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Silica particles enhance peripheral thrombosis: key role of lung macrophage-neutrophil cross-talk

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Abstract

Rationale— Inflammation and thrombosis are related via interactions between leukocytes, platelets, the vasculature and coagulation system. However, the mechanisms behind these interactions remain poorly understood.

Objectives— We have investigated the effects of the well-known pulmonary inflammation induced by silica for the development of peripheral thrombogenicity in a hamster model of thrombosis. In addition, the consequences of pulmonary macrophage and circulating monocyte and neutrophil depletion on the thrombogenicity were investigated.

Methods— Silica particles (2-200 µg/hamster) were intratracheally instilled, and experimental thrombosis in photochemically induced femoral vein lesions was assessed 24 h later, in association with cellular infiltration in the lung.

Measurements and Main Results— Intratracheally instilled silica particles (20 and 200 µg/hamster) triggered pulmonary inflammation, coupled to stimulation of peripheral platelet-rich thrombus formation. Both the selective depletion of lung macrophages by i.t. administration of clodronate-liposomes, and the combined depletion of circulating monocytes and neutrophils by i.p. injection of cyclophosphamide significantly reduced silica-induced influx of macrophages and neutrophils in BAL, and reduced peripheral thrombogenicity. Silica-induced lung inflammation was accompanied by increased neutrophil elastase levels in BAL and also in plasma. Specific neutrophil elastase inhibition in the lung did not affect lung inflammation but reduced peripheral thrombogenicity.

Conclusions— These findings uncover pulmonary macrophage-neutrophil cross-talk releasing neutrophil elastase into the blood circulation. Elastase, triggering

activation of circulating platelets, may then predispose platelets to initiate thrombotic events on mildly damaged vasculature.

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Key words*:* Silica particles, Lung, macrophage, neutrophil, Thrombosis

Introduction

Both inflammation and thrombosis play a central role in the development of atherothrombosis, the underlying cause of approximately 80 % of all sudden cardiac deaths [1;2]. There is growing evidence of extensive cross-talk between inflammation and thrombosis, not only for inflammation leading to activation of thrombotic events, but also showing that thrombosis affects inflammatory activity. During these processes, a multitude of interactions are triggered, involving different types of cells such as platelets, leukocytes, endothelial cells and the coagulation/anti-coagulation cascades [2;3].

Upon platelet activation in pathological vascular conditions, polymorphonuclear leukocytes (PMN) may adhere to the growing thrombus, amplifying the thrombotic process by additionally activating platelets [4]. Neutrophil adhesion can be accompanied by monocyte/macrophage accumulation, in turn amplifying the inflammatory process [2]. Platelet-leukocyte interactions further support vascular inflammation [5;6]. These inflammatory cellular interactions may take place not only in the systemic circulation, e.g. after contact with infectious agents, such as in sepsis [7], or with non-self cells, such as during transplant vasculopathy [8], but they also occur in the lung following exposure to environmental insults, such as particulate air pollution [9;10]. In this context, it has been reported that pulmonary exposure to particles triggers fibrinogen elevation [11;12], enhances atherosclerosis [13] and increases the risk for platelet-rich thrombosis [14-18].

We have recently shown that diesel exhaust particles (DEP) cause lung inflammation accompanied by the development of a peripheral vascular thrombogenic tendency due to platelet activation. We have also shown that

histamine release by pulmonary mast cells plays a major role in triggering these processes [14;18].

Experimentally, acute exposure to silica particles produces sustained pulmonary inflammation in animal models, characterized by increased macrophage and neutrophil numbers and by damage of lung tissue [19]. Therefore, this well-established model appeared to be appropriate for the study of the possible consequences of pulmonary macrophage and neutrophil inflammation for extrapulmonary events, such as vascular inflammation and platelet activation.

The hypotheses of this study were that 1) instilled silica particles enhance peripheral vascular thrombosis in a manner similar to that by other particles studied previously [14-18]; 2) thrombotic effects depended both on pulmonary macrophages and neutrophils. These questions were studied by depleting animals from macrophages or neutrophils by clodronate or cyclophosphamide pretreatments, respectively. Finally, the roles of neutrophil elastase, as mediator of platelet activation by neutrophils [20;21], and histamine were also assessed.

Material and Methods

This project was reviewed and approved by the Institutional Review Board of the University of Leuven and experiments were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee.

Silica particles

Crystalline SiO_2 (Min-U-Sil), kindly provided by Prof. B. Fubini (Facoltà di Farmacia, Università di Torino, Italy), was suspended in sterile pyrogen-free saline (NaCl 0.9 %). The median size of particles was around 2 µm, as measured by means of a Coulter LS particle size analyzer at the VITO (Vlaamse Instelling voor Technologisch Onderzoek), Belgium.

To minimize their aggregation, particle suspensions were always sonicated (Branson 1200, VEL, Leuven, Belgium) for 15 min and vortexed immediately (< 1 min) before their dilution and prior to intratracheal administration. Control hamsters received saline.

Intratracheal instillation of particles

Male or female hamsters (Pfd Gold, Iffacredo, Brussels, Belgium) weighing 100-110 g were anesthetized with sodium pentobarbital (60 mg/kg, i.p.). The tracheal zone was shaved and desinfected with ethanol (70%), and the trachea was exposed for the intratracheal (i.t.) administration of 120 µl of saline or silica particles (2, 20 or 200 µg/hamster), as well as for the i.p or i.t. pretreatment of hamsters with cell-depleting or elastase inhibitor agents.

Experimental thrombosis model

Twenty-four hours after i.t. instillation of particles or saline, *in vivo* thrombogenesis was assessed, as recently described [17;22]. Following induction of anesthesia, hamsters were placed in a supine position on a heating pad at 37°C. A 2F venous catheter (Portex, Hythe, UK) was inserted in the right jugular vein for the administration of Rose Bengal. Thereafter, the right femoral vein was exposed from the surrounding tissue and mounted on a transilluminator. Mild endothelial injury was produced in the hamster femoral vein [17;22] and thrombus formation/disappearance were monitored for 40 min under a microscope at 40 times magnification [17;22]. The size of the thrombus was expressed in arbitrary units (A.U.) as the total area under the curve, when plotting light intensity against time [23]. The hamsters were euthanized at the end of the recording.

Bronchoalveolar lavage (BAL) fluid analysis

Twenty-four hours following the i.t. instillation of particles or vehicle, hamsters were killed with an overdose of sodium pentobarbital. The trachea was cannulated and lungs were lavaged three times with 1.5 ml of sterile NaCl 0.9%. The recovered fluid aliquots were pooled. No difference in the volume of collected fluid was observed between the different groups. BAL fluid was centrifuged (1,000 g x 10 min, 4°C). Cells were counted in a Thoma hemocytometer after resuspension of the pellets and staining with 1% gentian violet. The cell differentials were microscopically performed on cytocentrifuge preparations fixed in methanol and stained with Diff Quick (Dade, Brussels, Belgium). The supernatant was stored at - 80 °C until further analysis.

