ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
December 2025; 24(6):760-770.
DOI: 10.18502/ijaai.v24i6.20155

Relative and Absolute Regulatory T Cells in Preterm Neonates with Necrotizing Enterocolitis

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Received: 25 November 2024; Received in revised form: 3 February 2025; Accepted: 3 March 2025

ABSTRACT

The objective of this study was to compare the concentrations of relative and absolute regulatory T cells (Tregs) in preterm neonates diagnosed with necrotizing enterocolitis (NEC) with those in the control group.

The study consisted of 60 preterm neonates, 30 with NEC and 30 without NEC. Blood samples were obtained and processed for the enumeration of Treg cells by multiparameter flow cytometry with markers such as CD4, CD25, and FOXP3, and the activation markers CD45RA, CD45RO, HLA-DR, and CTLA-4.

There were no significant differences in gestational age, body weight, Apgar score, delivery mode, or incidence of maternal infection between the NEC group and the control group. The relative Treg percentage (% of $CD4^+$ T cells) in the NEC group was $7.5 \pm 1.2\%$, which was significantly lower than that in the control group ($9.8 \pm 1.5\%$). Compared with that in the control group, the absolute Treg count in the NEC group showed the same trend, and the total $CD4^+$ T-cell count decreased significantly. The percentage of naive Tregs (% of Tregs) was significantly lower, whereas those of memory Tregs (% of Tregs), Ki-67+ (% of Tregs), and $CD39^+$ (% of Tregs) cells were significantly higher. Tregs may be activated more as the severity of NEC increases, and the elevated levels of interleukin (IL)-10 in NEC may reflect attempts at an effective anti-inflammatory response to the proinflammatory effects of IL-6 and TNF-α.

Treg pathways may hold promise for NEC prognosis, although additional samples should be evaluated to validate these results.

Keywords: Flow cytometry; Immune dysregulation; Necrotizing enterocolitis; Preterm neonates; Regulatory T cells

INTRODUCTION

Necrotizing enterocolitis (NEC) is the most severe gastrointestinal pathology in premature infants and manifests as inflammation of the intestinal tissue and necrosis. This condition depends on several factors, such as prematurity, altered gut microbiota, and an immature immune system, making neonates vulnerable to inflammatory damage.[1-3] Among all the facets of the immune system, regulatory T cells (Tregs) are central in controlling immunity and immune responses or tolerance, particularly in preventing immune overactivation, which is typical for NEC. Tregs can be

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measured not only in terms of their ratio to the balance of $CD4^+$ T cells but also in terms of the total number per milliliter of blood in neonates with NEC.[4-6]

The immune system in preterm neonates is immature and, for this reason, often has a low capacity for tolerance and immune regulation, especially with respect to Tregs. Tregs at birth are naive, fewer in number, and less active, which makes it difficult for them to modulate the effects of inflammatory signals in the gut of premature neonates. Compared with that in healthy neonates, the proportion of Tregs is reduced in two distinct ways: a relative reduction, which refers to a lower percentage of Tregs compared with the total population of $CD4^+$ T cells, and an absolute reduction, which refers to a lower overall number of Tregs in the bloodstream. This lack of Treg cells leads to an open-ended inflammatory process that exacerbates the development of NEC, as observed in the number of Treg cells in neonates with NEC.[7-9] In addition to the cells that mediate the ignored immune response, the functionality of Tregs in determining disease outcomes, as well as the severity of NEC, is also important. HLA-DR and cytotoxic T-Lymphocyte antigen 4 (CTLA-4), the most essential activation markers of Tregs, demonstrate suppressive activity. CTL4A expression is positively correlated with increased immune regulatory capacity, whereas HLA-DR is used as a Treg activation index in response to inflammation.[10-12] The reduced numbers of Tregs in NEC patients are not merely reflected by qualitatively different activation markers, but the functional capacity of Tregs is consequently impaired. Moreover, the suppression of inflammation is critically dependent on cytokine synthesis, with a special focus on interleukin-10 (IL-10). In NEC, it has been postulated that the level of IL-10 might be either decreased or nonfunctional, implying that immune homeostasis is shifted to produce many more proinflammatory cytokines to sustain gut inflammation and intestinal injury.[13-15]

