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Circulation 2010;121:80-90; originally published online Dec 21, 2009;
DOI: 10.1161/CIRCULATIONAHA.109.880187
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Myocardial Ischemia/Reperfusion Injury Is Mediated by Leukocytic Toll-Like Receptor-2 and Reduced by Systemic Administration of a Novel Anti–Toll-Like Receptor-2 Antibody

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Background—Reperfusion therapy for myocardial infarction is hampered by detrimental inflammatory responses partly via Toll-like receptor (TLR) activation. Targeting TLR signaling may optimize reperfusion therapy and enhance cell survival and heart function after myocardial infarction. Here, we evaluated the role of TLR2 as a therapeutic target using a novel monoclonal anti-TLR2 antibody.

Method and Results—Mice underwent 30 minutes of ischemia followed by reperfusion. Compounds were administered 5 minutes before reperfusion. Cardiac function and dimensions were assessed at baseline and 28 days after infarction with 9.4-T mouse magnetic resonance imaging. Saline and IgG isotype treatment resulted in 34.5/3.3% and 31.4/2.7% infarction, respectively. Bone marrow transplantation experiments between wild-type and TLR2-null mice revealed that final infarct size is determined by circulating TLR2 expression. A single intravenous bolus injection of anti-TLR2 antibody reduced infarct size to 18.9/2.2% (P=0.001). Compared with saline-treated mice, anti-TLR2–treated mice exhibited less expansive remodeling (end-diastolic volume 68.2±2.5 versus 76.8±3.5 µL; P=0.046) and preserved systolic performance (ejection fraction 51.0±2.1% versus 39.9±2.2%, P=0.009; systolic wall thickening 3.3±6.0% versus 22.0±4.4%, P=0.038). Anti-TLR2 treatment significantly reduced neutrophil, macrophage, and T-lymphocyte infiltration. Furthermore, tumor necrosis factor-α, interleukin-1α, granulocyte macrophage colony-stimulating factor, and interleukin-10 were significantly reduced, as were phosphorylated c-jun N-terminal kinase, phosphorylated p38 mitogen-activated protein kinase, and caspase 3/7 activity levels.

Conclusions—Circulating TLR2 expression mediates myocardial ischemia/reperfusion injury. Antagonizing TLR2 just 5 minutes before reperfusion reduces infarct size and preserves cardiac function and geometry. Anti-TLR2 therapy exerts its action by reducing leukocyte influx, cytokine production, and proapoptotic signaling. Hence, monoclonal anti-TLR2 antibody is a potential candidate as an adjunctive for reperfusion therapy in patients with myocardial infarction. (Circulation. 2010;121:80-90.)

Key Words: immune system ■ inflammation ■ myocardial infarction ■ reperfusion ■ Toll-like receptors

Early restoration of blood flow through the occluded coronary artery is currently the most effective therapy to limit infarct size and to preserve cardiac function and geometry after acute myocardial infarction (MI). Nevertheless, reperfusion alone is insufficient to save endangered myocardium because complications resulting from loss of viable myocardium are still common after MI even after restoration of blood flow. Furthermore, studies have clearly demonstrated that reperfusion after ischemia causes additional cell death and increases infarct size (IS), called myocardial ischemia/reperfusion (I/R) injury. Many interventions aiming at reducing myocardial I/R injury have been proven to be successful in experimental studies but have failed in clinical settings. Administration of experimental compounds before the ischemic period, rather than in the late ischemic period before reperfusion, is one reason for the failure of successful translation in the clinic.© 2009 American Heart Association, Inc.
Clinical Perspective on p 90

Myocardial I/R injury is characterized by a rapid increase in cytokines and chemokines and influx of leukocytes into the endangered myocardial region. Inflammatory responses after myocardial I/R injury are detrimental for cell survival and extracellular matrix integrity via enhanced activation of proapoptotic signaling pathways. The extent of cardiomyocyte apoptosis determines IS and subsequent heart function. Mitogen-activated protein kinases c-Jun N-terminal kinase (c-JNK) and p38 mitogen-activated protein kinase (p38-MAPK) are well documented as proapoptotic mediators of myocardial I/R injury. Indeed, cardiac infarct healing and post-MI remodeling are also processes in which leukocytes, cytokines, and chemokines play both a beneficial and a detrimental role. For example, administration of methylprednisolone results in more frequent cardiac ruptures despite IS reduction in animal models. Hence, the challenge lies in ameliorating the detrimental inflammatory response while not affecting the tissue repair response.

