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Small Intestinal Intraepithelial TCRγδ+ T Lymphocytes Are Present in the Premature Intestine but Selectively Reduced in Surgical Necrotizing Enterocolitis

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Abstract

Background: Gastrointestinal barrier immaturity predisposes preterm infants to necrotizing enterocolitis (NEC). Intraepithelial lymphocytes (IEL) bearing the unconventional T cell receptor (TCR) γδ γδ IEL maintain intestinal integrity and prevent bacterial translocation in part through production of interleukin (IL) 17.

Objective: We sought to study the development of γδ IEL in the ileum of human infants and examine their role in NEC pathogenesis. We defined the ontology of γδ IEL proportions in murine and human intestine and subjected tcrδ−/− mice to experimental gut injury. In addition, we used polychromatic flow cytometry to calculate percentages of viable IEL (defined as CD3+ CD8+ CD103+ lymphocytes) and the fraction of γδ IEL in surgically resected tissue from infants with NEC and gestational age matched non-NEC surgical controls.

Results: In human preterm infants, the proportion of IEL was reduced by 66% in 11 NEC ileum sections compared to 30 non-NEC controls (p<0.001). While γδ IEL dominated over conventional αβ IEL early in gestation in mice and in humans, γδ IEL were preferential decreased in the ileum of surgical NEC patients compared to non-NEC controls (50% reduction, p<0.05). Loss of IEL in human NEC was associated with downregulation of the Th17 transcription factor retinoic acid-related orphan nuclear hormone receptor C (RORC, p<0.001). TCRδ-deficient mice showed increased severity of experimental gut injury (p<0.05) with higher TNFα expression but downregulation of IL17A.

Conclusion: Complimentary mouse and human data suggest a role of γδ IEL in IL17 production and intestinal barrier production early in life. Specific loss of the γδ IEL fraction may contribute to NEC pathogenesis. Nutritional or pharmacological interventions to support γδ IEL maintenance in the developing small intestine could serve as novel strategies for NEC prevention.

Introduction

A critical, yet understudied, area in neonatology is the development of intestinal immune regulation in preterm infants, who are prone to exaggerated inflammatory host responses to bacterial antigens [1]. One example is necrotizing enterocolitis (NEC), a common, potentially lethal disease, primarily affecting preterm infants. Epidemiologic studies indicate that NEC incidence peaks at 32 weeks postmenstrual age, suggesting that there is a developmental window of susceptibility [2,3]. NEC is characterized by uncontrolled intestinal inflammation that can culminate in bowel necrosis [4–6]. Approximately 9,000 infants...
develop NEC in the United States each year, with reported mortality rates of 10–50% [7,8].

Intraepithelial lymphocytes (IEL) bearing the T cell receptor (TCR) γδ (γδ IEL) are the first type of T cell to colonize the epithelium during embryogenesis providing important immunoprotective and immunoregulatory activities in the perinatal period when conventional TCRαβ T cell responses are not yet fully mature [9]. While the precise role of γδ IEL is not yet clearly defined, they appear to be critical for the maintenance of epithelial integrity through antibacterial defense, tight junction preservation, recognition of epithelial stress, regulation of inflammatory responses and epithelial growth factor production [10–15]. The postnatal development of γδ IEL in the human preterm intestine is unknown. Given the immaturity of the intestinal epithelial barrier and its postulated role in NEC [16–20], we hypothesized that the developmental regulation of γδ IEL may relate to the window of NEC susceptibility in preterm infants and could represent a new target for disease prevention.

Here we report that γδ IEL are developmentally the prominent IEL subtype in the immature murine and human gut. However, we observed a specific reduction of γδ IEL proportions in the preterm ileum of NEC patients compared to gestational age matched preterm intestine resected for other indications. Loss of γδ IEL resulted in more severe experimental gut injury and inhibited gene expression of IL17 in mice while IEL reduction in preterm ileum of NEC patients compared to gestational age was confirmed by an immunologist (MTR) who was blinded to the indication for tissue resection, age at time of tissue resection, gestational age, and sex. We only analyzed viable cells. We only analyzed viable cells.

