Lung injury during LPS-induced inflammation occurs independently of the receptor P2Y₁

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Abstract Disruption of the lung endothelial and epithelial barriers during acute inflammation leads to excessive neutrophil migration. It is likely that activated platelets promote pulmonary recruitment of neutrophils during inflammation, and previous studies have found that anti-platelet therapy and depletion of circulating platelets have lung-protective effects in different models of inflammation. Because ADP signaling is important for platelet activation, I investigated the role of the ADP-receptor P2Y₁, a G protein-coupled receptor expressed on the surface of circulating platelets, during lipopolysaccharide (LPS)-induced inflammation and lung injury in P2Y₁-null and wild-type mice. Systemic inflammation was induced by a single intraperitoneal dose of LPS (3 mg/kg), and the mice were analyzed 24 h posttreatment. The data show that the LPS-induced inflammation levels were comparable in the P2Y₁-null and wild-type mice. Specifically, splenomegaly, counts of circulating platelets and white blood cells (lymphocytes and neutrophils), and assessments of lung injury (tissue architecture and cell infiltration) were similar in the P2Y₁-null and wild-type mice. Based on my results, I conclude that lung injury during LPS-induced inflammation in mice is independent of P2Y₁ signaling. I propose that if a block of purinergic signaling in platelets is a potential lung-protective strategy in the treatment of acute inflammation, then it is more likely to be a result of the disruption of the signaling pathway mediated by P2Y₁₂, another G protein-coupled receptor that mediates platelet responses to ADP.

Keywords P2Y₁ receptor · Inflammation · Platelets · Lung

Introduction

Acute lung injury is a condition of acute inflammation that results from a disruption of the lung endothelium and excessive transepithelial neutrophil migration [1]. There is growing evidence that platelets contribute to lung injury during inflammation by promoting pulmonary recruitment of neutrophils [2, 3]. For instance, in a study of sepsis-induced lung injury, mice that were depleted of platelets were found to have significantly lower levels of both neutrophil activation and lung damage [2].

Platelets, which contribute to hemostasis through their roles in thrombus formation, play important roles in inflammation by interacting with other cells of the immune system and by secreting inflammatory mediators [4]. Upon vessel injury, circulating platelets are activated, which leads to platelet aggregation and platelet secretion of second mediators (such as ADP) that recruit other platelets [5].

ADP-induced platelet aggregation is mediated by two members of the P2Y receptor family, P2Y₁ and P2Y₁₂, both of which are present on the surfaces of platelets [6, 7]. P2Y₁ is a G_q-coupled receptor. Stimulation of P2Y₁ causes PLCβ activation and calcium mobilization, resulting in platelet shape change and aggregation [8]. In addition to platelets, P2Y₁ is found in a wide range of cells and tissues, including vascular endothelial cells, leukocytes, and monocytes [9]. Previous studies have shown that P2Y₁ on endothelial cell surfaces modulates leukocyte recruitment into the endothelium, suggesting a role for P2Y₁ in vascular inflammation [10]. Other studies have investigated the role of P2Y₁ in neuroinflammation. For example, in a study of neuroinflammation induced by cerebral stroke, it was found that P2Y₁-null mice exhibited

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significantly less altered cognitive decline [11]. In another study, peripheral pretreatment with a selective P2Y₁ antagonist resulted in improved nociception in a model of formalin-induced inflammatory pain [12]. However, my colleagues and I have found that a deficiency of P2Y₁ did not influence inflammation levels or lung injury in a murine model of sepsis [13]. The discrepancy between the results of different studies suggests that the role of P2Y₁ in inflammation may depend on the type of inflammation.

P2Y₁₂ is a G_i-coupled receptor. Stimulation of P2Y₁₂ leads to inhibition of adenylyl cyclase and potentiation of platelet secretion. P2Y₁₂ has been found in platelets [14], microglia [15], and other cells of the immune system, including dendritic cells, monocytes, and lymphocytes [16, 17]. P2Y₁₂ has been shown to play a predominant role in inflammation. Both a deficiency of P2Y₁₂ and a blockade of the P2Y₁₂ signaling pathway have been found to reduce inflammation in a variety of models, including myocardial infarction [18], pancreatitis [19], and sepsis [2]. For example, pretreatment with the P2Y₁₂ antagonist clopidogrel was found to result in diminished cell infiltration in the lung in a rat model of lipopolysaccharide (LPS)-induced systemic inflammation [20].

