Expression of OLFM4 and Lysozyme During Necrotizing Enterocolitis and Volvulus in Newborns: a Comparative Observational Research Study

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Abstract

**Background:** In neonatal patients with necrotizing enterocolitis (NEC) and volvulus the inflammatory response is mediated by a plurality of different proteins. The proteins olfactomedin 4 (OLFM4) and lysozyme (LYZ) are part of the intestinal mucosal defense and especially OLFM4 has rarely been evaluated in neonatal gastrointestinal diseases. The aim of this study was to compare the expression levels of OLFM4 and lysozyme during NEC and volvulus in neonates.

**Methods:** Intestinal tissues of patients with NEC and patients with volvulus were examined using immunohistochemical staining of OLFM4 and lysozyme of formalin-fixed and paraffin-embedded sections of resected tissue. Staining-positive tissues were semi-quantitatively scored from 0 (no staining), 1 (weak staining), 2 (moderate staining) to 3 (highly intense staining) by two individual investigators.

**Results:** Both applied antibodies against OLFM4 showed different staining patterns with higher staining intensity of the antibody OLFM4 (D1E4M). OLFM4 (median score of the antibody OLFM4 (D1E4M): 3.0) and lysozyme (median score: 3.0) are highly expressed in intestinal and immune cells during NEC. The expression of OLFM4 and lysozyme in tissue with intestinal volvulus was also observable (median score of the antibody OLFM4 (D1E4M): 1.25) and median score of the antibody against LYZ: 2.0), but lower levels could be seen in comparison to tissue with NEC (p=0.033 and p=0.037, respectively).

**Conclusions:** Both proteins, OLFM4 and lysozyme, may play a role in the pathogenesis of NEC and volvulus in neonatal patients, but the exact mechanisms of OLFM4 and lysozyme function and their role in immunological responses have not yet been resolved. These observations add new insights as basis for further large-scale population research.

Background

Necrotizing enterocolitis (NEC) is a severe and life-threatening complication in the treatment of preterm infants [1]. It can also be diagnosed in term infants, especially with concomitant diseases such as congenital cardiac malformations [2]. It is characterized by an intestinal inflammation, leading to a systemic inflammatory response syndrome with the need of intensive care medicine including mechanical ventilation, catecholamine therapy and broad antibiotic treatment. Volvulus is another severe disease of the gastrointestinal tract during the neonatal period. It often results in a similar severe clinical picture due to its obstructive and ischemic pathophysiology: the intestinal twist causes a disruption of intestinal perfusion, which leads to ischemia, to bowel necrosis and destruction of the intestinal barrier, to secondary intestinal inflammation and to clinical symptoms similar to systemic inflammatory response syndrome and NEC [3]. In each case, the massive (NEC) [4] or moderate (volvulus) inflammatory response is mediated by a plurality of different proteins as part of the intestinal innate immune system. Further two of these proteins, olfactomedin 4 (OLFM4) and lysozyme (LYZ) are of major interest in the presented study.
OLFM4 is expressed under physiological conditions especially in the small intestine, colon, and prostate. It also moderately occurs in the stomach and bone marrow [5]. OLFM4 is found in macrophages, and mature human neutrophils express OLFM4 within the specific granules [6]. Its central function is determined in the mucosal defense of the stomach, small intestine and colon [7] and plays an important role in the innate immunity against bacterial infections [8]. The influence of OLFM4 was confirmed by experiments with OLFM4 deficient mice, showing a severe inflammation and proliferation in intestinal crypts of the small intestine and additional mucosal damage in the colon [9]. It is centrally discussed in pathogenesis of inflammatory bowel diseases (IBD), in which OLFM4 mRNA and protein expression levels are significantly increased in the intestinal epithelium [7, 10]. This upregulation may serve as a marker of the intestinal inflammation in IBD [8]. Most recently, TNF-α promoted OLFM4 secretion by human intestinal epithelium cells and cytoplasmic accumulation of OLFM4 in intestinal epithelium cells was observed to be promoted by Notch and TNF-α signaling, confirming a cell protective role in the inflamed mucosa of IBD patients [11]. It is additionally discussed as a robust stem cell marker for humans [12]. However, the exact regulatory mechanisms within these inflammatory conditions remain elusive and are controversially discussed [11]. In general, OLFM4 expression is highly increased during inflammatory diseases, such as sepsis, sepsis-induced acute respiratory distress syndrome, and respiratory syncytial virus infection [13–15]. In summary, OLFM4 binds to various proteins in inflammation signaling pathways and has an essential role in the innate immunity against bacterial infections, gastrointestinal inflammation, and cancer [8].

