Immunohistochemical study of inflammatory responses in septa arising from type I intestinal atresia

Authors: Xuelai Liu, Peiyu Hao, Xianghui Xie, Yingchao Li, Suolin Li, Yianbiao Song, Cuizu Feng, Mao Ye, Yandong Wei, Peng Zhao, Long Li and Zhe-Wu Jin

DOI: 10.14670/HH-18-364
Article type: ORIGINAL ARTICLE
Accepted: 2021-07-27
Epub ahead of print: 2021-07-27
Immunohistochemical study of inflammatory responses in septa arising from type I intestinal atresia

Xuelai Liu¹, Peiyu Hao², Xianghui Xie¹, Yingchao Li³, Suolin Li³, Yianbiao Song⁴, Cuizu Feng¹, Mao Ye¹, Yandong Wei¹, Peng Zhao², Long Li¹*, Zhe-Wu Jin²*

¹ Department of Surgery, Capital Institute of Pediatrics affiliated Children Hospital, Beijing, 100020
² Department of Anatomy, Wuxi School of Medicine, Jiangnan University, WuXi, Jiangsu, 214122
³ Department of Pediatric Surgery, The Second Hospital of Hebei Medical University, Shijiazhuang city, 050000.
⁴ Department of Central Laboratory, The Second Hospital of Hebei Medical University, Shijiazhuang city, 050000.

ORCID: 0000-0003-0793-8379 (Xuelai Liu)

*Corresponding author: Prof. Long Li
Department of Surgery, Capital Institute of Pediatrics affiliated Children Hospital, No.2, Yabao Rd, Chaoyang district, Beijing, 100020 China
Tel: +86) 10-8569-5669; Fax: +86) 10-8562-8194
E-mail: lilong23@126.com

*Corresponding author: Prof. Zhe-Wu Jin
Department of Anatomy, Wuxi School of Medicine, Jiangnan University, WuXi, 214122 China
E-mail: zwjin@jiangnan.edu.cn
Summary

The purpose of this study was to evaluate defensive functional cells in intestinal septa during recanalization in the embryonic period, and to access immune responses in septa arising from type I intestinal atresia and normal intestinal walls. Tissue samples were of septa located in the intestinal wall at a distance < 15cm from the ligament of Treitz, and normal intestine walls obtained from seven neonates who underwent surgery. Following serial tissue sectioning, the samples were subjected to hematoxylin and eosin (HE), periodic acid-Schiff (PAS) and immunohistochemical staining to determine the morphological features and markers of functional cells and immune responses in the septa and normal intestinal walls. Quantitative analysis was conducted to compare differences between them. Compared with normal intestinal wall, the mucosal layer of septa arising from type I intestinal atresia had fewer misaligned villi and no classic epithelial crypts. Immunohistochemical staining showed that the mucosal layer of septa arising from type I intestinal atresia had fewer Paneth cells and goblet cells and lower amounts of lysozyme and MUC2, than normal intestinal walls. The concentration of pro-inflammatory cytokines, including interleukin (IL)-6 and tumor necrosis factor (TNF) –α, as well as macrophage inflammatory protein 3α (MIP-3α) and its receptor, CCR6, were higher in the mucosal layer of septa arising from type I intestinal atresia than in normal intestinal walls. Moreover, the numbers of mature dendritic cells and CD4+ T lymphocytes were higher in the mucosal layer of septa than in normal intestinal walls. The defensive activity of septa arising from type I intestinal atresia is weaker than that of normal
intestinal walls. This weaker activity may correlate with increases in mature dendritic cells and CD4+ T lymphocytes, as well overexpression of proinflammatory cytokines.

