Myocardial infarction (MI) is closely associated with major alterations in cardiac structure and function that subsequently lead to the development of MI-induced cardiac failure. The ventricular remodeling post MI is characterized by 3 main phases: the inflammatory phase, the proliferative/adaptive phase, and the maturation phase. The main characteristic of the early inflammatory phase (3–72 hours post MI) is the activation of chemokines and cytokines such as interleukin-6 (IL-6) that recruit neutrophils to the infarcted area. In the adaptive/proliferative phase (72 hours to 7 days post MI), activated myofibroblasts produce extracellular matrix (ECM) proteins including collagen 1 and 3 as part of the healing process, and a microvascular network is built up to circumvent the ischemia-induced tissue damage. In the maturation phase (7–14 days), the collagen-based scar is formed, which is necessary to replace the extensive loss of cardiac myocytes and maintain ventricular integrity. Importantly, expression of collagen 1 and 3 is increased also in the nonischemic remote myocardium, and accelerated collagen cross-linking by lysyl oxidases (LOXL) subsequently increases the degree of passive ECM stiffness. These findings clearly demonstrate MI-induced matrix remodeling of the viable myocardium.

Rationale: Myocardial infarction (MI) increases the wall stress in the viable myocardium and initiates early adaptive remodeling in the left ventricle to maintain cardiac output. Later remodeling processes include fibrotic reorganization that eventually leads to cardiac failure. Understanding the mechanisms that support cardiac function in the early phase post MI and identifying the processes that initiate transition to maladaptive remodeling are of major clinical interest.

Objective: To characterize MI-induced changes in titin-based cardiac myocyte stiffness and to elucidate the role of titin in ventricular remodeling of remote myocardium in the early phase after MI.

Methods and Results: Titin properties were analyzed in Langendorff-perfused mouse hearts after 20-minute ischemia/60-minute reperfusion (I/R), and mouse hearts that underwent ligation of the left anterior descending coronary artery for 3 or 10 days. Cardiac myocyte passive tension was significantly increased 1 hour after ischemia/reperfusion and 3 and 10 days after left anterior descending coronary artery ligation. The increased passive tension was caused by hypophosphorylation of the titin N2-B unique sequence and hyperphosphorylation of the PEVK (titin domain rich in proline, glutamate, valine, and lysine) region of titin. Blocking of interleukine-6 before left anterior descending coronary artery ligation restored titin-based myocyte tension after MI, suggesting that MI-induced titin stiffening is mediated by elevated levels of the cytokine interleukine-6. We further demonstrate that the early remodeling processes 3 days after MI involve accelerated titin turnover by the ubiquitin–proteasome system.

Conclusions: We conclude that titin-based cardiac myocyte stiffening acutely after MI is partly mediated by interleukine-6 and is an important mechanism of remote myocardium to adapt to the increased mechanical demands after myocardial injury. (Circ Res. 2016;119:1017-1029. DOI: 10.1161/CIRCRESAHA.116.309685.)

Key Words: cardiac output ■ chemokines ■ interleukin-6 ■ myocardial infarction ■ myofibroblasts
which is required to adapt to the increased mechanical stress caused by the functional loss of ischemic ventricular tissue. However, collagen 1 and 3 expression and cross-linking have been reported for the proliferative phase after MI, and elevated mRNA and protein expression levels were detected not earlier than 3 to 4 days post MI. At that time, left ventricular (LV) ejection fraction is already significantly reduced and end-systolic volume increased, indicating substantial loss of ventricular function and increasing hemodynamic load. This results in increased mechanical stress of the remaining myocardium that needs rapid adaptation to maintain cardiac output. Because fibrotic matrix reorganization of the infarct zone manifests later than 3 days post MI, the increased mechanical stress must be compensated by modification of the viable cardiac myocytes in the remote area. To date, only little is known about functional changes of sarcomeric proteins 1 to 3 days post MI and about the involvement of MI-induced sarcomere dysfunction in the transition to cardiac failure. Early changes in sarcomere function have recently been investigated in a mouse model with ligature of the left anterior descending (LAD) artery. Avner et al demonstrated that 3 days post MI, proteins of the contractile machinery underwent complex post-translational modification, including altered protein phosphorylation and glutathionylation. The detected changes significantly increased myofilament Ca2+ sensitivity and elevated myofilament force production in the physiological range of Ca2+ concentrations.

Whether the early adaptive remodeling processes post MI also involves modulation of the passive mechanical properties of the surviving cardiac myocytes has not been investigated to date. Passive cardiac myocyte stiffness is mainly defined by the giant protein titin (3.0–3.3 MDa), which spans a half-sarcomere from the Z-disk to the M-line and resembles the third filament structure of the mammalian sarcomere. Because of its size and central position in the sarcomere, titin displays important roles in striated muscle function. Titin closely associates to myosin in the A-band region of the sarcomere and thereby helps to keep the A-band in the middle of the sarcomere during each contraction–relaxation cycle. Several titin–protein interactions link titin to hypertrophic signaling pathways and to the protein quality control system. Finally, the I-band region of titin contains 2 main elastic regions that can reversibly extend in response to mechanical stretch and thereby form the basis for titin’s role as a molecular spring. Titin stiffness thereby determines passive myocyte distensibility, which has been recognized as an important modulator of systolic and diastolic myocardial functions. Titin stiffness is mainly defined by the sarcomeric composition of the 2 main cardiac titin isoforms N2BA (3.2–3.3 MDa, long and compliant) and N2B (3.0 MDa, shorter and stiffer). Titin properties can also be modulated in short term by post-translational modification of the elastic I-band domains N2-B unique sequence (N2-Bus) and PEVK (titin domain rich in proline, glutamate, valine, and lysine). Phosphorylation within the cardiac-specific N2-Bus by cAMP-dependent protein kinase, cGMP-dependent protein kinase, extracellular signal–regulated kinase 1/2 (Erk1/2), and Ca2+/calmodulin-dependent protein kinase II delta (CaMKIIδ) decreases titin-based cardiac myocyte stiffness, whereas phosphorylation of the PEVK domain by Ca2+-dependent protein kinase C α (PKCα) increases stiffness.

Because of its gigantic size, titin is highly susceptible to fragmentation and degradation and is among the first proteins to be degraded after ischemia-induced sarcomere breakdown. In the ischemic myocardium, titin degradation is at least partly performed by matrix metalloproteinase 2 (MMP-2). MMP-2 is typically active in the ECM, but recent evidence suggested activation and sarcomeric localization under ischemic conditions with subsequent degradation of titin in the infarct region.

In our present study, we aimed to characterize early MI-induced changes in titin performance and to elucidate the role of titin in ventricular remodeling of ischemic and the surviving remote myocardium in the early phase after MI. To address these questions, we analyzed tissue samples from Langendorff-perfused hearts subjected to 20-minute ischemia and 60-minute reperfusion (I/R) and remote myocardium from mice 3 or 10 days after permanent ligature of the left anterior descending coronary artery. Our data indicate a significant role of titin in the maintenance of ventricular stability in the acute phase after myocardial ischemia by rapidly increasing cardiac myocyte stiffness. These alterations are based on hyperphosphorylation of the titin PEVK region and hypophosphorylation of the titin N2-Bus. Blocking of IL-6 inhibited the MI-induced cardiac myocyte stiffening in the remote myocardium, suggesting that the observed titin changes are at least partly induced by increased levels of IL-6. Finally, our study reports a significant increase in proteasomal activity in the viable myocardium after acute MI, which includes accelerated titin turnover via the ubiquitin/proteasome system.

