CHAPTER 5

# RP105 deficiency aggravates cardiac dysfunction after myocardial infarction in mice

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## Abstract

Toll-like receptor 4 (TLR4), a receptor of the innate immune system, is suggested to have detrimental effects on cardiac function after myocardial infarction (MI). RP105 (CD180) is a TLR4 homolog lacking the intracellular signalling domain that competitively inhibits TLR4-signalling. Thus, we hypothesized that RP105 deficiency, by amplifying TLR4 signalling, would lead to aggravated cardiac dysfunction after MI.

First, whole blood from RP105$^{-/-}$ and wild-type (WT) male C57Bl/6N mice was stimulated with LPS, which induced a strong inflammatory TNFα response in RP105$^{-/-}$ mice. Then, baseline heart function was assessed by left ventricular pressure-volume relationships which were not different between RP105$^{-/-}$ and WT mice. Permanent ligation of the left anterior descending coronary artery was performed to induce MI. Infarct sizes were analysed by (immuno)histology and did not differ. Fifteen days post MI heart function was assessed and RP105$^{-/-}$ mice had significantly higher heart rate (+21%, $p<0.01$), end-systolic volume index (+57%, $p<0.05$), end-systolic pressure (+22%, $p<0.05$) and lower relaxation time constant Tau (-12%, $p<0.05$), and a tendency for increased end-diastolic volume index (+42%, $p<0.06$), compared to WT mice. In the area adjacent to the infarct zone, compared to the healthy myocardium, levels of RP105, TLR4 and the endogenous TLR4 ligand fibronectin-EDA were increased as well as the number of macrophages, however this was not different between both groups.

Deficiency of the endogenous TLR4 inhibitor RP105 leads to an enhanced inflammatory status and more pronounced cardiac dilatation after induction of MI, underscoring the role of the TLR4 pathway in post-infarction remodelling.

# Introduction

Cardiovascular diseases remain the leading cause of death in the western countries, which is mainly accounted for by the high incidence of myocardial infarction (MI). Although survival after MI has improved significantly due to novel medical strategies and interventions, the incidence and prevalence of MI related morbidity is increasing which is mainly due to development of congestive heart failure (CHF).[1, 2] CHF is the result of a remodelling response of the ventricle upon reduced contraction capacity after cardiac damage such as MI.[2] During the last decades the immune system was demonstrated to a play major role in myocardial repair and remodelling. Evidence accumulates that prolongation of the post-infarction inflammatory response leads to increased remodelling and thereby CHF progression.[3-5] Therefore, new strategies to intervene in the pathogenesis of CHF may be worthwhile, which can be achieved by immediate anticipation and tackling of the inflammatory- and matrix degeneration processes that have been initiated by the immune system.[6]

Toll like receptors (TLR) are part of the innate immune system and are capable of recognizing Pathogen Associated Molecular Patterns (PAMPs) as well as Damage Associated Molecular Patterns (DAMPs). PAMPs are parts of exogenous pathogens such as bacteria while DAMPs, like fibronectin-extra domain-A (EDA) or Heat Shock Protein, may become available after cell stress or tissue damage/injury without the involvement of exogenous pathogens.[7] They have been widely associated with atherosclerotic plaque formation, restenosis and vein graft failure; all processes that may initiate ischemia or MI, resulting in cardiac remodelling.[8-10] One of the most studied TLRs is TLR4, which is present on circulating cells and cardiomyocytes. Cardiac expression of TLR4 was shown to be upregulated in cardiomyopathy.[11] In preclinical studies TLR4 was shown to

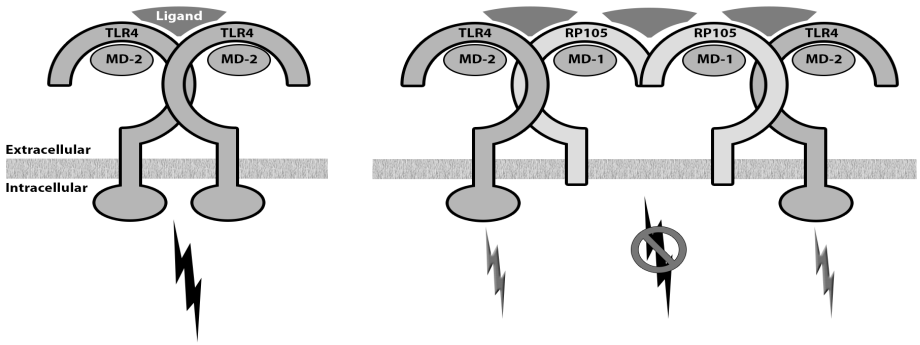


Figure 1 **Schematic model of the TLR4-MD2 and RP105-MD1 complex.** Activation of the TLR4-MD2 heterodimer by ligand binding results in activation of the intracellular signaling domain thereby initiating a downstream signaling cascade (left panel). The formation of the unusual 2:2 homodimer by TLR4-MD2 and RP105-MD1 alters the TLR4 signaling cascade, whereas RP105 dimerization by itself has no signaling capacity at all as it lacks the intracellular Toll Interleukin Receptor (TIR) domain (right panel). Adapted from Otho *et al.*[25]

