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$P2Y_{12}$ Receptor Modulates Sepsis-Induced Inflammation

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Abstract

Objective—Platelets modulate hemostasis and immune responses via interactions with immune cells, through secretion of immune-modulators and cell-cell interactions. The $P2Y_{12}$ receptor mediates ADP-induced aggregation and secretion in platelets.

Approach—using a mouse model of intra-abdominal sepsis and acute lung injury, we investigated the role of the $P2Y_{12}$ receptor in neutrophil migration and lung inflammation in $P2Y_{12}$ null mice and in mice pre-treated with the $P2Y_{12}$ antagonist clopidogrel.

Results—our data show a decrease in circulating white blood cells and a decrease in platelet activation and platelet-leukocyte interactions in treated mice compared to untreated. Additionally, lung injury and platelet sequestration were diminished in clopidogrel-treated mice compared to their untreated septic littermates. Similar results were observed in $P2Y_{12}$ null mice: platelet activation and platelet-leukocyte aggregates were decreased in septic $P2Y_{12}$ null mice compared to wild-type. $P2Y_{12}$ null mice were refractory to lung injury compared to wild-type. Lastly, to evaluate $P2Y_{12}$ independent effects of clopidogrel, we pre-treated $P2Y_{12}$ null mice. Interestingly, the number of circulating neutrophils was reduced in treated septic $P2Y_{12}$ null mice, suggesting neutrophils as a target for clopidogrel pleiotropic effects. No difference was observed in $P2Y_1$ null mice during sepsis, indicating that the $P2Y_{12}$ receptor is responsible for the effects.

Conclusions—$P2Y_{12}$ null mice are refractory to sepsis-induced lung injury, suggesting a key role for activated platelets and the $P2Y_{12}$ receptor during sepsis.

Keywords

sepsis; platelets; $P2Y_{12}$ receptor

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Conflict of interest disclosures

None

Introduction

Platelets regulate thrombus formation and hemostasis and play important roles during inflammation. Upon activation, platelets activate immune cells through cell-cell interactions and by secreting inflammatory mediators and second mediators, such as ADP, that recruit other platelets [1]. ADP-induced aggregation is mediated by two members of the P2Y receptor family: $P2Y_1$ and $P2Y_{12}$ [2]. Both are G protein-coupled receptors that are expressed on platelet membranes [3]. $P2Y_1$ is coupled to G_q protein, whose activation causes platelet shape changes and a weak, transient aggregation [4]. $P2Y_{12}$ is coupled to G_i protein, whose activation leads to platelet aggregation and potentiation of granule release, indicating a role for the $P2Y_{12}$ receptor in platelet secretion [5]. Signaling events downstream of the $P2Y_{12}$ receptor also potentiate agonist-induced dense granule release, pro-coagulant activity, and thrombus formation [6].

Platelets activate leukocytes through cell-cell interactions involving adhesion molecules such as P-selectin, a glycoprotein that, upon cell activation, is rapidly translocated from cytoplasmic α-granules to the cell surface [7]. $P2Y_{12}$ activation is required for α-granule release and subsequent expression of P-selectin on activated platelets [7]. P-selectin binds P-selectin glycoprotein ligand 1 (PSGL-1) on leukocytes, which activates leukocytes and promotes their infiltration into inflamed tissue [8].

Platelet-leukocyte interactions are important in the pathogenesis of sepsis [9], and leukocyte-platelet aggregates and P-selectin secretion are altered in septic patients [10]. In a model of sepsis induced by cecal ligation and puncture (CLP), neutrophil infiltration in the lungs was reduced following platelet depletion [11], 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 [12, 13]. In a rat model of LPS-induced inflammation, induction of pro-inflammatory cytokines (IL-6 and TNF-α), lung damage, and liver damage were attenuated upon treatment with clopidogrel, a drug that antagonizes the $P2Y_{12}$ receptor [14]. Both $P2Y_{12}$ receptor antagonism and platelet depletion reduced inflammation during sepsis, suggesting a role for platelets in regulating the inflammatory response in sepsis.

$P2Y_{12}$ receptor gene variants correlate with pulmonary inflammation and asthma [15]. Also, $P2Y_{12}$ receptor deficiency and platelet depletion abrogate dust mite-induced airway inflammation [16], suggesting a role for the $P2Y_{12}$ receptor in pulmonary inflammation. The mechanisms that trigger neutrophil infiltration are poorly understood but may result from the development of a pro-inflammatory phenotype in the lung. These observations suggest a regulatory role for the $P2Y_{12}$ 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 $P2Y_{12}$ antagonist, ticagrelor suggesting a regulatory role for $P2Y_{12}$ receptor signaling in sepsis [17]. However, several studies indicate that ticagrelor may have pleiotropic effects in addition to its anti-platelet properties [18]. To establish a role

for P2Y12 signaling in sepsis and sepsis-induced acute lung injury, we examined the effects of receptor deficiency ($P2Y_{12}$ null mice) and $P2Y_{12}$ antagonism (clopidogrel) in the CLP model of sepsis. We further pre-treated $P2Y_{12}$ null mice with clopidogrel to investigate $P2Y_{12}$ independent effects. Lastly, we studied $P2Y_1$ null mice in the same sepsis model to determine whether these effects were due to $P2Y_{12}$ deficiency or to altered responses to ADP. Our findings show that inflammation was decreased in $P2Y_{12}$ null mice but not in $P2Y_1$ null mice. Interestingly, $P2Y_{12}$ deficiency and receptor antagonism in this model of sepsis provided similar results, suggesting that modulation of the $P2Y_{12}$ 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 (WBCs) did not increase following clopidogrel treatments in septic mice

To study $P2Y_{12}$ receptor antagonism in sepsis, mice were treated with the $P2Y_{12}$ receptor antagonist clopidogrel (loading dose: 30 mg/kg; maintenance dose: 10 mg/kg) prior to surgery in a model of sepsis and lung injury. Both sham and CLP mice were treated. First, we analyzed the number of circulating WBCs in blood samples (Fig. 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 (Fig. 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 in the sham control ($^{*}p < 0.05$). When we analyzed the cells more specifically, we noticed that lymphocytes were increased following sepsis, whereas no difference was noted in neutrophils. However, following clopidogrel treatment, both cells were significantly reduced (Fig. 1B and C; $^{*}p < 0.05$; 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).

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 following CLP in treated and untreated mice using flow cytometry (Fig. 2 A and B). P-selectin expression was increased after CLP (Fig. 2A and B; $^{**}p < 0.01$; CLP versus sham), but no elevation was noted in mice pretreated with clopidogrel compared to the treated sham control (Fig 2A and B $^{**}p < 0.01$; untreated CLP versus treated CLP). Next we investigated the effect of clopidogrel treatment on leukocyte-platelet aggregate formation (Fig. 2C and D). Aggregate formation was elevated in samples from CLP mice compared to sham mice (Fig. 2C and D; $^{*}p < 0.05$ CLP versus sham). However, in clopidogrel-treated mice, aggregate formation was significantly reduced compared to untreated mice (Fig. 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 mice after CLP surgery (Fig. 2D, left panels, $n = 4$), but it was not noted in clopidogrel-treated mice (Fig. 2D, right panels).

Clopidogrel treated mice are refractory to sepsis-induced lung injury

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

Sepsis-induced increase in circulating white blood cells was not noted in $P2Y_{12}$ null mice

To compare receptor antagonism with receptor deficiency, we analyzed $P2Y_{12}$ null mice in the same model of sepsis-induced inflammation. First we investigated the number of WBCs in blood samples of $P2Y_{12}$ null mice 24 hours after either CLP or sham surgery (Fig. 4). No increase in circulating WBCs in septic mice was noted compared to the sham control (Fig. 4A). Specifically, no differences in neutrophils (Fig.4B) and lymphocytes (Fig. 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 $P2Y_{12}$ null mice

We investigated whether $P2Y_{12}$ deficiency influences platelet activation during sepsis-induced inflammation. P-selectin expression was not increased after CLP in both CLP and sham mice (Fig. 4E). Leukocyte-platelet aggregate formation was not elevated in samples from CLP $P2Y_{12}$ null mice compared to sham $P2Y_{12}$ null mice (Fig. 4F).

