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Platelet regulation of myeloid suppressor of cytokine signaling 3 accelerates atherosclerosis

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Abstract

Platelets are best known as mediators of hemostasis and thrombosis; however, their inflammatory effector properties are increasingly recognized. Atherosclerosis, a chronic vascular inflammatory disease, represents the interplay between lipid deposition in the artery wall and unresolved inflammation. Herein we reveal that platelets induce monocyte migration and recruitment into atherosclerotic plaques, resulting in plaque platelet-macrophage aggregates. In *Ldlr*^{-/-} mice fed a western diet, platelet depletion decreased plaque size and necrotic area and attenuated macrophage accumulation. Platelets drive atherogenesis by skewing plaque macrophages to an inflammatory phenotype, increasing myeloid suppressor of cytokine signaling 3 (SOCS3) expression and reducing the *Sox1:Sox3* ratio. Platelet-induced *Sox3* expression regulates plaque macrophage reprogramming by promoting inflammatory cytokine production (*Il6*, *Il1b*, *Tnfa*) and impairing phagocytic capacity, dysfunctions that contribute to unresolved inflammation and sustained plaque growth. Translating our data to humans with cardiovascular disease, we found that women with, versus without, myocardial infarction have upregulation of *SOCS3*, lower *SOCS1:SOCS3*, and increased monocyte platelet aggregate. A second cohort of patients with lower extremity atherosclerosis demonstrated that *SOCS3* and the *SOCS1:SOCS3* ratio correlated with platelet activity and inflammation. Collectively, these data provide a causative link between platelet-mediated myeloid inflammation and dysfunction, *SOCS3*, and cardiovascular disease. Our findings define an atherogenic role of platelets and highlight how, in the absence of thrombosis, platelets contribute to inflammation.

One Sentence Summary:

Platelet-induced myeloid *Sox3* expression in humans and mice contributes to unresolved atherosclerosis inflammation and sustained plaque growth.

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Competing Interests: JSB serves on advisory boards for Astra Zeneca, Janssen, and Amgen. KJM acts as a consultant to Intracellular Therapies Inc. EAF serves as an expert witness in patent dispute cases involving lipid-lowering agents.

Data and methods availability: All data associated with this study are in the paper or supplementary materials

Introduction

Platelets are critical mediators of plaque rupture and atherothrombosis [1–3]. Activated platelets locally release a host of inflammatory mediators that support the chemotaxis, adhesion, and transmigration of leukocytes to sites of inflammation [4–6]. Platelet activation results in an increase in circulating platelet-leukocyte aggregates, protagonists of inflammatory reactions in the vessel wall [7]. Data from our group and others demonstrated an increase in monocyte-platelet aggregation (MPA) across the spectrum of cardiovascular and other inflammatory diseases [8–10]. Subsequently, we considered the immunomodulatory role of platelets to monocytes in the context of vascular disease, and established that platelet activation promotes a proinflammatory monocyte phenotype in patients with lower extremity atherosclerosis [11]. However, whether these platelet-mediated events contribute to atherogenesis development is less established, despite their documented immune effector cell properties, interactions which we hypothesize to modulate plaque progression and inflammation.

Recruitment of monocytes to the subendothelium and their subsequent differentiation to macrophages are key steps in atherosclerotic plaque formation and progression. Macrophages, critical effectors of inflammation and innate immunity, are key pathogenic drivers of vascular diseases. Modulated primarily by their microenvironment, macrophages undergo phenotypic switching to adapt to changing conditions within tissues and tailor their phenotype and function to mediate an appropriate response [12, 13]. Macrophage functional subsets are broadly classified into inflammatory M1 and tissue-reparative M2 macrophages [14–17], although it is likely that a spectrum of activation states can exist in vivo. M1 macrophages express high amounts of inflammatory cytokines [interleukin (IL)-6, IL-1 β], and increased production of reactive oxygen species [18]. In contrast, M2 macrophages participate in tissue remodeling, immune regulation, and are highly phagocytic [19]. The suppressor of cytokine signaling (SOCS) proteins, SOCS1 and SOCS3, have recently been shown to regulate M1 and M2 macrophage polarization [20, 21]; the expression ratio of *Socs1:Socs3* is an indicator of macrophage inflammatory status in atherosclerotic plaques [22, 23].

Imbalances between M1 and M2 macrophages are common to various inflammatory diseases, including atherosclerosis, with unstable lesions largely dominated by M1-like macrophages [24, 25]. However, the plaque environmental cues which dictate macrophage phenotype and function remain to be comprehensively described. Despite well-documented platelet-monocyte interactions in patients with cardiovascular diseases [8], the contribution of platelets to plaque macrophage phenotype and function remains poorly characterized. Herein we investigated the role of platelets in the development of atherosclerosis with a focus on their interaction with macrophages, given their ability to recruit their precursors, monocytes, to sites of inflammation and tailor their immune response. Our study demonstrates that platelets induce monocyte migration and recruitment into (but not from) atherosclerotic plaques, resulting in macrophage-platelet aggregates in atherosclerotic plaque. In *Ldlr*^{-/-} mice, platelet depletion decreased plaque size and necrotic area and attenuated macrophage accumulation in plaques. Platelets interact with plaque macrophages, inducing a proinflammatory phenotype characterized by increased expression of *Socs3* and

decreased *Socs1:Socs3* ratio. This inflammatory skewing promotes the production of cytokines (IL-6, IL-1B), and impairs the phagocytic capacity of macrophages, an essential reparative function that attenuates plaque development and inflammation. Additionally, we found increased expression of *SOCS3* and *IL-1B* in subjects with cardiovascular diseases, as well as a positive association between platelet activity and *IL-1B* and an inverse association with *SOCS1:SOCS3*.

Results

Hypercholesterolemia promotes platelet-myeloid interactions in atherosclerosis

Lipids play a fundamental role in regulating platelet structure, signaling, and function [26]; activated platelets readily bind to immune cells in the circulation and tissues. However, the interaction between different subsets of leukocytes and platelets within atherosclerotic plaques is mostly unknown. In hypercholesterolemic mice, we found increased platelet activation as assessed by increased circulating leukocyte-platelet aggregates ($P < 0.01$, Fig. 1, A to B, and fig. S1A), monocyte-platelet aggregates (MPA) ($P < 0.01$, Fig. 1B), and proatherogenic Ly6C^{hi} MPA ($P < 0.01$, Fig. 1B). Single cell RNA-sequencing (scRNA-seq) of CD45⁺ leukocytes from the aortas of atherosclerotic mice and subsequent t-stochastic neighbor embedding (t-SNE) facilitated identification of atherosclerosis-associated immune cell populations (Fig. 1C). Notably, we found a subset of plaque macrophages enriched in the platelet-specific transcript platelet factor 4 (*Pf4*) (Fig. 1D), suggesting that macrophage-platelet aggregates accumulate in atherosclerotic plaques. Additionally, compared to chow-fed mice we found an increase in plaque macrophage-platelet aggregates in hypercholesterolemic mice as assessed via the increased expression of platelet markers *Pf4* and pro-platelet basic protein (*Ppbbp*) in this macrophage cluster (fig. S1B).

