

# Unexpected Macrophage Activity Alters the Inflammatory Process in Necrotizing Enterocolitis

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## Abstract

**Objective:** Macrophages play a role in clearing bacteria from the gut in the initial stages of necrotizing enterocolitis (NEC). Macrophage Inflammatory Protein-1 $\alpha$  (MIP-1 $\alpha$ ), also known as CCL3, is a chemokine produced by macrophages that enhances the immune response. It acts as both a recruiter of immune cells and a coactivator of other macrophages. Interleukin-12 (IL-12) is a pro-inflammatory chemotactant produced by several types of phagocytic cells such as dendritic cells and neutrophils, but primarily by macrophages. We hypothesized that the pro-inflammatory state associated with NEC pathophysiology would induce increased expression of MIP-1 $\alpha$  and IL-12 that would be detectable in serum and intestinal tissue.

**Methods:** Timed pregnant Sprague-Dawley rats were randomized by litter. Controls were delivered vaginally and dam-fed. NEC pup groups were delivered 12 h prematurely *via* Cesarean section, formula fed, given a single oral dose of lipopolysaccharide, and subjected to intermittent cold and hypoxia as part of a proven NEC rat model protocol. Animals were sacrificed at 0, 12, 24, 48, 72, and 96 h of life and serum and intestinal tissue samples were collected. Samples were analysed *via* western blot using antibodies with affinity to MIP-1 $\alpha$  and IL-12 respectively.

**Results:** Serum and ileal levels of MIP-1 alpha were increased from 48 to 72 h in NEC animals when compared with controls. Serum and ileal IL-12 was downregulated in NEC groups at 12 and 24 h compared to controls.

**Conclusion:** Macrophage function plays an important role in the first 48 h of NEC pathophysiology. Downregulation of IL-12 in the setting of increased MIP-1 $\alpha$  expression may represent deranged or inhibited macrophage function in the context of NEC pathogenesis. Further work to elucidate the significance of these findings is warranted.

**Keywords:** Necrotizing enterocolitis; Macrophage; Cytokine; Chemokine; MIP-1 alpha; CCL3; IL-12

## Introduction

Necrotizing enterocolitis (NEC) is a devastating gastrointestinal illness primarily affecting premature infants. NEC is characterized by epithelial destruction leading to gut barrier failure, and requires surgery in 20-40% of those affected. Of infants requiring operative intervention, 20-50% will die from their disease [1-3]. Despite years of research into the cause, prevention, and treatment of NEC, the mortality rate has remained the same over the last three decades, and no definitive cause has been found [4,5].

Macrophages have been shown to play an important role in the clearance of bacteria in the initial stages of NEC [6]. Activation of macrophages is characterized by the release of many intercellular mediators including cytokines, growth factors, and chemokines. Macrophage inflammatory protein 1-alpha (MIP-1 $\alpha$ ) (also known as CCL3) is a chemokine that is upregulated in macrophages in response to stressors such as exposure to bacterial lipopolysaccharide (LPS) [7,8].

Interleukin-12 (IL-12) is a pro-inflammatory cytokine produced by macrophages, dendritic cells, and B cells, and works to activate macrophages and recruit phagocytic cells to sites of infection. Mice that are deficient in IL-12 or its receptor are more susceptible to bacterial infections than their normal counterparts [9-11].

We hypothesized that the pro-inflammatory state associated with NEC would lead to an up-regulation of both MIP-1 $\alpha$  and IL-12.