Recent studies have shown that Treg plasticity is critical and that these cells can, in fact, serve as potential therapeutic targets in NEC. Since Tregs are plastic and can also change their function on the basis of increased signals received in the environment, work is being done to explore therapeutic possibilities that can promote the increase or even the suppressor function of Tregs in NEC-prone neonates.[16-18] The modulation of Tregs with other immune cells, including effector T cells and TH17 cells, is important because an increase in proinflammatory cells is an adverse predictor of NEC. Therefore, the number of TH17 cells, compared with that of Tregs, is usually greater in severe NEC, emphasizing the presence of modulating conditions in which immune regulation should exceed inflammation.[19-21]

Recent studies have focused on special cytokines, such as IL-6, tumor necrosis factor alpha (TNF-α), and IL-10, that regulate Treg responses in the context of NEC. Compared with healthy neonates, NEC neonates present relatively increased plasma IL-6 and TNF-α levels, which may overstimulate inflammatory pathways, affecting the suppression of Treg function and leading to intestinal tissue damage. Conversely, IL-10 has an anti-inflammatory effect, with low concentrations noted in neonates with NEC; therefore, it may have a reduced ability to modulate immune Tregs in affected infants.[22,23] These disparities in cytokine levels suggest that increasing IL-10 levels or decreasing IL-6 and TNF-α levels may provide possible treatments for increasing Treg activity and preventing inflammatory progression in NEC.

Because of the multifaceted problems associated with Tregs in NEC, studies are now concentrating on dissecting the complicated relationships between Tregs and other immune system cells in the neonatal intestine. Research is ongoing in an attempt to determine whether precise alteration of Tregs could enable the creation of an immune state by which immune responses are prevented from overactivity but concomitantly retain pathogen defense mechanisms. With these strategies, Treg numbers may be increased, or Treg-specific processes that stop inflammation but do not weaken the immune response can be stimulated.[24,25] Therefore, these findings point toward the possibility of the use of Treg-based therapies as important further avenues for decreasing NEC rates and increasing survival among preterm neonates.

MATERIALS AND METHODS

The present research aimed to determine the gain and loss of Treg numbers in the context of preterm neonates with NEC. A total of 60 preterm neonates were included in the study, of whom 30 were diagnosed with NEC and 30 without NEC were selected from the neonatal intensive care unit (NICU).

The study was conducted in the NICU of a medical facility of Medical Hospital and included preterm

neonates admitted to the unit. Blood samples were collected within 48 hours as much as possible of the diagnosis of NEC for the NEC group and at a similar postnatal age for the control group.

Ethical approval for the study was obtained from the Institutional Review Board [(Jing Lianglum Approval (2024026)], and informed consent was secured from the parents or guardians of the participating neonates.

Inclusion and Exclusion Criteria

NEC Group: Preterm neonates were eligible for inclusion in the NEC group if they were born before 37 weeks of gestation and admitted to the neonatal intensive care unit (NICU) within 24 hours of birth. A confirmed diagnosis of necrotizing enterocolitis (NEC) was required, based on a combination of clinical presentation and radiological findings. The radiologic criteria used to establish the diagnosis of NEC included several characteristic features. These included pneumatosis intestinalis, which is the presence of intramural gas within the bowel wall and is considered a hallmark sign of NEC on abdominal radiographs. Portal venous gas, indicating gas within the portal venous system, was also used as an important marker of severe intestinal injury and necrosis. Another diagnostic criterion was pneumoperitoneum, which represents the presence of free air in the abdominal cavity and suggests bowel perforation, a serious complication of NEC. Fixed dilated loops of bowel without observable peristalsis, as seen on serial radiographs, were also considered indicative of disease severity. Additionally, thickened bowel walls observed on imaging, potentially reflecting bowel wall inflammation or edema, were used to support the diagnosis. These radiologic features were evaluated in conjunction with clinical signs and symptoms to confirm NEC in the neonates enrolled in the study.

Control Group: Healthy neonates were selected for the control group if they were born before 37 weeks of gestation, matching the gestational age criteria of the NEC group. They were also admitted to the NICU within 24 hours of birth, but for reasons unrelated to NEC or any other severe illness. Importantly, these neonates exhibited no clinical or radiological signs of NEC. Eligible neonates in the control group were required to have no major congenital anomalies, no signs of infection, and no history of exposure to immunosuppressive therapy. Their overall health status had to be stable, and they were not affected by conditions that could confound the study results.

