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## **Toll-like receptor 4 signal transduction in platelets: novel pathways**

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## Toll-like receptor 4 signal transduction in platelets: novel pathways

In addition to their roles in haemostasis and thrombosis, platelets are important players in several other processes and their role in inflammation is also recognized (Weyrich & Zimmerman, 2004). Platelets express transcription factors involved in non-genomic functions, including the positive and negative regulation of platelet activation. Recent studies (Malaver *et al*, 2009; Spinelli *et al*, 2010) have proposed that inhibition of platelet nuclear factor  $\kappa$ B (NF $\kappa$ B) leads to the modulation of platelet function and that NF $\kappa$ B may be a novel mediator of platelet response. NF $\kappa$ B is the major transcription factor that regulates the genes involved in immune responses. The thrombin versus “immune” stimulation of platelets and

their subsequent differential effects on the activation of signalling pathways as well as on specific protein-protein interactions and alpha granule release have recently been partly examined (Rex *et al*, 2009). Platelets express the Toll-like receptor (TLR) 2, 4, and 9 molecules (Andonegui *et al*, 2005; Cognasse *et al*, 2005). TLRs are hallmarks of innate immunity and usually signal responsive cells to induce genes that produce defence proteins. However, the mechanisms involved in anucleated cells, such as platelets, remain unclear. In eukaryotic cells, activated TLRs recruit adapter molecules (e.g. MyD88 and TRIF) within the cytoplasm of cells in order to propagate a signal including the I $\kappa$ B kinase kinase (IKK)

complex that phosphorylates I $\kappa$ B and subsequent NF $\kappa$ B activation. NF $\kappa$ B is then translocated to the nucleus where it upregulates the transcription of various inflammatory cytokines genes.

In addition to membrane expression of TLRs, platelets also contain molecules with known immunomodulatory competences, and these molecules may either be accumulated mostly or released upon signalling and, depending on the nature of the molecule and prevailing environmental conditions.

Studies by our group and others have furnished evidence of a functional role for TLR2 and 4 on platelets (Clark *et al*, 2007; Blair *et al*, 2008; Cognasse *et al*, 2008). However, the intracellular signalling pathways that mediate TLR2- and TLR4-induced platelet activation have not been investigated in detail. The aim of this study was to examine the platelet TLR4 machinery pathway.

Peripheral blood was collected from healthy donors in endotoxin-free 3.2% sodium citrate tubes (Vacutainer<sup>®</sup>, Becton-Dickinson, San Jose, CA, USA). Platelet-rich plasma (PRP) was prepared by centrifuging the blood at 150 g for 12 min at room temperature. Contamination (CD3-T cells, CD19-B cells and CD14-monocytes), determined by flow cytometry, was undetectable in PRP (data not shown). Peripheral blood mononuclear cells (PBMCs) were prepared as described previously (Hamzeh-Cognasse *et al*, 2008), and cell lysates were prepared for use as positive controls in the Western blotting assay.

Platelet protein extract (1  $\mu$ g) was loaded in each well and separated on a 10% acrylamide gel (Sigma-Aldrich, Saint Quentin Fallavier, France). The gel was then transferred onto a 0.45- $\mu$ m nitrocellulose membrane (Amersham Pharmacia, Orsay, France) by electroblotting using transfer buffer supplemented with 20% methanol (Sigma-Aldrich). The blots were blocked overnight at 4°C in phosphate-buffered saline containing 0.1% Tween 20 and 1% bovine serum

albumin (I.D. Bio, Limoges, France). These were then incubated for 60 min at room temperature with the primary antibody against TLR4 (1/500; Imgenex, San Diego, CA, USA) and TLR4 signalling pathway molecules as described in Table I. A histone H3 antibody (diluted 1/1000; Abcam, Cambridge, MA, USA) was used as the negative control for the platelet protein extract. The blots were washed three times for 10 min with blocking buffer and again incubated for 60 min with the secondary antibody (horseradish peroxidase-linked goat anti-rabbit or anti-mouse antibody, diluted 1/100 000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Next, the blots were incubated with a chemiluminescent substrate according to the manufacturer's instructions (enhanced chemiluminescence [ECL]; Amersham Pharmacia, Piscataway, NJ, USA) and exposed to radiographic film (Sigma-Aldrich).

