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ORIGINAL ARTICLE

Platelets regulate leucocyte responses to Toll-like receptor stimulation

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Abstract

Objectives. Platelets are important regulators of vascular thrombosis and inflammation and are known to express Toll-like receptors (TLRs). Through TLRs, platelets mediate a number of responses by interacting with leucocytes. Here, we report the extent to which platelets modulate *in vitro* peripheral blood mononuclear cells (PBMCs) and granulocyte responses to TLR4, TLR2/1 and TLR2/6 stimulation in healthy subjects. **Methods.** Peripheral blood mononuclear cells and granulocytes from 10 healthy volunteers were cultured alone or cocultured with platelets. Cultures were left unstimulated or stimulated with 1 or 100 ng mL^{-1} of either LPS (TLR4 agonist), Pam3CSK4 (TLR2/1 agonist) or fibroblast-stimulating lipopeptide (FSL)-1 (TLR2/6 agonist). Neutrophil activation (CD66b expression), monocyte activation (HLA-DR), granulocyte elastase production and PBMC cytokine and chemokine production were examined. **Results.** Platelet coculture decreased neutrophil CD66b expression in response to LPS, Pam3CSK4 and FSL-1, and modestly decreased monocyte HLA-DR expression in response to low-dose LPS. Platelets reduced granulocyte elastase secretion in response to low doses of all TLR agonists tested. In response to LPS, platelet coculture reduced IL-6, tumor necrosis factor (TNF)-α and MIP-1β production, and increased IL-10 production by PBMCs. In response to FSL-1, platelets increased IL-6, IL-10 and MIP-1β production, but reduced TNF-α production. Platelet coculture did not alter PBMC cytokine/chemokine production in response to Pam3CSK4. **Conclusion.** This study challenges the notion that platelets act solely in a pro-inflammatory manner. Rather, platelets are complex immunomodulators that regulate leucocyte responses to TLR stimulation in a TLR agonist-specific manner. Platelets may modulate leucocyte responses to dampen inflammation, and this platelet effect may play an important role in reducing inflammation-mediated host damage.

Keywords: leucocytes, monocytes, neutrophils, platelets, Toll-like receptors

INTRODUCTION

Platelets are multi-functional cells that, aside from their role in thrombosis, are increasingly appreciated as mediators of vascular inflammation. In infection-related vascular injury, platelets are important effectors of antimicrobial host defence[1] and platelets also enhance leucocyte effector functions in a number of inflammatory pathologies which are characterised by sterile vascular injury.[2–4] Platelets express a range of Toll-like receptors (TLRs),[5,6] which are responsible for mediating early immune responses to both infection and sterile injury. Activating platelet-TLRs causes platelet activation and aggregation[7,8] alongside a number of pro-inflammatory, antimicrobial responses.[9–11] Platelet–leucocyte aggregation is a well-characterised response to TLR stimulation,[9,12] and these platelet TLR-mediated antimicrobial responses are facilitated, in the main, by their interaction with leucocytes.

Interestingly, the notion that platelets act only in a pro-inflammatory manner has recently been challenged.[13] Platelets have been shown to dampen leucocyte activation and pro-inflammatory cytokine production in both mouse models of sepsis[14] and *in vitro* cell models of infection,[15,16] and this platelet effect is associated with host defence and survival in sepsis.[17,18] The regulatory effect of platelets has largely been characterised in peripheral blood mononuclear cells (PBMCs) *in vitro* exposed to lipopolysaccharide (LPS; a TLR4 agonist), or in mouse models of sepsis.[14,17,19,20] However, the ability of platelets to affect the leucocyte response to a wider range of TLR agonists has not been fully characterised. To more broadly investigate this platelet effect, we aimed to determine the extent to which platelets modulate PBMC and granulocyte responses *in vitro* to TLR4, TLR2/1 and TLR2/6 stimulation in healthy subjects.

RESULTS

Neutrophil activation in response to TLR stimulation is reduced in the presence of platelets

To determine the effect of platelets on neutrophil activation in response to TLR stimulation, we examined neutrophil CD66b expression by flow cytometry in the absence of platelets (granulocytes + platelet-poor plasma [PPP]) and in the presence of platelets (granulocytes + platelet-rich plasma [PRP]) under the following conditions: (1) unstimulated, (2) 1 or 100 ng mL^{-1} LPS (a TLR4 agonist), (3) 1 or 100 ng mL^{-1} Pam3CSK4 (a TLR2/1 agonist) and (4) 1 or 100 ng mL^{-1} fibroblast-stimulating lipopeptide (FSL)-1 (a TLR2/6 agonist). The gating strategy for identifying neutrophils by flow cytometry is shown in Supplementary figure 1. Neutrophil CD66b expression increased in response to both 1 and 100 ng mL^{-1} of all three TLR agonists, as expected (Figure 1a, Supplementary table 1). Neutrophil CD66b expression for each of the culture conditions without and with platelets is shown in Figure 2, and the relative change in CD66b expression upon the addition of platelets is given in Table 1. The addition of platelets did not reduce CD66b expression in unstimulated neutrophil cultures, but platelet coculture differentially reduced neutrophil activation in response to all three TLR agonists. The increase in CD66b expression seen in response to low-dose LPS was reduced by 11% ($P < 0.05$) with platelet coculture. With platelets, the increase in CD66b expression was reduced by 15% at high-dose Pam3CSK4 ($P < 0.01$) and the increase in response to FSL-1 stimulation was reduced by 19% at a low dose and 14% at a high dose (all $P < 0.001$). This platelet effect on neutrophil CD66b expression was also shown to be platelet concentration-dependent (Supplementary figure 2).