My colleagues and I have previously shown that a loss of P2Y₁ signaling had no effect on inflammation levels or lung injury in a murine model of sepsis, as no difference was detected when septic P2Y₁-null mice were compared with the septic wild-type (WT) control [13]. However, these observations could be specific to sepsis and sepsis-induced lung injury. No studies have yet analyzed the role of P2Y₁ in LPS-induced inflammation and lung injury. Hence, I investigated the level of lung injury in P2Y₁-null mice in which inflammation was induced by a single intraperitoneal (i.p.) dose of LPS (3 mg/kg; 24-h time point). My findings revealed for the first time that P2Y₁ deficiency does not alter the level of LPS-induced inflammation, which I evaluated in terms of circulating white blood cells or acute lung injury. My results indicate that lung injury during LPS-induced inflammation occurs independently of P2Y₁ signaling.

Material and methods

Materials

All reagents were analytical grade and obtained from Thermo Fisher Scientific (Waltham, MA), unless stated otherwise. Lipopolysaccharides from *Salmonella enterica* serotype Enteritidis (L7770; protein content ≤1%) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO). Hank's balanced salt solution (HBSS) and PBS were purchased from Mediatech, Inc. (Manassas, VA).

Animals and treatments

All procedures for the care and handling of the animals adhered to the National Institutes of Health standards and were approved by the Institutional Animal Care and Use Committee at Temple University School of Medicine (Philadelphia, PA). The age-matched, pathogen-free C57BL/6 male mice (weight 25–30 g) used in this study were selected from either a wild-type strain or a P2Y₁-null strain that was generated by subcontract with Lexicon Genetics Inc. (Woodlands, TX) using knockout constructs as previously described [21, 22]. The mice were housed in a climate-controlled facility and given free access to food and water.

Wild-type and P2Y₁-null mice were treated with a single intraperitoneal dose of LPS (3 mg/kg) dissolved in a saline solution. Control mice received the saline solution only (vehicle). The specific LPS dose was chosen on the basis of previous studies that were carried out in our laboratory [23] and by other groups [24, 25].

At 24-h post-LPS treatment, mice were anesthetized and blood samples were collected by cardiac puncture (10:1 ratio of blood in 3.8% sodium citrate) for hematology studies that were performed using the Hemavet® Multispecies Hematology System (Drew Scientific Inc., Oxford, CT). The mice were euthanized by cardiac puncture and exsanguination. Lungs and spleens were weighed and the results are presented as organ weight (milligrams) per body weight (grams), as previously described [23, 26–28].

Lung histopathology

Lung sections were embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin (H&E). Morphological analysis of random fields ($n = 6$) from each section ($n = 3$ sections/animal) was performed by a second independent and blinded observer using previously described methods [29]. Acute lung injury (ALI) was scored on the basis of four parameters: (a) alveolar capillary congestion, (b) hemorrhage, (c) infiltration or aggregation of neutrophils in the airspace or the vessel wall, and (d) thickness of the alveolar wall. Each parameter was graded from 0 to 4 based on the damage present as follows: 0 indicates no or little damage, 1 indicates less than 25% damage, 2 indicates 25–50% damage, 3 indicates 50–75% damage, and 4 indicates more than 75% damage. The overall degree of ALI was determined by adding the scores of all four parameters.

Myeloperoxidase peroxidation

Lungs were homogenized and sonicated in cold PBS (pH 7.4). After centrifugation at $10,000 \times g$ for 10 min at 4 °C, myeloperoxidase peroxidation (MPO) levels were detected using an MPO assay kit (Cayman, USA).

Statistical analysis

Differences among the groups were analyzed using a one-way ANOVA statistical test. Bonferroni's Multiple Comparison Test was used for posttest analyses. $p < 0.05$ was considered to be significant. Data are reported as mean $\pm$ standard error of the mean (SEM) for each group.

