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*J Immunol* 2011; 186:4361-4366; Prepublished online 28 February 2011; doi: 10.4049/jimmunol.1002857

http://www.jimmunol.org/content/186/7/4361

Supplementary Material

http://www.jimmunol.org/content/suppl/2011/02/28/jimmunol.1002857.DC1

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Platelets Participate in Synovitis via Cox-1–Dependent Synthesis of Prostacyclin Independently of Microparticle Generation

Eric Boilard,*† Katherine Larabee,* Ruslan Shnayder,* Kathleen Jacobs,* Richard W. Farndale,‡ Jerry Ware,§ and David M. Lee*∗,†

In addition to the well-described role of platelets in thrombosis, a growing body of evidence implicates platelets in diverse inflammatory responses. We recently showed platelets can contribute to the pathophysiology of inflammatory arthritis via IL-1–containing microparticles. In this study, we demonstrate that platelets, and not platelet microparticles, actively contribute to synovitis via production of proinflammatory prostacyclin in an autoimmune arthritis model. Using both genetic and pharmacologic approaches, we establish that paracrine production of prostacyclin proceeds in the absence of cyclooxygenase-2. Furthermore, we also demonstrate that prostacyclin generation can arise via transcellular collaboration between platelets and fibroblast-like synoviocytes. In addition to shedding light on an unappreciated pathway of lipid synthesis in arthritis, we further delineate a novel effector activity by which platelets can contribute to inflammatory disease. The Journal of Immunology, 2011, 186: 4361–4366.

Platelets are most appreciated for their function in thrombosis, responding rapidly to interruption of vascular integrity to promote hemostasis and stem loss of blood. Thereafter, they contribute to wound repair after tissue trauma and vascular injury (1). In addition to these well-established platelet functions, there exists growing evidence for their role in other physiologic and pathophysiologic processes, including promotion of inflammation (2, 3). Platelet participation in inflammatory disease has been studied most extensively in the context of atherosclerosis (4, 5), in which activated platelets promote endothelial cell activation as well as leukocyte adhesion and transmigration via release of an extensive arsenal of mediators that includes IL-1, soluble CD40L, matrix metalloproteinases 2 and 9, amine serotonin, platelet-derived growth factor, and the prostanoid thromboxane (Tx)A2 (for review, see Ref. 2).

Recent studies have uncovered a novel platelet contribution to disease pathophysiology in inflammatory arthritis (6). Mechanically, we have already established that platelet activation via the collagen receptor GPVI stimulates production of microparticles shed from the platelet membrane. These platelet-derived microparticles are detectable at high levels in the synovial fluid that bathes joint tissues and are thought to amplify joint inflammation via elaboration of cytokines such as IL-1. However, the mechanisms elucidated to date explain only a fraction of the net platelet contribution to arthritis observable in an in vivo preclinical arthritis model. Thus, it is likely there exist numerous other mechanisms by which platelets may participate in synovitis.

In this study, we demonstrate a previously unappreciated contribution from platelet-dependent prostanooid generation via cyclooxygenase (Cox)-1 to disease pathogenesis in autoantibody-driven inflammatory arthritis. More specifically, we uncover Cox-1–dependent prostacyclin generation via transcellular metabolism between intact platelets and synovial fibroblasts as a relevant disease pathway in experimental arthritis. Moreover, we find human platelets and primary synovial fibroblasts demonstrate congruent activity upon interaction. Interestingly, this disease pathway proceeds in the absence of microparticle generation, demonstrating a novel independent contribution from platelets to disease.

Materials and Methods

Mice

We used 6- to 9-wk-old mice for all of our studies. All procedures were approved by the Institutional Animal Care and Use Committee of the Dana-Farber Cancer Institute (Boston, MA). Mice were housed in the specific pathogen-free animal facility of the Dana-Farber Cancer Institute. C57BL/6J were obtained from The Jackson Laboratory (Bar Harbor, ME). FcγR null (7), Cox-1 null (8), Cox-2 null (9), and their congenic wild-type (WT) mice were obtained from The Jackson Laboratory. GPVI null mice were generated and maintained as described (10).

Radiation chimeric mice

Recipient mice were irradiated (split dose, 500 and 450 cGy) and transplanted with donor bone marrow, as previously described (11). Mice were supported with oral antibiotics (Baytril) for 8 wk during bone marrow engraftment prior to initiating arthritis experiments.

