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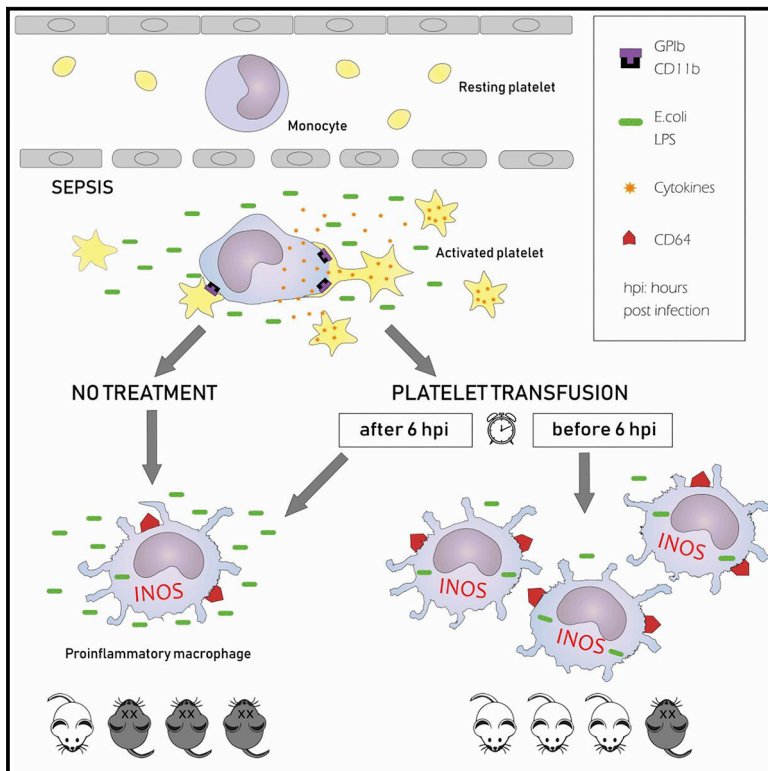
Platelets Promote Macrophage Polarization toward Pro-inflammatory Phenotype and Increase Survival of Septic Mice

Graphical Abstract

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In Brief

Carestia et al. describe the beneficial role of platelet transfusion for animal survival during sepsis by reprogramming phagocytic cells and fostering antimicrobial functions. The cross-talk between platelets and monocytes that promotes pro-inflammatory macrophages depends on the intimal cellular contact between platelet GPIb and CD11b in the monocyte surface.

Highlights

- Platelets sequester pro- and anti-inflammatory cytokines released by monocytes

- In the presence of LPS, platelets skew monocytes toward a pro-inflammatory phenotype

- During sepsis, platelet transfusion increases iNOS+ macrophages and bacterial clearance

- Platelet transfusion increases septic mice survival in a narrow time frame

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Platelets Promote Macrophage Polarization toward Pro-inflammatory Phenotype and Increase Survival of Septic Mice

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SUMMARY

We investigated the contribution of human platelets to macrophage effector properties in the presence of lipopolysaccharide (LPS), as well as the beneficial effects and time frame for platelet transfusion in septic animals. Our results show that platelets sequester both pro-(TNF-α/IL-6) and anti-(IL-10) inflammatory cytokines released by monocytes. Low LPS concentrations (0.01 ng/mL) induced M2 macrophage polarization by decreasing CD64 and augmenting CD206 and CD163 expression; yet, the presence of platelets skewed monocytes toward type 1 macrophage (M1) phenotype in a cell-contact-dependent manner by the glycoprotein Ib (GPIb)-CD11b axis. Accordingly, platelet-licensed macrophages showed increased TNF-α levels, bacterial phagocytic activity, and a reduced healing capability. Platelet transfusion increased inducible nitric oxide synthase (iNOS)$^+$ macrophages, improving bacterial clearance and survival rates in septic mice up to 6 h post-infection, an effect that was abolished by CD11b and GPIb blockade. Our results demonstrate that platelets orchestrate macrophage effector responses, improving the clinical outcome of sepsis in a narrow but relevant time frame.

Q1 Q2 INTRODUCTION

Beyond their critical role in hemostasis, platelets can regulate inflammation and infection through multiple mechanisms. Platelets may act as sentinels in the circulation, rapidly bind blood-borne pathogens, and activate several mechanisms that control innate resistance to infection (Semple, Italiano and Freedman, 2011; Kapur et al., 2015; Kapur and Semple, 2016). Platelets express major innate immune-related molecules, including Toll-like receptors (TLRs), complement receptors, adhesion molecules, and mediators essential for recruiting neutrophils and monocytes (Kral et al., 2016; Li, Zarbock and Hidalgo, 2017). Although lipopolysaccharides (LPSs) have limited effects on platelet activation, platelet-derived TLR4 appears to be critical for sensing LPS levels and promoting interaction with neutrophils and monocytes (Deppermann and Kubes, 2016).

Whereas platelet interactions with monocytes-macrophages play a relevant role in response against bacterial infections, it remains unclear whether this interaction is beneficial or harmful for the host. In vitro studies are limited and controversial, showing the inhibition or induction of either pro- or anti-inflammatory cytokines during co-culture of platelets with monocytes-macrophages followed by stimulation with LPS (Scull, Hays and Fischer, 2010; Gudbrandsdottir, Hasselbalch and Nielsen, 2013; Xiang et al., 2013). Furthermore, in vivo studies also showed controversial results. Although formation of circulating platelet-monocyte aggregates appears to amplify the inflammatory response and predicts mortality in older septic patients (Rondina et al., 2015), several studies have demonstrated that platelets contribute to bacterial clearance and resolution of inflammation by regulating macrophage responses (Xiang et al., 2013; de Stoppelaar et al., 2014; Wuescher, Takashima and Worth, 2015; Ali et al., 2017). In this sense, in a murine model of septic shock induced by intraperitoneal injection of bacteria, platelets inhibit the release of macrophage-derived pro-inflammatory molecules and rescue mice from death, highlighting the beneficial therapeutic role of platelets in severe septic patients (Xiang et al., 2013). However, given the differences between mouse and human macrophages, the biological relevance of platelet-macrophage interactions to human innate immunity remains uncertain.

Depending on prevalent stimuli in inflammatory microenvironments, human macrophages can be differentiated from circulating monocytes in vitro and polarized toward pro- or anti-inflammatory macrophages (typically recognized as classically activated-type 1 macrophage (M1) and alternatively activated-M2 macrophages, respectively). Microbes, damaged tissues, as well as the tissue microenvironment shape

macrophage polarization both spatially and temporarily, highlighting the plasticity of this process. This plasticity is critical for the initiation and resolution of the inflammatory response (Martinez and Gordon, 2014; Murray et al., 2014; Murray, 2017).

The biology of macrophage differentiation and polarization has mainly focused on soluble stimuli (such as cytokines and TLR ligands); however, the influence of cellular interactions in macrophage reprogramming has been poorly explored. In this regard, co-culturing human monocytes with regulatory T cells skews the balance toward differentiation of M2 macrophages (Tiemessen et al., 2007). Moreover, a recent study demonstrated that apoptotic cells are key players in the acquisition of an M2 phenotype when exposed to interleukin-13 (IL-13) and IL-4 (Bosurgi et al., 2017).

We hypothesized that platelets may skew the monocyte program toward a pro-inflammatory M1 phenotype in the presence of LPS, a key point to restrict bacterial dissemination, and that cell-contact-dependent crosstalk is critical for inducing the effector response. To further understand the role of platelets under septic conditions, we performed platelet transfusions at different time points after infection and evaluated outcomes in infected mice, as well as bacterial clearance and macrophage responses. The present work provides new insights into platelet biology, highlighting the role of these cells in promoting leukocyte reprogramming and enhancing immunity during critical life-threatening infections such as those associated with sepsis.

RESULTS

Platelets Sequester Both Pro- and Anti-inflammatory Cytokines Released by Monocytes

Human platelets were co-cultured with sorted CD14^{+} monocytes and stimulated with LPS for 24 and 48 h to evaluate the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α) and IL-6 and the anti-inflammatory cytokine IL-10, respectively. Monocyte stimulation with LPS (0.0001–1 ng/mL) resulted in a significant release of TNF-α, IL-6, and IL-10, whereas no cytokines were observed in LPS-stimulated platelets (Figures 1A–1C). The presence of platelets in the co-culture significantly reduced the amount of TNF-α and IL-6 released (Figures 1A and 1B). Surprisingly, we also observed that platelets significantly dampened IL-10 levels (Figure 1C), suggesting that cytokine reduction by platelets is not selective for pro- or anti-inflammatory mediators. Given that monocytes are the main source of cytokines after LPS stimulation and that they did not show significant differences in cell death after culture in the presence or absence of platelets (83.08% ± 4.48% versus 81.67% ± 4.53% of viable cells respectively), we first evaluated if platelets induced an alteration of cytokine synthesis in monocytes. The intracellular levels of TNF-α in monocytes, after 24 h of LPS stimulation, was similar regardless of whether monocytes had been co-cultured in the absence or presence of platelets (Figures 1D and S1A). We then sought to examine the possibility that platelets could sequester cytokines released by LPS-stimulated monocytes. Platelets were removed from the 24-h co-culture by fast washing with 10 mM EDTA buffer, and intracellular TNF-α was determined in platelets by flow cytometry. As shown in Figure 1E, TNF-α was detected inside platelets that had been

co-cultured with LPS-stimulated monocytes, indicating that platelets were loading cytokines (see Figure S1B for gating strategy). This observation was confirmed by analyzing cytokine localization in the 24-h co-culture of platelets and LPS-stimulated monocytes by confocal microscopy. Figure 1F shows co-localization images of the platelet-specific marker CD41 and cytokines (TNF-α, IL-6, and IL-10). The observation that the addition of platelets even at later time points reduced the amount of cytokines in supernatants of co-cultures indicated that reduction of cytokines was not due to platelet scavenging of LPS (Figure S1C).

Platelets Polarize Monocytes toward M1 Pro-inflammatory Profile in the Presence of LPS

Pro-inflammatory (M1) macrophages, characterized by the secretion of pro-inflammatory cytokines, high expression of CD64, and low expression of CD206 and CD163, are responsible for pathogen phagocytosis and killing. Anti-inflammatory (M2) macrophages, which are involved in tissue repair and provide regulatory signals, express high levels of CD206 and CD163 and lower levels of CD64 antigens (Martinez and Gordon, 2014). Given the functional relevance of these macrophage populations, we evaluated whether platelets could modulate differentiation, polarization, and function of monocyte-derived macrophage (M-DM) after 5 days co-culture in the presence of a broad range of LPS concentrations. Interestingly, stimulating monocytes with very low LPS concentrations (0.0001–0.01 ng/mL) induced differentiation to M2 macrophages by decreasing CD64 and augmenting CD206 and CD163 expression levels. In contrast, a highly pronounced pro-inflammatory M1 phenotype was observed when cells were exposed to increasing LPS concentrations (Figures 2A–2D). Remarkably, in the presence of platelets, monocytes were predominantly polarized toward the M1 phenotype, independent of LPS concentrations, by upregulating CD64 and decreasing CD206 and CD163 expression (Figures 2A–2D). Moreover, AXL and MERTK, which are tyrosine kinase receptors that are differentially expressed in pro-inflammatory M1 versus anti-inflammatory M2 macrophages (Zagórska et al., 2014), showed a clear dichotomous expression pattern, as in the presence of platelets and LPS, macrophages predominantly expressed AXL, thus reinforcing the notion that platelets polarize monocytes toward the M1 macrophage phenotype (Figure 2E).

