Platelets Amplify Inflammation in Arthritis via Collagen-Dependent Microparticle Production

Eric Boilard,1 Peter A. Nigrovic,1,2 Katherine Larabea,3 Gerald F. M. Watts,1 Jonathan S. Coblyn,3 Michael E. Weinblatt,1 Elena M. Massarotti,1 Eileen Remold-O’Donnell,3 Richard W. Farndale,2 Jerry Ware,9 David M. Lee1*

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—using cytokines and reactive oxygen species 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, but not in joint fluid from patients with osteoarthritis.

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 athero-sclerosis, 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 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/α2bß3) (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 × 10⁵ 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 hematopoietically 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 contained CD41+ platelets (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 histocompatibility complex class II 1-Aβ*, driving high-titer arthritis-associated autoantibody production [reviewed in (14)]. Arthritis can also be induced by passive transfer of immunoglobulin G (IgG) autoantibodies 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, Fe 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 insights 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 incubated 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 (Pigs1β−/−), thromboxane, GPIIbIlla (Iggb−/−), GPIbα (Gpibα−/−), 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 Fcγ–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 incubation 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−/− mice 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 chemoattractant IL-8 (Fig. 4A and fig. S2). Consistent with this observation, incubation of MPs isolated from RA SF induced FLS to release substantial quan-
mice were co-cultured 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.

Platelet-mediated activation of resident synoviocytes. 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.

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-1α was predominant (IL-1α, 87 ± 7 pg/mg protein; IL-1β, 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-1α, which as in murine MPs predominated over IL-1β (Fig. S4). Similarly, human platelet MPs elicited by in vitro collagen stimulation expressed both IL-1α and IL-1β (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-1β 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-1α and IL-1β (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...
pernantes were assayed for KC release by ELISA. Recombinant TNF (10 ng/ml) was added to FLS to
potency of human MP stimulation of FLS. FLS were exposed to graded concentrations of IL-1
presence of IL-1.

other, we have observed direct binding of MPs by platelet mediator in the synovial environment. Fur-
plete, and IL-1 is unlikely to be the only relevant

generating MPs and suppressing arthritis is incom-
tial constraints on the efficacy of IL-1β-specific
agents. Our results provide compelling evidence that platelets play an amplifying role in the patho-
physiology of inflammatory arthritis, liberating proinflammatory MPs that represent the most
abundant cellular element in SF.

Fig. 4. MPs activate FLS in an IL-1-dependent manner. (A) MPs generated by collagen stimu-
lization 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. (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 (Illr1−/−) 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-1γ, 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.

The relevance of these results for human dis-
ease 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-1β blockade in RA (31), and the prominence of IL-1α within MPs poses potential constraints on the efficacy of IL-1β–specific

References and Notes
32. Financial support for this work was provided by NIH grant PO1 AI065858, an Arthritis Foundation Investigator Award, and the Cogan Family Foundation (D.M.L.); an Arthritis Foundation Postdoctoral Fellowship Award (E.R.-O.); the British Heart Foundation and Medical Research Council, UK (R.W.F.); and the National Heart, Lung, and Blood Institute grant HL50545 (J.W.). D.M.L., E.R.-O., and J.W. have filed a provisional patent application for glycoprotein VI as a target for treatment for inflammatory arthritis.

Supporting Online Material
www.sciencemag.org/cgi/content/full/327/5965/580/DC1
Materials and Methods
Figs. S1 to S5
Table S1
References
14 September 2009; accepted 8 December 2009
10.1126/science.1181928

www.sciencemag.org SCIENCE VOL 327 29 JANUARY 2010 583
Downloaded from www.sciencemag.org on September 9, 2010.