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Serum transfer protocol and arthritis scoring

Arthritogenic K/B×N serum was transferred to recipient mice via i.p. injection (150 μl K/B×N serum) on experimental days 0 and 2 to induce arthritis, as described (12). The clinical index of arthritis was graded on a scale 0–12, as described previously (13).

Platelet isolation

Mouse blood was drawn by cardiac puncture using acid citrate dextrose (ACD) anticoagulant (0.085 M sodium citrate, 0.0702 M citric acid, 0.111 M dextrose [pH 4.5]). Blood was diluted by addition of 400 μl Tyrode’s buffer (pH 6.5) (134 mM NaCl, 2.9 mM KCl, 0.34 mM Na3HPO4, 12 mM NaHCO3, 20 mM HEPES, 1 mM MgCl2, 5 mM glucose, 0.5 mg/ml BSA) and centrifuged at 600 × g for 3 min. The pellet was discarded and the supernatant was centrifuged for 2 min at 400 × g to pellet contaminating RBC. The resulting supernatant (containing platelet-rich plasma [PRP]) was further centrifuged for 5 min at 1300 × g to pellet platelets. Human platelets were obtained from healthy volunteers under an Institutional Review Board-approved protocol using ACD as coagulant and isolated, as described above. Platelets were resuspended in Tyrode’s buffer at pH 7.4 and quantified cytofluorometrically using anti-CD41 staining (human CD41, clone M148 [Abcam] and mouse CD41: MWReg30 [BD Pharmingen]) and known amounts of 15 μm polystyrene microsphere Polybeads (Polysciences).

Adoptive transfer of platelets

Donor mice were bled by cardiac puncture using syringes loaded with ACD anticoagulant (140 μl: ACD: 0.085 M sodium citrate, 0.0702 M citric acid, 0.111 M dextrose [pH 4.5]). Blood was centrifuged 600 × g for 3 min, and PRP were collected, as described above. For controls, PRP fractions were centrifuged twice at 1300 × g for 5 min, and supernatants (platelet-poor plasma [PPP]) were collected. PRP (containing 2 × 10^7 platelets, 10% of the endogenous platelet number) and PPP were injected i.v. to the tail vein daily (five donor mice per recipient per day).

Fibroblast-like synovioyte coculture with platelets

Platelets in DMEM (3 × 10^7/ml) were added to washed early passage (P−7) primary fibroblast-like synoviocytes (FLS) (14) cultured to confluence in 6-well plates at 37°C for 4 h in absence of serum to allow platelet activation and release of sufficient substrate PGH2. When Cox inhibitors were used, 5 μM dual Cox-1/Cox-2 inhibitor indomethacin (INDO) or 0.5 μM selective Cox-2 inhibitor rofecoxib was added to the culture media. Supernatants were collected, intact platelets were cleared by centrifugation (1300 × g, 5 min) in presence of INDO (1 μM) and EDTA (5 μM), and PGs were quantified by ELISA (Cayman Chemical), per manufacturer protocol. For PGH2 and TXA2 concentration quantifications, an ELISA detecting the stable degradation products 6-ketoPGF1α and TxB2 was used. When the capacity of FLS to employ exogenous PGH2 was monitored, PGH2 (Cayman Chemical) freshly resuspended in DMEM containing 0.5% FBS was quickly added to wells containing FLS (and empty wells as negative controls), and supernatants were searched for presence of 6-ketoPGF1α by ELISA. In experiments in which platelet microparticles were added to FLS, platelet microparticles were generated using collagen as stimuli and were purified by centrifugation and incubated in presence of FLS (2 × 10^6 microparticles/ml), as described previously (6).

Statistical analysis

Mouse arthritis experiments are presented as mean ± SEM. The statistical significance for comparisons between groups was determined using two-way ANOVA, followed by Bonferroni correction using Prism software package 4.00 (GraphPad Software, San Diego, CA). The p values <0.05 were considered significant.

