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Inhibition of IP6K1 Suppresses NPC Formation via Platelet Short-Chain Polyphosphate Polymers in Necrotizing Enterocolitis

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Running title: GSDMD Inhibition Reduces NETs in NEC

Abstract

Necrotizing enterocolitis (NEC) is a life-threatening intestinal inflammatory disease in preterm infants. Inositol hexakisphosphate kinase 1 (IP6K1) regulates platelet polyphosphate (polyP) homeostasis, yet their roles in NEC remain unclear. This study aimed to investigate the regulatory mechanism of platelet IP6K1 in neutrophil-platelet aggregate (NPA) formation and NEC pathogenesis, as well as the therapeutic potential of IP6K1 inhibition. Clinical analyses revealed significantly elevated circulating mitochondrial levels, anticardiolipin IgG concentrations and NPAs in NEC patients. Platelets in NEC patients exhibited reduced counts, abnormal morphological parameters and pyroptosis as the primary cell death mode. In the mouse NEC model, LPS-induced NPA formation, neutrophil infiltration and neutrophil extracellular trap (NET) release were significantly attenuated in IP6K1$^{-/-}$ mice and TNP-treated mice. IP6K1 deficiency and TNP treatment significantly improved the survival rate of NEC mice, ameliorated intestinal mucosal structural damage, reduced disease severity scores. Mechanistically, IP6K1 regulated NPA formation and subsequent inflammatory responses by mediating platelet short-chain polyP secretion, and exogenous polyP supplementation could rescue the impaired NPA formation in IP6K1$^{-/-}$ mice. P-selectin neutralization not only inhibited NPA formation but also reduced NET production in inflamed intestinal tissues. In conclusion, platelet IP6K1 promotes NPA formation and NET release as a novel critical regulator of NEC

pathogenesis and a promising therapeutic target for attenuating local and systemic inflammation in severe NEC.

Key words: Necrotizing enterocolitis; IP6K1; platelets; polyphosphate; neutrophil-platelet aggregates; neutrophil extracellular traps

Introduction

Necrotizing enterocolitis is a disease with unpredictable clinical manifestations and a broad spectrum of clinical outcomes, ranging from mild, reversible lesions to life-threatening severe cases characterized by severe intestinal inflammation [1, 2, 3]. Due to limited understanding of its underlying pathophysiology, current therapeutic strategies for NEC remain largely restricted to supportive care.

In addition to their established roles in hemostasis and coagulation, mounting evidence indicates that platelets exert proinflammatory effects in various inflammatory models, including NEC [4, 5, 6]. Multiple studies have confirmed that platelets promote neutrophil recruitment to inflammatory sites through secretion of factors such as CD40L, CCL5, and CXCL4, leading to the formation of neutrophil-platelet aggregates, thereby demonstrating the proinflammatory role of platelets in systemic inflammation [7, 8]. Furthermore, platelet IP6K1 has been reported to regulate neutrophil-platelet aggregate formation in endotoxin-induced intestinal inflammation [9]. Compelling evidence also suggests that platelets promote neutrophil extracellular trap formation against bacterial and viral components [10]. Recent studies have further indicated that neutrophil-platelet aggregates exacerbate pulmonary inflammation in the context of acute lung injury [6]. Although the roles of such aggregates in systemic vascular occlusion and acute lung injury have received increasing attention in recent years, their potential role in the pathophysiology of NEC remains unexplored.

Inositol hexakisphosphate kinase 1 is a critical enzyme regulating polyphosphate homeostasis [11]. Previous studies have demonstrated that platelets are the primary source of inorganic polyphosphates in mammalian cells and can secrete short-chain polyphosphate polymers with potent procoagulant activity [12]. Moreover, recent literature indicates that polyP can exert various proinflammatory effects, such as enhancing NF-κB signaling, increasing vascular permeability, and activating the complement system [13]. Elucidating the cellular and molecular mechanisms by which IP6K1 drives platelet-induced neutrophil-platelet aggregate formation will lay the foundation for developing novel therapies to prevent thromboinflammation in NEC.

In this study, we further investigated the regulatory role of IP6K1 in neutrophil-platelet aggregate formation during the pathophysiological process of NEC. We found that LPS-induced NPA formation and neutrophil accumulation in the

inflamed intestine during bacterial infection depend on IP6K1 expression in platelets. The role of IP6K1 in NPA formation is primarily mediated by inorganic polyphosphates.

Methods

Study Subjects and Ethical Approval

This study retrospectively analyzed clinical data from preterm infants diagnosed with necrotizing enterocolitis (NEC) at the Neonatal Intensive Care Unit (NICU) of Chongqing Health Center for Women and Children between June 2023 and December 2025 (Table 1). Inclusion criteria were: preterm infants (gestational age < 35 weeks), low birth weight (≤2000 g), postnatal age 1-31 days, and diagnosed with NEC at Bell stage ≥2 during the study period. Exclusion criteria included: pneumoperitoneum on abdominal radiographs, recurrent NEC, incomplete data, or non-compliance with management recommendations.

Eligible patients were divided into two groups based on treatment modality: those receiving conservative treatment (NEC stage II) and those undergoing surgical intervention (NEC stage III). The decision for surgery was made by the surgical team based on clinical judgment and disease severity. Indications for surgery included clinical deterioration or hemodynamic instability despite optimal medical management. Surgical interventions included exploratory laparotomy with resection of necrotic intestinal segments and either enterostomy or anastomosis in the presence of necrosis.

Intestinal tissue samples were collected from infants undergoing emergency exploratory laparotomy for confirmed NEC. Control tissue samples were obtained from infants undergoing surgery for congenital conditions unrelated to NEC, such as duodenal stenosis or ileal atresia (Table 1). All clinical data and demographic characteristics were reviewed by the principal investigator, who made the final inclusion or exclusion decisions for each case.

