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Heme oxygenase-1 confers protection and alters T-cell populations in a mouse model of neonatal intestinal inflammation

Stephanie Schulz[1–3], Karen M. Chisholm[4], Hui Zhao[1], Flora Kalish[1], Yang Yang[5], Ronald J. Wong[1] and David K. Stevenson[1]

BACKGROUND: Necrotizing enterocolitis (NEC), an intestinal inflammatory disease affecting premature infants, is associated with low regulatory T (Treg) to effector T (Teff) cell ratios. We recently demonstrated that heme oxygenase-1 (HO-1) deficiency leads to increased NEC development. Here, we investigated the effects of HO-1 on T-cell proportions in a murine NEC-like injury model.

METHODS: Intestinal injury was induced in 7-d-old wild-type (WT) or HO-1 heterozygous (HO-1 Het) pups by formula-feeding every 4 h for 24–78 h by oral gavage and exposures to 5%O_2. Controls remained breastfed. HO-1 was induced in WT pups by administering heme preinjury induction. Lamina propria T cells were identified by flow cytometry. For adoptive transfer studies, WT splenic/thymic Tregs were injected intraperitoneally into HO-1 Het pups 12–24 h preinduction.

Results: Het mice showed increased intestinal injury and decreased Treg/Teff ratios. Genes for pattern recognition (Toll-like receptor-4, C-reactive protein, MyD88) and neutrophil recruitment increased in Het pups after NEC induction. Inducing intestinal HO-1 decreased NEC scores and incidence, and increased Treg/Teff ratios. Moreover, adoptive transfer of Tregs from WT to HO-1 Het pups decreased NEC scores and incidence and restored Treg/Teff ratios.

Conclusion: HO-1 can change Treg proportions in the lamina propria of young mice under inflammatory conditions, which might, in part, confer intestinal protection.

NEC is a devastating disease of premature infants. Characterized by intestinal inflammation, it can progress rapidly to intestinal necrosis. Its incidence correlates inversely with low birth weight and gestational age, reaching ~7% in infants weighing 500–1,500 g. Unfortunately, 20–40% of NEC patients need to undergo surgery, which increases mortality up to 50% and places these infants at higher risk for developing long-term sequelae, such as short bowel syndrome and neurodevelopmental impairments (1).

Although advances in the understanding of its pathophysiology have been made in the last decades, the pathogenesis of NEC remains incompletely understood. Thus, current prevention and treatment strategies are limited. Three factors are generally present when NEC occurs: (i) an immature intestinal barrier and immune system; (ii) enteral feedings; and (iii) a putative bacterial component. A genetic predisposition, an *in utero* maternal or fetal insult (hypoxia), and/or a highly immune-reactive intestinal mucosa may then cause the initial inflammation that precedes NEC (2).

Several studies have demonstrated the importance of the innate immune system in the pathophysiology of NEC. For example, a significant component is the pattern recognition of pathogens through Toll-like receptor (TLR)-4 (3,4). Also, a reduction in Paneth and in mucin-producing goblet cells (5,6) and an increase in proinflammatory mediators (i.e., TNF-α, IL-1, -6, -8, and platelet-activating factor) are associated with NEC (7).

However, less attention has been given to the adaptive immune system. The fact that NEC usually occurs at least a week after birth and after the initiation of enteral feedings is consistent with the idea that the adaptive immune system is also of significance. In a recent human study, premature infants with NEC were shown to have a significantly reduced ratio of T regulatory cells (Tregs) to effector T cells ($CD4^+$ and $CD8^+$, Teffs) compared to gestational age-matched controls (8). Furthermore, adoptive transfer of Tregs harvested from adult rats have been shown to protect NEC-induced pups against severe intestinal damage (9). The intestine has to not only cope with harmful pathogens; but also, it must possess the capacity to tolerate dietary and bacterial antigens. Tregs are a distinct population of $CD4^+$ cells that are characterized by the constitutive expression of the IL-2α chain ($CD25^{hi}$) and the transcription factor FOXP3 (10). It is well documented that Tregs are essential in regulating effector immune cells and play a pivotal role in controlling both autoimmune and inflammatory diseases. Treg activation via oral antigen administration points toward a role in oral tolerance (11). Moreover, Tregs are capable of inhibiting (12) and reversing established colitis (13), which suggests that they mediate microbial tolerance. In humans, Tregs are already present in the gut at 20 wk

[1]Department of Pediatrics, Stanford University School of Medicine, Stanford, California; [2]Vlaams Instituut voor Biotechnologie Inflammation Research Center, Gent, Belgium; [3]Department of Biomedical Molecular Biology, Ghent University, Ghent, Belgium; [4]Department of Pathology, Stanford University School of Medicine, Stanford, California; [5]Department of Genetics, Stanford University School of Medicine, Stanford, California. Correspondence: Ronald J. Wong (rjwong@stanford.edu)

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of gestation; however, in rodents, Treg development is significantly delayed (14).

Heme oxygenase-1 (HO-1) is the inducible isoform of HO, and catalyzes heme breakdown to produce equimolar amounts of bilirubin, free iron, and carbon monoxide (CO) (15). HO-1 plays a significant role in tissue protection especially under stress conditions. Crucial for its protective effects are its antioxidative, antiapoptotic, and anti-inflammatory functions, mediated by the heme degradation products bilirubin and CO (16). The original HO-1 KO mouse showed a progressive chronic inflammatory and proinflammatory state as demonstrated by enlarged lymph nodes, high white blood cell counts, hepatic inflammatory cell infiltrates, and a shift in T-helper (Th)-2 to Th1 cytokine response (17,18). Several authors have reported that HO-1 confers immune suppression by affecting Tregs (19,20), but these data are controversial, as others have shown that HO-1 does not affect the development and/or suppressive function of Tregs (21,22). Overall, the importance of HO-1 for Treg-mediated immune regulation has not been clearly characterized. It is conceivable that it is highly dependent on the immunological circumstance studied, e.g., at baseline or under inflammatory conditions. For the latter, differences between the early, acute, or chronic phases of an inflammatory state are likely to have an effect as well.

