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Complete reversal of acid-induced acute lung injury by blocking of platelet-neutrophil aggregation

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Introduction

Acute lung injury (ALI) is a common disease with an incidence of 79 per 100,000 person-years in the United States (1). Despite the use of state-of-the-art treatment, this disease is associated with high mortality of up to 38% (1). Sepsis, trauma, and massive transfusion are all extrapulmonary causes of ALI whereas pneumonia and acid aspiration are typical causes of intrapulmonary ALI. Aspiration is a regular complication of general anesthesia (2, 3) and can also occur in critically ill patients and unconscious patients (4). Acid aspiration in WT mice, which mimics human ALI (5–7), can be induced experimentally by inflicting HCl-induced chemical damage of the alveolar-capillary membrane (8), resulting in polymorphonuclear neutrophil (PMN) recruitment (9), pulmonary edema, and impairment of gas exchange (7).

Recruitment of PMNs into the lung is a key event in the development of ALI (10). PMN activation, sequestration, and emigration into the lung proceed in a sequence of overlapping events (11). In contrast with the well-characterized multistep adhesion cascade of leukocyte recruitment in systemic microcirculation, the molecular requirements of leukocyte recruitment into the lung are incompletely defined. Due to the anatomic and molecular properties of the lung, PMN recruitment is only partially β2 integrin dependent (12).

Neutrophils can interact with adherent platelets and leukocytes in a process called secondary capture, which is often followed by neutrophil-endothelial interactions (13, 14). Platelet glycoprotein Ibα (GPIbα) (15) and GPIIb/GPIIIa (CD41/CD61, αIIbβ3 integrin) (16), endothelial P-selectin (17), and von Willebrand factor (18) have been shown to play important roles in platelet-endothelial interactions. Platelet depletion leads to diminished leukocyte recruitment in many models (19, 20). Neutrophil-platelet interactions are mainly mediated by P-selectin GPs (PSGL-1), which is expressed on neutrophils and which binds to platelet P-selectin (21, 22). Firm adhesion of PMNs to platelets is mediated by the β3-integrin macrophage antigen-1 (Mac-1, also known as CD11b/CD18), which binds platelet GPIIbα, and the simultaneous binding of fibrinogen to platelet GPIIb/IIIa and CD11b/CD18 on PMNs. These interactions lead to PMN activation by a process of outside-in signaling through integrins (23, 24) in addition to the presentation of chemokines and lipid mediators by platelets to PMNs (25–27).

Platelets can be activated by thrombin, ADP, and endothelial-neutrophil interactions in a murine model of ALI (28). TXA2 is an arachidonic acid metabolite that is generated in several cell types and tissues, such as platelets, inflammatory cells, and pulmonary tissue, by the enzymes cyclooxygenase, hydroperoxidase, and tissue-specific isomerases (29, 30). The binding of TXA2 to the G protein–coupled thromboxane receptors (TPs) leads to a broad range of cellular responses, including integrin activation, platelet aggregation, contraction of smooth muscle cells, and increased vascular permeability, and can be involved in the development of ALI (31). Platelets express TPs, and endothelial cells express both TPs and TPβ (29). Stimulation of endothelial TPs induces an activation of G protein–dependent pathways, leading to recruitment of numerous downstream effector targets that increase the surface expression of adhesion molecules such as ICAM-1 in a PKC-dependent manner (29).

In the present study, we investigated the role of platelet-neutrophil interactions and TXA2 production in murine models of HCl- or sepsis-induced ALI and in vitro in a human pulmonary endothelial cell culture system. In order to investigate the mol-
ecules responsible for platelet-neutrophil interactions, chimeric mice were used that expressed P-selectin on hematopoietic cells, nonhematopoietic cells, both, or neither. The role of TXA₂ was investigated by a specific receptor antagonist. Our in vivo and in vitro data show that platelet-neutrophil interactions promote the formation of TXA₂ and are major contributors to acid-induced PMN recruitment and lung damage. Blocking P-selectin–dependent platelet-neutrophil interactions was highly protective in this model of acid-induced ALI.

