Heart disease is one of the major causes of morbidity and mortality and the leading cause of death in Western countries. \textsuperscript{1} Cardiovascular disease, in particular myocardial infarction (MI), results from coronary atherosclerosis, a chronic disease with stable and unstable periods.\textsuperscript{2} Unstable periods are characterized by activated inflammatory processes. In the plasma membrane they are degraded by phospholipases that are responsible for the degradation of phospholipids in the plasma membrane. PLD catalyzes the phosphatidylcholine and phosphatidylethanolamine remodeling endocytosis and, thereby, augmenting cardiac left ventricular function after ischemia/reperfusion. (Am J Pathol 2014, 184: 2450–2464; http://dx.doi.org/10.1016/j.ajpath.2014.06.005)

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It has been shown that PLD1 is coupled to tumor necrosis factor-\(\alpha\)-mediated activation of monocytes after myocardial ischemia and reperfusion using a mouse model of myocardial infarction. PLD1 expression was highly up-regulated in the myocardium after ischemia/reperfusion. Genetic ablation of PLD1 led to defective cell adhesion and migration of inflammatory cells into the infarct border zone 24 hours after ischemia/reperfusion injury, likely owing to reduced tumor necrosis factor-\(\alpha\) expression and release, followed by impaired nuclear factor-\(\kappa\)B activation and interleukin-1 release. Moreover, PLD1 was found to be important for transforming growth factor-\(\beta\) secretion and smooth muscle \(\alpha\)-actin expression of cardiac fibroblasts because myofibroblast differentiation and interstitial collagen deposition were altered in \textit{Pld1} \(\text{−/−}\) mice. Consequently, infarct size was increased and left ventricular function was impaired 28 days after myocardial infarction in \textit{Pld1} \(\text{−/−}\) mice. Our results indicate that PLD1 is crucial for tumor necrosis factor-\(\alpha\)-mediated inflammation and transforming growth factor-\(\beta\)-mediated collagen scar formation, thereby augmenting cardiac left ventricular function after ischemia/reperfusion. (Am J Pathol 2014, 184: 2450–2464; http://dx.doi.org/10.1016/j.ajpath.2014.06.005)
hydrolysis of phosphatidylcholine into phosphatidic acid (PA) and choline. PA, as well as its metabolites lysophosphatidic acid (LPA) and diacylglycerol, are important second messengers. PA directly stimulates the synthesis of phosphatidylinositol 4,5-bisphosphate by activating phosphatidylinositol-4-phosphate 5-kinase and can result from the phosphorylation of diacylglycerol by diacylglycerol kinase.

Different studies have provided evidence that PLD activity is strictly controlled by complex regulatory mechanisms. Although PLD1 has low basal activity and is activated readily by protein kinase C and small GTPases of the ADP-ribosylation factor and Rho family, PLD2 shows high basal activity and is strictly controlled by complex regulatory mechanisms. PLD plays an important role in neutrophil chemotaxis, cell migration, and modulates integrin-mediated cell adhesion. The analysis of Pld1−/− mice identified PLD1 as a critical regulator of platelet activity essential for integrin interaction and shear-dependent thrombus formation. Furthermore, the lack of PLD1 leads to protection against arterial thrombosis and ischemic brain infarction. Different studies have shown that oxidized low-density lipoprotein mediates the accumulation of PA and LPA mediated by the activation of PLD, thereby promoting the proliferation of smooth muscle cells and atherosclerosis.

By using antisense oligonucleotides, PLD1 was shown to be involved in inflammatory processes. PLD1 activity contributes to increased expression of tumor necrosis factor-α (TNF-α) and matrix metalloproteinases such as matrix metalloproteinase 9. However, the function and regulation of PLD1 in inflammatory cardiovascular diseases, especially in processes of myocardial ischemia/reperfusion (I/R) as well as in myocardial repair, have not been explored to date.

Here, we show that PLD1 is crucial for the TNF-α−induced inflammatory response that mediates NF-κB activation, the release of cytokines, cell adhesion, and migration of neutrophils and monocytes/macrophages.

Materials and Methods

Animals

Specific pathogen-free C57BL/6J mice were obtained from Charles River (Sulzfeld, Germany). Pld mutant mice were described previously. Animal studies were performed in accordance with the guidelines for the use of living animals in scientific studies, in accordance with German law for the protection of animals, and was approved by the Regional Council Tübingen (Regierungspräsidium Tübingen).

Real-Time Quantitative RT-PCR

Real-time RT-PCR analysis was performed using FastStart Universal SYBR Green Master (Rox; Roche, Basel, Switzerland) as a fluorogenic probe as described previously. Briefly, for the assessment of endogenously expressed PLD1, TNF-α, and IL-1, respectively, total RNA from the left ventricle of the heart of healthy mice and of mice that underwent ischemia and 24 hours of reperfusion was extracted. After reverse transcription, quantitative PCR amplification was performed using the following oligonucleotide primers: forward 5′-TGGCTGGAATCCGCTCAACCTTAG-3′, reverse 5′-CCCGCCAGGCTGGAACAG-3′ (PLD1); forward 5′-GCCCCCACCCTGCACCCCT-3′, reverse 5′-GGGGCGTGGCTGCTGTG-3′ (TNF-α); and forward 5′-ACCTTCGCTTTCAAAATGCCAGTT-3′, reverse 5′-CACCTAGAAAAACCTCTGCAGCA-3′ (IL-1α). Expression was normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA expression levels as an internal control.