<p>| Table 1. Demographics of NEC and control patients. |
|-------------------------------|-------------------------------|-------------------------------|-------------------------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>Acute NEC (N = 11)</th>
<th>Controls (N = 30)</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age (weeks)</td>
<td>26.9 (25.4; 28.4)</td>
<td>27.0 (26.0; 3.6)</td>
<td>.330</td>
</tr>
<tr>
<td>Age (days)</td>
<td>30.0 (17.0; 54.0)</td>
<td>50.0 (5.3; 71.8)</td>
<td>.487</td>
</tr>
<tr>
<td>Postmenstrual age (weeks)</td>
<td>32.0 (30.9; 35.5)</td>
<td>37.0 (33.2; 39.6)</td>
<td>.065</td>
</tr>
<tr>
<td>Female</td>
<td>36.4% (4)</td>
<td>53.3% (16)</td>
<td>.484</td>
</tr>
</tbody>
</table>

Continuous data are summarized with median (quartiles), and categorical data with percent (frequency). P values are computed with Wilcoxon rank sum test (continuous) and Fisher’s exact test (categorical).

doi:10.1371/journal.pone.0099042.t001

Isolation of human intraepithelial lymphocytes

Patient demographics and surgical indications for the non-NEC control tissues are shown in Table 1 and Table 2 respectively. All samples (NEC and controls) were from the ileum and patients were matched for gestational age. We isolated IEL from surgical ileum specimens as previously described [21]. Briefly, the dissected mucosa was washed in HBSS media without Ca2+ and Mg2+ containing antibiotics, 5 mM EDTA (Sigma-Aldrich, St. Louis, MO), and 5% heat-inactivated fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) for 20 min on a gentle rocker at room temperature. Cell suspensions were pelleted from the supernatant and washed twice in complete HBSS prior to counting using trypsin blue exclusion. Cells were resuspended in freezing medium containing 50% Dulbecco’s Modified Eagle’s medium (DMEM), 40% heat-inactivated fetal bovine serum, and 10% dimethyl sulfoxide (DMSO) (Merck KGaA, Darmstadt, Germany). Cells were frozen in liquid nitrogen for storage until analysis at a concentration of approximately 1×10^6 cells/ml.

Flow cytometric analysis and sorting of IEL

We performed 7-color flow cytometric analysis of IEL using an LSRII flow cytometer (BD). IEL were thawed and washed in PBS and counted prior to staining with a PE-TexasRed-conjugated amine viability dye (Invitrogen, Grand Island, NY) for 20 min at room temperature. Cells were then washed with FACS buffer [PBS containing 1% bovine serum albumin (Sigma-Aldrich) and 0.1% sodium azide (Sigma-Aldrich)] and stained with titrated amounts of PE-TexasRed-conjugated anti-CD14 and anti-CD19 (‘‘dump channel’’), PerCP-Cy5.5-conjugated anti-CD3 (BD), PE-Cy7-conjugated anti-CD8 (BD), PE-Cy5 (Tricolor)-conjugated anti-RORC (eBioscience, clone AFKJS-9), and APC-conjugated anti-TCRγδ (eBioscience, clone A008-8). Flow data were analyzed with FlowJo software version 9.3 (Tree Star, Ashland, OR). IEL were identified as CD3+CD103−, and characterized as γδ IEL if cells were also TCRγδ+ and TCRδβ−. To confirm the purity of the IEL populations, we performed flow cytometry analysis on the remaining tissue following IEL preparation (lamina propria cells) and did not detect any CD103− TCRγδ+ cells. We only analyzed viable surgical margins with adequate numbers of viable lymphocytes (“dump channel negative”). All flow cytometric gating/analysis was confirmed by an immunologist (MTR) who was blinded to the sample origin. Fluorescent Minus One (FMO) was used to control for nonspecific signal.
Table 2. Origins of control small intestinal tissue samples.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
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</thead>
<tbody>
<tr>
<td>Reanastomosis after NEC surgery</td>
<td>9</td>
</tr>
<tr>
<td>Reanastomosis after SIF repair</td>
<td>4</td>
</tr>
<tr>
<td>Reanastomosis after congenital volvulus repair</td>
<td>2</td>
</tr>
<tr>
<td>Stricture removal after medical NEC</td>
<td>2</td>
</tr>
<tr>
<td>Reanastomosis after gastrochisis repair</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>30</td>
</tr>
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</table>