Results

Platelets control neutrophil recruitment into the lung. In order to investigate the role of platelets in ALI, we depleted platelets by injecting mice with busulfan, which reduced blood platelet counts by 40% without affecting other blood cells (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI29499DS1). Platelet depletion significantly (P < 0.05) prevented the deterioration of gas exchange following HCl application (Figure 1A). To investigate the PMN recruitment pattern, including intravascular accumulation, transendothelial migration, and transepithelial migration, PMNs in lung homogenates were identified by flow cytometry (11). Two hours after acid instillation, HCl-treated mice showed a significant accumulation of PMNs in the intravascular and interstitial compartments compared with control mice (data not shown). Platelet depletion prior to the induction of ALI diminished the accumulation of PMNs in the intravascular, interstitial (data not shown), and alveolar compartments (Figure 1B). Furthermore, the depletion of platelets reduced protein leakage as determined by bronchoalveolar lavage (BAL) fluid protein levels (Figure 1C). Both findings were confirmed by depleting platelets with an anti-platelet Ab, which reduced platelet counts by 85% without affecting leukocytes. Histological analysis of the lungs following HCl application revealed alveolar septal thickening, accumulation of cells within the interstitial compartment, and influx of protein-rich fluid into the alveolar space (Figure 1E) compared with control mice (Figure 1D). Platelet depletion by busulfan prior to the induction of ALI led to a reduction of histological changes (Figure 1G) compared with the control group (Figure 1F).

Acid aspiration–induced ALI induces platelet-neutrophil interaction. Flow cytometric analysis of whole ventricular blood revealed the presence of platelet-neutrophil aggregates 30 minutes after acid application, as detected by a significant increase in the percentage of PMNs (CD45⁺, Gr-1⁺, 7/4⁺) positive for the platelet-specific marker CD41 (Figure 2, A and B). Depletion of platelets by Abs led to a significant reduction of platelet-neutrophil aggregates in the blood 30 minutes after induction of ALI (Figure 2C). The application of a P-selectin Ab 15 minutes after induction of ALI diminished platelet-neutrophil aggregates in the blood to baseline levels, suggesting that platelet-neutrophil aggregate formation is P-selectin dependent (Figure 2B). Flow cytometry data were confirmed by transmission electron microscopy showing numerous platelet-neutrophil aggregates in pulmonary microvascular vessels 30 minutes after initiation of acid-induced ALI (Figure 2D).

Hematopoietic P-selectin mediates the development of ALI. The increase in P-selectin–dependent platelet-neutrophil aggregates upon intratracheal acid application suggested that P-selectin blockade may lead to improvement of functional and morphological parameters. Mice treated with a P-selectin Ab 15 minutes after induction of ALI displayed a significant improvement in the oxygenation index (P < 0.05) compared with the control mice (Figure 3A). P-selectin blockade reduced PMN migration into the interstitial (data not shown) and alveolar compartments (Figure 3B) as well as protein leakage into the BAL fluid (Figure 3C). In accordance with these findings, the histological analysis of the lung after P-selectin blockade displayed reduced pathological changes (Supplemental Figure 1A).

To determine whether platelet or endothelial P-selectin was responsible for PMN recruitment and the development of ALI, we generated BM chimeric mice. Consistent with the findings in mice treated with P-selectin Abs, mice lacking platelet and endothelial P-selectin were protected from ALI. This protection was accompanied by an improvement of gas exchange (Figure 3D) and a reduc-
Acid-induced ALI causes platelet-neutrophil interactions. (A) Flow cytometry analysis of platelet-neutrophil aggregates after initiation of HCl-induced ALI. CD45, Gr-1, and 7/4 mAbs were used to identify PMNs (data not shown). Neutrophil-platelet aggregates were identified as neutrophils that were also positive for the platelet-specific marker CD41. (B) Thirty minutes after initiation of HCl-induced ALI, platelet-neutrophil interaction in the blood increased significantly. P-selectin Abs almost completely prevented the formation of platelet-neutrophil aggregates (n = 6–9 mice per group). (C) Platelet depletion by Abs prior to initiation of acid-induced ALI reduced the amount of platelet-neutrophil interactions in the blood. (D) Platelet-neutrophil interactions 30 minutes after initiation of acid-induced ALI in pulmonary microvasculature visualized by electron microscopy. Platelets (arrows) attached directly to the endothelium and a neutrophil. Scale bar: 1 μm. Original magnification, ×8000. *P < 0.05. P-sel, P-selectin.