Results

Bone marrow-derived Cox-1 activity suffices for establishment of K/B×N serum transfer arthritis

High levels of PGs are present in synovial fluid from patients suffering from rheumatoid arthritis (RA) and other forms of inflammatory arthritis (15, 16), and multiple preclinical in vivo arthritis models demonstrate a contribution from these inflammatory lipid mediators to disease (17−22). Our recent examination of the role of PGs in the K/B×N serum transfer model of inflammatory arthritis revealed a significant role for prostacyclin in disease pathogenesis with a predominance of Cox-1 over Cox-2 as the proximal enzyme in prostanoid generation (23). In this model, a distal symmetric erosive polyarthritids results upon passive transfer of pathogenic autoantibodies directed against the autoantigen glucose-6-phosphate isomerase to otherwise healthy recipient mice (12, 24). Numerous mechanisms have been implicated in the pathogenesis of the IgG-driven effector phase of K/B×N serum transfer arthritis, including neutrophils, mast cells, platelets, Fcγ receptors, and other soluble mediators (IL-1, TNF, complement C5a/C5aR, leukotriene B4, tryptase, and secreted phospholipase A2) (11, 13, 14, 23, 25−33).

In the present work, we proceeded with a goal of identifying the Cox-1−expressing cellular lineage(s) necessary to promote synovitis in vivo. As Cox-1 is ubiquitously expressed (34, 35), we used a radiation chimera approach to establish that Cox-1 activity was attributable to a bone marrow-derived lineage (hematopoietic) and not to a radioresistant mesenchymal (tissue) lineage (Fig. 1A).

Platelets can contribute to arthritis via their Cox-1 activity

In considering candidate bone marrow-derived lineages whose Cox-1 activity suffices to promote arthritis, we surveyed Cox-1−
protein levels in numerous hematopoietic lineages. Although all cells examined expressed substantial levels of Cox-1 (Fig. 1B), platelets were unique in that they do not express Cox-2 even after stimulation (36–38), a phenotype congruent with the Cox-1 predominant prostanooid contribution to the K/B×N serum transfer model. Using an adoptive transfer approach, we observe that platelets as the sole source of Cox-1 are sufficient to restore arthritis to the resistant Cox-1−/− strain (Fig. 1C). Interestingly, because platelets express exclusively Cox-1, their contribution to prostanooid production in synovitis necessarily proceeds in an obligate Cox-1–dependent manner.

**Platelet–synovial fibroblast transcellular synthesis of PGI2**

Having demonstrated that platelets can contribute to establishment of arthritis via Cox-1–dependent function, we sought to elucidate the pathway by which platelets produce arthritogenic PGs. Cox-1 coordinates the production of PGH2, which is subsequently metabolized to mature prostanooid species via terminal synthase enzymes (39). Previous studies demonstrate that platelets predominantly convert PGH2 to TxA2 (Supplemental Fig. 1) and 12-hydroxy-5,8,10-heptadecatrienoic acid, a ligand of the leukotriene B4 receptor 2 (BLT2) (40), in an equimolar ratio via the activity of thromboxane synthase (41). The only other prostanooid known to be synthesized by platelets is PGE2, albeit in small amounts (42) (Supplemental Fig. 1). Previous examination of prostanooid dependence in K/B×N serum-induced arthritis revealed that PGE2, TxA2, and 12-hydroxy-5,8,10-heptadecatrienoic acid are dispensable for development of disease (6, 23). In contrast, mice deficient in the PGI2 (prostacyclin) receptor IP are substantially resistant to the development of both K/B×N serum-induced arthritis (23) and collagen-induced arthritis (20). These observations led to the conclusion that PGI2 is an important Cox-1–derived PG involved in the development of autoimmune synovitis in those preclinical models. We therefore assessed platelet capacity for PGI2 synthesis. These analyses reveal that PGI2 synthase protein expression (Fig. 2A) and PGI2 release (Supplemental Fig. 1) upon ionophore activation are undetectable in platelets.

How then can platelets, incapable of autonomously generating the relevant proinflammatory eicosanoid, promote synovitis in a Cox-1–dependent manner? Earlier studies demonstrated that endo-

![Figure 2](http://www.jimmunol.org/)
thelial cells can use platelet-derived PGH2 to generate PGI2 (43–45). Given the predominance of the FLS in the synovial lining of the joint (46), we turned our attention to this major mesenchymal lineage. This lineage plays a significant role in both joint inflammation and the destruction of joint tissue via formation of locally invasive pannus (14, 47). Consistent with another report that demonstrated that the FLS bear the synthetic machinery to produce high levels of PGI2 synthase (Fig. 2A) in addition to both Cox-1 and Cox-2 (Fig. 3A). Interestingly, FLS generate PGI2 in a Cox-2–dependent manner after cytokine stimulation (Fig. 3). Thus, cytokine-dependent PGI2 generation by FLS does not account for the Cox-1–dependent prostacyclin synthesis relevant in this model.

