Integrative analysis links ferroptosis to necrotizing enterocolitis and reveals the role of ACSL4 in immune disorders

**Highlights**
- We first identified the involvement and role of ferroptosis in NEC.
- ACSL4 might exert an important effect on ferroptosis in NEC.
- ACSL4 was associated with multiple types of regulated cell death in NEC.
- ACSL4 could be a critical regulator between ferroptosis and immune dysfunction.

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Integrative analysis links ferroptosis to necrotizing enterocolitis and reveals the role of ACSL4 in immune disorders

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SUMMARY
Multiple types of regulated cell death (RCD) have been demonstrated to cause gut barrier dysfunction in necrotizing enterocolitis (NEC); however, whether and how ferroptosis is involved in NEC remains unknown. Here, ferroptosis was identified to play a role in NEC using bioinformatics analyses and wet experiments. Inhibition of ferroptosis significantly alleviated NEC in newborn mice. ACSL4 expression levels were augmented and positively correlated with ferroptosis in NEC. Surprisingly, ACSL4 was critically correlated with multiple types of RCD, including autophagy, apoptosis and pyroptosis, as well as hypoxia and inflammation. Besides, ACSL4 was positively correlated with the abundance of multiple types of immune cells, including macrophage, activated dendritic cell, neutrophil and regulatory T cell. Conclusively, we first identified the involvement and role of ferroptosis in NEC and revealed the potential effects of ACSL4 on immune disorders, which could provide a rationale for future research on ferroptosis in NEC.

INTRODUCTION
Necrotizing enterocolitis (NEC) is the most common gastrointestinal disease in newborns, affecting 5–12% of very low birth weight infants, with mortality up to 50% in NEC neonates requiring surgical management (Meister et al., 2020; Niño et al., 2016). NEC can cause diverse complications, reduce life quality and increase the global disease burden (Bisquerra et al., 2002; Stey et al., 2015; Zhu et al., 2021). However, the mechanisms of gut injury in NEC are largely unclear (Hackam and Sodhi, 2022).

Several types of regulated cell death (RCD), including apoptosis, pyroptosis and autophagy has been recently reported to be involved in gut barrier dysfunction in NEC (Chen et al., 2019; Jilling et al., 2004; Subramanian et al., 2020; Yin et al., 2020), whereas the effects of ferroptosis on NEC is unknown. Ferroptosis is a form of RCD characterized by iron-dependent and unrestricted lipid peroxidation (Chen et al., 2021), and involved in multiple biological processes in several diseases such as cancer and ischemia/reperfusion (I/R) injury (Li et al., 2020; Wang et al., 2021b; Zheng et al., 2021). Long chain acyl CoA synthetase 4 (ACSL4) is a pivotal lipid metabolism enzyme which converts AA/AdA to PE-AA-OOH/PE-AdA-OOH and has been recognized as a driver of ferroptosis (Doll et al., 2017; Lei et al., 2020; Müller et al., 2017). ACSL4 inhibition has been proven to be a viable therapeutic approach to prevent ferroptosis-related cancer, neuro-inflammation and I/R injury (Cui et al., 2021; Doll et al., 2017; Xu et al., 2020b). In addition, ACSL4 inhibition could protect ischemia/reperfusion-induced intestinal cell ferroptosis (Li et al., 2019). Nevertheless, it remains unknown whether and how ferroptosis and ACSL4 could affect the development of NEC in neonates.

Furthermore, multiple types of RCD are reported to contribute to tissue inflammation in various diseases (Mohammed et al., 2021; Sun et al., 2020; Van Opdenbosch and Lamkanfi, 2019); for example, ferroptosis-related immune therapy has been used in cancer (Chen et al., 2021; Song et al., 2021). Immune disorders have been proved to play a major role in the development of NEC, and neutrophils (Klinke et al., 2020), dendritic cells (Emami et al., 2011), macrophages (Wei et al., 2019) and T cells (Pang et al., 2018) have been implicated in NEC-related gut injury; however, there is a lack of a comprehensive profiling of immune cells in NEC, and the association of ferroptosis with immune response in NEC is presently unclear.
Here, we performed an integrative analysis using in silico and in vitro experiments to investigate the effects and potential mechanisms of ferroptosis and ACSL4 on NEC in neonates. The present research would clarify the association between ferroptosis and NEC, and ascertain the effects of ACSL4 on ferroptosis and immune disorders in NEC, which could provide new insights into future research on ferroptosis and NEC.

RESULTS

Discovery and confirmation of the involvement of ferroptosis in NEC

Recent studies have showed that reactive oxygen species (ROS), a product of ferroptosis, accumulate in damaged gut tissues of NEC infants (Zhang et al., 2019); antioxidant enzymes, which protect tissues against ferroptosis, are of lower activity in neonates than in adults (Osiak et al., 2020). In view of this, we hypothesized that ferroptosis may be involved in NEC. To demonstrate this hypothesis, we analyzed microarray data of eight intestinal tissues from GSE46619, including four NEC samples and four normal intestinal tissues. As we expected, heatmap analysis revealed that marker genes for ferroptosis were apparently up-regulated in NEC compared with that in normal tissues (Figures 1A and 1B), suggesting that ferroptosis is involved in NEC. Consistent with this, principal component analysis (PCA) also demonstrated a distinct expression pattern of marker genes for ferroptosis between NEC and normal intestinal tissues (Figure 1C), further underlining the involvement of ferroptosis in NEC.

