Neuroenteric axis modulates the balance of regulatory T cells and T-helper 17 cells in the mesenteric lymph node following trauma/hemorrhagic shock

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Submitted 30 March 2015; accepted in final form 27 May 2015

Morishita K, Coimbra R, Langness S, Eliceiri BP, Costantini TW. Neuroenteric axis modulates the balance of regulatory T cells and T-helper 17 cells in the mesenteric lymph node following trauma/hemorrhagic shock. Am J Physiol Gastrointest Liver Physiol 309: G202–G208, 2015. First published June 4, 2015; doi:10.1152/ajpgi.00097.2015.—CD103+ dendritic cells (DCs) continuously migrate from the intestine to the mesenteric lymph nodes (MLNs) and maintain tolerance by driving the development of regulatory T cells (Treg) in the gut. The relative expression of Treg and T-helper 17 (Th17) cells determines the balance between tolerance and immunity in the gut. We hypothesized that trauma/hemorrhagic shock (T/HS) would decrease the CD103+ DC population in the mesenteric lymph and alter the Treg-to-Th17 ratio in the MLN. We further hypothesized that vagus nerve stimulation (VNS) would promote tolerance to inflammation by increasing the Treg-to-Th17 ratio in the MLN after injury. Male rats were assigned to sham shock (SS), trauma/sham shock (T/SS), or T/HS. T/HS was induced by laparotomy and 60 min of HS (blood pressure 35 mmHg) followed by fluid resuscitation. A separate cohort of animals underwent cervical VNS after the HS phase. MLN samples were collected 24 h after resuscitation. The CD103+ DC population and Treg-to-Th17 cell ratio in the MLN were decreased after T/HS compared with SS and T/SS, suggesting a shift to an inflammatory response. VNS prevented the T/HS-induced decrease in the CD103+ DC population and increased the Treg-to-Th17 ratio compared with T/HS alone. VNS alters the gut inflammatory response to injury by modulating the Treg-Th17 cell balance in the MLN. VNS promotes tolerance to inflammation in the gut, further supporting its ability to modulate the inflammatory set point and alter the response to injury.

vagus nerve; gut inflammation; mesenteric lymph; systemic inflammatory response

THE GUT HAS LONG BEEN BELIEVED to be the driving force in the systemic inflammatory response to injury [systemic inflammatory response syndrome (SIRS)] (11, 28). The gut normally plays an important role as a barrier against potentially harmful bacteria in the lumen of the intestine and relies on an integrated system of epithelial cells and immune cells to prevent gut inflammation (41). After injury, a combination of epithelial barrier failure and immune cell activation and mobilization serves as the “motor” for the SIRS response that leads to distant organ injury (34).

This gut inflammatory response is transmitted systemically via the mesenteric lymph (ML). This has been confirmed in studies demonstrating that either ML diversion or mesenteric duct ligation prevents neutrophil activation and distant organ injury in models of severe injury (10, 44). Numerous studies over the past several decades have sought to identify the inflammatory mediators that are carried through the lymph and are responsible for transmission of the inflammatory response to the systemic circulation. The specific mediators within the ML that lead to its biological activity have yet to be fully elucidated. Free fatty acids (42), lipid mediators such as arachidonic acid (17), biologically active lipids (35), and pancreatic proteases (13) have been considered lymph-derived factors that lead to SIRS. We recently focused on the mobilization of immune cells as a contributor to the proinflammatory response after acute injury (36).

CD103+ dendritic cells (DCs) are found in the intestine and gut-associated lymphoid tissues and are considered key factors involved in the induction of regulatory T cells (Treg) cells that promote tolerance to inflammation in the gut (2, 6). The balance of expression of Treg and T-helper 17 (Th17) cells is essential for maintaining immune homeostasis and has long been thought to be one of the important factors in development of the gut inflammatory response (9, 24).

Vagus nerve stimulation (VNS) modulates the inflammatory set point by altering the gut inflammatory response to acute injury, independent of splenic TNF-α production (7, 29, 47). Our previous studies demonstrated that VNS prevents injury-induced gut barrier failure and alters the DC profile in the ML after trauma/hemorrhagic shock (T/HS) (36). In this study we hypothesized that T/HS would decrease the CD103+ DC population migrating in the ML that would be associated with a decreased Treg-to-Th17 ratio in the ML nodes (MLNs). We further postulated that VNS would promote tolerance to inflammation by increasing the Treg-to-Th17 cell ratio in the MLN after injury.

