FIBROSIS

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Platelet microparticles sustain autophagy-associated activation of neutrophils in systemic sclerosis

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Endothelial cell damage and platelet activation contribute to sustained vasculopathy, which is a key clinical characteristic of systemic sclerosis (SSc), also known as scleroderma. Microparticles released from activated platelets in the blood of SSc patients (SSc-microparticles) are abundant and express the damage-associated molecular pattern (DAMP) HMGB1. SSc-microparticles interacted with neutrophils in vitro and in immunocompromised mice and promoted neutrophil autophagy, which was characterized by mobilization of their granule content, enhanced proteolytic activity, prolonged survival, and generation of neutrophil extracellular traps (NETs). Neutrophils migrated within the mouse lung, with collagen accumulation in the interstitial space and the release of soluble E-selectin by the vascular endothelium. Microparticle-neutrophil interaction, neutrophil autophagy and survival, and generation of NETs abated in the presence of BoxA, a competitive inhibitor of HMGB1. Consistent with these results, neutrophils in the blood of SSc patients were autophagic and NET by-products were abundant. Our findings implicate neutrophils in SSc vasculopathy and suggest that platelet-derived, microparticle-associated HMGB1 may be a potential indicator of disease and target for novel therapeutics.

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INTRODUCTION

Systemic sclerosis (SSc) or scleroderma is an autoimmune disease with a chronic and progressive course. Tissue fibrosis interferes with the architecture and function of organs, and tissues and the mechanisms initiating and sustaining the disease are poorly understood. Thus, SSc remains a poorly understood medical condition with few effective therapies (*1*, *2*).

Dysfunctional and dysregulated responses to tissue injury contribute to the pathogenesis of SSc. Activated fibroblasts and myofibroblasts are effectors of fibrosis, and their heterogeneous properties, regulatory pathways, and origins contribute to clinical diversity among patients (*3*).

Activation of endothelial cells, loss of capillaries, and exposure of subendothelial connective tissues are early events in SSc (*4*–*6*). At later stages, vessels undergo remodeling with thickening of the medial layer, hyperplasia of the intima, and eventual obliteration of the vascular lumen (*7*, *8*). This is accompanied by abnormal expression of transcription factors, cytokines and growth factors, and defects in angiogenesis and vasculogenesis (*1*, *9*–*13*). The microvascular damage results in tissue hypoxia and oxidative stress and contributes to myofibroblast activation and fibrogenesis. The mechanisms underpinning endothelial injury are not well understood (*14*–*18*).

Concomitant to the changes in the SSc endothelial lining, platelets undergo activation (*19*–*21*) and release vascular endothelial growth factor, platelet-derived growth factor, transforming growth factor-β, and serotonin (*15*). Platelets are also a source of the high-mobility group box 1 (HMGB1) protein (*22*), a damage-associated molecular pattern (DAMP) (*23*–*27*). HMGB1 promotes fibrogenesis after endothelial cell damage and favors tissue remodeling (*28*). These actions depend on the ability of HMGB1 to influence cell metabolism inducing autophagy, a process by which stressed cells ingest organelles and protein aggregates to provide anabolic substrates to feed their own bioenergetics (*29*). Blood concentration of soluble HMGB1 is elevated in patients with SSc (*30*), whereas HMGB1 in SSc platelets is depleted (*31*). Platelet-derived microparticles in the blood of patients with SSc are abundant and contain bioactive HMGB1 (*32*–*35*). We thus hypothesized that platelets might be a source of HMGB1 in SSc patients and could be critical to sustaining and driving vascular damage.

Platelet HMGB1 activates neutrophils and commits them to generate neutrophil extracellular traps (NETs) (*34*, *36*–*38*). NETs are a source of autoantigens and a player in the pathogenesis of autoimmune diseases like systemic lupus erythematosus (SLE) and rheumatoid arthritis (*39*–*41*). Little is known about the potential involvement of neutrophils in SSc. Neutrophilia in bronchoalveolar lavage and neutrophilic infiltration within alveoli and interstitium are indicators of lung fibrosis and correlate with disease severity (*42*–*45*), which suggests the involvement of neutrophils in tissue damage. We hypothesized that neutrophils may also play a role in SSc, similar to what has been suggested for other systemic autoimmune diseases like SLE (*46*). We therefore verified the activation and function of neutrophils from patients with SSc, as well as the possible role of microparticles released by platelets in SSc vasculopathy and tissue fibrosis. This pathway might contribute, through the presentation of HMGB1 to neutrophils and to endothelial cells, to key features of SSc.

RESULTS

Microparticles in the blood of SSc patients express the DAMP HMGB1

Platelet-derived microparticles, identified by size and by the expression of the platelet-specific CD61 antigen, were more abundant in both whole blood and platelet-free plasma from SSc patients than from healthy volunteers or from patients with SLE (Fig. 1, A and B). Microparticles of SSc patients were more likely to express the DAMP HMGB1 than microparticles of healthy volunteers or of SLE patients, as assessed by flow cytometry (Fig. 1B). $HMGB1^{+}CD61^{+}$

