Contribution of platelet P2Y12 receptors to chronic Complete Freund’s adjuvant-induced inflammatory pain

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Essentials

- The role of platelet P2Y12 receptors in the regulation of chronic inflammatory pain is unknown.
- Complete Freund’s Adjuvant (CFA)-induced chronic inflammatory pain model was used in mice.
- Gene deficiency and antagonists of P2Y12 receptors attenuate hyperalgesia and local inflammation.
- Platelet P2Y12 receptors contribute to these effects in the chronic phase of inflammation.

Summary. Background: P2Y12 receptor antagonists are widely used in clinical practice to inhibit platelet aggregation. P2Y12 receptors are also known to regulate different forms of pain as well as local and systemic inflammation. However, it is not known whether platelet P2Y12 receptors contribute to these effects. Objectives: To explore the contribution of platelet P2Y12 receptors to chronic inflammatory pain in mice. Methods: Complete Freund’s adjuvant (CFA)-induced chronic inflammatory pain was induced in wild-type and P2ry12 gene-deficient (P2ry12−/−) mice, and the potent, direct-acting and reversible P2Y12 receptor antagonists PSB-0739 and cangrelor were used. Results: CFA-induced mechanical hyperalgesia was significantly decreased in P2ry12−/− mice for up to 14 days, and increased neutrophil myeloperoxidase activity and tumor necrosis factor (TNF-α) and CXCL1 (KC) levels in the hind paws were also attenuated in the acute inflammation phase. At day 14, increased interleukin (IL)-1β, IL-6, TNF-α and KC levels were attenuated in P2ry12−/− mice. PSB-0739 and cangrelor reversed hyperalgesia in wild-type mice but had no effect in P2ry12−/− mice, and PSB-0739 was also effective when applied locally. The effects of both local and systemic PSB-0739 were prevented by A-803467, a selective NaV1.8 channel antagonist, suggesting the involvement of NaV1.8 channels in the antihyperalgesic effect. Platelet depletion by anti-mouse CD41 antibody decreased hyperalgesia and attenuated the proinflammatory cytokine response in wild-type but not in P2ry12−/− mice on day 14. Conclusions: In conclusion, P2Y12 receptors regulate CFA-induced hyperalgesia and the local inflammatory response, and platelet P2Y12 receptors contribute to these effects in the chronic inflammation phase.

Keywords: cytokines; inflammation; P2Y12 purinoceptor; pain; platelet activation.

Introduction

The P2Y12 receptor (P2Y12R) is the central receptor for ADP on platelets and an established clinical drug target for inhibition of platelet aggregation [1]. P2Y12R belongs to a family of Gi/o-protein-coupled P2Y receptors [2,3], and its activation inhibits adenylate cyclase and reduces cAMP levels [4]. This process elicits rapid platelet aggregation, and the inhibition of this process by P2Y12R antagonists, i.e. thienopyridine prodrugs (clopidogrel) or direct-acting drugs (ticagrelor and cangrelor), is used to
prevent thrombosis, myocardial infarction, and stroke [5,6].

P2Y₁₂Rs are also expressed in dendritic cells, leukocytes, and microglia, the resident immune cells of the central nervous system (CNS). Following injury, nucleotides are released from damaged cells and activate P2Y₁₂Rs on platelets and nearby cells. Activation of platelet P2Y₁₂R is a major amplification mechanism in the process leading to aggregation, and also regulates inflammation, inducing the formation of proinflammatory platelet–leukocyte aggregates and subsequent activation of leukocytes [7]. Microglial P2Y₁₂Rs participate in chemotaxis [8], and microglia process extension and convergence [9,10], which represent a defensive mechanism against injury.

P2Y₁₂Rs also participate in different forms of pain regulation. P2Y₁₂R mRNA is overexpressed in microglia, and genetic invalidation and pharmacological blockade of P2Y₁₂Rs inhibited allodynia in neuropathic pain models [11,12] and acute inflammatory pain in rodents [13,14]. As an underlying mechanism, we showed that P2Y₁₂R inhibition time-dependently alleviates the cytokine response in the inflamed hind paw and spinal cord [14].