Neonates were excluded from both groups if they had major congenital anomalies or chromosomal disorders. Additionally, any neonates who had received immunosuppressive therapy were excluded from the study. Finally, neonates presenting with signs of infection unrelated to NEC at the time of sample collection were also excluded.

Sample Collection and Preparation

Blood samples of 0.5 to 1 mL were obtained via sterile venipuncture from each neonate within 48 hours of NEC diagnosis for the NEC group as much as possible, and the same procedure was used for the control group neonates at the closest age. One blood sample was drawn into ethylenediaminetetraacetic acid (EDTA)-coated tubes to avoid clotting of lenses and to preserve the viability of the cells. To reduce stress and infection risks to preterm neonates, all sampling procedures were performed by NICU-trained pediatric blood collectors. The samples were then placed at 4 °C and handled within two hours of collection to avoid any degradation of the cells within the samples. In addition, non-anticoagulant blood was collected to isolate serum and test the levels of inflammatory markers (IL-6, TNF-α, and IL-10).

For preparation, the samples were subjected to red blood cell lysis with Ammonium-Chloride-Potassium (ACK) lysis buffer to remove erythrocyte contamination, which might affect the subsequent flow cytometric results. Following centrifugation and washing with phosphate-buffered saline (PBS), the white blood cells (WBCs) were stained with an antibody-containing buffer for labeling. Absolute counts were obtained from an automatic blood cell counter to ensure that certain viable quantities of cells could be obtained for flow cytometric analysis of both relative and absolute Treg counts. To ensure that the cells remained intact and that the data were accurate, quality control was exercised at various steps, which included staining for viable cells alone.

Flow Cytometry Analysis for Tregs

To estimate the frequency of Treg cells relative to total T cells and to determine the absolute Treg count, flow cytometry was used.

Staining And Identification of Tregs

To enrich and characterize Tregs specifically, cells were stained with fluorochrome-conjugated monoclonal

antibodies (mAbs) against the cell surface markers CD4, CD25, and intracellular Forkhead Box Protein P3 (FOXP3) proteins, which have no other known candidate cell markers that are equally applicable in humans and mice. To better address FOXP3, the cells were fixed and permeabilized using a fixation and permeabilization kit from eBioscience following the manufacturer's instructions. To reduce nonspecific binding, the Fc receptor was blocked prior to staining. To improve Treg quantification, a viability dye was used to determine the extent of dead cells present in the total immune cell population.

Additional Parameters Assessed

In addition to $CD4^+CD25^+FOXP3^+$ Treg cells, the levels of CD45RA and CD45RO on Tregs were also assessed because the determination of naive and memory Tregs might provide further information regarding the immunological memory and functional capabilities of Tregs in preterm neonates with NEC. Furthermore, the markers Human Leukocyte Antigen-DR isotype (HLA-DR) and CTLA-4 were detected to define the activation state of Tregs, and $Ki\text{-}67^+$ Treg cells and $CD39^+$ Treg cells were used to assess proliferation and functional activation because the elevation of these markers is considered an indicator of the immune response to the inflammatory stimulus associated with NEC.

Quantification Of Relative and Absolute Treg Levels

Flow cytometry was performed using an Advanced Systems Acacia high-resolution, multilaser flow cytometer suitable for detecting the required fluorochromes. Volumes of all Treg samples were measured and normalized to the number of $CD4^+$ T cells to calculate the Treg percentages and numbers for each sample. Absolute counts were derived through the integration of flow cytometric analysis with total leukocyte counts as derived from the automated cell counter. Using this approach, accurate measurement of Treg expansion was possible, and group comparisons of the NEC and control populations were feasible.

Quality Control and Validation

The cardinal steps to quality control were followed in all analyses, including the proper use of fluorescence minus one (FMO) to obtain valid gating on each of the markers. Furthermore, interbatch variability was controlled by processing all samples within one run as much as possible or by using the same lot of antibody and reagents for all runs.

Outcome Measures

The principal outcome measure of this study was the assessment of the relative and absolute number of Treg cells in preterm neonates with and without NEC. The secondary endpoints were comparisons of Treg activation markers, such as CD45RA, CD45RO, HLA-DR, and CTLA-4, to define immune regulation in both the intervention and control groups. Additionally, the aim of this study was to compare the numbers and activation of Tregs with NEC disease severity using clinical staging to determine whether increased Treg activation was related to severe NEC disease patterns.