To further assess the relationship between ferroptosis and NEC, we quantified the ferroptosis levels using single-sample gene set enrichment analysis (ssGSEA) based on transcriptional expression values of marker genes for ferroptosis (Table S1), and compared ferroptosis levels between NEC and normal intestinal tissues. In accordance with the previous findings, we found that ferroptosis levels were significantly elevated in NEC than in normal tissues (t-test; p = 0.002921; Figure 1D), repeatedly highlighting the participation of ferroptosis in NEC.

Considering ferroptosis is a type of iron- and lipid ROS-triggered RCD, we next investigated the ferroptosis levels in NEC tissues through measuring intracellular iron and ROS levels. Consistently, the results showed that iron concentration was critically higher in NEC tissues than normal intestinal tissues (t-test, p = 0.0155; Figure 1E), and the MDA levels were also apparently higher in NEC tissue than normal intestinal tissue (t-test, p = 0.003; Figure 1F), further suggesting the role of ferroptosis in NEC.

Ferroptosis is characterized by small mitochondria with concentrated membrane density, decreased or vanishing mitochondrial cristae, and outer mitochondrial membrane rupture (Xu et al., 2021); thereby we then observed the mitochondria morphology using transmission electron microscope (TEM). Consistent with the previous results, we found that NEC tissues displayed a typical ferroptosis-related mitochondria morphology, whereas normal intestinal tissues showed a normal mitochondria morphology (Figure 1G), further highlighting the implication of ferroptosis in NEC.

Investigation of the role of ferroptosis in NEC

Although we had observed the involvement of ferroptosis in NEC, we were not sure whether it was a concomitant phenomenon or a causal relationship. To make clear the role of ferroptosis in NEC, we compared the severity of NEC between different conditions of ferroptosis in vivo. First, we established an in vivo NEC model by treating newborn mice with hypoxia-hypothermia-LPS method for four days. We observed that NEC newborn mice were malnourished than their counterparts (Figure 2A) and gained less weight than control newborn mice (1.49 ± 0.21 g VS. 1.70 ± 0.07 g; t-test, p = 0.0448; Figure 2B). Gross appearance of intestinal tissues showed hyperemia, poor elasticity, edema, pneumatosis intestinalis and even focal/transmural necrosis in NEC group, whereas intestine of the control group was in normal condition (Figure 2C). Further, HE staining showed impaired intestinal villus, focal/transmural necrosis,
inflammation, submucosal edema/separation, and interstitial hemorrhages (Figure 2D); injury grade (MohanKumar et al., 2019) was significant higher in NEC group than in control group (t-test, p< 0.0001; Figure 2E). All these findings proved that an animal model for NEC was successfully established.

Consistent with our previous findings based on RNA-seq data and human NEC tissues, ferroptosis was also found in NEC mice model. We found that levels of iron concentration and MAD were significantly higher in...
NEC group than in control group (t-test, p = 0.0003 and 0.0010, respectively; Figures 2G and 2H); TEM of intestinal epithelia cells showed typical ferroptosis appearance including mitochondrial volume shrinkage, membrane thickening and decreasing mitochondrial cristae (Figure 2F). Moreover, IHC showed that the ACSL4, a driver gene of ferroptosis, was overexpressed in the intestine tissue of NEC group (t-test, p = 0.0002; Figures 2I and 2J).

Then, to compare the role of ferroptosis in the severity of NEC, we classified NEC mice into two groups: NEC group (n = 6) and Lip-1-treated NEC group (n = 6) which was treated with ferroptosis inhibitor Lip-1. Accordingly, mitochondrial morphology change was retrained and the protein level of ACSL4 did not increase in the NEC + Lip-1 group (Figure 2F). As we expected, gut injury was alleviated in Lip-1-treated NEC group than NEC group both in gross and histologic appearance (Figures 2C–2E).

Collectively, we successfully established a newborn NEC mouse model and revealed the effect of ferroptosis on NEC by administrating ferroptosis inhibitor Lip-1 in NEC mouse.

**Observation of ACSL4 upregulation in NEC**

Because we had identified the involvement of ferroptosis in NEC, we next sought to find out key genes associated with ferroptosis in NEC. We first performed a differential expression analysis using microarray data of four normal intestinal tissues and four NEC tissues, and obtained 1571 differentially expressed genes (DE-Gs) including 539 upregulated and 1032 downregulated (|logFC| > 1, p < 0.05; Figure 3A and Table S2). Then, we intersected the 1571 DE-Gs and 259 known ferroptosis-related genes consisting of markers, inducers and suppressors, and obtained 35 ferroptosis-related DE-Gs in NEC (Figure 3B and Table S3). Consistent with this, heatmap analysis also manifested a distinct expression pattern of ferroptosis-related genes between NEC and normal intestinal tissues (Figure 3C).
ACSL4 is an important inducer of ferroptosis in cancer and I/R injury (Lei et al., 2020; Li et al., 2019; Xu et al., 2020b), however its role in NEC has not been reported. Here, we noticed that ACSL4 was among the 35 important ferroptosis-related genes; thus, we next sought to investigate its potential role in NEC. We compared the mRNA expression levels of ACSL4 based on microarray data of four NEC samples and four control intestinal samples, and intriguingly found that ACSL4 was significantly elevated in four NEC tissues than in four normal intestinal tissues (t-test; p = 0.002937; Figure 3D). Scatterplot also showed that ACSL4 expression levels were markedly positively correlated with ferroptosis levels in NEC (Pearson correlation test; $R$ = 0.96, p = 0.00016; Figure 3E). These findings strongly suggested that ACSL4 could be a key gene of ferroptosis in NEC.