We incubated PMNs, platelets, or both with HPMECs and determined endothelial ICAM-1 expression mRNA by quantitative real-time RT-PCR. Incubation of activated PMNs, platelets, or both resulted in a significant induction of endothelial ICAM-1 mRNA expression (Figure 4C). This was further enhanced when activated or resting platelets were added. Activated platelets together with resting PMNs induced as much endothelial ICAM-1 expression as did activated PMNs (Figure 4C). In order to show that the TXA2 release by platelet-neutrophil aggregates is responsible for the endothelial ICAM-1 mRNA expression, we incubated endothelia with a specific TP antagonist before we added the cells. The TP antagonist blocked the ICAM-1 mRNA expression by approximately 40% (data not shown).

To determine whether platelet interaction with neutrophils was sufficient to induce TXA2 synthesis, isolated human platelets were incubated with isolated human neutrophils for 2 hours at 37°C. This resulted in a 2.5-fold increase in TXB2 measured in the supernatant, which was further enhanced to 5.5-fold when either PMNs or platelets were activated by TNF-α or thrombin, respectively (Figure 4D).

Blocking TXA2 prevents acid-induced ALI. In order to test the biological significance of our in vitro findings in vivo, we measured TXB2, a stable degradation product of TXA2, in mouse plasma under baseline conditions and after platelet depletion. Two hours after intratracheal HCl application, TXB2 concentration in the plasma increased significantly, consistent with a previous study (7). The depletion of platelets by busulfan or Abs prior to induction of ALI significantly decreased plasma TXB2 (Figure 5A and data not shown).

To determine whether TXA2 is an important mediator of ALI, we pretreated acid-exposed mice with a specific TP antagonist. This improved gas exchange (Figure 5B) and reduced intravascular, interstitial (data not shown), and intraalveolar (Figure 5C) PMN infiltration of intravascular (Figure 3E) and interstitial PMN accumulation (data not shown) and permeability (Figure 3G) in response to HCl instillation. When WT mice were reconstituted with BM from selectin P−/− (Selp−/−) mice, gas exchange was significantly improved (Figure 3D), and intravascular (Figure 3E), interstitial (data not shown), and intraalveolar PMN accumulation (Figure 3F) as well as permeability (Figure 3G) were reduced. When Selp−/− mice were reconstituted with BM from WT mice, intravascular PMN accumulation was reduced, but all other functional and morphological parameters were similar to those seen in WT mice. These results show that platelet and not endothelial P-selectin is responsible for the pathology of acid-induced ALI.

Neutrophil-platelet aggregates interact with endothelial cells. In order to elucidate the molecular mechanisms responsible for our in vivo results, we investigated the role of different cell populations and activation states in PMN adhesion to human pulmonary microvascular endothelial cells (HPMECs). Incubation of activated PMNs or the combination of unstimulated PMNs and platelets induced a small increase in PMN adhesion. The combination of activated PMNs with activated or resting platelets or resting PMNs with activated platelets induced a further 3-fold increase (P < 0.05) of PMN adhesion to endothelial cells (Figure 4A).

Platelet-neutrophil interaction is known to promote the production of TXA2 (32). To differentiate between the importance of endothelial and platelet TPs, we incubated endothelia with a specific TP antagonist for 2 hours. Blocking of endothelial TPs reduced PMN adhesion (Figure 4B). These data suggest that the interaction of platelets and PMNs augments adhesion of PMNs to endothelial cells by TXA2 effects on the endothelia.