To determine whether phenotypic changes in monocytes-platelet-derived macrophages (M/P-DM) correlated with the expected functions, intracellular TNF-α production and GFP-Escherichia coli phagocytosis assays were performed. Classical M1 responses and the healing response of human umbilical vein endothelial cells (HUVECs) challenged with conditioned media from platelet-induced macrophages were assessed as a readout of the anti-inflammatory M2 response. We observed that stimulation of CD14^{+} monocytes with 0.01 ng/mL of LPS for 5 days induced a macrophage phenotype characterized by the production of intermediate levels of TNF-α (Figure 2F), with a lower capacity of bacterial phagocytosis but increased wound-healing properties, consistent with an M2 phenotype (Figures 2G and 2H). However, in the presence of platelets and LPS, macrophages showed significantly higher production of intracellular
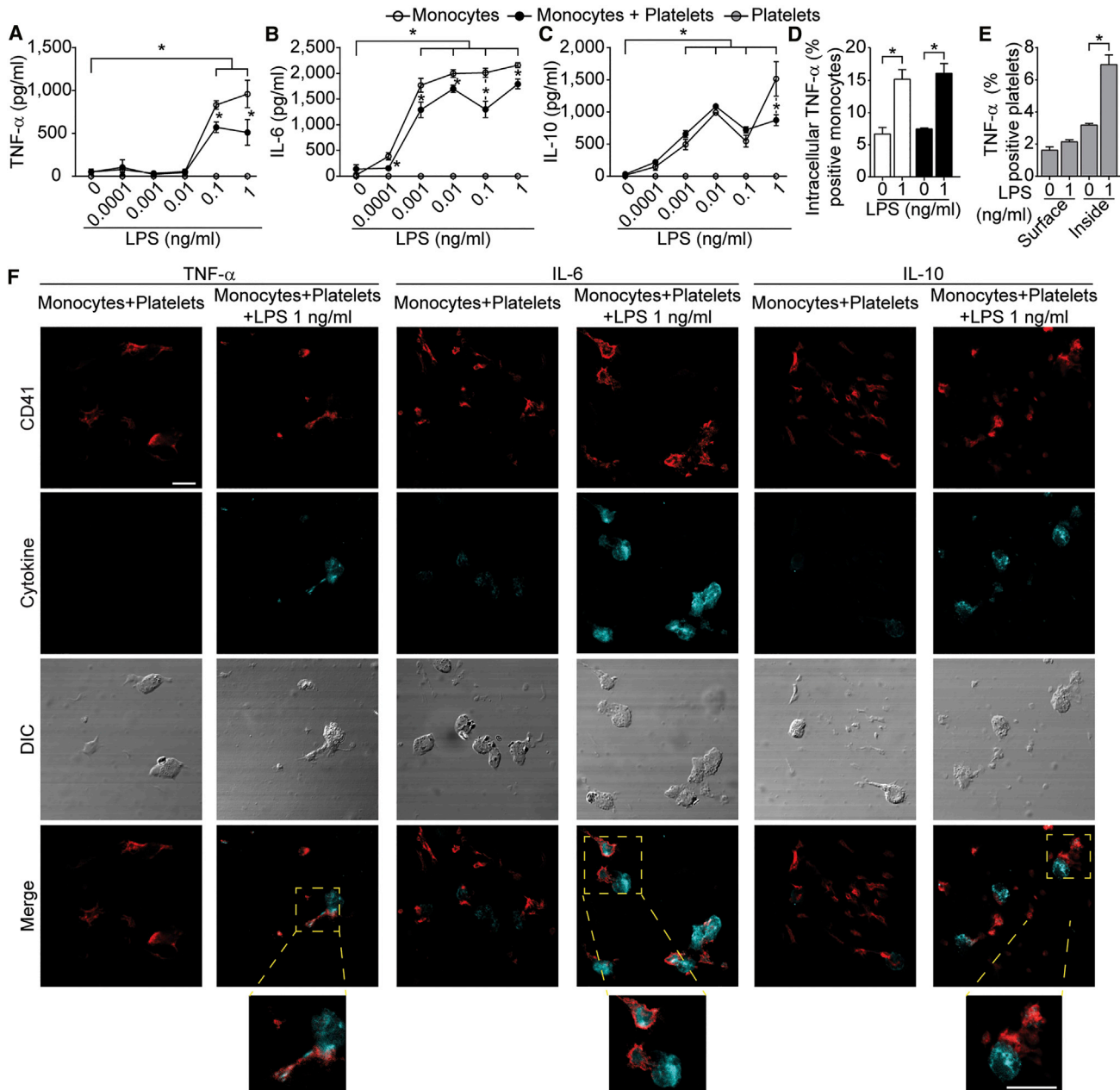
Figure 1. Platelets Decrease Pro- and Anti-inflammatory Cytokines Released by Monocytes

(A–C) Monocytes were stimulated with different concentrations of LPS in the presence or absence of platelets. Pro-inflammatory (A) TNF-α and (B) IL-6 and the (C) anti-inflammatory cytokine IL-10 were measured in the supernatants after 24 and 48 h of co-culture, respectively.

(D) Intracellular levels of TNF-α were analyzed in monocytes by flow cytometry after 24 h of co-culture.

(E) After 24 h of culture, platelets were harvested from the co-culture by adding 10 mM EDTA, stained with a PE-conjugated Ab against TNF-α, and analyzed by flow cytometry.

(F) The specific platelet marker CD41 (red) and the pro-inflammatory TNF-α and IL-6 and anti-inflammatory IL-10 (cyan) cytokines were analyzed by confocal microscopy after 24 h of monocyte + platelet co-culture. Representative images (60×, zoom 3×) of platelet and cytokine staining, co-localization, and differential interference contrast (DIC) of co-culture are shown (scale bar: 10 μm).

Independent data from each experiment, n = 4–6. Two-way ANOVA using the Fisher least significant difference (LSD) test, one-way ANOVA with the Tukey multiple comparison test, or two-tailed paired Student's t test were used. Statistical significance was set at $p < 0.05$. *$p < 0.05$. Data are represented as mean ± SEM.

See also Figure S1.
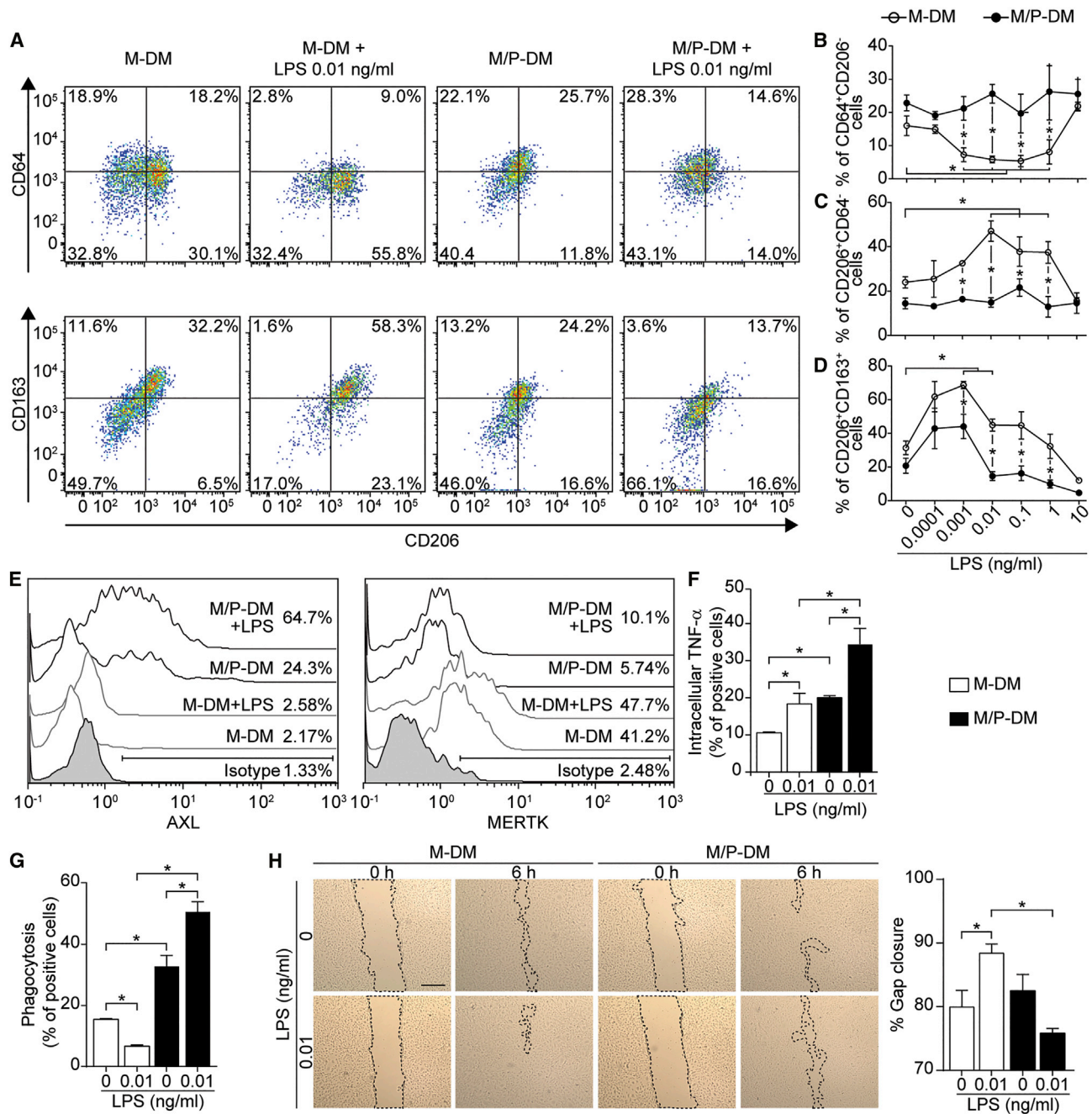
Figure 2. Platelets Polarize Monocytes to Pro-inflammatory Macrophages

Monocytes were cultured in the presence or absence of platelets and stimulated with different concentrations of LPS for 5 days to obtain monocyte-derived macrophages (M-DM) and monocyte-platelet-derived macrophages (M/P-DM).

(A) Representative dot plot showing CD64 versus CD206 and CD163 versus CD206, after gating on viable cells and CD11b^{+} macrophages, are shown.

(B–D) The percentages of (B) CD64^{+}CD206^{−}, (C) CD206^{+}CD64^{−}, and (D) CD206^{+}CD163^{+} cells are graphed.

(E) Representative levels of AXL and MERTK expression in macrophages are shown.

(F) Macrophage intracellular levels of TNF-α were analyzed by flow cytometry, and the percentage of positive cells after 5 days of co-culture is graphed.

(G) Macrophage phagocytosis of E. coli-GFP was assessed, and the cells were analyzed by flow cytometry. The percentages of GFP-positive cells are shown.

(H) After 5 days of macrophage differentiation in the presence or absence of platelets and LPS, the cells were re-stimulated with phorbol 12-myristate 13-acetate (PMA; 50 nM) for 1 h, and the supernatant was used to stimulate confluent HUVEC monolayers that had been scratched. Cell migration into the scratched area was calculated by analyzing images with ImageJ software at 0 and 6 h. Representative images (4×) of the kinetics of gap closure. (scale bar: 200 μm).

Independent data from each experiment, n = 6–10. Two-way ANOVA using the Fisher LSD test and one-way ANOVA with the Tukey multiple comparison test were used. Statistical significance was set at p < 0.05. *p < 0.05. Data are represented as mean ± SEM.
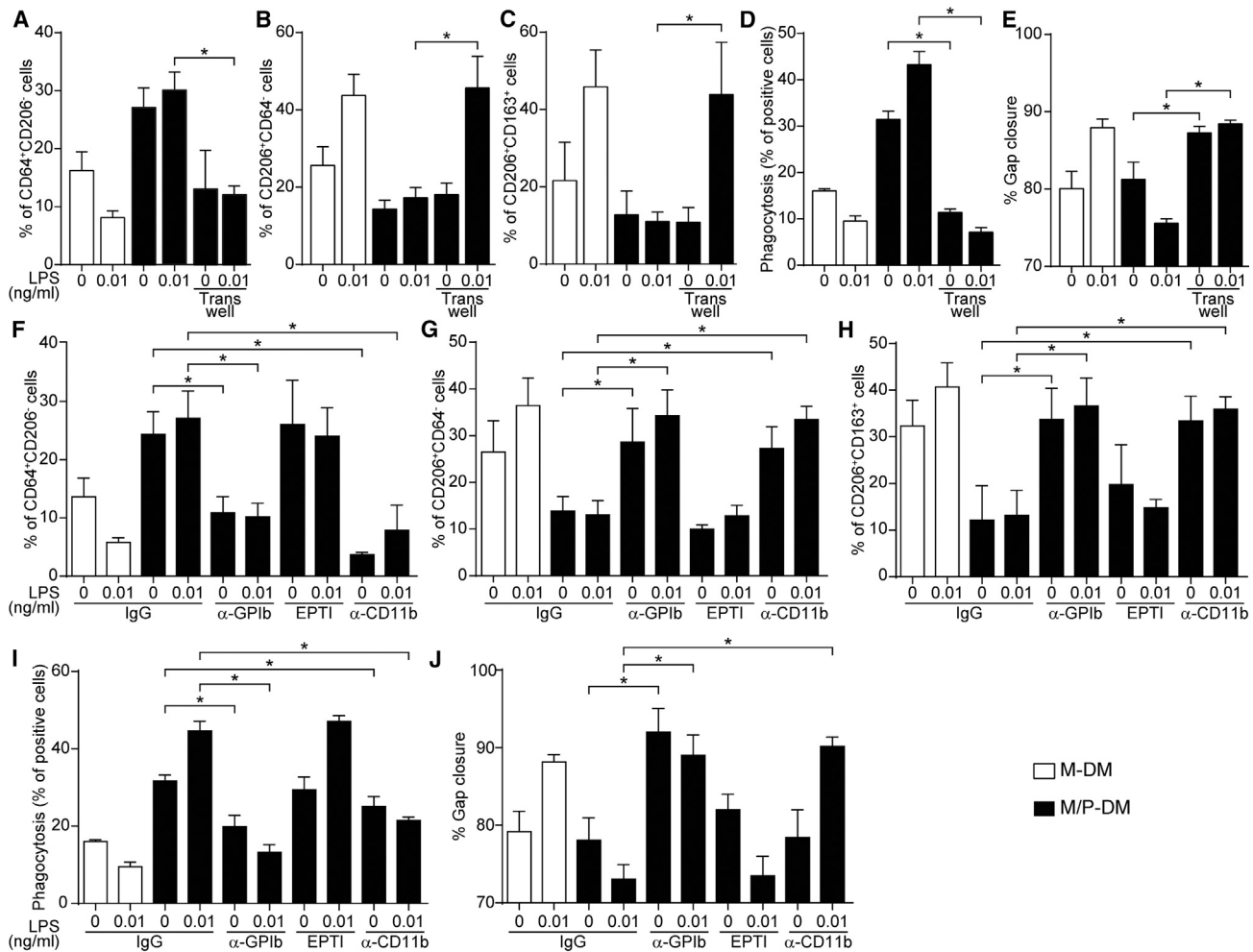
Figure 3. Platelet-Mediated Polarization of M1 Macrophages Is Dependent on Cell-Cell Contact and the GPIb-CD11b Partnership