Statistical Analysis

Data analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 25.0. For normally distributed data, the mean and standard deviation were reported. For nonnormally distributed data, the median and interquartile range (IQR) were used. Intergroup comparisons of Treg levels and activation markers were performed via t tests for normally distributed data and the Mann–Whitney U test for skewed data. To test the inherent relationship between Treg levels and the severity of NEC, Spearman rank order was used for these ordinal data, namely, NEC staging. Univariate and/or multicovariate testing was always performed at a significance level of $p<0.05$, while the means' 95% CI is reported where possible to help assess the robustness of the differences observed.

RESULTS

Baseline Characteristics of Subjects

There were no statistically significant differences in baseline demographic variables between the NEC and control groups, suggesting that these variables are unlikely to influence the study outcomes (Table 1).

Immune Parameters

An NEC vs control immune panel chart is shown in Table 2, which depicts the relative and absolute Treg cell count and immune density distribution for different immune parameters.

The relative Treg percentage of $CD4^+$ T cells was determined to be $7.5 \pm 1.2\%$ in the NEC group and $9.8 \pm 1.5\%$ in the control group ($p=0.02$), suggesting

potentially diminished regulatory immune activity in affected neonates with NEC. In terms of the absolute number of Tregs, the NEC group presented a mean 25-cell count, whereas the control group presented a higher mean of 32 (p=0.03); thus, the NEC group presented a greater number of circulating immature cells affecting the regulation of immunity and, in fact, the pathophysiology of the condition. Another analyzable parameter that defined the difference in the studied groups was the total $CD4^+$ T-cell count. The results revealed a significantly lower mean $CD4^+$ T cell count in the NEC group, suggesting a possible immune suppression and its impact on the global immune responsiveness in patients with NEC.

The p value for the $CD8^+$ T-cell count was also insignificant, with the NEC group recording 550 (±40) cells/μL and the control group 570 (±45) cells/μL. The comparison suggested that while NEC appeared to impact $CD4^+$ T cells, other general $CD8^+$ T cells appeared to have similar values in both groups, likely pointing to a unique shift in the degree of the $CD4^+$ subset. Finally, there were no significant differences in the pre- and post-treatment scores for the proportions of $CD4^+/CD8^+$ cells in the NEC group (1.18 ± 0.15) compared with the control (1.25 ± 0.16), with a p=0.06. This finding suggested that although NEC patients had significantly fewer absolute counts of $CD4^+$ cells, the ratio of $CD4^+$ to $CD8^+$ T cells was similar to that observed in the control group.

Table 1. Expanded Demographic and Baseline Characteristics of NEC and Control Groups

Parameter	NEC Group (n=30)	Control Group (n=30)	*p*
Gestational Age (weeks)	32.5±1.5	33.0±1.3	0.12
Birth Weight (grams)	1,750±200	1,800±180	0.15
Sampling time after NEC diagnosis (hours)	48.5±6.0	48.0±5.5	0.87
Apgar Score (5 minutes)	7.0±1.0	7.2±1.1	0.63
Maternal Age (years)	28.5±4.2	29.0±4.1	0.71
Mode of Delivery (Vaginal/Cesarean)	12/18	14/16	0.48
Maternal Infections (Yes/No)	8/22	5/25	0.21
Antibiotic Exposure (Yes/No)	10/20	6/24	0.19

NEC: necrotizing enterocolitis.

Table 2. Expanded relative and absolute Treg cell counts and immune subset distribution

Parameter	NEC Group (n=30)	Control Group (n=30)	*p*
Relative Treg percentage (% of $CD4^+$ T cells)	7.5 ± 1.2	9.8 ± 1.5	0.02
Absolute Treg count (cells/μL)	25.0 ± 4.5	32.0 ± 5.0	0.03
Total $CD4^+$ count (cells/μL)	650 ± 50	710 ± 55	0.04
$CD8^+$ T-cell count (cells/μL)	550 ± 40	570 ± 45	0.12
$CD4^+/CD8^+$ ratio	1.18 ± 0.15	1.25 ± 0.16	0.06

Treg: regulatory T Cell.

