Toll-like receptor 4 ligand can differentially modulate the release of cytokines by human platelets

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Summary

Blood platelets link the processes of haemostasis and inflammation. This study examined the immunomodulatory factors released by platelets after Toll-Like Receptor 4 (TLR4) engagement on their surfaces. Monoclonal anti-human FcγRII Ab (IV.3)-treated human platelets were cultured with TLR4 ligands in the presence or absence of blocking monoclonal antibody to human TLR4. The release of sCD62p, epidermal growth factor (EGF), transforming growth factor β (TGFβ), interleukin (IL)-8, platelet activating factor 4 (PAF4), platelet-derived growth factor, α, β polypeptide (PDGF-AB), Angiogenin, RANTES (regulated upon activation, normal T-cell expressed, and presumably secreted) and sCD40L were measured by specific enzyme-linked immunosorbent assay. TLR4 ligand [Escherichia coli lipopolysaccharide (LPS)] bound platelet TLR4, which differentially modulates the release of cytokines by platelets. It was noted that (i) sCD62p, IL-8, EGF and TGFβ release were each independent of platelet activation after TLR4 engagement; (ii) RANTES, Angiogenin and PDGF-AB concentration were weaker in platelet supernatant after TLR4 engagement; (iii) sCD40L and PAF4 are present in large concentration in the releaseate of platelets stimulated by TLR4 ligand. The effects of LPS from E. coli on the modulation of secretory factors were attenuated by preincubation of platelets with an anti-TLR4 monoclonal antibody, consistent with the immunomodulation being specifically mediated by the TLR4 receptor. We propose that platelets adapt the subsequent responses, with polarized cytokine secretion, after TLR4 involvement.

Keywords: platelets, Toll-like receptor 4, lipopolysaccharides, cytokines, inflammation.

Lipopolysaccharide (LPS), a cell wall component of all Gram-negative bacteria, is composed of an amphipathic lipid A moiety, core oligosaccharides and a variable O-antigen polysaccharide domain. It is the natural ligand for Toll-like receptor 4 (TLR4), the presence of which is essential for the cell response to LPS. A link between TLR4 and innate immunity has been described (Cook et al, 2004). The recognition of LPS by the innate immune system results in an inflammatory response characterized by the production of cytokines, such as tumour necrosis factor alpha, interleukin (IL)-1β, IL-6 and IL-8 (Akira & Takeda, 2004). Thus, TLR4 appears to be an important factor for the detection of pathogens and the induction of an adaptive immune response (Medzhitov et al, 1997).

Blood platelets, which are central to haemostasis, also have profound effects on the regulation of the immune system, linking innate (including inflammation) and adaptative immunity (Tang et al, 2002; Elzey et al, 2003; Yeaman & Bayer, 2006; von Hundelshausen & Weber, 2007). Furthermore, several studies have implicated LPS as a modulator of platelet function. Shibazaki et al (1996) showed that LPS from Escherichia coli induces a biphasic, organ- and strain-specific accumulation of murine platelets, and proposed that this effect is involved in the development of septic shock. Endo and
Nakamura (1992) reported the LPS-stimulated accumulation of 5-hydroxytryptamine (5-HT) in the liver, which was temporally associated with both a fall in the circulating platelet count and a reduction in the concentration of 5-HT in the blood.

We have shown the presence of certain Toll-Like Receptors (TLR) on human platelets, and that the percentage of TLR expression was significantly increased following activation, in both permeabilized and intact platelets (Cognasse et al, 2005). In addition, we have shown that stored platelets contain molecules with known immunomodulatory competences and differentially secrete them over time during storage (e.g. for transfusion purposes) (Cognasse et al, 2006).

Finally, several groups (including ours) have recently demonstrated that the TLR4 on platelets is functional (Andonegui et al, 2005; Aslam et al, 2006; Patrignani et al, 2006; Cognasse et al, 2007a; Semple et al, 2007), prompting us to study further the release of the secretory/immunomodulatory factors after the engagement of TLR4 on platelets.

Materials and Methods

Preparation of platelet concentrates

Platelet concentrates were prepared essentially as previously described (Cognasse et al, 2005). Platelet concentrate bags of 290 ml (containing approx. 3.9 x 10¹¹ platelets) were prepared as pools of five buffy coats of whole blood samples from ABO-identical donors, with a single white blood cell (WBC) filtration step according to routine blood bank manufacturing procedures. The pooling of five buffy coats from ABO-identical donors to obtain platelet concentrate bags does not allow conclusions to be drawn about the impact of gender or genetic factors on the present findings.

