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Impairment of regulatory T cells in patients with neonatal necrotizing enterocolitis

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ABSTRACT

Necrotizing enterocolitis (NEC) is a life-threatening condition that can occur in about 7% of pre-term infants, and approximately 20% to 30% of the cases will end in death. An overactive immune response is thought to be a primary instigator of many symptoms during NEC. Hence, we hypothesized that NEC patients might present impairment in regulatory T (Treg) cells that limited their capacity to contain the excessive inflammation-induced damage. To investigate this, peripheral blood mononuclear cells were collected from NEC and non-NEC infants with matching age and weight. Treg cells, identified as $CD3^+CD4^+CD25^{+/hi}Foxp3^+$ T cells, were present at significantly lower frequency in NEC infants than in non-NEC infants. We also observed that the frequency of $IL\text{-}17^+$ $CD4^+$ T cells was significantly higher in NEC infants, while the frequencies of $IL\text{-}10^+$ and $TGF\text{-}\beta^+$ $CD4^+$ T cells were significantly lower in NEC infants. The $CD4^+CD25^{+/hi}$ Treg cells from NEC infants were capable of suppressing $CD4^+CD25^-$ T conventional cell proliferation, but with significantly reduced potency than the $CD4^+CD25^{+/hi}$ Treg cells from non-NEC infants. In addition, the $CD4^+CD25^{+/hi}$ Treg cells from non-NEC infants, but not those from NEC infants, were capable of suppressing IL-17 expression. Furthermore, the $CD4^+CD25^{+/hi}$ Treg cells from NEC infants displayed reduced expression of CTLA-4, LAG-3, and Helios, compared to those from non-NEC infants. Overall, these results demonstrated that Treg cells from NEC infants displayed a multitude of functional impairments, and suggested that Treg cells might serve as a treatment target in NEC.

1. Introduction

Necrotizing enterocolitis (NEC) is a life-threatening condition characterized by excessive inflammation-mediated damages in the intestinal tract. Early symptoms include bloody stool and abdominal distention, which may develop into intestinal perforation, peritonitis, and systemic hypotension. NEC can occur in about 7% of premature infants, with 20% to 30% of the affected ending in death [1, 2]. Those that survive NEC are facing increased risks of other complications, including difficulties in feeding, delayed development in multiple growth parameters, microcephaly, late sepsis, and short bowel syndrome [3]. The direct causes of NEC remain unclear. Epidemiological investigations suggest that genetic predisposition, abnormal microbial colonization, aberrant immune responses, and intestinal immaturity are likely causes.

An overactive immune response is thought to be a primary instigator of many symptoms during NEC. Compared to the adult intestine, the preterm infant intestine presents higher expression of the LPS sensing molecule TLR4 and lower expression of the NF-κB regulator IκB, and tends to mount a more excessive inflammatory response to luminal microbial materials [4, 5]. Compared to non-NEC infants, NEC infants present an altered cytokine expression profile [6, 7]. In NEC serum samples, IL-6, IL-8, and IL-10 are strongly upregulated, while IL-2, IL-4, IL-5, and IFN-γ are weakly upregulated [7]. In contrast, TGF-β levels are lower in NEC subjects, both in the blood and in the necrotizing tissue [8]. The overexpression of proinflammatory cytokines in NEC subjects happens after NEC onset, suggesting that cytokine imbalance may not be a direct cause of NEC; however, this cytokine imbalance can still contribute to NEC by exacerbating inflammation and tissue damage [8]. In NEC animal models, the release of proinflammatory cytokines can induce septic shock in the absence of bacterial infections [9]. Investigations in mice demonstrated that $Rag1^{-/-}$ mice were protected against NEC development, and that NEC was transferable from NEC mice to non-NEC mice *via* intestinal lymphocytes

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[10]. In addition, TLR4 signaling increased the development of murine Th17 cells, while inhibition of IL-17 receptor or STAT3 attenuated NEC development in mice [10].

Th17 cells and regulatory T (Treg) cells are functionally diverse subsets of $CD4^+$ T cells. Th17 cells express the characteristic cytokine IL-17, and present critical roles in defense against microbial infections [11]. In recent years, it becomes increasingly clear that Th17 cell participate in the pathogenesis of multiple autoimmune diseases and chronic inflammatory conditions. Treg cells, on the other hand, are characterized by the expression of the transcription factor Foxp3, together with several immunosuppressive molecules, including CTLA-4, LAG-3, TGF-β, and IL-10 [12]. Treg cells are critical to the suppression of excessive inflammation, maintenance of tolerance, and protection and remodeling of tissues. Despite these differences, Treg and Th17 cells share several similarities. Both could be induced by TGF-β and IL-6, and both are present at high levels in the intestinal tract [13]. Interestingly, plasticity between Treg cells and Th17 cells was observed in mice. $Foxp3^+$ cells may develop into IL-17-producing cells under the influence of IL-6 and in the absence of TGF-β [14, 15].

Based on the observations that overactive immune responses are prevalent in NEC, we hypothesized that Treg cells, the cell type that would normally restrict the extent of inflammation, were impaired in NEC infants. To this end, we obtained peripheral blood mononuclear cells (PBMCs) from NEC infants and non-NEC infants, and examined the frequency and function of Treg cells, as well as other $CD4^+$ T cell subsets. Our results demonstrated that Treg cells from NEC infants were reduced in frequency and presented multiple functional deficiencies, including an inability to suppress T conventional (Tconv) cell proliferation and inhibit IL-17 expression. The expression of Treg-associated genes was also reduced in Treg cells from NEC infants. Overall, these results suggest that Treg cells might serve as a treatment target in NEC.