Platelets reduce monocyte activation in response to low-dose LPS

We also examined the effect of platelets on monocyte activation in response to TLR stimulation, under the same conditions as described for neutrophil activation. Monocytes were identified by flow cytometry as shown in Supplementary figure 3. Similarly to neutrophils, monocyte HLA-DR expression was elevated in response to TLR stimulation (Figure 1b, Supplementary table 1). Monocyte HLA-DR expression with and without the addition of platelets is shown in Figure 3, and the relative change in expression with platelets is given in Table 1. Following coculture with platelets, the increase in HLA-DR expression in response to low-dose LPS was reduced by 5% ($P < 0.05$). However, monocyte activation was not changed in the presence of platelets under unstimulated

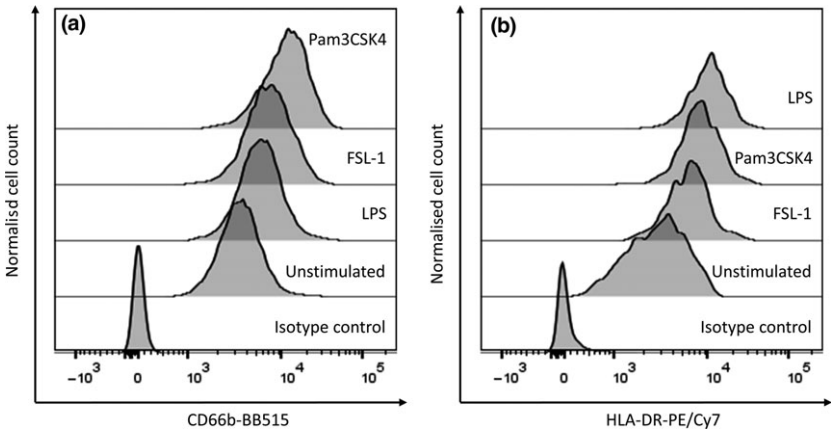

Figure 1. Neutrophils and monocytes became activated in response to Toll-like receptor (TLR) stimulation. Representative flow cytometry plots of neutrophil CD66b expression **(a)** and monocyte HLA-DR expression **(b)** in unstimulated cultures or following stimulation with 100 ng mL^{-1} of each TLR agonist, in ascending order of recorded geometric mean fluorescent intensity (MFI). Representative plots are from leucocyte cultures in the absence of platelets (leucocytes + platelet-poor plasma).

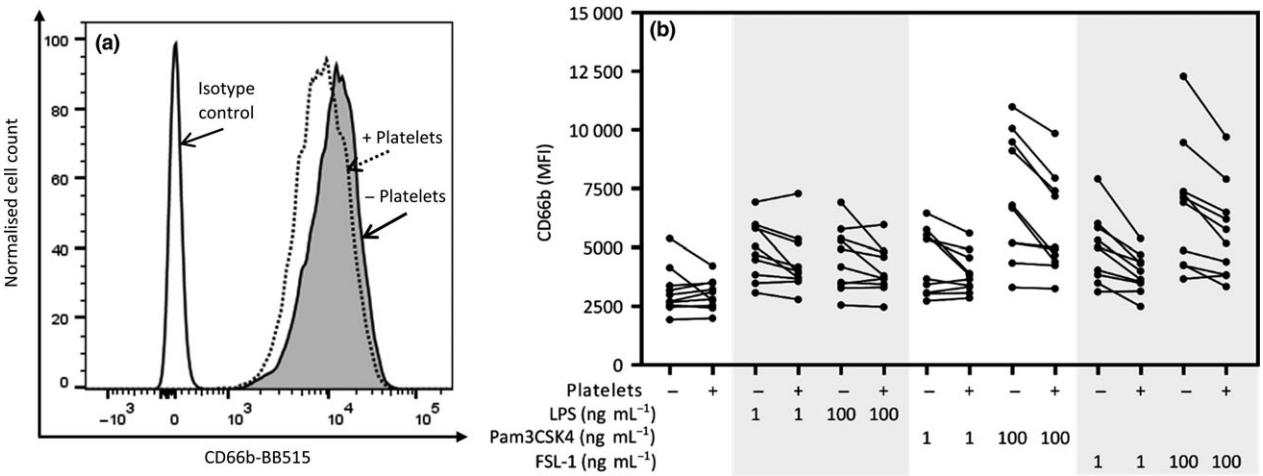

Figure 2. Platelets modulate expression of neutrophil CD66b in response to Toll-like receptor stimulation. **(a)** Representative flow cytometry plot of neutrophil CD66b expression in response to 100 ng mL^{-1} Pam3CSK4 in the absence and presence of platelets. **(b)** CD66b expression was measured by geometric mean fluorescent intensity (MFI) without platelets (granulocytes + platelet-poor plasma) and in granulocyte–platelet coculture (granulocytes + platelet-rich plasma) with no stimulation and in response to 1 and 100 ng mL^{-1} lipopolysaccharide (LPS), Pam3CSK4 and fibroblast-stimulating lipopeptide-1 (FSL-1).

conditions or in response to high-dose LPS, or any dose of Pam3CSK4 or FSL-1.