We have recently shown that HO-1-deficient (*Hmox*1$^{+/-}$, HO-1 Het) mice are more susceptible to NEC-like intestinal injury (23), and hypothesized that the anti-inflammatory and immune modulatory effects of HO-1 play a role to dampen intestinal injury induced by our experimental NEC model. We now provide evidence that HO-1-deficient mice have a compromised immune suppressive function under stress conditions, which may be responsible, at least in part, for their increased susceptibility to develop NEC.

RESULTS

High Treg/Teff Ratios Are Associated With Intestinal Protection in WT Mice

Since it has been previously reported that a low Treg/Teff ratio is associated with the development of human NEC (8), we first determined whether and how NEC induction affects the lymphocyte population in the lamina propria of HO-1 Het mice. After induction of intestinal damage, HO-1 Het pups showed significantly higher damage scores (2.3 vs. 1.5) and NEC incidence (73 vs. 41%) compared to WT pups (**Figure 1a**,**b**), similar to our previous findings of 2.5 vs. 1.5 and 78 vs. 43%, respectively (23). Flow cytometry demonstrated no significant changes in total leukocytes (viable CD45$^+$ cells) of control and NEC-induced pups for each genotype and between genotypes (data not shown).

The total Treg population (gating strategy on **Supplementary Figure S1** online) increased in No NEC pups of both genotypes compared to pups with NEC, but was higher for WT No NEC vs. HO-1 Het No NEC pups (**Figure 1c**). When the Treg population was expressed as a percentage of the CD4$^+$ cells, we found a significantly higher percentage in pups (independent of the genotype) that did not develop NEC compared to controls as well as to those which developed NEC after injury induction (**Figure 1d**). When comparing only the pups that did not develop NEC, WT pups had a significantly higher percentage of Tregs/CD4$^+$ compared to HO-1 Het pups. This tendency was also found for the percentage of Tregs in CD8$^+$ cells for both genotypes, but was not statistically significant ($P = 0.07$) (**Figure 1e**). Also, by analyzing the proportions of Tregs in the CD3 gate, we found a significant increase in Tregs for No NEC WT pups compared to HO-1 Het No NEC pups (**Figure 1f**). However, single CD4- and CD8-positive T cells in the CD3 gate remained constant in WT and HO-1 Het pups during NEC induction (**Supplementary Figure S2a** online). These results suggest that a high Treg/Teff ratio is directly due to an increased Treg population because the Teff populations in both genotypes did not change under control, NEC, and No NEC conditions. Moreover, a high Treg/Teff ratio appears to be associated with an attenuation of intestinal damage, but HO-1 Het pups might have an impaired ability to enhance their Treg population.

PCR array analyses were performed on intestinal tissues from WT and HO-1 Het mice to compare expression levels of genes mediating the immune response. Expression levels of genes important for pattern recognition (TLR4, C-reactive protein), defense response (MyD88), and neutrophil recruitment (chemokine (C-X-C motif) receptor 3 or Cxcr3) were significantly higher in HO-1 Het pups; whereas, expression of FOXP3 (not significant), interferon regulatory factor 7 (Irf7), and RAR-related orphan receptor-γ (Rorc) were reduced (see **Table 1**). These data confirm that there is increased intestinal inflammation in HO-1 Het mice.

HO-1 Induction Protects Against Intestinal Damage and Increases Treg/Teff Ratios

Next, we hypothesized that the induction of HO-1 will dampen intestinal damage by increasing Treg/Teff ratios in the lamina propria. We previously have shown that oral administration of 30 μmol/kg body weight of heme (as methemalbumin) increases HO activity approximately twofold in the jejunum and ileum of 1-wk-old mice pups 24 h postadministration (23). Therefore, we orally administered 30 μmol/kg body weight of heme at 24 and 3 h before the initiation of NEC induction. Heme administration reduced intestinal damage scores (1.0 vs. 1.8; WT+Heme vs. WT NEC, respectively) and NEC incidences (**Figure 2a–c**). In fact, no pup pretreated with heme developed intestinal damage scores ≥2. Flow cytometry showed that heme treatment enriched Tregs in the CD4$^+$ gate (**Figure 2d**) and increased CD25 expression of CD4$^+$FOXP3$^+$ Tregs (**Supplementary Figure S3a** online). Treg/Teff ratios of WT+Heme pups were significantly higher than those of WT+Heme Con and WT NEC pups and comparable to Treg/Teff ratios of WT No NEC pups (**Figure 2e**,**f**). CD4 cells in the CD3 gate remained similar among all group (**Supplementary Figure S2b** online). But CD8 cells in the CD3 gate were significantly reduced after heme treatment,