Platelets promote a pro-inflammatory monocyte phenotype primed for migration

Circulating monocyte count and phenotype are predictive of atherosclerosis plaque size and inflammation [27]. Given their central role to atherogenesis, we considered the effect of platelets on monocytes in vivo. *Ldlr*^{-/-} mice were fed a high fat, high cholesterol diet for 7 weeks to allow for the development of atherosclerotic plaques. Mice were then split into two groups, and treated with either α -CD42b or an antibody control every 4 days over a 2 week period (study outline, Fig. 2A). Antibody-mediated platelet depletion resulted in a >75% reduction in circulating platelet count (850 ± 150 versus $213 \pm 40 \times 10^9/L$ in antibody control-treated mice, $P < 0.0001$) without changes in circulating monocyte counts (Table 1) nor plasma total cholesterol, high density lipoprotein cholesterol (HDL-C), or triglycerides (Table 1, fig. S2A-D). In platelet-competent mice, the circulating monocyte Ly6C^{hi}:Ly6C^{lo} ratio was significantly elevated compared to platelet-depleted mice (Fig. 2, B to C, $P < 0.01$), demonstrating that platelets promote a proinflammatory monocyte phenotype in vivo. Skewing to a heightened inflammatory state was supported by increased monocyte CD11b surface expression in platelet competent mice ($P < 0.01$, Fig. 2D), and higher expression of inflammatory transcripts *Ccl2*, *Il1b*, and *Cd11b* in monocytes ($P < 0.01$, Fig. 2E). Further, the circulating platelet count and monocyte inflammatory transcript expression were significantly correlated (*Ccl2*, $P = 0.01$; *Il1b*, $P = 0.008$; *Cd11b*, $P = 0.02$, Fig. 2F).

We next assessed the potential of platelets and their released mediators to modulate the migratory capacity of monocytes. Ly6C^{hi} monocytes were isolated from the bone marrow of C57BL/6 (WT) mice, and their chemotaxis to platelet releasate or vehicle control monitored in real-time. Monocyte migration increased significantly in response to platelet releasate when compared to vehicle control (Fig. 2, G to H), indicating a direct chemoattractive capability of platelets. Additionally, we found that the recruitability of monocytes isolated from platelet competent and deficient mice to be significantly altered. Monocytes isolated from platelet-deficient mice had significantly reduced motility to a range of chemoattractants, including oxLDL, CCL2, and C5a (Fig. 2I, $P < 0.01$ for all chemoattractants). Additionally, monocytes from platelet-deficient mice were less adhesive (fig. S3). Collectively, these data demonstrate that platelets skew circulating monocytes to a proatherogenic, inflammatory phenotype, with enhanced adhesive properties and increased migratory capacity to chemoattractants known to promote atherosclerosis.

Platelets alter leukocyte trafficking to, but not from, plaques

Leukocyte migration to (recruitment) and from (egress) atherosclerotic lesions are key trafficking events governing plaque size and macrophage content [24]. We, therefore, considered the contribution of platelets to monocyte recruitment to, and macrophage egress from, established atherosclerotic plaques using in vivo cell tracking assays [28, 29] (Fig. 3A). To monitor monocyte recruitment to plaques, circulating monocytes were labeled in vivo with green fluorescent beads 2 days before harvest [28]. In platelet-deficient mice, monocyte recruitment was reduced by 58% compared to platelet competent mice (Fig. 3, B to C, $P = 0.04$). To assess macrophage egress from lesions, 5 days before the injection of the platelet depleting or control antibodies, the same mice were injected with 5-ethynyl-2'-deoxyuridine (EdU) to label circulating monocytes [30]. Platelets did not affect macrophage egress from plaques (Fig. 3, D to E). These data demonstrate that platelets alter the recruitment of monocytes to atherosclerotic plaques; however, they do not affect macrophage egress from plaques.

Platelets promote macrophage accumulation in plaques

Next, we quantified and characterized the atherosclerotic plaques in platelet competent and deficient mice. Platelet depletion reduced aortic root plaque size (Fig. 4, A to B, 31% reduction, $P = 0.03$) and CD68⁺ macrophage content (Fig. 4, C to D, 38% reduction, $P = 0.01$) versus the control treatment group. Additionally, we found decreased plaque size and macrophage content in the brachiocephalic artery in mice with reduced platelet counts (Fig. 4, E to F, 80% reduction in both plaque content and CD68%, $P = 0.02$). Plaque collagen content, a marker of lesion stability [31], was significantly increased in plaques from platelet-deficient mice compared to plaques isolated from platelet competent mice (Fig. 4, G to H). Immunohistochemical analysis of Ki67 demonstrated no significant difference for plaque proliferating macrophages in the absence or presence of platelets (fig. S4A) [32, 33]. Together, these data demonstrate that platelets promote atherogenesis and plaque macrophage accumulation, and skew lesions towards an unstable-like phenotype.

Macrophage efferocytotic capacity is impaired by platelets

The accumulation of apoptotic and necrotic debris has long been associated with advanced atherogenesis and plaque vulnerability [34, 35]. Characterization of aortic root plaques of platelet-depleted mice revealed they contained less necrotic area compared to control mice ($P < 0.005$, Fig. 5, A to B). Given that plaque necrotic core formation is considered to result from the defective clearance of apoptotic cells (defective macrophage efferocytosis [33]), we next quantified these parameters. Anti-cleaved caspase 3 immunohistochemistry noted a trend towards an increased content of apoptotic bodies in plaques from platelet-competent mice (fig. S5A, $P = 0.05$), suggesting defective apoptotic cell clearance by efferocytosis.

To assess efferocytosis capacity, peritoneal macrophages were treated with platelet releasate and their ability to engulf apoptotic cells assessed. Macrophages exposed to platelet releasate had significantly impaired efferocytotic ability, as evidenced by a reduced uptake of fluorescent apoptotic jurkats, confirming that platelets inhibit the ability of macrophages to effectively clear apoptotic debris ($P < 0.001$, Fig. 5, C to D). Macrophage phagocytic capacity, as determined by their ability to engulf fluorescently labeled *Escherichia coli* (*E. coli*), was also found to be significantly inhibited upon macrophage exposure to platelets ($P < 0.001$, Fig. 5, E to F). These data demonstrate that platelets in vivo and ex vivo reduce the efferocytotic and phagocytic capacity of macrophages, a defect that is likely to contribute to both unresolved plaque inflammation and enhanced expression of macrophage inflammatory cytokines, which are hypothesized to promote lesion progression.

Platelets promote a proinflammatory plaque macrophage phenotype via NF- κ B activation

To directly examine phenotypic changes in plaque macrophages, we analyzed their transcript expression profiles from platelet-competent and -deficient mice. Lesional macrophages were isolated by laser-capture microdissection (LCM), RNA was extracted, and RT-qPCR was performed. In support of a platelet-induced M1-like macrophage phenotype, we found significantly higher macrophage expression of the inflammatory transcripts (*Il6*, *Il1b*, *Tnfa*) from platelet-competent mice (Fig. 6A). Platelet count significantly correlated with plaque macrophage *Il6* and *Il1b* expression (Fig. 6B), suggesting that platelets are one of the drivers of plaque inflammation. Furthermore, *Il6* expression positively correlated with plaque macrophage area (Fig. 6C).

Platelet-mediated macrophage polarization to the M1 pro-inflammatory state was confirmed by treatment ex vivo of peritoneal macrophages with platelets or platelet releasate. Platelet exposure significantly increased macrophage mRNA expression of *Il6*, *Ccl2*, and *Il1b* (Fig. 6D), and release of IL-6 (Fig. 6E). In support of a pro-atherogenic phenotype, macrophages exposed to platelets were more lipid-rich after exposure to acetylated low-density lipoprotein (Fig. 6F).

Consistent with the in vivo setting (Fig. 6B), in vitro platelet-induced macrophage inflammation was platelet concentration-dependent (Fig. 6G). Furthermore, in agreement with previous studies in human monocytes [36], we found that increased macrophage inflammatory gene expression was linked to nuclear factor- κ B (NF- κ B)-signaling. Macrophage exposure to platelets induced p65 nuclear localization (Fig. 6H), and

pretreatment of macrophages with an NF- κ B inhibitor (10 μ M, Bay 11–7082) prior to platelet exposure abrogated inflammatory transcript expression (Fig. 6I).

