

Phosphoinositide 3-kinase gamma controls inflammation-induced myocardial depression via sequential cAMP and iNOS signalling[†]

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Aims

Sepsis-induced myocardial depression (SIMD), an early and frequent event of infection-induced systemic inflammatory response syndrome (SIRS), is characterized by reduced contractility irrespective of enhanced adrenergic stimulation. Phosphoinositide-3 kinase γ (PI3K γ) is known to prevent β -adrenergic overstimulation via its scaffold function by activating major cardiac phosphodiesterases and restricting cAMP levels. However, the role of PI3K γ in SIRS-induced myocardial depression is unknown. This study is aimed at determining the specific role of lipid kinase-dependent and -independent functions of PI3K γ in the pathogenesis of SIRS-induced myocardial depression.

Methods and results

PI3K γ knockout mice (PI3K $\gamma^{-/-}$), mice expressing catalytically inactive PI3K γ (PI3K $\gamma^{KD/KD}$), and wild-type mice (P3K $\gamma^{+/+}$) were exposed to lipopolysaccharide (LPS)-induced systemic inflammation and assessed for survival, cardiac autonomic nervous system function, and left ventricular performance. Additionally, primary adult cardiomyocytes were used to analyse PI3K γ effects on myocardial contractility and inflammatory response. SIRS-induced adrenergic overstimulation induced a transient hypercontractility state in PI3K $\gamma^{-/-}$ mice, followed by reduced contractility. In contrast, P3K $\gamma^{+/+}$ mice and PI3K $\gamma^{KD/KD}$ mice developed an early and ongoing myocardial depression despite exposure to similarly increased catecholamine levels. Compared with cells from P3K $\gamma^{+/+}$ and PI3K $\gamma^{KD/KD}$ mice, cardiomyocytes from PI3K $\gamma^{-/-}$ mice showed an enhanced and prolonged cAMP-mediated signalling upon norepinephrine and an intensified LPS-induced proinflammatory response characterized by nuclear factor of activated T-cells-mediated inducible nitric oxide synthase up-regulation.

Conclusions

This study reveals the lipid kinase-independent scaffold function of PI3K γ as a mediator of SIMD during inflammation-induced SIRS. Activation of cardiac phosphodiesterases via PI3K γ is shown to restrict myocardial hypercontractility early after SIRS induction as well as the subsequent inflammatory responses.

Keywords

Myocardial contractility • Autonomic nervous system • Acute inflammation • Phosphoinositide 3-kinase γ • NFAT • iNOS

1. Introduction

Sepsis-induced myocardial depression (SIMD) is a frequent event that corresponds to the severity of sepsis and is reversible in survivors.¹

SIMD occurs early in sepsis since a considerable part of septic patients express features of myocardial dysfunction at admission.^{2,3} A reduced myocardial performance may further aggravate the consequences of an already exaggerated systemic host response to infection and finally

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contribute to sepsis-induced cardiocirculatory shock.⁴ However, SIMD is frequently associated with an improved outcome of septic patients,⁵ indicating that an adaptive myocardial hypocontractility may maintain cell viability by down-regulating oxygen consumption, energy requirements, and ATP demand.⁶

Infection-associated SIMD is the result of complex host–pathogen interactions leading to activation of pathophysiological signalling pathways.⁷ Initiation of SIMD is mainly triggered by microbial toxins and proinflammatory mediators, which are excessively released during the innate immune response. As a consequence, β -adrenergic receptor (β -AR) responsiveness and signalling are attenuated despite enhanced catecholamine availability, which in turn leads to reduced myocardial contractility (MC).⁸ Among a plethora of exogenous and endogenous mediators that may interfere with myocardial function, agonists of the Toll-like receptor 4 (TLR4) are known to activate the phosphatidylinositol 3-kinase (PI3K γ) pathway and to provoke MC depression.^{9–11}

In cardiomyocytes, catecholamine-induced stimulation of the G-protein-coupled β -AR regulates cardiac contraction via cAMP/protein kinase A (PKA) activation and phosphorylation of effectors of the cardiac excitation–contraction coupling such as the L-type Ca²⁺ channel (LTCC), the ryanodine receptor (RyR), phospholamban (PLB), and troponin I. Although multiple PI3K isoforms are expressed in the heart, PI3K γ is particularly involved in controlling heart contractility.¹² It coordinates via its scaffold function the activation of the major cardiac phosphodiesterase isoforms, PDE3 and PDE4, thus creating a feedback loop to prevent pathological overstimulation of cAMP/PKA signalling by appropriate cAMP degradation.¹³ The PI3K γ pathway is involved in decreasing MC in sepsis-induced systemic inflammatory response syndrome (SIRS);^{10,11} however, the underlying mechanisms remain unclear.

The aim of this study was to examine the specific role of lipid kinase-dependent and -independent functions of PI3K γ in inflammation-induced myocardial dysfunction at the levels of the organism, the individual organ, and isolated cells. Cardiac autonomic nervous system (ANS) response and left ventricular performance were examined in PI3K γ wild-type (P3K $\gamma^{+/+}$), knockout (PI3K $\gamma^{-/-}$), and kinase-dead mice (PI3K $\gamma^{KD/KD}$) after intraperitoneal endotoxin/lipopolysaccharide (LPS) administration. To analyse molecular mechanisms, primary adult cardiomyocytes isolated from P3K $\gamma^{+/+}$, PI3K $\gamma^{-/-}$, and PI3K $\gamma^{KD/KD}$ mice were employed. The results identify the suppressive effect of PI3K γ on cAMP and inducible nitric oxide synthase (iNOS) signalling via its scaffold function and characterize PI3K γ as a critical mediator of cardiomyocyte immune response during acute systemic inflammation.

2. Methods

2.1 Animals and LPS-induced SIRS

For a full description of all applied methods, see Supplementary material online, Methods. Briefly, PI3K γ knockout mice (PI3K $\gamma^{-/-}$)¹⁴ and mice carrying a targeted mutation in the PI3K γ gene causing loss of lipid kinase activity (PI3K $\gamma^{KD/KD}$)¹⁵ were on the C57BL/6J background for >10 generations. Age-matched C57BL/6 mice were used as controls. The animals were maintained at 12 h light and dark cycles with free access to food and water. Ambient temperature was 29 \pm 1°C during the whole experimental period. The animal procedures were performed according to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Experiments were approved by the committee of the Thuringian State Government on Animal Research.

Mice (12–15 weeks old) received LPS (10 mg/kg) from *Escherichia coli* serotype O55:B5 (Sigma-Aldrich, St Louis, USA, Lot #032M4082V) as a single intraperitoneal injection. Control mice received saline. In addition, 500 μ L of saline was injected subcutaneously immediately after LPS administration and after 24 and 48 h to ensure appropriate fluid resuscitation. Experiments were performed after 3 h, 24 h, 3 days, or 1 week. Mice developed SIRS with tachycardia and decline of the clinical status¹⁶ with a maximum at 24 h after administration. Mortality rate remained <10% irrespective of the genetic background of mice.

2.2 Assessment of cardiac autonomic nervous system functions

Cardiac ANS functions were assessed by telemetric monitoring of electrocardiography, body temperature, and motor activity recording and subsequent estimation of respective heart rate variability (HRV) parameters. (Detailed description of data processing and HRV parameter calculation is given in Supplementary material online.)

2.3 Measurement of left ventricular performance

Measurement of left ventricular function was performed using the pressure–volume conductance catheter technique¹⁷ (detailed description is given in Supplementary material online).

