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Platelets Amplify Inflammation in Arthritis via Collagen-Dependent Microparticle Production

Eric Boilard[1], **Peter A. Nigrovic**[1,2], **Katherine Larabee**[1], **Gerald F. M. Watts**[1], **Jonathan S. Coblyn**[1], **Michael E. Weinblatt**[1], **Elena M. Massarotti**[1], **Eileen Remold-O'Donnell**[3], **Richard W. Farndale**[4], **Jerry Ware**[5], and **David M. Lee**[1,*]

[1]Division of Rheumatology, Immunology and Allergy, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA

[2]Division of Immunology, Children's Hospital Boston, Boston, MA 02115, USA

[3]Immune Disease Institute, Harvard Medical School, Boston MA 02115, USA

[4]University of Cambridge, Department of Biochemistry, Downing Site, Cambridge CB2 1QW, UK

[5]University of Arkansas for Medical Sciences, Little Rock, AR 72205–7199, USA

Abstract

In addition to their pivotal role in thrombosis and wound repair, platelets participate in inflammatory responses. We investigated the role of platelets in the autoimmune disease rheumatoid arthritis. We identified platelet microparticles—submicrometer vesicles elaborated by activated platelets—in joint fluid from patients with rheumatoid arthritis and other forms of inflammatory arthritis, but not in joint fluid from patients with osteoarthritis. Platelet microparticles were proinflammatory, eliciting cytokine responses from synovial fibroblasts via interleukin-1. Consistent with these findings, depletion of platelets attenuated murine inflammatory arthritis. Using both pharmacologic and genetic approaches, we identified the collagen receptor glycoprotein VI as a key trigger for platelet microparticle generation in arthritis pathophysiology. Thus, these findings demonstrate a previously unappreciated role for platelets and their activation-induced microparticles in inflammatory joint diseases.

Platelets are highly abundant hematopoietic cells, outnumbering leukocytes in the peripheral circulation by almost two orders of magnitude (1). The role of platelets in hemostasis and wound repair after vascular injury is well known (2); however, there is a growing appreciation for their role in inflammation. This role has been studied most carefully in atherosclerosis, a chronic inflammatory disease of the blood vessels in which platelets release a broad range of inflammatory mediators that support endothelial cell activation, leukocyte adhesion and transmigration, monocyte maturation, and elaboration of cytokines and reactive oxygen species [reviewed in (3)].

The growing literature regarding the proinflammatory capacity of platelets prompted us to investigate whether they could participate in another common inflammatory condition, inflammatory arthritis. Of the inflammatory arthritides, rheumatoid arthritis (RA) is the most common (4). RA manifests as chronic inflammation of the synovial lining of the joint, resulting in pain, swelling, and ultimately destruction of cartilage and bone (5). Although evidence has implicated lymphocytes, innate immune cells such as neutrophils and mast

*To whom correspondence should be addressed. dlee@rics.bwh.harvard.edu.

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cells, and synovial tissue cells in the evolution of RA, to date platelets have no known functional role. We first sought to determine whether platelets are present in RA synovial fluids (SF) by use of the platelet-specific marker CD41 (GPIIb/α2b from the platelet-specific integrin GPIIbIIIa/α2bβ3) (6,7). Flow cytometric analyses detected a substantial number of CD41-positive events in RA SF (Fig. 1, A and B). Unexpectedly, most CD41-positive events were smaller in size than intact leukocytes or platelets (fig. S1), suggesting that these particles are platelet microparticles (MPs). Platelet MPs are intact vesicles (0.2 to 1 μm in diameter) that form by budding from the membranes of activated platelets (8–10). We found on average just under 2×10^5 $CD41^+$ MPs per microliter of SF from patients with RA (Fig. 1C). In contrast to RA SF, where MPs were present in all samples, $CD41^+$ MPs were undetectable in 19 out of 20 osteoarthritis (OA) SF samples (Fig. 1C). We also detected platelet MPs in other inflammatory arthritides (Fig. 1D). We further investigated the presence of MPs originating from other hematopoeitically derived cell types. Consistent with previous observations (11), MPs expressing neutrophil, T cell, or macrophage markers were present albeit in substantially smaller amounts than observed for platelet MPs in RA SF (Fig. 1E). Recognizing that platelets can adhere to migrating leukocytes (12,13), we also assessed the presence of platelets associated with SF leukocytes. Intriguingly, we found that a substantial number of SF leukocytes costained with CD41 and the leukocyte marker CD45 (fig. S1, D and E). Microscopic examination revealed that CD41 staining in these cells was restricted to discrete particles of MP size adherent to the leukocyte surface; we could not detect intact platelets associated with SF leukocytes (fig. S1D).