Platelet-induced *Socs3* enhances the proinflammatory effects of macrophage IL-6-signaling

Members of the suppressor of cytokine signaling (SOCS) family are important inhibitors of cytokine receptor-mediated signaling and regulators of macrophage polarization [22]. SOCS1 promotes macrophage polarization to the M2 state, whereas SOCS3 suppresses M2 polarization and reciprocally promotes M1 polarization [21]. The SOCS3-mediated proinflammatory effects are due, in part, to its role in controlling nuclear accumulation of NF- κ B [20], and suppressing STAT3-signaling. Consistent with this, silencing of macrophage *Socs3* suppresses the macrophage inflammatory profile (fig. S6). Macrophage *Socs1:Socs3* mRNA is an in vivo biomarker of macrophage polarization: a lower ratio indicates a pro-inflammatory M1-type macrophage [20, 21]. The lower *Socs1:Socs3* mRNA promotes IL6-signaling by blocking STAT1 or STAT3 activation [22]. Although treatment of macrophages with platelets induced upregulation of both *Stat1* and *Stat3*, upregulation was greater for *Stat3* (fig. S7). Thus, we hypothesized that the macrophage *Socs1:Socs3* ratio is a driver of platelet-mediated macrophage polarization. Consistent with an inflammatory phenotype, plaque macrophages from platelet-competent mice had a significantly lower *Socs1:Socs3* compared to macrophages from platelet-deficient mice (Fig. 7A). The decrease in the ratio is driven by a reduction in macrophage *Socs3* expression from platelet-deficient mice (Fig. 7A). Consistent with a platelet-mediated proinflammatory effector role, the plaque macrophage *Socs1:Socs3* inversely correlated with platelet count (Fig. 7B). As noted above (fig. S1B), scRNA-seq of leukocyte populations from atherosclerotic plaques from hypercholesterolemic mice revealed increased macrophage-platelet aggregates (*Pf4* and *Pbbp* expression) compared to chow-fed mice. Consistent with this, *Socs3* expression was increased and the *Socs1:Socs3* ratio decreased in macrophages from atherosclerotic plaques under conditions of hypercholesterolemia (fig. S8). Further support of decreased platelet-driven macrophage *Socs1:Socs3* was confirmed in vitro, where treatment of peritoneal macrophages led to a platelet concentration-dependent increase in *Socs3* mRNA expression (Fig. 7C), and a significantly suppressed *Socs1:Socs3* ratio (Fig. 7D).

To confirm that platelet-mediated increases in *Socs3*, and a subsequent decreased *Socs1:Socs3* ratio, enhanced the inflammatory nature of macrophages, we blocked macrophage IL-6 signaling, a cytokine linked to *Socs3* induction (fig. S9). We found that antibody blocking in vivo of the receptor for IL-6 signaling, gp130, suppressed platelet-mediated increases in *Socs3* (Fig. 7E). Additionally, antibody-blocking of IL-6 signaling reduced macrophage *Il1b* mRNA expression, also indicative of reduced macrophage inflammation. Furthermore, siRNA suppression of *Socs3* attenuated platelet-mediated macrophage inflammation (Fig. 7F). Accordingly, blocking of macrophage gp130, or silencing of *Socs3*, corrected platelet-mediated macrophage efferocytosis deficiencies (Fig. 7, G to H and fig. S10, respectively).

SOCS1:SOCS3 in subjects with CVD correlates with IL1B and platelet activity

In our preclinical model, we discovered that platelets induced myeloid inflammation by upregulation of *Socs3* and suppression of the *Socs1:Socs3* ratio. We previously reported increased MPA across the spectrum of atherosclerotic CVD [8]. Here, we explored the association between *SOCS1:SOCS3* and platelet activity in two populations of CVD. In a cohort of women presenting with myocardial infarction (MI), we found platelet count, MPA, *SOCS3*, *SOCS1*, and *IL1B* to be significantly increased compared to women without MI referred for cardiac catheterization matched for age and race (Table 2). Women with, versus without, MI also had a significantly lower *SOCS1:SOCS3* ratio ($P < 0.01$). As expected, an inverse correlation was present between the platelet transcript *PF4* and the *SOCS1:SOCS3* ratio, and the inflammatory transcript *IL1B* and *SOCS1:SOCS3* (fig. S11, A to B). In a second cohort of patients with peripheral artery disease (PAD) with lower extremity atherosclerosis (table S1), we confirmed the link between platelets and SOCS3-mediated activation. In this larger cohort, we found the *SOCS1:SOCS3* ratio to inversely correlate with *IL1B* expression (Fig. 8A), and most notably with multiple markers of platelet activation including MPA, surface P-selectin and CD40 expression (Fig. 8, B to D). Additionally, MPA% positively correlated with atherosclerosis severity in both cohorts of subjects and was unaffected by aspirin or clopidogrel therapy (fig. S11, C to D, fig. S12 A to B, fig. S13, A to B). Together, these findings in our acute model of MI and stable model of lower extremity atherosclerosis reflect those seen in our preclinical model and suggest that the same platelet-mediated effector properties on myeloid cells are conserved in humans.

Overall, these data demonstrate that platelets directly contribute to the inflammatory profile of both circulating monocytes and plaque macrophages in vivo and in vitro. The finding that platelets mediate myeloid activation via induction of SOCS3 and subsequent inflammatory cytokine release highlights an unexplored mechanism by which platelets contribute to atherogenesis and the maintenance of unresolved plaque inflammation.

Discussion

Platelets are critical mediators of plaque rupture and thrombosis and can act both locally and systemically to promote inflammation [37–39]. Recently, we considered the effector role of platelets on monocytes in vascular disease and established that they promote a proinflammatory circulating monocyte phenotype in patients with lower extremity atherosclerosis [11]. In our present study, we report that platelets act as key immune regulators of atherosclerosis progression by promoting monocyte migration into lesions, and by inducing a proinflammatory M1-like macrophage phenotype. We have uncovered a host of previously unrecognized functions by which platelets contribute to plaque progression, which serve to reconcile clinical observations that platelets influence atherogenesis in the absence of thrombosis [40, 41]. We find that: 1) hypercholesterolemia primes platelet activity, increasing circulating MPA and plaque macrophage-platelet aggregates; 2) platelets promote a proinflammatory myeloid phenotype by inducing *Socs3* expression; 3) platelets skew plaque macrophage polarization to a proinflammatory M1-like state with impaired efferocytotic and phagocytic capacity; and 4) patients with CVD have heightened platelet activity which inversely correlates with peripheral blood *SOCS1:SOCS3* expression and

positively with *IL-1B*. Our major findings highlight how platelets are key regulators of myeloid inflammation within both the circulation and atherosclerotic lesions.

Hypercholesterolemia is a risk factor for atherothrombosis, largely attributed to its impact on lesional macrophages [24, 42]. However, we find that hypercholesterolemia also activates platelets leading to an increased abundance of MPA, a product of platelet activation linked to atherosclerosis development [40, 43]. Consistent with this, in the absence of platelets, circulating monocytes have reduced expression of inflammatory mediators and reduced migratory capacity to both atherogenic stimuli and to lesions - factors that are unequivocally linked to atherosclerosis progression [44]. Our data indicate that platelets act as important immune mediators of monocyte phenotype, particularly under conditions of high cholesterol, and represent a major factor of ongoing monocyte recruitment to plaques and unresolved inflammation.

Using scRNA-seq we found enrichment in *Pf4* in plaque macrophages, indicative of macrophage-platelet aggregates in plaques. Supporting the role of high cholesterol in platelet activation and subsequent immune cell modulation, we found a significant reduction in *Pf4* expression in macrophages isolated from plaques in chow fed mice. Given the evidence that platelet-monocyte interactions skew the monocyte towards a proinflammatory phenotype [36, 45, 46], we considered the contribution of platelets to plaque macrophage phenotype. Lesion staining for collagen from platelet-competent and -deficient mice revealed that, in the absence of platelets, plaques were collagen enriched with reduced necrotic area, indicative of “stable” plaque phenotype. These metrics are hallmarks of reduced lesional macrophage inflammation, and indeed direct transcriptome profiling of plaque macrophages revealed that macrophages isolated from platelet-deficient mice have reduced expression of the proinflammatory mediators, *Il6*, *Il1b* and *Tnfa*. Consistent with platelet-induced macrophage inflammation, macrophage inflammatory transcript expression positively correlated with both circulating platelet count and lesion macrophage content.

