# *SIGIRR* Genetic Variants in Premature Infants With Necrotizing Enterocolitis

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abstract

Necrotizing enterocolitis (NEC) is a severe form of bowel disease that develops in premature infants. Although animal data and human studies suggest that aberrant activation of the intestinal immune system contributes to NEC, the pathogenesis remains unclear. We hypothesized that inherited defects in the regulation of Toll-like receptor signaling can contribute to NEC susceptibility in premature infants. A forward genetic screen done in an infant with lethal NEC using exome sequencing identified a novel stop mutation (p.Y168X) and a rare missense variant (p.S80Y) in *SIGIRR*, a gene that inhibits intestinal Toll-like receptor signaling. Functional studies carried out in human embryonic kidney cells and intestinal epithelial cells demonstrated that *SIGIRR* inhibited inflammation induced by lipopolysaccharide, a cell wall component of Gram-negative bacteria implicated in NEC. The genetic variants identified in the infant with NEC resulted in loss of *SIGIRR* function and exaggerated inflammation in response to lipopolysaccharide. Additionally, Sanger sequencing identified missense, stop, or splice region *SIGIRR* variants in 10 of 17 premature infants with stage II+ NEC. To the best of our knowledge, this is one of the first reports of a phenotype associated with *SIGIRR* in humans. Our data provide novel mechanistic insight into the probable causation of NEC and support additional investigation of the hypothesis that inherited defects in the regulation of innate immune signaling can contribute to NEC susceptibility in premature infants.

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Necrotizing enterocolitis (NEC), a severe form of bowel disease that develops in 5% to 14% of premature infants, has a mortality rate of 25% to 40%.[1] Although risk factors that contribute to mucosal injury (patent ductus arteriosus, hypoxia) or aberrant intestinal colonization (formula feeds) are implicated in NEC, its pathogenesis remains unclear.[1–4] Animal data and human studies suggest that aberrant activation of intestinal immune responses by gut bacteria can trigger inflammation and mucosal injury in NEC.[3–5] In the immature intestine, the Toll-like receptor (TLR) family of pathogen recognition receptors maintains the critical balance between bacterial tolerance and intolerance.[3,6] We hypothesized that loss of function variants in genes that inhibit intestinal TLR signaling will contribute to NEC pathogenesis. In this study, we identified a potential candidate TLR pathway gene that can contribute to NEC susceptibility.

## METHODS

### Infant Recruitment

Infants were recruited under the auspices of an institutional review board–approved study to investigate the impact of genetic variation on diseases of prematurity. After informed consent was obtained, a blood sample was collected for DNA extraction.

Deidentified clinical data were stored in a password-protected database.

### Exome Capture and Analysis

We extracted DNA from blood samples by using the FlexiGene DNA kit (Qiagen, Inc, Alameda, CA). We performed exome capture by using 5 $\mu$g of genomic DNA with the Agilent v4 Exome capture kit (Agilent, Inc, Santa Clara, CA). The captured fragments were sequenced on the HiSeq 2000 with Illumina's TruSeq technology (Illumina, Inc, San Diego, CA) to an average depth of >40 reads per target base. Image files were processed into binary read files, transferred to an analysis server, and aligned to the human reference sequence hg19/GRCh37 via the Illumina CASAVA pipeline. Variants with respect to GRCh37 were identified and imported into our clinical laboratory's variant annotation tool "Carpe Novo" and then annotated for functional impact.[7]

### Statistical Analysis

Data are represented as mean ± SD. Changes in nuclear factor $\kappa$-light-chain-enhancer of activated B cells (NF-$\kappa$B) activation with lipopolysaccharide (LPS) were expressed relative to controls and compared between groups through analysis of variance (ANOVA). For interleukin-8 (IL-8) protein levels, absolute values were compared between groups through ANOVA. Interleukin-6 (IL-6) and inducible nitric oxide synthase (iNOS) RNA levels were expressed as a fold-change relative to controls and analyzed with ANOVA. The post hoc Tukey test was used in conjunction with ANOVA to correct for multiple comparisons. A $P < .05$ was considered significant.

