Decreased development of necrotizing enterocolitis in IL-18-deficient mice

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Halpern MD, Khailova L, Molla-Hosseini D, Arganbright K, Reynolds C, Yajima M, Hoshiba J, Dvorak B. Decreased development of necrotizing enterocolitis in IL-18-deficient mice. Am J Physiol Gastrointest Liver Physiol 294: G20–G26, 2008.—Necrotizing enterocolitis (NEC) is a devastating gastrointestinal disease predominantly of prematurely born infants, characterized in its severest form by extensive hemorrhagic inflammatory necrosis of the distal ileum and proximal colon. Proinflammatory cytokines have been implicated in the development of NEC, and we have previously shown that IL-18 is significantly elevated in the well-established neonatal rat model of NEC. To determine whether IL-18 contributes to intestinal pathology in NEC, we subjected IL-18 knockout mice to the protocol used to develop experimental NEC in newborn rats. Newborn B6.129P2-Ile18tm1Aki/J (NEC IL-18−/−) and wild-type (NEC WT) mice were hand fed every 3 h with cow’s milk-based formula and exposed to asphyxia and cold stress twice daily. After 72 h, animals were killed and distal ileum and liver were removed. Disease development was determined via histological changes in the ileum as scored by a blinded evaluator. The number of TNF-α-, IL-12-, and IL-1β-positive cells and macrophages were determined in both ileum and liver via immunohistology. IκB-α and IκB-β were determined from protein extracts from both ileum and liver using Western blot analysis. The incidence and severity of NEC was significantly reduced in NEC IL-18−/− mice compared with NEC WT. Furthermore, mean ileal macrophages and hepatic IL-1β were significantly reduced in NEC IL-18−/− mice subjected to the NEC protocol. There were no statistically significant changes in Kupffer cells, hepatic TNF-α, ileal IL-1β, or IL-12. IκB-α and IκB-β were significantly increased in NEC IL-18−/− mice ileum and liver, respectively. These results confirm that IL-18 plays a crucial role in experimental NEC pathogenesis.

interleukin-18; inflammation

NECROTIZING ENTEROCOLITIS (NEC) is a devastating gastrointestinal disease, predominantly of prematurely born infants, characterized in its severest from by extensive hemorrhagic inflammatory necrosis of the distal ileum and proximal colon. NEC affects thousands of newborns in the U.S. every year, with death occurring in 10–50% of affected individuals (27, 32). Survivors of a severe occurrence frequently suffer the effects of short bowel syndrome, resulting in prolonged medical expenses and chronic gastrointestinal difficulties (24, 25). As more premature infants are born in this country each year (22), the effects of this disease are likely to contribute to greater morbidity and mortality. The pathophysiology of this disease remains poorly understood; however, prematurity, enteral feeding, intestinal hypoxia-ischemia, and bacterial colonization are considered major risk factors (4, 20, 39). A better understanding of the mechanisms involved in the development of NEC and elucidation of early pathological changes in tissues at the molecular level will help to improve therapy and could lead to prevention of this disease.

The major risk factors for NEC likely promote an inflammatory cascade that results in the pathology associated with this disease. Of the many cytokines that play important roles in inflammation, proinflammatory IL-18 has been implicated in inflammatory diseases of the small intestine (23, 30, 34, 43). IL-18 is capable of inducing interferon-γ (IFN-γ) alone, and together with IL-12, it can work synergistically to induce greater quantities of IFN-γ. IL-18 is also capable of promoting an inflammatory cascade by enhancing the release of proinflammatory TNF-α. TNF-α, in turn, can stimulate production of IL-12. IL-18, IL-12, and TNF-α can also activate NF-κB, which initiates transcription of a variety of proinflammatory genes.

We have previously shown that IL-18 is significantly increased in the ileum (16) and liver (15) of neonatal rats with NEC. Furthermore, there is a positive correlation between production of IL-18 and the progression of ileal damage during disease development (16). To determine whether IL-18 contributes to intestinal pathology in experimental NEC, we subjected IL-18−/− mice to the protocol currently utilized in our laboratory and others to develop NEC in neonatal rats: enteral feeding of newborn, never-suckled pups with cow’s milk-based formula coupled with twice daily exposure to asphyxia and cold stress (9, 12, 13, 15–17). We evaluated the incidence and severity of disease as well as specific parameters known to be altered in experimental NEC: proinflammatory cytokine production, infiltration of macrophages, and IκB-α and IκB-β in the ileum and liver.