Myocardial Ischemia and Reperfusion in Mice

Ten- to 12-week-old Pld+/+ and Pld−/− mice were anesthetized by intraperitoneal injection of a solution of 5 mg midazolam, 0.5 mg medetomidine, and 0.05 mg fentanyl per kilogram of body weight. Myocardial ischemia was induced in Pld+/+ and Pld−/− mice by ligation of the left anterior descending artery (LAD) for 30 minutes. After 1 day of reperfusion the ischemic area (area at risk) was defined by negative staining with 4% Evans Blue (after re-ligation of the LAD at the level marked by the suture left in place), and the infarcted area (infarct size) was detected by triphenyltetrazolium staining (Sigma-Aldrich, St. Louis, MO). The ratio of infarct size/area at risk is an accurate measure to analyze infarct size within ischemic myocardium and is the primary end point, which determines the effect of the treatment strategy. Areas were quantified digitally by video planimetry. After 28 days of reperfusion, the infarct size was determined by Gomori’s One Step Trichrome staining (Dako, Glostrup, Denmark). Animals were sacrificed and serial sections of the hearts (10 per mouse, 250 μm apart, up to the mitral valve) were fixed in 4% formalin, embedded in paraffin, and stained with Gomori’s One Step Trichrome staining. Determination of infarct size was performed using Diskus software version 2005 (Hilgers, Königswinter, Germany) and was expressed as the percentage of total left ventricular (LV) volume. Twenty-eight days after MI an echocardiography was performed, as described previously. Analysis of fractional shortening and ejection fraction was performed with Vevo2100 software version 1.5.0 (VisualSonics, Toronto, ON, Canada).

Echocardiography

Echocardiography was performed using a VEVO 2100 ultrasound machine and a MS400 (18 to 38 MHz) linear transducer (both from VisualSonics) as described recently.

Immunohistochemistry of Cardiac Sections

Paraffin-embedded cardiac sections taken 1 day after MI were stained with hematoxylin and eosin. Immune staining of infarcted cardiac sections was performed with a streptavidin-biotin-immunoperoxidase method (Dako). The total number

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of cells was referred to a certain tissue area; the area was divided into grids, and data are shown per mm². Paraffin-embedded cardiac sections were stained with an anti-Mac3 monoclonal antibody (BD Biosciences, San Diego, CA), an antipolymorphonuclear monoclonal antibody (GeneTex, Irvine, CA), and isotype control antibodies according to standard protocols. The percentage of Mac3-positive and polymorphonuclear-positive cells was counted from six random areas in the infarcted myocardium of each heart sample (n = 4) in a blinded manner (N.A.).

For the analysis of PLD1 expression in monocytes of infarct areas, paraffin-embedded cardiac sections were stained with an anti-Mac3 monoclonal antibody (BD Biosciences) and a polyclonal PLD1 antibody (Cell Signaling, Danvers, MA).

**PLD Activity Measurements**

PLD activity was measured in an enzymatically coupled fluorescent *in vitro* assay (Amplex Red Phospholipase D Assay Kit; Molecular Probes, Eugene, OR) as described previously. Briefly, cell lysates were mixed with 100 μL of the Amplex Red reaction buffer. The PLD activity was determined for each sample by measuring fluorescence activity after 1 hour of incubation at 37°C in the dark with the GloMax-Multi detection system (Promega, Madison, WI). A standard curve with different concentrations ranging from 0 to 250 mU/mL was performed using purified PLD from *Streptomyces chromofuscus* (Sigma-Aldrich).

**Monocytes**

Murine monocytes were freshly isolated from whole blood of *Pld*+/+ and *Pld*−/− mice. Four hundred grams of whole blood was centrifuged for 20 minutes at room temperature. Lymphocytes were separated using Biocoll Separating solution (Biochrom, Berlin, Germany) and centrifugation for an additional 10 minutes. Lysis of erythrocytes was followed by cell labeling using 10 μL CD11b Micro Beads (Miltenyi Biotec, Bergisch Gladbach, Germany) per 10⁷ cells. Monocytes were separated with a Vario Macs Separator (Miltenyi Biotec) and counted.

**TNF-α, IL-1α, and TGF-β Enzyme-Linked Immunosorbent Assay**

For the quantification of TNF-α and IL-1α in plasma after MI and in 1 μg/mL lipopolysaccharide (LPS)-stimulated monocytes from *Pld*+/+ and *Pld*−/− mice, supernatant was taken and the release of TNF-α and IL-1α, respectively, was measured following the manufacturer’s protocol (Quantikine Mouse TNF-α Immunoassay, Quantikine Mouse IL-1α Immunoassay; R&D Systems, Minneapolis, MN). Transforming growth factor (TGF)-β in plasma of *Pld*+/+ and *Pld*−/− mice was quantified 3 days after MI following the manufacturer’s protocol (Quantikine Mouse TGF-β Immunoassay; R&D Systems).

**Immunocytochemistry**

Freshly isolated murine monocytes from whole blood of *Pld*+/+ and *Pld*−/− mice were seeded onto uncovered wells and stimulated with LPS for 6 hours to induce an immune response by the generation of cytokines such as TNF-α. Cells were stained with an anti-p65 antibody (Santa Cruz Biotechnology, Dallas, TX) according to a standard protocol.

**Chemotaxis**

Chemotaxis was performed using a 48-well modified Boyden chamber (Neuro Probe, Inc., Gaithersburg, MD). Monocytes were added to the upper chamber and medium containing monocyte chemotactic protein 1 or medium alone was added to the lower compartment. The cultures were incubated for 4 hours at 37°C. Finally, cells in the lower chamber were counted using CellTiter-Glo (Promega).

**Dynamic Adhesion Assay**

For adhesion experiments under flow conditions, 2 × 10⁵ cells/mL activated murine endothelial 5-T cells were allowed to adhere onto glass coverslips until they were confluent and then were activated overnight (20 hours) with 100 ng/mL TNF-α (Pepro Tech, Hamburg, Germany). Monocytes were perfused over glass coverslips using arterial shear rates (flow rate, 7.53 mL/hour; shear rate, 1000 seconds⁻¹). After perfusion, all experiments were recorded in real time and evaluated offline.

