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The P2X₁ Receptor Is Required for Neutrophil Extravasation during Lipopolysaccharide-Induced Lethal Endotoxemia in Mice

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Extracellular ATP is becoming increasingly recognized as an important regulator of inflammation. However, the known repertoire of P2 receptor subtypes responsible for the proinflammatory effects of ATP is sparse. We looked at whether the P2X₁ receptor, an ATP-gated cation channel present on platelets, neutrophils, and macrophages, participates in the acute systemic inflammation provoked by LPS. Compared with wild-type (WT) mice, P2X₁⁻/⁻ mice displayed strongly diminished pathological responses, with dampened neutrophil accumulation in the lungs, less tissue damage, reduced activation of coagulation, and resistance to LPS-induced death. P2X₁ receptor deficiency also was associated with a marked reduction in plasma levels of the main proinflammatory cytokines and chemokines induced by LPS. Interestingly, macrophages and neutrophils isolated from WT and P2X₁⁻/⁻ mice produced similar levels of proinflammatory cytokines when stimulated with LPS in vitro. Intravital microscopy revealed a defect in LPS-induced neutrophil emigration from cremaster venules into the tissues of P2X₁⁻/⁻ mice. Using adoptive transfer of immunofluorescently labeled neutrophils from WT and P2X₁⁻/⁻ mice into WT mice, we demonstrate that the absence of the P2X₁ receptor on neutrophils was responsible for this defect. This study reveals a major role for the P2X₁ receptor in LPS-induced lethal endotoxemia through its critical involvement in neutrophil emigration from venules. The Journal of Immunology, 2015, 194: 739–749.

S ystemic inflammatory response syndrome (SIRS) is a serious condition related to systemic inflammation that results in multiple organ dysfunctions; acute lung injury (ALI) represents the most common and earliest organ failure. SIRS may occur as a consequence of various pathogenic events, with sepsis syndrome being the most prevalent and lethal cause (1).

Resident macrophages and neutrophils constitute two important factors in the acute inflammatory response (2). Macrophages release reactive oxygen species and proinflammatory chemokines and cytokines that recruit and activate circulating neutrophils. Although activation of neutrophils is usually vital for host defense, in the case of SIRS, their overenrollment recruitment leads to pathologic tissue damage due to the massive transmigration of these cells to the site of inflammation, and their subsequent activation results in the release of aggressive defense molecules, ultimately leading to profound tissue injury and organ failure (3). In addition, the inflammatory response activates blood coagulation and leads to a prothrombotic state that exacerbates the tissue damage and lethality (4).

Among the various factors involved in inflammation, extracellular ATP and its receptors are major regulators. Accordingly, ATP constitutes a “danger signal” or damage-associated molecular pattern that is massively released from stressed or dying cells in damaged tissues or in response to inflammatory stimuli (5–7). ATP elicits a variety of proinflammatory responses in macrophages and neutrophils, including the release of cytokines and chemokines, the production of reactive oxygen species, phagocytosis, and chemotaxis (8–13). Recently, the importance of ATP in the pathogenesis of ALI was highlighted. ATP accumulated in the lungs of LPS-treated mice, whereas enhancing the catabolism or blocking the production of ATP attenuated pulmonary neutrophil accumulation and tissue injury (14).

Two families of membrane receptors mediate the effects of ATP: P2X receptors, ligand-gated ion channels comprising seven known subtypes, and P2Y receptors, G protein–coupled receptors comprising eight subtypes (15). Among these, only a few have been clearly identified as regulators of inflammation. On neutrophils, the P2Y₂ receptor mediates cell responses by amplifying chemotactic signals (8, 16), thereby contributing to the influx of neutrophils into lung tissues and lethality during sepsis (17). On macrophages, activation of the P2X₇ receptor induces the release of proinflammatory cytokines and the initiation of cell death (18), whereby P2X₇ was shown to modulate the disease pathogenesis in several animal models of inflammatory and autoimmune conditions (19). On endothelial cells, the P2Y₂, P2Y₁, and P2Y₆ receptors positively regulate expression of the adhesion molecules ICAM-1 and VCAM-1, which are necessary for tissue infiltration by leukocytes (20–22).
Among the other P2 receptor subtypes likely to play a role in acute inflammation, the P2X<sub>1</sub> receptor deserves attention because it is expressed on numerous cell types involved in inflammation, such as neutrophils, macrophages, platelets, and vascular smooth muscle cells, but it appears to be absent from vascular endothelial cells (23–26). Notably, it was reported recently that the P2X<sub>1</sub> receptor facilitates W-peptide–induced neutrophil chemotaxis in Boyden chamber assays (27).

