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ORIGINAL ARTICLE

$P2X_1$ receptor blockers reduce the number of circulating thrombocytes and the overall survival of urosepsis with haemolysin-producing *Escherichia coli*

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Abstract

Urosepsis is a severe condition often caused by *Escherichia coli* that spontaneously have ascended the urinary tract to the kidneys causing pyelonephritis and potentially bacteraemia. The number of sepsis cases has been steadily increasing over the last decades, and there are still no specific, molecular supportive therapies for sepsis to supplement antibiotic treatment. $P2X_1$ receptors are expressed by a number of immune cells including thrombocytes, which presently have been established as an important player in the acute immune response to bacterial infections. $P2X_1$ receptor-deficient mice have been shown to be relatively protected against urosepsis, with markedly reduced levels of circulating proinflammatory cytokines and intravascular coagulation. However, here we show that continuous intravenous infusion with $P2X_1$ receptor antagonist markedly accelerates development of a septic response to induced bacteraemia with uropathogenic *E. coli*. Mice exposed to the $P2X_1$ receptor antagonists die very early with haematuria, substantially elevated plasma levels of proinflammatory cytokines, massive intravascular coagulation and a concomitant reduction in circulating thrombocytes. Interestingly, infusion of $P2X_1$ receptor antagonists causes a marked acute reduction in circulating thrombocytes and a higher number of bacteria in the blood. These data support the notion that the number of functional thrombocytes is important for the acute defence against bacteria in the circulation and that the $P2X_1$ receptor potentially could be essential for this response.

Keywords Sepsis · *E. coli* · $P2X_1$ · Antagonist · Thrombocytes

Introduction

Urinary tract infections (UTI) are exceedingly common and affect one out of three women under 24 years of age [1]. UTIs usually manifest as simple cystitis but can, in severe cases, particularly after instrumentation of the urinary tract, cause pyelonephritis [2] and potentially sepsis. Urinary tract infections are often caused by *Escherichia coli* (*E. coli*) [3] that are serotypically different from those found in the normal intestinal flora [4–6]. The *E. coli* that successfully invade the urinary tract produce several virulence factors [4–8], of which α-haemolysin (HlyA) is the most abundant. Notoriously, HlyA is more frequently isolated from patient samples with severe urinary tract infection [9]. In vitro biological effects of HlyA are intimately associated with ATP release and P2 receptor activation. ATP is released to the extracellular phase directly upon insertion of HlyA into biological membranes [10] and in the instances where HlyA cause fulminant cellular lysis. The HlyA induced cellular effects, including cellular damage, is to a very high extent, secondary to activation of P2 receptors in several cell types [11–14]. In vivo, P2 receptor activation is exceedingly important during urosepsis [15], and the $P2X_1$ receptor abundance have been shown to

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be corelated to the degree of reduction in circulating erythrocytes during sepsis [16].

Interestingly, both $P2X_4$ and $P2X_7$ receptor-deficient mice exhibit an extreme sensitivity towards HlyA-producing *E. coli* and develop fulminant sepsis with majorly elevated proinflammatory cytokines and disseminated intravascular coagulation in a model of urosepsis [15]. Opposed to these phenotypes, $P2X_1$ receptor-deficient mice are relatively protected against urosepsis. The survival is similar to the wild types, but the $P2X_1$ receptor-deficient mice exhibit lower levels of proinflammatory cytokines in plasma and a marked reduction in the intravascular coagulation [15]. The notion that reduced $P2X_1$ receptor activation is beneficial during a systemic response to bacterial infections is supported by an earlier study that demonstrated longer survival and reduced intravascular coagulation in $P2X_1$ deficient mice after injection of lipopolysaccharide (LPS) from *E. coli* (O55:B5) as a model of endotoxemia [17]. In both instances, the tested $P2X_1$ mice were on a C57bl/6j background and, thus, had a loss of function mutation in the $P2X_7$ in the splice variant expressed in T lymphocytes [18] and known to lack nucleotide-binding oligomerisation domain, leucine-rich repeat and pyrin domain-containing family member 1b (*Nlrp1b)* susceptibility-allele, which is important for activation of caspase-1 in certain types of infection [19]. Mice on C57bl/6j background are less prone to develop sepsis in response to intravenous injection of uropathogenic *E. coli* [15] and are potentially a less favourable background to test whether the $P2X_1$ receptor has any influence on the prognosis of sepsis. One could assume that a given effect of interference with the $P2X_1$ during bacteraemia could be more pronounced in mice that do not have any of the mentioned genetic variants and thus, are more susceptible to uropathogenic *E. coli* as the Balb/c background. The viability among the outcome of $P2X_1$ receptor knockout mice in various sepsis-like models could in theory result from using a relative immune resistant background. Thus, it is striking that another study that uses a different type of LPS from *E. coli* O111:B4 showed that $P2X_1$ receptor knockout mice had reduced survival and increased intravascular coagulation after LPS exposure compared with wild type [20] also on a C57bl/6 J background.

Since the $P2X_1$ receptor-deficient mouse is not available on Balb/c background, we instead tested the effect of continuous infusion of $P2X_1$ receptor blockers on the outcome of induced bacteraemia with uropathogenic *E. coli*. Here, we show that mice, infused with the $P2X_1$ receptor antagonists NF449 and NF279, as opposed to the $P2X_1$ receptor-deficient mouse, die very early of urosepsis compared with vehicle controls. Surprisingly, infusion of both substances alone caused a marked and immediate reduction in circulating thrombocytes, which was paralleled by a reduced ability to limit the number of circulating bacteria.

Methods

Escherichia coli

The uropathogenic, HlyA-producing *E. coli* strain ARD6 (serotype: O6:K13:H1) was obtained from Statens Serum Institute (Copenhagen, Denmark). The bacteria were grown on agar plates containing lysogeny broth (LB) medium and kept for up to 1 month at 4 °C. For each experiment, a fresh liquid preparation of *E. coli* was cultured overnight at 37 °C at 250 rpm by transferring one colony to 4 ml LB medium. The following morning, the culture was centrifuged twice and resuspended in sterile saline. *E. coli* was counted by flow cytometry (Accuri C6, BD Biosciences). In all experiments, isolated bacteria were transferred to sterile saline (150 μl) and given as an injection into one of the lateral tail vein (iv) in a total volume of 150 μl.

