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The Role of P2Y₁₂ Receptor and Activated Platelets During Inflammation

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Abstract

Platelets play an important role not only during thrombosis, but also in modulating immune responses through their interaction with immune cells and by releasing inflammatory mediators upon activation. The P2Y₁₂ receptor is a Gi-coupled receptor that not only regulates ADP-induced aggregation but that can also dramatically potentiate secretion, when platelets are activated by other stimuli. Considering the importance of P2Y₁₂ receptor in platelet function, a class of anti-platelet drugs, thienopyridines, have been designed and successfully used to prevent thrombosis. This review will focus on the role of activated platelets in inflammation and the effects that P2Y₁₂ antagonism exerts on the inflammatory process. A change in platelet functions was noted in patients treated with thienopyridines during inflammatory conditions, suggesting that platelets may modulate the inflammatory response. Further experiments in a variety of animal models of diseases, such as sepsis, rheumatoid arthritis, myocardial infarction, pancreatitis and pulmonary inflammation have also demonstrated that activated platelets influence the inflammatory state. Platelets can secrete inflammatory modulators in a P2Y₁₂-dependent manner, and, as a result, directly alter the inflammatory response. P2Y₁₂ receptor may also be expressed in other cells of the immune system, indicating that thienopyridines could directly influence the immune system rather than only through platelets. Overall the results obtained to date strongly support the notion that activated platelets significantly contribute to the inflammatory process and that antagonizing P2Y₁₂ receptor can influence the immune response.

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CONFLICT OF INTEREST

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Keywords

Inflammation; P2Y₁₂ receptor; platelets and thienopyridines

INTRODUCTION

Blood platelets are anucleate cells derived from megakaryocytes [1], which under physiological conditions circulate in blood vessels. However upon vessel injury, the endothelial layer is disrupted, and circulating platelets are activated, upon exposure to collagen, Von Willebrand factor (vWF) and vitronectin [2]. Platelets contain different cytoplasmic granules and upon stimulation their contents are released into the surrounding environment and/or become incorporated in their plasma membrane. Three types of granules can be found in platelets: protein-containing α -granules, dense granules rich in ADP and serotonin, and lysosomes [3]. Therefore, platelet activation causes shape change, fibrinogen receptor activation, as well as granule release and thromboxane A₂ (TXA₂) generation, which will recruit other platelets. As a result, blood vessel integrity will be restored and rapid cessation of bleeding will occur. Similar events occur at the site of atherosclerotic plaque rupture, when platelet activation leads to thrombus formation and possible vessel occlusion [4].

Interestingly, platelets not only regulate thrombus formation and hemostasis, but also play an important role in modulating innate and adaptive immune responses [5]. They are able to bind and capture bacteria [6] and also to interact with immune cells, such as neutrophils, dendritic cells and macrophages [5]. As a result, platelets play a key role in orchestrating cell interactions during inflammation. Furthermore, platelet granules contain pro-inflammatory and anti-inflammatory cytokines such as RANTES, Interleukin (IL)-1 β , and platelet factor 4 (PF4) [7] that are released upon stimulation into the milieu activating other blood cells and endothelium [7]. Exocytosis also results in surface expression of P-selectin, which is important for the initial tethering of leukocytes to activated platelets [8] and CD40L that can be shed into the circulation and activate endothelial cells and leukocytes [9]. P2Y₁₂ is a Gi-coupled receptor expressed on platelets, which regulates ADP-induced aggregation and it has been shown to dramatically potentiate granule release and amplify platelet aggregation, upon activation by other agonists [10, 11]. Therefore, P2Y₁₂ receptor activation is fundamental for platelet contribution to the inflammatory process. As a result, P2Y₁₂ receptor antagonism not only reduces the risk of thrombosis, but also alters inflammatory levels [12–14].

In this review, we will summarize recent findings about the contribution of activated platelets to the inflammation process and we will discuss current views about the effects that P2Y₁₂ antagonism exerts on the inflammation process.

ACTIVATED PLATELETS DURING INFLAMMATION

Although platelets are anucleate cells that are crucial mediators of hemostasis, they also play an important role in modulating innate and adaptive immune responses [5, 7]. Platelets bind

and capture bacteria [6] and also interact with a wide variety of immune cells. As a result, platelets play a key role in orchestrating cell interactions during the inflammatory response.

