P2X$_1$, P2X$_4$, and P2X$_7$ Receptor Knock Out Mice Expose Differential Outcome of Sepsis Induced by α-Haemolysin Producing Escherichia coli

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α-haemolysin (HlyA)-producing Escherichia coli commonly inflict severe urinary tract infections, including pyelonephritis, which comprises substantial risk for sepsis. In vitro, the cytolytic effect of HlyA is mainly mediated by ATP release through the HlyA pore and subsequent P2X$_1$/P2X$_7$ receptor activation. This amplification of the lytic process is not unique to HlyA but is observed by many other pore-forming proteins including complement-induced haemolysis. Since free hemoglobin in the blood is known to be associated with a worse outcome in sepsis one could speculate that inhibition of P2X receptors would ameliorate the course of sepsis. Surprisingly, this study demonstrates that P2X$_7^{-/-}$ and P2X$_4^{-/-}$ mice are exceedingly sensitive to sepsis with uropathogenic E. coli. These mice have markedly lower survival, higher cytokine levels and activated intravascular coagulation. Quite the reverse is seen in P2X$_1^{-/-}$ mice, which had markedly lower cytokine levels and less coagulation activation compared to controls after exposure to uropathogenic E. coli. The high cytokine levels in the P2X$_7^{-/-}$ mouse are unexpected, since P2X$_7$ is implicated in caspase-1-dependent IL-1β production. Here, we demonstrate that IL-1β production during sepsis with uropathogenic E. coli is mediated by caspase-8, since caspase-8 and RIPK3 double knock out mice show substantially lower cytokine during sepsis and increased survival after injection of TNFα. These data support that P2X$_7$ and P2X$_4$ receptor activation has a protective effect during severe E. coli infection.

Keywords: sepsis, P2X, caspase-8, uropathogenic, E. coli
INTRODUCTION

Sepsis is the major cause of death in intensive care units worldwide. Urinary tract infections (UTI), often caused by uropathogenic *E. coli*, have been identified as the prime source in ~10–30% of severe sepsis or septic shock (Wagenlehner et al., 2007). Sepsis in general can result in multiple organ failure and death as a consequence of uncontrolled activation of the innate immune system with high circulating levels of pro-inflammatory cytokines such as interleukin 6 (IL-6), IL-8, IL-1β and tumor necrosis factor α (TNFα) (Nupponen et al., 2001; Santana et al., 2015). In addition to cytokines, adenosine triphosphate (ATP) comprises one of many host damage-associated-molecular-patterns (DAMPs) molecules released to the extracellular space during cell injury in response to invasive pathogens (Land et al., 2016; Ousingawat et al., 2017). The level of extracellular ATP is sensed by P2Y and P2X receptors and serves a wide range of physiological functions including thrombocyte aggregation, taste, pain, and chemo-sensing (Burnstock, 2009). In terms of infection, ATP is a well-recognized signaling molecule released at sites of inflammation/cell injury, and plays a central role in immune cell migration, chemotaxis, and cytokine release (Juenger, 2008; Bours et al., 2011). In particular, the P2X7 receptor has attracted considerable attention for its importance in immune cell communication (Di Virgilio and Vuerich, 2015), release of pro-inflammatory cytokines (Suzuki et al., 2004; Clark et al., 2010; Shieh et al., 2014; Trubiani et al., 2014), and recruitment of macrophages and lymphocytes (Moncao-Ribeiro et al., 2011; da Silva et al., 2013). Therefore, the P2X7 has emerged as a potential anti-inflammatory therapeutic target.

We have previously established that the effect of the pore-forming virulence factor α-haemolysin (HlyA), secreted from certain *E. coli* strains, is mainly secondary to ATP release and P2X receptor activation (Skals et al., 2009). *E. coli* strains that produce HlyA are commonly isolated from patients with severe urinary tract infections (Johnson, 1991; Bien et al., 2012) and contribute to the pathogenesis of urosepsis. *In vitro*, we have demonstrated in erythrocytes that ATP is released immediately after HlyA is inserted into the membrane (Skals et al., 2014) and potentiates haemolysis by activation of mainly P2X1 in mice and mainly P2X7 receptors in humans (Skals et al., 2009). Importantly, the P2X7-dependent amplification of cell damage is not specific to HlyA but is seen in response to many types of cytolytic proteins such as α-toxin from *S. aureus* (Skals et al., 2011), LtxA from *A. actinomycetemcomitans* (Munksgaard et al., 2012), ApxIA haemolysin from *A. pleuropneumoniae* (Masin et al., 2013), β-toxin from *C. perfringens* (Nagahama et al., 2015), and membrane attack complex formed after complement activation (Heij et al., 2012). Based on this, we speculated that P2X receptor antagonists may ameliorate the symptoms of urosepsis.

Urosepsis is not easily modeled experimentally as installation of bacteria in the urinary tract is unable to produce reproducible septic events in rodents. However, direct injection of uropathogenic bacteria intravenously has been proven to be a beneficial model for urosepsis (Barber et al., 2016). The chosen uropathogenic bacterium (ARD6, O6:K13:H1) commonly causes urinary tract infection in humans and, in addition to HlyA, also expresses other virulence enhancing proteins such as P-fimbriae (Zingler et al., 1992).