**Methods**

**Isolated Mouse Hearts**

All animal procedures were conducted in accordance with the animal care and use committee of the responsible authorities (Landesamt für Natur, Umwelt- und Verbraucherschutz Nordrhein-Westfalen) that reviewed and approved the experimental protocols (Az. 84-02.04.2013.A122).
C57/BL6J mice were injected intraperitoneally with 250 IU heparin. After cervical dislocation, the heart was rapidly excised and transferred for preparation of the aortic trunk to ice-cold Krebs–Henseleit buffer. The aorta was cannulated, and the heart was perfused in a nonrecirculating Langendorff mode at constant pressure (80 mm Hg) with a modified Krebs–Henseleit buffer containing (in mmol/L): NaCl, 116; KCl, 4.63; MgSO₄, 1.1; NaHCO₃, 24.9; CaCl₂, 2.52; KH₂PO₄, 1.18; glucose, 8.32; pyruvate, 2; and EDTA, 0.5, equilibrated with 95% O₂ and 5% CO₂ (pH 7.4; 37 °C). Hearts were allowed to equilibrate for 30 minutes until a constant coronary flow was reached. A fluid-filled balloon was inserted into the left ventricle, and the end-diastolic pressure was set to 2 at 5 mm Hg. Left ventricular pressure, heart rate, and coronary flow were recorded continuously. Hearts of the ischemia group underwent 20 minutes of global ischemia (37 °C) followed by 60 minutes of reperfusion. Control hearts underwent 80 minutes of perfusion. At the end of the experiments, hearts were snap-frozen in liquid nitrogen and stored at −80 °C until further analysis.

**Permanent Myocardial Ischemia**

C57/BL6J mice were anesthetized by intraperitoneal injection of ketamine (60 mg/kg body weight) and xylazine (10 mg/kg body weight) and ventilated as previously described. After left lateral thoracotomy between the third and fourth rib, the pericardium was dissected, and a 7-0 surgical suture was cautiously passed underneath the LAD artery at a position 1 mm from the tip of the left auricle. MI was produced by suture occlusion. The correct position of the suture was confirmed by blanching of the apex and characteristic change in ECG (ST-segment elevation). Permanent MI was performed for 3 and 10 days, respectively. Animals received buprenorphine (0.05–0.1 mg/kg body weight, SC) every 8 hours after induction of MI as described previously.

**Echocardiography**

Cardiac images were acquired using a Vevo 2100 high-resolution ultrasound scanner with 18 to 38 MHz linear transducer (VisualSonics, Inc). Echocardiography was performed as previously described: before MI and 3 and 10 days post MI, respectively. LV end-systolic volumes, end-diastolic volumes, and heart rate were measured; LV ejection fraction, stroke volume, and cardiac output were calculated.

**Isolation of Adult and Embryonic Rat Cardiac Myocytes**

Hearts were obtained from embryos (gestational day 18) of pregnant adult Wistar rats. Animals were anesthetized by 2% isoflurane and euthanized by decapitation. Cells were isolated by enzymatic digestion and plated in 6-well plates at a density of 5×10⁵ cells/well and cultured for 24 hours before induction of MI as described previously.

**Force Measurements on Isolated Cardiac Myocytes**

Passive tension (PT) measurements were performed on mouse cardiac myocytes isolated from tissue biopsies as previously described. For each tissue, 6 cells were measured. Briefly, single cardiac myocytes were selected and mounted between a piezoelectric motor and a force transducer (403A; Aurora Scientific). Cells were then step-wise stretched from slack sarcomere length (SL; average, 1.9 µm) to a maximum SL of 2.4 µm. Passive force and SL were recorded for each step and related to cardiac myocyte cross-sectional area.

**SDS-PAGE and Western Blot Analysis**

Titin isoform composition analysis and standard SDS-PAGE were performed as previously described. For Western blot analysis of titin domain phosphorylation, general protein and titin ubiquitination, and kinase activities, proteins were transferred onto polyvinylidene fluoride membrane and processed as previously described.

**Proteasome Activity Measurements**

Trypsin-like activity of the 26S proteasome in the mouse heart tissues was measured in a GloMax-Multi Detection System using Proteasome-GloTM–Trypsin-Like Cell-based Assay (Promega). Heart tissue lysates containing 10 µg of protein were mixed 1:1 with the Proteasome-Glo Reagent and measured after 5 minutes of incubation at room temperature. For determination of the final proteasome activity that is correlated to the amount of proteasome in the lysates, the calculated values of proteasomal activity were normalized by immunoblot analysis of the proteasome subunit α type 2.

**RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction**

For gene expression, septum samples were homogenized using the gentleMACS Dissociator (Miltenyi Biotec), followed by trizol/chloroform extraction and RNA precipitation with isopropanol. One thousand nanograms of RNA were transcribed (QuantiTect Reverse Transcription Kit; QIAGEN) followed by quantitative real-time polymerase chain reaction (qPCR) using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). LOXL1 AND LOXL2 expression was detected using the following primers: 5′-CTTGAGTTCGAGGCTGAAGGAAA-3′ and 5′-TTCACTGACCTGCAAGGCTGAAG-3′; 5′-ATGTTGCCTGGAGGACTA-3′ and 5′-TGAAGTGTCGCCACATCGTGC-3′; respectively, and normalized to 18S RNA. Coll1a1 and Col3a1 expression was detected using the following primers: 5′-GAGGGGAGCTGATCGC-3′ and 5′-GGTCCCAGCTGTAGTAC-3′ and 5′-TACTCT CCTTGGCTGT-3′, respectively, and normalized to GAPDH RNA.

**Histology**

Immunofluorescence staining was performed as described below. Raw hearts were snap-frozen at −40 °C and cryosectioned at −22 °C in 8-µm slices. Shown slices are 500 µm apical from the ligation in MI hearts; in sham-operated, hearts slices from comparable regions were taken. The sliced tissue was fixed in 4% paraformaldehyde in 0.1 mol/L sodium phosphate buffer pH 7.4 for 10 minutes. Primary antibodies were incubated over night at 4 °C. Secondary antibodies and wheat germ agglutinin were incubated together for 3 hours at room temperature in the dark. Primary antibodies: collagen I (ab34710) and 3 (ab7778) (Abcam). Secondary antibody: Cy3-AffiniPure Goat Anti-Rabbit IgG (H+L; #111-165-144), Jackson Immunoresearch Laboratories, Inc. Wheat germ agglutinin (Alexa Fluor 488 conjugated; #W11261, Invitrogen; 10 µg/mL).

