OPEN ACCESS Freely available online

PLOS ONE

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

Jörn-Hendrik Weitkamp[1]*, Michael J. Rosen[2], Zhiguo Zhao[3], Tatsuki Koyama[3], Duke Geem[4], Timothy L. Denning[4], Michael T. Rock[1], Daniel J. Moore[1], Melissa D. Halpern[5], Pranathi Matta[1], Patricia W. Denning[6]*

1 Department of Pediatrics, Vanderbilt University School of Medicine and Monroe Carell Jr. Children's Hospital at Vanderbilt, Nashville, Tennessee, United States of America, **2** Division of Pediatric Gastroenterology, Hepatology, and Nutrition, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, United States of America, **3** Department of Biostatistics, Vanderbilt University School of Medicine, Nashville, Tennessee, United States of America, **4** Center for Inflammation, Immunity, and Infection, Institute for Biomedical Sciences, Georgia State University, Atlanta, Georgia, United States of America, **5** Department of Pediatrics and Steele Children's Research Center, University of Arizona, Tucson, Arizona, United States of America, **6** Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia, United States of America

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 ontogeny 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 resections 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.

Citation: Weitkamp J-H, Rosen MJ, Zhao Z, Koyama T, Geem D, et al. (2014) Small Intestinal Intraepithelial TCRγδ$^+$ T Lymphocytes Are Present in the Premature Intestine but Selectively Reduced in Surgical Necrotizing Enterocolitis. PLoS ONE 9(6): e99042. doi:10.1371/journal.pone.0099042

Editor: Josef Neu, University of Florida, United States of America

Received March 11, 2014; **Accepted** May 9, 2014; **Published** June 6, 2014

Funding: This project was supported by award number K08HD061607 (to JHW) and R01HD05922 (to PWD) from the Eunice Kennedy Shriver National Institute of Child Health & Human Development (NICHD). This work was also supported by the Vanderbilt Physician Scientist Development Program Award (to JHW), NIH Award K23DK094832 (to MJR), the Vanderbilt CTSA grant UL1 RR024975-01 from NCRR/NIH (now NCATS/NIH grant 2 UL1 TR000445-06), 5K08DK090146-03 (to DJM) and by a Vanderbilt DRTC Pilot Grant P30DK20593 (to DJM). Support for flow cytometry experiments and biostatistics was provided through the Vanderbilt University Medical Center's Digestive Disease Research Center sponsored by NIH grant P30DK058404 and Emory University's Digestive Diseases Research Development Core sponsored by NIH grant DK 063399. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Jörn-Hendrik Weitkamp and Patricia Denning are authors and also PLOS ONE Editorial Board members. This does not alter the authors' adherence to all PLOS ONE policies on sharing data and materials.

* E-mail: hendrik.weitkamp@Vanderbilt.Edu (JHW); pllin@emory.edu (PWD)

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

Table 1. Demographics of NEC and control patients.

	Acute NEC	Controls	
	N = 11	N = 30	P values
Gestational age (weeks)	26.9 (25.4; 28.4)	27.0 (26.0; 3.6)	.330
Age (days)	30.0 (17.0; 54.0)	50.0 (5.3; 71.8)	.487
Postmenstrual age (weeks)	32.0 (30.9; 35.5)	37.0 (33.2; 39.6)	.065
Female	36.4% (4)	53.3% (16)	.484

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

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 human samples correlated with downregulation of IL17 transcription factor RORC. This first report on γδ IEL in the preterm gut suggests a novel target for prevention of severe intestinal complications of prematurity.

Materials and Methods

Ethics Statement

Fresh ileum tissue specimens from infants with NEC or non-NEC diagnoses were provided from the Vanderbilt Children's Hospital pathologist under a protocol approved by the Vanderbilt University Institutional Review Board. Informed consent was waived because all samples were de-identified and only demographic data pertinent to the study design (diagnosis and indication for tissue resection, age at time of tissue resection, gestational age, and sex) were collected from patient records prior to tissue release.

C57BL/6J and TCRδ-deficient ($tcr\delta^{-/-}$) mice (originally obtained from Jackson laboratories) were bred at an animal facility at Emory University and all studies were approved by the Emory University Institutional Animal Care and Use Committee (IACUC).

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 Ca^{2+} and Mg^{2+} 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 trypan 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 in the dark. 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") (Invitrogen), PerCp-Cy5.5-conjugated anti-CD3 (BD), PE-Cy7-conjugated anti-CD8 (BD), PE-Cy-5 (Tricolor)-conjugated CD103 (Invitrogen), FITC-conjugated anti-TCRαβ (BD), PE-conjugated anti-TCRγδ (BD), and APC-conjugated anti-RORC (eBioscience, clone AFKJS-9). Flow data were analyzed with FlowJo software version 9.3 (Tree Star, Ashland, OR). IEL were identified as $CD3^+$, $CD103^+$, $CD8^+$ cells and characterized as γδ IEL if cells were also $TCR\gamma\delta^+$ and $TCR\alpha\beta^-$. 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 $CD103^+$ $TCR\gamma\delta^+$ 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.

	N
Reanastomosis after NEC surgery	9
Resection for congenital intestinal atresia	7
Spontaneous intestinal perforation (SIP)	5
Reanastomosis after SIP repair	4
Reanastomosis after congenital volvulus repair	2
Stricture removal after medical NEC	2
Reanastomosis after gastroschisis repair	1
Total	30

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 RNeasy Mini Kit or from six 10-micron sections of formalin-fixed, paraffin-embedded tissue pieces using the RNeasy FFPE Kit (Qiagen Valencia, CA). Total RNA was reverse transcribed using the RT^2 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 RT-PCR analysis based on intercalation of SYBR® Green on an ABI 7300 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 $\Delta\Delta C_t$ 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 weeks old C57BL/6J or TCRδ-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 subequently 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) AlexaFluor 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 $CD103^+$, $CD3\varepsilon^+$ and characterized as γδ IEL if cells were also $TCR\gamma\delta^+$ and $TCR\beta^-$.

