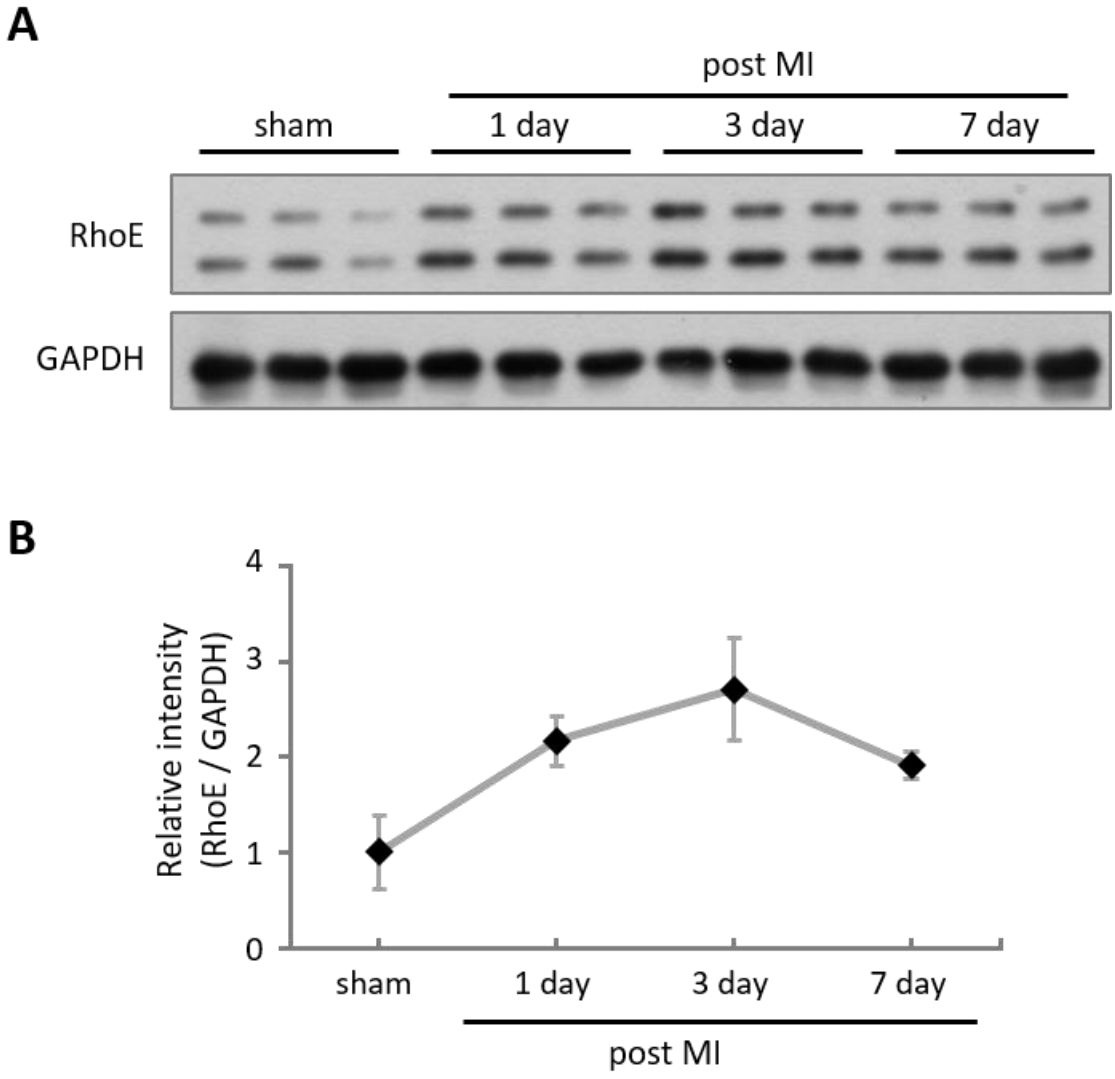

Figure 9. Acute myocardial infarction induces RhoE expression in the heart.

(A) Wild-type mice were subjected to sham operation or acute myocardial infarction surgery. The hearts were collected on day 1, 3, and 7 post myocardial infarction. Heart lysates were immunoblotted for RhoE and GAPDH.

(B) Quantification of immunoblot densities for RhoE relative to GAPDH in (A).

III.2 RhoE Negatively Regulates NF-κB Activity

III.2.1 RhoE deficiency promotes NF-κB activation in the heart

NF-κB is one of the most important regulators of inflammatory response in the heart. Overaction of NF-κB signaling triggers excessive inflammatory response during the cardiac healing process post MI, which contributes to adverse outcomes[74-76]. Here we examined if RhoE modulates post-MI inflammation via NF-κB signaling. Nuclear p65 and p50 protein levels were measured to determine the activation of NF-κB in the heart. We found that RhoE haploinsufficient ($RhoE^{flox/+}$;MHC-Cre) mouse showed markedly increased NF-κB activation compared to the control ($RhoE^{flox/+}$) mouse on day 3 post MI (Fig. 10A). Overactivation of NF-κB was also revealed in the cardiomyocytes isolated from control mouse and RhoE deficient mouse. More nuclear p65 and p50 were observed in the isolated RhoE-deficient cardiomyocytes under both resting and TNFα stimulation conditions (Fig. 10B), indicating that RhoE deficiency in cardiomyocytes promotes NF-κB activation to exaggerate inflammation in the heart.

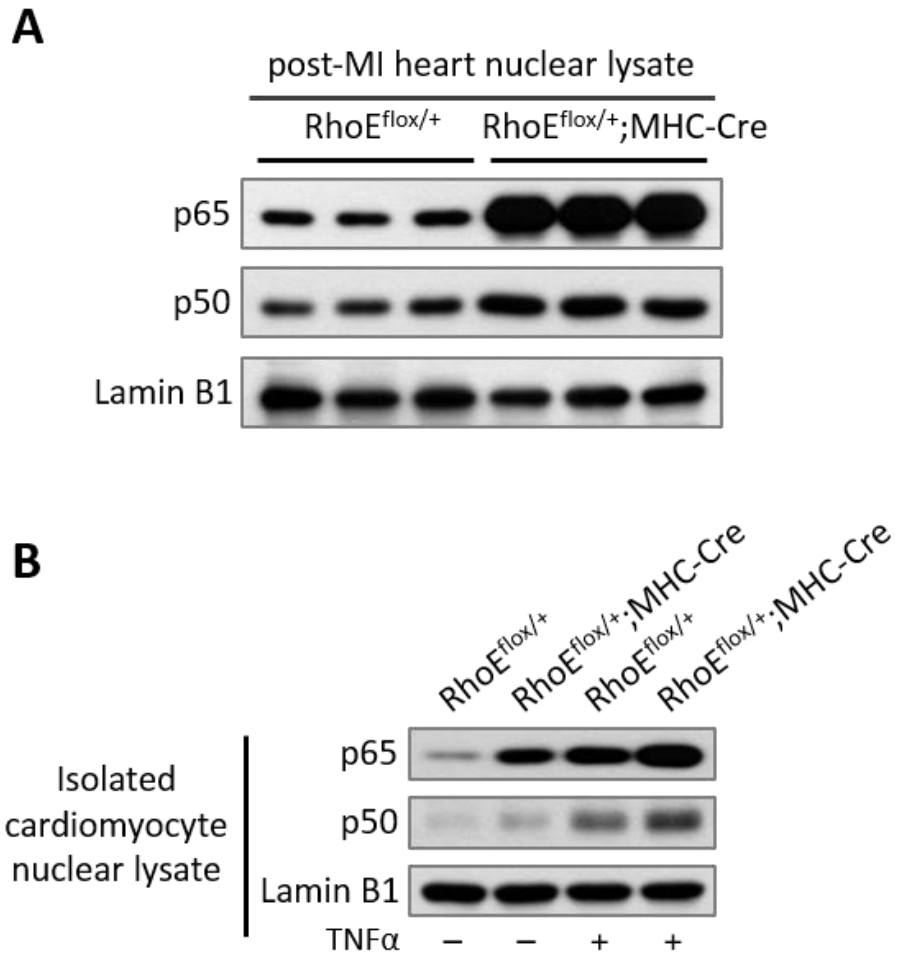

Figure 10. RhoE deficiency promotes NF-κB activation in the heart and isolated cardiomyocyte.

(A) Immunoblot for nuclear p65 and p50 in RhoE$^{flox/+}$ and RhoE$^{flox/+}$;MHC-Cre mouse hearts on day 3 post MI.

(B) Immunoblot for nuclear p65 and p50 in the adult cardiomyocytes isolated from RhoE$^{flox/+}$ and RhoE$^{flox/+}$;MHC-Cre mice. The cells were either untreated or stimulated with 40 ng/ml of TNFα for 15 minutes.

III.2.2 RhoE negatively regulates NF-κB in vitro

To examine whether RhoE is a new regulator of NF-κB, in vitro experiments were performed. NF-κB-dependent luciferase reporter assay was used to detect NF-κB activity. We found that RhoE knockdown induced about 10-fold increase in the NF-κB-dependent reporter luciferase activity (Fig. 11A). The increase was also revealed under TNFα stimulation. On the contrary, enforcing RhoE expression led to the opposite result: the luciferase activity gradually declined in parallel with incremental increases in the RhoE expression level (Fig. 11B). The inhibitory effects of RhoE for NF-κB were also verified by the electrophoretic mobility shift assay (EMSA). A strong band-shift of NF-κB-DNA complex was observed under RhoE knockdown condition in C2C12 cells (Fig. 12A), while an opposite result was detected when RhoE is overexpressed (Fig. 12B). Collectively, these in vitro results suggest that RhoE is a new suppressor of NF-κB, which is consistent with the results in the post-MI heart and the isolated cardiomyocytes.