In this study, we used a model of acute sepsis in P2X1, P2X4, and P2X7 receptor deficient mice under anesthesia using these uropathogenic *E. coli*, in accordance with Danish legislation for animal research. We establish that mice lacking P2X7 and P2X4 are significantly more susceptible to sepsis inflicted by uropathogenic *E. coli*. P2X7−/− mice did more quickly and showed massively increased plasma cytokine levels, intravascular haemolysis and activation of the coagulation system. Strikingly, we found a markedly smaller spleen in P2X7−/− mice compared to P2X7+/− even though the spleen just like the control enlarged during sepsis. The P2X1−/− mice seemed relatively protected against sepsis with uropathogenic *E. coli* with prominently lower plasma cytokine levels. The unexpected, high IL-1β-production in the P2X7−/− mice is likely to result from P2X7−/− independent activation of caspase-8 (casp8), since casp8/RIPK3 double knock out mice exhibit markedly lower cytokine levels compared to controls during sepsis with uropathogenic *E. coli*. These data support that P2X7 and P2X4 receptor activation protects against severe infection either by limiting the number of bacteria in the blood or by diminishing the casp8 dependent cytokine storm.

MATERIALS AND METHODS

*Escherichia coli*

The uropathogenic *E. coli* strain ARD6 (serotype: O6:K13:H1) and the non-pathogenic strain D2103 (serotype OR:H48) were obtained from Statens Serum Institute (Copenhagen, Denmark). The bacteria were grown on agar plates containing LB media and kept for up to 1 month at 4°C. For each experiment a fresh liquid preparation of *E. coli* was cultured overnight by transferring one colony to 4 ml LB medium at 37°C for 250 rpm. The following morning, the culture was centrifuged twice and re-suspended in sterile saline. Live and dead bacteria in this preparation were distinguished by a cell viability kit (BD biosciences) and approximately 10% dead cells were present in this preparation were distinguished by a cell viability kit (BD biosciences) and approximately 10% dead cells were present in this preparation. *E. coli* was counted by flow cytometry (Accuri C6, BD Biosciences) and different concentrations of bacteria were used depending on the specific mouse strain the protocol used (see section “mouse model of sepsis” below). In all experiments, isolated bacteria were injected into mice via a lateral tail vein in 150 µl saline.

**Animals**

P2X7−/− mice on a balb/cj background (over 10 generation back crossed) were bred at the Institute of Biomedicine, Aarhus University and matched with either P2X7+/+ littermates from heterozygous breeding or balb/cj mice from Janvier Labs (Saint-Berthevin, France). The P2X7−/− mice were originally developed by GlaxoSmithKline and bred into the balb/cj background. Animal experiments with the P2X antagonists, BBG, were performed on balb/cj mice from Janvier Labs.

P2X1 and P2X4 wild type and knockout mice were bred at the Institute of Biomedicine, Aarhus University, by heterozygous
breeding and litters were used. P2X<sub>1</sub> mice were on a C57BL/6J background and P2X<sub>4</sub> were on a mixed background (C57BL6.B6129s). All P2X mice used in this study were 8–10 week old males with a weight of 25.1 ± 0.8 g.

Caspase-8/RIPK-3<sup>−/−</sup> mice were bred in the Kiel facility, Germany, as published (Linkermann et al., 2013), and matched with C57BL/6N mice from either Charles River, Sulzfeld or Janvier Labs. The authors would like to thank NR Jorgensen for providing the P2X<sub>7</sub> mice, J Leipziger for providing the P2X<sub>4</sub> mice, D Green for providing the casp8/RIPK3<sup>DKO</sup> (Oberst et al., 2011), V. Dixit and K. Newton (Genentech) for providing RIPK3 deficient mice (Newton et al., 2004) and R Hakim for casp8 heterozygous mice (Salmena et al., 2003).

**Blood Samples**

Immediately before the mice were euthanised, blood was drawn from the abdominal vena cava into a heparinised syringe and centrifuged at 1,000 g for 10 min to obtain plasma. Plasma was used for measurements of intravascular haemolysis, levels cytokines and thrombin-antithrombin complexes.

**Haemolysis**

Haemolysis was measured immediately as the absorbance at 410 nm (dilution 1:32) on a spectrophotometer (Ultraspec III, LKB Biochrom) and the value evaluated by reference curve. The remaining plasma was stored at −20°C for later evaluation of cytokines and levels of thrombin-antithrombin complexes. Plasma was stored and used within 30 days.

**Reagents**

Brilliant Blue G (BBG) was from Sigma-Aldrich and NF449 was from Tocris Bioscience (Bristol, UK). Purified murine TNFα was purchased from BioLegend (Uithoorn, Netherlands). 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 complexes from Abcam (Cambridge, UK).

**Cytokines**

TNFα, IL-1β, KC (murine equivalent of IL-8 in humans), IL-6 were measured on stored plasma samples (−20°C) on a flow cytometer (BD Accuri C6, BD Biosciences) according to manufactures instructions.

**Thrombin-Antithrombin (TAT) Complexes**

TAT were measured in heparin-anticoagulated plasma samples with TAT Complexes Mouse ELISA Kit according to manufactures instructions.