However, it is unknown how P2Y₁₂R blockade alters chronic inflammatory pain, and the contribution of platelets to P2Y₁₂-driven actions has also remained unclear. Previous data are incongruent concerning P2Y₁₂R-mediated regulation of the cytokine response to chronic or systemic inflammation. P2ry12 gene deficiency showed a proinflammatory profile in a severe, lipopolysaccharide (LPS)-induced systemic inflammation model [15], similarly to clopidogrel in peptidoglycan polysaccharide-induced polyarthritis in mice [16]. In contrast, LPS-induced increases in plasma cytokine levels were significantly attenuated by both ticagrelor and clopidogrel, demonstrating anti-inflammatory effects of P2Y₁₂ inhibition [17]. A potential explanation for this discrepancy is that P2Y₁₂Rs on platelets and other cell types are differentially expressed and contribute to several forms and stages of inflammation.

Emerging data suggest a role of platelets in different forms of chronic inflammatory diseases, such as atherosclerosis. The benefit of antiplatelet agents has previously been attributed to their direct antiaggregation impact. However, recent results have demonstrated that platelets also act as repair and inflammatory cells, and link hemostatic and inflammatory processes [18–20]. During adhesion, platelets are activated and release potent inflammatory and mitogenic substances into the local microenvironment, altering endothelial cell function [21]. These alterations support chemotaxis, adhesion and transmigration of monocytes to the site of inflammation [22].

Activated platelets release an array of proteins with immunomodulatory properties, including cytokines and chemokines, which contribute to leukocyte activation and/or have a direct bactericidal effect. Furthermore, they form complexes with circulating leukocytes, which enhance the activation status of both leukocytes and themselves [23]. In rheumatoid arthritis, proinflammatory platelet microparticles elicit cytokine responses from synovial fibroblasts that could be identified in joint fluid from patients. Consistently, platelet-depleted mice show a marked reduction in arthritis [24].

It is not clearly known how platelets influence different forms of pain. Earlier studies showed that platelet injection elicits hyperalgesia [25], and carrageenan-evoked and snake venom-evoked inflammatory hyperalgesia was dramatically reduced following platelet depletion [26]. These findings suggest that a soluble mediator released from platelets contributes to hyperalgesia. However, the identity of this mediator and its molecular signaling pathway remain to be established.

In the present study, we examined whether genetic deletion and blockade of P2Y₁₂Rs affect chronic pain and inflammation, and, if so, whether this is attributable to platelet-mediated actions. We show that both genetic ablation and pharmacological blockade of P2Y₁₂Rs inhibit chronic mechanical hyperalgesia by reducing local cytokine production, and platelets contribute to this effect in the chronic phase.

Methods

Animals

All animal care and experimental studies were approved by the local Animal Care Committee of the IEM HAS (PEI/001/775-6/2015). All efforts were made to minimize animal suffering and reduce the number of animals used.

The experiments were performed on male wild-type (C57/B6) and P2ry12 gene-deficient (P2ry12/−−) mice weighing 25–30 g. B6;129-P2ry12Δtm1Dgen/H knockout mice (Deltagen, San Mateo, CA, USA; EMMA, EM:02301) were bred and genotyped as described in our previous study (MGTU, IEM HAS) [14]. Mice were housed under a 12-h light/dark cycle in a temperature-controlled room with food and water available ad libitum.

Chronic inflammatory pain model

Mice were randomly assigned to experimental groups, and lightly anesthetized with isoflurane (Forane; AbbVie, Budapest, Hungary), and freshly prepared complete Freund’s adjuvant (CFA) (15 μL in saline; Sigma-Aldrich, Budapest, Hungary) was injected intraplantarly into the right hind paw. The mechanical sensitivity was measured with a dynamic plantar aesthesiometer (37400; Ugo Basile, Comerio, VA, Italy) before and 3, 4, 7, 10 and 14 days after CFA injection, as described previously [13].