doi:10.1371/journal.pone.0099042.t002

Human RORC and occludin gene expression

Total RNA was extracted from 25 mg of either fresh NEC and non-NEC ileum using the RNAeasy Mini Kit or from six 10-micron sections of formalin-fixed, paraffin-embedded tissue pieces using the RNAeasy FFPE Kit (Qiagen Valencia, CA). Total RNA was reverse transcribed using the RT² First Strand Kit (Qiagen) per manufacturer’s instructions. The cDNA-containing reaction mixture was added to each well of a 96-well-plate PCR array for quantitative real-time (RT) PCR (RORC: Th17 for Autoimmunity and Inflammation PCR Array, occludin: cat no. PPH02571B RT2 Profiler PCR Array; Qiagen). PCR cycles were performed according to the manufacturer’s instructions. Expression levels of cytokine genes were quantified using quantitative real-time PCR analysis based on intercalation of SYBR® Green on an ABI 7500 Real-Time PCR system (Life Technologies, Carlsbad, CA). The relative level of mRNA expression for each gene in each sample was normalized to the expression level of reference gene GAPDH and the data were analyzed using the ΔΔCt method [22].

Human immunohistochemistry

Immunohistochemistry of IEL in formalin-fixed paraffin-embedded tissue sections was performed as recently described [23]. Briefly, 5 μm paraffin embedded sections were cut and placed on charged slides. After epitope retrieval and protein blocking, slides were incubated for 20 minutes with anti-human CD3 (1:125) (DakoCytomation). A streptavidin-biotin detection system was used followed by application of DAB. The murine Envision+ System, DAB/Peroxidase (DakoCytomation) was employed to produce localized, visible staining. The slides were counterstained with hematoxylin, dehydrated, and cover-slipped.

Intestinal injury model

To induce intestinal injury, we injected 2 week old C57BL/6j or TCRγδ-deficient mice with 100 μg/kg platelet activating factor (PAF, Sigma Aldrich, St. Louis, MO) and 1 mg/kg E. coli 0128:B12 lipopolysaccharide (LPS, Sigma Aldrich) intraperitoneally as previously reported [24–26]. Control animals were injected with PBS vehicle control. Pups were sacrificed two hours later and the distal small intestine was isolated. A portion of the distal small intestine was fixed in 10% formalin (Fisher Scientific, Pittsburgh, PA) for paraffin embedding, sectioning and hematoxylin and eosin (H&E) staining for intestinal injury severity scoring (see below). The remainder was collected in Trizol (Invitrogen, Grand Island, NY) for RNA isolation analysis of cytokine gene expression (see below).

Murine IEL isolation and analysis

To examine the ontogeny of γδ IEL, small intestines were harvested from 1 week old, 2 weeks old, 3 weeks old, and adult mice (6–8 weeks old). To examine frequencies of γδ IEL in mice subjected to experimental intestinal injury, small intestines were harvested from 2 weeks old mice subjected to experimental intestinal injury as described above. Intestines were cut longitudinally and rinsed of luminal contents and subsequently cut into 1 cm pieces and shaken at 250 rpm for 20 min at 37°C in HBSS (Ca/Mg-free) with 5% fetal bovine serum and 2 mM EDTA. The cell suspensions were passed through a 100 μm cell strainer then through glass wool columns and centrifuged at 1500 rpm. The cell pellets were resuspended in 45% isotonic Percoll, underlain with 70% Percoll, and centrifuged at 2000 rpm for 25 min. The IEL at the interface of 44% and 70% Percoll were collected and washed for flow cytometric analysis. This technique for IEL isolation has been shown to be valid for both neonatal and adult murine intestines [27,28].

Surface staining was performed at 4°C for 20 min in PBS with 5% FBS. Fc receptors were blocked with anti-FcγRIII/II (2.4G2) and the following antibodies were used: APC-conjugated TCRβ (clone H57–597), PE-Cy7-conjugated CD3ε (clone 145-2C11) AlexaFlour 700-conjugated CD4 (clone RM4–5) from eBioscience; PE-Texas Red-conjugated CD8α (clone 5H10) from Invitrogen; FITC-conjugated anti-TCRγδ (clone UC7-13D5), PerCP-Cy5.5-conjugated CD4 (clone RM4–5), and PE-conjugated CD103 (clone M290) from BD Pharmingen.