## Materials and Methods

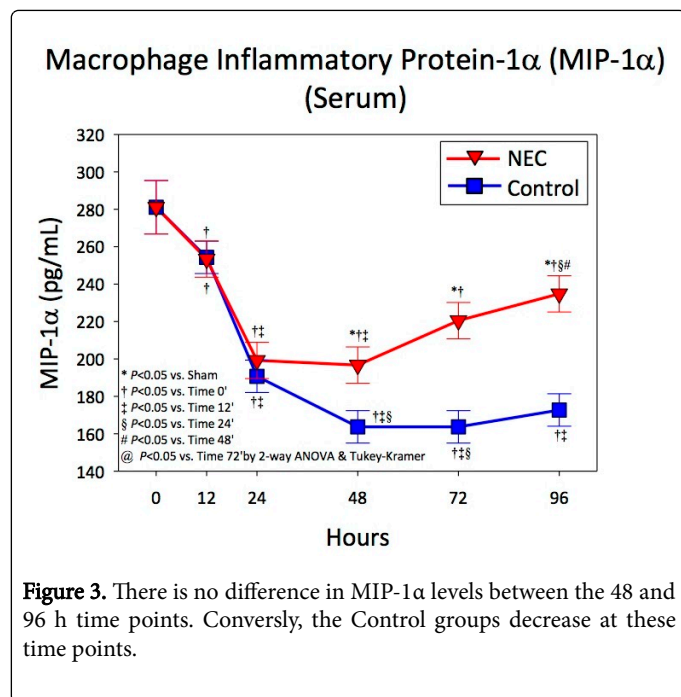
Approval for the study was granted by the Institutional Animal Care and Use Committee (IACUC), the Biohazard Safety Committee, and the Research and Development Committee of the Robley Rex Veterans Affairs Hospital in Louisville, KY. Timed pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN) were housed for one week prior to initiation of experimental protocol, and allowed water and rat chow ad lib.

Dams were then randomized to the Control or NEC group and their pups were assigned accordingly after birth. Control dams delivered their pups vaginally. Control pups were kept with their mothers and allowed to nurse on demand until the time of sacrifice for tissue and

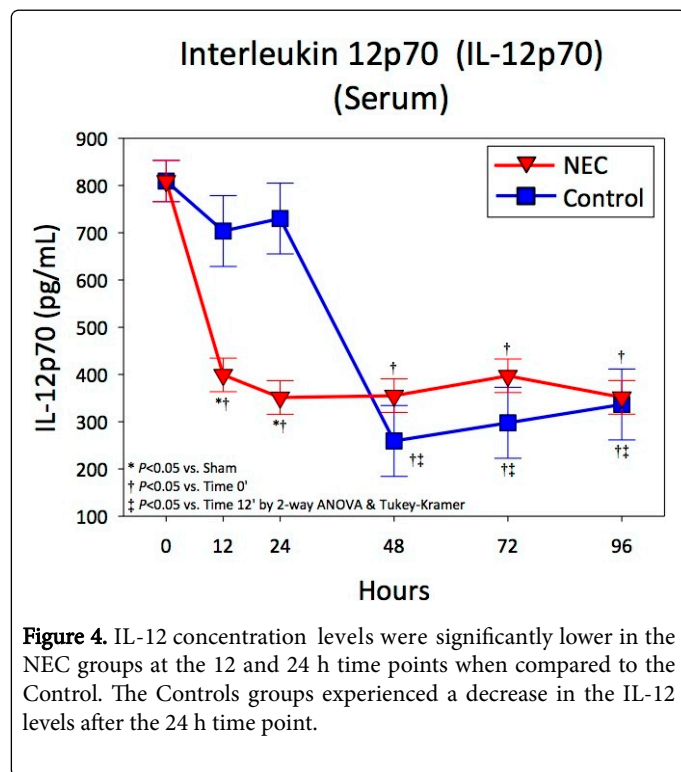


both groups. After 24 h, the Control animals continued to experience a decrease in MIP-1 $\alpha$ , whereas the NEC animals showed elevated levels.

After an initial drop, Interleukin-12 levels were steady in NEC animals throughout the NEC protocol (Figure 4). Levels of IL-12 were significantly lower in NEC animals when compared to controls at 12 and 24 h. Following this, the Control animals experienced a drop in IL-12 while the NEC animals remained unchanged.