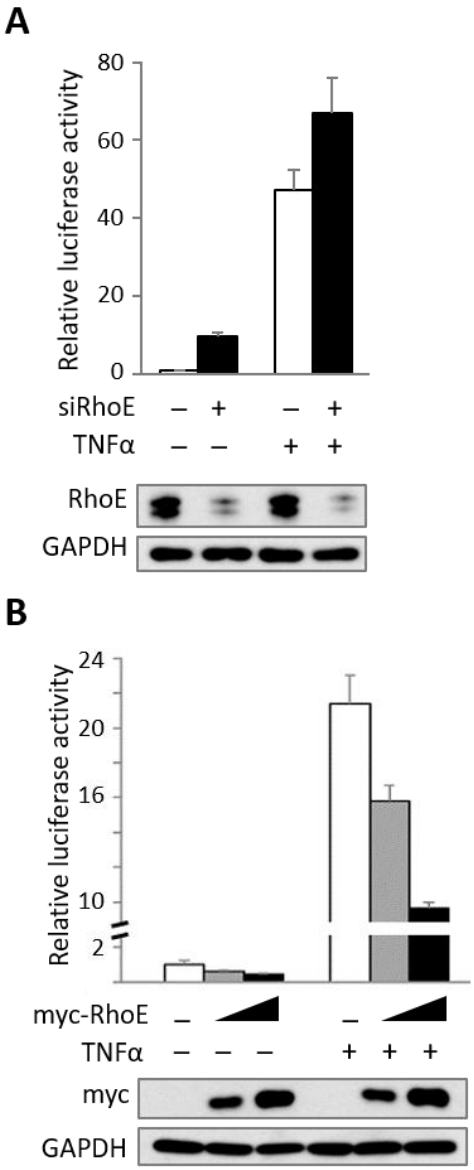

Figure 11. RhoE negatively regulates NF-κB transcriptional activity.

(A and B) NF-κB-dependent reporter luciferase assay in C2C12 cells transfected with control siRNA or RhoE-specific siRNA **(A)**, and in C2C12 cells transfected with empty vector or myc-RhoE expression construct **(B)**. The cells were either untreated or stimulated with 40 ng/ml of TNFα for 4 hours. The expression levels of RhoE were assessed by immunoblot.

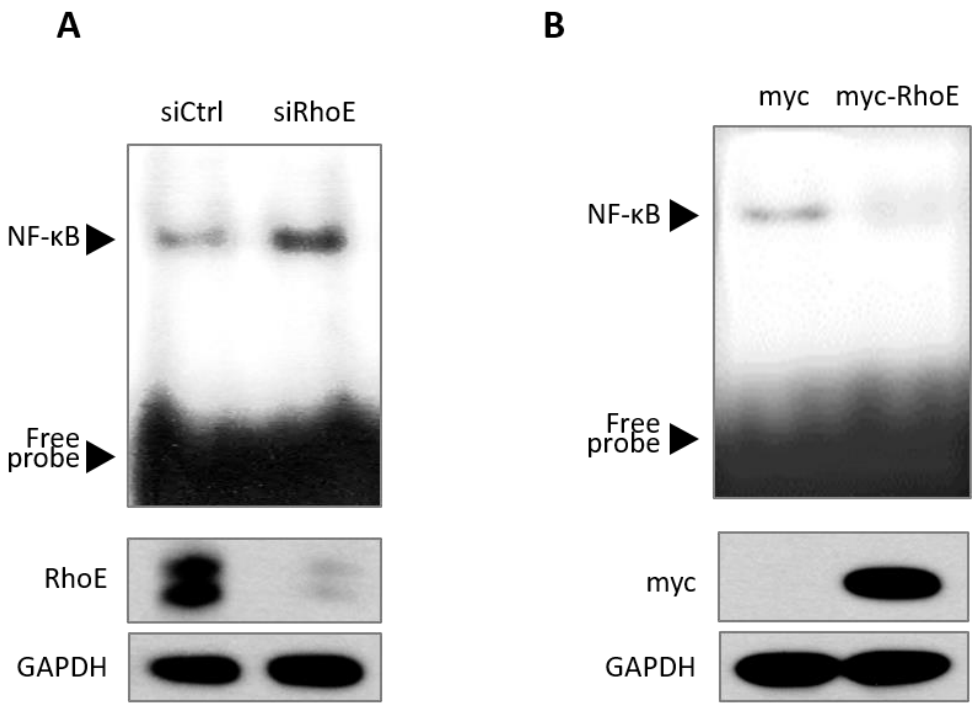

Figure 12. RhoE negatively regulates NF-κB DNA binding activity.

(A and B) EMSA assay for NF-κB activity in C2C12 cells transfected with control siRNA or RhoE-specific siRNA **(A)**, or in C2C12 cells transfected with empty vector or myc-RhoE expression construct **(B)**. The expression levels of RhoE were assessed by immunoblot.

III.3 RhoE Sequesters NF-κB in the Cytosol

III.3.1 RhoE binds to p65 and p50 in GST-pull-down

To study RhoE's regulatory mechanisms on NF-κB, we assessed the associations of RhoE with the NF-κB components: p65 and p50. GST and GST-RhoE recombinant proteins were first expressed in E. coli bacterial. The recombinant proteins were released by ultrasonication and bound by glutathione sepharose. The recombinant proteins were further purified by high-performance liquid chromatography and subsequently used to examine the binding with p65 and p50. In vitro GST pull-down showed strong bindings of GST-RhoE recombinant protein to His-p65 and His-p50, respectively (Fig. 13, A and B).

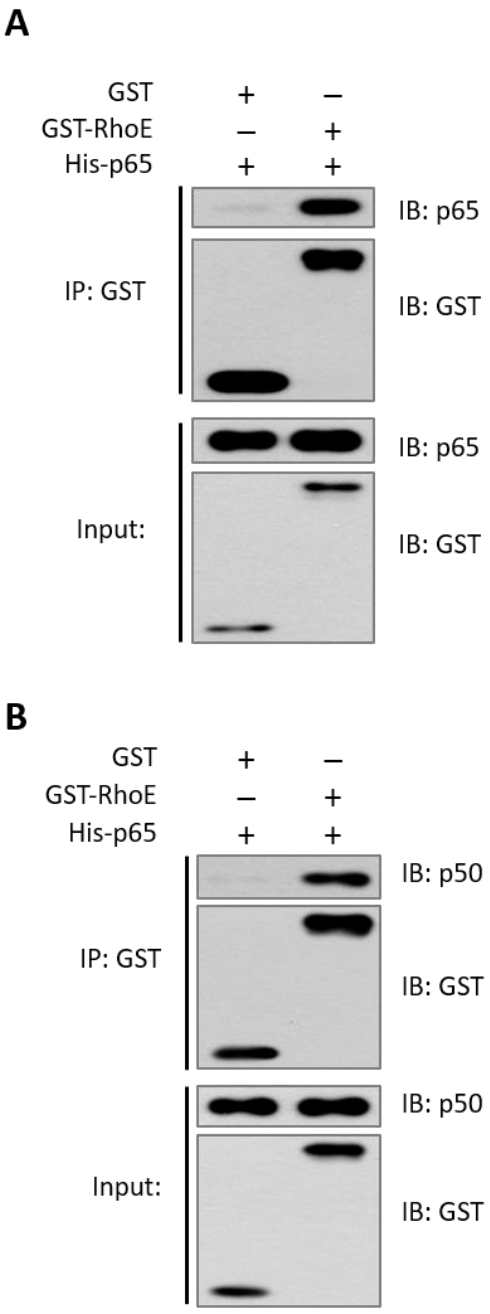

Figure 13. RhoE physically binds to p65 and p50 individually.

(**A**) In vitro GST pull-down assay of GST and GST-RhoE fusion proteins with His-p65 fusion protein.

(**B**) In vitro GST pull-down assay of GST and GST-RhoE fusion proteins with His-p50 fusion protein

III.3.2 RhoE binds to p65 and p50 in co-immunoprecipitation

We also performed co-immunoprecipitations to further verify the interaction of RhoE with p65 and p50. The HEK293T cells were transfected with myc-RhoE expression construct, and the mutual physical interaction of ectopic RhoE with endogenous p65 and p50 were analyzed. myc-RhoE showed strong binding to p65 and p50, individually (Fig. 14, A and B).

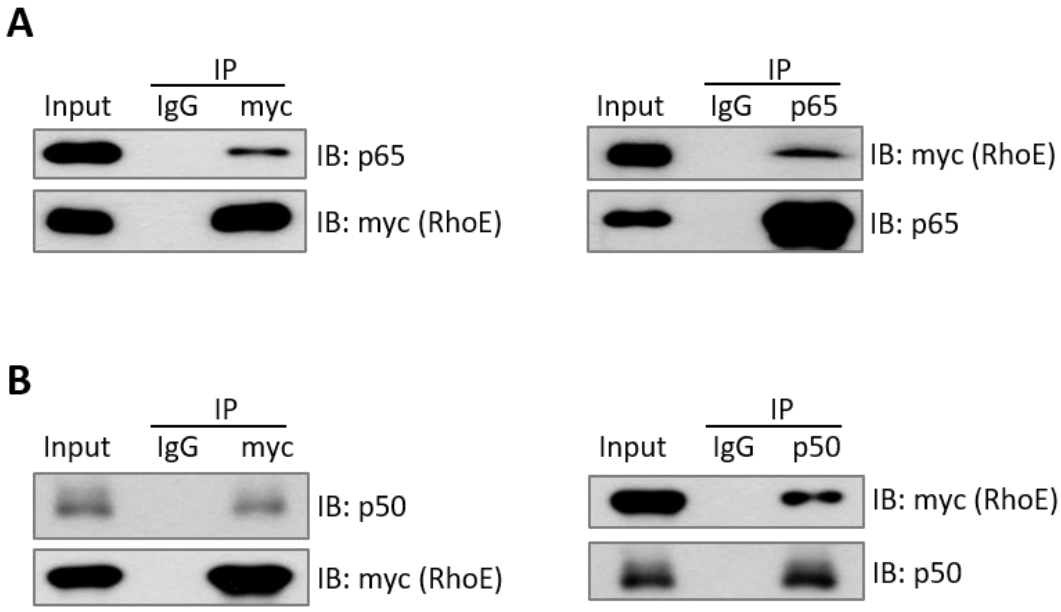

Figure 14. Mutual interactions of RhoE with p65 and p50.