Animals

Animal experiments with P2X antagonists (NF279 and NF449) were performed on Balb/cJ mice from Janvier Labs (Saint Berthevin Cedex, France). All mice were males, 8–10 weeks of age and with an average weight of 23.81 ± 0.16 g. $P2X_1$ wild-type ($P2X_1^{+/+}$) and knockout mice ($P2X_1^{-/-}$) were bred at the Department of Biomedicine, Aarhus University by heterozygous breeding, and littermates were used. $P2X_1^{+/+}$ and $P2X_1^{-/-}$ mice were on a mixed background (C57/BL6J/Balb/cJ). The $P2X_1^{+/+}$ and $P2X_1^{-/-}$mice used in this study were 7- to 10-week-old mice of mixed sex with a weight of 23.8 ± 0.7 g. $P2X_1^{-/-}$ mice were originally developed by Richard J. Evans, University of Leicester, UK, who have kindly supplied us with breeder pairs.

Murine model of urosepsis

Sepsis was induced in mice on a Balb/cJ background according to previously described procedures [15]. For all experiments, mice were anaesthetised by ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}) in sterile NaCl (0.9%) as subcutaneous injection and placed on a heating plate at 38 °C. The initial bolus injection was topped up every 30–45 min to sustain full anaesthesia for up to 6 h. *E. coli* were injected in one of the lateral tail veins in a total volume of 150 μl sterile saline. To monitor survival, the number of bacteria was adjusted to be fatal within 3 h for around 50% of the mice exposed to the uropathogenic bacteria alone (165×10^6 HlyA-producing *E. coli* iv). Our previous data demonstrated that this bacterial load caused a ceiling effect in terms of circulating proinflammatory cytokines [15]. Therefore, we harvested blood for further analysis in parallel experiments, where the bacterial load was reduced by a factor 0.25 corresponding to 41.3×10^6 HlyA-producing *E. coli*. The quality of blood samples from

animals that have died spontaneously could not be compared with samples collected in live animals. Therefore, we collected the blood samples well ahead of fatal events in all of the compared groups. For both protocols, NF279, NF449 and saline were given as a bolus (40 mg kg^{-1}) immediately before bacteria or saline were injected. Thereafter, the mice received a continuous infusion of $P2X_1$ receptor antagonist (40 mg kg^{-1} h^{-1}) or the corresponding sterile salt solution using a syringe pump from Harvard Apparatus (Holliston, Massachusetts, USA) with a flow of 100 μl h^{-1}. The experiments performed in this study were approved by the Danish ethic committee for animal research "Dyreforsøgstilsynet" (2014-15-0201-00316).

Blood samples

Murine blood Immediately before the mice were euthanised, blood was drawn from the abdominal inferior vena cava into a heparinised syringe and either used directly as a whole blood sample or after centrifugation at 1000*g* for 10 min at 4 °C to isolate plasma. Whole blood samples were used for thrombocyte count, and bacterial load is evaluated by counting the colony-forming units (CFU). Plasma was used for measurements of intravascular haemolysis, cytokine levels and thrombin-antithrombin (TAT) complexes.

Human blood Blood was drawn from healthy volunteers in 5 ml EDTA-containing tubes on the day of the experiment. All subjects gave their written consent and the Danish Scientific Ethics Committee had approved the sampling procedure (Videnskabsetisk Komité-RegionMidt, M201100217). The blood was washed three times in 0.9% NaCl (twice at 1162*g*, 3 min, 4 °C), and once at 581*g*, 2 min, 4 °C), and the upper buffy coat containing the white blood cells was removed. The isolated erythrocytes were then washed once in HEPES buffered salt solution (HBS, 1162*g*, 3 min, 4 °C), and diluted in HBS for a 2.5% solution of washed erythrocytes, which was kept at 4 °C until use.

Haemolysis

Erythrocyte solution and HlyA were added to 96-well plates with or without agonists or antagonists for a final erythrocyte concentration of 1.25%. The plate was placed in an incubation chamber for 60 min at 37 °C under a constant swirl of 250 rpm. Thereafter, the plates were centrifuged (1162 g, 3 min, 4 °C), and the haemolytic activity was measured by transferring the supernatants to a clean 96-well plate and detecting the light absorbance (optical density, OD) at 410 nm on a spectrophotometer (Ultraspec III, LKB Biochrom). Haemolytic activity was calculated as a percentage of the maximal haemolysis induced by deionised water. In all experiments, a control without HlyA was subtracted from the samples. For measuring intravascular haemolysis, plasma was diluted (1:16) and measured as OD_{410}. The remaining plasma was stored at − 20 °C for later evaluation of cytokines and levels of thrombin-antithrombin complexes.

Cytokines

TNF-α, IL-1β, KC (murine equivalent of human IL-8) and IL-6 were all measured on stored plasma samples (− 20 °C) on a flow cytometer (BD Accuri C6, BD Biosciences) according to the manufacturer's instructions. Plasma was stored for a maximum of 30 days.

Thrombin-antithrombin (TAT) complexes

TAT complexes were measured in heparin-anticoagulated plasma samples with TAT Complexes Mouse ELISA Kit according to manufactures instructions (Abcam, Cambridge, UK). Plasma was stored for a maximum of 30 days.

Thrombocytes

The platelet-specific, hamster anti-mouse and anti-rat CD42d antibody and the FITC-conjugated secondary mouse anti-hamster antibody were from BD Biosciences. A whole blood sample (5 μl) was transferred to 60 μl PBS containing 2 μl antibody and incubated for 15 min. Afterwards, after 2 μl, secondary antibody was added and incubated for another 15 min. Then, a 20 μl sample was transferred to 1500 μl formaldehyde (0.02%), and the cells were investigated by flow cytometry (C6 Accuri) detecting 488 nm fluorescence, forward and side scatter. The cell count was back calculated to give the percentage of thrombocytes in the whole blood sample.

Bacterial load

Colony forming units (CFU) were determined in blood samples isolated from the animals before euthanasia. Whole blood was diluted 1/10, and 5 μl was plated on a blood agar plate and cultured overnight at 37 °C. The number of colonies was counted and expressed as CFU μl $blood^{-1}$.

Materials

The $P2X_1$ antagonists NF279, NF179, NF449 and Ro0437626 and the $P2X_1$ agonist MRS2219 were purchased from Tocris Bioscience (Bristol, UK). Apyrase was from Sigma-Aldrich. All substances were dissolved in sterile isotonic saline (0.9% NaCl). CBA flex sets for measuring cytokines were from BD Biosciences. TAT Complexes Mouse ELISA Kit for measuring levels of thrombin-antithrombin was from Abcam. HBS is consisted of (in mM) the following:

[Na^+] 138, [K^+] 5.3, [Cl^-] 132.9, [Ca^{2+}] 1.8, [Mg^{2+}] 0.8, [SO_4^{2-}] 0.8, [glucose] 5.6, [HEPES] 14, pH 7.4 at 37 °C. Phosphate-buffered saline (PBS): [Na^+] 158, [K^+] 4.5, [Cl^-] 139.7, [HPO_4^{2-}] 10, [$H_2PO_4^-$] 1.8, pH 7.4 at 37 °C.