A previous study demonstrated that acid-induced ALI is partially ICAM-1 dependent (33). We therefore studied whether platelet-neutrophil interactions induced endothelial ICAM-1 expression.
accumulation ($P < 0.05$). TP blockade almost completely normalized permeability (Figure 5D), suggesting that TXA$_2$ is the main mediator of permeability in this model of ALI. Pretreatment with the TP antagonist before induction of ALI reduced alveolar septal thickening, accumulation of cells within the interstitial compartment, and influx of protein-rich fluid into the alveolar space in the histological analysis of the lungs (Supplemental Figure 1B). Acetylsalicylic acid (ASS) at a dose that completely blocks cyclooxygenase inhibited permeability (Figure 5D), suggesting that TXA$_2$ is the major mediator of permeability in this model of ALI. Pretreatment with the TP antagonist or platelet depletion by busulfan prior to induction of ALI caused a prolongation of survival. Remarkably, P-selectin Abs given as a therapeutic approach 15 minutes after induction of ALI led to survival of all mice until the end of the study (Figure 6C).

**Platelets influence sepsis-induced ALI**. In order to address the question of whether platelet-neutrophil interactions are also involved in development of ALI caused by sepsis, we investigated the role of this cell interaction in a zymosan/LPS-induced ALI model. Four hours after induction of ALI, mice showed significantly reduced gas exchange (Figure 7A), increased accumulation of neutrophils in the intravascular (E), interstitial (data not shown), and alveolar compartments (F), and diminished permeability (G) compared with mice expressing hematopoietic P-selectin. $^\dagger P < 0.05; ^\ddagger P < 0.05$ versus platelet Selp$^{-/-}$.

**Discussion**

The results of our study suggest that platelet-neutrophil interactions play a crucial role in the development of acid-induced ALI. Our model is severe and lethal, as opposed to a milder injury in mice ventilated with a high concentration of inspiratory oxygen (6). Depletion of platelets or disruption of platelet-neutrophil interactions reduced PMN migration and permeability and improved gas exchange after acid application, resulting in prolonged survival. Increased neutrophil adhesion and activation of endothelia was caused by TXA$_2$ produced by platelet-neutrophil aggregates. These observations indicate that platelet-neutrophil interactions are critical to the development of ALI, to which they contribute by enhancing their respective activations, which trigger the production of TXA$_2$ and other inflammatory mediators.
Pulmonary aspiration of gastric content induces ALI in 26%–36% of all cases and is associated with a high mortality (34, 35). Aspiration of acid may damage the alveolar-capillary membrane and induce a release of inflammatory mediators, inflammatory cells, expression of adhesion molecules, and enzymes, including TNF-α, IL-8, cyclooxygenase and lipoxygenase products, and reactive oxygen species (4). Furthermore, these inflammatory processes may also lead to a coagulopathy, which results from activation of coagulation and inhibition of fibrinolysis (36). Here we show that preventing platelet-neutrophil interactions can completely reverse this process. First, platelets control neutrophil recruitment into the lung in this and a second, sepsis-induced model. Second, induction of ALI leads to a significant increase of platelet-neutrophil aggregates. Third, blocking P-selectin by Abs after induction of ALI as a therapeutic approach reduces formation of platelet-neutrophil aggregates and development of ALI and leads to prolonged survival. Platelet but not endothelial P-selectin is responsible for the development of ALI.

Our in vitro studies show that activation of either PMNs or platelets alone has the same effects as simultaneous activation of both cell types, suggesting that PMNs and platelets activate each other. This interpretation is consistent with a previous study in a
different disease model showing that platelets present proinflammatory mediators on their surface to leukocytes and endothelia upon activation (37). When the interaction of platelets with endothelial cells or leukocytes is disturbed, mediator production and presentation are reduced.