**α-SMA and Collagen Staining of Cardiac Sections**

α-Smooth muscle actin (α-SMA) staining of paraffin sections was performed using anti-α-SMA antibody (1:200, ab5649; Abcam, Cambridge, UK), antirabbit horseradish peroxidase as a secondary antibody (1:200; Santa Cruz Biotechnology, Dallas, TX), and 3,3’-diaminobenzidine reagent (Zytomed, Berlin, Germany) as a chromogen. Nuclei were stained with hemalaun solution (Merck KGaA, Darmstadt, Germany). Collagen accumulation was detected by Masson’s trichrome, Gomori, and picrosirius red staining, celestine blue solution was used for nuclei staining. Fibrillar collagen was analyzed by polarized light microscopy.

Images were captured at the indicated magnifications by using an AxioImager.M2 microscope with AxioCam HRC and AxioVS40V 4.8.2.0 software (all from Zeiss, Jena, Germany).

**Primary Cardiac Fibroblasts**

Cardiac fibroblasts were isolated from 6- to 8-week-old female *Pld*−/− mice or their respective controls as described previously. Cells were grown in high-glucose Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) containing 20%
fetal calf serum (Invitrogen) and 8 ng/mL basic fibroblast growth factor (BPS Bioscience, San Diego, CA) and used for experiments in passage 2.

Cardiac fibroblasts were stimulated with 100 nmol/L angiotensin II (Sigma-Aldrich) or 10 ng/mL TGF-β (Sigma-Aldrich) and harvested for gene expression analysis 24 hours after stimulation with peqGOLD TriFast (PEQLAB, Erlangen, Germany) according to the manufacturer’s instructions. cDNA was synthesized and analyzed as described previously. For quantitative real-time RT-PCR the following oligonucleotide primers were used: forward 5'-CAGGCATGGATCATCAATTAC-3', reverse 5'-TGTCAAGTGTGCTGCG-3' (Acta2), and forward 5'-CTAATGTCGGACCGCAACA-3', reverse 5'-CTTGTTCCCGAATGCTTGA-3' (Tgfb1). Glyceraldehyde-3-phosphate dehydrogenase was chosen as an endogenous control: forward 5'-TGGCAAAGTGGAGATTGCCTG-3', reverse 5'-AAGATGGTGATGGCGCTCC-3'.

For TGF-β secretion, cells were stimulated with 100 nmol/L angiotensin II and secreted TGF-β was measured after 48 hours using a murine TGF-β enzyme-linked immunosorbent assay (R&D Systems) according to the manufacturer’s protocol.

Cell migration was analyzed using a modified Boyden chamber. After 6 hours the migration was stopped, migrated cells were stained with Coomassie, and the cells subsequently were analyzed with the Odyssey Near Infrared Imaging System (Li-Cor Bioscience, Lincoln, NE).

Statistical Analysis

Data are shown as means ± SD from at least three individual experiments (n represents the number of experiments). All

Figure 1  PLD1 is expressed strongly in infarcted myocardium in mice. Cardiac sections of wild-type mice 24 hours after I/R injury were immunostained for PLD1 at the indicated time points and compared with healthy controls (non-MI), isotype control. A: Representative images are shown. B: Quantification of PLD1 expression after MI at indicated time points. C: The quantification of mRNA expression of PLD1 in the myocardium 24 hours after I/R injury in mice compared with healthy controls as determined by quantitative RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase was used as a reference gene. Agarose gel resolves RT-PCR products. n ≥ 3 to 5 (A); n = 4 (C). Scale bar = 100 μm.
data were tested for significance using the paired or unpaired Student’s t-test and the U-test. \( P < 0.05 \) was considered statistically significant.

For quantification of α-SMA and picrosirius red staining, ImageJ version 1.45s software (NIH, Bethesda, MD) was used. Statistical analysis was performed using GraphPad Prism software version 6.0 (La Jolla, CA). The comparison of two groups was accomplished with an unpaired t-test; a P value <0.05 was considered significant.

**Results**

PLD1 Expression Is Strongly Enhanced after Ischemia and Reperfusion in Mice

Cardiac sections from C57BL/6J mice that underwent experimental I/R (ligation of the LAD artery) were immunostained with anti-PLD1 at different time points and compared with healthy (control) mice without MI (Figure 1A). PLD1 was highly expressed in the myocardium after MI. PLD1 accumulation occurs in the infarcted ischemic area and the border zone at 1 and 7 days after MI. PLD1 levels peaked 21 days after MI and were still fivefold higher than in healthy mice at day 28 (Figure 1, A and B). This was supported further by quantitative RT-PCR, in which we quantified the mRNA of PLD1 of the left cardiac ventricle after MI and compared the mRNA level with that of healthy mice (Figure 1C). PLD1 was strongly up-regulated 24 hours after I/R in mice, suggesting an important role in cardiovascular inflammatory disease and/or repair.

Reduced Migration of Pld1\(^{-/-}\) Immune Cells into Infarct Border Zone One Day after MI

To analyze the role of PLD1 in the processes of I/R injury, we tested Pld1\(^{-/-}\) mice in a model of myocardial I/R (Figure 2). After ligation of the LAD for 30 minutes, myocardial damage was assessed by 2,3,5-triphenyltetrazolium chloride staining to differentiate between metabolically active and inactive tissue.