(**A and B**) Reciprocal immunoprecipitations of overexpressed myc-RhoE with endogenous p65 **(A)** or with endogenous p50 **(B)** in HEK293T cells.

III.3.3 RhoE interacts with p65 and p50 in the cell cytosol

Furthermore, we examined the interaction localization of RhoE with p65 and p50 by bimolecular fluorescence complementation (BiFC). BiFC is an imaging technology used to visualize protein-protein interaction within a cell. The assay is based on re-formation of a functional fluorescent protein when two non-fluorescent fragments are brought together by two interacting proteins. The result provides the location and the intensity of the interaction between the two proteins[77]. Here, we fused RhoE to the N-terminus of fluorescent protein Venus (termed VN-RhoE); and p65 or p50 to the C-terminus of fluorescent protein Venus (termed VC-p65 or VC-p50). The constructs used in the assay were listed in Table 6.

In the BiFC assay, no fluorescent signal was detected when two non-interactive proteins were co-expressed in C2C12 cells, which presented as negative controls (Fig. 15). However, clear fluorescent signal was exhibited in cells co-transfected of VN-RhoE and VC-p65, or VN-RhoE and VC-p50, further confirming the physical interactions of RhoE with p65 and p50, respectively (Fig. 16). Importantly, the fluorescent signal was solely detected in the cell cytosol, suggesting that RhoE interacts with p65 and p50 individually only in the cell cytosol to suppress NF-κB activation.

Table 6 Constructs used in BiFC assay.

Construct name	Vector	Insert
VN	**N**-terminus of fluorescent protein Venus	—
VN-RhoE	**N**-terminus of fluorescent protein Venus	RhoE
VC	**C**-terminus of fluorescent protein Venus	—
VC-p65	**C**-terminus of fluorescent protein Venus	p65
VC-p50	**C**-terminus of fluorescent protein Venus	p50

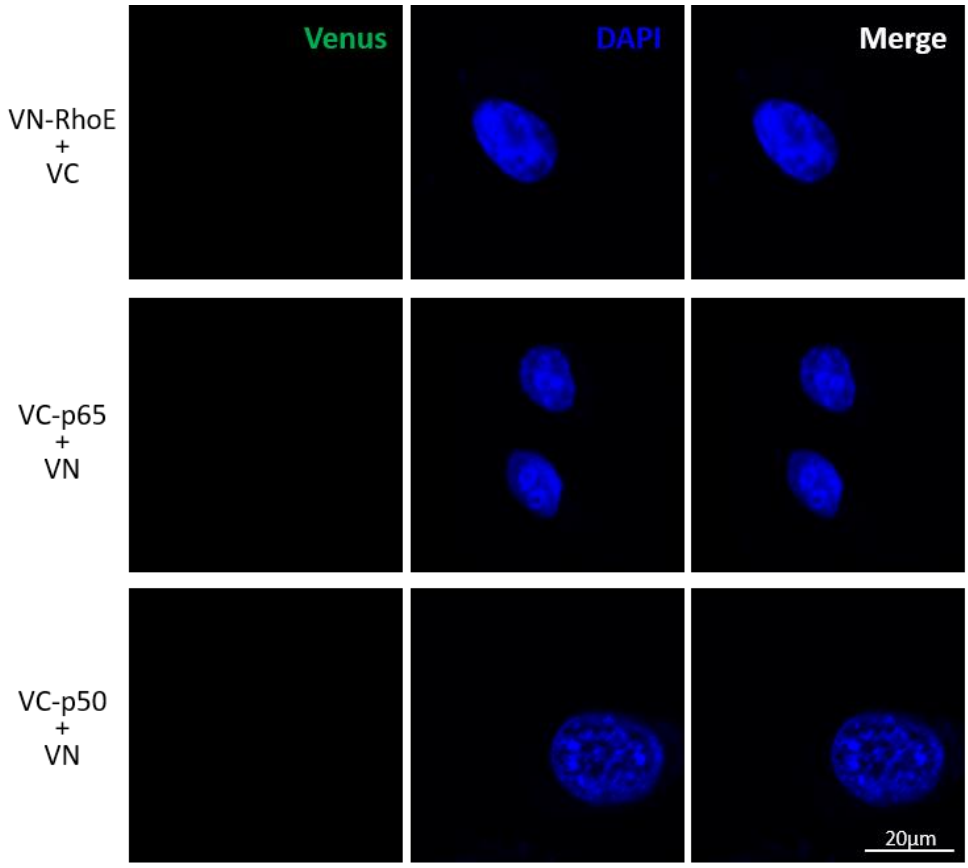

Figure 15. Negative controls in the BiFC assay.

C2C12 cells were co-transfected with a pair of VN-RhoE and VC, VC-p65 and VN, or VC-p50 and VN, respectively. The nucleus was counterstained with DAPI dye.

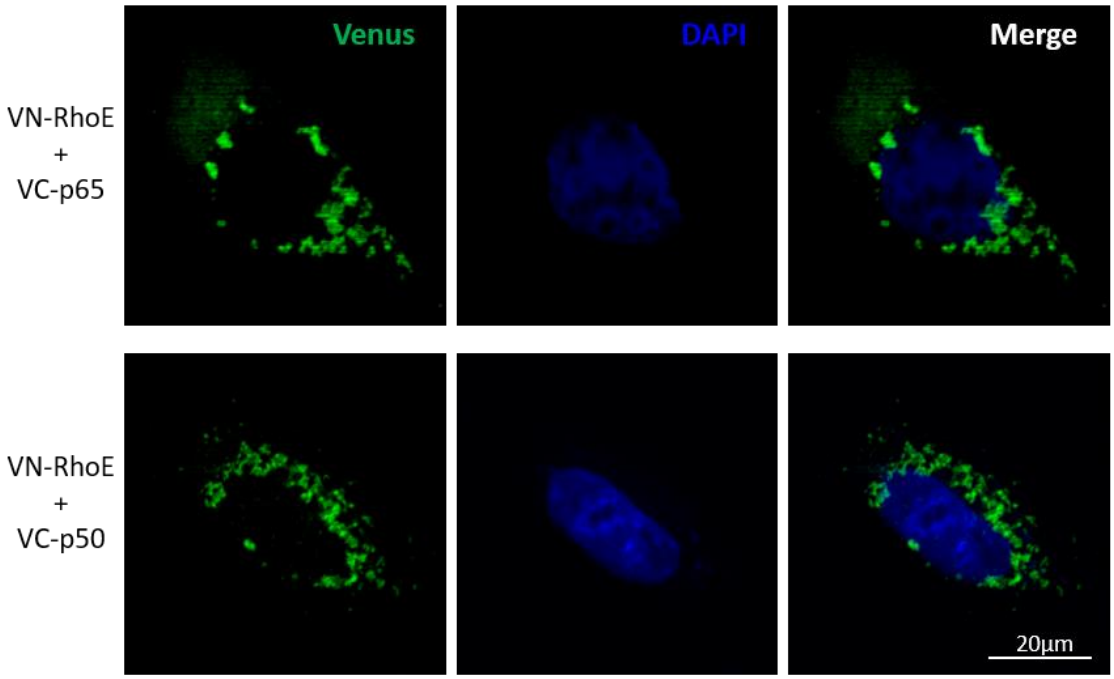

Figure 16. RhoE interacts with p65 and p50 in BiFC assay.

C2C12 cells were co-transfected with expression constructs of VN-RhoE and VC-p65, or VN-RhoE and VC-p50, respectively. 24 hours after transfection, the cell nucleus was counterstained in blue with DAPI dye. Venus and DAPI were examined by confocal fluorescent microscope.

III.3.4 RhoE does not act upstream of IκBα to regulate NF-κB

Known cytosolic suppressors for NF-κB exert their inhibitory effects mainly through stabilizing IκBα, promoting p65 and p50 degradation, inhibiting p65 phosphorylation, and blocking NF-κB nuclear translocation[78-81]. We examined these regulatory mechanisms to look for how RhoE suppresses NF-κB in the cytosol. Silencing or enforcing RhoE expression did not influence the phosphorylation and degradation of IκBα (Fig. 17, A and B), indicating that RhoE does not act in the upstream of IκBα and does not influence the stabilization of IκBα. Manipulation of RhoE expression also had no effect on the total level of p65 and p50 as well as the phosphorylation level of p65 at Ser536 site (Fig. 17, A and B), suggesting that RhoE does not affect p65 and p50 degradation as well as the phosphorylation of p65.

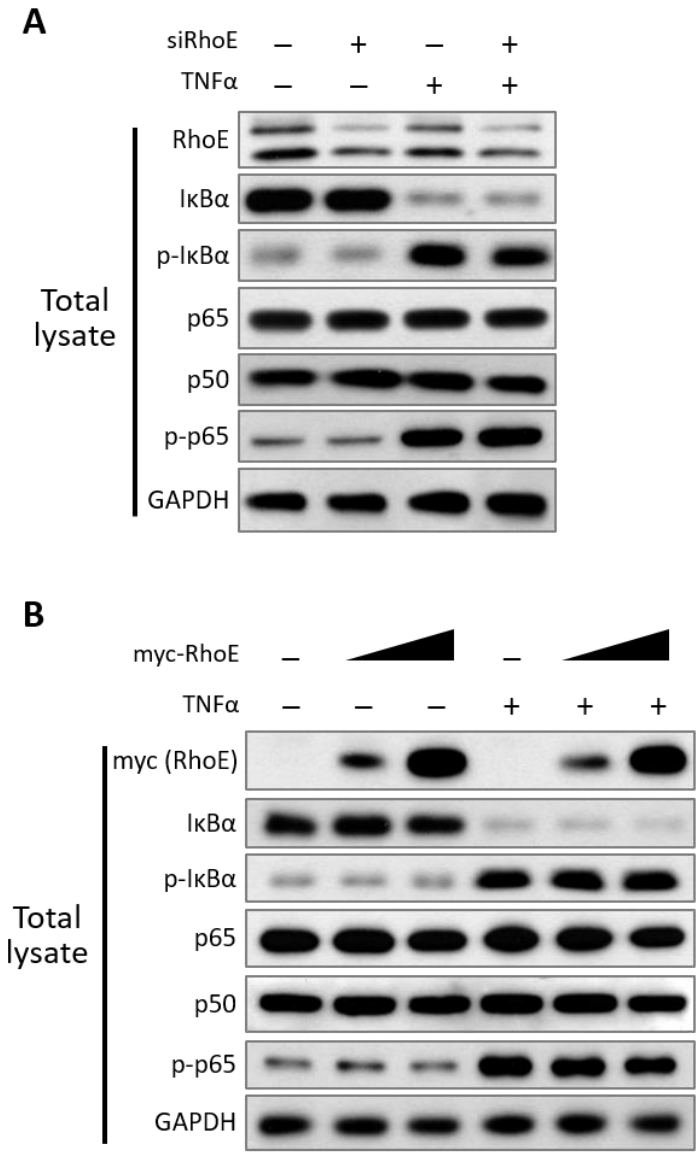

Figure 17. RhoE does not act upstream of IκBα.