During the interaction of activated platelets with leukocytes and endothelial cells, the platelets deposit platelet-derived mediators, including the chemokines CXCL4, CXCL7, and CXCL8. These mediators induce PMN activation, chemotaxis, adhesion, degranulation of primary and secondary granules, and production of reactive oxygen species (38–40). TXA2, an important lipid mediator of platelets, is involved in platelet-neutrophil interactions (41) and plays a critical role in acid-induced ALI (7, 42). TXA2 induces increased permeability (42) and PMN adhesion to pulmonary endothelial cells (43). TP blocking or thromboxane synthesis inhibition was previously shown to protect from acid-induced PMN sequestration and protein leakage (42, 44). In our in vivo study, we demonstrate that platelets are a main source of TXA2 upon intratracheal acid application. Our in vitro studies show that the interaction of platelets with PMNs increases F-actin polymerization in endothelial cells and augments adhesion of PMNs to endothelial cells by TXA2 effects on the endothelia.

Currently, there are no drugs to effectively manage pulmonary edema and PMN sequestration and to improve survival rates in ALI. The present data suggest that blocking TPs or P-selectin represents potential therapeutic approaches to controlling acid-induced ALI. Both TP antagonists and P-selectin blocking drugs are under development. Our data show that the cyclooxygenase inhibitor ASS significantly improved oxygenation and reduced neutrophil recruitment and edema in ALI. However, this treatment was less effective than blocking TPs. ASS would be expected to reduce biosynthesis of all prostanoids, including antiinflammatory arachidonic acid derivatives such as prostacyclin, whereas TP inhibitors would selectively block the action of TXA2. A recent clinical trial using ketoconazole showed no benefit (45). This study included patients with different causes of directly (pneumonia, aspiration of gastric contents) and indirectly (e.g., sepsis, transfusion) induced ALI. Importantly, the measured TXB2 concentration in patients treated with ketoconazole was not reduced compared with the control group. Furthermore, the drug was administered a long time after the onset of ALI.

Our data provide what we believe is the first direct evidence for a contribution of circulating platelets in the development of acid- and sepsis-induced ALI. P-selectin–dependent platelet-neutrophil interactions induced presentation of platelet-derived proinflammatory mediators to the endothelia and neutrophils, resulting in increased neutrophil adhesion and recruitment, activation of endothelial cells, and development of ALI. Therapeutic inhibition

Figure 6

Endothelial cell response to TP activation as reflected by F-actin localization and content. (A) Human pulmonary endothelial cells were treated with 75 or 150 nM of TXA2 analogue (SQ 29548), and F-actin was localized by phalloidin staining. Images are representative of 3 experiments with similar results. Original magnification, ×175. (B) Activated platelets and activated PMNs induced a significant increase of F-actin polymerization. *P < 0.05; †P < 0.05 versus control. (C) Platelet depletion with busulfan or TP antagonist SQ 29548 prior to induction of ALI led to significant prolongation of survival compared with that of HCl-treated mice (n = 4 per group). All mice treated with a P-selectin Ab survived until termination of the experiment (300 min), as did control mice (data not shown). The HCl group was significantly different from the other groups. P = 0.0002 by log rank test.
of platelet neutrophil aggregates and/or neutralization of platelet-derived proinflammatory mediators may provide a novel therapeutic approach to ALI that should be explored in clinical trials.

Methods

Animals. We used 8- to 12-week-old C57BL/6 mice (The Jackson Laboratory) and P-selectin-deficient mice (C57BL/6 for at least 10 generations). Mice were housed in a barrier facility under specific pathogen-free conditions. The Animal Care and Use Committee of the University of Virginia approved all animal experiments.