Mice had 10 individual consecutive measurements performed on both hind paws. The paw withdrawal threshold (PWT) was calculated from the average, and expressed in grams.
Mice were treated with P2Y₁₂R antagonists, or with their vehicle (sterile saline), intraperitoneally (dichloro-[[2R,3S,4R,5R]-3,4-dihydroxy-5-[6-(2-methylsulanyethylamino)-2-(3,3,3-trifluoropropylsulfonyl)purin-9-yl]oxolan-2-yl]methoxy-hydroxyphosphoryl]-oxyhydroxyphosphoryl]methyl]phosphonic-acid, cangrelor, 3 mg kg⁻¹; The Medicines Company, Parsippany, NJ, USA), intraperitoneally or intrathecally (1-amino-4-[4-phenylamino-3-sulfo-phenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate, PSB-0739, 0.3 mg kg⁻¹, selective P2Y₁₂R antagonist synthesized by Y. Baqi and C. E. Müller [27,28] on days 3, 4, 7, 10 and 14 after CFA injection. The doses were chosen on the basis of our previous experiments [14]; the pKₐ values of PSB-0739 and cangrelor at human P2Y₁₂Rs (hP2Y₁₂Rs) were 9.8 and 8.6, respectively, whereas, in the doses applied in the present study (PSB-0739, 0.3 mg kg⁻¹ intraperitoneally; cangrelor, 3 mg kg⁻¹ intraperitoneally), they reversed acute inflammatory pain for up to 96 h. Taking into account that the approximate blood volume of a 25-g mouse is 1700 μL [29], these doses correspond to 5 μM and 50 μM, indicating maximal target inhibition. As a reference compound, aspirin, an alternative platelet antagonist, was used at a low dose (2-acetyloxybenzoic acid, 20 mg kg⁻¹ intraperitoneally; Sigma-Aldrich) [30]. The mechanonociceptive thresholds of hind paws were measured 15 min or 30 min after intrathecal/intraperitoneal or intraplantar injections, with the exception of day 3, when PWT measurements were performed after drug administration. 5-(4-chlorophenyl)-N-(3,5-dimethoxyphenyl)-2-furancarboxamide (A-803467, 30 mg kg⁻¹; Tocris Cookson, Bristol, UK), a potent and selective NaV1.8 sodium channel antagonist or its vehicle (polyethylene glycol and dimethyl sulfoxide [9 : 1]) was administered intraperitoneally 5 min before the respective PSB-0739/saline injection. The dose of A-803467 was chosen on the basis of a previous study [31], and a submaximal dose (30 mg kg⁻¹ intraperitoneally) in the reduction of mechanical allodynia was selected to reveal any additive interactions between PSB-0739 and A-803467. In some experiments, paw edema was also volumetrically quantified by plethysmometry (7140; Ugo Basile).

In vivo imaging

In vivo imaging studies were performed 3 and 10 days after CFA injection. Myeloperoxidase (MPO)-dependent bioluminescence of luminol was used as an indicator of neutrophil activation [32,33]. Plasma extravasation was monitored by fluorescence imaging of indocyanine green (ICG).

Wild-type and P2ry12−/− mice received an intraperitoneal injection of luminol sodium salt (150 mg kg⁻¹ in phosphate-buffered saline [PBS]-based solution; Sigma-Aldrich), and were imaged 10 min after injection by the use of an IVIS Lumina II optical imaging system (Perkin-Elmer, Waltham, MA, USA). Data were analyzed with living image (Perkin-Elmer), and the regions of interest (ROIs) were drawn around the hind limbs. Luminescence was expressed as total radiance (total photon flux s⁻¹).