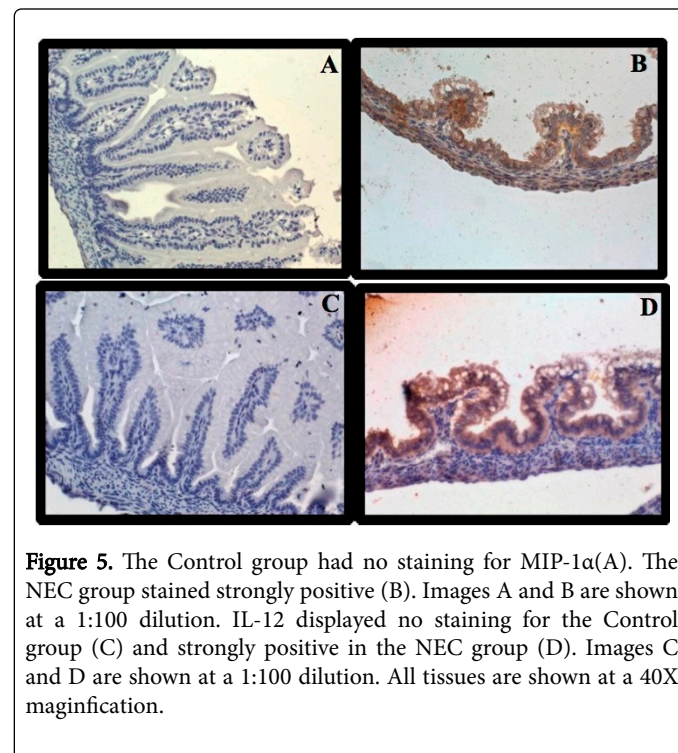


**Figure 3.** There is no difference in MIP-1 $\alpha$  levels between the 48 and 96 h time points. Conversely, the Control groups decrease at these time points.



**Figure 4.** IL-12 concentration levels were significantly lower in the NEC groups at the 12 and 24 h time points when compared to the Control. The Controls groups experienced a decrease in the IL-12 levels after the 24 h time point.

Immunohistochemistry of Control and NEC groups at the 48 h time point revealed similar results to serum levels at the same time point. The Control group (Figure 5A) showed no significant staining for MIP-1 $\alpha$  while the NEC group stained strongly positive for MIP-1 $\alpha$  (Figure 5B). Similarly for IL-12 the Control group displayed no significant staining (Figure 5C). The NEC group at the same time point stained strongly positive for IL-12 (Figure 5D).



**Figure 5.** The Control group had no staining for MIP-1 $\alpha$ (A). The NEC group stained strongly positive (B). Images A and B are shown at a 1:100 dilution. IL-12 displayed no staining for the Control group (C) and strongly positive in the NEC group (D). Images C and D are shown at a 1:100 dilution. All tissues are shown at a 40X magnification.

## Discussion

The exact pathophysiology of NEC continues to be unclear despite years of research into the cause. Many risk factors have been identified as contributing to the pathogenesis of NEC, including prematurity, enteral feeding, hypothermia, bacterial colonization, and hypoxia [16-23]. Many believe NEC to be secondary to a deranged or uncontrolled inflammatory response. Therefore, researchers have sought to elucidate the cascade of inflammatory cytokines found in NEC [24]. As a part of the innate immune system, the macrophage plays an important role in the recognition and clearance of bacteria from sites of infection. Present in tissues, macrophages are one of several cell types known as antigen presenting cells (APCs), capable of recognizing and engulfing foreign antigens for presentation to other immune cells to stimulate the clearance of infection.

Macrophages not only act as APCs and phagocytes, however. They also secrete cytokines, growth factors, and chemokines (cytokines that are chemoattractants) to recruit additional monocytes (macrophage precursors), neutrophils, and dendritic cells to the site of inflammation. Prior work by Emami et al. demonstrated the importance of macrophages in NEC caused by infection with the bacteria *Cronobacter sakazakii* [6]. Animals subjected to the bacteria that were depleted of macrophages and polymorphonuclear leukocytes (PMNs) were found to have higher intestinal bacterial load, higher pathologic scores for intestinal damage, and earlier mortality than those with intact macrophages and PMNs.