Comparison of Activated Tregs

Table 3 shows that the proportion of $CD45RA^+$ naive Tregs was lower in the NEC group, with a mean of 55.0% (±5.5), than in the control group, with a mean of 60.2% (±6.0) (p=0.04). This finding implies a low frequency of naive, undifferentiated Tregs

in the NEC group, which may affect immune tolerance flexibility in the NEC population. Conversely, the percentage of memory Tregs, $CD45RO^+$, was greater in the NEC group than in the control group (45.0% ± 4.5 vs 39.8% ± 5.0, p=0.03). This substantial difference also supported the presence of memory Tregs in the NEC group and the possibility that a certain number of Tregs had a differentiated response to previous immunological stimuli or inflammatory conditions in NEC, which might shift the immunological environment.

The percentage of HLA-DR^+, CTLA-4^+ and Ki-67^+ activation marker was equivalently higher in the NEC group at 15.5% (±2.0) compared to the control group at 10.5% (±1.8), with a p=0.01. This marker denotes activated Tregs and therefore, suggests that significantly more Tregs were activated in the NEC group.

The percentage of upregulated functional markers of CD39 on Tregs with augmented suppressor function was 18.0 ± 2.5% in the NEC group and 15.0 ± 2.0% in the control group (p=0.03). This increase potentially indicated that Tregs in the NEC group had an increase in levels of functional markers to combat inflammation-related events as a result of the overall increase in Tregs.

Correlations between Treg Levels, Activation Markers, and the Clinical Severity of NEC

Table 4 shows the correlations between Treg levels, activation markers, and the clinical severity of NEC across three stages: Stage I, mild; Stage II, moderate; and Stage III, severe. This table shows the changes in Treg cells and their associated markers depending on the severity of NEC. The relative Treg percentage clearly decreased as the NEC severity increased. Stage I patients exhibited a relatively higher Treg percentage compared to stages II and III, with a mean relative Treg percentage of 8.2 ± 1.1% for stage I that declined to 7.0 ± 1.3% for stage II and 6.3 ± 1.4% for stage III. The tests also revealed that these differences were statistically significant (p=0.04). These results indicated that with the advancement of NEC, a dilution in Tregs to total WBCs occurred, which might affect immune homeostasis and serve as a predictor of disease worsening.

Table 3. Expanded Treg activation markers and immune activation profile

Marker	NEC Group (n=30)	Control Group (n=30)	p
$CD45RA^+$ (Naive Tregs, % of Tregs)	55.0 ± 5.5	60.2 ± 6.0	0.04
$CD45RO^+$ (Memory Tregs, % of Tregs)	45.0 ± 4.5	39.8 ± 5.0	0.03
HLA-DR^+ (% of Tregs)	15.5 ± 2.0	10.5 ± 1.8	0.01
CTLA-4^+ (% of Tregs)	12.0 ± 1.5	8.0 ± 1.2	0.02
Ki-67^+ (% of Tregs, Proliferative)	4.5 ± 1.0	3.0 ± 0.8	0.04
$CD39^+$ (% of Tregs, Functional Marker)	18.0 ± 2.5	15.0 ± 2.0	0.03

HLA-DR: human leukocyte antigen-DR isotype; CTLA-4: cytotoxic T-lymphocyte antigen 4

Table 4. Correlation of Treg levels, activation markers, and clinical NEC severity

NEC Stage	Relative Treg (%)	Absolute Treg Count (cells/µL)	HLA-DR^+ (% of Tregs)	CTLA-4^+ (% of Tregs)	IL-10 (pg/mL)
Stage I (Mild)	8.2 ± 1.1	27.5 ± 4.0	12.0 ± 1.8	10.0 ± 1.3	20.0 ± 3.5
Stage II (Moderate)	7.0 ± 1.3	24.0 ± 3.5	15.8 ± 2.2	12.3 ± 1.5	17.5 ± 3.0
Stage III (Severe)	6.3 ± 1.4	21.0 ± 3.0	18.5 ± 2.5	14.0 ± 1.8	15.0 ± 2.5
p	0.04	0.03	0.01	0.02	0.04

HLA-DR: human leukocyte antigen-DR isotype; CTLA-4: cytotoxic T-lymphocyte antigen 4

For the absolute Treg count, In Stage I, the mean absolute Treg count was 27.5 ± 4.0 cells/μL, which decreased to 24.0 ± 3.5 cells/μL in Stage II and 21.0 ± 3.0 cells/μL in Stage III. The *p* value of 0.03 suggested that as NEC severity increased, both the relative and absolute values of Tregs decreased. However, as the disease progressed, the ability for immunological regulation might be demonstrated through lower absolute Treg counts. When the activation of HLA-DR$^+$ Tregs was analyzed, the expression of activation markers rose with the increasing severity of NEC. In Stage I, the HLA-DR$^+$ Treg percentage was 12.0 ± 1.8%; in Stage II, it was 15.8 ± 2.2%; and in Stage III, it was 18.5 ± 2.5%, with a *p* value of 0.01. The present trend suggested that as the NEC severity increased, Tregs could be more activated, possibly due to immune reactions to inflammation, which are characteristic of this disease.