**Validation of ACSL4 upregulation in ferroptosis based on human tissue and in vivo and in vitro experiments**

Because we had found that ACSL4 was significantly overexpressed and positively correlated with ferroptosis in NEC using bioinformatics analysis, we next wanted to investigate its protein levels in 10 human tissue and an erastin-induced intestinal epithelia cell ferroptosis model. The baseline characteristics of five NEC and five control infants were shown in Table 1. Immunohistochemistry (IHC) findings showed that the protein levels of ACSL4 was significantly upregulated in NEC tissues compared with their counterparts (t-test; p< 0.0001; Figures 4A and 4B), further supporting that ACSL4 could have an important role in NEC.

We then established an intestinal epithelial cell ferroptosis model using Caco-2 cell lines by adding erastin which is a small molecule that can cause ferroptosis (Gai et al., 2020). We examined the viability of intestinal epithelial cells using Cell Counting Kit-8 (CCK-8), and found that the cell viability in the ferroptosis group was significantly reduced than in the control group (one-way ANOVA, p< 0.001; Figures 4C and 4D). Western blot also demonstrated that the protein levels of ACSL4 were apparently increased in the ferroptosis group compared with the control group. Collectively, these findings indicated that ACSL4 could exert an important role in NEC.

**Investigation of the relationship between ACSL4 and NEC**

To assess the relationship between ACSL4 and NEC, we obtained the downstream effector gene set of ACSL4 and checked whether it was significantly enriched in NEC using GSEAPre-ranked approach. First, we obtained the downstream gene set of ACSL4 (Table S4) by differential expression analysis based on GSE181842 consisting samples with or without ACSL4 knockdown (Figures 5A and 5B). Then, we performed a GSEA preRank analysis using the downstream effector gene set of ACSL4 and microarray data from GSE46619, and found that the downstream gene set of ACSL4 was enriched in NEC patients (NES = 1.36, FDR = 0.11, p = 0.05; Figure 5C). Furthermore, we validated these results in another independent human NEC cohort (GSE64801) and observed a consistent result. That is, ACSL4 was significantly elevated in NEC (t test, p< 0.05; Figure 5D) and positively correlated with ferroptosis (R = 0.82, p = 0.013; Figure 5E). Also, the downstream gene set of ACSL4 was significantly enriched in NEC cohort from GSE64801 (NES = 3.18, FDR = 0.00, p = 0.00; Figure 5F). All these findings suggested that ACSL4 could have an effect on NEC.

Subsequently, we performed a functional enrichment analysis for the downstream gene set of ACSL4 using DAVID online tool to identify ACSL4-mediated downstream pathways. We found that pathways such as

| Table 1. Characteristics of NEC and control neonates |
|-----------------------------------------------|
| **NEC**          | **Control**          |
| Number of patients (n) | 5                  | 5                  |
| Gender (female/male)   | 3/2                | 2/3                |
| Mean birth weight      | 1.354              | 3.528              |
| Average gestational age at birth (in days) | 209 ± 23.274       | 278.2 ± 6.34       |
| DOL at diagnoses of NEC (days) | 18.86               | NA                 |
| Mode of delivery (vaginal/cesarean)  | 3/2                | 1/4                |
| Feeding model (breast milk/formula milk) | 2/3                | NA                 |
| DOL, Day of life; NA, not applicable.       |                     |
response to lipopolysaccharide, cytokine-mediated signaling pathway, cellular response to calcium ion and cytokine-cytokine receptor interaction were significantly enriched in NEC tissues (FDR <0.05). Then, we searched for pathogenic pathways in NEC in PUBMED and finally found 9672 NEC-related papers (up to April 26, 2022). Through reading these papers, we obtained 97 pathogenic pathways reported in NEC (Tables 2 and S5). We checked these pathogenic signaling pathways in functional enrichment results. Surprisingly, we found that several NEC-related signaling pathways were present in enriched signaling pathways for downstream gene set of ACSL4 (Figures 5G and 5H). Collectively, these results suggested that ACSL4 expression was associated with NEC-related signaling pathways, suggesting its potential role in NEC.

The association of ACSL4 with multiple types of RCD, hypoxia and inflammation in NEC

Because the above findings indicated that ACSL4 was implicated in ferroptosis in NEC, we wondered if ACSL4 participated in other types of RCD, including autophagy, apoptosis and pyroptosis. We first profiled the expression levels of autophagy, pyroptosis and apoptosis using ssGSEA based on transcriptional expression levels of corresponding marker genes from 4 normal intestinal tissues and 4 NEC neonates (Table S6). Then we conducted a heatmap analysis for RNA-derived levels of autophagy, pyroptosis and apoptosis, and found that RNA-derived levels of autophagy, pyroptosis and apoptosis were apparently increased in NEC than in control group (Figure 6A). Consistent with results of heatmap analysis, boxplot also revealed enhanced RNA-derived levels of autophagy (t-test; p = 0.0002065), apoptosis (t-test; p = 0.01583) and pyroptosis (t-test; p = 0.06) in NEC than in control group (Figure 6B). Furthermore, we revealed that ACSL4 was significantly associated with autophagy (R = 0.97, p = 0.000079), apoptosis (R = 0.95, p = 0.00028) and pyroptosis (R = 0.81, p = 0.015) (Pearson correlation test; Figures 6D–6F). All these results suggested that ACSL4 expression is significantly associated with other types of RCD including autophagy, apoptosis, and pyroptosis.
Figure 5. Investigation of the relationship between ACSL4 and NEC

(A) The expression levels of ACSL4 were significantly decreased in ACSL4-knockdown group compared with control group from GSE181842 consisting samples with or without ACSL4 knockdown (t test, p< 0.05).