C2C12 cells were transfected with control siRNA or RhoE-specific siRNA **(A)**; and empty vector or myc-RhoE expression construct **(B)**. Cells were either untreated or stimulated with 40 ng/ml TNFα for 15 minutes. The total lysates were immunoblotted for RhoE, IκBα, phosphor-IκBα at Ser32, p65, p50, phosphor-p65 at Ser536, and GAPDH.

III.3.5 RhoE blocks NF-κB nuclear translocation

Then we measured nuclear p65 an p50 protein levels to examine whether RhoE affects nuclear translocation of the NF-κB complex. Knockdown of RhoE significantly augmented nuclear p65 and p50 protein levels in both resting and TNFα-stimulated C2C12 cells (Fig. 18A), while overexpression of RhoE effectively reduced nuclear p65 and p50 (Fig. 18B), suggesting that RhoE functions in the cell cytosol to inhibit the nuclear translocation of NF-κB.

IκBα is recognized as the major inhibitor of NF-κB nuclear translocation[82]. We examined if IκBα is responsible for RhoE-mediated inhibition of NF-κB nuclear translocation. Knockdown of IκBα augmented nuclear NF-κB as expected (Fig. 19). However, overexpression of RhoE remained to attenuate NF-κB nuclear translocation under the IκBα knockdown condition (Fig. 19), indicating that RhoE inhibits NF-κB nuclear translocation independently of IκBα.

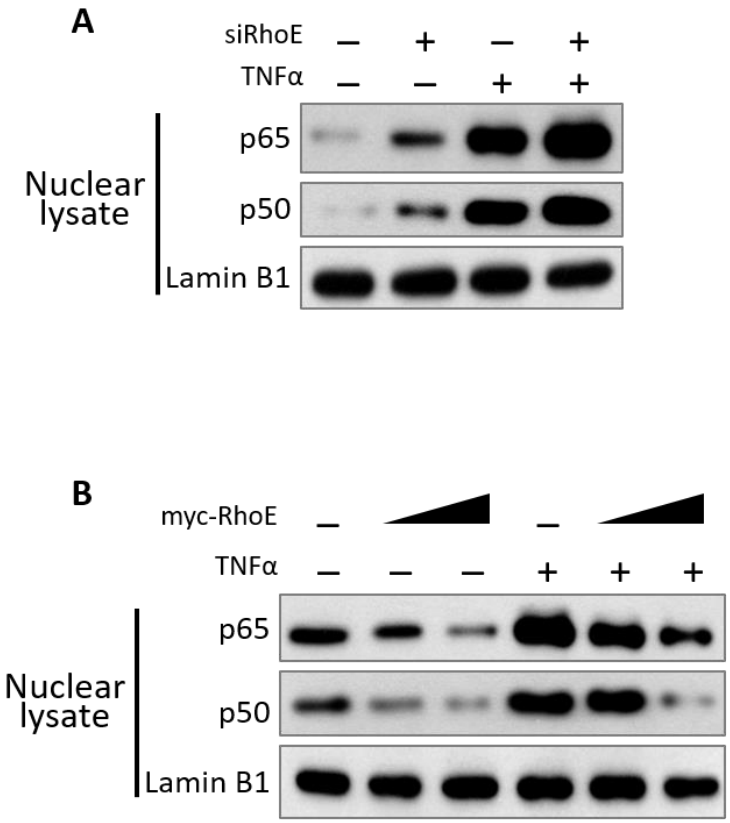

Figure 18. RhoE inhibits NF-κB nuclear translocation.

C2C12 cells were transfected with control siRNA or RhoE-specific siRNA **(A)**, and empty vector or myc-RhoE expression construct **(B)**. Cells were either untreated or stimulated with 40 ng/ml TNFα for 15 minutes. The nuclear lysates were extracted and immunoblotted for p65, p50 and Lamin B1.

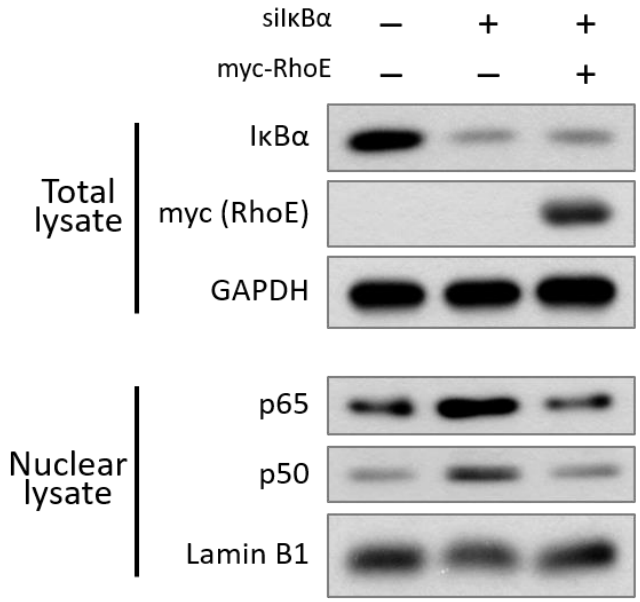

Figure 19. RhoE inhibits NF-κB nuclear translocation independent of IκBα.
C2C12 cells were transfected with control siRNA or IκBα-specific siRNA together with empty vector or myc-RhoE expression construct. Total lysates were immunoblotted for IκBα, myc and GAPDH. Nuclear lysates were immunoblotted for p65, p50 and Lamin B1.

III.4 RhoE Impedes the Heterodimerization of p65 and p50

III.4.1 RhoE binds to p50 on multiple interaction sites

We further identified the critical interaction regions of p50 and p65 with RhoE. A series of p50 and p65 truncated mutants were generated for co-immunoprecipitation assay. We found that both the N-terminus (1-245 aa) and C-terminus (246-443 aa) of p50 bound to RhoE (Fig. 20), indicating that RhoE associates with p50 via multiple interaction sites.

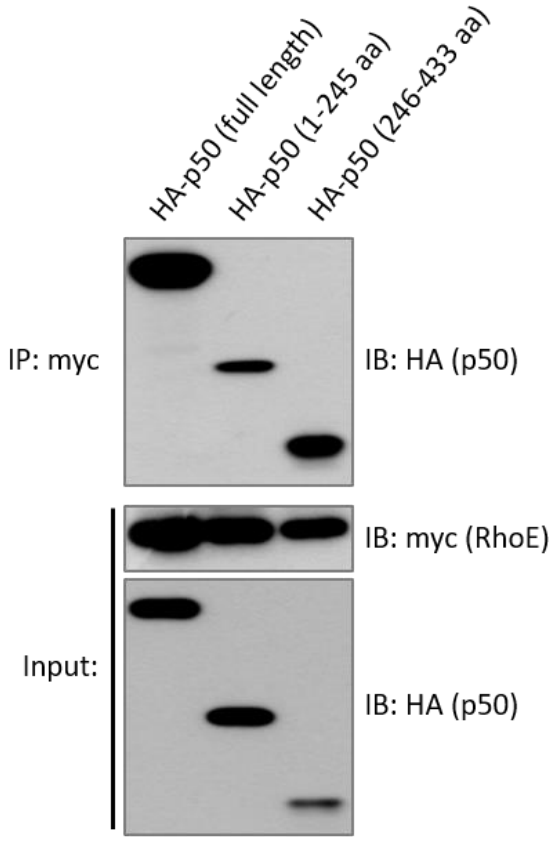

Figure 20. RhoE binds to p50 on multiple interaction sites.

HEK293T cells were transfected of myc-RhoE expression construct together with HA-p50, HA-p50 (1-245 aa), or HA-p50 (246-433 aa) expression construct. Co-immunoprecipitation assay was performed with anti-myc antibody.

III.4.2 RhoE binds to the dimerization domain of p65

Next, we examined the interaction region of p65 with RhoE. A series GFP-tagged p65 mutants were generated and used for co-immunoprecipitation assay (Fig. 21). We found that RhoE only bound to p65 N-terminus (1-286 aa) but not to its C-terminus (286-551 aa) (Fig. 22). The N-terminus of p65 contains two functional domains, the N-terminal domain (NTD, 1-186 aa), responsible for DNA binding activity; and the dimerization domain (DD, 187-286 aa), required for homo- or hetero-dimerization of p65 with other NF-κB subunits[83]. We found that the dimerization domain, but not the N-terminal domain of p65, is responsible for binding to RhoE (Fig. 22).