Blood samples (3 mL) were collected via central venous catheter on day 1 of enrollment using tubes containing 3.8% trisodium citrate (w/v). Twenty-two healthy volunteers provided blood samples as controls. Platelet parameters, including PDW and MPV, were obtained from routine clinical complete blood count (CBC) testing using an automated hematology analyzer.

This study was approved by the Institutional Review Board (IRB) of Chongqing Health Center for Women and Children (Approval No. WCHMU2024-066). Written informed consent was obtained from the parents or legal guardians of all enrolled infants prior to study inclusion.

Experimental Methods

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Chongqing Health Center for Women and Children. C57BL/6 mice were provided by Dr. Wenli Han from the Laboratory Animal Center of Chongqing Medical University, and IP6K1 knockout (IP6K1-/-) mice were provided

by Dr. Hongbo Luo from Boston Children's Hospital. This study used neonatal mice within 24 hours of birth, with a mean body weight of 1.36 ± 0.10 grams. These mice were exposed to stress conditions known to induce NEC, following established laboratory protocols including formula feeding, cold exposure, and hypoxic treatment. Pups were monitored daily for signs of NEC, including abdominal distension, apnea, and rectal bleeding. Clinical outcomes such as NEC incidence, body weight changes, time to NEC onset, and survival were meticulously recorded.

To evaluate the effect of the IP6K1 inhibitor TNP [N2-(m-trifluorobenzyl), N6-(p-nitrobenzyl)purine] in the NEC model, TNP (20 mg/kg body weight) was administered via intraperitoneal injection once daily for 5 days prior to NEC induction. TNP (Tocris Bioscience) was dissolved at a concentration of 2 mg/mL in a DMSO/Tween80/water mixture (1/1/8) and injected without further dilution.

On postnatal day 14, surviving animals were humanely euthanized with carbon dioxide. The entire intestine was removed for gross evaluation, followed by detailed microscopic examination to assess histological characteristics. NEC was confirmed by histological examination of the terminal ileum using criteria established by the research team. To observe morphological changes and neutrophil extracellular trap (NET) formation, intestinal tissues were fixed in 4% paraformaldehyde for 24 hours and subjected to hematoxylin and eosin (H&E) staining and immunofluorescence staining. Specific segments of the terminal ileum were preserved for further detailed analysis according to previously published methods.

Morphological and Histological Assessment

On postnatal day 14, following humane euthanasia of surviving animals, terminal ileum segments were collected and examined microscopically after H&E staining. Histological indicators of mucosal injury were assessed by a pathologist blinded to experimental conditions using a standardized grading system, as previously described.

Statistical Analysis

Data analysis was performed using GraphPad Prism software for Windows (version 4.0). The Shapiro-Wilk test was used to assess the normality of data distribution. Quantitative data with normal distribution were expressed as mean ± standard deviation (SD), and comparisons between two groups were performed using two-tailed Student's t-test, while comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Non-normally distributed data were expressed as median (25th-75th percentile) and analyzed using the Mann-Whitney U test. A P value <0.05 was considered statistically significant. Sample sizes were determined using PASS 11 software (NCSS, LLC, Kaysville, UT, USA), and power analysis was performed to ensure statistical validity. Correlation assessments were performed using Spearman's partial correlation coefficient. Survival data were analyzed using the log-rank (Mantel-Cox) test, and survival curves were plotted using the Kaplan-Meier method. Statistical significance is indicated in the figures and their corresponding legends.

Results

Increased Levels of Circulating Mitochondria in Patients with NEC

We first compared the concentration of mitochondrial particles in cell-free plasma samples from patients with NEC (n=62) and control subjects (n=26). The demographic and clinical characteristics of NEC patients with sepsis (NEC stage III), NEC patients without sepsis (NEC stage II), and control subjects are summarized in Table 1 and described in the Methods section. We found that plasma mitochondrial levels were significantly elevated in patients with NEC, particularly in those with severe disease (NEC stage III) (Fig. 1A). Furthermore, mitochondrial concentrations in both NEC patient groups were higher than those in the control group. These results collectively suggest that serum mitochondrial levels are closely correlated with the degree of inflammation and disease severity in patients with NEC. The numbers of circulating free mitochondria detected in plasma samples from healthy control subjects were very similar to values previously reported. Mitochondria are the sole source of cardiolipin, and we hypothesized that the presence of circulating mitochondria might induce the production of anticardiolipin antibodies. Quantitative analysis of anticardiolipin IgG levels in plasma samples from 32 patients with sepsis, 30 patients without sepsis, and 26 control subjects revealed that anticardiolipin IgG concentrations were significantly higher in patients with sepsis compared to those without sepsis, and both patient groups exhibited higher levels than the control group (Fig. 1B). We examined whether mitochondrial levels correlated with anticardiolipin IgG levels but found no significant correlation (Fig. 1C).

Neutrophils and Platelets Are Key Players in Host Defense and Inflammation-Induced Intestinal Injury in NEC Patients

Previous studies have established that NEC is characterized by substantial neutrophil infiltration into the intestinal tissue. We quantified neutrophils, platelets, and monocytes in both peripheral blood and intestinal tissue. Platelet counts progressively decreased after the onset of NEC in patients compared to healthy subjects (Fig. 2A). Patients with NEC exhibited thrombocytopathy, characterized by increased mean platelet volume, elevated platelet distribution width, and a higher proportion of large platelets (Fig. 2B). These results collectively indicate that platelet counts and morphology are closely associated with inflammation and disease severity in NEC patients. This raises critical questions regarding whether aberrant platelets could serve as an additional biomarker for assessing NEC severity and their role in exacerbating NEC pathophysiology.

