

## Original Article

# Severe traumatic hemorrhagic shock induces compromised immune barrier function of the mesenteric lymph node leading to an increase in intestinal bacterial translocation

Jian Zhang<sup>1,2\*</sup>, Yun Zhang<sup>1,2\*</sup>, Tao Xu<sup>1,2</sup>, Sheng-Jing Pan<sup>1,2</sup>, Gang Nie<sup>1,2</sup>, Xiao-Yan Miao<sup>1</sup>, Jun-Yu Qiu<sup>1,2</sup>, Wen-Qiao Yu<sup>1,2</sup>, Shao-Yang Zhang<sup>1,2</sup>, Ting-Bo Liang<sup>1,2,3</sup>

<sup>1</sup>Department of Hepatobiliary and Pancreatic Surgery, The Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang, China; <sup>2</sup>Zhejiang Provincial Key Laboratory of Pancreatic Disease, China; <sup>3</sup>Zhejiang University; Collaborative Innovation Center for Cancer Medicine, Guangzhou, China. \*Equal contributors.

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**Abstract:** Critically ill patients have increased susceptibility to translocation of gut bacteria. However, the mechanisms are complicated and remain unclear, and the aim of this study was to explore these mechanisms. Rats exposed to different levels of shock were orally administrated with bioluminescent *Citrobacter*. We found that severe shock caused an increase in bacterial translocation to the visceral organs, such as liver, spleen and blood, compared with mild shock. Surprisingly, bacterial translocation to mesenteric lymph node (MLN) was unchanged between the two shock groups. Various methods, including flow cytometry, a co-culture model and western blots, were used to evaluate MLN-associated immune function. Specifically, we focused on mesenteric lymph node dendritic cells (MLN-DCs), the critical antigen presenting cells involved in the construction of the immune barrier in MLN. We also found that severe shock impaired the phenotypic maturation of MLN-DCs and induced a tolerogenic phenotype. Furthermore, co-culture assays of DCs with naive CD4<sup>+</sup> T cells showed that DCs subject to severe shock were more inclined to polarize native CD4<sup>+</sup> T cells into Th2 and Treg cells. This study successfully reproduced the clinical phenomenon of severe shock resulting in increased bacterial translocation to extraintestinal tissues, and this may be related to the compromised immune barrier function of MLN, as maturation and function of MLN-DC's were badly impaired.

**Keywords:** Traumatic hemorrhagic shock, intestinal immune barrier, intestinal bacterial translocation, mesenteric lymph node dendritic cells, T cell polarization

## Introduction

Critically ill patients, such as those experiencing traumatic hemorrhagic shock (THS), experience extreme immune dysregulation characterized by an initial innate immune-driven inflammatory response followed by a compensatory anti-inflammatory response. This response is associated with suppressed adaptive immunity [1-3]. Subsequent sepsis and multi-organ dysfunction syndrome (MODS) are the major causes of deaths in the middle and late stages of severe trauma [4].

The gastrointestinal (GI) tract was firstly recognized 20 years ago as the “motor” driving systemic sepsis and MODS [5]. Some of the pro-

posed mechanisms behind this include production of pro-inflammatory cytokines induced by gut hypoperfusion [6], injury to the intestinal mucosa caused by epithelial apoptosis [7], hydroxyl radicals generated by xanthine-oxidase [8], injured tight junction proteins [9, 10], and translocation of intestinal bacteria [11]. Bacterial translocation from the gut to the visceral organs is thought to play a vital role in the systemic infections following THS [11] since gut-derived bacteria can usually only be detected in extraintestinal tissues of critically ill patients.

Impaired host immune defense, physical damage to the intestinal mucosa, and intestinal bacterial overgrowth are the three major fac-

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tors promoting bacterial translocation from the GI tract [12]. The injection of immunosuppressive agents into mice and thymectomies of neonatal mice [13] have been shown to increase bacterial translocation to the MLN and extraintestinal visceral organs. What's more, THS has been shown to damage the intestinal mucosa and enable bacterial translocation from the GI tract [11]. Previously, we demonstrated differentiation towards a Th2 phenotype among the T cell population in a rat model of THS [14, 15]. However, the exact mechanisms of bacterial translocation are complicated and remain unclear.

Here, we used rat models of THS to simulate different severities of shock and examine the effects of THS on intestinal bacterial translocation.