Preparation of liposome-encapsulated clodronate and depletion of alveolar macrophages

Liposomes composed of phosphatidylcholine and cholesterol (molar ratio, 6/1), with or without added dichloromethylene diphosphonate (clodronate, a gift of Roche Diagnostics GmbH, Mannheim, Germany), were produced as previously described [24]. Briefly, 86 mg of phosphatidylcholine and 8 mg of cholesterol were dissolved in 10 ml of chloroform and dried to a film by low vacuum rotary evaporation. The lipids were rehydrated in 10 ml of saline or in a solution of 2.5 g of clodronate in 10 ml of saline and incubated at room temperature. The liposome suspension was then diluted in 100 ml of saline and centrifuged at 100,000 x g for 30 min to remove free clodronate, after which liposomes were resuspended in 4 ml of saline.

Alveolar macrophage depletion was achieved by the i.t. instillation of 150 µl of a liposome-encapsulated clodronate suspension (CL), as described by Koay et al. [25]. Control hamsters received empty (saline-containing) liposomes (SL). Then, 24 h later, hamsters were i.t. instilled with silica particles (20 µg/ hamster) or saline. Still 24 h later, BAL was done and thrombosis experiments performed as described above, i.e. 48 h after SL/CL administration. The extent of lung macrophage and circulating monocyte depletion was assessed by differential cell counting in BAL and blood, respectively.

Depletion of neutrophils and circulating monocytes

In vivo depletion of circulating monocytes and neutrophils was achieved, as described by Lardot et al. [26], by a single i.p. injection of cyclophosphamide (CP, 20 mg/animal suspended in 100 µl of sterile saline) 3 days prior to the administration of silica particles or saline. Twenty-four hours after the i.t. administration of silica

particles or saline, i.e. 96 h after CP administration, the extent of cell depletion was assessed in the BAL and blood by differential cell counting. Platelets were counted on a CELL-DYN 1800 (Abbott Laboratories, Abbott Park, Illinois, USA) and thrombosis experiments were performed as described above.

Histamine determination in BAL and in plasma

Histamine concentrations in BAL and in plasma were determined by means of a commercially available radioimmunoassay kit (Immunotech, Marseille, France). The lower limit of detection of this assay was 0.2 nM.

Venous blood samples collected from the abdominal vena cava on EDTA (5mM) were centrifuged (1,000 g x 10 min, 4°C) and plasma samples were stored at - 80 °C.

Elastase determination in BAL and in plasma

Neutrophil elastase activity in BAL and in plasma was determined using the highly neutrophil elastase specific chromogenic substrate N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide (Sigma, St. Louis, MO) [27]. Briefly, samples were incubated in 0.1 M Tris–HCl buffer (pH 8.0) containing 0.5 M NaCl and 1 mM substrate for 24 h at 37 °C. After incubation, p-nitroaniline was measured spectrophotometrically at 405 nm and absorbance, corrected for baseline activity, was taken as an index of neutrophil elastase activity.

Elastase inhibition during lung inflammation and thrombosis

To assess the role of neutrophil elastase on lung inflammation and peripheral thrombosis, hamsters were i.t. instilled with methoxysuccinyl-alanyl alanyl-prolyl-valine-chloromethylketone (MeOSuc-AAPV-CMK, Calbiochem, Darmstadt, Germany) at a dose of 250 µg/animal 10 min before silica particle or

saline administration. Lung inflammation and thrombosis were assessed as outlined above.

Statistics

Data are expressed as means ± SEM. Comparisons between groups were performed by one way analysis of variance (ANOVA), followed by Newman-Keuls multiple range tests, two-way ANOVA, followed by Bonferroni multiple range tests or unpaired Student's t-tests, as indicated. P values <0.05 are considered significant.

The experiments were carried out over a number of weeks. The total numbers of hamster for the control groups and for each treatment group represent pools of hamsters over the entire experimental interval.

Results

Silica particles induce lung inflammation and enhance peripheral thrombosis

Following i.t. instillation of saline or silica particles, cells in BAL consisted mainly of macrophages and polymorphonuclear neutrophils (PMN), the remainder of the cells (<1%) being lymphocytes. The i.t. instillation of silica particles resulted in a marked cellular influx at doses of 20 and 200 µg/hamster but not at 2 µg/hamster (Figure 1). Macrophages increased to a comparable degree at 20 µg/hamster (4-fold, $p<0.05$) and 200 µg/hamster (5 fold, $p<0.01$) (Figure 1a). However, PMN numbers increased 30-fold at 20 µg/hamster ($p<0.05$) and 230-fold at 200 µg/hamster ($p<0.01$) (Figure 1b).

The i.t. instillation of silica particles enhanced the thrombus mass formed in a mildly photochemically injured hamster femoral vein 2.7-fold at 20 µg/hamster ($p<0.05$) and 3.7-fold at 200 µg/hamster ($p<0.001$) (Figure 2), i.e. at these doses that also triggered measurable lung inflammation.

Alveolar macrophage depletion reduces the silica-induced peripheral thrombogenicity

The i.t. pretreatment of control hamsters with empty (saline-containing) liposomes (SL) did not significantly affect the baseline amount of lung macrophages, as measured in BAL (Figure 1a and 3a). Similarly, i.t. pretreatment of hamsters with SL did not affect the macrophage infiltration induced by silica particles (20 µg/hamster) (Figure 1a and 3a). Correspondingly, pretreatment with SL had no effect on baseline or silica-induced PMN numbers in BAL (Figure 1b and 3b).

In contrast, the i.t. administration of clodronate-liposomes (CL) resulted in a reduction by 70 % in the baseline macrophage numbers (SL, n = 4) in BAL fluid compared to saline-liposomes (n = 4, $p < 0.001$) (Figure 3a). Pretreatment with CL did not block the silica-triggered macrophage lung infiltration entirely, but after silica exposure, macrophage numbers in CL-pretreated hamsters only reached values comparable to baseline values in controls (Figure 3a). CL-pretreatment did not deplete monocytes from the circulation: monocyte numbers in circulating blood were similar after SL-pretreatment ($2.6 \pm 0.9 \times 10^5$/ml blood, n=4) and CL-pretreatment ($2.8 \pm 0.6 \times 10^5$/ml blood, n=4) at the time of thrombosis induction. However, pretreatment with CL reduced significantly the influx of PMN in BAL fluid after i.t. silica (Figure 3b). Because circulating numbers of PMN were not affected by the i.t. pretreatment with CL (PMN numbers after SL-pretreatment: $1.6 \pm 0.4 \times 10^5$/ml blood, n=4; after CL-pretreatment: $1.5 \pm 0.3 \times 10^5$/ml blood, n=4, p=NS), these findings implicate that PMN-influx in the lung is secondary to the activation of pulmonary macrophages [28;29], which attract fewer PMN, when reduced in number.

Whereas no effect of pre-treatment with SL or CL was observed on peripheral thrombus formation in saline-treated hamsters, the pretreatment of hamsters with CL strongly reduced the prothrombotic effects induced 24 h after the i.t. silica particle administration (Figure 4).

Depletion of systemic PMN and monocytes inhibits peripheral thrombogenicity

Figure 5a shows that, in the saline-treated group, the number of macrophages in BAL was not affected by pretreatment with cyclophosphamide (CP), whereas the expected increase in the numbers of macrophages following i.t.

silica instillation was completely inhibited. Following i.p. CP injection, the low PMN numbers in saline-treated hamster lungs were unaffected. However, the PMN influx caused by silica particle administration was strongly reduced after CP pretreatment (Figure 5b).