Furthermore, we assessed the presence of granulocytes (identified by anti-Ly6G monoclonal antibody), macrophages (identified by anti-F4/80 monoclonal antibody), and lymphocytes (CD3-positive) in intestinal samples. As shown in the bar graph in Figure 2C, NEC was associated with pronounced granulocyte infiltration and a modest increase in macrophages within the small intestine. However, as illustrated in Figure 2C, there was no significant difference in lymphocyte counts between the NEC and control groups. Neutrophil numbers in the intestinal tissue progressively increased during NEC progression.

To elucidate the platelet changes associated with NEC, we performed flow cytometric analysis to determine the predominant cell death pathways in platelets from septic

patients. As shown in Figure2D, approximately 36% of platelets underwent apoptosis (caspase 3/7 positive), 5% underwent autophagy (LC3B positive), and 53% underwent pyroptosis (caspase 1 positive). Platelet pyroptosis was predominantly observed in NEC, especially in severe cases (with septic shock). These data indicate that pyroptosis is the primary form of platelet death in patients with NEC. Moreover, caspase 1 activation was significantly elevated in platelets from patients with severe sepsis compared to healthy subjects (Fig. 2D).

Platelet-Neutrophil Aggregates in NEC

Leukocyte-platelet aggregate formation is a common feature of inflammatory states, and NPA formation promotes neutrophil accumulation in various acute inflammatory conditions. Previous studies have established that NEC is characterized by significant neutrophil infiltration. We hypothesized that the formation of these complexes might influence neutrophil activation and NETs formation during NEC progression. We studied NEC patients with sepsis, NEC patients without sepsis, and control subjects. Elevated levels of circulating neutrophil-platelet aggregates were detected in patients with NEC compared to healthy controls, with 45% of patients having more than 40% of their neutrophils involved in aggregate formation (Fig. 3A). In contrast, this increase was not observed in the control group. We also confirmed that neutrophil-platelet aggregates were significantly higher in NEC patients with a history of sepsis compared to those without sepsis (Fig. 3A). Accompanying these findings, we observed a significant increase in the platelet activation marker P-selectin (CD62P) in patients with NEC compared to healthy controls (Fig. 3B). Notably, this increase was positively correlated with neutrophil-platelet aggregates (Fig. 3C), suggesting P-selectin as a potential mediator of neutrophil-platelet aggregation in NEC. Next, we examined whether P-selectin-mediated platelet-neutrophil contact is important for NETs formation. We found that immunological neutralization of P-selectin significantly reduced NEC-induced PNA formation (Fig. 3D). Furthermore, P-selectin inhibition significantly decreased levels of DNA-histone complexes in the plasma (Fig. 3E) as well as histone 3 and histone 4 levels in the pancreas (Fig. 3F, 3G) of NEC animals.

IP6K1 Deficiency Attenuates NPA Formation and NET Release During NEC Development

These findings also suggest that IP6K1 could be a rational therapeutic target for infection and inflammation-induced intestinal injury, providing a mechanism for controlling neutrophil accumulation during intestinal infection and inflammation. To determine whether IP6K1 deficiency or treatment with the specific IP6K1 inhibitor TNP attenuates inflammatory cell recruitment and subsequent NETosis in a mouse model of NEC, we quantified NPAs in the peripheral blood of live animals (Fig.4A). The percentage of NPAs was increased in the blood of LPS-challenged mice compared to untreated mice, but this increase was attenuated in TNP-treated NEC mice and IP6K1-deficient NEC mice. Consistent with the genetic knockout model, TNP treatment significantly reduced NET levels in both plasma and intestinal tissue compared to vehicle-treated controls(Fig.4B).

To directly assess whether IP6K1 disruption reduces neutrophil accumulation, we examined neutrophils in an experimental NEC model. IP6K1 deficiency hindered the infiltration of inflammatory cells, primarily neutrophils, into the intestine compared to wild-type neonatal mice(Fig.4C). Additionally, IP6K1 deficiency reduced MPO and ROS production(Fig.4D). IP6K1 is known to promote the release of chemokines and cytokines, thereby enhancing neutrophil infiltration. Based on this, we analyzed the levels of various cytokines and chemokines in peripheral blood and intestinal tissue. IP6K1 deficiency reduced serum inflammatory cytokine levels (IL-1β and IL-6) and minimized intestinal tissue damage(Fig.4E). NEC development significantly upregulated the expression of the chemokines CXCL1, CXCL2, and CCL3. Notably, these elevated chemokine levels, particularly CXCL2 and CCL3, were significantly reduced in IP6K1-/- mice (Fig.4F), highlighting the critical role of IP6K1 in modulating the inflammatory response.

IP6K1 Deficiency Confers Protection Against NEC

Survival analysis revealed that IP6K1-/- mice exhibited significantly higher survival rates following NEC onset compared to wild-type mice (Fig.5A). Further investigation into the protective effect of IP6K1 deficiency on NEC severity revealed that while NEC neonatal mice showed compromised microvilli integrity and structural damage to mucosal epithelial cells, IP6K1-/- neonatal mice displayed marked improvement in these histological alterations, rendering them nearly indistinguishable from control specimens (Fig.5B). Pathology scores by blinded observers also indicated lower disease severity in IP6K1-deficient NEC neonates (Fig.5C). Moreover, IP6K1 deficiency not only prevented bacterial translocation but also reduced levels of the bacterial translocation marker lipopolysaccharide, underscoring the significant physiological importance of IP6K1-mediated signaling in intestinal health. Bacterial translocation assays showed that IP6K1 deficiency effectively inhibited bacterial translocation across the intestinal barrier into mesenteric lymph nodes and reduced LPS levels (Fig.5D,E), suggesting a beneficial effect of IP6K1 deficiency in ameliorating NEC.