**Figure 2** Infarct size is comparable in Pld1\(^{+/+}\) and Pld1\(^{-/-}\) mice, but migration of Pld1\(^{-/-}\) immune cells is reduced in the infarct border zone 24 hours after I/R in mice. Mice were analyzed 1 day after reperfusion. A: Representative transversal cardiac sections are mapped from Pld1\(^{+/+}\) and Pld1\(^{-/-}\) mice. B: Quantitative analysis of the infarct size in the percentage of area at risk (% Inf/AaR) shows no differences between Pld1\(^{+/+}\) and Pld1\(^{-/-}\) mice 24 hours after I/R. Determination of the area at risk and infarct as the percentage of the left ventricle shows no differences between both groups. C: Cardiac sections were stained with hematoxylin and eosin 24 hours after MI to analyze the migration of inflammatory cells into the infarct border zone. Representative images of Pld1\(^{+/+}\) and Pld1\(^{-/-}\) mice are shown. A quantitative analysis of migration of inflammatory cells was performed. Bar graphs depict means ± SD. D: Cardiac sections from mice were immunostained for Mac-3 and polymorphonuclear (PMN) cells 24 hours after myocardial I/R injury. Representative images of Pld1\(^{+/+}\) and Pld1\(^{-/-}\) mice. E: Quantitative analysis shows a markedly reduced number of macrophages (Mac-3) and neutrophils (PMN) in the infarcted myocardium of Pld1\(^{-/-}\) mice. Bar graphs depict means ± SD. \( n \geq 8 \) (B); \( n = 7 \) (C); \( n = 8 \) (E). **P < 0.01, ***P < 0.001. Scale bars: 1 mm (A); 25 \( \mu m \) (D). Black bars, Pld1\(^{+/+}\); gray bars, Pld1\(^{-/-}\).
Infarct areas after 24 hours of reperfusion were determined (Figure 2, A and B). No differences in infarct size between mice were observed. However, the analysis of cardiac sections from Plhd1/+/ and Plhd1−/− mice 24 hours after MI showed approximately 50% reduced migration of cells into the infarct border zone in Plhd1−/− mice compared with Plhd1+/+ mice as seen by hematoxylin and eosin staining (Figure 2C). Previous reports have indicated that PLD1 is important for the adhesion and migration of neutrophils and macrophages. Based on our findings that PLD1 is up-regulated 24 hours after MI and a strongly reduced number of migrating cells into the infarct border zone of Plhd1−/− mice was detected 24 hours after MI, we decided to study the influence of PLD1 on the migration and chemotactic activity of specific inflammatory cells.

The analysis of macrophage recruitment into the infarct border zone 24 hours after MI showed a significant reduction of macrophage recruitment after 24 hours of reperfusion on PLD1 deficiency (0.9 ± 0.17 mm² versus 0.6 ± 0.1 mm²; P < 0.01; anti-Mac-3) (Figure 2, D and E). Similarly, neutrophil recruitment also was reduced (0.9 ± 0.1 mm² in Plhd1−/− mice compared with 2.4 ± 0.1 mm² in Plhd1+/+ mice; P < 0.001; antipolymorphonuclear) (Figure 2, D and E).

PLD1 Regulates TNF-α Release

TNF-α is one of the crucial cytokines involved in inflammation and acute phase reaction. Its major role is the regulation of immune cells. Thus, we decided to explore the release of TNF-α on PLD1 deficiency to analyze if PLD1 is able to modulate TNF-α release. Indeed, a lack of PLD1 led to a prominent reduction of TNF-α expression in cardiac sections and in blood plasma 24 hours after MI (Figure 3, A and B). Besides reduced TNF-α levels, we found reduced TGF-β levels in plasma of Plhd1−/− mice 72 hours after MI (Figure 3C). Monocytes and macrophages are the major source of TNF-α. Thus, we decided to analyze TNF-α signaling in PLD1-deficient monocytes and macrophages in further detail. First, we measured PLD activity in CD34+ cells and macrophages using a nonradioactive enzymatically coupled assay, in which PLD-mediated PA production was investigated with a fluorescent in vitro assay (Figure 3D). These results were confirmed by immunostaining for PLD1, showing that PLD1 accumulates in macrophages of the left ventricle (Figure 3E). The stimulation of monocytes isolated from

![Figure 3](ajp.amjpathol.org/articles/2455)
whole blood of $Pld1^{+/+}$ and $Pld1^{-/-}$ mice showed that LPS treatment led to a strong increase of TNF-α in $Pld1^{+/+}$ cells that was strongly reduced in $Pld1^{-/-}$ cells, showing that PLD1 is crucial for the release of TNF-α (986.7 ± 72.4 pg/mL versus 144.9 ± 31.3 pg/mL; $P < 0.001$) (Figure 3F). In control experiments, normal Toll-like receptor 4 expression on the plasma membrane of $Pld1^{-/-}$ monocytes was detected by flow cytometry, showing that differences in TNF-α release is not caused by alterations in LPS-receptor expression (Supplemental Figure S1).

**Reduced TNF-α Expression Affects the Expression and Release of the Acute Phase Cytokine IL-1α**

TNF is an endogenous pyrogen and is able to induce IL-1 production on inflammation.28 Consequently, we analyzed

![Figure 4](https://example.com/figure4.png)