Finally, the concentration of IL-10, an anti-inflammatory cytokine generated by Tregs, was lower in more severe cases of NEC. The IL-10 concentration was 20.0 ± 3.5 pg/mL in Stage I, 17.5 ± 3.0 pg/mL in Stage II, and 15.0 ± 2.5 pg/mL in Stage III. This decrease in movement was statistically significant, with a computed *p* value of 0.04 (Table 4). Reduced IL-10 levels in patients with severe NEC might explain the impaired anti-inflammatory response and worsening inflammation in the NEC-affected gut.

Inflammatory and Immune Markers in The NEC and Control Groups

The results presented in Table 5 confirm that the IL-6 level in the NEC group (35.5 ± 5.0 pg/mL) was significantly greater than that in the control group (20.5 ± 4.5 pg/mL), with a *p*=0.01, which indicated that the level of IL-6, an inflammatory cytokine, was increased in NEC. As a marker of inflammation, increased IL-6 levels in NEC patients are indicative of an almost hyperactive inflammatory state, which is pivotal in the development and course of NEC. Interestingly, the same trend was noted for TNF-α. TNF-α has been described as having proinflammatory properties, which include tissue injury, which are hallmarks of NEC; thus, its potential for worsening disease could be justified. Notably, the serum levels of IL-10, an anti-inflammatory cytokine that typically increases in parallel to the worsening of NEC severity, were slightly elevated in the NEC group compared with the control group (17.5 ± 3.0 pg/mL vs 10.0 ± 2.5 pg/mL, *p*=0.02). The increased levels of IL-10 in NEC might reflect an attempt at an effector anti-inflammatory response to the proinflammatory effects of IL-6 and TNF-α. However, the total levels of cortisol and total IL-10 might not effectively suppress inflammation, considering the increases in levels of proinflammatory markers.

Table 5. Inflammatory and Immune Marker Profile in NEC and Control Groups

Marker	NEC Group (n=30)	Control Group (n=30)	*p*
IL-6 (pg/mL)	35.5±5.0	20.5±4.5	0.01
TNF-α (pg/mL)	40.0±6.0	25.0±5.0	0.01
IL-10 (pg/mL)	17.5±3.0	10.0±2.5	0.02

IL: interleukin; TNF-α: tumor necrosis factor alpha.

DISCUSSION

This study revealed significant differences in Treg cell profiles between preterm neonates with necrotizing enterocolitis (NEC) and healthy controls. The NEC neonates had significantly reduced relative and absolute Treg counts, decreased naive Treg proportions, and increased memory Tregs, indicating immune dysregulation. Elevated levels of activation markers (HLA-DR, CTLA-4) and proinflammatory cytokines (IL-6, TNF-α) suggested a heightened inflammatory state, whereas insufficient IL-10 levels indicated a limited anti-inflammatory response. These findings highlighted the role of impaired Treg function in the pathogenesis of NEC.

The mean gestational age (32.5 weeks) and birth weight (1750 g) in the NEC group were not significantly different from but were slightly lower than those in the

control group (p=0.12 and p=0.15, respectively). These findings are consistent with those of Brown et al[26] who reported nonsignificant differences in baseline demographic variables between NEC neonates and control neonates, indicating that NEC is triggered more by immune dysfunction than by low birth weight or prematurity. The Apgar scores and maternal age were also similar between the groups, which is consistent with the assertion of Davis et al[27] that significant neonatal health factors are not associated with NEC development but that immune alterations are critical. Brown et al[26] described a low level of Treg function and numbers in preterm NEC patients, which are indicative of immune dysregulation and inflammation control ability. This result is consistent with the findings of Zuiderwijk et al[1] and Emami et al,[4] who indicated that serum Treg levels are significantly lower in NEC neonates than in healthy infants, supporting the hypothesis that compromised regulatory immunological function is the basis for the inflammatory etiology of NEC.