play an important role in MI healing, left ventricular (LV) remodelling and functional impairment following MI.[12] Additionally, intervention with a specific TLR4 antagonist Eritoran was demonstrated to be protective against remodelling.[13]

TLR-signalling is mainly regulated by accessory molecules[14], and no role for these accessory molecules in cardiac remodelling has been defined yet. RP105, one of these molecules, resides on the cell surface and has a high extracellular structural similarity to TLR4 (figure 1).[15] In addition, like TLR4, whose signalling depends on association with the extracellular accessory protein MD-2, RP105 surface expression depends on co-expression of the MD-2 homolog: MD-1. However, in contrast to TLR4, RP105 lacks the intracellular Toll Interleukin Receptor (TIR) domain that is essential to initiate cellular activation. As such RP105 is an inhibitor of the TLR4 signaling pathway.[16, 17] Whereas no direct ligands have been found for RP105, strong indications exist that it can bind TLR4 ligands, thereby influencing TLR4 signalling and dampening the inflammatory responses induced by TLR4 activation.

As TLR4 activation has been shown to enhance cardiac remodelling after MI, we hypothesized that RP105 deficiency would aggravate these effects, through reduced inhibition (thus by stimulation) of TLR4 signalling. In this study we demonstrate that RP105 deficiency indeed has a functional role in cardiac remodeling, since systolic and diastolic cardiac function indices are affected after MI.

# Materials and methods



## Animals and experimental design

Studies were performed with 10-12 week old, male RP105-deficient ($RP105^{-/-}$) mice bred in our animal facility and wild-type (WT) mice (Charles River, Maastricht, the Netherlands), both on a C57Bl/6N background. Animals were housed in a temperature and humidity controlled room on a 12:12-h light-dark cycle with *ad libitum* access to water and normal chow diet. Body weights were measured weekly. MI was induced by coronary ligation (see below) at day 0 in $RP105^{-/-}$ (n=12) and WT (n=12) mice. Subsequently, cardiac function assessments by pressure-volume loops (PV-loops) were performed at day 15. To obtain baseline cardiac function, additional PV-loop measurements were performed in a separate group of animals ($RP105^{-/-}$, n=4; WT, n=4) without MI. After PV-loop measurements the mice were sacrificed and hearts were isolated for further examination. The protocol was approved by the Animal Ethics Committee from the Leiden University Medical Center and was conform to the *Guide for Care and Use of Laboratory Animals* (NIH publication No.85-23, Revised 1996).

## Whole blood TNFα stimulation assay

In order to investigate the inflammatory response at baseline of the different groups, venous blood via a tail vein cut was collected and suspended 1:25 with RPMI 1640 (Gibco 52400-025, Paisley, UK) supplemented with non-essential amino acids (PAA M11-003, Pasching, Austria)

and glutamax (Gibco 35050). Blood from both WT and RP105$^{-/-}$ mice was incubated overnight at 37°C in absence and presence of LPS in the concentrations 25 ng/mL and 50 ng/mL. Subsequently, TNFα levels were measured by ELISA (BD Biosciences).

## Induction of myocardial infarctions

Mice were anesthetized by an intraperitoneal injection of a mixture of dormicum (0.7 mg/kg BW), dexdomitor (7.2 mg/kg BW) and fentanyl (0.07 mg/kg BW). Body temperature was maintained at 37°C using a temperature controlled, automatic heating pad. Mice were artificially ventilated using a dedicated mouse ventilator (model 845, Harvard Apparatus, Holliston, MA). The left anterior descending coronary artery (LAD) was ligated with a 7-0 ethilon suture (Johnson and Johnson, New Brunswick, NJ, USA), just distal to the left atrial appendage. Ischemia was visually confirmed by bleaching of the LV. The thorax was closed and the mice received an intraperitoneal injection of anexate (0.5 mg/kg BW), antisedan (2.5 mg/kg BW), naloxon (1.2 mg/kg BW) to antagonize the anesthesia. Analgesic temgesic (1.5 μg in 50 μL PBS) was administered subcutaneously. Thereafter, the mice were allowed to recover on a temperature controlled heating pad.