(A–E) A transwell system was used to evaluate cell contact dependency.

(A–C) The percentage of (A) CD64$^+$CD206$^-$, (B) CD206$^+$CD64$^-$, and (C) CD206$^+$CD163$^+$ cells revealed phenotype characterization.

(D and E) Percentage of $E. coli$ phagocytosis (D) and percentage of gap closure (E) revealed functional assays of macrophage differentiated in the presence or absence of platelets (M/P-DM and M-DM, respectively) and 0.01 ng/mL of LPS.

(F–J) An isotype IgG control, blocking Abs against glycoprotein (GP)Ib and CD11b, or eptifibatide was added to the co-culture, and phenotype phenotype (CD64$^+$ in F; CD206$^+$ in G; CD206$^+$CD163$^+$ in H) and functional (phagocytosis in I and Gap closure in J) analyses were performed at day 5.

Independent data from each experiment, n = 6. One-way ANOVA with the Tukey multiple comparison test was used. Statistical significance was set at p < 0.05. *p < 0.05. Data are represented as mean ± SEM.

See also Figures S2 and S3.

TNF-α, increased bacterial phagocytic activity, and reduced ability to induce endothelial wound healing (Figures 2F–2H), which typically delineates an M1 signature.

Platelet-Mediated Polarization of M1 Macrophages Is Dependent on Cell-Cell Contact and the Glycoprotein Ib (GPIb)-CD11b Partnership

Cell-contact dependency was first determined using a Transwell system. When platelets and monocytes were physically separated, phenotypic and functional M1 polarization was eliminated even in the presence of LPS, (Figures 3A–3E). CD41$^+$ positive platelets, attached to the surface or located inside of monocytes-macrophages were evaluated after 12, 24, 48, 72, and 96 h of initial co-culture. We observed that 40%–60% of monocytes had surface-bound or internalized platelets during the first 24 h (Figure S2A). Remarkably, when platelets were removed from co-culture after 24 h washing, M1 macrophage polarization still occurred. This result highlights the relevance of an early engagement of a macrophage differentiation program by platelets (Figure S2B). Furthermore, adding platelets 24 or 48 h after monocytes started differentiation was not effective in tilting the balance toward macrophage M1 polarization, suggesting that differentiation was already in progress (Figure S2C). These results reinforce our hypothesis that the initial contact between platelets and monocytes is critical for defining the fate of monocyte-macrophage differentiation and polarization reprogramming.
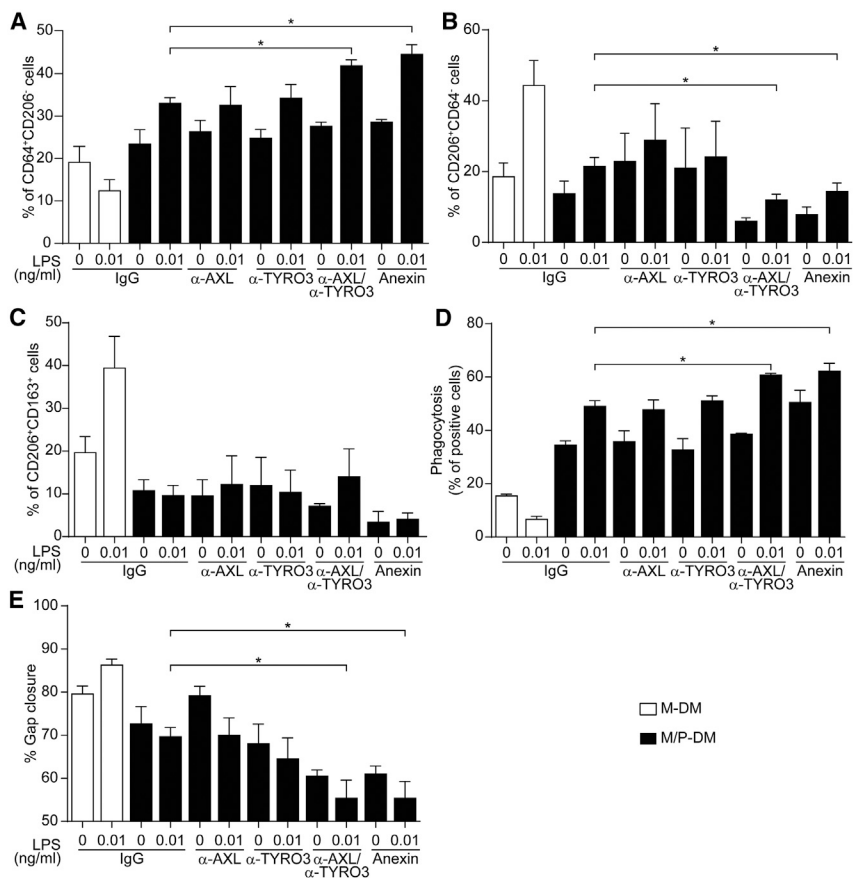
Figure 4. Platelet-Mediated Polarization of M1 Macrophages Is Tempered by Tyrosine Kinase TYRO3 and AXL Receptors

(A–E) Monocytes were pre-treated with Abs against AXL or TYRO3 or both and then co-incubated with platelets. Additionally, recombinant annexin V was added into the co-culture to block PtSer. Phenotype analysis revealed the percentages of (A) CD64⁺CD206⁻, (B) CD206⁺CD64⁻, and (C) CD206⁺CD163⁺ cells, and functional assays revealed (D) the percentage of *E. coli* phagocytosis and (E) the percentage of gap closure after 5 days of macrophage differentiation in the presence or absence of platelets (M/P-DM and M-DM, respectively) and 0.01 ng/mL of LPS. (n = 6).

One-way ANOVA using the Fisher LSD test was performed. Statistical significance was set at $p < 0.05$. *$p < 0.05$. Data are represented as mean ± SEM.

See also Figure S4.

phenotype was confirmed using a blocking antibody (Ab) against PF4 (Figures S3F–S3H).

TAM Receptors Regulate Platelet-Mediated Macrophage M1 Polarization

TYRO3, AXL, and MERTK (TAM) are key negative regulatory players in innate cells activated by two endogenous ligands: protein S (PROS1) and growth-arrest-specific 6 (GAS6). Phosphatidylserine (PtSer) exposure is crucial for TAM-ligand engagement (Rothlin et al., 2015), and this pathway has been shown to be critical in macrophage polarization (Bosurgi et al., 2017). For this reason and given that platelets contain PROS1 and expose PtSer after activation as shown in Figure S4 and in previous studies (Bevers, Comfurius and Zwaal, 1983; Schwarz et al., 1985) and monocytes express mainly AXL and TYRO3, we evaluated this negative regulatory signal by blocking both receptors. Simultaneous inhibition of both AXL/TYRO3 receptors enhanced the M1 macrophage signature (Figures 4A–4E). Furthermore, adding annexin V, a competitor of PROS1 for PtSer binding, from the initiation of co-culture, recapitulated the phenotype observed after AXL and TYRO3 blockade (Figures 4A–4E).

Platelet Transfusions Protect Mice from Septic Shock by Inducing Inducible Nitric Oxide Synthase (iNOS)⁺ Macrophages

Having demonstrated that platelets reprogram monocytes toward the M1 pro-inflammatory phenotype, we hypothesized that platelet transfusion might be beneficial at early time points during sepsis development by promoting monocyte skewing toward the M1 phenotype. To address this issue, a sepsis model was induced by intraperitoneal (i.p.) injection of 1 × 10⁹ GFP-*E. coli* in male C57BL/6J mice. As expected, infected animals showed thrombocytopenia as early as 2 h post-infection (hpi),
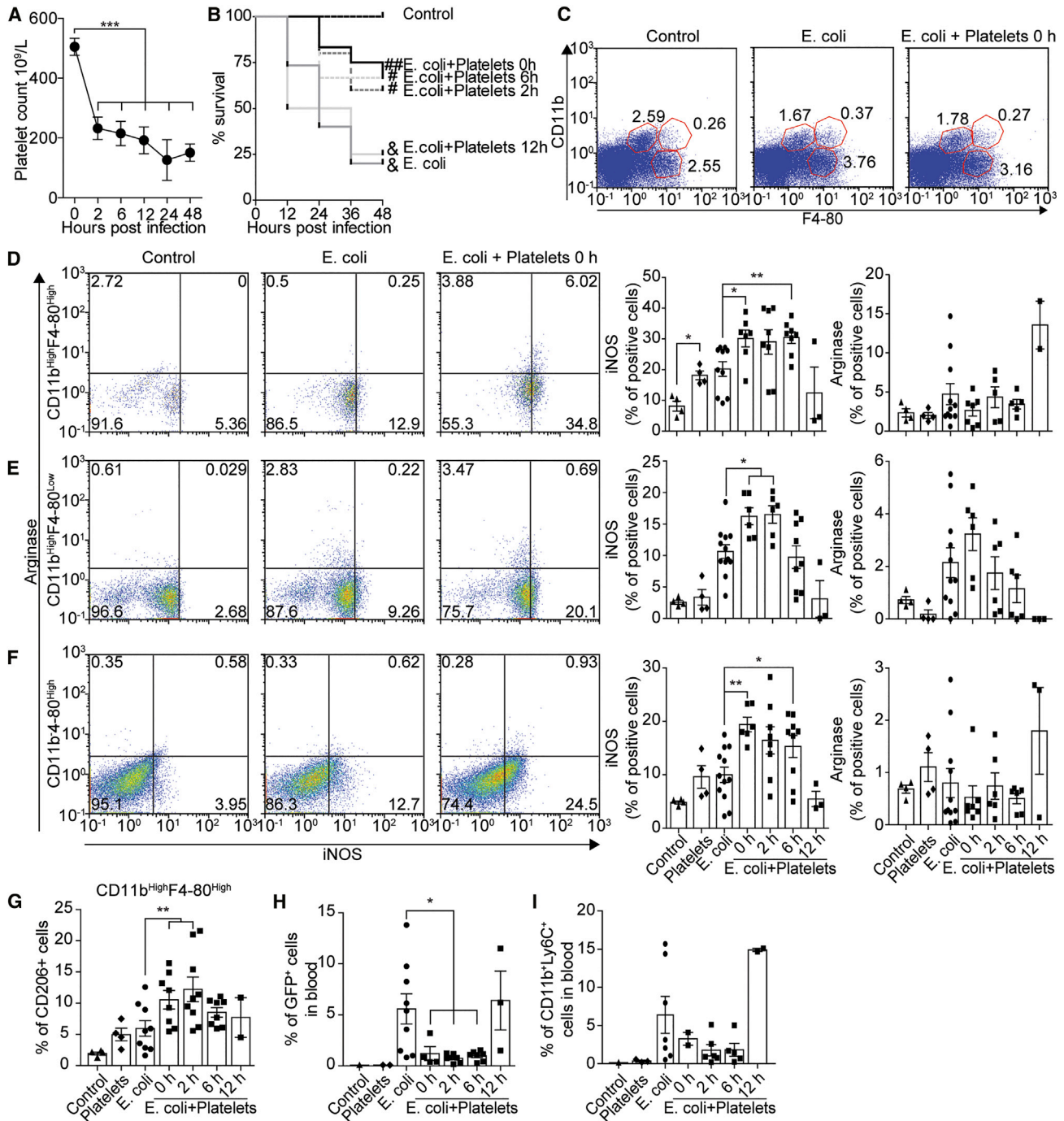
To further analyze the contribution of cell-cell interactions, we then evaluated the role of glycoprotein (GP) Ib and integrin αIIbβ3, two of the major platelet surface receptors. Polarization of monocytes toward the M1 phenotype was prevented when co-culture was performed in the presence of an anti-GPIb-blocking monoclonal antibody (mAb) but not when eptifibatide (integrin αIIbβ3 antagonist) was used (Figures 3F–3J). Considering that CD11b is a counter-receptor for GPIb (Simon et al., 2000), we then investigated the role of this molecule in macrophage polarization. Blocking CD11b completely prevented the switch toward M1 macrophages (Figures 3F–3J). Taken together, these results highlight the role of the GPIb-CD11b axis in platelet-mediated macrophage polarization.