We further assessed the impact of IP6K1 deficiency on intestinal immune barrier function by examining β-defensin-2 expression levels and transepithelial electrical resistance (TER). β-defensin-2 was significantly increased while TER levels were significantly decreased in the NEC group compared to controls (Fig.5F,G). These alterations were significantly mitigated in IP6K1-deficient mice, indicating enhanced immune barrier function. Furthermore, intestinal mucosal permeability was elevated in NEC mice compared to controls, and this elevation was partially rescued in IP6K1-deficient mice (Fig.5H). Concurrently, levels of Muc2 and secretory immunoglobulin A were decreased in NEC neonatal pups compared to controls (Fig.5I-K). However, IP6K1 deficiency significantly ameliorated these changes, suggesting enhanced immune barrier function upon IP6K1 deletion.

Treatment with the IP6K1 Inhibitor TNP Attenuates Intestinal Inflammation and Injury Associated with Bacterial Pneumonia

The data above suggest that IP6K1 could be a rational therapeutic target for infection

and inflammation-induced intestinal injury, as demonstrated in the IP6K1-KO mouse model. Therefore, we next investigated whether the specific IP6K1 inhibitor TNP could also attenuate intestinal injury in a mouse model of NEC. Consistent with the observations in IP6K1-deficient mice, TNP-treated mice showed significantly improved morphological indices assessed by histomorphometric analysis of intestinal tissue (Fig.6A). Additionally, IP6K1-/- and TNP-treated mice showed significantly improved survival rates following NEC onset compared to wild-type mice (Fig.6B). Furthermore, in taurocholate-treated animals, TNP administration resulted in a reduction in plasma DNA-histone complex levels, and reductions in pancreatic histone 3 and histone 4 levels, respectively (Fig.6C-E). TNP treatment effectively reduced polyP production in platelets; platelets isolated from TNP-treated mice displayed reduced intracellular polyP levels and decreased LPS-induced polyP secretion compared to platelets isolated from DMSO-treated controls(Fig.6F-G). Consistent with observations in IP6K1-deficient mice, TNP-treated mice showed significantly improved bactericidal activity and exhibited inhibited neutrophil accumulation in the inflamed intestinal(Fig.6H), as assessed by neutrophil counts in bronchoalveolar lavage fluid(Fig.6I).

Discussion

The present study demonstrates that necrotizing enterocolitis induces the formation of platelet-neutrophil aggregates, which subsequently accelerate neutrophil accumulation and activation, promote neutrophil extracellular trap formation, and amplify proinflammatory cytokine production. We further demonstrate that IP6K1 serves as a critical regulator of organ dysfunction progression during NEC pathogenesis by modulating inorganic polyphosphate generation in platelets and influencing NPA formation. Genetic deletion of IP6K1 or pharmacological inhibition with TNP in a mouse NEC model not only prevented NPA formation but also attenuated organ injury, improved survival in the NEC mouse model, and reduced NET-mediated excessive inflammatory responses. Furthermore, we confirmed that plasma concentrations of neutrophil-platelet complexes were significantly elevated in NEC patients.

NEC is a serious acute condition that can rapidly progress to severe sepsis. Early recognition is crucial; however, due to limited understanding of the underlying pathophysiological mechanisms and lack of specific therapeutic approaches, mortality remains as high as 20% in NEC patients who develop systemic complications [14, 15, 16]. Numerous studies have demonstrated that circulating platelet-leukocyte complexes are a common phenomenon in various inflammatory states, including reperfusion injury, abdominal sepsis, pulmonary infection, and acute cardiac disease [17, 18, 19]. Although the concept of NPA has existed for more than a decade, direct evidence regarding NPA-mediated neutrophil accumulation remains insufficient. Previous studies by Asaduzzaman et al. also indicated that platelets play a critical role in regulating pulmonary neutrophil infiltration through upregulation of Mac-1 in cecal ligation and puncture-induced sepsis [20]. Extensive research has confirmed the

ability of platelets to promote neutrophil accumulation [21]. During acute pulmonary inflammation, NPA formation facilitates neutrophil accumulation in the alveolar space. Furthermore, intravascular NPAs may directly damage pulmonary capillary endothelium, exacerbating lung injury. In mouse models of transfusion-related acute lung injury as well as LPS-induced or acid-induced acute respiratory distress syndrome, both neutrophils and platelets become sequestered in the pulmonary vascular system. Therapeutic depletion of platelets or disruption of NPAs significantly attenuates inflammation-induced lung injury [6, 22]. Although the potential functions of such aggregates in inflammation have received considerable attention recently, the specific role of platelet-leukocyte complexes in the pathophysiology of NEC remains unclear. We report for the first time that platelet-neutrophil complexes increased 7-fold in experimental NEC mice, consistent with observations in NEC patients. Notably, recent studies have reported that IP6K1 plays a critical role in platelet-mediated neutrophil activation and pulmonary recruitment during systemic inflammation by promoting neutrophil-platelet aggregate formation. We observed that inhibition of P-selectin not only abolished platelet-neutrophil aggregate formation but also significantly reduced NET formation in inflamed small intestinal tissue. These findings suggest that physical contact between platelets and neutrophils is essential for platelet-mediated NPC formation.