Platelets and leukocyte interactions have been shown to be a crucial link between inflammation and thrombosis, as first described in 1882 by Bizzozero [15]. Upon activation, platelets adhere to damaged endothelium or sub-endothelial matrix, where they recruit leukocytes to sites of inflammation [16]. In particular, platelet-leukocyte interaction is regulated by the expression of adhesion molecules, such as P-selectin, CD40 receptor and its ligand (CD40L). Platelet P-selectin can bind p-selectin glycoprotein ligand (PSGL) - 1 on leukocyte and activate these cells, favoring their infiltration in the inflamed tissue [17]. CD40 and CD40L are expressed on activated platelets and can modulate both inflammation and thrombosis by interaction with different cells [18]. Soluble CD40L is able to activate platelets [19], to enhance monocyte tissue factor expression [20] and to bind to CD40 expressed on endothelial cell membrane. It can also enhance the release of pro-inflammatory cytokines and chemokines, such as IL-6 and monocyte chemoattractant protein (MCP)-1 [21]. Indeed, plasma levels of soluble CD40L are elevated in patients with acute coronary syndrome and inflammatory conditions [19]. Studies examining the link between the neutrophil activity and platelet functions *in vivo* in two models of sepsis indicated that platelet-depletion or P2Y₁₂ antagonism significantly reduced neutrophil infiltration in the lungs [13, 22]. Similar observations were made in mice following myocardial infarction, where leukocyte infiltration in the heart was significantly reduced, when animals were platelet-depleted [23]. Furthermore, liver damage following acute pancreatitis was decreased in platelet-depleted mice as compared with controls [24]. These results suggest that platelets are crucial for neutrophil transmigration during inflammation in key organ systems.

Cross-talk between platelets and monocytes is reflected by the formation of platelet-monocyte aggregates that cause monocyte activation resulting in increased cytokine production, expression of cell-adhesion molecules and release of metalloproteinases [25]. An increase in platelet-monocyte aggregates has been observed in patients with heart failure [26].

Moreover, several studies evaluated whether platelet activation could influence dendritic cells (DC) functions, since DCs participate in both the innate and adaptive immune system and represent highly specialized antigen presenting cells [27]. It has been reported that activated platelets are able to inhibit DC differentiation and decrease secretion of pro-inflammatory cytokines such as IL-12p70 and tumor necrosis factor (TNF)- α , but enhanced IL-10 production by mature DC [28]. Another study indicated that adhesion of DC to injured carotid arteries in mice was mediated by platelets, namely by interaction of PSGL-1 on DC [29], suggesting that DC-platelet interaction could be important for the atherosclerotic process. Furthermore, this study also indicated that activated platelets were able to increase DC maturation and proliferation; Mac-1 and platelet JAM-C were identified as mediators of DC-platelet interaction [29].

Platelet/endothelium interaction plays a central role in inflammation within the vessel wall. Activated platelets can up-regulate endothelial secretion of MCP-1, a fundamental chemotactic molecule for monocytes [30], and can also increase endothelial expression of

intracellular adhesion molecule (ICAM) [30], which is critical for leukocyte binding and extravasation to sites of inflammation.

Furthermore, platelet functions appeared to be altered during inflammatory conditions also in patients. For example, during sepsis, platelet aggregation was impaired depending on sepsis severity in a number of studies [30–32], although platelet adhesion was preserved [33]. P-selectin secretion was increased in platelets from septic patients, compared with healthy controls [34]. On the contrary, in other studies no changes in P-selectin were noted, suggesting that the outcome may depend on septic conditions and severity [33]. Alpha-granule secretion was preserved, but the content of the granules, including vascular endothelial growth factor (VEGF), was significantly altered during sepsis [33], suggesting a variation of platelet secretion during inflammation. Furthermore, platelets-monocyte cross-talk and aggregate formation are increased in circulating blood of cardiovascular disease patients with heart failure [26]. As a result, aggregate analysis has been used as a diagnosis event to evaluate inflammatory diseases and their severity [35].

