Platelet Glycoprotein Ib-IX as a Regulator of Systemic Inflammation

Adam Corken, Susan Russell, Judith Dent, Steven R. Post, Jerry Ware

Objective—The platelet glycoprotein Ib-IX (GP Ib-IX) receptor is a well-characterized adhesion receptor supporting hemostasis and thrombosis via interactions with von Willebrand factor. We examine the GP Ib-IX/von Willebrand factor axis in murine polymicrobial sepsis, as modeled by cecal ligation and puncture (CLP).

Approach and Results—Genetic absence of the GP Ib-IX ligand, von Willebrand factor, prolongs survival after CLP, but absence of the receptor, GP Ib-IX, does not. Because absence of either von Willebrand factor or GP Ib-IX significantly impairs hemostasis and thrombosis, we sought to define additional GP Ib-IX–dependent pathways impacting survival in the CLP model. We document that the absence of GP Ib-IX leads to reduced platelet–neutrophil and platelet–monocyte interactions. Twenty-four hours after CLP, absence of GP Ib-IX coincides with an alteration in cytokine levels, such as tumor necrosis factor-α secreted by monocytes, and increased macrophage-1 antigen expression by neutrophils.

Conclusions—In contrast to the well-characterized proinflammatory properties of platelets, we describe in the CLP model an anti-inflammatory property associated with platelet GP Ib-IX. Thus, a single platelet receptor displays a dual modulatory role in both the thrombotic and inflammatory pathways associated with polymicrobial sepsis. In sharing leucine-rich motifs with toll-like receptors, platelet GP Ib-IX can be considered a multifunctional participant in hemostasis, thrombosis, and the inflammatory cascade. The results highlight a dynamic role for platelets in systemic inflammation and add to the complex pathophysiologic events that occur during the dysregulated coagulation and inflammation associated with sepsis. (Arterioscler Thromb Vasc Biol. 2014;34:996-1001.)

Key Words: cytokines • monocytes • neutrophils • platelet glycoprotein GPIb-IX complex • sepsis

The well-established role of circulating blood platelets is to support hemostasis and thrombosis. Platelets constantly survey the vasculature and on recognition of vessel injury aggregate to form a platelet-rich hemostatic plug and limit blood loss.1,2 The ability of platelets to aggregate also contributes to cardiovascular diseases because the platelet-rich thrombus obstructs blood flow.3,4 More recently, platelet function beyond hemostasis and thrombosis is being recognized with relevance to disease pathways associated with inflammation and cancer.5–7 Relative to inflammation, platelets affect a wide range of immune functions including leukocyte migration, release of proinflammatory mediators, release of antimicrobial proteins, promotion of neutrophil extracellular trap formation, and expression of toll-like receptors.8–13 Thus, platelets are an active extension of the immune system most likely reflecting the ancestral origins of the mammalian platelet to the thrombocyte of lower vertebrates.14–17

See accompanying editorial on page 962

Sepsis is a dynamic syndrome bridging several seemingly distinct pathophysiologic events, most notably inflammation and coagulation.18 Indeed, the complexity of sepsis pathophysiology has made it one of the most difficult clinical conditions to model in animals.19 However, given the complexity and potential inter-relationships of different pathways, animal models are indispensable and have been valuable to characterize the role of specific genes and pathophysiologic processes in the progression of the syndrome.19 For the current study, we used mice deficient in platelet glycoprotein Ib-IX (GP Ib-IX) with well-characterized defects in hemostasis and thrombosis mimicking the human disorder, the Bernard–Soulier syndrome.20,21 These mice lack the platelet adhesion receptor for von Willebrand factor (VWF), an essential ligand for tethering platelets to damaged or altered endothelial surfaces.22 Although the best characterized function of GP Ib-IX is binding to VWF, GP Ib-IX has a diverse array of other ligands.23 One such ligand is the leukocyte integrin macrophage-1 antigen (Mac-1; CD11b/CD18), which plays an integral role in promoting the migration of immune cells into extravascular tissues.24,25 Although there have been limited data addressing the in vivo consequences of this interaction, it establishes a potential involvement for GP Ib-IX in the process of inflammation.