**Mouse Model of Sepsis**

Sepsis was induced in mice on three different backgrounds (balb/cj, C57BL/6j and mixed). The number of bacteria required to investigate survival rates within 6 h were adjusted to an optimal number of 165·10<sup>6</sup> in balb/cj mice. However, we observed that mice on a C57BL/6j and mixed background required a higher number of bacteria to die within the observation period. Thus, the number of bacteria was increased by a factor 1.5, corresponding to 248·10<sup>6</sup>. These concentrations will be referred to as high doses in the result section. The high doses were decreased by a factor 0.25 corresponding to ~ 41·10<sup>6</sup> for balb/cj and 62·10<sup>6</sup> for C57bl/6j and mixed backgrounds and will be referred to as the low doses in the result section. All mice were anesthetized by a subcutaneous injection of ketamine (100 mg kg<sup>−1</sup>) and xylazine (7.5 mg kg<sup>−1</sup>) and placed on a heating plate at 38°C. E. coli was injected in a lateral tail vein in a volume of 150 μl sterile saline and mice were monitored carefully for either 2.5 or 6 h according to the protocol used. For both protocols, additional anesthesia was administered approximately every 45 min. Body temperature was measured continuously by a rectal thermometer (Bioseb, Florida, USA). Blood pressure was measured every 30 min by determining the tail blood volume with a volume-pressure recording sensor and an occlusion tail-cuff (Kent Scientific Corporation, Connecticut, USA) and respiratory rate (RR) was visually monitored every 30 min. BBG, NF449 or saline were given 2 h before and 2 and 4 h after E. coli injection. BBG was given subcutaneously and NF449 was given iv.

The following 3 protocols were used in this study:

**Survival—6 h:** Mice were continuously observed after E. coli injection in the tail vain. Mice were given injections of BBG, NF449 or saline. Body temperature, blood pressure and respiratory rate were monitored. 165·10<sup>6</sup> ARD6 was given to the mice on balb/cj background and 248·10<sup>6</sup> ARD6 was given to C57BL6 and mixed background. In the following these concentrations will be referred to as the high dose of E. coli.

**Harvesting blood and organs—2.5 h—high dose:** Mice were continuously observed after E. coli injection and organs and blood were harvested after 2.5 h. Mice were given injections of BBG, NF449 or saline. Body temperature, blood pressure and respiratory rate were monitored. 165·10<sup>6</sup> ARD6 was given to mice on balb/cj background and 248·10<sup>6</sup> ARD6 was given to C57BL/6j and mixed background. In the following these concentrations will be referred to as the low dose of E. coli.

**Survival—6 h:** Mice were continuously observed after E. coli injection in the tail vain. Mice were given injections of BBG, NF449 or saline. Body temperature, blood pressure and respiratory rate were monitored. 165·10<sup>6</sup> ARD6 was given to mice on balb/cj background and 248·10<sup>6</sup> ARD6 was given to C57BL/6j and mixed background. In the following these concentrations will be referred to as the low dose of E. coli.

**Caspase-8/RIPK-3<sup>−/−</sup>**—The model of TNFα-induced shock has been described in detail previously (Cauwels et al., 2003). In our experiments, C57BL/6N, RIPK3-deficient and casp8/RIPK3<sup>DKO</sup> mice received a single iv-injection of 25 mg kg<sup>−1</sup> murine TNFα (in 200 μl PBS) via the tail vein. Animals were under permanent observation and survival was checked every 15 min in accordance to the authorisation of the local committee for the preservation of animals act.

**Colony Forming Units (CFU)**

CFU were determined in blood after the animals were sacrificed. Whole blood (10 μl) was diluted 1/100 and 5 μl was plated on a blood agar plate and cultured overnight at 37°C and the number of colonies were counted and expressed as CFU μl blood<sup>−1</sup>.

**Histology**

Organs were isolated after euthanasia. Lungs, liver, spleen, kidneys and heart were immersion fixed in 4% paraformaldehyde for at least 24 h and stored at 4°C until further preparation.
For preparation, the organs were dehydrated in a series of three ethanol solutions (70, 96, and 99.9%) xylene and then imbedded in paraffin for haematoxylin eosin (HE) staining.

**Spleen Weight**

The spleens were dissected free from connecting tissues. The weight of each spleen was determined and the result expressed as percentage of body weight.

**Ethics**

The experiments performed in this study were approved by Danish ethic committee for animal research “Dyreforsøgstilsynet” (2014-15-0201-00316) and by the local committee for the preservation of animal act of Christian-Albrechts-University Kiel, Germany.

**Statistics**

Statistical analysis was performed using GraphPad Prism software. Survival studies were analyzed by Kaplan-Meier curve and log-rank test. All other data was reported as mean ± SEM and analyzed using Student t-test. A *p* < 0.05 was considered statistically significant and marked by *.

**RESULTS**

The present study was undertaken to determine the in vivo effects of uropathogenic *E. coli* during sepsis. Specifically, we were interested in the role of P2X1 and P2X7 because these receptors are predominantly responsible for the cytotoxic effects of HlyA *in vitro*. Moreover, we included the P2X4 receptor because it is expressed in most bone marrow derived cells and because it is hard to distinguish pharmacologically from P2X7. To this end, we used mice deficient of the given receptors and or pharmacological blockage of P2X1 and P2X7 receptors. Sepsis was induced in anesthetized mice by *iv*-injection of the HlyA-producing and uropathogenic *E. coli* strain ARD6. Mice were kept under anesthesia to follow regulations by the Danish ethic committee for animal research.