In summary, platelet functions are altered during inflammatory conditions in patients, depending on the diseases, suggesting a direct role for these cells in these inflammatory conditions that need to be further evaluated. In addition, platelets are also able to modulate the inflammatory response by interacting with other cells of the immune system, such as neutrophil, monocytes and DC.

PLATELET RELEASE OF INFLAMMATORY MEDIATORS

Platelets granules contain various pro-inflammatory and anti-inflammatory cytokines and chemokines that, upon stimulation, are released into the milieu activating other blood cells and endothelium. These small molecules are RANTES, IL-1 β , MCP-1, PF4, platelet activator factor (PAF) and transforming growth factor (TGF)- β . All these molecules contribute to and support inflammation by activating and interacting with other cell types.

The chemokine RANTES can bind to microvascular endothelium and trigger monocyte arrest under flow condition. Conveyance of RANTES by platelets and its deposition on the endothelial surface can trigger monocyte arrest to inflamed endothelium of microvascular or arterial origins [36]. TGF- β is a multifunctional cytokine that is able to regulate a variety biologic and pathologic functions, including cell proliferation and differentiation, the immune response, and tissue fibrosis [37]. It has shown to be abundant in platelets [38] and secretion of TGF- β upon activation significantly contributes to TGF- β plasma levels [39], although they are not the only source of this molecule. PF4 is an abundant platelet protein that plays a role in angiogenesis and thrombosis, but it can also mediate the pro-inflammatory response of vascular smooth muscle cells to injury [40].

Activated platelets also have IL-1 β activity [41, 42]. Upon activation, they are able to secrete this pro-inflammatory cytokine that can bind and activate endothelial cell and promote neutrophil adhesion [43]. Similarly, upon stimulation with lipopolysaccharide (LPS), platelets were shown to synthesize IL-1 and shed microparticles containing IL-1 that stimulates endothelial cells [44]. Further studies have shown that platelets are positive for

IL-1 β mRNA and that activation induces rapid and sustained synthesis of this inflammatory mediator, a response that was abolished by translational inhibitors [45].

THE PHARMACOLOGY OF P2Y₁₂ RECEPTOR

P2Y₁₂ Receptor

ADP-induced aggregation is mediated by two members of the P2Y receptor family, P2Y₁₂ and P2Y₁ [46]. They are G protein-coupled receptors expressed on platelet membranes [47]. P2Y₁ is a G_q-coupled protein whose activation leads to intracellular calcium mobilization, protein kinase C (PKC) activation and causes platelet shape change, resulting in a weak and transient aggregation [48]. On the other hand, the P2Y₁₂ receptor is coupled to G_i protein, and its activation leads to inhibition of adenylyl cyclase activity hence decreasing cAMP intracellular levels. This receptor has also a critical requirement for lipid rafts [49]. In particular, P2Y₁₂ receptor couples primarily to G α_{i2} and less prominently to other members of the G_i family [50]. Furthermore, G_i signaling leads to activation of the (phosphatidylinositol 3 PI3) kinase pathway and potassium channels [51]. Studies from our laboratory have identified the PI3 kinase β as the isoform involved in this signaling [52]. PI3 kinase β activation is required for Rap1b [53] and Akt activation [54] that causes a dramatic potentiation of granule release, indicating a critical role for the P2Y₁₂ receptor in platelet secretion [55]. Indeed, signaling events downstream of the P2Y₁₂ receptor also potentiate agonist-induced dense granule release, pro-coagulant activity and thrombus formation [56]. In addition, α -granule release and subsequent expression of P-selectin on activated platelets are dependent on P2Y₁₂ activation [8].

Previous studies have shown that G_i signaling mediated by the P2Y₁₂ receptor is dependent on the cholesterol rich lipid rafts [57] and that a high fat diet enhances platelet activation induced by other agonists [58]. In addition to chronic hypercholesterolemia, other pathologic conditions ranging from diabetes [59] to hypertension may increase P2Y₁₂ receptor functions and hence the risk of thrombosis.