Using a model of GP Ib-IX dysfunction (human interleukin-4 receptor [hIL-4R]/Ibcr), we present studies to determine the physiological consequence of platelet GP Ib-IX in a mouse model of dysregulated inflammation, the cecal ligation and
puncture (CLP) model. Mice devoid of VWF have improved survival after CLP, whereas mice deficient in the VWF receptor, GP Ib-IX, do not have improved survival. We identify GP Ib-IX contributions to a platelet/neutrophil and platelet/macrophage axis with significant consequences to the innate immune response as evidenced by cytokine levels and increased Mac-1 expression. Our results illustrate a platelet interface between coagulation and inflammation involving the GP Ib-IX complex.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**Platelet GP Ib-IX/VWF Axis in CLP**

Previously, VWF knockout mice have been reported to have prolonged survival after a CLP surgery to induce severe sepsis. Because VWF serves as the primary ligand for the platelet GP Ib-IX receptor, we hypothesized that the absence of the ligand binding subunit (GP Ib-IX) of the receptor would improve septic survival in a similar manner. The CLP procedure was performed on male cohorts of wild-type (WT; C57BL/6J), VWF knockout, and hIL-4R/Ibα mice (Figure 1). Interestingly, survival of hIL-4R/Ibα mice after CLP was not statistically different from the WT group, whereas survival for VWF knockout mice was improved. Thus, eliminating the ligand portion of the GP Ib-IX/VWF axis improves survival in this model, whereas eliminating the receptor portion of the axis was not beneficial. Twenty-four hours after CLP, all strains displayed an ≈60% reduction in circulating platelet counts, typical of the consumptive coagulopathy associated with septic shock (not shown).

**Platelet GP Ib-IX/Neutrophil and Monocyte Axes**

The leukocyte integrin Mac-1 (CD11b/CD18) has been reported to be a counter-receptor for GP Ibα. To further understand the role of GP Ib-IX in potentially influencing outcome in murine CLP, we assessed whether disrupting GP Ib-IX altered the extent of platelet association with neutrophils and monocytes. Whole blood collected from WT, hIL-4R/Ibα, and VWF knockout mice was stained using a platelet-specific marker (CD41), a neutrophil/macrophage marker (CD11b), and von Willebrand factor (VWF) knockout (Gr-1), and a monocyte/macrophage marker (CD11c). The number of platelet–neutrophil/macrophage interactions was analyzed via flow cytometry. The surface marker Gr-1 is an indicator of granulocytes, with neutrophils being the most predominant granulocyte. Therefore, Gr-1 staining is interpreted loosely as a marker for the neutrophil population.

Figure 2A displays representative flow cytometry profiles identifying the Gr-1+/CD115+ population that was further analyzed for CD41+ signals (Figure 2B). After blood collection, ≥80% of WT Gr-1+/CD115+ events were also CD41+ with no statistical difference between WT and VWF knockout samples (P=0.54). However, a statistically significant reduced number

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>CLP</td>
<td>Cecal ligation and puncture</td>
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<td>GP Ib-IX</td>
<td>Glycoprotein Ib-IX</td>
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<tr>
<td>hIL-4R</td>
<td>Human interleukin-4 receptor</td>
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<tr>
<td>Mac-1</td>
<td>Macrophage-1 antigen</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
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<tr>
<td>VWF</td>
<td>Von Willebrand factor</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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**Figure 1.** Kaplan–Meier survival curves after cecal ligation and puncture (CLP). Severe sepsis induced by CLP generated varying mortalities observed during a 5-day period. All wild types (WTs) succumbed to septic burden before the 72-hour mark. Deletion of the gene encoding von Willebrand factor (VWF knockout [KO]) significantly alleviated septic burden because the rate of mortality was reduced with several mice surviving past 5 days (P=0.003). Conversely, ablation of a functional platelet glycoprotein Ib-IX axis (human interleukin-4 receptor [hIL-4R/Ibα]) resulted in no discernible differences in survival when compared with WT (P=0.014). n=8 for each group. P values were determined by a log-rank analysis.