To examine relative frequencies of γδ IEL in wild-type mice subjected to intestinal injury, IEL were isolated from dam-fed wild-type 2-week-old mice or mice subjected to intestinal injury as described above. IEL were subsequently isolated, stained, and flow cytometric analysis was conducted on a BD LSR II (BD Biosciences, Franklin Lakes, NJ). IEL were defined as CD103+, CD3ε+ and characterized as γδ IEL if cells were also TCRγδ+ and TCRβ-.

Intestinal injury scoring

H&E sections were scored by a blinded reviewer on a 5 point scale: grade 0: no injury; grade 1: mild intestinal dilatation, mild submucosal edema or lamina propria separation, epithelial apoptosis; grade 2: moderate submucosal or lamina propria separation, submucosal edema, epithelial sloughing or necrosis, epithelial mucus depletion; grade 3: severe submucosal or lamina propria separation, severe edema, villous sloughing; grade 4: severe villous sloughing, transmural necrosis. This scale is a compilation of scales used in similar intestinal injury models [20,25,29–32].

Murine mRNA isolation and cytokine gene expression

Distal small intestinal samples were homogenized and total RNA isolated and reverse transcribed from random hexamer primers using the Quant iT Re reverse Transcription Kit (Qiagen, Carol Stream, IL). The resulting cDNA products were analyzed by real-time quantitative RT-PCR (qSYBR Green Supermix on MyiQ real time PCR detection system, Biorad, Hercules, CA) for IL17A, TNFα and GAPDH mRNA. The relative level of mRNA expression for each gene in each sample was normalized to the expression level of reference gene GAPDH and the data were analyzed using the ΔΔCt method [22].

Primer information:
- GAPDH-forward: TGG CAA AGT GGA GAT TGT TGC C
- GAPDH-reverse: AAG ATG GTG ATG GCC TTC TCG
- IL17A-forward: CAG CAG CGA TCA TCC CTC AAA G
- IL17A-reverse: CAG GAC GAT CAC TTC CTG

TCRγδ Small Intestinal Intraepithelial Lymphocytes in the Preterm Gut
IEL within epithelial CD3⁺ isolated in tissue epithelium preparations. The mean fraction of life CD3⁺ sizable proportion of cells were 0.001). Within the IEL compartment of the control group, a controls was 64% compared to 23% in NEC, Figure 1B, p = 0.02). Therefore surgical NEC was characterized by a significantly decreased in NEC patients (mean 15%) (Figure 1C, p = 0.478).

We considered the possibility of sample contamination from conventional lymphocytes in the lamina propria. We performed flow cytometry analysis on the remaining lamina propria tissue (LPL) following IEL preparation and did not detect any CD103⁺ TCRγδ⁺ cells supporting the purity of IEL and LPL preps. In addition, the mean total number of viable CD3⁺ cells isolated from the epithelium of NEC samples was 50% of cells identified in non-NEC samples (5,128 vs. 10,228 cells, p = 0.189), suggesting that the reduced IEL fraction in NEC is not explainable by significant influx of CD3⁺ cells from other compartments.

Statistical analysis

**Human studies (Vanderbilt).** Gene expression and flow cytometry cell type data followed skewed distributions and underwent logarithmic transformation. Data were compared between independent groups using Student’s t test. Lamina propria lymphocytes (LPL) and IEL RORC gene expression from the same set of subjects were compared using the paired t test. Associations between TCRγδ⁺ IEL and RORC mRNA expression and age parameters were explored using Pearson’s correlation coefficient after logarithmic transformation of the skewed variables. The relationship between the proportion of TCRγδ⁺ IEL and gestational age in non-NEC surgical control samples followed a non-linear distribution. Thus, a model was fitted to a second order polynomial equation using non-linear regression and plotted with 95% confidence bands. Goodness of fit was evaluated by the R² parameter. The runs test was performed to determine whether the curve deviated systematically from the data.

**Animal studies (Emory).** Data are reported as mean ± standard error of the mean (SEM). Statistical differences were determined by one-way analysis of variance (ANOVA) or Student’s t test as appropriate. A p < 0.05 was considered significant.