**Establishing a Sepsis Model in Mice**

Mice exposed to ARD6 develop bacteraemia demonstrated by colony forming units on blood culture. This was not seen in control mice injected with saline. In addition, balb/cj mice subjected to *iv*-injection of a high dose of ARD6 (165·10⁶) showed an increase in body temperature over the observation period of 2.5 h (Figure S1A). Moreover, animals exposed to bacteria developed haematuria and clearly showed acute tubular necrosis. The inner renal medulla showed obvious protein deposits in the lumen of the renal tubules in the animals exposed to ARD6 (Figure S2). Taken together these observations indicate septic shock. To distinguish the effect of uropathogenic *E. coli*, we included a non-pathogenic control strain of *E. coli* (D2103, OR:H48), which in contrast to ARD6 does not cause pyelonephritis in mice after injection into the urinary bladder (unpublished observations). When balb/cj mice were injected with an equal amount D2103 (165·10⁶), all mice survived the 6-h observation period (Figure S3A). Moreover, mice subjected to D2103 did not show intravascular haemolysis and only very slight changes in plasma cytokine levels (Figures S3B,C).

**Role of P2X7 Receptors in ARD6 Sepsis**

We investigated the role of the P2X7 receptor in this sepsis model over a 6-h observation period. Surprisingly, we found a significant reduction in the survival of the P2X7−/− mice compared to P2X7+/+ (Figure 1A). The average survival time was 323.3 ± 18.5 min for P2X7+/+ and 214.9 ± 24.2 min for P2X7−/− mice after a high dose of ARD6 (*p* = 0.0027). We also observed a significantly higher intravascular haemolysis in P2X7−/− mice compared to P2X7+/+ controls (Figure 1B), corresponding to approximately 12 and 3% in P2X7−/− and P2X7+/+ mice, respectively (Figure S1B). The bacterial load was

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**FIGURE 1 | P2X7 deficiency in *E. coli*-induced sepsis.** ARD6 was injected into anesthetized P2X7+/+ and P2X7−/− mice (high dose—165 million bacteria). (A) Kaplan-Meier plot shows survival after ARD6 injection, *n* = 6 for controls and 10–12 for ARD6 for each genotype. (B) Absorbance of plasma as an indication of plasma haemolysis 2.5 h after ARD6 injection, *n* = 5 for control and 10–12 for each genotype. (C) Colony forming units (CFU) in the blood 2.5 h after ARD6 injection, *n* = 4 for controls and 11 for both genotypes. *p* < 0.05.
seemingly, higher in blood drawn from P2X_7^-/- mice at the end of the experiment (Figure 1C). This did, however, not reach statistical significance compared to P2X_7^+/+, because of a large inter-animal variation. This does, however, support that P2X_7 receptor activation has been associated with increased bacterial macrophage-mediated bacterial clearance during sepsis (Csoka et al., 2015). Animals exposed to bacteria clearly showed acute renal tubular necrosis (Figure S2) in 80% of the mice exposed to ARD6 with no marked difference between P2X_7^+/+ and P2X_7^-/- mice. Haematuria was observed in 90% of the P2X_7^-/- mice and 30% of the P2X_7^+/+ mice following ARD6 exposure. This could be a simple consequence of higher haemolysis in P2X_7^-/- mice, however, it potentially suggests damage to the filtration barrier. In support of this notion, amorphous protein was observed in the tubular lumen of 40% P2X_7^+/+ and 63% P2X_7^-/- mice exposed to ARD6; this was most obvious in the renal medulla (Figure S2B). None of the P2X_7^+/+ or P2X_7^-/- mice exposed to saline showed any evidence of tubular necrosis or debris in the tubular lumen (Figure S2). Interestingly, this sepsis model caused quite dramatic changes in the morphology of the spleen (Figure 2A). In the mice injected with saline, the spleen appeared normal, dominated by large blue areas following HE staining of the white pulp. After ARD6 treatment, the ratio between white and red pulp changed markedly, apparently with a reduction of the marginal zone reducing the overall diameter of the white pulp. This could result from mobilization of B and T cells from the spleen during infection, since this has been demonstrated in spleens from septic patients (Hotchkiss et al., 2001; Gunia et al., 2005). However, it could potentially also reflect increased binding and phagocytosis of damaged red blood cells in the red pulp overshadowing the white pulp and thus, changes the ratio between the two. The low white/red pulp is seen in 80% of the spleen from P2X_7^+/+ mice and in 63% of the spleen from P2X_7^-/- mice. This could theoretically suggest that splenic monocytes/macrophages are unable to recognize HlyA-induced erythrocyte damage in P2X_7^-/- mice, as previously suggested (Fagerberg et al., 2013). We also measured spleen to body weight ratio and found a substantial increase in spleen mass after injection of ARD6 both in the P2X_7^+/+ and in P2X_7^-/- (Figure 2B). The mean increase was 0.06% of body mass in P2X_7^+/+ and 0.12% of body mass in P2X_7^-/- mice. Mice pre-treated with BBG did not exhibit any enlargement of the spleen upon exposure to ARD6. Remarkably, the saline treated P2X_7^-/- mice had a significantly smaller spleen compared to saline-injected P2X_7^+/+ mice. Therefore, we measured spleen sizes in untreated P2X_7^+/+ and P2X_7^-/- mice and found that the P2X_7^-/- mouse indeed had a significantly smaller spleen compared to P2X_7^+/+ (Figure 2C).