Our in vitro studies confirmed our in vivo findings and highlighted that platelets mediate macrophage polarization to an inflammatory M1-like state. We found that activation of macrophages was mediated by both platelet released factors, and by direct platelet-macrophage contact. Skewing macrophages toward the proinflammatory M1 phenotype, or enhancing the M1:M2 ratio, is likely to induce a relative deficit of macrophages with a high phagocytic capacity [47]. Indeed, plaques from platelet-competent mice contained a higher content of apoptotic cells and larger necrotic area. Further analyses found these observations to be a result of defective cell clearance. Treatment of macrophages with platelets impaired macrophage phagocytic and efferocytotic capacity by over 50%. Given that defective efferocytosis contributes to unresolved plaque inflammation and expansion of the necrotic core, these data highlight an undescribed mechanism by which platelets propagate lesion growth [33]. Further, this finding is of heightened importance when considered in conjunction with recent evidence supporting a role for macrophage efferocytosis in regulating migration away from sites of inflammation [48]. In the context of atherosclerosis, impaired macrophage efferocytosis would translate to increased macrophage accumulation and sustained plaque growth.

SOCS3 is an essential regulator of gp130-mediated macrophage signaling pathways and can block or promote inflammation depending on the local cell environment [49]. The effects of SOCS3 are primarily associated with IL-6 family cytokines and their subsequent interaction with gp130. In the absence of SOCS3, IL-6 acts like an immunosuppressive cytokine promoting a M2 macrophage phenotype, however when SOCS3 is upregulated STAT3-mediated signaling events are blocked, facilitating IL-6 inflammatory events [20, 21]. In addition to modulating STAT activity, SOCS3 restrains signaling through the PI3K/Akt pathway, which in turn augments NFkB activity to drive efficient expression of proinflammatory mediators [22, 50]. Consistently, we found that platelet activation of macrophages induced *Socs3* expression with concurrent NFkB activation and expression of inflammatory cytokines *Il6* and *Il1b*. The ability of platelets to reprogram macrophages for inflammatory activation by increasing *Socs3* expression is of high relevance in the milieu of the atherosclerotic lesions and provides mechanistic insight into how platelets may facilitate atherogenesis and plaque inflammation, thereby leading to a vulnerable plaques. Previous studies have reported that *Socs3* expression is increased at sites of acute and chronic inflammation [51] and in the unstable shoulder regions of human plaques where platelets are known to interact [52–55].

Various studies have highlighted that IL-6 is an upstream inflammatory cytokine that plays a central role in propagating the downstream inflammatory response in atherogenesis [56]. In support of this, individuals with a variant in the IL-6 receptor that impairs IL-6 signaling via membrane bound gp130 (“classical signaling”) have a decreased risk for coronary heart disease [57]. SOCS3 has recently been shown to positively regulate toll-like receptor 4 (TLR4) signaling by inhibiting TGFβ/Smad3 signaling [21, 51, 58]. This is likely to be pertinent in the context of atherosclerosis, given the major contributory role of macrophage TLR4 activation to atherosclerosis progression [59]. The synergistic upregulation of SOCS3 and enhanced TLR4 signaling likely represents a mechanism by which platelets mediate inflammatory effects to target cells in various inflammatory diseases. For example, we have previously shown that platelets from patients with lower extremity atherosclerosis produce the TLR4 ligand S100A9; thus, the effect of platelet-derived or local ligands is likely magnified by platelets [11]. *Socs3* induction is necessary to direct the inflammatory effects of IL-6, our study provides evidence of how platelets enhance proinflammatory IL-6 signaling in macrophages, and likely represent a source of heightened inflammation in plaques.

To further explore the clinical translational potential of these findings, human studies in acute MI and stable PAD were performed. Consistently, we observed that subjects with versus without MI had higher platelet counts, MPA, *SOCS3*, and *IL-1B* and lower *SOCS1:SOCS3* ratio. Moreover, the *SOCS1:SOCS3* ratio inversely correlated with the platelet transcript *PF4* and the inflammatory transcript *IL-1B*. Interestingly, many studies suggest an association between platelet count and cardiovascular disease [60–63]. In our larger PAD cohort, we further demonstrated that the *SOCS1:SOCS3* ratio inversely correlated with different platelet activation markers, including MPA, P-selectin expression, and CD40 expression. These clinical findings in an acute model of MI and lower extremity atherosclerosis reflect those seen in our preclinical model and suggest that the same platelet-

mediated inflammatory effector properties on myeloid cells are conserved in humans with CVD.

Suppression of platelet aggregation by aspirin, clopidogrel, or cilostazol in atherogenic mice has been shown in some, although not all, studies to reduce atherosclerotic size and inflammation [64–69]. Clinical studies in humans assessing the effect of aspirin or clopidogrel (and other P2Y₁₂ inhibitors) have found beneficial effects on myocardial infarction and stroke, proxies for atherothrombosis. Whether the clinical benefit of antiplatelet therapy in high-risk individuals is derived from the attenuation of platelet-mediated macrophage inflammation is currently unknown. We found that current antiplatelet therapies (aspirin and clopidogrel) did not affect circulating MPA in subjects with established atherosclerosis. Given the platelet-myeloid axis is a likely inflammatory pathway contributing to atherogenesis, further investigation is warranted to explore therapeutic targets to suppress these interactions.

Although the data we obtained from subjects with CVD supported the results from our mouse study, we acknowledge that our murine model of atherosclerosis is not directly translatable to all aspects of human atherosclerosis. Additionally, our murine model of platelet depletion is not clinically translatable. Further studies are ongoing to identify the factor(s) released by platelets, which reprogram macrophages towards a pro-inflammatory state by amplification of IL6 via SOCS3 signaling. Finally, our study did not assess whether this mechanism occurs across different phenotypes of vascular disease, nor did it determine whether targeting the platelet-monocyte/macrophage axis represents a viable therapeutic target for the prevention of CVD.

Collectively, our data define atherogenic roles for platelets and highlight how platelets mediate inflammatory immune reactions in the circulation and within plaques. We demonstrate that in the absence of thrombosis, platelets affect the necrotic area, apoptotic cell content, and plaque collagen content to alter plaque stability. Platelets skew plaque macrophages to an M1 inflammatory phenotype by induction of *Socs3* in macrophages, impairing the ability of plaque macrophages to effectively undertake efferocytosis and contributing to unresolved plaque inflammation and atherogenesis. We provide clinical context for our murine studies by demonstrating that platelet activity correlates with myeloid inflammation (*SOCS3* and *IL1B*) and atherosclerosis severity in two separate clinical cohorts. These data demonstrate the immune effector function of platelets during hypercholesterolemia and suggest that therapeutic targeting of platelet-myeloid interactions is likely to suppress atherogenesis.

Methods

Study design

The overall objective of our study was to investigate the role of platelets in the progression of atherosclerosis. To investigate this, we used an atherogenic mouse model (*Ldlr*^{-/-}) fed a western diet. After 7 weeks of western diet feeding, mice were randomly assigned to receive injections of either a control IgG or anti-CD42b to deplete circulating platelets. After 2 weeks of platelet depletion mice were harvested, and the aortic root and brachiocephalic

artery (BCA) collected. To assess the effect of platelet depletion atherosclerotic plaques were quantified and characterized in the aortic root and BCA. Sources of mouse data included the following: analysis of harvested tissues and cell by RT-qPCR, RNA-seq, flow cytometry, histology; and experiments performed on mouse cell lines cultured in vitro. Mouse injections were not blinded. Investigators were blinded for the analysis of plaque size and characteristics and during the quantification of efferocytosis. Sample processing and statistical analysis were performed concurrently on experimental and control groups using identical conditions. For all the experiments, sample sizes were determined by our previous data, prior literature, and power calculation to ensure sufficient sample sizes to allow the detection of statistically significant differences. Numbers of replicates and statistical tests are indicated in the figure legend.

Animal studies.