**Statistical Analysis**

If not otherwise noted, data are presented as means±SEM. Statistical significance of PT–SL relation was tested using 2-way ANOVA, followed by Bonferroni post hoc test. Normalized data were tested using Student t test. P<0.05 was set as threshold of significance.

Detailed descriptions of the experimental procedures are provided in the Online Methods and Material section.

**Results**

**Animal Models and Echocardiographic Examination**

Two models were used: (1) Langendorff-perfused mouse hearts with 20-minute ischemia and 60-minute reperfusion periods (I/R); (2) 3 and 10 days of permanent LAD artery ligation (3 and 10 days of MI). From LAD artery ligation groups, two models were used: (1) Langendorff-perfused mouse hearts with 20-minute ischemia and 60-minute reperfusion periods (I/R); (2) 3 and 10 days of permanent LAD artery ligation (3 and 10 days of MI). From LAD artery ligation groups, nonischemic myocardial areas were used.

Cardiac performance of animals that underwent LAD artery ligation and the appropriate sham animals were examined by echocardiography before (basal) and after the procedure (3 and 10 days; Figure 1A through 1F). Basal analysis did not otherwise noted, data are presented as means±SEM. Statistical significance of PT–SL relation was tested using 2-way ANOVA, followed by Bonferroni post hoc test. Normalized data were tested using Student t test. P<0.05 was set as threshold of significance.

Detailed descriptions of the experimental procedures are provided in the Online Methods and Material section.
not show differences between sham and MI animals. Sham operation did not significantly affect cardiac performance in the observed time period. After 3 days, stroke volume (sham=28.67±0.78 µL; 3 days of MI=16.18±2.07 µL), cardiac output (sham=14.86±0.32 mL/min; 3 days of MI=8.35±0.71 mL/min), and ejection fraction (sham=47.97±2.76%; 3 days of MI=29.11±3.63%) were significantly reduced in MI mice. Ten days after MI, stroke volume and cardiac output were partly recovered, whereas ejection fraction was still significantly reduced compared with sham-operated animals (sham=47.23±1.05%; 10 days of MI=26.06±5.33%).

Passive Myocyte Stiffness Is Significantly Increased in Response to I/R and MI

We next studied the effect of ischemia-induced modification of the elastic titin domains on cardiac myocyte stiffness and determined passive myocyte tension (PT) in the physiological SL range from 2.0 to 2.4 µm, with attention to 2.0 to 2.2 µm at which titin is the main contributor to passive stiffness. In Langendorff-perfused hearts, PT of skinned single cardiac myocytes was increased 1.71-fold at 2.0 µm, 1.67-fold at 2.1 µm, and 1.68-fold at 2.2 µm SL in response to I/R compared with controls (Figure 2A and 2B). Three days after MI, PT was significantly elevated 2.23-fold at 2.0 µm, 2.15-fold at 2.1 µm, and 2.09-fold at 2.2 µm compared with sham hearts (Figure 2C and 2D). Ten days after MI, PT was significantly increased compared with sham hearts, with 1.59-fold increased PT levels at 2.0 µm and 1.43-fold at 2.1 and 1.48-fold at 2.2 µm (Figure 2D). Importantly, PT levels at 10 days were significantly lower than that at 3 days post MI (Figure 2C and 2D).

Titin Isoform Composition Is Unchanged in the Acute Phase After I/R or MI

To determine effects of myocardial ischemia on titin isoform composition, LV tissue samples were analyzed by Coomassie-stained 2.1% SDS-PAGE (Figure 3A). Langendorff-perfused hearts. PT of skinned single cardiac myocytes were determined for sham and MI animals before (basal, n=10 per group) and 3 or 10 d after LAD ligature (n=3-4 per group).

Lines represent mean values. *Statistically significant differences (2-way ANOVA with Bonferroni post hoc test, P<0.05).

Figure 1. Echocardiographic analysis of mice 3 and 10 d after sham operation (sham) or left anterior descending (LAD) ligature (myocardial infarction [MI]). A, Ejection fraction. B, End-systolic volume. C, End-diastolic volume. D, Cardiac output. E, Stroke volume. F, Heart rate were determined for sham and MI animals before (basal, n=10 per group) and 3 or 10 d after LAD ligature (n=3-4 per group). Lines represent mean values. *Statistically significant differences (2-way ANOVA with Bonferroni post hoc test, P<0.05).

Figure 2. Passive cardiac myocyte tension in ischemia/reperfusion (I/R) mouse hearts and 3 or 10 d after left anterior descending artery ligature (myocardial infarction [MI]). Passive tension (PT)–sarcomere length (SL) relationship of skinned single cardiac myocytes from (A) I/R mouse hearts (ctrl: n=6 tissues; I/R: n=6 tissues) and (C) 3-d sham and MI (sham ctrl: n=6 tissues; MI: n=6 tissues) and 10-d sham and MI (sham ctrl: n=6 tissues; MI: n=6 tissues) hearts. Lines represent polynomial fits to the data (means±SEM; *statistically significant difference in the overall PT–SL relationship). Relative differences at sarcomere lengths of 2.0, 2.1, and 2.2 µm in (B) I/R samples, (D) 3-d sham and MI and 10-d sham and MI mouse hearts. Data were normalized to control hearts and are given as means±SEM. *Statistically significant differences (P<0.05 in Student t test and 2-way ANOVA).
Figure 3. Titin isoform composition, titin domain phosphorylation, and kinase activity after ischemia/reperfusion (I/R) or myocardial infarction (MI). A. Titin isoform composition in I/R hearts or mouse hearts 3 or 10 d after left anterior descending (LAD) ligature (MI) and in respective control hearts (sham [Sh]). Average titin isoform composition is given as N2BA+N2B=100%. (Continued)
hearts that underwent I/R were always compared with Langendorff-perfused control hearts. Isolated mouse hearts that underwent I/R showed no differences in titin isoform composition (79.8±1.25% N2B) compared with Langendorff-perfused control hearts (78.8±1.17%). Likewise, 3 and 10 days after MI, titin isoform composition remained unchanged in sham (3 days: 78.7±1.32%; 10 days: 78.7±0.98%) and MI mice (3 days: 78.1±1.57%; 10 days: 78.8±0.93%), indicating that the ischemia-related increase in myocyte stiffness is not caused by a decreased amount of the more compliant N2BA isoform. We further determined the ratio (relative OD) of the titin degradation product T2 to T1 (N2BA+N2B) in the Langendorff I/R model and 3- and 10-day MI tissues. The results show a significantly higher abundance of the T2 band at 3 and 10 days after MI, compared with sham animals. We did not detect significant differences in T2/T1 ratio in the Langendorff I/R model (Online Figure I).

Titin Domain Phosphorylation Is Significantly Altered in Response to I/R and MI

We next determined site-specific phosphorylation of the elastic titin I-band domains using phosphosite-directed antibodies detecting S4010 (targeted by Erk1/2 and cAMP-dependent protein kinase) and S4099 (targeted by cGMP-dependent protein kinase) in the N2-Bus and S11878 and S12022 (targeted by PKCα and CaMKIIα) in the PEVK region. Because the N2BA isoform is only weakly expressed in mouse hearts, only phosphorylation levels of the predominant N2B isoform were analyzed.