Without CP pretreatment, the mean numbers (± SEM) of total blood leukocytes in the saline and silica-treated groups were 20 ± 2 x10^5/ml blood (80 % lymphocytes, 12 % monocytes and 8 % neutrophils) and 22 ± 3 x10^5/ml blood (75 % lymphocytes, 13 % monocytes and 12 % neutrophils), respectively, i.e. silica administration by itself had no impact on circulating monocyte and PMN numbers (p=NS) 24 hours later. Cyclophosphamide pretreatment led to an 80 % reduction in circulating leukocytes in both the CP + saline group (3.7 ± 0.5 x10^5/ml blood) and the CP + silica group (4.5 ± 0.6 x10^5/ml blood). There were no quantifiable neutrophils among the circulating leukocytes remaining after cyclophosphamide pre-treatment. Circulating monocyte numbers in the saline-treated group (0.25 ± 0.03 x10^5/ml blood, n=4) were not different from those in the silica-treated group (0.24 ± 0.03 x10^5/ml blood, n=4) but they were reduced 10-fold, as a consequence of the CP-pretreatment.

Platelet numbers did not change significantly after pretreatment with CP (216 ± 15 x10^3/µl blood in the saline group; 220 ± 5 x10^3/µl in the CP + saline treated group; 195 ± 19 x10^3/µl blood in the silica group; 178 ± 30 x10^3/µl in the CP + silica group).

CP pre-treatment did not affect the extent of thrombosis in saline-treated hamsters, despite the strong reduction of circulating leukocyte numbers. However, pretreatment with CP significantly reduced the silica-induced stimulation of thrombosis (Figure 6).

Histamine and elastase determination in BAL and in plasma

Silica particle administration had no effect on the concentrations of histamine (mean ± SEM, n = 4-5) in BAL (2.5 ± 0.9 nM *vs.* 2.2 ± 0.7 nM, in controls), and these levels were not affected by pretreatment with CL (2.4 ± 0.8 nM) or with CP (2.5 ± 0.7 nM). Similarly, in plasma, no effect of silica on histamine levels was observed (26.6 ± 6.7 nM *vs.* 25.5 ± 8.0 nM, in controls), neither after pre-treatment with CL (29.5 ± 3.6 nM), nor with CP (24.3 ± 2.5 nM).

Silica particles induced a significant increase in neutrophil elastase (NE) activity in BAL and in plasma, compared to control hamsters. Pretreatment of hamsters with CL or with CP significantly reduced this increase in BAL and in plasma (Figure 7).

Neutrophil elastase inhibition in lung inflammation and thrombosis

The i.t. pretreatment of control hamsters with MeOSuc-AAPV-CMK did not significantly affect total cell numbers in BAL (figure 8a and 8b) nor the thrombosis *in vivo*. No effect of this pretreatment was observed on the silica-induced increase of macrophage or PMN numbers in BAL (figure 8a and 8b). However, the i.t. administration of MeOSuc-AAPV-CMK partially but significantly mitigated the silica-induced elevation of the thrombotic response (figure 8c).

Discussion

We have demonstrated that the i.t. instillation of silica particles in hamsters leads to significant dose-dependent increases of macrophage and neutrophil numbers in BAL, and the development of a prothrombotic tendency in circulating blood. By specifically depleting lung macrophages with clodronate-liposomes, we found that both the influx of PMN in BAL and the peripheral thrombotic tendency were abrogated. The depletion of circulating PMN and monocytes by cyclophosphamide, also abolished both the cellular influx in BAL and the peripheral thrombotic tendency, in spite of normal numbers of lung macrophages. While silica particles did not affect histamine concentrations in BAL or plasma, they caused an increase in neutrophil elastase activity in plasma.

Polymorphonuclear neutrophils, through the oxidant species and mediators they release, contribute to vessel injury not only by their adherence to endothelium and by diapedesis, but also through interactions with platelet receptors such as P-selectin [3;5;30]. The majority of studies have investigated the impact of inflammation on tissue injury, using isolated cells [31;32], whole blood, [33] or at sites of vascular damage linked to the presence of thrombi [34]. However, previously reported population-based studies have established that reduced lung function is associated with cardiovascular morbidity and mortality [35;36]. Also, it has been recently shown that particulate air pollution can cause lung inflammation and promotes systemic inflammation, atherosclerosis and thrombosis [11;13-17;37]. In the present study, we have investigated the relationship between lung inflammation and thrombosis via the study of interactions between lung macrophages, monocytes, PMN and platelets, operating in two different compartments, i.e. the respiratory and cardiovascular system. As a cardiovascular endpoint, we used a

recently established and validated model of acute thrombosis in the hamster [38]. In this photochemical injury model of platelet-rich thrombosis, prothrombotic tendencies can be approached experimentally [17;23;39].

To investigate the role of macrophages and PMN in priming platelet activation and thrombus formation, we selected silica particles as a tool to produce lung inflammation within 24 h [19]. In contrast to ultrafine particles (UFPs, diameter < 0.1 µm), which may translocate from the lung into the blood [40-42], the silica particles used (2 µm) were too large to translocate. It is known that extra-thoracic structures, such as the liver and spleen, may be affected by exposure to silica, however, these features have been described in the clinical and pathological literature after long term exposure to silica particles. Such extrathoracic silicosis is always associated with pulmonary silicosis, and it is generally believed to be "metastatic" through a possible lymphatic spread [43;44]. However, such extrathoracic spread is unlikely to have occurred in the present study because of the time window (24 h) investigated. Therefore, any systemic effect produced by this type of particles in our model must have resulted predominantly, if not exclusively, from lung inflammation and the passage of mediators released from the lung into the systemic circulation.

Silica particles caused a dose-dependent increase in the number of macrophages and neutrophils in BAL, along with enhanced thrombus formation, most likely due to peripheral platelet activation as indicated above.

An effect of silica particles on peripheral thrombosis has not been reported previously, but using other types of particles, we have previously reported that polystyrene UFPs and diesel exhaust particles (DEP) cause lung inflammation and the development of peripheral thrombogenicity resulting from circulating platelet activation [14-17]. Therefore, within the time window investigated (24 h), lung

inflammation, following exposure to silica particles, appears to be a common initiating step with other particles such as DEP.

Alveolar macrophages are the principal phagocytes mediating uptake and degradation of organisms in the lung. In addition to locomotion, phagocytosis, and microbiocidal activities, resident and infiltrating macrophages secrete a variety of chemokines and cytokines responsible for PMN recruitment [45]. To assess the role of macrophages in the influx of PMN in the lung and in the development of peripheral thrombosis in response to silica particles, hamsters were depleted by i.t. pretreatment with liposome-encapsulated clodronate (CL). Lung macrophage depletion in the present study was comparable to that described by Koay et al. [25] after i.t. administration of CL in mice. Our results demonstrate that the selective depletion of pulmonary macrophages leads to significant inhibition of monocyte and PMN influx upon administration of silica. This result confirms a primary role for macrophages in the PMN recruitment.

