Paneth cell ablation in the presence of *Klebsiella pneumoniae* induces necrotizing enterocolitis (NEC)-like injury in the small intestine of immature mice

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**SUMMARY**

Necrotizing enterocolitis (NEC) is a leading cause of morbidity and mortality in premature infants. During NEC pathogenesis, bacteria are able to penetrate innate immune defenses and invade the intestinal epithelial layer, causing subsequent inflammation and tissue necrosis. Normally, Paneth cells appear in the intestinal crypts during the first trimester of human pregnancy. Paneth cells constitute a major component of the innate immune system by producing multiple antimicrobial peptides and proinflammatory mediators. To better understand the possible role of Paneth cell disruption in NEC, we quantified the number of Paneth cells present in infants with NEC and found that they were significantly decreased compared with age-matched controls. We were able to model this loss in the intestine of postnatal day (P)14-P16 (immature) mice by treating them with the zinc chelator dithizone. Intestines from dithizone-treated animals retained approximately half the number of Paneth cells compared with controls. Furthermore, by combining dithizone treatment with exposure to *Klebsiella pneumoniae*, we were able to induce intestinal injury and inflammatory induction that resembles human NEC. Additionally, this novel Paneth cell ablation model produces NEC-like pathology that is consistent with other currently used animal models, but this technique is simpler to use, can be used in older animals that have been dam fed, and represents a novel line of investigation to study NEC pathogenesis and treatment.

**INTRODUCTION**

Neonatal necrotizing enterocolitis (NEC) is the leading cause of gastrointestinal mortality and morbidity in premature infants. In the United States, NEC affects 7% of all infants born less than 1500 g, causes the death of approximately 16–42% of those infants depending on their birth weight (Fitzgibbons et al., 2009) and increases the chance of developmental delays in survivors (Hintz et al., 2005; Fitzgibbons et al., 2009). Although the exact pathophysiology of NEC is still unclear, several lines of clinical and basic science observations suggest that an exaggerated inflammatory response is involved. The leading hypothesis for pathophysiology of NEC is that intestinal bacteria that are normally restricted to the intestinal lumen somehow penetrate through the immature epithelial barrier and gain access to the epithelial cells and underlying lamina propria of the small intestine. This insult causes an exaggerated inflammatory response that is in turn followed by tissue destruction (Nanthakumar et al., 2000; Lin et al., 2008). The small intestinal epithelium is normally protected through an extensive defense network of innate immunity including secreted mucus, IgA, pattern recognition molecules and antimicrobial peptides (McElroy and Weitkamp, 2011). However, the intestinal innate immune system of premature infants is fundamentally different from that of more mature infants, with functional or quantitative reductions in innate immune components such as tight junctions, goblet cell mucins, the number of Paneth cells and IgA concentrations (Salzman et al., 2007; McElroy and Weitkamp, 2011). Notably, our previous data demonstrated a decrease in the Paneth-cell-produced antimicrobial peptide lysozyme in infants with NEC (McElroy et al., 2011).

Paneth cells, which are located in the crypts of Lieberkühn, are one of the four types of epithelial cells derived from intestinal epithelial stem cells (Garabedian et al., 1997), and are characterized by their large secretory granules that are rich in antimicrobial peptides and proinflammatory mediators (Bevins and Salzman, 2011). Paneth cells are important in mucosal development, host defense and regulation of the intestinal microbiota (Bevins and Salzman, 2011). Although Paneth cells can be found as early as the first trimester of gestation in humans (Moxey and Trier, 1978; Ouellette, 2010), they are mature by the age of viability (22-24 weeks), but at this stage are present in low numbers compared with that found in their term counterparts (Mallow et al., 1996; Salzman et al., 2007). Ablation of these cells can have significant consequences in the immature intestine, such as decreasing the ability to clear bacterial pathogens (Sherman et al., 2005).

To help understand whether Paneth cells disruption plays a role in the development of NEC, we wanted to create a model of Paneth cell ablation to determine whether we could induce NEC-like injury in immature intestine. We treated 14-to 16-day-old mice with dithizone, a chemical chelator of heavy metals that has been shown to selectively ablate Paneth cells (Sawada et al., 1991; Sherman et al.,...
followed by lumenal infection with *Klebsiella pneumoniae* (hereafter referred to as *Klebsiella*). This novel two-hit treatment significantly and consistently induced small intestinal injury that is consistent with human NEC.