The aforementioned observations prompted us to examine transcellular biosynthesis of PGI2 as a possible mechanism active in arthritis pathophysiology. We hypothesized that upon activation, platelets generate excess PGH2 in an obligate Cox-1–dependent manner. This PGH2 diffuses locally to tissue FLS and is converted by this lineage to proinflammatory prostacyclin (please see this model illustrated in Fig. 4). To test this hypothesis, we first assessed FLS competency to convert exogenous PGH2 to PGI2 in a Cox-independent manner and found they are abundantly capable of prostacyclin synthesis from exogenous PGH2 (Fig. 2B). Because previous analyses point to local activation of platelets in the arthritic joint and because coculture with FLS results in robust platelet activation (6), we used FLS–platelet coculture to assess for transcellular metabolism of PGH2 obligately derived from platelet Cox-1. Utilizing a genetic approach with mouse platelets and primary FLS, we observe substantial prostacyclin production induced by platelet–FLS coculture (Fig. 2C). This prostacyclin production demonstrates a dependence on platelet Cox-1 activity (Fig. 2C). To validate this activity proceeds in human cells, we used human platelets and primary FLS in combination with Cox-selective and nonselective pharmacologic inhibitors. As in the mouse system, we demonstrate that platelet–FLS coculture results in prostacyclin generation in a platelet Cox-1 activity-dependent manner (Fig. 2D).

Platelets, not platelet microparticles, participate in Cox-1–derived PGI2 synthesis

Previous findings from mechanistic studies on platelet contributions to inflammatory arthritis identify collagen receptor GPVI–dependent stimulation of IL-1–containing microparticles as a relevant disease pathway in synovitis (6). In this study, we extended our examination of platelet contributions to disease pathophysiology by examining whether transcellular prostacyclin generation by platelet interaction with FLS also proceeds through platelet microparticles. Consistent with an earlier report that demonstrated that platelet microparticles can stimulate the Cox-2 expression and PGI2 release in endothelial cells (49), we find that platelet microparticles promote the Cox-2–dependent production of PGI2 by FLS. Interestingly, only intact platelets induce a Cox-1–dependent PGI2 generation when cocultured with FLS (Fig. 5A, 5B). Moreover, although GPVI ligation can stimulate platelet thromboxane production (Supplemental Fig. 2), we observe that the platelet–FLS collaboration for prostacyclin production proceeds in the absence of GPVI and its associated common γ-chain of the Fc receptor (Fig. 5C). These findings thus identify platelet Cox-1–dependent prostanooid transcellular metabolism as a novel inflammatory pathway distinct from the GPVI-dependent pathway identified in previous studies (6).

Discussion

Utilizing a Cox-1–dependent mouse model of inflammatory arthritis, we show that platelet Cox-1 expression is sufficient to promote arthritis development and that platelets can interact with

![FIGURE 3.](http://www.jimmunol.org/Downloaded_from/hp://www.jimmunol.org/)

**FIGURE 3.** FLS produce PGI2 in a Cox-2–dependent manner. **A**. Cox-2, Cox-1, PGIS, and GAPDH protein expression in FLS. Proteins from mouse FLS stimulated with IL-1β (10 ng/ml) or TNF (10 ng/ml) were separated by SDS-PAGE, and expression of indicated proteins was assessed by Western blot. Results presented are representative of three independent experiments. **B** and **C.** FLS synthesize PGH2 in a Cox-2–dependent manner. WT, Cox-2 null, and Cox-1 null FLS (B) and human RA FLS (C) were exposed to the Cox-2 inhibitor rofecoxib (RFX) and were stimulated with IL-1β (10 ng/ml) and TNF (10 ng/ml) for 4 h. PGI2 production was monitored by ELISA. Data are mean ± SEM pooled from three independent experiments.

