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Platelets regulate P-selectin expression and leukocyte rolling in inflamed venules of the pancreas

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ABSTRACT

Recent data suggest that platelets regulate inflammatory changes and tissue damage in acute pancreatitis although the role of platelets in leukocyte–endothelium interactions in the pancreatic microcirculation is not known. The aim of this study was to define the impact of platelets on leukocyte rolling and adhesion in acute pancreatitis. Acute pancreatitis was induced in C57BL/6 mice by caerulein challenge. Mice were treated with an anti-GP1bα (CD42b) antibody, which depletes platelets, or a control antibody before caerulein. Leukocyte rolling and adhesion were determined by the use of intravital fluorescence microscopy 18 h after the last dose of caerulein. In separate experiments, leukocyte–endothelium interactions were determined before and after administration of an anti-P-selectin, anti-PSGL-1 and a control antibody in mice with caerulein pancreatitis. Circulating platelet–neutrophil aggregates and pancreatic P-selectin mRNA were quantified 1 and 6 h respectively after caerulein challenge. Caerulein administration increased leukocyte and platelet interactions in the pancreatic microvasculature, increased tissue damage and expression of P-selectin mRNA in the pancreas as well as platelet–neutrophil complexes in the circulation. Notably, platelet depletion markedly reduced caerulein-provoked leukocyte rolling and adhesion in postcapillary venules. Interestingly, depletion of platelets significantly decreased caerulein-induced gene expression of P-selectin in the pancreas. Moreover, immunoneutralization of P-selectin and PSGL-1 abolished leukocyte rolling in the pancreatic venules triggered by caerulein. Our novel findings demonstrate that platelets regulate leukocyte rolling in acute pancreatitis *via* induction of P-selectin, which was critical in supporting leukocyte rolling in inflamed venules of the pancreas.

1. Introduction

The clinical course of acute pancreatitis includes a broad spectrum of presentations ranging from transient pain to the development of local and systemic complications. The majority of pancreatitis patients experience a mild and simple type whereas close to 20% develop severe pancreatitis associated with a mortality rate around 30% (Appelros et al., 2001; Mann et al., 1994). In spite of significant research efforts, the treatment options for patients with acute pancreatitis are still limited to supportive care and pose a major challenge to clinicians, which is largely due to our incomplete understanding of the pathophysiology in acute pancreatitis. In general, trypsinogen activation and neutrophil infiltration in the pancreas are considered to be central features in the initiation and progression of pancreatitis. A hallmark of pancreatitis is the accumulation of neutrophils in the pancreas (Abdulla et al., 2011c; Gukovskaya et al., 2002; Kyriakides et al., 2001). Several studies have shown that neutrophil infiltration is a rate-limiting step in the tissue injury associated with acute pancreatitis. For example, targeting specific adhesion molecules, such as LFA-1 and ICAM-1, not only attenuate neutrophil recruitment but also protect against tissue damage in pancreatitis (Awla et al., 2011; Zhang et al., 2009). However, the detailed mechanisms orchestrating leukocyte accumulation in acute pancreatitis are not well understood.

The extravasation process of leukocytes is generally considered to be a multistep process supported by specific molecules, such as the selectin and integrin family of adhesion molecules, although the details of the cascade have not been examined in the pancreas. At first, leukocyte recruitment is initiated by a rolling adhesive interaction followed by subsequent firm leukocyte adhesion and transmigration. In this cascade, rolling along the endothelium reduces the velocity of circulating leukocytes allowing them to detect chemoattractants expressed on the endothelium or leaking between endothelial cells from the tissues. In fact, this rolling adhesive interaction is known to be a precondition for subsequent firm adhesion in the microcirculation (Klintman et al., 2002; Lindbom et al., 1992; Mansson et al., 2000). Numerous studies have shown that P-selectin supports leukocyte rolling in postsinusoidal venules, including the liver, intestine, striated muscle, and brain (Ishikawa et al., 2004; Klintman et al., 2002; Thorlacius et al., 1997; Wan et al., 2002). Accumulating data suggest a pro-inflammatory role of platelets beyond their well-

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known function in haemostasis and thrombosis (McNicol and Israels, 2008; Nurden, 2011). Although clinical studies have not found any correlation between levels of circulating platelets and severity of pancreatitis, low platelet counts, as a feature of disseminated intravascular coagulation, is frequently observed in severe and complicated acute pancreatitis (Salomone et al., 2003). A recent study reported that platelets play a key role in pancreatitis by regulating neutrophil accumulation in the pancreatic tissue (Abdulla et al., 2011a), but the mechanisms regulating platelet dependent leukocyte recruitment in the pancreas are not known.

Based on the considerations above, we hypothesized herein that platelets may regulate leukocyte–endothelium interactions in inflamed venules of the pancreas and that P-selectin might be involved in this platelet-dependent leukocyte rolling in acute pancreatitis.

2. Material and methods

2.1. Animals

All experiments were done in accordance with the legislation on the protection of animals and were approved by the Regional Ethical Committee for animal experimentation at Lund University, Sweden. All experiments were performed using male C57BL/6 mice (Taconic Europe, Ry, Denmark) weighing 20–26 g (6–8 weeks old) were maintained in a climate-controlled room at 22 °C and exposed to a 12:12-h light–dark cycle. Animals were fed standard laboratory diet (R3, Lactamin AB, Kimstad, Sweden) and given water *ad libitum*. Mice were anesthetized by intraperitoneal (i.p.) administration of 7.5 mg of ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 2.5 mg of xylazine (Janssen Pharmaceutica, Beerse, Belgium) per 100 g body weight in 200 μl of saline. Analgesia was obtained by subcutaneous injection of buprenorfin hydrochloride 0.1 mg/kg (Schering-Plough Corporation, New Jersey, USA).