**Figure 2.** Platelet–neutrophil/macrophage interactions. Whole blood was collected from wild-type (WT), human interleukin-4 receptor (hIL-4R/Ibα), and von Willebrand factor (VWF) knockout (KO) mice under static conditions. A, Representative flow analysis of samples identifying the neutrophil population (Gr-1+/CD115−) within WT, hIL-4R/Ibα, or VWF KO blood is shown. Gating exclusively onto a Gr-1+/CD115+ population (upper left gray box in A) allowed for a second gating of associated CD41+ (platelet) events within the neutrophil gate. B, The CD41+/Gr-1+/CD115+ population analyzed as a percent of total Gr-1+/CD115+ events illustrates platelet–neutrophil interaction based on glycoprotein Ib-IX (GP Ib-IX) expression. WT and VWF KO express endogenous GP Ib-IX and are indistinguishable from one another (P=0.54), but both exhibit significantly higher platelet–neutrophil levels compared with hIL-4R/Ibα (P<0.001). This establishes a GP Ib-IX role in augmenting platelet binding to neutrophils. C, Using CD115+ fluorescence to gate exclusively onto monocytes (lower right gray box in A), the GP Ib-IX–dependent association with platelets is presented. Comparison of the sample groups showed the WT and VWF KO strains to be indistinguishable (P=0.24). Blood from hIL-4R/Ibα mice had significantly lower platelet–monocyte associations relative to WT (P=0.003). This indicates a significant GP Ib-IX–dependent modulation of platelet binding to monocytes in the circulation. n=6 for each group. *P<0.05.
of CD41+ events within the Gr-1+/CD115− gate was observed comparing WT and hIL-4R/ιβα samples (P=0.0008). Focusing on the monocyte population (Figure 2A and 2C), a statistically significant reduction of CD41+ events within the CD115+ gate was also observed comparing WT and hIL-4R/ιβα samples (P=0.003), but no difference was observed between WT and VWF knockout samples (P=0.244). These preliminary findings demonstrate a reduction of CD41+ (platelet events) within neutrophil and monocyte populations in the hIL-4R/ιβα strain with no dependence on the presence or absence of VWF.

To further support the hIL-4R/ιβα findings, an alternative analysis was performed whereby CD41+ events were first identified in whole blood and then further gated for Gr-1+/CD115− cells and CD115+ cells (Figure I in the online-only Data Supplement). Only 2.5% of CD41+ events were present in the Gr-1+/CD115− population in WT samples, and this was reduced to 0.5% analyzing blood from the hIL-4R/ιβα strain. A similar reduction within the CD115+ population was also observed using hIL-4R/ιβα blood. No statistically significant difference was observed in comparing WT with VWF knockout samples. Thus, WT and VWF knockout blood contained a significantly higher percentage of the platelet population associated with neutrophils and monocytes when compared with hIL-4R/ιβα blood samples, further validating that platelet GP Iba-IX contributes to a platelet interaction with these leukocyte subclasses.

Platelet GP Iba-IX/Neutrophil and Monocyte Axes After CLP

The induction of severe sepsis significantly alters the ability to maintain homeostasis of both the inflammatory and hemostatic pathways. This global dysregulation of both physiological mechanisms could significantly alter the ability of platelets and leukocytes to interact because membrane receptor expression is rapidly modified via cellular activation events. To investigate the GP Iba-IX–dependent platelet–neutrophil/monocyte axis under septic-like conditions, we conducted the same flow cytometry analysis as described above with blood collected from WT and hIL-4R/ιβα mice 24 hours after CLP (Figure 3A). Overall, CD41+ events within the Gr-1+/CD115− and CD115+ population for both WT and hIL-4R/ιβα blood samples were reduced after CLP compared with sham-treated controls (Figure 3B and 3C). hIL-4R/ιβα blood samples still displayed reduced platelet–neutrophil and platelet–monocyte interactions compared with WT samples after CLP, similar to that observed under normal conditions. In addition, we observed in both WT and hIL-4R/ιβα samples a positive shift in monocyte (CD115+) Gr-1 populations after CLP (comparing Figures 2 and 3).