Platelets reduce granulocyte elastase secretion in response to low-dose TLR stimulation

Elastase secretion in response to TLR stimulation with and without platelets was assessed in the granulocyte culture supernatant. In this study, an average of 93% of the granulocytes in culture were identified as CD16+ neutrophils, and we suggest that neutrophils are the predominant source of elastase secretion in granulocyte cultures. As expected, elastase secretion increased under each TLR stimulation condition (Supplementary table 1). Here, we show that platelets reduced elastase secretion in response to low doses of each TLR agonist (Figure 4, Table 2). In the presence of platelets, the increase in elastase production was reduced by 17% in response to low-dose LPS, reduced by 21% in response to low-dose Pam3CSK4 and reduced by 17% in response to low-dose FSL-1 (all $P < 0.01$). Of note, the relative change with platelet coculture that was recorded for low-dose TLR

stimulation was larger than the relative change recorded for high-dose TLR stimulation.

Platelets differentially modulate PBMC cytokine and chemokine production in response to LPS and FSL-1, but not Pam3CSK4

The production of interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-10 and macrophage inflammatory protein (MIP)-1β was assessed for PBMC ± platelet cultures in response to TLR stimulation. When compared to unstimulated cultures, the production of these cytokines and chemokines increased upon stimulation with both doses of LPS, Pam3CSK4 and FSL-1 (Supplementary table 1). Cytokine and chemokine production in response to TLR stimulation ± platelets is given in Figure 5, and the relative changes with the addition of platelets are given in Table 3. The increase in IL-6 and TNF-α production seen in response to both doses of LPS was reduced in the presence of platelets, and MIP-1β production following low-dose LPS stimulation was also reduced with platelets (all $P < 0.05$; Table 3). Conversely, the increase in IL-10 production in response to high-dose LPS was elevated by 19% ($P < 0.01$) with the addition of platelets. In contrast to LPS stimulation, the IL-6 response to high-dose FSL-1 was further increased with platelet coculture ($P < 0.05$) and the MIP-1β response to both doses of FSL-1 was further increased with platelets (both $P < 0.05$). Similarly to LPS stimulation, the increase in TNF-α production in response to both doses of FSL-1 was reduced with the addition of platelets (both $P < 0.01$) and the increase in IL-10 production in response to FSL-1 was further elevated with platelets (both $P < 0.05$). In response to Pam3CSK4, no change in the production of any cytokine or chemokine was seen when platelets were added to PBMC cultures.

Table 1. Relative change in leucocyte activation markers in leucocyte –platelet coculture (+ platelets) and following Toll-like receptor stimulation

		+ platelets[a]	
Agonist	ng mL^{-1}	Neutrophil CD66b	Monocyte HLA-DR
Unstimulated	–	0.99 (0.15)	0.97 (0.09)
Lipopolysaccharide (LPS)	1	0.89 (0.12)*	0.95 (0.06)*
	100	0.92 (0.12)	0.96 (0.06)
Pam3CSK4	1	0.91 (0.14)	0.99 (0.10)
	100	0.85 (0.12)**	0.97 (0.08)
Fibroblast-stimulating lipopeptide-1 (FSL-1)	1	0.81 (0.11)***	0.93 (0.10)
	100	0.86 (0.09)***	0.97 (0.07)

[a]All leucocyte-only measurements (– platelets) were normalised to 1, and all coculture (+ platelets) measurements were compared to this normalised response and reported as relative change. Differences between these measurements were examined by paired *t*-tests. Mean (SD) for 10 subjects is shown.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

DISCUSSION

In this study, we show that platelets reduced neutrophil expression of CD66b in response to stimulation with Pam3CSK4 (a TLR2/1 agonist), LPS

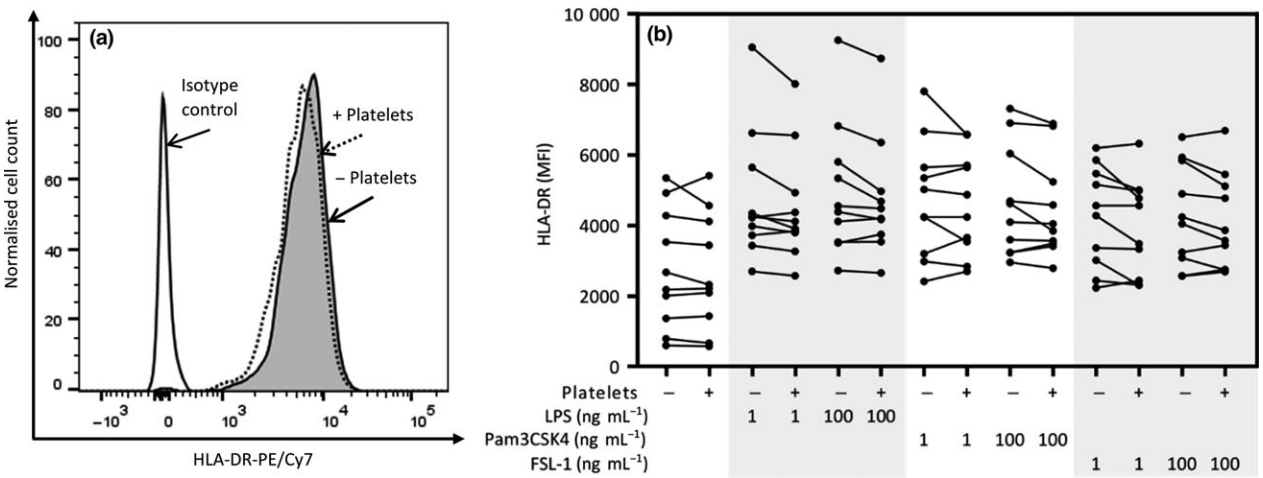

Figure 3. Platelets modestly reduce monocyte HLA-DR expression only in response to low-dose lipopolysaccharide (LPS). **(a)** Representative plot of monocyte HLA-DR expression in response to 1 ng mL^{-1} LPS. **(b)** HLA-DR expression was measured by MFI without (–) and with (+) platelets in unstimulated cultures and in response to LPS, Pam3CSK4 and fibroblast-stimulating lipopeptide-1 (FSL-1).