Modulating Toll-like receptor (TLR) activation may enhance the “good” and blunt the “bad” of the inflammatory response after myocardial I/R injury. TLRs are expressed by leukocytes and recognize pathogen-associated molecular patterns to defend the host from invading microorganisms. TLRs are also capable of recognizing endogenous “danger signals” released during cell death. More intriguing is the fact that TLRs are also expressed on cells with no direct role in host innate immune responses, like endothelial cells and cardiomyocytes. On activation, TLRs exert their inflammatory response through nuclear factor-κB (NF-κB) translocation to the nucleus. Therefore, inhibition of TLRs may provide new therapeutic options after MI. This view is supported by recent observations in TLR-knockout mice. For example, ex vivo experiments show that TLR2-knockout (TLR2−/−) hearts perform better than wild-type (WT) hearts after myocardial I/R injury. TLR2−/− mice are protected against endothelial dysfunction after myocardial I/R injury, whereas TLR2 stimulation impairs cardiomyocyte contractility via NF-κB. Furthermore, deficient TLR2 or TLR4 signaling in mice prevents adverse cardiac remodeling, resulting in preserved cardiac function and geometry after MI. This evidence suggests that preventing TLR activation may be beneficial after MI through the prevention of myocardial I/R injury and enhancement of tissue repair responses. In the present study, we show in vivo the relative contribution of TLR2 in myocardial I/R injury and preserves cardiac function and geometry.

Methods

Animals and Experimental Design

TLR2−/− animals were backcrossed for 6 generations into a C57Bl6 background. Male C57Bl6/J and C57Bl6-TLR2−/− mice (age, 10 to 12 weeks; weight, 25 to 30 g) received standard diet and water ad libitum. Experimental compounds were administered via the tail vein 5 minutes before reperfusion. Mice were given 250 μL stock with either phosphate-buffered saline or IgG isotype 10 mg/kg as a negative control (R&D Systems, Minneapolis, Minn), SB239063 at 0.5 mg/kg (a p38-MAPK inhibitor) as a positive control (Alexis Corp, Lausen, Switzerland), and OPN-301 at 10 mg/kg (kindly provided by Opsona Therapeutics Ltd). When possible, recommendations from the National Heart, Lung, and Blood Institute Working Group on the Translation of Therapies for Protecting the Heart From Ischemia were applied; the surgeon was blinded to treatment. Digital photos of infarcts were encrypted before being analyzed by the researcher. Heart function and geometry assessment was done by a technician blinded to treatment. All animal experiments were performed in accordance with the national guidelines on animal care and with prior approval by the Animal Experimentation Committee of Utrecht University.

Myocardial I/R Injury In Vivo

Mice were anesthetized with a mixture of Fentanyl (Jansen-Cilag) 0.05 mg/kg, Dormicin (Roche) 5 mg/kg, and medetomidine 0.5 mg/kg through an intraperitoneal injection. Atropine 0.05 mg/kg SC was administered after coronary ligation. A core body temperature of ~37°C was maintained during surgery by continuous monitoring with a rectal thermometer and automatic heating blanket. Mice were intubated and ventilated (Harvard Apparatus Inc, Holliston, Mass) with 100% oxygen. The left coronary artery was ligated for 30 minutes with an 8-0 Ethilon (Ethicon) suture with a section of polyethylene-10 tubing placed over the left coronary artery. Ischemia was confirmed by bleaching of the myocardium and ventricular tachyarrhythmia. Reperfusion was initiated by releasing the ligature and removing the polyethylene-10 tubing. In sham-operated animals, the suture was placed beneath the left coronary artery without ligation. Reperfusion of the endangered myocardium was characterized by typical hyperemia in the first few minutes. A piece of the loosened suture was left in place to determine ischemic area during termination. The chest wall was closed, and the animals subcutaneously received Antisedan (Pfizer) 2.5 mg/kg, Anexate (Roche) 0.5 mg/kg, and Temgesic (Schering-Plough) 0.1 mg/kg.

Generation of Chimeric Mice

We generated chimeric mice to study the relative contribution of TLR2 expression in blood and parenchymal cells to myocardial I/R injury. Donor bone marrow (BM) cells were collected from WT C57Bl6 and TLR2−/− mice by flushing humerus, femurs, and tibiae with RPMI-1640 medium. Recipient mice received 5×106 BM cells after receiving a single dose of 7 Gy. Mice recovered for 6 weeks to ensure stable engraftment of the donor BM cells. Hereafter, chimerization was confirmed by phenotyping TLR2 expression on peripheral blood samples with Cytoomcs FC500 (Beckman Coulter, Fullerton, Calif) analysis. Successful chimerization (>95% circulating donor cells) was achieved in all mice (data not shown). Irradiated WT mice with TLR2−/− BM are referred to as WT/TLR2−/− BM mice, and TLR2−/− mice with WT BM are called TLR2−/−/WT BM mice.