Statistics

Statistical analysis was performed using GraphPad Prism software. Survival studies were illustrated by Kaplan-Meier plot and analysed by a log-rank test. All other data were reported as mean ± standard error of the mean (SEM), and *n* indicated the number of individuals or mice unless otherwise stated in the figure legend. The data were tested for normal distribution by Kolmogorov-Smirnov test. Normally distributed data were tested by Student's *t* test for single comparison and one-way ANOVA test for multiple comparisons to determine significant differences. In the few cases where the data did not meet the criteria of a normal distribution, the Mann-Whitney Wilcoxon matched pairs test for single comparison was used. A p value less than 0.05 was considered statistically significant.

Results

Our previous study on experimentally induced urosepsis revealed that the global lack of $P2X_7$ or $P2X_4$ receptor rendered mice much more susceptible to bacteraemia. They developed fulminant sepsis very rapidly after exposure to a bacterial load that caused 50% lethality in wild-type controls. The $P2X_7^{-/-}$ and $P2X_4^{-/-}$ mice died with massively elevated proinflammatory cytokine levels in plasma, acute tubular necrosis in the kidney and overstimulation of the coagulation system associated with a marked drop in circulating thrombocytes. In contrast, $P2X_1$ receptor-deficient mice were relatively protected against the massive septic development. $P2X_1$ receptor knockout mice did not have a statistically significant longer survival, but they did show markedly lower levels of circulating proinflammatory cytokines and substantially reduced activation of intravascular coagulation compared with wild-type controls [15]. The C57/bl6J background of $P2X_1^{-/-}$ mice is known to have a T lymphocyte-related loss of function mutation in $P2X_7$ [18], and to be deficient in NLRP-1b susceptibility allele [19]. Overall, this made the C57/bl6J mice less disposed to bacteraemia [15], and we speculated that acute inhibition of $P2X_1$ receptors would be more pronounced in a Balb/cJ model.

Thus, we tested the effect of the renowned $P2X_1$ receptor antagonist NF449 in our murine model of urosepsis. Urosepsis is not easily modelled since mice do not readily develop sepsis after installation of bacteria in the urinary tract. Therefore, we used a previously established model that mimics a fast-developing urinary tract infection as seen after instrumentation of the urinary tract [15, 21]. We injected uropathogenic *E. coli* directly into the tail veins of the mice of anaesthetised mice and observed them in a period of 6 h. Since NF449 is known to have a relatively short half-life ($T_{1/2}$), we chose to give the substance as continuous iv infusion in the mice during the observation period. We tested three infusion levels of NF449: 10 mg kg^{-1} h^{-1}, 20 mg kg^{-1} h^{-1} and 40 mg kg^{-1} h^{-1}. At 10 and 20 mg NF449 kg^{-1} h^{-1}, the cytokine response to bacterial infection (2.5 h) was similar to mice infused with saline (Suppl. Figs. 1 and 2), and moreover, the survival during infection was similar to saline infusion at an NF449 infusion of 10 mg kg^{-1} h^{-1} (Suppl. Fig. 1). Thus, we used 40 mg kg^{-1} h^{-1} for the experiment, which corresponds to the dose of NF449 needed to increase tail bleeding in mice in a previous study [22]. Figure 1 clearly shows that NF449 did not protect the mice against developing sepsis during bacteraemia. Instead, mice that were continuously infused with NF449 died markedly faster upon exposure to HlyA-producing *E. coli* than the mice infused with saline after exposure to HlyA-producing *E. coli* ($p = 0.003$). Notably, infusion with NF449 alone did not affect the survival (Fig. 1a). In our experience, mice that die early in our model of urosepsis tend to have severely elevated proinflammatory plasma cytokines. Therefore, we measured the cytokine levels in the plasma 2.5 h after the onset of infection. Injection of HlyA-producing *E. coli* alone resulted in a statistically significant increase in plasma levels of IL-1β, TNF-α and the mouse equivalent of IL-8, keratinocyte chemoattractant (KC). Strikingly, this was substantially intensified in mice exposed to NF449 in addition to the HlyA-producing *E. coli*, which now also showed a statistically significant increase in IL-6 compared with control. Notably, NF449 alone did not have any influence on the baseline cytokine production.

Normally, our model of urosepsis increases circulating levels of proinflammatory cytokines immensely. This elevation is closely associated with an increased activation of the coagulation system and a drop in circulating thrombocytes. We found a very similar pattern in the case of NF449 infusion. Figure 2a confirms our previously reported drop in thrombocyte number after exposure to HlyA-producing *E. coli* alone. This fall was intensified by NF449 infusion to a level where only approximately 1% of the circulating blood cells are platelets. Surprisingly, infusion of NF449 alone caused a statistically significant reduction of circulating thrombocytes, which is comparable to the fall observed in response to HlyA-producing *E. coli* alone. Figure 2b shows that in this experimental series the exposure to HlyA-producing *E. coli* alone did not cause a statistically significant increase in the plasma levels of TAT complexes. However, combined with NF449, HlyA-producing *E. coli* caused a statistically significant elevation of TAT. Parallel to the data on circulating thrombocytes, NF449 alone surprisingly increased the TAT complex

