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Role of Platelets in Neonatal Necrotizing Enterocolitis

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Abstract

Necrotizing enterocolitis (NEC) is an inflammatory bowel necrosis of premature infants, and is a leading cause of morbidity and mortality in infants born between 23–28 weeks of gestation. Fifty to 95% of all infants with NEC develop thrombocytopenia (platelet counts $<150 \times 10^9$/L) within 24–72 hours of receiving this diagnosis. In many patients, thrombocytopenia is severe and is treated with one or more platelet transfusions. However, the underlying mechanism(s) and biological implications of NEC-related thrombocytopenia remain unclear. This review presents current evidence from human and animal studies on the clinical features and mechanisms of platelet depletion in NEC. Anecdotal clinical experience is combined with evidence from laboratory studies and from an extensive literature search in databases PubMed, EMBASE, Scopus, and the electronic archives of abstracts presented at the annual meetings of the Pediatric Academic Societies. To avoid bias in identification of existing studies, key words were short-listed prior to the actual search both from anecdotal experience and from PubMed's Medical Subject Heading (MeSH) thesaurus.

1. Introduction

Necrotizing enterocolitis (NEC) is an inflammatory bowel necrosis of premature infants, and a leading cause of mortality in infants born between 22–28 weeks' gestation (1, 2). Histopathologically, NEC is characterized by coagulative necrosis, inflammation, interstitial hemorrhages, and reparative changes. These changes typically begin in the mucosa and progress outward towards the serosa (3). The etiology of NEC remains uncertain; there are associations with hypoxia and hypothermia, inadequate anti-microbial defenses due to immature Paneth cells, mucosal inflammation in severely anemic infants that may worsen with red blood cell (RBC) transfusions, or following enteral exposure to immunological stimulants (4–7). Premature infants who develop enteric dysbiosis with a predominance of Gram-negative bacteria and have bacterial overgrowth may also be at enhanced risk of NEC (8–11)(8–11)(8–11)(8–11).

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Fifty to 95% of all infants with NEC develop thrombocytopenia (platelet counts <150 × 10^9/L) within 24–72 hours of receiving this diagnosis (11). This review focuses on thrombocytopenia in acute NEC, summing up anecdotal clinical experience, laboratory studies, and mechanistic information from an extensive literature search using the databases PubMed, EMBASE, and Scopus, and the electronic archive of abstracts submitted for the annual meetings of the Pediatric Academic Societies. To avoid bias, key words were short-listed from personal experience and from PubMed's Medical Subject Heading (MeSH) thesaurus before the actual search.

2. Low platelet counts in NEC

Thrombocytopenia (platelet counts <150 × 10^9/L) is a frequently encountered hematological abnormality in infants with NEC (11). Patients with confirmed NEC typically show decreased platelet counts within 24 hours of disease onset (Figure 1a). This thrombocytopenia may worsen until 72 hours, and the depth of the nadir correlates with the severity and extent of bowel injury. In an occasional infant, thrombocytopenia may precede abdominal signs of NEC by up to 24 hours (12, 13). In our institutional reviews, we have noted these drops in platelet counts to correlate with Bell's clinical stage of NEC (Figure 1b). Infants with advanced NEC needing surgery may show platelet counts as low as 30–60 × 10^9/L (11).

NEC is a leading cause of acute and subacute thrombocytopenia in premature and critically ill infants admitted to tertiary referral centers (11). Several reports describe a 50–90% drop in platelet counts in acute NEC (14–17). Some of the variability in the observed fall in platelet counts at diagnosis could be rooted in the proportion of infants admitted with Bell's stage 1/feeding intolerance, who may have some delay or uncertainty in the diagnosis of NEC (11). In patients who have a confirmed diagnosis, thrombocytopenia seems to correlates better with the severity and extent of bowel injury. A rapid drop in platelet counts below 100 × 10^9/L within 12–24 hours of disease onset may indicate a high likelihood of bowel necrosis and need for surgical intervention (14). In a review of 58 infants with NEC, Ververidis *et al.* (14) reported 54 (93%) infants with platelet counts <100 × 10^9/L within 24 hours of disease onset. Kenton *et al.* (15) detected thrombocytopenia in 47/91 (52%) of their patients with NEC. Nearly a third of enrolled patients in these two studies who developed severe thrombocytopenia (<50 × 10^9/L) within 24 hours of diagnosis, needed surgery. Ragazzi *et al.* (16) noted thrombocytopenia in 129/232 (56%) patients and the magnitude of drop in platelet counts reflected the extent of disease. Similar observations were reported by Hutter (18), Patel (17), and O'Neill *et al.* (19).