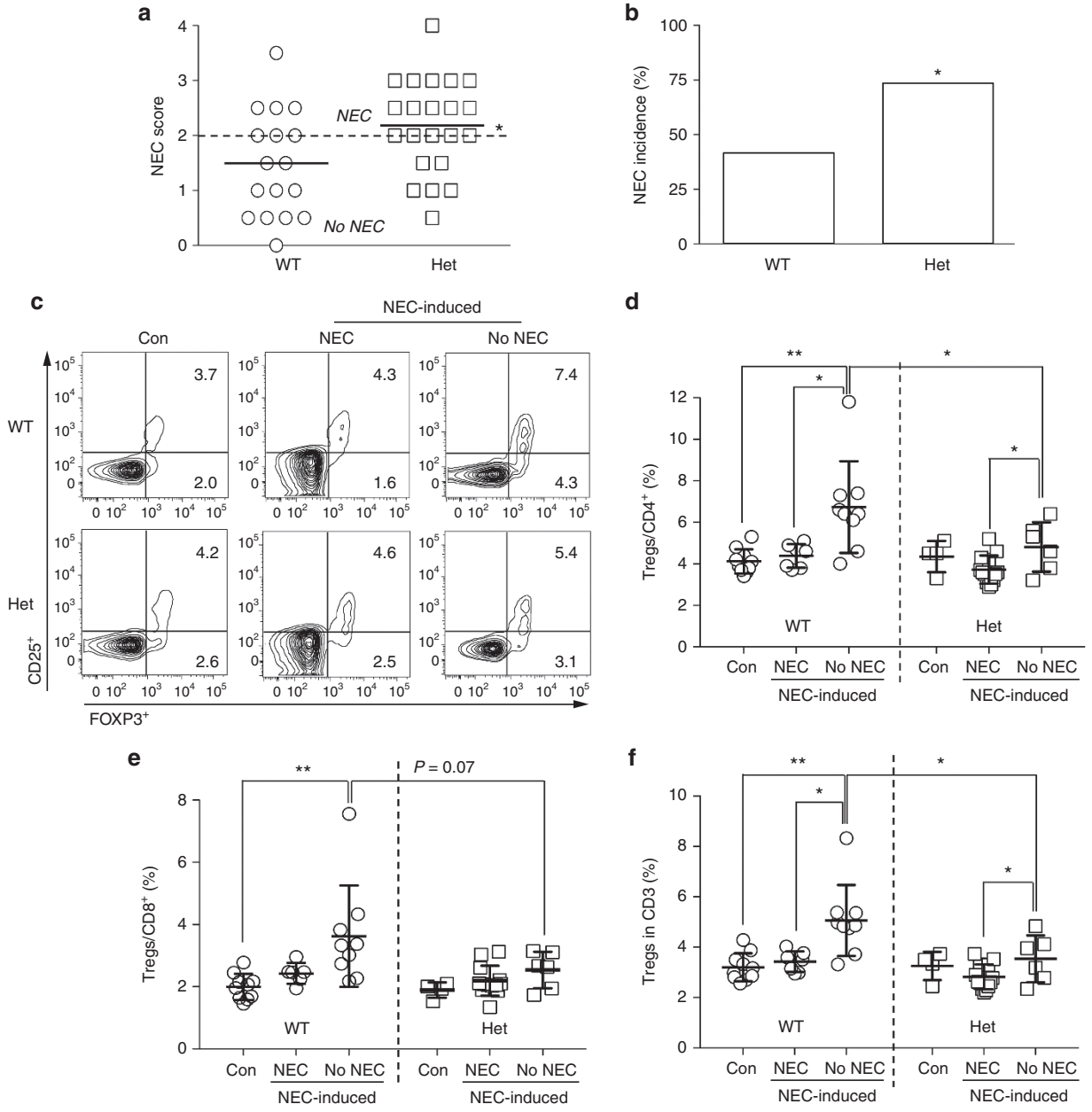

Figure 1. Intestinal damage and Treg/Teff ratios of WT pups compared to HO-1 Het pups after NEC induction. Summary of damage scores (**a**) and NEC incidences (**b**). Representative flow cytometric analyses of $CD25^+FOXP3^+$ T cells in lamina propria lymphocytes (gated on viable $CD4^+$ T cells) from WT and HO-1 Het controls (Con) and NEC-induced pups with scores ≥ 2 (NEC) and < 2 (No NEC) (**c**). Percentages of Tregs ($CD4^+CD25^+FOXP3^+$) to Teffs ($CD4^+$ (**d**) and $CD8^+$ (**e**)) were then calculated for WT (○) and HO-1 Het (□) Con and NEC-induced pups, and subcategorized as NEC or No NEC. Percentages of Tregs ($CD4^+CD25^+FOXP3^+$) in the CD3 population (**f**) are shown for WT (○) and HO-1 Het (□) Con and NEC-induced pups. Data are from 3 or more independent litters and expressed as medians for NEC scores, means for NEC incidences, and mean ± SD for %Tregs to Teffs ratios. $^*P \le 0.05$, $^{**}P \le 0.01$.

which could indirectly increase the Treg/CD8 ratio in this group (Figure 2f). However, since the CD8 single- and double-positive cells were a small population compared to the CD4 cells (S1), the increased Tregs in the CD3 gate still indicated a direct enhancement of $CD4^+CD25^+FOXP3^+$ cells after NEC induction in pups pretreated with heme (Figure 2g).

Flow cytometry also revealed a strong decrease in $CD11b^+Gr1^+$ neutrophil infiltration into the lamina propria of NEC-induced WT pups pretreated with heme compared to pups not pretreated, confirming decreased inflammation in heme-pretreated pups (**Supplementary Figure S3b** online). Together with our previously published data (23), which demonstrated an increase in HO-1 mRNA after NEC induction and increased HO activity after heme treatment, these data indicate that augmentation of HO-1 protein can positively affect Treg/Teff ratios during NEC induction and might mediate intestinal protection.

Adoptive Transfer of WT Tregs to HO-1 Het Pups Reduces Intestinal Damage and Increases Treg/Teff Ratios

We then investigated whether Tregs isolated from WT pups and transferred to HO-1 Het pups (Het+wTregs) could confer intestinal protection against NEC development. To this end, we injected ~5×10^4 thymic or splenic $CD4^+CD25^+CD45RB^-$ Tregs from 6-d-old WT pups into HO-1 Het pups 12–24 h prior to NEC induction. Adoptive transfer of WT Tregs to HO-1 Het pups decreased intestinal damage scores from 3.0 for Het pups to 1.8 for Het+wTregs and lowered NEC incidence from 78 to 50% (**Figure 3a–c**). Flow cytometry showed an increase in lamina propria Tregs in NEC-induced Het+wTreg pups compared to Het NEC pups (**Figure 3d**). Significantly higher Treg/$CD4^+$ (**Figure 3e**) and Treg/$CD8^+$ (**Figure 3f**) ratios were found for Het+wTreg pups with intestinal damage scores <2 compared to HO-1 Het NEC (not for Treg/$CD8^+$), and HO-1 Het No NEC pups. The Teff cells (CD4 and CD8) remained constant in the CD3 gate during NEC induction between NEC-induced Het and Het+wTregs (**Supplementary Figure S2c** online); whereas, Tregs were significantly increased in Het+wTregs compared to HO-1 Het pups (**Figure 3g**). These data indicate that adoptive transfer of WT Tregs led to an increase in Tregs in the lamina propria of HO-1 Het pups. Moreover, we also found that after NEC induction, CD4- and CD8-single-positive cells

Table 1. Expression profiles of innate and adaptive immunity genes in the small intestine of HO-1 Het and WT pups

Function	Gene	Fold change HO-1 Het NEC/ WT NEC	*P* value
Pattern recognition	Tlr-4	3.0	≤ 0.05
	C-reactive protein	6.0	≤ 0.05
Defense response	Myd88	2.0	≤ 0.05
Neutrophil recruitment	Cxcr3	4.3	≤ 0.05
Type 1 IFN response	Irf7	−24.5	≤ 0.005
Treg marker	Foxp3	−3.7	0.196
Th17 marker	Rorc	−1.9	≤ 0.05

The expression profiles of 84 genes involved in innate and adaptive immune responses were analyzed in small intestinal tissue samples from NEC-induced HO-1 Het and WT pups. Fold changes in expression over NEC-induced WT pups were calculated by $\Delta\Delta C_t$ analyses using the web-based software from Qiagen.