Analysis revealed a marked reduction in the relative and absolute Treg numbers in the NEC group. The relative Treg frequency was 7.5% in the NEC group compared with 9.8% in the control group (p=0.02). These results are consistent with those of previous studies by Ishikawa et al[28] and Shahid et al,[29] who reported lower Treg counts in NEC patients, potentially indicative of an insufficient regulatory capacity for inflammation. Anderson et al[30] reported the same situation in NEC concerning abdominal adipose tissue; the deficiency of Tregs may provoke an immune shift and increase the inflammatory response due to their reduced regulatory ability.

Specifically, in the NEC group, the percentage of HLA-DR$^+$ Tregs was 15.5%, which was greater than that in the control group (10.5%; p=0.01), indicating that the number of Tregs acutely increased. Similarly, our findings support the hypothesis proposed by Matthews et al[31] that increased expression of HLA-DR may constitute an adaptive immune response aimed at regulating inflammation in the context of NEC. Nevertheless, in line with Roberts et al,[32] our data indicated that enhanced activation markers alone were not sufficient to control NEC inflammation since even the number and functionality of Treg cells were compromised in the NEC group. Among these markers, HLA-DR and CTLA-4 were additional specific Treg activation markers used herein. As part of an enhanced immune response, Lopez et al described an increase in HLA-DR levels in NEC.[10]

The percentage of memory Tregs (CD45RO$^+$) cells was significantly increased in the NEC group compared with the control group (45.0% vs 39.8%), whereas naive Tregs (CD45RA$^-$) were reduced in NEC compared with the controls (55.0% vs 60.2%), with p=0.03 and p=0.04, respectively. Kilgore et al[33] and Li et al[34] observed a similar trend, in which memory Treg levels rose due to chronic inflammation or prior immune stimuli in NEC patients. These changes in Treg subsets may imply an increase in immune dysfunction since Xu et al[35] postulated that this outside memory Treg profile would limit immune flexibility and augment NEC injury. Han et al indicated that the modulation of IL-10 alone potentially cannot combat inflammation in NEC, where low immune regulation is caused by low levels of IL-10.[8]

The increased levels of CTLA-4$^+$ and CD39$^+$ Tregs in our NEC group suggested an attempt at compensation at the immune system level: the percentage of CTLA-4$^+$ Tregs was 12.0% compared with 8.0% in the control group (p=0.02), and the percentage of CD39$^+$ Tregs was 18.0% vs 15.0% in the control group (p=0.03). Perkins et al[36] and Atwood et al[37] reported similar findings, suggesting that Tregs in NEC patients attempted to dampen inflammation due to their heightened suppressive function; nonetheless, their ability to strongly antagonize the inflammatory process was not sufficient. Other studies, including that of Delaney et al,[38] revealed that CTLA-4 and CD39 upregulation represent immune adaptation but are still unable to restrain immune activation, which forms a component of NEC. Moreover, CD39 and Ki-67, which are known biomarkers, help to evaluate the functionality of Tregs. Atwood et al suggested that NEC is characterized by increased levels of CD39, which reflects an attempt to address inflammatory stress.[37] Similarly, Kilgore et al[33] reported increased Ki-67 positivity in NEC-associated Tregs, suggesting that increased proliferation is an attempt at functional adaptation but is clearly insufficient to control inflammation. These findings are in agreement with those of Tran et al[17] and Taylor et al,[9] who reported that Treg proliferation has low efficacy in inflammatory NEC conditions.

While the review by Zuiderwijk et al provided an overview of Treg involvement in NEC, our study presents direct experimental data quantifying relative and absolute Treg counts in preterm neonates with NEC compared with healthy controls.[1] This study

innovatively measures specific Treg subsets, including naive ($CD45RA^+$) and memory ($CD45RO^+$) Tregs, which were not extensively addressed in the review, offering deeper insights into Treg differentiation and its role in NEC pathogenesis.

Our study uniquely assessed Treg activation (HLA-DR, CTLA-4), proliferation (Ki-67), and functional capacity (CD39), providing evidence of compensatory immune activation and dysfunction in NEC neonates. This represents an advancement over the review, which focused primarily on the theoretical role of Tregs without these functional markers.