During acute NEC, thrombocytopenia predicts adverse clinical outcomes. Hutter *et al.* (18) noted significantly lower platelet nadirs in infants with fatal NEC than in survivors (46.5 × 10^9/L *vs.* 69.3 × 10^9/L). In the study described above, Ververidis *et al.* (14) reported 16/58 (28%) deaths; these patients had lower nadirs in platelet counts than the survivors. Infants who survived maintained platelet counts >100 × 10^9/L during the course of the disease. Ragazzi *et al.* (16) recorded thrombocytopenia in 86% infants with fatal disease. Similar results were reported by Kenton *et al.* (15), who observed higher platelet counts in survivors (median 203 × 10^9/L *vs.* 33 × 10^9/L in non-survivors; $p<0.001$). Severe thrombocytopenia

was a significant predictor of mortality (adjusted odds ratio, OR 6.39) and of complications such as cholestasis and short bowel syndrome (adjusted OR 5.47). Amongst survivors, platelet counts recovered to >150 × 10^9/L within 7 to 10 days (14, 15, 17). In another study, Baer *et al.* (20) reviewed 11,281 neonates treated in level III NICUs, and identified 273 (2.4%) with platelet counts <50 × 10^9/L. NEC was noted in 52 (14%) of these infants. The same group identified NEC as the leading diagnosis in very high users of platelet transfusions (≥20 platelet transfusions). Interestingly, infants who develop NEC may show altered thrombopoiesis with a lower platelet mass index (platelet count × mean platelet volume) than controls before the onset of NEC, even within the first postnatal week (21).

3. Pathophysiology of thrombocytopenia in NEC

The exact mechanism of NEC-related thrombocytopenia remains unclear. The following sections provide an overview of evidence from human and murine studies.

(a) Human studies

Patients with confirmed NEC may show a rapid drop in platelet counts and only brief, limited corrections in platelet counts following transfusions. The site of platelet consumption are unclear, although microthrombotic events in the diseased intestine remain an important possibility (22, 23). Conceptually, platelet activators such as bacterial products, platelet-activating factor, arachidonic acid metabolites, and coagulation factors may be important in NEC pathogenesis (11, 24). These mediators can stimulate endothelial cells and macrophages in concert with thromboplastin released from the gangrenous bowel, to induce secondary mediators such as inflammatory cytokines and nitric oxide. Together, these stimuli can promote platelet activation and aggregation in the microvasculature (11, 19).

The kinetic basis of thrombocytopenia in human NEC remains a subject of investigation. Brown *et al.* (25, 26) evaluated thrombopoiesis in 20 critically ill infants with sepsis or NEC by measuring serial platelet counts, blood thrombopoietin (Tpo) levels, reticulated platelets (RPs), and megakaryocyte progenitors. Elevated Tpo levels were associated with increased megakaryopoiesis and platelet release. The authors speculated that the severity of illness and specific platelet and/or bacterial products could downregulate thrombopoiesis. In another study, Cremer *et al.* (27) followed platelet counts and immature platelet fractions (IPFs) in 21 infants with surgical NEC or late-onset sepsis. Platelet counts correlated with IPFs; infants with fatal NEC had lower platelet counts and IPFs than survivors. The reasons for this dampened thrombopoiesis during severe NEC were unclear, but could include known inhibitors of megakaryopoiesis such as platelet factor (PF)-4, released from activated platelets (28). Kampanatkosol *et al.* (29) also found fewer circulating reticulated platelets in infants with late-onset NEC than controls, possibly due to decreased or failing thrombopoiesis. However, Cekmez *et al.* (30) have noted that infants who went to develop NEC had higher mean platelet volumes, possibly reflecting increased thrombopoiesis, even in their early neonatal period.