### Modeling Approach to Demonstrate Increased Prevalence of *SIGIRR* Variants in Infants With NEC

To determine whether there is a greater prevalence of potentially deleterious *SIGIRR* variants in infants with NEC when compared with the general population, we used the strategy recommended by MacArthur et al.[8] We constructed a variation site frequency spectrum (SFS) of *SIGIRR* variants from the exome variant server control cohort. The null model of the SFS considers identifying the pathogenic classes of variant present in that gene in the control cohort and calculating the probability of sampling variants of the same class of pathogenicity in the study population. Specifically, we evaluated the rate of predicted "null mutations," splice region mutations (ie, a variant occurring within 10 bases of the canonical splice site), and missense variants predicted by PolyPhen-2 to be damaging to the *SIGGR* function. Among the 18 cases of NEC we identified 5 potentially deleterious variants (Table 1). Performing the same estimates on the exome variant server cohort, we identified 380 variants (Table 2). In the exome variant server cohort, not all loci had the same allele counts. Therefore, for statistical analysis we took the site with the lowest number of cases as the denominator. Performing a $\chi^2$ test ($P < .001$, Pearson's $\chi^2$) we obtained a significance result.

### Other Methods

These methods are described in the Supplemental Material.

## RESULTS AND DISCUSSION

### *SIGIRR* Mutations in Proband With NEC

The proband was born at 23 weeks and 4 days' gestation with a birth weight of 560 g. On day of life (DOL) 50, while on full enteral feeds, the infant developed apneas of increasing severity, with 1 large gastric residual warranting increase in respiratory support. On DOL 51, he was noted to have abdominal distension, and radiography showed diffuse pneumatosis intestinalis. Despite intensive medical care there was clinical deterioration warranting surgery, which revealed NEC totalis with pan-pneumatosis and necrosis. We performed whole exome sequencing followed by variant identification by using the Illumina Hi-Seq 2000 and a custom bioinformatics tool developed in our institution.[7] We screened >19 000 exonic variants to identify complete loss-of-function variants. Variant prioritization based on gene function, relevance to immune signaling, and gastrointestinal disease identified 2 potentially deleterious variants in *SIGIRR* (NM_021805.2), a gene that inhibits TLR signaling.[9,10] The stop variant (p.Y168X, c.504C>G) is novel because it is not found in >6200 individuals belonging to the National Heart, Lung, and Blood Institute exome cohort (snp.gs.washington.edu/EVS). The other variant (p.S80Y, c.239C>A, rs117739035) has a mean allele frequency (MAF) of ~0.02. We performed Sanger sequencing to confirm the presence of both *SIGIRR* variants in our proband.

### *SIGIRR* Variants Result in Unregulated TLR4-Mediated Inflammation

Activation of TLR4, which senses LPS from Gram-negative bacteria, is posited to be a central event in NEC pathogenesis.[3,5,11] Therefore, we investigated whether *SIGIRR* variants dysregulated TLR4-mediated inflammation. We carried out functional analyses in human embryonic kidney cell line (HEK293) by using LPS. LPS-induced NF-$\kappa$B activation (a sign of inflammation) and IL-8 protein expression at 20 hours was strongly inhibited in cells transfected with wild-type *SIGIRR* (SIG-wT) (Fig 1). Similarly, *SIGIRR* attenuated LPS-induced IL-6 and iNOS RNA expression at 8 hours (Fig 1). Transfection with mutant *SIGIRR* (SIG-dM) encoding both variants (p.Y168× and p.S80Y) identified in the infant with NEC abolished *SIGIRR* function and restored TLR4-mediated NF-$\kappa$B activation, IL-8, IL-6, and iNOS expression (Fig 1). These data show