### Materials and methods

#### *Animals*

Male Sprague-Dawley rats, aged 8-10 weeks and weighing 250-300 g, were purchased from the Animal Resource Center of Zhejiang University School of Medicine. Animals were housed in a clean vivarium and fed standard rat chow. All animal research protocols used in this study were approved by the Institutional Laboratory Review Board and were in accordance with the principles of the Guide for the Care and Use of Laboratory Animals-Eighth Edition (National Institutes of Health publication, 2011). All rats were euthanized via cervical dislocation before collection of tissue samples.

#### *THS model*

We used a previously described THS rat model [7, 14, 15]. In brief, rats were preconditioned in a stress-free normothermic environment for more than 7 days before experiments. The rats were anesthetized via intraperitoneal injection of sodium phenobarbital (100 mg/kg). The right jugular vein and the right carotid artery were catheterized for fluid resuscitation and bleeding, respectively, via a small (20-30 mm) incision. The mean arterial pressure (MAP) was monitored using a tail-cuff system (BP-98A, Softron, Tokyo, Japan) [16, 17]. An open mid-diaphyseal transverse fracture was created in the left femur to cause trauma, and subsequently the animal was bled to achieve an

MAP of  $30 \pm 5$  mmHg. And 30 (mild THS) or 90 (severe THS) minutes later, the animals were then resuscitated over a period of 20 minutes with lactated Ringer's solution (about twice the volume of the blood lost) at a constant rate to control shock. Finally, the catheters were removed, the vessels were ligated, and the incisions were sealed. The normal group did not receive any anesthetic or surgical procedures. The sham group underwent the same anesthetic and surgical procedures, as well as fluid resuscitation, but was not subject to femoral fracture or bleeding. All procedures were performed while animals were respiring spontaneously, and their temperature was maintained at 36-37°C using a controlled metal surgical heat plate.

#### *Microvascular tissue perfusion*

Microvascular tissue perfusion in the intestine (lamina muscularis) during the THS phase and fluid resuscitation was evaluated by laser Doppler imaging (Moor Instruments, Axminster, England) according to the manufacturer's instructions.

#### *Bacterial translocation evaluation*

We investigated bacterial translocation using bioluminescent *Citrobacter* (provided by Professor Gad Frankel, Imperial College London) as previously described [18]. The bacteria was grown at 37°C in Luria-Bertani (LB) medium supplemented with kanamycin (50 µg/ml) and observed under in vivo imaging system (IVIS, Kodak, New York, USA). At 48 hours post THS, rats were intragastrically administered with approximately  $3 \times 10^{10}$  CFUs of an overnight culture of *Citrobacter* in 200 µl PBS. Rats were sacrificed by cervical dislocation 5 hours after administration of bacteria. Homogenates of MLN, liver, spleen and blood (200 µl) were plated on LB agar culture plates and grown overnight under an aerobic condition at 37°C. The bacterial counts were determined under IVIS.

#### *In vivo intestinal permeability test*

Permeability of the intestinal barrier was tested in vivo using FITC-dextran (Sigma-Aldrich, St. Louis, MO, USA) as a marker for permeability. A 4 cm loop of the distal ileum was ligated (without disrupting the mesenteric vascular arcades or blood supply) and 100 µl of FITC-dextran

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(100 mg/mL) was injected into the lumen. 300  $\mu$ l of blood was obtained by cardiac puncture after 5 hours, centrifuged (12,000 rpm, 4°C, 10 minutes), the plasma (50  $\mu$ l) was mixed with 150  $\mu$ l of PBS, and the concentration of fluorescein was determined by fluorometry (excitation at 485 nm and emission at 520 nm) (Beckman Coulter DTX 880, Brea, CA, USA).

### *Ex vivo test of intestinal resistance*

The intestinal barrier resistance was measured *ex vivo* in a segment from the distal ileum mounted in an ussing chamber slider (Physiologic Instruments, San Diego, CA, USA). Segments of the intestine (2-3 cm) were opened along the mesenteric border, the serosa was carefully peeled off and the segments were rinsed with Krebs-Ringers bicarbonate buffer (pH 7.4), and intact sheets were pinned between siliconized using half chambers (0.5 cm<sup>2</sup> exposed surface area). The chambers were equipped with two calomel voltage-sensitive electrodes and two Ag-AgCl current-passing electrodes. Both the mucosal and serosal reservoirs of the chamber contained 5 ml of oxygenated Krebs-Ringer bicarbonate buffer and were equilibrated for 120 minutes. The buffer was continuously oxygenated (95% O<sub>2</sub> and 5% CO<sub>2</sub>) during the experiment and kept at 37°C using a water-jacket. The resistance of the intestinal segment, which is an indicator of tissue integrity, was obtained using Analyze & Acquire Revision II (Physiologic Instruments, San Diego, CA, USA).