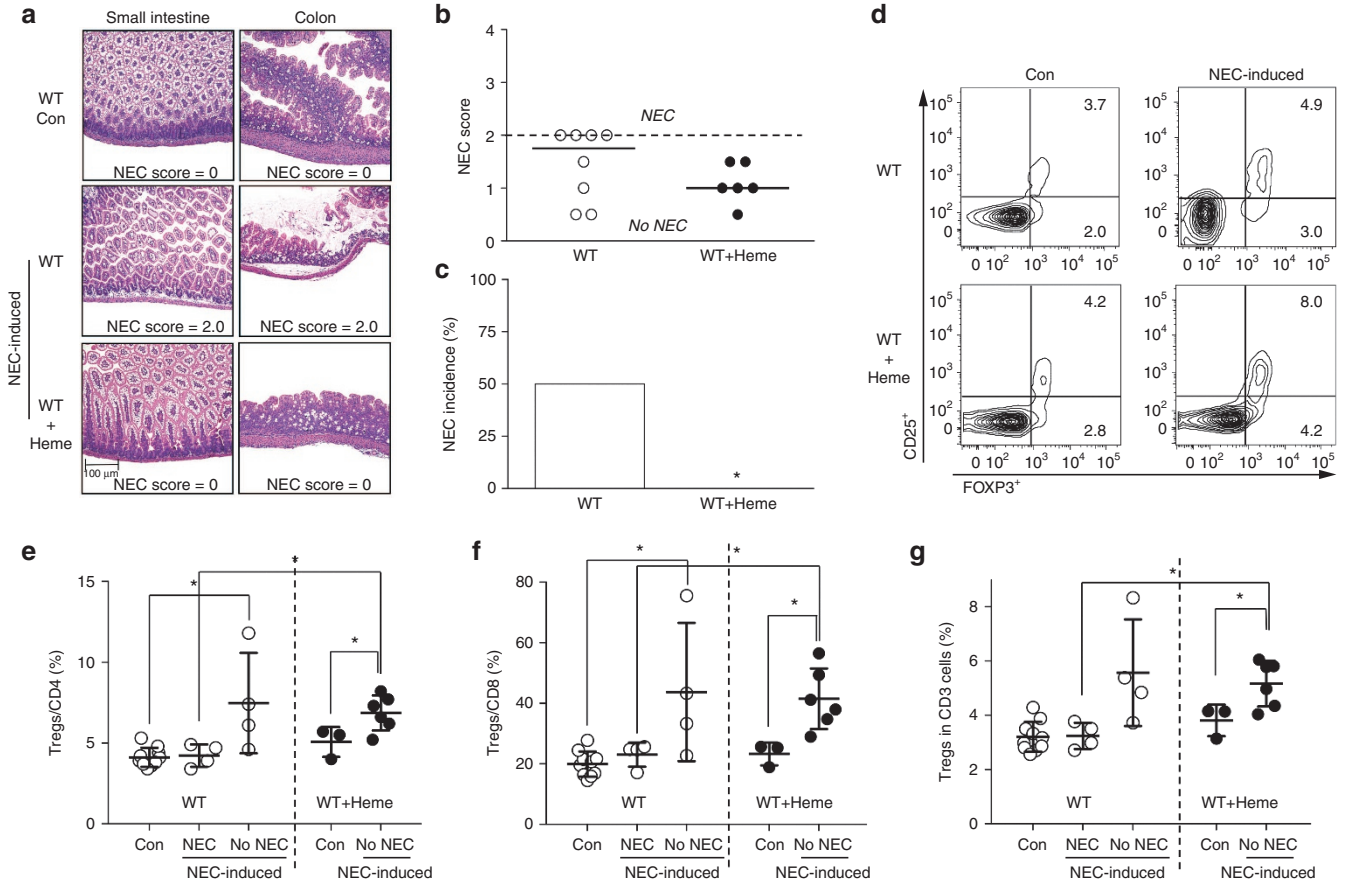

Figure 2. Intestinal damage and Treg/Teff ratios of WT pups compared to heme-treated WT (WT+Heme) pups after NEC induction. Representative images of H&E-stained small intestines and colons are shown with associated NEC scores (**a**). Magnification: 10X. Summary of damage scores (**b**) and NEC incidences (**c**). Representative flow cytometric analyses of $CD25^+FOXP3^+$ T cells in lamina propria lymphocytes (gated on viable $CD4^+$ T cells) from WT and WT+Heme (Con) and NEC-induced pups with scores ≥ 2 (NEC) and < 2 (No NEC) (**d**). Percentages of Tregs ($CD4^+CD25^+FOXP3^+$) to Teffs ($CD4^+$ (**e**) and $CD8^+$ (**f**)) were calculated for WT (○) and WT+Heme (●) Con and NEC-induced pups, and sub-categorized as NEC or No NEC. Percentages of Tregs ($CD4^+CD25^+FOXP3^+$) in the CD3 population (**g**) are shown for WT (○) and WT+Heme (●) Con and NEC-induced pups. Data are from 3 or more independent litters and expressed as medians for NEC scores, means for NEC incidences, and mean±SD for %Tregs to Teffs ratios. *$P \le 0.05$. Note: no WT+Heme pups developed NEC following intestinal injury induction.

