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# Platelet Toll-like receptor (TLR) expression and TLR-mediated platelet activation in acute myocardial infarction

Kathryn E. Hally [a,b,*], Anne C. La Flamme [a,b], Peter D. Larsen [a,b,c], Scott A. Harding [a,b,d]

[a] School of Biological Sciences, Victoria University of Wellington, Wellington, New Zealand
[b] Wellington Cardiovascular Research Group, Wellington, New Zealand
[c] Department of Surgery and Anaesthesia, University of Otago, Wellington, New Zealand
[d] Department of Cardiology, Wellington Hospital, Wellington, New Zealand

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ABSTRACT

Both platelets and Toll-like receptors (TLRs) contribute to acute myocardial infarction (AMI). Platelet activation can occur post-AMI and despite treatment with anti-platelet therapy. TLRs may represent an alternative platelet activation pathway, although the role of platelet-TLRs in AMI is poorly characterized. The aim of this study was to examine platelet-TLR expression and TLR-mediated platelet activation in healthy and AMI subjects. Here, we report that platelets from AMI patients exhibit upregulation of some, but not other, TLRs. When examined by western blotting, platelet-TLR1 and TLR4 were significantly upregulated in AMI subjects compared to healthy subjects (both $p < 0.05$). Platelet-TLR2 was slightly, but non-significantly, upregulated in AMI patients and platelet-TLR6 expression did not change across cohorts. Platelets from both healthy and AMI subjects exhibited distinct activation patterns in response to various TLR agonists (0.1–100 μg/mL), as determined by flow cytometry. Healthy and AMI platelets became dose-dependently and directly activated in response to Pam3CSK4, a TLR2/1 agonist, but were directly potently activated only in response to the highest dose (100 μg) of lipopolysaccharide (LPS), a TLR4 agonist. Platelet activation in response to both of these agonists was similar across cohorts, despite treatment with anti-platelet therapy in the AMI cohort. At all doses used in this study, platelets were unable to become directly activated by FSL-1, a TLR2/6 agonist. We conclude that the platelet-TLR2/1 activation pathway is functional post-AMI and despite treatment with anti-platelet therapy. The platelet-TLR4 pathway appears to be less likely, and the platelet-TLR2/6 pathways unlikely, to contribute to post-AMI platelet activation.


## 1. Introduction

Platelet activation and aggregation play a central role in the pathogenesis of acute myocardial infarction (AMI), complications following percutaneous coronary intervention, and exacerbating myocardial ischaemia/reperfusion (I/R) injury [1,2]. Dual anti-platelet therapy (DAPT) is the standard of care following AMI as this treatment has been shown to reduce the risk of recurrent adverse cardiovascular events [3,4]. However, recurrent thrombotic events continue to occur in some AMI patients despite treatment with DAPT [5,6] and this has been linked to persistent residual platelet activation [7]. It is possible that alternative platelet activation pathways that are not targeted by currently available anti-platelet agents may promote recurrent thrombosis in these settings.

Toll-like receptors (TLRs) are critically important innate immune receptors that have been shown to be expressed on and within platelets [8–10] and are capable of mediating platelet activation [11–13]. TLRs respond to exogenous pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs), and TLR activation in response to both types of ligands has been described in AMI. Local release of DAMPs during AMI is known to trigger pro-inflammatory TLR activation which can aggravate myocardial I/R injury [14,15]. Furthermore, various PAMPs can induce platelet activation [16] and may provide a link between infection and the onset of AMI [17,18]. It is possible that increased TLR expression and TLR-mediated activation of platelets during AMI may enhance coronary thrombosis, contribute to myocardial I/R injury, and provide a pathway for ongoing platelet activation post-AMI.

*Abbreviations:* AMI, acute myocardial infarction; DAMPs, damage-associated molecular patterns; DAPT, dual anti-platelet therapy; FSL-1, fibroblast-stimulating lipopeptide-1; I/R, ischaemia/reperfusion; LPS, lipopolysaccharide; MFI, mean fluorescent intensity; PAMPs, pathogen-associated molecular patterns; PMA, platelet-monocyte aggregation; PRP, platelet-rich plasma; TLRs, Toll-like receptors; TRAP6, thrombin receptor activator peptide 6; WB, whole blood.