There are currently two established methods of inducing NEC in neonatal mice. The first is based on the hypoxia and hypothermia (hypoxia-hypothermia) model of NEC originally developed in newborn rat pups (Barlow et al., 1974). This model requires Cesarean-section delivery of pups, gavage formula feeding, and twice-daily treatments with hypoxia and hypothermia for 72 hours. The second model uses lipopolysaccharide (LPS) and platelet activating factor (PAF) treatments to induce an NEC-like injury (Maheshwari et al., 2011). To further define our novel Paneth cell ablation model of NEC, we compared it to these two currently established animal models of NEC and found that our model produced NEC-like pathology comparable to or better than both methods. Additionally, our Paneth cell ablation model offers some important advantages to these models: it is simpler to use, can be used in older animals and works consistently in animals that have been dam fed. In addition to inducing tissue damage, our Paneth cell ablation model also induced an inflammatory response that is consistent with NEC, as tested by protein expression levels. Lastly, this method of inducing NEC was effective in vivo and in an in vitro small intestine explant model in which we determined that pretreatment with zinc can block the effects of dithizone alone, but not the combination of dithizone with *Klebsiella*.

In conclusion, we have developed a novel mouse model for investigating neonatal NEC: this model utilizes developmentally appropriate mice and employs a novel method for inducing the disease, which can then be further studied to develop new understanding and therapeutic interventions for NEC.

### RESULTS

**Infants with surgical NEC lack Paneth cells**

Recently, we described a loss of mucus-filled goblet cells in infants with NEC compared with age-matched infants with spontaneous intestinal perforations (SIP) (McElroy et al., 2011). In that study, we also observed that infants with surgical NEC were lacking lysozyme-positive Paneth cells. To better understand the role that Paneth cells might play in NEC, in the current study we obtained human surgical samples (mean gestational age of 27 weeks) from infants who developed either NEC or SIP (*n*=10 per group). All samples were obtained from the leading edge or marginal zone of the surgical sample to prevent use of necrotic tissue. Additionally, all tissue samples were examined for live cells and villous architecture by immunohistochemistry (IHC), and half of the samples were additionally measured for tissue viability through use of flow cytometry for lymphocytes. Any samples not having viable cells were deemed to be necrotic and discarded. Individual samples were de-identified so that the individual severity of each sample was unknown; however, it can be assumed that all NEC samples were Bell stage 3 because they required surgical intervention. Samples were stained with anti-lysozyme antibodies, phloxine/tartrazine and Alcian Blue/periodic acid Schiff (AB/PAS) to mark Paneth cells in multiple ways, and positive cells were counted per 100 total epithelial cells by a single blinded investigator. Paneth cell numbers were significantly decreased compared with age-matched controls (graph; *P*<0.001). Scale bars: 0.1 mm.
are affected in NEC, not just lysozyme as previously described (Coutinho et al., 1998; McElroy et al., 2011).

Dithizone selectively ablates Paneth cells in immature small intestine
A previous report used the chemical dithizone to selectively ablate Paneth cells in 4-day-old rats (Sherman et al., 2005). Our hypothesis for these previous studies was that Paneth cells play a role in the development of NEC. NEC is primarily a disease of premature infants (Fitzgibbons et al., 2009; Bajwa et al., 2011). With regards to intestinal morphology and enzyme production, small intestines from postnatal day (P)14-P16 (immature) mice are roughly equivalent to that of a human fetus, at 24 weeks of gestation, that is predisposed to develop NEC (Henning, 1985; Montgomery et al., 1999; Sanderson and Walker, 1999; McElroy and Weitkamp, 2011). To determine whether dithizone could ablate Paneth cells in P14-P16 mice, CD-1 mice of this age were treated with 75 mg/kg dithizone or an equivalent volume of Li2CO3 buffer (Sherman et al., 2005). Mice were observed for 16 hours following injection and monitored hourly. Ileal segments were harvested from dithizone-treated mice and controls, and were stained with AB/PAS to detect Paneth cells. Positive cells were counted per crypt by a single blinded investigator. Mice treated with dithizone had significantly fewer Paneth cells per crypt than did controls (\( n \) = 4/group; \( P \) = 0.008; Fig. 2). To determine whether dithizone had effects on other intestinal epithelial cell lines, ileal samples were harvested from dithizone-treated mice and controls, and were stained with AB/PAS to detect Paneth cells. Positive cells were counted per crypt by a single blinded investigator. Mice treated with dithizone had significantly fewer Paneth cells per crypt than did controls (\( n \) = 4/group; \( P \) = 0.008; Fig. 2). To determine whether dithizone had effects on other intestinal epithelial cell lines, ileal samples were harvested from dithizone-treated mice and controls, and were stained with AB/PAS to detect Paneth cells. Positive cells were counted per crypt by a single blinded investigator. Although dithizone significantly decreased Paneth cell counts by ~50% compared with controls, it had no effect on the number of mucin-positive Goblet cells (Fig. 2), which confirms the previous findings of dithizone causing selective Paneth cell ablation (Sawada et al., 1991).