(B) Downstream gene set of ACSL4 was obtained by differential expression analysis based on GSE181842 consisting samples with or without ACSL4 knockdown.

(C) Downstream gene set of ACSL4 was enriched in NEC patients from GSE46619 (NES = 1.36, FDR = 0.11, p = 0.05), indicating the relationship of ACSL4 in NEC.

(D) ACSL4 was significantly elevated in NEC than in control group (t test, p< 0.05) in NEC patients from GSE46619.

(E) ACSL4 was positively correlated with ferroptosis in NEC (R = 0.82, p = 0.013) in NEC patients from GSE46619.
Hypoxia and inflammation have been known as two essential factors in the development of NEC. Nevertheless, it is unknown whether ACSL4 is implicated in hypoxia and inflammation and thus induce NEC. We first calculated the expression levels of hypoxia and inflammation using ssGSEA based on microarray data from four normal intestinal tissues and four NEC neonates (Table S6) and found that RNA-derived levels of hypoxia (t-test; p = 0.003936) and inflammation (t-test; p = 0.0009731) were indeed augmented in NEC tissues compared with the control tissues (Figures 6A and 6B). Intriguingly, ACSL4 was also markedly correlated with hypoxia (R = 0.96, p = 0.00011) and inflammation (R = 0.96, p = 0.0002) (Pearson correlation test; Figures 6C and 6G). Moreover, to further investigate the relationship between ACSL4 and RCDs. We analyzed their relationship in another independent NEC cohort (GSE64801), and the results consistently demonstrated that ACSL4 was associated with these types of RCDs (Figure S1). These findings strongly indicate that ACSL4 could be also involved in hypoxia and inflammation and thus induce NEC.

**Effect of ACSL4 and ferroptosis on immune dysfunction of NEC**

Previous studies have implicated a role for intestinal immune cells in the pathogenesis of NEC (Emami et al., 2011; Klinke et al., 2020; MohanKumar et al., 2019; Pang et al., 2018; Wei et al., 2019), and we have previously found that ACSL4 was highly associated with inflammation (R = 0.96, p = 0.0002; Figure 6G). Therefore, we sought to investigate the association of ferroptosis and ACSL4 with intestinal immune cells in NEC. First, we calculated the abundance of immune cells in NEC tissues and normal tissues using ssGSEA based on microarray data from four normal intestinal tissues and four NEC neonates (Table S7), and surprisingly found that an overwhelming majority of immune cells were activated in NEC compared with normal intestinal tissues (Figure 7A and Table S8), suggesting an uncontrolled inflammatory storm present in NEC, which could induce resulting epithelial cell injury and intestinal barrier injury. Among these immune cells, several of them were reported to be activated in NEC including macrophages, regulatory T cells, neutrophils and activated dendritic cells (Emami et al., 2011; Klinke et al., 2020; Pang et al., 2018; Wei et al., 2019), whereas their association with ferroptosis in NEC is still unclear.

To ascertain the relationship of ferroptosis with intestinal immune cells, we performed a correlation analysis between ferroptosis and immune cells. We found that ferroptosis was significantly correlated with macrophage (R = 0.93, p = 0.00072), activated dendritic cell (R = 0.96, p = 0.0002), neutrophil (R = 0.95, p = 0.00023) and regulatory T cell (R = 0.75, p = 0.031) (Pearson correlation test; Figures 7B–7E). Surprisingly, the correlation coefficients were unbelievable high ranging from 0.75 to 0.96, which strongly suggested that there is a close connection between ferroptosis and immune dysfunction.

Because we have revealed a correlation between ferroptosis and immune response, we next explored if pro-ferroptosis driver ACSL4 has a role in immune disorders. We analyzed the relationship of ACSL4 with these immune cells. As expected, the results showed a positive relationship of ACSL4 with macrophage (R = 0.95, p = 0.000003), activated dendritic cell (R = 0.97, p = 0.000051), neutrophil (R = 0.98, p = 0.0000363) and regulatory T cell (R = 0.86, p = 0.0015) (Pearson correlation test; Figures 7F–7I), implying that ACSL4 could play a role in immune dysfunction and might be a critical regulator in the crosstalk between ferroptosis and immune dysfunction.

**Potential mechanisms underlying the effects of ACSL4 on NEC**

As the above results suggested that ACSL4 could have a substantial effect on multiple types of RCD and immune disorders in NEC neonates, we sought to interrogate the molecular mechanisms underlying the function of ACSL4 in NEC. To investigate the signaling pathways that correlated with NEC, we first performed differential expression analysis between NEC and control groups and obtained 1571 DE-Gs including 539 upregulated genes and 1032 downregulated genes. We then performed GO and KEGG enrichment analysis based on 539 upregulated genes between normal intestinal and NEC tissues using the website tool DAVID. The results showed that multiple immune-related signaling pathways were enriched in NEC, including inflammatory response, chemokine signaling pathway, TNF signaling pathway and neutrophil chemotaxis (p< 0.05; Figures 8A and 8B; Table S9). Also, we performed GSEA analysis based on microarray data of eight newborns to identify critical signaling pathways associated with NEC,
as well as signaling pathways correlated with ACSL4. GSEA results showed that multiple immune-related signaling pathways are significantly enriched in NEC group, including nod-like receptor signaling pathway, cytokine-cytokine receptor interaction and toll-like receptor signaling pathway (Table S10).