2.2. Experimental model of caerulein-induced pancreatitis

Acute pancreatitis was induced by seven hourly i.p. injections of a supramaximal dose of the pancreatic secretagogue caerulein (50 μg/kg, C9026, Sigma-Aldrich, City, St. Louis, MO, USA) in 200 μl of 0.9% saline as described previously. Animals were randomly divided into four groups. One sham group (n = 6), which only received repetitive i.p. injections of saline. The other three groups were challenged with caerulein and pretreated i.p. for 2 h with phosphate buffered saline (PBS, n = 6), a platelet-depleting antibody directed against GPIbα (CD42b, clone polyclonal anti-GPIb alpha, 1.0 mg/kg, rat IgG, Emfret Analytics GmbH & Co. KG. Eibelstadt, Wurzburg, Germany, n = 6) or a control antibody (1.0 mg/kg, clone polyclonal non-immune rat immunoglobulins, rat IgG, Emfret Analytics GmbH & Co. n = 6). Animals were sacrificed at 1 h, 6 h or 18 h after the last dose of caerulein injection. Blood was collected from the tail vein for systemic platelet and leukocyte differential counts and from the inferior vena cava for amylase levels.

2.3. Intravital fluorescence microscopy

In separate animals, intravital fluorescence microscopy was used for studying the role of P-selectin (n = 6) and PSGL-1(n = 6) in mediating leukocyte–endothelium interactions in the pancreatic microcirculation 18 h after the last dose of caerulein injection. Mice were anesthetized and a polyethylene catheter inserted in the jugular vein prior to intravital microscopy. The pancreas was exteriorized *via* a midline abdominal incision and fixed gently over a specific pancreatic holder using two stay sutures. The microcirculation was observed using a modified Olympus microscope (BX50WI, Olympus Optical Co. GmbH, Hamburg, Germany) equipped with a florescent lamp source and CCD camera. An equilibration time of 5 min was allowed before videos were recorded digitally for later analysis. Fluorescein-isothiocyanate-labeled dextran 150000 (0.05 ml, 5 mg/ml, Sigma-Aldrich) and rhodamine 6-G (0.1 ml, 0.5 mg/ml, Sigma-Aldrich) were injected into the jugular vein, enabling visualization of leukocyte–endothelium interactions. Analysis of leukocyte rolling and adhesion in the pancreas was performed in postcapillary venules before and 5 min after i.v. administration of a control antibody (clone R3-34, IgG_1, 40 μg/mouse, BD Bioscience Pharmingen), an anti-P-selectin antibody (clone RB40-34, IgG_1, BD Bioscience Pharmingen, San Diego, CA, U.S.A.) or an anti-PSGL-1 antibody (clone 4RA10, IgG_1, 40 μg/mouse, BD Bioscience Pharmingen). For quantification of leukocyte rolling and adhesion, two to five postcapillary venules were analyzed. Leukocyte rolling was defined as the number of rolling cells along a defined part of the endothelium for 20 s and is expressed as cells/min. Leukocyte adhesion were measured by counting the number of cells that adhered and remained stationary for more than 20 s during the observation time and is expressed as cells per mm.

2.4. Quantitative PCR

Pancreatic samples were obtained 6 h after the last dose of caerulein injection in mice treated with the anti-GP1bα (n = 6) or a control antibody (n = 4). Total RNA was isolated from samples using RNeasy® Mini Kit (Qiagen, West Sussex, Great Britain) and treated with RNase-free DNase (DNase I; Amersham Pharmacia Biotech, Sollentuna, Sweden) to remove potential genomic DNA contaminants. RNA concentrations were determined by measuring the absorbance at 260 nm. Each cDNA was synthesized by reverse transcription from 10 μg of total RNA using StrataScript® First-Strand Synthesis System and random hexamers primer (Stratagene, AH diagnostics AB, Stockholm, Sweden). Real-time PCR was performed using a Brilliant® SYBR® Green QPCR Master Mix and MX 3000P™ Detection System (Stratagene). The specific primers were as follows: P-selectin (f) 5′-ACG AGC TGG ACG GAC CCG-3′; P-selectin (r) 5′-GGC TGG CAC TCA AAT TTA CAG-3′; β-actin (f) 5′-ATG TTT GAG ACC TTC AAC ACC-3′; (r) 5′-TCT CCA GGG AGG AAG AGG AT-3′. Standard PCR curves were generated for each PCR product to establish linearity of the RT-PCR reaction. PCR amplifications were performed in a total volume of 50 μl, containing 25 μl SYBR Green PCR 2× master mix, 2 μl 0.15 μM each primer, 0.75 μl reference dye and one 1 μl cDNA as a template adjusted up to 50 μl with water. PCR reactions were started with 10 min denaturing temperature of 95 °C, followed by a total of 40 cycles (95 °C for 30 s and 55 °C for 1 min) and 1 min of elongation at 72 °C. The relative differences in expression between groups were expressed using cycling time (Ct) values. Ct values for the specific target genes were first normalized with that of β-actin in the same sample, and then relative differences between groups were determined.

