ORIGINAL CONTRIBUTION

Left ventricular dilation in toll-like receptor 2 deficient mice after myocardial ischemia/reperfusion through defective scar formation

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Abstract Restoration of myocardial blood flow after ischemia triggers an inflammatory response involving toll-like receptors (TLRs). TLR2$^{-/-}$-mice show short-term advantages upon reperfusion injury as compared with WT controls. Accordingly, it has been shown that transient TLR2-blockade prior to reperfusion is associated with improved left-ventricular performance after myocardial scar formation. We present here adverse myocardial remodeling due to a chronic lack of TLR2 expression. Myocardial ischemia/reperfusion (MI/R) was surgically induced in C3HeN-mice by ligation of the left anterior descending coronary artery for 20 min, followed by 24 h or 28 days of reperfusion. TLR2$^{-/-}$-mice and TLR2-Ab treated (T2.5) WT-mice displayed a reduction of infarct size, plasma troponin T concentrations, and leukocyte infiltration as compared with untreated controls after 24 h of reperfusion. After 28 days, however, magnetic resonance imaging revealed a marked left ventricular dilation in TLR2$^{-/-}$-animals, which was associated with pronounced matrix remodeling characterized by reduced collagen and decorin density in the infarct scar. Our data show adverse effects on myocardial remodeling in TLR2$^{-/-}$-mice. Although interception with TLR2 signaling is a promising concept for the prevention of reperfusion injury after myocardial ischemia, these data give cause for serious concern with respect to the time-point and duration of the potential treatment.

Keywords Toll-like receptor 2 · Myocardial ischemia · Reperfusion · Cardiac remodeling · Decorin · Biglycan

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Introduction

Myocardial ischemia is one of the leading causes of death and morbidity in the western industrialized world. The consequences of myocardial ischemia and subsequent reperfusion (MI/R) can be modified by different preconditioning and therapeutic strategies, albeit the gross of compounds that have been reported to be cardioprotective in animals have not yet been transferred into human therapy [3, 5, 20].

This is also true for preconditioning, blockade, or genetic targeting of toll-like receptors (TLRs). TLRs are receptors of the innate immune system, which in addition to their recognition of bacterial wall fragments and other pathogen-associated patterns (PAMPs) [1, 4, 26] are responsible for the inflammatory response to a number of

"danger associated patterns" (DAMPs) [25] that are released from host tissue itself upon sterile insult.

Of the family of TLRs specifically TLR2 and TLR4 are expressed in the heart. Pharmacologic preconditioning by application of TLR2 agonists such as Pam_3CSK_4 or lipoteichoic acid (LTA) or TLR4 agonist lipopolysaccharide (LPS) has been shown to provide cardioprotection in terms of reduction of infarct size or leukocyte infiltration [6, 17, 23, 30, 31]. Comparable effects have been observed in TLR2- or TLR4-deficient animals [7, 11, 19]. In the case of $TLR4^{-/-}$ mice, however, the initially observed infarct size reduction does not transfer into improved ventricular function after myocardial wound healing [15]. $TLR2^{-/-}$ mice show a blunted endothelial dysfunction and a reduction in infarct size after MI/R [11]. However, it is not known whether chronic TLR2 deficiency affects myocardial wound healing.

In a recent study, Arslan et al. [2] applied a TLR2-blocking antibody (T2.5) and reported subsequent infarct size reduction that was associated with an improved left ventricular function after 28 days.

In the present study, we provide evidence for an adverse course of myocardial wound healing in $TLR2^{-/-}$ mice despite the presence of analogous short-term effects in comparison with T2.5-treated animals. To identify potential underlying mechanisms we targeted extracellular matrix deposition in the infarct scar.

Materials and methods

Animals

C3HeN adult male wild-type (WT) and $TLR2^{-/-}$ mice [16] were used for the experiments. Animals were housed under specific pathogen-free conditions and given a standard diet and water ad libitum. All procedures were performed in accordance with the national guidelines on animal care and approved by the local government body.

Anti-TLR2 monoclonal antibody

A blocking monoclonal antibody (T2.5) directed against the extracellular domain of murine TLR2 cross-reacting with human TLR2 has been used as previously described [16]. Mice received an intravenous injection of 16 μg/g bodyweight T2.5 in 300 μl phosphate-buffered saline (PBS) 30 min prior to coronary artery ligation.

Myocardial ischemia/reperfusion (MI/R)

Anesthesia was induced by intra-peritoneal (i.p.) injection of pentobarbital (90 mg/kg). For postoperative analgesia

buprenorphine (0.05 mg/kg) was administered subcutaneously. Mice were subjected to myocardial ischemia (20 min) and reperfusion (24 h, 3, 7, or 28 days) via occlusion of the left anterior descending coronary artery (LAD) as previously described [17, 20]. Successful occlusion was confirmed by the myocardium turning notably pale. After 20 min, the ligation was loosened and blood flow was restored. The chest was closed using two circular 6–0 polyvinyl sutures. When spontaneous breathing was sufficient, animals were disconnected from the ventilator and were allowed to breathe 100% oxygen. All animals received saline (16 μL/g bodyweight i.p.) to prevent dehydration.