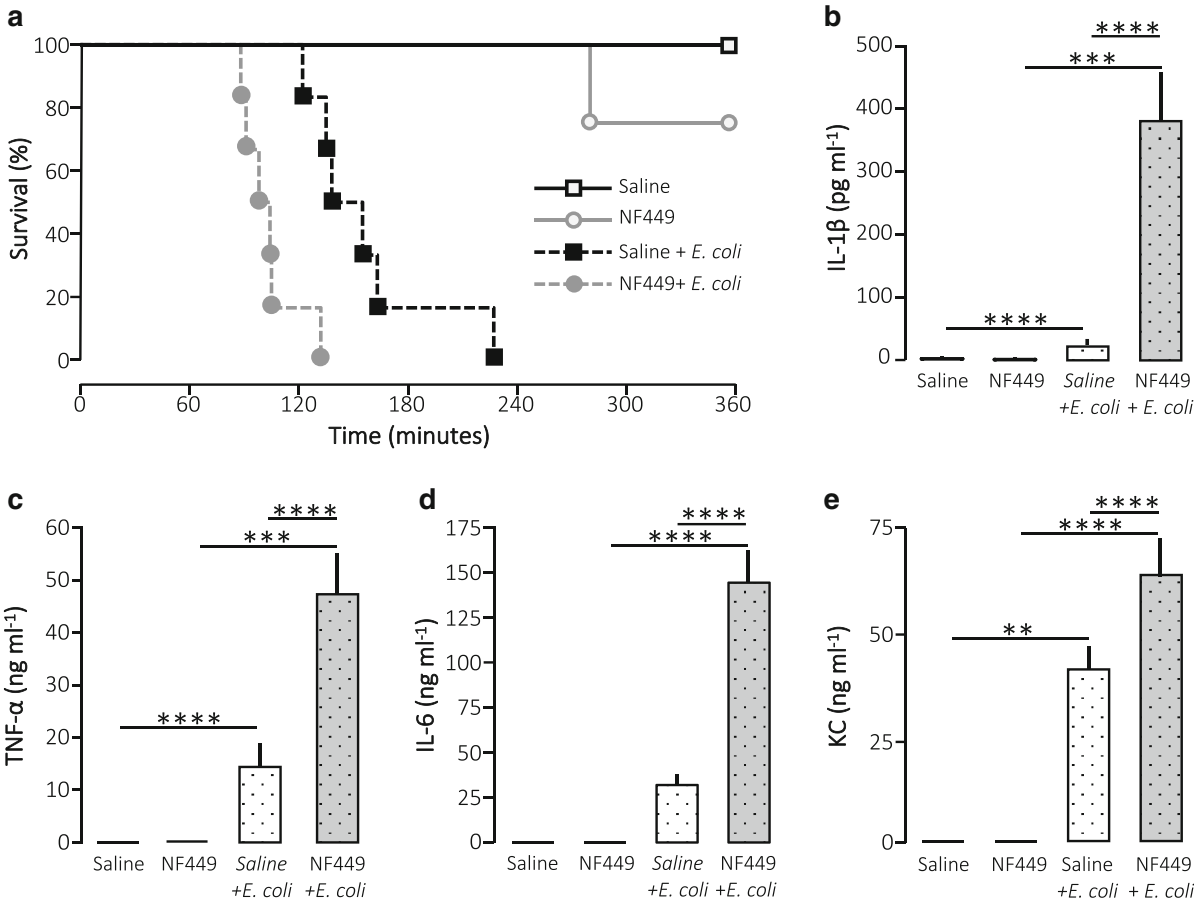

Fig. 1 Acute inhibition of $P2X_1$ receptor (NF449; 40 mg kg^{-1} h^{-1}) during ***E. coli***-induced sepsis. **a** Kaplan-Meier plot shows survival over 6 h after subjection to HlyA-producing *E. coli*. Anesthetised mice were continuously infused iv with either saline or NF449. In addition, the mice were either injected iv with HlyA-producing *E. coli* (165 million) or saline, $n = 2$ for saline infusion, 4 for NF449 infusion, 8 for HlyA-producing *E. coli* during saline infusion and 8 for HlyA-producing *E. coli* during NF449 infusion. There was a significant difference ($p < 0.05$) between all four groups. The corresponding levels of **b** IL-1β, **c** TNF-α,**d** IL-6 and **e** KC 2.5 h after injection of HlyA-producing *E. coli*. The cytokine levels were measured in parallel in mice 2.5 h after injection 41.25 million HlyA-producing *E. coli*. The data are given as mean ± sem, $n = 6$ for saline infusion, 5 for NF449 infusion, 12 HlyA-producing *E. coli* during saline infusion and 10 HlyA-producing *E. coli* during NF449 infusion, $*p < 0.05$, $**p < 0.01$,$***p < 0.001$, $****p < 0.0001$

level consistent with that the drug directly activating thrombocytes and the coagulation cascade in parallel.

These data suggest that either the mice in the presence of NF449 have a much more substantial response to the bacterial load or the bacterial load becomes more pronounced in the presence of NF449. We tested this by plating out whole blood collected from the mice 2.5 h after injection of the HlyA-producing *E. coli*. Figure 3a shows that the presence of NF449 markedly affected the number of bacteria in the blood. Infusion of NF449 massively increases the number of circulating living bacteria compared with infusion of saline. Even though P2 receptors or equivalents have not been described in bacteria, NF449 may potentially show off-target effects that influence bacterial proliferation. Figure 3b illustrates that the presence of NF449 caused a marked increase in the number of bacteria when these were grown in LB medium in vitro. Even though that this effect was only seen at very high concentrations of NF449, they prevent us from concluding with certainty that the observed effects are indeed caused by inhibition of the murine $P2X_1$ receptors.

Therefore, we set out to identify a $P2X_1$ receptor antagonist that does not affect bacterial growth. Initially, we tested three other $P2X_1$ receptor antagonists and one agonist in vitro. Figure 4 shows the previously demonstrated effect of $P2X_1$ receptor inhibition of HlyA-induced haemolysis, which was observed for all antagonists tested, whereas the $P2X_1$ receptor agonist MRS2211 slightly potentiated the HlyA-induced haemolysis in lower concentrations. The estimated the half maximal inhibitory concentration (IC_{50}) was lowest for NF279, with approximately 7 μM, whereas Ro0437626 did not show full inhibition of the haemolytic process. Thus, we tested if any of the $P2X_1$ receptor antagonists that fully inhibited the haemolytic process influenced the bacterial growth in vitro. Figure 4e shows the growth of HlyA-producing *E. coli* in LB medium alone or in the presence of either of the two $P2X_1$ receptor blockers that caused complete

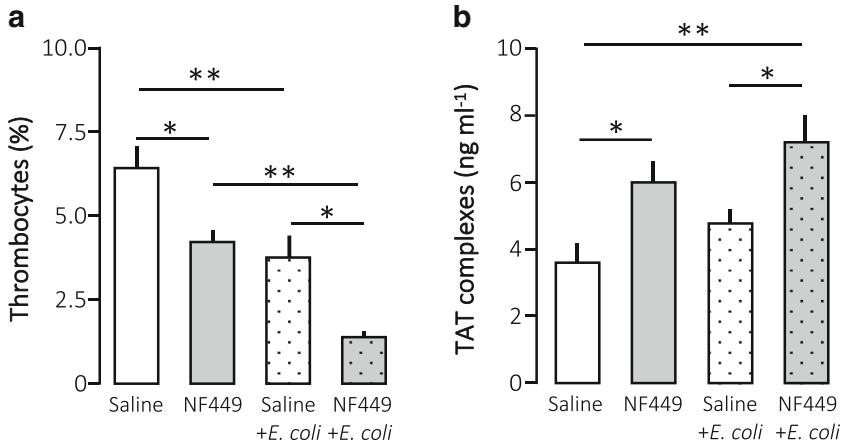

Fig. 2 The effect of $P2X_1$ receptor inhibition (NF449; 40 mg kg^{-1} h^{-1}) on thrombocyte and coagulation activation and erythrocyte damage in ***E. coli***-induced sepsis. Anaesthetised mice were infused iv with sterile saline or NF449 (40 mg kg^{-1} h^{-1}). In addition, the mice were either injected 41.3 millions of HlyA-producing *E.coli* or NF449 combined with HlyA-producing *E.coli*. A blood sample was collected after 2.5 h to determine **a** number of thrombocytes in blood, **b** intravascular coagulation measured as thrombin-antithrombin (TAT)-complexes. The bars indicate mean ± sem, $n = 7–10$, $^{*}p < 0.05$, $^{**}p < 0.01$,$^{***}p < 0.001$, $^{****}p < 0.0001$

