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A3 AND P2Y2 RECEPTORS CONTROL THE RECRUITMENT OF NEUTROPHILS TO THE LUNGS IN A MOUSE MODEL OF SEPSIS

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Abstract

We have recently shown that A3 adenosine receptors and P2Y2 purinergic receptors play an important role in neutrophil chemotaxis. Chemotaxis of neutrophils to sites of infections is critical for immune defense. However, excessive accumulation of neutrophils in the lungs can cause acute lung tissue damage. Here we assessed the role of A3 and P2Y2 receptors in neutrophil sequestration to the lungs in a mouse model of sepsis. Sepsis was induced by cecal ligation and puncture (CLP) using adult male C57BL/6J mice (wild type [WT]), homozygous A3 receptor knockout (A3KO) mice, and P2Y2 receptor knockout (P2Y2KO) mice. Animals were killed 2, 4, 6, or 8 h after CLP, and peritoneal lavage fluid and blood were collected. Lungs were removed, and neutrophil infiltration was evaluated using elastase as a marker. Leukocyte and bacterial counts in peritoneal lavage fluid and blood samples were determined. Survival after sepsis was determined in a separate group. Leukocyte counts in the peritoneum were lower in A3KO and P2Y2KO mice than in WT mice. Conversely, initial leukocyte counts in the peripheral blood were higher in KO mice than in WT mice. Neutrophil sequestration to the lungs reached a maximum 2 h after CLP and remained significantly higher in WT mice compared with A3KO and P2Y2KO mice ($P < 0.001$). Survival after 24 h was significantly lower in WT mice (37.5%) than in A3KO or P2Y2KO mice (82.5%; $P < 0.05$). These data suggest that A3 and P2Y2 receptors are involved in the influx of neutrophils into the lungs after sepsis. Thus, pharmaceutical approaches that target these receptors might be useful to control acute lung tissue injury in sepsis.

Keywords

A3 adenosine receptor; P2Y2 purinergic receptor; cecal ligation and puncture; sepsis; acute lung injury; neutrophil elastase

INTRODUCTION

Acute lung injury (ALI) is a serious complication that raises mortality after trauma, sepsis, and major surgical procedures (1). Acute lung injury results in acute respiratory distress syndrome, a prominent form of end-organ damage that is frequently observed in patients

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even in the absence of direct mechanical trauma to the lungs (2, 3). The events leading to ALI are not fully understood. However, it has been shown that activated neutrophils play an important role in this process. Neutrophils infiltrate lung tissues, occlude pulmonary capillaries, release cytotoxic mediators such as neutrophil pro-teases, and thus destroy lung tissues (2, 4–6).

A key feature of neutrophil activation is their ability to migrate toward infected or otherwise compromised tissues by following a concentration gradient of chemotactic substances that are released from bacteria and inflamed tissues. Chemotaxis is a process that involves gradient sensing to detect the orientation of a chemotactic gradient field, cell polarization within the gradient field, and finally directed migration toward the source of the chemoattractant (7–10). Recently, we have found that the polarized release of cellular adenosine triphosphate (ATP) from neutrophils, the formation of adenosine in the extracellular space near the leading edge, and feedback signal amplification through P2Y2 and A3 receptors at the leading edge of migrating cells play important roles in controlling gradient sensing and migration, respectively (11).

Here we investigated the roles of A3 or P2Y2 receptors in the infiltration of neutrophil into the lungs and in the development of ALI in a mouse model of sepsis.

MATERIALS AND METHODS

Animals

The use of laboratory animals was in accordance with National Institutes of Health guidelines and was approved by the Institutional Animal Care and Use Committee. We used adult male C57BL/6J wild-type (WT) mice aged 8 to 10 weeks, weighing 20 to 25 g. The animals were obtained from Jackson Laboratory (Bar Harbor, Me). Homozygous $A3^{-/-}$ mice (A3KO) were a generous gift of Dr Francisco Villarreal (University of California, San Diego, La Jolla, Calif). Homozygous $P2Y2^{-/-}$ mice (P2Y2KO) were obtained from Dr Beverly H. Koller (University of North Carolina, Chapel Hill, NC).