MATERIALS AND METHODS

T/HS model. Male Sprague-Dawley rats (280–300 g body wt) were obtained from Harlan Laboratories (Placentia, CA). All animal experiments were approved by the University of California San Diego Animal Care and Use Committee. Animals had free access to food and water prior to the experimental protocol. Animals were anesthetized with ketamine (75 mg/kg ip; Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (10 mg/kg ip; Sigma Chemical, St. Louis, MO), and the left femoral artery and vein were cannulated with polyethylene (PE-50) tubing. Trauma was caused by a 3-cm midline laparotomy incision to create tissue injury as previously described (18). HS was induced by withdrawal of blood until the mean arterial pressure (MAP) was reduced to 35 mmHg and maintained for 60 min. At the end of the shock period, animals in the T/HS group were resuscitated...
with shed blood and isotonic sodium chloride solution (2 times shed blood volume). MAP was continuously monitored using the femoral arterial catheter (model no. V24/26, Philips, Andover, MA). Trauma/sham shock (T/SS) animals were anesthetized as described for the T/HS group, and a midline laparotomy incision was created, but blood was not withdrawn. The sham shock (SS) group was anesthetized, cannulated, and observed. Each animal’s body temperature was maintained at 37°C.

VNS. A cohort of animals underwent right cervical neck incision followed by right cervical VNS immediately after T/HS insult. The vagus nerve was stimulated using a square-wave generator at 5 V, with a frequency of 5 Hz for 10 min as previously described (36). After VNS, the incision was closed with 4-0 silk suture. Sham animals underwent right cervical incision and exposure of the vagus nerve but did not receive electrical stimulation.

Collection of ML and MLNs. The ML duct was exposed and cannulated with polyethylene (PE-50) tubing. ML was collected on ice during the pre-HS phase (30 min), HS phase (60 min), and post-HS phase (120 min) by definition. Cell pellets were prepared for flow cytometric analysis as previously described (33, 36). CD103+ DCs were magnetically sorted using anti-CD103-conjugated magnetic beads and sequential passage over LS columns (Miltenyi Biotec), according to the manufacturer’s protocol (1, 21, 27). Purity of the prepared cells was monitored by flow cytometric analysis. MLNs were harvested at 24 h following resuscitation, cut into pieces, and incubated for 20 min under agitation at 37°C in the presence of Collagenase/Dispase (Roche Diagnostic, Indianapolis, IN) and DNase I (New England BioLabs, Ipswich, MA). The tissue was then passed through a 70-μm membrane to generate single-cell suspensions and prepared for flow cytometric analysis. Cell count and viability were obtained using a hemocytometer (Countess automated cell counter, Invitrogen, Grand Island, NY).

Histological evaluation. At 24 h postinjury, segments of distal ileum were removed and fixed in 10% buffered formalin, embedded in paraffin, and sectioned. Hematoxylin and eosin staining of the intestine was performed by University of California San Diego Histology Core Services. An investigator blinded to experimental groups analyzed tissue sections based on previous reports (43). At 24 h after T/HS, the histological appearance of distal ileum showed mucosal injury, consistent with previous reports (Fig. 1B) (36). In addition, the histological injury score of the T/HS group was significantly higher than that of the T/SS group (2.7 ± 0.4 vs. 0.9 ± 0.2, P < 0.05; Fig. 1C).

Surface phenotype of ML and MLN cells. Murine lymph DCs typically express MHC II, CD103, and CD11c on their surface (15), and T cells express CD4 on their surface (36). To determine the surface expression on ML and MLN cells from sham animals, cells were stained with DC markers (MHC II, CD103, CD11b/c, and CD4) and isotype-matched control antibodies for each of the surface marker antibodies and assessed using flow cytometry. As shown in Fig. 2, MHC II, CD103, CD11b/c, and CD4 expression was determined based on Chiu et al. (5).