Acid-induced ALI. Mice were anesthetized with i.p. injections of ketamine (125 μg/g body weight; Sanoﬁ-Aventis), xylazine (12.5 μg/g body weight; Phoenix Scientiﬁc Inc.), and atropine sulfate (0.025 μg/g body weight; Fuji-sawa) and were placed on a heating pad to maintain body temperature. In order to effect acid-induced ALI, mice received 2 intratracheal instillations of 1 M HCl (pH = 1.5) followed by a bolus of air (30 m/l/g). Following a tracheotomy, mice were ventilated with a respirator (MiniVent, Type 845; Hugo Sachs Elektronik) at respiration rate, 140/min; fraction of inspiratory oxygen [FiO2], 0.21). Control animals received saline instead of HCl in the same manner. Sepsis-induced ALI. Mice were anesthetized and mechanically ventilated. One minute prior to induction of ALI, 3 deep inhalations (3 × tidal volume) were applied. Subsequently, mice were injected with 3 μg/g of LPS i.v. (Escherichia coli O111:B4; Sigma-Aldrich), and 2 hours later, they received 7 μg/g zymosan A (Saccharomyces cerevisiae; Sigma-Aldrich) i.v. The control group received saline instead of LPS and zymosan A in the same manner. The experiment was terminated 4 hours after induction of ALI.

Platelet depletion. In order to investigate the role of platelets in the recruitment of PMNs into the lung and the development of ALI, platelets were depleted as previously described (46). Briefly, mice received 2 injections of busulfan (20 mg/kg dissolved in polyethylene glycol 400) or vehicle (polyethylene glycol 400) on days 0 and 3. Experiments were performed on day 14. Immediately before experiments, blood counts were determined (HEMAVET 850 FS; CDC Technologies Inc.). Platelet depletion by busulfan led to a significant reduction of platelets without affecting leukocytes (Supplemental Table 1A). In another set of experiments, we used a rabbit anti-mouse platelet serum (Accurate) to deplete platelets. Two hours prior to induction of ALI, mice were injected i.p. with 25 μl of the Abs. This method led to a significant reduction (85%) of platelets in the systemic circulation without affecting leukocytes (data not shown). Control mice received preimmune serum.

PMN recruitment into the lung. After euthanasia, BAL ﬂuid was collected (5 × 1 ml phosphate-buffered saline). After BAL ﬂuid was centrifuged, PMNs in the BAL ﬂuid were counted using Kimura stain. The protein concentration of the supernatant was determined by the Lowry method. In order to distinguish between the localization of intravascular and interstitial PMNs, we used a recently developed flow cytometry–based method to determine pulmonary PMN extravasation (11). In brief, Alexa Fluor 633–labeled Gr-1 Abs (clone RB6-8C5, puriﬁed from the supernatant of the Gr-1 hybridoma [ATCC] at the biomolecular facility of the University of Virginia; Alexa Fluor 633 Protein Labeling Kit [Invitrogen]) were injected 5 minutes prior to euthanasia. After collecting BAL ﬂuid, the inferior vena cava was opened and nonadherent PMNs were dislodged from the pulmonary vasculature by flushing 10 ml of PBS at 25 cm H2O through the right ventricle. Lungs were removed and minced, and the samples were incubated with 125 U/ml collagenase type XI, 60 U/ml hyaluronidase type I-s, 60 U/ml DNase I, and unlabeled anti–Gr-1 at 37°C for 60 minutes in order to prevent possible binding of the injected Abs to extravascular PMNs. A cell suspension was made by passing the digested lungs through a 70-μm cell strainer (BD Biosciences — Falcon). Erythrocytes were lysed and remaining leukocytes were resuspended and counted. The fraction of PMNs in the suspension was determined by flow cytometry (FACSCalibur; BD). PMNs were labeled with phycoerythrin–conjugated anti–mouse IgG (Pharmingen) and were analyzed using FACSCalibur. PMNs were identified by their typical appearance in the forward/side scatter profiles.
and their expression of CD45 (clone 30-F11; BD Biosciences — Pharmingen), 7/4 (clone 7/4; BD Biosciences — Pharmingen), and Gr-1 (clone RB6-8C5). Isotype controls were used to compensate for nonspecific Ab binding. The labeled Gr-1 Ab was used to differentiate between intravascular (CD45^+7/4^+Gr-1^+^) and interstitial (CD45^+7/4^+Gr-1^-^) PMNs.

**Pulmonary function — oxygenation.** Arterial blood was obtained from an arterial catheter, and standard arterial blood gas analyses were accomplished (Rapidlab 800 System; Bayer HealthCare). To normalize the partial pressure of arterial oxygen (PaO_2) to the PaO_2 to the ratio of inspiratory oxygen (FiO_2), the ratio of PaO_2 to FiO_2 (PaO_2/FiO_2, mmHg, oxygenation index) was calculated.