To investigate signaling pathways that correlated with ACSL4 in NEC, we divided eight neonates into the high-ACSL4 and the low-ACSL4 groups based on the median expression level of ACSL4, and performed GSEA analysis between groups. Intriguingly, most immune-related signaling pathways enriched in the NEC group were also significantly enriched in high-ACSL4 group, including nod-like receptor signaling pathway (NES = 2.52, FDR = 0.000), toll-like receptor signaling pathway (NES = 2.48, FDR = 0.000), JAK-STAT signaling pathway (NES = 2.03, FDR = 0.000), and chemokine-signaling pathway (NES = 2.40, FDR = 0.000) (Figures 8C–8F and Table S11), suggesting that ACSL4 could play a crucial role in the development of NEC through activating these signaling pathways.

Consistent with our findings, previous studies have also reported that toll-like receptor signaling pathway is a pivotal signaling pathway involved in NEC (MohanKumar et al., 2019; Ninño et al., 2016; Yu et al., 2019). We next investigated the effect of ACSL4 on the key components of toll-like receptor signaling pathway by comparing the expression levels of these pivotal components of toll-like receptor signaling pathway between the low-ACSL4 and high-ACSL4 groups. As expected, the main components of toll-like receptor signaling pathway, including TLR4, NFKB1, TLR5, CXCL8, IL6, IL1B, and CD14, were significantly increased in the high-ACSL4 group than in the low-ACSL4 group (Figure 8G).

### DISCUSSION

NEC is a devastating and life-threatening inflammatory disease in neonates worldwide. Although evidence shows that various risk factors are involved in the pathogenesis of NEC, the provocative events leading to NEC remain largely unclear (Ninño et al., 2016). In this study, ferroptosis was discovered in NEC (Figures 1, 2, and 3), and we are surprised to find that ACSL4, as a key regulator of ferroptosis execution, significantly induced ferroptotic cell death (Figures 3, 4, and 5) and correlated with overwhelming immune dysfunction in NEC (Figure 7). Further investigation revealed that ACSL4 may take part in NEC through activating NEC related signaling pathways (Figure 8). Besides, we also uncovered that ACSL4 could participate in autophagy, pyroptosis, apoptosis, hypoxia and inflammation in NEC (Figure 6), suggesting that ACSL4 plays a more extensive role in gut injury of NEC.

Discovery of ferroptosis in NEC tissues is of great importance, and will provide a new insight into NEC research. In the last two years, studies have revealed the role of ferroptosis in intestinal diseases (Xu et al., 2021) including ulcerative colitis (Tang et al., 2021a; Xu et al., 2020a), Crohn’s disease (Mayr et al., 2020) and intestinal I/R injury (Deng et al., 2021; Li et al., 2019), illustrating that ferroptosis could exist in gut injury. Compared with adults, neonates have a reduced activity of antioxidant enzymes, which makes infants vulnerable to oxidative stress (Osiak et al., 2020). ROS accumulation because of oxidative stress is believed to play a key pro-inflammatory role in NEC (Zhang et al., 2019) as well as ferroptosis. Based on the theories above, it is quite reasonable that ferroptosis is present in NEC neonates.

| No. | Key molecular mechanism                                      |
|-----|-------------------------------------------------------------|
| 1   | TLR4 related signaling pathway                              |
| 2   | NF-κB related signaling pathway                             |
| 3   | Cytokine-cytokine signaling pathway                         |
| 4   | Chemokine-chemokine signaling pathway                       |
| 5   | SIRT1 related signaling pathway                             |
| 6   | Tight junction related signaling pathway                    |
| 7   | LPS induced signaling pathway                               |
| 8   | MAPK related signaling pathway                              |
| 9   | NOS related signaling pathway                               |
| 10  | ERK1/2 related signaling pathway                            |

Table 2. Key molecular mechanisms in NEC
Because lipid peroxidation is a determining factor of ferroptosis, we next explored the mechanisms of ferroptosis in NEC tissues by investigating the role of ACSL4 in our study. ACSL4 is a member of acyl-CoA synthetases (ACS) family that convert fatty acids to fatty acyl-CoA esters and has a substrate selectivity for arachidonic acid (AA). After catalyzed by ACSL4 and other enzymes, AA/Ada is converted to PE-AA-OH/PE-AdA-OH to promote ferroptosis (Li and Li, 2020). Our study showed that ACSL4 was significantly upregulated and consistent with ferroptosis and NEC through both in vivo and in silico experiments. Moreover, ACSL4 may also play a role in other forms of RCD. Previous studies have showed a causality association between ACSL4 and apoptosis mostly in cancer related studies (Wang et al., 2020) (Kwon et al., 2021); several studies just linked ACSL4 with autophagy, for ACSL4 is a ferroptosis regulators and ferroptosis may participant in autophagy (Hu et al., 2021; Sha et al., 2021; Tang et al., 2021b); however, whether ACSL4 has a link with pyroptosis remains elusive. More research is needed to clarify the relationship between ACSL4 and RCDs. Nevertheless, we found that ACSL4 plays a crucial role in NEC probably through multiple types of RCD, and could be a potential target for NEC treatment.
We also analyzed various types of immune cell infiltration in NEC tissues and normal intestinal tissues using bioinformatics approaches based on microarray data, which is difficult to perform using wet experiments. To our astonishment, we found that more than ten types of immune cells were activated and infiltrated in NEC compared with normal intestinal tissues.