2.4 Heart tissue catecholamine and cytokine assessment

The heart tissue content of catecholamines (epinephrine and norepinephrine) and cytokines (TNF- α and IL-6) was determined by ELISA following the manufacturer's protocol [Antibodies-online GmbH, Aachen, Germany; ELISA kit (BioLegend[®], San Diego, USA)]. Detailed description is given in Supplementary material online.

2.5 Cell culture

Primary adult cardiomyocytes were isolated from hearts of adult mice as described.¹⁸ Cardiomyocytes were exposed to norepinephrine (30 μ M) or a mixture of norepinephrine (30 μ M), LPS (1 μ g/mL), and cytokines (TNF- α , 50 ng/mL and IL1- β , 50 ng/mL). Modifications are given in Supplementary material online.

2.6 SDS–PAGE and western blotting

These procedures were done using standard methods (detailed description is given in Supplementary material online).

2.7 cAMP assay

These procedures were done using standard methods (detailed description is given in Supplementary material online).

2.8 Histopathology and immunohistochemistry

These procedures were done using standard methods (detailed description is given in Supplementary material online).

2.9 Statistics

Data are reported as mean \pm S.D. Numbers of animals as indicated in legends refer to each group and time point. Comparisons between groups were made with one-way or two-way analysis of variance (ANOVA), if appropriate. In case of repeated measurements, two-way ANOVA with repeated measures was used. *Post hoc* comparisons were made with the Holm–Sidak test. Applicability of the statistical procedures used was verified using the calculation of the Bayes factors,¹⁹ if required. Differences were considered significant at *P*-values of <0.05.

3. Results

3.1 PI3K γ -deficient mice display a delayed myocardial depression after LPS induces SIRS

To investigate the role of PI3K γ in LPS-induced myocardial depression, we used PI3K $\gamma^{-/-}$ and PI3K $\gamma^{KD/KD}$ mice, which allows assignment of observed effects to either lipid kinase function of PI3K γ ²⁰ or kinase-independent 'scaffold' function such as PDE stimulation and cellular cAMP control.^{13,15} First, inflammatory alterations in heart tissue in response to LPS-induced SIRS were characterized. Early after LPS administration, a marked elevation of TNF- α and IL-6 and, subsequently, a substantial polymorphonuclear neutrophil invasion, which was more pronounced in PI3K $\gamma^{-/-}$ mice 3 days after onset, occurred (see Supplementary material online, Table S1). Furthermore, a long-lasting PI3K γ up-regulation in wild-type cardiomyocytes was observed (see Supplementary material online, Figure S1).

To evaluate left ventricular function, the pressure–volume conductance catheter technique was applied. Figure 1 shows a considerable increase of MC in PI3K $\gamma^{-/-}$ mice 3 h after LPS administration, whereas wild-type mice exhibited a marked reduction of MC. In line with this, alterations in cardiac output (CO) and total peripheral resistance (TPR) indicate opposing myocardial reactions in wild-type and PI3K $\gamma^{-/-}$ mice (see Supplementary material online, Figure S2 and Table S2). Whereas global haemodynamics of PI3K $\gamma^{-/-}$ mice remained within physiological ranges during LPS-induced SIRS, pronounced myocardial depression during the early SIRS period in wild-type mice was accompanied by marked reduction of CO and increased TPR (see Supplementary material online, Table S2). To verify the importance of lipid kinase-dependent or -independent signalling reactions for the observed hypercontractility in PI3K $\gamma^{-/-}$ mice, we studied the response to LPS in PI3K $\gamma^{KD/KD}$ mice. In these mice, LPS-induced myocardial depression was comparable to wild-type mice pointing to the importance of the scaffold protein activity of PI3K γ for suppressing MC.

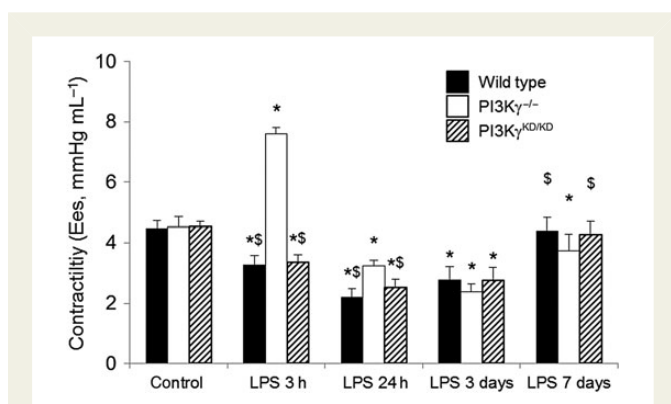


Figure 1 PI3K γ -deficient mice (PI3K $\gamma^{-/-}$) display early myocardial hypercontractility followed by delayed myocardial depression after LPS-induced SIRS. In contrast, wild-type and PI3K γ -kinase-dead mice (PI3K $\gamma^{KD/KD}$) developed myocardial depression early after LPS administration. Values are mean + SD, $n = 10-15$ per group and time point. * $P < 0.05$, *significant difference between the control and LPS-stimulated state within each group, $\$$ significant differences to PI3K $\gamma^{-/-}$ mice (two-way ANOVA, followed by the Holm–Sidak test for *post hoc* multiple comparisons).

The effects of PI3K γ on myocardial and haemodynamic response early after intraperitoneal injection of LPS were due to alterations in myocardial responsiveness since a difference in cardiac ANS activation could be excluded. As shown in Figure 2 and Supplementary material online, Table S3, the expected increase of sympathetic activation early after LPS administration was comparable between wild-type and PI3K $\gamma^{-/-}$ mice. The extent of tachycardia and the alteration of the obtained HRV indices demonstrate a similar enhancement of sympathetic input to the heart in all genotypes. In addition, the elevation of the myocardial catecholamine content was largely comparable (Figure 2E). However, reduced β 2-adrenoreceptor (β 2-AR) phosphorylation indicating diminished receptor internalization was evident in PI3K $\gamma^{-/-}$ cardiomyocytes stimulated with norepinephrine and cytokines (see Supplementary material online, Figure S3), whereas β 2-AR expression levels were similar between genotypes. As shown in Figure 2D, PI3K $\gamma^{-/-}$ mice also displayed enhanced myocardial contractile responsiveness upon intravenous norepinephrine stimulation. Taken together, these data indicate that the effect on MC observed in PI3K $\gamma^{-/-}$ mice early after LPS administration is independent from lipid kinase activity. The comparison of PI3K $\gamma^{-/-}$ and PI3K $\gamma^{KD/KD}$ mice clearly points to PI3K γ scaffold function and associated up-regulation of β -AR/cAMP signalling as a major cause for increased MC in PI3K γ deficiency.

3.2 Loss of PI3K γ activates myocardial Ca²⁺ trafficking by increased intracellular cAMP

To verify mechanisms underlying the lipid kinase-independent effects of PI3K γ on MC, we used primary cardiomyocytes isolated from PI3K $\gamma^{+/+}$, PI3K $\gamma^{-/-}$, and PI3K $\gamma^{KD/KD}$ mice. PI3K γ deficiency resulted in enhanced intracellular cAMP content as previously reported in heart tissue extracts,²¹ whereas the exclusive loss of the lipid kinase activity of PI3K γ did not alter cAMP levels when compared with wild-type cardiomyocytes (Figure 3C). Furthermore, norepinephrine provoked a long-lasting increase of cAMP in PI3K γ -deficient cardiomyocytes, whereas it failed to enhance cAMP levels in PI3K $\gamma^{+/+}$ and PI3K $\gamma^{KD/KD}$ cardiomyocytes. Intriguingly, LPS-induced up-regulation of PI3K γ expression (see Supplementary material online, Figure S1) suggests an enhanced contribution of PI3K γ -mediated control of cellular cAMP under the condition of SIRS.