We can appreciate that human data on the mechanisms of NEC-related thrombocytopenia are relatively limited, and sometimes conflicting. In the following sections, we describe our

ongoing efforts to develop a murine model to understand the changes in circulating platelets and bone marrow megakaryocytes during NEC-like intestinal injury.

(b) Murine model of NEC-related thrombocytopenia

We have developed a murine model of NEC-related thrombocytopenia where we administer an immunological stimulant, trinitrobenzene sulfonate (TNBS) in two doses of 50 mg/kg in 30% ethanol (w/v) by gavage and rectal instillation, on postnatal day (P) 10. These pups develop an acute necrotizing ileocolitis resembling human NEC within 15–18 hours (Figure 2) (12). These intestinal changes recapitulated the intestinal necrosis, the regional predilection for distal ileum and proximal colon (31), characteristic features of the mucosal inflammatory response and induction of specific cytokines (31, 32), monocyte/macrophage infiltration (31, 33), and specific changes in genome-wide transcription/inflammatory signaling in the intestine (7, 32). TNBS did not cause intestinal damage in germ-free pups (31), indicating that its inflammatory effects (a) required the presence of intestinal microflora; and (b) were not secondary to a direct chemical/corrosive action. TNBS-induced ileocolitis in pups was clearly distinct from its effects in adult mice, which were comprised of subacute/chronic inflammatory mucosal changes such as basal cryptitis and were most prominent in the distal colon (31).

To investigate the kinetic basis of NEC-related thrombocytopenia, we compared mice in control and NEC-like injury groups for serial platelet counts, platelet volume indices, and immature platelet fractions in blood, and the number/ploidy of megakaryocytes in the bone marrow (12). Pups with intestinal injury showed decreased platelet counts at 15–24 hours. The severity of thrombocytopenia correlated with the severity of intestinal injury and resembled the drop in platelet counts seen in human NEC (14, 16–19). We also noted increased platelet volume and IPF, platelet distribution width, platelet-large cell ratio, and the number/ploidy of bone marrow megakaryocytes, indicating increased platelet production (12). Pups with mild intestinal injury showed a reduction in platelet counts at 18 hours with a nadir at 24 hours. In moderate-severe injury, platelet counts were lower than controls at 15 hours and reached a minimum at 18 hours. These platelet counts then showed a time-dependent recovery between 24–48h (hazard ratio = 0.996, 95% confidence interval 0.995–0.997). The commitment of megakaryocytes and thrombopoiesis seen in this model resembled human NEC and favored increased peripheral consumption of platelets, not impaired production, as the likely mechanism of thrombocytopenia.

Our investigations of platelet indices in murine NEC-like intestinal injury show that thrombocytopenia develops despite increased platelet production (12):

- **i.** Mean platelet volume (MPV): quotient of the plateletcrit (ratio of platelet volume to whole blood volume, expressed as percentage) and the platelet count ($\times 10^9$/L) (34). At 18 hours, the median (range) MPV in mild, moderate, and severe intestinal injury at 7.5 (6.6–7.7), 7.2 (6.5–9.6), and 7.6 (6.4–9.9) fL, respectively, was significantly higher than 6.6 fL (range 6.1–8.3) in controls.
- **ii.** Platelet distribution width (PDW): range of platelet volumes at 20% frequency (peak of the frequency histogram = 100%) (35). PDW was increased in pups with intestinal injury.

iii. Platelet-large cell ratio (P-LCR): proportion of large platelets (>12 fL) in the total platelet population (35). P-LCR was increased in murine NEC-like injury, indicating increased number of larger, presumably younger platelets in the circulation (29).

iv. Immature platelet fraction (IPF): Pups with NEC-like injury showed more immature platelets, which are larger (forward light scatter) and carry more nucleic acid (side fluorescence intensity) (27, 34, 36). At 18 hours, pups with NEC-like injury had a larger IPF (more immature platelets in total platelet population; 13.4%, range 3.1–33.5% *vs.* controls, 7.4%; range 2.9–11.9%; $p<0.001$) (27).

v. Bone marrow megakaryocytes: Mouse pups with NEC-like injury carried more megakaryocytes ($5.34\pm0.2 \times 10^4$ *vs.* $13.45\pm0.36 \times 10^4$/mL of marrow preparation; Figure 3a) at the 18-hour time-point (12). Similar to human infants (37), pups had smaller megakaryocytes than adult mice (median area = 4749, range 2876–5107 μm^2 *vs.* 8906, range 6345–1345 μm^2; Figure 3b). In NEC-like injury, megakaryocytes showed increased nuclear ploidy (cells≥8N: median 14.8, range 12.4–18.2 in control *vs.* 30.9, range 18.1–36.4 in NEC-like injury), indicating increased megakaryocyte differentiation Figure 3c.