LYZ is a protein with antimicrobial and immune modulating characteristics. Its central role in the innate immunity has been proven [16]. It can be detected in different hematological cell types (macrophages, neutrophils, dendritic cells), and in the liver as well as in different secretions (saliva, urine, tears) and at mucosal surfaces. In parallel to OLMF4, LYZ influences a pro-inflammatory response and the resolution of inflammation at mucosal sites [16]. Within the first mentioned function, LYZ can hydrolyze cell wall peptidoglycan in bacteria, leading to bacterial destruction, which thereby activates pattern recognition receptors in host cells. In contrast, LYZ assists in the intestinal epithelial barrier protection to limit the invasion of the microbiota and contributes to the immune cell response in mice to resolve mucosal inflammation and to limit intestinal as well as systemic inflammation [17].

The aim of this study was to compare the expression levels of OLFM4 and LYZ during necrotizing enterocolitis and volvulus in newborns.

**Methods And Patients**

**4.1. Clinical data**

Intestinal tissues of seven patients with NEC and six patients with volvulus were examined. Patients’ demographical data were collected retrospectively by evaluation of medical reports of the University Hospital Heidelberg. The study was approved by the local Ethics Committee of the University of Heidelberg and the local Ethics Committee of the University of Erlangen-Nürnberg and was in accordance
with the Helsinki declaration (1964) and its later amendments. Gestational age was defined as time elapsed between the first day of the last menstrual period and the day of delivery. The resected intestinal tissues were exclusively gained in cases with a clinical indication for surgery due to NEC or volvulus and were received from surgery during acute NEC or acute volvulus. In the cases of multiple surgery only sections of one procedure were stained. As biopsies of intestinal tissue cannot be obtained from healthy infants, we compared expression of OLFM4 and LYZ between infants with NEC and volvulus.

4.2. Immunohistochemical analyses

The immunohistochemical staining of formalin-fixed and paraffin-embedded serial intestinal sections of tissue resected during NEC or volvulus were performed at the Institute of Pathology, University Hospital Heidelberg, Germany. The antigen retrieval was carried out with citrate buffer pH 6.1 (DAKO, Agilent, Waldbronn, Germany). Anti-OLFM4 antibody ab188812 (abcam, Berlin, Germany; rabbit polyclonal antibody against a synthetic 16 amino acid peptide of OLFM4) in a dilution of 1:100 and OLFM4 (D1E4M) XP® Rabbit mAb #14369 (Cell Signalling Technology, Frankfurt/Main, Germany; monoclonal antibody produced by immunizing animals with a synthetic peptide corresponding to residues surrounding Phe94 of human OLFM4 protein recognizing endogenous levels of total OLFM4 protein) in a dilution of 1:200 was used. We used these two antibodies with different targets to confirm OLFM4 expression with potentially different length or potentially modifications in neonatal tissue, as data on intestinal OLFM4 in these patients are still not available. LYZ was detected in intestinal tissue using Anti-LYZ antibody (BGN/06/961, ab36362, abcam, Berlin, Germany) in a dilution of 1:100.

Staining-positive tissues were scored semi-quantitatively from 0 (no staining), 1 (weak staining), 2 (moderate staining) to 3 (highly intense staining) and each section was scored independently by two investigators (SD, HM). The average score was used for statistics.

4.3. Statistical analyses

Data were analyzed using the Mann-Whitney-U-test. P-values < 0.05 were regarded as statistically significant.