2.5. Systemic leukocyte and platelet counts

At the end of the experiments, blood was sampled from the tail vein and diluted 1:500 in Stromatol solution (Mascia Brunelli S.p.A. Viale Monza, Italy) or 1:20 in Turks solution (0.2 mg gentian violet in 1 ml glacial acetic acid, 6.25% vol/vol) for quantification of platelets and leukocytes (monomorphonuclear and polymorphonuclear cells), respectively, in a Burker chamber.

2.6. Flow cytometry

For analysis of the number of platelet binding as well as PSGL-1 and Mac-1 expression on circulating neutrophils, blood was collected into syringes pre-filled with 1:10 acid citrate dextrose at 1 h post caerulein challenge. Immediately after collection, blood samples were incubated with an anti-CD16/CD32 antibody blocking Fcγ III/II receptors to reduce non-specific labeling for 10 min at room temperature. Next, samples were incubated with APC-conjugated anti-Mac-1 (clone M1/70, Integrin α_M chain, rat IgG_{2b}) or APC-conjugated anti-PSGL-1 (clone 4RA10, rat IgG_1), PE-conjugated anti-Gr-1 (clone RB6-8C5, Rat IgG_{2b}) and FITC-conjugated anti-CD41 antibodies (clone MWReg30, Integrin α_{IIb} chain,

rat IgG_1). By considering neutrophils as cells positive for Gr-1 and platelets as $CD41^+$ cells, the percentage of neutrophil–platelet aggregates and the expression of Mac-1 or PSGL-1 on neutrophils were quantified. Cells were fixed with 1% formaldehyde solution, erythrocytes were lysed using red blood cell lysing buffer (Sigma-Aldrich) and neutrophils were recovered following centrifugation. Flow cytometric determination of neutrophil–platelet aggregates was performed by first gating the neutrophil population of cells based on forward and side scatter characteristics and then was the percentage of neutrophils ($Gr\text{-}1^+$) binding platelets ($CD41^+$) analyzed in this population on a FACSort flow cytometer (Becton Dickinson, Mountain View, CA, USA). A viable gate was used to exclude dead and fragmented cells. All antibodies were purchased from BD Biosciences Pharmingen.

2.7. Statistics

Data are presented as mean and standard error of the mean (S.E.M.). Statistical evaluations were performed using Kruskal–Wallis one-way analysis of variance on ranks followed by multiple comparisons *vs.* control group (Dunn's method). $P<0.05$ was considered significant, and n represents the number of animals. SigmaStat® for Windows® version 3·5 software (Systat Software, Chicago, Illinois, USA) was employed.

3. Results

To evaluate the role of platelets in regulating leukocyte–endothelial cell interactions, we used an antibody directed against GP1bα expressed on platelets. To confirm that administration of the anti-GP1bα antibody effectively reduced platelets ($CD41^+$ cells) in the blood, we used flow cytometry (data not shown) as well as manual counting of blood platelets showing that administration of the anti-GP1bα antibody decreased systemic platelet counts by more than 83% ($P<0.05$ *vs.* Ctrl Ab + Caerulein, $n=6$). It is important to note that administration of the anti-GP1bα antibody had no effect on the levels of circulating leukocyte subtypes in acute pancreatitis (not shown). In order to determine the role of platelets in leukocyte rolling and adhesion, we used intravital fluorescence