We confirmed TLR4 expression on human platelets by using Western blotting (Fig 1). This technique was also used to detect the MyD88-dependent and MyD88-independent pathways in platelets (Fig 1). We ascertained that no TLR protein pathways had originated from non-platelet cells, as there was no PBMC in the platelet preparations (data not shown). PBMC contamination was consistently undetectable (data not shown).

In this study, we found that platelets expressed TLR4, MyD88, I $\kappa$ B- $\alpha$  and NF $\kappa$ B p65, confirming data that has been recently reported by other groups (Spinelli *et al*, 2010). Moreover, the presence of TRIF, TBK-1, IRAK-1, JNKs, MAPk, TRAF3, TRAF6, IRF-3 and IKK-I was also detected. To the best of our knowledge, this is the first report regarding the presence of these adapter proteins in platelets in general and their interactions with TLR4 in particular.

TLR4 ligands, such as lipopolysaccharide (LPS), bind to platelet TLR4, which differentially modulates the release of cytokines by platelets (Cognasse *et al*, 2008). LPS stimulates platelet secretion and aggregation via the TLR4/MyD88

**Table I.** TLR4 signalling pathway. Recognition of the ligand by TLR4 is aided by two adapter proteins MyD88 and TRIF. Monoclonal antibodies were used to study the proteins involved in signal transduction.

TLR4 signalling pathways	Adapter proteins	Proteins involved in signal transduction	Clone
MyD88-dependent pathways	MyD88 E-11 (Santa Cruz Biotechnology)	IRAK-1	C-2 (Santa Cruz Biotechnology)
		TRAF6	D-10 (Santa Cruz Biotechnology)
		JNKs	Polyclonal (Sigma-Aldrich)
		I $\kappa$ B- $\alpha$	Polyclonal (Millipore)
		NF- $\kappa$ B p65	F-6 (Santa Cruz Biotechnology)
		MAPk	Polyclonal (Sigma-Aldrich)
		TBK-1	108A429 (Imgenex)
		TRAF3	M-20 (Santa Cruz Biotechnology)
MyD88-independent pathways	TRIF Polyclonal (Alexis Enzo Life Sciences AG)	IRF-3	3-F10 (Santa Cruz Biotechnology)
		IKK-i	107A1458 (Santa Cruz Biotechnology)
		I $\kappa$ B- $\alpha$	Polyclonal (Millipore)
		NF- $\kappa$ B p65	F-6 (Santa Cruz Biotechnology)
		MAPk	Polyclonal (Sigma-Aldrich)

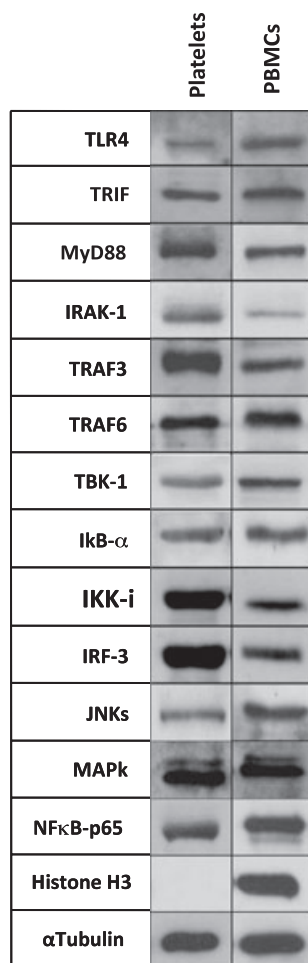


Fig. 1. Toll-like receptor 4 (TLR4) and expression of the molecular signalling pathway on platelets. Western blot analysis of the expression of TRIF, MyD88, TBK-1, IRAK-1, JNKs, MAPK, TRAF3, TRAF6, IRF-3, IKK-i, IκB-α and NF-κB p65 in platelets and peripheral blood mononuclear cells (PBMCs). α-Tubulin was used as the loading control. The histone H3 antibody was used as the negative control of the platelet protein extract. One representative experiment is shown. Western blotting was performed 15 times, and similar results were obtained in all runs.

receptor-signalling complex, as detailed recently. Our results are totally consistent with the finding that TLR4 is expressed on platelets (Andonegui *et al*, 2005; Cognasse *et al*, 2005). These present results also indicate that platelets contain the molecular machinery required for signalling via TLR4 in general (in nucleated eukaryotic cells).

