Ischaemia-induced up-regulation of Toll-like receptor 2 in circulating monocytes in cardiogenic shock

Simina Selejan, Janine Pöss, Felix Walter, Matthias Hohl, Ralf Kaiser, Andrey Kazakov, Michael Böhm, and Andreas Link

Klinik für Innere Medizin III, Universitätsklinikum des Saarlandes, D-66421 Homburg/Saar, Germany

Received 19 January 2011; revised 20 August 2011; accepted 13 September 2011; online publish-ahead-of-print 13 October 2011

Aims
To investigate the role of Toll-like receptor 2 (TLR2) in uncomplicated acute myocardial infarction (AMI) and in cardiogenic shock (CS).

Methods and results
In patients with uncomplicated AMI (n = 20), CS (n = 30) and in age-matched healthy controls (HC; n = 20), TLR2 expression on monocytes was assessed by flow cytometry. Tumour necrosis factor alpha (TNFα) and interleukin-6 (IL6) expression in monocytes was analysed by intracellular cytokine staining. TLR2 expression was increased in patients with AMI compared with HC [mean fluorescence intensity (MFI) 111.1 ± 8.2 vs. 66.9 ± 1.5, P < 0.001]. In patients with CS, TLR2 expression was further increased (132.8 ± 5.6 MFI, P = 0.009 vs. AMI). This was accompanied by an increased expression of the proinflammatory cytokines TNFα (4.3 ± 1.6% in AMI vs. 20.5 ± 5.9% in CS, P = 0.004) and IL6 (6.3 ± 1.6% in AMI vs. 20.6 ± 6.2% in CS, P = 0.032). Furthermore, in all patients with myocardial infarction (AMI + CS; n = 50), a strong correlation between the monocytic TLR2 expression and the symptom to reperfusion time (r² = 0.706, P < 0.001) was found, implying tissue hypoxia dependency. Symptom to reperfusion time is a main factor to influence TLR2 expression but not the presence of CS. TLR2 expression of mononuclear cells exposed in vitro to hypoxia was assessed by flow cytometry and western blot. In vitro measurements showed a hypoxia-mediated monocytic TLR2 expression up-regulation.

Conclusion
We demonstrate TLR2 up-regulation and increased proinflammatory cytokine expression in circulating monocytes in AMI/CS depending on disease severity, implying an important role of TLR2 expression in ischaemic injury.

Keywords
Myocardial infarction • Cardiogenic shock • Inflammation • Hypoxia • Toll-like receptor

Introduction
Acute myocardial infarction (AMI) is associated with high morbidity and mortality. Cardiogenic shock (CS) is the leading cause of death in patients with AMI. New evidence suggests that the development of a systemic inflammatory response syndrome (SIRS) not only plays an important role in the pathogenesis of CS but is also a predictor of poor outcome. Toll-like receptors (TLR), expressed in peripheral blood mononuclear cells (PBMCs), play a central role in innate immunity by recognizing invading microbial pathogens and inducing an enhanced proinflammatory cytokine expression such as interleukin-6 (IL6) and tumour necrosis factor alpha (TNFα). A large body of evidence suggests that TLRs seem to be crucial in ischaemia–reperfusion (I/R) injuries as they have been shown to recognize endogenous proteins released from damaged tissues. In patients with AMI, a rapid increase of TLR4 expression in PBMCs was found not only at the site of plaque rupture in thrombotic material but also in the peripheral circulation. In addition to TLR4, animal studies have also provided evidence for a deleterious role of TLR2 in myocardial ischaemia, demonstrating that survival of TLR2 knockout mice with myocardial infarction was significantly increased compared with wild-type mice. Consistently, TLR2 deficiency was associated with a smaller infarct size and reduced inflammation. In patients with ACS and early coronary intervention, a local increase of...
TLR2 on PBMCs was found locally in thrombi of the coronary infarction artery, but not on circulating PBMCs. Recently, murine studies of hypoxia revealed a co-ordinated induction of TLR2 expression. In the present study, we pursued the hypothesis that tissue hypoxia, as it occurs during CS, may modulate TLR2 expression in circulating PBMCs as a late onset of innate immunity. For this purpose, we investigated TLR expression in patients with AMI and CS after a long time period of myocardial I/R injury.