Results and discussion

To investigate the role of P2Y₁ in lung injury that occurs during LPS-induced inflammation, I treated WT and P2Y₁-null mice with either a single i.p. dose of LPS (3 mg/kg) or of the vehicle only (PBS), and samples were collected 24 h after treatment. All of the mice used in the experiment survived for 24 h following the LPS treatment. The changes in body weight for the groups included in the study are shown in Table 1. My results indicate that the LPS treatment resulted in a significant decrease in the body weight of the mice in the study ($p < 0.05$; LPS-treated vs. vehicle-treated), with no difference between WT and P2Y₁-null mice.

Next, I looked for possible changes in splenomegaly 24 h after the LPS treatment (i.p. dose of 3 mg/kg of LPS) (Fig. 1). The data are expressed as milligrams (mg) of organ weight per gram of animal weight. When I compared the results across the different treatment groups, I found that the LPS treatment resulted in a 1.5-fold increase in the average spleen weight of the WT mice ($p < 0.05$; LPS-treated WT vs. vehicle-treated WT) and a 1.75-fold increase in the average spleen weight of the P2Y₁-null mice ($p < 0.05$; LPS-treated P2Y₁-null mice vs. vehicle-treated P2Y₁-null mice, Fig. 1). Interestingly, the average spleen weight of the vehicle-treated P2Y₁-null mice was significantly lower than that of their WT counterparts ($p < 0.05$; vehicle-treated WT vs. vehicle-treated P2Y₁-null), suggesting that P2Y₁ plays an important role in spleen development. Previous studies have shown that P2Y₁ is expressed

Fig. 1 Effects of LPS exposure on splenomegaly and lung weight. Spleen weight was investigated in WT and P2Y₁-null mice treated with either a single i.p. dose of LPS (3 mg/kg) or of the vehicle only (PBS). Samples were collected 24 h after treatment. Data are expressed as organ weight (milligrams) per body weight (grams) (* $p < 0.05$; WT mice vs. P2Y₁-null mice, LPS-treated mice vs. vehicle-treated mice, and vehicle-treated WT mice vs. vehicle-treated P2Y₁-null mice; $n = 5$)

on rat splenic sinus endothelial cells [30], but specific roles for P2Y₁ in the development and function of the spleen have not yet been described.

The splenomegaly most likely results primarily from an increase in the immune cell count, which has been shown to occur in the course of inflammation [28]. Hence, I examined the number of circulating white blood cells (Fig. 2a). White blood cells were elevated in both the WT and P2Y₁-null mice that were treated with LPS ($p < 0.05$). Based on an analysis of cell populations, I noted that neutrophils were increased in both LPS-treatment groups compared with the vehicle-treated control groups ($p < 0.05$), and I observed no significant difference between the LPS-treated P2Y₁-null mice and the LPS-treated WT mice (Fig. 2b). Indeed, the lymphocyte count was significantly decreased in both LPS-treated WT and P2Y₁-null mice (Fig. 2c, $p < 0.05$) compared with that in their respective vehicle-treated controls. However, the decrease was more significant in LPS-treated WT mice than in LPS-treated P2Y₁-null mice (Fig. 2c, $p < 0.05$). Interestingly, P2Y₁ seems to be expressed in lymphocytes [9] but not in neutrophils [9]. It is possible that P2Y₁ deficiency prevents leukocyte release from the bone marrow or results in a decrease in the migration of lymphocytes into tissues. I found no significant difference when platelet counts were compared across all treatment groups (Fig. 2d).

To assess the functional consequence of the observed increase in the parameters of inflammation, I investigated whether P2Y₁ deficiency alters LPS-induced ALI ($n = 5$; Fig. 3). I found that mice treated with LPS (a single i.p. dose of 3 mg/kg of LPS; 24-h time point) exhibited an increase in lung weight ($p < 0.05$; LPS-

Table 1 Effects of LPS exposure on body weight, splenomegaly, and lung weight. WT and P2Y₁-null mice were treated with either a single i.p. dose of LPS (3 mg/kg) or of the vehicle only (PBS). Weight was recorded prior to initiation of treatment (pre-treatment) or prior to sacrifice (24-h post-treatment). Data are expressed in grams (g) and represent mean $\pm$ SEM