The Institutional Animal Care Use Committee of New York University Medical Center approved all animal experiments. C57BL/6 and *Ldlr*^{-/-} mice were from Jackson Laboratories (stock numbers, 000664 and 002207). All mice were housed in a pathogen-free facility at an ambient temperature of 22–25°C. For atherosclerosis studies, male 8 week old *Ldlr*^{-/-} mice were fed a Western diet (21% [wt/wt] fat, 0.3% cholesterol; Dyets Inc, #101977GI) for 9 weeks. At week 7, mice were randomized into two groups and received either control antibody injections (polyclonal non-immune rat immunoglobulins) or a GPIb-alpha antibody (Emfret) injections to deplete circulating platelets (3 µg/g). At sacrifice, mice were anesthetized with ketamine/xylazine, exsanguinated by cardiac puncture, and perfused with PBS, followed by 10% sucrose in PBS. The heart was separated from the aorta at the root and embedded in OCT medium and snap-frozen. To induce hypercholesterolemia in C57BL/6, mice were injected with an antisense oligonucleotide targeted to the low-density lipoprotein receptor (Ionis Pharmaceuticals) at a dose of 5 mg/kg once a week for 4 weeks [70].

Human studies.

The studies were conducted in accordance with policies of the New York University Langone Medical Center Institutional Review Board. Informed consent was obtained from each subject. Patients with MI or PAD were recruited into ongoing studies ([NCT03022552](#) and [NCT02106429](#)) investigating platelet activity and cardiovascular disease.

For the MI study, women presenting with MI were enrolled into the Heart Attack Research Program (HARP) study. Participants electively referred for invasive coronary angiography without acute coronary syndrome and did not have obstructive CAD were identified as disease controls. Patients and controls had blood collected at the time of coronary angiography. For the PAD study, men and women scheduled for lower extremity revascularization were enrolled into the Platelet Activity and Cardiovascular Events (PACE) study and had blood collected prior to their revascularization procedure. Both studies are described in detail elsewhere [8, 11, 71, 72]. Briefly, subjects >21 years old on aspirin were recruited from New York University Langone Medical Center, Bellevue Hospital, or the Veterans Affairs NY Harbor Healthcare System. Major exclusion criteria were use of NSAIDs (other than aspirin) in the past week, antithrombotic therapy, or any known

hemorrhagic diathesis. All patients had a blood collection during the index hospital admission for MI, or prior to undergoing lower extremity revascularization. In the case of controls, blood collection occurred at time of enrollment.

Atherosclerosis analysis.

Hearts were sectioned through the aortic root (6 μm) and stained with H&E for lesion quantification or used for immunohistochemical analysis. For morphometric analysis of lesions, 18 sections per mouse were imaged (Nikon Eclipse) with Image-Pro Plus, spanning the entire aortic root, and lesions and necrotic area were quantified using Image-Pro Plus software. For collagen analysis, 6 sections per mouse were stained with picrosirius red and imaged under polarized light using a Zeiss Axioplan microscope. For macrophage analysis, 6 sections per mouse were incubated with an anti-CD68 antibody (1:250, AbD Serotec) and antibody reactivity was visualized using the Vectastain ABC Alkaline Phosphatase Kit (Vector Laboratories) and the Vector Red Substrate Kit (Vector Laboratories). For apoptosis analysis, sectioned aortic roots were incubated with anti-cleaved caspase 3 (1:100, Cell Signaling) and counter stained with DAPI. Nuclei positively stained for the antibody and hematoxylin were considered apoptotic. Necrosis was quantified in aortic roots by measuring the acellular areas of plaques with Image Pro Plus software as previously described [28]. Cell proliferation in the plaques were analyzed in aortic roots by staining the plaques for Ki67 (1:100, Abcam). Nuclei positively stained for the antibody and DAPI were considered proliferating cells. To quantify proliferation in the plaque, the number of cells that stained positive for Ki67 per section was measured for each mouse. All analyses were performed by a blinded investigator.

Single cell RNA sequencing.

C57BL/6 mice were injected with 1×10^{12} particles of mPSC9AAV (UPenn Vector Core) and fed a Western diet for 20 weeks to induce hypercholesterolemia. A subset of mice were switched to a chow diet 20 weeks after Western diet feeding for an additional 4 weeks to reestablish normocholesterolemia. Aortic arches were harvested and digested in Liberase (Roche), Hyaluronidase (Sigma) and DNase I (Sigma) for 15 min at 37°C using the GentleMacs dissociator (Miltenyi). Aortic single cell suspensions from 5 mice were combined according to circulating cholesterol concentrations. Viable aortic plaque leukocytes were isolated and identified by CD45⁺ and viability staining (anti-CD45 PerCp-Cy5.5, BioLegend, and Fixable Viability Dye eFluor 780, eBioscience) on a FACS Aria II cytometer (BD) equipped with a 100 μm nozzle. After washing isolated aortic leukocytes were loaded into single cell gel beads (GEMs) and barcoded with a unique molecular identifier (UMI) using the Single Cell 3' reagent kit (10X Genomics) according to the manufacturer's protocol. Subsequently, cDNA was generated, libraries constructed and sequenced on a NovaSeq 6000 (Illumina). The data was demultiplexed, QC checked and aligned to the UCSC 10mm reference mouse genome using CellRanger software (10X). Outlier cells were then removed based on the number of genes expressed and the percent of expressed genes coming from the mitochondria, as outliers in these metrics are likely to represent either multiple captured cells or cells undergoing apoptosis. Using the R package Seurat, the samples were merged, canonical correlation analysis (CCA) performed, and

canonical correlation vectors (CCs) aligned, as previously described[73]. Using the aligned CCs, Louvain clusters were found and tSNE dimension reduction was performed.

Platelet activity in human blood.

MPA were identified in citrate anticoagulated blood as previously described [8]. Briefly, whole blood was fixed with 1% formaldehyde (Sigma) 15 min after blood collection. Fixed whole blood was stained with 5 μ L CD61-FITC (Dako) to identify platelets and 5 μ L CD14-APC (BD Biosciences) to identify monocytes. After lysis of red blood cells, monocytes were collected based on side-scatter properties and positive staining for CD14 using an Accuri C6 flow cytometer (BD Biosciences). MPA were identified as having a positive stain for CD14 and CD61, and were recorded as a percent of 2000 total monocytes collected.

Platelet activation was determined by platelet surface expression of P-selectin, and CD40 with whole blood flow cytometry, as previously described [8]. Briefly, P-selectin expression was determined with a FITC-conjugated anti-CD62P antibody (BD Biosciences), and CD40 expression was determined with a FITC-conjugated anti-CD40 antibody (BD Biosciences). Gates were established to include platelets with and without aggregation to monocytes. Monocytes were identified by staining with CD14-APC (BD Biosciences), and platelets were identified by staining with CD42-PE (BD Biosciences). The expression of platelet activation markers were assessed individually

Human Blood Transcriptome Profiling.

Whole blood was collected into PAXgene Blood RNA tubes (PreAnalytiX GmbH– BD Biosciences, Mississauga, ON, Canada), and RNA isolated. The quality and yield of the isolated RNA was determined with an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA) prior to RNA-sequencing (Illumina HiSeq4000 Sequencing). Transcript differential expression analysis was performed with DESeq.

Plaque macrophage profiling.

Laser capture microdissection (LCM) was performed as previously described using the Leica DM-6000B instrument and Leica LMD CC7000 camera (Leica Microsystems) [74]. Serially sectioned guide slides were prepared for each mouse by staining CD68⁺ macrophages, and the CD68⁺ areas in serial sections were collected by LCM. RNA was extracted from CD68⁺ plaque macrophages using the Arcturus Picopure RNA Isolation Kit (Life Technologies). Total RNA was amplified using the Ovation WT Pico Amp Kit (NuGen), purified using the QIAquick PCR Purification Kit (QIAGEN), and used for RT-qPCR.

In vivo monocyte & macrophage trafficking assays.

