P2Y<sub>12</sub> Receptor Modulates Sepsis-Induced Inflammation

Elisabetta Liverani, Mario C. Rico, Alexander Y. Tsygankov, Laurie E. Kilpatrick, Satya P. Kunapuli

Objective—Platelets modulate hemostasis and immune responses via interactions with immune cells through secretion of immunomodulators and cell-cell interactions. The P2Y<sub>12</sub> receptor mediates ADP-induced aggregation and secretion in platelets.

Approach and Results—Using a mouse model of intra-abdominal sepsis and acute lung injury, we investigated the role of the P2Y<sub>12</sub> receptor in neutrophil migration and lung inflammation in P2Y<sub>12</sub> null mice and in mice pretreated with the P2Y<sub>12</sub> antagonist clopidogrel. Our data show a decrease in circulating white blood cells and a decrease in platelet activation and platelet–leukocyte interactions in treated mice compared with untreated mice. Additionally, lung injury and platelet sequestration were diminished in clopidogrel-treated mice compared with their untreated septic littermates. Similar results were observed in P2Y<sub>12</sub> null mice: platelet activation and platelet–leukocyte aggregates were decreased in septic P2Y<sub>12</sub> null mice compared with wild-type mice. P2Y<sub>12</sub> null mice were refractory to lung injury compared with wild-type mice. Finally, to evaluate P2Y<sub>12</sub>-independent effects of clopidogrel, we pretreated P2Y<sub>12</sub> null mice. Interestingly, the number of circulating neutrophils was reduced in treated septic P2Y<sub>12</sub> null mice, suggesting neutrophils as a target for clopidogrel pleiotropic effects. No difference was observed in P2Y<sub>12</sub> null mice during sepsis, indicating that the P2Y<sub>12</sub> receptor is responsible for the effects.

Conclusions—P2Y<sub>12</sub> null mice are refractory to sepsis-induced lung injury, suggesting a key role for activated platelets and the P2Y<sub>12</sub> receptor during sepsis. (Arterioscler Thromb Vasc Biol. 2016;36:961-971. DOI: 10.1161/ATVBAHA.116.307401.)

Key Words: acute lung injury ■ neutrophils ■ platelets ■ P2Y<sub>12</sub> receptor ■ sepsis

Platelets regulate thrombus formation and hemostasis and play important roles during inflammation. On activation, platelets activate immune cells through cell-cell interactions and by secreting inflammatory mediators and second messengers, such as ADP, that recruit other platelets. ADP-induced aggregation is mediated by 2 members of the P2Y receptor family: P2Y<sub>1</sub> and P2Y<sub>12</sub>. Both are G protein–coupled receptors that are expressed on platelet membranes. P2Y<sub>1</sub> is coupled to G<sub>i</sub> protein, whose activation causes platelet shape changes and a weak, transient aggregation. P2Y<sub>12</sub> is coupled to G<sub>q</sub> protein, whose activation leads to platelet aggregation and potentiation of granule release, indicating a role for the P2Y<sub>12</sub> receptor in platelet secretion. Signaling events downstream of the P2Y<sub>12</sub> receptor also potentiate agonist-induced dense granule release, procoagulant activity, and thrombus formation.

Platelets activate leukocytes through cell-cell interactions involving adhesion molecules, such as P-selectin, a glycoprotein that, on cell activation, is rapidly translocated from cytoplasmic α-granules to the cell surface. P2Y<sub>12</sub> activation is required for α-granule release and subsequent expression of P-selectin on activated platelets. P-selectin binds P-selectin glycoprotein ligand 1 on leukocytes, which activates leukocytes and promotes their infiltration into inflamed tissue.

Platelet–leukocyte interactions are important in the pathogenesis of sepsis, and leukocyte–platelet aggregates and P-selectin secretion are altered in septic patients. In a model of sepsis induced by cecal ligation and double puncture (CLP), neutrophil infiltration in the lungs was reduced after platelet depletion, suggesting that platelets play a role in neutrophil activation during inflammation. Similar observations were made in other inflammation models, such as pancreatitis and ischemia reperfusion. In a rat model of lipopolysaccharide-induced inflammation, induction of proinflammatory cytokines (interleukin-6 and tumor necrosis factor-α), lung damage, and liver damage were attenuated on treatment with clopidogrel, a drug that antagonizes the P2Y<sub>12</sub> receptor. Both P2Y<sub>12</sub> receptor antagonism and platelet depletion reduced inflammation during sepsis, suggesting a role for platelets in regulating the inflammatory response in sepsis.

P2Y<sub>12</sub> receptor gene variants correlate with pulmonary inflammation and asthma. Also, P2Y<sub>12</sub> receptor deficiency and platelet depletion abrogate dust mite–induced airway inflammation, suggesting a role for the P2Y<sub>12</sub> receptor in pulmonary inflammation. The mechanisms that trigger neutrophil infiltration are poorly understood but may result from the development of a proinflammatory phenotype in the lung. These observations suggest a regulatory role for the P2Y<sub>12</sub> receptor.
receptor and activated platelets in regulating neutrophil influx into the lung.