In I/R hearts, PEVK phosphorylation at S11878 and S12022 was significantly increased by 57.5±13.6% and 39.1±15.4%, respectively. In contrast, N2-Bus phosphorylation was unchanged at S4010 and S4099 (Figure 3B). Three days after MI, PEVK phosphorylation was significantly increased by 37.9±5.5% at S11878 and by 91.2±22.3% at S12022 (Figure 3C). N2-Bus phosphorylation was significantly decreased at S4010 by 24.6±5% and unchanged at S4099. Ten days after MI, the relative phosphorylation of the PEVK domain at S11878 and S12022 and of S4099 in the N2-Bus was unchanged compared with sham-operated controls (Figure 3D). Relative phosphorylation of S4010 in the N2-Bus region was significantly reduced by 21.3±8.5%.

In a first attempt to analyze the molecular mechanisms that result in the altered phosphorylation of titin after MI, we examined the phosphorylation of Erk1/2 at T202/Y204, cardiac troponin I at S23/24 as an indicator for cAMP-dependent protein kinase and cGMP-dependent protein kinase activity, and PKCα at T497 as an indicator for PKCα activity using Western blot analysis (Figure 3E through 3G). Erk1/2 phosphorylation was unaltered in I/R and 10-day MI hearts but significantly decreased after 3 days of MI (Figure 3E). Cardiac troponin I phosphorylation at S23/24 was unchanged in all analyzed models (Figure 3F). In contrast, relative PKCα T497 phosphorylation was significantly increased in I/R hearts and 3 days after MI but was unchanged 10 days after MI (Figure 3G).

IL-6 Mediates Titin PEVK Phosphorylation and a Rapid Increase in Titin-Based Stiffness of Adult Cardiac Myocytes

Because IL-6 is strongly secreted after MI, we further tested whether IL-6 could account for the observed changes in titin modification and titin stiffness and treated isolated adult rat cardiac myocytes with 0.4 mM/L of purified recombinant IL-6. PT measurements of permeabilized adult rat cardiac myocytes showed a significant IL-6–induced increase in the PT–SL relation compared with untreated control cells (Figure 4A). Western blot analysis demonstrated that in adult rat cardiac myocytes treated with IL-6 for 15 minutes, the relative phosphorylation level of the N2-Bus at position S4099 remained unchanged, whereas relative phosphorylation of S11878 in the PEVK region was significantly increased 3.75-fold compared with untreated controls (Figure 4B). As internal control, IL-6 treatment induced phosphorylation of Signal transducer and activator of transcription 3 by +134±24%, indicating activation of the IL-6 signal transduction (Figure 4C). Finally, we observed a significant increase in the activation of the titin S11878-targeting kinase PKCα, as indicated by a +43±11% increase in the relative phosphorylation of T497 (Figure 4C). To test whether an early IL-6 response contributes to MI-induced titin stiffening, we analyzed PT in mice treated with an IL-6 antibody before LAD artery ligation. Three days after MI, PT was significantly decreased in IL-6–antibody–treated animals compared with MI animals without antibody treatment (Figure 4D). There was a trend toward reduced titin PEVK phosphorylation at residue S12022, which is targeted by PKCα (Figure 4E). Titin isoform composition was not altered in IL-6–antibody–treated animals (data not shown).

Reorganization of the Collagen I and III Network After MI

To check for alterations in the ECM after MI, real-time analysis has been performed for col1 and col3 and LOXL1 and LOXL2 (Figure 5). Real-time analysis of col1 and col3 was made in ischemic (LV) and remote (Septum, S) area. Sham animals did not show changes in the mRNA expression of col1 and col3. In LV and S col1 and col3 were highly upregulated starting 7 d and 3 d after MI, respectively, and were still increased 14d after MI (Figure 5A and 5B). LOXL1 and LOXL2 were analyzed in remote area and there was a trend toward an increased expression of LOXL2 3 d but not 10 d after MI (Figure 5C and 5D).

Cryosections were performed of heart tissues 3 and 10 d after MI and analyzed for col1 and col3 protein expression (Figure 6). In the infarcted area, col1 and col3 protein contents were strongly decreased 3 days after MI, whereas the remote area was unaltered (Figure 6A and 6B). Wheat germ
agglutinin staining was unchanged compared with sham animals. After 10 days, col1 and col3 were strongly expressed in the infarct region, but no obvious changes in the remote area were observed (Figure 6C and 6D).

**Titin Ubiquitination**

We next aimed to test whether the increased mechanical demand affects titin turnover in the remote regions after I/R or MI. In a first attempt to find evidences for titin turnover via the proteasome, we tested for titin ubiquitination in cultured embryonic rat cardiac myocytes. Under baseline conditions, ubiquitinated proteins are usually processed and degraded rather fast, and detection of such proteins by Western blot analysis is challenging. We therefore inhibited the proteasome for 4 hours with MG132, which led to accumulation of ubiquitinated proteins especially in the molecular weight range of 100 to 250 kDa (Figure 7A). Furthermore, blocking the proteasome with MG132 (starting at culture day 1) for ≤24 hours strongly increased the detectable levels of ubiquitinated titin. In the presence of MG132, first ubiquitination signals occurred after 8 hours of inhibition and become stronger after 16 and 24 hours (Figure 7B).

Myocardial ischemia can induce imbalanced protein homeostasis including changes of the proteasomal activity. 28 We, therefore, tested our models for alterations in total protein ubiquitination. In the Langendorff-perfused mouse hearts, the amount of ubiquitinated proteins seemed unchanged between controls and I/R. However, titin ubiquitination was lower in ischemic mice than that in control mice, whereas overall protein ubiquitination was unchanged (Figure 7D). In 3-day MI hearts, protein ubiquitination levels were increased in the molecular weight range of 100 to 260 kDa, and also titin ubiquitination was significantly increased compared with sham-operated controls (Figure 7D). After 10 days, total protein ubiquitination in sham-operated and ischemic animals was unchanged, whereas titin ubiquitination was still increased.

Accumulation of ubiquitinated proteins may indicate a dysfunction of the ubiquitin–proteasome system as in the MG132 experiments, but it may also indicate an increased protein turnover rate that is not entirely matched by...
ubiquitin–proteasome system activation. We therefore determined proteasomal activity by measuring the trypsin-like activity of the 26S proteasome (Figure 7E). In Langendorff-perfused hearts, trypsin-like activity was unchanged between control and ischemic hearts. In contrast, 3 days after MI, trypsin-like activity in the remote tissue was significantly higher (+75.7±33% relative light units) than that in sham animals. Ten days after MI, trypsin-like activity was unchanged compared with sham. These findings indicate increased turnover of cardiac proteins including titin, particularly in the phase of high myocyte stiffness 3 days after MI.