METHODS

Animal model. This protocol was approved by the Animal Care and Use Committee of the University of Arizona (A-324801-95081). Neonatal B6.129P2-Ile18tm1Aki/J (IL-18−/−) and C57BL/6J wild-type (WT) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Homozygous breeder pairs were utilized; IL-18−/− pups originating from 12 separate litters and WT mice originating from 10 separate litters were utilized in 3 different experiments. Newborn IL-18−/− (NEC IL-18−/−) and WT (NEC WT) mice were collected
immediately after birth to prevent suckling of maternal milk. All pups were hand fed 50 μl of rat milk substitute every 3 h for 3 days using the Hoshiba nipple, Yajima style (19, 44) developed and manufactured by Meiji Dairies (Tokyo, Japan). With the use of this device (Fig. 1), newborn mouse pups could be fed measured amounts of diet without potential physical damage inherent in the gavage method typically utilized in the much larger neonatal rat. All pups were stressed twice daily with asphyxia (breathing 100% nitrogen gas for 60 s) followed by cold (4°C for 5 min). Three-day-old dam-fed wild-type (DF WT; n = 10) and dam-fed IL-18−/− (DF IL-18−/−; n = 15) pups were used for comparison in all evaluations.

**NEC evaluation.** Pathological changes in intestinal architecture were evaluated using the NEC scoring system developed for use in neonatal rats. From each animal, 0.5-cm sections of tissue from the distal ileum and proximal and proximal colon were fixed overnight in 70% ethanol, paraffin embedded, microtome sectioned at 4–6 μm, and stained with hematoxylin and eosin for histological evaluation. Histological changes in the ileum were scored by a blinded evaluator on a scale of 0 (normal) to 4 (necrosis), using our previously published ileal damage scoring scale in neonatal rats (12–17).

**Immunohistology.** Ethanol-fixed, paraffin-embedded liver and ileum were serially sectioned at 4–6 μm. After deparaffinizing, sections were blocked with 1.5% appropriate serum (Vector Laboratories, Burlingame, CA) and then incubated with anti-mouse TNF-α (R&D Systems, Minneapolis, MN), anti-mouse macrophage (clone BM8; Biosource), or anti-mouse IL-1β, followed by biotinylated secondary antibody (Vector Laboratories), Vectastain Elite ABC reagent (Vector Laboratories), and diaminobenzidine (DAB), and counterstained with hematoxylin. No immunostaining was observed in the immunostaining controls. Sections from both experimental groups were stained for a specific primary antibody at the same time so that comparisons between groups could be assessed. Stained slides were evaluated by a blinded observer, and enumeration of positively stained cells was accomplished by counting ten ×40 microscopic fields.

**Western blot analysis.** Individual frozen ileum or liver samples were homogenized with a hand-held homogenizer (Pellet Pestle; Kimble/Kontes, Vineland, NJ) in a 5× volume of ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Na-deoxycholic acid, 1% Triton X-100, 50 mM DTT, 50 μg/ml aprotinin, 50 μg/ml leupeptin, and 5 mM PMSF). The homogenates were centrifuged at 10,000 rpm for 5 min at 4°C, and the supernatant was collected. Total protein concentration was quantified using the Bradford protein assay. For protein analysis, 50 g of protein were added to an equal volume of 2× Laemmli sample...
buffer and boiled for 5 min. The samples were run on a 10–20% gradient polyacrylamide gel (Bio-Rad, Hercules, CA) at 95 V for 1 h. Protein was transferred to Immuno-Blot polyvinylidene difluoride membranes (Bio-Rad) at 15 V for 1 h. Membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.1% Tween 20 (Sigma, St. Louis, MO) for 1 h at room temperature and then incubated with anti-IkB-α or IkB-β antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. After being washed, the membranes were incubated for 1 h at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody. Proteins were visualized with a chemiluminescence system (Pierce, Rockford, IL) and exposed to X-ray film. Membranes were stripped and probed with anti-β-actin to determine equal loading of protein. Densitometry was performed to compare protein expression between groups with Bio-Rad QuantityOne software. IkB-α and IkB-β optical density (OD) levels from each sample were normalized to the OD of the corresponding β-actin sample.