## Hemodynamic measurements

Thirteen days after induction of the MI, transthoracic, cardiac echocardiography was performed using a VisualSonics Vevo 770 with a 30 MHz ultrasound transducer (VisualSonics, Toronto, Canada) as described earlier.[18] The following parameters were obtained: heart rate (HR), stroke volume (SV), cardiac output (CO), end-diastolic volume (EDV) end-systolic volume (ESV), ejection fraction (EF), fractional area change (FAC) and area change. Briefly, mice were anesthetized with 3% isoflurane, and placed supine on a temperature-controlled animal platform. Parasternal long axis and short axis images were recorded in all animals. Analysis of the data was performed with software provided by VisualSonics. Subsequently, 2 days later (at day 15 post MI), LV function was assessed by invasive PV-loops as described earlier.[18] Mice were anesthetized with a starting dose of 4% isoflurane and a maintenance dose of 1.5% isoflurane. After intubation mice were ventilated and the jugular vein was cannulated for infusion of hypertonic saline to determine parallel conductance. Via the right carotid artery a 1.2F PV catheter (FTS-1212B-4518, Scisense Inc., London, Ontario, Canada) was placed into the LV. The catheter was connected to a Scisense ADV signal processor (Scisense Inc) to generate high-fidelity pressure and volume signals. Positioning of the catheter was guided by online pressure and volume signals. On-line display and acquisition of the signals (2000 samples/s) was performed with a PowerLab 8/30 data acquisition system and LabChart Pro software (AD Instruments GmbH, Spechbach, Germany). Off-line data analysis was performed with custom-made software (CircLab, P. Steendijk). The following parameters were measured: heart rate (HR), stroke volume (SV), cardiac output (CO), end-diastolic volume (EDV) end-systolic volume (ESV), ejection fraction (EF), end-diastolic pressure (EDP) and end-systolic pressure (ESP). Stroke work (SW) was determined as the area of the PV-loop and the maximal and minimal rate of LV pressure change, $dP/dt_{MAX}$ and $dP/dt_{MIN}$ were obtained. Effective arterial elastance ($E_A$) was calculated as ESP/SV. Relaxation time tau was calculated as the time-constant of mono-exponential pressure decay during isovolumic relaxation.

## Myocardial (immuno)histochemistry

Hearts were fixed overnight in paraformaldehyde and cut into five 1 mm-thick slices, perpendicular to the long axis of the heart. These slices were flat embedded in paraffin and 5 μm-thick sections

were prepared. To delineate LV area and infarct area the lower 3 sections were stained for collagen with Sirius Red. Total LV wall area (including septum) and infarct area were measured with cell^D imaging software (Olympus Soft Imaging Solutions, Tokyo, Japan). The infarct area was expressed as percentage (v/v) of the LV wall volume.

Additionally, sections were immunohistochemically stained for macrophages (rat anti-mouse MAC-3, 1:200; BD Biosciences, Erembodegem, Belgium) to count the infiltrating macrophages into the myocardium, TLR4 (rabbit anti-human TLR4, 1:150 SantaCruz, Heidelberg, Germany) to observe presence of TLR4 in the different areas of the myocardium, RP105 (rabbit anti-human, 1:250 SantaCruz) to verify the presence of the accessory molecule in WT mice, and fibronectin-EDA (mouse anti-human 1:800, Abcam, Cambridge, United Kingdom) whose presence precedes that of collagen and serves as an endogenous ligand for TLR4, which is frequently induced upon tissue injury and is known to play a role in cardiac remodeling. Images were scored by an observer blinded for the study groups. Per image three specific areas of the myocardium were distinguished: the infarcted area, the border zone and the remote, undamaged, healthy myocardium. MAC-3 staining was analyzed by counting the number of macrophages in three consecutive fields of view per specific area. For TLR4, RP105 and fibronectin-EDA stainings were scored in a semi-quantitative manner, using zero staining as grade 0, mild to moderate staining as grade 1 and a profound staining as grade 2.

### Statistical methods

Significance of differences between the groups was calculated non-parametrically using a Mann-Whitney U-test. Difference in survival rate after MI was calculated using a chi-squared test. P-values <0.05 were considered statistically significant. SPSS 17.0 for Windows (SPSS, Chicago, IL, USA) was used for statistical analysis. Values are presented as means ± SD.



