Published in final edited form as:

Gut. 2013 January ; 62(1): 73–82. doi:10.1136/gutjnl-2011-301551.

Necrotising enterocolitis is characterised by disrupted immune regulation and diminished mucosal regulatory (FOXP3)/effector (CD4, CD8) T cell ratios

Jörn-Hendrik Weitkamp¹, Tatsuki Koyama², Michael T Rock¹, Hernan Correa³, Jeremy A Goettel^{4,5}, Pranathi Matta¹, Kyra Oswald-Richter³, Michael J Rosen¹, Brian G Engelhardt⁶, Daniel J Moore¹, and D Brent Polk^{1,4,7,8}

¹Department of Pediatrics, Vanderbilt University School of Medicine, Monroe Carell Jr Children's Hospital at Vanderbilt, Nashville, Tennessee, USA

²Department of Biostatistics, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

³Department of Pathology, Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

⁴Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

⁵Division of Gastroenterology and Nutrition, Department of Pediatrics, Harvard Medical School, Children's Hospital Boston, Boston, Massachusetts, USA

⁶Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

⁷Department of Pediatrics, University of Southern California and The Saban Research Institute of Children's Hospital Los Angeles, Los Angeles, California, USA

⁸Department of Biochemistry & Molecular Biology, University of Southern California and The Saban Research Institute of Children's Hospital Los Angeles, Los Angeles, California, USA

Abstract

Background—Necrotising enterocolitis (NEC) is the most common gastrointestinal emergency in premature infants. Immaturity of gastrointestinal immune regulation may predispose preterm infants to NEC as FOXP3 T regulatory cells (Treg) are critical for intestinal immune homeostasis.

Correspondence to Dr Jörn-Hendrik Weitkamp, Division of Neonatology, Department of Pediatrics, Monroe Carell Jr. Children's Hospital at Vanderbilt, 2215 B Garland Ave., 1125 MRB IV/Light Hall, Nashville, TN 37232-0656, USA; hendrik.weitkamp@vanderbilt.edu.

Competing interests None.

Patient consent Since resected human tissue was used and de-identified before processing, the Vanderbilt IRB designated the study; non-human subject research; and waived the consent.

Ethics approval This study was conducted with the approval of the Vanderbilt University Medical Centre Ethics Committee. Vanderbilt Institutional Review Board.

Contributors JHW: conception and experimental design, experimental procedures, study supervision, data analysis and interpretation, funding acquisition and manuscript writing. TK: data analysis and manuscript writing. MR: data analysis and interpretation. HC: acquisition and quality control of tissue samples. JAG: provision of quantitative RT-PCR data, manuscript editing. PM: experimental procedures. KO-R: experimental support for Treg function data. MR: experimental procedures, data analysis and interpretation. BGE, DJM: data interpretation, manuscript editing. DBP: financial support, final approval of manuscript.

Provenance and peer review Not commissioned; externally peer reviewed.

Objective—To investigate the hypothesis that abnormal developmental regulation of lamina propria Treg would define premature infants with NEC.

Design—Lamina propria mononuclear cell populations from surgically resected ileum from 18 patients with NEC and 30 gestational age-matched non-NEC surgical controls were prospectively isolated. Polychromatic flow cytometry was performed to phenotype and analyse lamina propria T cell populations. The cytokine gene expression profile in NEC tissue was compared with that of non-NEC controls.

Results—The total number of Treg, CD4, or CD8 T cells in each ileum section was independent of gestational age, age or postmenstrual age and similar between patients with NEC and controls. In contrast, the ratio of Treg to CD4 T cells or Treg to CD8 T cells was significantly lower in NEC ileum than in infants without NEC (medians 2.9% vs 6.6%, $p=0.001$ and medians 6.6% vs 25.9%, $p<0.001$, respectively). For any given number of CD4 or CD8 T cells, Treg were, on average, 60% lower in NEC ileum than in controls. NEC tissue cytokine gene expression profiles were characteristic of inhibited Treg development or function. Treg/CD4 and Treg/CD8 ratios recovered between initial resection for NEC and reanastomosis.

Conclusion—The proportion of lamina propria Treg is significantly reduced in the ileum of premature infants with NEC and may contribute to the excessive inflammatory state of this disease.

INTRODUCTION

Necrotising enterocolitis (NEC) is the most common acquired gastrointestinal emergency in premature infants and a leading cause of death in the neonatal intensive care unit.¹ Despite advances in intensive care, mortality from NEC is up to 30%, causing over 800 deaths a year in the USA, and is on the rise given the increased initial survival of extremely premature infants.²⁻⁴ Surviving patients with NEC have significant morbidity, including parenteral nutrition dependence, feeding problems, bowel obstruction, short bowel syndrome, failure to thrive and neurosensory impairment.^{5,6} Although the pathogenesis of NEC and its associated complications remain undefined, a deregulated inflammatory response by the neonatal intestine to luminal bacteria is a unifying hypothesis that encompasses many of the factors that have been associated with the development of NEC.⁷⁻⁹ Findings supportive of the immunopathogenic theory of NEC include increased tissue and serum levels of inflammatory mediators, such as tumour necrosis factor (TNF) and platelet-activating factor in patients with NEC.¹⁰ Characterisation of proinflammatory cytokines associated with NEC may differentiate between infants likely to recover with little intervention from those who require surgical bowel resection.¹¹

Although T cell-mediated suppression of the early innate immune response is required to prevent death from acute infection,¹² T cells are traditionally not considered in the pathogenesis of NEC.^{4,7,9,13,14} However, T cells are present in the human fetal ileum at early gestation, accumulate after chorioamnionitis and can be activated in vitro.¹⁵⁻¹⁷ Single nucleotide polymorphism studies of genetic risk factors for NEC suggest that a Th1-mediated immune response is associated with more severe disease.¹⁸ In addition to alteration in effector T cell function, there may also be a role for deficient immune regulation in NEC. In humans and mice, a suppressor T cell population, termed T regulatory cells (Treg), expressing the transcription factor FOXP3 are critical for immune homeostasis in the intestinal tract.¹⁹⁻²¹ Studies using a T cell transfer model of colitis in mice show that infusion of FOXP3 Treg can be used to prevent the induction of colitis or treat established colonic inflammation.²² For disease prevention, the ratio between Treg and effector T cells is more important than the sole numerical change in Treg.²³ T cells that home to the gut in association with a host response can be distinguished through their expression of plasma

membrane proteins that bind intestinal tissue.²⁴ Homing markers identified on the surface of Tcells that home specifically to the small intestine include the integrin $\alpha 4\beta 7$.²⁵ Another integrin, $\alpha E\beta 7$ (CD103), has been involved in the retention of lymphocytes in the intestinal lamina propria.²⁶