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 QuantiTect Reverse Transcription Kit (Qiagen, Carol Stream, IL). The resulting cDNA products were analyzed by real-time quantitative RT-PCR (iQ SYBR 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 $\Delta\Delta C_t$ method [22].

Primer information:

GAPDH-forward: TGG CAA AGT GGA GAT TGT TGC C
GAPDH-reverse: AAG ATG GTG ATG GGC TTC CCG
IL17A-forward: CAG CAG CGA TCA TCC CTC AAA G
IL17A-reverse: CAG GAC CAG GAT CTC TTG CTG

TNFα-forward: CTA CTC CCA GGT TCT CTT CAA
TNFα-reverse: GCA GAG AGG AGG TTG ACT TTC

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 $TCR\gamma\delta^+$ 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 R^2 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 mucosa 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 as life $CD3^+$ $CD8^+$ $CD103^+$ lymphocytes and characterized as γδ IEL if cells were also $TCR\alpha\beta^-$ and $TCR\gamma\delta^+$. 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 αβ IEL.

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 $CD103^+$ $TCR\gamma\delta^+$ 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.

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 37% of the variance of the data ($R^2 = 0.37$). 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 $TCR\delta^{-/-}$ 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

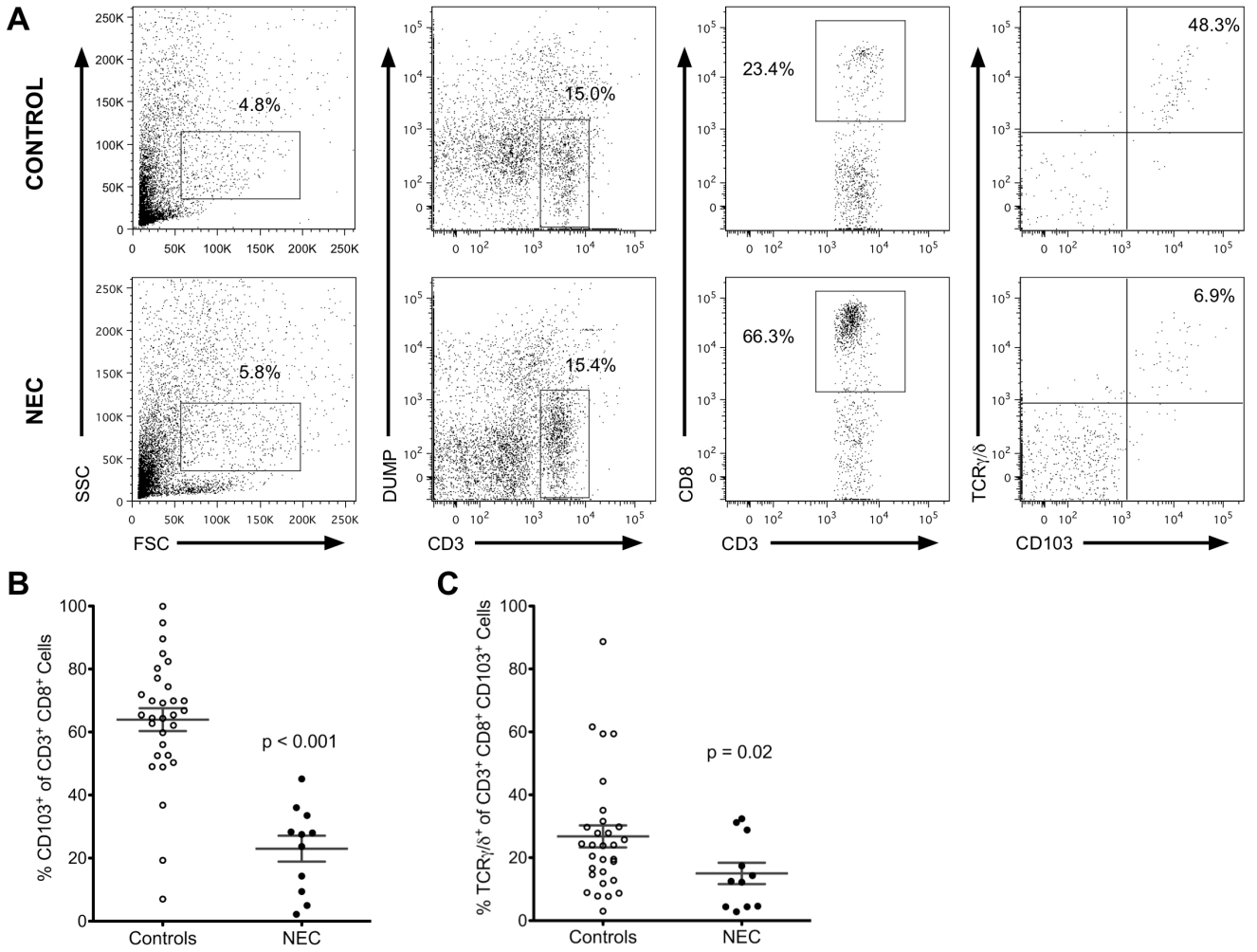

Figure 1. Reduced proportions of γδ IEL subsets in patients with NEC compared to non-NEC surgical controls. (A) Example of the gating strategy used to calculate proportions of γδ IEL subsets. The control sample shown is from a 4 days old 26 weeks gestation infant with spontaneous (focal) intestinal perforation 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 $CD3^+$ $CD103^+$ TCRαβ 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.
doi:10.1371/journal.pone.0099042.g001