Cell-contact dependency does not preclude a role for soluble factors after association occurs. Because LPS triggers the activation of both platelet cyclooxygenase (COX) and platelet factor 4 (PF4) (Carestia et al., 2016), we next evaluated the role of these pathways in the induction of the M1 program. We used aspirin-treated platelets, which were washed before co-culture to inhibit COX and heparin to block PF4 activity during the co-culture. Reprogramming of platelet-induced monocyte polarization toward the M1 phenotype was not altered by inhibition of platelet COX (Figures S3A–S3E). However, induction of a pro-inflammatory macrophage program during the co-culture was partially reversed by using heparin (Figures S3A–S3E). Furthermore, the partial role of platelet PF4 in the induction of an M1 macrophage

Figure 5. Platelet Transfusion Protects Mice from Septic Shock by Inducing iNOS+ Macrophages

Septic shock was induced in male C57BL/6 mice by i.p. injection of 1×10^9 E. coli, and the mice were monitored for 48 h.

(A) Platelet numbers were measured at different time points after infection.

(B) Survival curves of mice infected with E. coli (n = 15), and mice infected with E. coli plus a platelet transfusion immediately, indicated as 0 (n = 12), or 2 (n = 9), 6 (n = 9) or 12 (n = 4) hpi. Non-infected animals (n = 4) and animals that received only the platelet transfusion (n = 4) are indicated as a control.

(C–F) Representative dot plots of spleen CD11b/F4-80 subpopulations are shown after gating on live cells. The percentages of iNOS+ and arginase+ cells (C) were analyzed in the (D) CD11b^{high}F4-80^{high}, (E) CD11b^{high}F4-80^{low}, and (F) CD11b^{neg}F4-80^{high} subpopulations. Representative dot plot of iNOS versus arginase in each subpopulation and independent data are shown. The fluorescence minus one (FMO) controls of iNOS and arginase were used to set the threshold.

(G) The percentage of CD206+ cells in the spleen CD11b^{high}F4/80^{high} population is shown.

associated with an increased number of circulating granulocytes and a significant reduction of circulating lymphocytes (Figures 5A and S5A). Multi-organ failure was confirmed by measuring plasma concentrations of alanine aminotransferase enzymes (ALTs) and aspartate aminotransferase enzymes (ASTs), lactate dehydrogenase (LDH), and creatine kinase, which were significantly increased in the plasma of septic mice, indicating tissue damage and organ dysfunction (Figure S5C). Histo-pathological analysis of liver and lung confirmed the clinical findings (Figure S6D). At 12 hpi, animals started to die, reaching more than 80% mortality at 48 hpi (Figure 5B). To determine whether platelet transfusion ameliorates septic shock, washed platelets (0.25×10^9 per mouse) were infused by retro-orbital injection immediately or 2 h, 6 h, or 12 h after *E. coli* infection. Efficiency of platelet transfusion was determined by injecting carboxyfluorescein succinimidyl ester (CFSE)-labeled platelets and analyzing the percentage of labeled platelets in circulation at different time points after transfusion by flow cytometry (Figure S5B). Remarkably, platelet transfusions increased mice survival not only when performed immediately but also when given 2 or 6 h after *E. coli* infection (60%) compared with infected animals not receiving platelet transfusions (20%) (Figure 5B).

To confirm the hypothesis that platelet transfusion induces pro-inflammatory macrophages, iNOS and arginase were evaluated in three spleen subpopulations, namely, CD11b^high^/F4-80^low^, CD11b^high^/F4-80^high^, and CD11b^neg^/F4-80^high^, in infected animals (Figure 5C). Notably, transfusion of platelets performed immediately, 2, or 6 hpi increased the percentages of iNOS^+^ macrophages in the three cell subpopulations studied without changes in arginase expression (Figures 5D–5F). We also observed that platelet transfusion increased CD206^+^ cells in the CD11b^high^/F4-80^high^ spleen macrophage subpopulation (Figure 5G), suggesting the onset of the resolution process. Of note, a similar increase in iNOS^+^ cells in the CD11b^high^/F4-80^high^ subpopulation was observed in transfused non-infected animals (Figures 5D–5G).

Furthermore, infected mice that received a platelet transfusion showed reduced percentages of GFP^+^ *E. coli* bacteria (Figure 5H) and patrolling pro-inflammatory monocytes (CD11b^+^Ly6C^+^) in the peripheral blood (Figure 5I).

Then, we aimed to confirm the effects of platelets in macrophage polarization by using another model of sepsis. We observed that the high-grade sepsis model induced by cecal ligation puncture (CLP) reached 80% mortality after 5 days of surgical procedure, and a significant increased survival rate was observed after platelet transfusions (Figure S6A). Additionally, a significant drop in platelets number was observed in CLP animals compared to controls and CLP plus platelets (Figure S6B). Furthermore, mice receiving platelets augmented the percentage of spleen pro-inflammatory F4/80^high^CD11b^high/low^ iNOS^+^ M1 macrophages compared to CLP and controls (Figures S6C and S6D).

Our data indicate that platelet transfusion during sepsis induces a pro-inflammatory macrophage program that facilitates clearance of bacterial load and results in an increased survival rate.

In Vivo Blockade of CD11b or Platelet GPIb Suppresses the Protective Effect of Platelet Transfusion in Septic Mice

Finally, we evaluated the *in vivo* relevance of CD11b/GPIb interactions between myeloid cells and transfused platelets to the observed increased survival rate of septic mice by using two different strategies. First, we treated mice with anti-CD11b Ab prior to infection and then masked GPIb molecules in washed platelets prior to transfusion. Blocking CD11b 1 h prior to infection and platelet transfusion significantly reduced the survival rate of infected animals that received platelets (Figure 6A). Similarly, blocking GPIb on platelets before transfusion also decreased the survival rate of septic animals (Figure 6A). Furthermore, a significant increase in bacteraemia was observed after blocking both molecules (Figure 6B), and a significant reduction in iNOS^+^ cells was detected in splenic F4-80/CD11b macrophage when either CD11b or GPIb were blocked (Figures 6C–6D).

DISCUSSION

In this study, we have demonstrated that platelets regulate the inflammatory monocyte response by sequestering both pro- and anti-inflammatory cytokines and reprogramming monocytes toward the M1 phenotype. Moreover, the transfusion of platelets at the beginning of the *E. coli*-induced sepsis model reduced mortality by increasing iNOS expression and bacterial clearance.

Although previous studies mostly used platelets activated by classical agonists, such as collagen, thrombin, or adenosine diphosphate (ADP) (Weyrich et al., 1996; Halvorsen et al., 2014; Vogel et al., 2016; Linke et al., 2017; Pierre et al., 2017), scarce data have been documented regarding platelet-monocyte interaction after LPS stimulation. Xiang et al. (2013) recently showed that platelets reduce TNF-α production by murine bone-marrow-derived macrophages at high LPS concentrations (1 ng/mL) and that platelets induce an anti-inflammatory response in murine macrophages. Here, we show that platelets reduced not only TNF-α but also IL-6 and IL-10 in human platelet monocytes co-cultured in the presence of a broad range of LPS concentrations. Intriguingly, we observed that cytokine reduction was due to platelet sequestration of monocyte-derived cytokines. In fact it has been long recognized that platelets can load plasma molecules (Banerjee and Whiteheart, 2017) and can selectively take up angioregulatory proteins in microscopic tumors (Klement et al., 2009). Furthermore, it has been recently shown that platelets can be loaded with ADAMTS-13

(H and I) The percentages of GFP-positive cells in the blood (H) and CD11b+Ly6C+ patrolling monocytes (I) are shown. Survival was compared using the Kaplan-Meier method, followed by the log-rank test.

One-way ANOVA with the Fisher LSD test was performed to compare *E. coli*-infected mice and infected animals that had also received a platelet transfusion. Statistical significances are indicated as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

Survival curve: &$p < 0.05$ versus control; #$p < 0.05$ and ##$p < 0.01$ versus *E. coli*. Data are represented as mean ± SEM.

See also Figures S5 and S6.
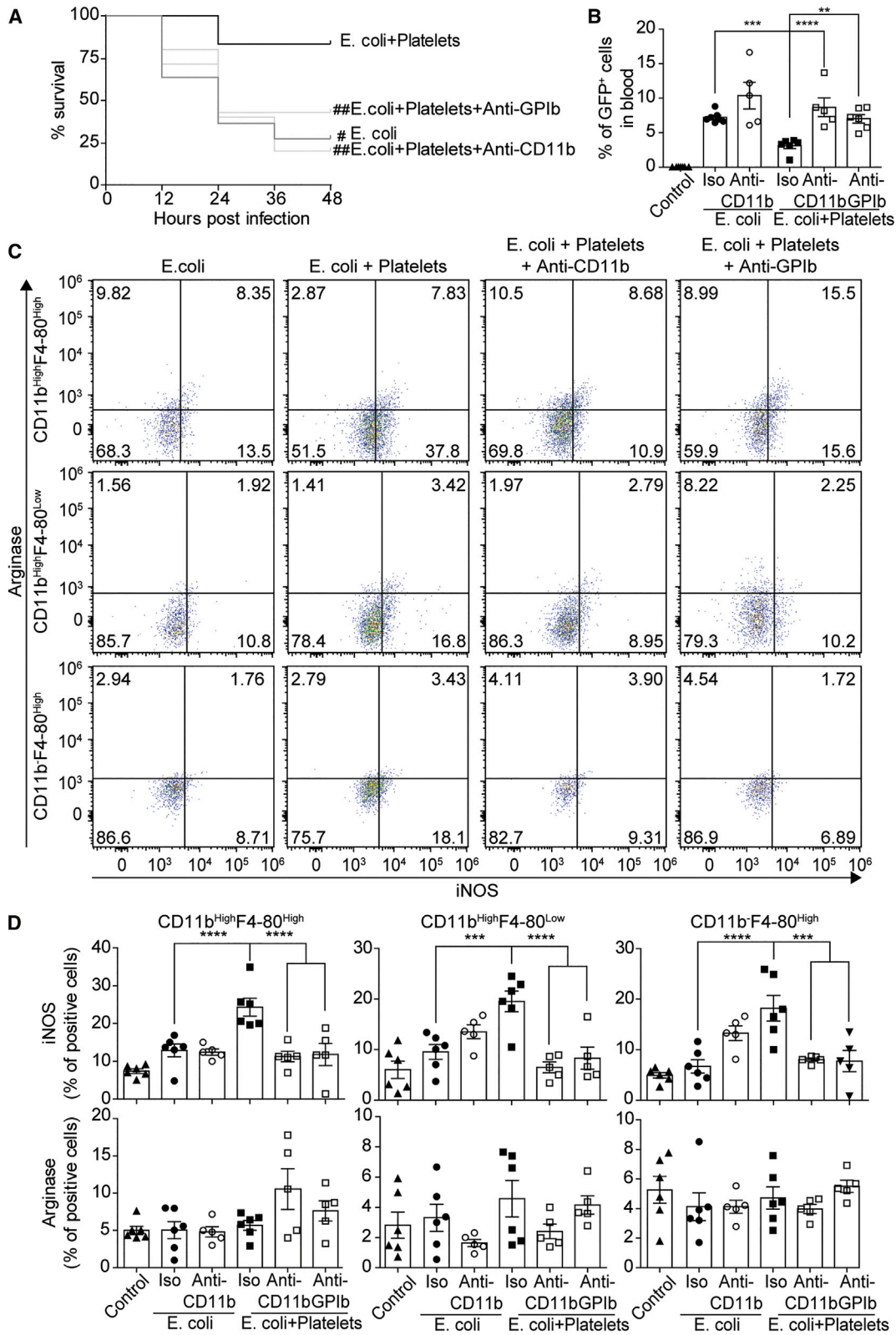
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(a disintegrin-like and metalloprotease with thrombospondin type 1 motif,13) for release upon activation (Abdelgawwad et al., 2018).It remains to be determined, whether, like ADAMTS-13, platelet-sequestered monocyte-derived cytokines upon LPS stimulation can be released at a distal site *in vivo*.