To further assess the role of lung macrophages, circulating PMN and monocytes were depleted, using cyclophosphamide. Cyclophosphamide did not affect the number and composition of cells (including macrophages) in BAL of control hamsters, nor did it affect the number of circulating platelets. The degree of thrombosis in saline-treated hamsters was not affected by the cyclophosphamide treatment, demonstrating that the acute thrombotic response to the photochemical injury is independent of leukocyte activation and platelet-leukocyte interactions. These results are in agreement with studies reported in mice [26], hamsters [46] and pigs [47]. However, depletion of monocytes and neutrophils with cyclophosphamide caused a strong inhibition of the silica particle-dependent peripheral thrombosis. These findings indicate that lung macrophage activation by silica [28;29] is required to trigger peripheral thrombogenicity, but that the simple activation of macrophages is

insufficient to do so, in the absence of a further macrophage-mediated influx of monocytes and PMN in the lung. Taken together, these results uncover the primary role of lung macrophages, which are responsible for PMN influx in the lung, but also the essential role of PMN which further contribute to additional monocyte infiltration. Our depletion approach thus revealed that macrophage-PMN cross-talk is an essential element in explaining the development of peripheral thrombotic events after instillation of silica particles.

Recently, we found that 24h following i.t. administration of DEP, histamine concentrations increased in BAL and in plasma and that the pretreatment of hamsters with diphenhydramine [14], a histamine H1-receptor antagonist, or with sodium cromoglycate [18], a mast cell and basophil stabilizer, abrogated the inflammatory and thrombotic effects, by antagonizing histamine H1 receptor or by blocking its release. In the present study, no histamine release was observed in BAL or plasma, thus excluding mast cell and basophil participation in the development of peripheral thrombogenicity. This striking difference between DEP and silica may be related to the specific surface chemistry of DEP, which carry organic compounds that may trigger histamine release in mast cells and initiate lung inflammation [48;49]. Therefore, to study the relation between pulmonary infiltrating cells and peripheral platelet activation, the silica model seems simpler, because it does not involve plasma histamine-dependent leukocyte activation, complicating the study of the relation between lung cells and peripheral platelets. Moreover, circulating leukocyte numbers were not affected following silica administration, excluding possible systemic leukocyte activation.

Neutrophil elastase activity was elevated in BAL and in plasma in response to silica particle administration. Neutrophil elastase activity has been shown to augment upon lung injury associated with neutrophil infiltration in alveolar spaces

[50]. In addition, neutrophil elastase and cathepsin G released from activated neutrophils were reported to contribute to platelet activation *in vitro*, via activation of the platelet receptor PAR-4 [21;51]. To assess the role of neutrophil elastase released in the lung on the observed peripheral thrombotic events, hamsters were i.t. instilled with MeOSuc-AAPV-CMK, a specific neutrophil elastase inhibitor [52], that has been shown to inhibit elastase-induced acute lung injury in hamsters [52]. Our results confirm a potential role for pulmonary elastase in peripheral platelet activation. Indeed, the i.t. pretreatment of hamsters with MeOSuc-AAPV-CMK while not affecting the silica particle-induced lung inflammation per se, partially but significantly inhibited the peripheral thrombotic tendency. Both neutrophil elastase and cathepsin G have been proposed as mediators of platelet activation by neutrophils [20;21]; the importance of each enzyme separately will have to be evaluated for the priming of platelets.

In conclusion, our findings provide novel evidence for a critical role of macrophage-neutrophil cross-talk during lung inflammation, leading to the release of neutrophil elastase into the systemic circulation. Neutrophil enzymes may be responsible for the priming of platelet activation and contribute to the development of a thrombotic tendency, when such primed platelets encounter a (mildly) injured vessel wall.

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Figure legends

Figure 1. Silica-induced lung inflammation. Numbers of macrophages (a) and polymorphonuclear neutrophils (PMN) (b) in bronchoalveolar lavage fluid, obtained 24 h after i.t. instillation of saline or silica particles (2, 20 or 200 µg/animal). Means ± SEM (n = 4-5 in each group). Statistical analysis by one-way ANOVA followed by Newman-Keuls multiple comparison test.

Figure 2. Silica-induced peripheral thrombogenicity in the femoral vein. Cumulative thrombus size, expressed as total light intensity over 40 min [in arbitrary units (A.U.)], after a mild photochemical damage to the endothelium of a femoral vein. Data were obtained 24 h after i.t. instillation of saline or silica particles (2, 20 or 200 µg/animal). Means ± SEM (n = 4-7 in each group). Statistical analysis by one-way ANOVA followed by Newman-Keuls multiple comparison test.

Figure 3. Silica-induced lung inflammation after pulmonary macrophage depletion. Numbers of macrophages (a) and polymorphonuclear neutrophils (PMN) (b) in bronchoalveolar lavage fluid obtained 24 h after i.t. instillation of saline or silica particles (20 µg/hamster) with or without i.t. pretreatment with clodronate-liposomes (CL, 150 µl) or saline liposomes (SL, 150 µl), 48 h earlier. Means ± SEM (n = 4 in each group). Statistical analysis by two-way ANOVA followed by Bonferroni multiple comparison test.

Figure 4. Silica-induced peripheral thrombosis after pulmonary macrophage depletion. Cumulative thrombus size, expressed as total light intensity over 40 min [in arbitrary units (A.U.)], after a mild photochemical damage to the femoral vein. Data were obtained 24 h after the i.t. instillation of saline or silica particles (20 µg/hamster) with or without i.t. pretreatment with CL (150 µl) or SL (150 µl), 48 h earlier. Means ± SEM (n = 4 in each group). Statistical analysis by two-way ANOVA followed by Bonferroni multiple comparison test.

Figure 5. Silica-induced lung inflammation after systemic PMN and monocytes depletion. Numbers of macrophages (a) and polymorphonuclear neutrophils (PMN) (b) in bronchoalveolar lavage fluid obtained 24 h after i.t. instillation of saline or silica particles (20 µg/hamster) with or without i.p. pretreatment with i.p. cyclophosphamide (CP, 20 mg/hamster), 96 h earlier. Means ± SEM (n = 4-5 in each group). Statistical analysis by one-way ANOVA followed by Newman-Keuls multiple comparison test. Data for non CP-pretreated hamsters are the same as in figure 1.

Figure 6. Silica-induced peripheral thrombosis after systemic PMN and monocytes depletion. Cumulative thrombus size, expressed as total light intensity over 40 min [in arbitrary units (A.U.)], after a mild photochemical damage to the femoral vein. Data were obtained 24 h after i.t. instillation of saline or silica particles (20 µg/hamster) with or without i.p. pretreatment with CP (20 mg/hamster), 96 h earlier. Means ± SEM (n = 4-7 in each group). Statistical analysis by one-way ANOVA followed by Newman-Keuls multiple comparison test. Data for non CP-pretreated hamsters are the same as in figure 1.

Figure 7. Neutrophil elastase release. Elastase concentrations (x 1 µmol of p-nitroanaline/ml sample) in BAL (a) and plasma (b), 24 h after i.t. instillation of saline or silica particles (20 µg/hamster) with or without i.t. pretreatment with clodronate liposomes (CL, 150 µl) or i.p. pretreatment with cyclophosphamide (CP, 20 mg/hamster). Means ± SEM (n = 4-5 in each group). Statistical analysis by one-way ANOVA followed by Newman-Keuls multiple comparison test.

Figure 8. Neutrophil elastase inhibition. Numbers of macrophages (a) and polymorphonuclear neutrophils (PMN) (b) in bronchoalveolar lavage fluid, and cumulative thrombus size, expressed as total light intensity over 40 min [in arbitrary units (A.U.)], after a mild photochemical damage to the femoral vein (c). after i.t. administration of MeOSuc-AAPV-CMK. Data were obtained 24 h after i.t. instillation of saline or silica particles (20 µg/animal) with or without i.t. pretreatment with MeOSuc-AAPV-CMK (250 µg/animal). Means ± SEM (n = 4-7 in each group). Statistical analysis by one-way ANOVA followed by Newman-Keuls multiple comparison test. Data for non MeOSuc-AAPV-CMK-pretreated animals are the same as in figure 1 and figure 2.