### *Isolation of MLN-DCs*

MLNs were minced and treated with Type 2 collagenase (2 mg/mL, Worthington, Lakewood, NJ, USA) and deoxyribonuclease I (50 mg/mL, Sigma, St. Louis, MO, USA) diluted in PBS with 2% fetal calf serum, and were then digested enzymatically for 45 min at 37°C. Cell suspensions were pooled and filtered through a 100  $\mu$ m cell strainer, and then enriched using anti-OX62 magnetic beads and a positive selection column (MS<sup>+</sup>) according to the manufacturer's instructions (MiltenyiBiotec, Cologne, Germany).

### *In vitro co-culture model*

Naïve CD4<sup>+</sup> T cells were sorted from healthy, untreated rat MLNs using a MACS naïve CD4<sup>+</sup>

T cell isolation kit (MiltenyiBiotec, Cologne, Germany). The culture medium was RPMI 16-40 supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin. CD4<sup>+</sup> T cells (1 $\times$ 10<sup>6</sup> cells/well) were co-cultured with OX62<sup>+</sup> MLN-DCs (1 $\times$ 10<sup>5</sup> cells/well) in the presence of LPS (10 ng/mL), recombinant rat IL-2 (Peprotech, Rocky Hill, USA), recombinant rat GM-CSF (Peprotech, Rocky Hill, USA), anti-rat CD3 (eBioscience, San Diego, CA, USA), and anti-rat CD28 (eBioscience, San Diego, CA, USA) for 3 days at 37°C with 5% CO<sub>2</sub> in a humidified incubator. We used a 1:10 ratio of stimulator cells to responder cells based on our pilot experiments and previous reports [19].

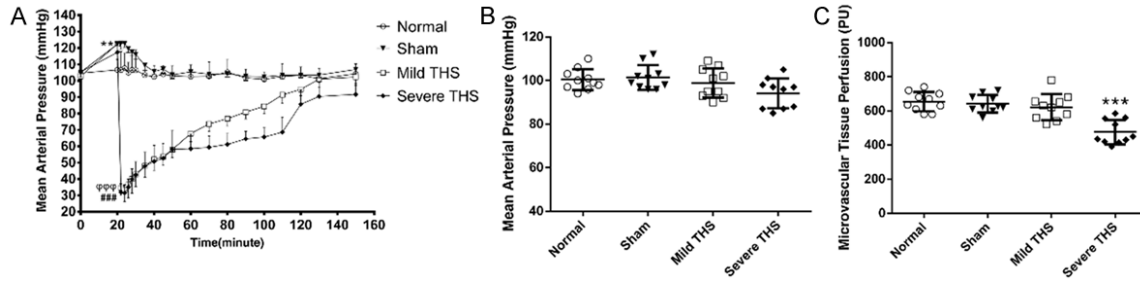
### *Flow cytometry and FACS sorting*

All antibodies were purchased from eBioscience unless specified otherwise. Enriched MLN-DCs were stained with fluorochrome-conjugated antibodies for CD80(3H5), CD86(24F), and MHC II(HIS19) or with an isotype antibody. Polarized T cells were stained with CD4(OX35) before FACS sorting. T cells from the co-culture system were either left unstimulated or stimulated at 37°C for 5 hours with Cell Activation Cocktail (Multisciences, Hangzhou, China). Cells were then stained with CD4 (OX35, BD Biosciences, Franklin Lakes, NJ, USA), CD25 (OX39, BD Biosciences, Franklin Lakes, NJ, USA), IFN $\gamma$  (DB1, BD Biosciences, Franklin Lakes, NJ, USA), IL-4 (OX81, BD Biosciences, Franklin Lakes, NJ, USA), and Foxp3(FJK-16s). Cell sorting was performed on a FACSAria II (BD Biosciences, Franklin Lakes, NJ, USA) and flow cytometric analysis was performed on a Canto-II (BD Biosciences, Franklin Lakes, NJ, USA). We used the Intracellular Cytokine Staining Kit (eBioscience, San Diego, CA, USA), and the Fix/Perm Kit (eBioscience, San Diego, CA, USA) was used to perform the transcriptional factor staining.