CLP-Induced Tumor Necrosis Factor-α Secretion

Platelets have been shown to be capable of influencing monocyte/macrophage secretion of tumor necrosis factor-α (TNF-α).25 Having established that the loss of GP Iba-IX reduces the number of platelet–monocyte interactions, we further hypothesized that GP Iba-IX absence would cause an alteration in monocyte/macrophage secretion during sepsis. Before CLP, serum levels of TNF-α were undetectable in WT, hIL-4R/ιβα, or VWF knockout strains (Figure 4A). TNF-α was detectable at 4 and 12 hours after CLP with no difference between WT and hIL-4R/ιβα sera (Figure 4A). However, at 24 hours after CLP, TNF-α levels in hIL-4R/ιβα serum were, on average, significantly higher than serum levels from WT or VWF knockout mice (Figure 4A and 4B).

After observing the effect of GP Iba-IX on modifying TNF-α serum levels after CLP, we investigated potential GP Iba-IX influence on the secretion of other inflammatory mediators. Using a Multiplex platform, we found significant differences in several cytokines and chemokines 24 hours after CLP induction. A representative sampling revealed increase in hIL-4R/ιβα serum for macrocyte chemotactic protein-1, IL-6, macrophage...
inflammatory protein-1β, and keratinocyte-derived chemokine while showing a reduction in IL-15 concentration (Figure 5). Interestingly, these effectors are primarily secreted by the monocyte and macrophage population of cells. Cytokines associated with neutrophils did not reveal significant differences between WT and hIL-4R/Ibα samples (not shown). These findings provide evidence that the platelet modifies the secretion pattern of monocyte lineage cells in a GP Ib-IX–dependent manner.

Mac-1 Expression After CLP
Mac-1 is upregulated on the surface of neutrophils and monocytes during inflammation. Reserve pools are stored within vessels that readily fuse with the plasma membrane on leukocyte activation. After tethering and rolling on the inflamed endothelium, the leukocyte must firmly adhere to the vessel wall before initiating a slow crawl to endothelial junctions before extravasation occurs. Thus, Mac-1 expression serves to indicate the migratory potential and activation state of the leukocyte.

Whole blood was collected as previously described and stained for neutrophils and monocytes. In addition, a marker for CD11b was added to identify Mac-1 (CD11b/CD18) expression. Under basal conditions, the Mac-1 expression by neutrophils did not differ between WT and hIL-4R/Ibα samples (Figure 6A). Furthermore, no significant difference was observed within the monocyte population (Figure 6B). Mac-1 expression was dramatically upregulated from basal conditions within the monocyte population for both WT and hIL-4R/Ibα samples collected 24 hours after CLP indicating that GP Ib-IX dysfunction did not directly lead to increased monocyte Mac-1 levels (Figure 6B). Interestingly, an increase in Mac-1 expression was observed in the hIL-4R/Ibα neutrophil population after CLP (Figure 6A). These results demonstrate that GP Ib-IX attenuates neutrophil Mac-1 expression during systemic inflammation.

Discussion
Platelets express receptors important in immunology, such as toll-like receptor 4, and this likely reflects the common origin for leukocytes and platelets, the thrombocyte, which functions in lower vertebrates in both hemostasis and inflammation. A common structural motif—the leucine-rich repeat—is present in each subunit of the GP Ib-IX complex and is a unifying motif for each of the 13 members of the toll-like receptor family. Thus, a participatory role for GP Ib-IX in both hemostasis and immune function could reflect an ancestral link between innate pattern recognition receptors and receptors dedicated to hemostasis. More importantly, defining a GP Ib-IX–dependent role in immune regulation complements its well-characterized role in hemostasis and thrombosis and represents a significant advancement in defining the complex pathophysiologic response and sequelae of events associated with septic shock and death.