Sepsis model

Sepsis was induced as described in Baker et al. (12) by subjecting mice to cecal ligation and puncture (CLP). Briefly, mice were anesthetized, restrained in supine position, and a 1-cm midline incision was made. The cecum was exposed, ligated distal to the ileocecal valve with 4-0 silk, avoiding intestinal blockade. The cecum was punctured twice with a 22-gauge needle, returned to the abdomen, and the abdominal wall was closed in layers. The mice were then returned to their cages and allowed access to a standard diet and water ad *libitum*. Animals were euthanized after 2, 4, 6, or 8 h, and leukocyte infiltration into the peritoneal cavity was assessed by lavage of peritoneal cavities with 3 mL ice-cold Hanks' buffered salt solution (HBSS) containing 1% mouse serum and counting peritoneal leukocytes.

Neutrophil recruitment to the lungs

We assessed neutrophil elastase activity in lung tissue samples as a marker of neutrophil infiltration because preliminary experiments have shown that elastase measurements provide

more sensitive results compared with myeloperoxidase. After *in situ* perfusion with normal saline to remove blood, lungs were extracted, weighed and placed in 5 mL of ice-cold potassium phosphate buffer (50 mM, pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (Sigma, St Louis, Mo). Tissues were homogenized on ice, and the homogenates were centrifuged at 17,000 rpm and 4°C for 30 min. The supernatants (20 μL) were added in duplicates into a 96-well plate followed by 80 μL/well of a buffer consisting of 50 mM Tris-HCl and 100 mM sodium chloride, pH 7.4, containing 0.05% (vol/vol) Triton X-100 (Sigma). Enzymatic reactions were started by the addition of the elastase-specific chromogenic substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide (Sigma) at a final concentration of 1 mM. After 30 min at room temperature, the change in optical density at a wavelength of 405 nm was measured using a UV-1201 spectrophotometer (Shimazu Corporation, Colombia, Md).

Bacterial cultures in peritoneal and blood samples

Peritoneal lavage fluids and heparinized blood samples were serially diluted in sterile saline, and 100-μL aliquots were plated onto trypticase soy agar plates (Becton Dickinson, Sparks, Md), cultured for 24 h at 37°C, and colonies were counted.

Lung histology

Lungs from each mouse strain were obtained for histological examination 24 hours after the CLP. Animals were anesthetized and killed as described previously. The trachea was exposed, cannulated with a dull 23-gauge needle (Monoject, Tyco Health Care Group, Mansfield, Mass), and 0.5 mL of fixative (10% paraformalde-hyde in HBSS) was instilled. The pulmonary circulation was flushed with 10 mL HBSS through the right ventricle, and the whole lung was carefully removed. Lungs were immersed in fixative solution for 72 h, dehydrated with graded alcohol, and embedded in paraffin. A series of 4-μm-thick tissue slices was obtained and stained with hematoxylin and eosin, and examined under the microscope.

Survival study

Survival after CLP was assessed in a separate set of eight animals per mouse strain.

Statistical analysis

All values were expressed as mean ± SD. Statistical analyses were performed with ANOVA followed by Bonferroni test or Kaplan-Meier tests. Differences between groups were considered statistically significant at $P < 0.05$.

RESULTS

A3 and P2Y2 receptors are required for neutrophil recruitment to the site of infection

Our previous data have shown that chemotaxis of neutrophils from A3KO and P2Y2KO mice is approximately 50% lower compared with WT mice, suggesting that A3 and P2Y2 receptors play important roles in neutrophil migration (11). Here we tested the roles of these receptors in neutrophil sequestration to the lungs of mice subjected to peritoneal sepsis.

Leukocyte counts in the peritoneal cavity of WT, A3KO, and P2Y2KO mice dropped significantly 2 h after CLP (Fig. 1A), probably caused by the rapid consumption of leukocytes during the immune defense. After 6 h, leukocyte counts in the peritoneal cavities of KO mice recovered, whereas cell counts in WT mice increased to levels that were significantly higher than those in KO mice ($P < 0.05$). Concomitantly, blood leukocyte counts in WT mice remained stable, whereas cell counts in KO mice were increased at 2 h ($P < 0.01$) and 4 h ($P < 0.05$) after CLP (Fig. 1B). These data are consistent with the notion that leukocyte recruitment to the site is diminished in A3KO and P2Y2KO mice.