### *Western blots*

Polarized CD4<sup>+</sup> T cells in the co-culture system were separated by FACS sorting. Cells were lysed in RIPA lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% Triton X-100; 1% sodium deoxycholate; 0.1% SDS; 1 mM PMSF) for 2 hours on ice. Sample proteins were quantified using the BCA Protein Kit (Thermo, Rockford, IL, USA). Equal amounts of proteins were separat-

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**Figure 1.** Haemodynamics and intestinal microcirculation. Mean arterial pressure (MAP) and microvascular tissue perfusion were continuously monitored throughout the experimental period. The MAP returned to normal levels only in the mild traumatic hemorrhagic shock (THS) group (A). There were no differences in MAP between the four groups 48 hours after fluid resuscitation (B). Microvascular tissue perfusion at 48 hours decreased substantially in the severe THS group (C). PU stands for perfusion unit, which is an output parameter of laser Doppler imaging. Data are expressed as mean  $\pm$  SD, and there were  $n = 10$  animals per group. \*\* $P < 0.01$  versus other groups;  $\phi\phi\phi P < 0.001$  versus the normal and sham groups; ### $P < 0.001$  versus the normal and sham groups.

ed on 10% SDS-PAGE and transferred onto PVDF membranes. Immunoblots were blocked with 5% milk and probed overnight at 4°C with anti-STAT1, anti-phospho-STAT1, anti-STAT6, anti-phospho-STAT6 and anti- $\beta$ -actin antibodies (Cell Signaling Technology, Danvers MA, US). Membranes were incubated with the corresponding secondary antibodies for 1 hour at room temperature before visualization.

### Statistical analysis

All the variables are given as mean  $\pm$  SD, and were analyzed in Prism 6.0 (GraphPad, San Diego, CA, USA). Multi-groups were initially analyzed via one-way ANOVA followed by a Tukey post-test to compare individual groups. Group and time effects for longitudinally monitored variables were determined by repeated measures ANOVA. Statistical significance was defined as  $P < 0.05$ .

### Results

#### *The effects of trauma and hemorrhage on the haemodynamics and intestinal microcirculation of rats*

Although the sham group showed a significant increase in MAP compared to baseline 20-30 minutes after anesthetic and surgical procedures, the MAP value remained relatively stable over the remaining time, and there were no significant differences between the normal and sham groups. The MAP values in mild THS and severe THS groups decreased significantly after rapid exsanguination, and this was followed by a gradual rise but only the mild THS

group reached the original level (**Figure 1A**). There were no differences in MAP between the four groups 48 hours after fluid resuscitation (**Figure 1B**). However, the blood flow in the lamina muscularis, which is an indicator of microvascular tissue perfusion, decreased dramatically in the severe THS group (**Figure 1C**).