Figure 1

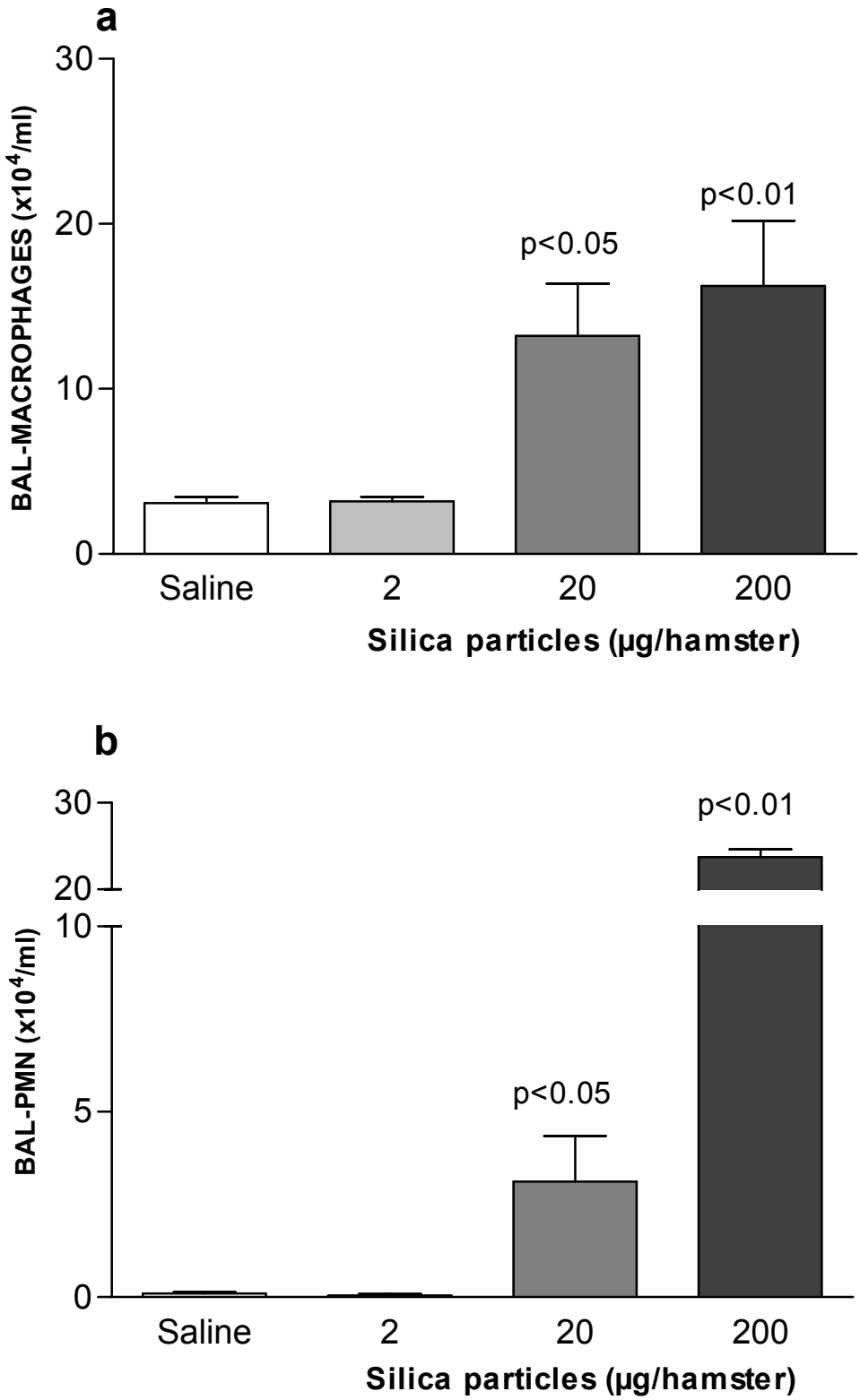

a
30
20
10
0
BAL-MACROPHAGES (x10^4/ml)
p<0.05
p<0.01
Saline
2
20
200
Silica particles (µg/hamster)
b
30
20
10
5
0
BAL-PMN (x10^4/ml)
p<0.05
p<0.01
Saline
2
20
200
Silica particles (µg/hamster)

Figure 2

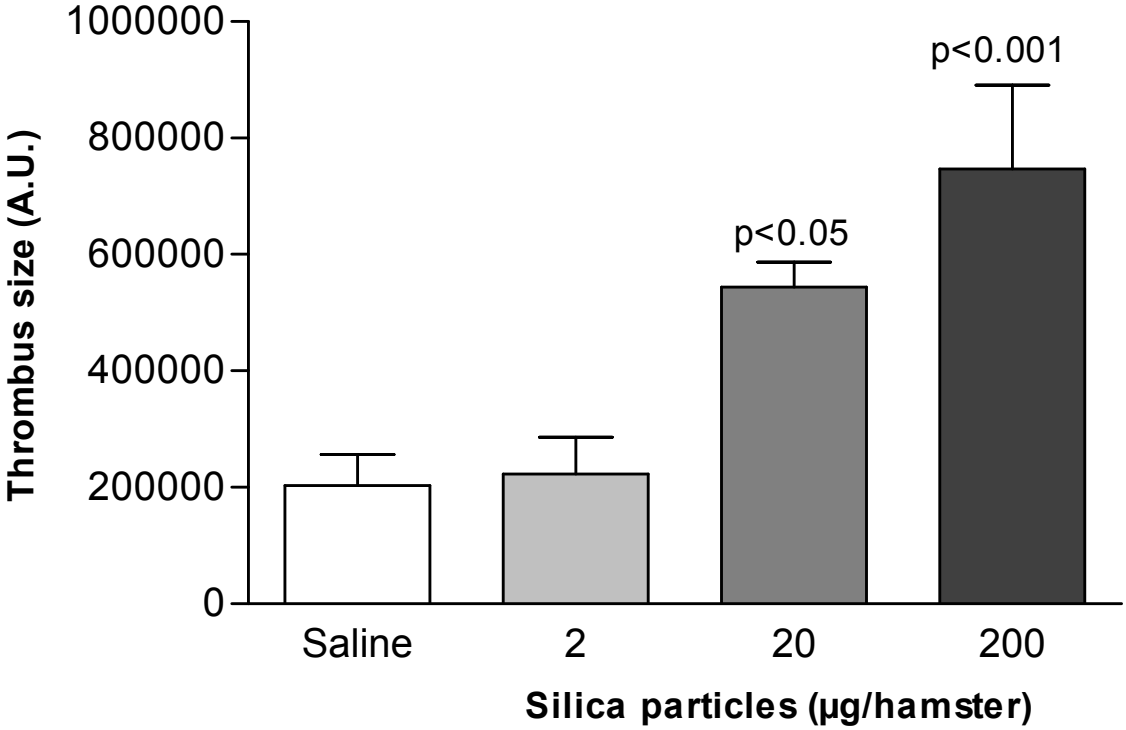
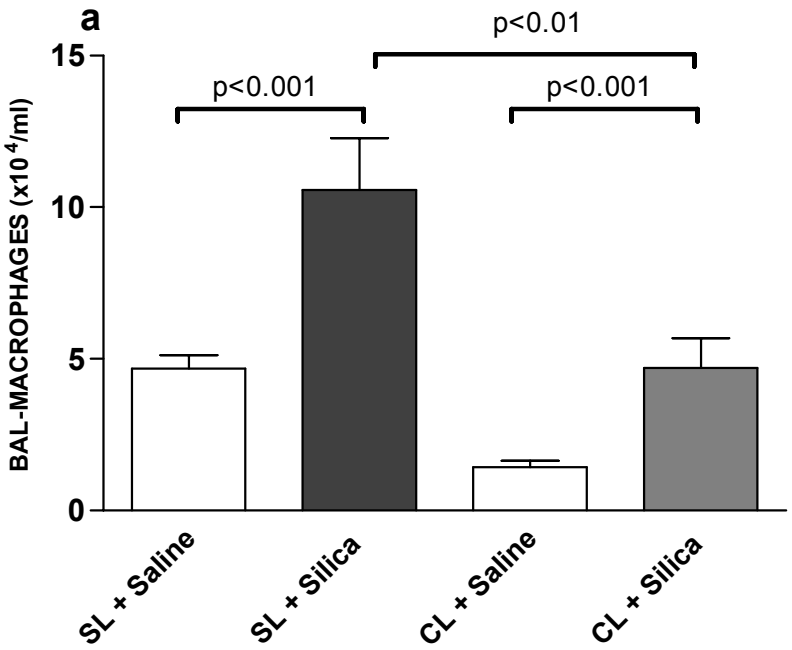