Treg ontogeny is significantly delayed in mice.²⁷ In the rodent small intestine Treg do not reach adult numbers until 4 weeks.^{27,28} In contrast, in the human fetus mesenteric lymph nodes show an abundance of Treg already at 20 weeks' gestation.²⁹ These profound differences in Treg ontogeny between mice and humans make it difficult to extrapolate the role of human intestinal Treg from murine NEC models. Using stored intestinal tissue samples from human infants, we recently reported the presence of FOXP3 T cells in small and large intestinal tissue of even extremely premature human infants shortly after birth.³⁰ Using immunohistochemical methods, we demonstrated that the proportion of FOXP3 T cells did not change with gestational or postnatal age but may be reduced in patients with NEC. However, the phenotypic characterisation of suppressive Treg in humans is complex and typically requires an extensive panel of cell surface markers, including CD45RO, the interleukin 2 receptor (IL2R α (CD25)) and the interleukin 7 receptor (IL7R (CD127)) in addition to CD4 and FOXP3.³¹ Therefore we developed a method of prospective isolation of live lamina propria Tcells from resected ileum from infants with and without NEC for polychromatic flow cytometry analysis.

We now report a significant decrease in functional lamina propria Treg proportions in patients with NEC compared with non-NEC gestational age-matched controls. Lamina propria Treg depletion in NEC samples was associated with a tissue-specific inflammatory gene expression profile known to inhibit Treg development. Overall, the role of Treg in NEC development represents a new mechanistic link in disease pathogenesis and a potential target for future therapeutic interventions.

MATERIALS AND METHODS

Tissue samples

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. All samples were deidentified 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 before tissue release. The patient demographics and surgical indications for the non-NEC control tissues are shown in tables 1 and 2, respectively. All samples (NEC and controls) were from the ileum and patients were matched for gestational age.

Isolation of human lamina propria mononuclear cells (LPMCs)

Mucosa was dissected and a representative aliquot was fixed in formalin for paraffin embedding and immunohistochemistry. LPMCs were isolated as previously described.^{32,33} Briefly, the intestinal mucosa was washed in complete *Hanks' Balanced Salt Solution* (HBSS) without Ca^{2+} and Mg^{2+} (Mediatech, Manassas, Virginia, USA) containing 5 mM EDTA (Sigma-Aldrich, St Louis, Missouri, USA) until all crypts and individual epithelial cells were removed (online supplementary figure 1). The residual lamina propria was digested in complete RPMI (Mediatech) with 1 mg/ml collagenase type 1A (Sigma-Aldrich) for 1 h. The tissue slurry was then passed through a 70 μm cell strainer (BD Biosciences, San Jose, CA, USA) to remove undigested tissue pieces. LPMCs were washed in complete media and counted using trypan blue exclusion. Cells were frozen in liquid nitrogen for storage until analysis at a concentration of 1×10^6 cells/ml.

T cell identification and phenotyping

T cell immunophenotyping was performed using 10-colour flow cytometric analysis (supplementary figure 2). LPMCs were thawed, washed in phosphate buffered saline (PBS) and counted before staining with a phycoerythrin -Texas Red-conjugated amine viability dye (Invitrogen, Grand Island, NY, USA). Cells were subsequently 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 antibodies as previously described.³⁴ Cells were then fixed and permeabilised according to the manufacture's recommendation, followed by intracellular staining with antibody to FOXP3 conjugated with Alexa647 (clone 259D/C7; BD Biosciences). After washing, cells were resuspended in 500 ml of PBS containing 1% paraformaldehyde. Data were acquired using the LSRII flow cytometer (BD Biosciences) and analysed with FlowJo software V.8.6.3. (Tree Star, Ashland, Oregon, USA). Treg were identified as live, 'dump'-negative, CD4 CD8⁻ CD25^{high} FOXP3^{high} CD127^{low} CD45RO cells and expressed as the percentage of positive cells in the total CD4 T cell gate. All flow cytometric gating/analysis was confirmed by an immunologist (MTR) who was blinded to the sample origin. Fluorescent Minus One was used to control for non-specific binding.

T cell suppression assay

To confirm that phenotypically identified Treg were indeed suppressive, we performed an in vitro T cell suppression assay from Treg isolated from a large segment of small intestine (supplementary figure 3). This tissue was processed as described above and Treg were sorted by flow cytometry using the gating strategy employed to identify Treg in preterm intestinal tissue (except for FOXP3). Allogeneic CD4 T cells were magnetically sorted according to the manufacturer recommendations (Dynabeads; Invitrogen) from peripheral blood of a healthy donor and labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen) as previously described.³⁵ Wells in a 96-well flat bottom microtitre plate were coated with GaM IgG (12 ng/ml) (Invitrogen) followed by coating with OKT-3 antibody (1 µg/ml). CFSE-labelled CD4 T cells were added at a concentration of 2×10^5 cells per well together with soluble anti-CD28 (1 µg/ml) (BD Biosciences). Sorted Treg were added to one well with activated CD4 T cells at a concentration of 4×10^4 cells per well (ratio Treg to responder T cells 1:5). Activated and non-activated CD4 T cells without Treg were used as controls. The proliferation of responder T cells as indicated by CFSE dilution was analysed by flow cytometry on day 5. Culture supernatant cytokine concentrations on day 2 were determined using Milliplex Map multiplex magnetic bead-based immunoassay kits (Millipore, Billerica, Massachusetts, USA) on a Luminex Flexmap 3D Platform (Luminex, Austin, Texas, USA).