(c) Platelet activation in murine NEC-like injury

Platelet activation was an early event during NEC-like injury, seen within 3 hours before any changes in platelet counts or histopathological evidence of mucosal injury (13). These activated platelets carried an activated conformation of the integrin GPIIb/IIIa (38, 39) and increased platelet endothelial cell adhesion molecule (PECAM)-1/CD31 (40). Neonatal platelets expressed P-selectin at low levels and upregulated it at 24 hours after induction of NEC-like injury (41).

Platelets collected 3 hours after induction of NEC-like injury showed increased aggregability with collagen. Pups in the NEC-like injury group showed evidence of platelet dense granule discharge as early as 3 hours. These platelets contained fewer dense granules, and released less ATP upon collagen stimulation. The release of α-granule contents such as platelet factor (PF)-4/CXC motif ligand (CXCL) was relatively delayed; plasma PF4/CXCL4 levels rose transiently at 6 hours but consistently only after 12 hours. These platelets showed consistent α-granule depletion beyond 18 hours after induction of NEC.

In murine NEC-like injury, the negative effects of early platelet activation are evident from the protective effects of platelet depletion to levels around $50–100 \times 10^9$/L (13). In our murine model, platelet depletion reduced the severity of bowel injury and improved survival (Figure 4), without increasing the severity of hemorrhages in the injured intestine (13). These studies support the possibility emerging from human studies that transfused, activated platelets could cause harm by releasing pre-formed vasoconstrictors and inflammatory mediators. (42) (43).

(d) Thrombin activates platelets in murine NEC-like injury

We investigated the role of platelet activators such as thrombin, thromboxane A2 (TxA2), endotoxin, and platelet activating factor (PAF) in NEC-like injury (13). Plasma thrombin activity and thrombin-antithrombin complexes were elevated at 3 hours into NEC-like injury (Figure 5). Endotoxin and PAF rose later, while no changes were seen in TxA2 (13). The role of thrombin was confirmed *ex vivo* when platelets from control pups were resuspended in plasma from pups with intestinal injury, which activated platelet GPIIb/IIIa. These changes were blocked by thrombin inhibitors such as bivalirudin or D-phenylalanyl-prolyl-arginyl chloromethyl ketone (44, 45). These thrombin inhibitors also blocked CD31 expression and dense granule discharge (35).

(e) Murine neonatal platelets are highly responsive to thrombin

Murine neonatal platelets responded to thrombin and release dense granule contents within 15 minutes. In contrast, adult platelets may take ≥30 minutes. Due to developmental differences, neonatal platelets express several mediators of thrombin-induced signaling at higher levels than in adults (46). In proteomic analysis, neonatal platelets express higher levels of platelet glycoprotein 1b; vasodilator stimulated phosphoprotein; guanine nucleotide-binding protein, alpha 13; guanine nucleotide-binding protein, g(q)-α; cytosolic phospholipase A-2 (PLA2); phospholipase A-2 activating protein; synaptosomal-associated protein 23; ras homolog gene family, member A (RhoA); Rho GDP-dissociation inhibitor 1, and Rho GDP-dissociation inhibitor 2.

Neonatal plasma showed less thrombin antagonistic activity than adults (47–50). These differences could be explained by lower expression of antithrombin and α2-macroglobulin in neonatal plasma, but not by the differences in the levels of α1-antitrypsin, tissue factor-pathway inhibitor, or heparin cofactor-II.