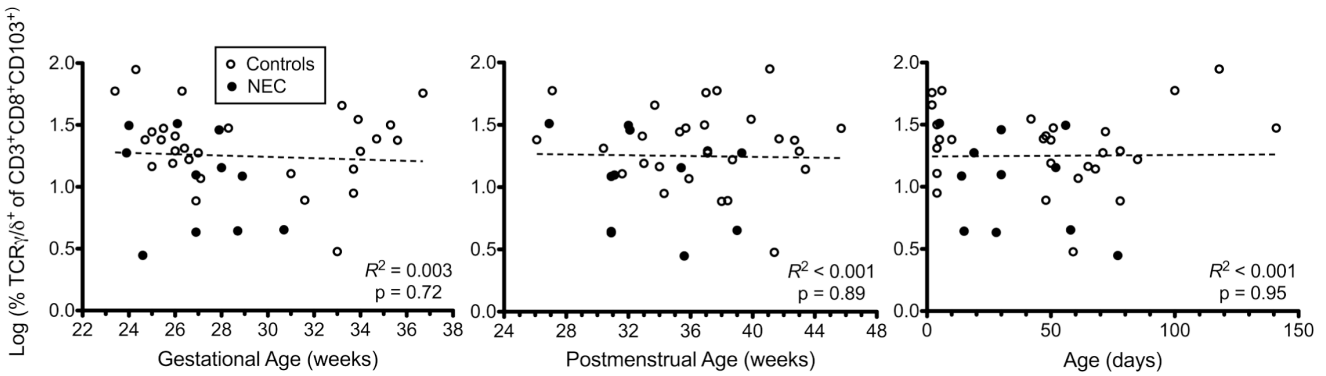

Figure 2. Developmental regulation of γδ IEL subsets in humans. Logarithmic transformed percentages of γδ IEL were plotted against gestational age (GA), postmenstrual age (PMA = gestational age plus chronological age) and age. Using Pearson's correlation coefficient we did not detect any association of γδ IEL proportions with GA, PMA or age in either NEC or non-NEC control patients.
doi:10.1371/journal.pone.0099042.g002

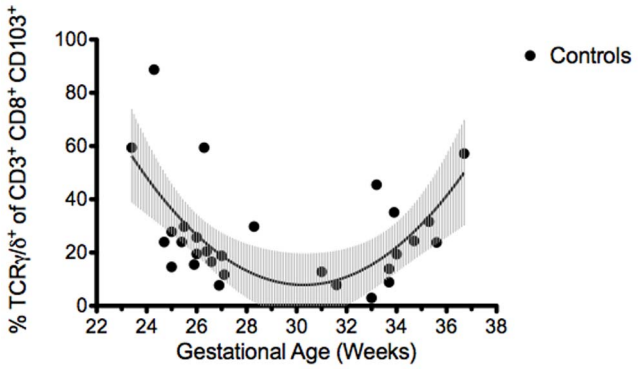

Figure 3. Window of susceptibility with low γδ IEL subsets in human neonates. Graph depicting the relationship between percentage of γδ IEL and gestational age in non-NEC surgical control samples. The black line shows the curve estimate of the U-shaped association as modeled by nonlinear regression with 95% confidence intervals of the model curve shaded in grey. The lowest percentage of γδ IEL occurs between 27 and 32 weeks gestation.
doi:10.1371/journal.pone.0099042.g003

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 (RORγt), which drives expression of IL17 in γδ 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 γδ 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 TCRγδ$^+$ IEL proportions and RORC gene expression (Pearson $R^2 = 0.41$, $p = 0.02$ (Figure 6C). Cumulatively, these data suggest that loss of γδ IEL in NEC may limit intestinal barrier defense through decreased production of IL17.

Intestinal injury in TCRδ-deficient mice is associated with increased TNFα but decreased IL17A gene expression

To investigate the role of γδ IEL in mucosal homeostasis and cytokine response, we measured mRNA expression of intestinal TNFα and IL17A in mice lacking γδ 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 TCRδ$^{-/-}$ mice (Figure 7A). When subjected to experimental gut injury, *TCR*δ$^{-/-}$ mice were found to have significantly worse disease scores compared to wild type mice (2.1 ± 0.1 versus 2.5 ± 0.1, $p<0.05$) (Figure 7B). TCRδ$^{-/-}$ mice also exhibited increased incidence of injury (defined as severity scores >2) when compared to wild-type mice (59% vs. 29%). Similarly, intestinal TNFα and IL17A mRNA expression was low in the steady state. In response to PAF-induced epithelial injury, intestinal mRNA expression of both TNFα and IL17A increased