* Corresponding author at: Clinical Research Laboratory, Victoria University of Wellington, Level 8, Clinical Services Block, Wellington Regional Hospital, Wellington 6021, New Zealand.
*E-mail address:* hallykath@myvuw.ac.nz (K.E. Hally).

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Platelet-TLR expression, TLR-mediated platelet activation and the effect of anti-platelet drugs on this pathway in AMI patients has not been fully characterized. In this study, we aimed to examine whether total protein expression of platelet-TLR 1, 2, 4 and 6 changed between healthy subjects and AMI subjects treated with DAPT. Furthermore, we compared the level of platelet activation in response to TLR4, TLR2/1 and TLR2/6 agonism in healthy subjects and in AMI subjects treated with DAPT.

## 2. Materials and methods

### 2.1. Subject recruitment

We recruited 12 healthy subjects and 12 AMI subjects to investigate platelet-TLR1, 2, 4 and 6 expressions by western blotting. Additionally, 5 healthy and 5 AMI subjects were recruited to examine platelet activation in response to TLR2/1, TLR4 and TLR2/6 agonism by flow cytometry. Exclusion criteria for healthy subjects were: known cardiovascular disease, diabetes mellitus, any inflammatory or platelet function disorder, a platelet count $<100 \times 10^9$/L, treatment with cardiovascular medication, anti-platelet therapies, immune-modulating medication or non-steroidal anti-inflammatory drugs within 7 days preceding recruitment. Studying healthy subjects was approved by the University of Otago Human Ethics Committee, New Zealand. Patients with AMI were recruited from Wellington Regional Hospital, New Zealand, following adequate pre-treatment with DAPT and prior to coronary angiography. AMI was defined according to the Third Universal Definition of Myocardial Infarction [19]. DAPT consisted of either a combination of aspirin and clopidogrel or aspirin and ticagrelor. Exclusion criteria for the AMI cohort were: administration with a fibrinolytic agent within 24 h of enrolment, administration of a glycoprotein IIb/IIIa receptor antagonist within 7 days prior to enrolment, any inflammatory or platelet function disorder and/or a platelet count $<100 \times 10^9$/L. Studying AMI subjects was approved by the Health and Disability Ethics Committee, New Zealand. All healthy and AMI subjects provided written informed consent. Demographic data, clinical characteristics and medications were collected for all subjects at the time of enrolment. Blood was collected from a peripheral vein for all healthy subjects. For AMI subjects, blood was collected in the cardiac catheterization laboratory from the arterial sheath immediately after insertion and before administration of heparin. Blood was drawn into tubes anticoagulated with hirudin (25 μg/mL; Dynabyte, Munich, Germany).

### 2.2. Whole platelet lysate preparation for western blotting

Hirudin-anticoagulated whole blood (WB) was centrifuged at 200 ×*g* for 12 min to produce platelet-rich plasma (PRP). Prostaglandin E1 (final concentration, 1 μM) was added to PRP, which was mixed and centrifuged at 100 ×*g* for 20 min to deplete leukocytes. Leukocyte-depleted PRP was centrifuged at 1000 ×*g* for 20 min to pellet platelets. The platelet pellet was resuspended in radio-immunoprecipitation buffer (10 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton-X-100, 0.1% SDS, 1% sodium deoxycholate, pH 7.4) with 10 μL/mL protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO, USA) for 60 min at 4 °C. Cell debris was removed from the whole platelet lysate by centrifugation at 14,000 ×*g* for 5 min. The protein concentration of each lysate was assessed with the bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA, USA) and all lysates were stored at −80 °C prior to western blot analysis.