Our aim in the present work was to assess the role of the P2X<sub>1</sub> receptor in acute systemic inflammation in vivo. To this end, we looked at the effect of P2X<sub>1</sub> receptor deficiency in a mouse model of LPS-induced endotoxemia. Our data show that this receptor is critically involved in the mortality of mice during endotoxemia, as well as in the associated coagulopathy, tissue damage, and systemic inflammation. Moreover, we provide direct evidence that the main way in which the P2X<sub>1</sub> receptor participates in endotoxemia is through its involvement in neutrophil emigration from venules. These findings suggest that P2X<sub>1</sub> could represent a target for new therapeutic strategies to reduce host tissue damage caused by neutrophils.

Materials and Methods

**Materials**

LPS (Escherichia coli, serotype O55:B5) was from Sigma-Aldrich (Saint Quentin Fallavier, France). Xylazine (Rompun) and ketamine (IMAGENE 1000) were from Bayer (Leverkusen, Germany) and Merial (Lyon, France), respectively. Carboxylic acid diacetate, succinimidyl ester (carboxy-DFFDA, SE) and DDAO-SE were from Molecular Probes (Life Technologies, Saint Quentin Fallavier, France). Xylazine (Rompun) and ketamine (IMALGENE) were from Bayer (Leverkusen, Germany) and Merial (Lyon, France), respectively. Carboxy-DFFDA was from Transgene (Illkirch-Graffenstaden, France).

**Animals**

Wild-type (WT) and P2X<sub>1</sub><sup>−/−</sup> mice had a pure C57BL/6 genetic background (29). Genotyping was performed on mouse tail DNA by a PCR-amplification method (30). Animals were housed under nonspecific pathogen–free conditions in the animal facilities of Etablissement Français du Sang–Arsacé (agreement no. E67-482-10). Experiments were performed with sex-matched 8–12-wk-old mice.

**Mouse model of endotoxemia**

Age-, sex-, and weight-matched WT and P2X<sub>1</sub><sup>−/−</sup> mice were injected i.p. with the specified dose of LPS in normal saline. The general condition and mortality were recorded for up to 5 d.

**Histopathology and immunohistochemistry**

At the end of the experiments, the vasculature was flushed with 0.9% normal saline via a needle inserted into the left ventricle. The lung and liver tissues of WT and P2X<sub>1</sub><sup>−/−</sup> mice were removed and fixed in 4% paraformaldehyde overnight. The fixed samples were washed in PBS, snap-frozen in OCT embedding medium, and cross sectioned (5 μm thick). Some lung and liver sections were stained with H&E for histopathological examination. Lung sections were stained with a rat anti-mouse GR-1 mAb (BD Pharmingen) and counterstained with hematoxylin to visualize hepatocytes and precisely visualize hepatic sinusoids and blood vessels. The effluents were layered onto a three-step Percoll gradient (72, 64, and 36% medium supplemented with 0.5% FBS, penicillin-streptomycin, and apyrase (0.5 U/ml). The effluents were layered onto a three-step Percoll gradient (72, 64, and 52%) that was centrifuged at 1600 × g for 30 min. Cells at the 72/64% interface were removed and washed once in the isolation buffer before use in experiments. The neutrophil preparations were analyzed by flow cytometry using GR-1 and CD11b double immunostaining and were found to be >80% pure. Neutrophils (5 × 10<sup>6</sup> cells/ml) were incubated in RPMI 1640 medium supplemented with 0.5% FBS, penicillin-streptomycin, and apyrase (0.5 U/ml), with or without LPS (1, 10, or 100 ng/ml), in a humidified incubator (37°C, 5% CO<sub>2</sub>, and 95% air). Supernatants were collected, and IL-6 and TNF-α levels were measured by enzyme immunoassay using murine ELISA kits (R&D Systems).