Dithizone mice treated with dithizone and enterally infected with "Klebsiella" developed NEC
Sherman et al. reported that rat pups that were pretreated with dithizone and then enterally infected with enteroinvasive Escherichia coli developed an NEC-like injury 10 hours after exposure (Sherman et al., 2005). To see if we could replicate similar findings in mice, P14-P16 mice were separated from their mothers and randomized into four groups: control, dithizone, Klebsiella and dithizone/Klebsiella (Dith/Kleb). Mice were given an intraperitoneal (i.p.) injection of 75 mg/kg dithizone dissolved in Li2CO3, or an equal volume of Li2CO3 buffer alone, and placed in a humidified, temperature-controlled incubator (34°C) for observation. At 6 hours after injection, mice were enterally infected with a gavage of 1×10¹¹ CFU Klebsiella/kg body weight or, for controls, were gavaged with an equal volume of sterile media. Following gavage, all mice were returned to the incubator for further observation. After 10 hours of observation, mice were euthanized and their distal small intestines were harvested for examination. Small intestinal samples were fixed and stained with hematoxylin and eosin (H&E) and scored by a single, blinded investigator on the 5-point scale used in currently accepted models of NEC (Musemeche et al., 1991; Dvorak et al., 2002; Maheshwari et al., 2011). Any samples with grade 2 or higher are considered to be consistent with the injury seen in human NEC. Mice treated with either Klebsiella or dithizone alone did not differ significantly from controls. However, mice treated with dithizone followed by Klebsiella had significantly higher injury scores than those in other groups (\( P < 0.001 \)), and had a mean score of 2.3 (Fig. 3). The injury produced was a colon-sparing, small-intestine-specific damage with the majority of the injury occurring at the ileum. On histological examination, injured areas showed mucosal edema and hemorrhage, loss of villous integrity, intramural air, and areas of...
transmural necrosis that were separated by areas of normal 'skip' tissue, which is consistent with a diagnosis of NEC in preterm infants (Kliegman and Fanaroff, 1984; Petty and Ziegler, 2005). Mice in the control, Klebsiella, and dithizone alone groups had 100% survival, whereas mice in the Dith/Kleb group had 20% mortality prior to euthanasia at 10 hours after Klebsiella infection. Mature Paneth cells are required for Dith/Kleb-induced NEC

Our data show that dithizone selectively induces Paneth cell ablation, and that the combination of Paneth cell ablation and abnormal intestinal flora induces the development of NEC. To determine whether mature granule-bearing Paneth cells were required for this model, we used our Paneth cell ablation model of NEC on P5 CD-1 mice that have not yet developed mature Paneth cells (Bry et al., 1994). P5 mice were randomized into three groups: control, Klebsiella and Dith/Kleb. Mice were given an i.p. injection of 75 mg/kg dithizone followed 6 hours later with a Klebsiella gavage (n=7). Using a blinded scoring system evaluating villus integrity, mice treated with Dith/Kleb were not significantly different from other groups and all scores were <2, which is considered as having no NEC-like damage. Scale bars: 0.1 mm.

Mature Paneth cells are required for Dith/Kleb-induced NEC

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The Paneth cell ablation model of NEC induces intestinal inflammation

One of the hallmarks of NEC is the increase in inflammatory mediators generated in the immature intestine. To measure the inflammatory response following Paneth cell ablation and Klebsiella
infection, we sampled pro- and anti-inflammatory mediators found in P14-P16 animals treated with Dith/Kleb and compared them with controls. Samples were homogenized, corrected for tissue weight, and then examined for protein concentration of IL-1β, IL-1, IL-6, IL-10, INF-γ, IL-12 and TNFα using a Meso-Scale Discovery 7-plex pro-inflammatory ultra-sensitive plate and compared with controls. All cytokines were significantly elevated except for IL-12 (P=0.5) and TNFα (P=0.1) (Fig. 5).

The novel Paneth cell ablation model of NEC is comparable to other published models of NEC
To determine whether our new model of NEC was comparable to currently used models, we compared our results with the two currently used mouse models of NEC: the hypoxia-hypothermia model (Barlow et al., 1974; Musemeche et al., 1991; Dvorak et al., 2002) and the LPS+PAF model (Maheshwari et al., 2011). We first compared our Paneth cell ablation method with the well-described hypoxia-hypothermia injury model. Both methods saw a significant increase in injury score in experimental groups (P<0.02 for hypoxia-hypothermia method and P<0.001 for Paneth cell ablation method). Both groups also had mean scores above 2 (the level of injury that is considered to be consistent with NEC-like injury in human infants; Fig. 6). Although both models produced histological injury consistent with NEC, it was interesting to note that the hypoxia-hypothermia model produced colonic as well as ileal injury, whereas our Dith/Kleb model produced ileal-specific injury. We also compared our Paneth cell ablation method with the injury model recently described that used LPS+PAF to induce an NEC-like injury (Maheshwari et al., 2011). Although the LPS+PAF-treated mice had significantly higher injury scores than controls (P=0.02), the mean injury score reached only 1.4, which was below the level of injury that is considered to be consistent with NEC-like injury and lower than what was seen in the hypoxia-hypothermia model and our Paneth cell ablation model (Fig. 6).