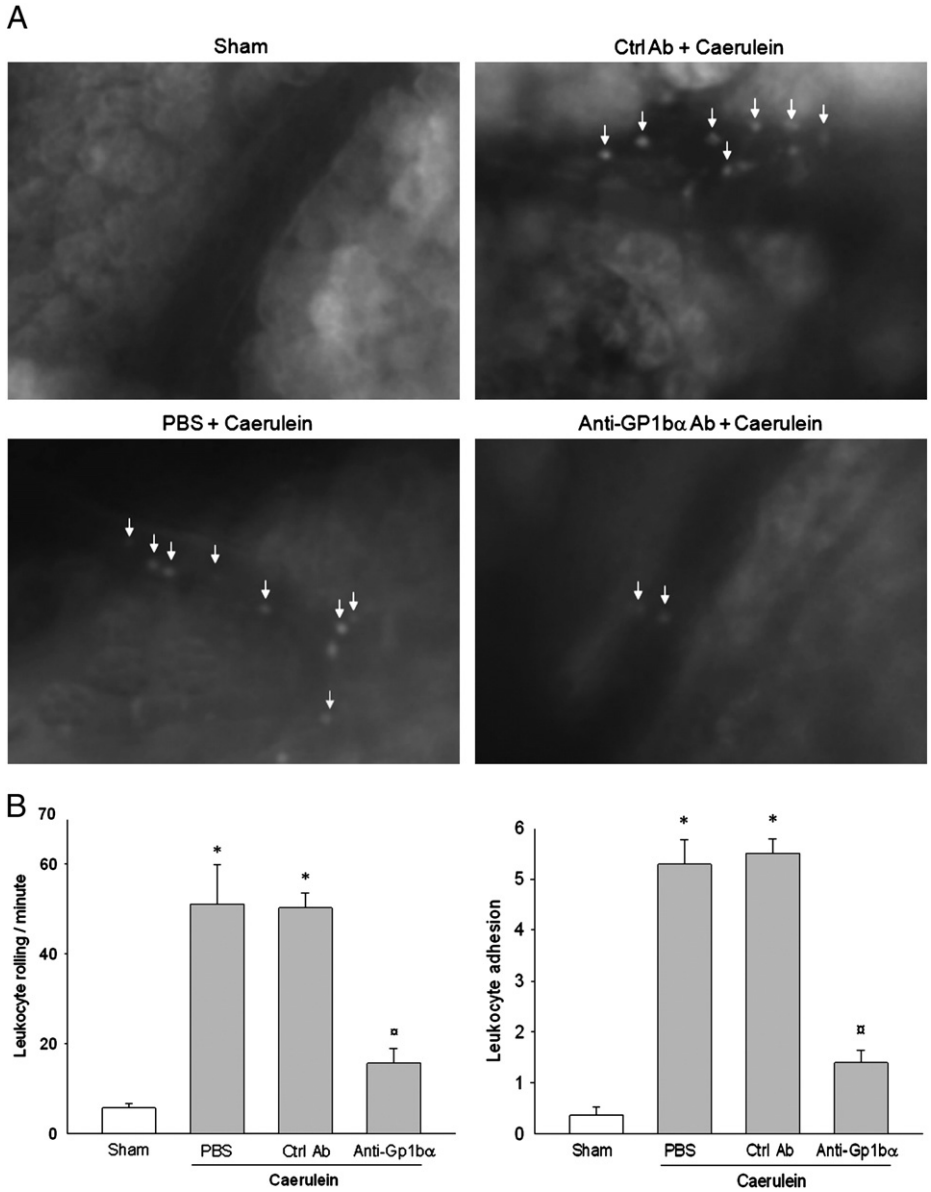

Fig. 1. A) Representative microphotographs from intravital fluorescence microscopy and B) aggregate data on leukocyte rolling and adhesion in acute pancreatitis. Animals were pretreated with PBS, a control antibody (Ctrl Ab) and a platelet depleting antibody (Anti-GP1bα) 2 h prior to pancreatitis induction. Pancreatitis was induced by repetitive i.p. caerulein (black bars) administration. Sham mice (white bars) received repetitive i.p saline injections. Data were quantified 18 h after the last dose of caerulein injection. Data represent means ± S.E.M. and $n=6$. $^*P<0.05$ *vs.* Sham, and $^{\text{¤}}P<0.05$ *vs.* Ctrl Ab + Caerulein.

microscopy 18 h after the last dose of caerulein injection (Fig. 1). We observed that caerulein challenge increased leukocyte–endothelium interactions (Fig. 1), *i.e.* leukocyte rolling was increased by nine-fold and leukocyte adhesion was increased by 14-fold (Fig. 1B, $P<0.05$ *vs.* Sham, $n=6$). Interestingly, we found that platelet depletion significantly reduced leukocyte rolling by 69% (Fig. 1B, $P<0.05$ *vs.* Ctrl. Ab + Caerulein, $n=6$). In addition, depletion of platelets reduced leukocyte adhesion by 74% (Fig. 1B, $P<0.05$ *vs.* Ctrl. Ab + Caerulein, $n=6$). As shown previously (Abdulla et al., 2011a), injection of the anti-GP1bα antibody significantly reduced caerulein-provoked myeloperoxidase activity (a marker of neutrophil accumulation) and tissue damage in the pancreas. Next, we examined whether platelets may regulate P-selectin expression in the pancreas. For this purpose, we used quantitative RT-PCR. We found that challenge with caerulein increased gene-expression of P-selectin by more than two-fold in the pancreas (Fig. 2, $P<0.05$ *vs.* Ctrl. Ab + Caerulein, $n=4$–6). Interestingly, it was observed that platelet depletion abolished the caerulein-induced increase in P-selectin mRNA expression in the pancreas (Fig. 2, $P<0.05$ *vs.* Ctrl. Ab + Caerulein, $n=4$–6). Moreover, we found that P-selectin was a critical adhesion molecule mediating leukocyte rolling but not adhesion in pancreatitis triggered by caerulein (Fig. 3). Notably, we also observed that administration of the anti-PSGL-1 antibody decreased caerulein-induced leukocyte rolling by 98% (Fig. 3, $P<0.05$ *vs.* Ctrl. Ab + Caerulein, $n=4$–6), suggesting that P-selectin-PSGL-1 interactions regulate leukocyte rolling in acute pancreatitis. Platelet–leukocyte aggregate formation is a common feature in inflammatory conditions and we hypothesized that such complex formation may influence neutrophil activation and consequently expression of PSGL-1 or Mac-1. We found that caerulein challenge markedly increased formation of platelet–neutrophil aggregates (Fig. 4A, $P<0.05$ *vs.* Sham, $n=6$). However, surface expression of PSGL-1 or Mac-1 on neutrophils was identical in neutrophils binding few compared to neutrophils binding many platelets (Fig. 4B, $P>0.05$ *vs.* Ctrl. Ab + Caerulein, $n=4$), suggesting that platelet–neutrophil complex formation do not modulate neutrophil activation in acute pancreatitis.

