Inducible cardiac ischaemia is related to a decrease in the whole-blood Toll-like receptor 2 and 4 response

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ABSTRACT

TLR (Toll-like receptor) activation-induced inflammatory responses are important in the progression of atherosclerosis. We previously showed that TLR-dependent leucocyte responsiveness is acutely attenuated following percutaneous coronary intervention or vascular surgery. Furthermore, cytokine release following whole-blood TLR-2 and TLR-4 stimulation is negatively correlated with fractional flow reserve, suggesting that chronic ischaemia can elicit an enhanced inflammatory response. In the present study, we assessed the association between leucocyte TLR-2 and TLR-4 responsiveness and pre-existent and inducible ischaemia in patients undergoing SPECT (single-photon emission computed tomography)-MPI (myocardial perfusion imaging). TLR-2, TLR-4 and CD11b expression on monocytes were measured in blood samples that were obtained from 100 patients with suspected coronary artery disease before and after myocardial stress testing for SPECT-MPI. IL-8 (interleukin-8) levels were determined after whole-blood stimulation with Pam3Cys (TLR-2) and LPS (lipopolysaccharide; TLR-4). On the basis of SPECT-MPI, patients were categorized into three groups: reversible defect, irreversible defect and no defect. Myocardial stress induced a reduction in TLR-4 expression (2.46 ± 0.21 compared with 2.17 ± 0.16 arbitrary units, P = 0.001) and CD11b expression (83.2 ± 1.73 compared with 76.0 ± 1.89 arbitrary units, P < 0.001). TLR-induced IL-8 production before myocardial stress induction was not associated with the results of SPECT-MPI. However, a significant decrease in IL-8 production following TLR stimulation was observed after stress, which was more pronounced in patients with a reversible defect. In conclusion, inducible ischaemia is associated with a decrease in whole-blood TLR-2 and TLR-4 response. These results point to a regulating role of TLRs in order to prevent excessive inflammatory events known to occur during acute ischaemia.

Key words: atherosclerosis, ischaemia, leucocyte, single-photon emission computed tomography (SPECT)-myocardial perfusion imaging (MPI), Toll-like receptor.

Abbreviations: DAMP, damage-associated molecular pattern; IL, interleukin; LH, lithium-heparin; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; MPI, myocardial perfusion imaging; PCI, percutaneous coronary intervention; SPECT, single-photon emission computed tomography; TLR, Toll-like receptor; WBC, white blood cell.

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INTRODUCTION

As an important component of the innate immune system, TLRs (Toll-like receptors) play a crucial role in the first line of defence against invading pathogens. Stimulation of TLRs leads to the activation of intracellular signalling pathways, which, via NF-κB (nuclear factor-κB), ultimately results in the production of pro-inflammatory cytokines, such as TNFα (tumour necrosis factor α) and IL-8 (interleukin-8) [1–3]. Various reports have revealed a role for TLRs, especially TLR-2 and TLR-4, in the pathogenesis of atherosclerosis and ischaemic disease [4–6]. Not only microbial components such as LPS (lipopolysaccharide), but also endogenous ligands are capable of activating the TLR pathway. Several studies have shown that TLRs also respond to factors, such as HSPs (heat-shock proteins), fibronectin containing EDA (extra domain A), fibrinogen and hyaluronic acid, released after stress or tissue damage as a consequence of hypoxia and ischaemia [7,8].

TLR-2 and TLR-4 are abundantly expressed by circulating leucocytes. Previously, we have shown that the production of pro-inflammatory cytokines following stimulation of both TLR-2 and TLR-4 on circulating leucocytes is significantly decreased after vascular surgery and PCI (percutaneous coronary intervention) [9,10]. Tissue damage and ischaemia accompanying both vascular surgery and PCI induce tolerance to TLR-2 and TLR-4 stimulation, potentially to prevent excessive production of pro-inflammatory cytokines in the acute phase.

In contrast with the TLR hypo-response following acute vascular damage, TLR-2 and TLR-4 hyperresponsiveness was found to be associated with a low FFR (fractional flow reserve) measurement [9]. Thus, in the presence of chronic haemodynamically significant stenosis, the inflammatory cells are more easily activated. This effect could be explained by the demand for collaterals, which is accelerated by local inflammation to restore the blood flow.