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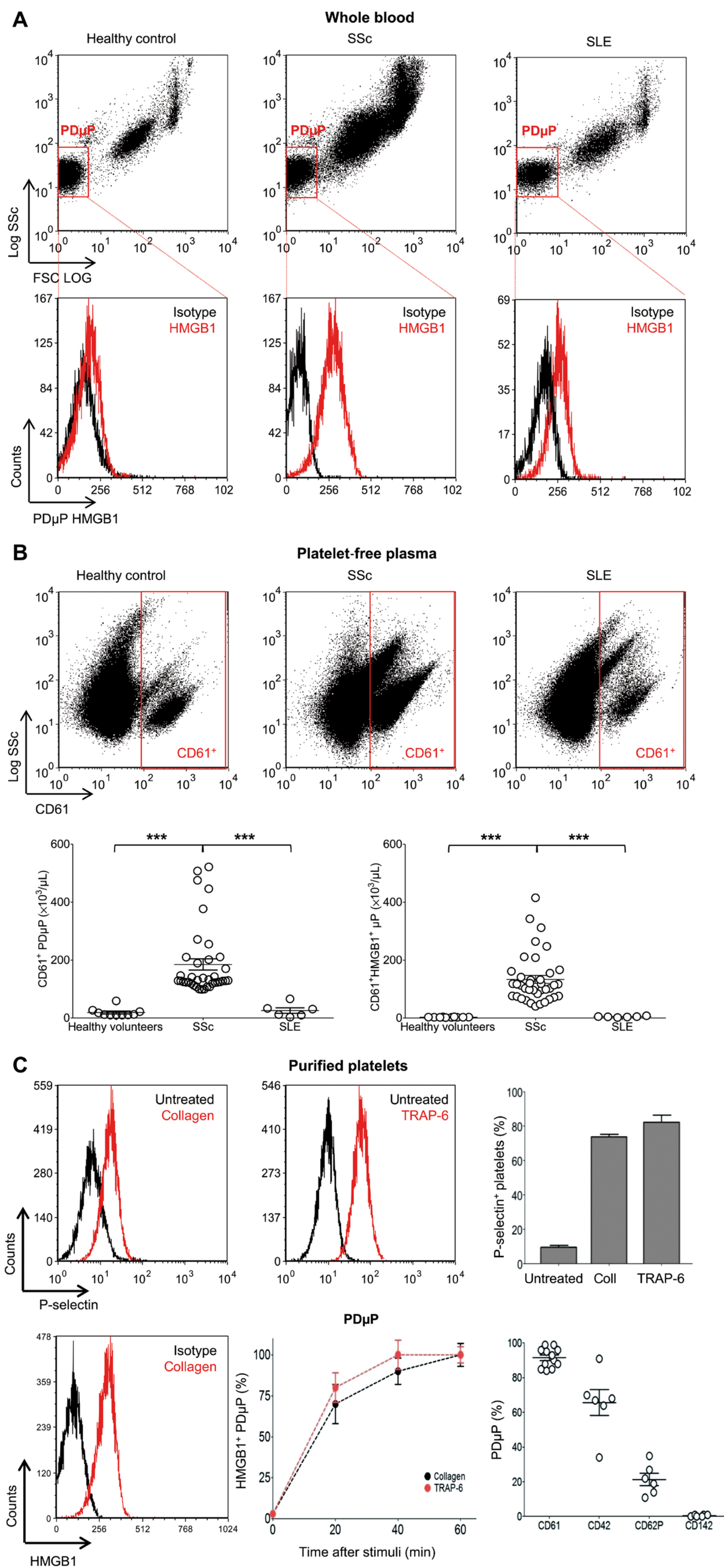

Fig. 1. Platelet microparticles from patients with SSc express HMGB1. (**A**) Whole blood from healthy controls (left) or patients (middle and right) was examined by flow cytometry. $CD61^+$ events were identified as microparticles based on low forward and side scatter. HMGB1 staining is shown in the lower row (red), with isotype staining in black. Images shown are representative of 53 healthy controls, 57 SSc patients, and 6 SLE patients. (**B**) (Top) Platelet-derived microparticles (PDμP) were identified in platelet-free plasma based on side scatter and CD61 expression. (Bottom) Concentration of platelet-derived $CD61^+$ microparticles and of $CD61^+$ microparticles expressing HMGB1. Each dot corresponds to a single subject, and lines indicate means ± SEM. ***$P < 0.0001$ by analysis of variance (ANOVA) and Bonferroni test. (**C**) (Top) Purified platelets from five healthy donors were stimulated with collagen or TRAP-6, and representative platelet P-selectin expression (red) is shown. Bars indicate means ± SEM (five different experiments carried out independently). (Bottom left) Expression of HMGB1 (red) on microparticles released by collagen-stimulated platelets (5 min). (Bottom middle) Fraction of $HMGB1^+$ microparticles released by collagen- or TRAP-6–stimulated platelets at various times. (Bottom right) Expression of CD61, CD42, CD62P (P-selectin), and CD142 (tissue factor) on collagen-microparticles. Each dot represents the result of a single independent assessment, and lines indicate means ± SEM.

microparticles were more numerous in patients with SSc than in patients with SLE or in healthy controls (Fig. 1B and Table 1). Purified platelets from healthy controls, activated with physiological stimuli (collagen or the thrombin receptor agonist, TRAP-6), exposed P-selectin (Fig. 1C) and released microparticles that expressed CD61, CD42, and HMGB1 in a time-dependent manner, as assessed by flow cytometry. The platelet-derived microparticles poorly expressed P-selectin (CD62P) and tissue factor (CD142) (Fig. 1C). In keeping with the scope of our work, we chose collagen, a constituent of the subendothelial layers of injured vessels, to commit platelets to the generation of microparticles (collagen-microparticles). Complexity, as assessed by side scatter, was similar in collagen-microparticles and SSc-microparticles (10 ± 2 and 11 ± 2 mean log side scatter, respectively; fig. S1).

SSc microparticles prompt healthy neutrophils to redistribute intracellular granule content, to undergo autophagy, and to generate NETs via HMGB1

We verified the bioactivity of microparticles by adding them to neutrophils freshly purified from the blood of healthy donors. The concentration of microparticles used to stimulate neutrophils correlated directly with granular content redistribution and expression of myeloperoxidase (MPO) on the neutrophil plasma membrane (Fig. 2A). The fraction of neutrophils expressing MPO on the surface also increased in a dose-dependent manner (Fig. 2A). Microparticle-stimulated neutrophils acquired the ability to proteolyse fibrin, which suggested that proteinases redistributed at the plasma membrane were biologically active. The extent of the fibrinolytic action correlated directly with the dose of $HMGB1^+$ microparticles used to challenge neutrophils (Fig. 2A). Both collagen- and SSc-microparticles were effective (Fig. 2A) at promoting fibrin

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Table 1. Microparticles from the blood of SSc patients trigger neutrophils and endothelial cell activation.

	Healthy controls	SSc	SLE
n	53	57	6
Leukocytes/μl	5793 ± 106	6922 ± 458*	5933.3 ± 113
Neutrophils/μl	3546 ± 83	4663 ± 536	3500 ± 543
Platelets (×10^3/μl)	200 ± 5	223 ± 17	248 ± 5
Whole blood†			
CD66b$^+$CD16$^-$ neutrophils (%)	1.0 ± 0.1	3.6 ± 1.2	21.6 ± 10.9*‡
Cyto-ID$^+$ neutrophils (%)	4.3 ± 1	78.9 ± 3.9*	33.4 ± 6.4*‡
P-selectin$^+$ platelets (%)	6.2 ± 0.3	20.9 ± 1.6*	20.1 ± 4.8*
TF$^+$ platelets (%)	4.9 ± 0.6	22.3 ± 3.8*	26.4 ± 6.6*
Platelet-free plasma§			
MPO-DNA complexes (a.u.)	0.2 ± 0.03	1.2 ± 0.08*	1.1 ± 0.39*
Platelet (CD61$^+$) microparticles (×10^3/μl)	19.1 ± 4.8	184.4 ± 19.3*	24.4 ± 11.4
Platelet (CD61$^+$) HMGB1$^+$ microparticles (×10^3/μl)	2.9 ± 0.3	133.4 ± 13.8*	4.7 ± 1.0‡
In vivo effects elicited by control-, SSC-, or SLE-microparticles‖			
Autophagic murine neutrophils (%)	6.5 ± 0.6	62.3 ± 3.6*	23.5 ± 6.5‡
mMPO murine neutrophils (%)	11.0 ± 0.6	77.8 ± 2.1*	17.7 ± 3.9‡
Citrullinated H4 (a.u.)	0.06 ± 0.01	0.14 ± 0.01*	0.07 ± 0.02‡
Soluble E-selectin (ng/ml)	10.7 ± 2.1	223.2 ± 74.6*	13.9 ± 1.2‡