As expected, cardiomyocytes derived from PI3K $\gamma^{-/-}$ mice show an enhanced and prolonged cAMP-mediated signalling via PKA activation with pronounced RyR and PLB phosphorylation (Figure 3A and B). These data indicate accelerated intracellular Ca²⁺ trafficking, leading to improved myocardial excitation–contraction coupling.²² Suppression of PDE3 or PDE4 activities in cardiomyocytes from PI3K $\gamma^{+/+}$ mice by pharmacological inhibitors reduced cAMP degradation and led to a similar increase in PLB phosphorylation as seen in PI3K $\gamma^{-/-}$ cardiomyocytes (Figure 3D). The enhanced MC early after LPS administration was not associated with an increased risk for cardiomyocyte death, because microscopic evaluation revealed similar apoptosis rates and no indication for necrotic cell loss at all (see Supplementary material online, Figure S4).

3.3 iNOS mediates delayed myocardial depression after LPS administration in PI3K γ -deficient mice

Haemodynamic measurements of the intact heart revealed that after an initial increase, the contractility of the left ventricle in PI3K $\gamma^{-/-}$ mice was progressively compromised. Three days after LPS administration,

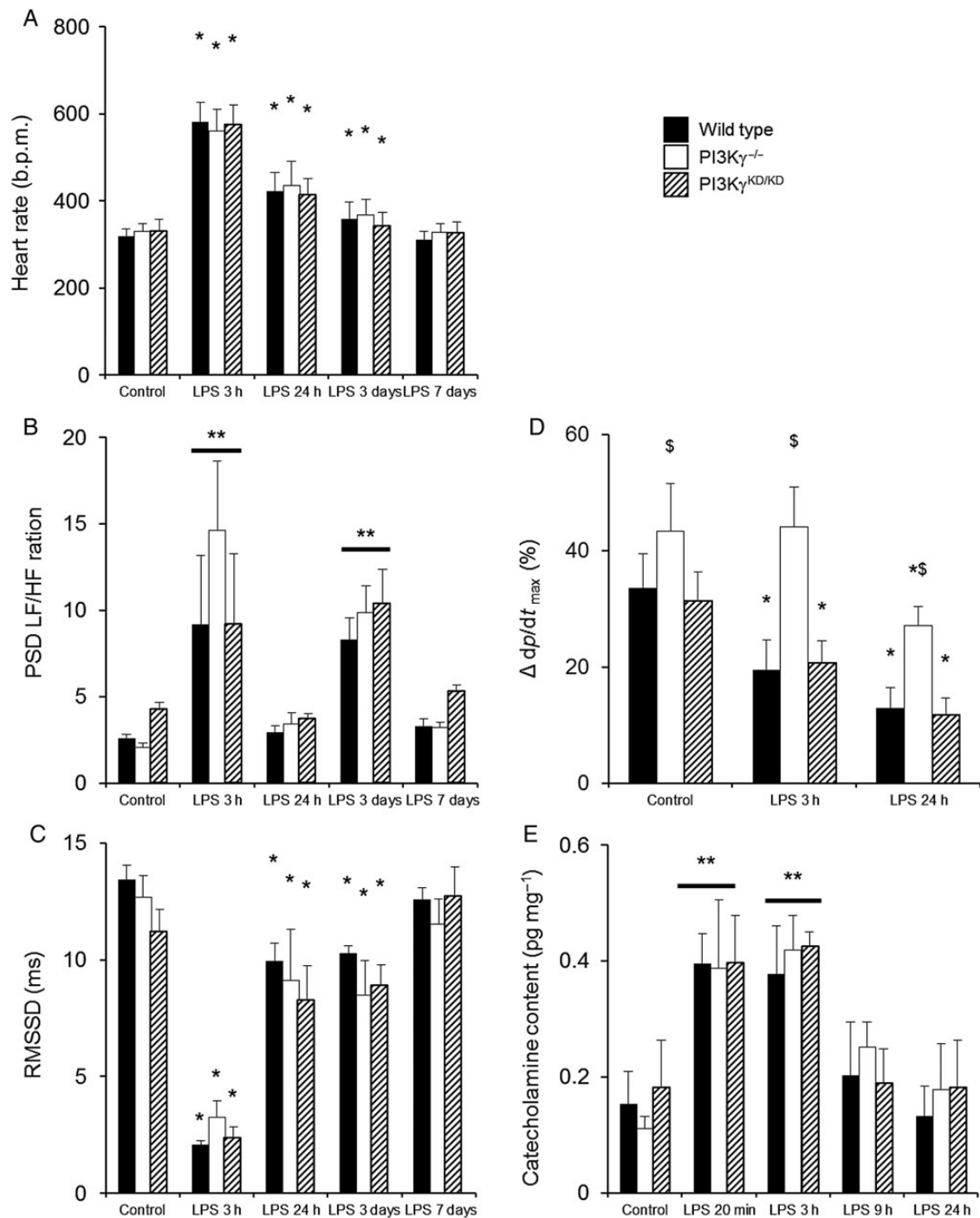


Figure 2 Autonomic nervous system control of heart function measured by telemetric assessment. (A–C) RMSSD: square root of the mean square successive differences between successive normal intervals; PSD LF/HF: ratio of low-frequency range (LF, 0.15–1.5 Hz) vs. high-frequency range (HF, 1.5–5 Hz) of the power spectral density (PSD) of the R–R interval time series; $n = 8–10$ per group and time point; (D) myocardial contractile responsiveness [$\Delta dp/dt_{max}$: percentage change of dp/dt_{max} induced by bolus injection of norepinephrine (100 $\mu\text{g}/\text{kg}$) into the jugular vein; $n = 4–5$ per group and time point]; (E) heart tissue analysis of catecholamine content ($n = 3–4$ per group and time point) in wild-type, PI3K $\gamma^{-/-}$, and PI3K $\gamma^{KD/KD}$ mice. Values are mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *significant difference between control and LPS stimulation, §significant differences between wild-type and PI3K $\gamma^{KD/KD}$ mice [two-way repeated-measures ANOVA (one-factor repetition), followed by the Holm–Sidak test for *post hoc* multiple comparisons].

a marked myocardial depression was seen, which was similar to the one that was detected in wild-type and PI3K $\gamma^{KD/KD}$ mice (Figure 1). Consequently, we investigated the mediators responsible for myocardial

depression in PI3K γ -deficient mice occurring at later times in LPS-induced SIRS. We screened for inflammatory responses in heart tissue that are known to be associated with compromised myocardial