(f) Tissue factor activates thrombin and promotes platelet consumption during NEC

In the adult intestine, TF is expressed in the perivascular smooth muscle, epithelia, leukocytes, and platelets (51). In contrast, the neonatal intestine expressed TF in resident macrophages but not in perivascular or epithelial cells. These macrophages showed TF expression in well-defined cytoplasmic compartments. *Ex vivo,* studies confirmed constitutive and LPS-induced expression of TF and its release in microvesicles. LPS-inducible macrophage TF expression was particularly interesting when we considered the emerging link between NEC and enteric dysbiosis with enrichment of Gram-negative bacteria.

Mouse pups with NEC-like injury showed increased plasma TF levels beginning at the 2-hour time-point, preceding the rise in thrombin activity at 3 hours (Figure 6). We first confirmed these findings in human infants; premature human infants with NEC had significantly higher plasma TF concentrations than controls (mean ± standard deviation 51.11±17.23 pg/mL compared to 4.48±1.26 pg/mL in controls). We then checked TF levels in murine intestine; there was a transient drop in TF at these time-points, suggesting that pre-formed TF stores may have been released from the injured intestine. This TF presumably formed TF-VIIa complexes to activate circulating prothrombin during the

pathogenesis of NEC-like injury. Inhibition of factor VII activation with PCI-27483 (*N*-aminoiminomethyl benzimidazol aminosulfonyl dihydroxy biphenyl acetyl aspartic acid) blocked this rise in plasma thrombin in NEC-like injury (52).

Mouse studies implicated thrombin-mediated platelet activation in a pathogenetic role in NEC-like injury. Unfortunately, systemic inhibition of thrombin is unpredictable in premature infants and may be either impaired or increase the risk of hemorrhagic complications (53, 54). Therefore, we evaluated antithrombin bivalirudin-tagged perfluorocarbon-core nanoparticles (NPs), which can bind thrombin in nascent blood clots and prevent progressive activation of the coagulation cascades without increasing the risk of systemic hemorrhagic complications (55–57). Considering the in-vivo half-life of about 4 hours for these NPs, we administered two doses, the first given 1 hour prior to induction of NEC-like injury and a second dose 4 hours later (55–57). Bivalirudin NPs prevented thrombocytopenia, improved survival, and reduced the severity of intestinal injury, without increasing hemorrhages into the injured intestine. There was some leukocyte infiltration and submucosal edema but no major mucosal damage (13).

4. Platelet transfusions in human infants

Current management protocols in human NEC emphasize careful monitoring of thrombocytopenia and aggressive treatment with platelet transfusions if needed, because of the risk of life-threatening hemorrhagic complications in these patients (58–70). Most platelet transfusions in the NICU are administered prophylactically to correct platelet counts, not actual bleeding, and data on the efficacy and safety of these transfusions remain scant. In the United States, transfusion thresholds may be relatively liberal compared to those in Europe, and nearly 50% of ELBW infants may receive ≥1 platelet transfusions during their hospital stay (65). An automatized audit system showed that 60% of all platelet transfusions were given outside of established guidelines, mostly at platelet counts higher than recommended (71).

There is some evidence linking platelet transfusions with adverse outcomes in NEC. Baer *et al.* (61, 72, 73) and Garcia *et al.* (74) showed that the number of platelet transfusions predicted mortality in infants with diagnosis of NEC. Kenton *et al.* (15, 75) noted that platelet transfusions predicted complications of NEC such as short bowel syndrome and cholestasis. Del Vecchio *et al.* (66) found an association between platelet transfusions and mortality in critically ill infants. Compared to infants who did not receive platelet transfusions, 1 platelet transfusion increased the relative risk of death by 10.4-fold, and >4 platelet transfusions increased it 29.9 times. The mechanism of these inferior outcomes, whether it represents direct harm from transfused platelets, or because these infants were sicker in the first place, is unclear (20, 61, 73). Sensitivity analysis on regression data suggested important effects of platelet transfusions on adverse outcomes (76). The evaluation of platelet transfusions remains difficult because of ethical concerns in withholding a potentially life-saving therapy from a critically ill infant. A few attempts sought to compare platelet transfusion practices with relatively conservative thresholds aiming to reduce platelet transfusions, but these efforts were only modestly ambitious with

minor reductions in platelet transfusion thresholds (61). These studies were not adequately powered to answer these questions and showed no difference in outcomes (20, 73).