1000000
800000
600000
400000
200000
0
Thrombus size (A.U.)
p<0.001
p<0.05
Saline
2
20
200
Silica particles (µg/hamster)

Figure 3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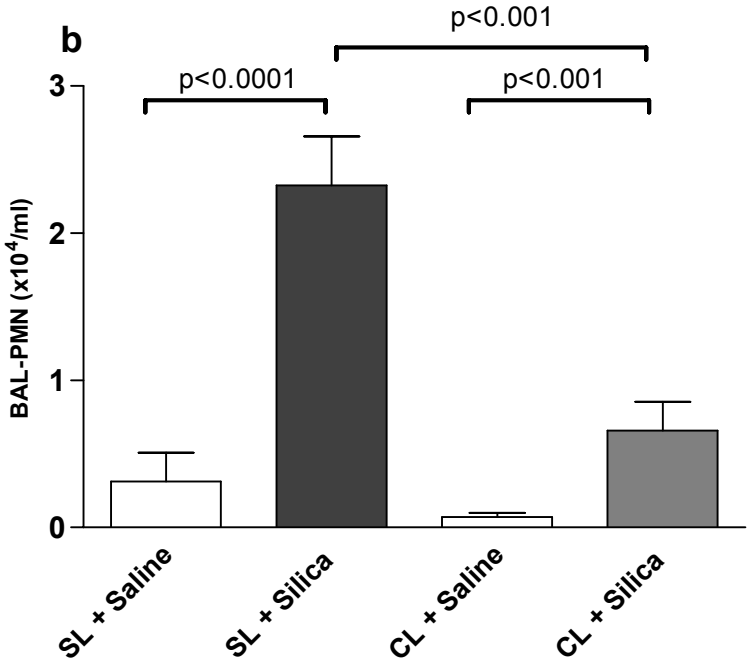
p<0.001
p<0.001
BAL-MACROPHAGES (x10^4/ml)
15
10
5
0
SL + Saline
SL + Silica
CL + Saline
CL + Silica


b
p<0.001
p<0.0001
p<0.001
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2
1
0
SL + Saline
SL + Silica
CL + Saline
CL + Silica

Figure 4

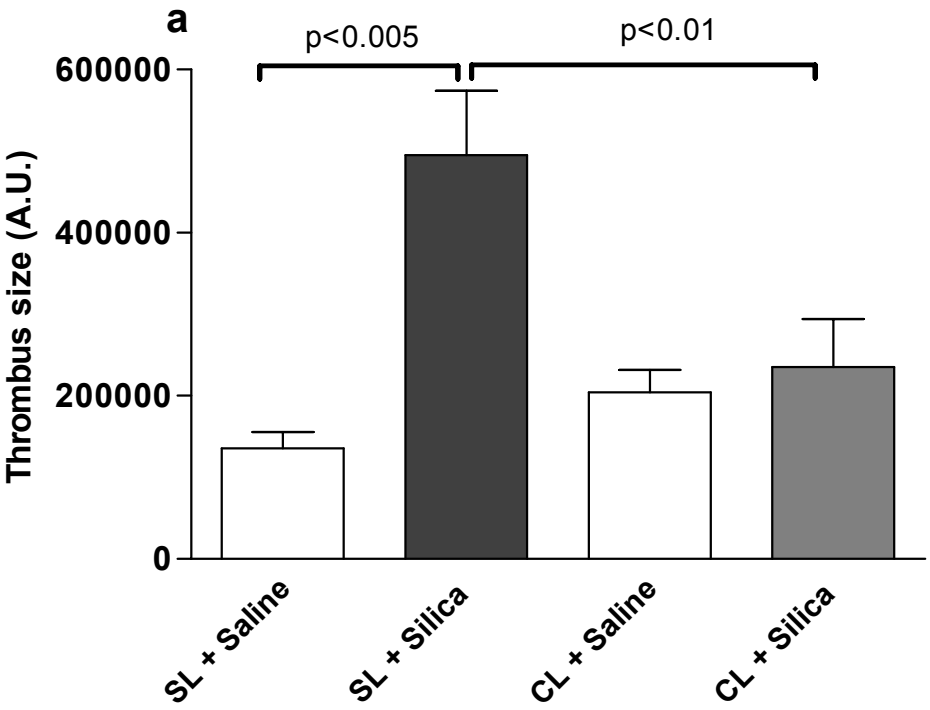
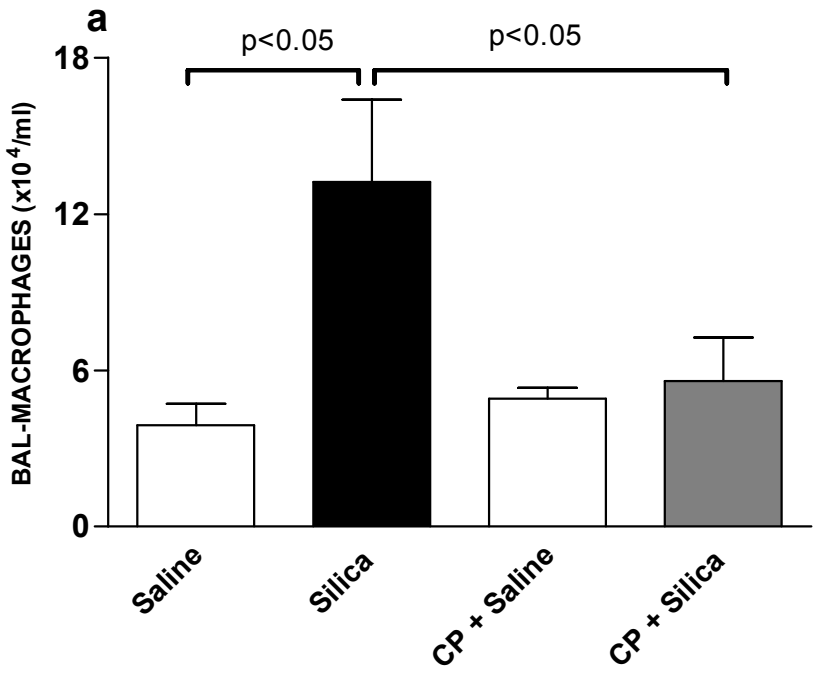

a
p<0.005
p<0.01
600000
400000
200000
0
Thrombus size (A.U.)
SL + Saline
SL + Silica
CL + Saline
CL + Silica