Myocardial perfusion defects as a result of ischaemia can be visualized using SPECT (single-photon emission-computed tomography)-MPI (myocardial perfusion imaging) [11]. Defects that arise during stress, but are not present at rest, are referred to as reversible and are considered to represent stress-induced ischaemia. In contrast, perfusion defects that arise both during stress and at rest are referred to as irreversible and indicate a persisting absence of perfusion which is the case after myocardial infarction. If no defect is present during both stress and resting condition, myocardial perfusion is considered normal (Figure 1).

In the present study, we assess the role of pre-existent (irreversible) and induced (reversible) myocardial ischaemia on TLR responsiveness and baseline TLR-2, TLR-4 and CD11b expression on circulating monocytes in patients undergoing SPECT-MPI.

MATERIALS AND METHODS

Subjects

Adult patients with suspected coronary artery disease, planned for SPECT-MPI in the University Medical Hospital Utrecht, Utrecht, The Netherlands, were included in this study. The study was approved by the medical ethical board of the hospital and all patients signed a written informed consent prior to study participation. Patients admitted to the hospital, suffering from malignant neoplastic disease, taking corticosteroid medication or participating in another non-observational study were excluded. Cardiovascular risk factors, sex, previous medical history and medication use were gathered from questionnaires and the patient medical records. Venous blood samples were taken immediately before and after stress testing and stored in both LH (lithium/heparin) anti-coagulated and EDTA anti-coagulated tubes. To prevent premature leucocyte activation all tubes were kept on ice until further processing.
Imaging and stress protocol
All gated SPECT studies were performed routinely according to a standard 2-day stress/rest protocol. In short, patients underwent scintigraphy 1 h after injection of 700 MBq Tc-99m-tetrofosmin (Myoview; GE Healthcare) during stress testing and at rest. Stress testing was performed either with a symptom-limited upright bicycle test or a pharmacologic test using adenosine or dobutamine. Image acquisition was performed with a triple detector gamma camera (Picker Prism 3000 XP). Tc-99m-tetrofosmin will accumulate in cardiomyocytes, but only when sufficient coronary flow is present. If Tc-99m-tetrofosmin is present in myocardial tissue at rest but not after myocardial stress, referred to as a significant reversible perfusion defect, it was considered to represent myocardial ischaemia.

Measurement of TLR-2, TLR-4 and CD11b expression
TLR-2 and TLR-4 expression on circulating CD14 positive monocytes were examined by flow cytometry (Cytomics FC500; Beckman Coulter) of EDTA anti-coagulated blood. The samples obtained before and after the stress testing were processed simultaneously. Whole-blood samples were stained for CD14 (PE (phycoerythrin)–Cy5] combined with either TLR-2 (FITC), TLR-4 (PE) or CD11b (FITC) (all from Serotec). TLR-2, TLR-4 and CD11b expression levels are referred to as MFI (mean fluorescence intensity) on CD14-positive monocytes.

TLR stimulation of whole-blood samples
A total of 100 µl of blood stored in LH from the samples drawn before and after stress testing was stimulated with 100 µl of 5, 50 and 500 ng/ml Pam3Cys, a synthetic TLR-2 ligand (Novabiochem), and 1, 10 and 100 ng/ml of the TLR-4 ligand LPS (Sigma). Samples were incubated overnight at 37°C in 5% CO2, centrifuged at 3000 g for 5 min and the resulting supernatant was transferred to a separate 96-well plate and stored at −20°C until further analysis.

As a surrogate of the TLR-2 and TLR-4 response, IL-8 was measured in the supernatants of the blood samples using an ELISA (PeliKine-compact; Sanquin), according to the manufacturer’s protocol. In all stimulated samples, a total WBC (white blood cell) count was performed using a haematology analyser (Celldyn 1700; Abbott Diagnostics).

Statistics
For baseline characteristics, a χ2, Fisher exact or Kruskal–Wallis test was used. After log-transformation, a one-way ANOVA was used to compare differences in the TLR response before stress testing. The Kruskal–Wallis test and the Dunn’s post-hoc test were performed to assess the percentage difference in TLR-induced IL-8 production between the three groups based on the SPECT-MPI results. The Wilcoxon signed rank test was used to compare differences in TLR-2 and TLR-4, CD11b and WBC count before and after stress testing. A P value of <0.05 was considered statistically significant. Statistical analysis was performed with SPSS version 17 and GraphPad Prism 5 software.