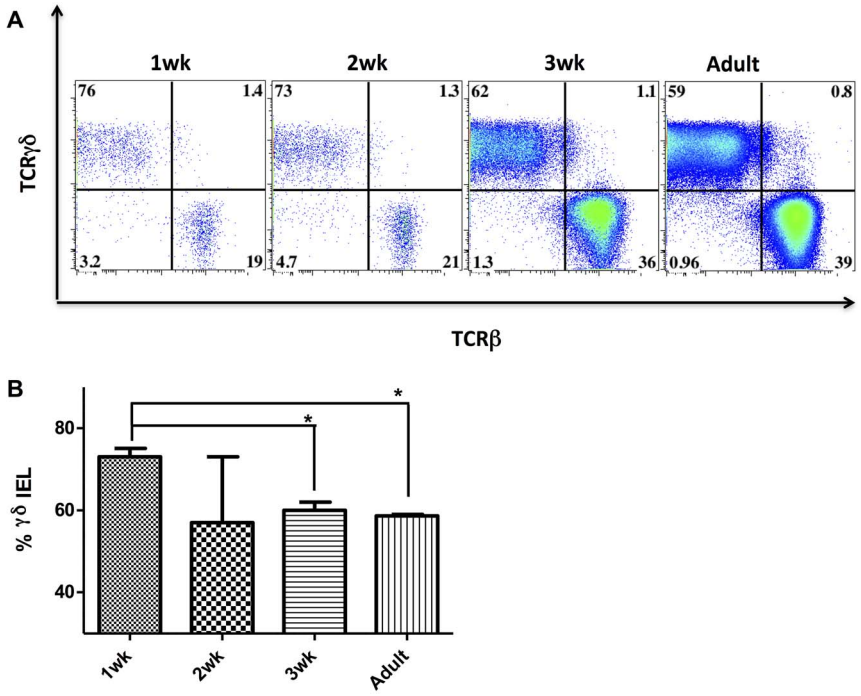

Figure 4. γδ IEL are the predominant IEL subtype in the immature murine small intestine. A) Flow cytometry of distal small intestinal intraepithelial cells from 1 week old (1 wk, n = 4), 2 weeks old (2 wk, n = 3), 3 weeks old (3 wk, n = 2) and adult (n = 1) C57BL/6J mice stained for CD3, CD8α, CD103, TCRγδ and TCRβ as described. Intraepithelial cells were pregated on CD103$^+$, CD3$^+$ to depict IEL and then further gated on TCRγδ and TCRβ as shown. B) Percent (mean ±SE) γδ IEL (defined as percent of total IEL that were TCRγδ$^+$, TCRβ$^-$ IEL) in the distal small intestines of 1 wk, 2 wk, 3 wk, and adult mice. Data are representative of 3 independent experiments (*$p<0.05$ when compared to 1 wk samples).
doi:10.1371/journal.pone.0099042.g004

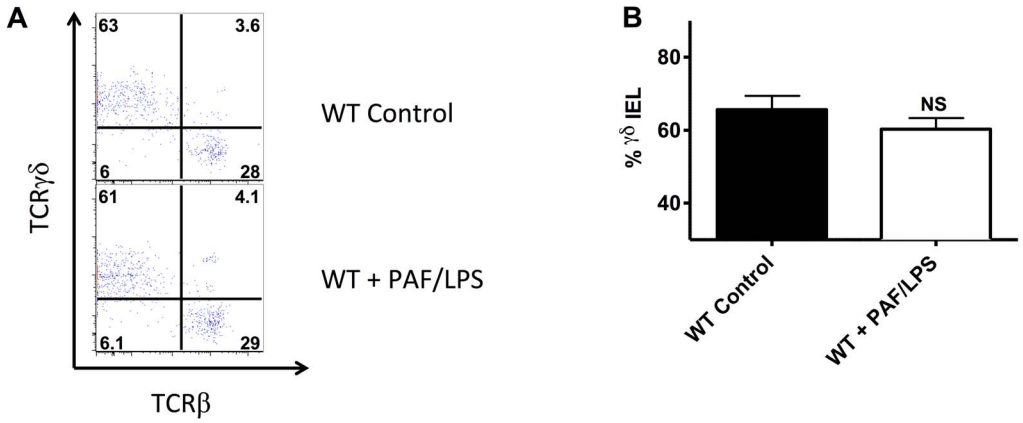

Figure 5. Intestinal injury in wild-type mice is not associated with a selective reduction in γδ IEL. A) Flow cytometry of small intestinal intraepithelial cells isolated from 2 weeks old dam fed wild-type (WT control, n = 2) or wild-type mice subjected to experimental gut injury as described (WT+PAF/LPS, n = 2). C57BL/6J mice stained for CD103, CD3, CD8α, TCRγδ and TCRβ as described above. Intraepithelial cells were pregated on $CD103^+$, $CD3^+$ to depict IEL and then further gated on TCRγδ and TCRβ as shown. B) Percent (mean ±SE) γδ IEL (defined as percent of total IEL that were $TCR\gamma\delta^+$, $TCR\beta^-$ IEL). Data are representative of 3 independent experiments (NS indicates no statistical difference between groups).
doi:10.1371/journal.pone.0099042.g005

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 TCR γδ 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