Area at risk and infarct size determination

Hearts were perfused with 1% Evans blue dye (Sigma–Aldrich) to detect the area at risk (AR) as previously reported [20]. Heart slices were incubated for 25 min in 0.5% *p*-nitro-blue tetrazolium (NBT, Sigma–Aldrich), resulting in viable myocardium staining purple. IS, AR, and the total area of the left ventricle (LV) were planimetrically assessed using SigmaScan Pro (SPSS Inc., Chicago, IL, USA) image measurement software. Surgical procedures and planimetric measurements were performed by an investigator blinded for genotype or treatment.
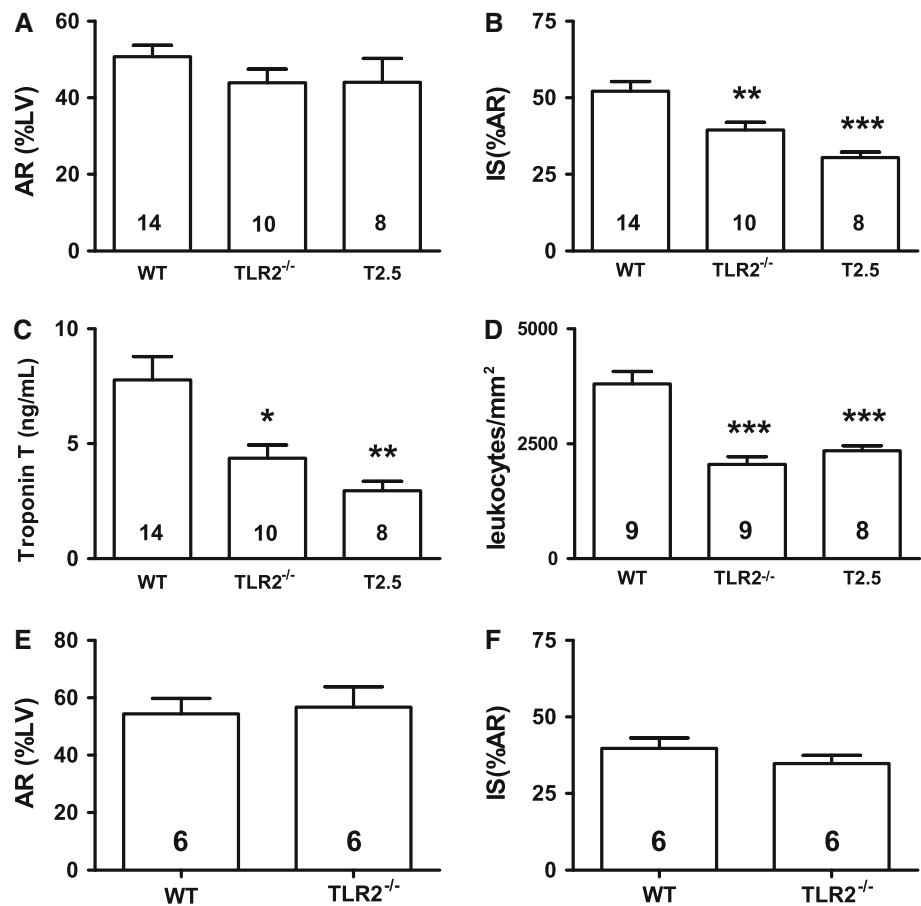
Troponin T

Heparinized blood was centrifuged to obtain plasma and stored at −30°C until assayed. Troponin T (TnT) was measured using the Cardiac reader system (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions.

Histology

Heart slices were fixed in 4% paraformaldehyde overnight, embedded in paraffin and cut into 5-μm sections. Hematoxylin and Eosin (H&E) staining was performed using standard protocols. Leukocyte accumulation in the AR was quantified on H&E stained sections. A total of ten microscopic fields covering 1 mm^2 of the AR were photographed and leukocytes were counted by a blinded investigator.

Sirius red (SR) staining was performed according to standard protocols. Photomicrographs were taken from bright field and under polarized light from identical regions of each section. Biglycan (Bcn) and decorin (Dcn) were detected after chondroitinase ABC digestion with polyclonal rabbit antisera against murine biglycan (1:1,000, LF 106) and murine decorin (1:1,000, LF 113) kindly provided by Larry Fisher (National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA).