Remarkably, platelets also reprogrammed monocytes toward the M1 pro-inflammatory phenotype in a cell-contact-dependent manner (GPIb-CD11b partnership) and independently of the pro-inflammatory cytokine milieu, indicating that cytokine scavenging and macrophage polarization are two independent platelet-mediated regulatory effects on monocyte-macrophage biology. In mice, platelet GPIbα engages integrin Mac-1 to orchestrate monocyte differentiation signals through the forkhead transcription factor FOXP1 (Shi et al., 2004, 2008; Wang et al., 2017), and platelets can encase bacteria through a mechanism that involves "touch-and-go" between GPIb and von Willebrand factor expressed on Kupffer cells in the liver sinusoids (Wong et al., 2013). This platelet surveillance mechanism has been proposed to contribute to rapid host defense and early survival against certain blood-borne pathogens. Our data highlight an alternative strategy through which platelets may contribute to rapid bacterial clearance.

It has been shown that PF4 controls monocyte survival and differentiation (Scheuerer et al., 2000) as well as the induction of a distinct subtype of macrophages termed M4 (Gleissner, 2012) and that platelet-derived PGE$_2$ can regulate monocyte inflammatory responses (Xiang et al., 2013; Linke et al., 2017). However, our data indicate that PF4 is required only to some extent to trigger platelet-mediated M1 reprogramming and aspirin-treated platelets did not trigger changes in M1 macrophage polarization. Importantly, these discrepancies could be related to mouse and human differences. In addition, although most studies on human platelets show that LPS has no direct effect *in vitro* but can synergise with other platelet agonists (Rivadeneyra et al., 2014; Vallance et al., 2017), monocytes are highly sensitive to LPS. Hence, we speculated that monocytes may trigger cross-activation of platelets, enabling a bi-directional synergism that might induce the synthesis and/or release of additional soluble factors that still remain elusive.

Interestingly, we also found that the AXL and TYRO3 tyrosine kinase receptors, which are involved in the control of innate immunity, negatively regulated platelet-mediated monocyte polarization to pro-inflammatory macrophages. Of note, this effect appears to play a major role during the initial monocyte contact because blocking Abs were washed away before co-culture to avoid any effect on platelets. The observation that administration of recombinant Gas6 in a murine sepsis model results in lower mortality and absence of TAM receptors increase susceptibility to endotoxin shock (Giangola et al., 2013; Rothlin et al., 2015) supports the relevance of this pathway in sepsis. However, because the expression of these receptors changes during macrophage differentiation, further studies are necessary to deeply understand the TAM pathway during platelet-induced macrophage polarization.

In two different mouse models of sepsis (bacterial infusion and CLP), we showed that platelet transfusions significantly reduced animal mortality in a specific time frame. This information could be extremely relevant for clinical application, given the window through which platelet transfusion may be beneficial for patients with sepsis.

Interestingly, increased animal survival induced by platelet transfusion was previously ascribed to platelet-dependent inhibition of macrophage-mediated inflammatory response (Xiang et al., 2013). In contrast, our data suggest that the beneficial effect of platelet transfusion is due to an increase in iNOS$^+$ macrophages, which contribute to efficient bacterial clearance.

There is a growing appreciation for the contribution of platelets to immunity, and it was demonstrated that mouse platelets enhance phagocytosis and killing capacity against bacteria (Ali et al., 2017; Kraemer et al., 2011; Hamzeh-Cognasse et al., 2015). Additionally, allogeneic platelet transfusion can induce potent antibody-dependent immunity in mice, dependent on iNOS and interferon (IFN)-γ (Semple et al., 1995; Bang et al., 2000; Sayeh et al., 2004). Likewise, circulating immune complexes allow platelets to live longer and recirculate after degranulation, playing a critical role as mediators of the inflammatory response (Cloutier et al., 2018). Supporting experimental studies, a consortium report has shown that admission thrombocytopenia is associated with enhanced mortality and a more disturbed host response during sepsis independently of disease severity (Claushuis et al., 2016).

Notably, we found that the positive effect of platelet transfusion was limited to a narrow time window, reinforcing the notion that platelets play a protective role in sepsis during early stages of the host response to infection when inflammatory stimuli are still low. In addition, our findings suggest that the persistence of platelet-mediated M1 macrophage differentiation would be harmful to the host.

Beyond their central roles in hemostasis and thrombosis, platelets are now acknowledged as essential players during infection, which disrupt tissue integrity and contribute to inflammation, pathogen killing, and tissue repair (Koupenova et al., 2018). This progress has opened a new perspective in our understanding of sepsis pathophysiology (Dewitte et al., 2017). Thus,

Figure 6. *In Vivo* Blocking of CD11b or Platelet GPIb Suppress the Protective Property of Platelet Transfusions in Septic Mice

Septic shock was induced in male C57BL/6 mice by i.p. injection of 1 × 10^9 *E. coli*, and the mice were monitored for 48 h.

(A) Survival curve of septic mice after blocking CD11b molecule 1 h previous to the infection and platelet transfusion. Platelets GPIb was blocked 30 min before transfusion, washed, and then transfused.

(B) Bacteremia was analyzed as the percentage of GFP$^+$ cells in blood.

(C) Representative dot plots showing iNOS and arginase expression after gating in the three spleen CD11b^{high}F4-80^{high}, CD11b^{high}F4-80^{low}, and CD11b$^-$F4-80^{high} macrophage subpopulations.

(D) Independent data showing the percentage of INOS$^+$ or arginase$^+$ are graphed for each subpopulation. Animals receiving the same amount of isotype intravenously (i.v.) or platelets treated with isotype were used as controls. Survival was compared using the Kaplan-Meier method, followed by the log-rank test. One-way ANOVA with the Fisher LSD test was used, and statistical significance is indicated as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Survival curve: #$p < 0.05$, ##$p < 0.01$ versus *E. coli* + platelets. Data are represented as mean ± SEM.

the function of platelets extends beyond direct encounters with pathogens. Our findings identify a central role of platelet-monocyte interactions, which can occur intra- and extravascularly, in reprogramming phagocytic cells, thus fostering antimicrobial function during sepsis. These findings may have implications in other blood-associated infections and a wide range of pathophysiologic conditions, including sepsis, chronic inflammation, atherosclerosis, and cancer.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.06.062.

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AUTHOR CONTRIBUTIONS

A.C. performed the experiments, analyzed the data, prepared figures, and wrote the manuscript. H.A.M., C.M.O., and J.M.O.W. performed the *in vivo* experiments, analyzed data, and prepared figures. S.N., A.E.E., and R.M.G. analyzed data and provided valuable input on the writing of the manuscript. C.N.J. provided animals for blocking *in vivo* experiments, read, and revised the manuscript. E.A.C.S. and M.S. conceived, designed, and supervised the research, analyzed data, and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

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KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
PE mouse anti-Human CD41a clone HIP8	BD Biosciences	Cat# 555467; RRID:AB_395859
APC mouse anti-human CD64 clone 10.1	BioLegend	Cat# 305013; RRID:AB_1595539
Alexa Fluor® 488 mouse anti-human CD206 clone 15-2	BioLegend	Cat# 321113; RRID:AB_571874
PerCP/Cy5.5 mouse anti-human CD163 clone GHI/61	BioLegend	Cat# 333607; RRID:AB_1134006
PE/Cy7 mouse anti-human CD14 clone HCD14	BioLegend	Cat# 325618; RRID:AB_830691
APC/Cy7 rat anti-mouse/human CD11b clone M1/70	BioLegend	Cat# 101226; RRID:AB_830642
PE mouse anti-human TNF-alpha clone MAb11	BioLegend	Cat# 502909; RRID:AB_315261
PE Streptavidin	BioLegend	Cat# 405203
Dylight 649 Streptavidin	BioLegend	Cat# 405224
Alexa Fluor® 488 Donkey anti-Rabbit IgG clone Poly4064	BioLegend	Cat# 406416; RRID:AB_2563203
Biotin rat anti-human IL-6 clone MQ2-39C3	BioLegend	Cat# 501202; RRID:AB_2249232
Purified mouse anti-human CD42b (GPIb) clone HIP1	BioLegend	Cat# 303902; RRID:AB_314382
PerCP/Cy5.5 rat anti-mouse/human CD11b clone M1/70	BioLegend	Cat# 101227; RRID:AB_893233
APC/Cy7 rat anti-mouse F4/80 clone BM8	BioLegend	Cat# 123117; RRID:AB_893489
Alexa Fluor® 647 rat anti-mouse CD206 clone C068C2	BioLegend	Cat# 141712; RRID:AB_10900420
PE Mouse IgG1 κ Isotype Ctrl clone MOPC-21	BioLegend	Cat# 400140; RRID:AB_493443
Purified Annexin V	BioLegend	Cat# 640902; RRID: AB_2562279
LEAF Purified rat anti-mouse/human CD11b clone M1/70	BioLegend	Cat# 101214; RRID: AB_312797
PE sheep anti-human/mouse Arginase 1	R&D Systems	Cat# IC5868P
APC mouse anti-human MERTK clone 125518	R&D Systems	Cat# FAB8912A; RRID:AB_357213
Biotin goat anti-human AXL	R&D Systems	Cat# BAF154; RRID:AB_2062559
Goat anti-human AXL	R&D Systems	Cat# AF154; RRID:AB_354852
Goat anti-human TYRO3/DKT	R&D Systems	Cat# AF859; RRID:AB_355666
Normal Goat IgG control	R&D Systems	Cat# AB-108-C; RRID:AB_354267
Normal Goat IgG Biotinylated Control	R&D Systems	Cat# BAF108; RRID:AB_355828
Rabbit anti-human/mouse TYRO3	Novus Biologicals	Cat# NBP1-28635; RRID:AB_1914103
Mouse anti-human CD42b (GP1b) clone VM16d	Novus Biologicals	Cat# NBP1-42151; RRID:AB_2113946
Rabbit IgG1 anti-human PF4	Abcam	Cat# ab9561; RRID:AB_308720
FITC rat anti-human PROS1 clone PS7	Abcam	Cat# ab81713; RRID:AB_1640157
Rat IgG2a (RTK2758) FITC Isotype control	Abcam	Cat# ab18446
Biotin rat IgG2a anti-human IL-10 clone JES3-12G8	eBioscience	Cat# 13-7109; RRID:AB_466921
PE/Cy7 rat anti-mouse iNOS clone CXNFT	eBioscience	Cat# 25-5920-82; RRID:AB_2573499
Rat IgG2b anti human/mouse CD11b clone 5C6	Fisher Scientific	Cat# PIMA-516527; RRID:AB_2538032
Bacterial and Virus Strains		
E. coli DH5α GFP tagged	Ricardo Martín Gómez	N/A
Chemicals, Peptides, and Recombinant Proteins		
BD Cytofix/Cytoperm	BD Biosciences	Cat# 554714
BD GolgiStop	BD Biosciences	Cat# 554724
eBioscience Fixable Viability Dye eFluor 780	Invitrogen	Cat# 65-0865-14
eBioscience Fixable Viability Dye eFluor 450	Invitrogen	Cat# 65-0863-14
eBioscience CFSE	Invitrogen	Cat# 65-0850-84
Ficoll-Paque PLUS	GE Healthcare - Life Sciences	Cat# 17144003
Lipopolysaccharides from *Escherichia coli* O111:B4 (LPS)	Sigma-Aldrich	Cat# L3024