**Neutrophil-platelet interactions.** In order to determine the amount of platelet-neutrophil aggregates, 100 μl acid-citrate dextrose (ACD) anti-coagulated blood was added to a saturating concentration of anti-CD41 (FITC), anti-CD45 (PerCP), anti–Gr-1 (Alexa Fluor 633), and anti-CD11b (PE). After incubation for 10 minutes at room temperature, cells were fixed using paraformaldehyde. The number of neutrophil-platelet interactions were determined by flow cytometry (FACSCalibur; BD) within 4 hours of blood withdrawal.

**Generation of P-selectin chimeric mice.** To distinguish the role of nonhematopoietic and hematopoietic P-selectin for the development of ALI, chimeric mice were generated following a previously described protocol (47). C57BL/6 and P-selectin–deficient mice were used as donors and/or recipients. Recipient mice were lethally irradiated in 2 doses of 6 Gy each (separated by 4 hours). BM was isolated from female donor mice under sterile conditions, and approximately 5 × 10^7 cells were injected i.v. into male recipient mice. Experiments were performed 6 weeks after BM transplantation. To confirm complete reconstitution of the transferred BM, DNA of leukocytes was investigated for an area specific to the Y chromosome by real-time RT-PCR (primers: forward, 5′-AGCAACACCTCTAC-TACCCCTCTA-3′; reverse, 5′-GGGCTCGCATCAATTTCAATCA-3′; probe, 5′-ACCTAACGTTGCCCCTGCGG-3′).

**Effects of intervention.** WT mice were used to assess the effects of P-selectin mAb (30 μg/mouse, clone RB40.34), specific TP antagonist (SQ 29548, 10 μg/ml) for 2 hours. After TP blocking, antagonist was removed by washing.

**PMN–endothelial cell adhesion assay.** Calcein AM–labeled cells (1 × 10^5 neutrophils and/or 1 × 10^5 platelets) were incubated with pulmonary endothelia for 30 minutes at 37°C, nonadherent cells were removed by gentle washing and aspiration, and adherent PMNs were lyzed and quantified in a fluorescence spectrometer. For blocking experiments, endothelial cells were incubated with specific TP antagonist (SQ 29548, 10 μM) for 2 hours. After TP blocking, antagonist was removed by washing.

**Quantitative real-time RT-PCR.** Two hours after incubation of endothelial cells with neutrophils and/or platelets, RNA was isolated using TRIzol reagent (Invitrogen). Total RNA (900 ng) was reverse transcribed with murine leukemia virus (MuLV) reverse transcriptase using the GeneAmp RNA PCR kit (Applied Biosystems) and oligo dT primers. The mRNA sequences of the investigated genes were obtained from GenBank. The primers for β_2 microglobulin were described previously (49). The primers for ICAM-1 were designed using Primer3 software from the Whitehead Institute for Biomedical Research. The following primers were used: 5′-GGTGGATGCTGTTTTGAGAC-3′ (forward) and 5′-ACGTGGTTCAACCTCTTG-3′ (reverse). Quantitative real-time RT-PCR was performed using iCycler iQ Real-Time Detection System technology (Bio-Rad). Quantification of target gene expression was performed using a mathematical model (50). The expression of the target molecule was normalized to the expression of β_2 microglobulin. To exclude contributions of ICAM-1 mRNA from neutrophils or platelets, these cell populations were isolated and RT-PCR was performed as mentioned above. Neither platelets nor PMNs expressed ICAM-1 mRNA (data not shown).

**Statistics.** Statistical analysis was performed with SPSS version 9.0 for Windows (SPSS) and included 1-way analysis of variance, Student-Newman-Keuls test, and 2-tailed Student’s t test where appropriate. Kaplan-Meier method was used for analyzing survival rates. All data are presented as mean ± SEM. P < 0.05 was considered significant.

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