## Results

### Increased inflammatory response in RP105$^{-/-}$ mice after TLR4 stimulation by LPS

To demonstrate whether an increased TLR4 mediated inflammatory response is present in RP105$^{-/-}$ mice as compared to WT mice, an *ex vivo* whole blood stimulation assay was performed in the presence and absence of LPS. After an overnight LPS stimulation with 25 ng/mL and 50 ng/mL LPS, TNFα levels were markedly increased in RP105$^{-/-}$ mice compared to WT mice for both concentrations, whereas no significant difference was observed under non-stimulated conditions (table 1).

### RP105 deficiency does not affect baseline cardiac function

To determine possible differences in baseline cardiac function between WT and RP105$^{-/-}$ mice, echocardiography and PV-loops were measured in animals without MI. Since body weight differed between the groups (25.7 ± 0.7 g in WT vs. 28.9 ± 2.7 g in RP105$^{-/-}$,

Table 1 **Effect of RP105 deficiency TLR4 mediated inflammatory response.**

| | TNFα (ng/mL) WT | TNFα (ng/mL) RP105$^{-/-}$ |
|---|---|---|
| **LPS dose (ng/mL)** | | |
| **25** | **20.0±4.1 ng/mL** | **108±24 ng/mL** |
| **50** | **28.7±7.0 ng/mL** | **128±25 ng/mL** |

Blood samples of WT and RP105$^{-/-}$ mice were diluted 1:25 with RPMI 1640, and incubated overnight with 25 or 50 ng/mL LPS. Subsequently TNFα levels were measured and expressed as ng/mL. Values represent means ± SD; n=5 per group.

p=0.036) all volumetric parameters were corrected for body weight. Results for the main PV-loop derived parameters show no significant differences between the WT and the RP105$^{-/-}$ mice (see supplemental table 1). These findings indicate that RP105 deficiency *per se* does not influence basal cardiac function.

## RP105 deficiency promotes cardiac dilatation after myocardial infarction

To investigate whether RP105 deficiency influences cardiac function after MI, the LAD was permanently ligated in 12 WT and 12 RP105$^{-/-}$ mice. No significant difference in survival rates was observed (92% in WT vs. 75% in RP105$^{-/-}$, p=0.273), suggesting that both strains were equally able to cope with the severe cardiac damage.

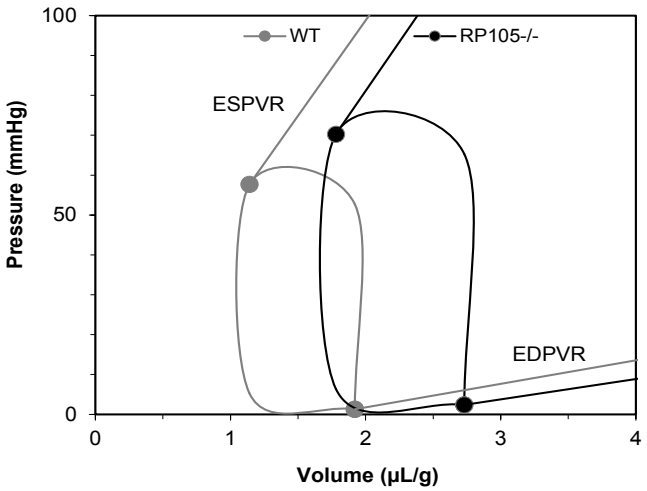


Figure 2 **PV-loops after MI.** WT and RP105$^{-/-}$ mice underwent ligation of the LAD, and 15 days post myocardial infarction (MI) pressure-volume loops (PV-loops) were recorded in each mouse. Average PV-loops and PV-relations, corrected for body weight, are shown per group. n=8 per group. ESPVR, end-systolic pressure volume relation; EDPVR, end-diastolic pressure volume relation.