Zinc treatment can prevent dithizone-induced injury but not Dith/Kleb-induced injury
Dithizone is an organic compound that complexes with metals, including zinc ions. Sawada previously described the formation of zinc-dithizonate complexes inside Paneth cells within 5 minutes following dithizone treatment (Sawada et al., 1991). At 0.5-1 hour after complex formation, Paneth cells become degenerative and necrotic and are expelled into the crypt lumen (Sawada et al., 1991). We hypothesized that having high levels of intraluminal zinc would prevent this complex formation and subsequent Paneth cell ablation. To test this hypothesis, we employed a modification of the organ culture system described by Yan (Yan et al., 2007). Ileal explants were harvested from P14-P16 CD-1 mice and cultured on Netwell inserts (Fig. 7A). All explants were cultured at 37°C and in 5% CO₂ for 30 minutes prior to treatment to allow for equilibration. All experiments lasted 120 minutes at 37°C and in 5% CO₂. Samples treated for 15 minutes were treated at the beginning of the experiment and then had their media replaced with DMEM for the remaining 105 minutes in all experiments. Explants cultured in DMEM media alone (Fig. 7B) or treated with L$_2$CO$_3$ buffer for 15 minutes (Fig. 7C) had no significant injury. Explants treated for 15 minutes with 0.05% dithizone also showed no injury (Fig. 7D); however, a tenfold higher dose (0.5%) caused injury that was characteristic of stage 3 NEC, as seen in our in vivo model (Fig. 7E). To determine whether the addition of Klebsiella would have an additive effect on our explants, $1\times10^7$ Klebsiella/ml was added in addition to 15 minutes of 0.5% dithizone treatment. This treatment induced damage in our explants that was similar to what was seen in explants treated with 0.5% dithizone alone (Fig. 7F). Addition of 15 $\mu$M zinc has previously been shown to protect against TNF-induced disruption of endothelial monolayers (Hennig et al., 1993). To test whether this dose of zinc would protect against dithizone- or Dith/Kleb-induced injury, 15 $\mu$M zinc was added to the media prior to treatment with either dithizone or Dith/Kleb. Zinc pretreatment was able to prevent dithizone-induced damage (Fig. 7G), but was not able to prevent the damage caused by the combination of dithizone exposure followed by Klebsiella infection (Fig. 7H). The measured pH difference was negligible between all groups (pH 8.3-8.5).

DISCUSSION
Neonatal NEC is the most devastating gastrointestinal problem facing premature infants today. NEC was described almost 50 years ago (MizrahI et al., 1965), but little has changed in the treatment or outcomes since. In this study we show evidence that Paneth cell markers are significantly decreased in infants who have surgical NEC compared with age-matched infants with surgical spontaneous intestinal perforations. We can experimentally mimic this Paneth cell loss using dithizone in immature mouse ileum without affecting other epithelial cell lines such as goblet cells. Treating P14-P16 mice with dithizone followed by exposure to Klebsiella significantly induced NEC-like injury in the small intestine, and this pathology is consistent with other currently used animal models. However, our model is simpler to use, can be used in dam-fed animals, and is used in animals at a similar intestinal
developmental stage to humans who develop NEC. Lastly, we show that Dith/Kleb treatment induces similar histological pathology in small intestinal explants as it does in vivo. These novel findings have important implications in the field of NEC because they provide new insights into the pathogenesis of the disease and open up new lines of study for potentially new therapeutic approaches.

Paneth cells play an important role in both the innate immune system and in maintaining homeostasis of the small intestinal stem cell niche (Ganz, 2000). Our data show significantly decreased numbers of granule-containing Paneth cells in infants with NEC compared with age-matched controls with SIP. Paneth cells can be detected as early as at 12 weeks of gestation in human fetuses (Moxey, 1978) and produce mature granules by the time fetuses are first viable (Mallow et al., 1996). However, the absolute number of Paneth cells is lower in fetuses than in term infants or adults (Mallow et al., 1996). Because of this, it is unclear whether Paneth cell destruction precedes NEC development. Targeting Paneth cells by using dithizone has allowed us to test the hypothesis that Paneth cell disruption predisposes immature intestine to develop NEC.