Methods

Study population and clinical study protocol

Fifty patients with AMI, comprising 20 patients with uncomplicated AMI and 30 patients with CS were prospectively enrolled from an intensive care unit (ICU) of a university hospital. All patients received early percutaneous coronary intervention (PCI) on admission. Patients were enrolled immediately after a TIMI-III flow was established by PCI. AMI was diagnosed on the basis of chest pain lasting >30 min, the presence of new ST-segment elevation (at least 0.1 mV in two or more standard leads or at least 0.2 mV in two or more contiguous precordial leads), or/and an increase in a biochemical marker of myocardial necrosis (creatinine kinase, troponin T) according to the ESC guidelines. Cardiogenic shock was defined as the simultaneous presence of all the following criteria before or at admission to the ICU: (1) arterial hypotension (systolic arterial blood pressure below 90 mmHg or mean arterial blood pressure below 70 mmHg for 30 min or longer with or without therapy), (2) need for a continuous infusion of inotropic drugs (any dose of dobutamine and norepinephrine), (3) requirement of an intra-aortic balloon pump, and (4) an acute decrease of the left ventricular ejection fraction below 40%. Exclusion criteria were resuscitation, blood transfusion, collagen diseases, advanced liver disease, malignancy, febrile conditions, or any other ongoing infectious diseases. Patients without definite coronary culprit lesions and patients who developed CS later during the ICU stay were also excluded from the analysis. A total of nine patients with CS died during the first 48 h on the ICU. Twenty age-matched healthy volunteers served as control group (healthy controls, HC). The study was approved by the institutional review board and written informed consent was obtained from all subjects. The demographic and clinical data of all patients are presented in Table 1.

Isolation of human PBMCs

Baseline blood samples were collected immediately after a TIMI-III flow was established by PCI. Peripheral blood mononuclear cells were isolated from arterial blood using Ficoll density gradient. Briefly, whole citrate blood was mixed 1:1 with a solution of phosphate-buffered saline (PBS) and layered onto 0.25 vol. of Ficoll. After centrifugation at 800 g for 20 min at room temperature (RT), the layer of mononuclear cells was removed. PBMCs were centrifuged three times in PBS and resuspended in RPMI 1640 with 10% foetal calves serum (FCS) and antibiotics (penicillin/streptomycin).

Isolation of CD14+ monocytes

Monocytes were isolated from PBMCs by magnetic cell sorting using microbeads in accordance with the instructions of the manufacturer (Dynabeads Monocyte, Invitrogen). Isolated monocytes were bead and antibody free with a purity of CD14+ monocytes of 95%. These cells were either further separated in cytokel and membrane proteins for western blot analysis or proceeded for mRNA isolation.

Cell fractionation

Cells were washed once with ice-cold PBS, pH 7.4, and resuspended at 500 g for 5 min at 4 °C. Cell pellets were suspended in hypotonic buffer [in mmol/L: Tris 5, ethylenediaminetetraacetic acid (EDTA) 1, MgCl2 5, pH 8.0, phenylmethylsulfonyl fluoride (PMSF) 1, leupeptin 1; aprotinin 5 μg/mL] for 15 min at 4 °C. The suspension was spun down for 5 min at 500 g at 4 °C and subjected to 100 000 g ultracentrifugation (1 h, 4 °C) to yield a “cytosolic” (supernatant) and “particulate” (pellet). The particulate fraction was resuspended in 100 μL hypotonic buffer containing the protease inhibitors mentioned above. Protein concentration was determined according to the method of Lowry using a detergent compatible kit (BioRad, Munich, Germany), using bovine serum albumin (BSA) as a standard. Particulate and cytosolic fraction samples were analysed by western blot as described below. Similar loading of total protein was controlled by Ponceau Red staining.

Flow cytometry

For cell surface staining, 3 × 10^6 PBMCs were collected and blocked with PBS 10% FCS for 30 min on ice. Monocytes were stained by the surface expression of anti-CD14-PE-Cy5 and the TLR2 expression by using a rabbit polyclonal anti-human-TLR2-FITC antibody diluted 1:1000 in PBS 10% FCS, 1% sodium azide for 45 min at in the dark. Cells were fixed with PBS 1% paraformaldehyde (PFA) to prevent deterioration. Analyses were performed on a FACS Calibur flow cytometer using the CellQuest software (BD Biosciences PharMingen, San Jose, CA, USA). Monocytes were identified by forward (FSC) and side scatter (SSC) and CD14+ TLR2+ positivity; 2000 events of CD14+ monocytes were gated (Figure 1A). Negative control consisted of isotype-matched antibodies. Data are shown as mean fluorescence intensity (MFI).

Intracellular cytokine measurement by flow cytometry

Cell fixation was done with 2 mM EDTA for 15 min. Cell membranes were reversibly permeabilized with Saponin (0.1%) (Sigma-Aldrich) in PBS containing 5% milk powder and 0.1% BSA (Sigma-Aldrich). Cell surface markers and intracellular cytokines were labelled with mouse anti-human antibodies conjugated to fluorescent dyes at saturating concentrations in permeabilization buffer. For staining of the cell surface markers, we used anti-CD-14-PE-Cy5. For staining of intracellular monocyte cytokines, we used anti-TNFα-FITC and anti-IL6-PE. After 45 min of incubation at RT, cells were washed three times in permeabilization buffer and twice in fluorescence-activated cell sorting (FACS) buffer. Subsequently, stained antigens were fixed with 1% paraformaldehyde. Monocytes were identified by a FSC and SSC and CD14+ positivity; 5000 events of CD14+ monocytes were gated. The data are shown as frequencies of TNFα- or IL6-producing CD14+ monocytes.