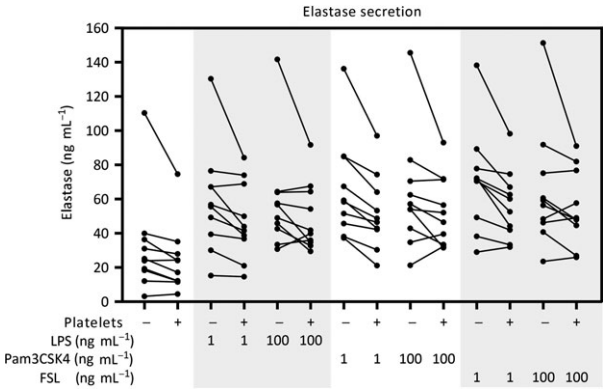

Figure 4. Platelets reduced granulocyte elastase secretion in response to low-dose Toll-like receptor (TLR) stimulation. Elastase secretion was measured in granulocyte only cultures (– platelets) and granulocyte–platelet cocultures (+ platelets) with no stimulation and in response to each TLR agonist.

Table 2. Relative change in granulocyte elastase secretion with platelet coculture and Toll-like receptor stimulation

Agonist	ng mL^{-1}	+ platelets[a] Elastase secretion
Unstimulated	–	0.85 (0.25)
Lipopolysaccharide (LPS)	1	0.83 (0.14)**
	100	0.89 (0.22)
Pam3CSK4	1	0.79 (0.11)***
	100	0.92 (0.27)
Fibroblast-stimulating lipopeptide-1 (FSL-1)	1	0.83 (0.14)**
	100	0.89 (0.20)

[a]All leucocyte-only measurements (– platelets) were normalised to 1, and all coculture (+ platelets) measurements were compared to this normalised response and reported as relative change. Differences between these measurements were examined by paired *t*-tests. Mean (SD) for 10 subjects is shown.
**$P < 0.01$, ***$P < 0.001$.

(TLR4) and FSL-1 (TLR2/6), and modestly reduced monocyte expression of HLA-DR in response to low-dose LPS. Furthermore, granulocyte elastase production, used here as a marker of neutrophil activation, was reduced in the presence of platelets following stimulation with a low-dose of each TLR agonist used. We also show that, in response to LPS, PBMC production of IL-6, TNF-α and MIP-1β decreased and IL-10 production increased with platelet coculture. With FSL-1 stimulation, the presence of platelets increased IL-6, IL-10 and MIP-1β production, but reduced TNF-α production by PBMCs. Conversely, platelets did not alter PBMC cytokine production in response to Pam3CSK4. These results indicate that platelets can regulate leucocyte function in a TLR agonist-specific manner.

Aside from their well-established role in thrombosis, platelets are increasingly recognised as participants in vascular inflammation[1] and contribute to inflammation, at least in part, via their TLRs. Platelets are known to express various TLRs,[5] and TLR stimulation can mediate a number of thrombotic[7,8,21] and inflammatory processes.[9,10] Platelets and leucocytes aggregate in response to TLR stimulation,[9,12] and platelets contribute to vascular inflammation via this interaction with leucocytes. This current study adds to the growing body of evidence which suggests that platelets can actively participate in limiting leucocyte functions, a mechanism which is postulated to prevent uncontrolled inflammation that would otherwise cause host damage.[13] In alignment with this thinking, others have shown that platelets can reduce a number of leucocyte-mediated pro-inflammatory processes,[15,19,22,23] and these effects are likely to redefine the role of platelets in sepsis, systemic inflammation and wound healing, to name a few processes. We add to this by suggesting that some, but not other, leucocyte subsets are more responsive to the presence of platelets, and these particular platelet–leucocyte interactions dampened the pro-inflammatory response to TLR stimulation.

We observed a significant increase in neutrophil expression of CD66b at baseline (in the absence of platelets) in response to all three TLR agonists used in this study. The addition of platelets modestly reduced this increase in CD66b expression at particular doses of each TLR agonist, and this platelet effect was more evident and consistent in response to stimulation with FSL-1. In addition, a reduction in elastase production in granulocyte cultures was seen in the presence of platelets in response to low-dose TLR stimulation. As neutrophils were the predominant cell type within granulocyte cultures, it is likely that neutrophils were the source of elastase release in this study. It is important to note that we saw large interindividual variability in the elastase measurements and this may have limited our ability to detect the platelet effect as significant under some culture conditions. Previous studies have also shown that platelets can suppress neutrophil function. Corken *et al.*[14] have shown that the loss of platelet glycoprotein (GP) Ib-IX enhances neutrophil expression of Mac-1 (CD11b/CD18) in a mouse model of sepsis. This highlights a platelet-dependent pathway that modulates neutrophil activation in the context of sepsis, which