4. Discussion

Accumulating studies implicate platelets in the pathophysiology of inflammatory diseases. Herein, we demonstrate that platelets play a significant role in regulating leukocyte–endothelium interactions in the pancreatic microvasculature. Indeed, it was found that platelet depletion reduced leukocyte rolling in postcapillary venules in the inflamed pancreas. In addition, we observed that depletion of platelets markedly reduced gene-expression of P-selectin, which is critical for leukocyte rolling and recruitment in acute pancreatitis. Thus, our findings may help to explain the pro-inflammatory role of platelets in the development of pathological inflammation in the pancreas.

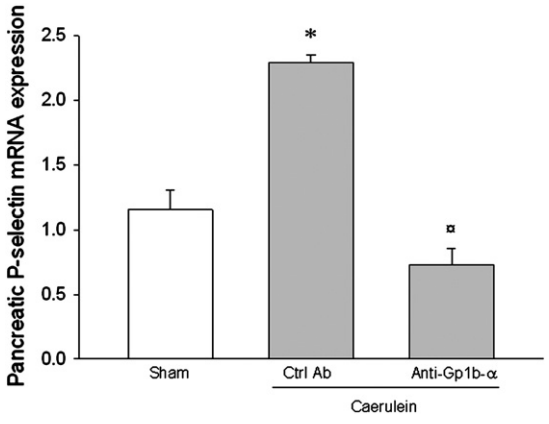

Fig. 2. P-selectin mRNA expression in the pancreas. Sham mice (white bars) received repetitive i.p. saline injection. Pancreatitis was induced by repetitive i.p. caerulein (black bars) administration. Mice were pretreated with a control antibody (Ctrl Ab) and a platelet depleting antibody (Anti-GP1bα) 2 h prior to pancreatitis induction. Samples were obtained 6 h after the last dose of caerulein injection. Data represent means ± S.E.M. and $n=4$–6. $^{*}P<0.05$ *vs.* Sham, and $^{¤}P<0.05$ *vs.* Ctrl Ab + Carulein.

The limited understanding of the pathophysiology behind acute pancreatitis poses major challenges to the management of patients with acute pancreatitis due to the restricted therapeutic options. New and emerging data in the literature forward a significant role of platelets in the initiation and progression of inflammatory processes in the pancreas (Abdulla et al., 2011a,b). In that study, it was shown that depletion of platelets had a significant influence on the extravascular accumulation of leukocytes in the pancreas (Abdulla et al., 2011a). Thus, it was of great interest in the present study to determine the role of platelets in supporting leukocyte–endothelial cell interactions in the pancreatic microcirculation. For this purpose, we used intravital fluorescence microscopy, which is the only method to directly examine temporal and spatial characteristics of leukocyte rolling and adhesion *in vivo* (Braun et al., 2008; Klintman et al., 2002; Mansson et al., 2000). Administration of an antibody directed against the platelet receptor CD42b reduced the circulating numbers of platelets by 83% suggesting that this protocol was an effective way to deplete animals of platelets. Notably, we found that platelet depletion reduced leukocyte rolling by 69% and firm adhesion by 74%, indicating for the first time that platelets indeed play a significant role in facilitating leukocyte–endothelium interactions during inflammation in the pancreas. Knowing that the rolling adhesive interaction is a compulsory precondition for subsequent firm adhesion of leukocytes in inflammatory reactions, which has been documented in numerous tissues (Klintman et al., 2002; Lindbom et al., 1992; Mansson et al., 2000), it may be proposed that the dominant effect of platelets on leukocyte–endothelial cell interactions is related to leukocyte rolling along the microvascular endothelium in the pancreas.

Convincing evidence have documented that leukocyte rolling is mediated by the selectin family of adhesion molecules, including L-, E-, and P-selectin. L-selectin has been shown to regulate lymphocyte rolling in secondary lymphatics (Steeber et al., 1996) and P-selectin has been shown to be the dominant molecule supporting rolling adhesive interactions of neutrophils in non-lymphatic tissues (Ishikawa et al., 2004; Klintman et al., 2002; Thorlacius et al., 1997; Wan et al., 2002) while E-selectin appears to be more important for cell activating events downstream of leukocyte rolling (Milstone et al., 1998; Ramos et al., 1997). A previous study suggested a role of P-selectin in acute pancreatitis (Hackert et al. 2009). Herein, we found that immunoneutralization of P-selectin abolished leukocyte rolling in venules of the pancreas. It was therefore of interest to examine whether platelets may influence P-selectin expression in acute pancreatitis. Indeed, we found that depletion of platelets significantly reduced caerulein-induced gene-expression of P-selectin in the pancreas suggesting that platelets regulate P-selectin expression in acute pancreatitis. Platelets secrete numerous substances, such as IL-1alpha (Thornton et al., 2010), platelet activating factor (Weber and Springer, 1997), platelet factor-4 (von Hundelshausen et al., 2007), and CD40L (Rahman et al., 2009), which have the capacity to activate endothelial cells and the identification of such platelet-derived mediators inducing P-selectin expression in the pancreas needs to be addressed in future investigations. In the pancreatic microvasculature in addition, we found that immunoneutralization of PSGL-1 reduced caerulein induced leukocyte rolling by 98%, suggesting that PSGL-1 is an important molecule regulating leukocyte rolling in acute pancreatitis. Interestingly, inhibition of P-selectin and PSGL-1 decreased leukocyte rolling to a similar degree and knowing that PSGL-1 is a high affinity ligand of P-selectin (Moore et al., 1995), it may be suggested that leukocyte rolling is regulated by interactions between P-selectin and PSGL-1 in acute pancreatitis. In this context, it is important to note that a previous report showed that depletion of platelets significantly reduce caerulein-induced formation of CXCL-2, which is a major CXC chemokine regulating neutrophil recruitment in the pancreas (Abdulla et al., 2011a).