We next explored the pathophysiologic importance of platelets and platelet MPs in inflammatory arthritis in vivo. Here, we used the K/BxN serum transfer model of inflammatory arthritis. The progressive distal symmetric erosive polyarthritis observed in K/BxN T cell receptor transgenic mice results from T cell recognition of a ubiquitous autoantigen, glucose-6-phosphate isomerase, presented by major histocompatability complex class II $I\text{-}A^{g7}$, driving high-titer arthri-togenic autoantibody production [reviewed in (14)]. Arthritis can also be induced by passive transfer of immunoglobulin G (IgG) autoanti-bodies from K/BxN mice into wild-type mice. Numerous effector mechanisms have been implicated in the pathogenesis of the IgG-driven effector phase of K/BxN serum-transfer arthritis, including neutrophils, mast cells, Fc gamma receptors, and soluble mediators [interleukin-1 (IL-1), tumor necrosis factor (TNF), complement C5a/C5a receptor, eicosanoids, and the mast cell protease tryptase] (14–16). To study the role of platelets in this system, we initiated K/BxN serum transfer arthritis in animals treated with a platelet-depleting antibody regimen that rapidly (within 60 min) reduces platelet numbers by >95% for at least 6 days (17). We found that platelet-depleted mice exhibited a marked reduction in arthritis as assessed by clinical scoring and by histological analysis (Fig. 2, A and B). These findings demonstrate that platelets are required for inflammatory arthritis development in vivo.

To gain further insight into the link between platelets and joint inflammation, we explored the mechanisms by which platelets are activated to release MP in the context of arthritis. Platelets can be triggered via several pathways, many of which have already been targeted for the prevention of thrombosis. Among these pathways we considered were thromboxane A2 stimulation of its receptor (TP) on platelets (blocked by TP antagonist SQ 29548), ligation of the P2Y12 receptor by adenosine 5′-diphosphate (inhibited by clopidogrel), and GPIb-IX, a platelet membrane glycoprotein complex that binds to von Willebrand factor. By using genetically deficient mice and pharmacologic blockade, we determined that interference with these pathways did not impede development of joint inflammation, suggesting that these pathways do not regulate platelet MP generation in inflammatory arthritis (Fig. 2, C to G).