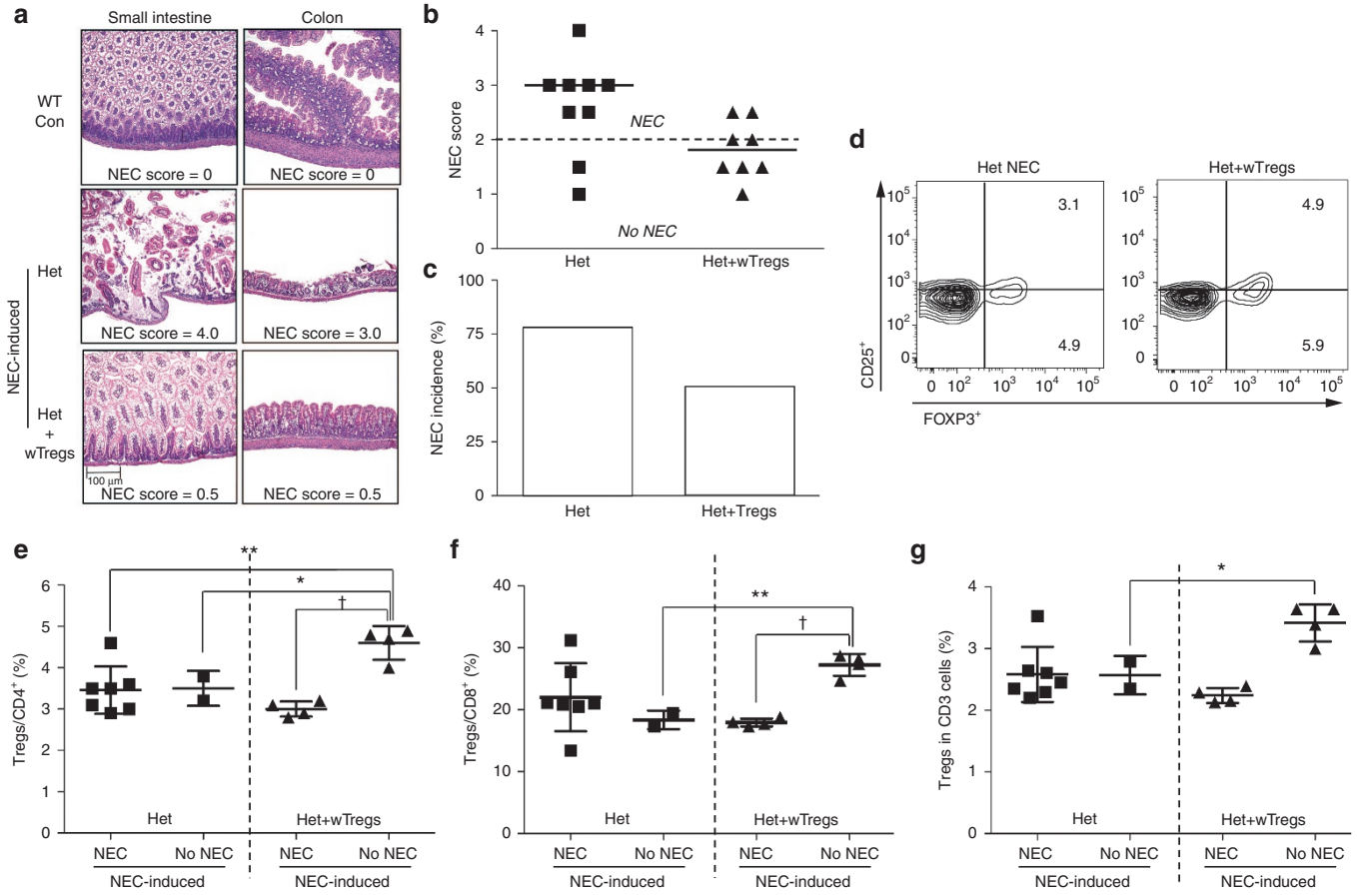

Figure 3. Intestinal damage and Treg/Teff ratios of NEC-induced HO-1 Het and HO-1 Het pups adoptively transferred with WT Tregs (Het+wTregs). Representative images of H&E-stained small intestines and colons are shown with associated NEC scores (**a**). Magnification: 10×. Summary of damage scores (**b**) and NEC incidences (**c**). Representative flow cytometric analyses of $CD25^+FOXP3^+$ T cells in lamina propria lymphocytes (gated on viable $CD4^+$ T cells) from NEC-induced HO-1 Het and Het+wTregs pups with scores ≥ 2 (NEC) and < 2 (No NEC) (**d**). Percentages of Tregs ($CD4^+CD25^+FOXP3^+$) to Teffs ($CD4^+$ (**e**) and $CD8^+$ (**f**)) were calculated for NEC-induced HO-1 Het (■) and Het+wTregs (▲) pups, and sub-categorized as NEC or No NEC. Percentages of Tregs ($CD4^+CD25^+FOXP3^+$) in the CD3 population (**g**) are shown for NEC-induced HO-1 Het (■) and Het+wTregs (▲) pups. Data are from 3 or more independent litters and expressed as medians for NEC scores, means for NEC incidences, and mean ± SD for %Tregs to Teffs ratios. $^*P \le 0.05$, $^{**}P \le 0.01$, $^{\dagger}P \le 0.001$.

were significantly reduced in the $CD45^+$ gate for Het+wTregs pups compared to HO-1 Het pups (data not shown), indicating that the transferred WT Tregs were indeed functional. Collectively, adoptive transfer of WT Tregs can augment Treg/Teff ratios in HO-1 Het pups and appears to protect against intestinal damage.

Correlation of Treg/Teff Ratios and NEC Scores

The collective data indicated that there is a strong correlation between NEC scores and Treg/Teff ratios, which might be dependent on HO-1 levels. We therefore plotted Treg/Teff ratios as a function of NEC scores for all groups (**Figure 4**). We found the highest correlation for WT+Heme pups ($r^2 = 0.643$ (CD4); 0.677 (CD8)), Het+wTregs ($r^2 = 0.583$ (CD4); 0.649 (CD8)), and WT pups ($r^2 = 0.355$ (CD4); 0.184 (CD8)); whereas, the correlation for HO-1 Het pups was very low ($r^2 = 0.156$ (CD4); 0.081 (CD8)) (**Table 2**). The mean percentage of Tregs/CD4 for NEC pups (3.78%) was significantly lower than for pups not developing NEC (5.97%, $P < 0.0001$). Taken together, these data show that NEC scores correlate with Treg/Teff ratios in conditions where HO-1 is induced or present (WT, WT+Heme, Het+wTregs). However, in an HO-1-deficient state, NEC scores and Treg/Teff ratios do not correlate. This supports our hypothesis that the development of intestinal injury in this model may be dependent on the level of HO-1 expression available to respond to inflammation.