inhibition in vitro (NF279 and NF157). Neither of the two substances had any influence on the growth of HlyA-producing *E. coli* (Fig. 4e). Moreover, apyrase up to 50 U ml^{-1} did not affect the bacterial growth, which led us to conclude that the effect of NF449 on the bacterial growth is unrelated to P2 receptors or P2-like receptors on the bacteria (Fig. 4f). Based on the concentration-response curve and the lack of effect on bacterial growth, we chose NF279 for the in vivo sepsis model.

Figure 5a shows that infusion of NF279, similar to the infusion of NF449, markedly reduced the survival of Balb/cJ mice exposed to HlyA-producing *E. coli*. However, exposure to NF279 alone, in fact, did reduce the survival in itself (Fig. 5a). Consistent to the data obtained with NF449, infusion with NF279 also markedly amplified the plasma levels of all the tested proinflammatory cytokines (IL-1β, IL-6, TNF-α and KC; Fig. 5b–e) whereas NF279 alone did not affect the plasma cytokine levels. Moreover, NF279, similar to NF449, amplified the reduction in circulating thrombocytes upon exposure to HlyA-producing *E. coli* (Fig. 6a). Additionally, NF279 alone caused an even more substantial acute thrombocytopenia in the mice compared with NF449. This sudden

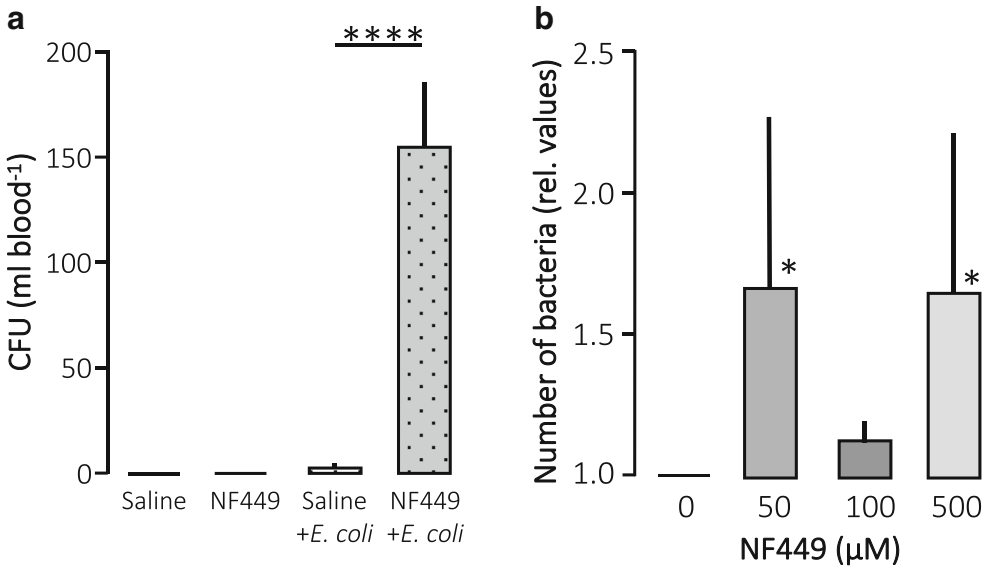

Fig. 3 Severity of infection after $P2X_1$ receptor inhibition during ***E. coli***-induced sepsis **a** shows number of colony forming units (CFU) in the blood collected 2.5 h after injection of 41.3 millions of HlyA-producing *E. coli* or saline to mice exposed to infusion with saline or NF449 (40 mg kg^{-1} h^{-1}). The colonies were counted after 24 h of culture on blood plates. The bars indicate mean ± sem, $n = 7–10$, **b** shows the effect of NF449 on the growth of HlyA-producing *E. coli* in LB medium in vitro. The bars indicate mean ± sem, $n = 5–7$ $^{*}p < 0.05$, $^{**}p < 0.01$,$^{***}p < 0.001$, $^{****}p < 0.0001$