ADP binding to platelets produces selective short term (5–10 min) desensitization of both the P2Y₁₂ and P2Y₁ receptors resulting in unresponsiveness to subsequent addition of agonists [60]. P2Y₁₂-mediated desensitization is mediated by G-coupled receptor kinases (GRK) 2 and 6, while P2Y₁-mediated desensitization is largely dependent on PKC activity [61]. Different PKC isoforms may have distinct roles in regulating platelet P2Y receptor function and trafficking [62].

Furthermore, this receptor is essential for platelet aggregation under shear conditions as P2Y₁₂ antagonism was able to significantly decrease shear-induced platelet aggregation [63], causing a diminished P-selectin expression and microparticle formation initiated by vWF activation [64]. However, greater inhibition was observed when both P2Y₁₂ and P2Y₁ were antagonized [65]. Similar results were observed in a mouse model of atherothrombosis, where pre-treatment with the P2Y₁₂ antagonists ticagrelor or cangrelor could not only inhibit thrombus formation, but also decrease its stability [66]. Similar results were observed in *ex vivo* thrombus formation with human platelets from coronary heart disease patients

treated with clopidogrel [67], further confirming the important role of P2Y₁₂ during thrombus formation and its stability.

Defects in the gene encoding the P2Y₁₂ receptor are responsible for a congenital bleeding disorder [68]: patients with defective P2Y₁₂ receptor functions have normal platelet shape change, but impaired abilities to inhibit adenylyl cyclase activity [69]. Dense granules are normal in both numbers and content, but granule release is generally decreased.

P2Y₁₃, another member of the P2Y receptor family, displays 48% amino acid sequence identity with the P2Y₁₂ receptor. P2Y₁₃ is a G_i-coupled receptor activated by ADP. It is more ubiquitously expressed than P2Y₁₂ [70], particularly in the brain and the spleen, suggesting roles in the nervous and immune system. P2Y₁₂ and P2Y₁₃ receptors have similar pharmacological profiles [56, 70, 71], although P2Y₁₃ is not antagonized by the active metabolite of clopidogrel [70]. This receptor does not appear to be expressed in platelets, although it is expressed in megakaryocytes [72] and may mediate ADP-induced pro-platelet formation.

Tissue Distribution of P2Y₁₂ Receptor

Although P2Y₁₂ receptor was thought to be expressed only in platelets [73] and microglia [74], recent studies suggest that it may have a wider expression than previously reported (as summarized in Table 1). Indeed, P2Y₁₂ mRNA has been detected in a number of human immune cells, such as lymphocytes and monocytes, but its protein expression has not been investigated [75]. Another group investigating the involvement P2Y₁₂ in CD45⁺ leukocyte functions demonstrated that 2-methyl-thio (MeS)-ADP-induced cell migration in P2Y₁₂-null mice was significantly decreased compared with that in wild-type controls [76]. In addition, dendritic cells express P2Y₁₂ mRNA, and these cells are functionally altered in P2Y₁₂-null mice [77]. These cells also express P2Y₁₃ mRNA, but cell functions are not altered in P2Y₁₃-null mice, suggesting that only P2Y₁₂ regulates dendritic cell function. As a result, the anti-platelet drugs designed to antagonize P2Y₁₂ receptor may directly influence inflammation by altering the functions of these immune cells as well as platelets. Furthermore, we have investigated a prasugrel metabolite mixture generated *in vitro* and analyzed its effects on neutrophils. This mixture was able to inhibit neutrophil functions, although this cell type does not appear to express the P2Y₁₂ receptor [78]. These data suggest that in addition to the direct effects on platelets and other cells of the immune system, prasugrel metabolites could also have off-target effects on neutrophils during inflammation.

Interestingly P2Y₁₂ has also been investigated in other cells that are not part of the immune system, such as smooth muscle cells (SMC), endothelial cells and osteoblasts. In SMC, both mRNA and protein expression was detected [76, 79]. Furthermore, Wilhborg *et al.* showed that AR-C67085, a P2Y₁₂ antagonist, was able to prevent 2-MeS-ADP induced contractions [79], suggesting that this receptor antagonism could alter smooth muscle cell functions. AR-C67085 is specific for P2Y₁₂ receptor at low concentrations (nM), but at higher doses (μM) it also binds P2Y₁₃ receptor [70, 80]. In this study, the lowest concentration effective was 0.1μM that is in the range of P2Y₁₂ specificity, but further studies are needed to completely rule out the involvement of P2Y₁₃, especially considering that P2Y₁₃ mRNA has also been

detected in SMC. Another group investigated 2-MeS-ADP-mediated SMC migration in P2Y₁₂-null mice, where migration was significantly inhibited in null mouse cells compared with wild type. Although interesting, these data should be supported by further analysis of intracellular changes in cAMP levels or Akt phosphorylation in order to demonstrate receptor functionality.