Table 2 **Effect of RP105 deficiency on cardiac function after an MI.**

| | WT | RP105$^{-/-}$ | P-value |
|---|---|---|---|
| **General** | | | |
| HR (beats/min) | 446±19 | 538±44 | **0.003** |
| SV (µL/g) | 0.8±0.3 | 0.9±0.4 | 0.490 |
| CO ((mL/g)/min) | 0.4±0.1 | 0.5±0.2 | 0.300 |
| SW (mmHg.(µL/g)) | 42±14 | 57±44 | 0.916 |
| $E_A$ (mmHg/(µL/g)) | 0.2±0.1 | 0.1±0.1 | 0.223 |
| $E_{ES}/E_A$ | 0.7±0.3 | 0.6±0.3 | 0.395 |
| **Systolic** | | | |
| ESP (mmHg) | 58±9 | 70±9 | **0.021** |
| ESV (µL/g) | 1.1±0.7 | 1.8±0.7 | **0.038** |
| EF (%) | 43±16 | 35±10 | 0.372 |
| $dP/dt_{MAX}$ (mmHg/ms) | 5±2 | 6±2 | 0.462 |
| $E_{ES}$ (mmHg/(µL/g)) | 48±10 | 49±27 | 0.955 |
| **Diastolic** | | | |
| EDP (mmHg) | 1±2 | 2±2 | 0.293 |
| EDV (µL/g) | 1.9±0.6 | 2.7±0.9 | 0.058 |
| Tau (ms) | 11.9±0.9 | 10.4±1.5 | **0.046** |
| $-dP/dt_{MIN}$ (mmHg/ms) | 4±0.8 | 5±2 | 0.093 |
| $E_{ED}$ (mmHg/(µL/g)) | 6±4 | 5±3 | 0.721 |
| $K_{ED}$ (1/(µL/g)) | 0.2±0.1 | 0.1±0.0 | 0.114 |

HR, heart rate; SV, stroke volume; CO, cardiac output; SW, stroke work; $E_A$, arterial elastance (afterload); $E_{ES}/E_A$, ventricular arterial coupling; ESP, end-systolic pressure; ESV, end-systolic volume; EF, ejection fraction; $dP/dt_{MAX}$, maximal rate of pressure increase; $E_{ES}$, end-systolic elastance (slope of ESPVR); EDP, end-diastolic pressure; EDV, end-diastolic volume; Tau, relaxation time constant; $-dP/dt_{MIN}$, maximal rate op pressure decline; $E_{ED}$, end-diastolic stiffness (slope of EDPVR); $K_{ED}$, diastolic stiffness constant. Values represent means ± SD; n=8 per group.



Fifteen days after induction of the MI, functional parameters were obtained by intraventricular PV-loop measurements. The results of these analyses are summarized in table 2. Based on mean values for end-diastolic and end-systolic pressures and volumes, average PV-loops were created for both groups (figure 2). Corresponding end-diastolic and end-systolic PV-relations were added, based on mean $E_{ES}$ and $E_{ED}$ values. These measurements revealed that RP105 deficiency significantly affected heart function after MI. Compared with WT mice, RP105$^{-/-}$ mice showed significantly higher heart rate (+21% p=0.003), ESV index (+42%, p=0.038), ESP (+22%, p=0.021) and lower Tau (-12%, p=0.046). Furthermore a clear tendency for a higher EDV (+35%, p=0.058) and higher $-dP/dt_{MIN}$ (+24%, p=0.093) was observed. Taken together, RP105$^{-/-}$ mice showed more prominent cardiac dilatation after MI, but general hemodynamics appeared to be relatively unaffected at this stage.

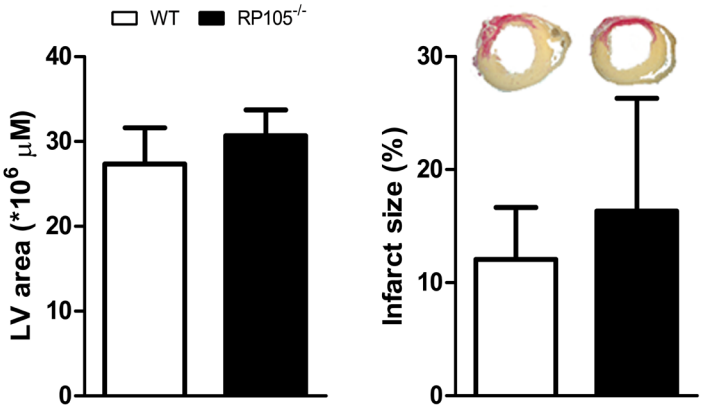


Figure 3 **Left ventricular area and myocardial infarct size in WT and RP105$^{-/-}$ mice.** Representative cross sections, after Sirius red staining, are shown above the corresponding bars for myocardial infarct size. Each bar represent mean ± SD; n= 8-10 per group.

## RP105 deficiency does not influence infarct size

Then it was investigated whether the enhanced dilatation of the RP105$^{-/-}$ mice could be secondary to an increased myocardial infarct size. Directly after the PV-loop measurements, 15 days after MI, mice were sacrificed and hearts were isolated. Figure 3 shows representative Sirius red stained cross sections of the infarcted heart. No differences in total LV area were observed ($27*10^6 \pm 4*10^6$ µM in WT vs $31*10^6 \pm 3*10^6$ µM in RP105$^{-/-}$, p=0.11) whereas the infarct area comprised 12 ± 5% of the total LV in the WT animals versus 16 ± 10% in the RP105$^{-/-}$ mice (p=0.53). The infarct sizes in both groups were thus similar, which may suggest that the more pronounced dilatation in RP105$^{-/-}$ mice reflects that the remote 'healthy' myocardium of the RP105$^{-/-}$ mice is less able to maintain cardiac function after MI as compared to WT mice.