The platelets used were tested for bacterial contamination in routine bacterial growth tests and proved to be bacterial-free, i.e., without bacteria growth capacity. The residual WBC count was 0.025 x 10⁶ ± 0.023 per test platelet pool (Good Manufacturing Practice procedure requires that WBC count is below 10⁶ per transfusion product, i.e. per platelet concentrate) (Council of Europe 2005).

Platelet concentrate mixes were obtained from the Auvergne–Loire Blood Bank and stored at 22 ± 2°C under constant agitation for 1 d prior to experimental investigation. It was recently suggested that platelets stored at 4°C retain superior in vitro characteristics than those stored at 22°C (Sandgren et al, 2006, 2007), however, this remains controversial. Other studies demonstrated that platelets undergo substantial changes in both in vivo viability and in vitro properties when they were exposed to temperatures below 20°C for short periods (Rao & Murphy, 1982; Bode & Knupp, 1994; Moroff et al, 1994). Traditionally, platelets are stored at 22°C and consequently, in this study, platelet concentrates were prepared according to routine blood bank manufacturing procedures. This enabled a better understanding of TLR4 involvement in platelet physiology under normal storage conditions.

Platelet stimulation

Platelets (3 x 10¹¹ platelets/l) were incubated (20 min, room temperature) with the anti-human FcRRII monoclonal antibody (MoAb) IV.3 (10 µg/ml; StemCell Technologies, Grenoble, France) to saturate the free FcRRII and block FcRRII engagement (Tomiyama et al, 1992). The platelets were then stimulated with the TLR4 ligand LPS from E. coli (LPS E. coli; 0.5 µg/ml; 15 min, room temperature) (Invivogen/Cayla, Toulouse, France), in the presence or absence of a blocking anti-human TLR4 MoAb (clone HTA125; 10 µg/ml; 30 min, room temperature) (Imgenex, San Diego, CA, USA), which blocked the activation of monocytes by LPS (Paik et al, 2003). The optimal concentration of LPS was determined by measuring their effects on platelets at concentrations ranging from 0 to 2 µg/ml. The anti-human TLR4 MoAb (clone HTA125, Imgenex) and the isotype control MoAb (IgG2a, clone eBM2a, Nordic Immunological Laboratories, Tilburg, The Netherlands) were each used at a final concentration of 10 µg/ml, and the TLR2 ligand Pam3CysSK4 (Invivogen/Cayla) was used at a final concentration of 1 µg/ml (Damas et al, 2006).

Flow cytometry and cytokine detection

Platelet surface expression was determined by flow cytometric analysis of all events that were positive for CD41 (a platelet surface characteristic) marker expression (gating). Platelets were labelled with a PE-conjugated anti-human CD63 MoAb (BD Biosciences, Le Pont de Claix, France) to determine platelet activation status. The platelet TLR4 expression in the absence of TLR4 ligand, as determined using the anti-human TLR4 MoAb, was defined as 100% and the effects of TLR4 ligands expressed as percentages of this control value (Fig 1). Figure 1 further shows that E. coli LPS specifically and reproducibly bound to TLR4 and not to e.g. TLR2.

The levels of soluble cytokines [sCD62p, epidermal growth factor (EGF), transforming growth factor β (TGFβ), IL-8, platelet activating factor 4 (PAF4), platelet-derived growth factor, α, β polypeptide (PDGF-AB), Angiogenin, RANTES (regulated upon activation, normal T-cell expressed, and presumably secreted) and sCD40L] were measured in triplicate from aliquots of unstimulated (control) or LPS-stimulated platelets by specific enzyme-linked immunosorbent assays (ELISA) obtained commercially (R&D Systems Europe Ltd., Lille, France or Abcyss, Paris, France).

Statistical analysis

Inter-experiment comparisons in cytokine concentrations for the different culture conditions were analysed by means of the Wilcoxon paired test. All values were reported as means ± SD and a P value <0.05 was considered to be significant.
The level of CD63 on the surface of unactivated, 1-d storage platelets was 18.4 ± 2.7%, consistent with a mild level of baseline activation that was probably a consequence of the preparation procedure (Sandgren et al, 2007). Following the addition of E. coli LPS (0.5 μg/ml for 15 min) there was a 36.2 ± 5.1% (P < 0.05) increase in CD63 expression (Fig 1A), indicating that the platelets were significantly activated by bacterial components. Increasing the concentration of E. coli LPS (1–2 μg/ml) did not induce significantly more CD63 expression on the platelet surface than that elicited by 0.5 μg/ml E. coli LPS (Fig 1A).