**Results**

**Surgical ileal mucosa from NEC patients was marked by decreased proportions of IEL and TCRγδ⁺ IEL ratios compared to non-NEC surgical controls**

To determine whether γδ IEL may play a protective role against intestinal injury in the premature human intestine, we studied the development, phenotype and distribution of these cells in relationship to total viable CD3⁺ CD8⁻ T cells in surgical ileum samples. We prospectively isolated IEL from fresh tissue obtained through medically indicated surgical resection for 11 NEC and 30 non-NEC patients. All tissue sections were ileum and were from infants of comparable gestational age (GA) (p = 0.330), age (p = 0.487), postmenstrual age (PMA) (p = 0.065), and sex distribution (p = 0.484) (Table 1). Non-NEC cases included resections for reanastomoses for various surgical indications (16), congenital intestinal bowel obstruction (7), spontaneous focal intestinal perforation (5), and tissue from stricture removal after medical NEC (2) (Table 2). Median mucosal weights for NEC and non-NEC tissues were similar (310 mg and 370 mg, respectively, p = 0.478).

We compared the proportions of total IEL and γδ IEL as demonstrated in Figure 1A. Using flow cytometry we defined IEL in life CD3⁺ CD8⁻ CD103⁻ lymphocytes and characterized as γδ IEL if cells were also TCRγδ⁻ and TCRγδ⁺. Compared to non-NEC surgical controls, NEC samples exhibited significantly lower numbers of total IEL (mean 2,342 versus 124 cells per tissue section, p<0.01). Because NEC is associated with necrosis and intestinal epithelium loss likely explaining reduction in total IEL, we calculated percentages of IEL based on total CD3⁺ CD8⁻ cells isolated in tissue epithelium preparations. The mean fraction of IEL within epithelial CD3⁺ CD8⁻ cells in non-NEC surgical controls was 64% compared to 23% in NEC, Figure 1B, p < 0.001). Within the IEL compartment of the control group, a sizable proportion of cells were γδ IEL (mean 27%), which was significantly decreased in NEC patients (mean 15%) (Figure 1C, p = 0.02). Therefore surgical NEC was characterized by a preferential reduction in γδ IEL over TCRγδ IEL.

γδ IEL are the predominant IEL subtype in the immature murine and human small intestine

Since NEC predominantly affects preterm infants, we examined whether γδ IEL are developmentally regulated in the preterm intestine. We examined the relationship between γδ IEL proportions and gestational age, postmenstrual age, and age. We did not observe a clear association between γδ IEL proportions and postmenstrual age or postnatal age, suggesting that even the most premature infants contain significant fractions of natural γδ IELs at birth [33] (Figure 2). Interestingly, the relationship between γδ IEL proportions and gestational age in non-NEC surgical control samples followed a U-shaped distribution as determined by nonlinear regression. This model accounted for 57% of the variance of the data (R² = 0.37). The observed data did not deviate significantly from the model curve as determined by the runs test (p = 0.31). This distribution suggests a possible window of vulnerability for NEC across gestation (Figure 3).

Young mice are frequently used for NEC-like injury models and correlating the maturity of the mucosal immune system between neonatal mice and humans is complex [33]. In addition, the human data on postnatal development may have been skewed, as neonatal intestinal tissue samples cannot be obtained from healthy neonates. Therefore we isolated epithelial-associated immune cells from the small intestines of wild type neonatal mice ages 1 week to adult (Figure 4A). γδ IEL were the predominant IEL subtype in younger mice (73% in 1 week old mice versus 59% in adult mice, p < 0.05), with frequency approaching adult levels by 3 weeks of life (60%, p < 0.05 vs. 1 week old) (Figure 4B).

Intestinal injury in wild-type mice is not associated with a selective reduction in γδIEL

For ethical reasons, it is not possible to determine definitively whether the selective reduction of γδ IEL in human NEC occurred prior to or as a result of intestinal injury. Therefore we sought to determine whether experimental intestinal injury in a murine model causes selective reduction in γδ IEL. To induce intestinal injury, we injected 2 weeks old C57BL/6j or TCRγδ⁻⁻⁻ mice intraperitoneally with 100 µg/kg PAF and 1 mg/kg E. coli 0128:B12 LPS or PBS vehicle control as described above. Pups were sacrificed two hours later and small intestinal epithelial-associated immune cells were isolated as stated above. We detected no differences in percentages of γδ IEL between control mice and those subjected to experimental intestinal injury (Figure 5). These data suggest that the selective reduction in γδ IEL associated with human NEC is not a secondary finding following injury but may indicate a specific risk factor.