**Isolation of mouse bone marrow neutrophils and LPS treatment in vitro**

Morphologically mature neutrophils were purified from mouse bone marrow by isotonic Percoll gradient centrifugation, as described previously (32). Briefly, mice were euthanized, and their tibiae and femurs were flushed with HBSS (without Ca<sup>2+</sup> or Mg<sup>2+</sup>) containing apyrase (0.5 U/ml). The effluents were layered onto a three-step Percoll gradient (72, 64, and 52%) that was centrifuged at 1600 × g for 30 min. Cells at the 72/64% interface were removed and washed once in the isolation buffer before use in experiments. The neutrophil preparations were analyzed by flow cytometry using GR-1 and CD11b double immunostaining and were found to be >80% pure. Neutrophils (5 × 10<sup>6</sup> cells/ml) were cultured in RPMI 1640 medium supplemented with 0.5% FBS, penicillin-streptomycin, and apyrase (0.5 U/ml), with or without LPS (1, 10, or 100 ng/ml), in a humidified incubator (37°C, 5% CO<sub>2</sub>, and 95% air). After 4 h, the supernatants were collected and quantified for TNF-α and IL-6 by enzyme immunoassay.

**Intravital microscopy of mouse cremaster muscle postcapillary venules**

WT and P2X<sub>1</sub><sup>−/−</sup> male mice, transiently anesthetized with inhaled isoflurane, were injected intracardially (i.v.s) with LPS (10 ng, 400 μl) or vehicle (saline). After 4 h, the mice were anesthetized with an i.p. injection of ketamine (100 mg/kg) and xylazine (20 mg/kg), and the cremaster muscle was surgically exteriorized to investigate neutrophil–vessel wall interactions (rolling or stable adhesion) and neutrophil extravasation in postcapillary venules (20–40 μm diameter) by intravital microscopy, as described (33). To visualize leukocyte–vessel wall interactions, circulating leukocytes were labeled by i.v. injection of 3,3′-dihexylcarbocyanine iodide (2.5 μl a 100-μl solution/g of body weight). For neutrophil extravasation studies, the mice were injected i.v. with a FITC-conjugated rat anti-mouse Ly6G mAb (clone 1A8; 0.25 mg/kg) 15 min before i.s. administration of LPS.

Neutrophil responses were monitored in real time under a fluorescent microscope (Leica Microsystems, Wetzlar, Germany) coupled to a charge-coupled device camera (CoolSNAP HQ2; Photometrics, Tucson, AZ). Rolling cells were defined as cells moving more slowly than the flowing erythrocytes, and the rolling neutrophil flux was calculated as the number of neutrophils passing a reference line perpendicular to the blood flow during a 1-min period. Firmly adherent neutrophils were defined as cells that remained stationary for ≥30 s within a 100-μm vessel segment. Extravasated neutrophils had left the blood vessel completely but were still within a distance of 50 μm of the vessel wall. Image analyses were performed with Metamorph software (Molecular Devices, Sunnyvale, CA), and results were averaged over six vessels in each animal.
Adaptive transfer of neutrophils

Neutrophils isolated from the bone marrow of WT or P2X1−/− male mice were labeled with DDAO-SE (5 μM, red) or carboxy-DFFDA, SE (5 μM, green), respectively, at 37°C for 15 min. The labeled cells (4 × 10^6 neutrophils in HBSS containing 0.5 U/ml apyrase) were mixed (1:1) and injected i.v. into recipient WT or P2X1−/− male mice under isoflurane anesthesia. After 15 min, the mice were injected with LPS (10 ng, i.s.). The extravasation of adoptively transferred WT or P2X1−/− neutrophils was evaluated 4 h later in cremaster postcapillary venules by intravital fluorescence microscopy. Neutrophil emigration was defined as the number of cells in the extracellular space within a 200 × 500-μm² area, adjacent to the observed venule. Image analyses were performed with Metamorph software (Molecular Devices), and the results were averaged over six vessels in each animal.

Study approval

Ethical approval for the animal experiments was received from the French Ministry of Research, in accordance with the guidelines of the European Union and the Guide for the Care and Use of Laboratory Animals.

Statistical analyses

Statistical analyses were performed with GraphPad software (Prism 5.0). Survival studies were analyzed using the log-rank test (Fig. 1, Supplemental Fig. 2). All other data are reported as the mean ± SEM, and statistical comparisons were calculated using the Student t test (Figs. 2B, 3C, 5A, 5C, Supplemental Fig. 1). A p value < 0.05 was considered statistically significant.

Results

P2X1−/− mice are protected against lethal endotoxemia

To determine whether the P2X1 receptor contributes to the in vivo responses provoked by endotoxemia, WT and P2X1−/− mice were given a single i.p. injection of 10 mg/kg LPS, and their survival was monitored. Following LPS administration, WT mice displayed a series of responses that included shivering, piloerection, hypothermia, lethargy, and, ultimately, death. Within 48 h, 40% of the mice survived (Fig. 1). P2X1−/− mice exhibited similar symptoms, but the animals appeared less lethargic; most importantly, the vast majority (85%) of the mice survived (n = 20, p = 0.0003, log-rank test) (Fig. 1). Thus, it appears that the P2X1 receptor plays an important role in the sequence of events leading to death during endotoxemia.