*P < 0.001, significantly different from healthy controls. †Whole blood was analyzed by flow cytometry, as described in the Supplementary Materials. CD66b$^+$CD16$^-$ refer to apoptotic neutrophils. Cyto-ID$^+$ accumulation identifies autophagic cells. CD62P and CD142 are markers of platelet activation. ‡P < 0.001, from patients with SSc. §MPO-DNA complexes were assessed by enzyme-linked immunosorbent assay (ELISA), and platelet-derived microparticles were assessed by cytometry in platelet-free plasma. ‖Microparticles from controls and from patients with SSc or with SLE were injected in NSG mice. Autophagy and membrane MPO expression (mMPO) were determined by cytometry in the mouse blood, and citrullinated histone H4 and soluble E-selectin were determined by ELISA in platelet-free plasma.

degradation by neutrophils, but neutrophils challenged with microparticles from healthy controls and untreated neutrophils minimally degraded fibrin. Recombinant soluble HMGB1 also activated neutrophils to degrade fibrin (Fig. 2A).

HMGB1 is a known inducer of autophagy. We confirmed that purified healthy neutrophils underwent autophagy upon stimulation with HMGB1$^+$ microparticles in vitro. Both SSc-microparticles and collagen-microparticles induced autophagy, as assessed by flow cytometry monitoring the intracellular accumulation of dye, which selectively labels autophagic vacuoles, or by evaluating the LC3 expression (Fig. 2B and fig. S2). Control-microparticles only marginally induced autophagy in neutrophils. Autophagy was dependent on HMGB1, because SSc- and collagen-microparticles did not induce autophagy in the presence of the HMGB1 competitive antagonist BoxA (Fig. 2B). The efficacy of microparticles at inducing neutrophil autophagy was concentration-dependent (fig. S2A).

Adherent neutrophils rely on autophagy to extrude decondensed chromatin threads containing citrullinated histones, referred to as NETs, and both SSc-microparticles and collagen-microparticles induced NET generation. Microparticles expressing the CD42 platelet antigen (red) were associated with NETting neutrophils (Fig. 2C) and soluble MPO-DNA complexes accumulated in the culture supernatant, as assessed by ELISA (Fig. 2C). Control-microparticles did not induce detectable NET generation. NET generation was reduced in the presence of BoxA (Fig. 2C); conversely, adherent neutrophils challenged with recombinant human HMGB1 and released NETs (Fig. 2C). Antibody blockade of the HMGB1-RAGE axis almost completely abrogated the induction of autophagy and the generation of NETs (Table 2).

Neutrophil autophagy and NETs are found in the blood of SSc patients

We reasoned that SSc-microparticles, which are abundant in the blood of the patients and express bioactive surface HMGB1, might act on circulating neutrophils. In keeping with previous reports, neutrophils of SSc patients appeared to be primed (*47*), having translocated granular enzymes to the plasma membrane (Fig. 3, A and B). Moreover, blood neutrophils of SSc patients, but not of healthy volunteers, exhibited features of autophagy, as assessed by confocal microscopy of autophagosomes (Fig. 3, C and D) and confirmed by electron microscopy (Fig. 3E). The fraction of autophagic blood neutrophils as assessed by flow cytometry was significantly higher in SSc patients than in controls (P < 0.0001; Fig. 3F). Markers of systemic inflammation, extent of cutaneous and pulmonary fibrosis, or any other clinical feature of the patients shown in table S1 did not correlate with the fraction of circulating neutrophils that accumulated the autophagy-indicating dye.

Platelet-derived HMGB1$^+$ microparticles, which are abundant in the blood of SSc patients, might also influence the ability of patient neutrophils to generate NETs. We found that the concentration of soluble DNA-MPO complexes or citrullinated H4 histones was correlated (r = 0.70, P < 0.0001; Fig. 3I) and significantly higher in the plasma of SSc patients than in that of healthy controls (both P < 0.0001; Fig. 3, G and H). MPO-DNA complexes were significantly more concentrated in the plasma of patients with an early and active scleroderma pattern compared with those with a late scleroderma pattern at the high-magnification assessment by nailfold videocapillaroscopy [1.13 ± 0.08 versus 0.67 ± 0.02 arbitrary units (a.u.), P < 0.002], suggesting that this approach reveals characteristics of the patients' microvasculature that correlate with the accumulation of NET by-products.

Platelet-derived microparticles prompt autophagy and survival of human neutrophils in NSG mice

Previous studies have demonstrated that autophagy induction correlates with inhibition of neoplastic cell apoptosis (*29*). We wondered whether microparticles could extend the survival of neutrophils via apoptosis inhibition. We exposed healthy human neutrophils to SSc-microparticles in the presence or absence of the blocker of the autophagic flux, wortmannin, and injected them in NSG mice. NSG mice