Results

5.1. Patients’ demographical data

The median birth weight of the seven infants with NEC was 800 g (range: 500 – 2940 g) and the median gestational age at birth was 26.0 weeks (range: 23.3 – 37.4 weeks). Surgery was done due to acute and high-staged NEC at a median postnatal age of 12 days (range: day 8 – 33 of life) and a median corrected gestational age of 29.6 weeks (range: 24.4 – 39.1 weeks). The 6 infants with volvulus showed a median birth weight of 688 g (range: 360 – 1960 g) and a median gestational age at birth of 24.5 weeks (range: 22.7 – 33.9 weeks). Surgeries due to volvulus were conducted at a median day 43 of life (range: day 2 – 70 of life) and at a median corrected gestational age of 33.6 (range: 24.2 – 34.9 weeks). Table 1 summarizes the patients’ demographic baseline data.
Table 1
Demographic data of participating patients

| Diagnosis | Birth weight [g] | Gestational age at birth [weeks] | Postnatal day at surgery [days] | Corrected gestational age at surgery [weeks] |
|-----------|------------------|----------------------------------|-------------------------------|---------------------------------------------|
| 1         | NEC              | 500                              | 23.3                          | 8                                           | 24.4                          |
| 2         | NEC              | 1490                             | 31.7                          | 11                                          | 33.3                          |
| 3         | NEC              | 800                              | 26.0                          | 24                                          | 29.6                          |
| 4         | NEC              | 650                              | 24.4                          | 30                                          | 28.7                          |
| 5         | NEC              | 2940                             | 37.4                          | 12                                          | 39.1                          |
| 6         | NEC              | 1630                             | 29.7                          | 33                                          | 34.4                          |
| 7         | NEC              | 630                              | 23.7                          | 11                                          | 25.3                          |
| 8         | volvulus         | 570                              | 22.7                          | 12                                          | 24.4                          |
| 9         | volvulus         | 1960                             | 33.9                          | 2                                           | 34.1                          |
| 10        | volvulus         | 740                              | 25.0                          | 69                                          | 34.9                          |
| 11        | volvulus         | 950                              | 27.9                          | 48                                          | 34.7                          |
| 12        | volvulus         | 635                              | 23.1                          | 70                                          | 33.1                          |
| 13        | volvulus         | 360                              | 24.0                          | 38                                          | 29.4                          |

Abbreviations: NEC: necrotizing enterocolitis

5.2. OLFM4 and LYZ expression during NEC

Both applied antibodies against OLFM4 showed partially different staining patterns with higher staining intensity of the antibody OLFM4 (D1E4M). High expression of OLFM4 and LYZ was observed in the infants with NEC (median score of the antibody OLFM4 (D1E4M): 3.0) and LYZ: 3.0). OLFM4 expression was observed in intestinal epithelial cells as well as in immune cells (neutrophils, macrophages) of the affected intestine in cases with NEC that were present due to severe inflammation (Figure 1). Furthermore, OLFM4 was detected within the mucus (Figure 1A, B; Table 2). Lysozyme expression was also high in NEC manifestation. Immune cells (neutrophils, macrophages) showed intensive LYZ expression (Figure 1C, F). Figure 2 illustrates the expression of OLFM4 (Figure 2A, B) and LYZ (Figure 2C) of neutrophils in the vessels of inflamed tissue and points to the source of staining-positive immune cells migrating from the vessel into intramural tissue.
Table 2
Description of each section concerning OLFM4 and LYZ staining