**Figure 7. Effect of ACSL4 and ferroptosis on immune dysfunction of NEC**

(A) An overwhelming majority of immune cells were activated in NEC compared with normal intestinal tissues. (B–E) Correlation analysis showed that ferroptosis were correlated with macrophage (Pearson correlation test; \( R = 0.93, p = 0.00072 \)), activated dendritic cell (Pearson correlation test; \( R = 0.96, p = 0.0002 \)), neutrophil (Pearson correlation test; \( R = 0.95, p = 0.00023 \)) and regulatory T cell (Pearson correlation test; \( R = 0.75, p = 0.031 \)) in NEC. (F–I) Correlation analysis revealed that ACSL4 was correlated with macrophage (Pearson correlation test; \( R = 0.95, p = 0.00003 \)), activated dendritic cell (Pearson correlation test; \( R = 0.97, p = 0.00051 \)), neutrophil (Pearson correlation test; \( R = 0.98, p = 0.0000036 \)) and regulatory T cell (Pearson correlation test; \( R = 0.81, p = 0.014 \)) in NEC.

We also analyzed various types of immune cell infiltration in NEC tissues and normal intestinal tissues using bioinformatics approaches based on microarray data, which is difficult to perform using wet experiments. To our astonishment, we found that more than ten types of immune cells were activated and infiltrated in NEC.
Figure 8. Potential mechanisms underlying the effects of ACSL4 on NEC
(A and B) GO and KEGG enrichment analysis suggested that multiple immune-related signaling were enriched in NEC.
(C–F) GSEA analysis showed that pathways including nod-like receptor signaling pathway (NES = 2.48, FDR = 0.000), JAK-STAT signaling pathway (NES = 2.03, FDR = 0.000), and chemokine-signaling pathway (NES = 2.40, FDR = 0.000) were significantly enriched in the high-ACSL4 group compared with the low-ACSL4 group.
(G) Main components of toll-like receptor signaling pathway, including TLR4, NFKB1, TLR5, CXCL8, IL6, IL1B, and CD14, were significantly increased in the high-ACSL4 group than in the low-ACSL4 group.
NEC tissues and positively correlated with ferroptosis. This illustrates that there is a catastrophic immune disorder in NEC. Among these highly activated immune cells were neutrophils, macrophages and dendritic cells, which are consistent with previous studies (Emami et al., 2011; Klinke et al., 2020; Pang et al., 2018; Wei et al., 2019). Different from previous study, anti-inflammatory regulatory T cells was increased in NEC in our study. Because regulatory T acts as an anti-inflammatory factor to balance the immune response in intestinal tissues, potential mechanism is a negative feedback loop. In addition, we discovered that activation of immune cells was closely related to ACSL4, and ACSL4 may also be derived from immune cells (Roelands et al., 2019). We also speculated that ACSL4 may be as a novel regulator of NEC through toll-like receptor signaling pathway. Collectively, our findings indicated that ferroptosis might be associated with extensive immune-related gut injury in NEC neonates.

Ferroptotic cell death is a warning signal of immune-mediated damage and associated with changes in multiple immune cells (Friedmann Angeli et al., 2019) (Proneth and Conrad, 2019). Here, we indeed found that ferroptosis was positively correlated with the abundance of multiple immune cells including macrophage, activated dendritic cell, and neutrophil and regulatory T cell. It is reported that inhibition of ferroptosis and ferroptosis-related molecules has been used in the treatment of cancer and intestinal inflammation (Wang et al., 2021a). For example, HMGB1 can be released to regulate immune response due to plasma member rupture in ferroptotic cells (Wen et al., 2019) (Kumar et al., 2019), and inhibition of HMGB1 can improve the intestinal inflammation in NEC via TLR4/NF-kB signaling pathways (Yu et al., 2019). However, whether anti-ferroptosis therapy could be applied to NEC deserves further investigation.

In summary, our results first link ferroptosis to NEC, and reveal that ACSL4 may induce ferroptosis and immune cell activation in NEC through NEC related signaling pathway. ACSL4 could be a key regulator of crosstalk between multiple types of RCD, hypoxia and inflammation in NEC. These findings could provide insights into current understanding of the role of ferroptosis and its translational use in NEC treatment.

Limitations of the study
Several limitations exist in this study, which are mainly due to rarely acquired tissue samples. According to disease severity of NEC, pediatric surgeons will offer primary peritoneal drainage or primary exploratory laparotomy (mainly enterostomy) for recovering bowel to maximize the length of viable intestine (Robinson et al., 2017). As a result, only a few patients will receive enterectomy. For this reason, sample sizes are small in both microarray data and in vitro experiment. Another limitation of this study is that there is a lack of application of anti-ferroptosis treatment in NEC. However, it is a promising treatment for NEC infants, warranting further research.

ETHICS STATEMENT
This study was approved by the Ethics Committee of First Hospital of Jilin University (No. 2021-0493).

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
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Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105406.