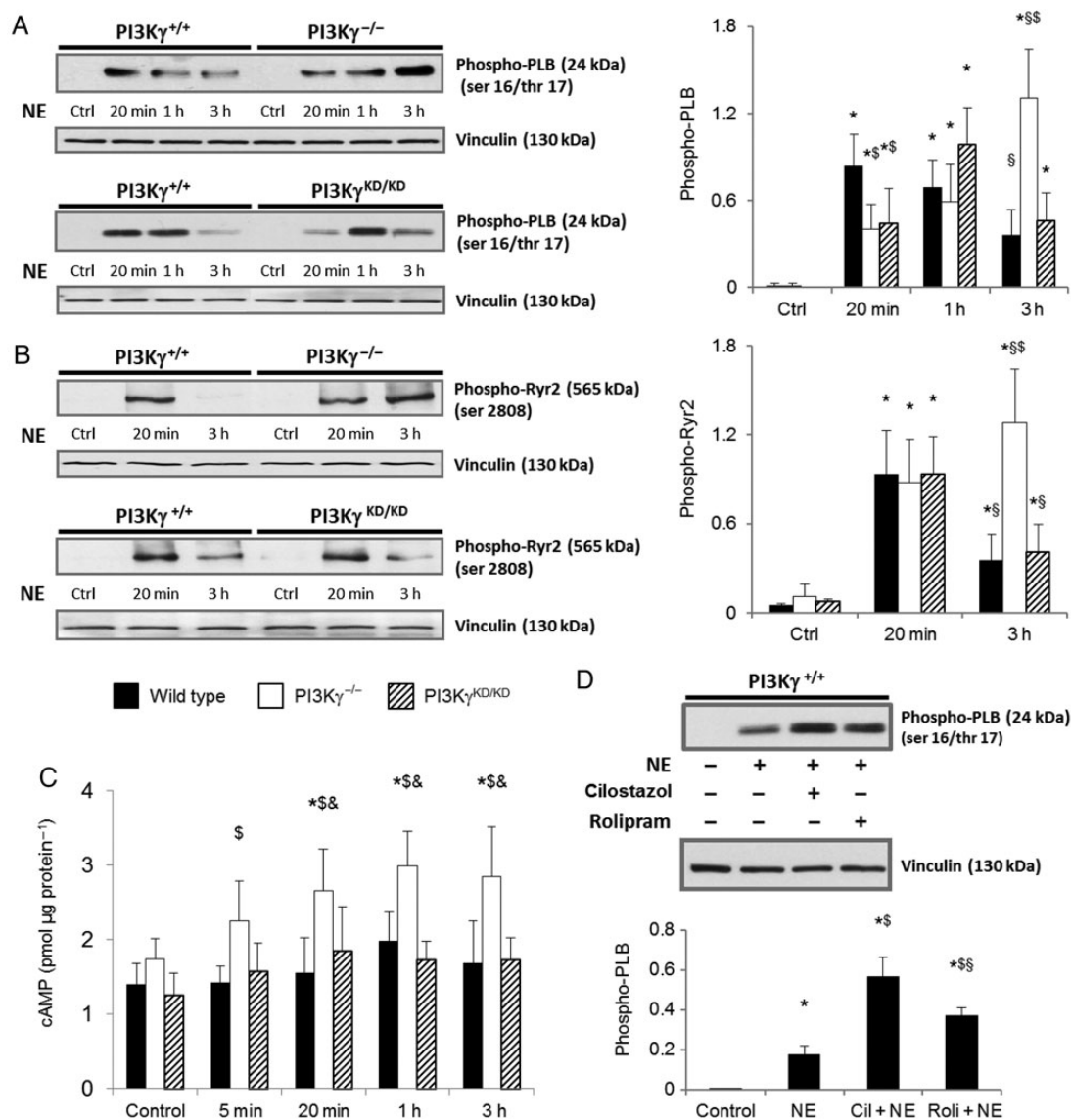


Figure 3 Cardiomyocytes isolated from PI3K $\gamma^{-/-}$ mice display enhanced and prolonged cAMP-mediated signalling during β -adrenergic stimulation. Norepinephrine (NE) leads to enhanced sarcoplasmic reticulum calcium trafficking in PI3K $\gamma^{-/-}$ cardiomyocytes indicated by (A) reinforced PLB phosphorylation (phospho-PLB, $n = 4-5$ per group and time point), (B) RyR phosphorylation (phospho-Ryr2, $n = 4$ per group and time point), and (C) a fast and sustained cAMP increase ($n = 4$ per group and time point). (D) Pharmacological inhibition of PDE3 (cilostazol, Cil.) or PDE4 (rolipram, Roli.) leads to PLB phosphorylation in wild-type cardiomyocytes ($n = 3$ per group and time point). Values are mean \pm SD. * $\$$ & $\$$ $p < 0.05$, *significant difference vs. control, $\$$ significant difference to wild-type cardiomyocytes, &significant difference to PI3K $\gamma^{KD/KD}$ cardiomyocytes, $\$$ significant difference to '20 min' (in A and B) and to 'Cil + NE' (in D). (For A–C: two-way ANOVA, followed by the Holm–Sidak test for *post hoc* multiple comparisons, was performed; For D: one-way ANOVA, followed by the Holm–Sidak test for *post hoc* multiple comparisons, was performed).

performance and found a profound and long-lasting up-regulation of iNOS expression in PI3K $\gamma^{-/-}$ mice. In contrast, wild-type mice showed only weak induction of iNOS expression (Figure 4A). Importantly, myocardial depression was largely rescued by pharmacological iNOS inhibition (Figure 4B). Subsequent studies in primary cardiomyocytes revealed a marked iNOS up-regulation induced by an LPS/proinflammatory cytokine mixture mimicking early SIRS conditions *in vivo*. iNOS expression was clearly more pronounced in cardiomyocytes isolated from PI3K $\gamma^{-/-}$ mice compared with cells from wild-type and PI3K $\gamma^{KD/KD}$ mice pointing to the importance of the PI3K γ scaffold function for iNOS up-regulation (Figure 4C).

To understand whether up-regulated iNOS interferes with intracellular Ca $^{2+}$ -trafficking, we first investigated PLB phosphorylation in cardiomyocytes from wild-type and PI3K γ mutant mice. Stimulation with norepinephrine alone or in combination with an LPS/cytokine mixture for 3 h led to a markedly enhanced PLB phosphorylation in PI3K $\gamma^{-/-}$ cells when compared with wild-type cardiomyocytes (Figure 4D). After prolonged β -adrenergic stimulation (24 h), PLB phosphorylation was reduced in wild-type cardiomyocytes regardless whether LPS/cytokine was administered in parallel. In contrast, PLB phosphorylation was considerably increased in PI3K $\gamma^{-/-}$ cardiomyocytes after 24 h norepinephrine stimulation, which was prevented by parallel LPS/cytokine