**Significant reduction in RORC expression in NEC tissue correlates with reduction of IEL**

TCRγδ cells have been attributed an important role in innate mucosal immune responses, partially mediated through the production of IL17 [35,36]. TCRγδ IEL have been specifically developed
Figure 1. Reduced proportions of γδ IEL subsets in patients with NEC compared to non-NEC surgical controls. (A) Example of the gating strategy used to calculate proportions of γδ IEL subsets. The control sample shown is from a 4 days old 26 weeks gestation infant with spontaneous (focal) intestinal perforation and the NEC sample is from a 15 days old 28 weeks gestation infant with surgical NEC. Gates were set on "live", CD14+, CD19- ("Dump" negative) and CD3+ cells before applying to sub-populations. Next we identified CD3+CD8+ T cells followed by differentiating conventional CD3+CD103+ TCRab from TCRγδ IEL (γδ IEL). The patient with NEC showed significant reduction in γδ IEL with a corresponding greater proportion of αE integrin (CD103) negative, conventional T cells. Dot plot of total IEL (B) and γδ IEL (C) proportions were statistically significantly reduced in NEC tissue compared to non-NEC controls, p<0.001 and p = 0.02, respectively.

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Figure 2. Developmental regulation of γδ IEL subsets in humans. Logarithmic transformed percentages of γδ IEL were plotted against gestational age (GA), postmenstrual age (PMA = gestational age plus chronological age) and age. Using Pearson’s correlation coefficient we did not detect any association of γδ IEL proportions with GA, PMA or age in either NEC or non-NEC control patients.

doi:10.1371/journal.pone.0099042.g002
shown to produce IL17 under inflammatory conditions [37,38]. To determine whether a similar mechanism may play a role in the human neonatal gut, we measured the gene expression of retinoic acid-related orphan nuclear hormone receptor C (RORC) in the small intestinal mucosa of 15 NEC patients compared to 7 surgical controls. Human RORC is an analogue to the murine retinoid orphan receptor (ROR\(_c\))t, which drives expression of IL17 in CD IEL [36]. Since expression of IL17 is dependent on cell stimulation and IEL numbers were too low to isolate sufficient cells for stimulation assays, we used RORC gene expression as a correlate for IL17 production [39]. By quantitative RT-PCR, RORC gene expression in NEC samples was reduced by a median of 10 fold (p<0.001, Figure 6A). Next, we sought to determine if the reduction of RORC expression in NEC could be explained by loss of \(\gamma\delta\) IEL. We measured RORC gene expression in LPL and IEL isolated from identical tissue sections from non-NEC controls. RORC gene expression was significantly higher in IEL compared to LPL (p = 0.01, Figure 6B). In addition, we found a statistically significant positive correlation between total TCR\(_{\gamma\delta}\) IEL proportions and RORC gene expression (Pearson \(R^2=0.41\), p = 0.02 (Figure 6C). Cumulatively, these data suggest that loss of \(\gamma\delta\) IEL in NEC may limit intestinal barrier defense through decreased production of IL17.

Intestinal injury in TCR\(_{\gamma\delta}\)-deficient mice is associated with increased TNF\(\alpha\) but decreased IL17A gene expression

To investigate the role of \(\gamma\delta\) IEL in mucosal homeostasis and cytokine response, we measured mRNA expression of intestinal TNF\(\alpha\) and IL17A in mice lacking \(\gamma\delta\) IEL and exposed to experimental gut injury as described above. At baseline, there was no difference in the histologic appearance of control dam fed wild type or TCR\(_{\gamma\delta}\)\(^/-\) mice (Figure 7A). When subjected to experimental gut injury, TCR\(_{\gamma\delta}\)\(^/-\) mice were found to have significantly worse disease scores compared to wild type mice (2.1\(\pm\)0.1 versus 2.5\(\pm\)0.1, p = 0.05) (Figure 7B). TCR\(_{\gamma\delta}\)\(^/-\) mice also exhibited increased incidence of injury (defined as severity scores >2) when compared to wild-type mice (59% vs. 29%). Similarly, intestinal TNF\(\alpha\) and IL17A mRNA expression was low in the steady state. In response to PAF-induced epithelial injury, intestinal mRNA expression of both TNF\(\alpha\) and IL17A increased.
in wild type mice. Interestingly, TCRδ-deficient mice demonstrated significantly reduced expression of IL17A (7-fold versus 22-fold induction in IL17A expression, \( p < 0.05 \)) (Figure 8). These data suggest that epithelial injury may induce TCRδ T cells to express IL-17 in order to protect the intestinal barrier.