Sepsis-induced increases in plasma cytokines are diminished in $P2Y_{12}$ KO mice

Next we investigated the role of $P2Y_{12}$ in regulating sepsis-induced elevations in plasma levels of cytokines (TNF-α, IL-10, IL-6 and MIP-1β) (Fig. 4G). As expected, the plasma concentration of each cytokine was elevated during sepsis in both WT and $P2Y_{12}$ null mice animals as compared to sham control. However, the sepsis-induced increase was significantly lower in $P2Y_{12}$ null mice as compared to WT mice, for all the cytokines analyzed ($**p < 0.01$ KO CLP model versus WT CLP). These data suggest a decreased level of inflammation in the absence of $P2Y_{12}$ receptor-mediated signaling.

Lung injury is decreased in septic $P2Y_{12}$ null mice

In $P2Y_{12}$ null mice 24 hours following CLP, no lung damage was observed as compared to the sham control (Figure 4H). Similarly, histology scores of ALI were not elevated. Furthermore, neutrophil infiltration (MPO levels) was not increased in $P2Y_{12}$ null mice following CLP (Fig. 4I). These data suggest that the $P2Y_{12}$ receptor plays a key role in pulmonary inflammation and inflammatory cell recruitment. To analyze how $P2Y_{12}$ 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 animals Sham mice, the lungs did not show cell infiltration, while 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, $P2Y_{12}$ null animals had decreased levels of platelets and neutrophils interaction in the lungs, suggesting a decrease in aggregation (Figure 4J, bottom panels).

Clopidogrel pre-treatment decreased circulating neutrophils in $P2Y_{12}$ null mice during sepsis

To determine whether clopidogrel has $P2Y_{12}$ independent effects, we treated $P2Y_{12}$ null mice with clopidogrel prior to surgery in the same model of sepsis (Fig. 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 $P2Y_{12}$ null mice compared to treated sham $P2Y_{12}$ null mice (Fig. 5A *$p < 0.05$;treated WT CLP versus treated WT Sham and treated CLP WT versus treated CLP $P2Y_{12}$ null mice). On the contrary, neutrophils were significantly lower in septic treated mice compared to the treated sham control for both WT and $P2Y_{12}$ null mice (Fig 5B, $p < 0.05$; treated WT sham versus treated WT CLP and treated $P2Y_{12}$ null mice Sham versus treated CLP $P2Y_{12}$ null mice). Lymphocyte count was not altered in treated $P2Y_{12}$ null mice compared with their respectively treated Sham control (Fig. 5C). The data show that clopidogrel has $P2Y_{12}$ 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 in $P2Y_{12}$ null mice during sepsis

We investigated whether clopidogrel treatment can influence platelet activation in $P2Y_{12}$ 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 $P2Y_{12}$ null mice (Fig. 5E). Leukocyte-platelet aggregate formation was not elevated in samples from treated CLP $P2Y_{12}$ null mice compared to treated sham $P2Y_{12}$ null mice (Fig. 5F). The data are similar to what observed in clopidogrel-treated sham and CLP WT. Then we investigated platelet sequestration in the lungs of septic mice. No CD41 staining was observed in treated $P2Y_{12}$ null mice after CLP surgery as well as in the treated sham control (data not shown). The data suggest that $P2Y_{12}$ independent effects of clopidogrel do not involve platelets.

Clopidogrel treated $P2Y_{12}$ null mice are still refractory to acute lung injury

We investigated acute lung injury in $P2Y_{12}$ null mice during sepsis following clopidogrel treatments. In treated $P2Y_{12}$ null mice 24 hours following CLP, no lung damage was observed as compared to the sham control (Fig. 5G). Similarly, histology scores of ALI were not elevated in the treated $P2Y_{12}$ null CLP group compared to the treated $P2Y_{12}$ null sham group (Fig. 5H). These data are similar to what observed in clopidogrel-treated sham and CLP WT. To confirm leukocyte infiltration, we investigated MPO activity in lung tissue samples (Fig. 5I). MPO levels was not increased in treated $P2Y_{12}$ null mice following CLP. These data suggest that clopidogrel has no $P2Y_{12}$ independent effects on lung tissue.

$P2Y_1$ deficiency does not influence sepsis-induced inflammation levels

The same model of sepsis was investigated in $P2Y_1$ null mice to investigate whether the data collected in $P2Y_{12}$ null mice were due to changes in cell responses to ADP or specifically to $P2Y_{12}$. First we analyzed circulating WBCs in blood samples (Figure 6). No significant difference in WBC number was observed in $P2Y_1$ null mice compared to wild type (Fig. 6A), with similar results for neutrophils (Fig. 6B) and lymphocytes (Fig. 6C). Platelet counts were similar among groups (Fig. 6D). Interestingly, the cell count in the sham group was significantly higher than the CLP and wild-type sham groups (Figure 6; $p < 0.05$; KO sham versus KO CLP).

We investigated lung injury in $P2Y_1$ null mice and wild-type mice following CLP. No significant differences in lung injury were observed (Fig. 6E and F). 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 $P2Y_1$ 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 $P2Y_{12}$ receptor plays a central role in various platelet functions, including secretion. $P2Y_{12}$ receptor antagonists prevent platelet aggregation and secretion [19, 20] and alter the inflammatory state in LPS-induced inflammation, myocardial infarction, and rheumatoid arthritis [13, 21, 22], suggesting that $P2Y_{12}$ modulation can influence inflammation by mechanisms which have yet to be fully elucidated. Pretreatment with the $P2Y_{12}$ 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 $P2Y_{12}$ signaling in sepsis. We investigated for the first time this model of sepsis in $P2Y_{12}$ null mice and WT mice treated with another $P2Y_{12}$ antagonist, clopidogrel. Interestingly, clopidogrel has shown to exhibit pleiotropic effects [23] as well as ticagrelor that can inhibit equilibrative nucleoside transporter 1 [18] Hence it is important to study sepsis in the $P2Y_{12}$ null mouse model to compare receptor deficiency and antagonism and clarify the mechanism of these anti-platelet drugs. Further, we examined the effect of clopidogrel treatment on $P2Y_{12}$ mice to identify $P2Y_{12}$ independent effects. Our studies demonstrate that clopidogrel-treated mice are more refractory to inflammation and lung injury compared with untreated animals, which is

similar to what we observed in $P2Y_{12}$ null mice, suggesting that the $P2Y_{12}$ receptor plays a central role during sepsis. Our studies demonstrate that $P2Y_{12}$ deficiency and receptor antagonism in this model of sepsis provided similar results, suggesting that the $P2Y_{12}$ 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, 25]. Recent studies have shown that interactions between platelets and leukocytes, in particular neutrophils [26], play a role during sepsis that can be relevant for the outcome of the disease [27]. Lung injury and neutrophil infiltration, for example, were decreased during anti-platelet therapy [17] 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, $P2Y_{12}$ receptor plays a role in regulating platelet secretion during ADP-induced aggregation and when platelets are activated by other agonists [2, 6]. Indeed, activated platelets not only secrete second messengers that recruit other platelets, but they also secrete inflammatory mediators such as IFN-γ, TGF-β and RANTES [1, 28]. Hence, antagonizing the $P2Y_{12}$ 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 TLR-4 cascade [29] Platelet-leukocyte interactions are regulated through secretion of inflammatory mediators and through cell-cell interactions mediated by adhesion molecules. Upon $P2Y_{12}$ mediated activation, platelets express P-selectin on the cell surface. P-selectin binds PSGL-1 on leukocytes, activating the leukocytes and promoting their infiltration into the inflamed tissue [8]. Furthermore, adhesion of dendritic cells to injured carotid arteries in mice is mediated by platelets, specifically by interaction with PSGL-1 on dendritic cells [30]. Our data show for the first time that during sepsis the expression of P-selectin was reduced in $P2Y_{12}$ null mice compared to their wild-type counterparts. As a result, platelet and 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 $P2Y_{12}$-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 $P2Y_{12}$ null mice, suggesting that platelet-leukocyte interaction was also modulated within organs as well as systemically. The decrease in inflammation levels is most likely due to changes in platelet activation instead of variation in cell number, as the numbers of circulating platelets were similar among the treatment groups. Interestingly, P-selectin secretion in plasma samples of septic patients was increased compared to healthy controls [9], indicating 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 $P2Y_{12}$ may be a new therapeutic option for sepsis, although the $P2Y_{12}$ independent effects need to be fully characterized.