RESULTS
Of the 100 patients included in the study, 96 were used for analyses. Two patients eventually did not undergo the stress test, one patient was excluded because of inaccurate sample handling, and another patient was excluded because of suspected global ischaemia. The baseline clinical characteristics of the studied population, divided into three groups based on the SPECT-MPI outcome, are reported in Table 1. A total of 39 of the included patients showed a significant, reversible perfusion defect, 32 patients showed an irreversible defect and 25 patients showed no defect. Patients with a reversible defect were significantly older and patients with any defect (reversible or irreversible) used ACE (angiotensin-converting enzyme) inhibitors and had a history of PCI or myocardial infarction.

TLR-2, TLR-4 response before and after myocardial stress testing in relation to SPECT-MPI outcome
Whole-blood IL-8 production following stimulation with LPS and Pam3Cys before stress testing (t = 1) were not significantly associated with the SPECT-MPI outcome (Figure 2). After myocardial stress, a significant decrease in whole-blood TLR response was observed in all patients for both Pam3Cys (P < 0.001) as well as LPS (P < 0.001) stimulation. Importantly, the percentage decrease in TLR response was more pronounced in the patients with a reversible perfusion defect as compared with the other groups (Figure 3). The average decrease in IL-8 release after stimulation with 10 ng/ml LPS was 34.9% for patients with a reversible defect compared with 16.7% for irreversible defect which was comparable with the decrease in patients with no defect (13.1%), P = 0.013 (baseline IL-8 expression after whole blood incubation without addition of TLR ligands: 23473 ± 274 pg/ml).

TLR-2, TLR-4 and CD11b expression in relation to SPECT-MPI outcome
A significant decrease in the expression of both TLR-4 (Figure 4B; 2.46 ± 0.21 compared with 2.17 ± 0.16; P = 0.001) and CD11b (Figure 4C; 83.2 ± 1.73 compared with 76.0 ± 1.89; P < 0.001), but not in TLR-2 expression (Figure 4A; 2.77 ± 0.09 compared with 2.74 ± 0.09; P = 0.392), was observed after myocardial stress. When specifically looking at the three different patient groups
Table 1 Baseline characteristics and medication use on the basis of the SPECT-MPI outcome

Values are means or numbers (%). BMI, body mass index; CABG, coronary artery bypass graft; CAD, coronary artery disease; MI, myocardial infarction; ACE, angiotensin-converting enzyme; ASA, acetylsalicylic acid.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Reversible defect (n = 39)</th>
<th>Irreversible defect (n = 32)</th>
<th>No defect (n = 25)</th>
<th>P value</th>
</tr>
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<tr>
<td>Male sex (n)</td>
<td>31 (79.5 %)</td>
<td>19 (59.4 %)</td>
<td>17 (68.0 %)</td>
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<td>Age (years)</td>
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<td>53.5</td>
<td>54.0</td>
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<td>BMI (kg/m²)</td>
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<td>25.8</td>
<td>26.7</td>
<td>0.749</td>
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<tr>
<td>Hypertension (n)</td>
<td>26 (66.7 %)</td>
<td>17 (53.1 %)</td>
<td>11 (44.0 %)</td>
<td>0.185</td>
</tr>
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<td>Diabetes (n)</td>
<td>8 (20.5 %)</td>
<td>2 (6.3 %)</td>
<td>1 (4.0 %)</td>
<td>0.103</td>
</tr>
<tr>
<td>Smoking (n)</td>
<td>7 (17.9 %)</td>
<td>6 (18.8 %)</td>
<td>4 (16.0 %)</td>
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<td>Hyperlipidaemia (n)</td>
<td>28 (71.8 %)</td>
<td>20 (62.5 %)</td>
<td>12 (48.0 %)</td>
<td>0.159</td>
</tr>
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<td>Family history of CAD (n)</td>
<td>17 (43.6 %)</td>
<td>14 (43.8 %)</td>
<td>9 (36.0 %)</td>
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<td>Prior CABG (n)</td>
<td>5 (12.8 %)</td>
<td>3 (9.7 %)</td>
<td>0 (0 %)</td>
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<td>Prior PCI (n)</td>
<td>20 (51.3 %)</td>
<td>11 (34.4 %)</td>
<td>5 (20.0 %)</td>
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<tr>
<td>Prior MI (n)</td>
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<td>3 (12.0 %)</td>
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<td>Medication</td>
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<td></td>
<td></td>
<td></td>
</tr>
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<td>β-Blocker (n)</td>
<td>27 (69.2 %)</td>
<td>17 (53.1 %)</td>
<td>12 (48.0 %)</td>
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<td>Calcium antagonist (n)</td>
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<td>7 (21.9 %)</td>
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<td>Nitrates (n)</td>
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<td>ACE inhibitor (n)</td>
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<td>10 (31.3 %)</td>
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<tr>
<td>A₂-antagonist (n)</td>
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<td>3 (9.4 %)</td>
<td>0 (0 %)</td>
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</tr>
<tr>
<td>Diuretics (n)</td>
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<td>10 (31.3 %)</td>
<td>2 (8.0 %)</td>
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<td>Anti-arythmics (n)</td>
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<td>ASA (n)</td>
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<tr>
<td>Coumarin (n)</td>
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<td>2 (6.3 %)</td>
<td>2 (8.0 %)</td>
<td>0.897</td>
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</tbody>
</table>