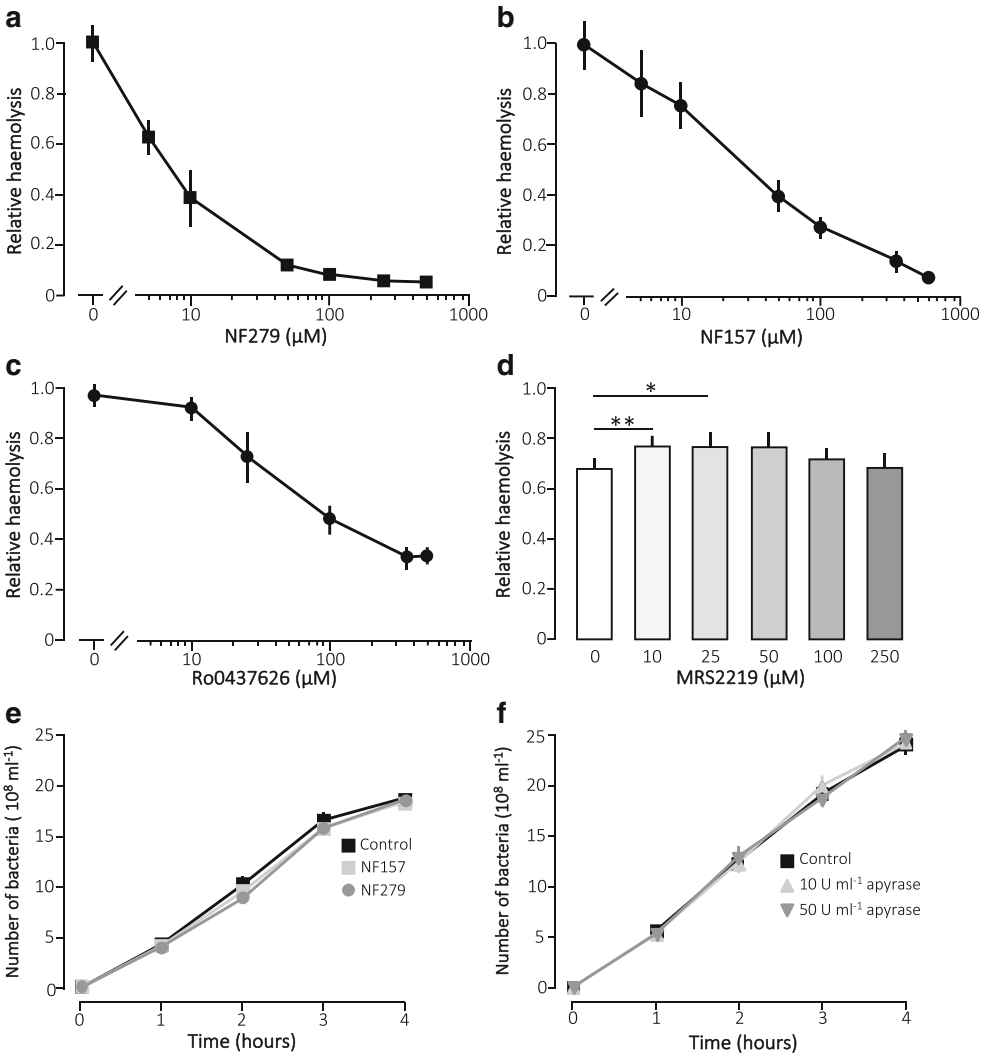

Fig. 4 The effects of $P2X_1$ modulations on HlyA-induced haemolysis in vitro in human erythrocytes and $P2X_1$ receptor antagonists and ATP scavenging and on *E. coli* growth in vitro. **a** NF279, $n = 5$; **b** NF157, $n = 5$; **c** Ro0437626, $n = 5$. All antagonists inhibit HlyA-induced haemolysis and the $P2X_1$ receptor agonist **d** MRS2219, $n = 5$ slightly potentiates haemolysis in human erythrocytes. ARD6 bacteria suspended in LB-growth medium and counted over time from 0 to 4.5 h in the presence or absence of **(e)** the $P2X_1$ antagonists NF279 or NF157, 250 μM or **(f)** the ATP scavenging enzyme apyrase, $n = 6$. *N* indicates number of repeats from 6 different cultures of ARD6. $*p < 0.05$, $**p < 0.01$

drop in circulating thrombocytes is unlikely to reflect sudden intravascular activation of the thrombocytes because there was no parallel increase in thrombin-antithrombin complex formation (Fig. 6b).

Interestingly, $P2X_1$ receptor inhibition with NF279, similarly to NF449, caused a marked increase in the number of circulating bacteria, as evident in whole blood samples taken from mice with experimentally induced bacteraemia. Since the whole blood sample is collected already after 2.5 h, the higher number of circulating bacteria is likely to reflect a reduced intravascular killing of the bacteria rather than an effect on proliferation. Thus, the data suggest that acute inhibition of $P2X_1$ receptors may impair the immune response to acute infection, potentially via a thrombocyte-related effect.

Discussion

Sepsis is a severe condition defined as life-threatening organ dysfunction that results from a dysregulated host response to an infection. The prognosis of sepsis has improved over the last 40 years, and the mortality is currently around 20% of all cases of sepsis [23]. Unfortunately, the number of sepsis cases has been steadily increasing over the last decades, and no specific molecular therapies for sepsis have yet been approved (for review see [24]). One of the major causes of sepsis is infections arising from the urogenital area and is often caused by *E. coli* that has spontaneously ascended the urinary tract to the kidneys causing pyelonephritis and potentially bacteraemia. Alternatively, the bacteria can spread from the urinary tract to the bloodstream, secondary to instrumentation of the urinary tract. The *E. coli* that successfully ascend to the kidney often produce the virulence factor HlyA. The biological effect of this pore-forming virulence factor has been demonstrated to be intimately associated with purinergic signalling [11, 25]. ATP is released immediately after insertion of HlyA to the plasma membrane [10], and many of the following biological effects of HlyA are a consequence of activation of P2 receptors in an auto and paracrine fashion. HlyA has recently been demonstrated to be a critical factor in the