DISCUSSION

Here, we demonstrate that HO-1 appears to mediate protection against neonatal intestinal injury. We found that: (i) HO-1 Het mice are more susceptible to develop NEC-like intestinal injury. This appears to be associated with decreased Treg/Teff ratios compared to mice in the experimental group not developing the disease; (ii) inducing HO-1 in the intestine prior to injury induction increases Treg/Teff ratios and appears to be protective against NEC development; and (iii) adoptive transfer of WT Tregs to HO-1 Het pups can attenuate intestinal injury. Additionally, our findings indicate that an augmentation of the Treg/Teff ratio might be a general mechanism that confers protection against NEC development, similar to recent findings in human neonates (8) and in a rat model of NEC (9).

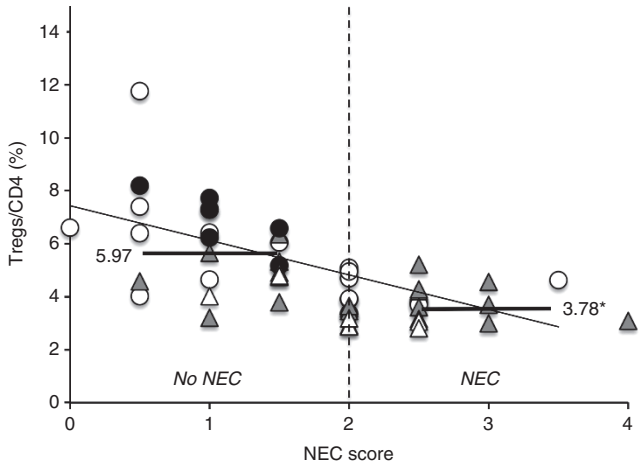

Figure 4. Correlations of the percentages of Tregs in the total CD4 populations with NEC scores are shown for WT (○), HO-1 Het (▲), WT pups pretreated with heme (WT+Heme, ●) and HO-1 Het pups adoptively transferred with WT Tregs (Het+wTregs, △) prior to NEC induction. Mean ± SD %Tregs/CD4 are shown. Data are from three or more independent litters. *$P \leq 0.05$.

Although HO-1 did not affect FOXP3 expression in $CD4^+CD25^+$ lamina propria T cells (data not shown), which agrees with previous reports for splenic and lymphatic tissues (22); we however did see an increased expression of CD25 in FOXP3 Tregs after heme administration. Several studies have demonstrated that CD25 expression is essential for Treg stability and survival (24,25). Yet, the specific molecular mechanism by which HO-1 enriches the Treg pool under stress conditions remains to be further clarified. Others have also reported that IL-10 may be involved in FOXP3 activation in lamina propria cells in the ileum (26,27). IL-10 is proposed to be one of the downstream mediators by which HO-1 confers its anti-inflammatory effects (28), and the induction of HO-1 by heme can increase IL-10. However, using PCR array analysis, we did not find a difference in IL-10 expression between WT and HO-1 Het NEC pups. It is also conceivable that HO-1 is able to increase the peripherally-derived Treg populations (often also referred to as inducible Tregs (iTregs)) (29). Our methodology used to identify $FOXP3^+$ Tregs in the intestine did not enable us to distinguish between thymus- and peripherally derived Tregs. FOXP3 expression of iTregs is transient after activation; therefore it is not a good marker for iTregs (30). This hypothesis should be further tested with new identified markers like CD49b and LAG-3 (30). However, we cannot exclude that the effects observed in pups pretreated with heme or with adoptively transferred Tregs are independent. Adoptive transfer of Tregs might reduce NEC severity simply by directly increasing the Treg population; while heme pretreatment might alter the immunoregulatory effects of Tregs through an induction of HO-1 expression in dendritic cells (DCs) as shown by George *et al.* (31).

Significant differences in Treg ratios between WT vs. HO-1 Het and WT vs. WT+Heme were only observed for pups that did not develop NEC. HO-1 is a stress enzyme and is upregulated in response to exposure to various stressors (16). We have previously shown that HO-1 mRNA increases during NEC induction, and that HO-1 expression may vary among WT pups (23). We also found that pups not developing NEC are able to significantly increase HO-1 mRNA during NEC induction; whereas, pups that develop NEC have low HO-1 expression (data not shown) (23). This may directly affect the Treg population. This hypothesis is supported by our HO-1 induction study. When HO-1 is induced prior to NEC induction, no WT pup developed NEC and all showed increases Treg/Teff ratios. Also, humans differ in their capacity to elicit an HO-1 response. There are two known human polymorphisms in the HO-1 gene promoter region: a (GT)n microsatellite and a functional single-nucleotide polymorphism: T(-413)A. Both can alter the basal level of HO-1 expression as well as in response to stressors (32). Individuals with short (GT)n repeat lengths are "high" HO-1 expressors compared to those individuals with long (GT)n) repeats (33). Several studies indicate that the ability to upregulate HO-1 expression is an important protective factor in some diseases (e.g., cardiovascular disease and renal transplantation) (32). However, no studies investigating an association between HO-1 polymorphisms and NEC have been published.

Table 2. Correlation of Treg/Teff ratios (%Tregs/CD4 and %Tregs/CD8) and NEC Scores

	Correlation coefficient, r^2	
Group	Tregs/CD4	Tregs/CD8
WT+Heme ($n = 6$)	0.642	0.677
HO-1 Het+wTregs ($n = 8$)	0.583	0.649
WT ($n = 18$)	0.355	0.183
HO-1 Het ($n = 19$)	0.156	0.081
All groups combined ($n = 51$)	0.386	0.270
All groups without HO-1 Het ($n = 32$)	0.437	0.300

Our comparison of the changes in innate and adaptive immunity genes after NEC induction revealed significant increases in the expression levels of genes important for pattern recognition and neutrophil recruitment in HO-1 Het pups, which developed NEC. Specifically, increases in the expression levels of TLR4 and MyD88 are well in line with previous data demonstrating that an activation of the TLR4/MyD88 signaling pathway is pivotal in NEC development (3,4). The increase in C-reactive protein indicates an advanced inflammatory stage with higher numbers of apoptotic and necrotic cells in HO-1 Het NEC compared to WT NEC pups. Expression of IRF7, which is a crucial for type I interferon production, was decreased in HO-1 Het pups. Interestingly, type I interferons have been shown to be essential for the regulation of intestinal homeostasis (34).