Figure 2 IL-8 levels following TLR stimulation before stress testing were not associated with SPECT-MPI outcome

Whole-blood samples were stimulated overnight with 1, 10 and 100 ng/ml LPS (left-hand graph) and 5, 50 and 500 ng/ml Pam3Cys (right-hand graph). IL-8 levels are log-transformed. Results are means ± S.E.M.

based on the SPECT-MPI outcome, TLR-4 expression (Figure 4B) was mainly decreased in patients with an irreversible defect, while CD11b (Figure 4C) expression was significantly decreased in all three groups. The stress-induced percentage changes in TLR-2, TLR-4 and CD11b expression did not differ between any of the groups (P = 0.381 compared with P = 0.979 compared with P = 0.256) (results not shown).

Leucocyte count

The total number of WBCs in the blood samples drawn after cardiac stress did not show a significant difference in comparison with the samples drawn before the procedure in either of the groups or combined (Figure 5A). Although we did observe a significant decrease in lymphocytes and monocytes, and a significant increase in granulocytes in all patients (P < 0.001), no differences in differential cell count were observed among the three groups (Figure 5B).

DISCUSSION

The innate immune system plays an important role in the pathogenesis of many cardiovascular diseases. The response of the innate immune system upon exogenous and endogenous stimulation may vary strongly within and between individuals. TLRs are the first line of defence of the immune system and can respond fast upon activation. In the present study, we describe the
We showed that whole-blood TLR responsiveness decreased following myocardial stress testing and that the attenuation of this response was more evident in the presence of reversible ischaemia. The lower release of IL-8 in patients with a reversible perfusion defect suggests that inducible ischaemia is a trigger for down-regulation of TLR responsiveness.

Previously, we demonstrated a similar reduction in cytokine release immediately following PCI [9]. PCI induces both vascular damage as well as short cardiac ischaemia during balloon inflation. In those experiments, it was not possible to discriminate between the effects of vascular damage and local acute ischaemia on TLR responsiveness. Previously, we confirmed the role of vascular damage as an important determinant that acutely influences TLR response [10]. The present study shows that inducible ischaemia is an additional factor that significantly influences the innate immune response.

The decrease in whole-blood TLR-2- and TLR-4-induced IL-8 release was accompanied by a decrease in both TLR-4 and CD11b expression. The observed reduction of TLR-4 expression could explain the decreased IL-8 release after LPS stimulation. However, the observation that TLR-2 responsiveness but not TLR-2 expression is reduced, indicates the involvement of other mechanisms in the ischaemia-induced down-regulation of TLR responsiveness. This is supported by other studies that have reported a down-regulated leucocyte response with preserved TLR expression [12–14]. An impaired release of cytokines upon TLR stimulation without alteration of receptor expression is, for example, also observed in pre-conditioning or tolerance, a hypo-responsive state that is induced by chronic stimulation of the TLRs [15–17]. We therefore assume that the changes in responsiveness after TLR activation cannot be explained by the expression of TLRs. In the present study, we found that the relative differences in IL-8 secretion between patients with no perfusion defect, patients with reversible perfusion deficits and patients with irreversible perfusion defects were similar for both TLR ligands (LPS and Pam3Cys). TLR-2 and TLR-4 share common intracellular signalling pathways which might explain this observation. In addition, it is well established that there is cross-talk between TLR-2 and TLR-4 signalling.