We have previously shown a significant expression of TLR4 on the surface of CD41⁺ platelets (59 ± 2.1% of platelets) (Cognasse et al, 2005). Therefore, the modulation of platelet TLR4 expression in the presence of the ligand was examined on CD41⁺ cells. Platelets were preincubated for 15 min with E. coli LPS and analysed for TLR4 by flow cytometry. Incubation of platelets with E. coli LPS led to a significant decrease of 49.2 ± 7.9% (P < 0.05) in the levels of TLR4 (Fig 1B). There was no significant modification of TLR4 expression on the platelet surface after stimulation with either the isotype control Ab IgG2a or the specific TLR2 ligand, Pam3CysSK4 (Fig 1B).

No modulation of platelet cytokine release after TLR4 engagement

CD62P (P-selectin) is a component of alpha granule membranes in resting platelets, which can be detected on the surface of activated platelets (Metzelaar et al, 1993) and is subsequently cleaved in a soluble form (sCD62P) (Divers et al, 1995). The function of platelet TLR4 in ligand-induced sCD62P release was examined, however incubation of platelets with E. coli LPS had no significant effect on sCD62P release (Fig 2A). Similarly there was no significant release of other cytokines such as EGF (Fig 2B), TGFβ (Fig 2C) and IL-8 (Fig 2D).

Diverse platelet cytokine release after TLR4 engagement

The engagement of TLR4 with E. coli LPS significantly decreased the level of RANTES (86.1 ± 2.4 ng/ml vs. 103.6 ± 2.3 ng/ml, P < 0.05) (Fig 3A), possibly a result of its sequestration in platelet alpha granules (Schmitt et al, 2000; El Golli et al, 2005). Platelet incubation with E. coli LPS resulted in a significant decrease of Angiogenin (51.8 ± 3.1 ng/ml vs. 57.8 ± 4.6 ng/ml, P < 0.05) (Fig 3B) and of PDGF-AB (83.3 ± 5.5 ng/ml vs. 94.8 ± 6.1 ng/ml, P < 0.05) (Fig 3C).

The decrease in RANTES, Angiogenin and PDGF-AB secretion may be the result of platelet cytokine or chemokine receptor modulation. Several reports indicated that platelet receptors bind and present cell-derived chemokines, and may be involved in platelet activation, inflammatory or another immune response. (Clemetson et al, 2000; Weber, 2005). Alternatively Hu et al (1993) have shown that the angiogenin binding protein is a cell surface actin. A large number of proteins can bind to actin and this may contribute to several cellular functions, particularly cytokinesis (Hu et al, 1993).
In contrast, platelet incubation with *E. coli* LPS resulted in a significant increase in sCD40L levels (11.4 ± 1.4 ng/ml vs. 8.2 ± 1.7 ng/ml, *P* < 0.04) (Fig 3D) and of PAF4 (150.4 ± 6.9 ng/ml vs. 132.1 ± 11.8 ng/ml, *P* < 0.05) (Fig 3E). Each of these effects of *E. coli* LPS on immunomodulatory factors was attenuated by preincubation with an anti-TLR4 MoAb (Fig 3), consistent with the effects being specifically mediated by TLR4.

**Discussion**

CD40-CD40L interactions have been involved in inflammation and thrombosis. Recent studies have showed clinical interest in the use of sCD40L levels as a marker of thrombotic risk, particularly for coronary syndrome (Heeschen *et al.*, 2003; Varo *et al.*, 2003, 2006). Although sCD40L is emerging as a promising marker of thrombotic risk, moderately little attention has been given to the impact of sample processing technique on the soluble CD40L assay (Varo *et al.*, 2003, 2006; Ahn *et al.*, 2004). Thus we investigated the effect of blood sampling techniques reported in the CD40L literature to help clarify these issues. Elzey *et al.* (2003) showed in transfusion studies that platelet-derived CD154 alone is sufficient to induce isotype switching and augments T lymphocyte function during viral infection, thereby leading to enhanced protection against viral rechallenge. Heddle (1999) demonstrated that many chemokines which accumulate in platelet concentrates after leucoreduction were associated with allergic transfusion reactions. In addition, the intradermal injection of supernatants from activated platelets induces a deferred inflammatory response, which is probably due to histamine release and is likely to be due to platelet factor 4 (PF4) and macrophage inflammatory protein alpha (MIP-1α) (Boehlen & Clemetson, 2001). It may be possible that, when transfused into patients, these mediators cause allergic symptoms that are associated with non-haemolytic transfusion reactions. Moreover, the authors suggested that the mediators may cause other adverse reactions; for example, the release of IL-8 during the preparation processes may synergise with other chemokines, such as PF4 or RANTES (Boehlen & Clemetson, 2001).