5. Recombinant peptides

Interleukin-11, Tpo, and Tpo-mimetic agents such as romiplostim and eltrombopag may not be useful in acute NEC (65, 77). Some of these peptides/growth factors could be used in selected infants with persistent thrombocytopenia in short bowel syndrome and cholestasis (77).

6. Summary

Thrombocytopenia is a common clinical finding in NEC, typically seen within 24–72 hours after developing NEC. The reduction in platelet counts is usually proportional to the severity and stage of NEC. The likely mechanism of this thrombocytopenia is the consumption of platelets in microthrombi formed in the intestinal microvasculature. Some neonates with severe NEC may also have concomitant suppression of the thrombopoietic response, resulting in prolonged thrombocytopenia. There is no consensus yet regarding the threshold for platelet transfusions in these infants with thrombocytopenia. There is a need for carefully designed studies to evaluate the safety and efficacy of platelet transfusions in NEC.

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Article impact

- Fifty to 95% of infants with necrotizing enterocolitis (NEC) develop idiopathic thrombocytopenia (platelet counts $<150 \times 10^9$/L) within 24–72 hours of disease onset
- Early clinical trials suggest that moderate thrombocytopenia may be protective in human NEC, although further work is needed to fully understand this relationship
- We have developed a neonatal murine model of NEC-related thrombocytopenia, where enteral administration of an immunological stimulant, trinitrobenzene sulfonate, on postnatal day 10 induces an acute necrotizing ileocolitis resembling human NEC
- In this murine model, thrombocytopenia is seen at 15–18 hours due to platelet consumption, and mildmoderate thrombocytopenia is protective

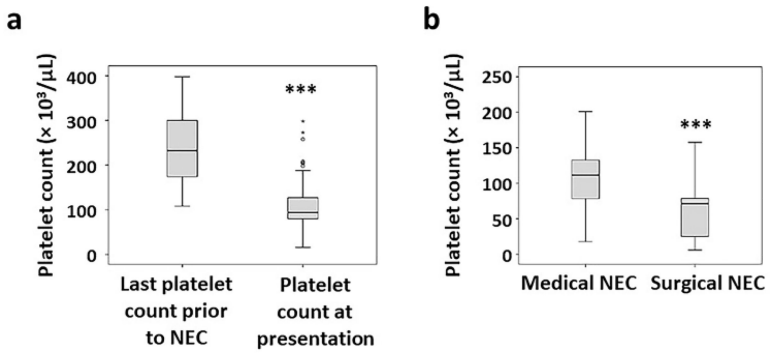

Figure 1. Diagnosis of NEC is associated with thrombocytopenia.

(a) Box-whisker plots depict the last available platelet counts prior to NEC and the platelet counts at presentation. *N*=76 cases of NEC, who were born at a gestational age (average ± SEM) of 26.6±0.3 weeks, had a birth weight of 845±63 grams, and age at onset of NEC was 21.2±4 days; (b) Box-whisker plots show lowest platelet counts in infants treated medically vs. those who needed surgery for NEC. *** $p<0.001$