Although sepsis is primarily characterized as systemic inflammation, it is also a state of hemostatic dysregulation. Indeed, the dysregulation of inflammation, coagulation, and the overlapping influence of each is a major challenge in accurately defining the septic syndrome. The platelet itself has been implicated as being an extension of the immune system because of its ability to elicit several inflammatory functions. Platelet adhesion to leukocytes has been speculated to form an interface where cross-talk between the cellular mediators of inflammation and coagulation occurs. The platelet’s ability to bind neutrophils and monocytes, in particular, has been implicated as a critical factor in generating vascular inflammation. More challenging has been to define the importance of cellular activation at the level of the platelet, neutrophil, or monocyte for whether the interaction facilitates a proinflammatory or anti-inflammatory state. Although under some conditions the platelet contributes to a proinflammatory state, our experiments identify a previously unidentified anti-inflammatory influence whereby an abrogated GP Ib-IX–dependent platelet interaction with monocytes results in elevated serum cytokine levels and reduces the inflammatory state.

Figure 5. Inflammatory cytokine and chemokine levels 24 hours after cecal ligation and puncture (CLP). Serum levels of 25 different inflammatory mediators were assessed after CLP surgery. Serum collected from mice was analyzed on an individual basis. A total of 6 different analytes (including tumor necrosis factor-α, not shown) secreted primarily by cells of monocyte/macrophage lineage had significantly different concentrations when comparing wild-type (WT) and human interleukin-4 receptor (hIL-4R)/Ibα serum. Four of the 6 analytes showed elevated levels in the hIL-4R/Ibα samples: macrophage inflammatory protein-1β (MIP-1β; P=0.0078), monocyte chemotactic protein-1 (MCP-1; P=0.0129), keratinocyte-derived chemokine (KC; P=0.0054), and IL-6 (P=0.0083). Serum levels of IL-15 were reversed with the WT strain exhibiting higher serum levels (P=0.0006). A horizontal bar indicates overall mean. n=10 for each group.

Figure 6. Macrophage-1 antigen (Mac-1) expression changes 24 hours after cecal ligation and puncture (CLP). Blood isolated from wild-type (WT) and human interleukin-4 receptor (hIL-4R)/Ibα mice under normal conditions or after CLP was stained using Gr-1 (neutrophils), CD115 (monocytes), and CD11b (Mac-1). Expression of Mac-1 for both the (A) neutrophil and (B) monocyte populations did not differ between strains in the absence of CLP (normal). Twenty-four hours after CLP, hIL-4R/Ibα displayed increased surface Mac-1 staining on the surface of neutrophils (P=0.0348). Monocyte Mac-1 did not differ between strains 24 hours after CLP. n=6 for normal samples and n=11 for CLP samples.
increased Mac-1 expression by neutrophils. Twenty-four hours after CLP, the majority of monocyte-derived cytokines in the hIL-4R/Ibα mouse are significantly elevated (Figure 5). This increase is reported from a single time point (24 hours) in a dynamic process. How this impacts the complex pathophysiology of sepsis and outcome is still unclear. However, the results are similar to a recent report of platelet depletion causing elevated serum levels of TNF-α and IL-6 in models of endotoxemia and Escherichia coli–induced sepsis.31

Our initial findings that VWF knockout displayed improved survival after CLP suggested to us that if the platelet/VWF thrombotic axis was aiding the improved survival, mice deficient in GP Ib-IX would have a similar improved outcome. Because this was not the case (Figure 1), we sought to explore possible mechanisms where platelet GP Ib-IX might influence inflammation. We do acknowledge that the improved survival in VWF knockout mice could be unrelated to VWF’s role in thrombosis and could reflect altered endothelial cell function, Weibel–Palade body formation, or even P-selectin expression. In fact, the concurrent dysregulated coagulation and inflammation of in vivo sepsis does complicate a simple explanation for why VWF knockout mice survive long and animals with dysfunctional GP Ib-IX die earlier. Despite an overwhelming inflammatory response in the hIL-4R/Ibα model, GP Ib-IX deficiency does drastically reduce the ability to generate occlusive thrombi. In contrast, WT mice have an intact GP Ib-IX complex participating in thrombosis, disseminated intravascular coagulation, and presumably organ failure during sepsis. Thus, although GP Ib-IX deficiency may protect against thrombosis, it subsequently contributes to excessive inflammation complicating the interpretation of survival as the single outcome.