Dithizone, a heavy metal chelator, has been shown to induce selective Paneth cell necrosis and loss (Sawada et al., 1991). This loss is temporary and Paneth cells recover 72 hours after treatment, implying that it does not affect the stem cell compartment. Interestingly, in the subsequent week after treatment, Paneth cells double in number, and staining of their granules becomes more intense (Sawada et al., 1991). This rebound increase in Paneth cells might be relevant, because some groups have described an increase in Paneth cells following NEC (Puiman et al., 2011), which implies that the timing of obtaining samples following the disease may be crucial. In our mouse experiments, dithizone injections significantly reduced the number of granule-containing Paneth cells without perturbing other epithelial cell lineages such as goblet cells.

Although no specific bacterium has been linked to NEC development, bacterial colonization with subsequent LPS induction of TLR4 is believed to be required for development of the disease (Jilling et al., 2006; Leaphart et al., 2007). *Klebsiella* has been implicated in producing the hydrogen-sulfide-rich gas pockets of pneumatosis that are characteristic of NEC (Hill et al., 1974) and was used in the original NEC animal models (Barlow et al., 1974).

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**Fig. 6. The Paneth cell ablation model of NEC is equivalent to other models of NEC.** Three models of murine NEC were compared. In the formula + stress (hypoxia-hypothermia) group, P4 mice were surgically given a gastrostomy tube or a sham surgery (controls). Control animals were placed back with their dams \((n=5)\). Control + stress animals were dam fed but given BID (twice daily) hypoxia and hypothermia \((n=8)\). Formula + stress animals were fed a prepared formula through a gastrostomy tube and given BID hypoxia and hypothermia treatments \((n=8)\). In the LPS+PAF group, P10 mice were treated with PAF followed by LPS \((n=14)\) or given an equivalent volume of saline for controls \((n=18)\). In the Dith/Kleb group, P14-P16 mice were divided into four groups. Dithizone mice were given an i.p. injection of dithizone \((75 \text{ mg/kg})\) and were monitored for 16 hours before sacrifice \((n=42)\). Control animals were given an equivalent volume i.p. injection of Li2CO3 buffer \((n=31)\). *Klebsiella* mice were given an i.p. injection of Li2CO3 and 6 hours later were given a gavage feeding of \(1 \times 10^{11} \text{ CFU} \text{ Klebsiella/kg body weight suspended in nutrient broth (n=13). Dith/Kleb mice were given an i.p. injection of dithizone followed 6 hours later with a Klebsiella gavage (n=30).**

(A) The formula + stress animals in the formula hypoxia-hypothermia group had significantly more NEC \((*P<0.02)\) than their control animals and had mean scores >2. In the LPS+PAF group, LPS+PAF mice had significantly higher scores than their control animals \((*P<0.001)\) but did not reach a mean score >2. In the Dith/Kleb group, animals treated with Dith/Kleb had significantly higher NEC scores than their controls \((*P<0.001)\) and reached a mean score >2. (B,C) Representative slides from the formula hypoxia-hypothermia group (B) and the LPS+PAF group (C). Scale bars: 0.1 mm.
Following dithizone treatment with exposure to *Klebsiella* significantly caused injury and inflammation resembling human NEC in our immature mice (Fig. 3). Additionally, this method has been shown to increase bacterial translocation across the intestinal wall, which is a hallmark of NEC (Edde et al., 2001; Sherman et al., 2005; Grootjans et al., 2011).

In addition to bacterial colonization, a major risk factor for development of NEC is intestinal immaturity; thus, it is important that animal models of NEC mimic the developmental stages at which infants are predisposed to developing disease as closely as possible. The most commonly used model of NEC is the hypoxia-hypothermia model (Barlow et al., 1974). This model uses hypoxia and hypothermia treatments on newborn (P0) mice along with gavaged formula feedings (Jilling et al., 2006). However, comparison of histology and enzyme ontogeny shows that newborn mice are developmentally similar to human fetuses at 16 weeks of gestation, which is developmentally less mature than the age at which humans develop NEC (McElroy and Weitkamp, 2011). Additionally, although breast milk reduces the incidence of NEC, it does not completely prevent it (Lucas and Cole, 1990). A second murine model of NEC has been described that uses LPS and PAF injections to induce intestinal injury (Maheshwari et al., 2011). Although the LPS+PAF model uses older dam-fed pups (P10 mice), the injury sustained is generally less severe than what is seen in the hypoxia-hypothermia model. Our Paneth cell ablation model provides a complementary approach to these two models, and offers unique advantages over both. Because our model requires mature Paneth cells, mice must be 14 to 16 days old for dithizone and *Klebsiella* to induce injury. This removes the need to gavage feed animals, as is required in the hypoxia-hypothermia model, and allows us to study intestine that is more similar in structure and function to preterm infants than is that of newborn (P0) mice, whose intestines are more similar to preivable small intestine (McElroy and Weitkamp, 2011). Unlike P14-P16 mice, P5 mice showed essentially no injury following Dith/Kleb treatment and were indistinguishable from controls (Fig. 4). A major difference between these ages is the absence of mature Paneth cells, which do not develop until around the tenth day of life in mice (Bry et al., 1994). This difference could be an important finding because the younger mice have less mature innate and adaptive immune systems and were theoretically more susceptible to *Klebsiella*- induced damage. It is possible that bacterial flora changes between day 5 and day 14 could contribute to these data. Mice are robustly colonized by enterococci and *Lactobacilli* as early as day 6 of life, but do not acquire coliforms, *Bacteroides* or anaerobes until the second week of life (Lee et al., 1971; Lee and Gemmell, 1972; Hirayama et al., 1995). However, our data showed a lack of injury in *Klebsiella*-only-treated P14-P16 mice, suggesting that bacterial flora is necessary but not sufficient to cause intestinal injury. These combined data suggest that mature Paneth cell disruption might play a significant role in NEC pathophysiology.