**Figure 4** PLD1-mediated TNF-α release is a prerequisite for the expression and release of the acute phase cytokines IL-1α and NF-κB activation in monocytes. Quantitative analysis of IL-1α on LPS-induced (A) and TNF-α-induced (B) stimulation of monocytes derived from whole blood of $Pld1^{+/+}$ and $Pld1^{-/-}$ mice. Bar graphs depict means ± SD. mRNA expression of TNF-α (C) and IL-1α (D) in the left ventricle of $Pld1^{+/+}$ and $Pld1^{-/-}$ mice 24 hours after I/R injury was determined using quantitative RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase was used as a control. E and F: Monocytes from whole blood of $Pld1^{+/+}$ and $Pld1^{-/-}$ mice were stimulated with LPS and translocation of NF-κB into the nucleus was observed by confocal immunofluorescent analysis. E: Cellular localization of NF-κB was monitored by p65 staining (red); overlay of nuclear (Draq 5, green) and p65 staining (merge, yellow) shows NF-κB localization in the nucleus and thus NF-κB activation. F: NF-κB activation on cell activation with recombinant TNF-α, $n = 6$ (A and B). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. Scale bars: 10 μm. Black bars, $Pld1^{+/+}$; gray bars, $Pld1^{-/-}$; R, Rest, basal.
the IL-1 expression of Pld1+/+ and Pld1−/− monocytes to investigate if defects in TNF-α release influences the activation of the proinflammatory cytokine IL-1 as a result of PLD1 deficiency (Figure 4A). Indeed, the level of IL-1 expression from Pld1−/− monocytes was strongly reduced after 24 hours after LPS stimulation (32.2 ± 3.0 pg/mL versus 5.0 ± 0.3 pg/mL) (Figure 4A). Furthermore, the stimulation of monocytes with TNF-α likewise showed reduced IL-1 levels in Pld1−/− monocytes compared with controls (Figure 4B). In the next step, we explored TNF-α expression after LPS treatment. TNF-α expression was strongly reduced after 6, 12, and 24 hours, and likely is responsible for the defective TNF-α and IL-1 release of Pld1−/− monocytes (Figure 4C). Furthermore, IL-1 expression was delayed because 6 hours after LPS treatment of monocytes, IL-1 expression of Pld1−/− monocytes was reduced compared with Pld1+/+ monocytes but was indistinguishable between both groups after 12 and 24 hours of LPS treatment. TGF-β1 and TGF-βRII expression in the infarcted heart (left cardiac ventricle) 72 hours after I/R was shown to be comparable between Pld1+/+ and Pld1−/− mice (Supplemental Figure S2). Thus, we conclude that PLD1-mediated TNF-α expression is responsible for the defective TNF-α and IL-1 release and also might be involved in reduced TGF-β secretion after I/R in Pld1−/− mice.

PLD1-Mediated TNF-α Release Is a Prerequisite for NF-κB Activation

TNF-α binds to TNF receptor type 1 and TNF receptor type 2, leading to the activation of NF-κB.29 NF-κB controls the expression of inducible chemokines, cell adhesion molecules, and anti-apoptotic proteins.30 Because of its central role in mediating vascular inflammation,31 we studied the functional consequences of defective TNF-α release on PLD1 deficiency with regard to NF-κB activation. Monocytes of Pld1+/+ and Pld1−/− mice were stimulated with LPS and translocation of NF-κB was investigated by co-staining of cells with anti-p65 and dra5 (nuclear staining) (Figure 4E). After LPS stimulation, NF-κB was observed in the nucleus of Pld1+/+ monocytes (Figure 4E), whereas NF-κB failed to transfer to the nucleus of Pld1−/− cells. To test if this is a secondary effect induced by defective TNF-α release, we examined NF-κB activation on stimulation with recombinant TNF-α (Figure 4F). Interestingly, we now found NF-κB in the nucleus of Pld1+/+ and Pld1−/− cells, suggesting that PLD1 is important for LPS-mediated NF-κB translocation to the nucleus, whereas it is not crucial for NF-κB activation upon TNF-α stimulation of monocytes.

Adhesion of Monocytes under Flow Conditions Is Impaired by the Absence of PLD1

To investigate the functional consequences of impaired TNF-α/IL-1 release and NF-κB activation on cell adhesion we performed adhesion studies under flow conditions using arterial shear stress. Here, we analyzed monocyte adhesion to activated murine endothelial 5-T cells (Figure 5, A and B). The lack of PLD1 led to a strongly reduced number of rolling and adherent monocytes under flow conditions (8.4 ± 4.5 versus 0.5 ± 0.6 adhesive monocytes/high-power field, and 4.5 ± 0.6 versus 0.75 ± 0.9 rolling monocytes/high-power field) (Figure 5, A and B), suggesting that PLD1 is crucial for TNF-α-mediated cell activation and adhesion.

Adhesion experiments using different extracellular matrix proteins showed that PLD1 is of great importance for cell adhesion under flow conditions, although it does not influence monocyte adhesion under static conditions as seen by in vitro studies using Pld1−/− monocytes that adhere to collagen or fibronectin, respectively, under static conditions comparable with Pld1+/+ monocytes (Supplemental Figure S3).

PLD1 Is Important for Monocyte Migration

Recent studies have provided strong evidence that PLD is required for phagocyte adhesion and migration.11,26,32 Based on our findings that PLD1 is crucial for cell adhesion under...
flow conditions we decided to study the relative contribution and relevance of PLD1 for monocyte chemotaxis using a modified Boyden chamber system. Here, we investigated the migration of monocytes along a monocyte chemotactic protein 1 gradient. We were able to show that migration of

$Pld1^{-/-}$ monocytes after a monocyte chemotactic protein 1 gradient was strongly reduced compared with the migratory potential of $Pld1^{+/+}$ monocytes (100% ± 26% versus 67.93% ± 27%) (Figure 5, C and D). Thus, we believe that strongly reduced TNF-α release and impaired NF-κB