In support of this hypothesis, studies by Rahman et al demonstrated a lung-protective effect in septic animals treated with the P2Y12 antagonist, ticagrelor, suggesting a regulatory role for P2Y12 receptor signaling in sepsis.17 However, several studies indicate that ticagrelor may have pleiotropic effects in addition to its antiplatelet properties.18 To establish a role for P2Y12 signaling in sepsis and sepsis-induced acute lung injury (ALI), we examined the effects of receptor deficiency (P2Y12 null mice) and P2Y12 antagonism (clopidogrel) in the CLP model of sepsis. We further pretreated P2Y12 null mice with clopidogrel to investigate P2Y12-independent effects. Finally, we studied P2Y1 null mice in the same sepsis model to determine whether these effects were caused by P2Y12 deficiency or by altered responses to ADP. Our findings show that inflammation was decreased in P2Y12 null mice but not in P2Y1 null mice. Interestingly, P2Y12 deficiency and receptor antagonism in this model of sepsis provided similar results, suggesting that modulation of the P2Y12 receptor offers a new therapeutic option for sepsis.

Materials and Methods

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

Results

Circulating White Blood Cells Did Not Increase Following Clopidogrel Treatments in Septic Mice

To study P2Y12 receptor antagonism in sepsis, mice were treated with the P2Y12 receptor antagonist clopidogrel (loading dose, 30 mg/kg; maintenance dose, 10 mg/kg) before surgery in a model of sepsis and lung injury. Both sham and CLP mice were treated. First, we analyzed the number of circulating white blood cells (WBCs) in blood samples (Figure 1) from treated and untreated mice. In septic mice, the WBC count was significantly increased compared with the sham control, but WBCs were not elevated in clopidogrel-treated CLP mice (Figure 1A; **P<0.01 sham versus CLP, *P<0.05 clopidogrel-treated CLP versus untreated CLP). Interestingly, the WBC count in the treated CLP animals was lower than that in the sham control (*P<0.05). When we analyzed the cells more specifically, we noticed that lymphocytes were increased after sepsis, whereas no difference was noted in neutrophils. However, after clopidogrel treatment, both cells were significantly reduced (Figure 1B and 1C; *P<0.05; clopidogrel-treated CLP versus clopidogrel-untreated CLP and treated sham versus treated CLP). No difference was reported in the platelet count among all groups (Figure 1D).

Nonstandard Abbreviations and Acronyms

<table>
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<th>Abbreviation</th>
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<tr>
<td>ALI</td>
<td>acute lung injury</td>
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<tr>
<td>CLP</td>
<td>cecal ligation and double puncture</td>
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<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
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<td>WBC</td>
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Figure 1. Circulating white blood cells (WBCs) did not increase in sepsis after clopidogrel treatments. Blood samples were collected by cardiac puncture in 3.8% sodium citrate (10:1), and hematology studies were performed. Graphs show counts of white blood cells (WBC; A), lymphocytes (LY; B), neutrophils (PMN; C), and platelets (D) in clopidogrel-treated or untreated mice. Both sham and cecal ligation and double puncture (CLP) samples were analyzed for treated and untreated mice. Values are expressed as 1×10⁶ cells/μL, mean±SEM (n=8; *P<0.05 wild-type [WT] sham vs CLP mice; **P<0.01 treated CLP vs untreated CLP).

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Clopidogrel Treatment Prevents Septic-Induced P-Selectin Increase and Platelet–Leukocyte Aggregate Formation

To determine whether clopidogrel exposure alters platelet activation during sepsis, we investigated P-selectin expression on platelet membranes after CLP in treated and untreated mice using flow cytometry (Figure 2A and 2B). P-selectin expression was increased after CLP (Figure 2A and 2B; **P<0.01; CLP versus sham), but no elevation was noted in mice pretreated with clopidogrel compared with the treated sham control (Figure 2A and 2B; **P<0.01; untreated CLP versus treated CLP). Next, we investigated the effect of clopidogrel treatment on leukocyte–platelet aggregate formation (Figure 2C and 2D). Aggregate formation was elevated in samples from CLP mice compared with sham mice (Figure 2C and 2D; *P<0.05 CLP versus sham). However, in clopidogrel-treated mice, aggregate formation was significantly reduced compared with untreated mice (Figure 2C; *P<0.05 CLP versus treated CLP). Then we investigated platelet sequestration in the lungs of septic mice. Lung samples were stained with the platelet marker CD41. Representative images are shown in Figure 2D, indicating that an increase in CD41 was observed in wild-type (WT) mice after CLP surgery (Figure 2D, left panels; n=4), but it was not noted in clopidogrel-treated mice (Figure 2D, right panels).