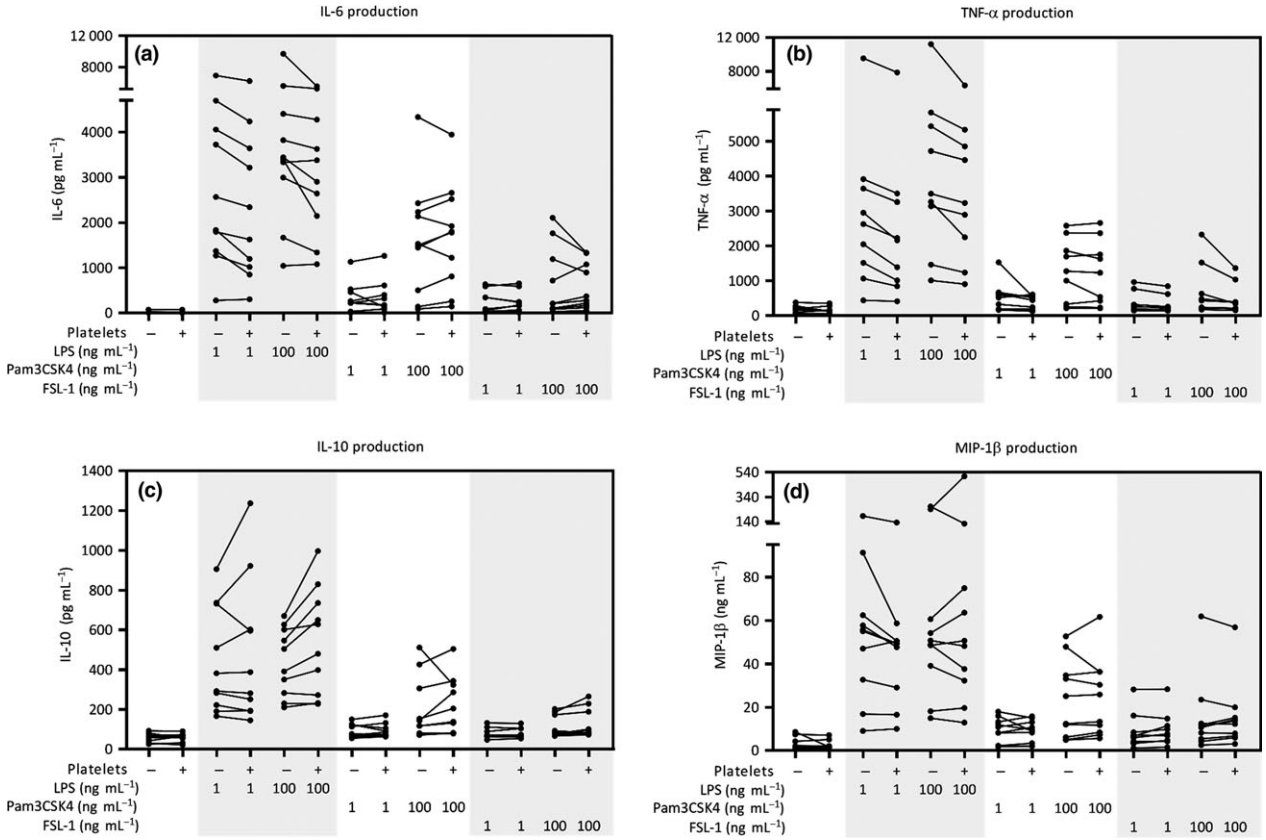

Figure 5. Distinct patterns of PBMC cytokine and chemokine production were seen following Toll-like receptor stimulation and platelet coculture. Levels of interleukin (IL)-6 **(a)**, tumor necrosis factor (TNF)-α **(b)**, IL-10 **(c)** and macrophage inflammatory protein (MIP)-1β **(d)** were measured in PBMC only cultures (− platelets) and peripheral blood mononuclear cell (PBMC)–platelet cocultures (+ platelets) with no stimulation and in response to each agonist.

Table 3. Relative change in peripheral blood mononuclear cell (PBMC) cytokine and chemokine production in platelet coculture and following Toll-like receptor stimulation

		+ platelets[a]			
Agonist	ng mL^{-1}	IL-6	TNF-α	IL-10	MIP-1β
Unstimulated	–	0.94 (0.30)	0.95 (0.38)	1.04 (0.26)	0.88 (0.30)
Lipopolysaccharide (LPS)	1	0.86 (0.14)**	0.81 (0.09)****	1.02 (0.19)	0.88 (0.15)*
	100	0.86 (0.16)*	0.85 (0.13)**	1.19 (0.17)**	1.01 (0.32)
Pam3CSK4	1	1.25 (0.66)	0.85 (0.26)	1.08 (0.11)	1.06 (0.27)
	100	1.24 (0.36)	0.90 (0.15)	1.19 (0.33)	1.09 (0.20)
Fibroblast-stimulating lipopeptide-1 (FSL-1)	1	1.53 (0.81)	0.84 (0.09)***	1.05 (0.07)*	1.23 (0.27)*
	100	1.38 (0.51)*	0.81 (0.16)**	1.14 (0.15)*	1.15 (0.20)*

[a]All leucocyte-only measurements (− platelets) were normalised to 1, and all coculture (+ platelets) measurements were compared to this normalised response. Differences between these measurements were examined by paired *t*-tests. Mean (SD) for 10 subjects is shown.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ***$P < 0.0001$.

is a potent source of TLR-triggering molecules. Hurley *et al.*[24] demonstrated that, in response to *Streptococcus pyogenes* M1 protein (another TLR-triggering molecule), platelet–neutrophil complexes formed in cell culture and, interestingly, these complexes exhibited reduced chemotaxis and

bacterial killing. It is likely that, in these models of sepsis, platelets and neutrophils are exposed to a multitude of stimulating molecules, including a mixture of TLR agonists. We are the first to demonstrate that platelets can also dampen neutrophil activation in response to very specific, singular TLR stimulation. Others have shown that platelets can limit neutrophil function in response to non-TLR agonists. Platelets have been shown to reduce elastase secretion[25] and reduce production of reactive oxygen species (ROS)[26,27] by neutrophils stimulated with the calcium ionophore, A23187, or the chemotactic protein, fMLP.