Flow cytometry identified a GP Ib-IX–dependent interaction for platelets with neutrophils and monocytes under normal physiological conditions and after CLP. This interaction most likely reflects the known GP Ib-IX/Mac-1 axis.25,42 However, even in the absence of GP Ib-IX, there still exist platelet/neutrophil and platelet/monocyte populations. Although the GP Ib-IX relevance in platelet–neutrophil and platelet–monocyte interaction seems modest, the effects on cytokine release and Mac-1 expression 24 hours after CLP seem robust. The molecular basis of this interaction is unknown but could be dependent on the activation state of the platelet with exposure of potential counter-receptors, such as P-selectin. A caveat yet to be addressed in this analysis is whether the CD41+ population is composed of platelets or platelet-derived microparticles also linked to various aspects of platelet-mediated inflammation.43

A variety of animal models have been developed to understand the molecular and genetic mechanisms of sepsis.19,44 The goal of each is to reproduce the pathophysiology observed in septic patients. The more simplistic approaches are the administration of exogenous toxins, such as lipopolysaccharide or viable pathogens. Both are relatively simple, reproducible, and restricted to toll-like receptor 4 pathways (lipopolysaccharide) or mechanisms of host response to pathogens. We have recently reported a prolonged survival after lipopolysaccharide administration in the absence of functional GP Ib-IX.45 This improved outcome most likely reflects the predominant effects of thrombosis occurring in the lipopolysaccharide model and the importance of GP Ib-IX in pathological thrombus formation. The CLP model differs from lipopolysaccharide-induced endotoxemia by using the host’s enteric bacteria to initiate the peritonitis followed by bacteria in the bloodstream, activation of an inflammatory response, and septic shock.28 Furthermore, reports indicate variation in both thrombus formation and cytokine secretion when comparing lipopolysaccharide administration with CLP.46,47 Thus, major differences in key pathways exist when interpreting lipopolysaccharide experimental outcomes to CLP outcomes.48 With respect to the GP Ib-IX/VWF axis, absence of ligand (VWF) is beneficial in CLP, whereas absence of the receptor (GP Ib-IX) is not. We conclude that platelet GP Ib-IX–dependent suppression of the inflammatory response is beneficial in CLP and less significant in the lipopolysaccharide model of endotoxemia.

Defining the importance of platelet GP Ib-IX to inflammation and thrombosis identifies opportunities to specifically target platelet function. Indeed, differentially targeting platelet function in inflammation and thrombosis to fit a specific pathophysiologic event or improve a clinical outcome would be a future goal. Recognizing an expanded functional influence for platelet GP Ib-IX in these processes is another piece to add to the complex pathophysiologic events of inflammation.

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Disclosures

None.

References


**Significance**

The intrinsic sticky nature of platelets is relevant in the pathophysiology of several seemingly unrelated diseases. However, major gaps in our understanding of platelet function in these other diseases still exist. Fortunately, the well-characterized mechanisms of hemostasis and thrombosis are providing new mechanistic insights into the relevance of platelets in these other diseases. Using a mouse model of polymicrobial sepsis, we provide data illustrating a modulatory role for platelet glycoprotein Ib-IX during systemic inflammation. Well characterized as a major platelet adhesion receptor, the studies illustrate a dynamic platelet/neutrophil and platelet/monocyte axis that is platelet glycoprotein Ib-IX receptor dependent. The consequences of this interaction are changes in the cytokine storm and upregulation of the neutrophil macrophage-1 antigen after induction of sepsis. Understanding the platelet's modulatory role in the immune response is an important advancement in the pathophysiology of sepsis.
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Materials and Methods