Clopidogrel-Treated Mice Are Refractory to Sepsis-Induced Lung Injury

We analyzed whether clopidogrel treatment alters sepsis-induced ALI. After CLP, mouse lung architecture was disrupted, signs of edema were apparent, and increased cell infiltration was notable (Figure 3A). However, inflammation levels were diminished in clopidogrel-treated mice compared with untreated CLP mice (Figure 3A; *P<0.05; CLP versus treated CLP). Similarly, histology scores (Figure 3B) were not increased in the CLP group pretreated with clopidogrel compared with the treated sham (Figure 3B). These data suggest a protective role for clopidogrel during lung inflammation. To determine neutrophil infiltration in the lungs, we investigated myeloperoxidase (MPO) activity. We observed a significant increase in MPO levels in septic mice compared with the sham group (Figure 3C; *P<0.05; CLP versus sham), whereas MPO decreased in septic mice pretreated with clopidogrel compared with untreated CLP mice (Figure 3C; *P<0.05; CLP versus treated CLP).

Sepsis-Induced Increase in Circulating WBCs Was Not Noted in P2Y12 Null Mice

To compare receptor antagonism with receptor deficiency, we analyzed P2Y12 null mice in the same model of sepsis-induced inflammation. First we investigated the number of WBCs in blood samples of P2Y12 null mice 24 hours after either CLP or sham surgery (Figure 4). No increase in circulating WBCs in septic mice was noted compared with the sham control (Figure 4A). Specifically, no differences in neutrophils (Figure 4B) and lymphocytes (Figure 4C) were observed in null mice in either the CLP or sham groups. Interestingly, platelet count was similar in all groups (Figure 1D).

Platelet Activation and Platelet–Leukocyte Interaction Is Not Elevated in P2Y12 Null Mice

We investigated whether P2Y12 deficiency influences platelet activation during sepsis-induced inflammation. P-selectin expression was not increased after CLP in both CLP and sham mice (Figure 4E). Leukocyte–platelet aggregate formation was not elevated in samples from CLP P2Y12 null mice compared with sham P2Y12 null mice (Figure 4F).

Sepsis-Induced Increases in Plasma Cytokines Are Diminished in P2Y12 Knockout Mice

Next we investigated the role of P2Y12 in regulating sepsis-induced elevations in plasma levels of cytokines (tumor necrosis factor-α, interleukin-10, interleukin-6, and macrophage inflammatory protein-1β) (Figure 4G). As expected, the plasma concentration of each cytokine was elevated during sepsis in both WT and P2Y12 null mice animals as compared with sham control. However, the sepsis-induced increase was significantly lower in P2Y12 null mice as compared with WT mice, for all the cytokines analyzed (***P<0.01 knockout CLP model versus WT CLP). These data suggest a decreased level of inflammation in the absence of P2Y12 receptor-mediated signaling.

Lung Injury Is Decreased in Septic P2Y12 Null Mice

In P2Y12 null mice 24 hours after CLP, no lung damage was observed as compared with the sham control (Figure 4H). Similarly, histology scores of ALI were not elevated. Furthermore, neutrophil infiltration (MPO levels) was not increased in P2Y12 null mice after CLP (Figure 4I). These data suggest that the P2Y12 receptor plays a key role in pulmonary inflammation and inflammatory cell recruitment. To analyze how P2Y12 receptor influences platelets/leukocytes interaction in the lung, we stained lung tissue for CD41 (platelet marker) and CD11b (leukocyte marker; Figure 4J). As expected, in WT sham mice, the lungs did not show cell infiltration, whereas in septic WT mice, both platelets and leukocytes infiltration were observed (Figure 4J, top panels). Co-localization was observed, suggesting leukocyte–platelet aggregation in the lungs as well as in the periphery during sepsis. In contrast, P2Y12 null animals had decreased levels of platelets and neutrophils interaction in the lungs, suggesting a decrease in aggregation (Figure 4J, bottom panels).

Clopidogrel Pretreatment Decreased Circulating Neutrophils in P2Y12 Null Mice During Sepsis