Monocytes were labeled as previously described [28, 75]. Briefly, 1 μ m Fluoresbrite FITC-dyed (YG) plain microspheres (Polysciences Inc.) were diluted in PBS (1:4), and 250 μ l injected retro-orbital into mice to label circulating monocytes. Bead labeling efficiency was assessed by flow cytometry, 24 h after bead injection. For the egress study, mice were injected with 1 mg (i.p) of EdU 5 days prior to control or antiplatelet injections. Macrophages retained in plaques were determined by performing a click reaction with Alexa

Fluor 647 nm-azide (Click-iT EdU Imaging Kit, Invitrogen) according to the manufacturer's instructions. Slides were imaged on a Leica SCN400F slide scanner, and EdU positive cells quantified.

Plasma analysis.

Total cholesterol, HDL-cholesterol (HDL-C), and triglyceride concentrations were measured using colorimetric assays (Wako Diagnostics, Richmond, VA). Plasma IL-6, IL-1 β , and MCP-1 were determined via flow cytometry with mouse IL-6 Enhanced Sensitivity Flex Set, IL-1 β Enhanced Sensitivity Flex Set, and a MCP-1 Flex Set, respectively (BD Biosciences).

Platelet isolation.

Citrated blood samples were allowed to rest at room temperature for 15 min post-phlebotomy. Platelet rich plasma (PRP) was obtained by centrifugation of blood at 200 g for 10 min. PRP was added to 1:10 (v/v) ACD-A [tris-sodium citrate (25 g/L), glucose (20 g/L), and citric acid (14 g/L)] and spun at 1000 g for 10 min. The platelet pellet was washed in Tyrode's buffer [137 mM NaCl, 2.8 mM KCl, 1 mM MgCl₂, 12 mM NaHCO₃, 0.4 mM Na₂HPO₄, 5.5 mM glucose, 10 mM HEPES, pH 7.4] and 1 μ M PGE₁ (Sigma), prior to centrifugation at 1000 g for 10 min and resuspension in Tyrode's buffer containing 2 mM CaCl₂. Platelets were counted on a COULTER AC^T diff2 Hematology Analyzer (Beckman Coulter Inc, Brea, CA) and adjusted to the desired concentration.

Monocyte isolation.

Monocytes were isolated from bone marrow using a monocyte Isolation Kit (BM) (Miltenyl Biotec). Briefly, femurs and tibias were harvested and flushed through with ice-cold phosphate-buffered saline (PBS). Bone marrow cell suspensions were passed through a 70- μ m cell strainer to obtain a single-cell suspension. Using the Monocyte Isolation Kit (BM), mouse, monocytes are isolated by depletion of non-target cells. Non-target cells are indirectly magnetically labeled with a cocktail of biotin-conjugated monoclonal antibodies, as primary labeling reagent, and anti-biotin monoclonal antibodies conjugated to microbeads as secondary labeling reagent. Unlabeled cells were depleted by retaining them within a MACS Column in the magnetic field of a MACS Separator. Unlabeled monocytes passed through the column. Monocytes were resuspended in migration buffer prior to assays.

Cell culture.

Peritoneal macrophages were isolated from mice by peritoneal lavage 4 days after i.p. injection of 3 ml of 5% thioglycollate to C57Bl/6 mice (male, 7–12 weeks of age), cells were cultured in DMEM with 10% FBS. For NF- κ B inhibition (BAY 11–7085) experiments cells were pretreated with each component for 30 min prior to exposure to washed platelets. The platelet to macrophage ratio for all experiments was 100:1 unless otherwise stated. Mouse peritoneal macrophages were transfected with 5 pmol Silencer Select siRNA inhibitors (*Socs3*, *Socs1* or control, Ambion) utilizing Lipofectamine RNAiMax Transfection reagent (Invitrogen). For gp-130 blocking experiments, macrophages were treated with either an antibody control (Anti-Mouse IgG, ab150107, Abcam) or anti-gp-130 (MAB4682-SP, R&D Systems) for 1 h prior to the addition of platelets or platelet releasate.

RNA isolation and quantitative PCR.

Total RNA was isolated using TRIzol reagent (Invitrogen) and Direct-zol RNA MiniPrep columns (Zymo Research). For mRNA quantification, cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR was performed using an iCycler Real-Time Detection System (Bio-Rad). mRNA were normalized to *Cyclophilin* or *Hprt* as a housekeeping gene. The following primer sequences were used: *Il6* 5'-TTCCATCCAGTTGCCTTCTT-3' and 5'-ATTCCACGATTCCCAGAG-3', *Il1b* 5'-GCAACTGTTCTGAACTCAACT-3' and 5'-ATCTTTTGGGGTCCGTCAACT-3', *Ccl2* 5'-GAAATGCCACCTTTTGACAGTG-3' and 5'-TGGATGCTCTCATCAGGACAG-3', *Socs3* 5'-ATGGTCACCCACAGCAAGTTT-3' and 5'-TCCAGTAGAATCCGCTCTCCT-3', *Socs1* 5'-CTGCGGCTTCTATTGGGGAC-3' and 5'-AAAAGGCAGTCGAAGGTCTCG-3', *Cd11b* 5'-ACCGGCTTGTGCTGTAGTC-3' and 5'-CCATGACCTTCCAAGAGAATGC-3', *Cyclophilin* 5'-GGCCGATGACGAGCCC-3' and 5'-TGTCTTTGGAAGTTTGTCTGCAA-3', *Hprt* 5'-TCAGTC AACGGGGGACATAAA-3' and 5'-GGGGCT GTACTGCTTAACCAG-3'.

Real-time cell migration and adhesion.

Monocytes were isolated from the femur and tibias of C57Bl/6 mice by use of a bone marrow monocyte isolation kit (MACS Miltenyi Biotec, 130–100629). Migration experiments were carried out with CIM-16 plates and an xCELLigence RTCA-DP instrument (ACEA, San Diego, USA) as previously described [76]. Chemoattractants were made to desired concentrations and loaded into the lower wells of the CIM-16 plate. Upper wells were filled with chemotaxis buffer and plates equilibrated for 30 min at RT. Elicited macrophages were resuspended in chemotaxis buffer incubated at 37°C, 5% CO₂ for 1 h prior to assay commencement. Cell suspensions were placed into the wells of the upper chamber, and the assay performed over 8 h (5 s data points). Adhesion assays were performed with E-plates and an xCELLigence RTCA-DP instrument (ACEA, San Diego, USA). Monocytes were isolated from platelet deficient or competent mice, and adhesion monitored over 8 h (2 min data points).

Phagocytosis assay.

Peritoneal macrophages were isolated from mice by peritoneal lavage 3 days after injecting them intraperitoneally with 2 ml of 3% thioglycollate and seeded in chamber slides at a density of 5×10^6 per well. FITC-labeled, heat-inactivated *E. coli* bacteria were added to the macrophages, and incubated for 1.5 hours. Media was removed, and cells were washed with ice cold PBS three times to remove non-phagocytized apoptotic cells. Macrophages were fixed by incubating in 10% formalin for 10 minutes and washed with PBS three times. Macrophages were then stained with Phalloidin Red (Molecular Probes) and slides were mounted with DAPI containing mountant (Molecular probes, P36935). To quantify the extent of phagocytosis, the number of *E. coli* internalized per macrophage was calculated and results expressed as phagocytotic capacity.

Efferocytosis Assay.

Peritoneal macrophages were isolated from mice by peritoneal lavage 3 days after injecting them intraperitoneally with 2 ml of 3% thioglycollate and seeded in chamber slides at a density of 5×10^6 per well. Jurkat cells were labeled with Cell Tracker Green (Molecular Probes) and rendered apoptotic by treating with cycloheximide (100 $\mu\text{g}/\text{mL}$) for 12 h. Apoptotic Jurkat cells in RPMI with 0.2 % BSA were added onto macrophages, at a 5:1 ratio of apoptotic cells: macrophages, and incubated for 1.5 hr. Media was removed, and cells were washed with ice cold PBS three times to remove non-phagocytized apoptotic cells. Macrophages were fixed by incubating in 10% formalin for 10 minutes and washed with PBS three times. Macrophages were then stained with Phalloidin Red (Molecular Probes) and slides were mounted with DAPI containing mountant (Molecular probes, P36935). To quantify the extent of efferocytosis, macrophages with internalized apoptotic bodies were scored by a blinded investigator, and results are expressed as % efferocytosis (number of macrophages with engulfed apoptotic cells / total number of macrophages $\times$ 100).