P2X1−/− mice display less tissue damage and neutrophil sequestration in response to LPS

We next investigated whether the survival of P2X1−/− mice resulted from reduced organ damage and weaker inflammatory responses. Mice were injected i.p. with 10 mg/kg LPS, and the lungs were recovered 24 h later for histological analyses. Histopathological features of ALI, including alveolar wall thickening, airspace collapse, and inflammatory cell infiltration into interstitial spaces, were observed in the lungs of WT mice but were much less pronounced in those of P2X1−/− mice (Fig. 2A).

The infiltration of lung tissues by neutrophils, a feature of endotoxin-induced injury, was quantified by immunohistochemical staining of neutrophils on lung sections isolated 24 h after LPS administration. In WT and P2X1−/− mice receiving saline, small numbers of neutrophils were detected in the lung tissues (Fig. 2B). Following LPS challenge, numerous neutrophils were present in the lungs of WT mice, but significantly fewer were noted in the lungs of P2X1−/− animals (p = 0.017, n = 9) (Fig. 2B).

Liver damage also was dampened in P2X1−/− mice, as demonstrated by measurement of AST levels in plasma (p = 0.0069, n = 10) (Fig. 2C). Histological assessment of liver injury in H&E-stained liver sections revealed damaged hepatic lobules with spotty areas of necrosis in WT mice, whereas P2X1−/− mice displayed reduced histological changes with minimal hepatocyte necrosis (Supplemental Fig. 3), consistent with decreased serum levels of AST. Overall, these results showed that disruption of the P2X1 receptor gene reduces neutrophil accumulation in tissues and multiple organ injury in mice experiencing endotoxemia.

P2X1−/− mice display attenuated coagulopathy in response to LPS

Because systemic activation of coagulation represents a major complication of endotoxemia, we examined thrombin generation by measuring concentrations of TAT in plasma. In the absence of LPS stimulation, TAT levels were 7.0 ± 0.7 ng/ml (n = 5) and 7.1 ± 0.5 ng/ml (n = 5) in WT and P2X1−/− mice, respectively (data not shown). In WT mice, TAT levels had increased to 57.9 ± 5.2 and 78.6 ± 11.9 ng/ml at 6 and 24 h after injection of LPS, respectively. In contrast, P2X1−/− animals exhibited significantly lower concentrations of TAT, which were 50% of the levels observed in WT mice at 6 h (p = 0.0016, n = 5) and 24 h (p = 0.0015, n = 10) after LPS administration (Fig. 3A).

Thrombocytopenia, due to platelet aggregation and sequestration in the microvasculature, is considered part of the consumptive coagulopathy induced by LPS. Accordingly, immunohistology of lung tissues using an anti-mouse platelet Ab indicated the presence of numerous platelet aggregates within the lung capillaries of WT mice 24 h after LPS injection compared with reduced numbers in LPS-treated P2X1−/− mice (p = 0.0313, n = 9) (Fig. 3B). Concomitantly, a marked decrease in the blood platelet count was observed in WT mice, whereas this drop was significantly less in P2X1−/− mice (p < 0.01, n = 10, two-way ANOVA for the genotype effect and p < 0.001, WT and P2X1−/− mice receiving LPS, two-way ANOVA, followed by the Bonferroni post hoc test) (Fig. 3C). Altogether, these data indicated that endotoxemia results in attenuated coagulopathy in P2X1−/− mice compared with WT animals.

P2X1−/− mice display a reduced systemic inflammatory response to LPS

Proinflammatory cytokines and chemokines play a fundamental role in the development of endotoxemia, because they activate a variety of cellular responses of the innate immune system and are implicated in neutrophil sequestration in tissues. We measured
plasma levels of the main proinflammatory cytokines TNF-α and IL-6 and of the chemokines MIP-1α (CCL3), MIP-1β (CCL4), and RANTES (CCL5) in WT and P2X1<sup>−/−</sup> mice after i.p. injection of LPS. In the absence of LPS stimulation, these molecules were undetectable in murine plasma (data not shown). At 6 and 24 h after i.p. administration of LPS, WT mice had elevated circulating levels of the tested cytokines and chemokines (Fig. 4). In contrast, P2X1<sup>−/−</sup> animals showed markedly reduced plasma levels of all of these inflammatory mediators at 6 h and an even more pronounced difference at 24 h after LPS injection (Fig. 4). Thus, disruption of the P2X<sub>1</sub> receptor gene appeared to diminish the systemic inflammatory response to LPS.