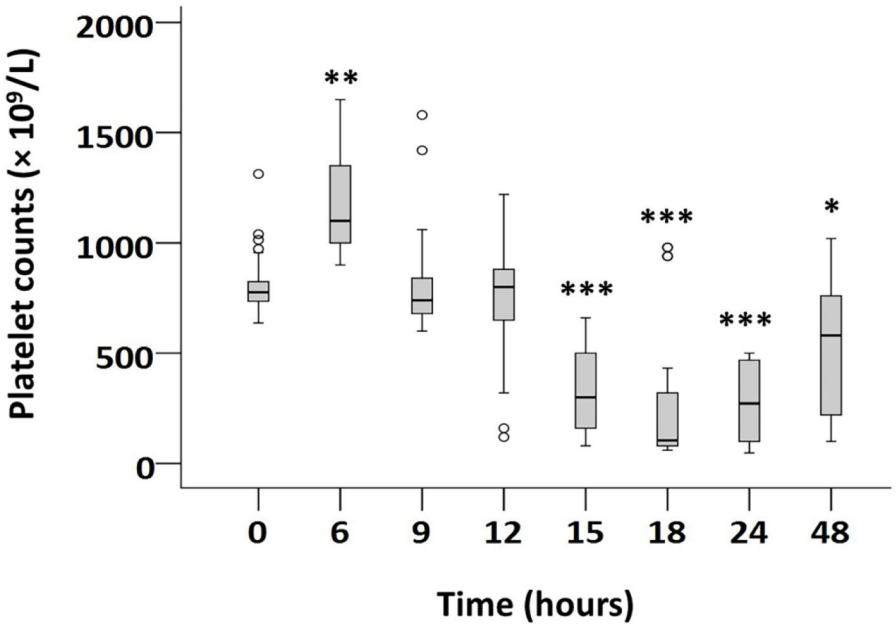

Figure 2. Blood platelet concentrations in severe murine NEC-like injury.

Boxplots show serial platelet concentrations in P10 mouse pups in control and severe NEC-like injury groups. Severe intestinal injury seen in 63 pups; 45 euthanized at earlier time-points for physical distress. Control mice (N=65) received vehicle (ethanol) alone and showed no distress or significant changes in platelet counts. Jonckheere-Terpstra test for ordered alternatives. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ *vs.* control.

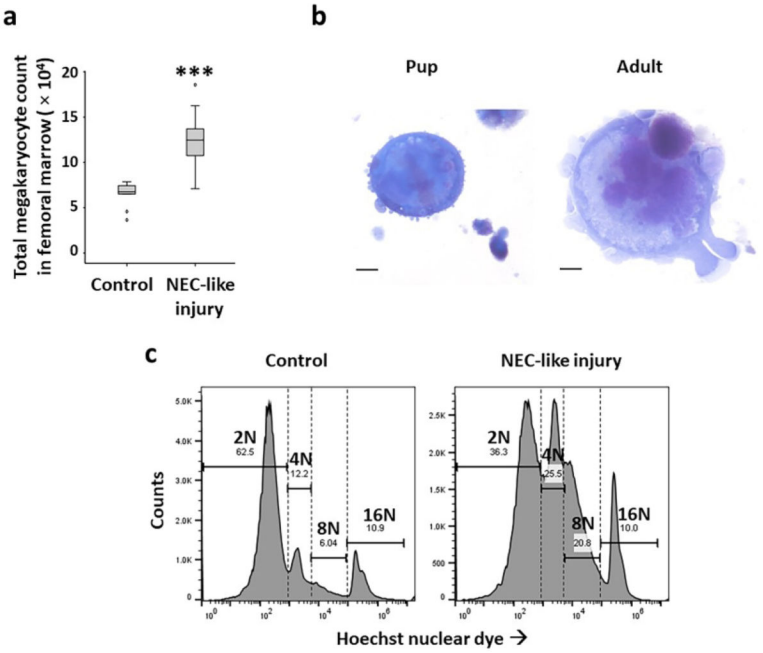

Figure 3. Megakaryocytes in murine NEC-like injury.

(a) Box-whisker plots show $CD41^+$ megakaryocyte counts in control and NEC-like injury; *N*=8 pups/group; *** $p<0.001$; (b) Mouse pups have smaller megakaryocytes (median area = 4749 μm^2) than adult mice (8906 μm^2). Data represent 5 mice/group; $p<0.001$ (c) Histograms show nuclear ploidy of megakaryocytes stained with the Hoechst nuclear dye, in control and NEC-like injury. Data represent *N*=8 pups/group.