Figure 5


a
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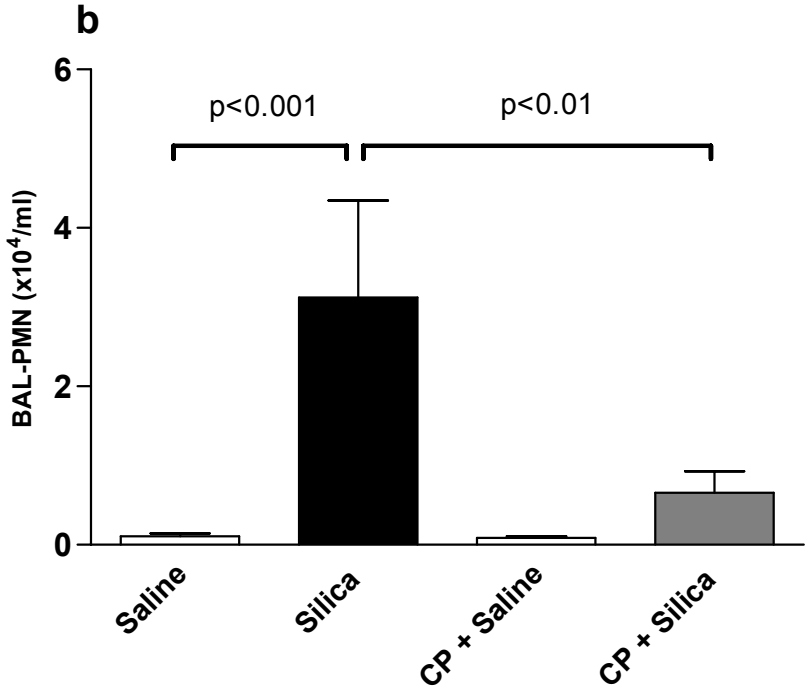
p<0.05
18
12
6
0
BAL-MACROPHAGES (x10 4/ml)
Saline
Silica
CP + Saline
CP + Silica


b
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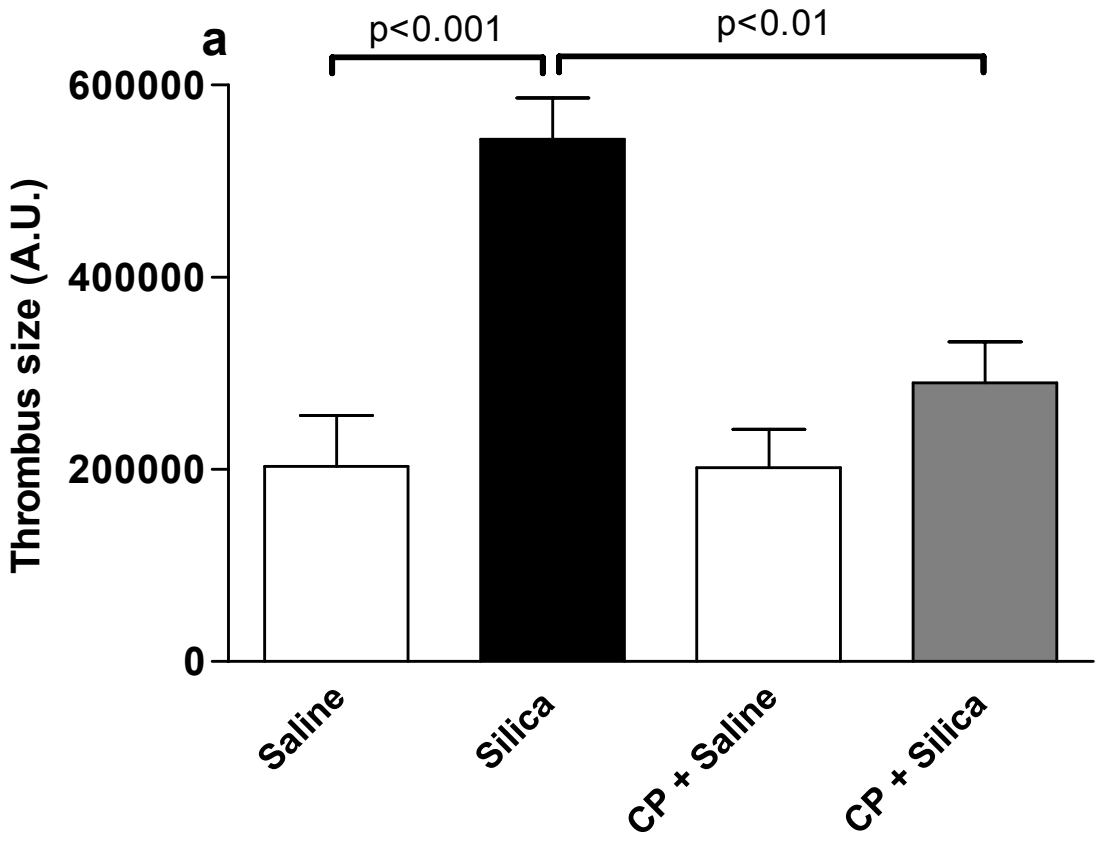
p<0.01
6
4
2
0
BAL-PMN (x10 4/ml)
Saline
Silica
CP + Saline
CP + Silica

Figure 6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p<0.01
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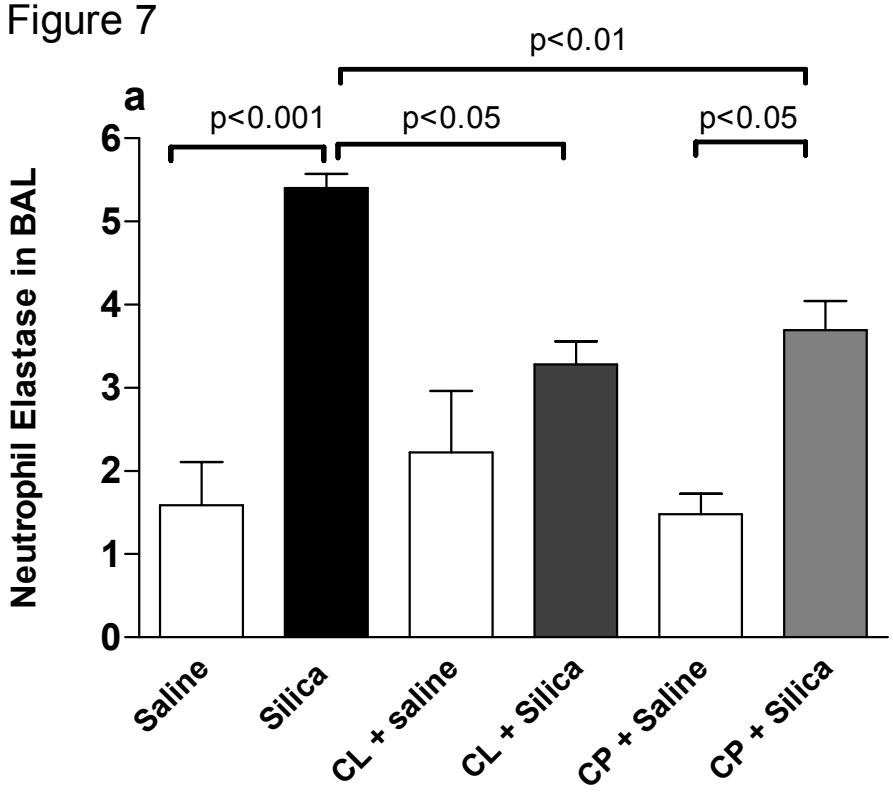
400000
200000
0
Thrombus size (A.U.)
Saline
Silica
CP + Saline
CP + Silica