Gene expression profile

Total RNA was extracted from 25 mg of either fresh NEC and non-NEC ileum using the RNeasy Mini Kit or from six 10 mm sections of formalin-fixed, paraffin-embedded tissue pieces using the RNeasy FFPE Kit (Qiagen Valencia, California, USA). Total RNA was reverse transcribed using the RT² First Strand Kit (Qiagen) according to the manufacturer's instructions. The cDNA-containing reaction mixture was added to each well of a 96-well-plate PCR array for quantitative PCR (Th17 for Autoimmunity and Inflammation PCR Array, RT2 Profiler PCR Array; Qiagen). PCR cycles were performed according to the manufacturer's instructions. Expression levels of cytokine genes were quantified using qRT-PCR analysis based on intercalation of SYBR Green on an ABI 7300 real-time PCR system (Life Technologies, Carlsbad, California, USA). The relative level of mRNA expression for each gene in each sample was normalised to the expression level of reference gene glyceraldehyde 3-phosphate dehydrogenase.

Statistical analysis

Data were summarised using descriptive statistics, including mean, median, quartiles and range, for continuous variables, as well as percentage and frequency for categorical variables. Comparison between independent groups was conducted using the Wilcoxon rank sum test for continuous variables and Fisher's exact test for categorical variables.

Association between two variables (eg, counts for Treg and CD4 Tcells) was assessed using a regression model, including an interaction term for the control/NEC group id. Data were transformed using logarithmic transformation, as necessary, to achieve normality and for ease of graphical presentation. Correlation between continuous variables was determined by Spearman's rank correlation. To identify genes differently expressed between the NEC and control groups from a set of available genes, a false discovery rate of 0.05 was used as a threshold. All reported p values were two-tailed and considered significant at $p < 0.05$. All analyses were carried out with R version 2.11.0.³⁶

RESULTS

Lamina propria mononuclear cells were isolated from fresh ileum sections from preterm infants with NEC and from non-NEC controls matched for gestational age

To improve our understanding of the immunological interactions in NEC, we studied the development, phenotype and contribution of ileum lamina propria Treg in relationship to total CD4 or CD8 Tcells. We prospectively isolated LPMCs from fresh tissue obtained through medically indicated surgical resection for 18 NEC and 30 non-NEC indications. Non-NEC indications included resections for spontaneous (focal) intestinal perforation (two), congenital intestinal atresia (five), small bowel obstruction (six), gastroschisis with bowel necrosis (one) and tissue from reanastomoses for various surgical indications (table 1).

All tissue sections were ileum and were from infants of matching gestational age (GA) (table 2). Continuous data are summarised with median (quartiles) and categorical data with percentage (frequency). p Values are computed with Wilcoxon rank sum test (continuous) and Fisher's exact test (categorical).

Patients with tissue sections obtained for non-NEC indications were significantly older at the time of surgery, which resulted in a higher postmenstrual age (PMA). The median mucosa weight of NEC and non-NEC tissues was similar (310 mg vs 370 mg, respectively, $p = 0.478$) and for all tissues combined we isolated an average of approximately 20×10^6 total lamina propria mononuclear cells per gram tissue with 87% viability. After recovery from cryopreservation, the median numbers of total cells analysed in the NEC and non-NEC groups were 102 882 and 179 489 ($p = 0.295$), respectively (table 3).

Reduction of Treg to effector T cell proportions in NEC versus non-NEC lamina propria

We evaluated whether NEC was associated with overall loss of T cells. Although the percentage of CD8 T cells was significantly higher in the NEC group than in the non-NEC group ($p = 0.039$), we only observed a trend in the total number of lamina propria CD4 T cells ($p = 0.71$), CD8 T cells ($p = 0.15$) or Treg ($p = 0.10$) per gram tissue specimen between groups (figure 1A). In contrast, proportions of Treg compared with CD4 and Treg with CD8 T cells were significantly lower in the NEC group than in controls (medians 2.94% vs 6.58% ($p = 0.001$) and 6.57% vs 25.90% ($p < 0.001$), respectively) (figure 1B). In comparison with the number of CD4 and CD8 T cells, Treg counts in the NEC group were reduced by 55.1% (95% CI 25.9% to 73.1%) and 65.2% (95% CI 36.3% to 81%), respectively (figure 1C). The reduction in Treg to CD4 and Treg to CD8 T cells ratios in NEC ileum remained highly statistically significant after exclusion of the eight post-NEC reanastomosis cases ($p = 0.008$).

and $p=0.002$, respectively). Thus, the decrease in the proportion of Treg to effector T cells in the mucosa of patients with NEC may contribute to the pathology and severity of the disease.

Ileum lamina propria Treg in NEC show less evidence of activation and gut homing by immunophenotyping

CD45RO, a T cell memory marker, is associated with immune suppressive effector Treg populations.³¹ Therefore, we included CD45RO in the Treg phenotyping flow cytometry panel. A median of 46.2% of CD4 T cells in the NEC group expressed the memory cell marker CD45RO compared with 65.5% in non-NEC controls ($p=0.008$, figure 1D). We wondered whether the younger age, and therefore lower PMA of patients with NEC, might explain the lower percentage of CD4 T cells expressing CD45RO. However, CD45RO expression did not change with gestational age or PMA (data not shown), and lower CD45RO expression was therefore independently associated with NEC. To assess whether gating on CD45RO-expressing cells biased the Treg results, we analysed the data after exclusion of CD45RO and confirmed the NEC-associated Treg decrease: the median percentage of CD25⁺FOXP3^{high} cells of CD4 T cells was 5.47% in NEC and 8.94% in non-NEC tissue ($p=0.009$).

We included the analysis of CD45RO⁻ (naïve) Treg and found the total numbers of naïve Treg per tissue sample was very low compared with memory Treg (approximately 1/10) and did not differ between patients with NEC and controls (table 3). Patients with NEC had a higher proportion of naïve Treg (30%) than controls (10%) but the ratios of naïve Treg to CD4 T cells and naïve Treg to CD8 T cells were not different between NEC and controls.