#### *Severe THS increased bacterial translocation*

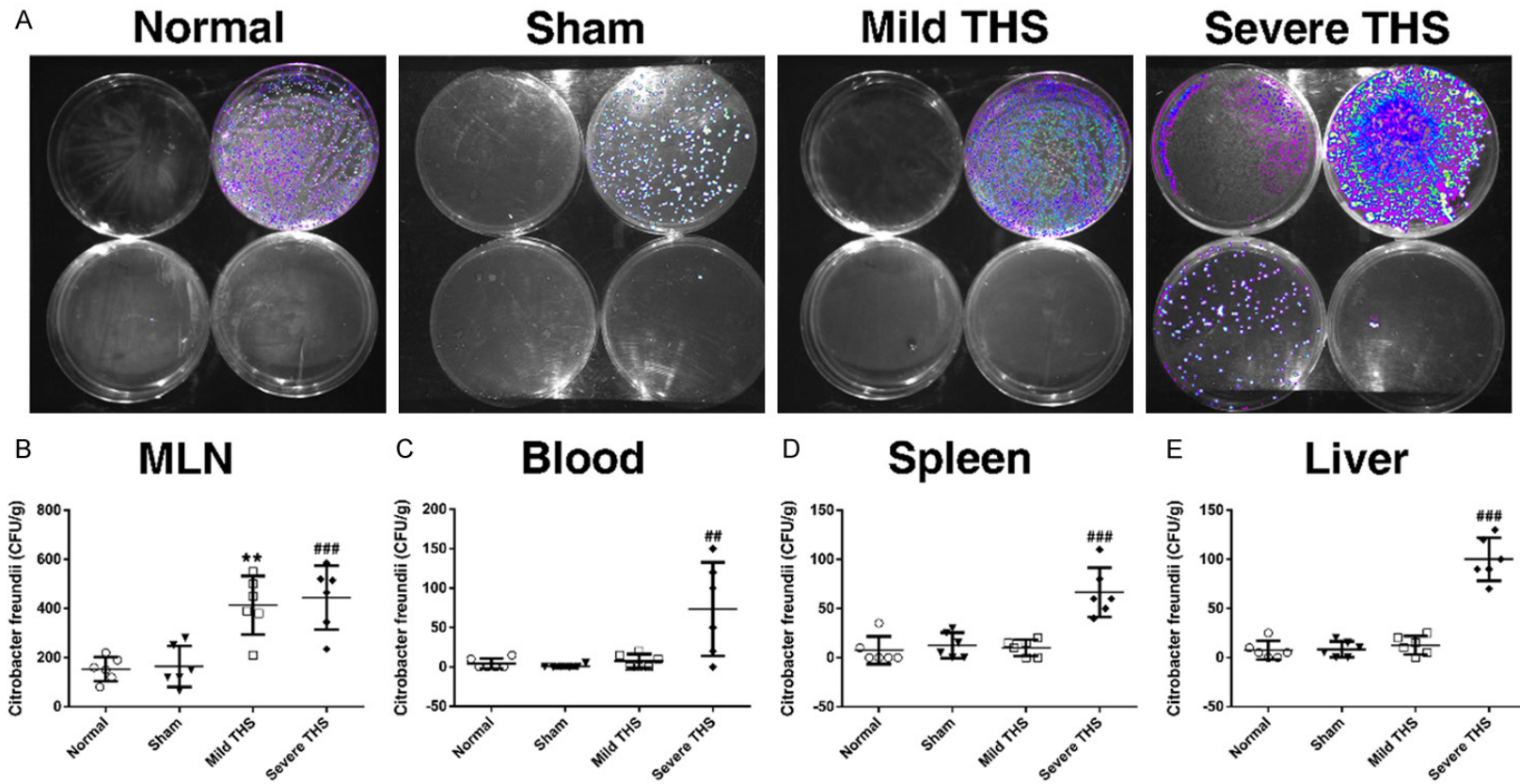
There were more *Citrobacter* in the MLN than in other organs or blood, especially in the THS group, but there were no significant differences between the mild THS and severe THS groups in bacterial translocation to MLN (**Figure 2A, 2B**). However, there were elevated bacterial counts in the liver, spleen, and blood of the severe THS group (**Figure 2A, 2C-E**).

#### *Mild and severe THS caused similar barrier injuries*

The mild and severe THS groups had significantly higher intestinal permeabilities and lower resistances than the normal and sham groups, however, there were no differences between the mild and severe THS groups (**Figure 3A, 3B**).