What other pathways to platelet activation could be operative in arthritis? Knowing that the concentration of platelet-derived MPs within RA SF (2×10^5 per microliter) greatly exceeds that in RA peripheral blood (600 per microliter) (18), we hypothesized that platelet activation likely occurs locally. The vasculature of the joint is in intimate contact with fibroblast-like synoviocytes (FLS) and the extracellular matrix (ECM) elaborated by these cells (19), and thus we modeled the interaction of platelets with synoviocytes and their ECM in vitro. Mouse platelets coincubated with primary mouse FLS promptly released MPs (Fig. 3A). To determine the trigger for MP release, we assessed MP formation after treatment with specific pharmacologic inhibitors or using platelets isolated from mice deficient in candidate genes. Consistent with our in vivo results, we found that cyclooxygenase (*Ptgs1*$^{-/-}$), thromboxane, GPIIbIIIa (*Itgb3*$^{-/-}$), GPIb (*Gp1ba*$^{-/-}$), and ADP-P2Y12 pathways were dispensable (Fig. 3B). We next explored whether the ECM generated by primary cultured FLS could be the relevant stimulus, particularly collagen. Previous studies have demonstrated that collagen can activate platelets to form MPs (8) and that glycoprotein VI (GPVI) is the predominant collagen receptor on platelets (20). GPVI is an immunoglobulin superfamily member expressed exclusively by megakaryocytes and platelets that signals via noncovalent interaction with the common γ-chain of the Fc receptor (21). Using platelets lacking either FcR–γ-chain (*Fcer1g*$^{-/-}$) or GPVI (*Gp6*$^{-/-}$), we found that generation of MPs by primary FLS was mediated predominantly via this pathway (Fig. 3B). After confirming that human platelets also released MPs upon coincubation with primary human FLS (Fig. 3, C and D), we validated the relevance of GPVI in MP release in humans using the GPVI-specific agonist collagen-related peptide (CRP) (22) (Fig. 3D). Further characterization of collagen-stimulated platelet MPs showed that their phenotype is congruent with that of platelet MPs from SF and distinct from that of intact platelets (table S1). Finally, we assessed whether GPVI is relevant for platelet activation in vivo by administering K/BxN serum to *Gp6*$^{-/-}$ and control mice and assessing the development of synovitis. Both clinical and histomorphometric assessment confirmed that arthritis in *Gp6*$^{-/-}$ mice was significantly reduced (Fig. 3, E and F). These results confirm that activation of platelets via the collagen receptor GPVI—a pathway resulting in MP generation—plays an important role in the pathogenesis of arthritis.

Having demonstrated platelet participation in inflammatory arthritis in vivo, we aimed to identify platelet MP effector activities that contribute to joint inflammation. The most abundant cell in the pathologic rheumatoid pannus tissue is the FLS (19). This lineage plays a substantial role in the perpetuation of joint inflammation and in the destruction of cartilage (19,23). We surveyed the capacity of collagen-stimulated human platelet MPs to elicit a range of cytokines and chemokines from FLS and observed prominent production of the broadly inflammatory cytokine IL-6 and the neutrophil chemoattract-ant IL-8 (Fig. 4A and fig. S2). Consistent with this observation, incubation of MPs isolated from RA SF induced FLS to release substantial quantities of both cytokines (Fig. 4B and fig. S3). We focused our further studies on MP stimulation of IL-8 by FLS because SF from the inflammatory arthritides is rich in neutrophils. To elucidate a mechanism by which platelet MPs stimulate FLS, we used a genetic approach with MPs generated from mice deficient in specific candidate genes. We found that platelet MPs generated from mice lacking both IL-lα and IL-lβ (*Illa/b*$^{-/-}$) were incapable of stimulating murine FLS to produce the murine IL-8 ortholog KC (Fig. 4C). Furthermore, FLS generated from mice deficient in the IL-1 receptor (*Illrf*$^{-/-}$) were unresponsive to platelet MPs, though release of KC remained intact after TNF stimulation (Fig. 4D). By contrast, MPs from mouse platelets deficient in prostaglandin synthesis capacity (*Ptgsl*$^{-/-}$) retained their ability to stimulate KC production from FLS (Fig. 4C).

Platelets exhibit membrane-associated IL-1 activity (24), and we confirmed that both forms of this cytokine were present in wild-type murine MPs, although IL-lα was predominant (IL-

lα, 87 ± 7 pg/mg protein; IL-lβ, 2 ± 0.2 pg/mg protein). Blocking both forms of IL-1 with neutralizing antibodies was necessary to fully blunt FLS activation by MPs (Fig. 4E). We obtained similar results in the human system. Platelet MPs from RA SF expressed surface IL-lα, which as in murine MPs predominated over IL-lβ (fig. S4). Similarly, human platelet MPs elicited by in vitro collagen stimulation expressed both IL-lα and IL-lβ (19.1 versus 0.1 pg/mg protein) and triggered RA FLS to release IL-8 in a dose-dependent manner, indeed, more robustly than either IL-lβ or TNF (Fig. 4F). Both forms of IL-1 participated in human FLS stimulation because neutralization of platelet MP IL-1 activity required blocking antibodies against both IL-lα and IL-lβ (Fig. 4G). Together, these results show that platelet MPs likely contribute to joint inflammation via mechanisms including highly potent IL-1–mediated activation of resident synoviocytes.