Infarct Size

IS, determined 24 hours after myocardial I/R, is expressed as a percentage of the area at risk (AAR). The ratio of AAR to left ventricle (LV) is a measure of the extent of myocardial tissue that underwent ischemia and reperfusion (ie, endangered area). The IS/AAR ratio is an accurate measure to determine IS within endangered myocardium and is the primary end point from which the efficacy of treatment is assessed. To determine the AAR, the left coronary artery was ligated once again (at the level marked by the suture left in place), and 4% Evans Blue dye was injected via the thoracic aorta in a retrograde fashion. Hearts were rapidly explanted, rinsed in 0.9% saline, and put in a −20°C freezer for 1 hour. Then, hearts were mechanically sliced into five 1-mm cross sections. Heart sections were incubated in 1% triphenyltetrazolium chloride (Sigma-Aldrich, St Louis, Mo) at 37°C for 15 minutes before being placed in formaldehyde for another 15 minutes. Viable tissue stains red; infarcted tissue appears white. Heart sections were digitally photo-
graphed (Canon EOS 400D) under a microscope (Carl Zeiss). IS, AAR, and total LV area were measured with ImageJ software (version 1.34).

**Magnetic Resonance Imaging**

Twenty-four mice (n=8 per group) underwent serial assessment of cardiac dimensions and function by high-resolution magnetic resonance imaging (9.4 T; Bruker, Rheinstetten, Germany) under isoflurane anesthesia before and 28 days after myocardial I/R injury. Long-axis and short-axis images with a 1.0-mm interval between slices were obtained and used to compute end-diastolic volume (EDV; largest volume) and end-systolic volume (ESV; smallest volume). Ejection fraction (EF) was calculated as 100 (EDV/ESV)/EDV. All magnetic resonance imaging data were analyzed with Qmass digital imaging software (Medis, Leiden, the Netherlands).

**NF-κB Activation Assay**

THP1-Blue-CD14 cells (Invitrogen, San Diego, Calif) were used for in vitro efficacy assessment of OPN-301. Detailed information on THP-1-Blue-CD14 cells is given in the online-only Data Supplement. The THP1-Blue-CD14 cells were incubated for 30 minutes with OPN-301 or IgG isotype, followed by 18 hours of incubation at 37°C with Pam-3-CSK4 (P3C; 100 ng/mL) or lipopolysaccharide (1 ng/mL). Quantification of NF-κB activity was done according to the manufacturer’s protocol.

**Murine Tumor Necrosis Factor-α Production Assay**

Mouse macrophage J774 cells (1×10⁶/mL) were used for in vitro efficacy assessment of OPN-301. Cells were coincubated with P3C (100 ng/mL) in the presence of a dose range of OPN-301 and IgG1 isotype control. Six hours after incubation at 37°C in 5% CO₂, supernatants were removed, and tumor necrosis factor-α (TNFα) was assayed by ELISA (Duoset, RnD Systems, Abingdon, UK).

**Immunohistochemistry**

On termination, hearts were excised and snap-frozen in liquid nitrogen. Frozen sections were stained for Ly-6G (for neutrophils: rat anti-mouse Ly-6G 1:200, Abcam, Cambridge, UK), MAC-3 (for macrophages: rat anti-mouse MAC-3 1:50, BD Pharmingen, Breda, the Netherlands), and CD3 (for T lymphocytes: rabbit anti-human CD3 1:100, Dako, Heverlee, Belgium) by overnight incubation with the first antibody at 4°C for MAC-3 or by 1-hour incubation at room temperature for Ly-6G and CD3. Detailed information on secondary
antibodies and incubation time is given in the online-only Data Supplement.

Quantification of scar area after 28 days of infarction was performed with Picrosirius red staining of 4% formalin-fixed and paraffin-embedded heart sections. Analysis was done with circularly polarized light and digital image microscopy. Total scar area was determined as the percentage positive staining of the LV wall.

Protein and RNA Isolation
Total RNA and protein were isolated from snap-frozen infarcted heart sections with 1 mL Tripure Isolation Reagent (Roche, Basel, Switzerland) according to the manufacturer’s protocol.

Caspase 3/7 Activity
The level of apoptosis after treatment was assessed with the Caspase-Glo 3/7 assay kit (G8090, Promega, Madison, Wis). A protein sample (1 mL) was diluted in 24 wells assay buffer. The remaining protocol followed manufacturer’s instructions.

Flow Cytometry
TLR2 and CD11b expression on circulating monocytes of EDTA anticoagulated blood was analyzed by flow cytometry (Cytomix FC500, Beckman Coulter). Whole blood was stained for TLR2 (FITC, eBioscience, San Diego, Calif), CD11b (FITC, Serotec, Oxford, UK), and F4/80 for monocytes (Alexa Fluor 647, Serotec, Oxford, UK). Peripheral blood composition was determined with fluorescent antibodies for monocytes (CD14-FITC, BioLegend, San Diego, Calif.), neutrophils (Ly6G-APC, RnD Systems), and lymphocytes (CD3-FITC, eBioscience).

TNFα, interleukin (IL)-1α, IL-10, and granulocyte macrophage colony-stimulating factor levels in isolated protein samples were measured with the Th1/Th2 multiplex (Bender MedSystems, Vienna, Austria). The protein samples were diluted 1:1 in assay buffer, and the remaining protocol followed manufacturer’s instructions.