Based on the survival curves, one would expect the cytokine levels to differ between the genotypes. However, although the level of TNFα, KC, IL-1β, and IL-6 were highly elevated 2.5 h after administrating 165·10^6 ARD6, they did not differ between the two genotypes (Figure S4). We speculated that the bacteria load may cause a ceiling effect, and masks any difference in cytokine levels between the genotypes. Therefore, we measured cytokine levels after exposing mice to a reduced dose of ARD6 (41·10^6). In this situation, IL-6 and IL-1β were distinctly higher in the P2X_7^-/- mice compared to controls (Figure 3A), whereas TNFα and KC was not statistically significantly different between the groups. Thus, the higher mortality in the P2X_7^-/- mouse is associated with higher cytokine release, supporting previous data that massive cytokine storm is associated with disadvantageous outcome of sepsis (London et al., 2016; Weber et al., 2015).

High cytokine levels are associated with the severity of sepsis and development of disseminated intravascular coagulation (for review see Gando et al., 2016). Interestingly, we found markedly higher levels of thrombin-antithrombin (TAT) complexes in plasma from P2X_7^-/- compared to P2X_7^+/+ mice (Figure 3B), which indicates enhanced activation of the coagulation cascade in these mice. Coincidently, we observed that the buffy coat in blood samples from P2X_7^-/- mice exposed to ARD6 was almost undetectable (data not quantified). This could potentially suggest infection-induced depletion of thrombocytes in P2X_7^-/- mice and support the notion of massively activated coagulation system in these mice.

To support the data from the P2X_7^-/- mouse, we tested the well-known P2X antagonist Brilliant Blue G (BBG) that has some selectivity toward P2X_7 in wild type balb/cj mice. BBG was chosen because the color allows us to directly measure the antagonist concentration in plasma. BBG was given subcutaneously (50 mg kg^-1) 2 h prior to the i.v.-injection of ARD6 and the mice were observed for 6 h under anesthesia, subsequently surviving mice were culled. At the end of the 6 h period the plasma level of BBG was determined to be 1.2 µM, well above the 1 µM needed to block P2X_7 receptors. BBG treated mice showed a tendency toward an increased survival rate but this was not statistically significant (Figure 4A). Similar to the in vitro experiments (Skals et al., 2009), BBG inhibited haemolysis in vivo (Figure 4B). BBG had no statistically significant effect on the number of bacteria in the blood (Figure 4C) but caused a significant decrease in TNFα and IL-1β after injection of 41 million ARD6 and no significant effect on KC or IL-6 (Figure 4D). Thus, pre-treatment with BBG does not mimic the phenotype of the P2X_7 receptor deficient mice.

**Role of P2X_1 and P2X_4 in ARD6 Sepsis**

BBG is not completely selective for P2X_7 receptors, and has been shown to antagonize both P2X_1 and P2X_4 receptors (Jiang et al., 2000; Seyffert et al., 2004). This may potentially explain the discrepancy between the P2X_7^-/- mice and the wild type mice treated with BBG. Therefore, we compared the outcome of sepsis in P2X_1 and P2X_4 deficient mice.

Mice on a C57BL/6 background required a markedly higher number of bacteria to develop lethal sepsis within the observation period. We did not observe any difference in mortality between P2X_1^+/+ and P2X_1^-/- mice after a high dose of ARD6 (Figure 5A). On average, however, the P2X_1^-/- survived 57 min longer compared to the P2X_1^+/+. The P2X_1 antagonist NF449 showed a tendency toward an increase in survival, but this was not statistically significant (Figure 5B). It must be
noted that NF449 is degraded quickly (Hechler et al., 2005) and thus, may not provide full P2X1R inhibition during the experiment. In a parallel series of experiments in P2X1+/+ and P2X1−/− mice, intravascular haemolysis, TAT and cytokine levels were measured in plasma 2.5 h after a low dose of ARD6. Intriguingly, TNFα, IL-1β, and IL-6 and TAT levels were all lower in the P2X1−/− compared to P2X1+/+ (Figures 5D,E), whereas intravascular haemolysis was similar in the two genotypes.
(Figure 5C). Notably, P2X<sub>1</sub> receptor expression has a positive impact on the cytokine storm inflicted by the bacterial infection and thus, any P2X<sub>1</sub>-antagonizing effect of BBG may potentially be responsible for the lower cytokine levels observed during sepsis in animals pre-exposed to BBG.

P2X<sub>4</sub>—Similar to our findings in P2X<sub>7</sub><sup>-/-</sup> mice, P2X<sub>4</sub><sup>-/-</sup> mice showed decreased survival when exposed to a high dose of ARD6 compared to P2X<sub>4</sub><sup>+/+</sup> controls (Figure 6A). Moreover, we found higher intravascular haemolysis, plasma IL-1β levels as well as higher TAT levels in the P2X<sub>4</sub><sup>-/-</sup> after a low ARD6 dose compared to control (Figures 6B–D). Thus, the data on this knock out mouse in many ways resembles data obtained in the P2X<sub>7</sub><sup>-/-</sup> mice and supports the notion that mice with high cytokine levels in plasma are more prone to die of sepsis. These data also support that mice deficient in P2X<sub>4</sub> and P2X<sub>7</sub> receptor are more sensitive to acute severe infection and that this sensitivity is not a result of reduced immune reaction.