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AUTHOR CONTRIBUTIONS

D.D.: Performed study concept and design, performed development of methodology, analysis and interpretation of data, writing – original draft, read and approved the final paper. C.Z.: Analysis and interpretation of data, statistical analysis, writing – original draft, read and approved the final paper. Z.M.: Performed study concept and design, provided technical and material support, read and approved the final paper. X.L.: Analysis and interpretation of data, read and approved the final paper. J.W.: Analysis and interpretation of data, read and approved the final paper. H.W.: Performed study concept and design, project administration, writing – review & editing, read and approved the final paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Rabbit monoclonal anti-ACSL4 | Abcam | Abcam Cat# ab155282, RRID:AB_2714020 |
| Goat monoclonal anti-β-tubulin | Abcam | Abcam Cat# ab6160, RRID:AB_305328 |
| Chemicals, peptides, and recombinant proteins | | |
| Liproxstatin-1 | Selleck | S7699 |
| Lipopolysaccharide | Sigma | L2630 |
| Critical commercial assays | | |
| Iron assay kit | leagene biotechnology | TC1015 |
| Lipid Peroxidation MDA Assay Kit | Beyotime | S0131S |
| Experimental models: Cell lines | | |
| Caco-2 | ATCC | ATCC® HTB-37™ |
| Software and algorithms | | |
| TEM system | Hitachi H7700 TEM system | Hitachi |
| Microscope | Olympus | BX50F4 |
| Electro Chemi Luminescence (ECL) detection kits | BIO-RAD | Chemi Doc XRS+ |

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hui Wu (wuhiu@jlu.edu.cn).

Materials availability
This study did not generate unique reagents.

Data and code availability
Data reported in this paper will be shared by the lead contact upon request.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human intestinal tissue samples
Intestinal tissues of 10 patients (5 male and 5 female) were collected in Neonatology Department in the First Hospital of Jilin University for immunohistochemistry (IHC) and transmission electron microscope (TEM). This study was approved by the institutional research ethics committee (No. 21K08-001). Written informed consents were obtained from parents of enrolled infants.

NEC mouse model induction and drug treatment
C57BL/6 mice at postnatal day 7 were purchased from QIANHE BIOTECH (http://www.qhbiotech.cn/#index) and kept in the specific pathogen-free conditions. Hypoxia-hypothermia-LPS were applied to establish NEC mouse model(Wang et al., 2019), and liproxstatin-1(Lip-1, Selleck, S7699) was used as a selective ferroptosis inhibitor. Mice of 7-day-old were divided into control group, NEC group and NEC+Lip-1.
To induce NEC gut injury, mice were exposed to hypoxia (5% O₂ and 95% N₂) for 5 minutes and cold stress for 10 minutes, three times daily for 4 days; Lipopolysaccharide (LPS, Sigma, L2630) was gavage fed 4 mg/kg after hypoxia and hypothermia, once daily. Before hypoxia-hypothermia-LPS feeding, Lip-1 was intraperitoneally injected with 10 mg/kg (dissolved in Ethanol warmed with 50°C water bath), once daily for 4 days in the NEC+Lip-1 group. We have not detected any sex differences related to our experiments. In vivo study was approved by the Ethics Committee of First Hospital of Jilin University (No. 2021-0493).

**Cell culture and drug treatment**

Caco-2 cells (donated by the Department of Translational Medicine Research Institute, First Hospital of Jilin University) are derived from a human colon adenocarcinoma and functionally resembling the small intestinal epithelium (Smetanová et al., 2011). Cells were cultured with minimum essential medium (Procell, PM150410, Wuhan, China) containing 20% fetal bovine serum, 100 μg/mL BIOMYC-3 antibiotic solution (Biological Industries, 03-038-1B, Israel) and 100 U/mL penicillin-streptomycin (TransGen Biotech, FG101-01, Beijing, China) at 37°C in a CO₂ incubator.

**METHOD DETAILS**

**Acquisition of microarray data**

Microarray normalized data of 13 newborns were downloaded from Gene expression omnibus (GSE46619), including five NEC neonates, four spontaneous intestinal perforation neonates (SIP) and four surgical controls with congenital intestinal conditions regarded as normal intestinal tissues. We excluded four SIP neonates and one NEC neonate with low quality of microarray data (GSM1133300) from this study. Eventually, microarray data of four NEC tissues and four congenital intestinal malformation tissues were used to analyze in this study.

A total of 259 ferroptosis-related genes, including 108 drivers, 69 suppressor and 111 markers, were downloaded from FerrDb database (Zhou and Bao, 2020) on February 15, 2021 (Table S1). Driver (108) + Suppressor (69) + Marker (111) = 288, which is larger than the Gene count (259) due to 28 multi-annotated genes.

**Principal component analysis**

Principle component analysis (PCA) is used in exploratory data analysis. It is commonly used for dimensionality reduction by projecting each data point onto only the first few principal components to obtain lower-dimensional data while preserving as much of the data’s variation as possible. The first principal component can equivalently be defined as a direction that maximizes the variance of the projected data. Thus, it can be shown that the principal components are eigenvectors of the data’s covariance matrix. Here, we determined whether the grouping information for microarray or RNAseq data of neonates was significant through observing the first two components using PCA.

**Differential expression analysis**

We performed differentially expressed genes (DEGs) analysis based on microarray or RNAseq data using R package limma (Ritchie et al., 2015), which implements a series of statistical methods including empirical Bayes estimation, exact tests, generalized linear models, and quasi-likelihood tests. Selection criteria for DEGs were as follows: |logFC| > 1 and FDR <0.05.

**Iron assay**

The intracellular iron concentration of intestinal tissues was detected by iron assay kit (Iron assay kit, TC1015, Leagene Biotechnology, Beijing) according to the manufacturer’s instructions.