To determine whether clopidogrel has P2Y12-independent effects, we treated P2Y12 null mice with clopidogrel before surgery in the same model of sepsis (Figure 5). Both sham and CLP mice were treated orally with clopidogrel (loading dose, 30 mg/kg; maintenance dose, 10 mg/kg). The decrease in WBC observed in treated WT CLP mice compared with treated sham was not noted in treated P2Y12 null mice compared with treated sham P2Y12 null mice (Figure 5A; *P<0.05; treated WT CLP versus treated WT sham and treated CLP WT versus treated CLP P2Y12 null mice). On the contrary, neutrophils were significantly lower in septic-treated mice compared with the treated sham control for both WT and P2Y12 null mice (Figure 5B; P<0.05; treated WT
Figure 2. P-selectin expression and leukocyte–platelet aggregates were not elevated in clopidogrel-treated mice during sepsis. A, and B, Blood samples were collected by cardiac puncture in 3.8% sodium citrate (10:1), and P-selectin expression on platelet surface was analyzed through flow cytometry. Representative flow cytometry histograms are shown for cecal ligation and double puncture (CLP) and sham controls in wild-type (WT) and knockout (KO) animals. Isotype control is shown in gray and P-selectin stained samples in black. C, Blood samples were labeled with antibodies against CD61 (platelet marker) and CD11b (leukocyte marker). Activated leukocytes were gated based on CD11b expression and cell shape, and data were analyzed as a percentage of aggregates expressing both CD41 and CD11b. Values are expressed as percentage of CD41+/CD11b+ cells, mean±SEM (*P<0.05; WT sham vs WT CLP and KO CLP vs WT, n=6). D, Representative images of CD41 staining (CD41: green; Nucleus: blue; 20×) for CLP and sham samples for both treated and untreated mice. Images are representative of 4 different experiments.
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sham versus treated WT CLP and treated P2Y12 null mice sham versus treated CLP P2Y12 null mice). Lymphocyte count was not altered in treated P2Y12 null mice compared with their, respectively, treated sham control (Figure 5C). The data show that clopidogrel has P2Y12-independent effects during sepsis and suggest that circulating neutrophils may be the target cells.

Clopidogrel Treatment Did Not Alter Platelet Activation and Platelet–Leukocyte Aggregate Formation in P2Y12 Null Mice During Sepsis
We investigated whether clopidogrel treatment can influence platelet activation in P2Y12 null mice during sepsis. Both sham and CLP groups were treated with clopidogrel. Data were compared with clopidogrel-treated WT sham and CLP. P-selectin expression was not increased after CLP in both treated CLP and sham mice for WT and P2Y12 null mice (Figure 5E). Leukocyte–platelet aggregate formation was not elevated in samples from treated CLP P2Y12 null mice compared with treated sham P2Y12 null mice (Figure 5F). The data are similar to that observed in clopidogrel-treated sham and CLP WT. To confirm leukocyte infiltration, we investigated MPO activity in lung tissue samples (Figure 5I). MPO levels were not increased in treated P2Y12 null mice after CLP. These data suggest that clopidogrel has no P2Y12-independent effects on lung tissue.

P2Y1 Deficiency Does Not Influence Sepsis-Induced Inflammation Levels
The same model of sepsis was investigated in P2Y1 null mice to investigate whether the data collected in P2Y12 null mice were because of changes in cell responses to ADP or specifically to P2Y1. First we analyzed circulating WBCs in blood samples (Figure 6). No significant difference in WBC number was observed in P2Y1 null mice compared with WT (Figure 6A), with similar results for neutrophils (Figure 6B) and lymphocytes (Figure 6C). Platelet counts were similar among groups (Figure 6D). Interestingly, the cell count in the sham group was significantly higher than those in the CLP and WT sham groups (Figure 6; P<0.05; knockout sham versus knockout CLP).

We investigated lung injury in P2Y1 null mice and WT mice after CLP. No significant differences in lung injury were observed (Figure 6E and 6F). In both groups, there was significant inflammatory cell infiltration, edema, and disruption of tissue architecture. No differences in MPO activity were observed (Figure 6F). The data suggest that the P2Y1 receptor does not contribute to sepsis-induced lung injury in this animal model.

Discussion
Platelets play a role in hemostasis and are increasingly recognized for their ability to modulate immune responses.1 The P2Y12 receptor plays a central role in various platelet functions,
including secretion. P2Y₁₂ receptor antagonists prevent platelet aggregation and secretion¹⁹,²⁰ and alter the inflammatory state in lipopolysaccharide-induced inflammation, myocardial infarction, and rheumatoid arthritis,¹³,²¹,²² suggesting that P2Y₁₂ modulation can influence inflammation by mechanisms which have yet to be fully elucidated. Pretreatment with the P2Y₁₂ antagonist ticagrelor was shown to be lung protective and decrease circulating levels of leukocyte–leukocyte aggregates during sepsis.¹⁷ The results presented here provide essential new information about the role of P2Y₁₂ signaling in sepsis. We investigated for the first time this model of sepsis in P2Y₁₂ null mice and WT mice treated with another P2Y₁₂ antagonist, clopidogrel. Interestingly, clopidogrel has shown to exhibit pleiotropic effects²³ as well as ticagrelor that can inhibit equilibrative nucleoside transporter 1.¹⁸ Hence, it is important to study sepsis in the P2Y₁₂ null mouse model to compare receptor deficiency and antagonism and clarify the mechanism of these antiplatelet drugs. Further, we examined the effect of clopidogrel treatment on P2Y₁₂ mice to identify P2Y₁₂-independent effects. Our studies demonstrate that clopidogrel-treated mice are more refractory