Animal Models

All animal procedures were performed following approval from the UAMS Institutional Animal Care and Use Committee. Breeding stocks of wild-type (C57BL/6J) mice and VWF KO mice (B6.129S2-Vwt<sup>tm1Wgr</sup>/J) were purchased from Jackson Laboratories. The hIL-4R/Ib<sub>α</sub> have been previously described and expresses a chimeric α-subunit of GPIb-IX which consists of a human IL-4 receptor extracellular fragment fused to the transmembrane and cytoplasmic domains of human GPIb<sub>α</sub> and is referred to as the hIL4R/Ib<sub>α</sub> strain.<sup>1</sup> The transgenic expression of the hIL-4R/Ib<sub>α</sub> cassette expresses in a strain lack the endogenous murine GP Ib<sub>α</sub> gene.<sup>2</sup> The hIL-4R/Ib<sub>α</sub> strain has been extensively backcrossed (>10 generations) onto the C57BL/6J background. The model retains surface expression of the smaller subunits of the GP Ib-IX complex, GP Ibβ and GP IX. Expression of a modified α-subunit confers a GPIb<sub>α</sub> knockout phenotype without the added giant platelet disorder (macrothrombocytopenia) associated with traditional GPIb<sub>α</sub> deletion.<sup>1</sup> Unlike a straight GPIb<sub>α</sub> knockout with a 30% level of circulating platelets, the platelet count in the hIL-4R/Ib<sub>α</sub> model is 70% of normal while still retaining a severe bleeding and anti-thrombotic phenotype.<sup>1,3</sup> The expression of the modified α-subunit has not altered the surface levels of the immune receptor TLR4 nor has it affected LPS binding as compared to wild-type mice via flow cytometry (not shown). The hIL-4R/Ib<sub>α</sub> mice have been backcrossed for more than 10 generations to represent a congenic strain in the C57BL/6J strain. All mice used in this study were males between the ages of 14 and 24 weeks.

Immunological Reagents

PE conjugated anti-mouse CD41 (12-0411), FITC-conjugated anti-mouse CD11b (11-0112), APC conjugated anti-mouse CD115 (11-1752), PE-Cy7 conjugated anti-mouse Gr-1 (25-5931) and Alexa 488 conjugated anti-mouse TLR4 (53-9041) were purchased from eBioscience. Additionally, BD FACS Lysing solution (349202) was purchased from BD Biosciences for fixation of FACS samples.

Bacterial Reagents

Alexa 488 conjugated LPS was purchased from Life Technologies. Serotype 055:B5 (L-23351).

Cecal Ligation and Puncture

CLP was performed as documented.<sup>4</sup> Briefly, mice were anesthetized with isoflurane distributed through a nose cone. The abdomen was shaven and sterilized with 70% ethanol. A midline incision of the epidermis was performed and excess skin was peeled back to allow for a subsequent incision of the abdominal wall exposing the content of the peritoneum. The cecum was isolated from the peritoneal cavity and ligated using a suture before being punctured with a 22-gauge needle. The cecum was gently massaged allowing for fecal matter to extrude out of the puncture sites. The cecum was returned to the peritoneal cavity and anchored to the abdominal wall before the incisions
in the muscle and epidermal layers were closed. 1 ml of isotonic saline (37°C) was administered intraperitoneally before revival.

**Whole Blood Collection**

Mice were anesthetized with Anafane and blood was drawn from a retro-orbital sinus with a heparinized capillary tube into tubes containing the anticoagulant sodium citrate. Blood samples were analyzed from individual animals and not pooled. Blood cell counts were determined with a Hemovet 950 (Drew Scientific, Waterbury CT).