P2Y₁₂ receptor expression has also been detected in rat splenic sinus endothelial cells [81] through western blotting and fluorescence microscopy. Further studies are needed to demonstrate whether the receptor is functional.

Recent studies indicate that extracellular nucleotides play a significant role in bone biology and in modulating the function of both osteoblasts and osteoclasts [82] suggesting a role for P2Y receptors in these cell types. Among others, P2Y₁₂ receptor mRNA has been detected in rat osteoblasts and murine osteoclasts [82], suggesting that the effects of ADP and ATP on bone biology could be regulated through this receptor. In addition, P2Y₁₂ receptor protein expression was demonstrated in cells of both lineages and clopidogrel treatment altered bone homeostasis both *in vivo* and *in vitro* [83]. Moreover, P2Y₁₂-deficient mice have decreased osteoclasts activity and are protected from age-associated bone loss [84]; this effect is similar to that observed after clopidogrel treatment.

P2Y₁₂ Receptor Antagonists

A variety of specific P2Y₁₂ receptor antagonists have been designed over the years [85, 86]. Among the most used are the AR-C compounds, competitive P2Y₁₂ antagonists that irreversibly and selectively bind this receptor preventing platelet aggregation. These compounds are AR-C67085, ARC66096 and AR-C69931MX. AR-C67085 is selective for P2Y₁₂ receptor, but at μ M concentrations can also bind P2Y₁. AR-C69931MX is known also as cangrelor and has been extensively studied in both animals and humans [87, 88]. 2-MesAMP is an antagonist for P2Y₁₂ receptor, but also binds the P2Y₁ receptor and hence is not very specific [10]. Other important and very selective antagonists are termed thienopyridines and will be discussed below.

It is worth mentioning that MRS2211, a commercially available antagonist of P2Y₁₃ [89], is able to distinguish between P2Y₁₂ and P2Y₁₃ receptors, but only when used at low concentrations. In fact, at high concentrations of MRS2211, nonspecific P2Y₁₂ receptor binding will likely occur. Hence it is difficult to discriminate between these receptors when the antagonist concentration required is high. On the contrary, P2Y₁ antagonists, such as A3P5P, A2P5P, A3P5PS and MRS2179, have shown higher specificity and they are less likely to interact with P2Y₁₂ receptor [90].

THIENOPYRIDINES: CLOPIDOGREL AND PRASUGREL

Considering the importance of P2Y₁₂ receptor in platelet activation, a class of anti-platelet drugs, named thienopyridines, has been designed to antagonize this receptor on platelet membranes [56]. Thienopyridine-receptor binding prevents ADP-induced aggregation and consequently thrombus formation [52, 56, 86]. Clopidogrel and prasugrel have been successfully used for years to prevent anti-thrombotic events in patients with acute coronary

syndrome and those undergoing cutaneous coronary intervention [91]. They are both orally administrated as a pro-drug, then metabolized in the liver to generate the active form that irreversibly inhibits P2Y₁₂ receptor [92]. Clopidogrel is extensively hydrolyzed by esterases in the blood, so that only 15% of the administered amount is metabolized by the cytochrome P450 system in order to generate the active form. This modification of the receptor site by the active form of clopidogrel follows the formation of a disulfide bridge between the reactive thiol group of the metabolite and the two extracellular cysteine residues (Cys 17 and Cys 270) of the P2Y₁₂ receptor. On the other hand, prasugrel has shown highly efficient metabolism to the active form. In fact, prasugrel is rapidly hydrolyzed and efficiently converted to the active derivative by CYP isozymes. The active form binds covalently to the P2Y₁₂ receptor *via* a disulfide bond similar to that formed by the clopidogrel active metabolite, irreversibly antagonizing the ADP P2Y₁₂ receptor. This more efficient and rapid action of prasugrel over clopidogrel was assessed in patient studies. However, prasugrel, while able to decrease the risk of myocardial infarction and stroke more efficiently, caused a significant increase in major and fatal bleeding, suggesting that it needs to be used cautiously [93]. However, previous studies carried out with human plasma have shown that the metabolism of the pro-drug, for both clopidogrel and prasugrel, results *in vivo* in generation of other metabolites [94, 95] that have been so far considered inactive [94].