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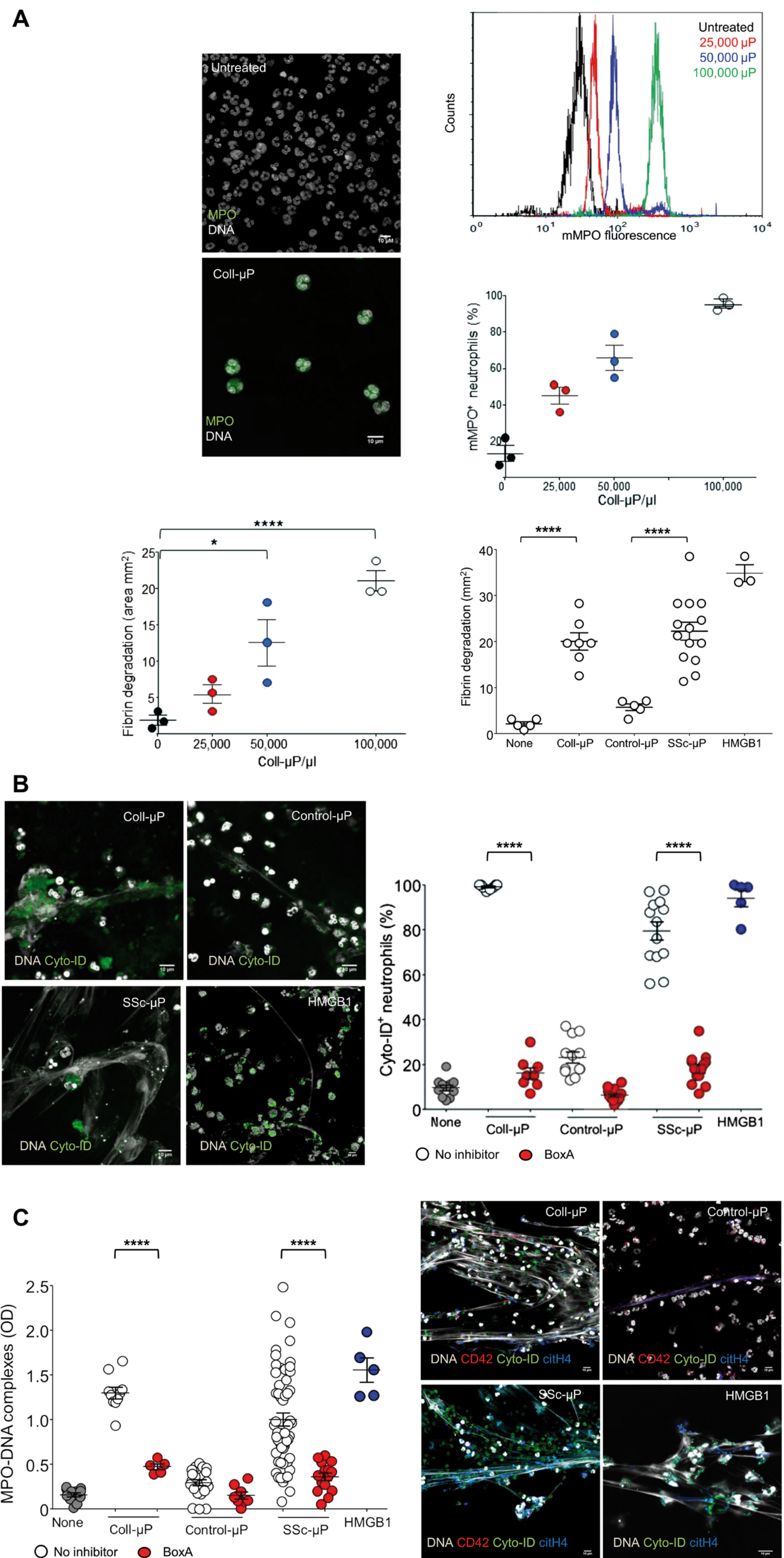

Fig. 2. Microparticles activate neutrophils via HMGB1. (**A**) Neutrophils from healthy donors were stimulated or not with collagen-microparticles (Coll-μP), and membrane MPO expression (mMPO) was assessed by confocal microscopy and flow cytometry (upper histogram: MPO fluorescence intensity; middle: fraction of neutrophils expressing mMPO). Representative of 10 experiments carried out independently. (Bottom) Fibrin proteolysis, assessed as actual area of proteolysis (mm^2), with increasing concentrations of collagen-microparticles. The effect of a given concentration of microparticles (100,000/μl) of different origin (Coll-μP or microparticles derived from SSc or healthy controls) on the ability to digest fibrin is also shown. Recombinant HMGB1 was used as positive control. Each dot represents a single donor. Lines indicate means ± SEM. *P < 0.01 and ****P < 0.0001 by ANOVA and Bonferroni test. (**B**) (Left) Neutrophils were stimulated with collagen-, control-, and SSc-microparticles or with HMGB1 and analyzed by confocal microscopy for the Cyto-ID autophagy tracer (green) or for DNA (white). (Right) Cyto-ID was quantified by flow cytometry in untreated (gray) or microparticle-stimulated neutrophils with (open circles) or without the HMGB1 inhibitor BoxA (red). HMGB1 (blue) is the positive control. (**C**) The concentration of the NET by-products, MPO-DNA complexes, released by neutrophils was assessed by ELISA (left), and NETs were visualized by confocal microscopy (right). White, DNA; red, platelet antigen CD42; blue, citrullinated H4 histone; green, Cyto-ID. ****P < 0.0001 by ANOVA and Bonferroni test. OD, optical density.

have an almost complete defect in adaptive immunity and are thus highly receptive to engraftment of human cells and tissues. Neutrophils either left untreated or committed to autophagy by treatment with rapamycin served as controls. Three hours after injection, there was no difference in the blood counts of human neutrophils, regardless of treatment (Fig. 4A). At later time points, the frequency of SSc-microparticle neutrophils in peripheral blood was significantly higher than that of untreated neutrophils (P < 0.001; Fig. 4A), and SSc-microparticle neutrophils were mostly autophagic (Fig. 4B). Autophagy induction by rapamycin had a similar effect, prompting both autophagy and survival in circulation (Fig. 4, A and B). Autophagy inhibition with wortmannin did not extend survival of SSc-microparticle–activated neutrophils (Fig. 4, A and B), suggesting that autophagy and survival may be linked.

The rapid reduction in blood counts of untreated neutrophils (Fig. 4 and fig. S3A) suggested earlier cell death and clearance. Human CD66b$^+$ neutrophils became detectable after 24 hours within murine spleen F4/80$^+$ macrophages, as assessed by flow cytometry. Human neutrophils were detectable only after cell permeabilization, as expected for apoptotic cells actively internalized by macrophages (fig. S3B). Phagocytosis of untreated neutrophils by spleen macrophages was significantly higher than that of neutrophils treated with microparticles (P < 0.001; fig. S3C). At later time points (72 hours), blood

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Table 2. Cellular adhesion molecule ligand pairs potentially involved in microparticle-neutrophil interaction. Freshly purified healthy neutrophils were stimulated with autologous collagen-microparticles. Collagen-microparticles were treated with blocking monoclonal antibodies (mAbs) before incubation with neutrophils to identify the involvement of P-selectin (CD62P), glycoprotein Ib (CD42), or HMGB1. Neutrophils were treated with blocking mAbs before incubation with autologous collagen-microparticles to identify the involvement of PSGL1 (P-selectin receptor), activated Mac-1 (GPIb receptor), or RAGE (HMGB1 receptor). Cyto-ID^{+} was used to track autophagosomes by flow cytometry, and the soluble MPO-DNA complexes were determined by ELISA as bona fide NET-derived signals. Results are expressed as means ± SEM of six independent experiments carried out with different donors. N/A, not applicable; Ag, antigen.