**Discussion**

The titin filament spans a half-sarcomere from the Z-line to the M-band and therefore plays an important role for the structural integrity of the sarcomeres, particularly in situations of increased mechanical stress. We reported here that I/R in isolated hearts and 3-day MI caused a rapid and profound increase in titin-based cardiac myocyte stiffness. Our data further imply that cardiac myocyte stiffening in the early phase after MI is mediated by (1) PKCa-induced hyperphosphorylation of the titin PEVK region, and (2) by Erk1/2/cGMP-dependent protein kinase–mediated hypophosphorylation of the titin N2-Bus region. Our data do not rule out the possibility of altered phosphorylation of different sites by other kinases, such as CaMKIIδ. Although CaMKIIδ can be highly activated in response to MI,29 a significant CaMKIIδ-mediated effect on titin-based cardiac myocyte stiffening seems unlikely, because CaMKIIδ-mediated titin phosphorylation has previously been shown to decrease PT.17 Importantly, in tissue from 10-day MI animals, titin phosphorylation levels of the PEVK region were completely restored, and cardiac myocyte stiffness, although still increased compared with sham animals, was significantly lowered compared with the 3-day MI tissues. This observation matches with the detected PKCa phosphorylation, which was significantly accelerated in the Langendorff I/R model and 3 days after LAD artery ligation but back to control levels in the 10-day MI tissues. Nonetheless, the reduced phosphorylation of...
of S4010 in the N2-Bus region at 10 days after MI can only in part explain the remaining increase in PT. We speculate that this increase is mediated by an altered phosphorylation status of residues other than the ones tested here or by additional titin modifications, for example, triggered by changes in the redox state of the cardiac myocytes. Oxidative stress has been reported to promote the formation of disulphide bonds within the elastic N2-Bus region and increase PT.33 Although titin is protected from degradation via association to the small heat shock proteins αB-crystallin and HSP27, there is a certain possibility that titin filaments aggregate under ischemic conditions, which would increase titin-based PT.34 Oxidative stress in response to cardiac ischemia has further been reported to promote the abundance of gluthationylated sarcomeric proteins, including titin. However, in vitro data have shown that titin glutathionylation inhibits Ig-domain refolding and elongates the titin spring and thereby reduces titin stiffness.35 Whether titin aggregation, disulfide bond formation, or altered glutathionylation occur in the viable cardiac myocytes in the early phase after MI and contribute to or counterbalance titin stiffening remains to be investigated.

We hypothesized that myocardial titin stiffening in response to acute MI is at least partly mediated by IL-6. IL-6 is a multifunctional cytokine involved in the regulation of immune responses3 and has been implicated in cardiac remodeling.30 IL-6 is highly expressed in the LV within the first 24 hours after MI, and its plasma and LV tissue levels remain elevated for the first 3 days post MI.3 Importantl, IL-6 is particularly expressed in the viable infarct border zone of reperfused hearts,31 indicating a role for IL-6 signaling in the noninfarcted region. Recent data suggested a role of IL-6 in mediating altered titin phosphorylation in a mouse model of experimental myocarditis.32 Here, using isolated adult rat cardiac myocytes, we demonstrated that IL-6 induced a rapid increase in PKCα-dependent phosphorylation of the elastic titin PEVK domain and significantly elevated titin-based cardiac myocyte stiffness. We, therefore, tested whether IL-6 mediates MI-induced cardiac myocyte stiffening and blocked the IL-6 response by application of anti-IL-6 antibodies before LAD artery ligation. Our results demonstrated that blocking of IL-6 indeed prevented cardiac myocyte stiffening in the viable nonischemic myocardium at 3 days after MI. It was recently demonstrated that at 3 days after MI, anti-IL-6–treated mice already show a trend toward impaired ventricular function that manifested after 3 weeks with decreased ejection fraction, increased end-systolic volumes, and dilation and thinning of the LV.7 In summary, these data highlight the functional importance of IL-6–induced remodeling in the acute phase after MI and demonstrate that inhibition of the early IL-6 response is detrimental.

The question arises whether the titin stiffening observed in our models is beneficial for LV function or whether the increased myocardial stiffness reduces LV distensibility and thus impairs diastolic filling. Several lines of evidence suggest a beneficial role of titin stiffening in maintaining ventricular function. Stiffening of the titin spring accelerates the retraction of myosin to the central position of the sarcomere and therefore increases the structural stability of the sarcomeres during situations with increased contraction–relaxation cycles. In addition, stiffer titin promotes length-dependent activation of the sarcomere,36 which is an essential mechanism for the infarcted LV to process the elevated volume load. Considering
the massive loss of functional myocardium in the infarcted region, the rapidly increased titin stiffness in the nonischemic region may prevent load-induced overstretching and disruption of nonischemic cardiac myocytes and thereby reduce sarcomeric and cellular damage in the first hours and days after myocardial injury. This process is sustained by the onset of ECM-based remodeling as indicated by increased mRNA levels of collagen 1 and 3 and a trend toward increased LOXL2 expression 3 days after MI. This idea is supported by the observation that titin modification and titin stiffness are partly restored in the later phase 10 days after MI. At this stage, there is an already progressed scar formation in the ischemic region and because of the ECM-based ventricular stiffening, titin’s role in promoting the structural integrity may become dispensable (Figure 8). However, our data do not exclude the possibility that excessive or prolonged titin-induced cardiac myocyte stiffening in remote areas may also exert mechanical strain and thereby destabilize the infarcted area.

Although ECM reorganization and maturation is important to compensate the ischemia-induced myocyte loss, inadequate ECM accumulation and massively elevated matrix stiffness have been associated with LV dilation and loss of cardiac function. Patients experiencing end-stage ischemic cardiomyopathy with severe LV fibrosis, titin isoform composition was shifted toward an increased amount of the more compliant N2BA isoform, which decreased myofilament tension. It has been suggested that these alterations aim to counterbalance the increased collagen-based ventricular stiffening in the terminally failing hearts, which again supports the idea of a precisely controlled interplay of ECM remodeling and titin stiffness.

In response to MI, matricellular proteins such as MMPs are upregulated to control post-MI remodeling and to orchestrate key molecular repair signals. MMPs have further been suggested to accelerate titin degradation in response to an ischemic insult, which results in elevated levels of the specific titin degradation band T2 and in a switch in titin isoform composition toward the stiffer N2B isoform in ischemic myocardium. These findings suggest that MMPs could be involved in titin degradation in the time frame 3 hours to 3 days after the ischemic event. In our present study, we did not detect changes in titin isoform composition in either of the models, but in the viable nonischemic myocardium of mice 3 and 10 days after MI, we found an increased abundance of the titin degradation band T2, indicated by increased T1/T2 ratio.
finding further supports our hypothesis of increased titin turnover in the early phase after MI.