For the fluorescence measurement of vascular leakage, ICG (0.5 mg kg⁻¹ in Kolliphor HS15-based solution; Sigma-Aldrich) was injected intravenously [34,35]. Mice were imaged 30 min after injection. The excitation and emission wavelengths were 745 nm and > 800 nm. Data were analyzed, and the ROIs were applied as described above. The calibrated fluorescence units and the radiant efficiency (photons s⁻¹ cm⁻² steradian⁻¹)[μW cm⁻²]) originating from the ROIs were used for further analysis.

Cytokine measurement

Samples from the hind paws of wild-type and P2ry12−/− mice were collected before (day 0) and after (days 3 and 14) the intraplantar injection of CFA or saline. After tissue homogenization and 15 min of centrifugation at 27000 × g, as described previously [36,37], supernatants were collected for measurement of the levels of the inflammatory mediators interleukin (IL)-1α, IL-1β, IL-6, IL-10, tumor necrosis factor (TNF)-α and CXCL1 (KC) with BD Cytometric Bead Array Flex Sets (BD Biosciences, San Jose, CA, USA). Measurements were performed on a BD FACSVerse flow cytometer, and data were analyzed with FCAP Array v5 software (Soft Flow, Burnsville, MN, USA). Cytokine levels were normalized to total protein levels measured by photometry with the BCA Protein Assay Kit (Thermo Scientific Pierce, Rockford, IL, USA). Absorbance was measured at 560 nm with a Perkin-Elmer Victor 3V 1420 Multilabel Counter (Perkin-Elmer).

Platelet depletion

A specific anti-CD41 antibody was applied to deplete platelets, as described previously [38]. Wild-type and P2ry12−/− mice were injected with CFA, and, 6 days later, platelets were depleted by an intraperitoneal injection of purified rat anti-mouse CD41 (25 μg in PBS; BD Biosciences). As a control, we used purified rat IgG₁, isotype antibody (25 μg in PBS; BD Biosciences). Platelet depletion was confirmed by flow cytometry with an anti-mouse/rat CD42d–phycoerythrin (PE) antibody (eBioscience, San Diego, CA, USA) on a BD FACSVerse instrument. The assumption that only platelets are affected by CD41 treatment was supported by use of the following anti-mouse antibodies: CD4–fluorescein isothiocyanate (FITC), CD8a–PE, CD3–allophycocyanin (APC), CD11b–FITC, Ly6c–PE-cyanine7, CD11b–APC, CD19–FITC, and major histocompatibility complex Class II–APC (eBioscience). Blood samples were taken from the tail vein or right ventricle before and 1, 4 and 8 days after CD41 treatment, with acid citrate dextrose solution as anticoagulant.
Assessment of platelet CD62P levels by flow cytometry

To investigate how P2Y12R antagonists and antiplatelet agents administered via different routes altered platelet activation, we measured ADP-induced changes in platelet CD62P levels *ex vivo*, in platelet-rich plasma (PRP) samples. Wild-type mice were treated with PSB-0739 (0.3 mg kg⁻¹ intrathecally), cangrelor (3 mg kg⁻¹ intraperitoneally), aspirin (20 mg kg⁻¹ intraperitoneally), or their vehicle. Blood samples were taken directly from the vena cava of anesthetized mice 15 min or 30 min after the treatment. Apyrase (1 U mL⁻¹) was added to the samples to prevent ADP receptor desensitization. After 10 min of centrifugation at 150 × g, PRP was collected. Platelet activation was induced by ADP (500 μM), and changes in platelet CD62P levels were assessed after 60 min of incubation. Platelets were stained with anti-human/mouse CD62P (Psel.KO2.3; eBioscience) antibody for 10 min. Samples were acquired with a BD FACSVerse machine, and analyzed with BD FACSUITE software (BD Biosciences). Changes in CD62P mean fluorescence intensity values were determined on CD42d-positive platelets.

Statistics

We used ANOVA with Tukey’s *post hoc* test for results obtained from aesthesiometry, plethysmometry, *in vivo* imaging, and platelet activation studies, and Fisher’s LSD *post hoc* test for the cytokine analysis. Two-way ANOVA was used for analysis of experiments investigating
treatment effects in two different genotypes. All data were expressed as means ± standard errors of the mean of \( n \) observations.