Group	Pre-treatment (g)	Post-treatment (g)
WT treated with vehicle only	26.7 $\pm$ 0.8	26.9 $\pm$ 2.5
P2Y ₁ -null treated with vehicle only	24.3 $\pm$ 0.2	25.1 $\pm$ 0.8
WT treated with LPS	28 $\pm$ 1	22.6 $\pm$ 0.5*
P2Y ₁ -null treated with LPS	26 $\pm$ 4	20.5 $\pm$ 2.6*

LPS-treated mice vs. vehicle-treated mice, $n = 5$ for each group

* $p < 0.05$

Fig. 2 Circulating white blood cell counts are elevated in both P2Y₁-null mice and WT mice during LPS-induced inflammation. Mice were treated with either a single i.p. dose of LPS (3 mg/kg) or of the vehicle only (PBS). After 24 h, blood samples were collected by cardiac puncture in 3.8% sodium citrate (10:1) and hematology studies were performed. The graphs show counts of white blood cells (WBC) (a), neutrophils (PMN) (b), lymphocytes (LY) (c), and platelets (d) in WT (black) and P2Y₁-null (white) mice for both the LPS-treated and vehicle-treated groups. Values are expressed as 1×10^3 cells/ μ L and plotted as the mean $\pm$ SEM (* p < 0.05 LPS-treated mice vs. vehicle treated mice, n = 5)

treated groups vs. untreated groups), with similar results in both the WT and P2Y₁-null backgrounds (Fig. 3a). I analyzed representative images of lung sections (H&E staining; Fig. 3b) and found that lung architecture was compromised in LPS-treated mice but not in the vehicle-treated mice. We found similar results in both the P2Y₁-null and WT backgrounds (p < 0.05; LPS-treated groups vs. untreated groups). Likewise, histology scores (Fig. 3c) based on alveolar capillary congestion, hemorrhage, infiltration or aggregation of neutrophils in the airspace or the vessel wall, and thickness of the alveolar wall were significantly higher in the LPS-treated WT and P2Y₁-null groups compared with those in their respective vehicle-treated groups (p < 0.05; LPS-treated groups vs. untreated groups). Again, I observed no difference in the lung injury between WT and P2Y₁-null mice in response to LPS exposure. Neutrophil infiltration was analyzed using MPO measurements, as shown in Fig. 3d. These data are consistent with the H&E findings in which neutrophil infiltration was higher in both the LPS-treated P2Y₁-null and

WT mice than in either of the vehicle-treated P2Y₁-null or WT mice (p < 0.05; LPS-treated groups vs. untreated groups). My results are consistent with previous findings in which sepsis-induced lung injury was not altered in P2Y₁-null mice compared with that in the septic WT group [13]. Interestingly, P2Y₁ seems not to be expressed in the lung [31] or on neutrophils [9], which could explain why lung injury is not affected by a loss of P2Y₁ signaling.

In contrast with my findings, P2Y₁ deficiency was protective in a model of neuroinflammation induced by cerebral stroke [11] and in a model of formalin-induced inflammatory pain [12]. Moreover, P2Y₁ on endothelial cell surfaces modulates leukocyte recruitment during vascular inflammation [10]. As P2Y₁ seems to be selectively expressed in certain immune cells (such as lymphocytes, monocytes, and platelets [9]) but not in others (such as neutrophils [9]), the differences in outcomes may be related to the immune cells mediating the biological/pathological phenomena observed while using a specific model.