| Diagnosis   | OLFM4 staining ab188812 | OLFM4 staining (D1E4M) | Lysozyme staining |
|-------------|-------------------------|------------------------|-------------------|
| 1 NEC       | 1 (immune cells)        | 3 (immune cells)       | 3 (immune cells)  |
| 2 NEC       | 3 (immune cells)        | 3 (immune cells)       | 3 (immune cells)  |
| 3 NEC       | 3 (intestinal epithelial cells, immune cells) | 3 (intestinal epithelial cells, immune cells) | 2 (immune cells, a few crypts) |
| 4 NEC       | 2.5 (intestinal epithelial cells, multiple immune cells) | 3 (intestinal epithelial cells, a few immune cells) | 3 (many crypts, a few immune cells) |
| 5 NEC       | 3 (immune cells, intestinal epithelial cells) | 3 (immune cells, intestinal epithelial cells) | 2.5 (immune cells and crypts) |
| 6 NEC       | 2.5 (immune cells)      | 3 (immune cells)       | 3 (immune cells)  |
| 7 NEC       | 1.5 (immune cells)      | Adequate tissue was not available | 0.5 (immune cells) |
| 8 Volvulus  | 3 (immune cells, a few intestinal epithelial cells in the crypts) | 3 (immune cells, a few intestinal epithelial cells in the crypts) | 2 (immune cells, a few crypts) |
| 9 Volvulus  | 1 (immune cells)        | 0.5 (immune cells)     | 2 (immune cells)  |
| 10 Volvulus | 0.5 (immune cells)      | 1 (immune cells)       | 0.5 (immune cells) |
| 11 Volvulus | 2.5 (immune cells)      | 3 (immune cells, intestinal epithelial cells) | 2.5 (immune cells) |
| 12 Volvulus | 2.5 (immune cells, intestinal epithelial cells) | 1 (intestinal epithelial cells) | 2.0 (crypts, a few immune cells) |
| 13 Volvulus | 1.5 (immune cells, a few intestinal epithelial cells in the crypts) | 1.5 (immune cells, a few intestinal epithelial cells in the crypts) | 0.5 (crypts, immune cells) |

Abbreviations: NEC: necrotizing enterocolitis. OLFM4: olfactomedin 4

### 5.3. OLFM4 and LYZ expression during volvulus

As already observed in the cases with NEC, the staining intensity of antibody OLFM4 (D1E4M) was partially different from that of Anti-OLFM4 antibody ab188812. The expression of OLFM4 and LYZ in tissue with intestinal volvulus was also obvious, but lower in comparison to tissue with NEC (median score of the antibody OLFM4 (D1E4M): 1.25 and lysozyme: 2.0) and the difference in staining between
infants with NEC or volvulus was statistically significant (OLFM4 (D1E4M): \( p=0.033 \), LYZ: \( p=0.037 \); see Figure 3A, B; Table 2). In tissue sections of patients with volvulus, OLFM4 was highly expressed in intestinal epithelial cells. Paneth cells in the crypt base showed high expression levels of LYZ (Figure 3C). Furthermore, macrophages and neutrophils were positive for LYZ (Figure 3C, Figure 4).

**Discussion**

We are presenting a study on the expression of OLFM4 and LYZ in neonatal patients with NEC and volvulus. In both, NEC and volvulus, a pronounced expression of these proteins could be observed.

Especially in NEC, immunological aspects play a central role in current research, aiming at the improvement of knowledge on inflammatory processes and the identification of potential new therapeutic targets. Recent studies confirm a lack of knowledge in this field while highlighting the interference of mechanisms of the innate and the adaptive immune system with its deficiencies in term and preterm neonates [18–20]. Both proteins, OLFM4 and LYZ, are proven to be a part of the innate immune system. However, associations of OFLM4 to NEC or volvulus have rarely been evaluated so far.

The role of OLFM4 in gastrointestinal diseases, such as IBD, gastric or colorectal cancer, has been confirmed in a plurality of publications [5], supporting its influence as sepsis regulator and confirming its survival benefit. As mentioned before, a cell protective role in the inflamed mucosa of IBD patients could be seen, regulated via the NF-kB pathway [7]. On the contrary, a subset of neutrophils expressing OLFM4 has been identified. Alder et al. confirmed an association of these OLFM4 neutrophils with a worse outcome during sepsis [14]. Moreover, Levinsky et al. observed that OLFM4 null mice are protected from death during sepsis and showed less intestinal barrier dysfunction, implicating that OLFM4 might contribute essential pathogenic aspects to the inflammatory process [21]. Furthermore, they described the central involvement of OLFM4-positive neutrophils in the pathologic process leading to intestinal damage and mortality after intestinal ischemia/reperfusion injury. The authors hypothesized a potential mechanism for this observation, in which activated neutrophils next to the injury secrete OLFM4 into the environment leading to enhanced iNOS production by macrophages and injured tissue. This leads to an impaired intestinal barrier [21]. We observed a moderate/high expression of OLFM4 in affected intestinal tissue and in immune cells of inflammatory intestinal regions in cases of NEC and volvulus and are therefore able to support these observations.