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**Figure 4.** Circulating white blood cell counts, platelet activation, and platelet–leukocyte aggregate formation are not increased in P2Y₁₂ null mice. Blood samples were collected by cardiac puncture in 3.8% sodium citrate (10:1) and hematology studies performed. Graphs show counts of white blood cells (WBC; A), neutrophils (PMN; B), lymphocytes (LY; C), and platelets (D) in wild-type (WT) P2Y₁₂ knockout (KO) mice. Both sham and cecal ligation and double puncture (CLP) samples were analyzed. Values are expressed as 1×10³ cells/µL, mean±SEM (n=8; **P<0.01 WT CLP vs KO CLP mice). E, Blood samples were collected by cardiac puncture in 3.8% sodium citrate (10:1), and p-selectin expression on platelet surface was analyzed through flow cytometry. Representative flow cytometry histograms are shown for CLP and sham control mice. Isotype control is shown in gray and P-selectin-stained samples in black. F, The percentage of aggregates is reported for all groups. Blood samples were labeled with antibodies against CD61 (platelet marker) and CD11b (leukocyte marker). Activated leukocytes were gated based on CD11b expression, and cell shape and data were analyzed as a percentage of aggregates expressing both CD41 and CD11b. Values are expressed as percentage of CD41+/CD11b+ cells, mean±SEM (*P<0.05; WT sham vs WT CLP and KO CLP vs WT, n=6). G, Plasma samples obtained from each animal were used for detection levels of tumor necrosis factor (TNF)-α, interleukin (IL)-10, IL-6, and macrophage inflammatory protein (MIP)-1β in WT (black) and KO (white) mice. Both Sham and CLP samples were analyzed for wild-type and KO animals. Values are expressed as pg/mL, mean±SEM (*P<0.05; **P<0.01; KO CLP model vs WT CLP, n=5). (Continued)
to inflammation and lung injury compared with untreated animals, which is similar to what we observed in P2Y12 null mice, suggesting that the P2Y12 receptor plays a central role during sepsis. Our studies demonstrate that P2Y12 deficiency and receptor antagonism in this model of sepsis provided similar results, suggesting that the P2Y12 signaling has an important role in the inflammation and tissue injury associated with sepsis. Platelets are important for the development of sepsis through an unknown mechanism.22–24 Recent studies have shown that interactions between platelets and leukocytes, in particular neutrophils,25 play a role during sepsis that can be relevant for the outcome of the disease.26 Lung injury and neutrophil infiltration, for example, were decreased during antiplatelet therapy17 or platelet depletion.11 Similar results were noted in a model of myocardial infarction,13 where platelet depletion lessened the inflammation levels in the heart. Our results are in line with these previous findings,11,13 suggesting that platelets are important mediators during inflammation. As previously reported, P2Y12 receptor plays a role in regulating platelet secretion during ADP-induced aggregation and when platelets are activated by other agonists.26

**Figure 4 Continued.** H. Photomicrographs of hematoxylin- and eosin-stained tissue sections obtained after CLP surgery. Representative images of lung tissue specimens are shown for sham and CLP samples (magnification 20× and 40×; n=5). Acute lung injury (ALI) score was assessed in KO mice. I. Myeloperoxidase (MPO) analysis was performed in lung samples of sham and CLP mice. Values are expressed as rfu/min per mg, mean±SEM (n=5). J. Representative images of CD41 and CD11b staining (CD41, green; CD11b, red; Nucleus, blue; 20×) for CLP and sham samples for both WT and KO mice. Images are representative of 3 different experiments.
Figure 5. Clopidogrel treatment alters circulating neutrophil in septic P2Y12 null mice. Blood samples were collected by cardiac puncture in 3.8% sodium citrate (10:1) and hematology studies performed. Graphs show counts of white blood cells (WBC; A), neutrophils (PMN; B), lymphocytes (LY; C), and platelets (D) in clopidogrel-treated wild-type (WT; black) and P2Y12 null mice (white). Both sham and cecal ligation and double puncture (CLP) samples were analyzed. Values are expressed as 1×10^3 cells/μL, mean±SEM (n=8; *P<0.05 WT CLP vs knockout [KO] CLP mice and *P<0.05 treated CLP WT vs treated CLP P2Y12 null mice). E, Blood samples were collected by cardiac puncture in 3.8% sodium citrate (10:1), and p-selectin expression on platelet surface was analyzed through flow cytometry. Representative flow cytometry histograms are shown for CLP and sham control mice in WT (black) and P2Y12 null (white) mice. Isotype control is shown in gray and P-selectin-stained samples in black. F, The percentage of aggregates is reported for CLP and sham control mice in WT (black) and P2Y12 null (white) mice. Blood samples were labeled with antibodies against CD61 (platelet marker) and CD11b (leukocyte marker). Activated leukocytes were gated based on CD11b expression, and cell shape and data were analyzed as a percentage of aggregates expressing both CD41 and CD11b. Values are expressed as percentage of CD41+/CD11b+ cells, mean±SEM (n=6). G, Photomicrographs of hematoxylin- and eosin-stained tissue sections obtained after CLP surgery. Representative images of lung tissue specimens are shown for sham and CLP samples (magnification 20, n=5) in CLP and sham control mice in WT (black) and P2Y12 null (white) mice. H, Acute lung injury (ALI) score was assessed in sham and CLP clopidogrel-treated WT (black) and P2Y12 null (white) mice. I, Myeloperoxidase (MPO) analysis was performed in lung samples of sham and CLP mice for sham and CLP clopidogrel-treated WT (black) and P2Y12 null (white) mice. Values are expressed as rfu/min per mg, mean±SEM (n=5).
Indeed, activated platelets not only secrete second messengers that recruit other platelets, but they also secrete inflammatory mediators, such as interferon-γ, transforming growth factor-β, and RANTES (Regulated on Activation, Normal T Expressed and Secreted).1,27 Hence, antagonizing the P2Y₁₂ receptor can directly influence the inflammatory process by altering platelet secretion of inflammation mediators. There are other pathways in platelet that are activated during inflammation and lead to increased secretion, such as the Toll-like receptor 4 cascade.28