Figure 6 Increased infarct size in $Pld1^{-/-}$ mice after 28 days of I/R injury. A: Representative transverse cardiac sections stained with Gomori’s One Step Trichrome show increased infarct size in $Pld1^{-/-}$ mice. B: Infarct size was determined using Diskus software (Hilgers) and was expressed as the percentage of total LV volume. C: Survival of $Pld1^{+/+}$ and $Pld1^{-/-}$ mice on challenge. D: Representative M-mode echocardiograms of $Pld1^{+/+}$ mice (upper panel) and $Pld1^{-/-}$ mice (lower panel). Echocardiographic analysis of ejection fraction (baseline versus 28 days after I/R) (E), cardiac output (F), and fractional area shortening (baseline versus 28 days after I/R) (G) of $Pld1^{+/+}$ and $Pld1^{-/-}$ mice as an indicator for LV systolic function. Bar graphs depict means ± SD. ΔEF (EF before I/R - EF 28 days after I/R) in percentage and ΔFS (FS before I/R - FS 28 days after I/R) in percentage of echocardiographic analysis before and after 28 days of I/R (n ≥ 6; basal versus MI; $Pld1^{+/+}$ versus $Pld1^{-/-}$). N = 11 to 13 mice per group (B); n = 16 (C). Scale bar = 100 μmol/L. *P < 0.05, **P < 0.01, and ***P < 0.001, basal versus MI; †P < 0.05, ‡P < 0.01, $Pld1^{+/+}$ versus $Pld1^{-/-}$. Black bars, $Pld1^{+/+}$; gray bars, $Pld1^{-/-}$. EF, ejection fraction; FS, fractional shortening.
activation of monocytes/macrophages that lack PLD1 likewise are responsible for reduced adhesion and migration of inflammatory cells into the infarct border zone after I/R in mice, which might influence cardiac repair.

Infarct Size Increases and Cardiac Function Decreases in Pld1−/− Mice after MI

To investigate the (patho)physiological consequences of reduced TNF-α release followed by reduced adhesion and migration of inflammatory cells into the infarct border zone 24 hours after myocardial ischemia in mice, we analyzed cardiac damage and repair of ischemic hearts in further detail. MI was induced in Pld1+/+ and Pld1−/− mice and the infarct areas 28 days after I/R was determined (Figure 6, A and B). Infarct areas 28 days after reperfusion were enhanced in Pld1−/− mice compared with control mice (100% ± 42.68% versus 150.99% ± 41.73% infarct size/LV as percentage of control; P < 0.01) (Figure 6B). Unexpectedly, our data showed a trend toward decreased mortality of Pld1−/− mice compared with controls, without statistical significance (Figure 6C). The increase in infarct size in Pld1−/− mice was functionally relevant because echocardiography 28 days after LAD ligation showed significantly decreased systolic LV function (Figure 6, D–G), as shown by the analysis of ejection fraction, fractional shortening, and cardiac output in Pld1−/− mice compared with Pld1+/+ mice (13.0% ± 3.9% versus 21.2% ± 1.8% delta ejection fraction; 4.5% ± 1.0% versus 7.8% ± 1.2% delta fractional shortening; cardiac output; 13.52 ± 0.89 mL/minute before MI versus 10.01 ± 0.5 mL/minute 28 days after MI in Pld1+/+ mice; and 12.32 ± 0.61 mL/minute before MI versus 7.96 ± 0.45 mL/minute 28 days after MI in Pld1−/− mice; P < 0.05) (Figure 6, E–G and Supplemental Videos S1 and S2). Additional hemodynamic parameters were measured and are presented in Table 1, indicating severe impairment of hemodynamic functions of Pld1+/+ and Pld1−/− mice 28 days after MI (eg, end systolic and end diastolic volume in microliters). These data clearly indicate that Pld1−/− mice develop impaired hemodynamic function in response to myocardial ischemia and reperfusion.

Matrix Remodeling 28 Days after MI

To investigate the mechanisms that, in addition to a reduced inflammatory response in Pld1−/− mice, led to a decreased hemodynamic function of Pld1−/− mice, collagen remodeling was characterized after 28 days of reperfusion. Representative hematoxylin and eosin–stained sections of infarcted hearts showed histology of infarct repair (Figure 7A) in Pld1+/+ and Pld1−/− mice 28 days after MI. Masson’s trichrome staining of cardiac sections confirmed the collagenous character of scar tissue in both groups (Figure 7B). To analyze the collagen organization in further detail we investigated fibrillar collagen fibril density by Sirius red staining 28 days after MI. Birefringence analysis of Sirius red staining showed a trend toward a decreased percentage of tightly packed collagen fibrils, suggesting that the collagen matrix in the scar tissue of Pld1−/− mice was different compared with controls (Figure 7C). Because fibroblasts are the cell type that is responsible for collagen deposition, we stained cardiac myocardial fibroblasts after MI with α-SMA. As shown by representative images and quantification, a reduced number of α-SMA–positive myofibroblasts were detected in the infarct border zone of Pld1−/− mice compared with Pld1+/+ mice 28 days after ischemia (Figure 7D). These differences prompted us to quantify interstitial fibrosis in the noninfarcted regions as well. Sirius red staining of the remote myocardium showed increased interstitial collagen in noninfarcted regions of the left ventricle of Pld1−/− mice compared with controls as shown by representative Sirius red–stained cardiac sections and quantitative analysis (Figure 7E). To identify a mediator responsible for altered matrix remodeling in Pld1−/− mice we examined if PLD1 deficiency leads to alterations in TGF-β expression and release, leading to impaired myofibroblast transdifferentiation and defective scar formation. Release of TGF-β at 48 hours after stimulation of cardiac fibroblasts with 100 nmol/L angiotensin II showed a reduction of secreted TGF-β into cell culture supernatants, whereas mRNA expression of TGF-β was not different between Pld1+/+ and Pld1−/− fibroblasts (Figure 7, F and G). Furthermore, we found decreased expression of α-SMA as verified by quantitative real-time RT-PCR when fibroblasts were stimulated with angiotensin II, whereas no differences were observed on stimulation with 10% fetal calf serum or 10 ng/mL TGF-β (Figure 7H). To analyze if reduced TGF-β release and α-SMA expression influence the migratory properties of fibroblasts we performed Boyden chamber experiments.