Western blotting

Particulate and cytosolic samples were mixed 2:1 with 2× SDS–PAGE loading buffer. The denatured (95 °C, 5 min) proteins (50 μg/lane) were separated on 10% SDS polyacrylamide electrophoresis gels, transferred to nitrocellulose membranes (Protran, Schleicher & Schuell GmbH, Dassel, Germany) by semi-dry electrophoretic blotting (0.8 mA/cm²) and subjected to western blot analysis. Total protein loading was controlled by Ponceau Red staining. Membranes were blocked with 0.1% Western Blocking Reagent (Roche, Mannheim, Germany) and 0.005% goat serum and probed with primary antibodies for TLR2 (ab47840, rabbit polyclonal to TLR2, 1:1000, Abcam, Cambridge, UK) over night.
at 4 °C. The goat anti-rabbit peroxidase-labelled secondary antibody was diluted 1:10 000 and incubated for 60 min at RT. Proteins were visualized by enhanced chemiluminescence according to the manufacturer’s guide-
lines (Amersham Pharmacia Biotech, Freiburg, Germany). Autoradio-
graphs were quantified by imaging densitometry and analysed by the
‘LabWorks 4.6’ Software (LabWorks Image Acquisition and Analysis
Software, UVP BioImaging Systems, Cambridge, UK). Data are presented
as arbitrary units in per cent of a control sample and normalized to
Ponceau Red, unless stated otherwise.

### mRNA isolation and polymerase chain reactions

After the indicated treatments, culture medium was aspirated and the
cells were lysed with 1 mL RNA-Clean and processed according to the
manufacturer’s protocol to obtain total cellular RNA. Isolated total
RNA (2 μg) was reverse transcribed using random primers and
MMLV reverse transcriptase for 60 min at 42 °C and 10 min at 75 °C.
cDNAs were amplified by polymerase chain reaction (PCR) using
TaqDNA polymerase. Sequences for sense and antisense primers
and PCR conditions (and amplification fragment lengths) were as
follows: TLR2_for: GGC CAG CAA ATT ACC TGT GT; TLR2_rev:
AGA AGG TGG TG; GAPDH_rev: CAT ACC AGG AAA TGA
CAG GAA TGA AGT CCC GCT TA; GAPDH_for: ATG ACA TCA
ATG ACA TCA

### Hypoxia chamber

PBMCs from healthy volunteers were placed in RPMI 1640 10% faetal
calf serum (FCS) medium that had been pre-equilibrated with 5%
CO2/95% N2 for 6 h. Cells were then placed in a Labotect incubator