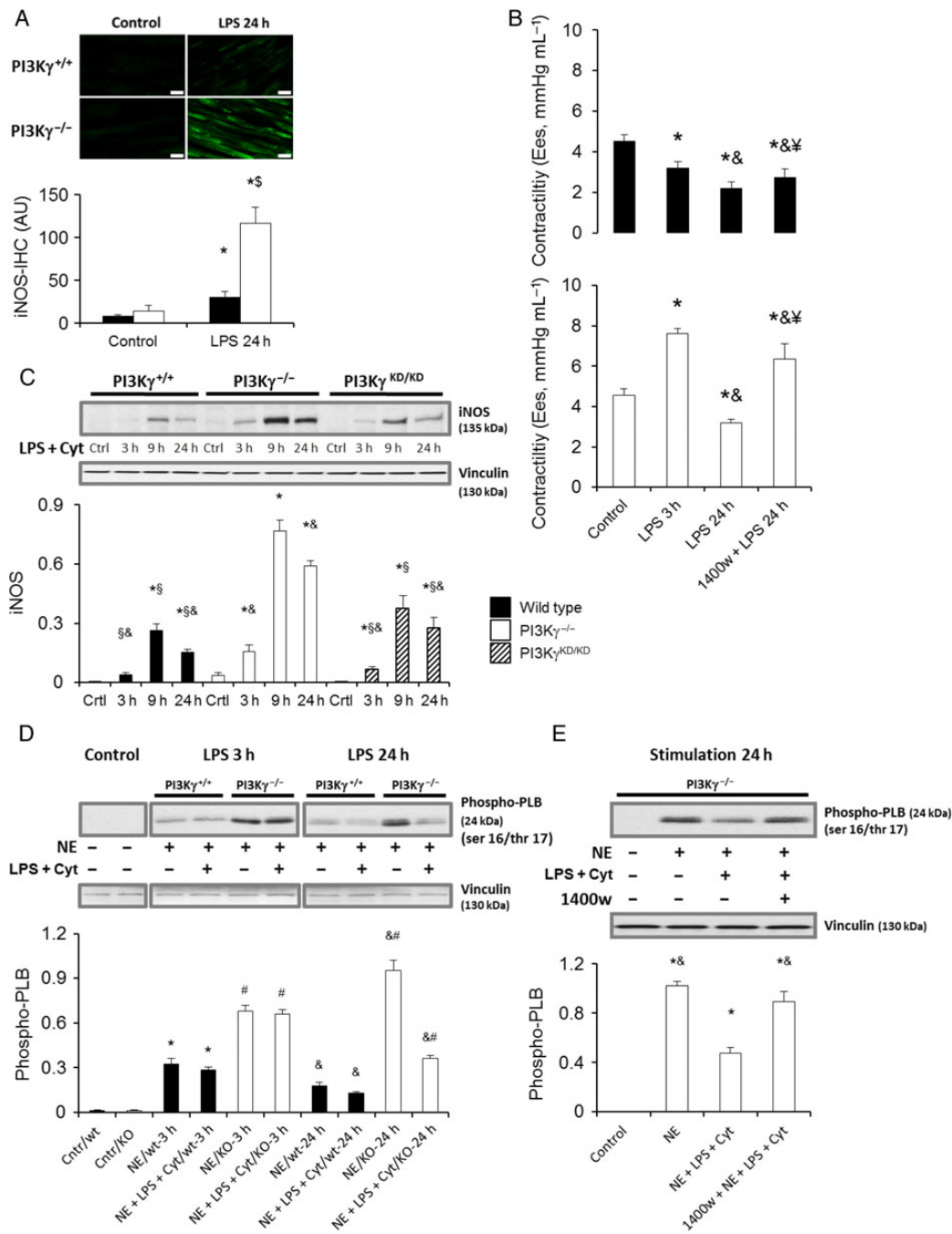


Figure 4 Loss of PI3K γ results in enhanced up-regulation of iNOS in heart tissue of mice obtained 24 h after LPS administration. (A) Upper panel: iNOS expression in heart tissue, magnification $\times 20$, bars 100 μm . Bottom panel: quantification of iNOS expression ($n = 4$ at each group and time point). (B) Myocardial depression is rescued by pharmacological iNOS inhibition in wild-type and PI3K $\gamma^{-/-}$ mice ($n = 4-8$ at each group and time point). (C) Primary cardiomyocytes derived from wild-type, PI3K $\gamma^{-/-}$, and PI3K $\gamma^{\text{KD/KD}}$ mice show an increased iNOS expression after LPS + cytokine (LPS + Cyt) stimulation. A response is markedly enhanced in PI3K $\gamma^{-/-}$ cells [top panel: representative western blots, stimulated with LPS + Cyt; bottom panel: densitometric quantification ($n = 4$ at each group and time point)]. (D) LPS + Cyt stimulation of cardiomyocytes derived from wild-type and PI3K $\gamma^{-/-}$ mice leads to suppression of norepinephrine (NE)-stimulated PLB phosphorylation [top panel: representative western blots; bottom panel: densitometric quantification ($n = 4$ at each group and time point)]. (E) Pharmacological iNOS inhibition rescues suppressed PLB phosphorylation in cardiomyocytes derived from PI3K $\gamma^{-/-}$ mice [top panel: representative western blots of pretreatment with 1400w (selective iNOS inhibitor); bottom panel: densitometric quantification of iNOS normalized to vinculin ($n = 3$ at each group and time point)]. Values are mean + SD. Wild-type mice, PI3K $\gamma^{-/-}$ mice, * $\&\$ \# \# P < 0.05$, *significant difference vs. control, $\$$ significant difference to wild-type heart tissue, $\&$ significant difference to PI3K $\gamma^{-/-}$ cardiomyocytes, $\&$ significant difference to 'LPS 3 h' (in B) '9 h' (in C), to '3 h' (in D), to 'NE + LPS + Cyt' (in E), $\#$ significant difference to 'LPS 24 h' (in B), $\#$ significant difference between wild-type and PI3K $\gamma^{-/-}$ cardiomyocytes at same treatment and time point. (For A, C, and D: two-way ANOVA, followed by the Holm-Sidak test for *post hoc* multiple comparisons, was performed; for B and E: one-way ANOVA, followed by the Holm-Sidak test for *post hoc* multiple comparisons was performed.).

stimulation (Figure 4D). To know whether the prevention of PLB phosphorylation by proinflammatory agents was attributed to iNOS up-regulation, PI3K $\gamma^{-/-}$ cells were treated with a selective iNOS inhibitor (1400w, Sigma-Aldrich Chemie GmbH, Munich, Germany) during nor-epinephrine/LPS/cytokine stimulation. As shown in Figure 4E, iNOS inhibition rescued the LPS/cytokine-mediated inhibition of PLB phosphorylation.

Taken together, these data reveal an important role of the PI3K γ scaffold function for the control of iNOS expression, which in turn affects intracellular PKA signalling and Ca²⁺-trafficking. Loss of PI3K γ resulted in enhanced iNOS expression in cardiomyocytes after LPS administration possibly contributing to myocardial depression occurring at later stages of LPS-induced SIRS.

3.4 Enhanced iNOS expression is mediated by NFAT activation in PI3K $\gamma^{-/-}$ cardiomyocytes

To examine PI3K γ -dependent regulation of iNOS expression in response to LPS/cytokines, we investigated pathways involved in the expression of inflammatory proteins in primary cardiomyocytes derived from PI3K $\gamma^{+/+}$, PI3K $\gamma^{-/-}$, and PI3K $\gamma^{KD/KD}$ mice. Whereas NF- κ B and mitogen-activated protein (MAP) kinase activation upstream of activator protein 1 (AP-1) did not show substantial genotype-specific differences, nuclear factor of activated T-cells (NFAT) was markedly activated in PI3K $\gamma^{-/-}$ cells compared with PI3K $\gamma^{+/+}$ and PI3K $\gamma^{KD/KD}$ cells as evidenced by NFAT dephosphorylation (Figure 5A and B).