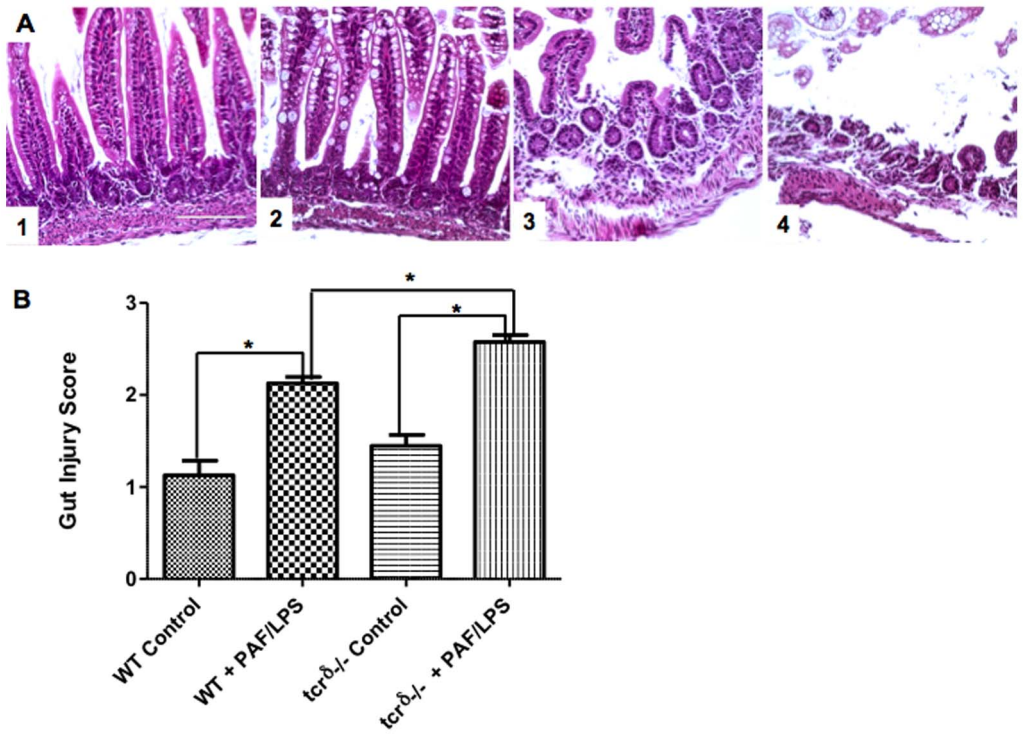

Figure 6. Retinoic acid-related orphan nuclear hormone receptor C (RORC) gene expression in intestinal lymphocyte subsets. (A) We measured gene expression levels of RORC by quantitative RT-PCR in 15 NEC tissue sections and 7 controls by quantitative RT-PCR array. RORC gene expression was significantly decreased in NEC samples versus controls ($p<0.001$). Relative level of mRNA expression of RORC in each sample was normalized to the expression level of reference gene GAPDH. (B) To determine whether loss of IEL contributed to reduction of RORC expression, we compared IEL with lamina propria lymphocytes (LPL) from the same tissue source using 10 non-NEC controls. RORC gene expression was significantly higher in IEL compared to LPL ($p = 0.01$). (C) RORC gene expression and proportions of total TCRγδ IEL correlated positively with each other ($p = 0.02$).
doi:10.1371/journal.pone.0099042.g006

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 αβ 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 $CD103^+$ $TCR\gamma\delta^+$ 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.

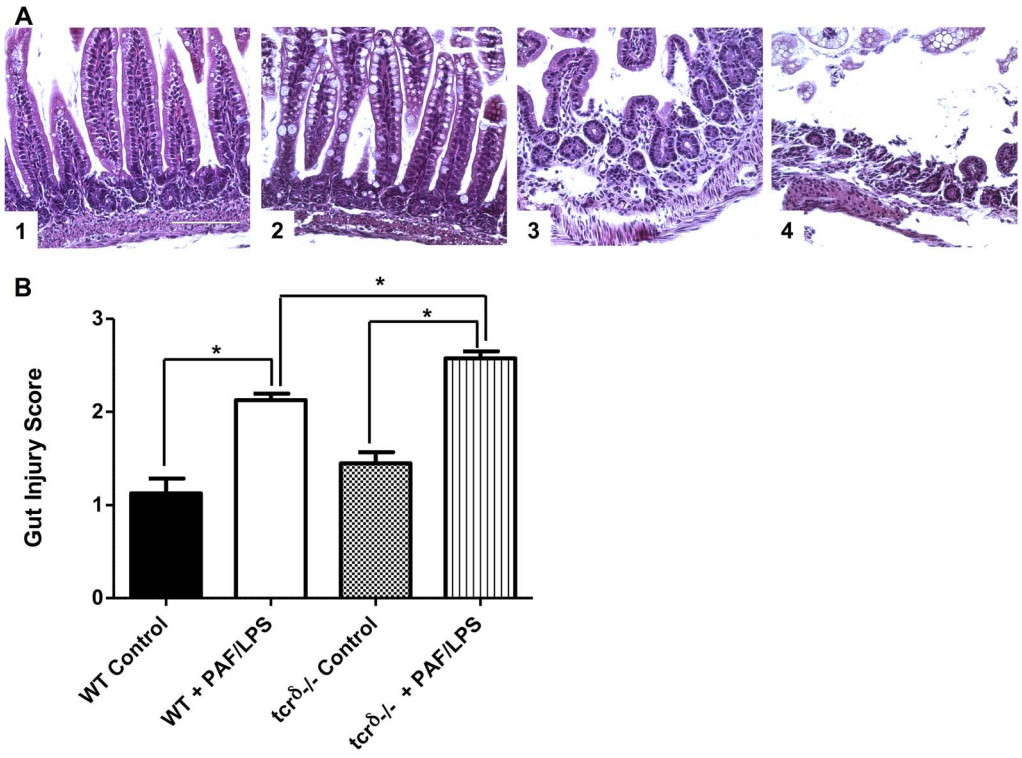

Figure 7. γδ T cells reduce experimental gut injury. A) Representative H&E staining of distal small intestines isolated from dam fed wild-type (1) or $TCR\delta^{-/-}$ (3) mice with normal histologic appearance; or wild-type (2) or $TCR\delta^{-/-}$ (4) mice subjected to experimental gut injury (PAF/LPS) 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 $TCR\delta^{-/-}$ PAF/LPS mice (4). B) Histologic severity score (mean ±SE) of distal small intestinal sections obtained from dam fed wild-type (WT control) or $TCR\delta^{-/-}$ ($tcr\delta^{-/-}$ control) mice; or wild-type (WT PAF/LPS) or $TCR\delta^{-/-}$ ($tcr\delta^{-/-}$ 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 (*$p<0.05$).
doi:10.1371/journal.pone.0099042.g007