Platelet–leukocyte interactions are regulated through secretion of inflammatory mediators and through cell–cell interactions mediated by adhesion molecules. Upon P2Y₁₂-mediated activation, platelets express P-selectin on the cell surface. P-selectin binds P-selectin glycoprotein ligand 1 on leukocytes, activating the leukocytes and promoting their infiltration into the inflamed tissue.4 Furthermore, adhesion of dendritic cells to injured carotid arteries in mice is mediated by platelets, specifically by interaction with P-selectin glycoprotein ligand 1 on dendritic cells.29 Our data show for the first time that during sepsis, the expression of P-selectin was reduced in P2Y₁₂ null mice compared with their WT counterparts. As a result, platelet and

Figure 6. Inflammation-induced elevation in circulating white blood cell, platelet counts, and lung injury are not altered in P2Y₁₂ null mice. Blood samples were collected by cardiac puncture in 3.8% sodium citrate (10:1), and hematology studies were performed. Graphs show counts of white blood cells (WBC; A), neutrophils (PMN; B), lymphocytes (LY; C), and platelets (D) in wild-type (WT; black) and P2Y₁₂ knockout (KO; white) mice. Both sham and cecal ligation and double puncture (CLP) samples were analyzed for wild-type and KO mice. Values are expressed as 1×10⁶ cells/μL, mean±SEM. E, Photomicrographs of hematoxylin- and eosin-stained tissue sections obtained after CLP surgery. Representative images of lung tissue specimens were obtained for sham and CLP in wild-type and KO mice (magnification 20× and 40×; n=5). G, Acute lung injury (ALI) score, based on alveolar capillary congestion, hemorrhage, infiltration, or aggregation of neutrophils in the airspace or the vessel wall and thickness of the alveolar wall, was assessed in wild-type (black bars) and KO (white bars) mice. F, Myeloperoxidase (MPO) analysis was performed in lung samples of sham and CLP in wild-type (black) and KO (white) mice. Values are expressed as rfu/min per mg, mean±SEM (*P<0.05; KO CLP model vs WT, n=5).
leukocyte aggregate formation was also reduced. The data suggest that a decrease in membrane P-selectin expression may be the mechanism through which platelets contribute to decreased leukocyte activation during inflammation. Because P-selectin expression is P2Y12-dependent, receptor antagonism may modulate platelet–leukocyte interactions. A decrease in the septic-induced co-aggregate formation was also observed in the septic lung of P2Y12 null mice, suggesting that platelet–leukocyte interaction was also modulated within organs, as well as systemically. The decrease in inflammation levels is most likely because of changes in platelet activation instead of variation in cell number because the number of circulating platelets were similar among the treatment groups. Interestingly, P-selectin secretion in plasma samples of septic patients was increased compared with healthy controls, suggesting that P-selectin regulation may play a role in sepsis. A decrease in platelet activation was also noted in clopidogrel-treated mice, and again, there was no change in platelet number. The alignment between receptor deficiency and blockage emphasizes that modulating P2Y12 may be a new therapeutic option for sepsis, although the P2Y12-independent effects need to be fully characterized.

Circulating lymphocyte levels during sepsis were elevated in WT mice but not in P2Y12 null mice, which is similar to what was observed in clopidogrel-treated mice. Lymphocyte abnormalities have been reported in septic patients, and they were related with the pathophysiology of the disease. P2Y12 receptor antagonist influenced circulating lymphocyte numbers over platelets and neutrophils. Previous studies indicate that expression of the P2Y12 receptor is not exclusive to platelets. For example, P2Y12 receptor mRNA was detected in lymphocytes and dendritic cells, and the decreased inflammation levels and lymphocyte numbers might have resulted from the P2Y12 receptor antagonist affecting the immune system directly instead of through platelets. Considering that sepsis increases lymphocyte apoptosis, it would be interesting to investigate whether P2Y12 receptor–deficient lymphocytes are less sensitive to apoptosis. However, although lymphocytes have shown to express P2Y12 mRNA, it has not been established that they express a functional P2Y12 receptor. In contrast, platelets are known to express a functional P2Y12 receptor that is crucial for platelet functions and biology. Hence, following our study and these previous observations, we can conclude that platelet P2Y12 plays a determinant role during inflammation.