A3 and P2Y2 receptors and bacterial clearance

Reduced leukocyte recruitment into the peritoneal cavity would be expected to decrease the ability of KO mice to clear bacteria from the site of infection and to prevent the spread of bacteria in the systemic circulation. Bacterial counts in the peritoneal cavity after CLP did not differ between WT mice and A3KO and P2Y2KO mice. However, bacterial counts in the systemic circulation rose less rapidly in WT mice than in A3KO and P2Y2KO mice (Fig. 2). These findings may be caused by the reduced availability of leukocytes at the infection site in KO mice. However, it is also possible that the absence of P2Y2 and A3 receptors may impair the ability of neutrophils to phagocytose and kill bacteria.

Neutrophil recruitment to the lungs after sepsis depends on A3 and P2Y2 receptors

Sepsis results in a systemic inflammatory response that causes neutrophil sequestration to host organs such as the lungs, which contributes to end-organ damage. Because of its fragile architecture, lung tissue is particularly prone to secondary tissue damage by sequestered phagocytes. We compared the time course of neutrophil accumulation in the lung tissues of WT with that of KO mice using elastase as a marker. As shown in Figure 3, sepsis in WT mice caused a greater than 10-fold increase in lung elastase activity within 2 h after CLP. These levels remained significantly elevated above control values for up to 4 h after the induction of sepsis ($P < 0.001$). In comparison, during the first 4 h after CLP, neutrophil accumulation in the lungs of A3KO and P2Y2KO mice was significantly lower than that in WT mice ($P < 0.01$). This result shows that A3 and P2Y2 receptors are important for neutrophil accumulation in lung tissues, but that the absence of one of these receptors does not completely prevent neutrophil sequestration. This is likely a result of the fact that both receptor types play different roles in the control of neutrophil chemotaxis (11). Thus, the absence of either one of these receptors diminishes chemotaxis, but it does not completely prevent chemotaxis.

Lung tissue damage in KO and WT mice

Lung tissue damage after sepsis was evaluated with histological samples obtained 24 h after CLP (Fig. 4). Focal atelectasis and hyperemia caused by blood congestion were observed in lung samples from WT mice. In addition, there was clear evidence of numerous neutrophils and debris in alveolar spaces of the lungs in WT mice subjected to CLP. These histological markers of lung injury were less evident in A3KO and P2Y2 KO mice.

Mortality after sepsis in A3 and P2Y2 receptor KO mice

Our previous work indicated that chemotaxis is reduced in A3KO and P2Y2KO mice compared with that in WT controls. Our current data suggest that neutrophil sequestration to the lungs of septic mice and lung tissue damage are decreased in A3KO and P2Y2KO mice compared with those in WT mice. Based on these observations, mortality would be expected to be lower in KO mice compared with WT animals, despite the fact that fewer neutrophils are available to prevent bacterial spreading beyond the site of infection.

To better understand the roles of P2Y2 and A3 receptors in the clinical course of sepsis, we evaluated survival of WT, A3KO, and P2Y2KO mice in a separate set of eight animals per mouse strain. Twenty-four hours after CLP, only 3 of the 8 animals in the WT group survived, whereas 7 of 8 were still alive in the A3KO and P2Y2KO groups. Whereas only 1 of the WT mice was still alive at 36 h, there were 4 survivors in the P2Y2KO group and in the A3KO group. Although A3KO mice and P2Y2KO mice showed significantly reduced mortality rates in the first 36 h after CLP (PG 0.05), these differences in survival were no longer significant at later time points (Fig. 5). Thus, our data suggest that the absence of A3 and P2Y2 receptors in KO animals delays mortality after sepsis, possibly by reducing neutrophil sequestration and damage of the lungs.

DISCUSSION

Secondary organ damage is a major complication after sepsis, which results in acute respiratory distress and multiple organ failure syndromes (3). Neutrophils sequestered to host organs are known to play an important role in organ damage (2, 13, 14). Thus, therapeutic approaches aimed at preventing neutrophil activation or the recruitment of neutrophils to host tissues hold promise to reduce organ damage (15, 16). Recently, we have shown that autocrine feedback mechanisms through A3 and P2Y2 receptors control neutrophil chemotaxis (11). We found that neutrophils release ATP at the leading edge, where adenosine is rapidly formed from the released ATP. Adenosine activates A3 adenosine receptors expressed predominantly at the leading edge of migrating cells. We found that the inhibition of A3 adenosine receptors with specific receptor antagonists impaired the ability of neutrophils to undergo chemotaxis toward bacterial products such as formyl peptides and inflammatory mediators, such as platelet activating factor, complement fragment C5a, and IL-8 (11). Others have reported that agonists of A3 receptors can attenuate neutrophil functions and myocardial reperfusion injury by decreasing polymorphonuclear leukocyteendothelial cell interactions (17).