Fig. 3 LPS-induced lung injury is not changed in P2Y₁-null mice. **a** Lung injury was evaluated in WT and P2Y₁-null mice treated with either a single i.p. dose of LPS (3 mg/kg) or of the vehicle only (PBS). Samples were collected 24 h after treatment. **a** Lung weights are expressed as organ weight (milligrams) per body weight (grams) (**p* < 0.05; LPS-treated WT mice vs. vehicle-treated WT mice, LPS-treated P2Y₁-null mice vs. vehicle-treated P2Y₁-null mice; *n* = 5). **b** Photomicrographs of hematoxylin- and eosin-stained tissue sections obtained after either a single dose i.p. of LPS or of the vehicle only (PBS). Representative images of lung tissue specimens are shown for LPS- and vehicle-treated groups in both WT (black) and P2Y₁-null

(white) backgrounds (magnification=20×; *n* = 5). **c** Acute lung injury scores are based on alveolar capillary congestion, hemorrhage, infiltration or aggregation of neutrophils in the airspace or the vessel wall, and thickness of the alveolar wall. ALI was evaluated in WT (black bars) and P2Y₁-null (white bars) backgrounds, with some groups treated with LPS and other groups treated with the vehicle only (**p* < 0.05; LPS-treated mice vs. vehicle-treated; *n* = 5). **d** MPO analysis was performed in lung samples of wild type (black) and P2Y₁-null (white) mice following either LPS or vehicle exposure. Values are expressed as relative fluorescent units per minute per milligram, mean ± SEM. (**p* < 0.05; LPS-treated mice vs. vehicle-treated mice, *n* = 5)

Activation of P2Y₁, a G_q-coupled receptor, causes calcium mobilization and activation of PLCβ [1]. PLCβ is a key regulator of cell functions [32] and seems to be activated during inflammation in leukocytes [33] and endothelial cells [34]. Hence, it is possible that the protective effects associated with a P2Y₁ deficiency that have been observed in other models of inflammation resulted from decreased PLCβ activity. In contrast, activation of PLCβ may not be a determinant for LPS-induced lung injury, especially considering that P2Y₁ seems not to be expressed in the lung [5]. In platelets, P2Y₁ signaling leads to platelet shape changes and calcium mobilization [1], whereas P2Y₁₂ signaling leads to inhibition of adenylyl cyclase and potentiation of platelet secretion of signaling molecules, including inflammatory mediators. As a result, P2Y₁₂ deficiency is expected to result

in decreased platelet secretion of inflammatory mediators, which would likely lead to reduced lung injury during inflammation. In contrast, P2Y₁ signaling in platelets does not potentiate secretion, which might explain why P2Y₁ deficiency offers no protection against lung injury during LPS-induced inflammation.

Conclusions

LPS-induced inflammation and lung injury were not altered by the loss of P2Y₁ signaling, which is consistent with what has been previously observed in sepsis-induced lung injury. These data suggest that lung injury is independent of the receptor P2Y₁. However, this is in contrast with what has been observed with vascular and neuroinflammation, indicating

that the role of P2Y₁ during inflammation may depend on the type of inflammation.

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Authorship E.L. designed and conducted the experiments described, carried out the data analysis and statistical tests presented, interpreted the results, and wrote the manuscript.

Compliance with ethical standards All procedures for the care and handling of the animals adhered to the National Institutes of Health standards and were approved by the Institutional Animal Care and Use Committee at Temple University School of Medicine (Philadelphia, PA).

Conflict of interest The author declares that she has no conflict of interest.