In an earlier study, we demonstrated that prasugrel metabolites inhibit neutrophil functions in vitro, even though neutrophils do not express the P2Y12 receptor. The data suggest a P2Y12-independent effect for this class of drugs. Interestingly, we found for the first time that circulating neutrophils in septic P2Y12 null mice treated with clopidogrel were decreased compared with untreated septic P2Y12 null mice. Hence, clopidogrel may have P2Y12-independent effects during sepsis, and neutrophils are the most likely target. Indeed, the P2Y12-independent effects observed in vitro may explain the altered neutrophil functions observed in vivo.

Our group investigated a murine model of systemic inflammation in which mice were treated with repeated doses of lipopolysaccharides for 4 days. We found that inflammation was more severe in P2Y12 null mice compared with their WT counterparts. In this model of inflammation, however, we observed that P2Y12 null mice are protected against sepsis. The discrepancy may be because of a different phase of inflammation (4 days versus 24 hours) or of a different disease. Interestingly, these different results have been previously observed during a model of rheumatoid arthritis. Specifically, in a peptidoglycan polysaccharide-induced arthritis model after clopidogrel treatment, the disease was exacerbated, whereas serum transfer arthritis was more severe in WT compared with P2Y12 null mice. Indeed, inhibiting platelet activation has different outcomes depending on the phase of inflammation and on the disease model. More investigations are needed to better understand how to safely administer this class of drugs during inflammation.

ADP plays an important role in hemostasis. When released by activated platelets, ADP acts as an aggregating agent and potentiates platelet responses to other agonists. ADP-induced aggregation is mediated by both the P2Y1 and P2Y12 receptors. Previous studies have shown that the P2Y1 receptor on endothelial cell surfaces modulates leukocyte recruitment, suggesting a role for P2Y1 during vascular inflammation. However, the role of the P2Y1 receptor during sepsis has not been previously investigated. Hence, we wondered whether our observations were because of P2Y1 receptor deficiency or of an altered platelet response to ADP. To address this question, we analyzed P2Y1 null mice in the same model of sepsis. We noted that the P2Y1 receptor did not influence the inflammation levels in the CLP model. Overall, our data suggest that P2Y12 receptor deficiency is responsible for the effects previously observed.

In conclusion, our data show that P2Y12 null mice are protected against sepsis-induced lung injury, which is similar to what we observed after P2Y12 antagonism with clopidogrel. Platelet activation and segregation in the lungs were diminished, suggesting that platelets play a role in inflammation both directly and through immune cell interactions and that their functions can be modulated through the P2Y12 receptor. This effect is specifically because of the P2Y12 receptor and not of an altered response to ADP. Taken together, the data indicate that during sepsis, activated platelets play a central role that is dependent on P2Y12 receptor activation.

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Disclosures
None.

References
Sepsis and sepsis-induced lung injury is one of the leading causes of death in the intensive care unit. Sepsis is characterized by a systemic inflammatory response leading to excessive neutrophil infiltration of the lungs producing tissue damage. There is growing evidence that platelets are involved in neutrophil recruitment and play a key role in neutrophil-mediated organ damage. Hence, targeting platelets could offer new therapeutic option for this disease. Our data show that P2Y12 null mice are refractory to sepsis-induced lung injury, which is similar to what we observed after P2Y12 antagonism with clopidogrel. This effect is specifically because of the P2Y12 receptor because receptor deficiency and antagonism provided similar results. Hence, targeting the P2Y12 receptor may offer a unique therapeutic strategy for the control of neutrophil migration and activation in sepsis-induced lung injury.
P2Y12 Receptor Modulates Sepsis-Induced Inflammation
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Material and Methods

Materials
All reagents, analytical grade, were obtained from Thermo Fisher Scientific (Waltham, MA) unless stated otherwise. Bovine serum albumin (BSA) was obtained from Sigma-Aldrich (St. Louis, MO). Clopidogrel was provided as 75 mg Plavix® tablets from Bristol-Myers Squibb/Sanofi Pharmaceutical partnership (New York, NY). Hank’s balanced salt solution (HBSS) and PBS were purchased from Mediatech Inc. (Manassas, VA). BD FACS™ lysing solution, PE-conjugated rat anti-mouse CD41 (clone MWReg30), FITC-conjugated rat anti-mouse CD41 (clone M1/70) and FITC-conjugated rat anti-mouse P-selectin (clone RB40.34) and were purchased from BD Bioscience (San Jose, CA). PE-conjugated rat anti-mouse CD11b (clone M1/70) and FITC-conjugated rat anti-mouse CD11b (clone M1/70.15) and the isotype control IgG2b were purchased from Invitrogen (Grand Island, NY). Isotype controls, IgG1κ and IgG1λ, were obtained from BD Bioscience (San Jose, CA).