Another class of anti-platelet drug targeting P2Y₁₂ receptor is the cyclopentyl-triazolo-pyrimidine; these drugs reversibly bind the receptor. Among them, ticagrelor is an oral drug that acts directly on P2Y₁₂ receptor and does not require metabolic activation. Interestingly, this compound does not prevent ADP binding, but rather inhibits ADP-induced receptor signaling by antagonizing ADP-induced receptor conformational change and G-protein activation.

THE P2Y₁₂ RECEPTOR ANTAGONISTS IN PATIENT STUDIES

In order to understand the role of platelets during cardiovascular diseases, inflammation biomarkers and possible interaction between platelets and leukocytes have been investigated in patients undergoing anti-platelet therapy. Results have shown that antagonizing P2Y₁₂ receptor with clopidogrel decreased P-selectin expression on platelets and reduced platelet-leukocyte aggregate formation [96]. In acute coronary syndrome patients a reduction in soluble CD40L and C-reactive protein was demonstrated [97], but no difference was noted between a decrease caused by ticagrelor and clopidogrel treatment [98]. Therefore, during atherogenesis, P2Y₁₂ receptors appear to regulate platelet-mediated release of pro-inflammatory mediators, underscoring the importance of platelet *et al.* granule release during the inflammatory process *in vivo*.

On the contrary, in patients with diabetes and acute coronary diseases, clopidogrel withdrawal caused an increase in C-reactive protein and P-selectin expression in platelets, showing that this drug has pro-inflammatory and pro-thrombotic effects as well as increase platelet reactivity during diabetes [99]. Further studies are needed to evaluate drug usage in these patients.

P2Y₁₂ RECEPTOR IN INFLAMMATION

P2Y₁₂ Receptor in Vascular Inflammation

In abdominal aortic aneurism (AAA) patients, increased platelet infiltration was observed in the aortic inflamed tissue [12]. In an animal model of Angiotensin-II induced AAA, clopidogrel treatment attenuated disease severity, causing a decrease in platelet-macrophage interaction [12]. On the other hand, another study investigating clopidogrel effects in coronary artery disease (CAD) patients reported that patients treated with clopidogrel had increased expression of RANTES and MIP-1 β in peripheral blood mononuclear cells (PBMC) as compared with patients receiving placebo, suggesting that this drug could increase inflammation [100]. It has not been investigated whether these effects were the result of a direct effect on PBMC or platelet-mediated.

The P2Y₁₂ receptor also plays a role in the development of transplant arteriosclerosis [76], a chronic rejection model of carotid artery allografts. In this model, P2Y₁₂-null mice are used as recipients to demonstrate that P2Y₁₂ deficiency caused a reduction in the development of transplant arteriosclerosis. Some of the cells involved in the neointima formation of arteriosclerosis, such as leukocytes and SMC, have been shown to express P2Y₁₂ receptor. Therefore, the absence of P2Y₁₂ receptor expression in these cell types could be the reason for the decreased levels of arteriosclerosis. However, the involvement of these cells *in vivo* has not been fully demonstrated yet.

P2Y₁₂ Receptor in Sepsis

Platelet-leukocyte interaction is important in the pathogenesis of sepsis [34]. As previously mentioned, platelet characteristics in septic patients appear to be altered in terms of leukocyte-platelet aggregate and P-selectin secretion [33]. In a model of sepsis induced by cecal ligation and puncture (CLP), neutrophil infiltration in the lungs was significantly reduced following platelet depletion [22], suggesting that platelets play a fundamental role in neutrophil activation during inflammation; this was similar to what was observed in other inflammation models, such as pancreatitis and ischemia reperfusion [23, 24]. Furthermore, in a rat model of LPS-induced inflammation induction of pro-inflammatory cytokines (IL-6 and TNF- α) as well as lung and liver damage were attenuated upon treatment with clopidogrel (10mg/kg, 5 days before LPS injection) [13]. These studies indicate that P2Y₁₂ receptor antagonism and platelet depletion are able to decrease inflammation during sepsis, suggesting that the effects observed are most likely exclusively platelet dependent. However, further experiments will be required to exclude the involvement of other cells.