The mechanisms of titin integration and degradation from the working sarcomere are still under investigation, and no information exists on whether changes in the activity of the protein turnover machinery after MI may affect titin turnover and thus cardiac myocyte function. In the ischemic myocardium, MI has been shown to rapidly reduce proteasomal activity and cause an accumulation of ubiquitinated proteins in the cytosol. However, the increased hemodynamic stress of the viable myocardium after MI could negatively affect the half-life of sarcomeric proteins including titin, and an accelerated turnover rate of sarcomeric proteins of this region therefore seems plausible. Polyubiquitination for downstream degradation often occurs in unstructured protein regions. These regions contain so-called PEST motifs, a classical protein degradation–targeting signal. Because of its amino acid composition and a missing secondary structure, the elastic titin PEVK domain is a likely target for polyubiquitination. For skeletal muscle, a MuRF-1–dependent ubiquitination of titin has already been shown. Here, we provide evidence that cardiac titin turnover is at least partly performed via the ubiquitin–proteasome system, as inhibiting the proteasome in embryonic rat cardiac myocytes caused a substantial increase in K48-dependent polyubiquitination of full-length titin. We, therefore, tested titin ubiquitination and proteasome activity in the Langendorff I/R model and in viable remote tissue 3 and 10 days post MI. Interestingly, we did not observe changes in titin ubiquitination and proteasome activity in the Langendorff-perfused hearts 1 hour after I/R. In contrast, in hearts 3 days after LAD artery ligation, titin ubiquitination and trypsin-like proteasome activity were substantially increased in the viable myocardium, suggesting that the proteasome activity is still not sufficient to process all ubiquitinated proteins. These aspects support our hypothesis of extensive remodeling and protein turnover in the remote myocardium 3 days post MI. Importantly, in hearts 10 days after LAD artery ligation, titin ubiquitination was less pronounced and proteasome activity was unchanged compared with sham-operated animals. This observation supports our hypothesis that the onset of ECM remodeling 3 to 10 days post MI reduces wall stress and cellular damage of the viable remote myocardium in response to MI.

Future studies will have to address the question, whether the excessive titin ubiquitination levels in hearts 3 days after MI may exert an additional influence on titin elasticity. A single ubiquitin molecule has a size of $\approx 8$ kDa, and depending on its localization, the sheer size of a polyubiquitin chain could potentially affect the persistence length of elastic titin domains and thus modulate titin stiffness.
We conclude from our observations that in response to myocardial ischemia, increased titin-based cardiac myocyte stiffness helps maintaining the ventricular integrity and performance of the viable myocardium. This effect may in part be mediated by the ischemia-induced release of the cytokine IL-6. We further suggest that the increased titin stiffness significantly elevates titin turnover within the first days after MI and involves degradation by the ubiquitin–proteasome system. In summary, our study provides evidence that titin is an important element of the early functional adaptation of the viable myocardium to meet the increased physiological demands acutely after MI.

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Disclosures
None.

References
What Is Known?

- Acute myocardial infarction (MI) leads to a substantial loss of functional cardiac myocytes in the ischemic ventricular tissue.
- Long-term adaptation of the extracellular matrix results in scar formation in the ischemic region, which could eventually cause fibrotic ventricular stiffening and heart failure.
- After MI, the viable myocardium experiences increased mechanical stress.
- MI triggers an acute inflammatory response, which is associated with an increase in production of cytokines such as interleukin-6.

What New Information Does This Article Contribute?

- In response to MI, cardiac myocyte stiffness rapidly increases in the nonischemic ventricle.
- MI-induced cardiac myocyte stiffening is attributable to altered modification of the giant sarcomeric protein titin and involves interleukin-6 signaling.
- The increased mechanical stress in the nonischemic ventricle increases proteasomal activity and accelerates the turnover of sarcomeric proteins (e.g., titin).

MI leads to complex ventricular remodeling that in the long-term often results in ventricular hypertrophy and cardiac failure. To date, little is known about the processes by which surviving cardiac myocytes adapt in the early aftermath of MI. We used Langendorff-perfused mouse hearts and a mouse model with permanent ligation of the left anterior descending coronary artery to characterize how passive tension of viable cardiac myocytes is affected in the first days after MI. We show that MI induces rapid changes in the phosphorylation status of the sarcomeric filament protein titin that it leads to increased cardiac myocyte passive tension. We also demonstrate that this increase involves interleukin-6 signaling. Finally, we demonstrate that the increased mechanical stress in the viable myocardium increases the activity of the ubiquitin–proteasome system and results in enhanced titin turnover. These results suggest that early titin-based cardiac myocyte stiffening is an important mechanism of the viable myocardium to adapt to increased mechanical stress after MI.
Titin-Based Cardiac Myocyte Stiffening Contributes to Early Adaptive Ventricular Remodeling After Myocardial Infarction

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All animal procedures were conducted in accordance with the animal care and use committee of the responsible authorities (Landesamt für Natur, Umwelt- und Verbraucherschutz Nordrhein-Westfalen) that reviewed and approved the experimental protocols (Az. 84-02.04.2013.A122).

Permanent Myocardial ischemia
C57/BL6J mice were anesthetized by intra-peritoneal injection of ketamine (60 mg/kg BW) and xylazine (10 mg/kg BW), as previously described1. After anesthesia was sufficient (confirmed by squeezing the paws) animals were intubated and connected to a rodent ventilator (Minivent Microventilator, Hugo Sachs, Germany). The mice were ventilated with a tidal volume of 200 μl at a rate of 140 strokes/min, with a mixture of two thirds air, one third oxygen and isoflurane 2.0-2.5 Vol.% (Forene®, Abbott GmbH, Germany). Mice were placed in a supine position on a water-warmed plate. Body temperature was maintained at 37°C and the electrocardiogram (ECG) (Hugo Sachs Apparatus) was monitored. After left lateral thoracotomy between the third and fourth rib, the pericardium was dissected and a 7-0 surgical suture was cautiously passed underneath the left anterior descending coronary artery (LAD) at a position 1 mm from the tip of the left auricle. MI was produced by suture occlusion. The correct position of the suture was confirmed by blanching of the apex and characteristic change in ECG (ST-elevation). The chest was closed with four interrupted stitches utilizing 5-0 prolene suture. Anesthesia was turned off while closing the skin. After mice regained spontaneous breathing they were extubated. Permanent myocardial infarction was performed for 3 d and 10 d, respectively. Animal received buprenorphine (0.05 – 0.1 mg/kg BW, s.c.) every 8 h up to five days. Additionally, for some experiments mice received a blocking IL-6 antibody (clone: MP5-20F3, 250 µg/mouse, In Vivo BioTechServices) intraperitoneally 16h prior to induction of MI as described previously2.

Echocardiography
Cardiac images were acquired using a Vevo 2100 high-resolution ultrasound scanner with 18-38 MHz linear transducer (VisualSonics Inc.) pre MI and 3 days post MI respectively 10 days post MI as previously described1,2. Echocardiography was performed under slight mask anesthesia by an inhaled mixture of 1.5% (v/v) isoflurane and 100% oxygen. ECGs were obtained with built-in ECG electrode-contact pads. Body temperature was maintained at 36,5 °C by a heating pad. All hair was removed from the chest using a chemical hair remover (Veet). Aquasonic 100 gel (Parker Laboratories, Hellendoorn, Netherlands) was applied to the thorax surface to optimize the visibility of the cardiac chambers. Parasternal long-axis was acquired. Left ventricular (LV) end-systolic and end-diastolic volumes (ESV and EDV) were calculated by identification of frames with maximal and minimal cross-sectional area and width. The system software employs a formula based on a cylindrical-hemilipsoid model3. LV ejection fraction (EF), stroke volume (SV) and cardiac output (CO) were calculated.