Occludin gene expression is decreased in NEC tissue

Occludin forms rings at sites of γδ IEL/epithelial contact and promotes γδ IEL migration into epithelial monolayers [40]. Enterocytes internalized occludin in experimental NEC but expression in human NEC was unchanged in the small intestine by immunohistochemistry [16]. We sought to determine occludin
expression in human NEC tissue to test the possibility that reduced expression may inhibit migration of γδ IEL into the intraepithelial compartment. We found statistically significant reduction in occludin gene expression in by quantitative RT-PCR in 16 NEC tissue sections compared to 13 controls (p<0.0001, Figure 9).

**Discussion**

Although the exact biological function of γδ IEL is elusive, these cells reportedly play an important role in innate mucosal immune responses by preventing invasion of pathogenic bacteria [41], partially mediated through the production of IL17 [35,36]. In addition, γδ IEL maintain epithelial barrier function through production of keratinocyte growth factor in mice [15,42] and protect from dextran sodium sulfate (DSS) induced colitis [11,43]. Furthermore, γδ IEL appear to be critical for immune homeostasis [44,45]. Since epithelial barrier disruption, invasion of pathogenic bacteria and exaggerated inflammation are key contributors to the development of NEC in the preterm infant [46], we sought to determine the developmental regulation of γδ IEL in the small intestinal mucosa of preterm infants and a possible role in NEC pathogenesis. We demonstrate here for the first time abundance of γδ IEL in the preterm gut but also a statistically significant reduction in acute NEC. Different subtypes of γδ IEL exist [34]; however we focused on CD8\(^+\)γδ IEL, because of their dominance in the small intestine [47]. The loss of CD8\(^+\)γδ IEL in NEC could represent a disproportional lack of immune regulatory IEL, which may be critical in the phase of precipitously increasing antigen exposure [10].

We do not know the reason for reduced IEL proportions in NEC. We considered the possibility that the reduction of IEL may be due to loss of epithelium through tissue necrosis. However, as shown in Figure S1, analyzed NEC tissue contained epithelium and IEL, although in lower numbers compared to non-NEC controls. We controlled for NEC-associated epithelium loss by calculating the fraction of IEL within the total number of epithelial CD3\(^+\)CD8\(^+\) cells. In addition, the preferential reduction of γδ IEL compared to γβ IEL cannot be explained by absence of enterocytes.

We contemplated the possibility of contamination from conventional lymphocytes in the lamina propria. We think this is unlikely since our protocol effectively separates IEL and LPL cells as previously published and shown in Figure 5B [21]. To further confirm the purity of the IEL populations, we performed flow cytometry analysis on the remaining tissue (LPL) following IEL preparation and did not detect any CD103\(^+\) TCRγδ cells. We have previously described an increase in non-regulatory T cells in NEC lamina propria [21] and therefore it is possible that reduction in IEL proportions in NEC is due to additional T cells entering the epithelium. However, as described above, non-NEC samples contained twice as many epithelial T cells in as NEC samples making data skew by contaminating cells unlikely. In addition, influx of CD3\(^+\) cells in NEC would not explain the specific reduction in the γδ IEL fraction.

**Figure 7.** γδ T cells reduce experimental gut injury. A) Representative H&E staining of distal small intestines isolated from dam fed wild-type (1) or TCRδ\(^−/−\) (3) mice with normal histologic appearance; or wild-type (2) or TCRδ\(^−/−\) (4) mice subjected to experimental gut injury (PAF/LPS) as described (scale marker = 100 μm). Note shortened villi and epithelial sloughing with inflammatory infiltrate in wild-type PAF/LPS mice (2) and submucosal edema with severe villous sloughing in TCRδ\(^−/−\) PAF/LPS mice (4). B) Histologic severity score (mean ±SE) of distal small intestinal sections obtained from dam fed wild-type (WT control) or TCRδ\(^−/−\) (tcrδ\(^−/−\) control) mice; or wild-type (WT PAF/LPS) or TCRδ\(^−/−\) (tcrδ\(^−/−\) PAF/LPS) mice subjected to experimental gut injury as described. Data are representative of 4 independent experiments with at least 3 mice per condition per experiment (*p<0.05).