Phosphorylated target protein for p38-MAPK (Thr180/Tyr182) and c-JNK (c-JNK; Thr 183/Tyr185) was measured with the Bio-Plex Multiplex Assay (Bio-Rad Laboratories, Hercules, Calif) according to the manufacturer’s instructions after 1:8 dilution in assay buffer.

Polymerase Chain Reaction
Gene expression levels of monocyte chemotactic protein-1, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 were quantified with quantitative polymerase chain reaction. Further information is available in the online-only Data Supplement.

Statistical Analysis
Data are represented as mean±SEM. One-way ANOVA with post hoc 2-sided Dunnett t test adjustment (saline was set as control) was used to compare IS between groups. Nonparametric t test for TLR2 expression data analysis and 1-way ANOVA post hoc least-significant-difference test were used for comparison of cytokine and protein levels between groups, and 1-way ANOVA post hoc Dunnett T3 test was used for mRNA data. All statistical analyses were performed with SPSS 15.1.1 (SPSS Inc, Chicago, Ill). Values of P<0.05 were considered significant.
Results

TLR2 on Hematopoietic Cells Mediates Myocardial I/R Injury

With the use of an in vivo mouse model of myocardial I/R injury (30 minutes of ischemia followed by reperfusion), saline treatment resulted in 34.5±3.3% IS/AAR (Figure 1). TLR2−/− mice showed an infarcted area of 23±2.9% (≈33% reduction; *P=0.029). Final IS was determined by hematopoiesis-derived TLR2. TLR2−/−/WT BM mice were not protected against myocardial I/R injury, as shown by an IS similar to that of saline-treated WT mice (33.9±3.2% versus 34.5±3.3%; †P=1.0). WT/TLR2−/− BM mice were protected against myocardial I/R injury to the same extent as completely TLR2−/− mice, resulting in an ≈34% reduction in IS (22.9±2.7% versus 34.5±3.3%; ‡P=0.024). The extent of endangered myocardium was similar in all animals. The mean AAR/LV ratio was ≈41% (Figure 1 of the online-only Data Supplement). BM transplantation itself after radiation did not affect final IS; no significant difference in IS occurred in WT/WT BM and in TLR2−/−/TLR2−/− BM mice.

OPN-301 Selectively Inhibits Human Mononuclear TLR2, Reduces IS, and Preserves Heart Function In Vivo

Systemic inhibition of TLR2 could prevent myocardial I/R injury because TLR2 on circulating cells mediates myocardial I/R injury. Hence, we studied the therapeutic potential of OPN-301, a novel monoclonal antibody against TLR2. OPN-301 is a mouse IgG1 antibody that inhibits TLR2-mediated responses in mouse, pig, monkey, and humans (data not shown), indicating that it is specific for a critical epitope. The IC$_{50}$ for OPN-301 in human peripheral blood monocytes

Figure 4. Cardiac function and geometry at baseline and 28 days after infarction. A, Saline treatment results in a 22.8±12.3% increase in ESV and a 13.9±6.9% increase in EDV. However, OPN-301 treatment preserves ESV and EDV. Sham operation does not affect cardiac function and geometry; n=8 per group. *P=0.021, †P=0.046 vs saline treatment. B, Scar area as a percentage of the LV in mice after 28 days of infarction. *P=0.003 vs saline treatment. C, Representative 4-chamber magnetic resonance images and scarred tissue (Picrosirius red staining) 28 days after myocardial I/R injury. Each bar represents mean±SEM.
Furthermore, OPN-301 prevents NF-κB activation during reperfusion (Figure 2B). These results demonstrate that OPN-301 selectively inhibits TLR2 signaling. NF-κB activation does occur after lipopolysaccharide stimulation in THP-1 cells pretreated with OPN-301 (Figure 2B). However, NF-κB activation does occur after lipopolysaccharide stimulation pretreated with OPN-301 (Figure 2B). These results demonstrate that OPN-301 selectively inhibits TLR2 signaling. Furthermore, OPN-301 prevents NF-κB activation in a dose-dependent manner (Figure 2A). TNFα production after P3C stimulation in a mouse macrophage cell line was also highly inhibited in cells pretreated with OPN-301 compared with IgG1 isotype control (Figure 2C). In vivo, a single dose of OPN-301 administered 5 minutes before reperfusion reduces IS to 18.9 ± 2.2% (45% reduction; *P = 0.001 vs saline; Figure 3A). In addition, the IS reduction reveals a dose-dependent correlation (Figure 3B). Neither IgG1 isotype nor SB239063 treatments reduce IS, resulting in 31.4 ± 2.7% (P = 0.931 versus saline) and 31.7 ± 2.4% (P = 0.956 vs saline) infarction within the AAR, respectively (Figure 3A). AAR/LV was similar between treatment groups (P > 0.937 versus saline treatment; Figure I of the online-only Data Supplement). Both TLR2 and CD11b expression in monocytes decreased until the end of the 30-minute ischemic period. However, IS of reperfusion was reduced in the AAR and CD11b expression (Figure 3A). In concordance with the extensive infarct development, saline treatment significantly deteriorated LV volumes and EF (Figure 4A and Movie I in the online-only Data Supplement). In contrast, OPN-301–treated animals showed preserved LV volumes and cardiac performance, as shown by significantly smaller ESV (P = 0.021) and EDV (P = 0.046) and higher EF (P = 0.009) compared with saline treatment (Figure 4A and Movie II in the online-only Data Supplement). Furthermore, OPN-301–treated animals showed preserved regional LV function. Systolic wall thickening in both remote and infarct areas and EF were significantly higher in OPN-301–treated animals (Table). In line with the prevention of reperfusion injury (Figure 3), mice treated with anti-TLR2 antibody exhibited reduced scar formation after 28 days of infarction (Figure 4B and 4C). No difference was observed in any of the hemodynamic parameters measured (ie, heart rate, blood pressure) between animals (data not shown).