### 2.3. Platelet-TLR expression by western blotting

Western blotting was used to assess expression of TLR1, 2, 4 and 6 in whole platelet lysates from healthy ($n = 12$) and AMI subjects ($n = 12$). TLRs 1, 2, 4 and 6 were chosen for this study as these are known to be expressed on and within healthy platelets [8–10], although there is little information on how expression of these TLRs changes during AMI. Western blotting was carried out as described previously [20]. Each whole platelet lysate was incubated with Laemmli buffer (62.5 mM Tris–HCl, 2% SDS, 20% glycerol, 1% bromophenol blue) with 10% β-mercaptoethanol and boiled at 95 °C for 5 min. 50 μg of lysate was loaded onto an 8% SDS-PAGE and run at 120 V for 120 min at room temperature in running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). Platelet proteins were blotted onto a PVDF membrane at 20 V for 17 h at 4 °C in transfer buffer (25 mM Tris, 192 mM glycine, 10% methanol). Following this, the membrane was blocked with 5% BSA in Tris-buffered saline (TBS, 50 mM Tris, 150 mM NaCl, pH 7.4) supplemented with 0.1% Tween 20 for 1 h at room temperature. The membrane was probed with either primary mouse anti-human TLR1 (clone GD2.F4; BioLegend, San Diego, CA, USA), TLR2 (clone TL2.1; BioLegend), TLR4 (clone HTA125; BioLegend) or TLR6 (clone TLR6.127; BioLegend) overnight at 4 °C. The membrane was washed and incubated with a secondary goat anti-mouse antibody conjugated to HRP (Santa Cruz Biotechnology, Dallas, TX, USA) and detected using an electro-chemiluminescent detection system (Bio Rad, Hercules, CA, USA). Each membrane was then stripped and probed with primary mouse anti-human β-actin (clone AC-74; Sigma Aldrich) for 2 h at room temperature. β-actin was used as a loading control. Membranes were imaged using an Amersham Imager 600 (GE Healthcare, Little Chalfont, United Kingdom), the density of the platelet-TLR bands were analysed using ImageJ (imagej.nih.gov/ij/) and expressed relative to the density of the corresponding β-actin band.

### 2.4. TLR-mediated platelet activation by flow cytometry

Flow cytometry was used to assess platelet activation in response to TLR4, TLR2/1 and TLR2/6 agonism in WB and PRP in both healthy ($n = 5$) and AMI subjects ($n = 5$). Incubation of these TLR agonists in PRP determines the component of platelet activation that is mediated by direct TLR agonism. Incubation in WB determines whether any component of platelet activation is mediated by indirect TLR agonism through 'off-platelet' TLR pathways. Following blood drawing, leukocyte-depleted PRP was prepared. Both WB and PRP were adjusted to $1 \times 10^8$ platelets/mL with phosphate-buffered saline (PBS; 145 mM NaCl, 8.7 mM $Na_2HPO_4$, 1.3 mM $NaH_2PO_4$). WB and PRP were incubated for 15 min at room temperature with 0.1–100 μg/mL of the following TLR agonists: lipopolysaccharide (LPS) from *Escherichia coli* serotype R515 (TLR4 agonist; Enzo Life Sciences, Farmingdale, NY, USA), Pam3CSK4 (TLR2/1 agonist; Tocris Bioscience, Bristol, UK), and fibroblast-stimulating lipopeptide (FSL)-1 (TLR2/6 agonist; Santa Cruz Biotechnology). As a positive control, PRP and WB were incubated with 2.5 μM and 10 μM Thrombin Receptor Agonist Peptide 6 (TRAP6; Tocris Bioscience) for 15 min at room temperature. As a negative control, PRP and WB were incubated with PBS. Following incubation with these TLR agonists, PRP and WB were incubated with anti-CD42a-PerCP (clone Beb-1; Becton Dickinson, San Jose, CA, USA), anti-CD62p-PE (clone AK4; BioLegend) and PAC1-FITC (clone PAC-1; Becton Dickinson) in staining buffer (2% fetal calf serum, 0.1% sodium azide in PBS) for 20 min in the dark at room temperature. In parallel, aliquots of PRP and WB were incubated with PerCP-mouse IgG1,κ (Becton Dickinson), PE-mouse IgG1,κ (BioLegend) or FITC-mouse IgM (Becton Dickinson) isotype controls. Antibody-stained PRP and WB were fixed with 1% paraformaldehyde and analysed on a Millipore Guava easyCyte 8HT flow cytometer (Merck Millipore, Darmstadt, Germany). Flow cytometric data was analysed using FlowJo software (v10.0.7, Tree star, Ashland, OR, USA). Platelets were identified, firstly, by their forward and side scatter properties and, secondly, by their CD42a-positivity (Supplementary Resource 1). The percentage of CD42a-positive platelets that were positive for CD62p and PAC1 was determined, as a distinct positive and negative platelet population for each marker was observed. Monocytes were incubated overnight with 0.01 to 100 ng/mL LPS and FSL-1 as a positive control for agonist activity. The methodology is described in Supplementary Resource 2.