**Keywords**: Intestinal atresia, Septum; Inflammatory, MIP-3α, D4+ T lymphocytes
Introduction

Intestinal atresia (IA) is a congenital gastrointestinal malformation and a common cause of neonatal intestinal obstruction. In type I IA, the continuity of the intestinal duct is interrupted, with a septum in the intestinal lumen causing stenosis or atresia (Guzman et al., 2011). Both complete and windbag-like septa in type I IA have been regarded as extensions of intestinal wall tissue towards the lumen. Although the etiology for IA remains incompletely understood, it may be due to recanalization failure in the intestinal duct during the embryonic period. This theory postulates that, at gestation age (GA) 6 to 7 weeks, many vacuoles start to appear, expand and fuse in embryonic lumen occlusion. It gradually recanalizes to form the intestinal lumen with vacuoles disappearing at embryonic GA 8 to 12 weeks (Adams et al., 2014; Subbarayan et al., 2015; Langer et al., 2017). Depending on extent, the failure of recanalization leads to IA or intestinal stenosis (Morris et al., 2016; Batra et al., 2017). Because immune responses induce almost all developmental processes in fetuses, and over-expression of proinflammatory cytokines delays the formation of normal tissue, resulting in deformity (Subbarayan et al., 2015; Langer et al., 2017), failure of recanalization during the formation of IA may be caused by abnormal inflammatory responses. Abnormalities in immune defensive cells, including goblet cells (GCs) and Paneth cells (PCs), as well as a weakened barrier during intestinal development (Riba et al., 2017), may induce inflammatory responses, affecting recanalization during fetal development.

The present study assessed the morphological and Immunohistochemical
characteristics of the septa and normal intestinal walls of the same neonates with type I IA. Immune defense barriers were compared histologically in the septa and intestinal walls during development, as were differences in inflammatory responses.
Materials and methods

Materials

HE solution, Masson staining kits, phosphate buffered saline (PBS), 4% percent paraformaldehyde (PFA), ethanol, and sodium chloride (NaCl) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All reagents were dissolved in double-distilled water, and the solutions were adjusted to pH 7 and stored at room temperature. Tissue morphology was assessed using an SZX7 light microscope (Olympus, Japan) and an Eclipse E600 digital camera (Nikon, Japan).

Tissue specimens

The use of human neonatal intestinal tissue samples for scientific research was approved by the Medical Ethics Committee of Capital Institute of Pediatrics affiliated Children’s Hospital (Beijing, China) and Hebei Medical University Affiliated Second Hospital. The parents of all enrolled subjects provided written informed consent. Human intestinal septum tissues were obtained from seven neonates aged 1-3 days who underwent surgery (longitudinal incision and transverse suture) for IA at the Department of Surgery of the two centers between September 2017 and March 2019. All patients had type I IA based on intra-operative and histopathological analyses. Of these seven patients, five (four boys and one girl) had jejuna atresia, and two (one boy and one girl) had jejunoileal atresia. Patients did not receive any other treatment prior to the surgery. A small incision, about 2 cm in length, was made on the surface of the abdominal wall, the intestinal wall using a scalpel, and the septum was exposed and
its identity as a circular windbag-like septum confirmed. Small fragments of normal intestinal wall tissue, close to the septum were removed, and the septum was completely detached from the normal intestinal wall. Both the septum and normal intestinal wall tissue samples were fixed in 10% PFA for 24 h at room temperature. The circular windbag-like septum was confirmed during surgery in all patients in this study.

**H&E staining**

Tissue specimens were fixed in formalin, and embedded in paraffin, and cut into 4-µm-thick sections along the longitudinal axis of the intestinal tract to expose both surfaces and the intestinal wall and hole edges of each septum. These tissue sections were subsequently dewaxed and rehydrated for hematoxylin and eosin (H&E) staining. In brief, sections were stained with hematoxylin (Zhongshan Golden Bridge Biotechnology Co., Ltd.) for 10 min, differentiated in a hydrochloride acid-ethanol mixture, and counterstained with eosin (Zhongshan Golden Bridge Biotechnology Co., Ltd.) for 3 min. The sections were dehydrated in ethanol and cleaned in xylene before viewing with a light microscope (Olympus BX40; magnification).