**TABLE 1** Distribution of *SIGIRR* Variants in Infants With Stage II+ NEC

| Infant | Variant rs Number | Protein Change | MAF | GA, wk | Bwt, g | Gender | Race | Feed Type | PDA | NEC Stage | DOL |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1[a] | Novel and rs117739035 | p.Y168X, p.S80Y | Novel, 0.02 | 23 | 560 | M | AA | BOTH | Yes | 3 | 51 |
| 2 | | | | 29 | 1240 | F | AA | FM | No | 3 | 11 |
| 3 | rs111819059 | p.P115R | 0.006 | 26 | 680 | F | Cau | BM | Yes | 2 | 64 |
| 4 | | | | 24 | 800 | M | Cau | BOTH | Yes | 2 | 30 |
| 5[b] | rs3210908 | p.Q312R | 0.18 | 30 | 1310 | M | Cau | BM | No | 2 | 18 |
| 6[b] | rs3210908 | p.Q312R | 0.18 | 30 | 1340 | M | Cau | BM | No | 2 | 14 |
| 7 | | | | 24 | 660 | M | Cau | BOTH | Yes | 2 | 64 |
| 8 | rs3210908 | p.Q312R | 0.18 | 29 | 1475 | M | Cau | BM | No | 2 | 12 |
| 9 | | | | 26 | 850 | M | AA | BOTH | No | 3 | 26 |
| 10[c] | Novel and rs117739035 | p.Y168×, p.S80Y | Novel, 0.02 | 23 | 630 | M | AA | BOTH | Yes | 2 | 61 |
| 11 | | | | 28 | 1140 | F | AA | BOTH | No | 3 | 10 |
| 12 | rs111819059 | p.P115R | 0.006 | 24 | 580 | M | Cau | BM | No | 3 | 16 |
| 13 | | | | 25 | 940 | M | Cau | FM | Yes | 3 | 14 |
| 14 | rs3210908 | p.Q312R | 0.18 | 24 | 780 | F | AA | BM | No | 3 | 13 |
| 15 | rs201897529[d] | Splice region variant | 0.002 | 25 | 780 | F | Cau | FM | Yes | 3 | 40 |
| 16 | rs111819059 | p.P115R | 0.006 | 24 | 300 | F | Cau | BM | Yes | 2 | 65 |
| 17 | | | | 27 | 1025 | F | AA | FM | No | 3 | 13 |
| 18 | rs3210908 | p.Q312R | 0.18 | 29 | 1320 | F | Cau | BM | No | 2 | 14 |

AA, African American; BM, breast milk; BOTH, breast milk and formula feeds; Bwt, birth wt; Cau, Caucasian; DOL, day of life for NEC onset; FM, formula milk; GA, gestational age; PDA, patent ductus arteriosus; rs number, reference single nucleotide polymorphism number.

[a] Infant 1 was the proband.

[b] Another pair of twins.

[c] Twin of infant 1.

[d] Variant predicted by SpliceAid (http://www.introni.it/splicing.html) to alter exon splicing.[16]

that although *SIGIRR* inhibits inflammation mediated by bacterial ligands, the variants identified in our proband result in loss of regulation of LPS-mediated inflammation.

To investigate whether *SIGIRR* variants alter intestinal signaling, we performed studies in IEC-18, a nontransformed small intestinal epithelial cell line.[12] Although *SIGIRR* (SIG-wT) inhibited LPS-induced NF-κB activation, IL-8, IL-6, and iNOS RNA expression, mutant *SIGIRR* (SIG-dM) abolished *SIGIRR* function and restored LPS responsiveness (Supplemental Fig 2). Furthermore, *SIGIRR* mediated inhibition of LPS-induced cleaved caspase-3 expression was abolished with mutant *SIGIRR* (Supplemental Fig 2). These data show that *SIGIRR* inhibits TLR4-mediated inflammation and apoptotic signaling in intestinal epithelial cells.

### *SIGIRR* Variants in Other Infants With NEC

We sequenced the coding region of *SIGIRR* in 17 premature infants with stage II+ NEC. The distribution of clinical and epidemiologic variables in infants with NEC is shown in Table 1. The twin of our proband who survived stage II NEC had both the p.Y168× and p.S80Y variants. Five infants had the missense p.Q312R variant (rs3210908, MAF = 0.18), 3 had the rare missense p.P115R variant (rs111819059, MAF = 0.006), 1 had the rare splice region variant (rs201897529, MAF = 0.002), and the other 7 did not have missense, splice, stop, or frameshift exonic *SIGIRR* variants. We modeled the SFS approach to compare the prevalence of potentially deleterious *SIGIRR* variants among NEC infants ($n$ = 18) with the exome variant server cohort.[8] This approach revealed a significant excess of deleterious *SIGIRR* variants ($P < .001$) in our cohort compared with the general population (Table 2). Additionally, we sequenced DNA from 20 preterm infants without NEC. We did not find the novel (p.Y168×), rare exonic (p.S80Y, p.P115R), or rare splice region variant (rs201897529). Only the benign, common missense variant (p.Q312R) was found in 4 infants. These data show that functional or rare *SIGIRR* variants are more common in infants with NEC.