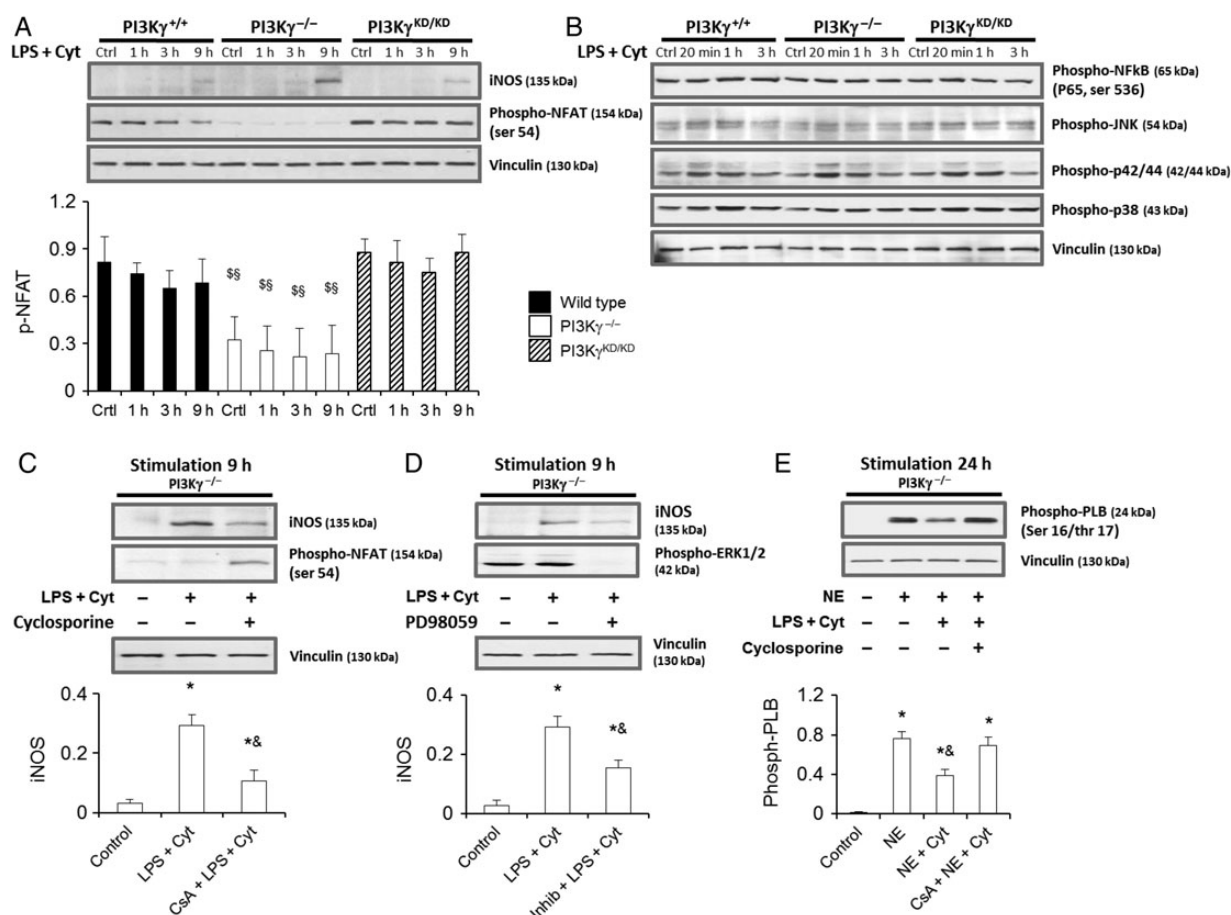


Figure 5 iNOS up-regulation in PI3K $\gamma^{-/-}$ cardiomyocytes in response to sustained proinflammatory stimulation requires NFAT/AP-1 co-activation. (A) LPS + Cyt stimulation of primary PI3K $\gamma^{-/-}$ cardiomyocytes leads to increased iNOS expression and NFAT activation. (B) LPS + Cyt stimulation of primary PI3K $\gamma^{-/-}$ cardiomyocytes leads to activation of MAPKs (JNK, ERK1/2, and P38) and NF- κ B activation. (C and D) Pharmacological inhibition of NFAT by cyclosporine and MEK by PD98059 prevents iNOS up-regulation. (E) PLB phosphorylation in PI3K $\gamma^{-/-}$ cardiomyocytes stimulated with nor-epinephrine (NE/LPS/cytokines) is rescued by cyclosporine. (A) Top panel: representative western blots of NFAT; bottom panel: densitometric quantification of NFAT phosphorylation ($n = 4$). (B) Top panel: representative western blots of MAPKs (JNK, ERK1/2, and P38) and NF- κ B activation ($n = 3$ at each group and time point). (C) Top panel: representative western blots of iNOS and NFAT in PI3K $\gamma^{-/-}$ cardiomyocytes. Bottom panel: densitometric quantification of iNOS expression intensity ($n = 4$ at each group). (D) Top panel: representative western blots of iNOS and ERK1/2 in PI3K $\gamma^{-/-}$ cardiomyocytes. Bottom panel: densitometric quantification of iNOS expression ($n = 4$ at each group). (E) Top panel: representative western blot of phospho-PLB in PI3K $\gamma^{-/-}$ cardiomyocytes. Bottom panel: densitometric quantification of phospho-PLB ($n = 4$ at each group). Values are mean + SD. * $\&\&\&p < 0.05$, *significant difference vs. control, $\&$ significant difference to wild-type cardiomyocytes, $\&$ significant difference to PI3K $\gamma^{KD/KD}$ cardiomyocytes, $\&$ significant difference to 'LPS + Cyt' (in C and D), to 'NE', and to 'CsA + NE + Cyt' (in E). (A): two-way ANOVA, followed by the Holm–Sidak test for *post hoc* multiple comparisons, was performed; C–E: one-way ANOVA, followed by the Holm–Sidak test for *post hoc* multiple comparisons, was performed.).

However, NFAT regulation appeared GSK3 β -independently (see Supplementary material online, *Figure S5*). Pharmacological inhibition of NFAT with cyclosporine A (by blockade of the calcium-dependent phosphatase calcineurin, which activates cytoplasmic NFAT by dephosphorylation²³) led to a markedly reduced iNOS expression (by 63%; *Figure 5C*), suggesting that iNOS is up-regulated via NFAT. LPS/cytokine-induced iNOS expression was also decreased by inhibition of MEK and the downstream extracellular signal-regulated kinases 1/2 (ERK1/2) pathway (*Figure 5D*). Of note, NFAT-mediated iNOS up-regulation seems to be causally involved in LPS/cytokine-induced suppression of myocardial excitation–contraction coupling since cyclosporine A rescued PLB phosphorylation (*Figure 5E*).

To test whether the data obtained in cardiomyocytes are valid *in vivo*, cardiomyocytes isolated from excised heart tissue and lysates from heart tissue extracts derived from mice treated with LPS were analysed. A marked up-regulation of iNOS was seen only in PI3K $\gamma^{-/-}$ animals early after SIRS onset and persisted until the end of our observation period of 7 days (*Figure 6A*). These findings confirm that cAMP/PKA/Ca²⁺ signalling controls NFAT, which, in turn, controls iNOS expression and may explain the different behaviour in recovery of MC between PI3K $\gamma^{-/-}$ compared with PI3K $\gamma^{+/+}$ and PI3K $\gamma^{KD/KD}$ mice. Pronounced and maintained iNOS up-regulation was accompanied by enhanced nitrotyrosine formation (*Figure 6B*) and matrix metalloproteinase 9 (MMP-9) expression (see Supplementary material online, *Figure S6*) in the heart tissue of PI3K $\gamma^{-/-}$ mice. Consequently, iNOS inhibition appears to be an option to improve myocardial performance without the need for β -adrenergic stimulation.

4. Discussion

Our study proposes PI3K γ as a major regulator of inflammation-induced functional alterations in heart tissue in a mouse model of LPS-induced SIRS. PI3K γ -deficient mice developed myocardial hypercontractility early after LPS administration followed by an aggravated proinflammatory response with concomitantly depressed MC from 24 h up to the third day of SIRS onset. In contrast, wild-type mice and mice expressing a catalytically inactive PI3K γ developed myocardial depression early after LPS administration and exhibited a lower inflammatory response despite similarly increased catecholamine contents in heart tissue. These data suggest that the scaffold function of PI3K γ is crucial to protect the heart against an exaggerated immune response caused by infection-induced SIRS.