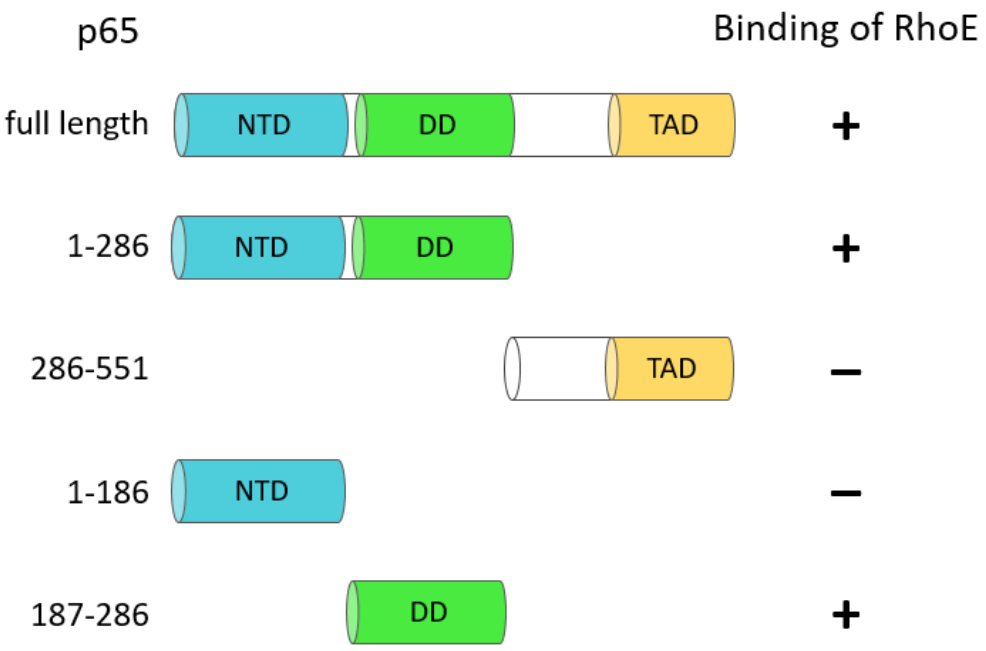

Figure 21. Schematic of p65 domains and truncated mutants.

NTD: N-terminal domain; DD: dimerization domain; TAD: transactivation domain.

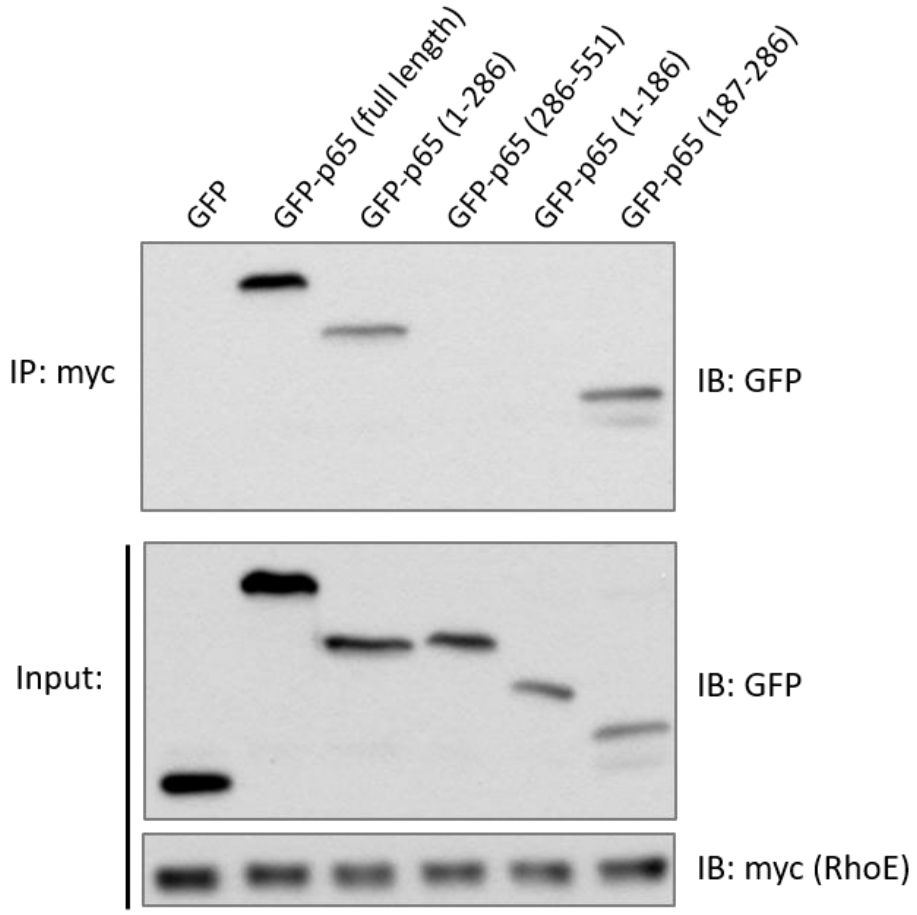

Figure 22. RhoE binds to the dimerization domain of p65.

HEK293T cells were co-transfected of myc-RhoE expression construct with GFP-tagged p65 or its truncated mutants. Cell lysates were used for co-immunoprecipitation assay.

Furthermore, we generated a series of VC-tagged p65 mutants to visualize the interaction between p65 fragments and RhoE in BiFC assay. Consistent with the Co-IP results, dimerization domain of p65 was showed to be essential for the interaction with RhoE (Fig. 23).

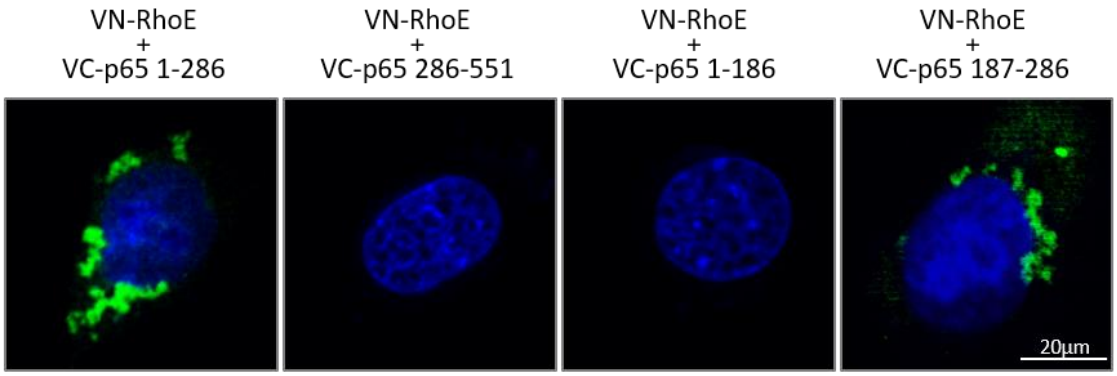

Figure 23. RhoE binds to the dimerization domain of p65 in BiFC assay.
C2C12 cells were co-transfected with VN-RhoE and VC-p65 truncated mutants. 24 hours after transfection, the cell nucleus was counterstained in blue with DAPI dye. Venus and DAPI were examined by confocal fluorescent microscope.

III.4.3 RhoE disrupts p65/p50 heterodimer

The dimerization domain of p65 is critical for its dimerization with other NF-κB subunits, which is an important step for NF-κB activation[83]. Thus, the binding of RhoE to the dimerization domain of p65 may impede the heterodimerization between p65 and p50. We used co-immunoprecipitation assay to determine the level of p65/p50 heterodimer. Enforcing RhoE expression led to decrease of p65-bound p50 (Fig. 24A) and p50-bound p65 (Fig. 24B). The data therefore suggest that RhoE also disrupt the heterodimerization of p65 and p50 to inhibit NF-κB activity.

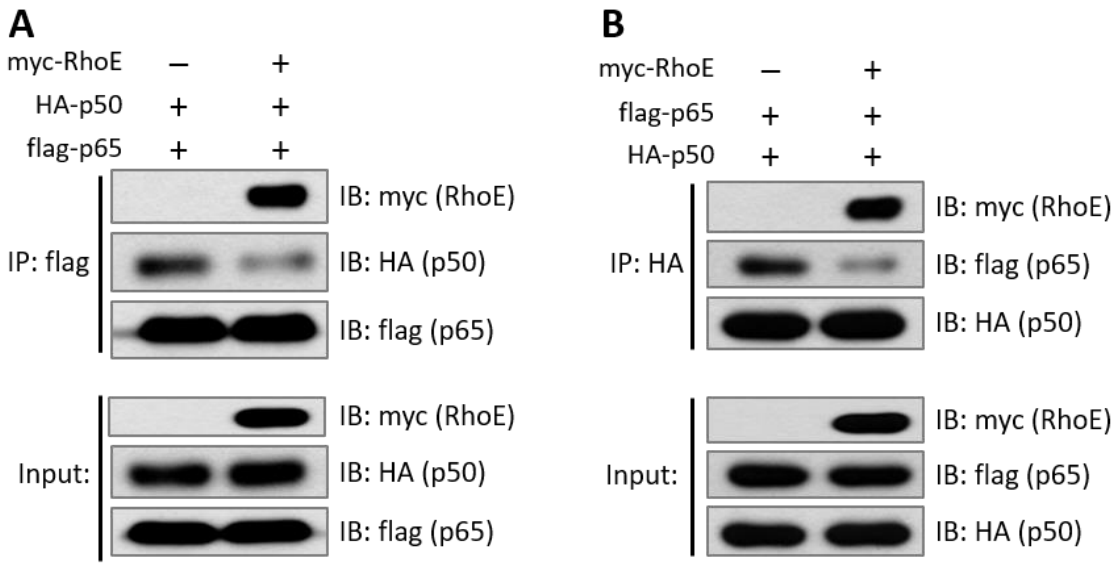

Figure 24. RhoE impede the heterodimerization between p65 and p50.

(A and B) HEK293T cells were transfected of HA-p50 and flag-p65 expression constructs together with empty vector or myc-RhoE expression construct. The interaction between p65 and p50 was analyzed by co-immunoprecipitation using anti-flag **(A)** or anti-HA **(B)** antibodies as the immunoprecipitation antibody.

III.5 RhoE Overexpression Protects Heart from MI Injury

III.5.1 RhoE overexpression in heart inhibits NF-κB activation

Transgenic mouse with cardiac-specific RhoE overexpression (RhoE-TG) was previously generated in our lab[65]. We took advantage of this gain-of-function genetic mouse to test whether upregulation of RhoE could diminish the cardiac inflammation. Decreased nuclear p65 and p50 were observed in the isolated cardiomyocytes from RhoE-TG mouse compared to wildtype mouse (Fig. 25A). Consistent with this result, RhoE-TG mouse showed augmented nuclear NF-κB protein levels in the heart post MI than wild-type mouse (Fig. 25B), which further confirmed RhoE's inhibitory role on NF-κB.