Circulating lymphocyte levels during sepsis were elevated in wild-type mice but not in $P2Y_{12}$ 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 [31]. $P2Y_{12}$ receptor antagonism influenced circulating lymphocyte numbers over platelets and neutrophils. Previous studies indicate that expression of the $P2Y_{12}$ receptor is not exclusive to platelets [32, 33]. For example, $P2Y_{12}$ receptor mRNA was detected in lymphocytes and dendritic cells [34, 35], and the decreased inflammation levels and lymphocyte numbers might have resulted from the $P2Y_{12}$ receptor antagonist affecting the immune system directly instead of through platelets. Considering that sepsis increases lymphocyte apoptosis, it would be interesting to investigate whether $P2Y_{12}$ receptor deficient lymphocytes are less sensitive to apoptosis. However, although lymphocytes have shown to express $P2Y_{12}$ mRNA [34, 35], it has not been established that they express a functional $P2Y_{12}$ receptor. In contrast, platelets are known to express a functional $P2Y_{12}$ receptor that is crucial for platelet functions and biology. Hence following our study and these previous observations we can conclude that platelet $P2Y_{12}$ play 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 $P2Y_{12}$ receptor [36]. The data suggest a $P2Y_{12}$ independent effect for this class of drugs. Interestingly, we found for the first time that circulating neutrophils in septic $P2Y_{12}$ null mice treated with clopidogrel were decreased compared to untreated septic $P2Y_{12}$ null mice. Hence, clopidogrel may have $P2Y_{12}$ independent effects during sepsis, and neutrophils are the most likely target. Indeed, the $P2Y_{12}$ independent effects observed *in vitro* [36] 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 LPS for 4 days[23]. We found that inflammation was more severe in $P2Y_{12}$ null mice compared with their wild-type counterparts. In this model of inflammation, however, we observed that $P2Y_{12}$ null mice are protected against sepsis. The discrepancy may be due to a different phase of inflammation (4 days versus 24 hours) or to 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 following clopidogrel treatment, the disease was exacerbated [21, 37], whereas serum transfer arthritis was more severe in wild-type compared to $P2Y_{12}$ null mice [38]. 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 $P2Y_1$ and $P2Y_{12}$ receptors [39]. Previous studies have shown that the $P2Y_1$ receptor on endothelial cell surfaces modulates leukocyte recruitment, suggesting a role for $P2Y_1$ during vascular inflammation [40]. However, the role of the $P2Y_1$ receptor during sepsis has not been previously investigated. Hence, we wondered whether our observations were due to $P2Y_{12}$ receptor deficiency or to an altered platelet response to ADP. To address this question, we analyzed $P2Y_1$ null mice in the same model of sepsis. We noted that the $P2Y_1$ receptor did not influence the inflammation levels in the CLP model.

Overall, our data suggest that $P2Y_{12}$ receptor deficiency is responsible for the effects previously observed.

In conclusion, our data show that $P2Y_{12}$ null mice are protected against sepsis-induced lung injury, which is similar to what we observed following $P2Y_{12}$ 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 $P2Y_{12}$ receptor. This effect is specifically due to the $P2Y_{12}$ receptor and not to an altered response to ADP. Taken together, the data indicate that during sepsis activated platelets play a central role that is dependent on $P2Y_{12}$ receptor activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ALI	acute lung injury
CLP	cecal ligation and double puncture
H&E	hematoxylin and eosin
PSGL-1	P-selectin glycoprotein ligand 1
MPO	Myeloperoxidase
WBC	white blood cells

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Significance

Sepsis and sepsis-induced lung injury are one of the leading causes of death in the ICU. 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 $P2Y_{12}$ null mice are refractory to sepsis-induced lung injury, which is similar to what we observed following $P2Y_{12}$ antagonism with clopidogrel. This effect is specifically due to the $P2Y_{12}$ receptor, as receptor deficiency and antagonism provided similar results. Hence, targeting the $P2Y_{12}$ receptor may offer a unique therapeutic strategy for the control of neutrophil migration and activation in sepsis-induced lung injury.