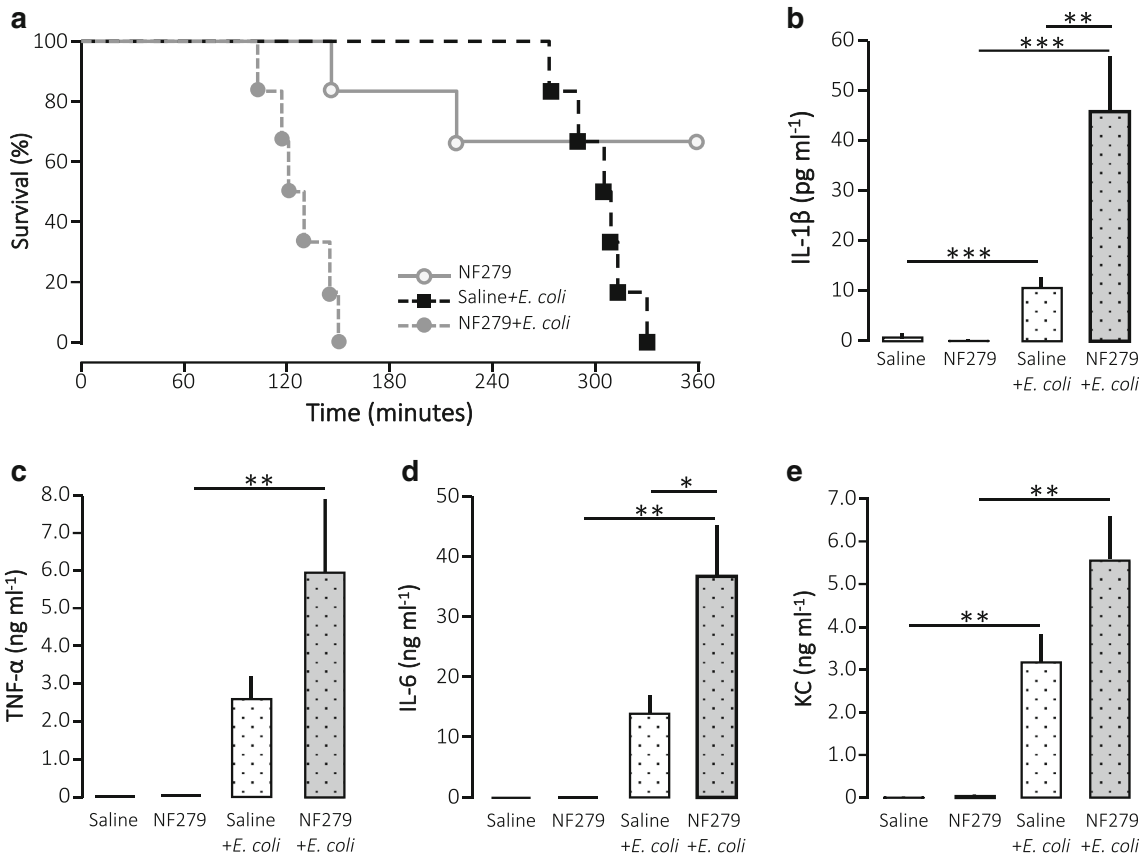

Fig. 5 The effect of the $P2X_1$ receptor inhibition NF279 (40 mg kg^{-1} h^{-1}) which do not affect bacterial growth in vitro in ***E. coli***-induced sepsis. **a** Kaplan-Meier plot shows survival over 6 h after subjection to HlyA-producing *E. coli*. Anesthetised mice were continuously infused iv with either saline or NF279. In addition, the mice were either injected iv with HlyA-producing *E. coli* (165 million) or saline, $n = 6$ for all three groups. There was a statistically significant difference ($p < 0.05$) between mice exposed to *E. coli* + saline and *E. coli* + NF279 and between mice exposed to *E. coli* + NF279 and NF279 alone. The corresponding levels of **b** TNF-α, **c** IL-1β, **d** IL-6 and **e** KC, 2.5 h after injection of HlyA-producing *E. coli*. The cytokine levels were measured in parallel in mice 2.5 h after injection of 41.3 million HlyA-producing *E. coli*. The data are given as mean ± sem, 5 mice were infused with saline and injected iv with saline, 5 mice were infused with NF279 and injected iv with saline, 12 mice infused with saline and injected with HlyA-producing *E. coli* and 10 mice were infused with NF279 and injected with HlyA-producing *E. coli*, $*p < 0.05$, $**p < 0.01$,$***p < 0.001$, $****p < 0.0001$

development of sepsis in response to circulating *E. coli* in mice [21]. In that study, *E. coli* transfected with a plasmid containing the full HlyA operon were compared with the exact same strain transfected with a parallel plasmid, which had a substantial deletion in the HlyA gene. The data were exceedingly clear and showed that if a strain expresses HlyA, the injected mice develop fulminant sepsis whereas if HlyA secretion is absent the mice exhibit little if any systemic response to the bacteraemia [21]. This allowed us to conclude that HlyA is a virulence factor during bacteraemia that pushes an induced bacteraemia towards sepsis.

Since ATP is an important damage-associated molecular patterns (DAMP) molecule and essential in immune cell (for review see [26]) and thrombocyte activation [27–29], it is likely that the HlyA-induced ATP release is central in the systemic reaction to bacteraemia. Previous results confirm that meddling with P2 receptors has substantial impact on the prognosis of sepsis. Lately, our group made a systematic review of induced bacteraemia with uropathogenic *E. coli* in mice globally knocked out for $P2X_1$, $P2X_4$ or $P2X_7$ receptors [15]. The $P2X_4$ and $P2X_7$ receptor-deficient mice showed a massive septic response to bacteraemia measured as extensive elevation in proinflammatory cytokines in plasma, drop in circulating thrombocytes, intravascular coagulation and haematuria [15]. Interestingly, we found that the complete opposite was the case in the $P2X_1$ receptor-deficient mice, which exhibited a very modest increase in proinflammatory cytokines after injection with HlyA-producing *E. coli* compared with control [15]. As mentioned, the $P2X_1$ receptor-deficient mouse was on a C57/bl6 background, which was remarkably immune resistant. Therefore, we tested whether we could observe a more pronounced phenotype by targeting the $P2X_1$ receptor on Balb/cJ background.

Here, we tested the effect of infusion of established $P2X_1$ receptor antagonists on the survival and immune response of Balb/cJ mice exposed to acute bacteraemia with

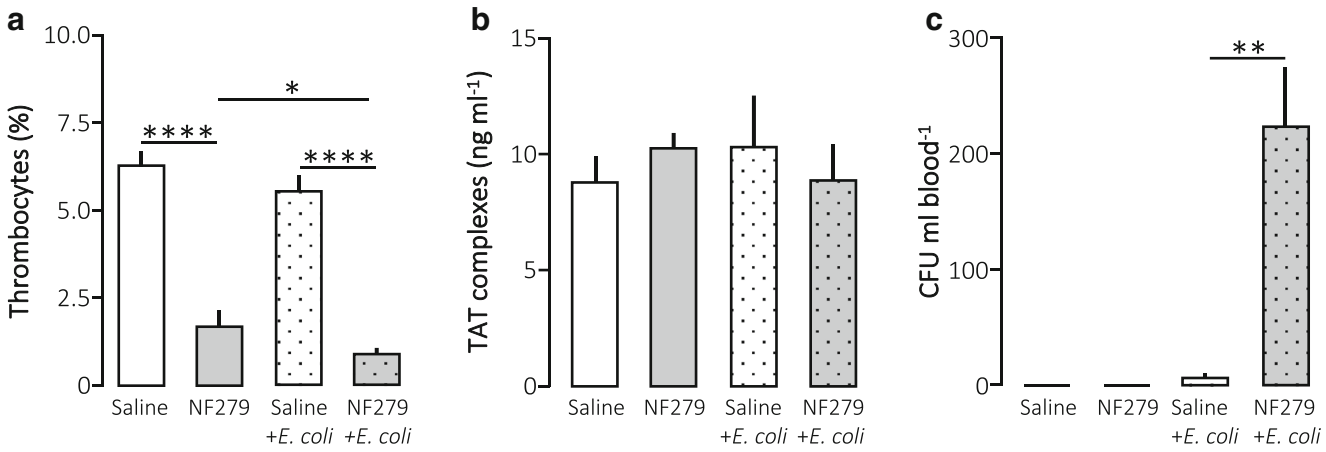

Fig. 6 $P2X_1$ receptor inhibition (NF279; 40 mg kg^{-1} h^{-1}) in ***E. coli***-induced sepsis. Anaesthetised mice were injected iv with 41.3×10^6 HlyA-producing *E. coli* for 2.5 h during constant infusion either saline or NF279. Blood sample was collected after 2.5 h to investigate whole blood and plasma. **a** Number of thrombocytes in blood, **b** intravascular coagulation measured as TAT-complexes, **c** colony forming units (CFU) in the blood collected 2.5 h after injection with HlyA-producing *E. coli* and cultured for 24 h, $n = 5$ for all four groups. $^*p < 0.05$

uropathogenic *E. coli*. Infusion of NF449 and NF279 both caused a marked reduction in the survival of mice exposed to HlyA-producing *E. coli*. The reduced survival was associated with substantial activation of the immune system measured as a sizable elevation of all the measured pro-inflammatory cytokines, early haematuria, reduction in circulating thrombocytes and increase in intravascular coagulation. All these findings are consistent with animals exposed to the $P2X_1$ receptor antagonists, which quickly develop a septic response to induced bacteraemia.