Fig. 1 Comparative analysis of MI/R effects in WT mice, TLR2 deficient mice (TLR2$^{-/-}$), or WT mice treated with anti-TLR2 mAb (T2.5). Area at risk (**a**), infarct size (**b**), TnT plasma levels (**c**), and leukocyte recruitment (**d**) were analyzed 24 h upon experimental MI/R. 28 days after MI/R no significant differences could be detected in area at risk (**e**) or infarct size (**f**) between WT and TLR2$^{-/-}$ animals. $* P < 0.05$, $** P < 0.01$, $*** P < 0.001$ compared with WT; one-way ANOVA/Bonferroni's, n-numbers are depicted in the columns

Using SigmaScan Pro (SPSS Inc., Chicago, IL, USA) image measurement software the intensity of SR staining or Bgn and Dcn immuno-staining was quantified in regions of myocardial infarct scar formation, in which no cardiomyocytes were interposed. The average intensity of the region of interest was calculated and subtracted from the background intensity.

PCR and real-time PCR

Three and seven days after MI/R total RNA was isolated from snap-frozen tissue collected from the ischemic and remote area of WT ($n = 7$) and TLR2$^{-/-}$ mice ($n = 7$), delineated as described above by standard Evans Blue staining, using Tri Reagent (Sigma–Aldrich, Deisenhofen, Germany).
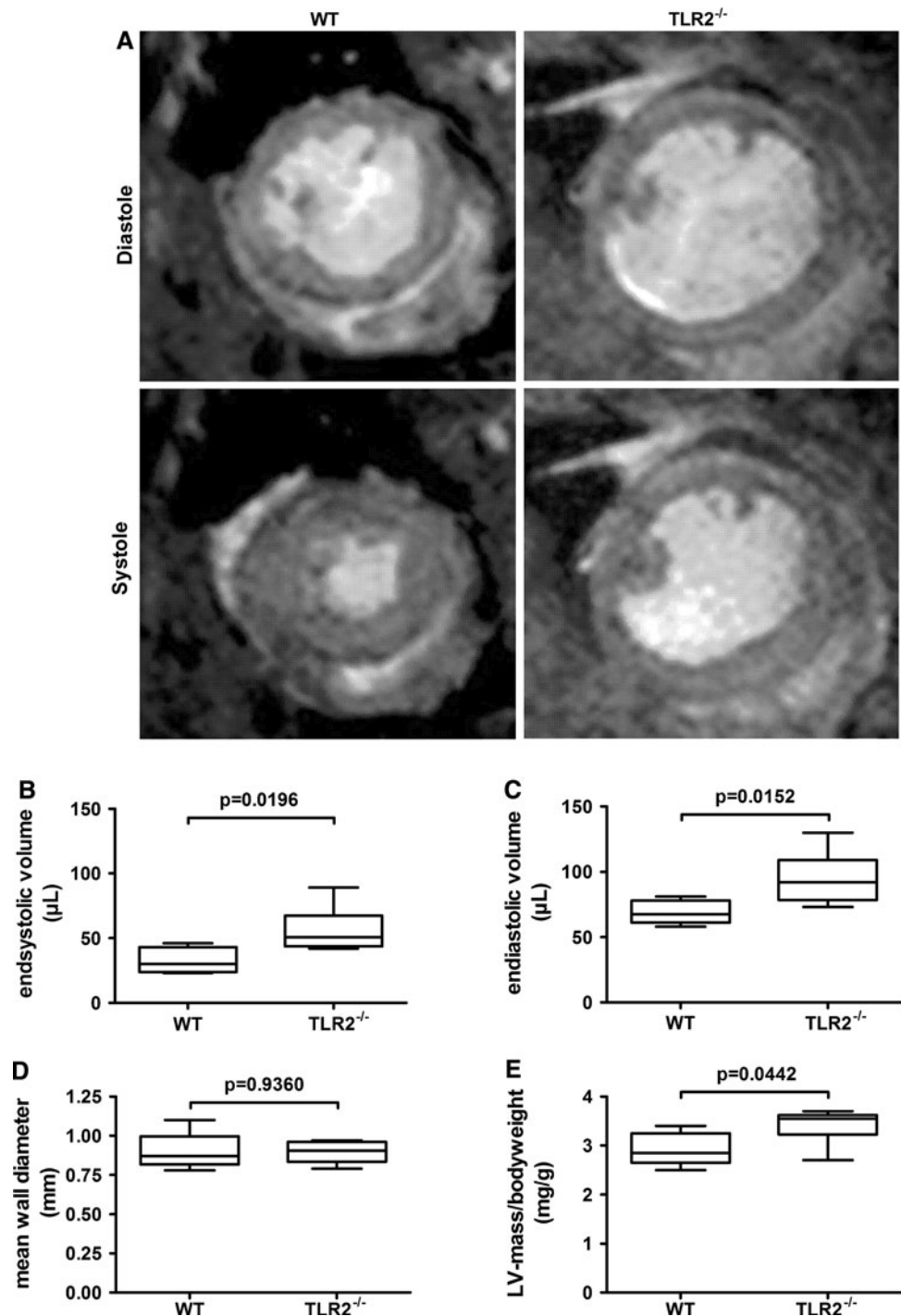
Reverse transcription of RNA was performed with SuperScript II Reverse Transcriptase (Invitrogen, Heidelberg, Germany), followed by amplification of the resulting cDNA (Taq PCR Core Kit; Qiagen, Hilden, Germany) according to the manufacturer's instructions. Primers used were synthesized by Sigma. Cycling parameters were as following: denaturation at 95°C for 3 min, followed by 24 cycles (18S) or 30 cycles (all other genes) of 30 s at 95°C, 45 s at 58°C and 1 min at 72°C with a final extension of 5 min