It is interesting to note that, for each TLR agonist used in this study, elastase production was relatively similar between the two doses used, but platelets significantly attenuated elastase production only in response to low-dose TLR stimulation. This would suggest that low-dose TLR stimulation is sufficient to induce a strong release of elastase by neutrophils, but it may be that the environment induced by high-dose TLR stimulation limits the ability of platelets to have the regulatory effect that is seen at low-dose TLR stimulation.

We also observed that platelets modestly reduced monocyte activation, as evidenced by HLA-DR expression, in response to low-dose LPS. This modest attenuation by platelets was not seen following stimulation with either high-dose LPS, or either dose of Pam3CSK4 or FSL-1. It has previously been shown that, in platelet–PBMC coculture, activated platelets slightly but nonsignificantly reduced monocyte HLA-DR expression in response to LPS.[19] Similarly, platelet microparticles can reduce HLA-DP, DQ and DR expression on monocyte-derived dendritic cells, with the authors suggesting that monocytes that come into contact with platelet microparticles may be less likely to develop into fully pro-inflammatory dendritic cells.[15] In our study, it is unclear why an effect of platelets was observed with low-dose LPS, but not observed under any other condition tested. Overall, we suggest that monocyte HLA-DR expression is only modestly influenced by platelets, and it is possible that this modulation may be secondary to modulation of other monocyte functions by platelets, such as cytokine and chemokine production.

In this study, platelets modulated cytokine and chemokine production by PBMCs in a complex manner, and this modulation differed between the three TLR agonists tested here. In the context of stimulating PBMCs with TLR agonists, it is likely that the cytokine and chemokine response is driven, in the main, by monocytes as this leucocyte subset is most responsive and sensitive to direct TLR stimulation. Platelets did not modulate the PBMC response to the TLR1/2 agonist, Pam3CSK4, for any cytokine or chemokine measured in this study. However, the cytokine/chemokine response to LPS was modulated by platelets. Platelet coculture lowered IL-6 and TNF-α levels (both traditional pro-inflammatory cytokines), increased IL-10 levels (an anti-inflammatory cytokine) to LPS and decreased MIP-1β levels (a pro-inflammatory chemokine) at low-dose LPS. These patterns indicate that platelets can amplify the production of anti-inflammatory cytokines and attenuate the production of pro-inflammatory cytokines and chemokines by PBMCs stimulated with LPS. Previous work has described this type of modulation by platelets in response to LPS. Activated platelets, or activated platelet supernatant, can reduce TNF-α and IL-6 production and induce IL-10 release by both resting[21,28] and LPS-stimulated human PBMCs[19] as well as LPS-stimulated mouse bone marrow-derived macrophages.[16,17] Similarly, platelets that have been exposed to high shear stress can induce IL-10 production in monocyte-derived immature dendritic cells in response to LPS.[29] These collective results suggest that platelets can push PBMCs away from pro-inflammatory and towards anti-inflammatory, cytokine production in response to LPS.

The response of PMBCs to FSL-1, a TLR2/6 agonist, in the presence of platelets was more complex. FSL-1-mediated production of IL-6, IL-10 and MIP-1β production was further elevated with platelets, but production of TNF-α was reduced. This picture, with the up- and downregulation of traditional pro-inflammatory (IL-6, MIP-1β and TNF-α) and anti-inflammatory (IL-10) cytokines and chemokines, does not clearly demarcate a pro- or anti-inflammatory influence of platelets on the PBMC phenotype in response to FSL-1. It is important to note that FSL-1 did not stimulate PBMC cytokine/chemokine production as effectively as LPS and, when examining relative changes when platelets are added, small absolute changes in response to FSL-1 may be represented as large relative changes. However, a small absolute change in cytokine/chemokine production in a low-cytokine/chemokine environment, such as

following FSL-1 stimulation, may still be physiologically important. These results suggest that platelets have a multifaceted role in balancing pro- and anti-inflammatory responses, which is agonist-specific and which may concurrently facilitate both types of inflammatory responses. To the best of our knowledge, modulation of PBMC cytokine production by platelets in response to FSL-1 has not been previously described.

The ability of platelets to modulate both pro- and anti-inflammatory responses of leucocytes also has implications in wound healing. External application of autologous PRP gel has often been used as an advanced wound healing therapy.[30,31] It is acknowledged that the role of platelets at the wound site can be twofold: platelets form an aggregate at the wound site and trigger the inflammatory phase of wound healing,[31] but platelets may also be key in the later stages of wound healing by modulating PBMC release of cytokines and growth factors.[21] The use of PRP has been successful in a number of clinical settings, particularly in chronic conditions that are characterised by persistently dysregulated inflammation.[32,33] We can hypothesise that a mechanism to promote good healing with PRP may be the ability of platelets to interact with resident leucocytes and dampen down their inflammatory responses at the wound site. Nami *et al.*[21] similarly conclude that platelets participate in many, including late, stages of wound healing and this is a previously unappreciated role for this cell type. We suggest that further defining the modulatory role of platelets in late-phase wound healing may advance the case for the use of autologous PRP in the clinical setting.