IP6K1 serves as a novel regulator of mammalian hemostatic function by controlling inorganic polyphosphate production in platelets. Polyphosphates also exert various proinflammatory effects, such as activation of the kallikrein-kinin system and complement system [23, 24]. IP6K1-deficient mice exhibit reduced platelet polyphosphate levels, slower platelet aggregation, and impaired platelet-mediated plasma coagulation [25]. We hypothesized that the reduced NPA formation, attenuated neutrophil accumulation, and ameliorated small intestinal injury observed in IP6K1-deficient mice were due to decreased polyphosphate generation. Indeed, in this study we observed reduced platelet polyphosphate levels in IP6K1-deficient mice. This study confirms that IP6K1 is a critical regulator of NPC formation during inflammatory progression in NEC. We demonstrate that IP6K1 deficiency results in reduced neutrophil accumulation in the small intestine of NEC mice, and that platelet-derived polyphosphates are essential for neutrophil accumulation. IP6K1 promotes NPA formation by maintaining high levels of PolyP in platelets [26, 11, 27]. Our data also indicate that platelet IP6K1 is a potential target for inhibiting NPC formation and tissue damage in NEC. The reduction in neutrophil accumulation in IP6K1-deficient mice is most likely attributable to decreased polyphosphate production by their platelets. Consistent with this, NET formation induced by thrombin stimulation was reduced in mixed systems of IP6K1-deficient platelets with wild-type neutrophils, which may be attributed to reduced polyphosphate production by deficient platelets, as this defect could be rescued by addition of exogenous polyphosphates [28]. Polyphosphate treatment rescued impaired LPS-induced NPA formation between IP6K1-deficient platelets and neutrophils, indicating that the function of IP6K1 in NPA formation is mediated by polyphosphates. Treatment of IP6K1-deficient mice with polyphosphates restored their LPS-induced small intestinal

neutrophil accumulation to levels comparable to wild-type mice. However, polyphosphate treatment alone was insufficient to induce NPA formation; this IP6K1-mediated process requires LPS stimulation. Therefore, other LPS-dependent factors are involved in NPA formation.

These results indicate that platelet IP6K1 serves as a novel regulator of NETosis through polyphosphate secretion, which has important implications not only for NEC but also potentially for other diseases characterized by NETs in their pathophysiology, such as sepsis, infection, and autoimmune diseases [29, 30, 31]. Combined with our current findings and the fact that polyphosphates are potent stimulators of the coagulation system, blocking IP6K1 may be considered a useful strategy for simultaneously antagonizing the pathological inflammation characteristic of severe NEC.

In conclusion, this study demonstrates for the first time that platelets regulate neutrophil accumulation and neutrophil-platelet complex formation during the course of necrotizing enterocolitis. Inhibition of NPC formation through IP6K1 knockout reduces NEC mortality. Our data also indicate that IP6K1, likely through platelet secretion of polyphosphates, is a critical regulator of NPC formation, inflammatory response, and tissue damage. Therefore, these findings establish a novel function for IP6K1 in NEC-associated inflammation and suggest that targeting IP6K1 may be beneficial for attenuating local and systemic inflammation in severe NEC.

Declarations section

Ethics Approval and Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and Materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

No potential conflicts of interest relevant to this article are reported.

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Author's Contributions

K.G., M.Z., Q.L., and Y.M. performed the research. Y.M. and C.G. designed the research study. H.X., and C.G. contributed essential reagents or tools. K.G., M.Z., Q.L. and Y.M. analysed the data. Y.M. and C.G. wrote the paper.

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Figure

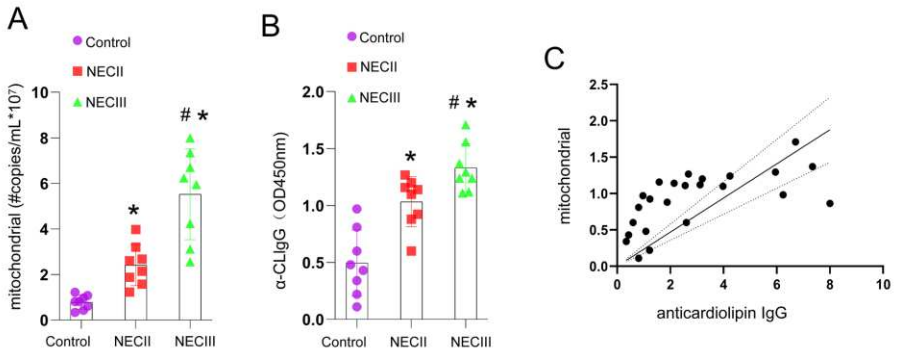


Figure 1. Circulating Mitochondrial Levels in NEC Patients
(A) Circulating mitochondrial levels in plasma samples from NEC patients and control subjects. Data are expressed as mean ± SD. *P < 0.01 versus control group; #P < 0.01 versus NEC stage II. (B) Anti-cardiolipin immunoglobulin G concentrations in plasma samples from NEC patients and control subjects were measured by enzyme-linked immunosorbent assay and expressed as optical density values. P values were calculated using two-tailed Student's t-test with Welch's correction. (C) Spearman correlation analysis between neutrophil-platelet aggregates and P-selectin expression in aPL-positive patients.