In addition, we incorporated staining for intestinal lamina propria-homing integrins $\alpha 4\beta 7$ and $\alpha E\beta 7$ (CD103) to examine any possible discrepancies in expression of homing receptors. Although the analysis was limited by the overall low number of lamina propria Treg, $\alpha 4\beta 7$ was less frequently expressed on NEC Treg (median $\alpha 4\beta 7^{+}$ Treg (lower quartile, upper quartile) in NEC ($n=15$) was 24.1% (20.8, 43.8) and in non-NEC ($n=28$) was (32.4, 72.0), $p=0.03$). 40.5% Therefore $\alpha 4\beta 7$ appears to be more frequently downregulated on lamina propria Treg in NEC. In contrast, the percentage of CD103-expressing Treg was not significantly different between NEC and controls (median CD103 Treg (lower quartile, upper quartile) in NEC ($n=15$) was 48.4% (41.2, 55.3) and in non-NEC ($n=28$) was (26.5, 36.1% 45.2), $p=0.067$).

To test whether the analysed cells are truly regulatory, we sorted intestinal Treg from resected ileum and confirmed their suppressive function in vitro (figure 2). Ileum lamina propria Treg suppressed the proliferation of stimulated allogeneic responder CD4 T cells and the production of proinflammatory cytokines by 50%.

Ontogeny of lamina propria Treg

Delayed intestinal Treg ontogeny is documented in rodents.²⁸ Since our population consisted of extremely premature infants and because the non-NEC population was significantly older at the time of surgical resection, we assessed the developmental regulation of lamina propria Treg. We plotted the total number of lamina propria Treg for each tissue specimen (figure 3A) and lamina propria Treg to CD4 ratios (figure 3B) against GA, age and PMA independent of surgical indication. Treg were present in the lamina propria of ileum specimens in patients with and without NEC at an early GA (23 weeks). No association was found between either total Treg numbers or Treg to CD4 ratios with GA, age, or PMA (figure 3A,B). These data indicate that the full numeric potential of Treg in the human fetal gut is not delayed as it is in mice.

Lamina propria Treg frequencies recover after resolution of NEC

If Treg were important mediators of homeostasis and protection in the preterm gut, we hypothesised that their numbers would recover in infants who healed postoperatively from NEC. We were able to compare Treg percentages between initial resection for NEC and reanastomosis after intestinal healing in four patients. We found that the Treg proportions increased in all four patients with an average rise of between 1.2% and 4.7% a month (figure 4). This suggests that the NEC immunopathology in these patients is probably not due to intrinsic alterations in Treg proportions before disease onset but rather due to changes within the microenvironment that result in Treg depletion.

Inflammatory gene expression profile from resected lamina propria NEC tissue

Treg induction in the periphery is suppressed by certain proinflammatory cytokines.³⁷³⁸ Therefore we hypothesised that local inflammation might prevent Treg development or maintenance in the preterm gut undergoing NEC. We determined cytokine expression profiles in 15 small-bowel specimens from 14 patients with surgical NEC with seven gestational ages and surgical control samples matched for anatomical site. Compared with surgical controls, NEC tissue exhibited overall unchanged levels of interleukin (IL)2 and transforming growth factor (TGF) β gene expression but significantly increased levels of IL1 β , IL6, IL8, IL10, IL18, matrix metalloproteinase (MMP)3, MMP9 and TNF α gene expression at the time of resection (figure 5). Overall, the cytokine gene expression profile in NEC tissue was characteristic of inhibited Treg development. These findings support the inflammatory pathogenesis in NEC that is characterised by relative depletion of mucosal Treg despite their intact ontogeny in the premature small intestine.

DISCUSSION

The development and role of tissue-specific Treg in human infants is largely unknown. Our approach is the first comprehensive investigation of effector T cell and Treg proportions in the small intestine of preterm infants with and without NEC using polychromatic flow cytometry analysis of surgical specimens. We found a robust presence of CD4 and CD8 effector T cells in human NEC tissue, which is in contrast to the attenuated mucosal T cell response described in a piglet model of NEC.¹⁴ In contrast, we discovered that although Treg are generally abundant in the intestinal mucosa of premature infants, patients with NEC exhibit a significant decrease in ileum lamina propria Treg ratios compared with surgical controls matched for gestational age. Although patients with NEC had significantly less CD45RO (memory) CD4 T cells in ileum tissue, the reduced ratio of Treg to effector T cells in NEC tissues was independent of the numbers of CD45RO T cells, CD4 T cells or naïve Treg.

Intestinal Treg depletion in NEC was associated with unchanged levels of IL2 and TGF β gene expression but increased levels of IL1 β , IL6, IL8, IL10, MMP3, MMP9 and TNF α gene expression at the time of resection in NEC tissue compared with non-NEC controls. IL2 and TGF β play a critical role in generation, function and stabilisation of Treg.³⁹ IL1 β can downregulate the Treg lineage transcription factor FOXP3 and induce conversion of Treg into Th17 cells.⁴⁰ TNF α and IL6 can inhibit Treg development and function.³⁷³⁸ MMP9 and fragmented hyaluronan can reduce Treg numbers in tissue and therefore promote inflammatory injury.⁴¹⁴² In our study Treg proportions recovered in patients with NEC once the inflammation had stopped, suggesting that the strong inflammatory response in NEC temporarily inhibits Treg development and that the healing premature intestine provides factors that help retrieve immune tolerance.⁴³ Alternatively, inflammation may be suppressed as Treg ratios are increasing with healing.