P2Y₁₂ Receptor in Pulmonary Inflammation

It has been reported that platelets promote pulmonary recruitment of neutrophils during inflammation, contributing considerably to the levels of lung injury [13, 22]. Studies investigating the mechanism through which platelets could regulate this process have shown that platelet-derived CD40L plays an important role in regulating neutrophil activation and recruitment. Similar results were observed in a model of abdominal sepsis, where platelet depletion was protective against lung damage and decreased neutrophil activation [22]. Clopidogrel pre-treatment effects were also studied in a rat model of LPS-induced systemic

inflammation [101], showing that antagonizing the P2Y₁₂ receptor diminished cell infiltration in the lung and decreased cytokine production.

The importance of platelets during asthma has also been evaluated, showing that asthmatic patients have increased number of circulating platelets expressing P-selectin as compared with control subjects. The consequences of platelet activation was also noted in formation of leukocyte and platelet aggregates, that were significantly higher in asthma patient blood samples compared with healthy controls, and increased inflammatory cells in the lungs of asthmatic patients [102]. Furthermore, receptor deficiency, as well as P2Y₁₂ antagonism, abrogated dust mite-induced airway inflammation in an animal model of pulmonary inflammation [14], suggesting that this receptor plays an important role during asthma and it could be considered as a target for future therapeutic options.

P2Y₁₂ Receptor in Rheumatoid Arthritis

Platelet activation also plays a key role in the pathology of rheumatoid arthritis (RA). Increased platelet reactivity has been observed during this disease, increasing the risk of cardiovascular complications. Indeed, aggregation was increased in RA patients compared with control [103]. Activated platelets have also been isolated in the inflamed joints of patients, where they contributed to inflammation and alternation of the synovial microcirculation by locally elevating P-selectin and CD40L [104]. However, plasma levels of platelet-derived pro-inflammatory markers were also increased, showing that activated platelets were not restricted to the joints. Interestingly, platelet aggregation was higher in RA patients than in healthy controls. The consequences of P2Y₁₂ receptor antagonism or deficiency have been investigated in animal models exhibiting different results in different models. In contrast with all the inflammation models previously reported, our group showed that during erosive arthritis induced by peptidoglycan polysaccharide the severity of the inflammation in the joints was augmented when animals were pre-treated with clopidogrel [14]. Similarly, another study demonstrated that platelets can amplify the pathophysiology of RA by liberating pro-inflammatory microparticles that were detected in human synovial fluid [105]. On the other hand, P2Y₁₂-deficient mice have shown decreased osteoclast activity; these mice are protected from age- and tumor-associated bone loss [84]. Similar results were obtained following clopidogrel treatment in wild-type animals, suggesting that P2Y₁₂ deficiency and antagonism have a protective role against bone loss. Furthermore, in contrast with the results of previous studies in other RA models following clopidogrel treatment [14, 105], serum transfer arthritis was more severe in wild-type compared with P2Y₁₂-null mice [84]. The observed discrepancy between studies suggests that more investigations need to be carried out to understand how P2Y₁₂ antagonism can alter RA in order to determine whether P2Y₁₂ antagonist exerts beneficial effects on this inflammatory condition.