**Casp8 and Receptor-Interacting-Protein-3 (RIPK3) in ARD6 Sepsis and TNFα Shock**

IL-1β production is surprisingly prominent in both P2X<sub>7</sub><sup>-/-</sup> and P2X<sub>4</sub><sup>-/-</sup> mice exposed to uropathogenic *E. coli*. This underscores that IL-1β production in this model can occur P2X<sub>7</sub>R-independently. Numerous studies have indicated that the NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome can be activated by casp8 leading to pro-IL-1β processing (Gringhuis et al., 2012; Gurung et al., 2014; Antonopoulos et al., 2015). Interestingly, activation of casp8 can occur P2X<sub>7</sub> receptor independently (Felley et al., 2016) and thus, may explain the IL-1β production in the P2X<sub>7</sub><sup>-/-</sup> mice. Casp8 deficiency in mice is embryonically lethal (Varfolomeev et al., 1998) because casp8 suppresses receptor-interacting protein kinase 3 (RIPK3) (Kang et al., 2013), which cause massive inflammasome activation in Casp8<sup>-/-</sup> mice. Mice lacking both casp8 and RIPK3 are, however, viable (Kaiser et al., 2011; Oberst et al., 2011) and showed similar survival during 6-h observation after iv-injection of ARD6 (high dose, Figure 7A). Strikingly, the cytokine levels were substantially lower in casp8/RIPK3<sup>DKO</sup> mice compared to controls following a low dose of ARD6 (Figure 7B). Therefore, we tested how these mice manage a less severe stimulus and mimicked severe inflammatory response syndrome (SIRS) by intravenous injection of TNFα (25 µg kg<sup>-1</sup>). In these experiments the mice were not anesthetised during the procedure and were carried out at Christian-Albrechts-University Kiel, Germany under German legislation for animal experiments and in accordance with the local committee for the preservation of animal act. This procedure resulted in death of all wild type mice within 36 h, whereas casp8/RIPK3<sup>DKO</sup> mice showed a striking 100% survival (Figure 7C). RIPK3 deficiency alone did not protect mice from dying of TNFα injection although they showed a marginal increase in survival compared to wild type. These data suggest an important upstream involvement of casp8 in this model. We conclude that P2X-receptors are crucial determinants for the outcome of sepsis induced by HlyA-producing *E. coli* in mice. The increased immunoreactivity

**FIGURE 3 | P2X<sub>7</sub> deficiency and *E. coli*-induced sepsis.** ARD6 was injected into anesthetized P2X<sub>7</sub><sup>+/+</sup> and P2X<sub>7</sub><sup>-/-</sup> mice (low dose—41 million). (A) plasma TNFα, KC, IL-1β, and IL-6 measured 2.5 h after ARD6 injection, n = 7–8 for controls and 8–9 for each genotype. (B) Plasma thrombin-antithrombin (TAT) complexes—measured 2.5 h after ARD6 injection, n = 6–7 for controls and 10–11 for both genotypes. *p < 0.05.
in P2X7 and P2X4 deficient mice is likely to be mediated via non-canonical activation of IL-1β via casp8.

DISCUSSION

Urinary infections are exceedingly common and often caused by E. coli, known as the dominant facultative bacterial agent in the normal intestinal flora. However, simple urinary infections can progress to severe pyelonephritis and sepsis. The invasive, more aggressive E. coli-strains responsible for these severe infections are serotypically distinct from facultative strains and frequently produce the virulence factor α-haemolysin (Cavalieri et al., 1984; Bhakdi et al., 1988). A recent study showed that specific fine-tuning of HlyA expression by the human cystitis isolate UTI189 alters the course of both acute and chronic urinary tract infection in mice (Nagamatsu et al., 2015). In the present study, we investigated acute sepsis in mice induced by an uropathogenic α-haemolysin producing E. coli strain (ARD6). We chose to induce sepsis by direct injection of live bacteria intravenously in mice, since this model allow us to specifically choose the sepsis-causing bacterium. The virulence factor HlyA is known to cause severe cell damage in a P2X-receptor dependent fashion and thus, we were interested in the role of three P2X receptors (P2X1, P2X4, and P2X7) in sepsis caused by HlyA-producing, uropathogenic E. coli.

IL-1β is a key cytokine in sepsis during which the plasma levels of the cytokine can become exceedingly high (for review see Dinarello, 2005). The NLRP3 inflammasome is essential in processing and activation of IL-1β during inflammation (Lamkanfi and Dixit, 2014) and a decrease in intracellular K+ concentration is a prime activator of the inflammasome (Cain et al., 2001; Munoz-Planillo et al., 2013). Stimulation of ionotropic P2X7-Rs on macrophages by ATP directly causes...
FIGURE 5 | P2X<sub>1</sub> deficiency and P2X<sub>1</sub> inhibition in E. coli-induced sepsis. ARD6 was injected into anesthetized P2X<sup>+/+</sup> and P2X<sup>−/−</sup> mice at a concentration of either low (61 million) or high (248 million) number of bacteria. (A) Kaplan-Meier plot shows survival (high dose), n = 6 for control and n = 12 for both genotypes. (B) Survival in the presence or absence of the P2X<sub>1</sub> antagonist NF449 (100 μg kg<sup>−1</sup>, iv) in balb/c mice. The mice were exposed to the high concentration of bacteria, n = 6 for control with or without NF449 and n = 10–12 for ARD6 with or without NF449. (C) Absorbance of plasma as an indication of plasma haemolysis after the low dose, n = 8 for both genotypes. (D) Levels of thrombin-antithrombin (TAT) complexes in plasma 2.5 h after the low dose, n = 8 for both genotypes. *p < 0.05.