One of the defining characteristics of NEC is the massive inflammatory response that is produced during damage of the intestinal epithelia (Caplan and MacKendrick, 1994; Ford et al., 1997; Nadler et al., 2001; Halpern et al., 2002; Frost et al., 2008; Lin et al., 2008). Dith/Kleb-treated mice had significantly increased tissue concentrations of multiple inflammatory mediators, including IL-1α, IL-1, IL-6, IL-10 and IFN-γ with non-significant increases in both IL-12 and TNFα. It is interesting to note that, although NEC is considered a proinflammatory disease, Dith/Kleb-induced injury resulted in an increase of both pro- and anti-inflammatory mediators. We speculate that NEC might well represent a disruption of the balance that exists in biological systems between pro- and anti-inflammatory mediators, and a more thorough kinetic characterization using this model might also provide new insight into how inflammatory mediators regulate intestinal inflammation and injury. A key example of this is TNFα, which has been linked to pathogenesis of NEC (Caplan and Hsueh, 1990), promotion of apoptosis and NFκB-induced inflammation (Baker and Reddy, 1998), and inhibition of trophic signaling in intestinal epithelial cells (McElroy et al., 2008). However, TNFα has a short half-life (Waage et al., 1989), so catching it at multiple time points is essential to better understand its role in pathogenesis. Because of this, we are currently further examining the timing and magnitudes involved in several of these important inflammatory mediators in our new model.

Zinc is an essential trace element in humans and is located relatively evenly throughout the body, where it plays an important role in the development and maintenance of Paneth cells. In our model, zinc administration was able to significantly prevent dithizone-induced injury (Fig. 7). This suggests that Zinc administration is a potential therapeutic strategy for NEC.
role in signal transduction, gene expression and cellular homeostasis including proliferation, growth, differentiation and apoptosis (Hambridge and Krebs, 2007). Paneth cells contain large amounts of zinc in their cytoplasmic granules (Seno et al., 2001) and dithizone-induced ablation of Paneth cells occurs via the formation of zinc-dithizionate complexes (Sawada et al., 1991). Because dithizone ablates Paneth cells through zinc chelation, it was unclear in our model whether NEC was induced through Paneth cell loss, or through loss of zinc. To examine this, we used dithizone and *Klebsiella* treatments on small intestinal cultured explants. It was interesting to note that, although dithizone treatment alone did not induce significant NEC injury in vivo, it could consistently produce NEC in a dose-dependent manner in our explants. This difference might be due to a higher local concentration of dithizone in our explant experiments than what we achieved in our in vivo studies, or it might have been secondary to the additional manipulation of intestines during ileal harvesting that is intrinsic to the explant experiments. Whichever the case, it is notable that zinc treatments were able to prevent the damage induced by dithizone alone. Importantly, this was not the case in explants that were also treated with *Klebsiella*. This suggests that dithizone-induced effects are zinc-dependent. However, it also suggests that the severe damage seen in NEC is not caused by a loss of zinc. We hypothesize that it is instead the loss of Paneth cells themselves that is the causative mechanism behind the induction of NEC.

It is still unclear whether induction of NEC requires loss of Paneth cells themselves or simply the expulsion of their granules. Paneth cells help to guard against bacterial translocation, and dithizone ablation causes increased bacterial translocation across the intestinal wall (Edde et al., 2001; Sherman et al., 2005). However, Paneth cells are also essential in maintaining the intestinal stem cell niche (Sato et al., 2011). Paneth cells reside at the base of the small intestinal crypts, interspersed between the intestinal stem cells (Snippert et al., 2010). Although Paneth cells play a role in providing antimicrobial protection for this stem cell niche (Salzman et al., 2007), they have also been shown to directly maintain the stem cells themselves through Wnt signaling (Sato et al., 2011). This intimate relationship with the microenvironment of the intestinal crypt might play an important role in the development of NEC, because one of the earliest damages seen in NEC models is separation of the mucosa from the lamina propria (Musemeche et al., 1991; Dvorak et al., 2002).