**Immunohistochemistry**

For immunohistochemistry staining, tissue sections were deparaffinized, rehydrated, and incubated with 3% hydrogen peroxide/methanol to quench endogenous peroxidase. The samples were subsequently incubated at room
temperature with blocking solution containing 5% normal goat serum (Dako Bioresearch, USA) prior to antigen retrieval. The sections were incubated overnight in solution containing primary antibody (Table 1), rinsed in PBS, and incubated with HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Positive signals were detected by incubation with DAB (3, 3′-diaminobenzidine) and the samples were counterstained in hematoxylin solution and photographed under a microscope. The degree of expression of proinflammatory cytokines was assessed by a researcher who was blinded to the experimental groups.

**Periodic acid-Schiff (PAS) staining**

Periodic acid-Schiff (PAS) stain kits were purchased from Baso Diagnostics Inc. (Zhuhai, China). Briefly, paraffin-embedded sections were dewaxed in xylene, rehydrated in ethanol, and rinsed in distilled water. After oxidation in periodic acid for 15 minutes, each section was stained with Schiff reagent for 10 minutes, rinsed with distilled water for 5 minutes, and counterstained with hematoxylin for 2 minutes. The sections were subsequently washed, dehydrated, and sealed with a neutral resin for microscopic examination.

**Morphometric evaluation**

Immunohistochemical staining of anastomotic intestinal tissues was quantified by Image-Pro plus 6.0 digital software (Media Cybernetics) (Kwan et al., 2011; Zhang et al., 2014; Liu et al., 2017). The average optical density in the stained area was
determined in five random images at 400X magnification. The numeric data obtained from the image analysis were exported for statistical analysis. The ratio between the average optical density of tissue in each group and that in normal ileum was calculated.

Statistical analysis

Results were expressed as mean ± standard deviation (SD) and compared using Student's paired t tests of SPSS 22.0 (IBM, USA). A $p$ value $<0.05$ was considered statistically significant.
Results

*Epithelial morphology and goblet cell distribution in septa.*

Epithelial morphology and the distribution of GCs were compared in septa and normal intestinal samples. HE staining showed that villi in normal intestinal walls were abundant and arranged tightly and in an orderly manner, whereas the septa lacked these characteristics, having a thinner mucosal layer and a reduced number of intact epithelial glands. In contrast to the normal mucosal layer in intestinal walls, GCs in septa were isolated and the number reduced in mucosal epithelial gland walls and glandular cavities, with these cells lacking intact morphology (Figure 1). Because GCs contain basophilic granules and can be stained scarlet by PAS stain, the density and distribution of GCs were evaluated by PAS staining. GCs were detected in mucosal epithelial gland walls and glandular cavities, in intestinal walls. Compared with the mucosa layer in normal intestinal walls, the mucosa layer of septa contained fewer and more condensed basophilic granules (Figure 2). The number of GCs in five random fields was significantly lower in septa than in normal intestinal walls (Figure 2).

The expression of secretion-type mucoprotein-2 (MUC2) by GCs in the mucosal layer in septa and normal intestinal walls was compared immunohistochemically. MUC2 expression was significantly lower in the mucosal layer of septa than in normal intestinal walls (Figure 2), further confirming that the number of GCs and the expression of secretory glycoproteins were lower in the mucosal layer of septa than of normal intestinal walls.
Paneth cells and lysozyme expression in septa

Although PCs are essential immune cells that form defensive barriers in the intestine, their abnormalities can induce inflammatory responses. PCs start to develop in the duodenum of human fetuses at GA 14 weeks, and in the jejunum at GA 17-18 weeks. Intestinal walls contain significantly fewer PCs than other functional cells, although the number of PCs in intestinal walls gradually increases with development. These cells, however, cannot be detected by HE or immunohistochemical staining (Riba et al., 2017). The location and distribution of PCs suggested that these areas should be targeted in normal intestinal wall tissue and septa collected from neonates 1-3 days after birth. Immunohistochemical staining with antibody to lysozyme showed that isolated PCs were present in the normal mucosa layer, with lysozyme being expressed in the mucosal epithelial gland walls and glandular cavities in the intestinal wall. In contrast, the basement of the glands beneath the mucosal layer in septa did not contain typical epithelial crypts and had fewer PCs with normal or intact morphology, as well as reduced lysozyme expression (Figure 3). Morphometric evaluation showed that the expression of lysozyme was significantly lower in septal than in normal epithelium (Figure 3).