This reduced survival is in many ways a surprise and contradicts our previous data from $P2X_1$ receptor-deficient mice, tested in the exact same model [15]. There are many obvious reasons as to why there may be a discrepancy between global knockout of a receptor and acute inhibition of the given receptor. In this instance, the substantial drop in circulating thrombocytes during infusion with NF449 comes to mind. This effect is in many ways peculiar because an inhibition of $P2X_1$ receptors if anything should prevent activation of the thrombocytes [30–32]. Previous studies have reported a moderate reduction in thrombocyte activation after bolus injection with 10 mg kg^{-1} of NF449 [22]. This was substantiated by injection of a higher concentration of 50 mg kg^{-1} where NF449 supposedly also affect the $P2Y_1$ and $P2Y_{12}$ receptors on the thrombocytes [22]. Similarly, we find that a low infusion of 10–20 mg kg^{-1} h^{-1} did not influence the outcome of or the immune response to sepsis opposed to 40 mg kg^{-1} h^{-1}. Thus, the observed effect on the thrombocytes may potentially be an effect of inhibition of all thrombocytic P2 receptors. Notably, we observed the same effect with infusion of NF279, where the result was even more pronounced. However, there are reports that other drugs that interfere with platelet function have been shown to cause thrombocytopenia. This is primarily true for acetylsalicylic acid, although the actions are not acute and rely mainly on stimulation of antibody formation and inhibition of megakaryocyte function [33]. Moreover, it has also been reported casuistically for $P2Y_{12}$ receptor antagonists [34–36].

Whatever the cause is for the thrombocytopenia, early fall in platelet count is a negative clinical predictor for sepsis [37–40]. We found in our model of urosepsis complete consistency in the reduction of circulating thrombocytes, immune activation and early death of animals exposed to *E. coli* [15, 21]. A recent paper supports the notion that thrombocytes are implicated in the acute response to bacteria during sepsis regarding the role of thrombocytes in the septic response. The article demonstrates that mice lacking the damage-associated molecular pattern protein high-mobility group box 1 (HMGB1) specifically in thrombocytes die earlier of sepsis with higher bacterial load and higher levels of proinflammatory cytokines in plasma [41].

Thrombocytes have been established to be important in the first line of defence against intruding pathogens (for review see [42, 43]). Recently, thrombocytes have been shown to bind and trap fibrin coated *E. coli* in the circulation [44], and thus, severe thrombocytopenia may reduce the capacity for scavenging and inactivation of bacteria in general. Since both NF449 and NF279 cause acute thrombocytopenia in the mice, they may simultaneously reduce the mice defence against the injected *E. coli*. This could potentially explain why the mice infused with either NF449 or NF279 have a staggering higher number of bacteria in the bloodstream 2.5 h after injection of the same bacterial load, particularly because NF449 and NF279 have a minor and no effect, respectively, on the bacterial growth in vitro. The data support that the $P2X_1$ antagonists increase the number of circulating bacteria and thereby have a more severe reaction to bacteraemia, possibly by compromising the number of functional circulating thrombocytes. It must be noted, however, that $P2X_1$ receptors are

expressed on neutrophil granulocytes, which in tissues are considered the first line of defence against intruding bacteria (for review see [45]). However, in the bloodstream, the slow process of phagocytosis has been challenged as the main route for neutralising invading bacteria [46]. Neutrophils have been shown to express $P2X_1$ albeit much less prominently than thrombocytes [47]. There have been a few studies that imply a functional role of $P2X_1$ receptors on neutrophils either promoting [48] or more recently, inhibiting neutrophil chemotaxis towards a pathogen target [49]. The notion that the $P2X_1$ receptor promotes chemotaxia was supported by a study that showed that $P2X_1$ receptor-deficient mice showed a lower degree of extravasation of neutrophils in response to LPS [17]. Since $P2X_1$ receptors, to our knowledge, never has been linked to phagocytosis and that bacterial phagocytosis is likely to be less critical for bacterial clearance in the bloodstream, it is unlikely that neutrophil $P2X_1$ receptors are responsible for the effects of NF449 and NF279 on the outcome of experimental urosepsis.

However, the theory that NF449 and NF279 reduced the first line defence against infectious agent via a marked reduction in the number of circulating thrombocytes could also potentially explain the discrepancy between the increased mortality of sepsis observed with $P2X_1$ antagonists and the relative protection seen in $P2X_1^{-/-}$ mice. We showed that the $P2X_1^{-/-}$ mice had the same percentage of circulating thrombocytes as the wild-type littermate controls and thus, potentially be less susceptible to infection.

Thus, we speculate that the $P2X_1$ receptor is essential for normal function of circulating thrombocytes and that an acute inhibition causes acute senescence or necrosis of the platelets. Since thrombocytes are implicated in binding bacteria in the bloodstream, it is reasonable to assume that a low circulating thrombocyte count will result in less sequestering of bacteria in the blood. This reduction of functional thrombocytes makes the animals more susceptible to the circulating bacteria that are now free in plasma because of the reduced thrombocyte binding. These findings have to be considered when considering interfering with P2 receptor signalling as a potential therapeutic target. Importantly, the data do certainly not support that $P2X_1$ receptor as an appealing target during severe infection.

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Compliance with ethical standards

Conflict of interest Marianne Skals declares that she has no conflict of interest.

Anne-Sofie Greve declares that he has no conflict of interest.

Nanna Johnsen declares that she has no conflict of interest.

Mette G. Christensen declares that she has no conflict of interest.

Helle A. Praetorius declares that she has no conflict of interest.

Ethical approval The experiments performed in this study were approved by the Danish ethic committee for animal research "Dyreforsøgstilsynet" (2014-15-0201-00316).

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platelets are mechano-scavengers that collect and bundle bacteria. Cell 171(6):1368–1382 **e1323**

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