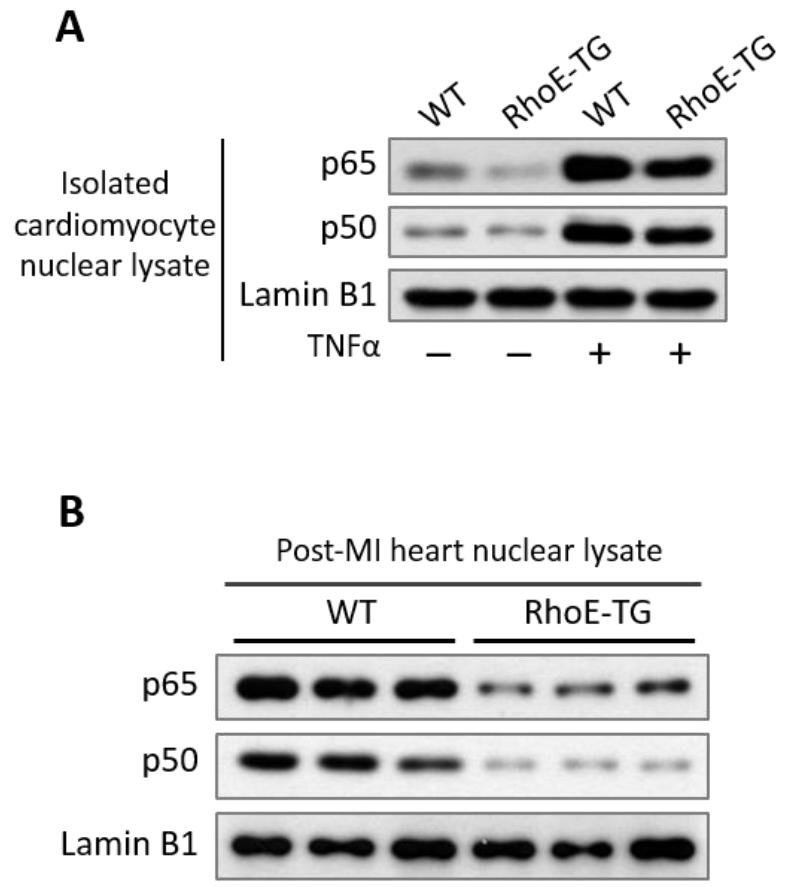

Figure 25. RhoE overexpression reduces nuclear p65 and p50 in cardiomyocyte and post-MI heart.

(**A**) Immunoblot for nuclear p65 and p50 in cardiomyocytes isolated from WT and RhoE-TG mice. The cells were either untreated or stimulated with 40 ng/ml of TNFα for 15 minutes. (**B**) Immunoblot for nuclear p65 and p50 in heart nuclear lysates from WT and RhoE-TG mice on day 3 after MI.

III.5.2 RhoE overexpression in heart diminishes post-MI inflammation

The degree of cardiac inflammation in the RhoE-TG mouse was also evaluated on day 3 post MI. Moderate neutrophil and macrophage infiltration with less production of pro-inflammatory cytokines (IL-1β and TNFα) and MMPs were observed in the RhoE-TG mouse heart compared to the WT mouse heart (Fig. 26 and 27). Furthermore, RhoE-TG mouse showed reduced infarct size, better preserved cardiac function, and higher survival rate post MI compared to WT mouse (Fig. 28 and 29). Together, these results have provided physiological evidence to support RhoE as an endogenous suppressor of NF-κB to diminish post-MI inflammation.

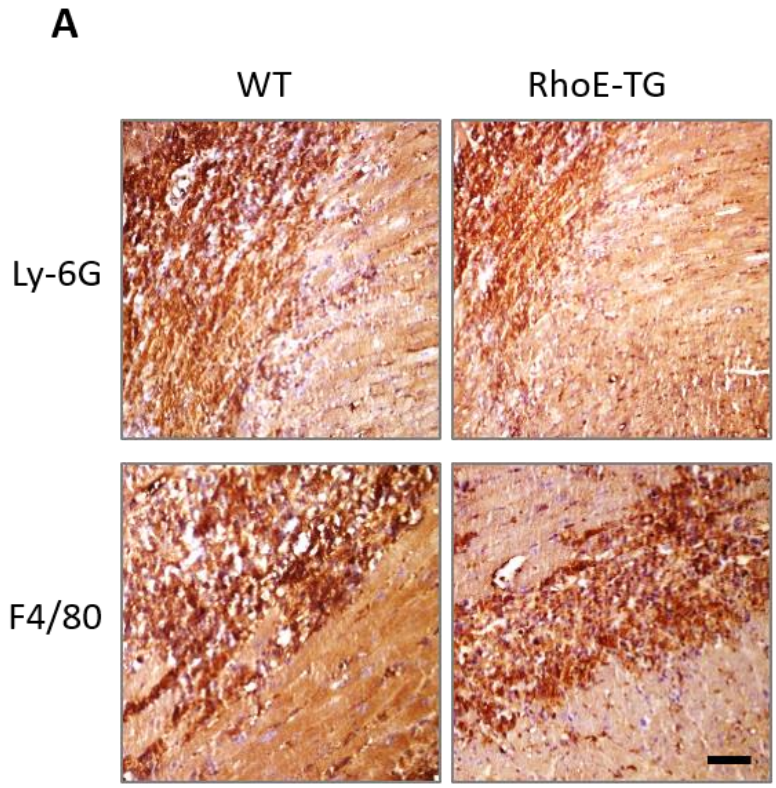

Figure 26. RhoE overexpression diminishes post-MI leukocyte infiltration.
(A and B) WT and RhoE-TG mouse heart were conducted for the following assays on day 3 post MI: immunohistochemistry staining **(A)** and immunoblot **(B)** for the neutrophil marker (ly-6G) and the macrophage marker (F4/80). Scale bar: 0.2 mm.

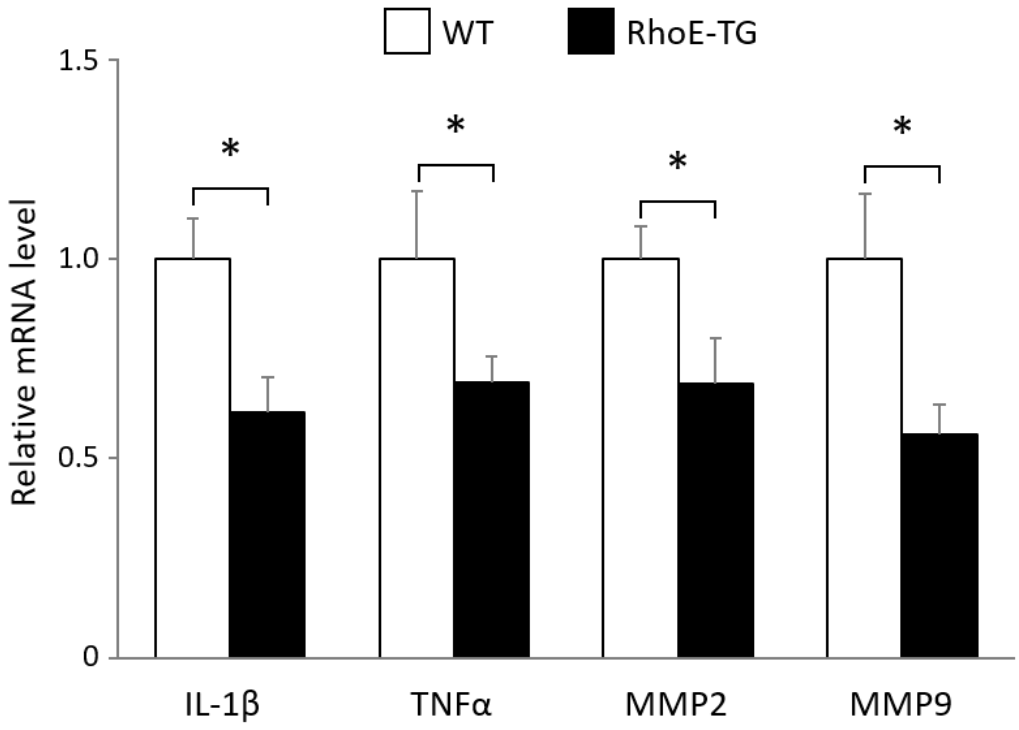

Figure 27. RhoE overexpression suppresses post-MI expression of cytokines and MMPs.

On day 3 post MI, WT and RhoE-TG mouse heart were analyzed for expression of IL-1β, TNFα, MMP2 and MMP9 by qRT-PCR.

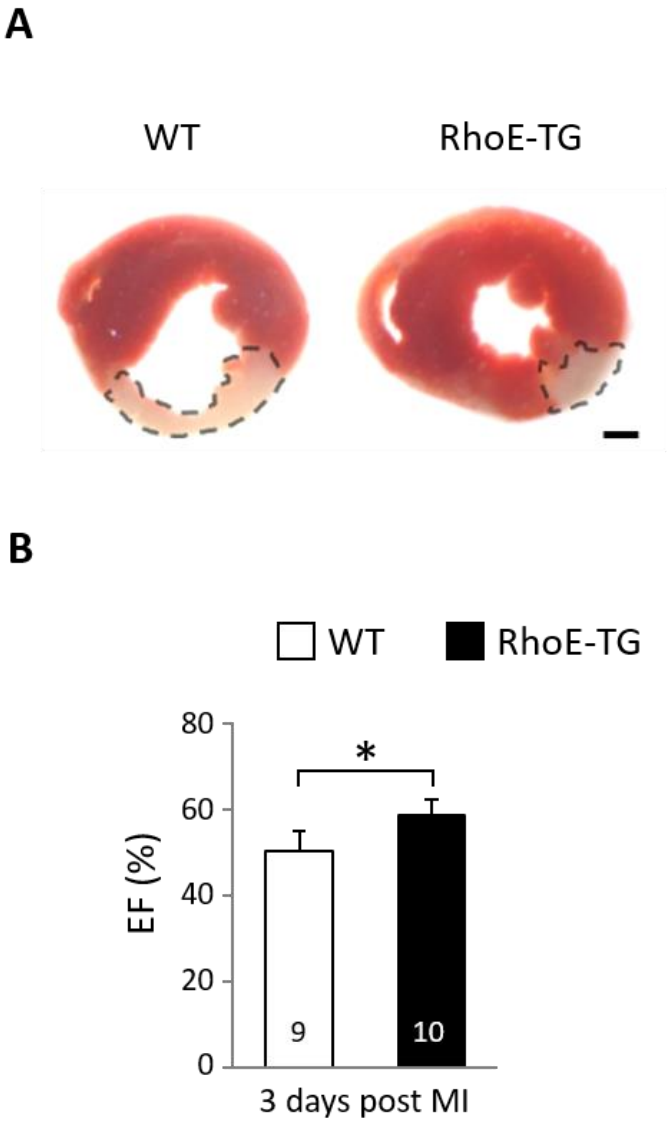

Figure 28. RhoE overexpression reduces infarct size and improves cardiac function post MI.