Expression of pro-inflammatory cytokines in septa

Because PCs and GCs are important immune cells, constituting a defensive barrier during intestinal development and after birth, we next assessed whether the
presence of fewer PCs and GCs in septal mucosa than in normal intestinal walls would weaken this biological barrier and local defense function, resulting in upregulated expression of proinflammatory cytokines. Immunohistochemical staining showed that the expression of IL-6 in the mucosal and submucosal/muscular layers was significantly higher in septa than in normal neonatal intestinal walls (Figure 4). In addition, the expression of TNF-α was significantly higher in septa than in normal intestinal walls (Figure 5).

**Expression of the dendritic cells receptor CCR6**

Because developing fetuses swallow sterilized amniotic fluid *in utero*, their intestinal villi and glands are in a relatively sterilized microenvironment during intestinal recanalization. We therefore assessed whether the up-regulated expression of pro-inflammatory cytokines in septa was induced by chemokine-mediated dendritic cells (DCs), which are essential antigen presenting cells in the intestine during embryonic development. Because CC-chemokine receptor 6 (CCR6) is the only receptor for chemokine-mediated DCs and macrophage inflammatory protein 3α (MIP-3α) is the only CCR6 ligand identified to date, we investigated the expression of MIP-3α and CCR6 in the mucosal layers of septa and normal intestinal walls. Immunohistochemistry showed that the levels of expression of MIP-3α and CCR6 were significantly higher in mucosal layers of septa than of normal neonatal intestinal walls (Figure 6) suggesting that DCs mediate immune inflammatory responses in septa.
Involvement of mature dendritic cells and CD4+ T lymphocytes in modulating inflammatory responses in septa

To determine the subtype of DCs involved in these immune inflammatory responses in septa, we assessed the expression in septa and normal neonatal intestinal walls of CD1 and CD83, biomarkers of immature DCs (imDC) and mature DCs (mDC), respectively. Immunohistochemical staining showed that CD1 was not expressed in the mucosal layers of septa and normal neonatal intestinal walls (Figure 7). In contrast, CD83 was expressed in septa, with the difference between septa and normal neonatal intestinal walls being statistically significant (Figure 7).

To determine the involvement of T lymphocytes in inflammatory responses in septa, we assessed the expression of the biomarker CD4 in septa and normal neonatal intestinal walls. Immunohistochemistry showed that the numbers of CD4+ T lymphocytes infiltrating the mucosal and submucosal layers were significantly higher in septa than in normal neonatal intestinal walls (Figure 7).
Discussion

IA is a type of congenital gastrointestinal malformation and a frequent cause of intestinal obstruction in neonates. Although the etiology for IA is not yet fully understood, it is thought to be due to recanalization failure of the intestinal duct in the embryonic period, although it also may be due to vascular dysplasia (Nichol et al., 2011; Reeder et al., 2012). According to the recanalization failure hypothesis, the embryonic intestine forms a lumen during GA week 5, but this lumen is obstructed by an epithelial plug. Subsequent over-proliferation of epithelial cells results in the formation of an occluded lumen (Marine et al., 2014; Koberlein et al., 2015), which condenses within a duct of the intestinal tract. This period probably ends at GA weeks 6-7, as many vacuoles start to appear in the occluded lumen. These vacuoles gradually expand and merge, followed by recanalization to form the intestinal lumen at GA weeks 8-12 (Adams et al., 2014; Subbarayan et al., 2015; Langer et al., 2017). Failure of recanalization due to abnormal molecular events in some intestinal segments during GA weeks 8-12 can lead to IA or intestinal stenosis (Morris et al., 2016; Batra et al., 2017). This may explain the morphological structure of atresia or stenosis that forms between the normal proximal and distal intestinal tracts, as well as the effects of septum resection on intestinal tract re-construction and restoration of normal intestinal function. In contrast, deformities can occur in any segment of the jejunum or ileum without being specific to the intestinal tract (Brinkley et al., 2016). This may also explain the morphological basis of multiple atresia or stenosis.