### Anti-TLR2 Treatment Reduces Inflammation After Myocardial I/R Injury In Vivo

To further elucidate the role of leukocytes in our treated animals, we studied the influx of neutrophils and macrophages after myocardial I/R injury. OPN-301 treatment resulted in reduced neutrophil influx after 1 hour, 1 day, and 3 days of reperfusion compared with both saline and IgG1 isotype treatment (Figure 5A). After 1 day, macrophages entered the myocardium to remove necrotic cells (eg, neutrophils, myocytes) and debris, contributing to post-MI extracellular matrix remodeling. OPN-301–treated animals showed a reduction of macrophage influx, consistent with our previous observation (Figure 5B). T-lymphocyte influx was also highly reduced in OPN-301–treated animals after 1 hour and 1 day of reperfusion (Figure 5C and 5D; remaining representative sections are shown in Figures II through IV in the online-only Data Supplement). The reduced influx of leukocytes after OPN-301 treatment may have been caused by changes in peripheral blood composition on treatment. Whole-blood analyses revealed no differences between saline and OPN-301 treatment after 1 and 3 days of reperfusion (Figure V in the online-only Data Supplement).

In addition, we studied cytokine and chemokine expression levels in ischemic/reperfused hearts. Mice treated with OPN-301 showed diminished tissue TNFα levels at all time points, whereas saline and IgG1 isotype treatment resulted in increased levels of TNFα (at 1 day reperfusion; Figure 6A). A significant reduction of OPN-301 treatment was also seen in the levels of IL-1α (at 1 day reperfusion; Figure 6B), IL-10 (at all time points; Figure 6C), and granulocyte macrophage colony-stimulating factor levels (responsible for monocyte maturation and activation) at all time points (Figure 6D). We performed quantitative polymerase chain reaction to investigate whether the highly reduced inflammation at tissue level depended on changes in chemotactic and adhesive factors in the heart. At the mRNA level, monocyte chemotactic protein-1, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 expression did not correlate with the observed decrease in leukocyte infiltration. Although expression levels were the same at 1 hour of reperfusion, monocyte chemotactic protein-1 and intercellular adhesion molecule-1 were significantly decreased on OPN-301 treatment after 1 day of reperfusion (Figure 6D).  

### Table: Wall Thickness, Systolic Wall Thickening, and EF 28 Days After Reperfusion Injury

<table>
<thead>
<tr>
<th></th>
<th>Saline</th>
<th>OPN-301</th>
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<tr>
<td></td>
<td>Baseline</td>
<td>28 Days After Myocardial I/R Injury</td>
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<tr>
<td>Wall thickness, septum (remote), mm</td>
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<tr>
<td>EF, %</td>
<td>42.6±2.1</td>
<td>39.9±2.2</td>
</tr>
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Data are given as mean±SEM; n = 8 per group. Mann–Whitney U test: *P < 0.001 versus baseline; †P = 0.021, ‡P = 0.038, and §P = 0.009 versus saline treatment.
reperfusion ($P=0.008$ and $P=0.059$, respectively, compared with saline; see Table I of the online-only Data Supplement).

**Anti-TLR2 Treatment Inhibits Proapoptotic Signaling Pathways After Myocardial I/R Injury In Vivo**

Having established that anti-TLR2 treatment with OPN-301 is a potent suppressor of key markers of inflammation, we next sought to examine the effects of OPN-301 on critical signaling pathways involved in cardiac function and remodeling. Anti-TLR2–treated mice exhibited less activation of proapoptotic signaling pathways than did mice treated with saline treatment. At all time points after reperfusion, the levels of phosphorylated c-JNK (Figure 7A) and phosphorylated p38-MAPK were significantly decreased (Figure 7B). In line with this observation, OPN-301–treated animals showed highly reduced caspase 3/7 activity levels after 1 hour and 1 day of reperfusion (Figure 7C).