References

- Johnson ER, Matthay MA (2010) Acute lung injury: epidemiology, pathogenesis, and treatment. *J Aerosol Med Pulm Drug Deliv* 23(4):243–252. doi:10.1089/jamp.2009.0775
- Asaduzzaman M, Lavasani S, Rahman M, Zhang S, Braun OO, Jeppsson B, Thorlacius H (2009) Platelets support pulmonary recruitment of neutrophils in abdominal sepsis. *Crit Care Med* 37(4):1389–1396. doi:10.1097/CCM.0b013e31819ceb71
- Hagiwara S, Iwasaka H, Hasegawa A, Oyama M, Imatomi R, Uchida T, Noguchi T (2011) Adenosine diphosphate receptor antagonist clopidogrel sulfate attenuates LPS-induced systemic inflammation in a rat model. *Shock* 35(3):289–292
- Semple JW, Italiano JE Jr, Freedman J (2011) Platelets and the immune continuum. *Nat Rev Immunol* 11(4):264–274. doi:10.1038/nri2956
- Semple JW, Freedman J (2010) Platelets and innate immunity. *Cell Mol Life Sci* 67(4):499–511
- Kunapuli SP, Ding Z, Dorsam RT, Kim S, Murugappan S, Quinton TM (2003) ADP receptors—targets for developing antithrombotic agents. *Curr Pharm Des* 9(28):2303–2316
- Jin J, Kunapuli SP (1998) Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. *Proc Natl Acad Sci U S A* 95(14):8070–8074
- Jin J, Daniel JL, Kunapuli SP (1998) Molecular basis for ADP-induced platelet activation II. The P2Y₁ receptor mediates ADP-induced intracellular calcium mobilization and shape change in platelets. *J Biol Chem* 273(4):2030–2034
- Jin J, Dasari VR, Sistare FD, Kunapuli SP (1998) Distribution of P2Y receptor subtypes on haematopoietic cells. *Br J Pharmacol* 123(5):789–794. doi:10.1038/sj.bjp.0701665
- Zerr M, Hechler B, Freund M, Magnenat S, Lanois I, Cazenave JP, Leon C, Gachet C (2011) Major contribution of the P2Y₁ receptor in purinergic regulation of TNF α -induced vascular inflammation. *Circulation* 123(21):2404–2413
- Barragan-Iglesias P, Mendoza-Garcés L, Pineda-Farías JB, Solano-Olivares V, Rodríguez-Silverio J, Flores-Murrieta FJ, Granados-Soto V, Rocha-González HI (2015) Participation of peripheral P2Y₁, P2Y₆ and P2Y₁₁ receptors in formalin-induced inflammatory pain in rats. *Pharmacol Biochem Behav* 128:23–32. doi:10.1016/j.pbb.2014.11.001
- Malin SA, Molliver DC (2010) Gi- and Gq-coupled ADP (P2Y) receptors act in opposition to modulate nociceptive signaling and inflammatory pain behavior. *Mol Pain* 6:21. doi:10.1186/1744-8069-6-21
- Liverani E, Rico MC, Tsygankov AY, Kilpatrick LE, Kunapuli SP (2016) P2Y₁₂ receptor modulates sepsis-induced inflammation. *Arterioscler Thromb Vasc Biol* 36(5):961–971. doi:10.1161/ATVBAHA.116.307401
- Wang L, Ostberg O, Wihlborg AK, Brogren H, Jern S, Erlinge D (2003) Quantification of ADP and ATP receptor expression in human platelets. *J Thromb Haemost* 1(2):330–336
- Sasaki Y, Hoshi M, Akazawa C, Nakamura Y, Tsuzuki H, Inoue K, Kohsaka S (2003) Selective expression of Gi/o-coupled ATP receptor P2Y₁₂ in microglia in rat brain. *Glia* 44(3):242–250
- Ben Addi A, Cammarata D, Conley PB, Boeynaems JM, Robaye B (2010) Role of the P2Y₁₂ receptor in the modulation of murine dendritic cell function by ADP. *J Immunol* 185(10):5900–5906
- Wang L, Jacobsen SE, Bengtsson A, Erlinge D (2004) P2 receptor mRNA expression profiles in human lymphocytes, monocytes and CD34+ stem and progenitor cells. *BMC Immunol* 5:16
- Liu Y, Gao XM, Fang L, Jennings NL, Su YQX, Samson AL, Kiriazis H, Wang XF, Shan L, Sturgeon SA, Medcalf RL, Jackson SP, Dart AM, Du XJ (2011) Novel role of platelets in mediating inflammatory responses and ventricular rupture or remodeling following myocardial infarction. *Arterioscler Thromb Vasc Biol* 31(4):834–841. doi:10.1161/ATVBAHA.110.220467
- Abdulla A, Awla D, Hartman H, Rahman M, Jeppsson B, Regner S, Thorlacius H (2011) Role of platelets in experimental acute pancreatitis. *Br J Surg* 98(1):93–103. doi:10.1002/bjs.7271
- Winning J, Claus RA, Pletz MW, Bauer M, Losche W (2011) Adenosine diphosphate receptor antagonist clopidogrel sulfate attenuates LPS-induced systemic inflammation in a rat model. *Shock* 36(3):317 author reply 317–318
- Fabre JE, Nguyen M, Latour A, Keifer JA, Audoly LP, Coffman TM, Koller BH (1999) Decreased platelet aggregation, increased bleeding time and resistance to thromboembolism in P2Y₁-deficient mice. *Nat Med* 5(10):1199–1202
- Leon C, Hechler B, Freund M, Eckly A, Vial C, Ohlmann P, Dierich A, LeMeur M, Cazenave JP, Gachet C (1999) Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y₁ receptor-null mice. *J Clin Invest* 104(12):1731–1737
- Liverani E, Rico MC, Yaratha L, Tsygankov AY, Kilpatrick LE, Kunapuli SP (2013) LPS-induced systemic inflammation is more severe in P2Y₁₂ null mice. *J Leukoc Biol* 95(2):313–323. doi:10.1189/jlb.1012518
- Kang S, Lee SP, Kim KE, Kim HZ, Memet S, Koh GY (2009) Toll-like receptor 4 in lymphatic endothelial cells contributes to LPS-induced lymphangiogenesis by chemotactic recruitment of macrophages. *Blood* 113(11):2605–2613
- Nagano I, Takao T, Nanamiya W, Takemura T, Nishiyama M, Asaba K, Makino S, De Souza EB, Hashimoto K (1999) Differential effects of one and repeated endotoxin treatment on pituitary-adrenocortical hormones in the mouse: role of interleukin-1 and tumor necrosis factor- α . *Neuroimmunomodulation* 6(4):284–292
- Chen Y, Wang W, Wang H, Li Y, Shi M, Li H, Yan J (2016) Rapamycin attenuates splenomegaly in both intrahepatic and prehepatic portal hypertensive rats by blocking mTOR signaling pathway. *PLoS One* 11(1):e0141159. doi:10.1371/journal.pone.0141159
- Kostyak JC, Bhavanasi D, Liverani E, McKenzie SE, Kunapuli SP (2014) Protein kinase C δ deficiency enhances megakaryopoiesis and recovery from thrombocytopenia. *Arterioscler Thromb Vasc Biol* 34(12):2579–2585. doi:10.1161/ATVBAHA.114.304492
- Tolosano E, Fagoonee S, Hirsch E, Berger FG, Baumann H, Silengo L, Altruda F (2002) Enhanced splenomegaly and