The early systemic response to infection-related SIRS involves enhanced myocardial sympathetic activation, which is mainly mediated by norepinephrine released from cardiac sympathetic nerve endings as a result of increased sympathetic outflow from the CNS driven by neurohumoral stimulation.²⁴ Accordingly, our results reveal an enhanced ANS response verified by HRV analysis and cardiac catecholamine release with no differences between the investigated mouse genotypes. However, myocardial responsiveness to the increased sympathetic tone exhibited remarkable differences between PI3K $\gamma^{-/-}$ mice on the one hand and PI3K $\gamma^{+/+}$ and PI3K $\gamma^{KD/KD}$ mice on the other hand. The strongly enhanced MC in PI3K γ -deficient mice appears to reflect an intensified β -AR signalling with elevated myocardial cAMP content and reinforced cardiac excitation–contraction coupling. In line with this, high cAMP level as a result of the missing PI3K γ scaffold function that mediates myocardial PDE activation¹² and elevated RyR and PLB phosphorylation indicating high PKA activity were observed. Our recent collaborative study revealed that the catalytic subunit p110 γ of

PI3K γ anchors PKA through a site in its N-terminal region. Anchored PKA activates PDE3B to enhance cAMP degradation. In addition, PKA was shown to phosphorylate p110 γ and to inhibit its lipid kinase activity, thus providing local feedback control of PIP3 and cAMP signalling events.¹³ Loss of PI3K γ in p110 γ -deficient mice impedes formation of this multiprotein complex resulting in enhanced cardiomyocytic cAMP signalling.

Our data show that LPS-induced myocardial TLR4 stimulation led to marked up-regulation of PI3K γ expression. Interestingly, PI3K γ was demonstrated to reduce β -AR density by enhanced receptor internalization and concomitant receptor desensitization.^{25,26} PI3K γ -related attenuation of β -AR signalling, which is shown here, appears to be an adaptive event with cardioprotective consequences due to reduced cardiac energy demands. The following indications support this interpretation: (i) pharmacological blockade with short-acting β_1 -AR antagonists improves haemodynamic data in different preclinical sepsis models and clinical trials.^{27,28} Our data support this finding since the systemic perfusion pressure remained stable despite reduced MC and CO in wild-type and PI3K $\gamma^{KD/KD}$ mice (see Supplementary material online, *Table S2*). (ii) Enhanced contractility in PI3K $\gamma^{-/-}$ mice was mirrored by increased CO indicating enhanced cardiac energy demands. Furthermore, attenuation of contractile function evidently affects systolic functions with preserved diastolic processes, which is indicated by maintained myocardial relaxation dynamics suggesting merely unaltered ATP availability.

Our data reveal for the first time that a single administration of LPS with adequate fluid resuscitation resulted in prolonged myocardial depression. We observed a reduced MC for a minimum of 3 days with temporarily attenuated haemodynamic performance shown by pronounced CO reduction and enhanced TPR, suggesting that general sympathetic stimulation modulates TPR even during LPS-induced SIRS. LPS-induced myocardial depression was reported in several previous studies performed in different species including men,^{29–31} but considered to be merely an acute response. Intriguingly, PI3K $\gamma^{-/-}$ mice displayed a progressive myocardial depression after initial hypercontractility in our study, whereas wild-type and PI3K $\gamma^{KD/KD}$ mice developed myocardial depression right after LPS administration. After 7 days, a complete recovery of myocardial performance was ascertained. Our new findings of iNOS expression control via cAMP/PKA/Ca²⁺ signalling pathways provide a novel strategy to improve myocardial performance without the need for β -adrenergic stimulation.

Whereas the early myocardial suppression in wild-type and PI3K $\gamma^{KD/KD}$ mice is clearly mediated by restriction of cAMP signalling via the PI3K γ -scaffold function, the attenuation of MC in PI3K $\gamma^{-/-}$ mice after a transient hypercontractility needed further consideration. Previous experimental studies report that intraperitoneal LPS injection in mice resulted in increased blood levels of LPS and TLR4 signalling for at least 3 h and normal values are reached again latest after 12 h.³² Proinflammatory responses of circulating cytokines released by immune cells display similar temporal patterns.³³ Prolonged myocardial TLR4 signalling after LPS may be caused by PI3K γ -dependent alarmin release.¹¹ Moreover, as shown here, the initially enhanced myocardial catecholamine content returned to baseline within 24 h (*Figure 2E*). Thus, the observed long-lasting myocardial depression in PI3K $\gamma^{-/-}$ mice was most probably not caused by extracellular stimuli but by an altered functional pattern of cardiomyocytes. In line with this, a recent transcriptomic analysis in septic heart tissue indicating up-regulation of several proinflammatory pathways and concomitant dysregulation in the β -AR/cAMP/PKA pathway proposes a causal relationship between