**Discussion**

Many advances in clinical cardiology for the treatment of acute MI have aimed at restoring epicardial flow in the occluded coronary artery. Indeed, restored epicardial flow is a prerequisite for cardiomyocyte salvage; however, injury to both endothelial cells and cardiomyocytes occurs as a result of reperfusion therapy. As a consequence of restored flow, both myocardial perfusion and cardiomyocyte survival are hampered, causing a paradoxical increase in cell death and deterioration in cardiac function. Since the phenomenon of myocardial I/R injury was first described by Jennings et al.\(^19\) in 1960, a significant amount of research has been conducted to elucidate the mechanisms that underlie myocardial I/R injury and to investigate cardioprotective interventions. Despite the increased knowledge about underlying mechanisms, none of the experimental interventions has proved to be effective in the clinic. Only a few have been shown to reduce surrogate markers of myocardial I/R injury\(^20\) or to have an effect on...
secondary end points. This highlights the fact that myocardial I/R injury is a complex pathological condition for which a clinical therapy remains a great challenge. The discovery of TLRs in the cardiovascular system has greatly contributed to our knowledge of how the inflammatory reaction initiates and enhances myocardial I/R injury and postinfarct remodeling. Furthermore, previous studies using TLR-knockout animals provide evidence for TLRs as novel therapeutic targets in the cardiovascular system.

We have shown that TLR2-dependent circulating blood components mediate myocardial I/R injury. Interestingly, our study is supported by earlier observations in which ex vivo Langendorff-perfused hearts showed similar IS after myocardial I/R injury. Feng et al and Sakata et al showed similar IS after global myocardial I/R injury in MyD88−/− (downstream of both TLR2 and TLR4) and TLR2−/− hearts compared with WT littermates. Our study shows that circulating, not parenchymal, TLR2 determines final IS, which explains the negative observations in Langendorff-perfused heart experiments.

Our experiments demonstrate that anti-TLR2 treatment could potentially be applied successfully in human patients with acute MI. First, we used a novel anti-TLR2 monoclonal antibody treatment to inhibit TLR signaling in vivo, which can potentially be used in humans. Second, we chose a clinically applicable time point for drug administration in the setting of acute MI. Finally, 28-day survival experiments demonstrate the long-term efficacy of an anti-TLR2 treatment. We have demonstrated that treatment of mice with OPN-301 reduces IS by ~45% and prevents subsequent deterioration of cardiac function and geometry after a single intravenous bolus just 5 minutes before reperfusion. Hence, it has the potential to be effective when administered to patients.
with acute MI in the ambulance or emergency room before reperfusion therapy through primary percutaneous transluminal coronary angioplasty. Our model also showed that the p38-MAPK inhibitor SB239063 did not reduce IS when given in the late ischemic period before reperfusion. We chose this compound as a positive control because of experimental data showing that it was effective when administered before ischemia.25 Our study emphasizes the importance of a clinically relevant model (ie, in which compounds are given in the late ischemic period) to test therapeutic drugs in the preclinical stage.

OPN-301 exerts its action by selectively inhibiting TLR2 signaling in leukocytes. It suppresses leukocyte infiltration in the post–ischemic/reperfused myocardium partly via CD11b downregulation without affecting peripheral blood composition. Levels of highly potent inflammatory cytokines involved in cardiomyocyte injury and cardiac dysfunction (reviewed in Reference 6) are substantially decreased or even diminished. The decreased levels of cytokines are consistent with the reduction in IS and preserved heart function. In addition, decreased neutrophil, macrophage, and T-lymphocyte infiltration occurs on OPN-301 treatment and is not likely due to decreased chemotaxis and adhesion but rather to decreased activation of these leukocyte subsets. Only the expression of monocyte chemotactic protein-1 is significantly decreased on day 1 after myocardial I/R injury. Decreased infiltration, however, is already seen after 1 hour of reperfusion, and an IS increase does not occur beyond the first day. This is in accordance with our notion that circulating TLR2 plays a crucial role in myocardial I/R injury and not parenchymal TLR2. The exact mechanism by which TLR2 inhibition leads to decreased leukocyte infiltration remains to be addressed. One possible mechanism is that TLR2 inhibition prevents activation of circulating cells by endogenous

![Proapoptotic signaling in hearts after myocardial I/R injury.](image)

The chart shows the expression levels of phosphorylated c-JNK, p38-MAPK, and Caspase 3/7 in different groups (saline, IgG isotype, OPN-301, Sham). The statistical significance is indicated by asterisks and daggers.