Dingle *et al.* (9) have recently shown that increasing the Treg/Teff ratio using adoptive transfer of Tregs from adult rats can protect against the development of severe intestinal inflammation to rescue NEC-induced rat pups. In our study, we also successfully rescued NEC-induced HO-1 Het pups by adoptively transferring WT Tregs from young (age-matched) mice. The reduction in NEC scores and incidence for

Table 3. NEC scoring system

Score	Histopathology
0	Normal
1	*"mild"* or slight submucosal and/or lamina propria separation
2	*"moderate"* separation of the submucosa and/or lamina propria layers and/or edema in the submucosa and muscular layers
3	*"severe"* separation of the submucosa and/or lamina propria layers and/or severe edema in the submucosa and muscular layers with regional villous sloughing
4	*"necrosis"* and loss of villi

Adapted with permission from Figure 3 in ref. (40), p. G159.
Data taken from refs. (23,40).

adoptively-transferred pups reported by Dingle *et al.* (9) was more dramatic than those in our study. This may most likely be due to the fact that they transferred ~20 times more Tregs (1×10^6 vs. 5×10^4). In addition, they transferred Tregs derived from adult rats (not age-matched pups), which may also have an impact as it is not well established if Tregs from adult animals have the same functional activity and expression levels of immune suppressive cytokines than Tregs from 1-wk-old pups (9). Although we did not find a difference in NEC scores and incidence between pups transferred with thymic compared to those transferred with splenic Tregs, a recent study showed that thymus-derived Tregs are the most important in mediating tolerance to commensal microbiota (35). Interestingly, FOXP3 expression is increased in $CD4^+CD25^+$ Tregs isolated from the thymus compared to those from the spleen (**Supplementary Figure S4a** online). Because the Treg population is very small, we sorted with a purity of 80–98% for the thymus and spleen (depending on the CD25 gate). Therefore, a difference in the protective ability between thymus- and spleen-derived Tregs, if present at all, could not be distinguished.

Since Tregs are $CD4^+$ T cells, they need to be activated by MHC class II-epitopes on DCs. In fact, DCs are not only indispensable for the activation of naive Teffs, but they are also essential for the activation of Tregs (36). For example, Tregs are often located at the interface of antigen-presenting cells (APCs) and Teffs supporting a role of APC-Treg interaction for Treg activation and/or migration and function (13). Interestingly, we found an increase in $CD45^+CD3^-CD11b^+MHC^{hi}$ APCs, which we further identified as $CD11c^+$DEC-205^+ lamina propria DCs (37) in the wTregs-transferred HO-1 + Het pups (**Supplementary Figure S3c** online). This increase in APCs indicates that Tregs injected intraperitoneally can indeed migrate to the intestine. It has also been shown that increasing the number of DCs in the lymph nodes and spleen can enhance Treg proliferation and requires MHC II expression by DCs (38). However, whether transferred allogeneic Tregs increase DCs and those further stimulate migration of Tregs to the intestine is speculative and beyond the scope of this study.

In conclusion, we provide evidence that HO-1 may mediate intestinal protection in response to inflammation by positively affecting Treg/Teff ratios. A deficiency in HO-1 may impair the ability to increase the Treg population in response to intestinal inflammation and thus leads to an increased susceptibility to damage. We speculate that enrichment of the Treg pool in the lamina propria might be a potential strategy for the prevention of NEC. A major limitation of a proposed therapeutic use of Treg cells is the difficulty in obtaining sufficient amounts of this rare cell population. Therefore, indirectly increasing the Treg/Teff ratio by pharmacologically inducing HO-1 expression in the context of inflammation (as indexed by an elevated C-reactive protein) could be an attractive strategy to mitigate intestinal damage.

METHODS

Animals

WT FVB/n (*Hmox1*$^{+/+}$) female and male mice (6–8-wk old) were obtained from Charles River Laboratories (Wilmington, MA). The original *Hmox1*$^{-/-}$ (HO-1 KO) mouse strain has a targeted deletion of a large portion of *Hmox1* gene, and was established on a C57BL/6 background (18). For establishment on a predominant FVB/n background, C57BL/6 *Hmox1*$^{-/-}$ mice were backcrossed with FVB/n WT mice for >6 generations. Animals were maintained in strict compliance with Stanford University institutional guidelines and procedures were approved by the Institutional Animal Care Committee of Stanford University.

Genotyping

Genomic DNA from tail clippings was isolated using the Tissue DNeasy kit (Qiagen, Valencia, CA) and analyzed by PCR. For *Hmox1*$^{+/+}$ and *Hmox1*$^{+/-}$ screening, two sets of primers specially designed for WT and HO-1 Het were used.

Induction of NEC-Like Intestinal Damage

To induce NEC-like intestinal injury, we used our previously described induction model consisting of formula feeding by oral gavage combined with hypoxic exposure (23). Control pups remained with their mothers in room air and breastfed. Fresh intestinal tissues were harvested at day 9 for flow cytometric analysis. 0.3–1 cm of the jejunum, distal ileum, and proximal colon were placed in 10% (vol/vol) neutral buffered formalin and RNAlater (Qiagen) for sectioning and H&E staining as well as RNA analyses, respectively. Intestinal tissues from pups of both genotypes, which developed signs of distress or imminent death, were included if they survived at least 24 h of NEC induction. Presence of NEC after 24 h of induction has been shown previously in an experimental rat model (39). For adoptive transfer studies, we used only offspring from HO-1 Het-to-Het breedings.

Heme Treatment and Induction of NEC-Like Intestinal Damage

We have shown that the oral administration of heme (as methemalbumin) to 6-d-old mice pups induces intestinal HO activity (23). To evaluate the effect of increased HO-1 protein during NEC induction, we fed WT pups with 30 μmol/kg body weight of heme by oral gavage 24 and 3 h prior to formula feeding and hypoxia. Because the above described NEC protocol led only to mild intestinal damage in WT pups (23), we extended the duration of formula feedings to 78 h with a total of five 2-min hypoxic exposures. Fresh intestinal tissues were harvested at day 10 for flow cytometric analysis. Approximately 1 cm of jejunum, distal ileum, and proximal colon were archived for sectioning and H&E staining.