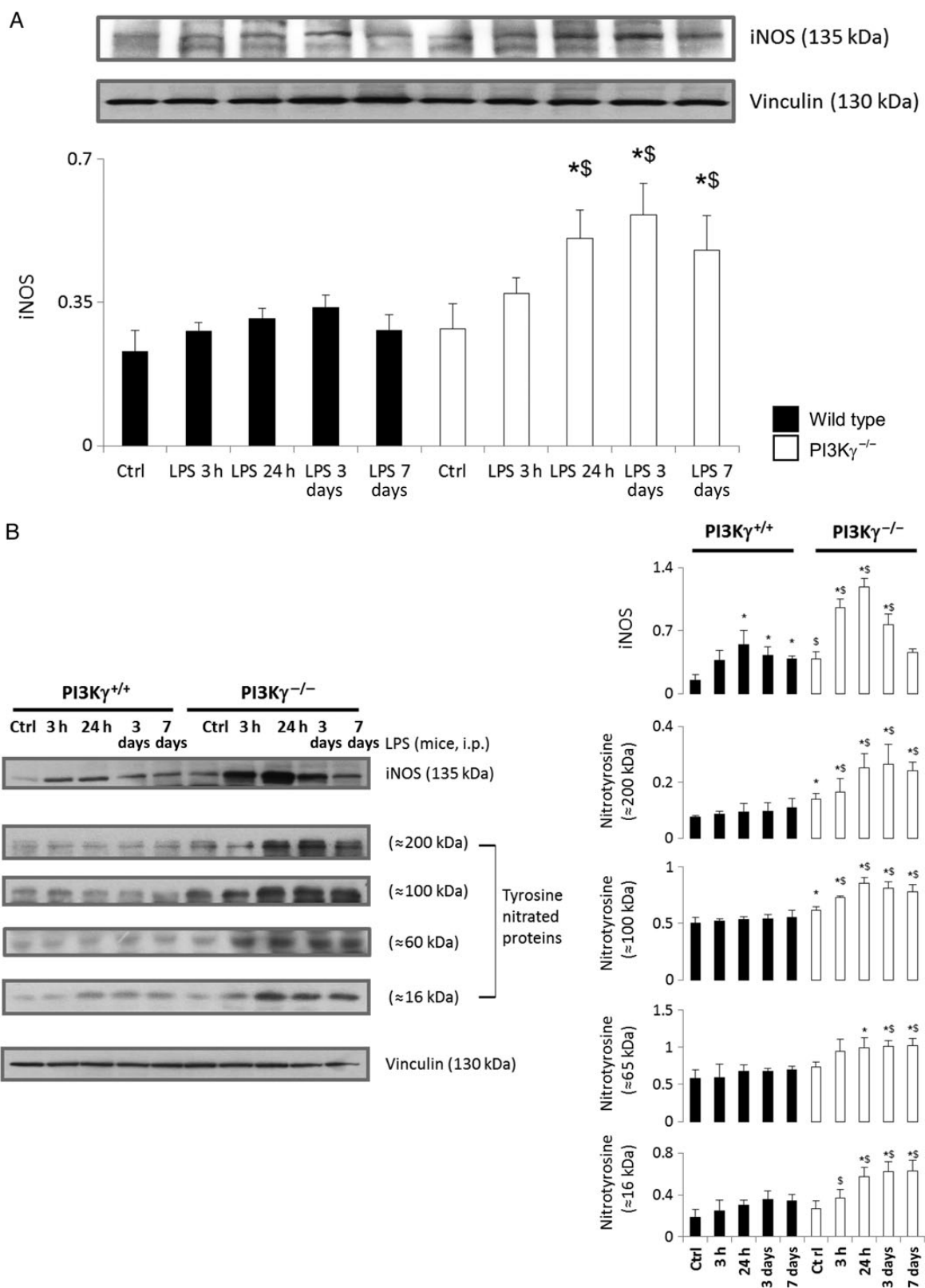


Figure 6 iNOS and nitrotyrosine levels are up-regulated in heart tissue extracts from PI3K γ ^{-/-} mice treated with LPS (10 mg/kg, i.p.). (A) Marked and long-lasting up-regulation of iNOS in cardiomyocytes derived from LPS-treated PI3K γ ^{-/-} animals compared with wild-type mice ($n = 3$ at each group and time point). (B) iNOS up-regulation is accompanied by increased nitrotyrosine formation in heart tissue of PI3K γ ^{-/-} mice ($n = 3-4$ at each group and time point). Values are mean \pm SD. * $P < 0.05$, *significant difference vs. control, \S significant difference to wild-type cardiomyocytes (two-way ANOVA, followed by the Holm-Sidak test for *post hoc* multiple comparisons).

activated myocardial immune response and suppressed contractile performance.³⁴ Our study supports this suggestion by demonstrating a link between proinflammatory activation and reduced contractile performance in adult cardiomyocytes. Our data disclose a so far unknown mechanism, showing that PI3K γ deficiency is able to trigger calcineurin/NFAT activation with subsequent iNOS up-regulation and concomitant attenuation of cardiac excitation–contraction coupling via altered Ca²⁺-trafficking (Figures 4 and 5). Using genetic and pharmacological approaches, we demonstrate that NFAT is activated in resting PI3K γ ^{-/-} cardiomyocytes and remains activated during LPS/cytokine stimulation mediating an enhanced and long-lasting iNOS up-regulation (Figure 4A and C). At the same time, PLB phosphorylation was markedly reduced indicating attenuated cardiac excitation–contraction coupling. Thus, PI3K γ -deficient cardiomyocytes develop myocardial hypocontractility during LPS-induced SIRS via enhanced iNOS up-regulation thereby achieving a similar down-regulation of cardiac performance as seen by cAMP signalling restriction in wild-type cells.

Intriguingly, NFAT activation in unstimulated PI3K γ ^{-/-} cardiomyocytes indicates a critically elevated intracellular Ca²⁺ content under resting conditions (Figure 5A). As shown by cyclosporine A inhibition (Figure 5C), increased Ca²⁺ may trigger Ca²⁺/calmodulin signalling via activated phosphatase calcineurin. This finding extends a recent report on PI3K γ ^{-/-} cardiomyocytes, demonstrating sequential β_2 -adrenergic receptor activation, abnormal cAMP accumulation, PKA-mediated hyperphosphorylation of LTCC (Ca_v1.2), and PLB finally leading to increased Ca²⁺ spark occurrence and amplitude.³⁵ Our data show that the missing scaffold function of PI3K γ provokes cAMP elevation and subsequent activation of the Ca²⁺/calmodulin/calcineurin/NFAT transcription pathway under resting conditions, which might play a central role in the heart's response to pathological stressors.³⁶ NFAT inhibition by phosphorylation is obviously GSK3 β -independent and is enhanced via the scaffold function of PI3K γ in cardiomyocytes under proinflammatory stimulation (see Supplementary material online, Figure S5).³⁷ Interestingly, ERK1/2 was involved in mediating enhanced iNOS expression in response to LPS/cytokine stimulation (Figure 5D). Previously, it has been shown that MEK1–ERK1/2 signalling enhances NFAT-dependent gene expression through an indirect mechanism involving induction of cardiac AP-1 activity, which functions as a necessary NFAT-interacting partner.³⁸ As a possible mechanism, MEK1–ERK1/2 and calcineurin–NFAT proteins may form a complex in cardiac myocytes, resulting in direct phosphorylation of NFAT within its C terminus. MEK1–ERK1/2-mediated phosphorylation of NFAT directly augmented its DNA-binding activity, while inhibition of MEK1–ERK1/2 signalling reduced NFAT DNA-binding activity.³⁸ Erk1/2 involvement for enhanced iNOS expression confirms previous reports about a cooperation of NFAT with the transcription factor AP-1 in the control of inducible genes like iNOS in adult cardiomyocytes.^{39,40}

Importantly, our data reveal that iNOS up-regulation is involved in down-regulation of MC since LPS/cytokine-induced inhibition of PLB phosphorylation is rescued either by direct iNOS blockade (Figure 4E) or by cyclosporine-induced calcineurin blockade (Figure 5E). NO is known to signal through at least two distinct pathways either via cGMP or independent from cGMP.⁴¹ cGMP-dependent signalling includes the activation of guanylate cyclase and protein kinase G (PKG), whereas cGMP-independent signalling primarily occurs via direct protein modification (e.g. S-nitrosylation).⁴² A number of different cGMP-dependent effects of iNOS signalling leading to decreased myocardial contraction, such as reduction in myofilament Ca²⁺ sensitivity, have been observed.⁴³ The latter was likely mediated by troponin

I phosphorylation via PKG⁴⁴ and reduced calcium current due to the phosphorylation of the LTCC by PKG.⁴⁵ cGMP-independent effects of iNOS expression are causally linked to peroxynitrite formation.⁴⁶ Peroxynitrite is most likely the major signalling molecule of iNOS⁴⁷ capable to directly inactivate SERCA at high concentrations by reducing cAMP-dependent PLB phosphorylation.⁴¹ As shown in Figure 6, the pronounced and persistent iNOS up-regulation provokes enhanced nitrotyrosine formation upon LPS administration in heart tissue of PI3K γ -deficient mice. Thus, our data indicate that the cGMP-independent pathway of iNOS signalling appears to be responsible for the delayed down-regulation of contractile function in PI3K γ ^{-/-} mice. In addition, proinflammatory MMP-9 (see Supplementary material online, Figure S6) was up-regulated in PI3K γ -deficient cardiac tissue and may further contribute to myocardial dysfunction and remodelling.^{48,49} In contrast to previous studies, MMP-2 up-regulation was not observed.^{48,49}

In conclusion, our data extend our knowledge about the control of MC under acute stress conditions such as infection-induced SIRS, which is of substantial clinical importance. We disclose a coordinative role of PI3K γ in restricting β -adrenergic signalling via its scaffold function thereby suppressing cAMP-dependent pathways and reducing iNOS expression. PI3K γ deficiency in cardiomyocytes leads to an early hypercontractility state and subsequent iNOS up-regulation via cooperative NFAT/AP-1 activation, which in turn triggers prolonged inflammation and myocardial depression. Our findings characterize lipid kinase-independent scaffold function of PI3K γ as a key mediator of cardiac excitation–contraction coupling early after LPS-induced SIRS and as an important negative regulator of iNOS expression. Taken together, the results describe a protective function of PI3K γ in cardiomyocytes and heart tissue during infection-induced SIRS. Furthermore, iNOS inhibition may represent a novel strategy to improve myocardial performance without the need for β -adrenal stimulation.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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