This comprehensive cytokine expression profile in the ileum of surgical patients with NEC correlates well with previously described cytokine levels in serum and tissue of patients with NEC.¹⁰¹¹ Concentrations of IL8 and IL10 were significantly higher in infants with stage 3 NEC than in infants with less severe disease.¹¹ TNF α is a potent cytokine whose activation results in apoptosis and induction of inflammatory responses and is important in the initiation and propagation of NEC.¹⁰⁴⁴ The role of IL18 in NEC has been suggested from studies in animal models, where decreased development of NEC in IL18-deficient mice was seen.⁴⁵ Interestingly, we found a significant downregulation of IL18 in NEC tissue, which might be explained by loss of intestinal epithelial cells.⁴⁶ We did not study the origin of proinflammatory cytokine in the resected small bowel samples but it is likely to originate from intestinal T cells.⁴⁷ Stimulated T cells can cause degradation of the lamina propria matrix by production of matrix MMPs.⁴⁸⁴⁹ Although we cannot conclude causation, future studies geared at modifying inflammatory responses in the premature intestine and other organ systems should consider Treg as important correlates since depletion of Treg may contribute to the inflammatory sequelae associated with NEC. Our data refutes prevailing dogma, as T cell populations are traditionally not considered in the pathogenesis of NEC.⁴⁷⁹¹³

Although our data highlight the potential benefit of therapeutic strategies that promote tissue-specific Treg development, the data must be considered within the context of the specimens collected. Since we examined T cell and Treg composition in surgical tissue specimens, we limit our findings to surgical NEC. Most patients with NEC do not have an operation, only those with evidence of perforation or necrosis. Therefore, our study may inherently introduce bias by the limitations associated with analysing cases of NEC requiring surgical intervention. However, in this prospective study of patients with intestinal tissue resection, disease definition is more reliable through direct inspection of the bowel and pathology interpretation. Many retrospective studies on human NEC have been limited by the inclusion of cases with other possible causes of feeding intolerance, intestinal ischaemia and intestinal perforation.⁵⁰

On average, control cases were older at the time of tissue resection. It is unknown how chronological age changes the frequency of lamina propria Treg in relation to other T cells but in this, as well as in our previous study, we did not find a correlation between GA, chronological age or PMA and intestinal Treg proportions.³⁰ For study feasibility informed consent was waived and tissue specimens were de-identified after collection of basic demographic and diagnostic information before processing. Therefore, we do not know whether patients with NEC had had different feeding regimens and exposures to food antigen than controls. Although controls received similar anaesthesia, surgery and antimicrobial protocols, potentially longer exposure to more broad-spectrum antibiotics might have led to a change in flora, which could affect Treg frequencies. However, studies in germ-free or antibiotic-treated mice indicate that Treg populations are largely unchanged or even increased, making antibiotic exposure an unlikely explanation for Treg depletion in NEC.²⁸⁵¹⁵²

In conclusion we provide the first evidence that although extremely premature infants, in general, display an abundance of Treg in the small intestinal lamina propria, infants with NEC exhibit a significant temporary Treg to T effector cell imbalance. Decreased gut-homing marker expression on local Treg and the specific proinflammatory microenvironment may have a role in the relative Treg depletion. Studies are underway exploring the capacity of breast milk, vitamin A and probiotics in induction, maintenance and function of intestinal Treg in the developing human intestine. Interventions designed to enhance Treg numbers and function by modifying the local Treg milieu may represent a new approach in preventing NEC and other inflammatory complications of prematurity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful for the technical support of Melissa Hill and Marium Ali. We are indebted to our surgical colleagues for their help in tissue acquisition.

Funding The project described was supported by award number K08HD061607 (to JHW) from the Eunice Kennedy Shriver National Institute of Child Health & Human Development (NICHD) and award numbers RO1DK56008 and RO1DK066176 (both to DBP) from The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). The content is solely the responsibility of the authors and does not necessarily represent the official views of NICHD, NIDDK or the National Institutes of Health (NIH). This work was also supported by the Vanderbilt Physician Scientist Development Program Award (to JHW) and the Vanderbilt CTSA grant UL1 RR024975-01 from NCRR/NIH. Support for flow cytometry experiments and biostatistics was provided through the Vanderbilt University Medical Centre's Digestive Disease Research Centre sponsored by NIH grant P30DK058404.

REFERENCES

1. Fanaroff AA, Stoll BJ, Wright LL, et al. Trends in neonatal morbidity and mortality for very low birthweight infants. *Am J Obstet Gynecol.* 2007; 196:147.e1–8. [PubMed: 17306659]
2. Guthrie SO, Gordon PV, Thomas V, et al. Necrotizing enterocolitis among neonates in the United States. *J Perinatol.* 2003; 23:278–85. [PubMed: 12774133]
3. Holman RC, Stoll BJ, Clarke MJ, et al. The epidemiology of necrotizing enterocolitis infant mortality in the United States. *Am J Public Health.* 1997; 87:2026–31. [PubMed: 9431297]
4. Lin PW, Stoll BJ. Necrotizing enterocolitis. *Lancet.* 2006; 368:1271–83. [PubMed: 17027734]
5. Hintz SR, Kendrick DE, Stoll BJ, et al. Neurodevelopmental and growth outcomes of extremely low birth weight infants after necrotizing enterocolitis. *Pediatrics.* 2005; 115:696–703. [PubMed: 15741374]
6. Rees CM, Pierro A, Eaton S. Neurodevelopmental outcomes of neonates with medically and surgically treated necrotizing enterocolitis. *Arch Dis Child Fetal Neonatal Ed.* 2007; 92:F193–8. [PubMed: 16984980]
7. Neu J, Walker WA. Necrotizing enterocolitis. *N Engl J Med.* 2011; 364:255–64. [PubMed: 21247316]
8. Grave GD, Nelson SA, Walker WA, et al. New therapies and preventive approaches for necrotizing enterocolitis: report of a research planning workshop. *Pediatr Res.* 2007; 62:510–14. [PubMed: 17667844]
9. Nanthakumar N, Meng D, Goldstein AM, et al. The mechanism of excessive intestinal inflammation in necrotizing enterocolitis: an immature innate immune response. *PLoS One.* 2011; 6:e17776. [PubMed: 21445298]
10. Caplan MS, Sun XM, Hseuh W, et al. Role of platelet activating factor and tumor necrosis factor- α in neonatal necrotizing enterocolitis. *J Pediatr.* 1990; 116:960–4. [PubMed: 2348301]
11. Edelson MB, Bagwell CE, Rozycki HJ. Circulating pro- and counterinflammatory cytokine levels and severity in necrotizing enterocolitis. *Pediatrics.* 1999; 103:766–71. [PubMed: 10103300]
12. Kim KD, Zhao J, Auh S, et al. Adaptive immune cells temper initial innate responses. *Nat Med.* 2007; 13:1248–52. [PubMed: 17891146]
13. Afrazi A, Sodhi CP, Richardson W, et al. New insights into the pathogenesis and treatment of necrotizing enterocolitis: toll-like receptors and beyond. *Pediatr Res.* 2011; 69:183–8. [PubMed: 21135755]
14. Anttila A, Kauppinen H, Koivusalo A, et al. T-cell-mediated mucosal immunity is attenuated in experimental necrotizing enterocolitis. *Pediatr Surg Int.* 2003; 19:326–30. [PubMed: 12764628]
15. Spencer J, Dillon SB, Isaacson PG, et al. T cell subclasses in fetal human ileum. *Clin Exp Immunol.* 1986; 65:553–8. [PubMed: 3536221]