at 72°C. Amplification products were separated in 1–2% agarose gels and visualized with SYBR safe DNA gel stain (Invitrogen). Primers used were CGATCGCTACCCGGC GTTCC (forward) and GGGGTTCGGGCACTGCTTCC (reverse) for transforming growth factor β (TGF-β), GAGT TTCCGTGCCTGGCCCC (forward) and ACCTCGGGGAC CCATCTGGC (reverse) for collagen α1, ACGAACTTCAC CTGGACCAC (forward) and CAGAGGATAAGGGCAG CAAG (reverse) for biglycan, TAAAAGGTCGTGAAAAT ACAT (forward) and GAAGTCAAATAAGCCTCTCTG (reverse) for decorin, CCCAGGTGTGGGGTGCCTGA (forward) and TGGTGTTGTTGCACCTGTTGGCT (reverse) for matrix metalloproteinase 1 (MMP-1), CCGGTCCTGCTG TGGCTGTG (forward) and TGCGCCAAAAGTGCCTG TCT (reverse) for MMP-3, CCCTACCCGAGTGGACG CGA (forward) and AAGGCGGAGTCCAGCGTTGC (reverse) for MMP-9, and GGCCCCCTTTGCATCT CTGGC (forward) and CGTTCCTTAGGCGGCCCGTG (reverse) for tissue inhibitor of matrix metalloproteinase 1 (TIMP1).

The resulting 18S values were used as standard for presentation of the mRNA data of the Cnx43 transcript.

In addition, real-time PCR for decorin was performed from the same samples ($n = 6$ for each group due
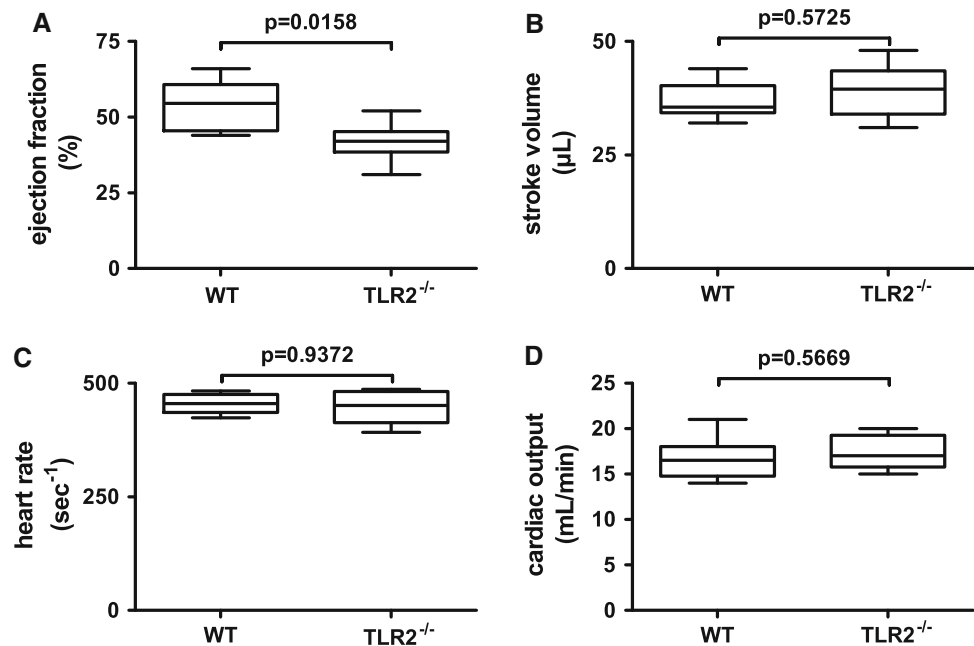
limitations of the 96-well plate) using SYBR Green
incorporation on a StepOnePlus Real-Time PCR system
(Applied Biosystems, Weiterstadt, Germany) according to
the manufacturer's instructions.

Magnetic resonance imaging (MRI)

Images were recorded with a 400-MHz DRX 9.4-T wide-bore
nuclear magnetic resonance spectrometer (Bruker Biospin

Corporation, Billerica, MA, USA) as previously described
[13]. Mice were subjected to 20 min of ischemia and 28 days
of reperfusion. For MRI mice were anesthetized with 1.2%
isoflurane in 30% oxygen and 70% nitrogen. Animals were
breathing spontaneously throughout the procedure. End-
systolic and end-diastolic volume (ESV/EDV), mean wall
diameter (mWD), and heart rate (HR) were measured. From
these parameters stroke volume (SV), ejection fraction (EF),
cardiac output (CO), and left ventricular mass per

bodyweight (LV-mass/bw) were calculated. The mean wall
diameter was computed as the average distance between the
inner and outer circumference of the left ventricular wall on
every virtual section of the heart.