**TABLE 2** Modeling Approach to Demonstrate Greater Prevalence of *SIGIRR* Variants in Infants With NEC

| Cohort | Total Tested Alleles | Missense Variants | Splice Region Variants | Stop Variants | Total Deleterious Alleles | Normal Alleles |
|---|---|---|---|---|---|---|
| NEC | 36 | 2 | 1 | 2 | 5 | 31 |
| Exome variant server | 12 317 | 352 | 27 | 1 | 380 | 11 937 |

SFS analysis was performed to compare frequency of deleterious *SIGIRR* alleles in our cohort with the exome variant cohort (see the Methods section for description). This revealed an increase in *SIGIRR* variants in the NEC cohort compared with the general population ($P < .001$).

### *SIGIRR* and NEC

*SIGIRR* is a major negative regulator of intestinal inflammation mediated by TLRs.[9,10,13] However, to the best of our knowledge this is one of the first reports of a phenotype associated with *SIGIRR* in humans. Of the 2 variants identified in our proband and his twin, the stop variant (p.Y168×) has not been found among the genome of >6000 adults.

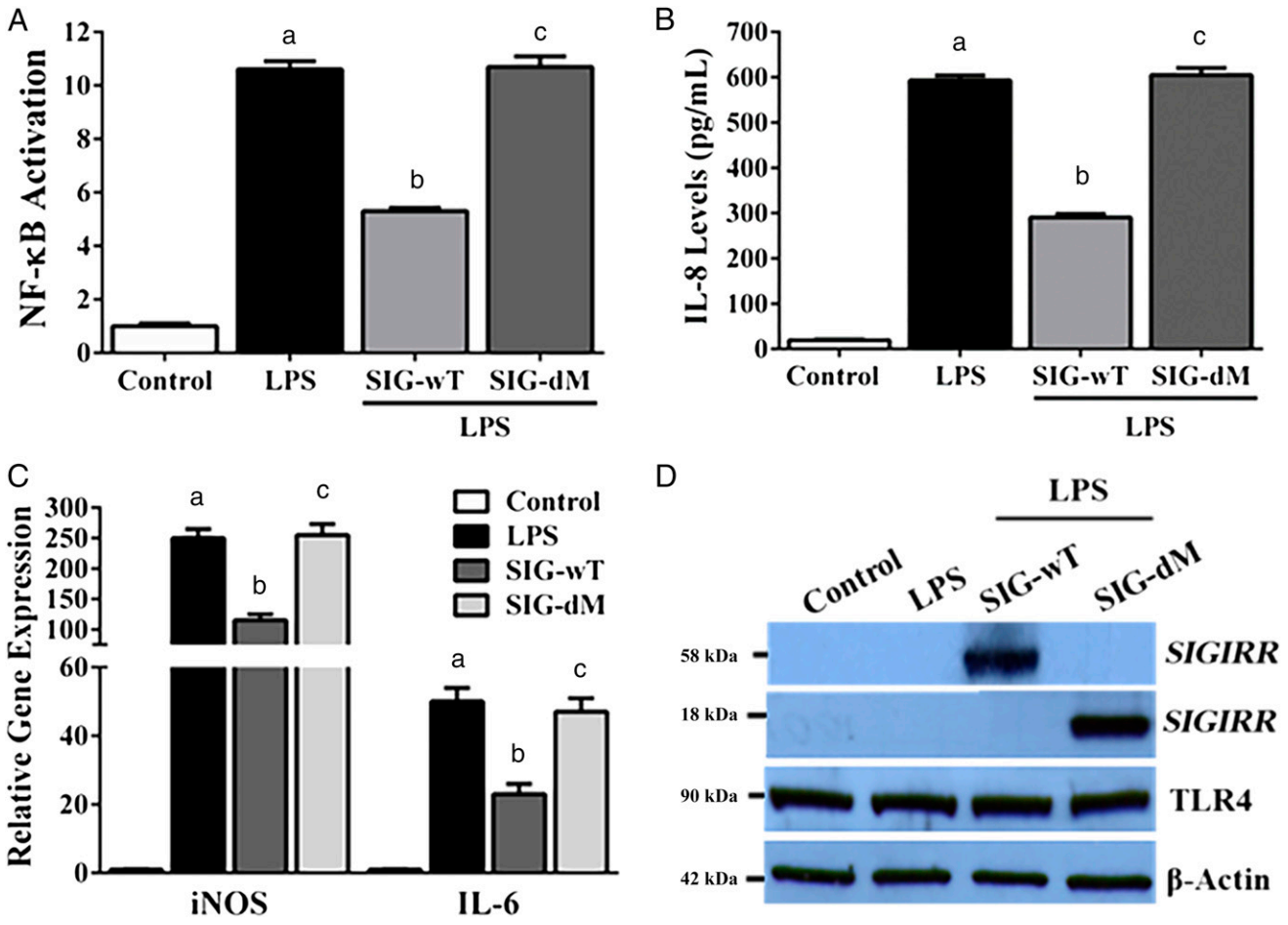


**FIGURE 1**

Effect of *SIGIRR* variants on LPS-induced inflammation; HEK293 cells transfected overnight with plasmids encoding empty plasmid, the reference allele (SIG-wT), and both *SIGIRR* variants (SIG-dM) were treated with LPS (100 ng/mL). Culture supernatants or cell lysate protein were used for experiments. A, NF-$\kappa$B activation was quantified in culture supernatants 20 hours after LPS, as described in the Methods section. Fold-increase in NF-$\kappa$B activation relative to control is shown ($n$ = 4). $P < .05$ for all comparisons. B, IL-8 protein was quantified 20 hours after LPS treatment in cell culture supernatants by enzyme-linked immunosorbent assay ($n$ = 4). $P < .05$ for all comparisons. C, IL-6 and iNOS RNA expression were quantified in cell lysates by quantitative reverse transcription PCR 8 hours after LPS treatment. Fold-increase in expression relative to control is shown ($n$ = 3). $P < .05$ for all comparisons. D, Whole cell-lysate protein (20 $\mu$g) obtained 20 hours after LPS treatment was immunoblotted for *SIGIRR*, TLR4, and $\beta$-actin ($n$ = 4). Note: The stop variant in mutant *SIGIRR* plasmid (SIG-dM) encodes a truncated *SIGIRR* protein (18 kDa). [a]Control versus LPS. [b]LPS versus LPS + SIG-wT. [c]LPS + SIG-wT versus LPS + SIG-dM.