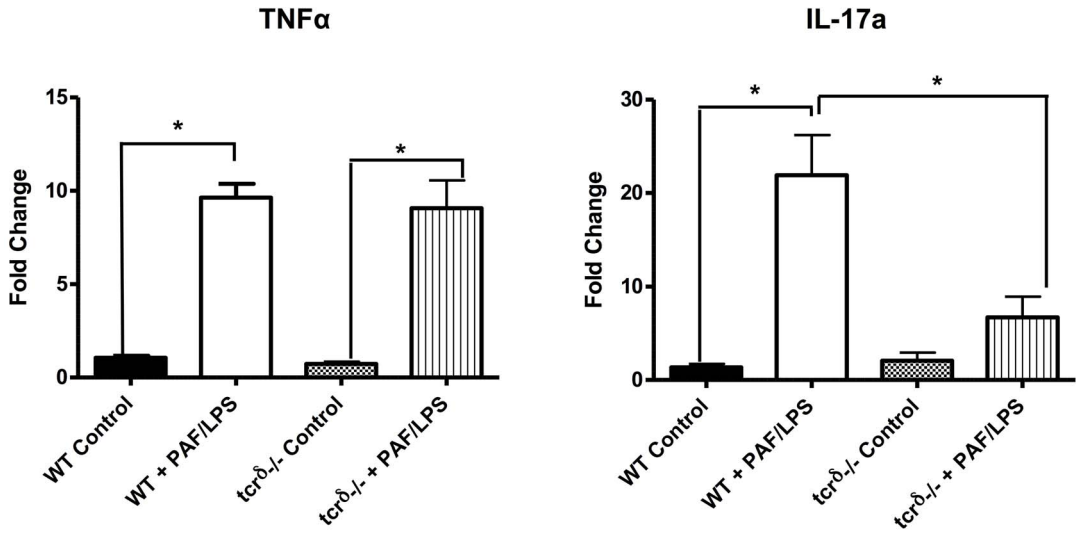

Figure 8. More severe intestinal injury in TCR$\delta^{-/-}$ mice was associated with increased TNFα and decreased IL17A gene expression. Gene expression of TNFα and IL17A as measured by quantitative RT-PCR in distal small intestinal sections obtained from dam fed wild type (WT control) or TCR$\delta^{-/-}$ (tcr$\delta^{-/-}$ control) mice; or wild type (WT PAF/LPS) or TCR$\delta^{-/-}$ (tcr$\delta^{-/-}$ PAF/LPS) mice subjected to experimental gut injury injury as described. Data are representative of 3 independent experiments with at least 3 mice per condition per experiment (*$p<0.05$).
doi:10.1371/journal.pone.0099042.g008

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].

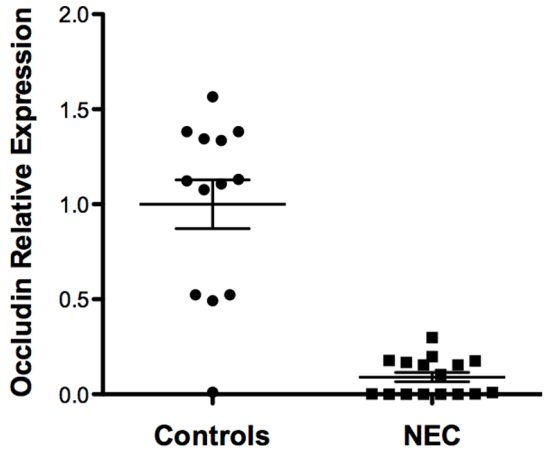

Figure 9. Occludin gene expression in human NEC versus control mucosa. (A) We measured gene expression levels of occluding by quantitative RT-PCR in 16 NEC tissue sections and 13 controls by quantitative RT-PCR array. Occludin gene expression was significantly decreased in NEC samples versus controls ($p<0.0001$). Relative level of mRNA expression of occludin in each sample was normalized to the expression level of reference gene GAPDH.
doi:10.1371/journal.pone.0099042.g009

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 [54]. 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 [59] 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 IEL 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 IEL in the ileum of premature infants is significantly reduced in surgical NEC. Complimentary animal and human data suggest a potentially important role of γδ IEL in IL17 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 representative 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).
(TIFF)

Acknowledgments

We are indebted to our surgical colleagues for their help in tissue acquisition.

Author Contributions

Conceived and designed the experiments: JHW TLD PWD. Performed the experiments: JHW PM DG. Analyzed the data: JHW MJR ZZ TK MTR PD DG. Contributed reagents/materials/analysis tools: MJR TLD MTR MDH DJM. Wrote the paper: JHW MJR PWD.