Statistics. Statistical analyses between groups were performed using ANOVA followed by Fisher’s protected least significant difference. Analysis of NEC score was accomplished using the Mann-Whitney test for nonparametric values, and the χ2 test was used to analyze differences in incidence of disease. All statistical analyses were determined using the statistical program StatView (Abacus Concepts, Berkeley, CA). All numerical data are expressed as means ± SE.

RESULTS

Incidence and severity of NEC in IL-18−/− mice. In Fig. 2, normal histology from 3-day-old DF WT (A) and typical ileal damage in 3-day-old formula-fed, asphyxia/cold-stressed NEC WT mice (B–D) is shown. For NEC IL-18−/− mice, the median histological ileal damage score was 1.5 compared with 2.5 for WT NEC WT mice (P < 0.05). Representative histology for both groups is shown in Fig. 3. Any animal with a histological damage score of 2 or greater was considered to have developed disease. We found that 4/34 (11.76%) NEC IL-18−/− mice developed NEC compared with 19/31 (61.29%) NEC WT mice (Fig. 4). The incidence of NEC in both DF WT and DF IL-18−/− groups was 0%; 0/10 and 0/15, respectively (data not shown).

Ileal inflammatory cytokines. To determine how the absence of IL-18 affects ileal inflammation during NEC, we stained ileal samples from each animal with antibodies directed against proinflammatory cytokines IL-1β and IL-12. There were no statistically significant differences in the number of ileal IL-1β- or IL-12-positive cells between NEC IL-18−/− and NEC WT or DF WT and DF IL-18−/− groups (data not shown). In addition, ileal macrophages were counted from ten ×40 microscopic fields from each animal after immunohistologically staining for the mouse macrophage marker BM8. Mean ileal macrophages were significantly decreased in NEC IL-18−/− mice (Table 1, Fig. 5) to numbers comparable to those in both DF WT and DF IL-18−/− mice.

Hepatic inflammatory markers. We have previously shown that hepatic inflammation is increased during experimental NEC in rats. To determine whether hepatic inflammatory mediators are altered in NEC IL-18−/− mice, we counted the number of Kupffer cells and IL-1β- and TNF-α-positive cells in immunohistologically stained liver tissue from each animal. There was no change in Kupffer cells or TNF-α-positive cells between groups, however, hepatic IL-1β-positive cells were significantly decreased in NEC IL-18−/− mice compared with NEC WT, with numbers similar to those in both the DF WT and DF IL-18−/− groups (Table 1, Fig. 6).

NF-κB in IL-18−/− mice subjected to the NEC protocol. Activation of NF-κB is a hallmark of the inflammatory cascade and has been implicated in NEC pathogenesis. Because of the

Table 1. Ileal macrophages and hepatic IL-1β

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean Ileal Macrophages</th>
<th>Mean Hepatic IL-1β</th>
</tr>
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<tbody>
<tr>
<td>NEC WT</td>
<td>30</td>
<td>28.9 ± 3.7*</td>
<td>32.3 ± 3.3*</td>
</tr>
<tr>
<td>NEC IL-18−/−</td>
<td>34</td>
<td>12.2 ± 2.3</td>
<td>10.5 ± 3.0</td>
</tr>
<tr>
<td>DF WT</td>
<td>10</td>
<td>15.9 ± 3.5</td>
<td>9.8 ± 4.1</td>
</tr>
<tr>
<td>DF IL-18−/−</td>
<td>10</td>
<td>13.2 ± 4.6</td>
<td>8.1 ± 2.5</td>
</tr>
</tbody>
</table>