The two different antibodies against OLFM4 showed in part different staining scores and this may have different causes: One possibility includes the presence of different OLFM protein variants as the antibodies had different targets within the protein. Furthermore, potential modifications might lead to changes at antibody binding regions and might thereby influence the staining. However, this must be analyzed by further studies.

LYZ has been evaluated regarding its role in the pathogenesis of NEC. Coutinho et al. confirmed an absence of lysozyme in Paneth cells of NEC patients compared to matched controls [22]. Furthermore, expression of LYZ was observed in macrophages of the lamina propria. They hypothesized that the loss
of Paneth cells enhances infections by the impairment of the mucosal barrier [22]. Further research by McElroy et al. supported this hypothesis: a decreased Paneth cell number was observed in infants with surgically treated NEC compared to age-matched controls with spontaneous intestinal perforation [23]. The loss of Paneth cells in different murine models resulted in a NEC-like pathology in the small intestine of immature mice [24, 25]. Markasz et al. supported also pathophysiologic mechanism describing the absence of alpha-defensins in Paneth cells as a central factor in the pathogenesis of NEC [26]. Our study contradicts the reduced LYZ expression, as we observed an obvious expression of LYZ in NEC and volvulus patients. This expression includes additional cells aside Paneth cells in regions of proximity to affected intestinal regions with NEC or volvulus. Schaart et al. described a weak expression of LYZ in Paneth cells, but explained a possible cause for different LYZ expression levels in NEC tissue: Paneth cells rapidly secreted lysozyme in response to epithelial injury to prevent translocation of bacteria and it depends on the timepoint whether low or higher LYZ expression levels were seen [27]. The absence of LYZ-positive Paneth cells reported in studies (e.g. [22]) might therefore be explained as a result of increased LYZ secretion [28]. Other studies described that Paneth cell antimicrobials were increased in response to NEC [29, 30]. Puiman et al. reported increased Paneth cell numbers and expression of Paneth cell antimicrobials only during recovery from NEC [28]. Despite the observation that antimicrobial proteins were up-regulated in response to NEC, the protein expression levels are low and may be inadequate to protect the immature gut from a bacterial invasion [30]. Surgical intervention within our study was early during NEC process and removed only intestinal tissue which was not vital and showed no amelioration during operation. In these very strongly affected regions during early NEC process, we observed the up-regulation of OLFM4 and LYZ in intestinal epithelial cells and immune cells. In some cases, intestinal sections included tissue with higher distance to the destroyed and very strongly affected regions showing low expression of OLFM4 in different cells. Furthermore, the intestinal tissue far away from the very strongly affected regions demonstrated a LYZ expression in a decreased number of immune cells as well as a LYZ expression restricted to Paneth cells compared to affected regions. We assume that the different surgical time-points and different grades of destroyed intestinal tissue may explain the contradictory findings of the studies. In analogy to OLFM4, LYZ is described in both, up- and downregulation during inflammation. Even a certain strengthening of the mucus barrier could be observed in rat pups fed with LYZ after exposition to NEC-stressors [31]. This leads to the hypothesis of a protective role of LYZ in the development of NEC explaining the sense of LYZ up-regulation. Unfortunately, this innate defense of LYZ was not sufficient to protect the intestine.

Blood monocyte count is low in NEC and the intestinal inflammatory response is rich in macrophages. Furthermore, differentiation of monocytes into intestinal macrophages and up-regulation of monocyte recruitment genes were induced by NEC [32]. We observed extensive OLFM4 and LYZ staining of macrophages in intestinal tissue of infants with NEC. This emphasizes the antimicrobial function of OLFM4 and LYZ during NEC.