(A) On day 3 post MI, WT and RhoE-TG mouse hearts were analyzed for the infarct size by triphenyltetrazollum chloride (TTC) staining. The infarct areas were highlighted by the dashed lines. Scale bar: 1.0 mm.

(B) On day 3 post MI, WT and RhoE-TG mice were analyzed for the cardiac function by echocardiography. n: number of the mice used for assessment. * $P < 0.05$.

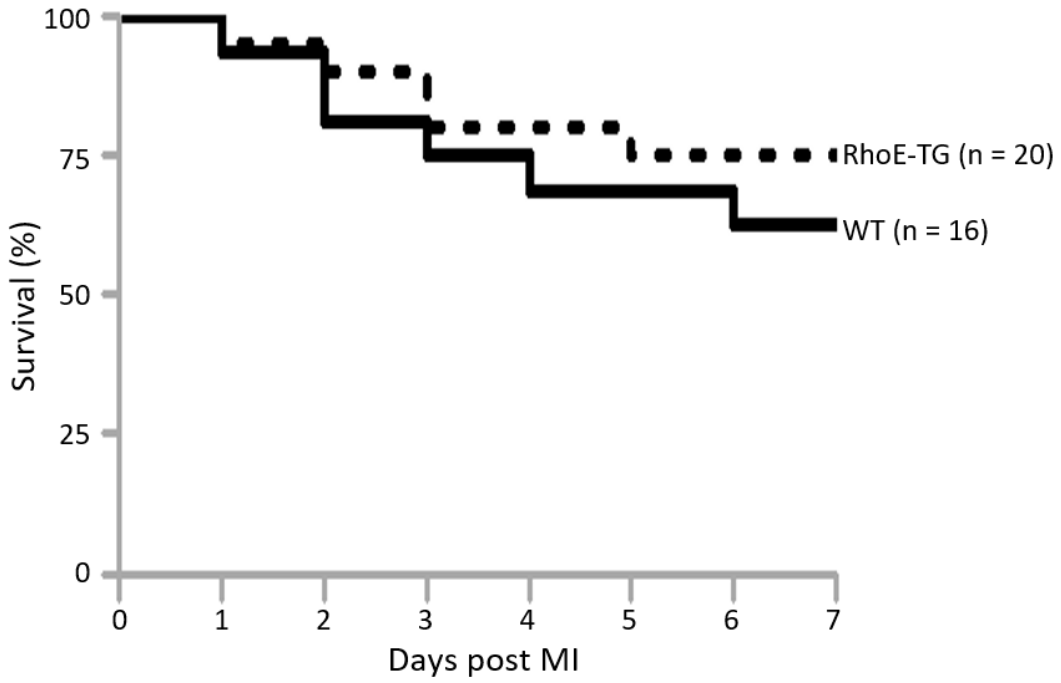

Figure 29. RhoE overexpression improves survival rate post MI.

Kaplan-Meier survival curves in the first week post MI. n: number of the mice used for assessment.

CHAPTER IV

SUMMARY

IV.1 Conclusions

Cardiac inflammation in the acute myocardial infarction is a prerequisite for healing and repairing of the infarcted myocardium, while tight control and timely suppression of this acute inflammatory response is important to restrain from inflammatory injury and excessive myocardium degradation[19]. In this study, RhoE is identified as a negative mediator of post-MI inflammation by acting on its upstream regulator, NF-κB. RhoE specifically inhibits NF-κB activation through two novel mechanisms: the blockage of NF-κB nuclear translocation, and the disruption of p65/p50 heterodimer (Fig. 30). Furthermore, RhoE overexpression in the heart protects heart against post-MI inflammatory injuries. RhoE, therefore, may provide a new anti-inflammatory target for the treatment of MI and other inflammatory diseases.

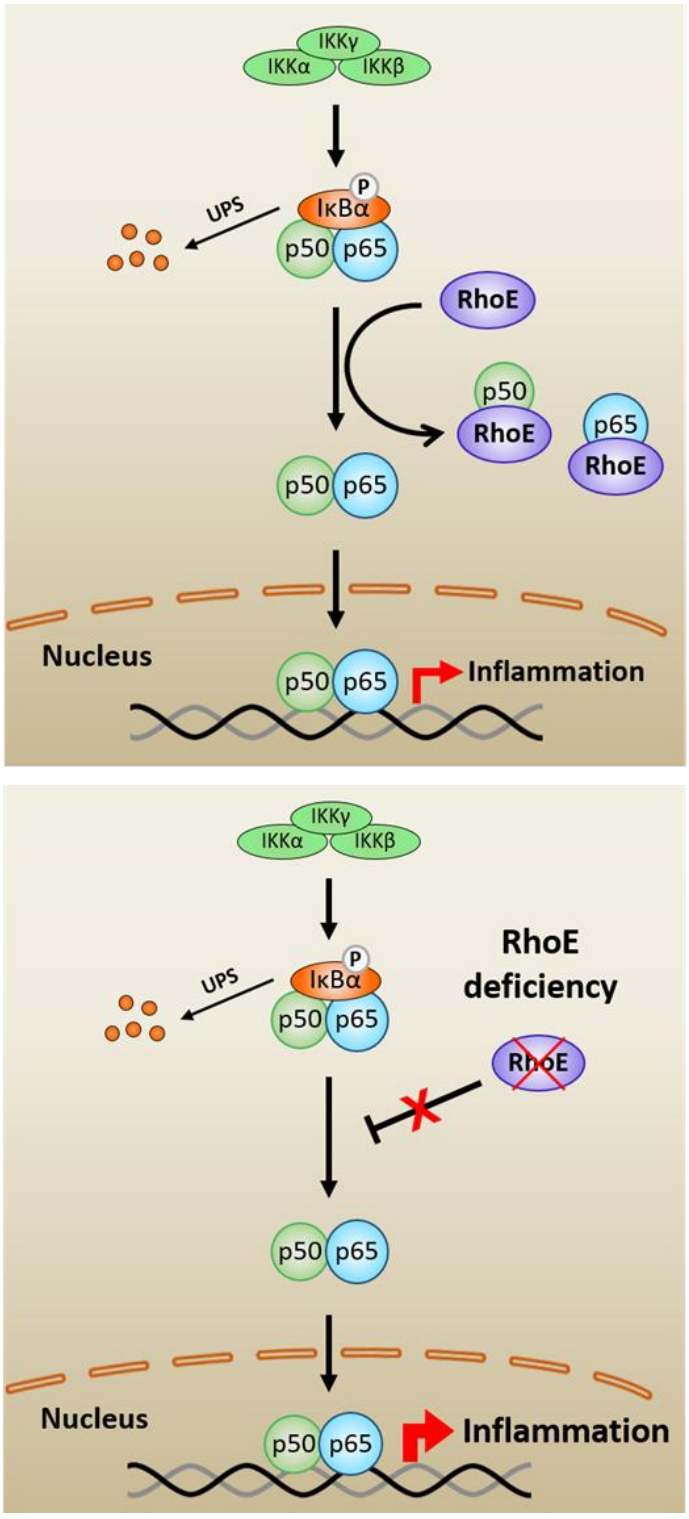

Figure 30. Schematic model for RhoE-mediated regulation of inflammation by suppressing NF-κB signaling.

IV.2 Role of NF-κB in the Heart

The NF-κB signaling has been indicated to play an important role in multiple pathological changes in the cardiovascular diseases[84, 85]. Here, I will discuss the role of NF-κB in ischemic preconditioning, ischemic-reperfusion injury and myocardial infarction.

Ischemic preconditioning refers to an experimental technique of short episode of ischemia and reperfusion[86]. Myocardial ischemic preconditioning in the experimental animals can be performed following a preconditioning protocol that lasts less than 2 hours before sustained ischemia in the anesthetized animals (classical preconditioning or first window of myocardial protection)[87, 88], or 24 to 72 hours in non-anesthetized animals (delayed preconditioning or second window of myocardial protection)[89]. Both strategies significantly reduce the infarct size and improve cardiac function. During the preconditioning treatment, increased expression of reactive oxygen species, adenosine, purines, and nitric oxide efficiently activates NF-κB[90-92]. Importantly, the activation of NF-κB is found to be required for the protective effects from preconditioning treatment since pharmacological inhibition of NF-κB abolished the beneficial effects in both classical and delayed preconditioning treatments[93]. Cardio-protection by NF-κB activation may be caused by NF-κB-mediated upregulation of cardioprotective factors, such as inducible cyclooxygenase, inducible nitric oxide synthase, and manganese superoxide dismutase[94-96]. Moreover, preconditioning treatment leads to a reduced NF-κB activation after sustained ischemia in the heart, which prevents excessive inflammatory response during the sustained myocardial ischemia[97].

Myocardial ischemia and reperfusion were proved to effectively activate the NF-κB signaling[98]. Though NF-κB activation induces a cardioprotective effect during preconditioning, overactivated NF-κB during myocardial ischemia and reperfusion is detrimental[75, 99]. NF-κB induces an excessive inflammation and concomitantly extended ischemic injury by promoting the expression of pro-inflammatory cytokines, chemokines, leukocyte adhesion molecules, and metalloproteinases. Blockage of NF-κB activation post myocardial ischemic injury has been proved to effectively improve the infarct size, cardiac function, and ventricular remodeling[74, 75, 100, 101].