- **A.** Phosphorylated c-JNK levels are decreased on OPN-301 treatment. *P* = 0.049, **P** = 0.024, ***P** < 0.001 vs saline; †P = 0.005, ††P = 0.045 vs IgG isotype treatment.
- **B.** Phosphorylated p38-MAPK levels. *P* = 0.044, **P** = 0.016, ***P** = 0.008 vs saline; †P = 0.006 vs IgG isotype treatment.
- **C.** Caspase 3/7 levels. *P* = 0.005, **P** = 0.005 vs saline; †P = 0.03, ††P = 0.024 vs IgG isotype treatment.

Each bar represents mean ± SEM; n = 6 per group per time point. Phosphoprotein levels are expressed as picograms per milliliter; caspase 3/7 activity, as relative light units (RLU). Both assessments are corrected for total protein concentration.
“danger signals.” Endogenous ligands released during cardiomyocyte necrosis (eg, HMGB1) may act as an activating ligand for TLRs.26

Our study also demonstrates that IgG isotype antibody, used as a negative control in our experiments, has several nonsignificant antiinflammatory effects. It does not reduce IS; however, compared with saline treatment, we do observe a slightly decreased neutrophil influx (Figure 5A) and slightly reduced IL-1α (at 3 days), IL-10 (at 1 and 3 days), and granulocyte macrophage colony-stimulating factor (at 3 days; Figure 6) levels. These observations point toward binding of IgG isotype antibodies to membrane-bound Fc receptor. This notion must be considered when isotype controls are used, especially in in vitro assays using single cells (lacking total organ functional assessments) with inflammatory markers as a readout.

Conclusions

Our study shows that myocardial I/R injury is mediated by TLR2-dependent blood components. Inhibition of TLR2 with OPN-301 reduces myocardial I/R injury and preserves cardiac function and geometry in vivo. The main mode of action of OPN-301 is the prevention of detrimental NF-κB–mediated inflammation by reducing leukocyte infiltration, reducing cytokine production, and reducing proapoptotic signaling and apoptosis. Moreover, our study demonstrates that OPN-301 is a good candidate as an adjunctive therapeutic for patients undergoing percutaneous transluminal coronary angioplasty because it has been shown to be effective when administered at a clinically applicable time point.

Acknowledgments

We thank the following persons for their assistance: Ben J. van Middelaar, Krista den Ouden, Chaylendria Strijder, Petra Homoet, Arjan Schoneveld, Gert-Jan Rood (all at the University Medical Center Utrecht, the Netherlands), Dr Christopher Locher (currently at Vertex Pharmaceuticals), Mary Reilly, and Dr Mark Hefferman (both at Opsona Therapeutics Ltd). We thank S. Akira (Osaka University, Osaka, Japan) and T. van de Poll (AMC Amsterdam, Amsterdam, the Netherlands) for providing us with the TLR2−/− mice.

Sources of Funding

This work is supported by research grants from the European Community’s 6th Framework Program (contract LSHMCT-2006–037400, IMMUNATH) and Utrecht University Mozaique grant (017.004.004 to Dr Arslan).

Disclosures

Professor O’Neill is a founder and shareholder of Opsona Therapeutics Ltd. Drs McGuirk and Keogh are employees and shareholders of Opsona Therapeutics Ltd. The other authors report no conflicts.

References


CLINICAL PERSPECTIVE

Over the past few decades, many molecular targets have been studied to limit the excessive tissue loss that occurs during the reperfusion phase after ischemia. This so-called myocardial ischemia/reperfusion injury limits the full potential of reperfusion therapy. Unfortunately, successful clinical translation of preclinical promises remains to be established. Our understanding of the pathogenesis of myocardial ischemia/reperfusion injury became much clearer with the discovery of Toll-like receptors (TLRs). The role of innate immunity in cardiac ischemia appeared to be more pivotal than we thought. More important, TLRs hold great promise as a therapeutic target within the innate immune system, also beyond cardiac ischemia, without affecting host defense or proper scar formation after infarction. In the present study, we show the first therapeutic application of an anti-TLR2 antibody after myocardial ischemia and reperfusion. We found that circulating TLR2 mediates myocardial ischemia/reperfusion injury. Administration of a TLR2 antagonist just 5 minutes before reperfusion reduces infarct size and improves cardiac performance and geometry. Furthermore, antagonizing TLR2 reduces inflammation and cell death after infarction. Our results reappraised the critical role of TLRs in cardiac ischemia and elucidated the mechanisms by which TLR2 mediates myocardial ischemia/reperfusion injury. Our results establish TLR2 as a novel therapeutic target for the treatment of acute myocardial infarction, even when it is initiated in the late ischemic period. For this reason, we provide a rationale for anti-TLR2 treatment initiated either in the ambulance or in the catheterization laboratory before reperfusion.
SUPPLEMENTAL MATERIAL

Methods

NF-κB activation assay
The THP1-Blue-CD14 are human peripheral blood monocytic cells, stably transfected with CD14-expression plasmid and a reporter plasmid expressing a secreted embryonic alkaline phosphatase (SEAP) gene under the control of a promoter inducible by NF-κB. Upon TLR stimulation, THP1-Blue™-CD14 cells activate NF-κB and subsequently the secretion of SEAP. In turn, SEAP activity is quantified using QUANTI-Blue™ (Invivogen, San Diego, USA), by reading the OD at 655 nm with a microplate reader.