Here we studied the roles of the A3 or P2Y2 receptors in leukocytes recruitment to sites of infection and the sequestration of neutrophils to the lungs in a mouse model of sepsis. Consistent with our previous results, our findings in the sepsis model indicate that the absence of A3 or P2Y2 receptors significantly impairs leukocyte migration into the peritoneal cavity of mice subjected to CLP. This may be one of the reasons for the differences in bacterial counts in the peripheral blood of KO and WT mice (Fig. 2). Diminished mobilization of leukocytes to the peritoneum of KO mice renders these animals more susceptible to sepsis compared with WT mice. Despite the diminished leukocyte response in KO mice, we observed reduced mortality in the early phase after sepsis in

A3KO and P2Y2KO compared in WT mice. These data imply that the absence of A3 or P2Y2 receptors can have certain benefits for the host, perhaps by reducing host organ damage by neutrophils sequestered to the lungs. This notion is supported by initial histological data that indicate diminished lung tissue injury in KO mice compared with WT mice (Fig. 4).

However, our study has certain limitations that must be considered. For example, the complicated dynamics of neutrophil recruitment from the bone marrow and the influx of neutrophils into the peritoneal cavity and their sequestration into the lungs make it difficult to determine which, if not all, of these events depend on A3 and P2Y2 receptors. Similarly, the numbers of bacteria in the peripheral blood and in the peritoneal cavity depend on the dynamics of neutrophil recruitment and flux mentioned previously. In addition, other factors might influence the numbers of bacteria. It is likely that the absence of A3 and P2Y2 receptors may not only affect the ability of neutrophils to undergo chemotaxis, but that it may also impair the cells' ability to killing and phagocytose bacteria.

Nevertheless, our current findings suggest an important role for A3 and P2Y2 receptors in leukocyte responses that are involved in the complex sequence of events that lead to lung tissue damage in response to acute sepsis. P2Y2 receptors, and in particular, A3 receptors, for which numerous specific pharmacological inhibitors are available, thus seem possible targets for therapeutic interventions to reduce the risk of host tissue damage during sepsis. Several reports have shown that inflammatory complications after stroke (18, 19), myocardial infarction (20, 21), asthma (22, 23), and rheumatoid disorders (24, 25) are diminished in A3 receptorYdeficient mice. A3 agonists have been shown to worsen I/R-induced renal failure (26) and to attenuate I/R injury of heart (21, 27) and lung tissues (28, 29). Moreover, A3 antagonists have been reported to reduce myocardial ischemic injury (30) and airway inflammation (31). Our findings presented here show improvements in the early survival after sepsis of A3KO and P2Y2KO mice. However, despite the initial survival benefits, long-term survival was not improved in A3KO or P2Y2KO mice.

In addition to A3 receptors, other members of the adenosine receptor family, in particular the A2 receptor subclass, also control neutrophil responses and they mediate lung tissue damage in response to endotoxemia, I/R, and trauma/hemorrhagic shock (28, 32, 33). The available pharmacological agents used to inhibit these receptors can vary considerably in their effectiveness to block human and murine receptors. This should be taken into account when considering therapeutic strategies to reduce lung tissue damage in sepsis.

To our knowledge, this study is the first to show that A3 and P2Y2 receptors control neutrophil sequestration to the lungs after peritoneal sepsis. Our findings conflict with those of a recent report by Lee et al. (29), who showed that the mortality after CLP is more severe in A3KO mice compared with WT controls. It is likely that these differences are caused by differences in the severity of the sepsis in the two models. Our sepsis model caused 100% mortality in 40 h. The CLP procedure described by Lee et al. (29) resulted in approximately 20% mortality within 24 h, whereas our model caused twice that mortality in the same period.

In summary, our results indicate that A3 and P2Y2 receptors contribute to the sequestration of neutrophils into the lungs after sepsis, suggesting that these receptors may represent suitable pharmaceutical targets to control neutrophil host tissue damage after sepsis.