	Blocking mAbs against	Cyto-ID (%)	MPO-DNA complexes (OD)
Neutrophils alone	N/A	15.8 ± 1.7	0.35 ± 0.12
Neutrophils + collagen-microparticles	Irrelevant Ag	81.3 ± 1.0	1.28 ± 0.46
	P-selectin	79.3 ± 2.2	1.23 ± 0.48
	PSGL1	80.5 ± 1.0	1.37 ± 0.53
	CD42	78.5 ± 1.5	1.24 ± 0.49
	Activated Mac-1	82.8 ± 4.0	1.21 ± 0.43
	HMGB1	15.0 ± 1.2	0.35 ± 0.15
	RAGE	21.5 ± 1.4	0.41 ± 0.12

counts of untreated and microparticle-treated neutrophils and phagocytosis by spleen macrophages were similar.

Human collagen-microparticles prompt neutrophil activation and NET generation in NSG mice

To further explore the pathophysiological relevance of these events, we injected purified microparticles intravenously in NSG mice and traced them using antibodies selectively recognizing the human platelet antigen CD61. The response of neutrophils from NSG mice to collagen-microparticles or to HMGB1 does not differ from the response of neutrophils from wild-type mice (fig. S4). The concentration of human microparticles was high 2 hours after injection (798,800 ± 85,000 microparticles/ml; Fig. 5A) and progressively decreased at later times (Fig. 5A). In parallel, the fraction of mouse neutrophils with adherent microparticles increased (Fig. 5B). Most blood neutrophils accumulated the autophagy dye after the injection of human collagen-microparticles (Fig. 5B), in contrast with sham-treated mice (87 ± 8% versus 7 ± 1%, $P < 0.0001$). Autophagy induction was associated with adherence of human microparticles to mouse neutrophils (fig. S5). Neutrophil vesicular fusion and degranulation depend on autophagy (*48*). Virtually all circulating autophagic neutrophils expressed on their plasma membrane the MPO enzyme, which in normal conditions is retained within primary granules (94 ± 3% versus 5 ± 3% in sham-treated mice; Fig. 5D). Liposome-encapsulated clodronate, which is selectively internalized by the phagocytes and jeopardizes their vitality, abrogated the interaction of neutrophils with microparticles, induction of autophagy, MPO membrane expression, and the formation of NETs while inducing exposure of phosphatidylserine and incorporation of vital dye (fig. S5, B to F), indicating that functional responses elicited by microparticles depend on neutrophil viability.

Human SSc-microparticles cause endothelial damage in NSG mice

Endothelial damage is a hallmark of SSc, and so we tested whether human SSc microparticles could cause endothelial damage in NSG mice. Injection of human collagen-microparticles caused lung inflammation, with infiltration of granulocytes expressing MPO in close contact with the vascular wall, including vessel endothelial linings, in the interstitial perivascular spaces, and occasionally within the alveoli (Fig. 5, E to H). Diffuse congestion and thickening of the alveolar walls were also detected by hematoxylin and eosin histochemistry (Fig. 5, F to I). Endothelial damage and lung neutrophil infiltration after injection of microparticles were associated with elevation of the collagen content in the septal and perivascular spaces, as assessed by Sirius red staining, suggesting incipient fibrosis (Fig. 5, G to J). Increased circulating soluble E-selectin, the specific marker of endothelial activation that is persistently elevated in the plasma of patients with SSc (*33*), also confirmed the endothelial response to microparticle injection (Fig. 5K). The citrullinated H4 histone concentration in cell-free plasma was also higher in microparticle-treated than in sham-treated mice (Fig. 5L), suggesting active generation and accumulation of NETs.

SSc-microparticles injected into NSG mice induced neutrophil autophagy and redistribution of MPO (62 ± 9% and 78 ± 5% of the total blood neutrophils, respectively, after 18 hours; Fig. 5, M and N) (Table 1). In contrast, the injection of microparticles retrieved from the blood of healthy donors (control-microparticles) did not influence the autophagic flux or the expression of MPO (10 ± 2% and 11 ± 1%, respectively; statistically different from SSc-microparticle–injected mice, both $P < 0.0001$, but not from sham-treated mice, 7 ± 1% and 5 ± 3%, respectively) (Fig. 5, M and N). The HMGB1 antagonist BoxA abrogated the induction of autophagy, the redistribution of MPO at the plasma membrane, and the accumulation in the blood of NET by-products elicited by SSc-microparticles (Fig. 5, M and N), indicating that HMGB1 was required for the in vivo effects of microparticles. The plasma concentration of NET by-products—soluble citrullinated histones and low–molecular weight DNA fragments—also increased significantly ($P < 0.001$) after the injection of SSc-microparticles. Control-microparticles did not exert detectable effects (Fig. 5, G and H). Microparticles retrieved from the blood of patients with SLE were weaker inducers of neutrophil activation compared with SSc-microparticles (Table 1), possibly because of the substantially lower fraction of microparticles expressing HMGB1 (Fig. 1 and Table 1).

DISCUSSION

We show here that platelet-derived microparticles from SSc patients recapitulate endothelial damage and initiate fibrosis when injected into mice, and these pathological correlates include neutrophil activation and NET production. In contrast, microparticles retrieved from the plasma of healthy controls did not cause endothelial damage in mice. Endothelial damage is an early and critical feature of SSc, and our findings suggest that platelet activation is an upstream factor that contributes to the pathogenesis of SSc. The cause of platelet activation in SSc patients, though, remains to be determined.

Previous studies have demonstrated that microparticles accumulate in the blood of patients with SSc and that most of them are derived from platelets (*32*, *33*). We show here that microparticles from SSc patients interact with neutrophils and prompt them to enter an autophagic state associated with the redistribution of their vesicular content and with the generation of NETs. Such microparticle-activated