## Structural analysis of the myocardium and infarct area

To investigate potential underlying mechanisms causing the observed differences in cardiac function after MI, (immuno)histochemical analyses on the myocardium of both groups were performed. We focused on inflammatory components in specific areas of the myocardium: the infarcted area, the border zone and the remote 'undamaged/ healthy' myocardium. Figure 4 shows representative images of the different stainings for Sirius red, TLR4, fibronectin-EDA, MAC-3 and RP105. All stainings showed little to no response in the undamaged myocardium remote from the infarct area. An upregulation of TLR4, fibronectin-EDA staining and the number of macrophages (figure 5) were observed in the area adjacent to the infarcted zone. As expected, the infarcted area consisted mainly of collagen (scar tissue) and macrophages. No differences in staining patterns were observed between WT and RP105$^{-/-}$ mice.

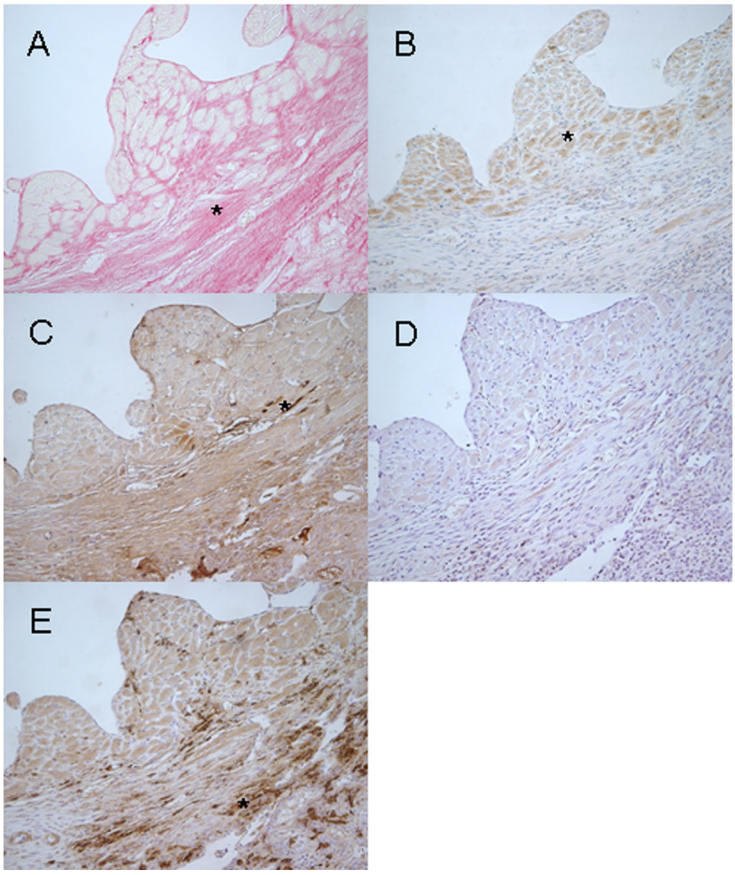


Figure 4 **Typical examples of (immuno)histological stainings**. Images are shown of (A) Sirius red staining indicating the collagen in the tissue adjacent to the infarcted area (*), (B) TLR4 staining in tissue adjacent to the infarcted area (*), (C) fibronectin-EDA staining in tissue adjacent to the infarcted area (*), (D) MAC-3 (macrophages) and (E) RP105 staining in tissue adjacent to the infarcted area (*). All images were photographed in sections adjacent to the infarcted area and this example is from a WT mouse. No differences were observed for Sirius red, TLR4, fibronectin-EDA or MAC-3 staining between the two groups. 200x magnification.



# Discussion

The innate immune system, and especially TLRs, plays a pivotal role in the remodeling process that is initiated after an MI. Since the role of TLR accessory molecules and especially the negative regulator of TLR4, RP105, in cardiovascular disease is still largely unknown, we investigated its role in post MI cardiac function and remodeling. Our results show, for the first time, that deficiency of the TLR accessory molecule RP105, results in hampered post MI repair and subsequent loss of cardiac function. This causal involvement of RP105 in the post MI inflammatory processes provides new opportunities for therapeutic approaches to reduce cardiac remodeling and thereby improving cardiac function after a myocardial event.