Table 1  Hemodynamic Measurements at 28 Days after MI

<table>
<thead>
<tr>
<th>n = 4–8</th>
<th>Heart rate, bpm</th>
<th>Stroke volume</th>
<th>End-systolic volume (μL)</th>
<th>End-diastolic volume (μL)</th>
<th>CO (mL/minute)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Pld1+/+</td>
<td>378.26 ± 45.16</td>
<td>34.66 ± 4.25</td>
<td>33.55 ± 14.6</td>
<td>4.69 ± 11.64</td>
<td>9.03 ± 1.82</td>
</tr>
<tr>
<td>Pld1−/−</td>
<td>363.23 ± 43.63</td>
<td>35.19 ± 5.21</td>
<td>26.23 ± 12.97</td>
<td>67.53 ± 13.24</td>
<td>13.87 ± 1.88²</td>
</tr>
<tr>
<td>MI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pld1+/+</td>
<td>396.82 ± 28.07</td>
<td>23.55 ± 7.78**</td>
<td>84.06 ± 51.67*</td>
<td>84.74 ± 42.79*</td>
<td>13.18 ± 1.27**</td>
</tr>
<tr>
<td>Pld1−/−</td>
<td>374.71 ± 37.45</td>
<td>21.27 ± 4.33***</td>
<td>20.21 ± 4.41¹</td>
<td>51.59 ± 3.93**¹</td>
<td>8.68 ± 2.26**¹</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01, ***P < 0.001 basal versus MI.

1P < 0.05 Pld1+/+ versus Pld1−/−.
Analysis of Pld1\textsuperscript{+/+} and Pld1\textsuperscript{−/−} fibroblast migration showed no significant differences between both groups but showed a trend toward reduced migration of Pld1\textsuperscript{−/−} fibroblasts 6 hours after stimulation with 10% fetal calf serum (Figure 7I).

**Discussion**

The present study provides strong evidence that PLD1 is an important mediator of the TNF-α-mediated inflammatory response and affects cardiac remodeling after I/R in mice. By
using a mouse model of I/R, we showed the following: i) strong enhancement in expression of PLD1 in infarcted myocardium after I/R injury in mice, ii) strong decrease in migration of neutrophils and macrophages into the infarct border zone of Plδ1−/− mice and reduced TNF-α and TGF-β plasma levels 24 and 72 hours after ischemia and reperfusion, iii) disordered NF-κB activation and reduced IL-1β release resulting from impaired TNF-α signaling in monocytes after PLD1 deficiency, iv) altered myofibroblast differentiation and interstitial collagen deposition 28 days after MI, and v) reduced TGF-β secretion and decreased mRNA expression of α-SMA in cultured cardiac fibroblasts, which all lead to impaired cardiac remodeling with increased infarct size and decreased cardiac function 28 days after I/R in Plδ1−/− mice.

These results strongly imply that PLD1 is crucial for myocardial repair to limit heart failure because PLD1 is important for an adequate inflammatory response that may lead to proper cellular repair and preserves cardiac function. 

Previously, a significant increase in PLD activity was observed in hearts that were subjected to I/R; stimulation of PLD was reported to be responsible for a significant improvement of cardiac function during reperfusion.33 Moreover, PLD was reported to be involved in the protection of ischemic preconditioning in the rabbit heart.34 Recent studies provided strong evidence that PLD and PLA2, an enzyme of the PA/LPA signaling pathway, play an important role in processes of myocardial I/R in vivo. Dent et al35 showed increased PLD1 protein contents in the viable left ventricle tissue after ligation of the LAD. Moreover, they detected PLD2 protein in scar tissue where PLD1 protein was undetectable. In the central nervous system, up-regulation of PLD in neuronal cells results in the selective delay of CA1 pyramidal cell death after transient forebrain ischemia, showing that PLD also influences cell survival of neuronal cells under ischemic conditions.36 The mechanism by which PLD expression was enhanced has not been explored to date. It is tempting to speculate that TNF-mediated NF-κB activation induces up-regulation of PLD1 because two studies using different small molecules to inhibit PLD showed that NF-κB activation is coupled to PLD expression.37,38

Figure 7 Myofibroblast differentiation and interstitial fibrosis are altered in Plδ1−/− mice 28 days after MI compared with Plδ1+/+ mice. Hematoxylin and eosin (A) and Masson’s trichrome (B) staining of infarcted hearts from Plδ1−/− and Plδ1+/+ mice. C: Analysis of fibrillar collagen fibril density using Sirius red staining and polarized light for quantification of scar-associated collagen. Proportion of red to green collagen, representative images and quantification are shown. D: α-SMA staining in Plδ1−/− and Plδ1+/+ mice to detect α-SMA-positive myofibroblasts (arrows) in the border zone, representative images and quantification are shown. E: Sirius red staining of interstitial collagen of remote myocardium, representative images and quantification are shown. F: mRNA expression of TGF-β1 24 hours after stimulation is not different between cardiac fibroblasts isolated from Plδ1+/+ and Plδ1−/− mice. G: TGF-β enzyme-linked immunosorbent assay showed lower levels of secreted TGF-β in medium of cultured cardiac fibroblasts from Plδ1−/− mice 48 hours after stimulation with 100 nmol/L AngII. H: mRNA expression of α-SMA 24 hours after stimulation with 100 nmol/L AngII was reduced in Plδ1−/− fibroblasts. I: Migration of cardiac fibroblasts using a 10% FCS gradient showed no differences between the two genotypes. N = 6 to 9 (C); N = 8 to 10 (D); N = 8 to 11 (E); N = 6 (F and G); N ≥ 6 (E–G); N = 5 to 6 (H); n = 4 to 5 (I). Original magnification: ×200 (C); ×400 (D); ×200 (E). *P < 0.05; **P < 0.01. Plδ1−/− versus Plδ1+/+; 1P < 0.05 stimulated cells versus unstimulated control. Black bars, Plδ1+/+; gray bars, Plδ1−/−. AngII, angiotensin II; FCS, fetal calf serum.
us to the hypothesis that PLD is not only an important mediator of the inflammatory response after MI, but a general and important player in the processes of inflammation.