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Ionomycin from Streptomyces conglobatus	Sigma-Aldrich	Cat# I9657
Phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich	Cat# P1585
Heparin sodium salt	Sigma-Aldrich	Cat# H3149
Acetylsalicylic acid (Aspirin)	Sigma-Aldrich	Cat# A5376
2,2,2-Tribromoethanol (Avertin)	Sigma-Aldrich	Cat# T48402
Gelatin from bovine skin	Sigma-Aldrich	Cat# G-9391
Paraformaldehyde Powder	Cicarelli	Cat# 1088211
Penicillin-Streptomycin	GIBCO	Cat# 15-140-122
Fetal Bovine Serum, qualified, New Zealand	GIBCO	Cat# 10091148
RPMI 1640 Medium	GIBCO	Cat# 11875119
Eptifibatide acetate	Santa Cruz Biotechnology	Cat# sc-205675
Prostaglandin I2 (PGI_2)	Cayman Chemical Company	Cat# 18220
Triton X-100	ICN Biomedicals	Cat# 807426
Luria Bertani Broth medium (LB)	Neogen Acumedia	Cat# 7290A
Agar	BritaniaLab	Cat# B0101406
EGM-2 Endothelial Cell Growth Medium-2 BulletKit	Lonza	Cat# CC-3162
Normal Goat Serum	ThermoFisher	Cat# O16201
Critical Commercial Assays		
EasySep Human CD14 Positive Selection Kit	StemCell Technologies	Cat# 18058
Human IL-6 ELISA Ready-Set-Go!	eBioscience	Cat# 88-7066; RRID:AB_2574993
Human IL-10 ELISA Ready-Set-Go!	eBioscience	Cat# 88-7106; RRID:AB_2575001
Human TNF alpha ELISA Ready-Set-Go!	eBioscience	Cat# 88-7346; RRID:AB_2575093
FITC Annexin V Apoptosis Detection Kit I	BD Biosciences	Cat# 556547
Software and Algorithms		
GraphPad Prism	GraphPad Software Inc.	RRID:SCR_002798
FlowJo	FlowJo LLC.	RRID:SCR_008520
FIJI ImageJ	NIH	RRID: SCR_002285
illustrator	Adobe	RRID:SCR_010279

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and request for reagents may be directed to and will be fulfilled by the Lead Contact, Eugenio Antonio Carrera Silva (carrerasilva@yahoo.com.ar).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Study subjects

Anti-coagulated blood samples were obtained from male and female healthy volunteer donors (age range: 23-58 with average of 36.7 years old) who had not taken any non-steroidal anti-inflammatory drugs for 10 days prior to sampling. This study was approved by the Institutional Ethics Committee, National Academy of Medicine, Argentina. Written consent was obtained from all subjects.

Mice

Eight- to ten-week-old male and female C57BL/6 (B6) mice were provided by the animal facility of IMEX and maintained in a specific pathogen-free environment (SPF). Animals were purchased from Charles River and they were breed in our animal facilities for several generations. These animals are tested every 6 months at Charles River for SPF. All mice were maintained under a strict 12 h light cycle (lights on at 7:00 a.m. and off at 7:00 p.m.), and given food and water available *ad libitum*.

Mouse experiments were performed according to the National Institutes of Health guidelines with the approval of the Institutional Animal Care and Use Committee (CICUAL) at IMEX.

METHOD DETAILS

Blood sample and purification of human monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated through Ficoll-Hypaque (GE) density gradient centrifugation, as described previously (Carrera Silva et al., 2013). CD14$^+$ monocytes were isolated from PBMCs by an EasySep Human CD14 Positive Selection Kit (StemCell Tech, Vancouver, Canada).

Preparation of human platelets

Blood was drawn into plastic tubes containing sodium citrate (3.8%). Platelet-rich plasma (PRP) was obtained by centrifugation of the blood samples (200 × g for 10 min). For washed platelet (WP) suspensions, PRP was treated with prostacyclin (PGI$_2$, 75 nM) and centrifuged (900 × g for 10 min), and the platelets were washed in washing buffer (140 mM NaCl, 10 mM NaHCO$_3$, 2.5 mM KCl, 0.5 mM Na$_2$HPO$_4$, 1 mM MgCl$_2$, 22 mM sodium citrate, 0.55 mM glucose, pH 6.5). Finally, the platelets were resuspended in RPMI supplemented with 10% FBS and 1% penicillin streptomycin (PS) (RPMI/FBS/PS) and kept at room temperature (RT) until the experiment was performed.

In some experiments, platelets were pre-incubated with aspirin (0.5 mM) and then washed in the presence of PGI$_2$ to remove excess aspirin.

Monocyte-platelet co-culture

CD14$^+$ monocytes (2x10^5) were seeded in 48-well flat-bottom plates, and washed platelets (2x10^7) were added for co-culture conditions. Stimulation was performed with different concentrations of LPS for 24 h, 48 h or 5 days.

Cytokine levels were evaluated in supernatants after 24 and 48 h of co-culture by ELISA.

Macrophages were harvested after 5 days of differentiation and polarization for phenotypic analysis or functional assays. Phenotypic characterization of macrophages was achieved by surface staining of CD11b, CD64, CD163, CD206 and CD14. Bacterial phagocytosis and cell migration into the wounded area were used as functional assays. E. coli expressing green fluorescent protein (GFP) (GFP-E. coli) was employed for phagocytosis assay and supernatant of re-stimulated macrophages (PMA for 4h) was added for scratched assay (Mena et al., 2014). Intracellular staining of cytokines (TNFα, IL-6 and IL-10) was determined after 24 h of monocyte-platelets co-culture by flow cytometry and confocal fluorescence microscopy.

Blocking experimental conditions in monocyte-platelet co-cultures

Co-cultures were carried out in a humidified incubator at 37°C with CO$_2$ (5%). In some experiments, the co-culture was incubated with anti-glycoprotein (GP) Ib, clone HIP1, (20 μg/ml); eptifibatide (20 μg/ml); annexin V (2.5 μg/ml); anti-CD11b, clone M1/70, (10 μg/ml); heparin (5 U/mL) or rabbit IgG anti-human PF4 (2 μg/ml). Cell contact dependence was analyzed using a Transwell 24-System with 0.4 μm pores (Transwell COSTAR, Corning, Merck, Darmstadt, Germany). AXL and TYRO3 receptors on monocytes were blocked for 1 h before the co-culture. After washing, the platelets were added to the monocytes and stimulated with LPS. Supernatants were collected 24 and 48 h after LPS stimulation, and macrophages were harvested after 5 days of differentiation and polarization for phenotypic analysis or functional assays. Cell harvesting was performed by a 20-min incubation with PBS/2% FBS and 1 mM EDTA on ice. The harvested cells were washed once with PBS/2% FBS.

Cytokine levels

Supernatants from the co-cultures were collected after 24 (TNF-α and IL-6) or 48 h (IL-10), centrifuged and stored at −80°C until cytokines were measured using commercial ELISA kits (eBioscience, Ready Set Go).

Surface and intracellular staining and flow cytometry analysis

The surface staining for APCCy7-CD11b, APC-CD64, PercP/Cy5.5-CD163, AF488-CD206 and PECy7-CD14 (phenotypic characterization of macrophages) was performed following standard protocol. Briefly, the harvested cells were washed with PBS and blocked in PBS/2% FBS on ice for 30 min. The cells were washed with PBS and the respective Abs cocktails (prepared in PBS/2% FBS) were added to the cell pellet and incubated for 30 min on ice. A fixable viability dye was used according to the manufacturer's instructions to gate on live cells. After washing, the cells were fixed with a Cytofix/Cytoperm Kit (BD Biosciences), washed again and analyzed in a FACS Canto I (Becton Dickinson). All analysis was carried out with FlowJo software (Tree Star). AXL and MERTK expression were evaluated after fixation and permeabilization (Cytofix/Cytoperm Kit) using biotin-conjugated goat anti-human AXL and APC anti-human MERTK mAb IgG1 from R&D. PE-Streptoavidin (Biolegend) was used as detection signal for AXL.

For intracellular staining of TNF-α in monocytes, cells were re-stimulated with ionomycin (1 μg/ml) plus PMA (50 nM) and GolgiStop for an additional 4 h. The cells were then harvested, fixed, permeabilised (Cytofix/Cytoperm Kit) and stained with a PE-conjugated antibody (Ab) against TNF-α. PE Mouse IgG1 κ Isotype Ctrl clone MOPC-21 were used.

Platelet bound and internalized TNF-α

To determine the presence of TNF-α inside platelets or bound to the platelet surface, after 24 h of co-culture, platelets were removed by the addition of 10 mM EDTA for 1 min. The platelets were centrifuged and stained with a PE-conjugated Ab against TNF-α according to the protocol for surface and intracellular staining.

Bacterial phagocytosis

E. coli DH5α expressing green fluorescent protein (GFP) (GFP-*E. coli*) were grown at 37°C under aerobic conditions with shaking in Luria Bertani medium overnight. When the bacterial culture reached an OD of 0.6 (spectrophotometry), bacteria were added to the macrophage cultures (1:2500 v/v) and incubated for 1 h at 37°C. Then, the macrophages were washed, fixed and analyzed by flow cytometry.

HUVEC isolation and cell migration into the wounded area

Umbilical cords were collected from healthy donors. All individuals provided written informed consent for the collection of samples and subsequent analysis. Primary Human Umbilical Vein Endothelial Cells (HUVEC) were obtained from human umbilical cord vein by digestion with collagenase type I and characterize by the presence of Weibel–Palade bodies and the combined expression of endothelial markers (Mena et al., 2014). The cells were grown in a T25 flask previously coated with gelatin 2% and cultured in EGM2. HUVEC were used between second and fourth passages for experiments.

For the healing assay, after 5 days of co-culture, macrophages were re-stimulated with PMA for 4 h. The supernatants were collected, centrifuged and added to confluent HUVEC monolayers that had been scratched. The extent of cell migration into the wounded area was measured after 6 h (Mena et al., 2014).

Fluorescence Imaging

CD14+ monocytes were seeded in 48-well plates containing a glass slide on the bottom, co-cultured with platelets (1:100) and stimulated with LPS. After 24 h, the cells were fixed in 4% PFA for 10 min, washed and permeabilised with 0.1% Triton X-100 for 10 min. The slides were washed and blocked using blocking buffer (3% of goat serum in PBS). Biotin-conjugated rat anti-human IL-6 (clone MQ2-39C3) or IL-10 (clone JES3-12G8) or mouse anti-human-TNFα (Mab11) was used for cytokine determination. Primary Abs against the cytokines were incubated in blocking buffer for 60 min and then washed 3 times. Dylight 649-Streptoavidin (Biolegend) was used as detection signal and incubated for 60 minutes. After 3 washes, the platelets were labeled with mouse anti-human PE-CD41 (BD Bioscience). After 3 washes, the slides were mounted and analyzed by confocal fluorescence microscopy using an FV-1000 microscope (Olympus, Tokyo, Japan).

In vivo sepsis models

Sepsis model was induced in male mice by intraperitoneal (ip) injection of 1×10^9 GFP-*E. coli* per mouse in 200 μL of PBS (adapted from Xiang et al., 2013), which results in a 50% of mortality at 24 h and 80% at 48h post injection. The inoculation amount of *E. coli* was established by dose-response assay. After the injection of bacteria, survival was assessed every 4 h for 48 h. All surviving mice were euthanised at the end of the 2nd day.

In selected experiments cecal ligation and puncture (CLP) sepsis model was used. Briefly, high-grade sepsis was induced by 75% of cecal ligation with one puncture using 25G needle, adapted from Rittirsch et al. (2009). Under this condition more than 80% of mortality was observed after 5 days of surgery. Sham animals underwent exactly to the same procedures except for the CLP.

Platelet preparation

Orbital blood from Avertin-anesthetised mice was drawn into plastic tubes containing 10% by volume 3.8% citrate. The anticoagulated blood was diluted 1:1 in PBS/citrate 0.38% and platelet rich plasma (PRP) was obtained by centrifugation (5 minutes [min] at 100 g at room temperature [RT]). Washed platelets (WP) were obtained by centrifuging PRP (5 min at 200 g at RT) in the presence of PGI2 (75 ng/ml) and EDTA (2 mM) to avoid platelet activation. After washing, WP were re-suspended in RPMI and maintained at RT for 30 min prior to transfusion (0.25×10^9/150 ul/mice injected into the retro-orbital plexus) (Rivadeneyra et al., 2015).

Haematological analysis

Platelets and white cell counts were performed using an Abacus Junior Vet veterinary automated analyzer (Vienna, Austria).