References

1. Claud EC, Lu L, Anton PM, Savidge T, Walker WA, et al. (2004) Developmentally regulated IkappaB expression in intestinal epithelium and susceptibility to flagellin-induced inflammation. Proc Natl Acad Sci U S A 101: 7404–7408.
2. Llanos AR, Moss ME, Pinzòn MC, Dye T, Sinkin RA, et al. (2002) Epidemiology of neonatal necrotising enterocolitis: a population-based study. Paediatr Perinat Epidemiol 16: 342–349.
3. Neu J (2005) Neonatal necrotizing enterocolitis: an update. Acta Paediatr Suppl 94: 100–105.
4. Fanaroff AA, Stoll BJ, Wright LL, Carlo WA, Ehrenkranz RA, et al. (2007) Trends in neonatal morbidity and mortality for very low birthweight infants.Am J Obstet Gynecol 196: 147 e1–8.
5. Holman RC, Stoll BJ, Clarke MJ, Glass RI (1997) The epidemiology of necrotizing enterocolitis infant mortality in the United States. Am J Public Health 87: 2026–2031.
6. Lin PW, Stoll BJ (2006) Necrotising enterocolitis. Lancet 368: 1271–1283.
7. Kliegman RM, Fanaroff AA (1981) Neonatal necrotizing enterocolitis: a nine-year experience. Am J Dis Child 135: 603–607.
8. Shah TA, Meinzen-Derr J, Gratton T, Steichen J, Donovan EF, et al. (2012) Hospital and neurodevelopmental outcomes of extremely low-birth-weight infants with necrotizing enterocolitis and spontaneous intestinal perforation. J Perinatol 32: 552–558.
9. Gibbons DL, Haque SF, Silberzahn T, Hamilton K, Langford C, et al. (2009) Neonates harbour highly active gammadelta T cells with selective impairments in preterm infants. Eur J Immunol 39: 1794–1806.
10. Bhagat G, Naiyer AJ, Shah JG, Harper J, Jabri B, et al. (2008) Small intestinal CD8+TCRgammadelta+NKG2A+ intraepithelial lymphocytes have attributes of regulatory cells in patients with celiac disease. J Clin Invest 118: 281–293.
11. Chen Y, Chou K, Fuchs E, Havran WL, Boismenu R (2002) Protection of the intestinal mucosa by intraepithelial gamma delta T cells. Proc Natl Acad Sci U S A 99: 14338–14343.
12. Groh V, Steinle A, Bauer S, Spies T (1998) Recognition of stress-induced MHC molecules by intestinal epithelial gammadelta T cells. Science 279: 1737–1740.
13. Ismail AS, Behrendt CL, Hooper LV (2009) Reciprocal interactions between commensal bacteria and gamma delta intraepithelial lymphocytes during mucosal injury. J Immunol 182: 3047–3054.
14. Komano H, Fujiura Y, Kawaguchi M, Matsumoto S, Hashimoto Y, et al. (1995) Homeostatic regulation of intestinal epithelia by intraepithelial gamma delta T cells. Proc Natl Acad Sci U S A 92: 6147–6151.
15. Yang H, Antony PA, Wildhaber BE, Teitelbaum DH (2004) Intestinal intraepithelial lymphocyte gamma delta-T cell-derived keratinocyte growth factor modulates epithelial growth in the mouse. J Immunol 172: 4151–4158.
16. Bergmann KR, Liu SX, Tian R, Kushnir A, Turner JR, et al. (2013) Bifidobacteria stabilize claudins at tight junctions and prevent intestinal barrier dysfunction in mouse necrotizing enterocolitis. Am J Pathol 182: 1595–1606.
17. Clark JA, Doelle SM, Halpern MD, Saunders TA, Holubec H, et al. (2006) Intestinal barrier failure during experimental necrotizing enterocolitis: protective effect of EGF treatment. Am J Physiol Gastrointest Liver Physiol 291: G938–G949.
18. Patel RM, Myers LS, Kurundkar AR, Maheshwari A, Nusrat A, et al. (2012) Probiotic bacteria induce maturation of intestinal claudin 3 expression and barrier function. Am J Pathol 180: 626–635.
19. Piena-Spoel M, Albers MJ, ten Kate J, Tibboel D (2001) Intestinal permeability in newborns with necrotizing enterocolitis and controls: Does the sugar absorption test provide guidelines for the time to (re-)introduce enteral nutrition? J Pediatr Surg 36: 587–592.
20. Shiou SR, Yu Y, Chen S, Ciancio MJ, Petrof EO et al. (2011) Erythropoietin protects intestinal epithelial barrier function and lowers the incidence of experimental neonatal necrotizing enterocolitis. J Biol Chem 286: 12123–12132.
21. Weitkamp JH, Koyama T, Rock MT, Correa H, Goettel JA et al. (2013) Necrotising enterocolitis is characterised by disrupted immune regulation and diminished mucosal regulatory (FOXP3)/effector (CD4, CD8) T cell ratios. Gut 62: 73–82.
22. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3: 1101–1108.
23. Weitkamp JH, Rudzinski E, Koyama T, Correa H, Matta P, et al. (2009) Ontogeny of FOXP3+ regulatory T cells in the postnatal human small intestinal and large intestinal lamina propria. Pediatr Dev Pathol 12: 43–449.
24. Hsueh W, González-Crussi F, Arroyave JL (1987) Platelet-activating factor: an endogenous mediator for bowel necrosis in endotoxemia. FASEB J 1: 403–405.
25. Maheshwari A, Kelly DR, Nicola T, Ambalavanan N, Jain SK, et al. (2011) TGF-beta2 suppresses macrophage cytokine production and mucosal inflammatory responses in the developing intestine. Gastroenterology 140: 242–253.
26. Sun X, Rozenfeld RA, Qu X, Huang W, Gonzalez-Crussi F, et al. (1997) P-selectin-deficient mice are protected from PAF-induced shock, intestinal injury, and lethality. Am J Physiol 273: G56–G61.
27. Kuo S, El Guindy A, Panwala CM, Hagan PM, Camerini V (2001) Differential appearance of T cell subsets in the large and small intestine of neonatal mice. Pediatr Res 49: 543–545.
28. Denning TL, Granger SW, Mucida D, Graddy R, et al. (2007) Mouse TCRalphabeta+CD8alphaalpha intraepithelial lymphocytes express genes that down-regulate their antigen reactivity and suppress immune responses. J Immunol 178: 4230–4239.
29. Khailova L, Dvorak K, Arganbright KM, Halpern MD, Kinouchi T, et al. (2009) Bifidobacterium bifidum improves intestinal integrity in a rat model of necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol 297: G940–G949.
30. Musemeche C, Caplan M, Hsueh W, Sun X, Kelly A (1991) Experimental necrotizing enterocolitis: the role of polymorphonuclear neutrophils. J Pediatr Surg 26: 1047–1049.
31. Tian R, Liu SX, Williams C, Soltau TD, Dimmitt R, et al. (2010) Characterization of a necrotizing enterocolitis model in newborn mice. Int J Clin Exp Med 3: 293–302.
32. Mirpuri J, Sotnikov I, Myers L, Denning TL, Yarovinsky F, et al. (2012) Lactobacillus rhamnosus (LGG) regulates IL-10 signaling in the developing murine colon through upregulation of the IL-10R2 receptor subunit. PloS One 7: e51955.
33. Cheroutre H, Lambolez F, Mucida D (2011) The light and dark sides of intestinal intraepithelial lymphocytes. Nat Rev Immunol 11: 445–456.
34. McElroy SJ, Weitkamp JH (2011) Innate immunity in the small intestine of the preterm infant. Neoreviews 12: e517–e526.
35. Asigbetse KE, Eigenmann PA, Frossard CP (2010) Intestinal lamina propria TcRgammadelta+ lymphocytes selectively express IL-10 and IL-17. J Investig Allergol Clin Immunol 20: 391–401.
36. Martin B, Hirota K, Cua DJ, Stockinger B, Veldhoen M (2009) Interleukin-17-producing gammadelta T cells selectively expand in response to pathogen products and environmental signals. Immunity 31: 321–330.
37. Park SG, Mathur R, Long M, Hosh N, Hao L, et al. (2010) T regulatory cells maintain intestinal homeostasis by suppressing γδ T cells. Immunity 33: 791–803.
38. Schaefer JS, Montufar-Solis D, Vigneswaran N, Klein JR (2010) ICOS promotes IL-17 synthesis in colonic intraepithelial lymphocytes in IL-10-/- mice. J Leukoc Biol 87: 301–308.
39. Unutmaz D. RORC2: the master of human Th17 cell programming (2009)Eur J Immunol 39: 1452–1455.
40. Edelblum KL, Shen L, Weber CR, Marchiando AM, Clay BS, et al. (2012) Dynamic migration of gammadelta intraepithelial lymphocytes requires occludin. Proc Natl Acad Sci U S A 109: 7097–7102.