Inflammatory responses strongly rely on TNF-α signaling.42 Our results show that PLD1 deficiency leads to strongly impaired TNF-α expression and release, and prohibits LPS-mediated NF-κB translocation and IL-1α release on stimulation. Recently, the use of antisense oligonucleotides to reduce PLD1 expression showed that PLD1 is coupled to TNF-α signaling and activation of NF-κB.15 It was reported that TNF-α activates PLD1 in a dose-dependent manner, and that PLD1 was required for the activation of NF-κB, sphingosine kinase, and cytosolic calcium.18 Moreover, knockdown of PLD1 decreased the levels of different cytokines (eg, IL-1 and IL-6 on stimulation with TNF).18 Here, LPS-mediated stimulation of cells showed that TNF-α and IL-1α release are impaired significantly by PLD deficiency, confirming recent results that the release of proinflammatory cytokines strongly depends on PLD1 signaling under inflammatory conditions. Furthermore, it was shown in a mouse model of TNF-α–induced peritonitis that induction of inflammation by TNF-α led to infiltration with phagocytes and increased vascular permeability, whereas knock-down of PLD1 by specific siRNA strongly reduced these inflammatory processes. Although Sethu et al18 provided evidence in vivo that PLD1 is important for TNF-α signaling they did not use PLD1-deficient cells or mice. Knock-down of PLD1 was achieved by using siRNA, leading to results that might differ from mice deficient in PLD1. Our results show for the first time that PLD1 deficiency leads to impaired TNF-α signaling under inflammatory conditions in vitro and in vivo, providing direct evidence that PLD1 is crucial for the TNF-α–mediated inflammatory response after I/R injury in mice. The finding that PLD1 deficiency results in altered TNF expression that causes defective TNF release was very surprising. However, in Jurkat cells, PLD stimulation can activate the transcription factor activator protein 1 (AP-1), which is known to be involved in LPS-mediated TNF expression,19,43 suggesting that PLD1 deficiency leads to inhibition of AP-1 and thus to decreased TNF formation and release in Pld1−/− monocytes.

Our data provide evidence that TNF-α signaling strongly depends on PLD1 signaling under inflammatory conditions because IL-1 release also was impaired in PLD1 deficiency. IL-1 is a key proinflammatory cytokine that recently was shown to be a promising target for the treatment of cardiovascular diseases. One-time intravenous administration of anakinra, an IL-1– receptor antagonist, can reduce infarct size in experimental ischemia and reperfusion injury.44 Moreover, IL-1 blockade is effective in improving outcomes in acute MI in animal studies and preliminary data in patients with acute MI are promising.45,46 By using the recombinant human IL-1– receptor antagonist, IL-1 blockade was found to protect the myocardium and preserve cardiac function.46 In contrast to all of these recently published studies in which only IL-1 was targeted, we found reduced IL-1 levels in cells lacking PLD1 together with decreased cardiac function. However, besides decreased IL-1 levels, multiple inflammatory defects including decreased TNF-α levels and impaired NF-κB activation were observed in Pld1−/− cells/mice, showing that inflammation after infarction is critical for the healing process. On the other hand, excessive inflammation could play a pivotal role in the development of LV remodeling.47,48 Thus, the inflammatory response after MI is a double-edged sword,47,48 indicating that it is important to control inflammation and to suppress pathologic remodeling.48

Indeed, TNF-α–mediated responses as well as anti-inflammatory therapy after MI not only modulate the inflammatory response, but also influence the matrix and LV collagen network to contribute to LV dysfunction.49,50 However, in this study we provide evidence that ablation of PLD1 does not lead to decreased LV function solely via perturbed TNF-α signaling but affects scar formation by alterations in fibroblast transformation and increased interstitial fibrosis, leading to aggravated LV dysfunction because the quality of scar formation seems to be poor with PLD1 deficiency. This was accompanied by decreased expression of α-SMA in cardiac fibroblasts and reduced secretion of the fibrogenic mediator TGF-β into cell culture supernatant, suggesting that altered matrix remodeling in Pld1−/− mice is not a result of decreased inflammation but is caused by direct modulation of myofibroblast differentiation by PLD1. Recent studies already have shown that PLD1 and PLD2 contribute to fibroblast transformation in cooperation with signals from epidermal growth factor receptor or Src kinases, making PLDs important target molecules for anticancer strategies.51–53 Increased expression of PLD-transformed rat fibroblasts overexpressing c-Src or epidermal growth factor receptor are reported to contribute to a malignant phenotype in cells with increased tyrosine kinase activity.53,54

Taken together, our data clearly indicate that PLD1 is not important for acute cardiomyocyte injury because we found comparable acute infarct size in both genotypes. However, PLD1 is crucial for scar formation and LV function 28 days after I/R because both the initial injury and the qualitative reparative response was altered in Pld1−/− mice, suggesting impaired myofibroblast activation and wound contraction after I/R in these mice.

However, we do not know if PLD1 affects cellular function besides TNF-α signaling and matrix remodeling, which contribute to the observed phenotype in those mice. In addition, different cell types are injured on I/R such as neutrophils, endothelial cells, cardiomyocytes, and so forth,55 which are related to TNF-α signaling and might contribute to the observed Pld1−/− phenotype in vivo. Thus, further studies are needed to define cell-type–specific and humoral effects mediated by PLD1 after myocardial infarction in mice.

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Supplemental Data

Supplemental material for this article can be found at http://dx.doi.org/10.1016/j.ajpath.2014.06.005.

References