These results provide an explanation for several disparate previous observations. Radiolabeled platelets localize to inflamed joints (25) and RA SF displays appreciable amounts of soluble platelet proteins (26), yet intact platelets are rare in arthritic SF. Whereas SF MP concentrations exceed those in the circulation of RA patients by several orders of magnitude (18), platelet activation appears to be primarily an articular process, wherein MPs disseminate platelet-derived cytokines into the arthritic joint. Subsynovial capillaries exhibit fenestrations and are prone to enhanced permeability after stimulation (27,28). We speculate that circulating platelets contact ECM via these fenestrations when local conditions favor permeability, activating GPVI. Alternatively, platelet “sampling” of ECM via fenestrations may be routine, activating platelets as ECM constituents are modified. Thus, synovium is enriched for collagen type IV (29), and we have observed that FLS deficient in collagen type IV demonstrate reduced capacity to stimulate platelet MP release (fig. S5).

Other pathways linking platelets to arthritis await discovery. The effect of GPVI deficiency on generating MPs and suppressing arthritis is incomplete, and IL-1 is unlikely to be the only relevant platelet mediator in the synovial environment. Further, we have observed direct binding of MPs by SF leukocytes (fig. S1, D and E), an interaction whose functional consequences remain unknown.

The relevance of these results for human disease is substantial. Because mice or humans lacking GPVI remain healthy (30), antagonism of this receptor represents a novel therapeutic approach. The observation that membrane-associated MP IL-1 is unusually difficult