Pre-existing differences in left ventricular dimensions or
function have been excluded by echocardiography. Ani-
mals were anesthetized with isoflurane (2% in room air)
and echocardiography was performed with a Vevo 770
High-Resolution In vivo Micro-Imaging System (Visual
Sonics, Amsterdam, The Netherlands).

Statistics

Data are presented as mean $\pm$ SEM of n observations.
Comparisons between groups were made using Prism 5.02
(GraphPad Software Inc., USA) after datasets passed nor-
mality testing—using the t test or ANOVA followed by
Bonferroni's post-hoc test. Comparisons between groups
that failed to pass normality testing were made using the
Mann–Whitney test. P values <0.05 were considered sta-
tistically significant. Correlations between parameters were
estimated using the Pearson method. Best-fit lines in
Figs. 5 and 6 were calculated by linear regression.

Results

TLR2 interception leads to reduced myocardial damage and inflammation

The area at risk [AR(%LV)] was not different between
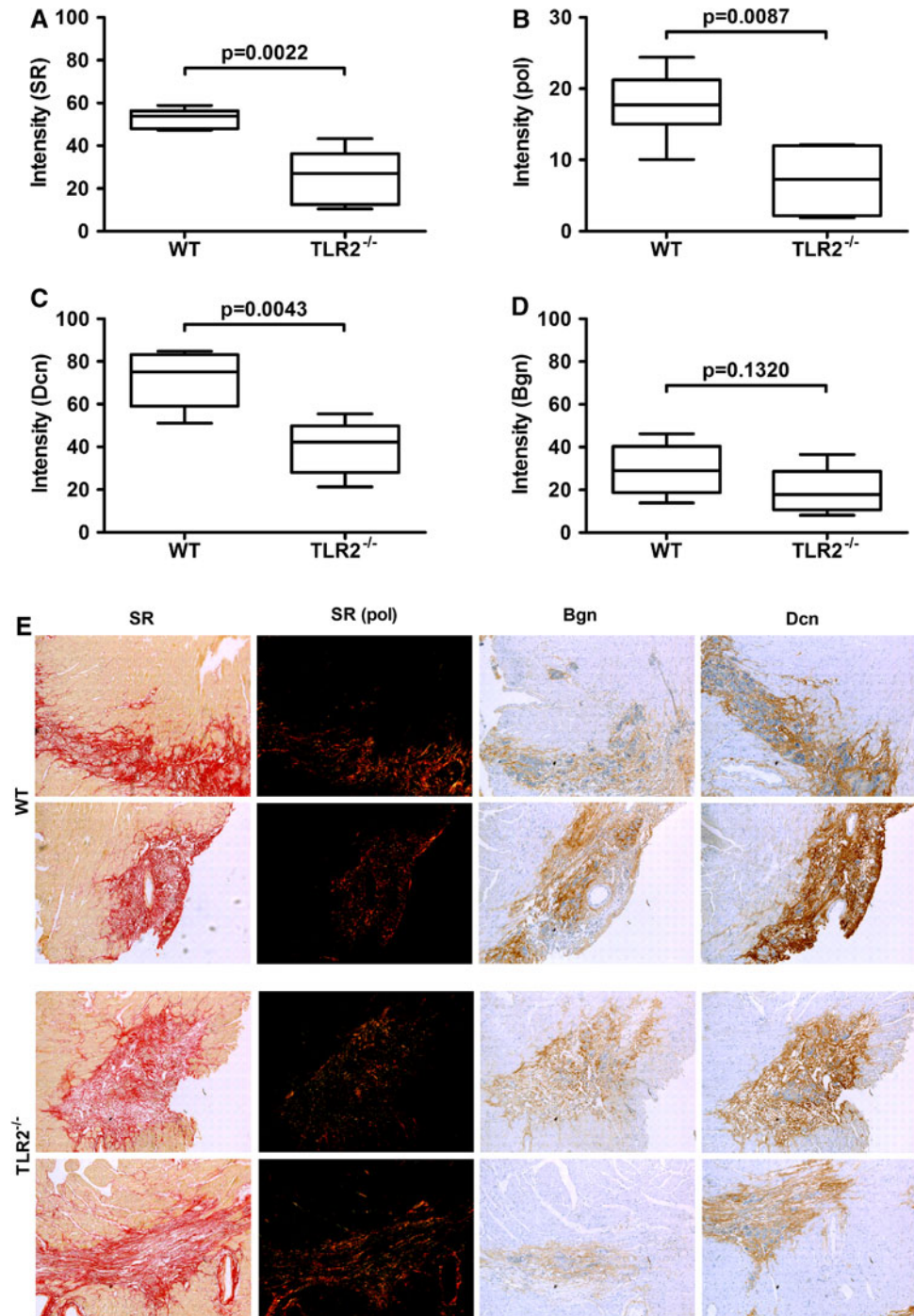study groups (Fig. 1a). 24 h after subjecting TLR2$^{-/-}$