Surprisingly, not only patients with a reversible defect, but all patients showed a down-regulation in their whole-blood TLR response following stress testing. This is likely inherent to the protocol as pharmacological and/or physical stress have been shown to decrease TLR responsiveness [18–22]. Possibly, cardiac stress itself, independent of whether it causes ischaemia, is responsible for the observed hypo-response. It has been described that exercise can elicit a decrease in TLR responsiveness. The same inhibitory mechanisms might also be involved during cardiac stress. Furthermore, in comparison with...
patients with an inducible (reversible) perfusion defect, the decrease in IL-8 release was less in patients with a pre-existent (irreversible) defect, but higher in patients with no perfusion defect. Although patients with irreversible ischaemia have a perfusion defect, this is not caused by the myocardial stress testing, suggesting that patients with irreversible and no perfusion defect both should have the same percentage decrease in TLR response. However, it is likely that border ischaemia might be present in all or several of the patients with pre-existent (irreversible) ischaemia, explaining the observed decrease in TLR response following exercise.

WBC counts showed no differences comparing pre- and post-exercise measurements. However, when specifically considering the different WBCs, lymphocyte and monocyte fractions were decreased, whereas the granulocyte fraction was increased. As we studied whole-blood stimulation, it could not be elucidated which cell type was predominately responsible for the IL-8 levels in our study set-up. However, significant differences in TLR-2- and TLR-4-induced IL-8 release were no longer observed after correction for WBC count, suggesting that the changes in differential cell counts may have contributed to the observed down-regulation in responsiveness. The fast fall in lymphocyte and monocyte counts after myocardial stress testing could have been caused by the infiltration of these circulating cells into the ischaemic tissue. It is well accepted that a high expression of adhesion molecules such as CD11b by circulating leucocytes promote the adhesion and transmigration through the endothelium. Although purely speculative, it might be possible that the decrease in monocyte count that is observed has resulted in a reduction in total CD11b expression on peripheral blood leucocytes.

During ischaemia, the challenged myocardial tissue releases small amounts of DAMPs (damage-associated molecular patterns) that activate the TLR signalling pathway, leading to the release of pro-inflammatory cytokines. Tolerance induced by subsequent TLR challenges by these endogenous ligands, might serve to protect the body from excessive damage. Hence, two other processes might account for the observed reduction in TLR responsiveness. First, the exposed DAMPs might be recognized by passing leucocytes, inducing TLR tolerance in those cells which can then be detected in the peripheral blood. Secondly, the DAMPs might be released into the systemic circulation, subsequently reducing TLR responsiveness in circulating leucocytes.

The exact mechanisms responsible for the down-regulation in TLR response have not yet been elucidated. Upon TLR activation, several negative regulators are induced to dampen the inflammatory response, such as anti-inflammatory cytokines (for IL-10 example) and decoy receptors, to block the effects of pro-inflammatory cytokines [16]. Furthermore, intracellular inhibitors such as ST2 (suppressor of tumorigenicity 2), MyD88s (myeloid differentiation 88s) and SOCS3 (suppressor of cytokine signalling 3) interfere with the intracellular cascade to inhibit the production of pro-inflammatory factors [16]. However, for most of these feedback inhibitors de novo protein synthesis is required. In the present study, and especially in a previous study [10], the down-regulatory effects on TLR responsiveness were already observed within minutes, so probably other inhibitory mechanisms are involved. One possible explanation might be the rapid disruption of membrane lipid rafts, thereby increasing membrane fluidity and suppression of inflammatory signalling [16]. Another possibility might be the involvement of the vagal nerve. Several publications have shown a role for autonomic vagal nerve activity in the regulation of inflammation [23,24]. Although speculative, an explanation for the rapid change in TLR response might therefore lie in vagal nerve activity, directly following myocardial stress.

In conclusion, whole-blood LPS- and Pam3Cys-induced TLR responsiveness, as measured by IL-8...
release, are decreased after myocardial stress testing with the strongest effect observed in patients with inducible, reversible ischaemia. These data suggest a rapid down-regulation of whole-blood TLR responsiveness to protect the body from an excessive inflammatory state during acute ischaemia.

**AUTHOR CONTRIBUTION**

Patient inclusion and interpretation of clinical results were performed by Dik Versteeg, Jan-Willem Sels, Pieter-Jan Vlaar, Monique Hobbelink, Maarten-Jan Cramer, René Tio and Bart de Smet; Ellen Eelsenberg and Dik Versteeg analysed the results; Ellen Eelsenberg, Dik Versteeg, Monique Hobbelink, Imo Hoefer, Dominique de Kleijn, Pieter Doevendans and Gerard Pasterkamp participated in the analysis, discussion and interpretation of the data; Ellen Eelsenberg, Imo Hoefer and Gerard Pasterkamp drafted the paper; the Figures were created by Ellen Eelsenberg and Monique Hobbelink. All authors were involved in finalizing the paper and approved the final version.

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