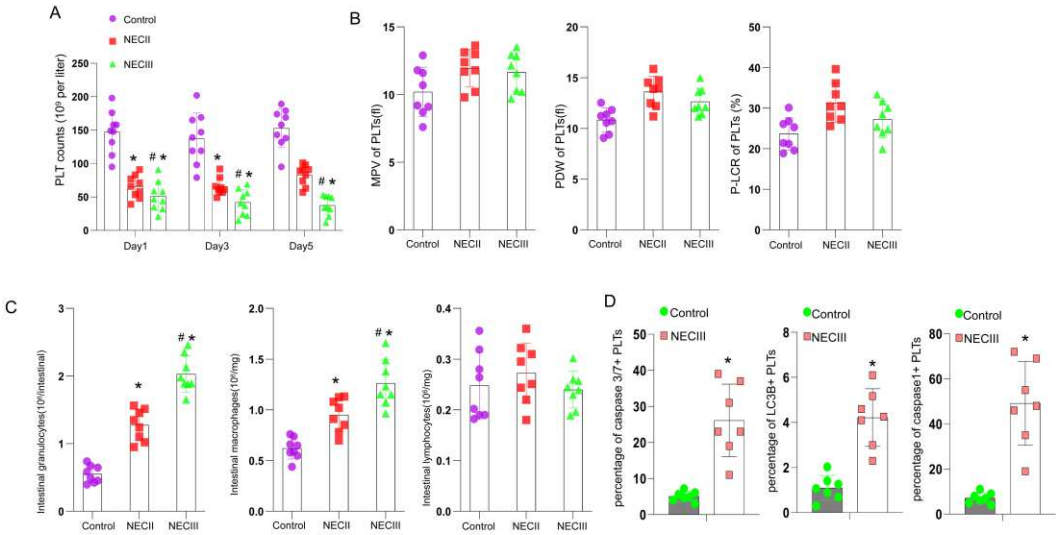


Figure 2. Evaluation of Neutrophils and Platelets in NEC Patients
(A) Platelet counts in NEC patients at different time points. Data are expressed as

mean ± SD. P < 0.01 versus control group; #P < 0.01 versus NEC stage II. (B) Platelet parameters in NEC patients versus control subjects: mean platelet volume, platelet distribution width, and platelet-large cell ratio. (C) Quantification of granulocyte, macrophage, and lymphocyte counts in neonatal micemice from NEC patients and control subjects. Bar graph data are expressed as mean ± SD. *, P < 0.01; #, P < 0.01 (one-way ANOVA). (D) Bar graph showing the percentage activation of caspase-3/7 (apoptosis), LC3B (autophagy), and caspase-1 (pyroptosis) in platelets measured by flow cytometry. Data are expressed as mean ± SD. *P < 0.01 versus control group.

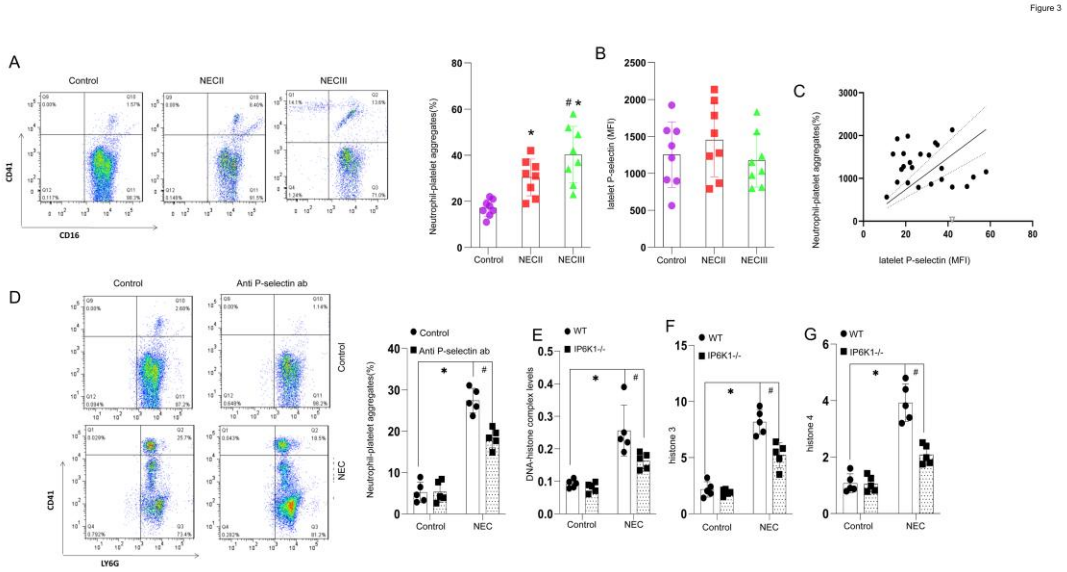


Figure 3. Evaluation of Platelet-Neutrophil Aggregates in NEC Patients
(A) Neutrophil-platelet aggregates in fresh blood from NEC patients and control subjects were measured by flow cytometry. Data are expressed as mean ± SD. *, P < 0.01; #, P < 0.01 (one-way ANOVA). (B) Platelet P-selectin (CD62P) expression in fresh blood from NEC patients and control subjects was assessed by flow cytometry. (C) Spearman correlation analysis between neutrophil-platelet aggregates and P-selectin expression in aPL-positive patients. (D) Flow cytometric analysis of NPA formation in peripheral blood. Data are mean ± SD (n = 5 mice). *P < 0.05 versus wild-type mice (one-way ANOVA). (E) Levels of DNA-histone complexes in plasma. (F) Levels of histone H3 in pancreatic tissue. (G) Levels of histone H4 in pancreatic tissue. Data are expressed as mean ± SD. *, P < 0.01; #, P < 0.01 (one-way ANOVA).

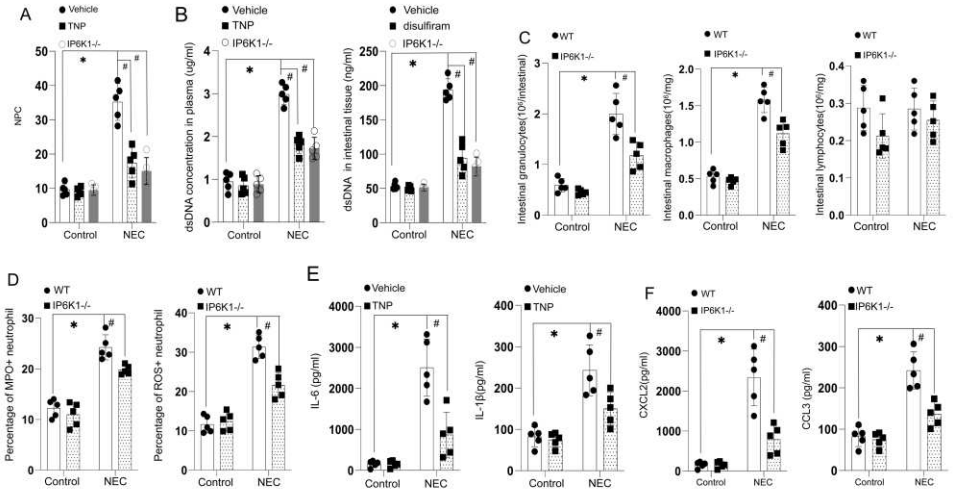


Figure 4. IP6K1 Deficiency Attenuates NPA Formation and NET Release During NEC Development