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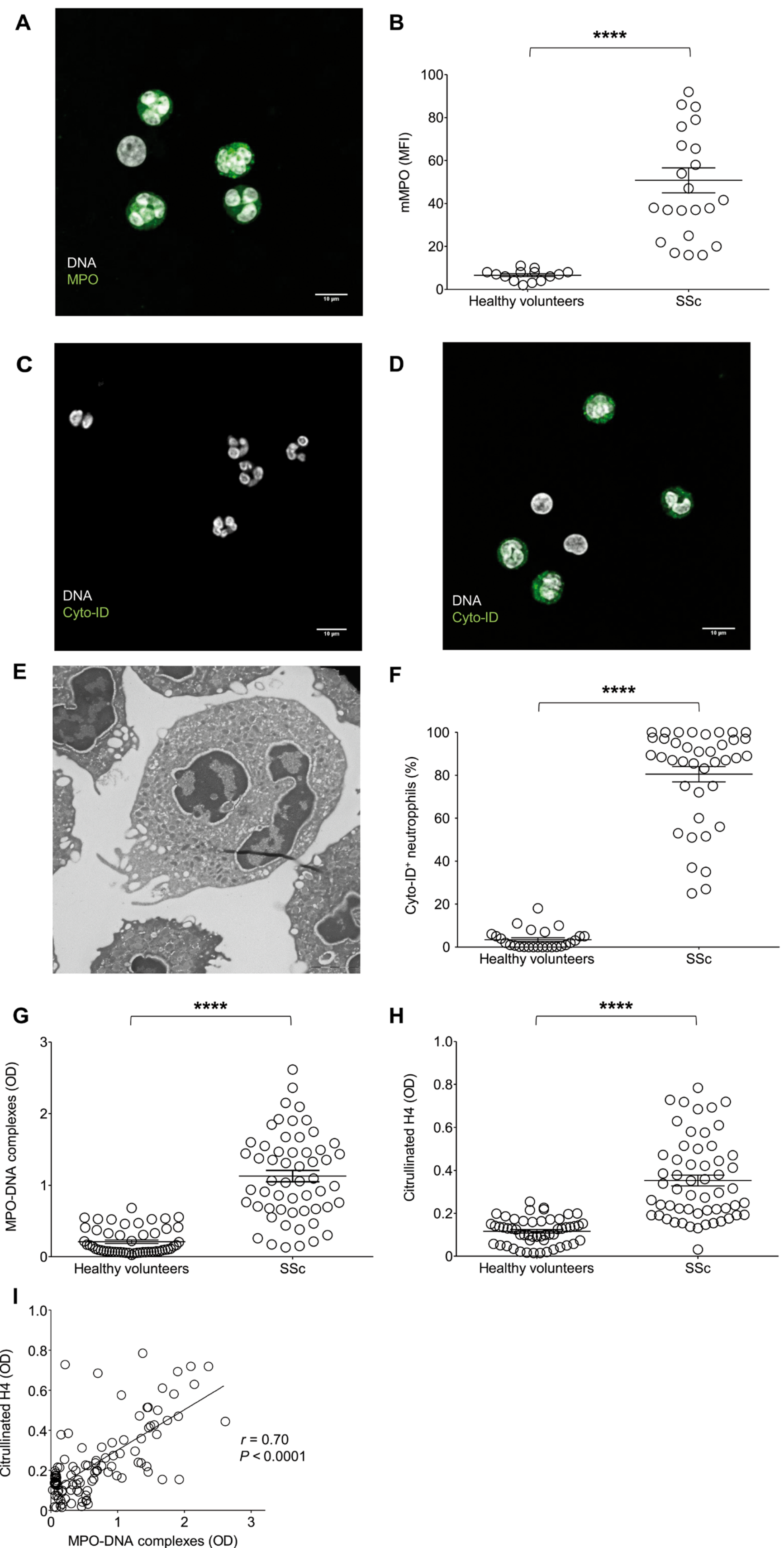

Fig. 3. Neutrophil activation, autophagy, and NETs are associated in the blood from SSc patients. (**A**) Representative image of neutrophils from patients with SSc. Green, MPO; white, nuclei. (**B**) Neutrophil membrane MPO expression (mMPO) from SSc patients and matched healthy volunteers was quantified by flow cytometry. Each symbol refers to a single subject. (**C** and **D**) Neutrophils from healthy volunteers (C) and SSc patients (D) were examined for autophagosomes by confocal microscopy (green). DNA was counterstained with Hoechst (white). (**E**) Transmission electron microscopy of neutrophils purified from the blood of SSc patients, immediately fixed, and processed. (**F**) The fraction of Cyto-ID$^+$ neutrophils in the blood of SSc patients and healthy controls was assessed by flow cytometry. Each symbol refers to a single subject. Bars indicate means ± SEM. ****$P < 0.0001$. (**G** and **H**) Concentration of by-products of NET generation/catabolism, including citrullinated H4 histone (G) and soluble DNA-MPO complexes (H), in the plasma of SSc patients and healthy volunteers. (**I**) Correlation of citrullinated H4 and MPO-DNA complexes in plasma. Symbols depict individual observations in each subject, and bars indicate means ± SEM. ****$P < 0.0001$ by ANOVA and Bonferroni test, significantly different from healthy subjects. MFI, mean fluorescence intensity.

neutrophils acquire the ability to jeopardize endothelial integrity by multiple mechanisms. Neutrophils redistribute granular enzymes to the plasma membrane and become proteolytically active. Neutrophils release NETs that can directly damage endothelial cells (*49*, *50*). NETs also influence the characteristics and the function of endothelial progenitor cells (*51*, *52*), and this may limit tissue repair and exacerbate SSc vasculopathy (*11*, *53*). NETs may also contribute to other disease features, including the activation of myofibroblasts (*54*) and the development of autoimmunity (*55*).

Activated autophagic neutrophils are abundant in the blood of SSc patients, corroborating our inference that they are involved in endothelial damage and fibrosis. Likewise, circulating citrullinated histones and MPO-associated DNA are elevated in SSc patients, in accordance with our proposal that activated neutrophils produce NETs. Consistently, neutrophils in NSG mice receiving human platelet-derived microparticles produce NETs, and neutrophil pharmacological depletion with clodronate prevents the accumulation of by-products of NET generation/catabolism in the peripheral blood.

The most relevant question is why microparticles from SSc patients, but not from healthy donors or patients with SLE, recapitulate early pathogenetic events of SSc in mice. Platelet-derived microparticles are abundant in plasma from SSc patients, but not in plasma from healthy donors or SLE patients. In SLE, decreased clearance of apoptotic cells is well established (*56*–*59*). The main source of blood microparticles consists of apoptotic endothelial cells in SLE (*60*) and platelets in SSc,