P2X<sub>1</sub><sup>−/−</sup> macrophages and neutrophils produce normal amounts of proinflammatory cytokines and chemokines in response to LPS

Macrophages contribute to initiation of the inflammatory response through the release of proinflammatory cytokines and chemokines...
that attract and activate other immune cells. To explore the mechanisms responsible for the attenuated inflammatory response to LPS in P2X1<sup>−/−</sup> mice, resident peritoneal macrophages were isolated from WT and P2X1<sup>−/−</sup> mice and stimulated with LPS (1 or 10 ng/ml) for 24 h. As measured by ELISA, IL-6 and TNF-α were secreted at slightly lower levels by P2X1<sup>−/−</sup> macrophages compared with WT macrophages (Fig. 5A). However, the difference was not statistically significant, suggesting that the proinflammatory responses of macrophages from WT and P2X1<sup>−/−</sup> animals were similar, at least in vitro.

Additional in vivo investigations during endotoxemia were performed by measuring plasma concentrations of the keratinocyte...
The P2X<sub>1</sub> receptor is required for neutrophil emigration from venules in response to LPS in vivo

Because the P2X<sub>1</sub> receptor was found not to directly modulate the synthesis of proinflammatory cytokines and chemokines by macrophages or neutrophils, we hypothesized that this receptor might indirectly control the production of these mediators through the recruitment of neutrophils at sites of inflammation. To test this hypothesis, we looked for the presence of neutrophils in the hepatic vasculature, either within liver sinusoids or in the hepatic parenchyma, 24 h after LPS challenge. Liver sinusoids are particularly well suited for such analyses, because they are larger than the pulmonary capillaries and allow the precise localization of neutrophils either inside or outside the vessels. In the livers of WT mice, neutrophils were predominantly located outside the venules, either in the liver parenchyma or around the vessels ($p < 0.001$, $n = 5$) (Fig. 6A). In contrast, in P2X<sub>1</sub>−/− mice, neutrophils were predominantly observed inside the venules, adherent to endothelial cells, whereas considerably fewer neutrophils infiltrated the liver parenchyma ($p < 0.001$, $n = 5$) (Fig. 6A). These findings pointed to impaired neutrophil emigration from the circulation into the tissue in P2X<sub>1</sub>−/− mice during endotoxemia.

To elucidate why neutrophil extravasation is impaired in P2X<sub>1</sub>−/− mice, we monitored the behavior of neutrophils in postcapillary venules of the mouse cremaster muscle for 4 h after i.s. injection of LPS. Epifluorescent intravital video microscopy showed that, in untreated animals, neutrophils had minimal adhesive and emigration capacity (data not shown). In LPS-stimulated mice, the numbers of rolling and adherent neutrophils were significantly increased but were similar in WT and P2X<sub>1</sub>−/− animals (Fig. 6B). In contrast, 4 h after LPS injection, transmigration of neutrophils across the endothelium was significantly reduced in P2X<sub>1</sub>−/− mice compared with WT mice ($p = 0.0293$, $n = 5$) (Fig. 6B). Collectively, these results demonstrated that disruption of the P2X<sub>1</sub> receptor gene reduces the transendothelial migration of neutrophils during endotoxemia.

This difference in emigration between WT and P2X<sub>1</sub>−/− neutrophils could arise from differences in the (peri)vascular inflammatory environment between the two types of animals (e.g., differences in the local production of cytokines and chemokines) or from an intrinsic altered capacity of P2X<sub>1</sub>−/− neutrophils to

FIGURE 4. Deletion of the P2X<sub>1</sub> receptor reduces systemic production of cytokines and chemokines in response to LPS. WT and P2X<sub>1</sub>−/− mice were injected i.p. with LPS (10 mg/kg). Blood samples were collected 6 or 24 h later, and plasma concentrations of cytokines and chemokines were measured by ELISA. WT mice displayed markedly higher plasma concentrations of TNF-α, IL-6, MIP-1α, MIP-1β, and RANTES compared with P2X<sub>1</sub>−/− animals. Data are mean ± SEM (n = 6/group). For 6 h: IL-6, **p = 0.0059; TNF-α, ***p = 0.0033; MIP-1α, *p = 0.0172; MIP-1β, ***p = 0.0002; RANTES, *p = 0.0450. For 24 h: IL-6, *p = 0.0115; TNF-α, ***p = 0.0004; MIP-1α, *p = 0.0291; MIP-1β, *p = 0.0214, RANTES, ***p = 0.0013.