to antagonize (Fig. 4) may help to explain the limited effect of IL-1 blockade in RA (31), and the prominence of IL-1α within MPs poses potential constraints on the efficacy of IL-1β–specific agents. Our results provide compelling evidence that platelets play an amplifying role in the pathophysiology of inflammatory arthritis, liberating proinflammatory MPs that represent the most abundant cellular element in SF.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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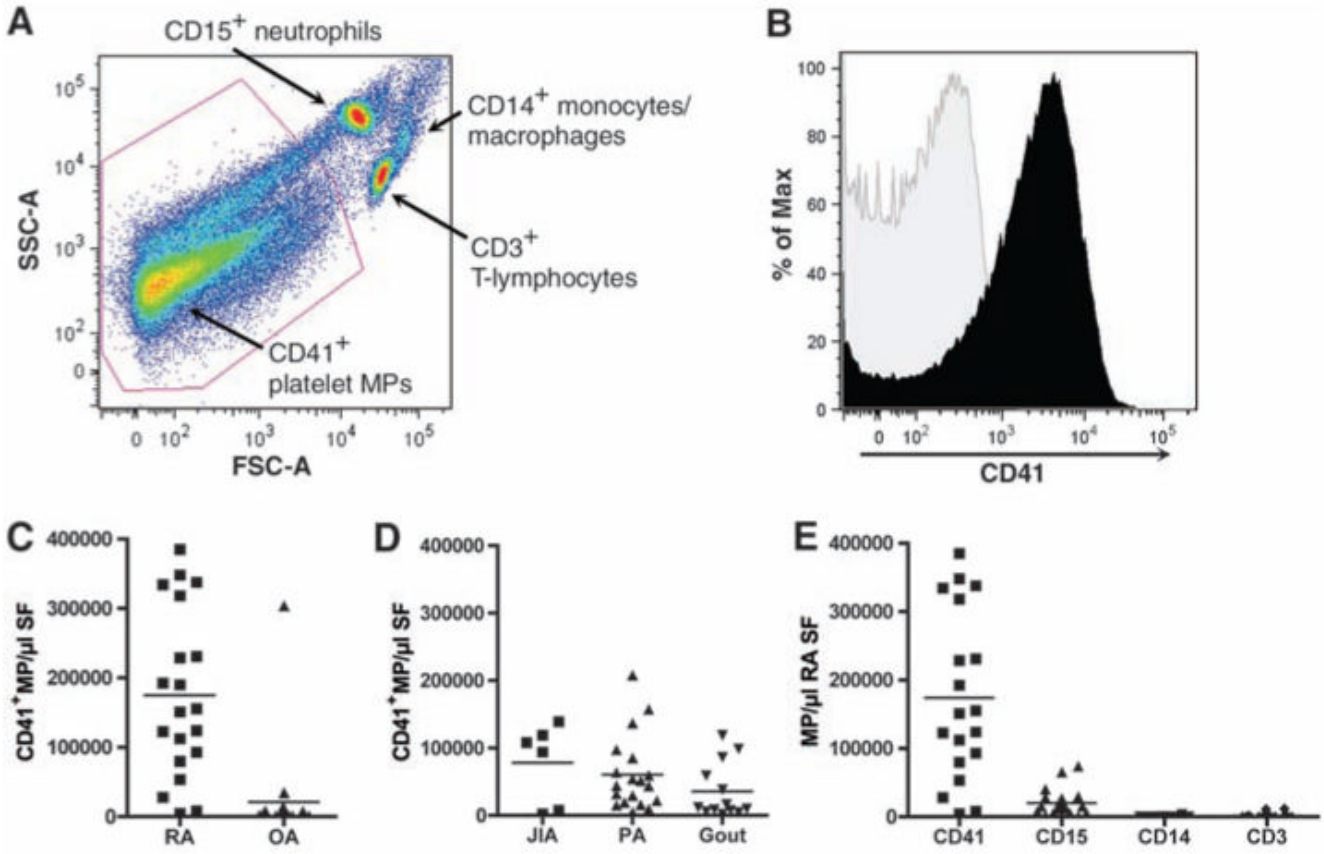

Fig. 1.

Platelet MPs are abundant in inflammatory SF. Cells in freshly isolated RA SF were stained with lineage markers: CD15 (neutrophils), CD3 (T cells), CD14 (monocytes and macrophages) and CD41 (platelets), or the appropriate isotype controls and analyzed by flow cytometry. (**A**) Forward- by side-scatter profiles of events in RA SF. Populations identified by further gating and lineage marker staining are labeled. (**B**) Representative histogram of $CD41^+$ (black fill) platelet MPs resident in RA SF. Events were gated based on the forward-scatter parameters indicated in (A). (Gray fill, isotype control.) Data are representative of profiles from eight RA patients. (**C**) Flow cytometric quantification of $CD41^+$ platelet MPs (<1 µm as determined by size calibration beads) in RA and OA SF after removal of leukocytes by centrifugation (n = 20 donors per group). (**D**) Flow cytometric quantification of platelet ($CD41^+$) MPs in SF from juvenile idiopathic arthritis (JIA, n = 6), psoriatic arthritis (PA, n = 19), and gout (n = 14). (**E**) Flow cytometric quantification of MPs in RA SF derived from the indicated cell types (n = 19 donors).