### Table 1  Baseline characteristics and therapeutic strategies

<table>
<thead>
<tr>
<th></th>
<th>HC</th>
<th>AMI</th>
<th>CS</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>63</td>
<td>68</td>
<td>67</td>
<td>0.238</td>
</tr>
<tr>
<td>Risk factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current tobacco use, n (%)</td>
<td>5 (25)</td>
<td>8 (40)</td>
<td>16 (53.3)</td>
<td>0.501</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>0</td>
<td>7 (35)</td>
<td>11 (36.7)</td>
<td>0.008</td>
</tr>
<tr>
<td>Hyperlipidaemia, n (%)</td>
<td>0</td>
<td>16 (80)</td>
<td>30 (100)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>0</td>
<td>7 (35)</td>
<td>12 (40)</td>
<td>0.008</td>
</tr>
<tr>
<td>SAPS-II score, points</td>
<td>–</td>
<td>28 (16; 44)</td>
<td>34 (16; 66)</td>
<td>0.093</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>STEMI/NSTEMI, n</td>
<td>10/10</td>
<td>20</td>
<td>10/20</td>
<td></td>
</tr>
<tr>
<td>Troponin T, μg/mL</td>
<td>–</td>
<td>2.59 (0.02; 8.24)</td>
<td>9.0 (0.02; 54.57)</td>
<td>0.055</td>
</tr>
<tr>
<td>Creatine kinase, U/L</td>
<td>–</td>
<td>1716 (53; 11392)</td>
<td>3725 (53; 29871)</td>
<td>0.201</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>–</td>
<td>36 (20; 72)</td>
<td>25 (12; 40)</td>
<td>0.045</td>
</tr>
<tr>
<td>Cardiac index, L/min/m²</td>
<td>–</td>
<td>2.42 (2.32; 2.87)</td>
<td>1.22 (0.67; 1.76)</td>
<td>0.025</td>
</tr>
<tr>
<td>CPI, mmHg/L/min/m²</td>
<td>–</td>
<td>0.55 (0.42; 0.58)</td>
<td>0.13 (0.07; 0.19)</td>
<td>0.025</td>
</tr>
<tr>
<td>Time delay</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symptoms to PCI, h</td>
<td>–</td>
<td>9.3 (3.0; 24.0)</td>
<td>9.3 (3.0; 24.0)</td>
<td>0.996</td>
</tr>
<tr>
<td>mvSO2, (%)</td>
<td>–</td>
<td>68 (60; 75)</td>
<td>59.7 (28.76)</td>
<td>0.013</td>
</tr>
<tr>
<td>Procalcitonin (μg/L)</td>
<td>–</td>
<td>4.7 (0.07; 71.8)</td>
<td>3.4 (0.01; 71.8)</td>
<td>0.759</td>
</tr>
<tr>
<td>Lactate, mmol/L</td>
<td>–</td>
<td>1.5 (0.7; 3.8)</td>
<td>2.5 (0.9; 6.3)</td>
<td>0.022</td>
</tr>
<tr>
<td>Infarct artery</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAD/CX/RCA, n</td>
<td>8/6/6</td>
<td>17/6/7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCI + Stent, n (%)</td>
<td>–</td>
<td>20 (100)</td>
<td>30 (100)</td>
<td></td>
</tr>
<tr>
<td>IABP, n (%)</td>
<td>–</td>
<td>0</td>
<td>30 (100)</td>
<td></td>
</tr>
<tr>
<td>Concomitant therapy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ASS + clopidogrel, n (%)</td>
<td>–</td>
<td>20 (100)</td>
<td>30 (100)</td>
<td></td>
</tr>
<tr>
<td>Dobutamine, dose, μg/kg/min</td>
<td>–</td>
<td>0</td>
<td>6.1 (0; 12.0)</td>
<td></td>
</tr>
<tr>
<td>Norepinephrine, dose, μg/kg/min</td>
<td>–</td>
<td>0</td>
<td>3.1 (0; 17.0)</td>
<td></td>
</tr>
</tbody>
</table>

Data are shown as number (n) or median and range. AMI, Acute myocardial infarction; CS, cardiogenic shock; NSTEMI, non-ST-elevation myocardial infarction; STEMI, ST-elevation myocardial infarction; CPI, cardiac power index (=Cardiac index × Mean arterial pressure × 0.0022); PCI, percutaneous coronary intervention; ASS, acetylsalicylic acid; mvSO2, mixed venous oxygen saturation. ANOVA t-test: AMI vs. CS. Fisher’s exact test for nominal parameters.
Figure 1  Toll-like receptor 2 expression on CD14⁺ monocytes measured by flow cytometry. (A) Representative dot blot of median fluorescence intensity. (B) Quantitative analysis of Toll-like receptor 2 expression on monocytes separated in healthy controls ($n=20$), patients with acute myocardial infarction ($n=20$), and patients with cardiogenic shock secondary to myocardial infarction ($n=30$). The mean fluorescence intensity was normalized to healthy control expression and expressed as the fold increase over controls. ANOVA was performed ($P$-value).
CA2 (MidAtlanticDiagnostics, Göttingen, Germany) and exposed to hypoxia (5% CO2, 94% N2, 1% O2) for 2–24 h followed by reoxygenation for 22–0 h. The pH of the medium remained unchanged during hypoxia and reoxygenation. Measurements of supernatant pH, lactate, and pO2 were performed in blood gas analysis tubes ‘safe storage for 22–0 h. The pH of the medium remained unchanged during hypoxia and reoxygenation. As all parameters were distributed normally, data were compared contribution of data was tested by Kolmogorov–Smirnov and Lillie-P wise stated. A two-sided Statistical analyses were performed using StatView (SAS Institute Inc., USA). Concentrations inducing 50% of the maximal effect (EC50) were calculated using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA).

**Materials**

Leupeptin, PMSF, PFA, aprotinin, and penicillin/streptomycin were purchased from Sigma Aldrich (Deisenhofen, Germany). Rabbit polyclonal against the extracellular domain of human TLR2 was from Abcam (ab47840, Cambridge, UK) and was used for western blotting. HRP-conjugated goat-anti-rabbit secondary antibodies were from Sigma (Deisenhofen, Germany). The following monoclonal antibodies were used for FACS analysis: anti-CD14-PE-Cy5 (Beckman Coulter, Krefeld, Germany), anti-TLR2-FITC (Abcam ab59711, Cambridge, UK), anti-TNF-FITC (BD Biosciences, Heidelberg, Germany), and anti-IL-6-PE (BD Biosciences, Heidelberg, Germany). Ficoll was purchased from Biochrom (Biocell Separating Solution, Biochrom, Berlin, Germany), RPMI 1640 medium, and FCS was provided by Gibco/Invitrogen (Karlsruhe, Germany). TaqDNA polymerase and nucleotides were purchased from Sigma. MMLV reverse transcriptase and RNA-Clean were obtained from Gibco. All other substances were used from Sigma, unless specified otherwise.