**Results**

Wild-type and \( P2ry12^-/- \) mice were subjected to an intraplantar CFA injection, and the PWT was determined on days 3, 4, 7, 10, and 14.

Three days after intraplantar CFA injection, the PWT values were significantly decreased in \( P2ry12^+/+ \) mice, indicating the development of mechanical hyperalgesia (Fig. 1A). This was accompanied by an increase in paw volume because of inflammatory edema (Fig. 1B). Both hyperalgesia and edema were maintained for up to 14 days. A robust increase in neutrophil MPO activity was also observed in wild-type mice on day 3, which was attenuated but remained detectable on day 10 (Fig. 1C,D). In addition, CFA treatment elicited increases in proinflammatory cytokine levels in hind paws (Fig. 2). IL-1\( \beta \), IL-6, TNF-\( \alpha \) and KC levels increased further by 14 days as compared with 3 days after CFA treatment in \( P2ry12^+/+ \) mice (Fig. 2B,C,E,F). Plasma extravasation did not differ in CFA-treated mice, so this parameter was subsequently not assessed.

In \( P2ry12^-/- \) mice, mechanical hyperalgesia was significantly decreased as compared with wild-type mice (Fig. 1A), and the difference between the genotypes was maintained 14 days after CFA injection. Likewise, the CFA-induced increase in neutrophil MPO activity was significantly less intensive in \( P2ry12^-/- \) mice on day 3 than in wild-type mice (Fig. 1C,D); however, no genotype-related difference was detected on day 10. Paw edema was not affected by the genotype at any of the examined time points (Fig. 1B), so paw swelling was subsequently not assessed. Genetic deletion of P2Y12Rs counteracted the proinflammatory cytokine response at both time points: on day 3, the increases in TNF-\( \alpha \) and KC levels were significantly attenuated, and a tendency for decreased IL-6 levels was also observed as compared with wild-type mice (Fig. 2C,E,F). IL-1\( \alpha \) remained undetectable in \( P2ry12^-/- \) mice but not in wild-type mice (Fig. 2A). The CFA-
induced increases in IL-1β, IL-6, TNF-α and KC levels were significantly alleviated in P2ry12<sup>−/−</sup> mice as compared with wild-type mice (Fig. 2B,C,E,F). CFA downregulated the anti-inflammatory cytokine IL-10 in wild-type mice, and this effect was not altered by the genotype on day 3 (Fig. 2D).

Next, we attempted to reproduce the antihyperalgesic effect of P2Y<sub>12</sub>R deficiency with specific antagonists (Fig. 3). At first, we used PSB-0739, a potent and selective antagonist of P2Y<sub>12</sub>Rs that has a nanomolar potency (Fig. 3). At first, we used PSB-0739, a potent and selective antagonist of P2Y<sub>12</sub>Rs that has a nanomolar potency (Fig. 3A). In contrast, hyperalgesia was not changed by intrathecal injection of PSB-0739 or aspirin. Aspirin had no effect on CFA-induced hyperalgesia. Interestingly, aspirin elicited a slight but significant prohyperalgesic effect in P2ry12<sup>−/−</sup> mice as compared with the saline-treated control group (Fig. 3D).

To compare the antihyperalgesic effect of P2Y<sub>12</sub>R agonists with that of an alternative platelet antagonist, the effect of low-dose aspirin (20 mg kg<sup>−1</sup> intraperitoneally) was also evaluated. In contrast to PSB-0739 and cangrelor, aspirin had no effect in the early phase (day 4), but a gradual antihyperalgesic effect developed in the later phase of inflammation, and became similar to the effect of cangrelor by day 14. This mild antihyperalgesic effect of aspirin was also detected in P2ry12<sup>−/−</sup> mice as compared with the saline-treated control group (Fig. 3D).