NEC WT, wild-type mice subjected to necrotizing enterocolitis (NEC) by formula feeding and cold stress; NEC IL-18−/−, IL-18 knockout mice subjected to NEC protocol; DF WT, dam-fed wild-type mice; DF IL-18−/−, dam-fed IL-18 knockout mice. *P < 0.01 vs. NEC IL-18−/−, DF WT, and DF IL-18−/− groups.
limited amount of ileal tissue obtainable from 3-day-old mouse pups, we did not have enough tissue to determine NF-κB activation using nuclear extracts. NF-κB is held inactive in the cell cytoplasm by the inhibitor protein IκB. Stimuli such as TNF-α or IL-18 induce selective IκB phosphorylation, which is then ubiquitinated and degraded. Activated NF-κB is then free to migrate to the nucleus (7, 26, 38). As activated NF-κB levels increase in the nucleus, cytoplasmic IκB-α and IκB-β levels should decrease (11, 33, 40). Therefore, we evaluated ileal and hepatic IκB-α and IκB-β levels using Western blot analysis. Ileal IκB-α levels were significantly lower in NEC WT mice compared with NEC IL-18−/−, DF WT, and DF IL-18−/− mice (Fig. 7), indicating greater activation of NF-κB in the NEC WT group. Liver IκB-α levels were similar in all groups (Fig. 8). Ileal IκB-β levels were significantly lower in NEC WT mice compared with NEC IL-18−/−, DF WT, and DF IL-18−/− mice. However, IκB-β levels were extremely variable in the NEC WT group; thus statistically significant differences were not achieved (Fig. 9). Hepatic IκB-β levels were significantly lower in NEC IL-18−/− mice compared with the other groups (Fig. 10).

DISCUSSION

Our previous studies suggested that IL-18 plays an important role in the pathogenesis of experimental NEC (13, 16). In the present studies, we have shown that IL-18 is a crucial component in the development of NEC, since IL-18 knockout mice have significantly lower incidence and severity of NEC than do WT mice, which are able to produce IL-18.

Our previous studies were conducted using the well-characterized neonatal rat model of NEC. In this model, NEC is developed in newborn rats by feeding milk-based formula coupled with exposure to hypoxia and cold stress. The major advantage of the neonatal rat NEC model is that many clinical and pathological changes are similar to those found in humans; the abdomen is distended, blood is detected in stool, and the ileum and proximal colon are the most affected parts of the intestine. In addition, the major risk factors for human NEC (intestinal immaturity, enteral formula feeding, and hypoxia/ischemia) are essential factors to develop disease in the rat model (4–6).

The main disadvantage of this model is the lack of knockout or genetically modified rat strains with which to investigate mechanisms of disease development and pathology. Currently, mechanistic evaluations in the rat model require inhibition of potential disease contributors with specific antibodies or specific chemical inhibitors. The paucity of congenic, knockout, or spontaneous mutant rat strains has severely hampered research into this disease. Thus the ability to utilize a neonatal mouse model of NEC increases the ability to investigate disease pathogenesis and the molecular mechanisms underlying these processes. By developing a mouse model of NEC that encompasses the major risk factors for human disease and applying this model to investigate the role of IL-18 in disease pathology, we have been able to show that IL-18 is critical for disease development.

Inflammation requires a cascade of events and the upregulation and activation of a number of proinflammatory media-
tors. Thus some disease pathology would be expected even in the absence of a specific proinflammatory mediator such as IL-18. Although disease development was not completely halted in NEC IL-18−/− mice, our finding that the incidence of NEC was decreased from 61 to 12% in the NEC IL-18−/− group offers proof that IL-18 is critical for disease pathology.

The complexities of inflammation are also illustrated in this model, given that some proinflammatory cytokines were decreased in NEC IL-18−/− mice while others were unchanged. The neonatal rodent intestine is immunologically immature. In fact, the majority of IL-18 in rat (16) and mouse (unpublished data) models of NEC is produced by intestinal epithelial cells rather than immune cells. We found essentially no TNF-α, IFN-γ, or IL-1β produced in the ileum during experimental NEC treatment but high levels of hepatic IL-1β and TNF-α (15). Thus the lack of decreased IL-1β and TNF-α in the ileum of NEC IL-18−/− mice is likely a reflection of the small quantities of these cytokines found in the ileum during disease development rather than a direct consequence of the lack of IL-18.