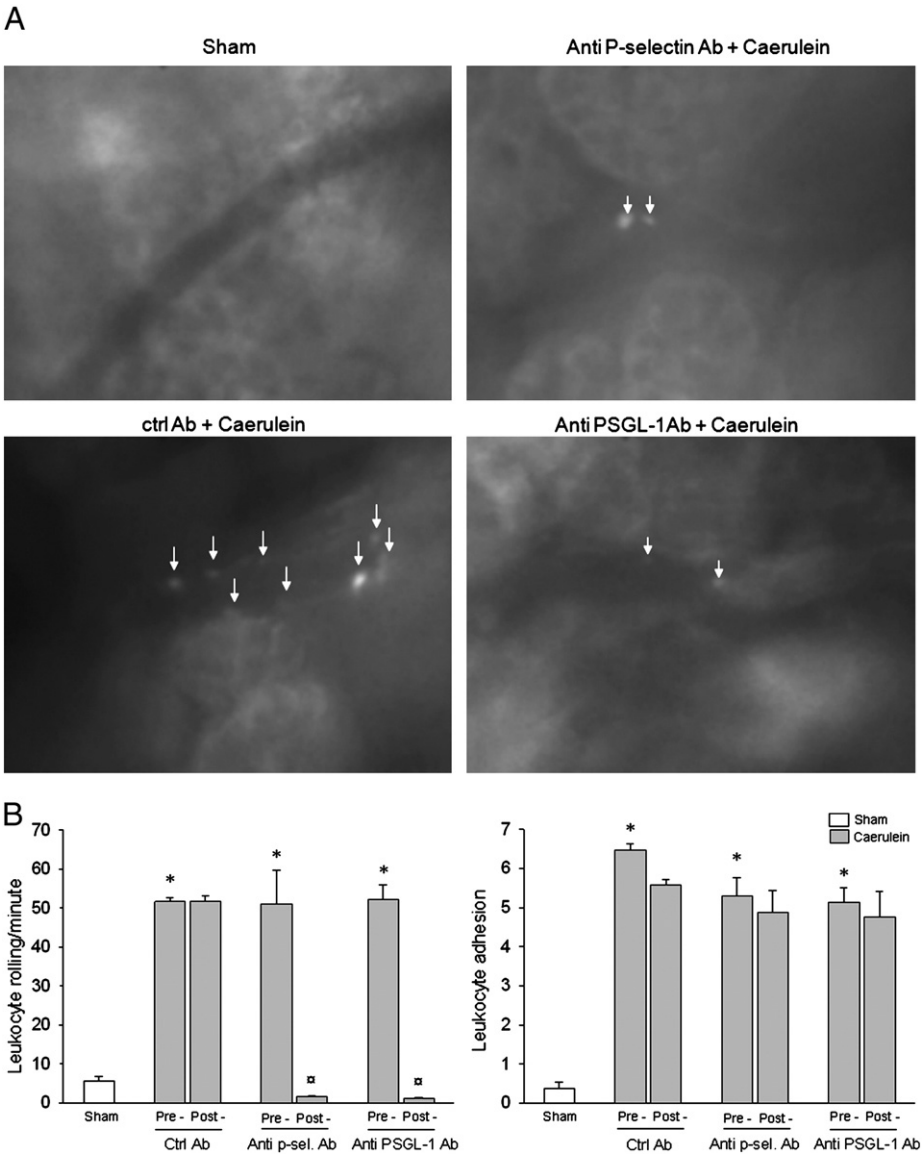

Fig. 3. A) Representative microphotographs from intravital fluorescence microscopy and B) aggregate data on leukocyte rolling and adhesion before and 5 min after treatment of specified antibodies. Animals were treated i.v. with a control antibody (Ctrl Ab), an anti-P-selectin Ab (Anti-P-sel. Ab) or an anti-P-selectin glycoprotein ligand-1 Ab (Anti-PSGL-1 Ab) 18 h after the last dose of caerulein injection. Pancreatitis (black bars) was induced by repetitive i.p. caerulein administration. Sham mice (white bars) received repetitive i.p. saline injection. Data represent means ± S.E.M. and $n = 4–6$. $^{*}P<0.05$ *vs.* Sham, and $^{¤}P<0.05$ *vs.* Ctrl Ab.