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We wondered if the immature mucosal immune system contributed to the reduced γδ IEL proportions in the small intestine of patients with NEC. While an inverse relationship between number of villus IEL and increasing age has been reported in adults [48], the postnatal developmental regulation of γδ IEL in preterm infants was unknown. We found robust proportions of γδ IEL early in life even at extreme prematurity. In addition, we defined the postnatal development of γδ IEL in human non-NEC infants showing a U-shaped distribution in the last trimester (Figure 3). TCRγδ IEL may be initially recruited to the immature gut as the predominant IEL subtype in order to protect against potential injury at a time when the gut barrier is immature and exposure to new bacterial antigens is rapidly growing [49].

One potential mechanism for the reduced γδ IEL fraction in preterm infants at risk for NEC may be in-utero exposure to inflammation. Histological chorioamnionitis with fetal involvement has been considered a possible risk factor for NEC [50] and inflammation associated with this pregnancy complication may lead to occludin endocytosis and therefore reduced migration of γδ IEL into the intraepithelial compartment [39]. Occludin internalization has been reported in experimental NEC [16] and we show that small intestinal occludin gene expression was significantly decreased in NEC tissue compared to non-NEC controls. We consider chorioamnionitis a more likely candidate for γδ IEL reduction than inflammation associated with NEC because our control group included infants with conditions that involved intestinal perforation with a significant inflammatory response.

Homing and/or retention of lymphocytes in the intestinal epithelium is maintained by expression of integrin αEβ7, which is regulated by TGFβ signaling [51,52]. We recently discovered overexpression of its negative regulator Smad7 in NEC tissue [53]. Inhibited TGFβ signaling reduces expression of integrin αE (CD103), which in conjunction with integrin beta 7 forms a complete heterodimeric integrin molecule that is thought to mediate retention of IEL in the epithelium [34]. Downregulation of TGFβ may also play a role in reduced expression of RORC [55] and enhanced T cell mediated inflammation in NEC tissue [21,56].

NEC occurs only in a subgroup of preterm infants and its risk is increased with lack of breast milk feeding and a microbiome with decreased diversity [6,46,57,58]. Expansion of intestinal γδ IEL in mice depends on bacterial interaction [36] and the altered microbiome in NEC may contribute to underdevelopment of γδ IEL. Dietary natural aryl hydrocarbon receptor (AhR) ligands are critical for normal intestinal immune development [39] and postnatal maintenance of IEL [60]. Lack of AhR signaling has been implicated in the pathogenesis of inflammatory bowel disease [61]. The role of AhR ligands in maintaining γδ IEL in preterm infants is unknown and should be explored in future studies.
In conclusion, we demonstrate for the first time the postnatal development of γδ TIE in the premature intestine and therefore contribute to the understudied area of human neonatal mucosal immune development [62]. We further show that the normally enriched fraction of γδ TIE in the ileum of premature infants is significantly reduced in surgical NEC. Complementary animal and human data suggest a potentially important role of γδ TIE in IL-17 production and intestinal barrier protection. Ways to recruit and maintain this likely important T cell population in the preterm gut could serve as a novel strategy to reduce or prevent NEC and other intestinal complications originating early in life.

Supporting Information

Figure S1 Immunohistochemistry of intraepithelial lymphocytes. Immunohistochemistry for CD3\(^+\) cells in repre-

sentative tissue sections. (A) Eleven days old 32 weeks gestation infant with NEC. (B) Four days old 33 weeks gestation infant with intestinal atresia. Arrows illustrate intraepithelial lymphocytes, which were reduced in NEC patients (200× magnification), (TIF)

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Author Contributions

Conceived and designed the experiments; JHW TLD PWD. Performed the experiments; JHW PM DG. Analyzed the data: JHW MRR ZZ PD. Contributed reagents/materials/analysis tools: MRR TLD MDR DMD JDM. Wrote the paper: JHW MRR PWD.

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