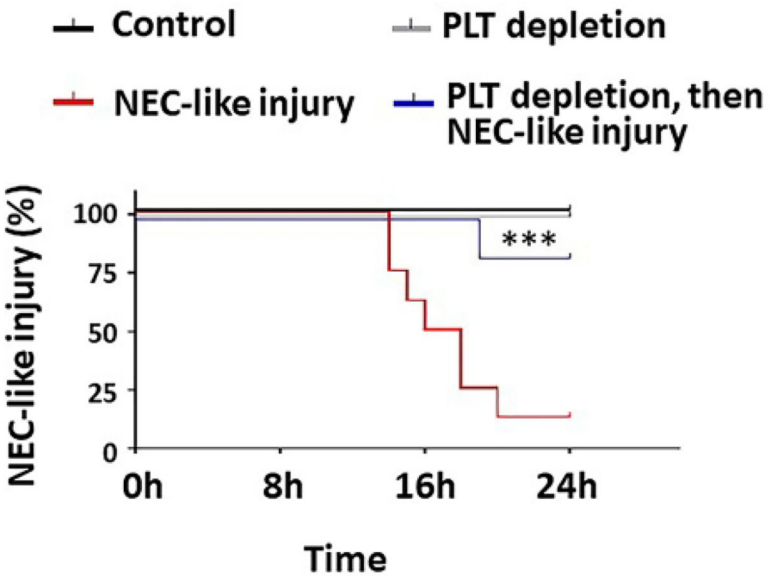

Figure 4. Platelet depletion protects against murine neonatal NEC-like injury.

Kaplan-Meier curves summarize survival data from animals in control ($N = 3$), intestinal injury ($N = 8$), platelet depletion ($N = 5$), and platelet depletion followed by intestinal injury ($N = 6$) groups. Mantel-Cox log-rank test, *** $P<0.001$.

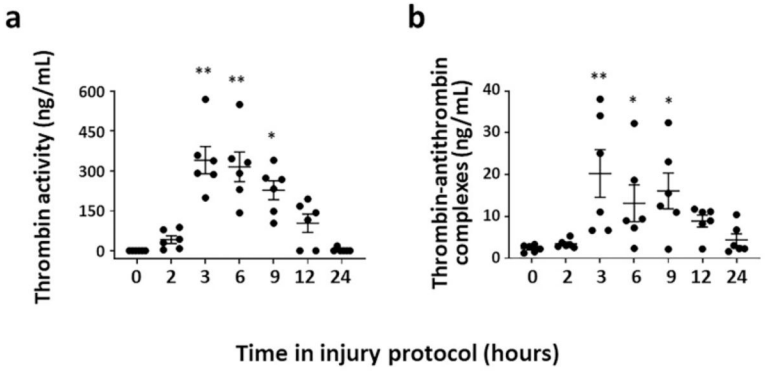

Figure 5. Thrombin activates platelets during murine NEC-like injury.

Scatterplots (means ± SEM; *top to bottom*) show (a) plasma thrombin activity and (b) thrombin-antithrombin complexes measured at various time-points between 0–24h after initiation of intestinal injury. $N = 6$ pups. Friedman's test for repeated measures, * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ *vs.* control

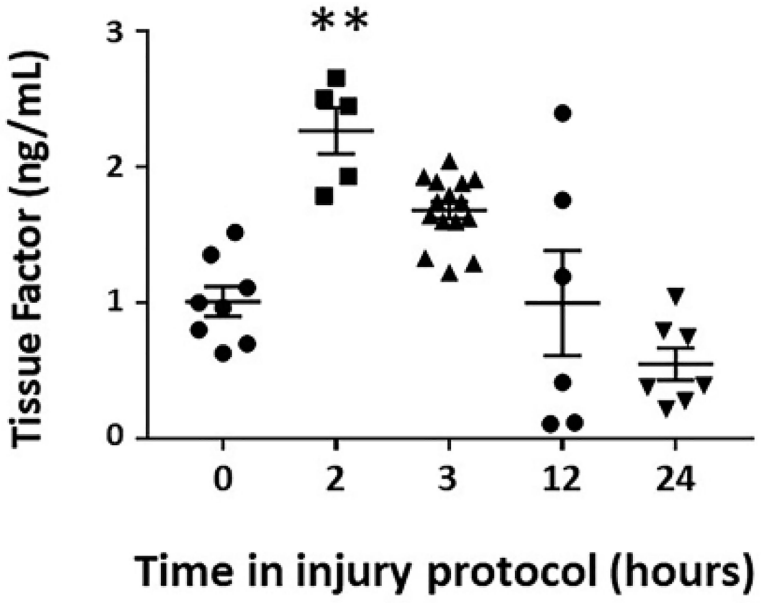

Figure 6. Plasma tissue factor (TF) concentrations during murine NEC-like injury.
Scatterplot (means ± SEM) show serial TF concentrations. $N = 6$ mice/group; Kruskal Wallis H test with Dunn's post-test, * $P<0.05$, ** $P<0.01$ *vs.* control