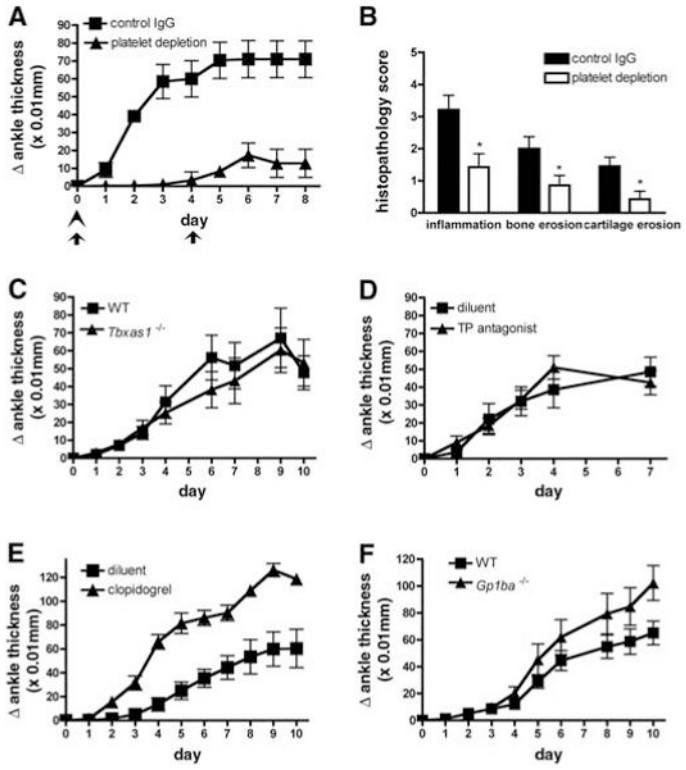

Fig. 2.

Platelets are involved in arthritis development. (**A**) Arthritis severity after K/BxN serum transfer in mice administered a platelet-depleting antibody (triangles) or isotype control (squares); $n = 10$ mice per group. Data are the mean ± SEM pooled from two independent experiments. $P < 0.001$. Arrows, parenteral administration of platelet-depleting antibody; arrowheads, K/BxN serum administration. (**B**) Histomorphometric quantification of arthritis severity in ankle joints of platelet-depleted and control mice at experiment termination; $n = 10$ mice per group. *$P < 0.01$. (**C** to **F**) Arthritis severity was measured after administration of K/BxN serum in mice (C) deficient in thromboxane synthase (*Tbxas1*$^{-/-}$), treated daily with (D) the thromboxane A2 (TP) antagonist SQ 29,548 or (E) ADP:P2Y12 inhibitor clopidogrel, or in mice deficient in (F) GPIb (*Gp1ba* $^{-/-}$). Data are the mean ± SEM, $n = 10$ mice per group. (C), (D), (F) P = not significant; (E) $P < 0.001$.