41. Li Z, Zhang C, Zhou Z, Zhang J, Zhang J, et al. (2012) Small intestinal intraepithelial lymphocytes expressing CD8 and T cell receptor gammadelta are involved in bacterial clearance during Salmonella enterica serovar Typhimurium infection. Infect Immun 80: 565–574.
42. Boismenu R, Havran WL (1994) Modulation of epithelial cell growth by intraepithelial gamma delta T cells. Science 266: 1253–1255.
43. Dalton JE, Cruickshank SM, Egan CE, Mears R, Newton DJ, et al. (2006) Intraepithelial gammadelta+ lymphocytes maintain the integrity of intestinal epithelial tight junctions in response to infection. Gastroenterology 131: 818–829.
44. Fu YX, Roark CE, Kelly K, Drevets D, Campbell P, et al. (1994) Immune protection and control of inflammatory tissue necrosis by gamma delta T cells. J Immunol 153: 3101–3115.
45. Roberts SJ, Smith AL, West AB, Wen L, Findly RC, et al. (1996) T-cell alpha beta + and gamma delta + deficient mice display abnormal but distinct phenotypes toward a natural, widespread infection of the intestinal epithelium. Proc Natl Acad Sci U S A 93: 11774–11779.
46. Gordon P, Christensen R, Weitkamp JH, Maheshwari A (2012) Mapping the new world of necrotizing enterocolitis (NEC): review and opinion. E J Neonatol Res 2: 145–172, 2012.
47. Camerini V, Panwala C, Kronenberg M (1993) Regional specialization of the mucosal immune system. Intraepithelial lymphocytes of the large intestine have a different phenotype and function than those of the small intestine. J Immunol 151: 1765–1776.
48. Istvanic S, Yantiss RK, Baker SP, Banner BF (2007) Normal variation in intraepithelial lymphocytes of the terminal ileum. Am J Clin Pathol 127: 816–819.
49. Romano-Keeler J, Moore DJ, Brucker R, Lovvorn H, Wang C, et al. (2014) Early life establishment of site-specific microbial communities in the gut. Gut Microbes 5: 1–10.
50. Been JV, Lievense S, Zimmermann LJ, Kramer BW, Wolfs TG (2013) Chorioamnionitis as a risk factor for necrotizing enterocolitis: a systematic review and meta-analysis. J Pediatr 162: 236–242 e232.
51. Cepek KL, Parker CM, Madara JL, Brenner MB (1993) Integrin alpha E beta 7 mediates adhesion of T lymphocytes to epithelial cells. J Immunol 150: 3459–3470.
52. Shibahara T, Si-Tahar M, Shaw SK, Madara JL (2000) Adhesion molecules expressed on homing lymphocytes in model intestinal epithelia. Gastroenterology 118: 289–298.
53. Namachivayam K, Blanco CL, MohanKumar K, Jagadeeswaran R, Vasquez M, et al. (2013) Smad7 inhibits autocrine expression of TGF-beta2 in intestinal epithelial cells in baboon necrotizing enterocolitis. Am J Physiol Gastrointest Liver Physiol 304: G167–G180.
54. Suzuki R, Nakao A, Kanamaru Y, Okumura K, Ogawa H, et al. (2002) Localization of intestinal intraepithelial T lymphocytes involves regulation of alphaEbeta7 expression by transforming growth factor-beta. Int Immunol 14: 339–345.
55. Zhang F, Fuss IJ, Yang Z, Strober W (2013) Transcription of RORγt in developing Th17 cells is regulated by E-proteins. Mucosal Immunol doi: 10.1038/mi.2013.69 [Epup ahead of print].
56. Fuss IJ, Boirivant M, Lacy B, Strober W (2002) The interrelated roles of TGF-beta and IL-10 in the regulation of experimental colitis. J Immunol 168: 900–908.
57. Neu J, Walker WA (2011) Necrotizing enterocolitis. N Engl J Med 364: 255–264.
58. Wang Y, Hoenig JD, Malin KJ, Qamar S, Petrof EO, et al. (2009) 16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis. ISME J 8: 944–954.
59. Kiss EA, Vonarbourg C, Kopfmann S, Hobeika E, Finke D, et al. (2011) Natural aryl hydrocarbon receptor ligands control organogenesis of intestinal lymphoid follicles. Science 334: 1561–1565.
60. Li Y, Innocentin S, Withers DR, Roberts NA, Gallagher AR, et al. (2011) Exogenous stimuli maintain intraepithelial lymphocytes via aryl hydrocarbon receptor activation. Cell 147: 629–640.
61. Monteleone I, Rizzo A, Sarra M, Sica G, Sileri P, et al. (2011) Aryl hydrocarbon receptor-induced signals up-regulate IL-22 production and inhibit inflammation in the gastrointestinal tract. Gastroenterology 141: 237–248.
62. Sharma AA, Jen R, Butler A, Lavoie PM (2012) The developing human preterm neonatal immune system: a case for more research in this area. Clin Immunol 145: 61–68.