Analysis of macrophages phenotype in spleen

A splenocyte suspension was obtained by mechanical separation employing a 60 μm mesh. After centrifugation, erythrocytes were lysed with hypotonic buffer, and after washing, the splenocytes (5×10^6) were blocked and stained for PercP/Cy5.5-CD11b, APCCy7-F4/80, and AF647-CD206 for 30 min on ice. Then, to determine intracellular iNOS and arginase, the cells were washed, fixed and permeabilised with the Cytofix/Cytoperm Kit (BD Bioscience). After 30 min of incubation with Abs (PE/Cy7 anti-mouse iNOS and PE anti-mouse/human Arginase-1) the cells were washed and analyzed in a Partec-Sysmex CyFlow flow cytometer (Görlitz, Germany).

Determination of GFP-*E. coli* in blood

Blood samples were obtained by ocular bleeding at the endpoint of the experiment. Plasma was separated by centrifugation (2500 rpm for 15 min), and red blood cells were lysed to obtain total leukocytes. The cells were fixed with 1% PFA, and the percentage of GFP-*E. coli* was evaluated directly in the total leukocytes by flow cytometry.

Blockade of CD11b and GPIb in mice

In vivo blockade of cellular contact mediated by CD11b and GPIb was performed by using anti-CD11b (clone 5C6, Thermo Fisher) and anti-GPIb (Emfred, Novus Biologicals) antibodies. Briefly, 100ug of anti-CD11b (5C6) antibody was injected intravenously (iv) per mouse, 1 h before *E. coli* infection and platelet transfusion. In other experiments, anti-GPIb (20 μg/ml) was added to washed platelets 30 minutes before transfusion, washed again and then transfused by retro-orbital injection. Animals receiving the same amount of isotype iv or platelets treated with isotype were used as controls.

QUANTIFICATION AND STATISTICAL ANALYSIS

In vitro

Data are expressed as the mean ± SEM. Significant differences between groups were determined using the two-tailed paired Student's t test, one-way ANOVA with the Tukey multiple comparison or Fisher LSD test or two-way ANOVA with the Fisher LSD test was performed. Bartlett's test for equal variances was also performed.

In vivo

One-way ANOVA with the Fisher LSD test was performed to compare *E. coli*-infected mice and infected animals that had also received a platelet transfusion. Survival was compared using the Kaplan-Meier method, followed by the log-rank test. Statistical significance was set at $p < 0.05$.