The parameters that are decreased in NEC IL-18−/− mice (hepatic IL-1β and ileal macrophages) are parameters that are increased during experimental NEC. The only exception is hepatic TNF-α, which is increased in NEC but unchanged in IL-18−/− mice subjected to the NEC protocol. Although the

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**Fig. 7.** A: representative Western blot for ileal IκB-α from NEC WT, NEC IL-18−/−, DF WT, and DF IL-18−/− mice. Ileal tissues from 3-day-old mice were probed using anti-IκB-α. B: mean optical density (OD) for ileal IκB-α taken from NEC WT (n = 10), NEC IL-18−/− (n = 10), DF WT (n = 10), and DF IL-18−/− (n = 10) mice. *P ≤ 0.05 vs. NEC IL-18−/−, DF WT, and DF IL-18−/−.

**Fig. 8.** A: representative Western blot for hepatic IκB-α levels from NEC WT, NEC IL-18−/−, DF WT, and DF IL-18−/− mice. Livers from 3-day-old mice were probed using anti-IκB-α. B: mean OD for hepatic IκB-α taken from NEC WT (n = 10), NEC IL-18−/− (n = 10), DF WT (n = 10), and DF IL-18−/− (n = 10) mice.

**Fig. 9.** A: representative Western blot for ileal IκB-β from NEC WT, NEC IL-18−/−, DF WT, and DF IL-18−/− mice. Ileal tissues from 3-day-old mice were probed using anti-IκB-β. B: mean OD for ileal IκB-β taken from NEC WT (n = 10), NEC IL-18−/− (n = 10), DF WT (n = 10), and DF IL-18−/− (n = 10) mice.

**Fig. 10.** A: representative Western blot for hepatic IκB-β levels from NEC WT, NEC IL-18−/−, DF WT, and DF IL-18−/− mice. Livers from 3-day-old mice were probed using anti-IκB-β. B: mean OD for hepatic IκB-β taken from NEC WT (n = 10), NEC IL-18−/− (n = 10), DF WT (n = 10), and DF IL-18−/− (n = 10) mice. *P ≤ 0.05 vs. NEC IL-18−/−, DF WT, and DF IL-18−/−.
reasons for this observation are unclear, it is not without prior precedent, since Sakao et al. (37) found elevated TNF-α levels in IL-18−/− mice after LPS challenge. They hypothesized that during sepsis, IL-18 can actually downregulate TNF-α. It is possible, therefore, that in the NEC IL-18−/− group, the lack of IL-18 prevents downregulation of TNF-α, resulting in similar levels of hepatic TNF-α in all groups.

NF-κB has been shown to play a role in intestinal inflammation in a number of models of colitis and intestinal epithelial cell lines (21, 33, 35, 41, 42). The importance of NF-κB is underscored by the NF-κB-induced expression of many cytokines, enzymes, and adhesion molecules potentially involved in the pathogenesis of NEC. NF-κB regulates transcription of and can be activated by a number of inflammatory mediators including TNF-α, IL-18, and IL-12 (8, 18, 29), which we have shown to increase during NEC (13, 15, 16). In addition, NOS2, a proinflammatory target gene of NF-κB, is increased in intestinal inflammation (28, 36) and NEC (10, 31) and has been implicated in the pathogenesis of gut-barrier failure (1–3). Therefore, a crucial regulatory role of NF-κB in the pathogenesis of NEC is likely. With the use of IκB-α and IκB-β as indirect identifiers, our data suggest that there is a significant decrease in NF-κB activation in IL-18−/− mice subjected to the NEC protocol. We speculate that hepatic TNF-α, which we have previously shown is the source of elevated TNF-α in the ileal luminal contents during NEC (12, 15), may be responsible for both the activated ileal and hepatic NF-κB.

In conclusion, we have shown that IL-18 is critical for the ileal pathology observed in experimental NEC. The results of these studies provide proof that IL-18 is crucial for disease development and suggest that neutralization of IL-18 could be utilized as a potential therapeutic approach to human NEC.

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