Sodium dodecyl sulfate-polyacrylamide gelelectrophoresis and western blot analysis
For titin isoform composition analysis and standard sodium dodecyl sulfate polyacrylamide-gelelectrophoresis (SDS-PAGE) samples were solubilized in modified Laemmli buffer4 and proteins separated on agarose strengthened 2.1 % PAGE5,6 or 12.5 % PAGE. Proteins were visualized by imperial protein staining solution (Thermo Scientific). For densitometrical analysis gels were scanned using a Fusion FX imager (Vilbert & Lourmat) and analysed densitometrically (Image J). Average titin-isoform composition was calculated from a minimum of n = 3 repetitions per experiment. Titin degradation was measured by calculating the ratio of full length total titin (N2BA + N2B) and the typical titin degradation product T2. Results were normalised to control levels and shown as relative T2/T1 ratio.
To study titin domain phosphorylation and titin ubiquitination proteins were transferred onto a PVDF membrane. Membranes were first incubated with phosphorylation specific antibodies
(α-pN2-Bus S4010/S4099 or α-pPEVK S11878/S12022 all Eurogentec) and second with a total titin antibody (α-PEVK pan) as previously described. Phospho-specific titin antibodies were raised against the following peptides: N2-Bus S4010 (VRIEEGKSLRFPC) and N2-Bus S4099 (QANLFSWLRNID); PEVK S11878 (CEVVLKSVLRKR) and PEVK S12022 (LRPGSGEKPP). For raising the non-specific pan antibodies an unmodified fragment was used. Recombinant titin fragments of the N2-Bus phosphorylated with PKA and PKG, respectively, and PEVK phosphorylated with PKCα were used to verify the specificity of the antibodies. Between the phospho-specific and the pan antibodies membranes were stripped as previously described. Briefly, membranes were incubated with stripping buffer (6M GnHCl, 0.2% Nonidet P-40 (NP-40), 0.1M β-mercaptoethanol, 20mM Tris-HCl, pH7.5) for 2 * 30 minutes to remove the primary and secondary antibodies. The blots were then washed several times with TBST to remove β- mercaptoethanol. Stripped membranes probed with secondary antibody did not show protein bands, thus confirming complete removal of the primary antibodies. Importantly, the blotted protein was not removed by this procedure. To detect differences in titin domain phosphorylation levels, we determined, for each sample and with each set of phosphosite-directed antibodies, the signal intensity of phospho titin and total titin. The ratio of phospho:total titin was then used to normalize the phosphorylation status of ischemic mouse hearts to the sham control samples. To study kinase activities/activation membranes were first incubated with phosphorylation specific antibodies (α-pPKCα T497 [Abcam, ab76016]; α-pTroponin I S23/24 [Cell Signalling, #4004]; α-pErk1/2 T202/Y204 [Cell Signalling, #4370]; α-pSTAT3 S705 [Cell Signalling, #9145]) and second with the corresponding pan antibodies (α-PKCα [Abcam, ab32376]; α-Troponin I [Cell Signalling, #4002]; α-Erk1/2 [Cell Signalling, #9102]; α-STAT3 [Cell Signalling, #9139]). Bands were visualized using a Fusion FX imaging system (Vilber and Lourmat, France) and signal intensity was analysed densitometrically (Image J). PKCα activity was determined by measuring the phosphorylation levels of T497 in the catalytic core. PKA and PKG activity was estimated by analysing the phosphorylation level of troponin I S23/24 which is a substrate of PKA and PKG. Erk1/2 activity determined by phosphorylation levels if T202/Y204. Again the ratio of phospho:pan of ischemic compared to sham control was calculated. Protein polyubiquitination was investigated using a K48-specific polyubiquitin antibody (Cell Signalling).

Force measurements on isolated cardiomyocytes
Passive tension (PT) measurements were performed on mouse cardiomyocytes isolated from tissue biopsies as previously described and on isolated rat cardiomyocytes (see below). For isolation of single mouse cardiomyocytes from biopsies, small tissue samples (3-6 mg) were obtained from the frozen muscle tissue and transferred into relaxing solution (7.8 mM ATP, 20 mM creatine phosphate, 20 mM imidazole, 4 mM EGTA, 12 mM Mg-propionate, 97.6 mM K-propionate, pH 7.0, 30 mM 2,3-butanediol monoxide (BDM), 1 mM dithiothreitol (DTT), 100 mM protease inhibitor cocktail, 50 mM phosphatase inhibitor cocktail). Samples were then repeatedly homogenized with an overhead stirrer at 750 rpm. The cardiomyocyte suspension was then centrifuged with 1000 rpm for 1 min., resuspended and permeabilized for 3 minutes in relaxing solution additionally supplemented with 3% Triton-X-100. Myocytes were washed in three centrifugation steps using relaxing solution without Triton-X-100 to remove the detergent. The final myocyte suspension was kept on ice until further experimental use. For passive force measurements a few microliters of the cardiomyocyte suspension were transferred to a cover slip mounted on an inverse phase contrast microscope (Nicon eclipse Ti). One single cell was selected and mounted between a piezoelectric motor and a force transducer (403A, Aurora Scientific) both covered with a mixture (ratio 2:1) of silicone glues (Dow Corning Glue 3140 and 3145-transparent). Cells were then stretched from slack SL (average, 1.9 µm) in five steps to a maximum sarcomere length (SL) of 2.4 µm. In the sarcomere length range of 2.0 – 2.2 µm titin is the main contributor to passive stiffness. In between the stretches a short hold period of 10 seconds was performed to wait for stress relaxation. Following the last stretch-hold, cardiomyocytes
were released back to slack SL to test for possible shifts of baseline force. During the stretch protocol sarcomere length was recorded using an IMPERX (CCD) camera (Aurora Scientific). From the recordings we analyzed the force at the end of each hold period. Passive force was related to cross-sectional area ("passive tension") determined from the diameter of the cardiomyocytes. Each cell was measured 3 times with 2 minutes in between the measurements.

Histology
Immunofluorescence staining was performed as described before\textsuperscript{11}. Raw hearts were snap frozen at -40°C and cryosectioned at -22°C in 8µm slices. Shown slices are 500µm apical from the ligation in MI hearts, in sham operated hearts slices from comparable regions were taken. The sliced tissue was fixed on slides in 4% paraformaldehyde (PFA) in 0.1M sodium phosphate buffer (PB) pH 7.4 for 10 minutes. Washing was performed in PBS and 0.2% Saponin/PBS. After blocking with 10% Normal Goat Serum (NGS) in 0.2% Saponin/PBS for one hour primary antibodies were incubated overnight at 4°C. Secondary antibodies and wheat germ agglutinin (WGA) were incubated together for 3 hours at room temperature in the dark. WGA binds to glycoproteins of the cell membrane and is commonly used for determination of myocyte cross sectional areas. In addition it labels fibrotic tissue after MI and can therefore be used as a marker for fibrosis\textsuperscript{11}. The sections were coverslipped with Pro long gold antifade reagent with DAPI (#P-36935, Invitrogen). Slides were analyzed with fluorescence microscope Keyence BZ 9000 (Keyence, Neu-Isenburg, Germany). Pictures were taken with 4x objective using the merge function.