**Statistical analyses**

Statistical analyses were performed using StatView (SAS Institute Inc., Cary, NC, USA). Data are expressed as the mean ± SEM unless otherwise stated. A two-sided P < 0.05 was considered significant. Normal contribution of data was tested by Kolmogorov–Smirnov and Lilliefors. As all parameters were distributed normally, data were compared between two groups using the unpaired t-test and correlations were done by Pearson’s. In order to compare the three groups (HC, AMI, CS), we used one-way ANOVA and Fisher’s PLSD test for categorical data and Fisher’s exact test for nominal data. Multiple linear regression analysis was performed by SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Concentrations inducing 50% of the maximal effect (EC50) were calculated using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA).

**Procalcitonin measurements**

At admission baseline procalcitonin (PCT) was measured in serum probes using the PCT-sensitive Kryptor assay from BRAHMS (Hennigsdorf, Germany). The assay sensitivity was marked by 0.06 μg/L. According to the manufacturer’s protocol, a cut-off of PCT > 0.5 μg/L is postulated for a high risk for a potential bacterial infection.

**Materials**

Leupeptin, PMSF, PFA, aprotinin, and penicillin/streptomycin were purchased from Sigma Aldrich (Deisenhofen, Germany). Rabbit polyclonal against the extracellular domain of human TLR2 was from Abcam (ab47840, Cambridge, UK) and was used for western blotting. HRP-conjugated goat-anti-rabbit secondary antibodies were from Sigma (Deisenhofen, Germany). The following monoclonal antibodies were used for FACS analysis: anti-CD14-PE-Cy5 (Beckman Coulter, Krefeld, Germany), anti-TLR2-FITC (Abcam ab59711, Cambridge, UK), anti-TNF-FITC (BD Biosciences, Heidelberg, Germany), and anti-IL-6-PE (BD Biosciences, Heidelberg, Germany). Ficoll was purchased from Biochrom (Biocell Separating Solution, Biochrom, Berlin, Germany), RPMI 1640 medium, and FCS was provided by Gibco/Invitrogen (Karlruhe, Germany). TaqDNA polymerase and nucleotides were purchased from Sigma. MMLV reverse transcriptase and RNA-Clean were obtained from Gibco. All other substances used were from Sigma, unless specified otherwise.

**Statistical analyses**

Statistical analyses were performed using StatView (SAS Institute Inc., Cary, NC, USA). Data are expressed as the mean ± SEM unless otherwise stated. A two-sided P < 0.05 was considered significant. Normal contribution of data was tested by Kolmogorov–Smirnov and Lilliefors. As all parameters were distributed normally, data were compared between two groups using the unpaired t-test and correlations were done by Pearson’s. In order to compare the three groups (HC, AMI, CS), we used one-way ANOVA and Fisher’s PLSD test for categorical data and Fisher’s exact test for nominal data. Multiple linear regression analysis was performed by SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). Concentrations inducing 50% of the maximal effect (EC50) were calculated using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA).

**Results**

**Healthy control and patient characteristics**

Demographic data and baseline characteristics are shown in Table 1. The CS group and the AMI group were age matched. There were no significant differences between the two groups regarding risk factors such as smoking, hypertension, hypercholesterolaemia, diabetes mellitus, and regarding previous medication but statins. Previous statin medication was more frequent in the CS group than in the AMI group (70% in CS vs. 40% in AMI). All patients received PCI on admission. Patients with CS shock had lower ejection fraction, lower cardiac index, lower SVO2 levels, and higher lactate levels compared with AMI patients. Furthermore, patients with CS had substantial circulatory assistance with vasopressors, inotropes, and intra-aortic balloon pumps.

**TLR2 expression on circulating monocytes**

FACS analysis (Figure 1A) demonstrated that the expression of TLR2 on CD14+ monocytes was significantly increased in patients with AMI compared with HC (111.1 ± 8.2 vs. 66.9 ± 1.5 MFI, P < 0.001, Figure 1B, Table 2). In patients developing CS, TLR2 expression was further increased (CS 132.8 ± 5.6 vs. AMI 111.1 ± 8.2 MFI, P = 0.009). Within the CS group, there was no significant difference in TLR2 expression between patients dying within 48 h after admission to the ICU (128.8 ± 13.7 MFI, n = 9) and subjects with long-term survival (134.7 ± 5.3 MFI, n = 21, P = 0.626).