![FIGURE 4.](http://www.jimmunol.org/Downloaded_from/hp://www.jimmunol.org/)

**FIGURE 4.** Proposed Cox-1–dependent transcellular biosynthesis of prostacyclin. Activated platelets promote the activation of phospholipase A2 (PLA2) activity that releases AA from membrane phospholipids. Once AA is processed by Cox-1, PGH2 can diffuse locally for metabolism by neighboring FLS that express PGIS into proinflammatory PGI2.
FIGURE 5. Platelets, not platelet microparticles, participate in Cox-1–dependent transcellular biosynthesis of prostacyclin. Murine (A) and human (B) platelets and microparticles were coincubated with FLS in the presence or absence of 0.5 μM Cox-2 inhibitor rofecoxib (RFX). PGI2 secreted into the supernatant was quantified by ELISA. Data are mean ± SEM pooled from three independent experiments. C. Platelets lacking GPVI or FcRγ were incubated in presence of FLS, and PGI2 secreted into the supernatant was quantified by ELISA. Data are mean ± SEM pooled from three independent experiments.

FLS to generate proinflammatory prostacyclin via transcellular metabolism of the prostanoi precursor PGH2. Furthermore, we elucidate a novel microparticle-independent mechanism by which intact platelets can participate in arthritis physiology. Taken together, these observations point to a previously unappreciated paracrine pathway wherein joint tissues synergize with a blood-borne element to promote local inflammation.

It was at first surprising that platelets are sufficient for generation of robust synovitis in vivo. In this context, it is important to highlight that platelets are particularly abundant cellular elements in circulation (150–400 WBCs/μl versus 4–10 × 105 WBCs/μl in humans, and 1400 × 106 platelets/μl versus 4 × 105 WBCs/μl in mice). Our studies thus provide a new paradigm whereby platelets represent a large reservoir of activity that is potently capable of participating in local disease pathophysiology upon recruitment.

Our studies reside in the context of previous work demonstrating intact platelets and endothelial cells can generate PGI2 via transcellular metabolism of Cox-1–generated PGH2 from intact platelets in vitro (44, 50). Others have also observed Cox-2–dependent PGI2 synthesis, including platelet microparticle contributions to vascular endothelium PGI2 production (49, 51). It deserves note that the results in this study do not preclude a role for Cox-2–dependent prostacyclin generation in vivo. Similarly, our analyses remain silent on the potential role of other prostanoid species in arthritis pathophysiology, such as PGD2 and PGF2, and the contribution of other lineages capable of generating PGI2 from platelet-derived PGH2, such as endothelial cells and leukocytes.

Our previous observations pointed to contributions from IL-1–rich platelet microparticles to inflammatory arthritis (6). The mechanisms described in these studies document a distinct pathway by which activation of intact platelets—and not microparticles—can contribute to synovitis. Although more precise understanding of the mechanisms governing this dichotomous activity awaits further investigation, our findings suggest that the process of platelet microparticle formation results in a subcellular fragment lacking either the activation receptors or the synthetic machinery needed to produce PGH2. Furthermore, given that previous analyses failed to uncover intact platelets in arthritic synovial fluid (6), we speculate that the transcellular generation of PGI2 in the joint microenvironment occurs near the vascular–tissue interface, where intact platelets have access. Taken together, the picture that emerges from these observations illustrates that platelet contributions to joint inflammation are multifold and that further examination of this lineage will demonstrate yet further mechanisms by which platelets can contribute to autoimmune and inflammatory pathophysiology. Indeed, a recent manuscript has pointed to a contribution from platelet CD40L to autoimmunity in systemic lupus erythematosus (52).

From a translational standpoint, there exist substantially elevated levels of PGI2 in joints of patients with RA (16). Multiple preclinical models of inflammatory arthritis point to PGI2 as a participant in disease (20, 23). Furthermore, elegant previous work documents FLS activation and inflammatory cytokine release by FLS after stimulation by prostacyclin (20). In addition to further delineating effector activities by which platelets can contribute to inflammatory disease, these studies uncover a robust prostacyclin synthetic pathway that proceeds via abundant circulating and synovial tissue cellular elements. We suggest that this pathway needs to be accounted for in interpreting the role of pharmacologic inhibition of prostanooid production in treatment of inflammatory arthritis and potentially in other inflammatory states.

Disclosures

D.M.L. is employed by Novartis Pharma, AG. The other authors have no financial conflicts of interest.

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