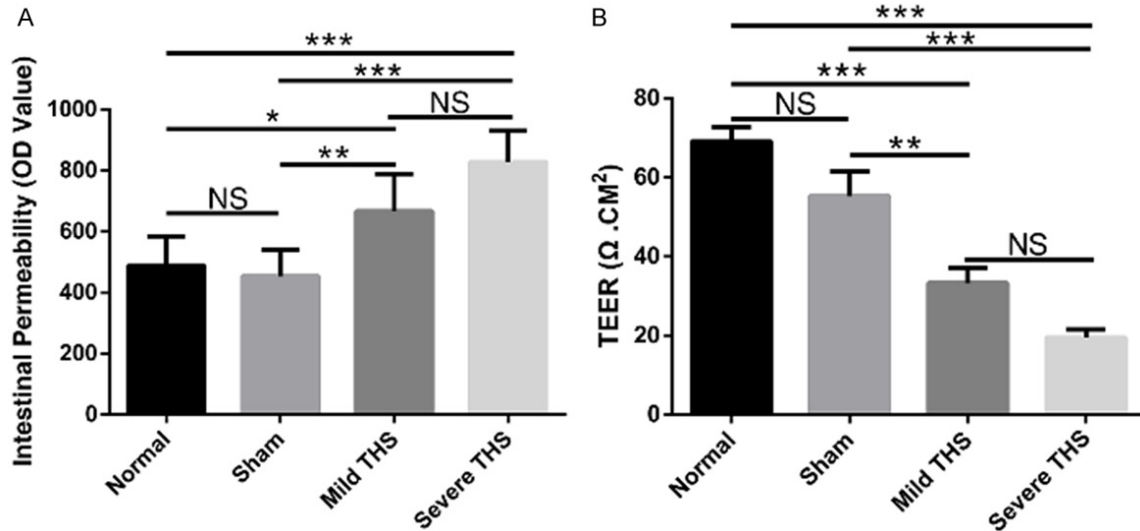
#### *Severe THS impaired phenotypic maturation of MLN-DCs*

The expression levels of maturation markers were elevated in both the mild and severe THS groups. However, only the severe THS group significantly impaired the phenotypic maturation of MLN-DCs (**Figure 4A-C**).



**Figure 2.** Bacterial translocation after THS. Blood (200  $\mu$ l) and homogenates from equal weights (0.2 g) of spleen, liver, and MLN of each group were plated on LB plates, cultured overnight at 37  $^{\circ}$ C, and observed via IVIS (A). In each figure, the upper left plate is blood, the upper right is MLN homogenate, the lower left plate is spleen homogenate, and the lower right is liver homogenate. The mild and severe THS groups had significantly higher bacterial translocation to MLN (B). However, only severe THS caused significantly elevated bacterial translocation to the blood (C), spleen (D), and liver (E). Data are expressed as mean  $\pm$  SD, and there were six animals per group. In (B)  $**P < 0.01$  or  $###P < 0.001$  versus the normal and sham groups; in (C)  $###P < 0.01$  versus other groups; in (D and E)  $###P < 0.001$  versus other groups.

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**Figure 3.** Mechanical barrier injury. The intestinal permeability was tested by using FITC-labeled dextran as a permeability marker. Intestinal resistance was measured with an ussing chamber to investigate barrier injury after shock. The mild and severe THS groups had significantly higher intestinal permeabilities (A) and lower resistances of the mucus layer (B) than the normal or sham group; however, there were no differences between the mild and severe THS groups. Data are expressed as mean  $\pm$  SD, and there were  $n = 6$  animals per group. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .

### *THS caused changes in the polarization of T cells induced by MLN-DCs, and pSTAT1 and pSTAT6 may be involved*

Co-culture experiments showed that there were no differences between the normal and sham groups (Figure 5A-C). Both the mild and severe THS groups had significantly higher percentages of Th1 and Th2, however, the mild THS group induced more Th1 differentiation, while the severe THS group showed increased polarization of CD4<sup>+</sup> T cells into Th2 cells (Figure 5A-C). Interestingly, there was a significant decrease in Th1/Th2 ratio in the severe THS group compared to the mild THS group (Figure 5D). CD4<sup>+</sup> T cells co-cultured with MLN-DCs from the severe THS group had a significantly higher percentage of Treg cells compared to the other groups (Figure 5E, 5F).

Western blots showed that both mild and severe THS increased STAT1 and STAT6 phosphorylation. However, mild THS induced more STAT1 activation and severe THS induced more STAT6 activation (Figure 5G).