Preactosome activity measurements
Trypsin like activity of the 26S-proteasome in the mouse heart tissues was measured in a GloMax® Multi Detection System by modifying the commercial available Proteasome-Glo\textsuperscript{TM} Trypsin-Like Cell-based Assay (Promega). The assay contains a substrate for trypsin-like activity (Z-leucine-arginine-arginine-aminoluciferin; Z-LRR-aminoluciferin), two inhibitors to reduce nonspecific protease activities and a thermostable luciferase. The substrate is cleaved by the trypsin-like peptidase activity and consumption of the aminoluciferin by the luciferase results in a luminescent signal (relative light units, RLU) that is proportional to the proteasome activity in the lysates.

Heart tissue (2-5 mg) was homogenized with an overhead stirrer for 5 seconds (s), sonicated for 10 s and centrifuged at 13000 rpm for 15 minutes. Protein content was determined by BCA assay (Thermo Scientific). Lysates (100µl) containing 10µg of protein were mixed 1:1 with the Proteasome-Glo\textsuperscript{TM} Reagent in black 96-well plates and measured after 5 min of incubation at room temperature (RT). All tissues were measured three times. The luminescent signal is proportional to the peptidase activity. For determination of the final proteasome activity that is correlated to the amount of proteasome, the calculated values of proteasomal activity were normalized by immunoblot analysis of the proteasome subunit alpha type 2 (PSMA2).

Preparation of embryonic (E18) rat cardiomyocytes
Adult pregnant Wistar rats were anesthetized with 2 % isofluran and killed by cervical dislocation. Embryos were decapitated before preparing the hearts. Hearts were minced and cardiomyocytes were isolated by enzymatic dissociation with collagenase Type II (Gibco,1 mg/ml) and trypsin (Gibco, 3 mg/ml) in dissociation buffer (137 mM NaCl, 11 mM D-Glucose, 2.7 mM KCl, 12 mM NaHCO\textsubscript{3}, 417 µM NaH\textsubscript{2}PO\textsubscript{4}·H\textsubscript{2}O, 56 µM phenol red). Enzymatic dissociation was performed at 37°C for 30 min. Additional mechanical dissociation was done by repeated mixing and pipetting of the solution. After the dissociation procedure the solution
was filtered through a 70µm cell strainer (BD Falcon) and the enzymatic reaction was stopped by adding ten volumes of culture medium standard DMEM (+ 20% fetal bovine serum (FBS), 1% non-essential amino acids, 1% Pen/Strep, 50 µM β-Mercaptoethanol). Cells were pelleted by 5 min centrifugation at 800 rpm, resuspended in culture medium, plated at a density of 5x 10^5 cells/well and cultured at 37°C and 5% CO_2. On the next day cells were treated with mitomycin (30 µM; Sigma M4287) for 1 hour to prevent further proliferation of fibroblasts. After the incubation the medium was changed to low FBS culture medium (DMEM+1% charcoal-filtered FBS). The medium was changed every day.

Preparation of adult rat cardiomyocytes

Adult rat CMs were isolated using a Langendorff perfusion system, as previously described^{12,13}. Adult male Wistar rat with an age of 8 to 12 weeks were terminally anesthetized with isofluran and killed by cervical dislocation. The thorax was then opened and the heart was injected with heparin solution (78U/ml heparin) to avoid coagulation and allow proper perfusion and then was removed by dissecting the aorta. Afterwards the heart was placed in a petri dish filled with cold heparin solution and weighed to calculate the appropriate perfusion time. The aorta was then cannulated and connected to a perfusion apparatus filled with perfusion buffer + heparin (130 mM NaCl, 5.4 mM KCl, 1.25 mM NaH_2PO_4, 25 mM HEPES, 20 mM glucose, 3 mM pyruvate, 5 mM creatine, 2 mM carnitine, 5 mM taurine, pH 7.3-7.4; heparin 11 U/ml). The heart was perfused with perfusion buffer at a flow rate of 7 ml/min. After approx. 5 minutes the perfusion buffer was switched to digestion buffer containing collagenase type II (85 U/ml, Worthington), protease from Streptomyces griseus (6 U/ml, Sigma) and CaCl_2 (50 mM) aerated with 100% oxygen and the heart was perfused for 25-35 min. The heart was carefully removed from the perfusion apparatus and transferred into digestion buffer (100 mM CaCl_2, 1.5 mM BSA). Here, the heart tissue was smoothly minced, transferred into a 50 ml falcón and incubated in a waterbath for 15 minutes at 37°C. The solution was mixed by pipetting every 5 min. Thereafter, the solution was filtered through a 200 µm filter to remove remaining non dissolved tissue. The cells were pelleted by centrifugation for 1 minute at 500 rpm and the supernatant was discarded. The cells were resuspended in low-calcium solution (0.2 mM CaCl_2, 1.5 mM BSA) and centrifuged as described above. This step was repeated with a high-calcium solution (0.4 mM CaCl_2, 1.5 mM BSA). The pellet was then resuspended and transferred into pre-warmed M199 Hanks cell culture medium (10 % FBS superior, 1% Penicillin/Streptomyacin, 5 mM creatine, 2 mM carnitine, 5 mM taurine, 10 mM HEPES). Cells were left for about 10 minutes at 37°C. Thereafter the cells were resuspended in M199 Hanks cell culture medium supplemented with blebbistatin (12.5 nM) to prevent contraction of the cells. Following a recovery period of 2 h intact isolated cardiomyocytes were treated with recombinant IL-6 (0.4 nM) for 15 minutes at 37°C. After this period cardiomyocytes used for PT measurements were centrifuged with 1000 rpm for 1 min., resuspended and permeabilized for 3 minutes in relaxing solution additionally supplemented with 3% Triton-X-100. Cardiomyocytes were then washed in three centrifugation steps using relaxing solution without Triton-X-100 to remove the detergent. The final myocyte suspension was kept on ice until further experimental use. Cardiomyocytes used for biochemical experiments were centrifuged with 1000 rpm for 1 min and immediately resuspended in modified Laemmli sample buffer as described above.
Supplemental References


Supplemental figures

Online figure I

Online Fig. I: Increased ratio of T2/T1 at 3 and 10 d after MI. Degradation was quantified by the ratio of the typical titin degradation product (T2) compared to total titin (T1; N2BA + N2B) in Langendorff hearts (Ctrl n = 5; I/R n = 6) and hearts 3 (sham n = 7; MI n = 8) and 10 days (sham n = 10; MI n = 9) after MI. Representative titin bands are shown below the bar graphs. Data were normalized to control hearts; Bar graphs show means ± SEM. Asterisks indicate statistically significant differences (p < 0.05 in student’s t-test).