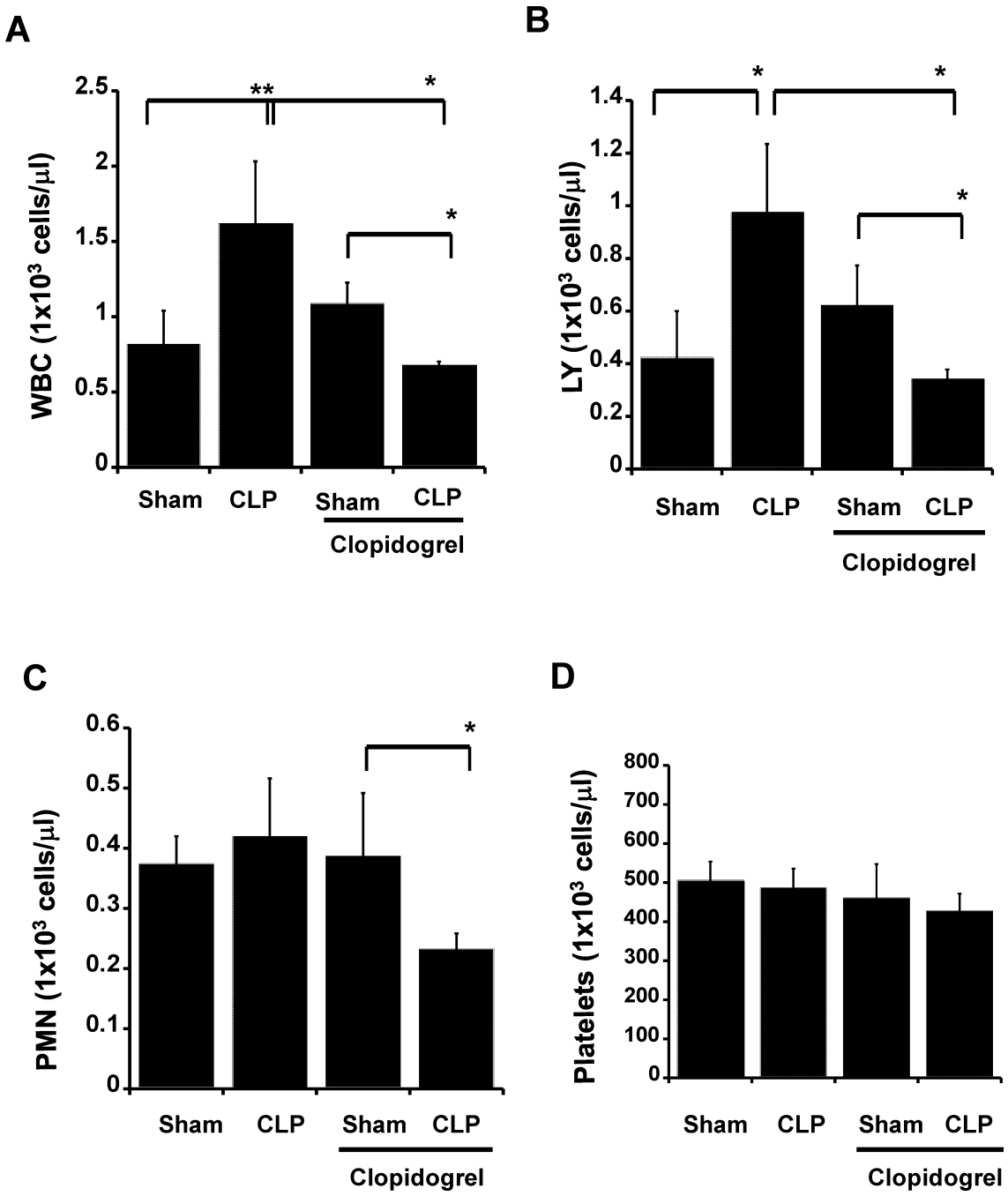
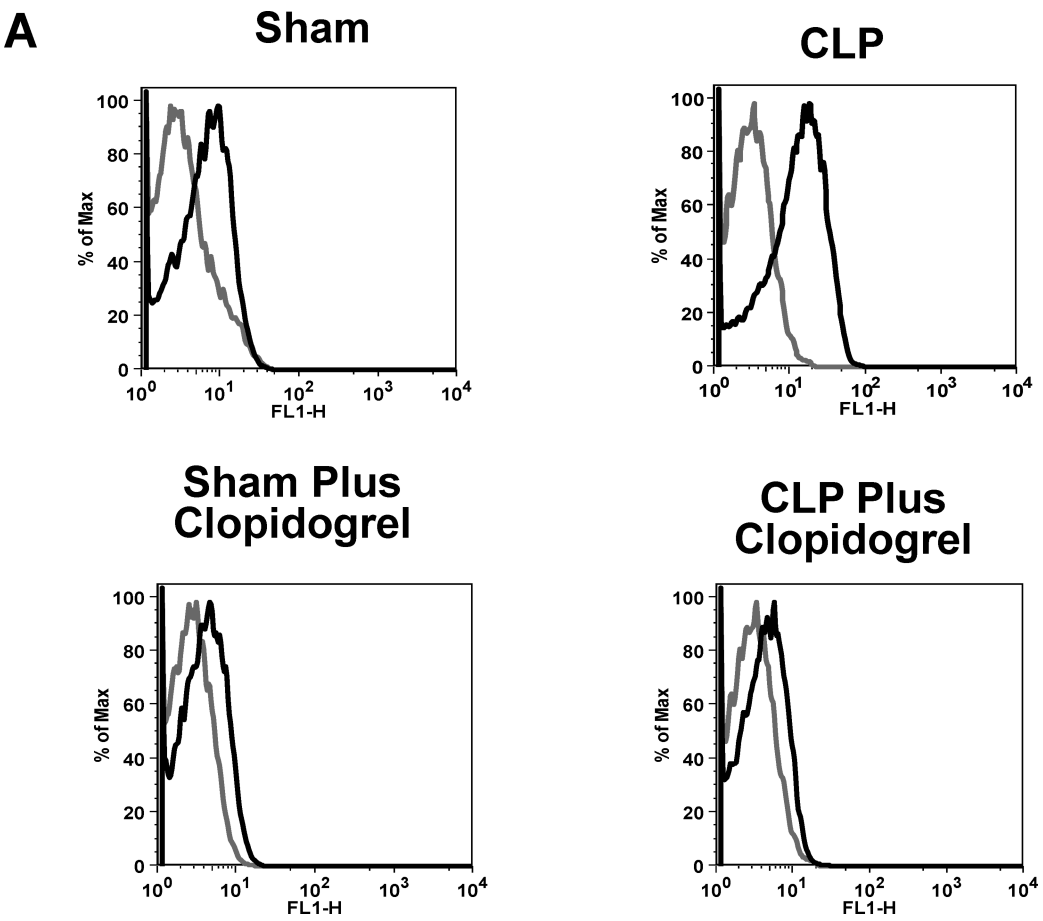
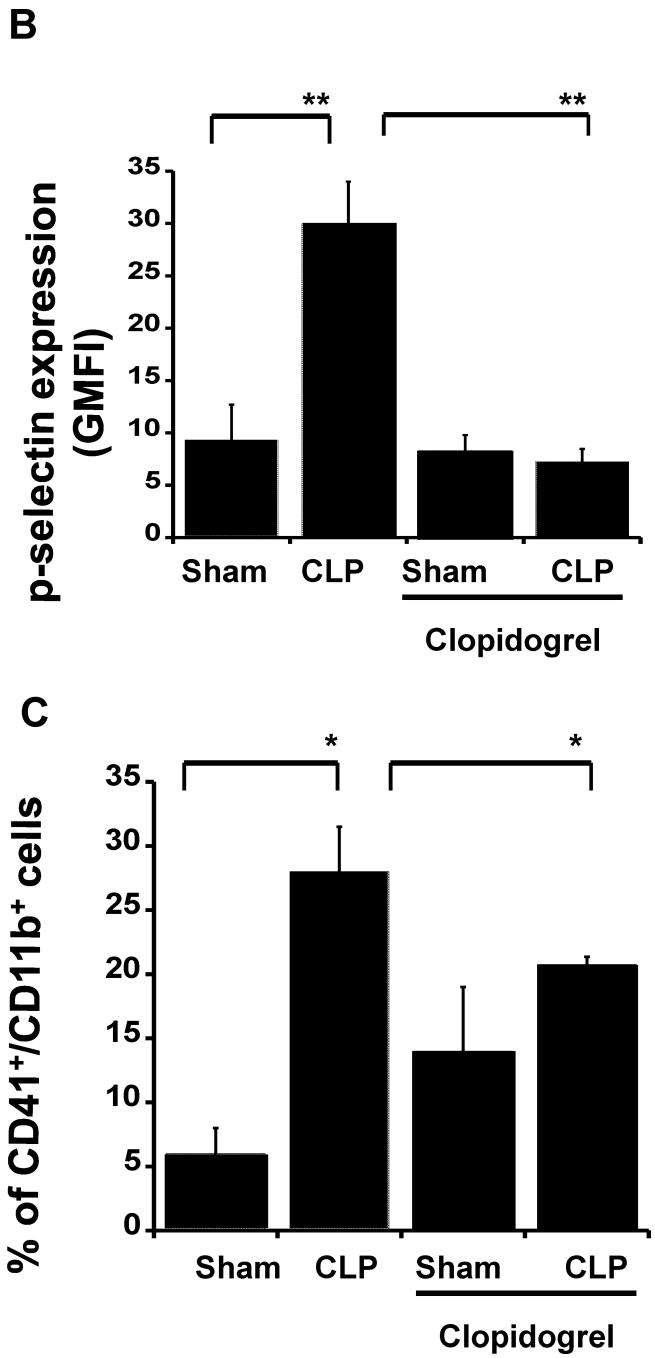


Figure 1. Circulating white blood cells did not increase in sepsis following clopidogrel treatments

Blood samples were collected by cardiac puncture in 3.8% sodium citrate (10:1) and hematology studies were performed. Graphs show counts of (**A**) white blood cells (WBC), (**B**) lymphocytes (LY), (**C**) neutrophils (PMN), and (**D**) platelets in clopidogrel-treated or untreated mice. Both sham and CLP samples were analyzed for treated and untreated mice. Values are expressed as 1×10^3 cells/μL, mean ± S.E.M., ($n = 8$; *$p<0.05$ WT sham versus CLP mice; **$p<0.01$ treated CLP versus untreated CLP).





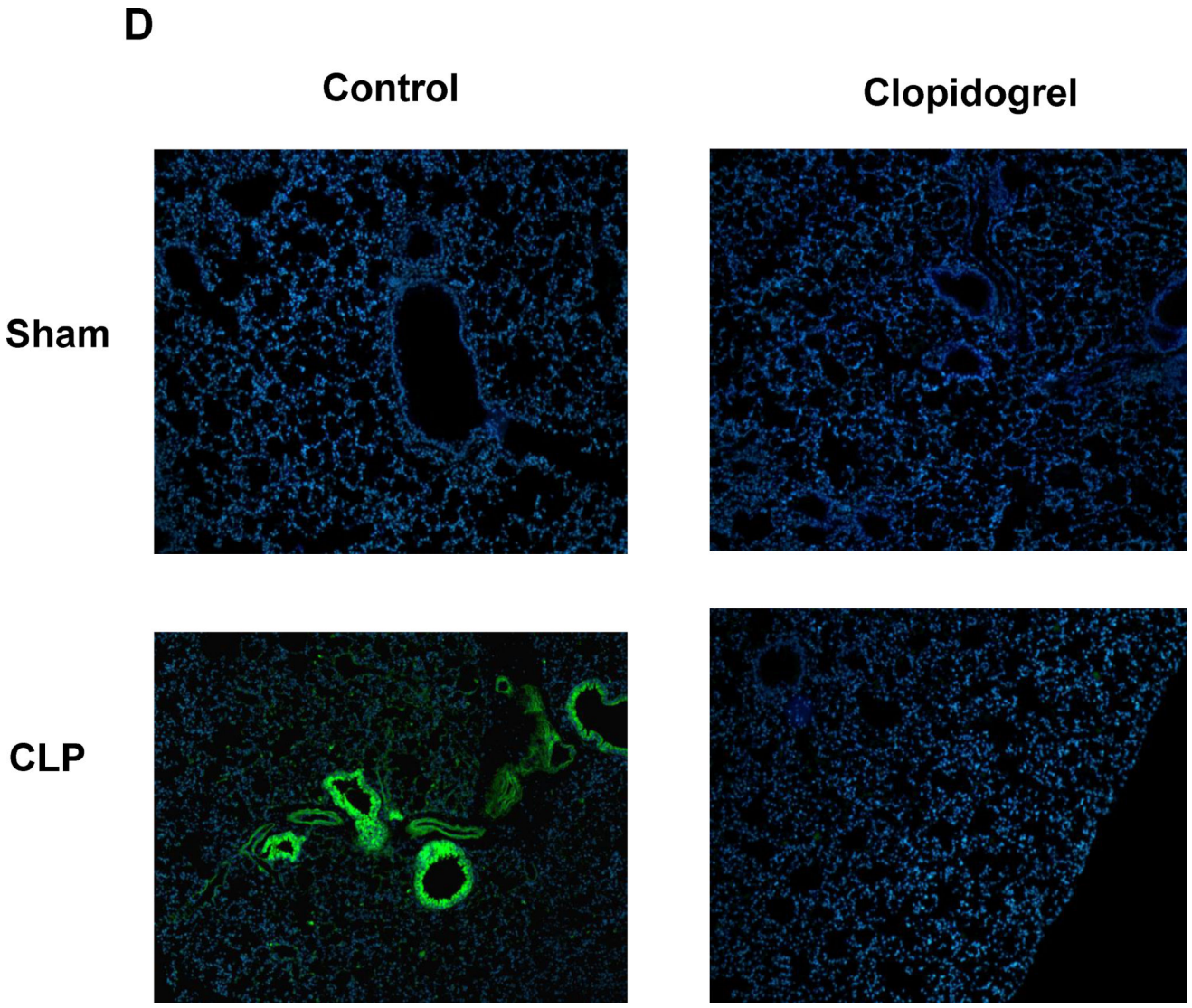


Figure 2. P-selectin expression and leukocyte-platelet aggregates were not elevated in clopidogrel treated mice during sepsis