Flow cytometric analysis. The expression of cell surface molecules on DCs, Treg cells, and Th17 cells was determined by flow cytometry. ML and MLN cells (1.0 × 10^6 cells/ml) were incubated with anti-rat RTB1 [myosin heavy chain (MHC) II-peridinin-chlorophyll protein complex (PerCP); clone OX-6, BD Bioscience, San Diego, CA], anti-rat CD4-allophycocyanin (APC; OX-35, BD Bioscience), PerCP mouse IgG1 k-isotype control antibody (MOPC-31C, BD Bioscience), anti-rat CD103-FITC (OX-62, BioLegend, San Diego, CA), anti-rat CD11b/c-APC (OX-42, BioLegend), FITC-mouse IgG1 k-isotype control antibody (MOPC-21, BioLegend), phycoerythrin (PE)-mouse IgG1 k-isotype control (FC) antibody (MOPC-173, BioLegend) for 30 min at 4°C. Intracellular staining for Foxp3 [Foxp3-Alexa Fluor 488 (150D), BioLegend] and IL-17A [FITC (eBio17B7), eBioscience, San Diego, CA] was performed using the Cytofix/Cytoperm kit (BD Biosciences). Flow cytometry was performed with a BD Accuri C6 (BD Bioscience, San Jose, CA), and 40,000 events were collected for analysis. Data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Statistical analysis. Values are means ± SE. ANOVA with Student-Newman-Keuls post hoc analysis or Student’s t-test was performed as appropriate. Gut injury grading was analyzed using Kruskal-Wallis nonparametric ANOVA. Statistical significance was determined based on P < 0.05.

RESULTS

T/HS model. Animals were subjected to nonlethal HS (35 mmHg for 60 min) and resuscitated with shed blood and isotonic sodium chloride solution. MAP was continuously monitored during the experiments (Fig. 1A). Histological analysis was performed on representative tissue samples based on the previously established effect of T/HS on the gut barrier (36, 43). At 24 h after T/HS, the histological appearance of distal ileum showed mucosal injury, consistent with previous reports (Fig. 1B) (36). In addition, the histological injury score of the T/HS group was significantly higher than that of the T/SS group (2.7 ± 0.4 vs. 0.9 ± 0.2, P < 0.05; Fig. 1C).

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Fig. 1. Trauma/hemorrhagic shock (T/HS) model. A: mean arterial pressure (MAP) throughout the experiment. Gray line and symbols, trauma/sham shock (T/SS); black line and symbols, T/HS. B: hematoxylin-eosin-stained sections of distal ileum harvested 24 h after T/HS. Original magnification ×4. C: gut injury scores (Chiu score). Values are means ± SE; n = 6. *P < 0.05 vs. T/SS.
detected on the surface of ML and MLN cells from sham animals.

CD103+ DCs in ML and MLNs. ML cells were collected during the pre-HS and post-HS phases from the same animal and enriched for CD103+ DCs using magnetic bead selection. Enriched CD103+ DCs in Giemsa-stained ML are shown in Fig. 3A. The absolute number of CD103+ DCs collected from ML by magnetic-activated cell sorting significantly decreased after T/HS (4.8 ± 2.3 × 10^5 vs. 1.8 ± 0.5 × 10^5 cells/ml, P < 0.05; Fig. 3B). Next, we examined whether expression of the
DC surface markers on CD103+ and CD103− DCs changed in response to injury. CD103+ and CD103− DCs in the ML were stained with MHC II, CD11b/c, and ICAM-1 antibodies and analyzed by flow cytometry. ML DCs were compared in the pre-HS (baseline) and post-HS phases from the same animals. As shown in Fig. 3, C–F, CD103+ and CD103− ML DCs demonstrated altered expression of MHC II, CD11b/c, and ICAM-1 after T/HS (P < 0.05).

At 24 h after T/HS, MLN cells were isolated and stained with CD103 and MHC II antibodies and analyzed by flow cytometry (Fig. 4A). MLN DCs, which are larger (increased forward scatter) and have slightly higher granularity (increased side scatter) than non-DCs (mainly T cells), were gated for the CD103+ DC population. MLN CD103+ DCs significantly decreased in the T/HS group compared with the SS and T/SS groups (P < 0.05; Fig. 4, B and C).