Next, we examined whether local P2Y<sub>12</sub>R blockade has any effect on CFA-induced hyperalgesia. Interestingly, PSB-0739 (0.3 mg kg<sup>−1</sup> intraplantarly), when administered to the plantar surface of the inflamed hind paw, had a significant antihyperalgesic effect as compared with the saline-treated control group (Fig. 4A). To identify the underlying neuronal signaling mechanism involved, we examined whether this antihyperalgesic effect could be...
attenuated by blocking axonal conduction in sensory nerves. To this end, A-803467, a selective and potent antagonist of NaV1.8 channels, was used (IC$_{50}$ = 8 nM for hNaV1.8), and showed no significant activity against TRPV1, P2X2/3, CaV2.2 and KCNQ2/3 channels [42]. Consistent with literature data [31,42], A-803467 (30 mg kg$^{-1}$ intraperitoneally) relieved hyperalgesia as compared with vehicle treatment, although complete reversal was not obtained at this dose (Fig. 4B). However, no further anti-hyperalgesic effect was obtained by either intrathecal or intraplantar injection of A-803467 or saline. The arrows indicate the respective P2Y$_{12}$ antagonist treatment (5 min before the PSB-0739 injection). The data are expressed as means ± standard errors of the mean of the paw withdrawal threshold (PWT) values measured on the right hind paw. Two-way ANOVAs followed by Tukey’s multiple comparison post hoc test were used (**P < 0.001). n = 8 per group.

Fig. 4. Effect of A-803467, a potent and selective NaV1.8 sodium channel blocker, on Complete Freund’s adjuvant (CFA)-induced mechanical hyperalgesia. (A) CFA-induced mechanical hyperalgesia in wild-type mice subjected to intraplantar injection of PSB-0739 or saline. (B) Effect of A-803467 pretreatment (5 min before the PSB-0739 injection) on CFA-induced mechanical hyperalgesia in wild-type mice subjected to intrathecal or intraplantar injection of PSB-0739 or saline. The arrows indicate the respective P2Y$_{12}$/saline administrations. The data are expressed as means ± standard errors of the mean of the paw withdrawal threshold (PWT) values measured on the right hind paw. Two-way ANOVAs followed by Tukey’s multiple comparison post hoc test were used (**P < 0.001). n = 8 per group.

Because the majority of peripheral P2Y$_{12}$Rs are expressed on platelets, we examined whether P2Y$_{12}$-driven actions in CFA-induced hyperalgesia require the presence of functional platelets. To this end, mice were treated with anti-mouse CD41 antibody (25 μg intraperitoneally) 6 days after CFA administration, which resulted in almost complete depletion of platelets from day 7 (11.75%) up to day 14 (8.54%) (Fig. 5A,B). Leukocyte populations were not affected by the antibody treatment. On days 7 and 10, platelet deficiency did not influence mechanical sensitivity; however, on day 14 (when the inflammatory cytokine responses peaked), platelet depletion reduced hyperalgesia in wild-type mice but not in P2ry$_{12}^{-/-}$ mice as compared with the control (anti-IgG$_{1}$-treated) groups (Fig. 5C). As shown above, P2Y$_{12}$R deficiency by itself significantly alleviated hyperalgesia at all time points in the control groups (Fig. 5C).

When the cytokine profile was analyzed in hind paw samples collected on day 14, both platelet depletion and genotype influenced the proinflammatory cytokine response. Similarly to the aesthesiometry findings, platelet depletion had a significant anti-inflammatory effect in the wild-type but not in the P2ry$_{12}^{-/-}$ mice, as shown by significant decreases in IL-1β, IL-6, TNF-α and KC levels in anti-CD41-treated mice as compared with anti-IgG$_{1}$-treated mice (Fig. 6B,C,E,F). Moreover, platelet depletion slightly increased IL-6 and KC levels in P2ry$_{12}^{-/-}$ mice as compared with wild-type mice (Fig. 6C,F). In contrast, IL-10 levels did not significantly change in platelet-depleted mice of either genotype, as compared with their controls (Fig. 6D). As observed earlier, in the absence of P2Y$_{12}$R, the IL-1β, IL-6, TNF-α and KC levels were lower than in their wild-type littermates (Fig. 6).