RP105 was originally identified as a B-cell specific molecule, but turned out to be also present on myeloid cells including macrophages and dendritic cells.[16, 19] Since the expression of RP105 mirrors that of TLR4, and TLR4 is an important player in the pathophysiology of cardiovascular disease processes, we hypothesized that RP105 might be an essential regulator in cardiovascular diseases. In this study we demonstrate that after an MI, RP105 deficiency results in an increased ESV and EDV which is associated with a more dilated LV. Since infarct size was similar in both groups, this effect suggests that the unaffected healthy myocardium of RP105$^{-/-}$ mice is less able to preserve cardiac function after MI as compared to WT mice.

After MI, loss of function in the infarct zone may be compensated by the unaffected myocardium, for example by hypertrophy, or by invoking the Frank-Starling mechanism via cardiac dilatation. In addition, cardiac output may be maintained by increased heart rate. No significant differences in cardiac output were observed between the two groups, but the RP105$^{-/-}$ mice showed a more pronounced dilatation and higher heart rate. This suggests that the intrinsic myocardial function in the undamaged myocardium was less in these mice, requiring more pronounced compensatory responses.

This conclusion is supported by the results regarding the end-systolic pressure volume relation (ESPVR) as shown in figure 2. The figure illustrates that the differences between the groups do not merely reflect altered loading conditions, but changes in intrinsic LV function as well reflected by a rightward shift of the ESPRV. Interestingly, in contrast to the ESPVR which indicates a depressed systolic LV function, the downward shift of the end-diastolic pressure volume relation (EDPVR) points towards an improved diastolic function in line with the positive effects on Tau and $dP/dt_{MIN}$.

Although macrophages in the infarcted zone are essential for the removal of necrotic tissue [20], they may also contribute to cardiac dysfunction by adherence to cardiomyocytes.[21]

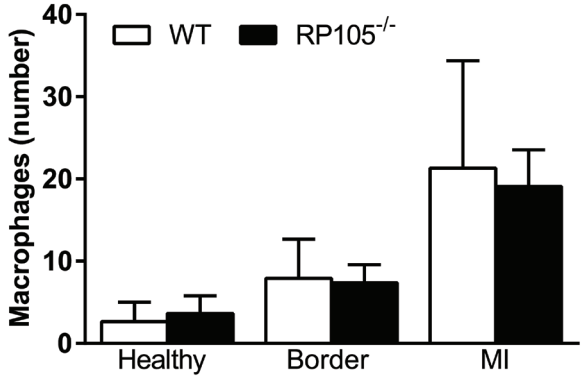


Figure 5 **Amount of macrophages per specific area of the myocardium in WT and RP105$^{-/-}$ mice.** the infarcted area, the border zone and the remote 'undamaged/healthy' myocardium Per image three specific areas of the myocardium were distinguished per image; the infarcted area, the border zone and the remote, undamaged, healthy myocardium. The number of macrophages was in three consecutive fields of view per specific area. Each bar represent mean ± SD; n= 8-10 per group.

However, as both WT and RP105$^{-/-}$ mice show a comparable density of macrophages in the border and infarcted areas, this cannot explain the differences in cardiac function.

Another factor that may contribute to the observed LV dilatation could be the availability of ligands for TLR4. Previously we showed presence of TLR4 and the endogenous TLR4 ligand fibronectin-EDA after MI resulting in deteriorating effects on cardiac remodeling.[12, 22] Similarly, in this study we observed an upregulation of fibronectin-EDA in WT mice when the damaged myocardium is compared to the undamaged myocardium. This was similar in the RP105 deficient mice showing equal fibronectin-EDA expression. We thereby demonstrated that RP105 does not influence local endogenous TLR ligand fibronectin-EDA expression.

In the current study we did not observe any effects of RP105 deficiency on histologic parameters. This could be explained by the lack of RP105 expression in the myocardium, since RP105 is known to be present on antigen presenting cells (APC)[16] but not on cardiomyocytes. APCs are well known to be involved in the cardiac remodeling process.

Alternatively it could be that RP105 has no direct signaling capacities. Since RP105 has no direct signaling function, but rather acts as a potent inhibitor of TLR4 signaling, it is more likely that the effects of RP105 deficiency are due to enhanced TLR4 signaling. In line with previously published results of Divanovic *et al.*[16, 23] we were able to demonstrate an enhanced inflammatory response in whole blood in RP105$^{-/-}$ mice. *Ex vivo* stimulation of whole blood samples of RP105$^{-/-}$ mice by the TLR4 ligand LPS resulted in strong upregulation of TNFα levels compared to the controls (table 1), a difference in TNFα levels that was not observed under unstimulated conditions. This supports the hypothesis that the TLR4 mediated inflammatory response is enhanced by RP105 deficiency.