Because abnormal immune mediation and aberrant lumen development are
simultaneous events, we hypothesized that the failure of recanalization during the formation of IA was probably caused by an abnormal immune inflammatory response. The present study therefore compared populations of defensive barrier and immune system cells, as well as the proinflammatory cytokines produced by these cells, in septa and normal intestinal walls of neonates. PCs and GCs are the main defensive barrier cells in intestinal walls. During the differentiation of intestinal gland cells, PCs differentiate later than GCs and endocrine cells. These differentiation processes are complicated by multiple signaling pathways and transcription factors. Thus, cells or tissues that begin to differentiate later are more susceptible to malformation or abnormality. PCs present in mucosal epithelial crypts during the intestinal recanalization may be more susceptible to malformation or abnormality, resulting in a greater change in local defense or barrier function. PCs are relatively rare in the intestines, as well as being cells specific to glands in the small bowel. These cells are located at the bottom of pyramid-shaped intestinal glands. Their cytoplasm contains large amounts of rough endoplasmic reticulum and abundant Golgi complex (Albores et al., 2015; Liu et al., 2016). Invasion of the intestinal walls by bacterial or other antigen triggers PCs to secrete naturally anti-microbial peptides, including lysozyme, α-defensin-5, and secretory phospholipase A2, into the lumen of the intestinal walls, resulting in the elimination of intestinal microbes. PCs function by providing host defenses, maintaining the intestinal barrier, remodeling intestinal microflora, and protecting intestinal stem cells, functions similar to those of neutrophils (Chen et al., 2017; Huang et al., 2017). Reductions in the number of PCs and of their secreted
antibacterial peptides have been associated with the onset of inflammatory bowel
diseases, including Crohn's disease and ulcerative colitis (Schaart et al., 2006), as well
as diseases caused by ectopic intestinal flora. PCs also provide indispensable support
for the intestinal innate immune system in neonates, findings confirmed in animal
models of neonatal necrotizing enterocolitis (Rodriguez-Colman et al., 2017; Seyde et
al., 2017). In humans, intestinal stem cells start to differentiate into PCs at GA week
14, at birth, all epithelial differentiation in the small bowel has been completed and
epithelial proteins have started to be expressed. In rodents, however, epithelial
differentiation in the small bowel starts after birth (Xian et al., 2017; Haber et al.,
2017).

The septa contained fewer villi, which were arranged in a disorderly fashion
without typical epithelial crypts, with no PCs or lysozyme expression observed in
mucosal epithelial gland walls and glandular cavities. These findings suggest that the
septa, which were remnants of embryonic intestinal walls, possess weaker mucosal
defensive and barrier function than normal intestinal walls in neonates. These findings
also suggest that the inflammatory responses of the mucosal layer of the septum in
response to embryonic intestinal stimulating factors will be more severe. PAS staining
showed that the number of GCs and the levels of MUC2 expression were lower in
septa than in normal mucosa. Taken together with the enhanced expression of pro-
inflammatory cytokines in septa, these findings suggest that immune defensive
barriers and functions are weaker in septa during embryonic development.

This study found that the levels of expression of the DC surface receptor CCR6
and its only ligand, MIP-3α, were higher in septa than in normal intestinal walls, suggesting that DCs are involved in the induction of immune inflammatory responses in septa during the embryonic period. DCs are antigen presenting cells (APCs) with typical dendritic morphology that can identify, process and present antigens, and induce and stimulate the proliferation of T cells. DCs initiate immune responses, especially cellular immune responses (Gil-Pulido et al., 2017; Waisman et al., 2017).