In summary, we have shown that loss of Paneth cells might play a key role in the intestinal injury and inflammation that occurs during NEC pathogenesis. Most importantly, this novel method of inducing NEC generates injury that is consistent other models but provides some unique advantages. It is less complicated to use, can be done in developmentally appropriate mice, and represents a novel line of investigation to study a disease that has shown little clinical progress over the past several decades.

METHODS

Mice

CD-1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were delivered vaginally and raised to age P14-P16 unless otherwise noted. Prior to experiments, mice were housed in standard rodent cages and were dam fed. After experiments, small intestine was harvested and fixed by snap freezing or emersion in 10% formaldehyde solution. Tissues prepared for IHC were paraffin-embedded and sectioned at 5 µm. Tissues prepared for quantitative PCR were homogenized and digested using the RNeasy mini kit (Qiagen). All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at Vanderbilt University, Nashville, TN.

Human intestinal samples

De-identified, archived human ileal sections from preterm infants with NEC (n=10; median gestational age: 27 weeks; median age at tissue collection: 20 days) and preterm infants with SIP (n=10; median gestational age: 27 weeks; median age at tissue collection: 47 days) were obtained under appropriate oversight by the Institutional Review Board of Vanderbilt University. Sections were prepared and stained for IHC as described below. NEC samples were obtained at the time of surgery and were taken from the leading edge of damaged tissue. Infants in this cohort were de-identified, so their severity of NEC is not precisely known; however, all infants required surgical intervention and can be assumed to be Bell stage 3. All tissue samples were examined for live cells and villous architecture by IHC, and half of the samples were measured for lymphocytes by flow cytometry, as a secondary measure of tissue viability. Any samples not having viable cells were deemed to be necrotic and were discarded. Traditionally, it is difficult to obtain suitable controls for NEC samples. To address this we used age-matched infants who developed SIP and required surgery.

Immunohistochemistry and staining

Ileal sections were deparaffinized, rehydrated, and antigen unmasked by boiling in a citrate-containing buffer (Vector Labs). Slides were stained with H&E (Newcomer Supply) to determine histological characteristics. To detect Paneth cells, samples were treated with 10% goat serum (Zymed) for 30 minutes followed by exposing samples to anti-lysozyme (Dako) antibody at 4°C overnight. Anti-rabbit horseradish peroxidase (Dako) was applied to slides for 30 minutes, and samples were developed using a DAB substrate kit (Vector Labs) and counterstained with methyl green or Meyer’s hematoxylin. Alternatively, Paneth cells were detected by staining with Alcian Blue (Sigma) followed by periodic acid Schiff (Sigma) or by phloxine/tartrazine (Newcomer Supply) staining. Positive Paneth cells were counted per crypt by a single blinded investigator to determine quantification. Sections were stained with Alcian Blue (Sigma) to detect mucus-positive goblet cells. Positive goblet cells were counted per 100 epithelial cells above the +4 position to determine quantification by a single blinded investigator.

Cytokine analysis

Ileal samples were weighed and homogenized in a 1:10 ratio of lysis buffer. Cytokine levels in samples were quantified using a Mesoscale Discovery 7-plex pro-inflammatory ultra-sensitive plate (Mesoscale, Gaithersburg, MD) according to the manufacturer’s instructions. Plates were read on a Sector Imager 2400 at 620 nm.

NEC Models

Dithizone model

P14-P16 CD-1 mice were separated from their mothers and maintained without feeds in a temperature- and humidity-
Controlled chamber throughout the experiment. On the day of experimentation, mice were given an i.p. injection with either 75 mg/kg dithizone (Sigma) dissolved in Li₂CO₃, or an equivalent volume of Li₂CO₃ buffer alone (Sherman et al., 2005). At 6 hours after injection, mice were enterally infected by gastric gavage with 1×10¹¹ CFU Klebsiella/kg body weight (ATCC) or given a gavage of an equivalent volume of sterile media (nutrient broth; ATCC) (Sherman et al., 2005). Mice were monitored for 10 hours after gavage and then the experiment was concluded.