CONCLUSIONS

Antagonizing the P2Y₁₂ receptor decreases platelet activity and, hence, thrombus formation, but also diminishes the levels of inflammation in a variety of animal models (as summarized in Table 2) as well as in patients. P2Y₁₂ receptor was thought to be expressed exclusively in

platelets and, therefore, the observed effects of its loss or antagonism were considered to be only platelet-mediated. However, the recent discovery that some other cells of the immune system express P2Y₁₂ suggests that receptor antagonism by thienopyridines could directly alter the immune response in inflammation (Table 1). Although both dendritic cells and CD45⁺ lymphocytes from P2Y₁₂ deficient mice have altered cellular responses compared with wild type, it is necessary to study receptor functionality in order to determine whether these cells are influenced by P2Y₁₂ antagonism. Furthermore, P2Y₁₂ expression may not be limited to the immune system, but it may also be expressed on endothelial cells and SMC, with further implication during inflammation (Table 1). Also in this case, receptor functionality needs to be characterized before drawing any further conclusions. Interestingly, other metabolites of these drugs, considered inactive so far, or the active metabolite itself may also have important effects on cell functions, considering their ability to alter neutrophil responses, despite the fact that these cells do not express the receptor. Therefore the possibility of off-target interactions need to be further evaluated in cells of the immune system and beyond. On the contrary, only in a model of erosive rheumatoid arthritis, clopidogrel treatments were able to increase inflammation levels, while anti-inflammatory properties were noted for this drug in serum transfer arthritis. Hence more information needs to be gathered in order to determine whether this drug is safe to use in RA patients. Taken together, the data obtained so far indicate that in addition to their anti-thrombotic properties, thienopyridines may also have anti-inflammatory characteristics.

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Table 1

Tissue distribution of P2Y₁₂ receptor.

Cell Type	mRNA and/or Protein Expression	Changes in Cell Functions in KO Mice	P2Y ₁₃ Expression	ADP Activation	Reference
Platelets	Protein expression (WB)	+	-	+	[46, 49, 73]
Dendritic cells	mRNA	+	+	+	[77]
Lymphocytes	mRNA	Not analyzed	+	Not analyzed	[75]
Monocytes	mRNA	Not analyzed	+	Not analyzed	[75]
CD45 ⁺ leukocyte	mRNA	+	Not analyzed	+	[76]
Smooth muscle cells	mRNA	+	+	+	[76, 79]
Endothelial cells	Protein expression (Fluorescence microscopy)	Not analyzed	Not analyzed	Not analyzed	[81]
Osteoblasts/Osteoclasts	mRNA	+	+	+	[82–84]

Table 2

P2Y₁₂ receptor in animal models of inflammation.

Animal Model	Time Points	Clopidogrel/Prasugrel Treatments	P2Y ₁₂ Deficiency	Platelet Depletion	References
Angiotensin-II induced abdominal aortic aneurism	4 weeks	30 mg/kg – 1 week before the Angiotensin II infusion then continue throughout the study	Not analyzed	Not analyzed	[12]
Chronic rejection model of carotid artery allografts	8 weeks	Not analyzed	Reduction of luminal occlusion and inflammation	Not analyzed	[76]
Myocardial infarction model	3 days	Treatment 2 hours after MI and lasted 3 days – 2doses: 50/15/15 and 15/5/5 mg/kg. Decreased severity of cardiac inflammation. Treatment 2 hours after MI and lasted 3 days – dose: 5/5/5 mg/kg Decreased severity of cardiac inflammation	Not analyzed	Decreased severity of cardiac inflammation.	[24]
Sepsis (Cecal ligation and puncture)	4, 6 and 24 hours	Not analyzed	Not analyzed	Decreased neutrophil infiltration in the lung.	[22]
LPS-induced systemic inflammation	3, 6, 9, 12, and 24 hours	10mg/kg (5 days before LPS injection) – decreased neutrophil infiltration in the lung and decreased liver injury.	Not analyzed	Not analyzed	[13]
Acute pancreatitis	24 hours	Not analyzed	Not analyzed	Reduced tissue damage.	[23]
Erosive arthritis induced by peptidoglycan polysaccharide	After 21 days	30 mg/kg daily – pro-inflammatory effects	Not analyzed	Not analyzed	[14]
Serum transfer arthritis	11 Days	Not analyzed	Decreased arthritis-associated Bone-loss	Not analyzed	[84]
Leukotriene E4-induced pulmonary inflammation	24 hours	500 µg/ml clopidogrel in drinking water for 3 days before the first intranasal dose of LTs. – decreased levels of pulmonary inflammation	Decreased levels of pulmonary inflammation	Decreased levels of pulmonary inflammation	[14]