Flow cytometry.

Leukocytes: Blood was collected via the tail vein with EDTA-coated capillaries. Red blood cells were lysed with RBL buffer (Sigma Aldrich), and blocking achieved with antimouse CD16/CD32 (eBioscience). Monocytes were identified by staining with anti-mouse CD45-PerCp/Cy5.5 (Biolegend), anti-mouse CD115 (CSF-1R)-Brilliant Violet 605 (Biolegend) and antimouse Ly-6G/Ly-6C-APC (Biolegend). Monocyte activation was determined via by CD11b expression (geo-mean fluorescence intensity [MFI]). Neutrophils were identified as CD45^{hi}CD115^{lo}Ly6-C/G^{hi}. Monocyte-platelet aggregates were identified by first gating on monocyte population of interest and identifying those positive for platelets with CD41. Flow cytometry was performed using a LSRII analyzer. Whole blood counts were recorded on a CBC (Oxford Sciences). Platelets: Blood was collected *via* retro orbital bleeding with EDTA-coated capillaries, and diluted in heparin. Antibodies against P-selectin-AlexaFluor 647 (BD Biosciences) or JON/A-PE (Emfret) were added to whole blood, and platelet agonists added in accordance with the figure legend. Samples were incubated for 15 min, prior to dilution in Tyrode's buffer, and immediately analyzed via flow cytometry (BD Accuri). Platelets were identified by forward scatter and side scatter characteristics.

Macrophage immunohistochemistry.

For immunofluorescence, cells were fixed in 4% PFA, blocked/permeabilized in 2.5% BSA/0.1% Triton X-100, and stained with an antibody directed against NF- κ B p65 (Sigma-Aldrich) at 1:100, overnight at 4°C.

Statistical analysis.

For atherosclerosis and immunohistochemical analyses, all comparisons were made using a two-tailed Student's *t* test or 1-way ANOVA ($P < 0.05$). Normality of data was assessed via a Shapiro-Wilk normality test. All data are expressed as mean $\pm$ SEM, unless otherwise noted. All data used to generate the figures are in data file S1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Hypercholesterolemia promotes platelet-myeloid interactions.

(A-B) Circulating leukocyte-platelet aggregates (LPA), monocyte-platelet aggregates (MPA), and Ly6C^{hi} monocyte-platelet (Ly6C^{hi}-PA) aggregates as determined via flow cytometry in control and hypercholesterolemic (LDLR ASO treated) mice. Data are expressed as mean $\pm$ SEM, $n = 5$ mice/grp, $*P < 0.05$ as determined by a two-tailed Student's t test. (C-D) t-Stochastic neighbor embedding (t-SNE) representation of aligned gene expression data in single cells ($n = 2540$) extracted from atherosclerotic aortic arches of hypercholesterolemic mice. (C) Identification of CD45⁺ plaque leukocyte clusters based on transcript expression,

and **(D)** Platelet factor 4 (*Pf4*) expression projected on leukocyte clusters of t-SNE plots (scale log-fold transformed gene expression).

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Fig. 2. Platelets prime monocytes for migration

(A) Experimental overview: Male *Ldlr*^{-/-} mice were fed a western diet (0.3% cholesterol) for 7 weeks. At week 6, mice were injected with EdU to label circulating monocytes. Mice were then split into groups, and over a 2 week period received 4 injections (3 µg/g, i.p.) of either α-CD42b (α-plt), or α-IgG (α-ctrl). (B) Representative flow cytometry plot to identify circulating monocyte subtypes, (C) ratio of Ly6C^{hi} to Ly6C^{lo} monocytes and (D) monocyte CD11b surface expression in *Ldlr*^{-/-} mice fed a western diet (0.3% cholesterol) for 7 weeks and subsequent treatment with an IgG control antibody (α-ctrl) or α-CD42b (α-

plt). Data are expressed as mean $\pm$ SEM, $n = 6$ mice/grp. (E) Inflammatory transcript expression on monocytes from mice treated as in (A), as determined by FACS sorting and RT-qPCR. Data are expressed as mean $\pm$ SEM, $n = 4-5$ mice/grp, * $p < 0.01$. (F) Correlation of platelet count with the expression of inflammatory transcripts in circulating monocytes, data are expressed as mean $\pm$ SEM, $n = 4$ mice/grp. (G) Monocyte migration to platelet releasate and (H) chemotaxis quantification, data are expressed as mean $\pm$ SEM, $n = 3$. (I) Migratory capacity towards chemoattractants of monocytes isolated from mice treated as in (A), data are expressed as mean $\pm$ SEM, $n = 6$ mice/grp. * $P < 0.05$, ** $P < 0.005$ as determined by a two-tailed Student's t test.

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Fig. 3. Platelets alter monocyte recruitment to atherosclerotic lesions

(A) Experimental overview: Male *Ldlr*^{-/-} mice were fed a western diet (0.3% cholesterol) for 7 weeks. At week 6, mice were injected with EdU to label circulating monocytes. Mice were then split into groups, and over a 2 week period received 4 injections (3 μ g/g, i.p.) of either α -CD42b (α -plt), or α -IgG (α -ctrl). 48 hours prior to harvest mice were injected with green fluorescent beads to track monocyte entry into plaques. At week 9 mice were harvested for plaque analyses. Representative images of (B) macrophage recruitment to the aortic sinus, and (C) quantification of monocyte recruitment in the aortic sinus. (D)

Representative image and (E) quantification of macrophage retention to the aortic sinus. Dashed lines highlight plaque area, insert is magnified area. Data are expressed as mean $\pm$ SEM, $n = 6$ mice/grp, * $P < 0.05$ as determined by a twotailed Student's t test, one dot per mouse. Scale bar: 100 μm .

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Fig. 4. Platelets promote atherosclerosis progression

Male *Ldlr*^{-/-} mice were fed a western diet (0.3% cholesterol) for 7 weeks. Mice were then split into groups, and over a 2 week period received 4 injections (3 ug/g, i.p.) of either α-CD42b (α-plt) or α-IgG (α-ctrl). At week 9 mice were harvested for plaque analyses. (A) Representative images (scale bar: 500 μm) and (B) quantification of aortic root lesions stained with H&E. (C) Representative images (scale bar: 100 μm) and (D) quantification of aortic root lesions after immunohistochemical staining for the macrophage marker CD68. (E) Representative images (scale bar: 100 μm) and (F) quantification of brachiocephalic arteries stained with H&E and CD68. (G) Representative brightfield (BF) and polarized light images (scale bar: 100 μm) and (H) quantification of aortic root lesions stained with picrosirius red to quantify plaque collagen content, data are expressed as mean ± SEM, n = 6 mice/grp, * *P* < 0.05 as determined by a two-tailed Student's *t* test.

Fig. 5. Platelets impair macrophage efferocytosis

Male *Ldlr*^{-/-} mice were fed a western diet (0.3% cholesterol) for 7 weeks. Mice were then split into groups, and over a 2 week period received 4 injections (3 μ g/g, i.p.) of either α -CD42b (α -plt) or α -IgG (α -ctrl). At week 9 mice were harvested for plaque analyses. **(A)** Representative images (scale bar: 100 μ m) and **(B)** quantification of aortic root necrotic area, data are expressed as mean $\pm$ SEM, $n = 6$ mice/grp. **(C)** Representative images and **(D)** quantification of peritoneal macrophage apoptotic jurkat uptake after treatment with platelets or vehicle control for 6 h, and jurkat exposure for 90 min. **(E)** Representative images and **(F)**

quantification of peritoneal macrophage FITC-labeled heat inactivated *Escherichia (E.) coli* uptake after treatment with platelets or vehicle control for 6 h, and subsequent *E. coli* incubation for 90 min. Macrophages were counterstained with DAPI, and the actin cytoskeleton stained with phalloidin. Data are from one experiment representative of 4 (D, F, mean $\pm$ SEM) independent experiments with similar results, * $P < 0.05$, and ** $P < 0.001$ as determined by a two-tailed Student's t test. Scale bar: 50 μm .