Conclusions
In conclusion, OLFM4 and LYZ are highly expressed in intestinal and immune cells during NEC and volvulus. However, the exact mechanisms of OLFM4 and LYZ function and their role in immunological responses have not yet been resolved. These observations of a comparative observational research study add new insights as basis for further large-scale population research and potential new therapeutic targets such as daily supplementation of LYZ [31, 33].

**Abbreviations**

IBD inflammatory bowel disease

LYZ lysozyme

NEC necrotizing enterocolitis

OLFM4 olfactomedin 4

**Declarations**

**9.1 Ethics approval and consent to participate**

This study was approved by the local ethical committees (ethics committee of the medical faculty of the Ruprecht-Karls-Universität of Heidelberg, Germany; ethics committee of the medical faculty of the Friedrich-Alexander-Universität Erlangen-Nürnberg, Germany) and was performed in line with the principles of the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Due to the retrospective analysis of anonymized data without further patient contact, both, the ethics committee of the medical faculty of the Ruprecht-Karls-Universität of Heidelberg and the ethics committee of the medical faculty of the Friedrich-Alexander-Universität Erlangen-Nürnberg approved informed consent waiver.

**9.2 Consent for publication**

Not applicable

**9.3 Availability of data and materials**

All data generated or analyzed during this study are included in this published article.

**9.4 Competing interests**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**9.5 Funding**
The study received no funding.

9.6 Authors' contributions

All the authors contributed to the realization of the study and its publication. Initially, the concept of the study was designed and adjusted in progress (SD, MB, HM). Patients’ data were acquired and included in a database (HM). Immunohistochemical analyses were conducted and analyzed (MR, VB, AH, SD, HM). Statistical analysis and interpretation of data was conducted (SD, HM) and finally, the manuscript was originally drafted by SD and revised and approved by all authors. The project was supervised by HM. Submission of manuscript was conducted by corresponding author SD.

9.7 Acknowledgments

Not applicable.

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**Figures**

**Figure 1**

**Expression of OLFM4 and LYZ in NEC tissue**

A-C: NEC in a preterm infant (patient 3 of Table 1). OLFM4 was highly expressed in intestinal epithelial cells (arrow in A and B) and in immune cells dispersed in the whole intestinal wall (A). LYZ was expressed in Paneth cells of the crypts (thin arrow in C) and in immune cells (e.g., macrophages, thick arrow in C). D-
F: NEC in a preterm infant (patient 2 of Table 1). Massive expression of OLFM4 and extensive OLFM4 and LYZ staining of macrophages (arrows in E and F).

**Figure 2**

**Expression of OLFM4 and LYZ in immune cells during NEC**

A: Neutrophils with expression of OLFM4 located in the intestinal wall. B: OLFM4 positive immune cells attached to the endothelial lining of a large vessel in the intestinal wall. C: LYZ-positive immune cells within intestinal wall migrated from the vessels into intramural tissue.

**Figure 3**

**Expression of OLFM4 and LYZ in intestinal tissue with volvulus**

A-C: Preterm infant with volvulus (patient 12 of Table 1). OLFM4 was expressed in intestinal epithelial cells (arrow in A, thin arrow in B, thick arrow in B). Staining of both used antibodies against OLFM4 showed sometimes differences in staining score and in stained structures (intestinal epithelium and endothelial cells). Paneth cells in the crypt base showed high expression levels of LYZ. LYZ was detected in the immune cells (e.g., macrophages, arrows in C).

**Figure 4**

**Expression of OLFM4 and LYZ in immune cells during intestinal volvulus**

OLFM4-positive neutrophils were observed in the vessels of gastrointestinal tissue affected by volvulus (arrows in A, thick arrow in B). The neutrophils also expressed LYZ (C), invading the crypts (thin arrow in C) after leaving the vessels (thick arrows in C). Additionally, an up-regulation of OLFM4 and LYZ in inflamed tissue of infants with volvulus can be confirmed. Intestinal cells expressed OLFM4 (thin arrow in B).