the K<sup>+</sup> efflux and maturation and release of IL-1β (Perregaux and Gabel, 1994; Kahlenberg and Dubyak, 2004; Lister et al., 2007; Qu et al., 2007; Pelegrin et al., 2008; Wiley et al., 2011). These findings prompted the concept that P2X<sub>R</sub> antagonists potentially could be used against inflammatory diseases of the kidney, in rheumatoid arthritis and pain disorders (Arulkumaran et al., 2011; Alves et al., 2013). Here, surprisingly we demonstrate that P2X<sub>7</sub><sup>−/−</sup> mice have an enhanced susceptibility to sepsis induced by uropathogenic E. coli with an increased mortality compared to wild type mice. The coagulation cascade is often activated alongside the immune system during sepsis and results in thrombin formation and platelet activation (for review see Brass, 2003). Thrombocytopenic mice are associated with a higher systemic bacterial load, increased plasma levels of pro-inflammatory cytokines (TNFα, IL-6, and IFN-γ) and accordingly a poorer outcome of sepsis induced by Gram-negative bacteria (van den Boogaard et al., 2015). In the present study, we found an increased plasma level of TAT-complexes in P2X<sub>7</sub><sup>−/−</sup> mice, which is associated with increase mortality during sepsis (van den Boogaard et al., 2015). Increased mortality and increased TAT were also found in the P2X<sub>4</sub> deficient mice. Thus, one may speculate that thrombocytopenia could be a part of an immune deficiency in P2X<sub>7</sub><sup>−/−</sup> and P2X<sub>4</sub><sup>−/−</sup> mice. Moreover, high plasma concentration of hemoglobin during sepsis is also associated with increased mortality (Larsen et al., 2010; Adamzik et al., 2012) and P2X<sub>7</sub><sup>−/−</sup> mice showed increased plasma levels of hemoglobin compared to wild type. Thus, several risk factors associated with poorer outcome was observed in our murine model of urosepsis.

Previous studies from our group demonstrate a sizable reduction of HlyA-induced cell damage by P2X receptor antagonists on human and murine erythrocytes (Skals et al., 2009, 2014) and monocytes (Fagerberg et al., 2016). Therefore, it was exceedingly surprising to find that P2X<sub>7</sub><sup>−/−</sup> mice were not relatively protected, but rather distinctively more sensitive ARD6-induced sepsis. This contrasts with in vivo studies that
have shown improved survival of P2X₇⁻/⁻ mice when sepsis was induced by coecal ligation and puncture (Santana et al., 2015), lipopolysaccharide (LPS)-injection (Yang et al., 2015) and adenovirus infection (Lee et al., 2012). However, like in the present study, decreased survival and a greater bacterial load in the blood of P2X₇⁻/⁻ mice subjected to coecal ligation and puncture has been reported (Csoka et al., 2015). Thus, P2X₇ receptors have a critical role during sepsis and potential survival of this critical condition is a fine balance of the degree of inflammasome-activation and subsequent cytokine production. Massive infection apparently reveals an immune deficiency in P2X₇⁻/⁻ mice, a notion generally supported by the markedly smaller spleen in P2X₇⁻/⁻ mice. Our study clearly reveals acute splenomegaly in septic mice, which is explained through a combination of the severe infection and resultant intravascular erythrocyte damage. The tentatively higher infection-induced splenomegaly in the P2X₇⁻/⁻ fits the lower survival rates, higher cytokine levels and intravascular haemolysis. Interestingly, a recent study showed clear up-regulation of the NLRP3 inflammasome pathway in primary microglial and macrophages from P2X₇⁻/⁻ mice (Franceschini et al., 2015). Such an up-regulation may explain the higher cytokine levels and the increased mortality in P2X₇⁻/⁻ mice if the NLRP3 inflammasome could be activated by an alternative pathway to casp-1. It must be noted that neither of the available P2X₇⁻/⁻ mice are complete knockouts (Nicke et al., 2009; Masin et al., 2012). This is the result of the many splice variations of the P2X₇ receptor. The splice variant still remaining in the P2X₇⁻/⁻ mice used in the current study is expressed in the spleen (Nicke et al., 2009). It is, however, unlikely that the difference between P2X₇⁻/⁻ and the P2X₇ antagonist would result from this splice variation, because the mice in which some P2X₇ receptor function is preserved show a worse outcome compared to general inhibition of the receptor.