Finally, we examined how identical treatments with P2Y$_{12}$R antagonists and the reference compound aspirin influence platelet activation, by assessing ADP-induced changes in platelet CD62P levels ex vivo in PRP samples (Fig. 7). ADP (500 μM) induced significant upregulation (296.47% ± 22.14% and 309.21% ± 12.99% in the intraperitoneal and intrathecal saline-treated groups, respectively, n = 8, P < 0.01) of CD62P on CD42d-positive platelets (Fig. 7A,B). Cangrelor (3 mg kg$^{-1}$ intraperitoneally) almost completely reversed this effect (Fig. 7B). Aspirin (20 mg kg$^{-1}$ intraperitoneally) and PSB-0739 (0.3 mg kg$^{-1}$ intrathecally) had no effect on ADP-induced platelet activation as compared with the respective controls (Fig. 7B).

Discussion

Here, we report for the first time that endogenous P2Y$_{12}$R activation aggravates hyperalgesia and local inflammation in a chronic CFA-induced inflammatory pain model in mice, and that P2Y$_{12}$Rs expressed by platelets contribute to this effect. We have also identified endogenous P2Y$_{12}$-mediated proinflammatory cytokine production as the underlying molecular mechanism.

The attenuation of mechanical hyperalgesia in P2ry$_{12}^{-/-}$ mice in the chronic phase confirms and extends previous data showing the role of P2Y$_{12}$Rs in acute inflammatory pain [13,14] and neuropathic pain [11,12]. In vivo imaging experiments demonstrated significantly decreased...
neutrophil activity in $P2\text{ry}12^{-/-}$ mice during the acute phase, consistent with decreased TNF-α and KC levels in the early phase. IL-1β, IL-6, TNF-α and KC levels were also significantly lower in $P2\text{ry}12^{-/-}$ mice on day 14, identifying the most likely underlying mechanism of the antihyperalgesic effect in the chronic phase. Regarding the acute phase, the inhibition of central sensitization by IL-1β and of peripheral cytokine responses by an α7-nicotinic acetylcholine receptor-mediated efferent pathway might play a role [14]. CFA-induced paw edema was not changed in $P2\text{ry}12^{-/-}$ mice. These data are in line with findings obtained with the acute CFA-induced model [14], and the fact that platelet depletion does not affect paw edema in rodents, even though the same manipulations profoundly alter inflammatory hyperalgesia [26].

$P2\text{Y}_{12}R$ antagonists attenuated inflammatory hyperalgesia in both the acute phase and the chronic phase. PSB-0739 did not affect nociception in $P2\text{ry}12^{-/-}$ mice, suggesting that this antagonist acts exclusively through the $P2\text{Y}_{12}Rs$. In contrast, the slight prohyperalgesic effect of cangrelor in $P2\text{ry}12^{-/-}$ mice may be a $P2\text{Y}_{12}$-independent pleiotropic effect. Cangrelor inhibits the $P2\text{Y}_{13}$ receptor with comparable in vitro activity (IC₅₀ = 4 nm) to its affinity for h$P2\text{Y}_{12}R$ [41]. Therefore, it probably antagonizes $P2\text{Y}_{13}$ at the level of plasma exposure expected from the dosing used.

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Interestingly, PSB-0739 was also effective intraplan- 
tarily, indicating that local, P2Y12R-mediated mecha- 
nisms also modulate inflammatory hypersensitivity. As PSB-
0739 cannot penetrate the BBB [14], any CNS effects 
were excluded in this experiment.

NaV1.8 sodium channels expressed by primary sensory 
neurons are important targets of inflammatory mediators,
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When A-803467 and PSB-0739 were administered together, 
no additive effect was observed, indicating that NaV1.8-
expressing primary sensory neurons mediated the antihy-
peralgesic effects of both local and central PSB-0739.