RP105 alters TLR4 signaling via the RP105-MD1 complex which forms an unusual 2:2 homodimer. Two possible mechanisms for signaling inhibition effects on TLR4-MD2 were suggested previously; a lateral binding of TLR4-MD2 to the RP105-MD1 complex or the formation of TLR4-MD2/RP105-MD1 complexes reminiscent of the usual ligand-induced TLR homodimers.[24] Therefore the final effects of same amount of endogenous ligands activating TLR signaling, and thereby initiating cardiac remodeling, may have been increased due to the lack of RP105. While TLR4 expression in the myocardium was also comparable in both groups, this supports the hypothesis that RP105 does not cause its effects in remodeling directly via endogenous ligand or modulation of TLR4 receptor expression but probably via its previously described alternation of TLR4 signalling. By demonstrating that deficiency of the TLR accessory molecule RP105 affects the remodelling process, we reveal that a non-signalling extracellular receptor may be a potential target in the prevention of cardiac remodelling.

In summary, this study provides the first evidence that RP105 is involved in mechanisms underlying the TLR4 pathway induced post-infarction healing process. We show that RP105 deficiency has deleterious effects on cardiac function after an MI compared to WT mice with similar infarct size. These results underscore the role of the TLR4-pathway in post-infarction remodelling and as a result modulating RP105 may be an interesting new therapeutic strategy. To elucidate the exact mechanism further investigations are necessary.

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# Supplemental data

Supplemental table 1 **Baseline cardiac function of WT and RP105$^{-/-}$ mice.**

| | WT | RP105$^{-/-}$ | P-value |
|---|---|---|---|
| **General** | | | |
| HR (beats/min) | 587 ± 51 | 545 ± 79 | 1.000 |
| SV (µL/g) | 0.7±0.3 | 0.8±0.3 | 1.000 |
| CO ((mL/g)/min) | 0.4 ± 0.1 | 0.4 ± 0.2 | 0.886 |
| SW (mmHg.(µL/g)) | 69±20 | 71±19 | 1.000 |
| $E_A$ (mmHg/(µL/g)) | 0.2±0.1 | 0.2±0.1 | 0.486 |
| $E_{ES}/E_A$ | 1.0±0.1 | 0.9±0.1 | 0.343 |
| **Systolic** | | | |
| ESP (mmHg) | 96 ± 14 | 90 ± 17 | 0.686 |
| ESV (µL/g) | 1.0 ± 0.3 | 0.9 ± 02 | 0.686 |
| EF (%) | 42.3 ± 5.7 | 45 ± 10.4 | 0.686 |
| $dP/dt_{MAX}$ (mmHg/ms) | 8.9±1.8 | 8.0±1.4 | 0.686 |
| Ees (mmHg/(µL/g)) | 138±57 | 116±66 | 0.686 |
| **Diastolic** | | | |
| EDP (mmHg) | 6 ± 5 | 3 ± 3 | 0.686 |
| EDV (µL/g) | 1.7 ± 0.6 | 1.7 ± 0.4 | 1.000 |
| Tau (ms) | 10±3 | 9±1 | 1.000 |
| $-dP/dt_{MIN}$ (mmHg/ms) | 8±2 | 7±1 | 1.000 |
| $E_{ED}$ (mmHg/(µL/g)) | 6±1 | 8±4 | 1.000 |
| $K_{ED}$ (1/(µL/g)) | 0.16±0.03 | 0.09±0.0 | 0.500 |

HR, heart rate; SV, stroke volume; CO, cardiac output; SW, stroke work; $E_A$, arterial elastance (afterload); $E_{ES}/E_A$, ventricular arterial coupling; ESP, end-systolic pressure; ESV, end-systolic volume; EF, ejection fraction; $dP/dt_{MAX}$, maximal rate of pressure increase; $E_{ES}$, end-systolic elastance (slope of ESPVR); EDP, end-diastolic pressure; EDV, end-diastolic volume; Tau, relaxation time constant; $-dP/dt_{MIN}$, maximal rate op pressure decline; $E_{ED}$, end-diastolic stiffness (slope of EDPVR); $K_{ED}$, diastolic stiffness constant. Values represent means ± SD; n=4 per group.