However, it is unclear whether the effect of LPS on *in vivo* platelet activation is indeed due to the direct response of platelets to LPS. Further research is required to determine the relative importance of the TLR4/MyD88-dependent and -independent (TRIF) signalling complexes in platelets and how each of these is involved in the development of the inflammatory response and - in that perspective - drugs that target platelet TLRs and the TLR signalling pathway may be valuable in clinical settings.

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## Disclosure of Conflict of Interest

The authors state that they have no conflict of interest.

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## Effect of withdrawal of zoledronic acid treatment on bone remodelling markers in multiple myeloma

Osteolytic bone disease is an important clinical manifestation of multiple myeloma (MM). The most widely used treatment is monthly intravenous (i.v.) infusion of potent bisphosphonates (BPs), typically pamidronate or zoledronic acid (ZOL) (Berenson *et al*, 1996; Rosen *et al*, 2003). The effect of BPs is ascribed to their potent activity against osteoclasts, the bone resorbing cells, and to their strong binding to the bone mineral. Following the observation that prolonged treatment with BPs could cause osteonecrosis of the jaw (ONJ) it has been recommended that treatment should be discontinued after 24 months, or even after 12 months (Lacy *et al*, 2006; Durie, 2007; Kyle *et al*, 2007; Terpos *et al*, 2009). However, the risk of progressive bone disease as a result of BP discontinuation is a major concern, since it is unknown (i) for how long bone resorption remains inhibited after BP discontinuation and (ii) to what extent bone resorption is affected by the duration of the previous BP treatment.

To address these questions we conducted a single centre retrospective analysis in an unselected patient cohort, who had been treated with ZOL at the dose of 4 mg given by a 15-min i.v. infusion every fourth week. Biochemical markers of bone remodelling were measured before the discontinuation of ZOL and thereafter monthly for 18 months. Bone resorption was evaluated by measuring levels of C-terminal telopeptide of collagen type I (CTX-I) in serum (Roche Diagnostic, Hvidovre, Denmark), and N-terminal crosslinked telopeptide of type-I collagen (NTX-I) in urine (Ostex International, Seattle, WA, USA). Urine NTX-I was expressed relative to urine creatinine. Bone formation was assessed by measuring bone-specific alkaline phosphatase (bALP) levels in serum (Quidel Corporation, San Diego, CA, USA) as previously described (Lund *et al*, 2010). Serum and urine samples were collected from fasting patients in the morning. Twenty-nine patients were treated for a median period of 12 months (Group “short”), and another 34 patients for a median period of 24 months (Group “long”) (Table I). The difference in treatment duration was due to changes in hospital guidelines

**Table I.** Patient characteristics.

	Group “short”	Group “long”
Number of patients	29	34
Sex (male/female)	16/13	13/21
Age* (years)	73 (55–90)	69 (51–87)
Treatment duration*†	12 (8–12)	24 (14–43)
M-component type		
IgG	22	18
IgA	2	9
Light chain only	5	7
CTX-I baseline value‡ (µg/l)	0.10	0.14
NTX-I baseline value‡	13	16

\*Age and treatment duration are shown as median values with ranges.

†Duration of preceding zoledronic acid treatment.

‡CTX-I and NTX-I are shown as mean values.

during the study period. Changes in bone remodelling markers were analysed using linear regression. Significant differences from the time of discontinuation were investigated using a paired *t*-test. All *P*-values are two-sided and the significance level was set at  $P \leq 0.05$ . The study was approved by the Danish Data Protection Agency and conducted according to the national ethical guidelines and the Helsinki Declaration.

Prior to ZOL discontinuation, the mean levels of the bone resorption markers CTX-I and NTX-I were at the lower limit of the normal range: CTX-I Group “short”: 0.10 µg/l, CTX-I Group “long”: 0.14 µg/l (normal range 0.1–0.5 µg/l); NTX-I Group “short”: 13, NTX-I Group “long”: 16 (normal range 13–78) (Fig 1A–B). After ZOL discontinuation, CTX-I and NTX-I levels both increased significantly in Group “short” ( $P < 0.0001$  and  $P = 0.0009$  respectively), whereas their increase in Group “long” did not reach significance. Furthermore, the increase in CTX-I levels occurred significantly faster in Group “short” compared to Group “long” ( $P = 0.02$ ), and