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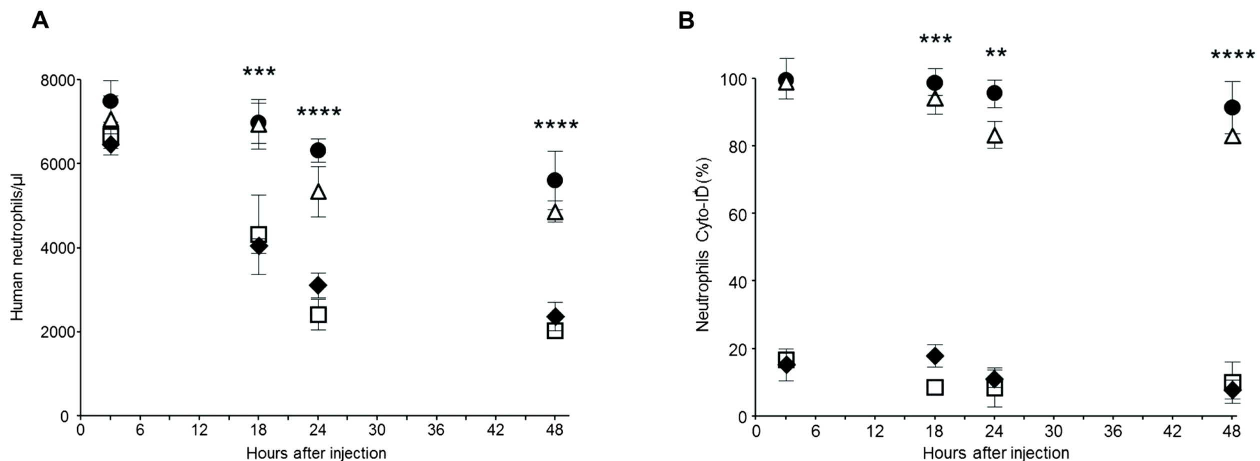

Fig. 4. Interaction with SSc-microparticles prompts neutrophil survival due to activation of autophagy. Human neutrophils from healthy donors were exposed (open triangles) or not (open squares) to SSc-microparticles and injected in NSG mice. (**A**) Human neutrophil counts in the mouse were assessed over time. Dependence of neutrophil survival on autophagy was assessed by treatment with the blocker of the autophagic flux, wortmannin, before the challenge with SSc-microparticles (black diamond) and the effect of pharmacological induction of autophagy by treatment with rapamycin (black circles). (**B**) The fraction of autophagic untreated and treated neutrophils, determined by the accumulation of Cyto-ID, was assessed at various times after injection. Symbols indicate the mean and bars indicate the SEM of three and four different experiments conducted independently. ****$P < 0.0001$, ***$P < 0.005$, and **$P < 0.05$, by ANOVA and Bonferroni test, significantly different from untreated neutrophils. Purified neutrophils were challenged (or not) with collagen microparticles from three different healthy donors. Independently purified neutrophils were injected in groups of three mice.

which could explain the different mechanism of action on vascular endothelium after injection into NSG mice.

Microparticles derived from collagen-activated platelets from healthy donors also interact with and activate neutrophils, and both microparticles from SSc patients and from collagen-activated platelets expose high amounts of HMGB1 on their surface. BoxA abrogated all events elicited by SSc-microparticles. Neutrophils can be activated in vitro by direct exposure to soluble recombinant HMGB1, and we thus argue that extracellular HMGB1 is ultimately responsible for the endothelial damage in SSc. Notably, HMGB1 actively promotes fibrosis (*61–63*), and platelet-derived HMGB1 has been recently found to coordinate the actions of monocytes and neutrophils in deep vein thrombosis (*38*). We suggest that HMGB1 might similarly coordinate microthrombosis in SSc patients and contribute to sustain the vasculopathy associated to the disease.

Limitations of the current study include the in vivo model in which human cells and microparticles were injected into mice. The pairs of ligand receptor involved can differ from mouse to man. We also relied on mice lacking acquired immunity to avoid recognition of xenoantigens that could influence the outcome of microparticle transfer and cells. We cannot evaluate the contribution of the acquired immunity in this system, which plays an important role in the pathogenesis of SSc.

In conclusion, the results presented here indicate a direct and notable role for signals associated with microparticles released from activated platelets as key features of SSc vasculopathy and possibly in facilitating interstitial collagen deposition. The initial trigger of platelet activation in SSc patients remains to be identified, but we note that endothelial damage exposes collagen from the subendothelial matrix, which, in turn, can activate platelets. Thus, a vicious circle of platelet activation is maintained. We also note that the players that we have identified in the pathogenesis of SSc vasculopathy, which include platelet microparticles, activated/autophagic neutrophils, and NET by-products, can discriminate together between SSc and SLE and may serve as potential SSc biomarker candidates. Further clarification of the role of platelet signals in the pathogenesis of SSc, including specific studies in other validated preclinical models of the disease, and identification of the ligand(s), besides HMGB1 responsible for promoting neutrophil activation and endothelial damage, will provide additional insights into the disease pathogenesis and may guide the development of new therapeutic strategies.

MATERIALS AND METHODS

Study design

We conducted a cross-sectional study of patients with SSc compared to controls. Fresh blood was collected at a single time point. Neutrophils, platelets, and microparticles were studied for their activation state, function, reciprocal interaction, and expression of HMGB1. Neutrophil activation was also verified by comparing the concentration of NET by-products in the plasma. Microparticles were characterized for their ability to influence neutrophil survival and function in vitro and upon injection in immunocompromised mice. Features reflecting in vivo endothelial cell activation and lung fibrosis were assessed in the presence or absence of inhibitors of HMGB1. The size of the sample to be studied was determined on the basis of results obtained in smaller groups of patients and controls that indicated that 50 subjects were sufficient to reach a test power of 0.80 for an effect size of $f = 0.290$ and a significance level of $\alpha = 0.05$. For mouse experiments, based on previous results, we estimated that groups of six mice per each condition were sufficient (80% power; 0.5 α error) to identify differences smaller than 3 SDs between sham-treated and microparticle-injected animals. Primary data are located in table S2.

Patients

The study group consisted of 57 patients classified according to the 2013 American College of Rheumatology (ACR)/European League