(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 CLP and sham controls in WT and KO animals. Isotype control is shown in gray and P-selectin stained samples in black. **(C)** Blood samples were labelled 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 versus WT CLP and KO CLP versus WT, $n = 6$). (**D**) Representative images of CD41 staining (CD41: green; Nucleus: blue; 20x) for CLP and sham samples for both treated and untreated mice. Images are representative of 4 different experiments.

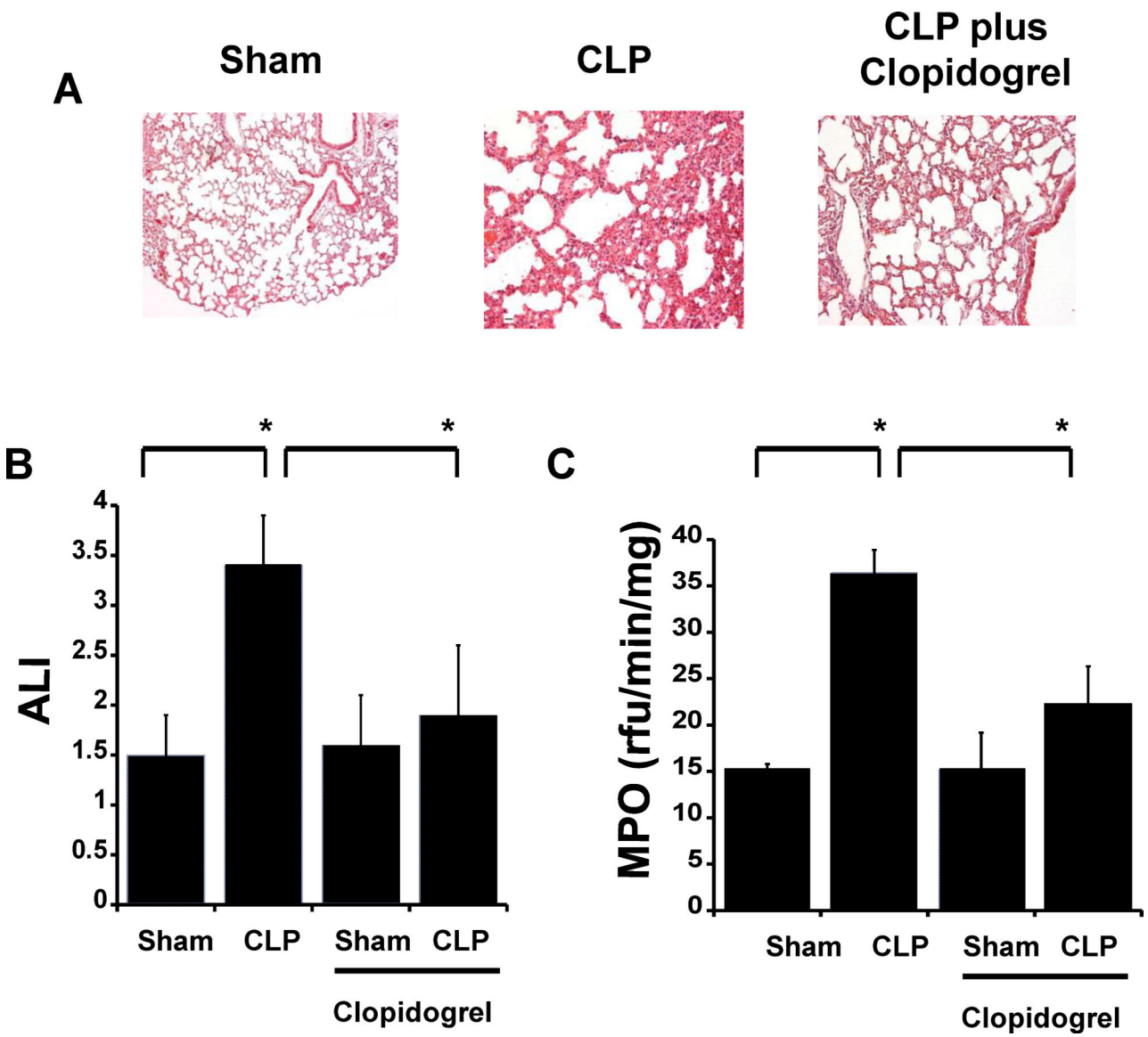
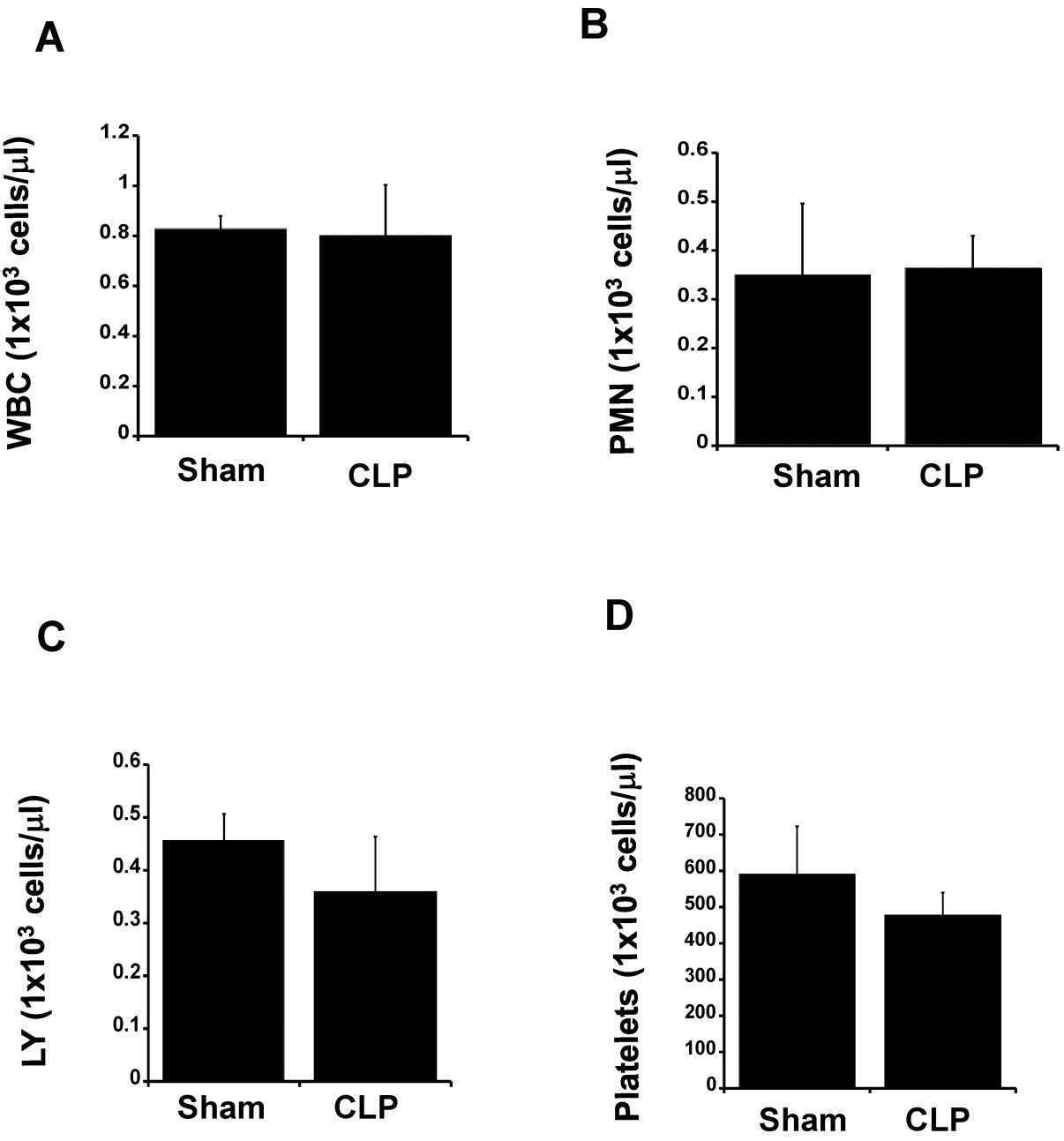
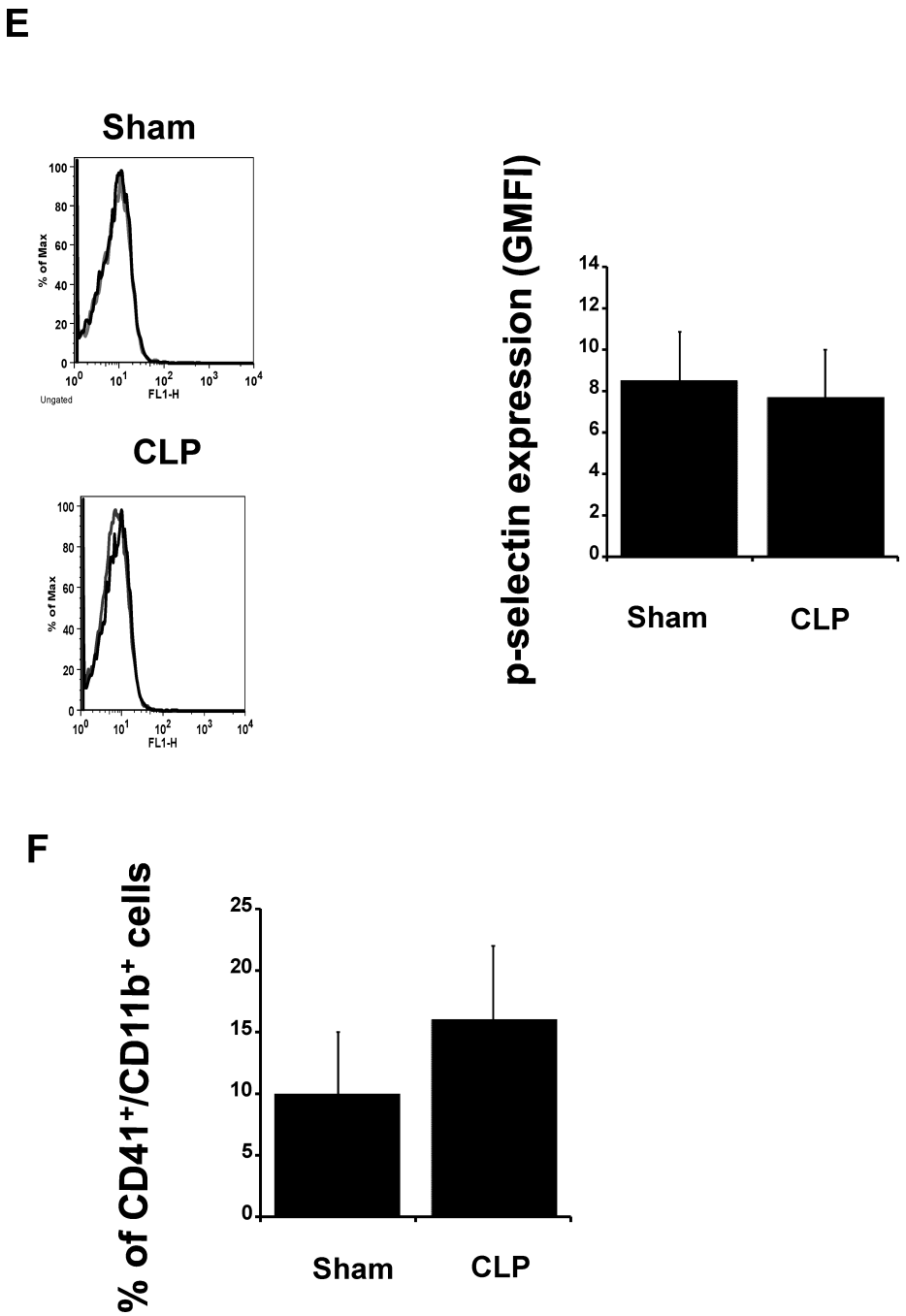