chemoattractants KC (CXCL1) and MIP-2 (CXCL2), two major chemokines that are rapidly released primarily by resident tissue macrophages and mastocytes after LPS administration (34, 35). Circulating levels of KC and MIP-2 were induced rapidly and peaked at similar concentrations in the blood of WT and P2X<sub>1</sub>−/− mice within 2 h of LPS injection (Fig. 5B), suggesting that the macrophages and/or mastocytes of WT and P2X<sub>1</sub>−/− mice display similar responses to endotoxemia in vivo. Nevertheless, after 24 h, P2X<sub>1</sub>−/− animals showed markedly reduced plasma concentrations of KC ($p = 0.0011$, $n = 7$) and MIP-2 ($p = 0.0045$, $n = 6$) compared with WT mice (Fig. 5B). We hypothesized that P2X<sub>1</sub> receptor–expressing cells other than resident cells might be responsible for this difference. Therefore, we looked at whether the P2X<sub>1</sub> receptor controls the production of proinflammatory cytokines in neutrophils. Bone marrow neutrophils were isolated from WT and P2X<sub>1</sub>−/− mice and stimulated with different concentrations of LPS. Analysis of the cell supernatants by ELISA showed that WT and P2X<sub>1</sub>−/− neutrophils secreted similar amounts of TNF-α and IL-6 in response to LPS (1, 10, or 100 ng/ml) in vitro (Fig. 5C). Overall, these results indicated that the P2X<sub>1</sub> receptor is unlikely to play a significant and direct role in regulating the release of proinflammatory cytokines and chemokines by macrophages and neutrophils in response to LPS.

This difference in emigration between WT and P2X<sub>1</sub>−/− neutrophils could arise from differences in the (peri)vascular inflammatory environment between the two types of animals (e.g., differences in the local production of cytokines and chemokines) or from an intrinsic altered capacity of P2X<sub>1</sub>−/− neutrophils to...
FIGURE 5. Deletion of the P2X1 receptor has no effect on proinflammatory cytokine synthesis in macrophages or neutrophils stimulated with LPS. (A) Resident peritoneal macrophages (2.5 × 10^6 cells/ml) from WT and P2X1^−/− mice were cultured or not with 1 or 10 ng/ml LPS for 24 h, after which supernatants were collected and assayed for TNF-α and IL-6 by ELISA. Results are mean ± SEM (n = 4). (B) WT and P2X1^−/− (Figure legend continues)
extravasate from blood vessels. To clarify this issue, we conducted an adoptive transfer of neutrophils isolated from WT and P2X1\(^{-/-}\) mice into WT or P2X1\(^{-/-}\) mice and compared the ability of the cells to extravasate from cremaster venules. WT and P2X1\(^{-/-}\) neutrophils were fluorescently labeled in vitro with DDAO-SE (red) or carboxy-DFFDA, SE (green), respectively, or vice versa. The two types of neutrophils were mixed (1:1) and then injected i.v. into WT or P2X1\(^{-/-}\) mice. The recipient animals were challenged i.v. with LPS (10 mg/kg); blood samples were collected 2, 6, or 24 h later; and plasma concentrations of CXCL1 and CXCL2 were measured by ELISA. Data are mean ± SEM. CXCL1: \(*p = 0.0011, n = 7\); CXCL2: \(*p = 0.0045, n = 6\). (C) Neutrophils (5 \(\times\) \(10^6\) cells/ml) from WT or P2X1\(^{-/-}\) mice were incubated or not with 1, 10, or 100 ng/ml LPS for 4 h. TNF-\(\alpha\) and IL-6 were quantified in the culture supernatants by ELISA, and results are mean ± SEM (n = 4).
were similar in WT and P2X$_{1}^{-/-}$ recipient mice ($p > 0.05$, $n = 5$); in contrast, the emigration of P2X$_{1}^{-/-}$ neutrophils was reduced in WT and in P2X$_{1}^{-/-}$ recipient mice ($p < 0.01$, $n = 5$) (Fig. 7). These observations revealed an intrinsic defect in the capacity of P2X$_{1}^{-/-}$ neutrophils to extravasate in vivo relative to WT neutrophils. In addition, these results indicated that the P2X$_{1}$ receptor from cell types other than neutrophils have no major role in the neutrophil emigration in response to LPS. Altogether, our results showed that the P2X$_{1}$ receptor of neutrophils acts as a positive regulator in the transendothelial migration of these cells from postcapillary venules during endotoxemia.