Peripheral DCs include imDCs and mDCs. At present, CD1a is recognized as the main characteristic marker of imDCs and has been widely used in DC isolation, purification and identification in vitro, whereas CD83 is the primary surface marker of activated mDCs (Gomes et al., 2016; Wolkow et al., 2018). Physiologically, imDCs have the ability to phagocytose and process antigens, but have weak T cell activating activity. Under pathological conditions or after stimulation by antigens, imDCs migrate from non-lymphoid tissue to secondary lymphoid organs and differentiate into mDCs. In addition, imDCs can spontaneously mature after phagocytosis of antigen and induction of a signaling pathway, resulting in the upregulation of expression of MHC class II molecules on mDC surfaces and the down-regulation of antigen uptake. In response to chemokines, these mDCs gradually migrate to peripheral blood and lymph, followed by homing to the T cell region of the secondary lymphoid organs (e.g. lymph nodes, spleen), activating T cells (Pabst et al., 2018). Endogenous antigens bind to MHC-I molecules on DCs and form antigen peptide-MHC-I molecular complexes, which are recognized by CD8+ T lymphocytes and activate immune responses to viruses or tumors mediated by cytotoxic T lymphocytes (El-
Murr et al., 2018; Wilson et al., 2018). However, exogenous protein antigen can be recognized and bound directly by imDCs, followed by their enzymatic hydrolysis to polypeptide fragments by proteases and degradation into short peptides with HLA-DM molecules, followed by combination with MHC- molecules and the formation of MHC- complexes, which are recognized by CD4+ T cells and activate immune responses, inducing the proliferation of CD4+ T cells and the generation of large amounts of IL-6 and IL-12 (Sekiya et al., 2016; Stone et al., 2016). Based on this, the activation of CD4+ T lymphocytes by mDC in septa was evaluated by assessing CD4 expression.

In summary, decreased numbers of PCs and GCs in the mucosal epithelial crypts of septa, along with the reduced expression of lysozyme and MUC2 and the increased expression of pro-inflammatory cytokines, suggest that the defensive barrier function of the mucosa is weaker and the inflammatory responses greater in septa than in normal intestinal walls. In addition, septal formation may involve the abnormal differentiation of embryonic intestinal stem cells into PCs and GCs.
Funding

This work was supported by Beijing Municipal Administration of Hospitals [grant number XTZD20180302 by Long Li] and Research Project of Wuxi Commission of Health [grant number Z201711 by Peng Zhao].
References


Gomes J.O., de Vasconcelos Carvalho M., Fonseca F.P., Fonseca F.P., Gondak R.O.,


Liu T.C., Gurram B., Baldridge M.T., Head R., Lam V., Luo C., Cao Y., Simpson P.,


Riba A., Olier M., Lacroix-Lamandé S., Lencina C., Bacquié V., Harkat C., Gillet M.,


Table 1. Primary monoclonal/polyclonal antibodies used in this study

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Abbreviation</th>
<th>Dilution</th>
<th>Species</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>Lysozyme</td>
<td>1:200</td>
<td>Rabbit</td>
<td>Proteintech</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>IL-6</td>
<td>1:200</td>
<td>Rabbit</td>
<td>Proteintech</td>
</tr>
<tr>
<td>Tumor necrosis factor-alpha</td>
<td>TNF-α</td>
<td>1:400</td>
<td>Mouse</td>
<td>Proteintech</td>
</tr>
<tr>
<td>Macrophage Inflammatory protein 3α</td>
<td>MIP-3α</td>
<td>1:200</td>
<td>Mouse</td>
<td>Abcam</td>
</tr>
<tr>
<td>CC-chemokine receptor 6</td>
<td>CCR6</td>
<td>1:300</td>
<td>Rabbit</td>
<td>Abcam</td>
</tr>
<tr>
<td>Immature dendritic cell</td>
<td>CD1a</td>
<td>1:200</td>
<td>Rabbit</td>
<td>Abcam</td>
</tr>
<tr>
<td>Mature dendritic cell</td>
<td>CD83</td>
<td>1:300</td>
<td>Rabbit</td>
<td>Abcam</td>
</tr>
<tr>
<td>CD4+ T lymphotoxic cell</td>
<td>CD4</td>
<td>1:300</td>
<td>Rabbit</td>
<td>Abcam</td>
</tr>
</tbody>
</table>
Figure legends

Figure 1. HE staining, showing the goblet cells in septa and normal intestinal walls of neonates aged 1-3 days. Upper panel, 100X; Lower panel, 400X.