16. MacDonald TT, Spencer J. Evidence that activated mucosal T cells play a role in the pathogenesis of enteropathy in human small intestine. *J Exp Med*. 1988; 167:1341–9. [PubMed: 2965735]
17. Wolfs TG, Buurman WA, Zoer B, et al. Endotoxin induced chorioamnionitis prevents intestinal development during gestation in fetal sheep. *PLoS One*. 2009; 4:e5837. [PubMed: 19503810]
18. Treszl A, Tulassay T, Vasarhelyi B. Genetic basis for necrotizing enterocolitis—risk factors and their relations to genetic polymorphisms. *Front Biosci*. 2006; 11:570–80. [PubMed: 16146753]
19. Veltkamp C, Ruhwald R, Giesem T, et al. CD4+CD25+ cell depletion from the normal CD4+ T cell pool prevents tolerance toward the intestinal flora and leads to chronic colitis in immunodeficient mice. *Inflamm Bowel Dis*. 2006; 12:437–46. [PubMed: 16775487]
20. Moes N, Rieux-Laucat F, Begue B, et al. Reduced expression of FOXP3 and regulatory T-cell function in severe forms of early-onset autoimmune enteropathy. *Gastroenterology*. 2010; 139:770–8. [PubMed: 20537998]
21. Torgerson TR, Linane A, Moes N, et al. Severe food allergy as a variant of IPEX syndrome caused by a deletion in a noncoding region of the FOXP3 gene. *Gastroenterology*. 2007; 132:1705–17. [PubMed: 17484868]
22. Mottet C, Uhlig HH, Powrie F. Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells. *J Immunol*. 2003; 170:3939–43. [PubMed: 12682220]
23. Luhn K, Simmons CP, Moran E, et al. Increased frequencies of CD4+ CD25(high) regulatory T cells in acute dengue infection. *J Exp Med*. 2007; 204:979–85. [PubMed: 17452519]
24. Agace WW. T-cell recruitment to the intestinal mucosa. *Trends Immunol*. 2008; 29:514–22. [PubMed: 18838302]
25. Hamann A, Andrew DP, Jablonski-Westrich D, et al. Role of alpha 4-integrins in lymphocyte homing to mucosal tissues in vivo. *J Immunol*. 1994; 152:3282–93. [PubMed: 7511642]
26. Schon MP, Arya A, Murphy EA, et al. Mucosal T lymphocyte numbers are selectively reduced in integrin alpha E (CD103)-deficient mice. *J Immunol*. 1999; 162:6641–9. [PubMed: 10352281]
27. Fontenot JD, Dooley JL, Farr AG, et al. Developmental regulation of Foxp3 expression during ontogeny. *J Exp Med*. 2005; 202:901–6. [PubMed: 16203863]
28. Atarashi K, Tanoue T, Shima T, et al. Induction of colonic regulatory T cells by indigenous *Clostridium* species. *Science*. 2011; 331:337–41. [PubMed: 21205640]
29. Mold JE, Michaelsson J, Burt TD, et al. Maternal alloantigens promote the development of tolerogenic fetal regulatory T cells in utero. *Science*. 2008; 322:1562–5. [PubMed: 19056990]
30. Weitkamp JH, Rudzinski E, Koyama T, et al. Ontogeny of FOXP3(+) regulatory T cells in the postnatal human small intestinal and large intestinal lamina propria. *Pediatr Dev Pathol*. 2009; 12:443–9. [PubMed: 19203136]
31. Miyara M, Sakaguchi S. Human FoxP3(+)CD4(+) regulatory T cells: their knowns and unknowns. *Immunol Cell Biol*. 2011; 89:346–51. [PubMed: 21301480]
32. Alford SK, Longmore GD, Stenson WF, et al. CD46-induced immunomodulatory CD4+ T cells express the adhesion molecule and chemokine receptor pattern of intestinal T cells. *J Immunol*. 2008; 181:2544–55. [PubMed: 18684945]
33. Bull DM, Bookman MA. Isolation and functional characterization of human intestinal mucosal lymphoid cells. *J Clin Invest*. 1977; 59:966–74. [PubMed: 323291]
34. Engelhardt BG, Jagasia M, Savani BN, et al. Regulatory T cell expression of CLA or alpha(4)beta(7) and skin or gut acute GVHD outcomes. *Bone Marrow Transplant*. 2011; 46:436–42. [PubMed: 20577222]
35. Oswald-Richter KA, Beachboard DC, Zhan X, et al. Multiple mycobacterial antigens are targets of the adaptive immune response in pulmonary sarcoidosis. *Respir Res*. 2010; 11:161. [PubMed: 21092305]
36. Team, RDC. R: A Language and Environment for Statistical Computing. Vienna, Austria: 2011. <http://www.R-project.org>
37. Korn T, Reddy J, Gao W, et al. Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. *Nat Med*. 2007; 13:423–31. [PubMed: 17384649]
38. Valencia X, Stephens G, Goldbach-Mansky R, et al. TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. *Blood*. 2006; 108:253–61. [PubMed: 16537805]