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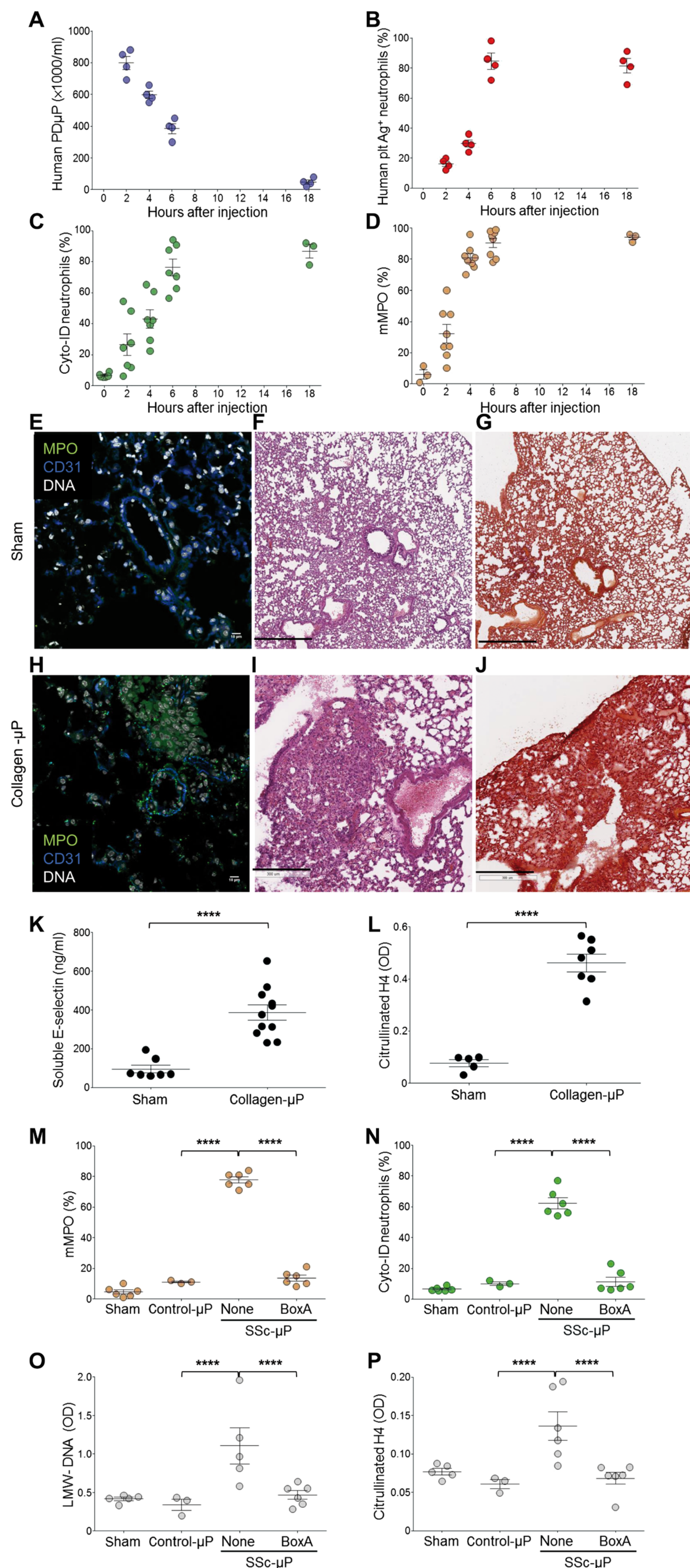

Fig. 5. Platelet microparticles trigger neutrophil and endothelial activation, lung inflammation, and fibrosis in NSG mice. (**A**) Human collagen-microparticles were injected into NSG mice, and their blood counts were assessed at various times based on the expression of the human CD61. (**B**) The fraction of murine neutrophils with adherent human microparticles, (**C**) the fraction of autophagic neutrophils identified based on the accumulation of Cyto-ID, (**D**) and the fraction of neutrophils that expressed MPO on the plasma membrane were assessed at various times after injection. (**E** to **G**) Lungs of NSG mice were harvested 24 hours after injection with phosphate-buffered saline (sham) (**H** to **J**) or with human collagen-microparticles. Sections were processed for confocal microscopy (E and H), and endothelial lining was identified by CD31 (blue), infiltrating neutrophils were identified by MPO (green), and nuclei were counterstained with Hoechst (white). Alternatively, sections were processed for histochemistry with hematoxylin and eosin (F and I) or Sirius red (G and J) and examined by immunofluorescence for infiltration of inflammatory cells. Scale bars, 300 μm. Concentrations of E-selectin (**K**) and of the NET by-product, citrullinated H4 histone, (**L**) assessed by ELISA in the plasma of mice injected with collagen-microparticles, to assess systemic endothelial activation and NET generation, respectively. Microparticles from the plasma of patients with SSc (μP) or of healthy volunteers (Control-μP) were injected into NSG mice in the presence or absence of the HMGB1 inhibitor BoxA. The fraction of murine neutrophils that expressed MPO on the plasma membrane (**M**) and the fraction of autophagic neutrophils (**N**) were assessed by flow cytometry, while the concentration in the plasma of low–molecular weight soluble DNA (**O**) or of citrullinated H4 histones (**P**) was assessed by ELISA. Symbols depict individual observations in each mouse injected with microparticles derived from a single donor. Lines represent the means ± SEM for each determination. ****$P < 0.0001$, significantly different from sham-treated mice (K and L) or from mice injected with control microparticles or with SSc-microparticles in the presence of BoxA (M to P) by ANOVA and Bonferroni test. LMW, low molecular weight.

Against Rheumatism (EULAR) criteria for SSc (*64*), recruited from November 2009 to December 2016. Patients with other systemic autoimmune disorders or overlap syndromes were excluded. Fifty-three age-matched healthy donors (median, 52 years old; range, 25 to 74; 36 females, 17 males) and 6 patients fulfilling ≥4 of the 1982 ACR SLE classification criteria served as controls. Table 1 summarizes some demographic and clinical characteristics of patients with SSc. The pattern at nailfold videocapillaroscopy was classified and categorized as early, active, and late, as described (*65*). All patients and controls gave their written informed consent to participate to the study. The Institutional Review Board approved the study and the biobanking of blood derivatives.

Statistical analysis

Results were reported as means ± SEM, unless otherwise indicated. The normal distribution of each continuous variable was assessed with the Kolmogorov-Smimov test. All patient data sets were found to be normally distributed. Statistical analyses were performed by one-way ANOVA, followed by Bonferroni multiple comparison test or Student's *t* test for comparison of two different groups. All tests were two-sided, and *P* values lower than 0.05 were considered statistically significant. In all analyses, GraphPad Prism 5.00 was used.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Fig. S1. Relative complexity of microparticles released from activated platelets.
Fig. S2. Collagen-microparticles elicit neutrophil autophagy.
Fig. S3. The kinetics of clearance of human neutrophils by splenic macrophages depends on their activation state.
Fig. S4. Similar function of neutrophils from wild-type and NSG mice.
Fig. S5. Viability is required for neutrophil response to platelet-derived microparticles.
Table S1. SSc patients' characteristics.
Table S2. Primary data.
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Platelet microparticles sustain autophagy-associated activation of neutrophils in systemic sclerosis

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Platelets nurture neutrophil damage
Systemic sclerosis, or scleroderma, is a complicated inflammatory fibrotic disease involving multiple organs. Different immune cells contribute to the pathology of systemic sclerosis. Maugeri *et al.* investigated mechanisms that could contribute to endothelial damage in patients. They observed that activated platelets from patients produced microparticles that induced neutrophil activation, including autophagy and neutrophil extracellular trap production. These microparticles expressed the damage-associated molecular pattern HMGB1, and accordingly, an HMGB1 inhibitor prevented this neutrophil activiation. Inhibiting microparticles to interrupt this platelet-neutrophil axis may be helpful to prevent endothelial damage, thereby disrupting disease pathogenesis.

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