### Hypoxia-hypothermia model

P4 CD-1 mice were separated from their mothers and maintained without feeds in a temperature- and humidity-controlled chamber throughout the experiment. On the day of experimentation, mice were randomly grouped into one of three groups: control, control + stress, and stress. All mice were anesthetized with isoflurane (Drobac et al., 2004) and a surgical gastrectomy procedure was performed in an operation in which a surgical gastrostomy tube was placed, but the tube was cut short and buried under the nape of the neck. Formula group mouse pups were fed continuously through the gastrostomy tube with a prepared rodent substitute formula (Dvorak et al., 2000). Mice randomized to stress groups were exposed twice daily to 100% oxygen in a temperature-controlled water bath (Fig. 8) (Beierle et al., 2004). Mice in the control group were given a sham operation in which a surgical gastrostomy tube was placed without feeds in a temperature- and humidity-controlled chamber throughout the experiment. On the day of experimentation, mice were given an i.p. injection with either 1 mg/kg LPS (Escherichia coli 5 endotoxin, Calbiochem Catalog #437620) and 50 mg/kg PAF (PAF-16, Catalog #511075, Calbiochem) or equivalent volumes of saline control (Maheshwari et al., 2011). Mice were sacrificed for 2 hours after injection (Maheshwari et al., 2011).

### Scoring

For all methods, mucosal injury was evaluated by a single blinded investigator and graded on a 5-point scale: grade 0, no injury; grade 1, mild separation of lamina propria; grade 2, moderate separation of sub-mucosa; grade 3, severe separation and/or edema in sub-mucosa; grade 4, transmural injury (Musemeche et al., 1991; Dvorak et al., 2002; Maheshwari et al., 2011).

### Results

This group previously reported that lysozyme-expressing Paneth cells are deficient in infants with NEC, suggesting that this cell type plays a role in the development of the disease. In this study, the authors verify that Paneth cells in general are lacking in infants with NEC, and show that this effect is specific to the disease, because age-matched infants with spontaneous intestinal perforation have normal numbers of Paneth cells. In mice, inducing selective Paneth cell ablation with the zinc chelator dithizone, followed by exposure to Klebsiella pneumoniae, causes intestinal pathology that closely resembles human NEC. Furthermore, treatment of small intestinal explants with dithizone or dithizone plus K. pneumoniae induces similar histological pathology as in vivo exposure to these agents. Using this organ culture system, the authors show that pretreatment of small intestinal explants with a zinc-rich medium inhibits the detrimental effects of dithizone, but not the combined effect of dithizone with K. pneumoniae. Thus, the pathology in this model is not directly caused by a loss of zinc, but by the loss of Paneth cells.

### Implications and future directions

These findings provide new insights into the potential role of Paneth cells in the pathogenesis of NEC. Importantly, this new mouse model of NEC exhibits intestinal pathology that is consistent with that of other currently used animal models of NEC, but it provides several unique advantages: it is simpler, and can be used in mice of a developmental stage that is relevant to human NEC. This new mouse model could lead to new therapeutic approaches.

### Organ culture model

P14-P16 CD-1 mice were sacrificed and the small intestine was opened and washed with sterile PBS and DMEM media (Invitrogen), and then cut into 2×0.5 cm pieces. The explants were laid on Netwell inserts (membrane mesh size of 500 µm; Corning Incorporated Life Sciences, Acton, MA) with the serosal layer facing the insert. DMEM containing 0.5% FBS was filled to a point just over the epithelium and incubated at 37°C with 5% CO₂ for 30 minutes before treatment (Yan et al., 2007). At the end of the experiment, intestinal tissue was fixed in 10% formaldehyde at 4°C overnight before sectioning. Small intestinal explants were treated for a total of 120 minutes with one
of the following seven conditions: DMEM media; Li$_2$CO$_3$ buffer (Sigma); 15 µM ZnCl$_2$ (Sigma) in DMEM; 0.05% dithizone in DMEM for 15 minutes followed by DMEM alone for 105 minutes; 0.5% dithizone in DMEM followed by DMEM alone for 105 minutes; 1×10$^5$ Klebsiella/ml DMEM; or 15 µM ZnCl$_2$ (Sigma) with 1×10$^4$ Klebsiella/ml DMEM in DMEM.

Replicates and statistical analysis

All data are representative of at least three independent experiments. For NEC models, differences between groups were assessed using the proportional odds logistic regression model. For quantitative PCR, gene expression was normalized to β-actin, and the 2$^{-ΔΔCT}$ method was used to compare gene expression levels between samples. Minimum level of significance was set at P<0.05, and error bars designate upper and lower confidence intervals.

COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

C.Z. performed experiments and analyzed the data; M.P.S. helped prepare the manuscript; L.S.P. performed experiments and prepared the manuscript; D.B. helped analyze the data; J.H.W. helped obtain human tissue samples; J.C.S. performed statistical analysis; and S.M.J. performed experiments, analyzed the data and prepared the manuscript.

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