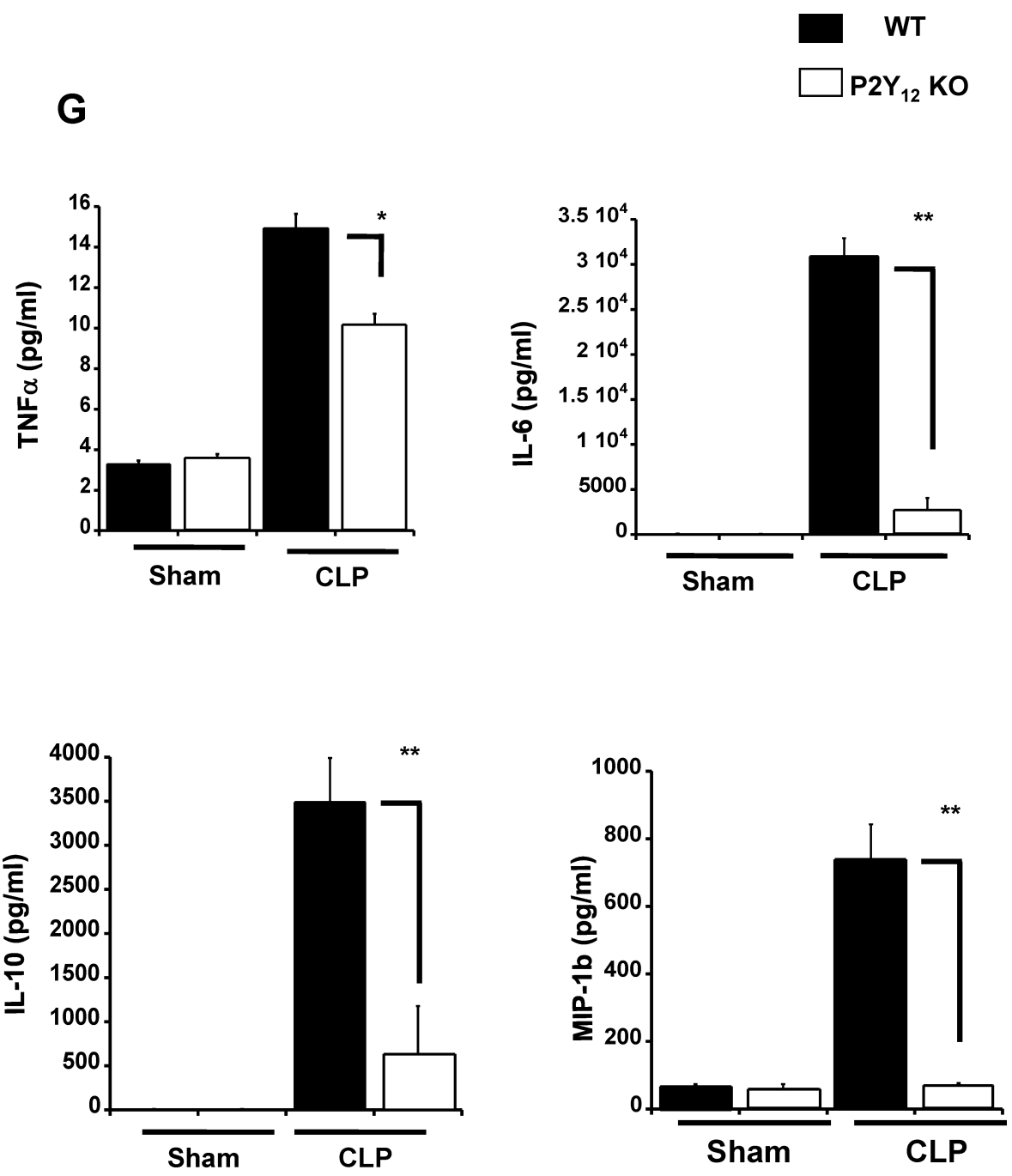


Figure 3. Sepsis-induced lung injury is ameliorated in clopidogrel-treated mice

(**A**) Photomicrographs of hematoxylin- and eosin-stained tissue sections were obtained after CLP surgery. Representative images of lung tissue specimens are shown for sham and CLP in clopidogrel-treated and untreated mice (Magnification 20 and 40x; $n = 5$). (**B**) Acute lung injury (ALI) scores were assessed in treated and untreated animals. (**C**) MPO analysis was performed in lung samples of sham and CLP mice. Values are expressed as rfu/min/mg, mean ± SEM (*$p < 0.05$; CLP versus sham, $n = 7$).