mice and WT animals treated with a TLR2-blocking
monoclonal antibody (T2.5) to MI/R the mice hearts
showed a significant reduction in infarct size in relation
to the area at risk [IS(%AR)] as compared with untreated
WT mice (Fig. 1b). Plasma troponin T (TnT) levels as
an indicator of cardiomyocyte damage were also signi-
ficantly lower in TLR2$^{-/-}$ and T2.5-treated WT animals
(Fig. 1c). Since accumulation of inflammatory cells is
one of the hallmarks of reperfusion injury, we quantified
leukocytes in the AR. Both TLR2$^{-/-}$ and T2.5-treated
WT mice displayed a significantly reduced tissue infil-
tration 24 h after MI/R compared with untreated WT
mice (Fig. 1d).

LV-dilation of TLR2$^{-/-}$ hearts upon completion of infarct scar formation

In order to address whether TLR2 deficiency also posi-
tively affects remodeling processes and cardiac function,
LV function was determined by magnetic resonance
imaging after a prolonged follow-up period. Surprisingly,
TLR2$^{-/-}$-mice showed a marked dilation of the left
ventricle 28 days after MI/R (Fig. 2a). End-systolic and
end-diastolic volumes were significantly increased com-
pared with WT hearts (Fig. 2b, c). The mean wall
diameter was not different between groups (Fig. 2d),
while the calculated LV mass/bodyweight was signifi-
cantly higher in TLR2$^{-/-}$-animals (Fig. 2e) as compared
to WT controls.

Although the ejection fraction was significantly
reduced in TLR2$^{-/-}$-hearts, stroke volume was not
affected by LV dilation (Fig. 3a, b). Under anesthetic

conditions no difference in heart rate between groups could be observed and therefore cardiac output was preserved (Fig. 3c, d).

Baseline characterization of LV function in healthy WT and TLR2$^{-/-}$ mice was performed by echocardiography in a different set of experiments ($n = 12$). No differences regarding left-ventricular dimensions, ejection fraction, or heart rate could be detected (data not shown).

Collagen, biglycan, and decorin expression within the infarct scar

The intensity of SR-stained collagen fibers in the infarct scars of WT animals was significantly higher as compared with TLR2$^{-/-}$-hearts (Fig. 4a, b). This finding was accompanied by a significantly increased intensity signal of decorin immuno-staining in infarct scars of WT animals

Fig. 5 Cardiomyocyte hypertrophy is more pronounced TLR2$^{-/-}$ hearts. Mean cardiomyocyte diameter was determined 28 days after MI/R ($n = 6$) and was significantly higher in TLR2$^{-/-}$ hearts in both ischemic and remote myocardium (**a**). A significant correlation between cardiomyocyte diameter and left ventricular mass/bodyweight could be observed (**b**). TLR2$^{-/-}$ animals are represented by *open circles*, *WT* animals by *filled circles*. *CM* cardiomyocyte, *LV* left ventricle

(Fig. 4c). Biglycan deposition, however, did not significantly differ between groups (Fig. 4d). Hypertrophy of left-ventricular cardiomyocytes is aggravated in TLR2$^{-/-}$ hearts. Cardiomyocyte diameter in TLR2$^{-/-}$ left ventricles was significantly higher compared to WT in both ischemic and remote myocardium (Fig. 5).

Decorin deposition correlates with collagen and biglycan density

As expected, collagen and decorin deposition correlated with each other in infarct scars, and likewise biglycan and decorin (Fig. 6a, b). No significant correlation was observed between collagen and biglycan deposition ($r^2 = 0.2288$, $P = $ ns, not shown).

Collagen and decorin deposition correlate with LV dilation and hypertrophy

We observed a strong correlation between collagen deposition and end-systolic and end-diastolic volumes and a weaker correlation with LV-mass/bodyweight (Fig. 6c–e). Decorin deposition also correlated with LV dilation and even more strongly with LV-mass/bodyweight (F–H). No significant correlation could be found between biglycan and LV dilation or hypertrophy ($r^2 = 0.1265$ vs. end-systolic volume, $r^2 = 0.1713$ vs. end-diastolic volume, $r^2 = 0.3301$ vs. LV-mass/bodyweight; all $P = $ ns, not shown).