Discussion

Compelling evidence points to an important role for ATP and its P2 receptors in regulating inflammatory responses (13). Some of these proinflammatory functions were shown to be mediated by P2X$_{7}$ receptors on macrophages and P2Y$_{2}$ receptors on neutrophils (16, 19). However, other P2 receptor subtypes are also likely to play a role in the complex phenomenon of inflammation. The studies reported in this article demonstrate that the P2X$_{1}$ receptor is critically involved in the lethality of mice during endotoxemia and in the associated tissue damage, systemic inflammation, and coagulopathy. Moreover, we provide direct evidence that the main way in which P2X$_{1}$ participates in inflammation might occur earlier and, thereby, contribute to the protection against death.

To explore the potential mechanisms responsible for the reduced inflammatory state in the absence of the P2X$_{1}$ receptor, we investigated whether P2X$_{1}$ directly mediates cytokine synthesis in macrophages or neutrophils. Our data indicated that this receptor did not play a significant role in the synthesis of proinflammatory cytokines in macrophages or neutrophils stimulated with LPS in vitro. In addition, during endotoxemia in vivo, systemic levels of KC and MIP-2, the two major chemokines primarily released by resident macrophages, were comparable soon after LPS administration in the presence or absence of P2X$_{1}$. Hence, it is unlikely that the attenuated inflammatory response to LPS in P2X$_{1}^{-/-}$ animals was due to an intrinsic defect in the ability of macrophages or neutrophils to synthesize proinflammatory cytokines and chemokines.

Therefore, we examined whether the P2X$_{1}$ receptor affects cytokine and chemokine levels by influencing the accumulation of cytokine-producing neutrophils at inflammatory sites. Intravital microscopy showed that, in P2X$_{1}^{-/-}$ mice, LPS provoked less transmigration of neutrophils across the walls of cremaster venules, whereas neutrophil rolling or adhesion was unaffected. This defect was intrinsic to the cells, because P2X$_{1}^{-/-}$ neutrophils adoptively transferred into WT mice also exhibited less transmigration. Thus, our results provide evidence that the P2X$_{1}$ receptor plays a role in the emigration of neutrophils from the circulation into tissues. Hence, in WT mice stimulated with LPS, larger...
numbers of neutrophils are recruited to the lungs and other organs and release proinflammatory mediators, in particular MIP-1α, which recruits more neutrophils, thereby amplifying and sustaining the inflammatory reaction (37–39). Consistent with these data, the reduction in systemic levels of cytokines and chemokines in P2X$_1^{-/-}$ mice was more pronounced 24 h after LPS injection than 6 h after receiving LPS, when the accumulation of P2X$_1^{-/-}$ neutrophils was impaired.

One mechanism potentially contributing to the impaired trans-endothelial migration of P2X$_1^{-/-}$ neutrophils is an alteration of the surface expression of cell adhesion molecules. However, the surface expression of CD11b (Supplemental Fig. 1), CD29, or CD31 (data not shown), the main adhesion molecules involved in neutrophil transmigration, was found to be similar in blood neutrophils from WT and P2X$_1^{-/-}$ mice under resting conditions or after LPS administration. In addition, we did not observe any differences in neutrophil rolling or adhesion in inflamed cremaster venules. Alternatively, P2X$_1^{-/-}$ neutrophils might have an alteration in the intracellular signaling pathway responsible for cell movement. This is consistent with earlier studies suggesting that the P2X$_1$ receptor facilitates W-peptide–induced neutrophil chemotaxis in Boyden chamber assays through activation of the RhoA/Rho kinase pathway and myosin L chain phosphorylation, which promote contraction and retraction of the trailing uropod (27). Interestingly, deficiencies in the intracellular signaling proteins Rac1, p38 MAPK δ (p38δ), PTEN, and protein kinase D1 (PKD1), which directly or indirectly regulate actomyosin contractility, were shown to inhibit (Rac1, p38δ) or exacerbate (PTEN, PKD1) the recruitment of neutrophils to inflammatory sites and tissue damage (40–43). Thus, PTEN and PKD1 deficiencies exacerbate the lung tissue damage provoked by intratracheal instillation of LPS (41, 42). In contrast, Rac1 deficiency protects against the emphysema induced by intratracheal administration of iMLP (40), whereas p38δ deficiency attenuates LPS-induced ALI (41). Altogether, these properties suggest that P2X$_1$ receptor–dependent RhoA/Rho kinase–mediated neutrophil migration might contribute to the tissue damage and lethality provoked by LPS-induced endotoxemia.