Strikingly, the P2X receptor antagonist BBG, did not reduce the survival of sepsis induced by uropathogenic E. coli. On the contrary, BBG showed a tendency toward prolonging survival of infected mice and statistically significantly reduce plasma IL-1β and TNFα following a low dose of ARD6. BBG was given at a dose, which resulted in a plasma concentration known to give maximal inhibition of both HlyA and complement induced haemolysis in mice (Hejl et al., 2012). Essentially, one
could imagine that acute inhibition of P2X7 may have other consequences. However, although BBG is primarily used as a P2X7-antagonist, it also inhibits P2X1 and P2X4 receptors (Jiang et al., 2000; Seyffert et al., 2004). Thus, if either P2X1 or P2X4 receptors oppose the effect of P2X7 in sepsis, it may potentially explain the discrepancy between the P2X7−/− and the results obtained with BBG. Interestingly, we found that specific lack of P2X1 receptors considerably reduced level of the cytokines TNFα, IL-1β, and IL-6 and lowered coagulation activation following the low dose of ARD6. Notably, the P2X1 mouse is on a C57BL/6 background, which has a loss of function mutation in the P2X7, at least in the splice variant expressed in T lymphocytes (Adriouch et al., 2002) and is deficient in NLRP-1 (Boyden and Dietrich, 2006), which both may oppose the effect of P2X1 deficiency. Two recent studies where sepsis-like conditions were induced in P2X1−/− mice show contradicting results. One study showed increased survival in P2X1−/− mice after LPS injection (10 mg kg−1) (Maitre et al., 2015), while another demonstrated decreased survival after injection of 20 mg kg−1 LPS (Lecut et al., 2012) in mice lacking P2X1 receptors. Since both these studies also had P2X4−/− on a C57BL/6 background and thus, a less functioning P2X7 receptor, this may explain the higher sensitivity in the mice at higher dose of LPS. In mice pre-treated with NF449 there was no difference in intravascular haemolysis or cytokine levels in plasma (data not shown), which is likely to result from inadequate blockage of the receptor as previously suggested from the substance pharmacokinetics (Hechler et al., 2005).

Thus, there may be room for a specific P2X1 antagonist with a prolonged effect in the supportive therapy of septic conditions, since it is very likely that the effect of BBG is mediated through P2X1 receptor inhibition.

The P2X4 receptor is also known to be expressed in monocytes and macrophages (Kawano et al., 2006) and to influence cytokine release and cell death via P2X7-dependent mechanisms (Kawano et al., 2006; Perez-Flores et al., 2015). Thus, activation of the P2X4 receptor in macrophages may have similar effects as the P2X7 receptor in terms of macrophage function including IL-1β release and apoptosis. This study supports a similar function of P2X7 and P2X4 receptors in the acute septic response. Similar to the P2X7 receptor, P2X4−/− mice died earlier upon exposure
to HlyA-producing *E. coli* and showed an increased level of IL-
1β. Though the finding from P2X<sub>7</sub><sup>−/−</sup> mice cannot explain the
discrepancy between the P2X<sub>7</sub><sup>−/−</sup> and BBG data, these data point
to an important function of P2X<sub>7</sub>Rs in acute severe infection.

The question is: what causes the high cytokine levels, particularly of IL-1β in P2X<sub>7</sub><sup>−/−</sup> mice? As previously mentioned P2X<sub>7</sub>
has a central role in activation of casp1 and the final
clavage of pro-IL-1β to the active form. However, natural killer
cells can induce P2X<sub>7</sub> receptor-independent monocyte IL-1β
release via activation of both casp1 and casp8 (Felley et al., 2016).
Casp8 plays an essential role during apoptosis, necroptosis and
NLRP3 activation (Mocarski et al., 2011) and has recently been
demonstrated to cause cytokine release in a murine model of
LPS-induced shock (Oliva-Martin et al., 2016). Mice deficient
of casp8 are not viable, which is most likely because casp8
normally suppresses RIPK3 depended necroptosis. Thus, we used
the viable casp8<sup>−/−</sup>RIPKP3<sup>−/−</sup> and demonstrate that the levels of TNFβ,
KC, IL-6, and IL-1β were markedly suppressed compared
to wild type controls. Despite that we only observed a tendency
toward increased survival after this acute, massive infection with
uropathogenic *E. coli*, a marked effect of lack of casp8 was
observed, if we used a milder model of septic shock— injection of
TNFβ. Thus, casp8 is readily activated during sepsis induced by
HlyA-producing *E. coli* and this pathway may very well explain
the cytokine production in the P2X<sub>7</sub> deficient mice.

In summary, our data demonstrate enhanced susceptibility to sepsis with HlyA-producing *E. coli* in mice lacking P2X<sub>7</sub>
and P2X<sub>4</sub> receptors, whereas mice lacking P2X<sub>4</sub> receptors exhibit
lower cytokine levels during this condition. Elevated plasma
levels of the pro-inflammatory cytokines, free hemoglobin and
activation of the coagulation system could potentially explain
the poorer outcome of sepsis in the P2X<sub>7</sub><sup>−/−</sup> and P2X<sub>4</sub><sup>−/−</sup>
mice. Interestingly, this sepsis model strongly activates the non-
canonical inflammasome pathway via casp8 short-circuiting the
classical P2X<sub>7</sub> dependent activation of casp1. Deficiency of this
pathway completely prevents the infection induced cytokine
response. These surprising and new results provide an additional
insight into the pathogenesis in sepsis and for new ways to
approach the condition pharmacologically.

**AUTHOR CONTRIBUTIONS**

Conceived and designed the experiments: AG, MS, AL, and HP.
Performed the experiments: AG, MS, SF, WT, AL. Analyzed the
data: AG, AL, and MS. Wrote the paper: mainly MS and HP, with
contribution from AG, RE, SE, and AL.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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