(A) Flow cytometric analysis of NPA formation in peripheral blood from mice subjected to different treatments. Data are mean ± SEM (n = 6 mice). , $P < 0.01$; #, $P < 0.01$ (one-way ANOVA). (B) Quantification of dsDNA (NET structures) in plasma (left) and intestinal tissue (right) from mice subjected to different treatments. $P < 0.01$; #$P < 0.05$, Mann–Whitney U test. Data are expressed as mean ± SD. , $P < 0.01$; #, $P < 0.01$ (one-way ANOVA). (C) Quantification of granulocyte, macrophage, and lymphocyte counts in neonatal micesubjected to different treatments. Bar graph data are expressed as mean ± SD. , $P < 0.01$; #, $P < 0.01$ (one-way ANOVA). (D) Bar graph describing MPO or ROS expression levels in neutrophils subjected to different treatments analyzed by flow cytometry. Data are expressed as mean ± SD (n = 7). *$P < 0.01$; #$P < 0.01$ (one-way ANOVA). (E) Bar graph showing levels of IL-1β and IL-6 in plasma from neonatal mice subjected to different treatments (n=7). Data are expressed as mean ± SD. *$P < 0.01$; #$P < 0.01$, one-way ANOVA. (F) Bar graph showing levels of CXCL2 and CCL3 in plasma from mice subjected to different treatments (n=7). Data are expressed as mean ± SD. *$P < 0.01$; #$P < 0.01$, one-way ANOVA.

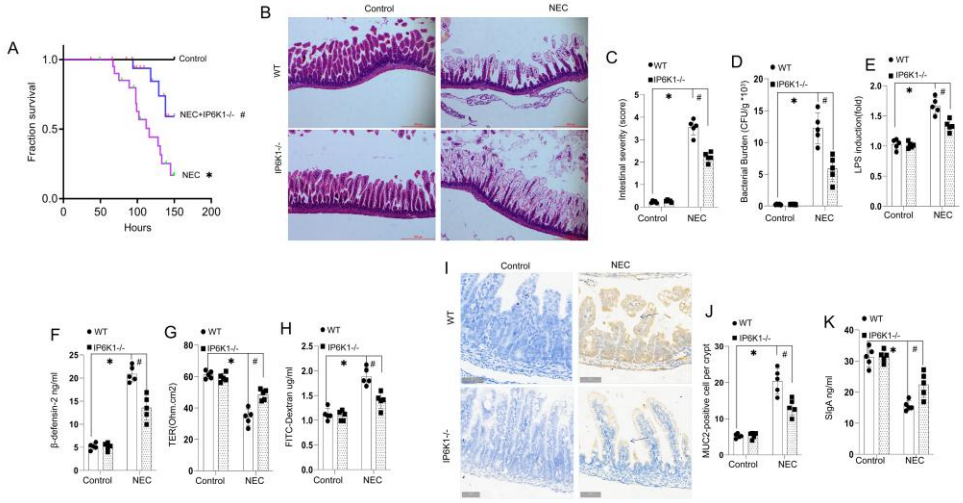


Figure 5. IP6K1 Deficiency is Protective Against NEC Phenotype
(A) Kaplan-Meier survival analysis of neonatal mice in different treatment groups. Data represent survival percentage (n = 10–18 mice per group). *P < 0.01, log-rank test. (B) Representative H&E staining of neonatal intestinal sections from neonatal mice subjected to different treatments (n = 6 animals per group, 12 fields per animal). Scale bar = 50 μm. (C) NEC severity score calculated based on morphological changes (n = 7). Data are expressed as mean ± SD. *P < 0.01; #P < 0.01 (one-way ANOVA). (D) Bacterial growth in the terminal ileum of neonatal mice under specified conditions. Data are expressed as mean ± SD. *P < 0.01; #P < 0.01, one-way ANOVA. (E) Serum endotoxin levels in neonatal mice from specified groups (n = 7). Values are expressed as mean ± SD. *P < 0.01; #P < 0.01 (one-way ANOVA). (F) Concentration of β-defensin-2 in the terminal ileum of neonatal mice subjected to different treatments (n=7). Data are expressed as mean ± SD. *P < 0.01; #P < 0.05, one-way ANOVA. (G) Assessment of TER levels in neonatal mice subjected to different treatments (n = 7). Experiments were repeated at least three times. Data are expressed as mean ± SD. *P < 0.01; #P < 0.01, one-way ANOVA. (H) Serum FITC-dextran levels in mice from specified treatment groups as an indicator of intestinal permeability (n = 7). Results are expressed as mean ± SD. (I) Representative Muc2 immunostaining in intestinal tissue from mice subjected to different treatments (n = 6 animals per group, 12 fields per animal). Scale bar = 50 μm. (J) Histogram of semi-quantitative analysis of Muc2-positive cells per crypt. Twelve fields per section were analyzed for each mouse. Data represent mean ± SD. *P < 0.01; #P < 0.05, one-way ANOVA. (K) Concentration of SIgA in the terminal ileum of mice subjected to different treatments (n=7). Data are expressed as mean ± SD. *P < 0.01; #P < 0.01, one-way ANOVA.