*Treg-Th17 cell balance in MLNs.* At 24 h following T/HS, MLN cells were isolated and stained with CD4, CD25, Foxp3, and IL-17A antibodies and analyzed by flow cytometry. Lymphocytes were gated with characteristic low forward- and side-scatter profile. As shown in Fig. 5A, the percentage of CD4+CD25+Foxp3+ lymphocytes was significantly lower in T/HS than SS and T/SS rats (P < 0.05). CD4+ T cells expressing IL-17 in each group is shown in Fig. 5B. The percentage of CD4+IL-17A+ lymphocytes was significantly higher in T/HS than SS and T/SS rats (P < 0.05). In addition, we simultaneously measured Treg and Th17 MLN cells from the same MLN sample, allowing for direct comparison of Treg and Th17 cell populations in each group. The ratio of Treg to Th17 cells was significantly lower in T/HS than SS and T/SS rats (P < 0.05; Fig. 5C).

**VNS modulates Treg-to-Th17 ratio in MLNs.** To determine the role of VNS in the Treg-Th17 cell balance, MLN cells were isolated and stained with CD4, CD25, Foxp3, and IL-17A antibodies and simultaneously analyzed by flow cytometry (Fig. 6). VNS prevented an injury-induced decrease in the CD4+CD25+Foxp3+ Treg cell population in the MLNs, whereas VNS prevented an injury-induced increase in the CD4+IL-17A+ Th17 cell population in the MLNs (P < 0.05), thus altering the balance between tolerance and immunity after injury.

**DISCUSSION**

DCs in the intestinal mucosa play a crucial role in directing immune responses to luminal antigens (20, 32). Intestinal DCs can efficiently migrate to MLNs, thereby providing a critical link to adaptive T cell immunity (51). Recently, the importance of CD103+ DCs in regulation of the homeostatic balance between mucosal immunity and tolerance in the gastrointestinal tract has been characterized (45). CD103+ DCs are thought to migrate through the ML to the MLN, where they play an important role in the differentiation of Foxp3+ Treg cells, supporting their ability to promote tolerance to inflammation (37). In our study the CD103+ DC population in the ML and MLN significantly decreased following T/HS. This suggests that the decrease in ML CD103+ DCs at early time points after acute injury may represent a signal to a shift to a proinflammatory phenotype.

Interestingly, intestinal CD103− DCs share important functional and phenotypic properties with CD103+ DCs. Intestinal CD103− DCs arise from the lamina propria of the gut and migrate through the ML to the MLN. CD103− DCs expand in response to inflammation and are capable of rapidly generating effector T-cell responses (3). Mucosal CD103− DCs respond to LPS by secreting proinflammatory cytokines and lead to the differentiation of effector Th17 cells (3, 12). Therefore, the balance of CD103− and CD103+ DCs is thought to be critical for maintaining intestinal immune homeostasis.

Naïve CD4+ T cells can be differentiated into various subsets, such as Th1, Th2, Th17, and Treg cells (9). While the Th1-Th2 paradigm provided a reasonable basis for exploration of the mechanisms of immunity to infection and autoimmune diseases, several lines of evidence suggest that Th1 cells are not the only T cell subset responsible for the induction and progression of immune responses, as originally proposed (24). Th17 cells are characterized by their expression of the proinflammatory cytokines IL-17 and IL-22 and reside mainly at barrier surfaces, particularly the mucosa of the gut, where they function to protect the host from microorganisms that invade through the epithelium (50). Treg cells are a separate subpopulation of immunosuppressive T cells; they develop in the thymus or can be induced in the periphery (4). The induced Treg cells are characterized by expression of the transcription factor Foxp3 and are also found mainly in the intestinal mucosa, where they function to restrain excessive effector T cell responses that might damage host tissues (50).
Neely et al. (39) demonstrated that Th17 cells in wound-draining lymph nodes, but not in the spleen, are spontaneously generated after burn injury. Dai et al. (9) demonstrated decreased percentages of Treg and Th17 cells in the peripheral blood in a model of polytrauma. Another study showed elevated numbers of Th17 cells combined with decreased Treg cells in the peripheral blood in inflammatory bowel disease (14). The contribution of Treg/Th17 cells in the gut following acute traumatic injury is poorly documented. Our study reveals that T/HS induces an increase in the percentage of Th17 cells, which together with a decrease in Treg cells, leads to a significant reduction of the Treg-to-Th17 ratio in the MLN.