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Baseline laboratory parameters of monocyctic Toll-like receptor 2 expression and cytokine expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HC</td>
</tr>
<tr>
<td>Leucocytes, 10^9/L</td>
<td>n = 20</td>
</tr>
<tr>
<td>Monocytes, 10^9/L</td>
<td>n = 20</td>
</tr>
<tr>
<td>TLR2 expression on CD14+ monocytes, MFI</td>
<td>n = 30</td>
</tr>
<tr>
<td>TNFα expression in CD14+ monocytes, %</td>
<td>n = 30</td>
</tr>
<tr>
<td>IL6 expression in CD14+ monocytes, %</td>
<td>n = 30</td>
</tr>
</tbody>
</table>

Data are shown as number (n) or median and range. HC, healthy control; AMI, acute myocardial infarction; CS, cardiogenic shock; MFI, mean fluorescence intensity. ANOVA and Fisher’s PLSD test (P-value).
Intracellular cytokine expression in monocytes

The spontaneous cytokine production in CD14+ monocytes was measured by intracellular cytokine staining by flow cytometry. TNFα expression in monocytes was significantly increased in patients with CS (20.6 ± 5.9%) compared with patients with uncomplicated AMI (4.3 ± 1.6%, P = 0.004, Figure 2A, Table 2). Furthermore, the intracellular IL-6 expression was significantly increased in CS (20.6 ± 6.2%) compared with AMI (5.5 ± 1.6%, P = 0.032, Figure 2B, Table 2). However, no differences in spontaneous monocytic cytokine production were observed between patients with AMI and HC (Figure 2A and B).

Association of TLR2 expression with procalcitonin

At admission, none of our study patients suffered from a known active infection nor showed any clinical signs of an infectious disease. Nevertheless, 18 of all 50 included patients had elevated PCT levels (>0.5 µg/L) at enrolment. However, there was no difference in the mean TLR2 expression in patients with elevated PCT compared with the group with normal PCT (120.3 ± 9.1 vs. 127.8 ± 5.2, P = 0.448, Figure 3). In the study population, no correlation was found between the TLR2 expression and PCT levels.

Association of TLR2 expression with symptom to reperfusion time and parameters for tissue hypoxia

There was no significant difference with regard to time delay from symptoms to PCI-mediated reperfusion of the infarct vessel
between the AMI (9.3 ± 1.8 h) and the CS group (9.3 ± 1.3 h, 
P = 0.996), nor between CS survivors and non-survivors (9.0 ± 1.4 vs. 9.9 ± 2.6 h, 
P = 0.740, Table 1). TLR2 expression on monocytes showed a significant and strong correlation with symptom to reperfusion time in the whole study population (r² = 0.706, 
P < 0.001, n = 50, Figure 4).

Additionally, there was a significant positive correlation between TLR2 density on monocytes and serum lactate levels (r² = 0.262, 
P = 0.001, n = 50, Figure 5A) and an inverse correlation between TLR2 and mixed venous oxygen saturation (r² = 0.209, 
P = 0.001, n = 50, Figure 5B), both indirect parameters for tissue oxygen lack and anaerobic metabolism.

Subsequently, a multiple regression analysis was performed including symptom to reperfusion time and other factors known or thought to have an impact on TLR2 expression (i.e. mixed venous oxygen saturation, lactate, PCT, troponin levels, and ejection fraction) in order to investigate if the observed difference in TLR2 expression between myocardial infarction and CS is independent from these parameters. In this analysis, only symptom to reperfusion time (P < 0.0001) and mixed venous oxygen saturation (P = 0.026) were significant predictors of an increased TLR2 expression (Table 3), whereas the group (AMI vs. CS) was not (P = 0.983).

Hypoxia-induced TLR2 expression in PBMCs and monocytes in vitro

PBMCs from healthy volunteers (n = 20) were exposed for up to 24 h to either hypoxia alone (Figure 6). The pH of the culture medium showed no significant difference between hypoxia-exposed and control PBMCs for up to 12 h of culture, while supernatant lactate levels increased significantly starting after 6–12 h of hypoxia. Throughout hypoxenation, partial oxygen pressure (pO₂) was kept stable at about 40 mmHg; pO₂ in the control medium was about 140 mmHg.

Hypoxia time-dependently increased TLR2 expression on monocytes starting after a period of 6 h and reaching a maximum after 12 h of culture (baseline: 66.9 ± 1.6 h; 85.6 ± 2.6 MFI, 12 h, P < 0.0001, Figure 6). Therefore, hypoxia had stimulatory effects of the TLR2 surface expression on CD14⁺ monocytes with an EC₅₀ value of 6 h and a maximum of inducible TLR2 expression after 12 h (Figure 6).