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Fig. 6. Platelets promote a pro-inflammatory plaque macrophage phenotype

Male *Ldlr*^{-/-} mice were fed a western diet (0.3% cholesterol) for 7 weeks. Mice were then split into groups, and over a 2 week period received 4 injections (3 μ g/g, i.p.) of either α -CD42b (α -plt) or α -IgG (α -ctrl). At week 9 mice were harvested for plaque analyses. Plaque macrophages were isolated by laser capture microdissection, and transcripts analyzed via RT-qPCR. (A) Expression of inflammatory transcripts in plaque macrophages isolated from mice. Correlation between inflammatory transcript expression and (B) circulating platelet count and (C) plaque macrophage area. Data are expressed relative to *Hprt* and

expressed as mean $\pm$ SEM, $n = 5$ mice/grp, * $P < 0.05$ as determined by a two-tailed Student's t test. **(D)** mRNA expression of peritoneal macrophages treated with either platelets or releasate of platelets for 6 h, * $P < 0.05$ as determined by 1-way ANOVA. **(E)** Inflammatory cytokine quantification in peritoneal macrophage supernatant after exposure to platelets for 6 hours. Data are expressed relative to *Cyclophilin*. *** $P < 0.002$ as determined by a two-tailed Student's t test. **(F)** Oil Red O staining of macrophages exposed to acetylated LDL (50 $\mu\text{g/mL}$) for 6 h in the presence or absence of platelets. **(G)** Dose-response of inflammatory gene expression of macrophages treated with increasing ratio of platelets to macrophages (0:1, 20:1, 50:1, 100:1). * $P < 0.05$ as determined by 1-way ANOVA. **(H)** Macrophage p65 staining as by microscopy, NFkB i = pretreatment of macrophages for 30 min with 10 μM Bay 11–7082 prior to platelet exposure. **(I)** Expression of proinflammatory genes in macrophages treated with platelets with or without inhibition of NFkB-mediated signaling (10 μM , Bay 11–7082) for 6 h. Data are expressed as mean $\pm$ SEM, $n = 4$ mice/grp, * $P < 0.05$ as determined by a two-tailed Student's t test. Scale bar: 20 μm .

Fig. 7. *Socs3* induction by platelets enhances the proinflammatory effects of macrophage IL-6 signaling

(A) *Socs1* and *Socs3* expression of plaque macrophages from platelet competent and deficient mice expressed as mean $\pm$ SEM, $n = 6$ mice/grp, * $P < 0.05$ as determined by a two-tailed Student's t test. (B) Correlation of *Socs1:Socs3* with circulating platelet count at time of harvest, data are expressed relative to *Hprt*. (C) Peritoneal macrophage expression of *Socs3* and *Socs1* after exposure to different doses of platelets. (D) *Socs1:Socs3* expression in peritoneal macrophages exposed to increasing platelet to macrophage ratio (0:1, 20:1,

50:1, 100:1) expressed as mean $\pm$ SEM, * $P < 0.05$ relative to the vehicle control as determined by 1-way ANOVA. (E) *Socs3* and *Il1b* expression in macrophages after exposure to platelets with and without inhibition of IL-6 signaling via α -gp130 (2 μ g/mL), as mean $\pm$ SEM, * $P < 0.02$ relative to the vehicle control or # $P < 0.02$ relative to platelet-treated cells as determined by 1-way ANOVA. (F) Macrophage *Il1b* expression after exposure to platelets with or without knockdown of *Socs3* for 6 h. * $P < 0.01$ relative to siRNA control platelet treated samples as determined by a 2-way ANOVA. Data are from one experiment representative of 3 (C-F mean $\pm$ SEM) independent experiments with similar results, data are expressed relative to *Cyclophilin*. (G) Representative images and quantification of peritoneal macrophage apoptotic jurkat uptake after treatment with α -gp130 and platelets or vehicle control for 6 h, and jurkat exposure for 90 min. (H) Representative images and quantification of peritoneal macrophage apoptotic jurkat uptake after treatment with platelets with or without knockdown of *SOCS3* or vehicle control for 6 h, and jurkat exposure for 90 min. Macrophages were counterstained with DAPI, and the actin cytoskeleton stained with phalloidin. Data are from one experiment representative of 4 (C-F, mean $\pm$ SEM) independent experiments with similar results, *** $P < 0.001$, * $P < 0.005$ as determined by a two-tailed Student's *t* test. Scale bar: 50 μ m.

Fig. 8. *SOCS1:SOCS3* in subjects with CVD correlates with *IL1B* and platelet activity. Correlation of whole blood *SOCS1:SOCS3* expression to (A) *IL1B* ($n = 99$), (B) circulating monocyte-platelet aggregates (MPA) ($n = 95$), (C) platelet surface P-selectin expression ($n = 100$), and (D) platelet CD40 expression ($n = 99$) in subjects with peripheral artery disease.

Table 1.
Lipid and cell count characteristics of mice after 2 weeks of platelet depletion.

Ldlr^{-/-} mice were fed a western diet (0.3% cholesterol) for 7 weeks. Mice were then split into groups, and over a 2 week period received 4 injections (3 µg/g, i.p.) of either α-CD42b (α-plt) or α-IgG (α-ctrl).

	α-control	α-platelet
Total cholesterol (mg.dL)	1156 ± 101	969 ± 49
HDL-C (mg.dL)	95 ± 4.5	89 ± 5.8
Triglycerides (mg.dL)	695 ± 53	573 ± 80
Weight (g)	35.7 ± 1.3	37.7 ± 2.1
Platelets (10 ⁹ .L)	850 ± 150	213 ± 40*
White blood cells (10 ⁹ .L)	9.8 ± 2.1	9.6 ± 2.6
Monocytes (10 ⁹ .L)	0.5 ± 0.1	0.6 ± 0.1
Neutrophils (10 ⁹ .L)	2.5 ± 0.7	2.3 ± 0.7
Lymphocytes (10 ⁹ .L)	6.3 ± 1.3	6.6 ± 1.8
Red blood cells (10 ⁹ .L)	7.4 ± 0.9	7.4 ± 1.1
Hematocrit (10 ⁹ .L)	36.8 ± 4.9	37.7 ± 5.7
Hemoglobin (10 ⁹ .L)	10.1 ± 1.4	9.1 ± 1.8

Data are expressed as mean ± SEM, *n* = 6 mice/grp

* *P* < 0.001 as determined by a two-tailed Student's *t* test. HDL-C: high-density lipoprotein-C.

Table 2.

Demographics and transcript expression of women with MI and controls.

	Control (<i>n</i> = 24)	MI (<i>n</i> = 61)
Age (median [IQR])	66.6 [57.8, 69.7]	63.1 [56.1, 71.9]
Race (White, %)	48.5	50.5
Platelets (10 ⁹ .L) (median [IQR])	223 [191, 291]	242 [210, 323]*
MPA (%) (median [IQR])	16.2 [12.3, 29.1]	24.3 [18.5, 43.0]*
Transcript Expression (median [IQR])	<i>n</i> = 9	<i>n</i> = 23
<i>SOCS1</i>	84.4 [61.6, 98.1]	136.1 [116.9, 181.3]*
<i>SOCS3</i>	624.4 [535.7, 961]	1557 [930.9, 2755]*
<i>SOCS1:SOCS3</i>	0.15 [0.09, 0.26]	0.08 [0.06, 0.13]*
<i>IL1B</i>	705.8 [683.1, 1261.0]	1372 [1113.0, 1769.0]*

* $P < 0.01$, as determined by a two-tailed Student's *t* test.