Although the twin of our proband also had both the *SIGIRR* variants, he survived stage II NEC. Whether differences in their outcomes were caused by presence of other clinical risk factors or the effect of modifier genes remains unclear. Additionally, we found both rare and common missense or splice region variants in 9 of 16 infants with NEC. By comparing the frequency of *SIGIRR* variants in our cohort with the general population, we found more *SIGIRR* variants in infants with NEC. Furthermore, among 20 premature infants without NEC, we did not find functional or rare *SIGIRR* variants.

NEC is a complex disease involving interactions between clinical variables, developmental immaturity, gut microbiota, and genetic factors.[1] Although our data support a role for *SIGIRR* variants in NEC, additional studies are needed to evaluate how *SIGIRR* variants interact with known risk factors to cause disease.

Our functional data suggest that *SIGIRR* variants may contribute to NEC through loss of inhibition of TLR4-mediated inflammation. Activation of TLR4-mediated inflammation has been implicated in NEC pathogenesis in rodent models and humans.[5,14] Though consistent with these studies, our data suggest that genetic factors can contribute to selective activation of innate immune pathways in infants with NEC. Similarly, a role for reduced *SIGIRR* function in NEC has been suggested by Nanthakumar et al,[4] who showed less *SIGIRR* expression in ileal tissue resected from infants with NEC. We speculate that mucosal injury and gut bacteria elicit unregulated TLR-mediated inflammation in infants with *SIGIRR* variants causing NEC.[15]

## CONCLUSIONS

Our data provide novel insight into the probable causation of NEC and support the hypothesis that inherited

defects in genes that inhibit intestinal innate immune signaling can contribute to NEC. Adequately powered studies are needed to study the impact of *SIGIRR* variants and variant–clinical variable interactions in the causation of NEC.

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