These hypoxia-mediated effects were confirmed by cell fractionation and western blotting of isolated CD14⁺ monocytes from healthy volunteers (Figure 7). Additionally, TLR2 mRNA levels in isolated monocytes were significantly increased after 2 h of hypoxia, indicating transcriptional activation (P = 0.002, Figure 8).

**Table 3** Independent parameters in regard to Toll-like receptor 2 expression on monocytes in patients with acute myocardial infarction (n = 20) and cardiogenic shock (n = 30): a multiple linear regression analysis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Regression coefficient</th>
<th>Standard error</th>
<th>Standard coefficient</th>
<th>t-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group (AMI vs. CS)</td>
<td>−0.186</td>
<td>8.898</td>
<td>−0.003</td>
<td>−0.021</td>
<td>0.983</td>
</tr>
<tr>
<td>Symptom reperfusion time</td>
<td>2.716</td>
<td>0.392</td>
<td>0.727</td>
<td>6.934</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mixed venous oxygen saturation</td>
<td>−0.513</td>
<td>0.218</td>
<td>−0.222</td>
<td>−2.349</td>
<td>0.026</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.656</td>
<td>2.144</td>
<td>0.081</td>
<td>0.773</td>
<td>0.446</td>
</tr>
<tr>
<td>Procalcitonin</td>
<td>−0.026</td>
<td>0.150</td>
<td>−0.015</td>
<td>−0.170</td>
<td>0.866</td>
</tr>
<tr>
<td>Troponin T</td>
<td>0.110</td>
<td>0.263</td>
<td>0.041</td>
<td>0.418</td>
<td>0.679</td>
</tr>
<tr>
<td>Ejection fraction</td>
<td>−0.208</td>
<td>0.231</td>
<td>−0.148</td>
<td>−0.902</td>
<td>0.374</td>
</tr>
</tbody>
</table>

AMI, acute myocardial infarction; CS, cardiogenic shock.
Discussion

TLRs play a crucial role in innate immunity as the first defence system not only against pathogen-associated molecular patterns in infectious diseases but also against damage-associated molecular patterns in I/R injuries.12,14,18 Previous studies concentrated on the role of TLRs in the myocardial I/R injury found an early onset of TLR4 expression on PBMCs in coronary thrombi and in the peripheral circulation16 and TLR4 expression reached its peak values within 2 h in reperfusion states or 6 h in non-reperfusion states.26 Furthermore, the TLR2 expression was locally elevated in coronary thrombi but not systemically.16 In animal models of myocardial I/R, TLR2 signalling also seems to be involved in a deleterious course.13,15,20,21,27,28 Accordingly, TLR2-deficient mice had less fatal arrhythmias,28 less ventricular remodelling,21 and a higher survival rate compared with wild-type mice.

TLR2 has been postulated as being essential for the sensing of oxidants during inflammation.29 Reperfusion-induced myocardial injury is associated with an accumulation of reactive oxygen species (ROS).30 Activation of TLR may trigger ROS generation,31 and TLR2 deficiency is associated with a smaller infarct size and reduced reperfusion-induced production of ROS.13,15 Recently, murine studies of hypoxia revealed a co-ordinated induction of TLR2 expression.22

We demonstrated for the first time that hypoxia leads to a time-dependent increase in TLR2 expression in CD14+ monocytes from healthy volunteers. Therefore, hypoxia had stimulatory effects on the TLR2 expression on monocytes with an EC50 of 6 h and a maximum of inducible TLR2 expression after 12 h. These results obtained by FACS analysis were confirmed on the protein level by cell fractionation and subsequent western blotting as well as on the mRNA level, indicating transcriptional activation.

An increasing duration of myocardial ischaemia is frequently associated with clinical signs of centralization, organ hypoperfusion, and tissue hypoxia and consecutively with a SIRS. We pursued the hypothesis that tissue hypoxia in AMI and CS may modulate TLR2 expression on circulating PBMCs as a late onset of innate immunity. For this purpose, we investigated TLR expression in patients with AMI and CS after a long time period of myocardial ischaemia.

In the data presented herein, we observed an increase of TLR2 expression on PBMCs in the peripheral circulation in AMI which was even further increased in the setting of CS. Furthermore, myocardial I/R, TLR2 signalling also seems to be involved in a deleterious course.13,15,20,21,27,28 Accordingly, TLR2-deficient mice had less fatal arrhythmias,28 less ventricular remodelling,21 and a higher survival rate compared with wild-type mice.