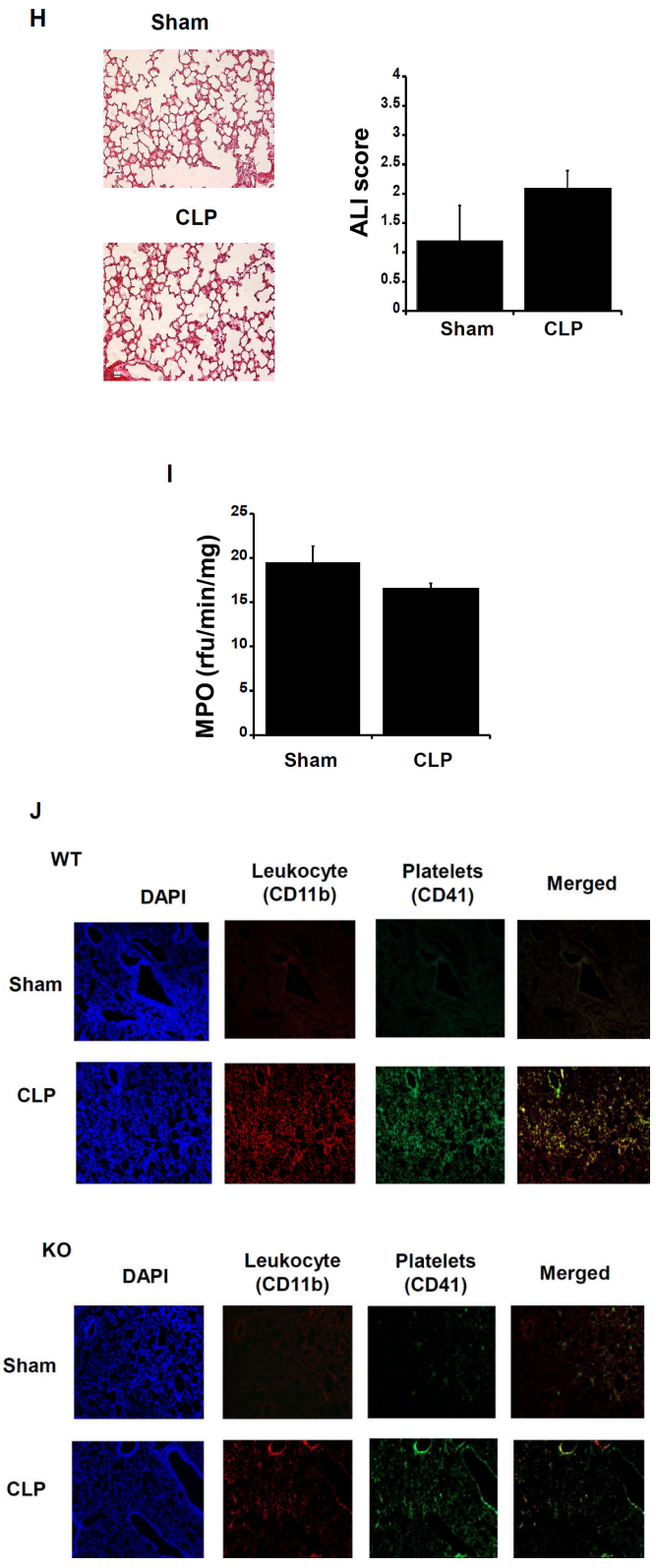
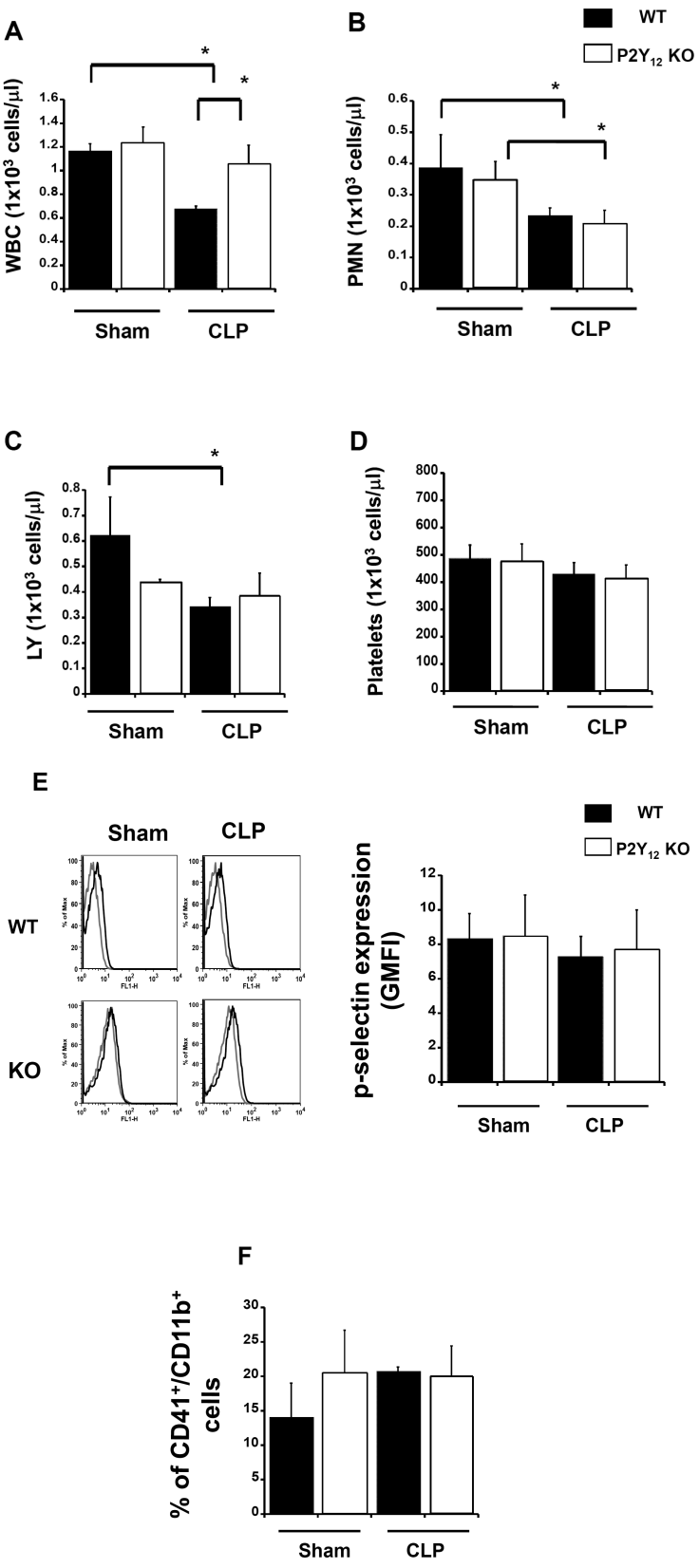


Figure 4. Circulating white blood cells counts, platelet activation, and platelet-leukocyte aggregate formation are not increased in $P2Y_{12}$ null mice

Blood samples were collected by cardiac puncture in 3.8% sodium citrate (10:1), and hematology studies performed. Graphs show counts of (**A**) white blood cells (WBC), (**B**) neutrophils (PMN), (**C**) lymphocytes (LY), and (**D**) platelets in WT $P2Y_{12}$ KO mice. Both sham and CLP samples were analyzed. Values are expressed as 1×10^3 cells/μL, mean ± S.E.M., ($n = 8$; $^{**}p<0.01$ WT CLP versus 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 labelled 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 versus WT CLP and KO CLP versus WT, $n = 6$). **(G**) Plasma samples obtained from each animal were utilized for detection levels of TNF-α, Il-10, IL-6 and MIP-1b 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 ± S.E.M. ($^{*}p < 0.05$; $^{**}p < 0.01$; KO CLP model versus WT CLP, n=5). (**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 40x; $n = 5$). Acute lung injury (ALI) score, was assessed in KO mice. (**I**) MPO analysis was performed in lung samples of sham and CLP mice. Values are expressed as rfu/min/mg, mean ± SEM ($n = 5$). (**J**) Representative images of CD41 and CD11b staining (CD41: green; CD11b: red; Nucleus: blue; 20x) for CLP and sham samples for both WT and KO mice. Images are representative of 3 different experiments.