Animals and treatments
Animal procedures and handling adhered to National Institutes of Health standards and were approved by the Institutional Animal Care and Use Committee at Temple University School of Medicine (Philadelphia, PA, USA). Male wild-type and P2Y12 deficient pathogen-free C57BL/6 mice (weight, 25-30 g) were obtained from Schering-Plough Corporation (Kenilworth, NJ)1-4. P2Y1 deficient pathogen-free C57BL/6 male mice were generated by subcontract with Lexicon Genetics Inc. (Woodlands, TX) through knockout constructs as described previously 5-7. Animals were housed in a climate-controlled facility and given free access to food and water.

The cecal ligation and double puncture (CLP) were performed on isoflurane-anesthetized animals as described previously 5-7. Sham control animals underwent a laparotomy without ligation or double puncture. Experiments were performed in P2Y12, P2Y1 KO, and wild-type mice that were randomly assigned to one of four groups for wild-type or KO: wild-type and KO sham control group (6 animals per group); wild-type and KO undergoing CLP (CLP group, 6 animals per group).

Clopidogrel was orally administrated to wild-type and P2Y12 KO (6 animals per group) with a loading dose of 30 mg/kg the day before surgery and a maintenance dose of 10 mg/kg two hours before surgery. Sham mice received the same doses of clopidogrel. After the procedure but prior to emergence, sham and CLP mice were fluid-resuscitated (1 ml/mouse sterile saline, subcutaneously). At 24 hours post-surgery, mice were anesthetized and blood samples were collected by cardiac puncture (10:1 in 3.8% sodium citrate) for hematology studies (Hemavet® Multispecies Hematology System, Drew Scientific, Inc. Oxford, CT). All mice were euthanized by cardiac puncture and exsanguination. Lungs were collected and fixed or frozen immediately in liquid nitrogen.

Platelet-leukocyte aggregate formation and P-selectin expression in whole blood
Murine blood samples were incubated with either FITC-conjugated anti-mouse CD11b and PE-conjugated anti-mouse CD41 or with FITC-conjugated anti-mouse P-selectin for
20 minutes at 25 °C. The reaction was stopped by adding BD FACSTM lysing solution (1:10 in PBS). Samples were kept at 4°C prior to analysis. Flow cytometry was performed using a FACSCalibur analyzer and data were analyzed with FlowJo software. Platelet and neutrophil aggregates were discriminated by forward and side light scatter and identified by their positive staining for PE-CD41 or FITC-CD11b, respectively. Events double positive for PE and FITC identified platelet–neutrophil aggregates and were recorded as a percentage of a total of 10,000 gated neutrophils.

**Lung histopathology**
Lung sections were paraffin-embedded, cut into 5 μm sections, and stained with hematoxylin and eosin (H&E). Morphological analysis of random fields (n = 6) from each section (n = 3 sections/animal) was performed by a second independent, blinded observer using previously described methods. Acute lung injury (ALI) was scored based on four parameters: a) alveolar capillary congestion, b) hemorrhage, c) infiltration or aggregation of neutrophils in the airspace or the vessel wall, and d) thickness of the alveolar wall. Each parameter was graded from 0–4 based on the damage present (0, no or little damage; 1, less than 25% damage; 2, 25–50% damage; 3, 50–75% damage; and 4, more than 75% damage). The degree of ALI was assessed by sum of scores of four parameters.

**Myeloperoxidase peroxidation (MPO)**
Lungs were also homogenized and sonicated. After centrifugation (10,000 for 10 minutes at 4°C), MPO levels were detected using a MPO assay kit (Cayman, USA).

**Fluorescence microscopy**
Murine lung tissue sections were deparaffinized, and antigen retrieval was achieved by microwaving the tissue slides for 4 min in citrate buffer pH 6.0. The slides were washed and incubated with FITC-conjugated rat anti-mouse CD41 or with FITC-conjugated rat anti-mouse CD41 and PE-conjugated rat anti-mouse CD11b. The slides were mounted in Vectashield with DAPI (DNA stain) and imaged using fluorescence microscopy.

**Cytokine profiles**
Plasma aliquots from each animal were obtained by blood centrifugation (2,000g for 10 minutes) and utilized for detection of TNF-α, IL-6, IL-10 and MIP-1b plasma levels by the Luminex® System (Allied Biotech, Inc. Ijamsville, MD).

**Statistical analysis**
Differences among groups were statistically analyzed using one-way ANOVA; Bonferroni’s Multiple Comparison Test was used as post-test analyses. P < 0.05 was considered to be significant. Data are reported as mean ± standard error of the mean (S.E.M.) for each group.
References