NEC Scoring and Histological Staining

Histological changes in intestines were scored blindly on a scale of 0–4 by a pathologist using the NEC scoring system previously published by Dvorak *et al.* (40) and defined in our previous study (23), with a score of 2 or higher being classified as experimental NEC (**Table 3**). Small intestine (jejunum and ileum) and colon were scored for each pup and the maximum score of either part was used for the summarized score. Intermediate scores of X.5 were also used to more precisely assign the degree of intestinal tissue damage.

Following NEC induction, intestinal sections were isolated and placed for 24 h in 10% (vol/vol) neutral buffered formalin. Tissues were fixed, embedded in paraffin, and then cut 6-µm thick. After deparaffinization, sections were stained with H&E.

Isolation of Intestinal Cells

Single-cell suspensions from small intestines were yielded using the Lamina Propria Isolation kit from Miltenyi Biotec (Auburn, CA) as previously described (23). Briefly, after pups were euthanized, small intestines were removed *in toto*, cleaned with phosphate-buffered saline, exposed longitudinally, and cut laterally into 0.5-cm pieces. Next, pieces were placed in tubes containing predigestion solution (Hank's Balanced Salt Solution (HBSS) (without Ca^{2+} and Mg^{2+}), 5 mmol/l ethylenediaminetetraacetic acid (EDTA), 5% fetal bovine serum, and 1 mmol/l DTT) and incubated three times at 37 °C for 20 min with fresh predigestion solution. Between incubations, epithelial cells and intraepithelial lymphocytes were removed from lamina propria by vortexing and filtering through a 100-µm strainer. Following predigestion, a solution of HBSS (with Ca^{2+} and Mg^{2+}), 5% fetal bovine serum, and a proprietary digestion solution (Miltenyi Biotec) was added to lamina propria cells and incubated for 30 min at 37 °C. Cells were further dissociated in MACS buffer using the gentleMACS Dissociator (Miltenyi Biotec), filtered (70-µm strainer), and washed with MACS buffer. The resultant single-cell lamina propria suspensions were stained with the appropriate antibodies for flow cytometry.

Flow Cytometry

The following antibodies were purchased from eBioscience (San Diego, CA): fixable viability dye eFluor 450 and eFluor 506, anti-CD45 PE-Cy7, anti-CD45 PerCP-Cy5.5, anti-CD3e AlexaFluor 700, anti-CD4 fluorescein isothiocyanate (FITC), anti-CD4 eFluor 450, anti-CD25 PE, anti-CD8α PerCP-Cy5.5, anti CD8α PE-Cy7, anti-FOXP3 APC, anti-CD11c PE-Cy5.5, anti-MHCII APC, anti-DEC205 PE, anti-F4/80 PE-eFluor 610, and anti-Gr1 eFluor 450. Anti-HO-1 FITC was purchased from Enzo Life Sciences (Farmingdale, NY). Single-cell lamina propria suspensions were incubated with fixable viable dye in azide-free phosphate-buffered saline for 30 min at 2–8 °C, followed by Fc blocking with anti-CD16/CD32 and antibody staining in MACS buffer. After fixation and several washes with permeabilization buffer, cells were incubated with FOXP3 and HO-1 antibodies. Flow cytometry was performed with a LSRII.2 (BD Bioscience, San Jose, CA) and results analyzed using FlowJo (Tree Star, Ashland, OR). Debris and doublets were excluded by sequential gating on forward scatter height vs. forward scatter area.

T-Cell Identification and Phenotyping

7-color flow cytometric analysis was used to phenotype T-cell populations. For Treg identification, we gated as followed: live cells, $CD45^+$ cells, $CD3^+$ cells, and $CD4^+$ cells. We identified the $CD25^+FOXP3^+$ Tregs, also including $CD4^+CD8^+FOXP3$-expressing Tregs without further identification of their anatomical origin (**Supplementary Figure S1** online). Thus, we did not distinguish between thymus- and peripherally derived Tregs. $CD4^+CD8^-$ and $CD4^+CD8^+$ gates were used to calculate Treg/CD4 ratios.

Adoptive Transfer of Thymus-Derived Tregs

Spleens and thymi from 6-d-old WT pups were pooled and single cell suspensions were obtained using gentleMACS Dissociator. After filtration through a 40-µm strainer, cells were stained with viability dye, followed by anti-CD45 PE-Cy7, anti-CD3e AlexaFluor 700, anti-CD4 eFluor 450, anti-CD45RB FITC, and anti-CD25 PE for 30 min at 2–8 °C. To ensure proper gating, an aliquot of the single cell suspension was fixed and stained with FOXP3. $CD4^+CD25^+CD45RB^{lo}$ cells were sorted using an Aria II (BD Biosciences). On average, 5×10^4 WT Tregs were sorted with purity between 81–98% (**Supplementary Figure 4b,c** online) from 8 to 10 pooled spleens and thymi, respectively. Tregs from spleens and thymi were separately suspended in sterile 0.9% saline and 50 µl containing 5×10^4 WT Tregs were injected intraperitoneally into 6-d-old HO-1 Het pups.

PCR Array Innate and Adaptive Immune Responses

Freshly harvested small intestines were placed in RNAlater (Qiagen) and stored at 80 °C. Total RNA was extracted using the RNeasy Mini Kit (Qiagen). RT2 First Strand Kit (Qiagen) was used to synthesize cDNA. Real-time PCR was performed with RT2 Real-Time SYBR Green/ROX PCR Master Mix (Qiagen) on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Three housekeeping genes glyceraldehyde 3-phosphate dehydrogenase, glucuronidase, and heat-shock protein 90 were used for normalization. Fold changes in gene expressions in NEC-induced HO-1 Het over NEC-induced WT small intestines were calculated using the web-based PCR Array data analysis software (Qiagen).

Statistical Analyses

Statistical comparisons of median NEC scores were performed using Mann–Whitney test. χ^2 test was used to assess differences in NEC incidences. All other comparisons were analyzed using Student's unpaired *t*-tests or two-way ANOVA. Data are expressed as medians and/or mean ± SD. Comparisons were considered significant when $P \leq 0.05$.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/pr

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