Indeed, we observed that caerulein-induced leukocyte adhesion was decreased in platelet-depleted mice. However, the influence of CXCL-2 in this reduced leukocyte adhesion is not likely significant since platelet depletion decreased leukocyte rolling, which is necessary for firm adhesion limiting the potential influence of CXCL-2 downstream of rolling in this situation. Thus, this observation excludes the possibility that a mechanism of platelet-mediated P-selectin-dependent leukocyte rolling is due to initial adhesion of platelets to endothelium followed by secondary selectin-mediated capture of leukocytes from the circulating blood, a mechanism suggested being in operation between platelets and lymphocytes in lymph nodes (Diacovo et al., 1996). Whether direct adhesion of platelets might contribute to leukocyte accumulation and tissue damage in acute pancreatitis remains elusive and, further studies are needed to delineate the molecular mechanism controlling platelet–endothelium interactions in the pancreas. Circulating platelet–leukocyte complexes is a frequent observation in a wide range of inflammatory conditions, including reperfusion injury (Kohler et al., 2011), abdominal sepsis (Salat et al., 1999), pulmonary infections (Schaub et al., 1981), and acute myocardial disease (Habazettl et al., 2000). Although, the potential function of such aggregates in inflammation has attracted a lot of interest recently the actual role of platelet–leukocyte complexes has not been identified. It was therefore of great interest to examine the role of aggregate formation in platelet–leukocyte interactions in the present study. We found that the percentage of platelet–leukocyte complexes increased by 5.6-fold in mice with pancreatitis, which is in line with previous observations in acute pancreatitis (Abdulla et al., 2011a,b). Notably, we observed that there was no difference in terms of surface expression of PSGL-1 and Mac-1 on neutrophils exhibiting low compared to high levels of platelet binding, indicating that physical contacts between circulating platelets and leukocytes cannot explain the platelet-mediated leukocyte–endothelium interactions observed in acute pancreatitis. In addition, a previous study showed that immunoneutralization of PSGL-1, which abolish aggregate formation had no concomitant effect on sepsis-induced Mac-1 expression on neutrophils, reinforcing the concept that platelet–leukocyte aggregate formation *per se* is not of major importance in platelet-dependent leukocyte recruitment. This notion is further supported by investigations demonstrating that there is no difference in the number of platelet–leukocyte aggregates in venous blood entering tissue and in