It is well known that platelets are algogenic agents 
[25,44] and play important roles in inflammatory 
responses [1,24,45]. To investigate whether P2Y12Rs on 
thrombocytes contribute to the observed antihyperalgesic 
effect, platelets were depleted by an anti-mouse CD41 
antibody. This treatment elicited a progressive antihyper-
algesic effect, which became significant on day 14.

Aspirin, an alternative platelet antagonist, had a signifi-
cant antihyperalgesic effect in the later phase of inflam-
mation, similarly to the effect of platelet depletion. This 
mild effect was also detected in P2y12−/− mice, indicating 
that it is independent from P2Y12Rs. The antihyperal-
gesic dose of cangrelor almost completely reversed ADP-
induced platelet activation ex vivo, whereas aspirin and 
intrathecal treatment with the non-BBB-permeable PSB-
0739 counteracted hyperalgesia, but did not affect ADP-
induced platelet activation. These data are compatible 
with the assumption that, in the earlier phases of inflam-
mation, platelet P2Y12Rs do not mediate pain sensation, 
whereas, in the chronic phase of CFA-induced inflamma-
tion, both platelet P2Y12Rs do not mediate pain sensation, 
and P2Y12Rs in the CNS, supposedly expressed on microglial 
cells (inhibited by aspirin), and complementary platelet 
activation pathways (inhibited by aspirin) could con-
tribute to CFA-induced hyperalgesia.

Regarding the cytokine response, platelet depletion had 
an anti-inflammatory effect that was absent in P2y12−/− 
mice. However, IL-6 and KC levels were higher in

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by Tukey's mean fluorescence intensity values after group. (C) aspirin-treated and intrathecal PSB-0739-treated inflammatory phenotype observed in responsible for the inflammation. However, the anti- and the glycoprotein (GP) VI pathway was found to be in the platelet-mediated chronic inflammation phase. affect inflammation [7]. Although we do not have experi-
tors. Our findings do not contradict these data, but point to an additional role of platelet P2Y12Rs in this process.

Nucleotides are known to be released at the site of inflammation [26], and might activate platelet P2Y12Rs to amplify hyperalgesia and local inflammation (Fig. 8). The source of the released nucleotides could be dense granules of platelets, which contain ATP and ADP; however, injured cells could also contribute as an initial trigger.

The clinical significance of our findings is underscored by the widespread use of P2Y12 antagonists, and indicates their use in inflammatory pain, similarly to aspirin, which is also used for both indications. However, depending on the conditions, P2Y12R antagonists might also have pleiotropic, i.e. extraplatelet and non-P2Y12R-mediated actions, including proinflammatory effects [52]. For instance, high-dose clopidogrel aggravated inflammation in a rat arthritis model, independently from platelet P2Y12Rs [16]. In our experiments, cangrelor also elicited a P2Y12R-independent prohyperalgesic response. Because central mechanisms also play a role in the antinociceptive effect of P2Y12R inhibition [14], compounds that cross the BBB and have a selective action on P2Y12Rs would be more advantageous.

In conclusion, P2Y12R inhibition is a potential thera-
peutic approach for chronic inflammatory pain, as it relieves mechanical hyperalgesia and the inflammatory response, and inhibition of platelet P2Y12Rs contributes to this beneficial action.
Addendum

K. Bekő performed the experiments, collected and analyzed data, and wrote the manuscript. B. Kováiny, F. Gölöncsér, and G. Horváth conducted experiments and analyzed data. Á. Dénes and Zs. Helyes helped to design experiments, provided reagents and intellectual input, and edited the manuscript. Zs. Környei supervised the flow cytometry experiments. B. Botz performed the in vivo imaging experiments and edited the manuscript. C. E. Müller synthesized, analyzed and provided PSB-0739. B. Sperlégh conceived the project, designed experiments, analyzed results, wrote the manuscript, and acquired the funding.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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