TLR2 has been postulated as being essential for the sensing of oxidants during inflammation.29 Reperfusion-induced myocardial injury is associated with an accumulation of reactive oxygen species (ROS).30 Activation of TLR may trigger ROS generation,31 and TLR2 deficiency is associated with a smaller infarct size and reduced reperfusion-induced production of ROS.13,15 Recently, murine studies of hypoxia revealed a co-ordinated induction of TLR2 expression.22

We demonstrated for the first time that hypoxia leads to a time-dependent increase in TLR2 expression in CD14+ monocytes from healthy volunteers. Therefore, hypoxia had stimulatory effects on the TLR2 expression on monocytes with an EC50 of 6 h and a maximum of inducible TLR2 expression after 12 h. These results obtained by FACS analysis were confirmed on the protein level by cell fractionation and subsequent western blotting as well as on the mRNA level, indicating transcriptional activation.

An increasing duration of myocardial ischaemia is frequently associated with clinical signs of centralization, organ hypoperfusion, and tissue hypoxia and consecutively with a SIRS. We pursued the hypothesis that tissue hypoxia in AMI and CS may modulate TLR2 expression on circulating PBMCs as a late onset of innate immunity. For this purpose, we investigated TLR expression in patients with AMI and CS after a long time period of myocardial ischaemia.

In the data presented herein, we observed an increase of TLR2 expression on PBMCs in the peripheral circulation in AMI which was even further increased in the setting of CS. Furthermore,
significant correlations of TLR2 expression with symptom to reperfusion time, serum lactate levels, and mixed venous oxygen saturation were found. Interestingly, in a multiple regression analysis, symptom to reperfusion time and mixed venous oxygen saturation, both being indirect indicators for tissue hypoxia, were shown to be independently associated with TLR2 expression. Of note, the classification of groups AMI vs. CS in the view of haemodynamics was not independently associated with TLR2 expression. These findings support our hypothesis that severeness of tissue hypoxia, clinically manifested by cool extremities and vital organ hypoperfusion and triggered by compensatory mechanisms of vasoconstriction or vasopressors, plays a key pathophysiological role in up-regulation of TLR2 on circulation PBMCs and in ongoing inflammatory response such as SIRS.

Increased numbers of circulating monocytes in AMI have been described before and may promote the development of heart failure. As shown by our data, not only the absolute number of monocytes but also the number of monocytes expressing TNFα and IL6 were significantly increased in CS. These findings support the role of monocytes as relevant production sites of cytokines and point out an additional mechanistic explanation for the increased plasma levels of TNFα and IL6 in AMI/CS.

It has been shown that proinflammatory cytokines, notably IL6 and TNFα, are elevated in AMI patients compared with healthy subjects and that plasma levels of these cytokines even further increase in CS. However, the production site of these cytokines remained unclear. We investigated spontaneous monocytic cytokine expression in AMI and CS and confirmed the increase of IL6 and TNFα in AMI/CS patients compared with HC on monocyte level. IL6 is known to be the main cytokine associated with TLR2 activity and seems to be implicated in myocardial injury and reperfusion. These results implicate that proinflammatory cytokines known to be elevated in the setting of AMI/CS might at least partly derive from circulating monocytes and that TLR2 on the surface of monocytes might be involved in this increase.

However, some limitations of this study should also be considered. The average symptom to reperfusion time in our patients (AMI/CS) was considerably long (mean 9.3 h). Previous studies showed that early revascularization was not associated with an increased TLR2 expression on circulatory PBMCs and TLR2 response decreased after PCI. These findings underline the importance of an early revascularization in patients with AMI/CS. Interleukin-6 and TNFα might also be induced by some other inflammation triggering receptors which were not investigated in this study. Furthermore, we cannot exclude the possibility that IABP therapy in CS might have influenced TLR2 expression levels, as there are no reports about this so far. However, a recent study of Prodzinsky et al. found no effects of IABP therapy on inflammation parameters such as IL6 levels. Then again, catecholamines are regarded as anti-inflammatory agents suppressing cytokine production. Thus, we cannot exclude the possibility that the observed cytokine expression in our CS patients might have even been attenuated by catecholine therapy. The same applies to the monocytic TLR2 expression and the higher number of patients with previous statin medication in the CS group (70% vs. 40% in the AMI group), as statins are known to down-regulate cytokine and TLR expression.

In summary, our data show that TLR2 expression on circulating monocytes might be relevant in patients with AMI and subsequent CS by increasing proinflammatory cytokines involved in I/R injury. Ischaemia/tissue hypoxia itself seems to be responsible for TLR2 up-regulation in this pathological setting. Immunotherapeutic intervention strategies targeting TLR2 may be beneficial to CS patients.

**Acknowledgements**

We are indebted to Nina Rebmann for excellent technical assistance.

**Funding**

This work was supported by a HOMFOR grant of the University of Saarland, Homburg/Saar, Germany, and grants of the Ministerium für Wirtschaft und Wissenschaft des Saarlandes (LFFP 09/09).

**Conflict of interest:** none declared.

**References**