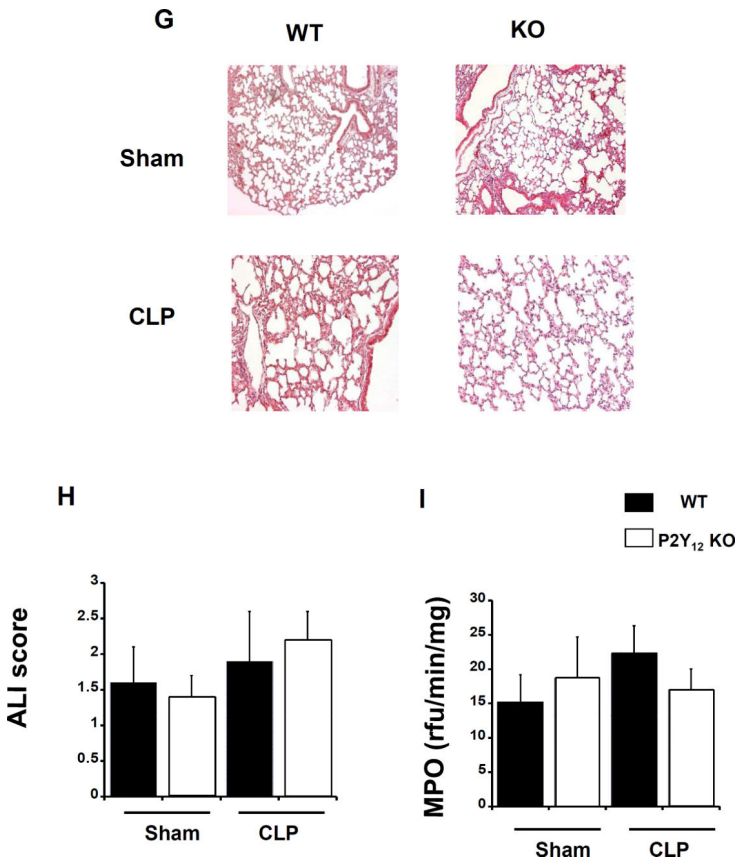
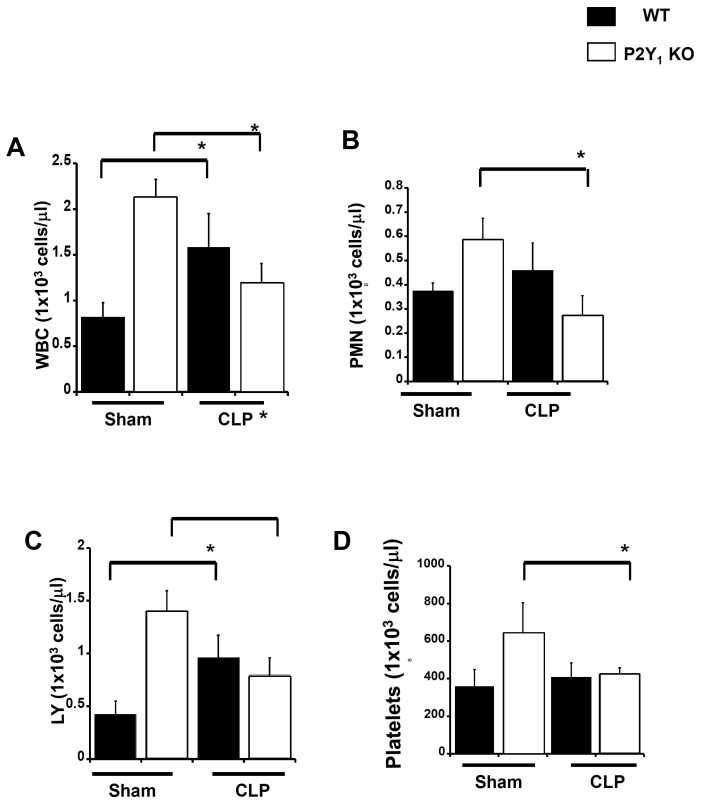


Figure 5. Clopidogrel treatment alter circulating neutrophil in septic P2Y$_{12}$ null mice

Blood samples were collected by cardiac puncture in 3.8% sodium citrate (10:1), and hematology studies performed. Graphs show counts of (**A**) white blood cells (WBC), (**B**) neutrophils (PMN), (**C**) lymphocytes (LY), and (**D**) platelets in clopidogrel-treated WT (black) and P2Y$_{12}$ null mice (white). Both sham and CLP samples were analyzed. Values are expressed as 1×10^3 cells/μL, mean ± S.E.M., ($n = 8$; *$p<0.05$ WT CLP versus KO CLP mice and *$p<0.05$ treated-CLP WT versus treated-CLP P2Y$_{12}$ 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 P2Y$_{12}$ 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 P2Y$_{12}$ null (white) mice. Blood samples were labelled 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 P2Y$_{12}$ null (white) mice. (**H**) Acute lung injury (ALI) score, was assessed in Sham and CLP clopidogrel-treated WT (black) and P2Y$_{12}$ null (white) mice. (**I**) MPO

analysis was performed in lung samples of sham and CLP mice for Sham and CLP clopidogrel-treated WT (black) and $P2Y_{12}$ null (white) mice. Values are expressed as rfu/min/mg, mean ± SEM ($n = 5$).



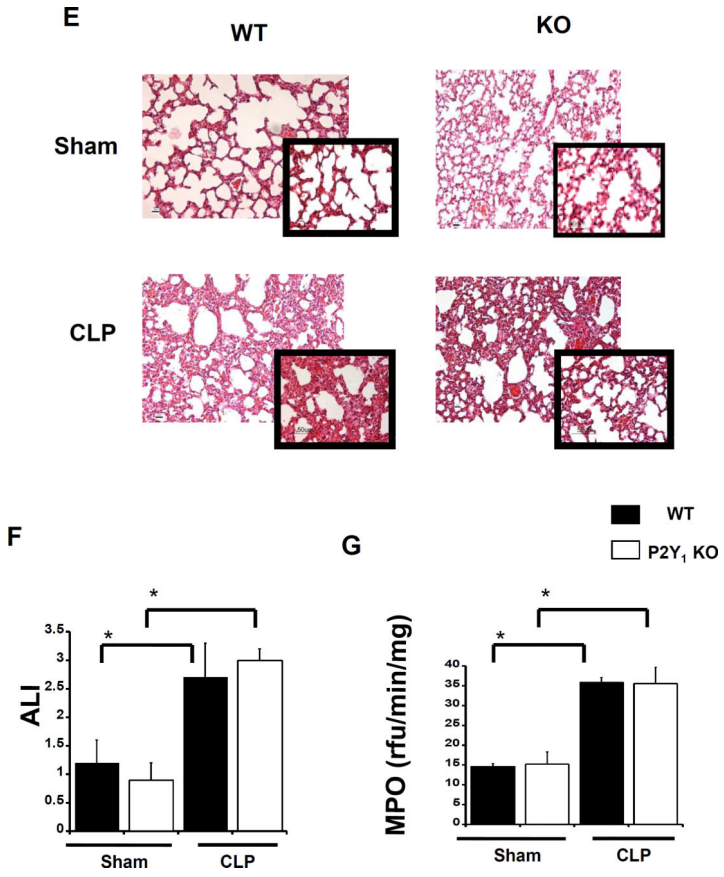


Figure 6. Inflammation-induced elevation in circulating white blood cell, platelet counts, and lung injury are not altered in $P2Y_1$ null mice

Blood samples were collected by cardiac puncture in 3.8% sodium citrate (10:1), and hematology studies were performed. Graphs show counts of (**A**) white blood cells (WBC), (**B**) neutrophils (PMN), (**C**) lymphocytes (LY), and (**D**) platelets in WT (black) and $P2Y_{12}$ KO (white) mice. Both sham and CLP samples were analyzed for wild-type and KO mice. Values are expressed as 1×10^3 cells/μL, mean ± S.E.M. (**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 40x; $n = 5$). (**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). (**F**) 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/mg, mean ± SEM (*$p < 0.05$; KO CLP model versus WT, $n = 5$).