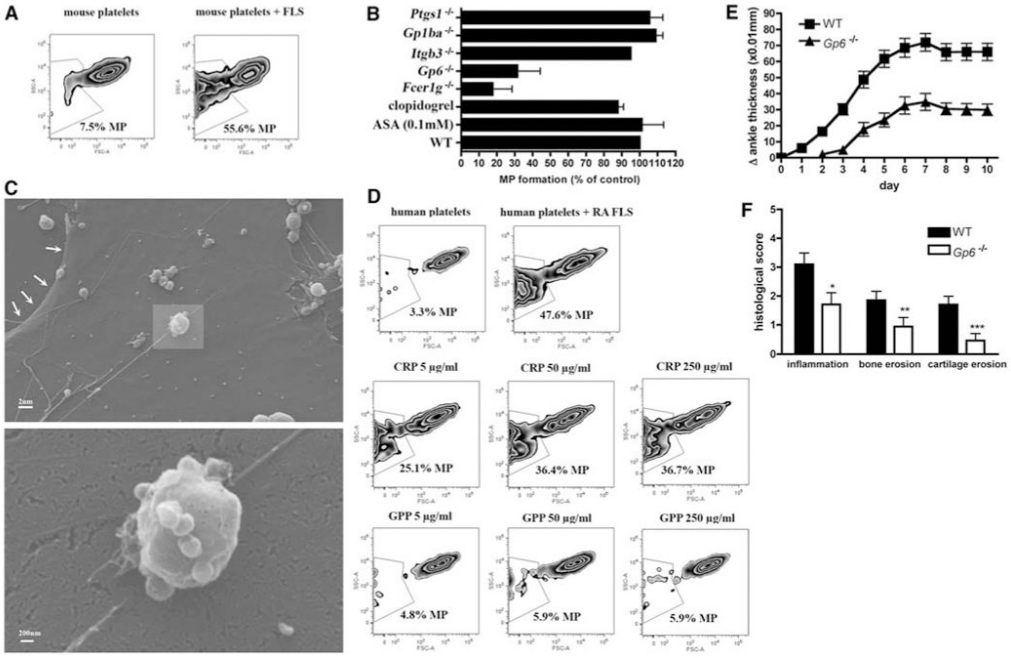

Fig. 3.

Platelets form MPs and participate in arthritis pathophysiology via stimulation of the collagen receptor GPVI. (**A**) Representative flow cytometry forward- and side-scatter plots of $CD41^{+}$ mouse platelets incubated in the presence or absence of FLS. (**B**) Examination of candidate stimuli of murine platelet MP formation upon co-culture with FLS. Mouse platelets incubated in the presence of cyclooxygenase inhibitor salicylic acid (ASA), isolated from mice treated with ADP:P2Y12 inhibitor clopidogrel, or from the indicated gene-targeted mice were coincubated with mouse FLS. MP formation was quantified by flow cytometry. Data are the mean ± SEM, $n = 3$ independent experiments in duplicate. (**C**) Scanning electron micrograph of human platelets exhibiting MP budding when incubated in the presence of FLS. Arrows indicate the edge of the fibroblast-like synoviocyte. Upper and lower panels are 9800× and 69,270× magnifications, respectively. (**D**) Human platelets form MPs when incubated with FLS and when exposed to a GPVI-specific peptide ligand (CRP) but not in the presence of a related control peptide (GPP). (**E**) Arthritis severity after K/BxN serum transfer was quantified in GPVI-null ($Gp6^{-/-}$) (triangles) or wild-type control (squares) mice. Data are the mean ± SEM pooled from three independent experiments; $n =$ 25 mice per group. $P < 0.001$. (**F**) Histomorphometric quantification of arthritis severity in ankle tissues from GPVI-null ($Gp6^{-/-}$) and WT mice at experimental termination. Data are the mean ± SEM; $n = 25$ mice per group. $^{*}P = 0.019$, $^{**}P < 0.05$, $^{***}P < 0.01$.