In this study, RhoE is found to negatively regulate NF-κB activation. RhoE deficiency in the mouse heart leads to increased NF-κB activation post myocardial infarction, on the contrary, RhoE overexpression in the mouse heart attenuates NF-κB activation. These results validate the cardioprotective role of RhoE in myocardial infarction via suppressing NF-κB activation.

IV.3 RhoE Negatively Regulates NF-κB Signaling

NF-κB pathway is so closely related to many critical physiological processes that the interest of discovering new selective and effective NF-κB suppressors has never faded. The prevailing view is that IκB proteins function as the major inhibitor to sequester NF-κB complex in the cytoplasm in an inactive state. Stimuli activates the IKK complex, which triggers the phosphorylation IκBα at Ser48 and subsequent proteasome-mediated degradation. Then NF-κB translocates into the nucleus to transcriptionally activate its target genes[57]. Indeed, most NF-κB regulators exert their effects through affecting this canonical IKK-IκB-NF-κB signaling[54, 76, 102, 103]. Several deubiquitinase (DUB) enzymes, for example, down-modulate NF-κB signaling via disrupting the IKK-activating complex[104]. A20 is a deubiquitinase that specifically targets on RIP1 and subsequently induces RIP1 degradation. Upregulated A20 reduces RIP1 level and RIP1-mediated IKK activating complex level as well, resulting in decreased IKK activation and suppressed NF-κB activity[105]. Furthermore, A20 also targets other IKK activators, including RIP2, Malt1 and TRAF6[106-108]. The cellular zinc finger anti-NF-κB (Cezanne) is another DUB enzyme that targeting on RIP1 to negatively modulate NF-κB signaling[109]. The expression of Cezanne is also induced by NF-κB through TNFR pathways, which further provides a negative feedback loop to modulate the NF-κB signaling. Cylindromatosis (CYLD) is another DUB enzyme that negatively regulates the activators of IKK. CYLD deubiquitinates TAK1, TRAF2 and TRAF6 and selectively blocks the TNFα-mediated NF-κB activation[110].

Whether there exist IκB-independent regulators to hold NF-κB in the cytoplasm is unknown[76, 103]. One of our findings is that RhoE inhibits NF-κB nuclear translocation independent of IκBα. RhoE sequesters NF-κB in the cytosol through directly interacting with p65 and p50 individually in the cytosol. It could be that the binding of RhoE to p65 and p50 masks their nuclear localization signal (NLS), and thus RhoE-bound p65 and p50 cannot translocate into the nucleus. Consistent with interpretation, we found RhoE binds to p50 at multiple sites; RhoE also binds to p65 dimerization domain, which is close to p65 NLS site. Furthermore, recent studies show that the nuclear accumulation of NF-κB is also regulated by nuclear degradation of p65 [80, 111] and nuclear export of p65 [79, 112]. Our results rule out these possibilities since RhoE has no effect on the expression levels of p65 and p50. Also, the interactions of RhoE with p65 and p50 occur in the cytosol, so RhoE may not act directly on the nuclear export of NF-κB.

Termination of NF-κB activity in the nucleus is another important mechanism to suppress the NF-κB signaling. IκB proteins can bind to and mediate the export of the nuclear NF-κB complex back into the cytosol, resulting in termination of NF-κB signaling[113]. Besides IκB proteins, there also exist some IκB-independent inhibitors that terminate NF-κB activity in the nucleus. The E3 ubiquitin ligase PDLIM2, for example, induces the degradation of nuclear p65 to suppress the LPS-stimulated NF-κB activity. PDLIM2 binds to nuclear p65 through its PDZ domain and subsequently triggers p65 polyubiquitination. The polyubiquitinated p65 is then targeted by PDLIM2 to discrete intracellular compartments for proteasomal degradation[80]. The COMMD1-Cullin2-SOCS1 complex is another E3 ligase that promotes degradation of p65 in the nucleus.

After TNFα-triggered NF-κB activation and nuclear translocation, COMMD1 bridges nuclear p65 to Cullin2 and SOCS1, which mediate the ubiquitination and degradation of nuclear p65[111]. In this study, we also examined whether RhoE modulates p65 degradation. However, we did not observe any change in total p65 protein level under RhoE knockdown or RhoE overexpression conditions, suggesting that RhoE regulates NF-κB not through modulating its degradation.

Post-translational modification (PTM) of NF-κB components, such as phosphorylation, acetylation and methylation of p65, and microRNA-induced inhibition of NF-κB signaling, are also emerging as alternative strategies to eliminate NF-κB activity[114, 115]. Recently, methylation of p65 is reported as an important PTM-regulatory mechanism to modulate NF-κB signaling. SETD6 specifically targets on p65 at Lys310 and induces mono-methylation of Lys310 (p65K310me1)[116]. Under basal conditions, p65K310me1 is recognized by the ankyrin repeat of the histone methyltransferase GLP, which induces methylation of H3 Lys9 at NF-κB target genes, resulting in a repressed chromatin state. Under stimulated conditions, protein kinase C-ζ (PKC- ζ) phosphorylates p65 at Ser311. This PTM modification blocks the binding of GLP to p65K310me1 and therefore relieve repression of NF-κB target genes. Furthermore, p65 is phosphorylated by protein kinase A (PKAc) at Ser276, which enhances the NF-κB transcriptional activity[117]. Also, Ser536 of p65 is targeted for phosphorylation by IKKs, ribosomal subunit kinase-1 (RSK1), and TANK binding kinase (TBK1)[118-120]. On the contrary, GSK3β, IKKβ and IKKε phosphorylate p65 on Ser468, resulting in reduced NF-κB transcriptional activity[121, 122]. The RhoE effector ROCK1 has been reported to be required

for NF-κB activation by phosphorylating p65 at Ser 536 [123, 124]. There is a possibility that RhoE negatively regulates NF-κB by the inhibition of ROCK1 activity. However, our results show that the phosphorylation level of p65 Ser536 is not affected by RhoE, indicating that RhoE exerts its inhibitory effects on NF-κB independent of ROCK1.

Assembly of individual subunit of Rel family into homo- and hetero-dimer is a critical step in the formation of functional NF-κB complex. p65/p50 heterodimer has been proved to be the most stable and abundant NF-κB [57, 125]. Heterodimerization of p65 and p50 enables p65/p50 heterodimer the DNA binding and transactivation activity. To our knowledge, there are few repots about the regulation of p65/p50 heterodimerization. Our observations suggest that RhoE also regulates p65/p50 heterodimerization. RhoE binds to the dimerization domain of p65 and therefore disrupts the dimerization surface of p65 with other NF-κB subunits. Indeed, decreased p65/p50 heterodimer was observed when RhoE is upregulated.

Collectively, this study demonstrates two regulator mechanisms of RhoE on NF-κB: 1) RhoE binds to p65 and p50 in the cytosol to block their nuclear translocation; 2) RhoE binds to the dimerization region of p65 and subsequently disrupts the heterodimerization between p50 and p65.

IV.4 RhoE Protects Heart from Post-infarction Inflammatory Injury

RhoE has been reported as a cardioprotective factor in preventing the apoptotic cardiomyopathy, promoting angiogenesis in response to pressure-overload stress, and in tightly handling cardiac calcium[64, 65, 67]. This study identified RhoE as a new negative regulator of NF-κB signaling. Since NF-κB is the major inflammatory regulator during myocardial infarction. Overactivated NF-κB triggers excessive inflammation, leading to expanded ischemic injury. In this study, RhoE deficiency in the heart was found to promote NF-κB activation and post-infarction inflammation, resulting in increased infarct size and higher mortality. On the contrary, overexpression of RhoE in the heart was able to suppress NF-κB activation and diminish post-infarction inflammation. Overexpression of RhoE remarkably reduced the infarct size and improved the cardiac function and survival rate. Thus, RhoE functions as a cardioprotective factor against the myocardial infarction injury.

IV.5 Limitations and Future Work

Given the complexity of the negative regulation of inflammatory responses, many important questions about RhoE in this study remain to be answered. The limitation of the study should be noticed.

Multiple cell types are involved in the development of cardia inflammation during myocardial infarction, including cardiac resident macrophages, infiltrated neutrophils and macrophages, endothelial cells, cardiomyocytes, and cardiac fibroblasts. The present study only used cardiomyocyte-specific RhoE deficient or overexpressed mice for the investigation of post-infarction inflammation. Though alternation of RhoE expression in cardiomyocytes is sufficient to modulate post-infarction inflammation, verification of RhoE in different tissues/cells, such as the immune system/cells, with different stimuli is necessary and important in future studies.

This study only explored the relationship between RhoE and the major NF-κB components, p50 and p65. Whether RhoE interacts with its upstream IKK complex is unknown. RhoE is also localized to the cell membrane when activated, whether RhoE influences the transduction of the NF-κB-stimuli is unclear.

Moreover, in this study we observed inhibited NF-κB nuclear translocation by RhoE. Though RhoE binds to dimerization domain of p65, which is close to the nuclear localization signal (NLS) of p65, it is still unknown whether RhoE could occupy the NLS of p65 and subsequently block NF-κB nuclear translocation. The crystal structure of RhoE with p65 may reveal more RhoE-p65 interaction information.

The NF-κB activity is also regulated by different post-translational modifications, including phosphorylation, acetylation, ubiquitination, and methylation. This study only examined the phosphorylation level of p65 at Ser536, which is uninfluenced by RhoE. Whether RhoE affects other post-translational modifications are unclear and may need further studies.

Finally, the NF-κB signaling includes the canonical pathway and alternative pathway. Canonical NF-κB pathway primarily regulates the inflammatory responses, while alternative NF-κB pathway plays an important role in lymphoid organogenesis and overactivated alternative NF-κB pathway promotes lymphatic cancers. Whether RhoE has a similar regulatory role in the alternative pathway as in canonical pathway remains unexplored as well.

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