2. Methods

2.1. Subjects

The study was approved by the ethics committee of the First People's Hospital of Jining (No. 650626). Written informed consent was obtained from legal guardians of each study subject, who were enrolled between Jan. 2015 and Feb. 2017. Blood samples were collected from infants displaying clinical symptoms and radiographic discoveries consistent with proven NEC, according to the modified Bell's criteria [16]. Surgical treatment was performed after collection of blood samples, and the diagnosis of NEC was further confirmed during operation. Blood samples were collected from Non-NEC infants with matching birth weight, gestational age, and age at sample collection. No sign of NEC or infection was observed in control infants. The neonatal characteristics of NEC and non-NEC infants are summarized in Table 1.

Table 1
Characteristics of the non-NEC and the NEC infants.

	Non-NEC	NEC
N	15	15
Female, N (%)	7 (47%)	9 (60%)
Male, N (%)	8 (53%)	6 (40%)
Gestational age (weeks), mean ± SD	31.8 ± 2.8	31.5 ± 2.6
Age at sample collection (days), mean ± SD	48 ± 25	40 ± 22
Weight at sample collection (g), mean ± SD	3098 ± 435	2985 ± 432
Early onset, N (%)	N/A	2 (13%)
Bell's stage, N (%)		
IIA	N/A	4 (27%)
IIB	N/A	8 (53%)
IIIA	N/A	3 (20%)

2.2. PBMC collection

Peripheral blood was layered on sterile Ficoll (Sigma) at 2 to 1 ratio (v/v) in a 15 mL conical tube (Corning). The tubes were centrifuged at 400*g* for 30 min and were stopped by natural friction. The murky layer containing PBMCs were aspirated. PBMCs were then washed twice in pre-warmed complete medium (RPMI 1640 + 10% heat-inactivated FBS + 1% L-glutamine +1% penicillin-streptomycin; all from Thermo Fisher Scientific), and resuspended in 10% DMSO (Sigma) + 90% FBS for storage at −150 °C for no longer than one month. Before use, cells were thawed in pre-warmed complete medium +1% DNase (Sigma), and washed twice.

2.3. Flow cytometry

The following anti-human monoclonal antibodies, including anti-CD3, CD4, CD25, Foxp3, IFN-γ, IL-17, IL-10, TGF-β, were purchased from eBioscience and used at concentrations recommended by the manufacturer. For the detection of Treg cells, thawed PBMCs were incubated with Live/Dead Violet Stain (Invitrogen) and surface antibodies for 30 min on ice, washed, and then stained with anti-human Foxp3 using the Foxp3/TF staining buffer set (eBioscience). For the detection of intracellular cytokines, thawed PBMCs were stimulated with 20 ng/mL PMA + 1 μg/mL ionomycin (Sigma) in the presence of 3 μg/mL brefeldin A and 3 μg/mL monensin (BD Pharmingen) for 5 h, washed and incubated with Live/Dead Violet Stain and surface antibodies for 30 min on ice. After washing, cells were fixed and permeabilized in CytoFix/CytoPerm (BD Pharmingen) for 15 min on ice, washed, and stained with intracellular antibodies in 1× Perm Wash (BD Pharmingen). Samples were fixed in 2% formaldehyde and acquired in a BD FACSCanto system. Analyses were performed in FlowJo. For sorting, thawed PBMCs were incubated with surface antibodies for 30 min on ice and washed twice to remove excess antibodies. The cells were then sorted in a BD FACSAria system.

2.4. Treg-Tconv coculture experiment and suppression assay

Sorted $CD4^+CD25^-$ Tconv cells were plated at 1×10^4 cells per well in a 96-well round-bottom plate. Sorted $CD4^+CD25^{+/hi}$ T cells were then added to each well at concentrations specified per experiment. T activator beads (Thermo Fisher scientific) were added at 1 bead per T cell, and recombinant human IL-2 (R&D Systems) was added at 20 U/mL. All experiments were topped up to 100 μL final volume in complete culture medium. For Tconv proliferation, cells were pulsed with 0.5 μCi/mL [^{3}H]-thymidine ([^{3}H]-T) for 8 h, and the cells were harvested and radioactivity was measured in a direct β counter. For measuring IL-17 expression, supernatant was collected after 3 h incubation. Cells were then resuspended in complete culture medium for 5 h additional incubation in the presence of 3 μg/mL brefeldin A and 3 μg/mL monensin. ELISA was performed on the supernatant and intracellular staining was performed on the cells. Two independent replicates were performed.

2.5. ELISA

ELISA kits for human IL-17A (range 3.12–100 pg/mL), IL-10 (range 2.34–150 pg/mL), and TGF-β1 (range 31–2000 pg/mL) were purchased from Abcam and used according to the manufacturer's instructions. Fresh supernatants were used for all experiments and the results were expressed as pg/mL.

2.6. RT-PCR

RNA was extracted using the RNeasy Mini kit (Qiagen) from PBMCs immediately after thawing, from which cDNA was synthesized using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems),

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