Of note, CD14, the common LPS receptor on the surface of mononuclear leucocytes (Jiang *et al.*, 2005), was undetectable on the surface of platelets by flow cytometry and the incubation of platelets with an anti-CD14 MoAb did not affect LPS binding (data not shown), as previously reported (Stahl *et al.*, 2006). However, this does not exclude the possibility that soluble CD14 can participate in platelet activation, as has been described for certain other cellular systems (Pugin *et al.*, 1993).

The delivery of proteins to the platelet plasma membrane, including alpha granule proteins, seems to be controlled by the nature of the ligand/receptor involved. The post-TLR4 regulation of platelets appears to be closely regulated; the current study has shown that *E. coli* LPS favours the sequestration of
RANTES, Angiogenin and PDGF-AB while enhancing sCD40L and PAF4 release. It therefore seems plausible that different sets of “polarized” cytokines are released after specific platelet TLR4 engagement. Previous studies in dendritic cells have shown that different LPS molecules may induce distinct patterns of immunity while acting on the same receptor (Pulendran et al., 2001). Thus, it can be hypothesized that the nature and/or intensity of engagement of platelet membrane TLR4 determines a preferential cytokine pattern production/release, an issue that deserves further exploration. Meanwhile, the data (Fig 3) suggests that sCD62p, IL-8, EGF and TGFβ release are each independent of platelet activation involving TLR4, while RANTES, Angiogenin and PDGF-AB, each of which are less abundant in the platelet releasate, are prone to sequestration after the engagement of TLR4. An important issue, with clinical implications in transfusion medicine (Khan et al., 2006), concerns the conditions after which PAF4 and sCD40L are present in large concentrations in platelet stimulated by TLR4 ligand. These cytokines exert potent effects on the vascular epithelia and on inflammation in general (Prescott et al., 2006).
et al., 2000; Phipps et al., 2001; Danese & Fiocchi, 2005; Fillon et al., 2006), and the amounts of sCD40L that are secreted during platelet storage are compatible with biological effects on immune circulating cells of the platelet concentrate transfusion recipient (Cognasse et al., 2006, 2007a,b,c; Corash et al., 2006a,b). To date, the platelet stimulus that connects vascular inflammation with vascular occlusion and thrombosis in sickle cell disease has been elusive (Bennett, 2006). However the current study suggests that the stimulus, LPS, is the same as for other inflammatory conditions involving platelets and CD40/CD40L, such as systemic lupus erythematosus (Delmas et al., 2005; Nagasawa et al., 2005) and atherosclerosis (Chakrabarti et al., 2005; Lee et al., 2006).

There has been a plethora of reports in recent years suggesting that a number of endogenous molecules can activate the innate immune system (Tsan & Gao, 2004), while TLRs and other Pattern Recognition Receptors sense external danger (Pasare & Medzhitov, 2004; Martinon & Tschopp, 2005). These endogenous molecules, particularly heat shock protein 70 (HSP70) (Dybdahl et al., 2002), can induce the release of proinflammatory cytokines. Taken together, there is now a clear indication that almost all sets of immune cells can exhibit, upon differential stimulation, patterns of polarized cytokine secretion (Mossman, 2000). Therefore, we propose that platelets can similarly respond by differentially releasing polarized cytokines. However in contrast to other immune cells, which differentially respond by gene reorganization, platelets must thus use a process of differential exocytosis that is as yet still unclear. It can be speculated that, in a given local environment, platelets may contribute with other cell types to the maintenance of a coherent cytokine pattern. In conclusion, we propose that TLR expression on platelets serves to sense TLR ligands, primarily bacterial, according to the infectious non-self model of revised danger theory (Gallucci & Matzinger, 2001).

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