Macrophage inflammatory proteins-1 $\alpha$ , 1 $\beta$ , and 2 (MIPs) are heparin binding proteins with inflammatory and immunoregulatory activity [8]. The MIPs were initially characterized as proteins secreted from endotoxin-stimulated mouse macrophages, but further research demonstrated that neutrophils, fibroblasts, epithelial cells, and T cells are also MIP-secreting cells. The three MIPs have distinct characteristics, but share a few overlapping roles. All MIPs act primarily in a pro-inflammatory nature and are chemotactic, acting to recruit and activate other cells at the sites of inflammation or injury. MIP-1 $\alpha$  acts with a broader specificity than MIP-1 $\beta$  in that it attracts B cells, cytotoxic T cells, and CD4+ T cells, whereas MIP-1 $\beta$  works preferentially on CD4+ T cells [25]. The macrophage is thought to be the primary source for MIPs, but Sherry et al. demonstrated that T cells are either a major source of MIPs or that they act to markedly upregulate the production of MIPs by other cell types [7]. In that study, levels of MIP-1 $\alpha$  were measured in mice *in vitro* after intraperitoneal injection of LPS, and were shown to peak at two hours, then fall to undetectable levels by eight hours.

In our study, there were similar amounts of MIP-1 $\alpha$  at the 12 and 24 h time points in both the NEC and Control groups, and that level was trending downward. By 48 h, we saw a significantly different increase in MIP-1 $\alpha$  in NEC animals compared to controls. This trend continued in the NEC animals and remained significant until 96 h-the end-point of our study. As one of the first responders to sites of infection and inflammation, it seems likely that the macrophage response to the induction of NEC would be nearly immediate, but we did not find that to be true. Pathological signs of NEC were first apparent at 24 h in NEC animals, but levels of MIP-1 $\alpha$  in those animals did not increase significantly until 48 h. Additionally, instead of peaking and then becoming undetectable, we show a sustained increase in MIP-1 $\alpha$ .

These findings are markedly different than those demonstrated in the prior work by Sherry et al. [7]. It is possible that the lag time between histopathological evidence of the disease compared to significantly elevated serum levels of MIP-1 $\alpha$  in NEC animals demonstrates a slow reaction of macrophages in this NEC model. A delay in macrophage activation might perpetuate further damage to the intestines, worsening NEC. The sustained increase in MIP-1 $\alpha$  in NEC animals demonstrates the continued inflammatory response as macrophages are attempting to fight off the intestinal inflammation.

IL-12, produced by macrophages, dendritic cells, and B cells, works synergistically with IL-18 to induce production of interferon-gamma (IFN- $\gamma$ ) from natural killer and B cells. Together, these pro-inflammatory factors can induce intestinal inflammation in mice [26]. Prior studies have demonstrated conflicting results regarding the production of IL-12 in rats undergoing a NEC-inducing protocol when compared to dam fed controls. While Nadler et al. showed decreased IL-12 mRNA expression in NEC animals [27], Halpern et al. showed an increase in IL-12 mRNA in NEC animals and a correlation between the number of IL-12 positive cells and the degree of tissue destruction [28]. The positive correlation between the number of IL-12 positive cells and the amount of tissue damage present led many to draw the conclusion that IL-12 is involved with the initial stages of NEC and may contribute to the intestinal necrosis associated with NEC [24].

Our findings (i.e., a relatively steady level of IL-12 in NEC animals compared to a marked elevation of IL-12 at 12 and 24 h in Control animals) suggest that macrophage function in NEC animals may be diminished. One might expect a reduced inflammatory reaction, but this is not confirmed histologically. There is clear progression of inflammation and necrosis of the intestine in the NEC animals despite

the decreased amount of IL-12. Therefore, although other studies have demonstrated the involvement of IL-12 in tissue inflammation in the intestine, our findings of low IL-12 with markedly inflamed intestine indicate that further investigation into the activity of IL-12 in NEC animals is warranted.

## Conclusion

Macrophages play a central role in the elimination of bacteria from the gut in the initial stages of NEC. Animals undergoing a NEC-inducing protocol experienced a delayed upregulation of MIP-1 $\alpha$  and no upregulation of IL-12. This delay in the upregulation of the macrophage-secreted pro-inflammatory cytokine MIP-1 $\alpha$  paired with a lack of response by IL-12 in NEC animals likely contributes to the pathophysiology of NEC. Enteral supplementation of IL-12, as we have previously done with Relaxin, may offer a mechanistic explanation of IL-12 and if this cytokine is contributing positively or negatively to the pathophysiology of NEC.

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