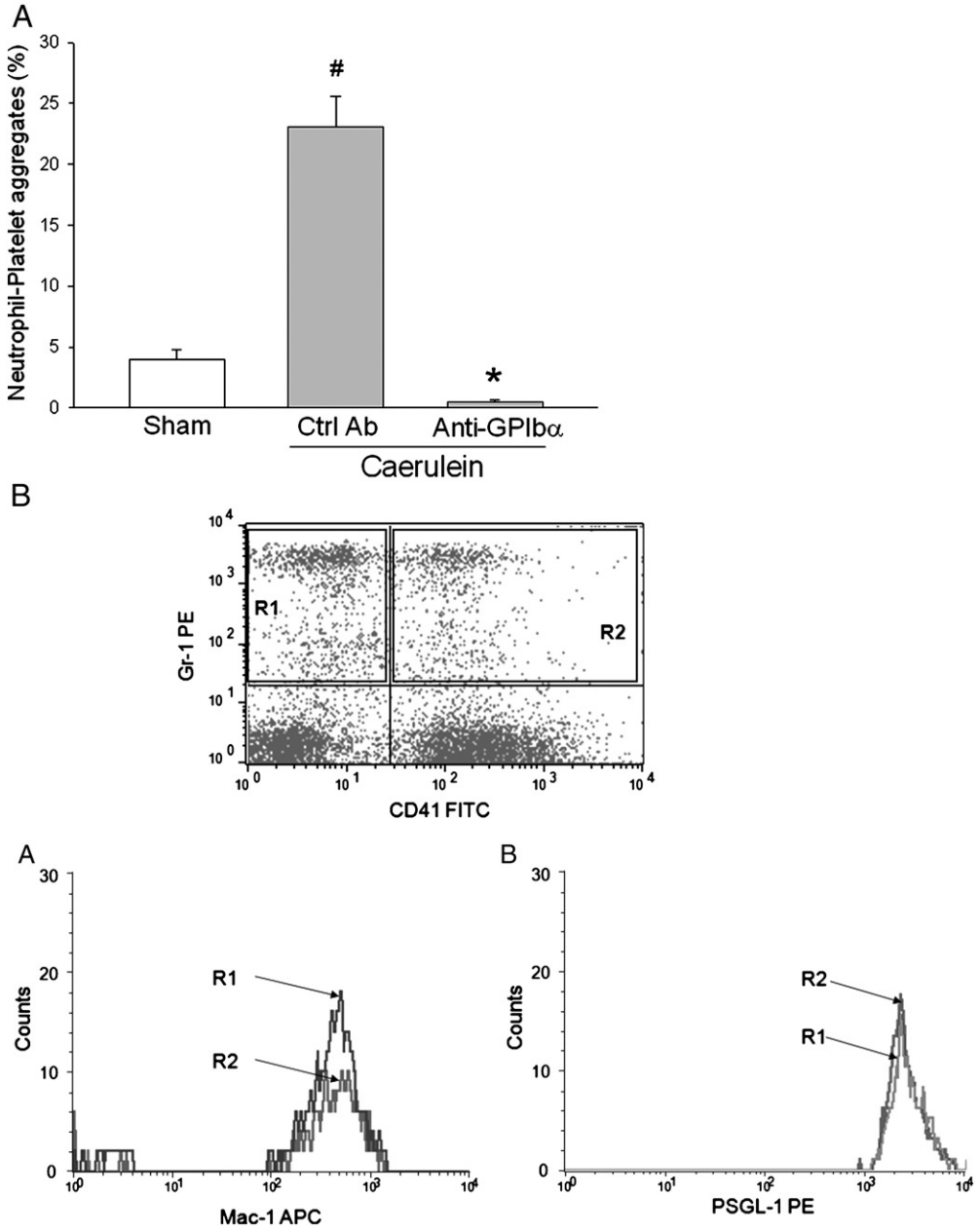

Fig. 4. Platelet–neutrophil aggregates formation and expression of PSGL-1 and Mac-1 on neutrophils. A) Platelet–neutrophil aggregates were quantified as the percentage of neutrophil (Gr-1$^+$) binding platelets (CD41$^+$) 1 h after the last dose of caerulein injection. Mice were pretreated with phosphate buffered saline (sham), a control antibody (Ctrl Ab) and a platelet-depleting Ab against GP1bα (Anti-GP1bα) prior to induction of acute pancreatitis. Data represents mean ± S.E.M. and $n = 6$. $^{*}P<0.05$ *vs.* Sham, $^{\alpha}P<0.05$ *vs.* Ctrl Ab + Caerulein. B) Dot plots showing the numbers of circulating neutrophils (Gr-1$^+$) and platelets (CD41$^+$) in mice 1 h after caerulein challenge and histogram showing PSGL-1 and Mac-1 expression on neutrophil binding no/low (R1 gate) or high numbers of platelets (R2 gate). Data are representative of four other experiments ($n = 4$). Pancreatitis was induced by repetitive i.p. caerulein (black bars) administration. Sham mice (white bars) received repetitive i.p. saline injections.

the arterial blood exiting tissue in models of pneumonia and abdominal sepsis (Asaduzzaman et al., 2009; Yoshida et al., 2006). Thus, we suggest that direct interactions between platelets and leukocytes in the circulation are not related to platelet-dependent leukocyte recruitment in acute pancreatitis. In this context, it is important to note that this does not exclude the possibility that other aspects of platelet–leukocyte contacts, including formation of pro-inflammatory mediators or reactive oxygen radicals might also contribute to inflammation and tissue damage in acute pancreatitis.

5. Conclusions

Taken together, these results demonstrate that platelets regulate leukocyte rolling *via* expression of P-selectin in acute pancreatitis. In addition, it was documented that PSGL-1 is a critical adhesion molecule supporting leukocyte rolling in the pancreas. However, we did not find evidence supporting a role of aggregate formation between platelets and leukocytes in platelet-dependent leukocyte–endothelium interactions in the pancreas. These findings elucidate the mechanistic role of platelets in regulating pathological inflammation in the pancreas as summarized in Fig. 5 and further support the idea to target platelet functions in order to protect against tissue damage in acute pancreatitis.

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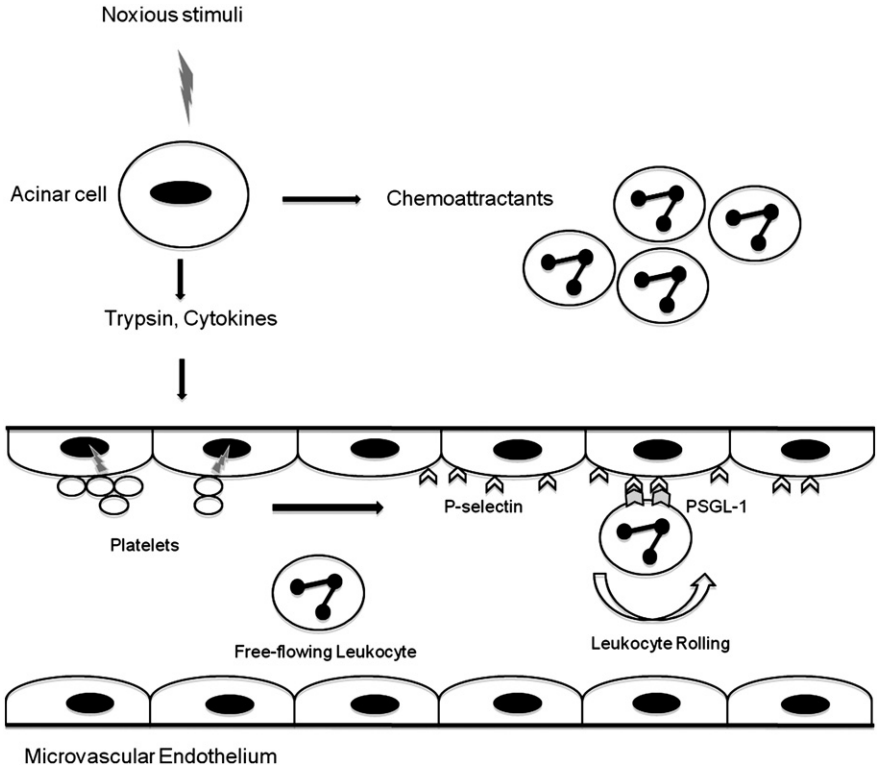

Fig. 5. The schematic diagram summarizes the proposed hypothesis behind platelet-dependent leukocyte recruitment in acute pancreatitis. During induction of pancreatitis, damaged acinar cells release trypsin and cytokines, which are known to activate endothelial cells and promote recruitment and activation of platelets. Platelets up-regulate P-selectin expression on the endothelium, which is recognized by PSGL-1 on circulating leukocytes promoting leukocyte rolling on the microvascular endothelium in the pancreas.

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