39. Horwitz DA, Zheng SG, Wang J, et al. Critical role of IL-2 and TGF-beta in generation, function and stabilization of Foxp3+CD4+ Treg. *Eur J Immunol.* 2008; 38:912–15. [PubMed: 18395858]
40. Deknuydt F, Bioley G, Valmori D, et al. IL-1beta and IL-2 convert human Treg into T (H)17 cells. *Clin Immunol.* 2009; 131:298–307. [PubMed: 19211307]
41. Brusko TM, Wasserfall CH, Hulme MA, et al. Influence of membrane CD25 stability on T lymphocyte activity: implications for immunoregulation. *PLoS One.* 2009; 4:e7980. [PubMed: 19956753]
42. Bollyky PL, Falk BA, Wu RP, et al. Intact extracellular matrix and the maintenance of immune tolerance: high molecular weight hyaluronan promotes persistence of induced CD4+CD25+ regulatory T cells. *J Leukoc Biol.* 2009; 86:567–72. [PubMed: 19401397]
43. Bollyky PL, Wu RP, Falk BA, et al. ECM components guide IL-10 producing regulatory T-cell (TR1) induction from effector memory T-cell precursors. *Proc Natl Acad Sci U S A.* 2011; 108:7938–43. [PubMed: 21518860]
44. McElroy SJ, Prince LS, Weitkamp JH, et al. Tumor necrosis factor receptor 1-dependent depletion of mucus in immature small intestine: a potential role in neonatal necrotizing enterocolitis. *Am J Physiol Gastrointest Liver Physiol.* 2011; 4:G656–66. [PubMed: 21737776]
45. Halpern MD, Khailova L, Molla-Hosseini D, et al. Decreased development of necrotizing enterocolitis in IL-18-deficient mice. *Am J Physiol Gastrointest Liver Physiol.* 2008; 294:G20–6. [PubMed: 17947451]
46. Takeuchi M, Nishizaki Y, Sano O, et al. Immunohistochemical and immuno-electron-microscopic detection of interferon-gamma-inducing factor (“interleukin-18”) in mouse intestinal epithelial cells. *Cell Tissue Res.* 1997; 289:499–503. [PubMed: 9232828]
47. Drakes ML, Blanchard TG, Czinn SJ. Colon lamina propria dendritic cells induce a proinflammatory cytokine response in lamina propria T cells in the SCID mouse model of colitis. *J Leukoc Biol.* 2005; 78:1291–300. [PubMed: 16204624]
48. Pender SL, Tickle SP, Docherty AJ, et al. A major role for matrix metalloproteinases in T cell injury in the gut. *J Immunol.* 1997; 158:1582–90. [PubMed: 9029093]
49. Pender SL, Fell JM, Chamow SM, et al. A p55 TNF receptor immunoadhesin prevents T cell-mediated intestinal injury by inhibiting matrix metalloproteinase production. *J Immunol.* 1998; 160:4098–103. [PubMed: 9558121]
50. Gordon PV, Swanson JR, Attridge JT, et al. Emerging trends in acquired neonatal intestinal disease: is it time to abandon Bell’s criteria? *J Perinatol.* 2007; 27:661–71. [PubMed: 17611610]
51. Ivanov II, Frutos Rde L, Manel N, et al. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. *Cell Host Microbe.* 2008; 4:337–49. [PubMed: 18854238]
52. Min B, Thornton A, Caucheteux SM, et al. Gut flora antigens are not important in the maintenance of regulatory T cell heterogeneity and homeostasis. *Eur J Immunol.* 2007; 37:1916–23. [PubMed: 17549737]

Significance of this study

What is already known about this subject?

- ▶ Necrotising enterocolitis (NEC) is the most common acquired gastrointestinal medical and surgical emergency in premature infants and characterised by an acute inflammatory cascade leading to bowel necrosis.
- ▶ NEC originates from immaturity of the intestinal barrier and mucosal immunity, followed by bacterial invasion and exaggerated inflammation due to immature immune regulation.
- ▶ FOXP3 T regulatory cells (Treg) are essential for intestinal immune homeostasis through suppression of innate and adaptive host responses. Treg development can be interrupted by a local proinflammatory cytokine milieu.
- ▶ The ontogeny of lamina propria Treg in the small intestinal tract is significantly delayed in rodents. Although intestinal FOXP3 cells can be identified immediately after birth in extremely premature infants, the effects of gestational age and postnatal maturation on functional Treg in the small intestine are unknown.

What are the new findings?

- ▶ Functional Treg are present in the human small intestinal mucosa at early age.
- ▶ Using polychromatic flow cytometry on surgical resected tissue, we discovered a significantly decreased Treg to effector T cell ratio in premature infants with NEC compared with gestational age-matched controls.
- ▶ Decreased Treg ratios in NEC tissue correlated with a mucosal cytokine expression profile characteristic of inhibited Treg induction. Clinical recovery from disease resulted in significant improvement in immune homeostasis.

How might it impact on clinical practice in the foreseeable future?

- ▶ Our data refute the prevailing dogma that T cells do not play a role in NEC pathogenesis. Therefore, our results may open a new path to early interventions for NEC aimed at augmenting or sustaining mucosal regulatory adaptive immune responses.

Figure 1.

Diminished lamina propria Treg ratios in patients with necrotising enterocolitis (NEC) versus non-NEC surgical controls. (A) Dot plot of total number of CD4 T cells, CD8 T cells and Treg. Total cells were counted as total number of events in the respective flow cytometry gate normalised for tissue weight and plotted comparing NEC (closed circles) and non-NEC (open circles) lamina propria. Comparing NEC tissue (closed circles) with control tissue (open circles): CD4 T cells: $p=0.71$, CD8 T cells: $p=0.15$, Treg: $p=0.10$. (B) Dot plot of Treg proportions expressed as percentage Treg of total CD4 and CD8 T cells, respectively. Comparing NEC tissue (closed circles) with non-NEC controls (open circles): Treg/CD4 T cells: $p=0.001$, Treg/CD8 T cells $p<0.001$. The reduction in Treg to CD4 and

Treg to CD8 T cells ratios in NEC ileum remained highly statistically significant after exclusion of cases of post-NEC reanastomosis ($p=0.008$ and $p=0.002$, respectively). (C) Ordinary regression analysis of lamina propria Treg numbers. Treg numbers increased with CD4 and CD8 T cell counts and Treg numbers in the NEC group (closed circles) were consistently lower (60e70%) than those of the non-NEC surgical control group (open circles), regardless of CD4 and CD8 T cell counts. (D) Plotting CD45RO/CD4 T cells against all CD4 T cells, the ratio of CD45RO/CD4 T cells to all CD4 T cells was lower in NEC (closed circles) than in non-NEC controls (open circles, $p=0.008$).

Figure 2.