No differences in mRNA expression of matrix regulators after 3 and 7 days of reperfusion

PCR analysis did not reveal significant differences in TGF-β, MMP-1, -3 and -9, TIMP1, biglycan, decorin, and collagen α1 chain mRNA expression between WT and TLR2$^{-/-}$ animals in both ischemic and remote myocardium 3 and 7 days after MI/R.
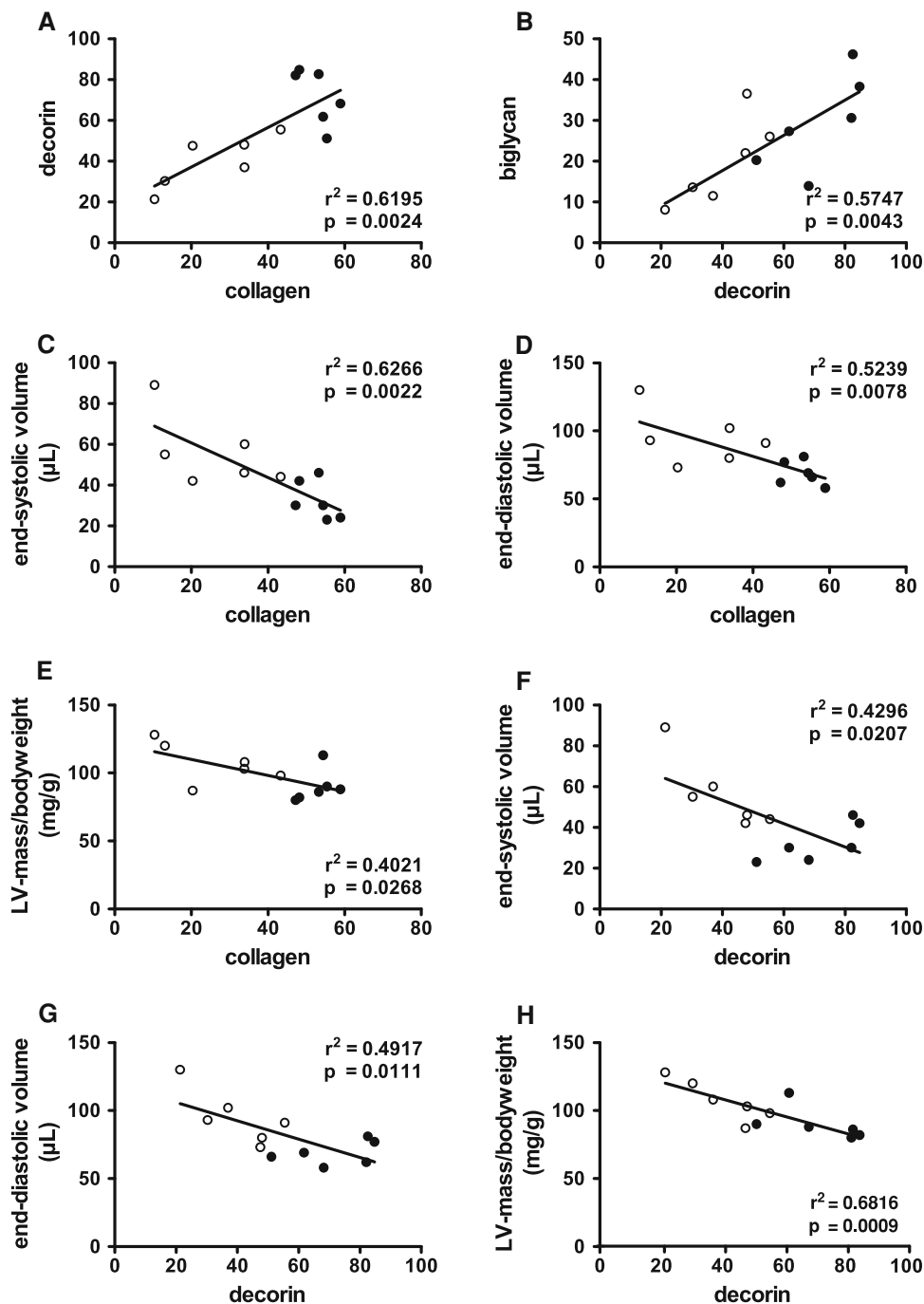
Discussion

Here, we demonstrate a transient cardioprotection capacity of TLR2 deficiency in experimental MI/R. However, timely limitation of TLR2 blockade might be critical to prevent adverse myocardial scar formation and remodeling resulting in LV-dilation.

We have reported previously that TLR2$^{-/-}$ mice are protected against lethal brady-arrhythmias within the first 24 h of reperfusion [18].

A report by Arslan et al. [2] demonstrated cardioprotection by means of TLR2 pathway interception through TLR2 blockade. These authors demonstrated a short-term and long-term benefit when the antibody was injected shortly prior to reperfusion. Due to a limited half-life of the antibody used [16] it is reasonable to assume that TLR2 blockade was effective during the initial 4 h of reperfusion within which the oxidative burst causing inflammation and promoting infarct formation occurs. During the later phase of reperfusion when myocardial wound healing is initiated, TLR2 function had been recovered to support myocardial wound healing and scar formation. The observed improved left ventricular function 28 days after MI/R is interpreted by us as a result of a smaller infarct size as compared with controls.