Although the adoptive transfer of neutrophils definitely demonstrated that disruption of the P2X$_1$ receptor in these cells is at least partially responsible for the weaker inflammatory reaction in P2X$_1^{-/-}$ mice, one cannot exclude the involvement of other cell types expressing this receptor (e.g., platelets). Platelets are becoming increasingly recognized to contribute to the innate immune response to LPS. Thus, endothoxin infusion leads to thrombocytopenia mediated by neutrophil-dependent sequestration of platelets in the pulmonary microvasculature (44, 45). Activated platelets exacerbate the immune response to LPS by enhancing neutrophil activation, with the subsequent release of inflammatory cytokines and proteolytic material, thereby aggravating tissue damage (45). Interestingly, we observed less platelet sequestration in the microvasculature and less peripheral thrombocytopenia in LPS-treated P2X$_1^{-/-}$ mice, suggesting reduced platelet activation. The possibility that the platelet P2X$_1$ receptor, which participates in platelet activation (29, 46, 47), also plays a role in these processes cannot be ruled out. Studies of acute inflammation in mice presenting a platelet-specific deletion of the P2X$_1$ receptor should enable us to clarify this issue.

There is extensive cross-talk between inflammation and coagulation. Indeed, expression of tissue factor (TF), which triggers coagulation, is induced on leukocytes and vascular cells by endothoxin and inflammatory cytokines and contributes to LPS-induced coagulation, inflammation, and mortality (48). We observed less production of TAT complexes and fewer microvascular platelet aggregates in P2X$_1^{-/-}$ mice following LPS injection, indicating reduced systemic coagulopathy in these animals. This might be a consequence of the lower level of systemic inflammation in P2X$_1^{-/-}$ mice, which would lead to reduced induction of TF expression within the vasculature and in blood cells. Alternatively, we cannot exclude the possibility of a direct role for the P2X$_1$ receptor in TF exposure on leukocytes. These two mechanisms are not mutually exclusive, and both might contribute to the exacerbation of tissue injury and increased lethality.

Others investigators reported that P2X$_1$ receptor deficiency failed to protect against, and rather slightly increased, mortality in LPS-induced endotoxemia (49). The reasons for these discrepancies are not clear. One could argue that they might be related to differences in the severity of the endotoxemia. These investigators used a dose of 20 mg/kg LPS, whereas we used a lower dose (10 mg/kg). However, even at 20 mg/kg LPS, we observed a significant prolongation of the survival of P2X$_1^{-/-}$ mice compared with WT mice ($p = 0.0191$, log-rank test, $n = 30$), even though the overall mortality at 5 d was identical in the two groups (Supplemental Fig. 2). Alternatively, the differences could be related to the experimental design (e.g., monitoring of age, sex, and weight matching) or to the serotype of LPS. Further investigations will be necessary to clarify this issue.

Overall, our findings point to an important role for the P2X$_1$ receptor in neutrophil extravasation leading to tissue damage and lethality during endotoxemia. Hence, pharmaceutical approaches targeting this receptor might be useful to reduce neutrophil emigration and accumulation in tissues and, thereby, avoid excessive tissue injury in endotoxemia. The next step in this work is the development of selective P2X$_1$ receptor antagonists and their evaluation in mice in vivo. NF449 (4,49-imino-5,1,3-benzenetriyl[carbonylbis(imino-5,1,3-benzenetriyl-bis(carbonylimino))]tetraakisbenzene-1,3-disulfonic acid, octasodium salt), the only P2X$_1$ antagonist available, recently was shown to protect mice from ALI related to transfusion (50). Unfortunately, the short half-life of NF449 in vivo (46) precludes its evaluation in endotoxemia.

In summary, our results indicate that the neutrophil P2X$_1$ receptor plays a critical role in the sequestration of neutrophils in tissues, leading to tissue damage and lethality during endotoxemia. This receptor could represent a suitable new pharmaceutical target to control host tissue damage due to neutrophils. Given the implication of these cells in other pathological processes responsible for chronic inflammatory diseases, including rheumatoid arthritis, cystitis, inflammatory bowel disease, and asthma, further studies are awaited to evaluate the importance of the P2X$_1$ receptor in these pathologies.

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References