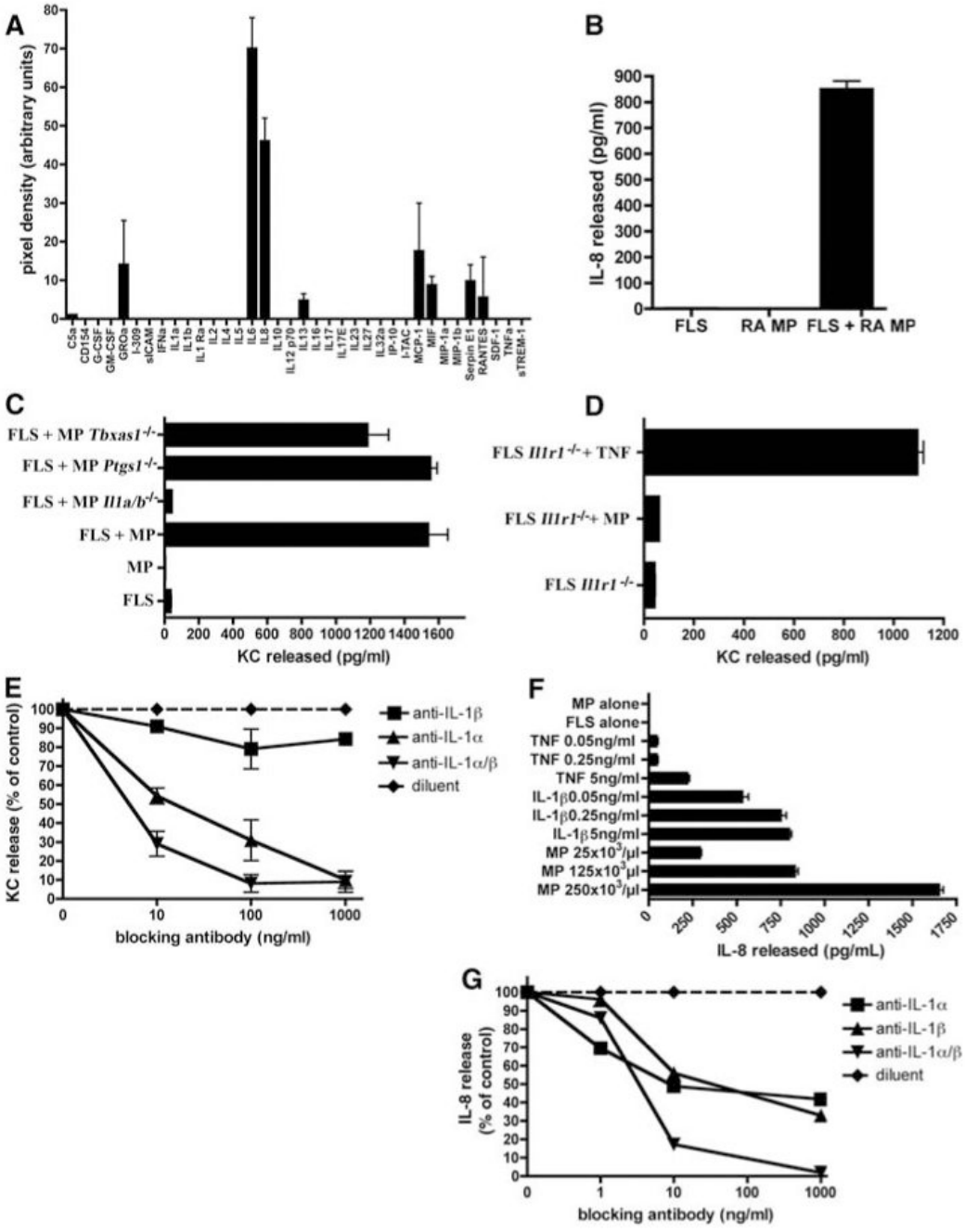

Fig. 4.

MPs activate FLS in an IL-1–dependent manner. (**A**) MPs generated by collagen stimulation of human platelets were coincubated with human FLS, and cytokine release was quantified by Proteome Profiler. Data are representative of three independent experiments. (**B**) MPs isolated from RA SF were coincubated with FLS, and supernatants were assayed for IL-8 release by ELISA (enzyme-linked immunosorbent assay). (**C**) Mouse platelet MPs generated by collagen stimulation of platelets from the indicated genotypes were coincubated with mouse FLS, and supernatants were assayed for KC release by ELISA. (**D**) Mouse MPs generated by collagen stimulation of WT platelets were coincubated with IL-1R1–null (*Il1r1*$^{-/-}$) FLS, and supernatants were assayed for KC release by ELISA. Recombinant TNF

(10 ng/ml) was added to FLS to induce KC release as a positive control. (**E**) Mouse platelet MPs were coincubated with FLS in the presence of IL-1–neutralizing antibodies, and supernatants were assayed for KC release by ELISA. (**F**) Potency of human MP stimulation of FLS. FLS were exposed to graded concentrations of IL-1β, TNF, or platelet MPs, and IL-8 release was quantified in culture supernatants by ELISA. (**G**) Human platelet MPs were coincubated with FLS in the presence of IL-1–neutralizing antibodies, and supernatants were assayed for IL-8 release by ELISA. Data for (B) to (G) are the mean ± SEM of three independent experiments.