Acknowledgments

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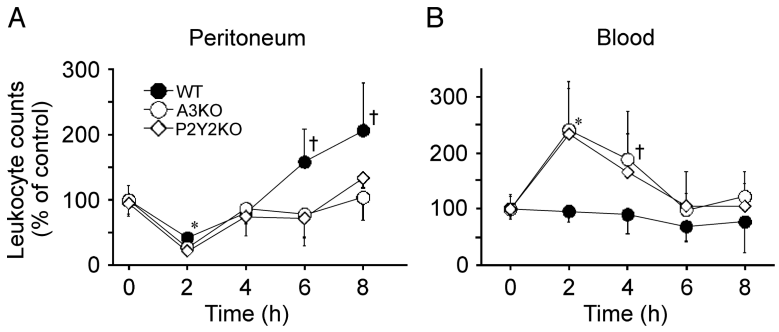

Fig 1. Leukocytes in the peritoneum and peripheral blood after CLP

The WT, A3KO, and P2Y2KO mice were subjected to intraperitoneal sepsis by CLP with a 22-gauge needle, and the time course of leukocyte recruitment in the peritoneal cavity (A) and the peripheral blood (B) was measured after CLP. Data are expressed relative to leukocyte counts of controls without CLP. Data points represent mean ± SD of 5 animals per group. Statistical analyses were performed with ANOVA followed by Bonferroni test, $*P < 0.01$, $^{\dagger} P < 0.05$ compared with WT control.

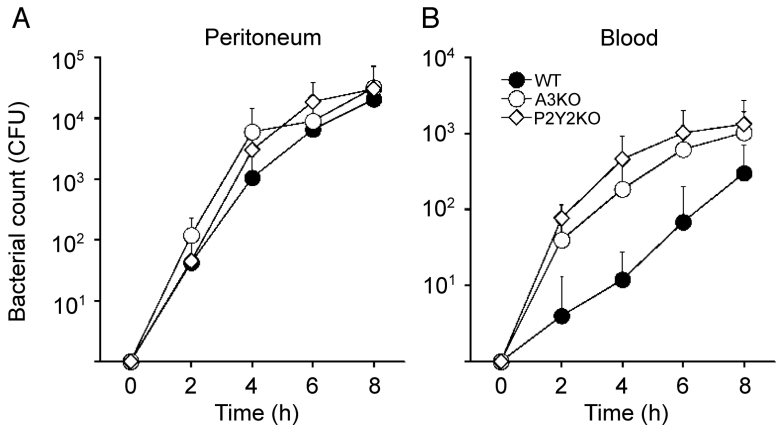

Fig 2. Bacterial counts in peritoneum and peripheral blood after CLP

Bacterial counts in peritoneal lavaged fluids (A) and peripheral blood (B) of WT, A3KO, and P2Y2KO mice were assessed at different times after CLP. Data show mean ± SD of colony-forming units determined in five animals for each time point and mouse strain. CFU indicates colony-forming units.

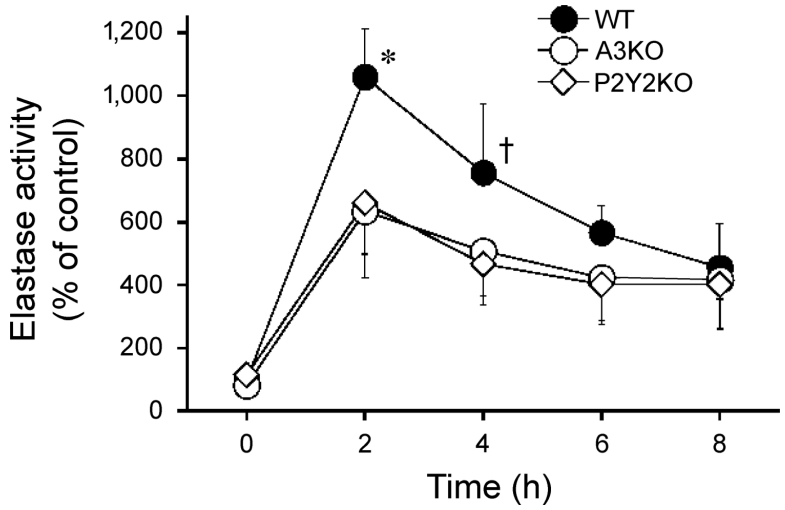

Fig 3. Neutrophil recruitment to the lungs after CLP

Neutrophil recruitment to the lungs of WT, A3KO, and P2Y2KO mice after CLP was assessed by measuring elastase activity in lung tissue homogenates. Data are expressed relative to control values in untreated animals. Values are mean ± SD of five animals in each group and at each time point. Statistical analysis between WT, A3KO, or P2Y2KO was performed with 2-way ANOVA followed by Bonferroni test, $^{*}P < 0.001$, $^{\dagger}P < 0.01$.