Figure 7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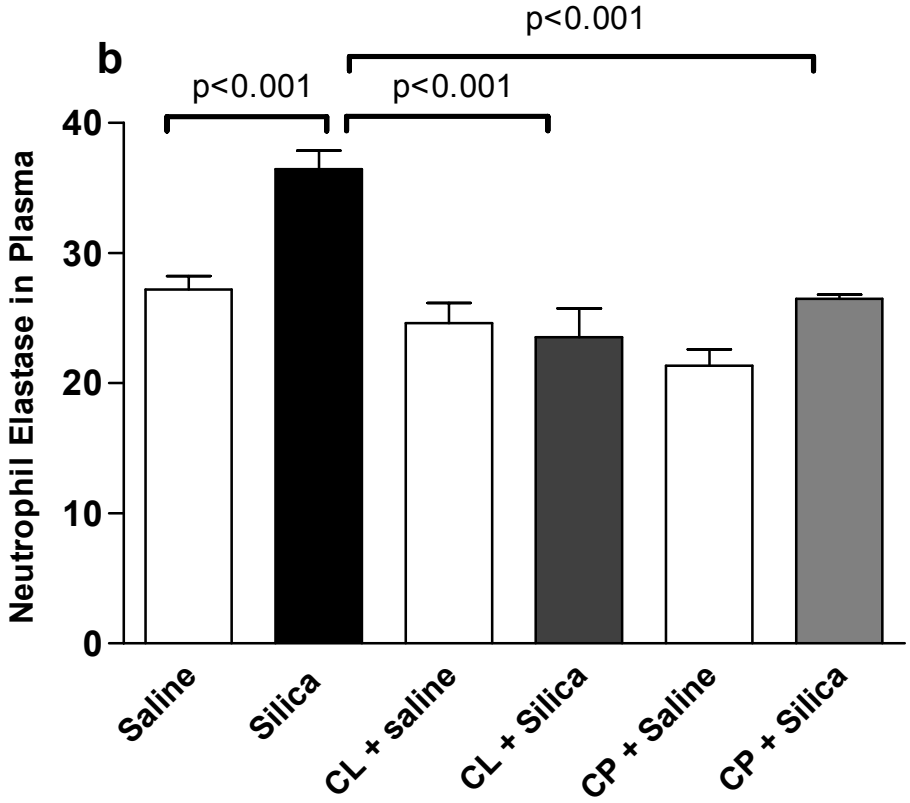
p<0.001
p<0.05
p<0.05
Neutrophil Elastase in BAL
0
1
2
3
4
5
6
Saline
Silica
CL + saline
CL + Silica
CP + Saline
CP + Silica


b
p<0.001
p<0.001
p<0.001
Neutrophil Elastase in Plasma
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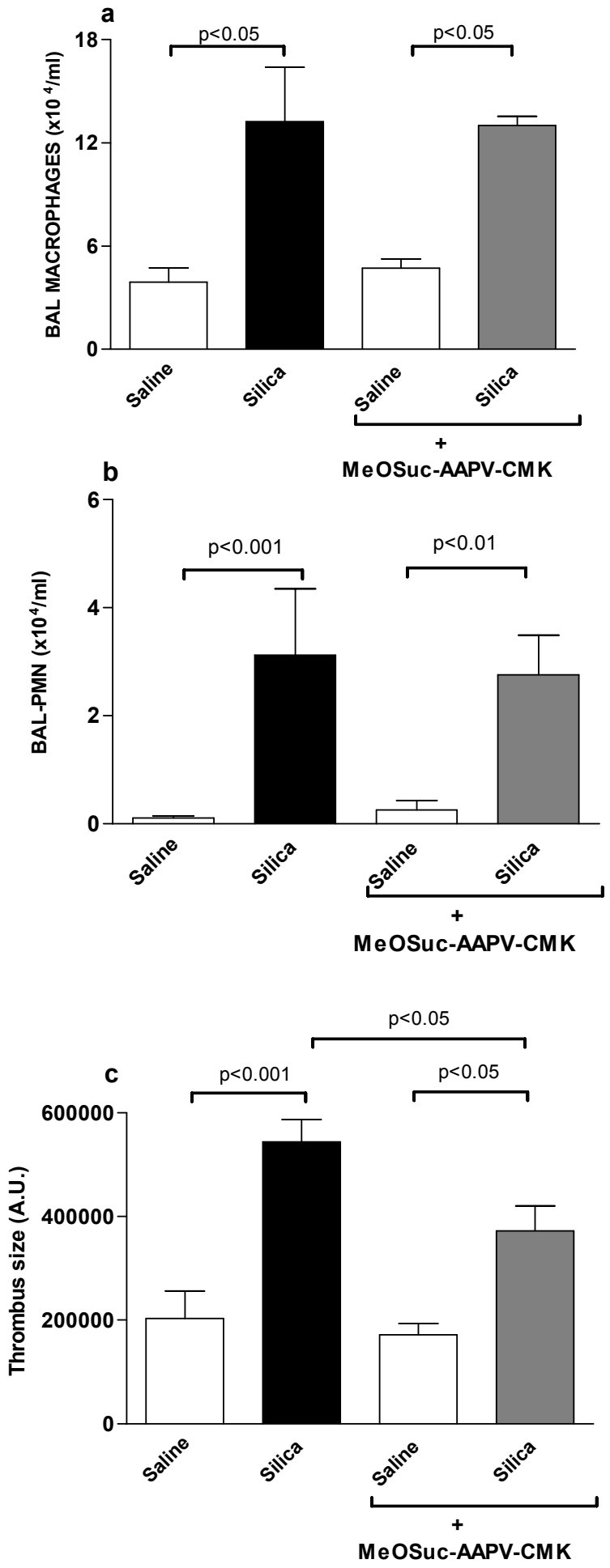
10
20
30
40
Saline
Silica
CL + saline
CL + Silica
CP + Saline
CP + Silica

Figure 8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p<0.05
p<0.05
BAL MACROPHAGES (x10 4/ml)
18
12
6
0
Saline
Silica
Saline
Silica
+
MeOSuc-AAPV-CMK
b
p<0.001
p<0.01
BAL-PMN (x10 4/ml)
6
4
2
0
Saline
Silica
Saline
Silica
+
MeOSuc-AAPV-CMK
p<0.05
c
p<0.001
p<0.05
Thrombus size (A.U.)
600000
400000
200000
0
Saline
Silica
Saline
Silica
+
MeOSuc-AAPV-CMK