We have previously shown that platelets exhibit very different activation profiles and also aggregate with monocytes differently in response to a range of TLR agonists.[7] More specifically, we have demonstrated that platelets can dose-dependently and directly activate in response to Pam3CSK4 and in response to high-dose LPS, but do not become directly activated in response to FSL-1. In this current study, we also show that platelets elicit an effect on leucocytes in response to some, but not other, TLR agonists. This is particularly evident when examining the platelet effect on PBMC cytokine and chemokine production in response to TLR agonism: the presence of platelets modulates the PBMC response to LPS and FSL-1, but not Pam3CSK4. Similarly, platelets reduce neutrophil CD66b expression to a greater extent in response to FSL-1 than in response to LPS or Pam3CSK4. From the results from this study, and our previous study,[7] we can conclude that both platelet thrombotic and inflammatory responses are TLR agonist-specific.

Although we did not assess platelet activation in this study, it is interesting to hypothesise the role that activation plays in eliciting the platelet effect that we observe in this study. As described above, we have previously shown that platelets can become directly potently activated only in response to some TLR agonists.[7] However, the concentration of TLR agonists used ($\mu g\ mL^{-1}$ versus $ng\ mL^{-1}$) and the time of incubation with these agonists (35 min versus 4 or 24 h) are important differences between our previous work and this current study. Particularly given the use of much lower concentrations of TLR agonists in this study, it is conceivable that direct TLR-mediated platelet activation may not play as important a role in eliciting these platelet-dampening effects as other mechanisms. Rather, we hypothesise that the complex and intense cross-talk between platelets and leucocytes is likely to contribute significantly to the platelet effect that we observed in this study. Leucocytes became strongly and differentially activated in response to TLR stimulation, and we suggest that this activation is likely to drive the platelet response. Thus, a lack of enough direct platelet activation by these agonists is compensated by an indirect effect of TLR agonist-stimulated leucocytes.

If this is the case, the complexity of the cross-talk becomes evident here: leucocytes are able to become activated in response to TLR stimulation and are able to mediate platelet responses, and platelets, in turn, act to dampen leucocyte activation. It must be recognised that the dampening platelet effect is likely a result of very complex, nuanced and subtle cross-talk between these cell types.

It is important to consider the results from this study in the context of *in vitro* leucocyte work: platelets may confound, or contribute to variability in, experimental read-outs if the methodology for their removal from leucocytes is suboptimal. Platelets are routinely 'removed' from leucocyte suspensions by centrifugation, but this is far from sufficient to eliminate platelets[34] and, as such, platelet contamination is likely to be commonplace. Depending on the attention that is paid to these 'platelet removal' steps,

contaminating platelets may outnumber particular leucocyte subsets within the final cell suspension. Our results suggest that investigators should take precautions to minimise platelet contamination when preparing leucocyte suspensions for use in *in vitro* experimentation. McFarland *et al.*[35] similarly highlight the need to reduce platelet contamination for flow cytometry experiments, particularly when considering the use of markers that are expressed both on leucocytes and platelets. Others[34,36–38] have also identified that measurement levels can be confounded by platelet contamination in various experimental set-ups.

The following limitations of our study were identified. We have designed this study as a broad 'survey' to determine the effect of platelets on a range of different responses across different leucocyte subsets. An inherent limitation of this approach was that we have not wholly characterised the platelet effect on any particular leucocyte subset. For example, we limited the number of measurements of leucocyte function that were assessed and did not investigate these platelet–leucocyte interactions in isolated leucocyte subsets (for example, isolated monocytes as opposed to PBMCs). Our intent was to broadly characterise these platelet effects in order to determine those effects that warrant further investigation. Additionally, we did not assess whether these leucocyte responses were affected by direct platelet interaction or by the indirect release of platelet immunomodulators. However, the literature suggests that the combination of both direct and indirect interactions facilitates the platelet effect.[19,21] As a result of the *in vitro* study design, we are not able to conclude whether the same platelet effect is observed *in vivo*. We hypothesise that platelets may play a regulatory (rather than purely pro-inflammatory) role *in vivo*, and elucidating this platelet effect in various *in vivo* models of inflammatory diseases is required to translate this research into a more clinically applicable space. We did not anticipate the large variability in some of the measurements of this study. Combined with the moderate relative change in these measurements with the addition of platelets, this may have limited our ability to detect statistically significant differences for some conditions.

We show in this study that platelets differentially regulate leucocyte responses to TLR stimulation in a TLR agonist-specific manner. We suggest that platelets can limit some leucocyte pro-inflammatory processes in response to TLR stimulation, and this platelet effect may play an important role in dampening host damage in response to infectious or sterile inflammation.

METHODS

Subject recruitment and blood sampling

Blood was collected via venepuncture from 10 healthy subjects (five male, 29 ± 5 years) with no known cardiovascular disease, diabetes mellitus, or inflammatory or platelet function disorder. Other exclusion criteria were a platelet count $< 100 \times 10^9$ L^{-1}, pregnancy and/or treatment with cardiovascular medication, antiplatelet therapies, immune-modulating medication or nonsteroidal anti-inflammatory drugs within 7 days preceding recruitment. Recruitment was approved by the University of Otago Human Ethics Committee, New Zealand. Blood was collected into hirudin-anticoagulated tubes (Dynabyte, Munich, Germany) and EDTA-anticoagulated tubes (Becton Dickinson, San Jose, CA, USA).