The short-term effects of TLR2 antibody blockade was confirmed in the present study. Furthermore, TLR2$^{-/-}$ mice developed infarcts, troponin T levels, and leukocyte infiltration of similar sizes and degrees as compared with antibody-treated mice. However, contrasting the improved LV-function reported for antibody-treated animals [2], LV-function in TLR2$^{-/-}$ animals was impaired.

Subsequent to myocardial ischemia/reperfusion injury, cardiac fibroblasts are activated to produce extracellular matrix molecules to preserve mechanical stability of the ventricular wall [29]. The most prominent extracellular matrix molecules are collagens which are subject to post-

Fig. 6 Correlations between collagen, biglycan, and decorin deposition, and LV dilation. Collagen and decorin deposition correlated in infarct scars (**a**). Likewise was the deposition of decorin correlated with biglycan (**b**). No significant correlation can be found between collagen and biglycan (not shown). Of note, a strong correlation between collagen deposition and end-systolic and –diastolic volumes and a weaker correlation with LV-mass/bodyweight was detected (**c–e**). Decorin deposition also correlates to a smaller degree with LV dilation but strongly with LV-mass/bodyweight (**f–h**). No significant correlation was found between biglycan and LV dilation or hypertrophy (not shown). TLR2$^{-/-}$ animals are represented by *open circles*, WT animals by *filled circles*

translational modification by small leucine-rich proteoglycans such as biglycan and decorin. The latter mediate collagen fibril organization thereby increasing the

mechanical strength of the infarct scar. Decorin nullmutant mice display loosely packed and less organized collagen fibrils in myocardial infarct scars leading to

ventricular dilation and impaired left ventricular systolic function [27]. We have previously shown that biglycan deficiency leads to disturbed collagen deposition after myocardial infarction with subsequent ventricular rupture [28]. TLR2 signaling has been connected to biglycan by Schaefer et al. [22] who identified biglycan as a ligand of TLR2 in macrophages inducing the production of inflammatory cytokines via the NF-κB pathway. A more recent study provided evidence for a regulatory role of TLR2 on phospholipid transfer protein expression after co-localization with biglycan in human aortic valve stenosis [10]. However, Csont et al. [8] observed cardioprotective/anti-inflammatory properties of biglycan when primary rat cardiomyocytes were treated with biglycan prior to hypoxia/reoxygenation. To our knowledge no direct link between TLR2 and decorin has been established to date.

In the present study—in parallel with impaired hemodynamics—decorin and collagen deposition within the infarct scar were reduced in TLR2$^{-/-}$-animals. In fact, decorin and collagen density negatively correlated with left ventricular dilation (end-systolic and end–diastolic volume). Decorin binds to collagen fibrils and is thought to control the lateral fusion of fibrils and the structure of three-dimensional collagen networks [9, 12, 14]. We therefore assume that the mechanical strength of the ventricular wall is impaired due to down-regulation of decorin and a subsequently decreased collagen network compaction in post-ischemic TLR2$^{-/-}$ hearts, and that dilation may be a direct result of this. Subsequently, cardiomyocyte hypertrophy compensates for the loss of ventricular wall integrity, which might be the reason for the observed negative correlation between LV-mass/bodyweight and collagen or decorin. Of note, no significant difference in biglycan density could be observed between TLR2$^{-/-}$ and WT infarct scars. But although biglycan and decorin are thought to synergistically facilitate collagen organization, one does not compensate for the lack of the other [27, 28].

TGF-β being an early activator of extracellular matrix production [21, 24] was not differentially regulated in TLR2$^{-/-}$ animals. Nor could differences in gene expression of matrix metalloproteinases 1, 3, and 9, and their inhibitor TIMP1 be detected, which mediate collagen turnover after deposition. MMPs are, however, subject to posttranslational activation. So the finding of equal MMP and TIMP synthesis does not exclude different activities with a subsequent increased degradation or turnover of collagen fibrils in TLR2$^{-/-}$ hearts. This might furthermore be supported by the fact that the transcription products of collagen α1, decorin and biglycan were also not changed in TLR2$^{-/-}$ hearts, besides the observed differences in collagen and decorin deposition in the infarct scar.

In summary, the results presented here underline that interference with TLR2 signaling in the reperfused myocardium is capable of limiting infarct size and initial cardiomyocyte necrosis. Furthermore, they imply undisturbed TLR2 signaling as mandatory for scar formation. TLR2 pathway interception should therefore be tightly limited in time in order to minimize myocardial damage and abrogate lethal arrhythmias while permitting TLR2 signaling during myocardial wound healing.

Conflict of interest The authors declare that they have no conflict of interest.

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