- severe liver inflammation in haptoglobin/hemopexin double-null mice after acute hemolysis. *Blood* 100(12):4201–4208. doi:[10.1182/blood-2002-04-1270](https://doi.org/10.1182/blood-2002-04-1270)
29. Chen F, Liu Z, Wu W, Rozo C, Bowdridge S, Millman A, Van Rooijen N, Urban JF Jr, Wynn TA, Gause WC (2012) An essential role for TH2-type responses in limiting acute tissue damage during experimental helminth infection. *Nat Med* 18(2):260–266
 30. Uehara K, Uehara A (2011) P2Y1, P2Y6, and P2Y12 receptors in rat splenic sinus endothelial cells: an immunohistochemical and ultrastructural study. *Histochem Cell Biol* 136(5):557–567. doi:[10.1007/s00418-011-0859-2](https://doi.org/10.1007/s00418-011-0859-2)
 31. Communi D, Painsavoin P, Place GA, Parmentier M, Boeynaems JM (1999) Expression of P2Y receptors in cell lines derived from the human lung. *Br J Pharmacol* 127(2): 562–568. doi:[10.1038/sj.bjp.0702560](https://doi.org/10.1038/sj.bjp.0702560)
 32. Litosch I (2002) Novel mechanisms for feedback regulation of phospholipase C-beta activity. *IUBMB Life* 54(5):253–260. doi:[10.1080/15216540215673](https://doi.org/10.1080/15216540215673)
 33. Wu D (2005) Signaling mechanisms for regulation of chemotaxis. *Cell Res* 15(1):52–56. doi:[10.1038/sj.cr.7290265](https://doi.org/10.1038/sj.cr.7290265)
 34. Pober JS, Sessa WC (2007) Evolving functions of endothelial cells in inflammation. *Nat Rev Immunol* 7(10):803–815. doi:[10.1038/nri2171](https://doi.org/10.1038/nri2171)