**MDA assay**

High intracellular malondialdehyde (MDA) levels indicates lipid peroxidation. The intracellular MDA concentration of intestinal tissues was assessed using Lipid Peroxidation MDA Assay Kit (Lipid Peroxidation MDA Assay Kit, S0131S, Beyotime, Shanghai) according to the manufacturer’s instructions.

**Transmission electron microscope**

TEM was used for the morphological examination of epithelial cell death. Samples of intestinal tissues were acquired softly and immediately after loss of blood supply. Tissues less than 1 mm × 1 mm × 1 mm was fixed in
glutaraldehyde, washed by 0.1M phosphate buffer (PH 7.4), fixed in 1% osmic acid, dehydrated in ethanol, saturated by acetone and SPI-pon812(SPI, 90529-77-4), and embedded in SPI-pon812. Finally, the samples were sliced with a thickness of 60–80nm, double-stained with 2% uranyl acetate and lead citrate and detected using a Hitachi H7700 TEM system (Hitachi, Japan) at 80 kV.

**Immunohistochemistry**

IHC was used to detecting the protein levels of ACSL4 in NEC and normal tissues. Intestinal tissues were paraffin-embedded after fixed by 10% formalin. The wax blocks were sliced continuously with a thickness of 4 μM and then baked the blank slices in an oven. The slides were deparaffinized, hydrated, processed for antigen retrieval (Tris/EDTA buffer pH 9.0, Absin, 9342) under high pressure, and blocked endogenous activity by perhydrol liquid. The slides were then incubated overnight at 4°C with primary antibodies (dilution 1:400; Abcam, ab155282). Secondary antibodies (goat anti-rabbit IgG-HRP, dilution 1:20000; Absin, 20040ss) were incubated for 1 hour at room temperature. Finally, the slides were 3',3'-diaminobenzidine (DAB) for 3–10min and retained by Haematoxylin before observation and analysis under a microscope (Olympus BX50F4).

**Western blot**

Caco-2 cells (1.8×10⁵/1mL) were seeded in 12-well plates overnight. Cells were collected 40 hours after erastin were added. Total intracellular proteins were extracted in cold RIPA Lysis Buffer (Solarbio, R0010), and protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Catalog No. 23227). Samples were isolated by 12% SDS-PAGE gel electrophoresis and then isolated proteins were transferred onto a PVDF membrane. The PVDF membrane was sealed for 1 h in 5% skimmed milk. Anti-ACSL4 (dilution 1:20000; Abcam, ab155282) or anti-Tubulin (dilution 1:5000; Abcam, ab6160) was added and incubated at 4°C overnight. The membrane was thoroughly washed using tris buffered saline tween (TBST) three times for 10 min each time. The PVDF membrane was sealed into the HRP-IgG antibody (dilution 1:10000; Abcam, ab6721). The membrane was incubated in a shaker at room temperature for 1 h and then washed with TBST three times for 8 min each time. Protein bands was visualized by the Electro Chemi Luminescence (ECL) detection kits (Chemi Doc XRS+, BIO-RAD, USA).

**GSEAPreranked**

GSEAPreranked is another type of Gene Set Enrichment Analysis (GSEA) based a priori defined set of genes and a user-supplied, ranked list of genes, and determines whether a priori defined set of genes show statistically significant enrichment at either end of the ranking. A statistically significant enrichment indicates that the biological activity (e.g., ACSL4-mediated biological activity) characterized by the gene set is correlated with the user-supplied ranking. Here, we acquired a priori defined set of ACSL4-mediated genes based on an in vivo experiment of ACSL4-knockdown, and ranked all genes in NEC based on their correlation with ACSL4. Then, we performed a GSEAPreranked analysis to determine whether the downstream genes of ACSL4 was significantly enriched in NEC. According to the protocol of GSEA, eligible criteria are as follow: NES>1, FDR <0.25 and p < 0.05.

**Calculation of the abundance of diverse immune cells in NEC tissues**

To quantify the abundance of various types of immune cells in NEC tissues, we performed ssGSEA analysis using R package “GSVA” (Hänzelmann et al., 2013). Gene set variation analysis (GSVA) is a non-parametric, unsupervised method to calculate variation of gene set enrichment through the samples of an expression data set. The gene sets of marker genes for distinct immune cells are obtained from previous literature (Charoentong et al., 2017), and available in Table S7.

**Functional annotation**

We performed gene annotation using the Database for Annotation, Visualization and Integrated Discovery (DAVID, v6.8) (Huang et al., 2009). Gene annotation includes gene oncology (GO) and kyoto encyclopedia of genes and genomes (KEGG) pathway analyses. GO comprises three independent categories: biological process (BP), molecular function (MF) or cellular component (CC). Terms with FDR <0.05 are considered as significantly enriched.

To re-examine the findings of DAVID, we performed gene set enrichment analysis (GSEA, v3.0) (Subramanian et al., 2005) using microarray data for 8 neonates from GSE46619. GSEA is a computational approach...
that determines whether a priori defined gene set shows statistically significant between two biological states (e.g. phenotypes). The key parameters were set as follows: the number of permutations at 1000, weighted enrichment statistic, metric for ranking genes (Signal2Noise), max size (500), and min size (15). The selection criteria include FDR <0.05 and |NES| > 1.

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical analyses were performed using GraphPad Prism 9.0 software (GraphPad Software, San Diego, CA, USA). The statistical significance between or within groups was determined using One-way ANOVA or t-tests. Differences were considered statistically significant with a two-tailed p value <0.05.