Immunohistochemistry
For MAC-3 staining, the sections were incubated at RT with a biotin labeled secondary antibody for 1 hour and subsequent incubation with streptavidin-horseradish peroxidase at RT for 1 hour, the next day. For Ly-6G and CD3 staining, the sections were incubated with a secondary antibody (polyclonal rabbit anti-rat 1:750 or pure for CD3) for 30 minutes and subsequently with for 30 minutes with Powervision poly-HRP anti-rabbit IgG (ImmunoVision Technologies, Daily City, USA). The stainings were immediately visualized with Vector NovaRED™ substrate kit following the manufacturer's instructions (Vector Laboratories Inc., Burlingame, USA). All sections were rinsed in deionized water and counterstained with Mayer's haematoxylin stain for 10 sec.

Polymerase chain reaction
After DNase treatment, 500 ng total RNA was used for cDNA synthesis using the iScript™ cDNA synthesis kit (Bio-Rad). Amplification was performed using 10 µl iQ™ SYBR Green supermix and 10 µl cDNA. Quantities are determined by comparison with known quantities of cloned MCP-1, ICAM-1, VCAM-1 and 18S PCR products. All mRNA expression levels are normalized for 18S mRNA. Specificity of amplification for the detection with Cybergreen is visually checked on PAGE gels or a melting curve after the PCR. Used oligonucleotide primers for RT-PCR are: MCP-1 (forward: 5’- gatcggaaccaaatgagatcag-3’; reverse: 5’-gtggaaaaggtaggtgatgc-3’), ICAM-1 (forward: 5’-cagtgaggaggtgaatgtataag-3’; reverse: 5’-gatgtggaggagcagagaac-3’) and VCAM-1 (forward: 5’-caccctccaccttaattgtcgtg-3’; reverse: 5’-cgctcagaacaacggaatcc-3’).
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TLR2KO / TLR2KO BM
WT / WT BM

15mg/kg
5mg/kg
10mg/kg

IgG isotype
SB239063
Saline

AAR / LV, %

Error Bars: +/- 1 SE
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**Neutrophils**

- **Sham**
- **OPN-301**
- **Saline**

**Monocytes**

- **Sham**
- **OPN-301**
- **Saline**

**Lymphocytes**

- **Sham**
- **OPN-301**
- **Saline**
Table 1. MCP-1, ICAM-1 and VCAM-1 mRNA expression levels (normalized for 18S) in heart tissue after MI/R injury. *p<0.05 compared to saline †p<0.05 compared to IgG treatment. N=6/timepoint/group

Figure 1. Area at risk as a percentage of the left ventricle. No differences are observed in area of endangered myocardium between different study groups. Each bar represents Mean±SEM; n=10/group; TLR2 KO=Toll-like receptor 2 knock-out; WT/TLR2KO BM=wild-type mice with TLR2 KO bone marrow; TLR2 KO/WT BM=TLR2 KO mice with WT bone marrow. WT/WT BM=wild-type mice with wild-type bone marrow; TLR2 KO/TLR2 KO BM=TLR2 knock-out mice with TLR2 knock-out bone marrow.

Figure 2. Neutrophil influx in the myocardium after MI/R injury. Representative images from mice treated with saline, IgG isotype, OPN-301 and sham operation. Hearts are stained for neutrophils (Ly-6G staining; red cells with blue nuclei).

Figure 3. Macrophage influx in the myocardium after MI/R injury. Representative images from mice treated with saline, IgG isotype, OPN301 and sham operation. Hearts are stained for macrophages (MAC3 staining; red cells with blue nuclei).

Figure 4. Lymphocyte influx in the myocardium after MI/R injury. Representative images from mice treated with saline, IgG isotype, OPN301 and sham operation. Hearts are stained for T-lymphocytes (CD3 staining; red cells with blue nuclei).

Figure 5. Peripheral blood composition after MI/R injury. Neutrophil (Ly-6G+ cells), monocyte (CD14+ cells) and lymphocyte (CD3+ cells) counts are performed on whole blood samples upon treatment.). No differences were observed in any of the leukocyte subsets between saline and OPN-301 treated animals. Bars represent Mean±SEM.

Video 1. Four-chamber cine-MRI of saline treated mouse after 28 days post-reperfusion injury. Note the expansive remodeling of the left ventricle and decreased systolic function.

Video 2. Four-chamber cine-MRI of OPN-301 treated mouse after 28 days post-reperfusion injury. Both left ventricle geometry and systolic function are preserved upon anti-TLR2 treatment.