We have found that changes in the cell populations mobilized into the ML and MLN occur at early time points following severe, acute injury. T/HS has been shown to alter splenic DC maturation at 2 h following resuscitation, supporting the potential for injury to cause rapid changes in tissue DCs (7). Studies evaluating mobilization of intestinal CD103+ DCs, Treg, and Th17 cells have focused mostly on inflammatory bowel disease models, where the inflammatory insult is sub-acute and less dramatic than in models of acute injury such as T/HS (16, 31). A previous study demonstrated that CD103+ DCs are absent from inflamed areas of the ileum in a model of enteritis during the acute stage of inflammation (46). Prior studies have characterized the central role of the injured gut, and the large immune system resident in the gut, as a driver of the systemic inflammatory response to injury that begins in the hours after the insult and supports our findings that the Treg-Th17 balance is altered within hours of the insult (11, 28).

In this study, VNS altered the gut inflammatory response to injury by modulating the CD4+CD25+Foxp3+ Treg-CD4+IL-17A+ Th17 cell balance in the MLNs. Several immune cells, such as macrophages, DCs, and T cells, express various nicotinic acetylcholine receptor (nAChR) subtypes, including the α3-nAChR (22)hR, which mediates the anti-inflammatory effects of VNS in the spleen-dependent (19) and spleen-independent (7, 30) pathways. The anti-inflammatory effect of VNS is lost in α3-nAChR knockout mice, can be blocked by specific

![Fig. 5. Regulatory T (Treg) and T-helper 17 (Th17) cells in MLNs. At 24 h following T/HS, MLN cells were isolated and stained with CD4, CD25, Foxp3, and IL-17A antibodies and analyzed by flow cytometry. A: Treg cells as percentage of CD4+ cells that are CD25+Foxp3+. B: Th17 cells as percentage of CD4+ cells that are IL-17A+. Black trace, FITC-isotype control; gray histogram, FITC-IL-17A antibody (T/HS). C: percent changes in Treg-to-Th17 cell ratio in SS, T/SS, and T/HS. Values are means ± SE; n = 7–8. *P < 0.05.](image)

![Fig. 6. Vagus nerve stimulation (VNS) modulates Treg-to-Th17 cell ratio in MLNs. Treg and Th17 cells from the same rat sample were simultaneously measured, allowing for direct comparison of Treg and Th17 cell populations in 4 groups. A: Treg cell populations as percentage of CD4+CD25+Foxp3+ cells. B: Th17 cell populations as percentage of CD4+IL-17A+ cells. C: Treg and Th17 MLN cells from the same rat MLN sample were simultaneously measured to evaluate Treg-to-Th17 cell ratio. Values are means ± SE; n = 4–8. *P < 0.05.](image)
functional properties of DCs and Treg cells in the gut after colitis mice (40). The effects of cholinergic signaling on the nerve modulates the gut immune cell response to injury. response to acute injury and the mechanism by which the vagus further define the role of intestinal DCs and T cells in the gut immune cell expression. Continued studies are needed to tolerance to inflammation after injury by altering the balance of MLNs by VNS suggests that the vagus nerve may promote responses. Modulation of the Treg-to-Th17 cell ratio in the Treg-Th17 balance that alters local and systemic inflammatory inflammation in the gut. T/HS decreased the CD103
expressed on DCs and CD4
expression on CD4
CD4
alters the migration of gut-derived CD103
injury and its effect on the balance between tolerance and
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K.M. prepared the figures; K.M. drafted the
GRANTS
This work was funded by the American College of Surgeons C. James Carrico Faculty Research Fellowship for the Study of Trauma and Critical Care (T. W. Costantini).
DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.
AUTHOR CONTRIBUTIONS
K.M., B.P.E., and T.W.C. developed the concept and designed the research; K.M. performed the experiments; K.M., B.P.E., and T.W.C. analyzed the data; K.M., R.C., S.L., B.P.E., and T.W.C. interpreted the results of the experiments; K.M. prepared the figures; K.M. drafted the manuscript; K.M., R.C., S.L., B.P.E., and T.W.C. approved the final version of the manuscript; R.C., S.L., B.P.E., and T.W.C. edited and revised the manuscript.
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