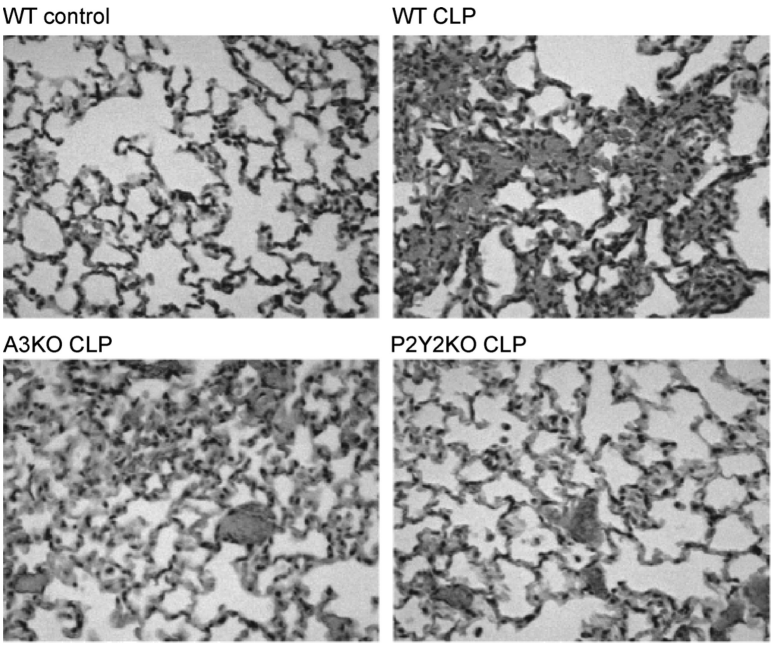

Fig 4. Lung histology

Representative micrographs of histological sections of lung tissue from untreated control WT mice (WT control) and WT mice, A3KO mice, and P2Y2 KO mice 24 h after CLP (hematoxylin-eosin, original magnification [notdef]200). Lung sections of WT mice after CLP showed characteristic abnormalities including focal atelectasis, hyperemia with congestion, and infiltration of erythrocytes and neutrophil within the widened alveolar septae, and with alveolar spaces containing fluid, debris, and inflammatory cells.

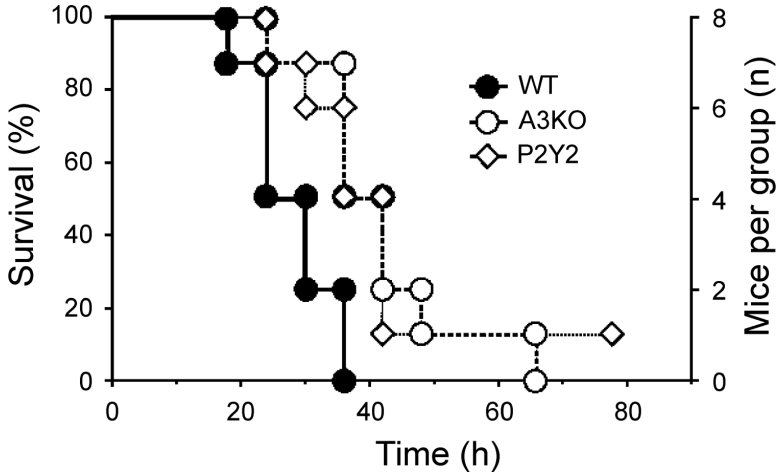

Fig 5. Mortality from sepsis is delayed in A3KO and P2Y2KO mice

Mortality of WT, A3KO, and P2Y2KO mice was monitored for 80 h after CLP. The survival curves for each mouse strain were analyzed and compared using Kaplan-Meier statistics (n = 8 per strain). At the 24-h time point, mortality rates in the A3KO and P2Y2KO groups were significantly lower than in the WT group ($P < 0.05$).