Lamina propria Treg suppress proliferation and cytokine production of activated CD4 T cells. Sorted lamina propria Treg (4×10^4) isolated from a surgical ileum specimen were co-cultured with 2×10^5 magnetically sorted peripheral blood CD4 T cells from a healthy donor. Responder CD4 T cells were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and stimulated with OKT-3 antibody and anti-CD28. Activated and non-activated CD4 T cells without Treg were used as controls. (A) The proliferation of responder T cells as indicated by CFSE dilution was analysed by flow cytometry on day 5. Treg presence suppressed the percentage of proliferating cells by 50% compared with responder cells without Treg. (B) Culture supernatant cytokine concentrations on day 2 were determined using Milliplex Map multiplex magnetic bead-based immunoassay kits on a Luminex Flexmap 3D Platform. Except for IL10, Th1 and Th2 cytokine production by responder cells was suppressed by approximately 50%. IL, interleukin, TNF α , tumour necrosis factor α .

Figure 3.

Proportions of lamina propria Treg do not increase with postnatal development in premature infants. Total Treg per ileum mucosa specimen (A) and Treg to CD4 T cell ratios (B) were plotted for necrotising enterocolitis (NEC) (closed circles) and non-NEC (open circles) samples against gestational age (GA), age and postmenstrual age (PMA gestational age plus chronological age). Lamina propria Treg were present at an early gestational age (as early as 23 weeks). There was no association between either total Treg numbers or Treg to CD4 ratios with GA, age, or PMA (Pearson's correlation (95% CI) with GA: 0.072 (−0.22 to 0.35), $p=0.63$, age: −0.0057 (−0.29 to 0.28) $p=0.97$ and PMA: 0.047 (−0.24 to 0.32) $p=0.75$).

Figure 4.

Increase in ileum Treg proportions in patients with necrotising enterocolitis (NEC) between initial surgery and bowel reanastomosis. Scatter plot of four preterm patients with NEC (26 weeks' gestation) with pair of dots representing one patient. The slope represents the rate of change. The average increase in Treg proportion between initial surgery (open circles) and bowel reanastomosis (closed circles) was 0.11% per day for Treg/CD4 T cell ratios and 0.53% per day for Treg/CD8 T cell ratios.

Figure 5.

Cytokine gene expression profile of necrotising enterocolitis (NEC) ileum compared with non-NEC surgical controls. Cytokine gene expression as determined by quantitative real-time PCR from 15 small bowel specimens of 14 patients with surgical NEC (closed circles) compared with seven tissue-site and gestational age-matched surgical control specimens (open circles) expressed as normalised ratio to glyceraldehyde-3-phosphate dehydrogenase. IL, interleukin; TGF, transforming growth factor; MMP, matrix metalloproteinase; TNF, tumour necrosis factor.

Table 1
Origins of control small intestinal tissue samples

Origin	N (%)
Reanastomosis after NEC surgery	8 (27)
Stricture removal after medical NEC	5 (17)
Resection for congenital intestinal atresia	3 (10)
Reanastomosis after congenital volvulus repair	3 (10)
Reanastomosis after SIP repair	3 (10)
Spontaneous intestinal perforation (SIP)	2 (7)
Reanastomosis after gastroschisis repair	2 (7)
Reanastomosis after meconium perforation repair	1 (3)
Gastroschisis with bowel necrosis	1 (3)
Reanastomosis congenital bowel obstruction repair	1 (3)
Adhesion removal after congenital intestinal atresia repair	1 (3)
Total	30 (100)

NEC, necrotising enterocolitis.

Table 2

Demographics of patients with necrotising enterocolitis (NEC) and controls

Demographics	Acute NEC (N=18)	Controls (N=30)	p Values
Gestational age (weeks)	27.3 (26.0, 29.9)	27.0 (25.4, 34.1)	0.94
Age (days)	17.0 (10.0, 29.5)	56.5 (48.5, 84.8)	0.002
Postmenstrual age (weeks)	30.8 (30.0, 39.2)	38.1 (35.4, 42.9)	0.004
Female	44% (8)	53% (16)	0.77

Continuous data are summarised with median (quartiles) and categorical data with percentages (frequency).

p Values are computed with Wilcoxon rank sum test (continuous) and Fisher's exact test (categorical).

Table 3

T cell subclasses, total numbers and ratios comparing samples from patients with necrotising enterocolitis (NEC) with those from non-NEC surgical controls

	Acute NEC (n=18)	Controls (n=30)	p Values*
Total cells	102 882 (56 028; 236 800) [†]	179 489 (107 768; 262 712)	0.30
Live CD3	3344 (1551; 6086)	2800 (1448; 6525)	1.00
CD3CD4	1438 (571; 4000)	1242 (650; 3049)	0.94
CD4CD45RO	538 (182; 1190)	906 (428; 2274)	0.27
CD3CD8	559 (288; 1448)	333 (172; 932)	0.36
Naive Treg	11 (6; 20)	9 (3; 23)	0.64
Treg [‡]	44 (18; 80)	100 (33; 164)	0.12
Live CD3 (%)	2.3 (1.1; 5.5)	1.9 (0.6; 4.2)	0.42
CD4/CD3 (%)	46.9 (40.9; 56.6)	48.57 (39.7; 57.3)	0.77
CD45RO/CD4 (%)	46.2 (30.2; 64.6)	65.5 (51.0; 82.3)	0.01
CD8/CD3 (%)	20.5 (11.3; 31.3)	14.5 (10.0; 18.0)	0.04
Naive Treg/CD4 (%)	0.9 (0.6; 1.0)	0.6 (0.2; 1.4)	0.36
Treg/CD4 (%)	2.9 (2.0; 4.6)	6.6 (4.0; 11.8)	0.001
Naive Treg/CD8 (%)	1.5 (0.1; 3.2)	1.2 (0.5; 4.9)	0.62
Treg/CD8 (%)	6.6 (4.5; 9.3)	25.9 (11.0; 47.6)	<0.001

* Wilcoxon rank sum test.

[†] Median (first quartile; third quartile).

[‡] Treg defined as viable CD4 CD25^{high} FOXP3^{high} CD127^{low} CD45RO cells.