Isolation of platelets and leucocytes

Hirudin-anticoagulated blood was spun at 200 *g* for 12 min to produce PRP or at 1500 *g* for 12 min to produce PPP. PRP was diluted in phosphate-buffered saline (PBS; 145 mM NaCl, 8.7 mM Na_2HPO_4, 1.3 mM NaH_2PO_4) to 2.5×10^8 platelets mL^{-1}. PPP was diluted in PBS in the same ratio as PRP. EDTA-anticoagulated blood was layered over Polymorphprep (Axis-Shield, Dundee, UK), spun at 650 *g* for 30 min, and the PBMC and granulocyte layers were isolated and washed in PBS. To lyse erythrocytes, PBMCs were resuspended in erythrocyte lysis buffer (Qiagen, Hilden, Germany) and granulocytes were resuspended in ice-cold water followed by the addition of PBS in excess. Finally, both cell suspensions were washed and resuspended in isolation buffer (0.1% BSA, 2 mM EDTA in PBS, pH 7.4).

Platelet depletion from leucocytes

Leucocytes were incubated with 10 μg anti-human CD42b conjugated to biotin (clone AK2; Thermo Fisher Scientific, Waltham, MA, USA) for 30 min at 4°C, washed and finally incubated with 100 μl Biotin Binder Dynabeads (Thermo Fisher) for 30 min at 4°C. Platelets were then removed by magnetic separation. Leucocytes were resuspended in cell culture media (10% FCS, 2 mM L-glutamate, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 0.01 M HEPES buffer, 0.1% β-mercaptoethanol and 0.01 nM nonessential amino acids) to 1×10^6 cells mL^{-1} and kept on ice for 60 min prior to stimulation.

In vitro stimulation of PBMCs and granulocytes

A portion of PBMCs and granulocytes were cocultured with PRP in a leucocyte:platelet ratio of 1:250. As a control

condition, PPP was added to a separate portion of leucocytes, and these leucocytes were cultured alone. Cells were kept at 37°C/5% CO_2 for 60 min prior to TLR stimulation. Leucocytes ± platelets were then either left unstimulated or stimulated with 1 and 100 ng mL^{-1} of the following TLR agonists: LPS from *Escherichia coli* serotype R515 (TLR4 agonist; Enzo Life Sciences, Farmingdale, NY, USA), Pam3CSK4 (TLR2/1 agonist; Tocris Bioscience, Bristol, UK) and FSL-1 (TLR2/6 agonist; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Granulocytes were stimulated for 4 h, and PBMCs were stimulated for 24 h at 37°C/5% CO_2.

Flow cytometry

Following TLR stimulation, all samples were spun at 400 *g* for 12 min, and the cell culture supernatant was collected and stored at −80°C. Leucocytes ± platelets were resuspended in Fixable Viability Stain (FVS) 620 (Becton Dickinson) diluted in PBS and incubated for 15 min. Samples were washed twice in staining buffer (2% foetal calf serum and 0.1% sodium azide in PBS) and resuspended in Fc receptor blocking solution (BioLegend, San Diego, CA, USA) diluted in staining buffer. Granulocytes ± platelets were incubated with anti-CD16-BV421 (clone 3G8), anti-CD45-PE/Cy7 (clone HI30) and anti-CD66b-BB515 (clone G10F5). PBMCs ± platelets were incubated with anti-CD64-BV421 (clone 10.1) and anti-HLA-DR-PE/Cy7 (clone G46-6). All antibodies were sourced from Becton Dickinson. Antibodies were diluted in staining buffer, and all samples were stained for 50 min at 4°C. Corresponding isotype controls, all sourced from Becton Dickinson, were run in parallel. Antibody-stained leucocytes ± platelets were fixed with 1% paraformaldehyde, spun and finally resuspended in staining buffer. All samples were analysed on a FACSCanto II flow cytometer (Becton Dickinson). Flow cytometric data were analysed using FlowJo software (v10.0.7; Tree Star, Ashland, OR, USA). Live cell subpopulations were identified by their FVS620-negativity and by their characteristic forward and side scatter properties. The flow cytometry gating strategies are shown in Supplementary figures 1 and 3. The geometric mean fluorescent intensity (MFI) of CD66b and HLA-DR was used to determine CD16 + neutrophil activation and CD64+ monocyte activation, respectively.

Analysis of cell culture supernatant

The concentration of elastase in granulocyte cell culture supernatant was measured using a human polymorphonuclear elastase ELISA kit (Abcam, Cambridge, UK), as per the manufacturer's instructions. The concentrations of TNF-α, IL-6, IL-10 and MIP-1β in PBMC cell culture supernatant were measured using a Luminex multiplex assay (R&D Systems, Minneapolis, MN, USA), as per the manufacturer's instruction.

Statistical analysis

For examining leucocyte activation in response to TLR stimulation, differences in raw measurements were examined using one-way ANOVA with *post hoc* Sidak multiple comparisons tests. For analysing the effect of platelets on the leucocyte response to TLR stimulation, each leucocyte-only measurement (− platelets) was normalised to 1. Each platelet coculture (+ platelets) measurement was then reported as mean (standard deviation) relative change, compared to the paired leucocyte-only (− platelets) measurement (+ platelets measurement/− platelets measurement). Differences between these measurements were examined using paired *t*-tests. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.