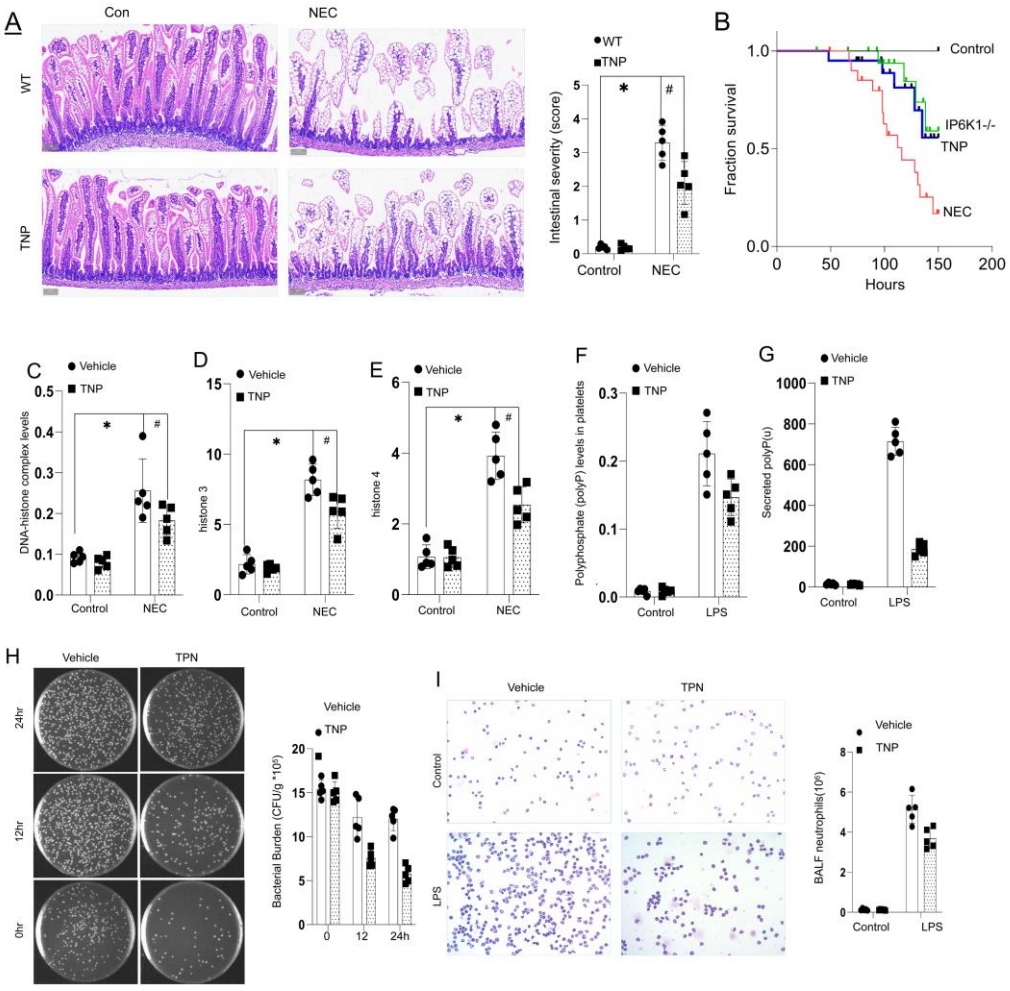


Figure 6. TNP Alleviates Intestinal Inflammation Through the NPA Pathway
(A) Representative H&E staining of neonatal intestinal sections from neonatal mice subjected to different treatments (n = 6 animals per group, 12 fields per animal). Scale bar = 50 μm. NEC severity score was calculated based on morphological changes (n = 7). Data are expressed as mean ± SD. *P < 0.01; #P < 0.01 (one-way ANOVA). (B) Kaplan-Meier survival analysis of neonatal mice in different treatment groups. Data represent survival percentage (n = 10–18 mice per group). *P < 0.01, log-rank test. (C) Levels of DNA-histone complexes in plasma. Data are expressed as mean ± SD. P < 0.01; #P < 0.01 (one-way ANOVA). (D) Levels of histone H3 in intestinal tissue. Data are expressed as mean ± SD. P < 0.01; #P < 0.01 (one-way ANOVA). (E) Levels of histone H4 in intestinal tissue. Data are expressed as mean ± SD. *P < 0.01; #P < 0.01 (one-way ANOVA). (F) Polyphosphate levels in platelets from neonatal mice subjected to different treatments (n = 6 animals). Data are expressed as mean ± SD. *P < 0.01; #P < 0.01 (one-way ANOVA). (G) Platelets were treated with TNP (20 μM) at room temperature for 4 days to induce LPS-stimulated polyP secretion. PolyP levels in supernatant were measured. Data are mean ± SEM from four experiments, expressed as mean ± SD. *P < 0.01; #P < 0.01 (one-way ANOVA). (H) Mice were treated with TNP or dimethyl sulfoxide alone for 10 days (20 mg/kg body weight, once daily). Mice were then given a single intraperitoneal injection of 5.13 × 10^8 CFU

Staphylococcus aureus and sacrificed at specified time points. Bacterial killing capacity was quantified as CFU per mouse. Data are shown as mean ± SEM (n=4 mice). *p<0.01; #p<0.01 (one-way ANOVA). (I) Representative smear test of total cells from peritoneal lavage fluid from cells subjected to specified treatments and stained with modified Wright-Giemsa. Experiments were repeated at least 3 times. Quantitative assessment of neutrophils was performed based on cytological smear staining data. Data are expressed as mean ± SD. *p<0.01; #p<0.01 (one-way ANOVA).

Supplementary Files

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