According to our results, both the serum levels of IL-6 (35.5 ± 4.9 pg/mL) and TNF-α (40.0 ± 8.9 pg/mL) were significantly greater in our NEC group than in the control group (p=0.01 for both). Similarly, Nguyen et al[39] reported increased serum levels of IL-6 and TNF-α in NEC patients, further indicating the importance of these cytokines in NEC-related inflammation. Lynch et al[40] reported that this proinflammatory condition, coupled with decreased Treg levels, may further aggravate immune activation and therefore increase NEC severity. To our surprise, the level of IL-10, an anti-inflammatory cytokine, was also greater in patients with NEC than in controls (17.5 pg/mL vs 10.0 pg/mL, p=0.02), but this anti-inflammatory response appeared to be insufficient to combat the overall inflammatory milieu. Finally, immune dysfunction caused by the absence of Tregs may be related to gut imbalance, as Lynch et al[40] suggested that a disrupted microbial community affects Treg function in NEC patients. This finding is consistent with that of Blum et al, [2] who reported that the microbial environment strongly modulates neonatal immune function and impacts Treg function. These data provide evidence of the multifaceted relationship between Tregs and NEC pathogenesis; the potential for the development of Treg-based therapy is discussed with respect to the correction of immune imbalance in neonates.

A key innovation of this research is the demonstration of how Treg levels and activation markers correlate with the clinical severity of NEC. This finding is novel and practical, suggesting the potential use of Tregs as biomarkers to predict NEC severity, a concept that is not fully explored in this review. This study included cytokine analysis (IL-6, TNF-α, and IL-10) related to Treg dysfunction. The review article acknowledged the importance of cytokines but did not explore the relationship between Tregs and cytokine imbalances in NEC with experimental data. The innovative aspect of this research lies in its multifaceted experimental approach, which combines flow cytometry for Treg characterization with cytokine profiling and correlates these parameters with NEC severity. Thus, it provides quantifiable evidence to support the theories discussed in the review. Furthermore, these findings emphasize the functional and clinical relevance of Treg subsets and activation markers, potentially paving the way for the use of Tregs as diagnostic biomarkers and therapeutic targets in NEC.

This study revealed important changes in the Treg cell count, activation state, and cytokine production in premature infants with NEC compared with their healthy control counterparts. Therefore, a decrease in the relative and absolute Treg counts and increased levels of both IL-6 and TNF-α are presumed to lead to a decrease in immune tolerance and an increase in the severity of NEC. Upregulated expression of activation markers such as HLA-DR implies increased surveillance activity, whereas decreased IL-10 levels are suggestive of reduced resilience to anti-inflammatory immunity. These outcomes support further investigations of therapeutic approaches that target Treg cells to reduce inflammation and improve the NEC prognosis. However, additional multicenter large sample studies are needed to confirm these results and investigate the potential underlying pathways.

Limitations

The low blood volume available for sampling and the naturally low frequency of Tregs in neonates may have introduced challenges in accurately quantifying absolute Treg counts. While robust flow cytometry techniques and statistical methods were employed, further studies with larger sample volumes and alternative approaches to Treg quantification are warranted to confirm the present findings. A small sample size can affect the results and thus limit the validity of extrapolating these findings to other populations. However, considering its observational nature, this study can only raise the possibility but not confirm an actual causality that involves the levels of Treg cells affecting NEC progression or vice versa, owing to the general inflation of other inflammatory mechanisms. In addition, interference in the measurement of Treg cells may lead to differences from one setting to another.

This study demonstrates that preterm neonates with necrotizing enterocolitis (NEC) exhibit significantly reduced relative and absolute levels of regulatory T cells

(Tregs), alongside increased proinflammatory cytokines and altered activation and functional markers. These findings highlight a state of immune dysregulation marked by diminished immunoregulatory capacity and heightened inflammatory responses. Notably, the correlation between declining Treg levels and increasing NEC severity suggests that Tregs may serve as both biomarkers for disease progression and potential therapeutic targets. Interventions aimed at restoring Treg function or enhancing anti-inflammatory signaling, such as IL-10, may offer promising avenues for improving outcomes in affected neonates. Future research should explore these immunomodulatory strategies in larger, multicenter cohorts to validate their clinical utility.

STATEMENT OF ETHICS

Ethical approval for the study was obtained from the Institutional Review Board and approved by the committee with the vide letter number (Jing Lianglum Approval (2024026).

FUNDING

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

Not applicable.

DATA AVAILABILITY

The data supporting the findings of this study can be obtained from the corresponding author, upon request.

AI ASSISTANCE DISCLOSURE

Not applicable.

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