**Flow Cytometry Analysis**

Whole blood was diluted 1:1 with Tyrode’s buffer and 100μl aliquots were distributed to 5ml polystyrene round bottom tubes. A combination of 0.2μg anti-mouse CD41, CD115, Gr-1 and/or antibodies diluted in 25μl Tyrode’s buffer was added to each sample. Samples were shaken for 10 minutes, removed and 400μl FACSLyse solution was added to each sample before shaking an additional 10 mins. An extra 500μl Tyrode’s buffer was added to each tube and samples were collected using a BD LSRFortessa. Data is presented from the collection of 25,000 leukocyte events. Analysis of the samples was conducted using FCS Express software version 3.0. For determination of leukocyte populations, CD115 fluorescence was displayed on the x-axis and Gr-1 fluorescence on the y-axis of a dot plot. Though Gr-1 is a general granulocyte marker, we utilized it as an indicator of neutrophils due to the predominance of neutrophils within the granulocyte population. Cells fluorescing positively for Gr-1 but negative for CD115 (Gr-1+/CD115-) were gated and interpreted to be the neutrophil population because the Gr-1 antibody can weakly bind the monocyte surface protein Ly-6C. The CD115 positive population was gated and interpreted as the monocyte population. CD115 has not been reported to cross-react with granulocytes. The forward and side scattering of the Gr-1+ and CD115+ gates further verified the identity of these populations (not shown).

Once the neutrophil and monocyte populations had been identified, the presence of platelets (CD41+) was determined in each gate. We began by observing the CD41 fluorescence within the pre-determined neutrophil gate. CD41 positive staining within the Gr-1+ gate was utilized as an indicator of platelets associating with neutrophils. This technique alone is not able to discern the ratio of platelets and neutrophils; therefore a CD41 fluorescent event within the neutrophil gate was interpreted as a neutrophil associated with at least one platelet. This method was repeated for the monocyte population with the number of CD41 fluorescent events observed and recorded as a percentage of the total number of events within the specified CD115+ gate.

To analyze Mac-1 expression, whole blood was collected and prepared as previously described. Treatment with anti-Gr-1 and anti-CD115 antibodies identified the neutrophil and monocyte populations, respectively. Additionally, blood samples were treated with anti-CD11b to stain for the α-subunit of Mac-1. Data was generated in the same manner as platelet-neutrophil/monocyte analysis by collecting 25,000 leukocyte events. Using FCS Express software, the neutrophil and monocyte populations were established using gates. Within the gated populations, the CD11b mean fluorescent
intensity (geometric mean) was identified using statistical analysis tools within the software.

**TNFα ELISA**

Analysis of serum TNFα was conducted at various time points following CLP. Whole blood was collected from anesthetized mice via the retro-orbital sinus. Blood was collected in un-citrated centrifuge tubes using un-heparinized capillary tubes. Blood was given adequate time to coagulate (~2 hours) before spinning at 2,000 rcf for 20 mins to pellet the clot. Aliquots of serum were rapidly frozen in liquid nitrogen and stored at -80°C for later ELISA analysis. The eBioscience Mouse TNFα ELISA Ready-SET-Go! Kit was utilized for TNFα quantification.

**Cytokine and Chemokine Multiplex**

Serum before or following CLP was collected in the same manner as described for the TNFα ELISA protocol. Blood was allowed to clot and serum isolated from the pelleted clot. Analysis was conducted using the MilliplexMAP Mouse Cytokine/Chemokine Magnetic Premixed Bead Panel Immunoassay purchased from Millipore, which allowed for the quantification of 25 different serum analytes. A Luminex 200 instrument was used for data collection.

**Statistical Analysis**

SigmaPlot scientific software was used to analyze all data. A Logrank test was conducted for CLP-induced sepsis survival experiments and graphed on a Kaplan-Meier plot. The remaining data was presented using the mean with standard deviation bars analyzed using a two-tailed unpaired t test to generate p values.

**References**


Supplemental Figure I. **CD41** gated events. (A) Platelet and platelet-associated events (CD41⁺) were identified by flow cytometry and gated as outlined by the gray box (left panel of A). Parameters switched to and Gr-1⁺ or CD115⁺ fluorescence (right panels of A) to identify monocyte and neutrophil populations within the platelet pool. (B) The number of neutrophil and monocyte gated events graphed as percentages of the total platelet population are presented. Significantly reduced levels of platelets associated with each leukocyte sub-class are shown in the hIL-4R/Ibα strain as compared to wild-type (WT) were observed (neutrophil and monocyte p < 0.001). VWF KO samples did not differ statistically from wild-type. N = 6 for each group.