Figure 2. PAS staining, showing the location of goblet cells, and immunohistochemical staining, showing the expression of MUC2, cells in septa and normal intestinal wall tissue collected from neonates aged 1-3 days. A: PAS staining, Upper panel, 100X; Lower panel, 400X. B: Staining with anti-MUC antibody. Upper panel, 100X; Lower panel, 400X. C: Semiquantitative comparison of numbers of goblet cells and MUC2 expression in septa and normal intestinal walls.

Figure 3. Immunohistochemical staining, showing the expression of lysozyme generated by PCs in septa and normal intestinal wall tissue collected from neonates aged 1-3 days. Upper panel, 100X; Middle panel, 400X; Lower panel, Semiquantitative comparison of lysozyme expression in mucosal layers from septa and normal intestinal walls.

Figure 4. Immunohistochemical staining, showing the expression of IL-6 in septa and normal intestinal wall tissue collected from neonates aged 1-3 days. Upper panel, 40X; Lower panel, 200X. Semiquantitative comparison of IL-6 expression in mucosal and submucosal/muscular layers of septa and normal intestinal walls.
**Figure 5.** Immunohistochemical staining, showing the expression of TNF-α in septa and normal intestinal wall tissue collected from neonates aged 1-3 days. Upper panel, 40X; Lower panel, 200X. Semiquantitative comparison of TNF-α expression in mucosal and submucosal/muscular layers of septa and normal intestinal wall tissue.

**Figure 6.** Immunohistochemical staining, showing the expression of MIP-3α and CCR6 in septa and normal intestinal wall tissue collected from neonates aged 1-3 days. A: Staining with anti-MIP-3α antibody, Upper panel, 100X; Lower panel, 400X. B: Staining with anti-CCR6 antibody. Upper panel, 100X; Lower panel, 400X. C: Semiquantitative comparison of MIP-3α and CCR6 expression in septa and normal intestinal wall tissue.

**Figure 7.** Immunohistochemical staining showing the expression of CD1a, CD83, and CD4 in septa and in normal intestinal wall tissue collected from neonates aged 1-3 days. A: Staining with anti-CD1a antibody, a marker for imDCs. Upper panel, 100X; Lower panel, 400X. B: Staining with anti-CD83 antibody, a marker for mDCs. Upper panel, 100X; Lower panel, 400X. C: Staining with anti-CD4 antibody, a marker for T lymphocytes. Upper panel, 100X; Lower panel, 400X. D Semiquantitative comparison of CD1a expression in septa and normal intestinal wall tissue. E: Semiquantitative comparison of CD83 expression in septa and normal intestinal wall tissue. F, G: Semiquantitative comparison of CD4 expression in (F) mucosal and (G) submucosal/muscular layers in septa and normal intestinal wall tissue.
Expression comparison of lysozyme in mucosa in two groups

- Normal intestinal wall
- Septum

![Image showing normal intestinal wall and septum with lysozyme expression comparison](image)

**Optical density (OD) value**

![Graph showing comparison of optical density values between normal intestinal wall and septum](graph)

- Normal intestinal wall: 0.35
- Septum: 0.1

**Statistical significance:**

\[ p = 0.0005 \]
Normal intestinal wall

Expression comparison of IL-6 in mucosa in two groups

![Graph showing optical density (OD) values](image)

Septum

Expression comparison of IL-6 in muscular layer in two groups

![Graph showing optical density (OD) values](image)
Expression comparison of TNF-α in mucosa in two groups

Expression comparison of TNF-α in muscular layer in two groups
Expression comparison of MIP-3α in mucosa in two groups

\[ P = 0.0130 \]

Expression comparison of CCR6 in mucosa in two groups

\[ P = 0.0027 \]
Normal intestinal wall  Septum

Expression comparison of CD1a in mucosa in two groups

Expression comparison of CD83 in mucosa in two groups

Comparison of CD4+ cells in mucosa in two groups

Comparison of CD4+ cells in muscular layer in two groups