Supplemental Information

Platelets Promote Macrophage Polarization

toward Pro-inflammatory Phenotype

and Increase Survival of Septic Mice

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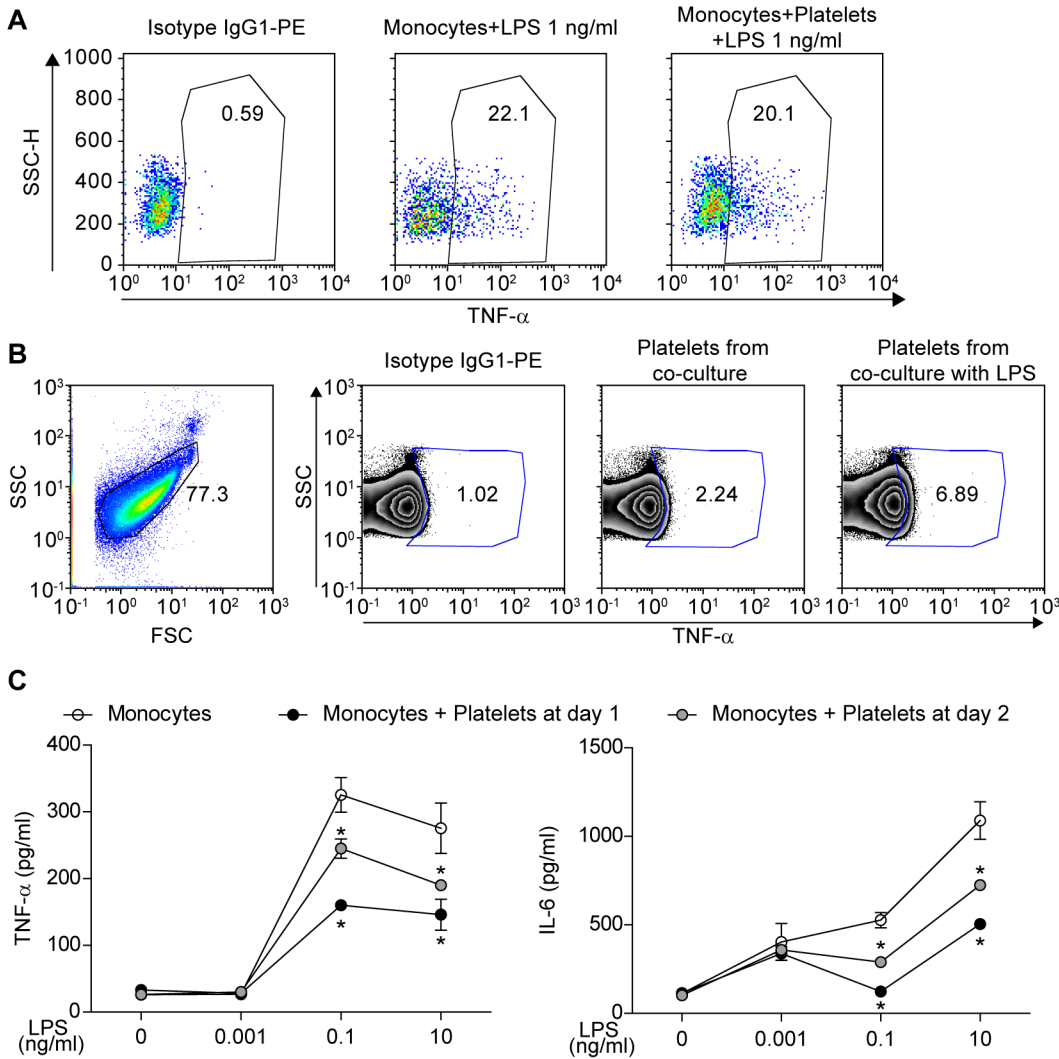
Supplementary figure 1. **Cytokine measurement in monocytes and platelets, Related to Figure 1.**
A) Representative dot plot showing intracellular TNF-α staining in cells pre-gated as monocytes after 24 h of culture in the presence or absence of platelets and LPS stimulation (1ng/ml). B) Gating strategy showing FSC vs SSC in log scale and TNF-α staining in platelets after 24 h of been co-cultured with monocyte stimulated or not with 1ng/ml of LPS. C) Monocytes were cultured and stimulated with LPS for 24 or 48 h and then platelets were added for an additional 24 h. TNF-α and IL-6 were measured in the supernatant of these co-cultures. Two-way ANOVA, *p<0.05 vs Monocytes with the same concentration of LPS.
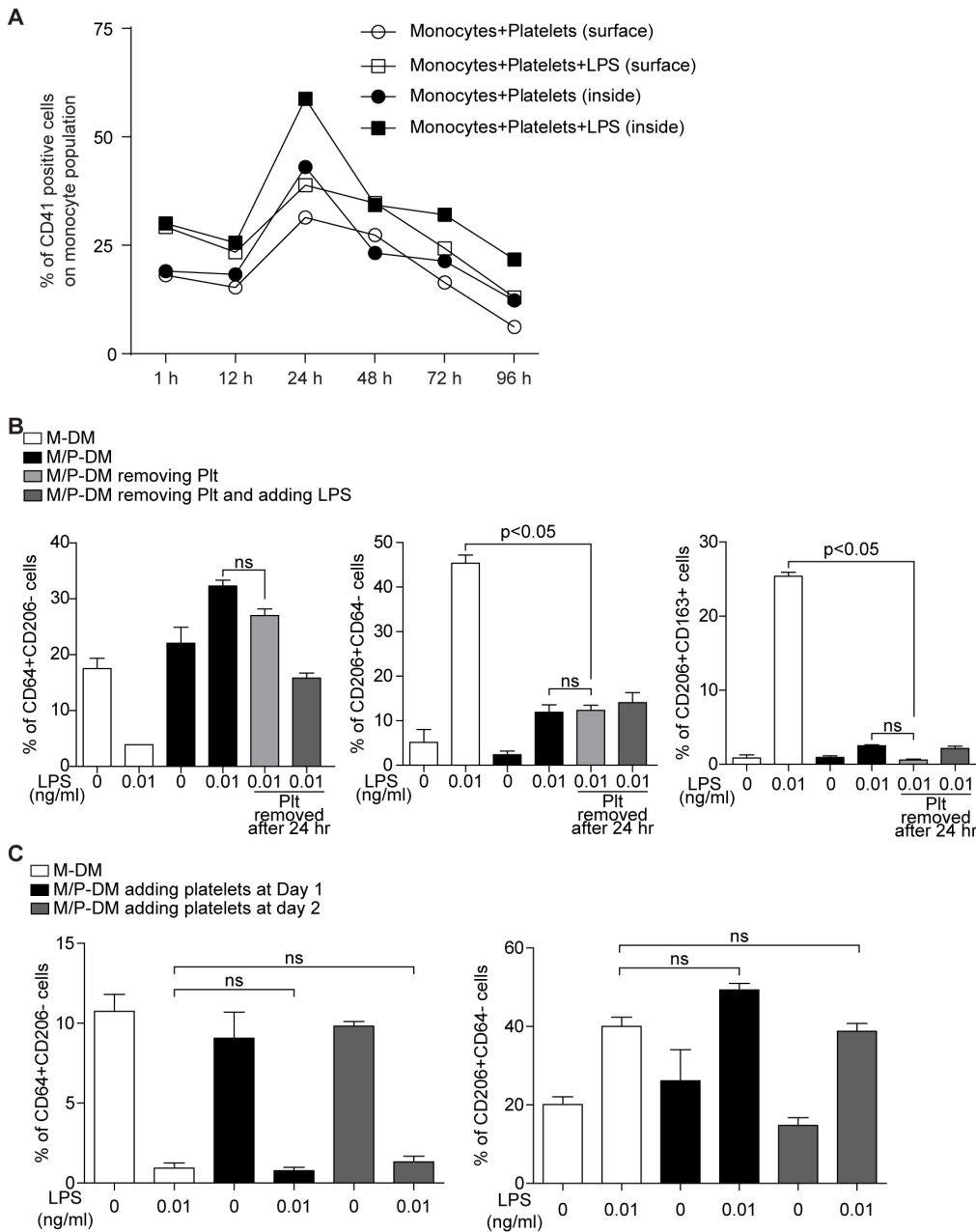
Supplementary figure 2. Early platelet-monocyte interaction is needed to induce pro-inflammatory macrophage, Related to Figure 3. (A) Kinetic of platelets bound to the cell surface (non permeabilised cells) or located inside of monocytes (permeabilised cells) during differentiation and polarisation of macrophage. Data represent the percentage of monocytes with CD41^{+} platelets associated to the surface (white box and circle) or inside of monocytes (black box and circle) after 12, 24, 48, 72 and 96 h of initial co-culture. N=3. B) Platelets were removed from monocyte co-culture after 24 h of starting, by washing, and monocyte-derived macrophages (M-DM) or monocyte/platelet-derived macrophages (M/P-DM) were analysed after 5 days of culture using the phenotype markers CD64, CD206 and CD163. C) Monocytes were cultured and stimulated with LPS for 24 or 48 h; then platelets were added and cultured together for additional 3 days. Macrophages were harvested at day 5 and phenotype markers were analysed by flow cytometry. One way ANOVA with the Tukey multiple comparison test. Results represent the mean ±SEM of at least 3 independent donors.
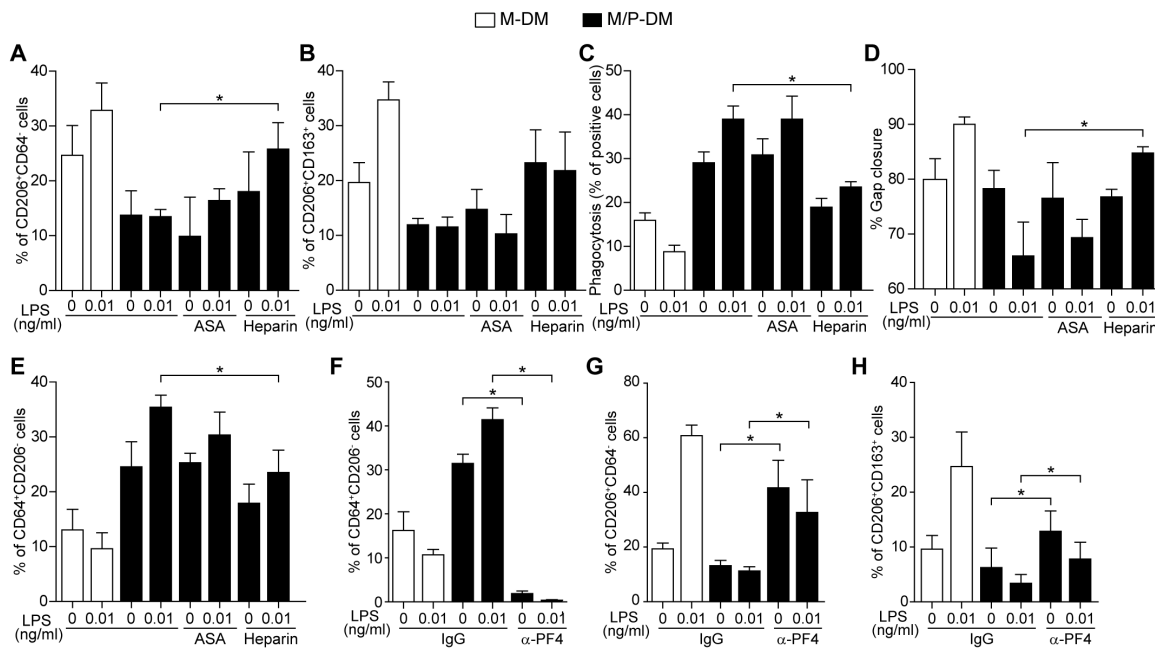
Supplementary figure 3. Pre-treatment of platelets with heparin or blocking PF4 partially reverses the effect of platelets on macrophage polarization, Related to Figure 3. (A-E) The platelets were pre-treated with ASA (0.5 mM), washed and then added to monocyte cultures. Alternatively, heparin (5 U/ml) was added to the co-culture and phenotype and functional analyses were performed on day 5 on monocyte-derived macrophages (M-DM) and monocyte/platelet-derived macrophages (M/P-DM). (F-H) A specific blocking anti-PF4 antibody was added to the platelet-monocyte co-culture, and the macrophage phenotype was analysed after 5 days. n=4, one-way ANOVA with the Tukey multiple comparison test were used. Statistical significance was set at p<0.05. *p<0.05.
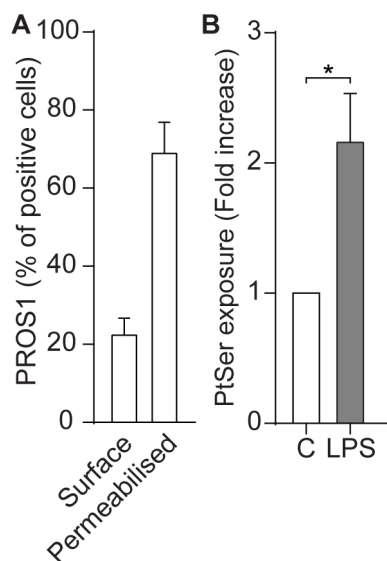
Supplementary figure 4. Resting platelets express protein S (PROS1) and expose phosphatidylserine (PtSer) after activation, Related to Figure 4. (A) PROS1 was evaluated on the surface or intracellularly in resting platelets using FITC anti-human PROS1 (PS7). The percentage of PROS1+ platelets is graphed. (B) PtSer exposure was evaluated in LPS-stimulated (1 μg/ml) platelets after 30 min using FITC-Annexin V kit and the fold induction is shown. n=4, Two-tailed, paired Student's t-test was used, *p<0.05.
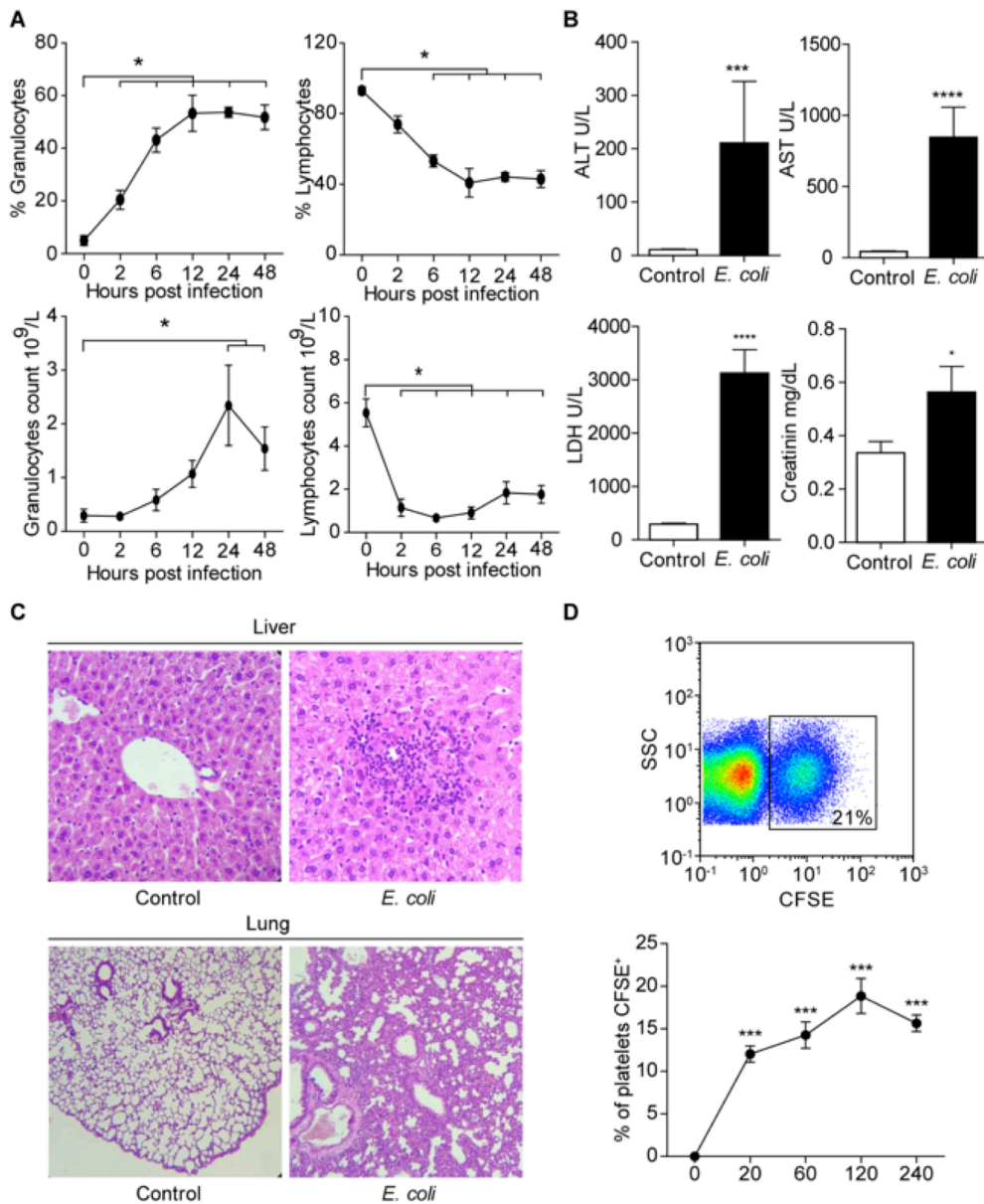
Supplementary figure 5. Sepsis and platelets transfusion validation, Related to Figure 5. A) Hematological analysis was performed at different time point after infection and % and absolute number of granulocytes and lymphocytes are graphed. Septic animals were followed up for 48 h or until animal decease (n=4). B) Plasma concentrations of alanine and aspartate aminotransferase enzymes (ALT and AST), Lactate dehydrogenase (LDH) and Creatine kinase in septic mice (n=8) during the first 24h of infection with 10^9 *E. coli* and compared to non-infected control mice (n=11). C) Histo-pathological sections of liver and lung from control and septic mice are shown. Spread inflammatory foci are found in *E. coli* infected mice compared to control mice in both tissues analysed (n=5). D) Platelet transfusion efficiency. Mouse platelets were isolated and labelled with CFSE and then transfused by retororbital injection. Circulating CFSE-label platelets were measured after 20, 40, 60, 120 y 240 minutes post transfusion by flow cytometer (n=4) and compared to 0 (previous transfusion). Results represent the mean ±SEM. One-way ANOVA with the Tukey multiple comparison test were used in A and D. Two-tailed Student's t-test was used in B and statistical significance is denoted as *p<0.05, ***p<0.001, ****p<0,0001.
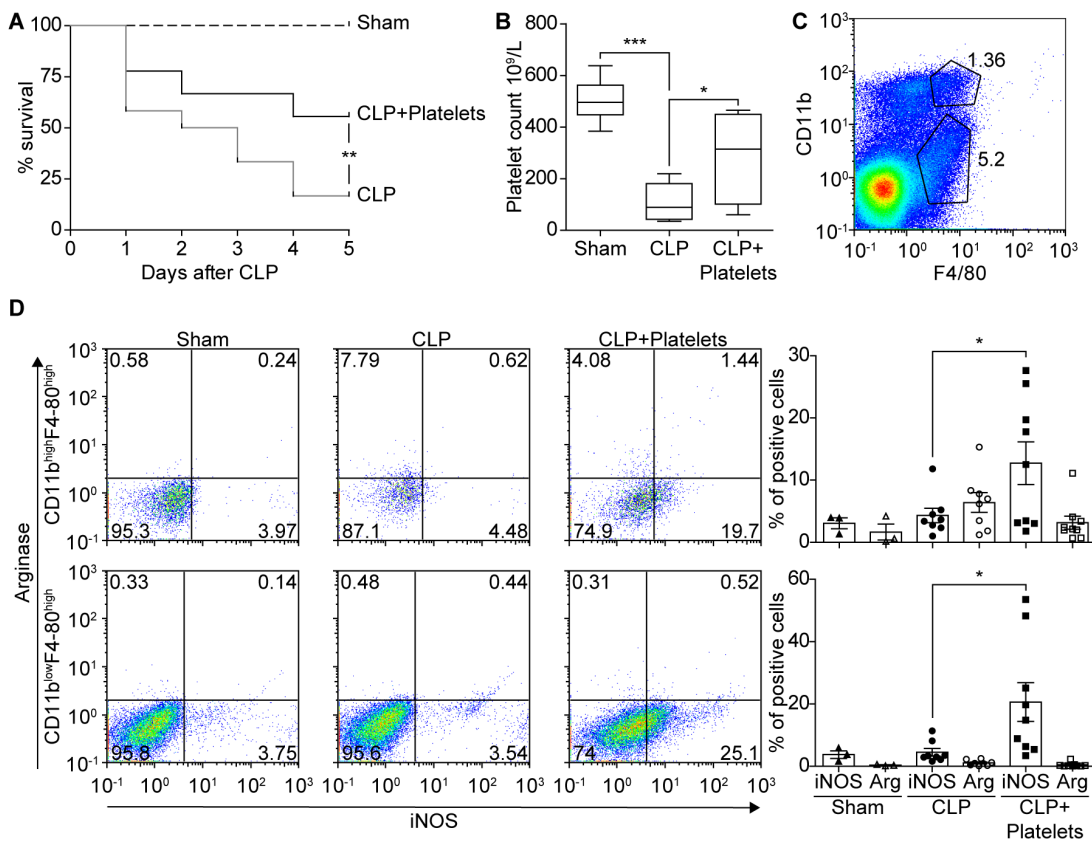
Supplementary figure 6. Platelet transfusion increases survival and induces pro-inflammatory macrophage in a CLP model of sepsis, Related to Figure 5. A) Survival curve of CLP mice (N=12) compared to CLP plus transfused platelets (N=9) and sham (N=3) mice after 5 days of the chirurgical procedure. B) Platelets number was analyzed after 48h of surgery by submandibular bleeding. C) Representative dot plot showing spleen macrophages gate based on F4/80 high expression and CD11b high or low. D) Representative dot plots showing iNOS and Arginase expression after gating on F4-80^{high}CD11b^{high} and F4-80^{high}CD11b^{low} macrophages. Independent data are shown on the side of dot plots. Survival was compared using the Kaplan-Meier method, followed by the log-rank test. One-way ANOVA with the Fisher LSD test was used to compare Sham, CLP and CLP plus platelets. Statistical significance was set at $p < 0.05$. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.