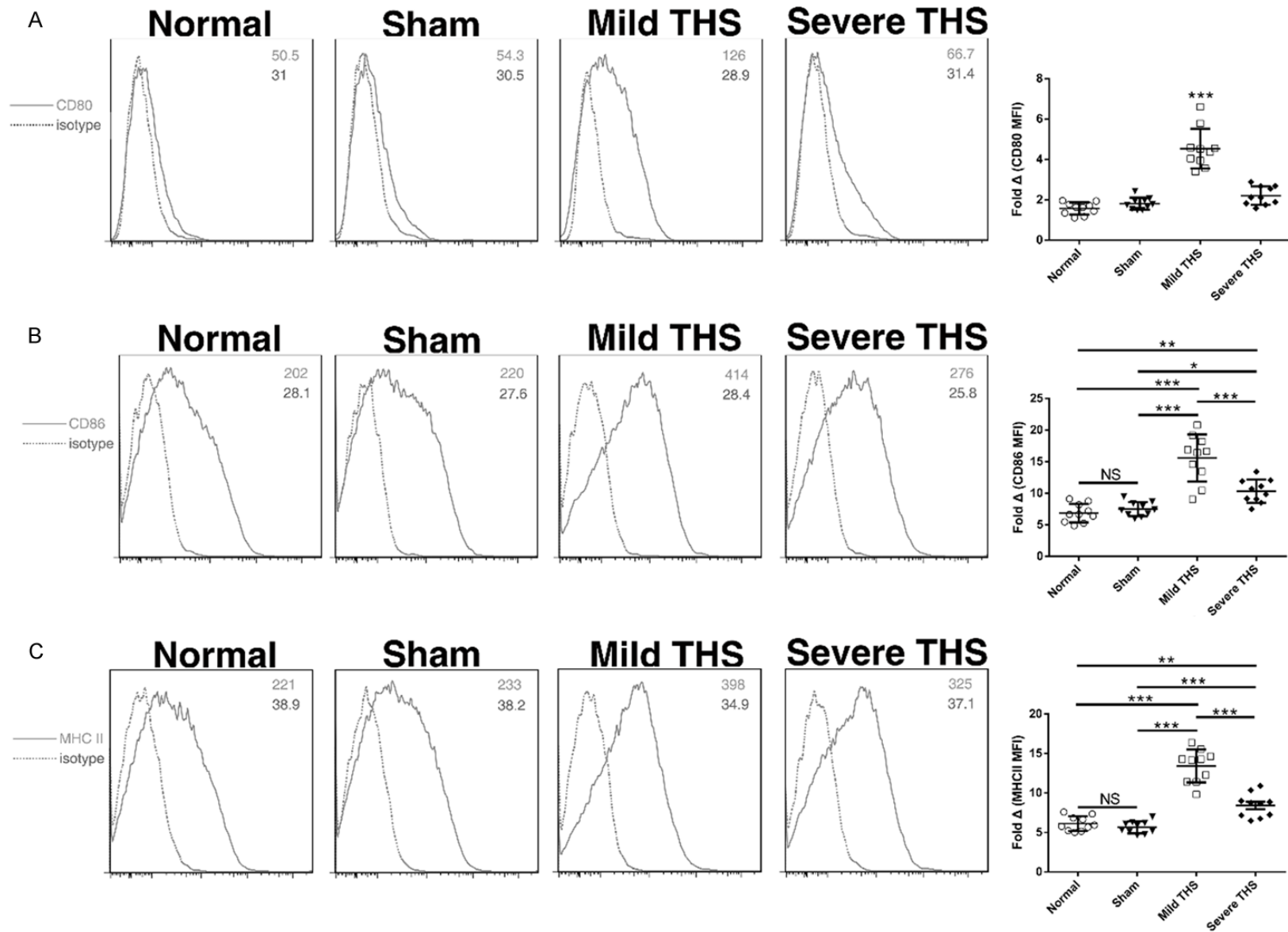
### Discussion

We discover that severe THS increases bacterial translocation to the visceral organs, such

as liver, spleen and blood, but not MLN. To explore this phenomenon, we focused on MLN, especially MLN-DCs, which play a crucial role in the construction of immune barrier of MLN. We find that the phenotypic maturation of MLN-DCs is badly impaired by severe THS. Furthermore, MLN-DCs from the severe THS group are more inclined to polarize the differentiation of naive CD4<sup>+</sup> T cells into Th2 and Treg cells, which may increase bacterial translocation to the visceral organs.

Intestinal bacterial overgrowth, intestinal mucosal injury, and compromised immune defense are considered as the three primary factors driving bacterial translocation, and these factors can exist individually or in combination [12]. In our study, rats from all groups were co-housed and fed the same food from birth until the start of experiments. All groups received equal quantities of bacteria by oral gavage in an effort to minimize differences in gut microbiota composition. Histology results showed intestinal villi damage and mucosal edema after THS (data not shown), and additionally, we detected a higher intestinal permeability and lower resistance after THS. However, there were no differences between the mild and severe THS groups. This could explain the similar bacterial translocation to MLN in these two

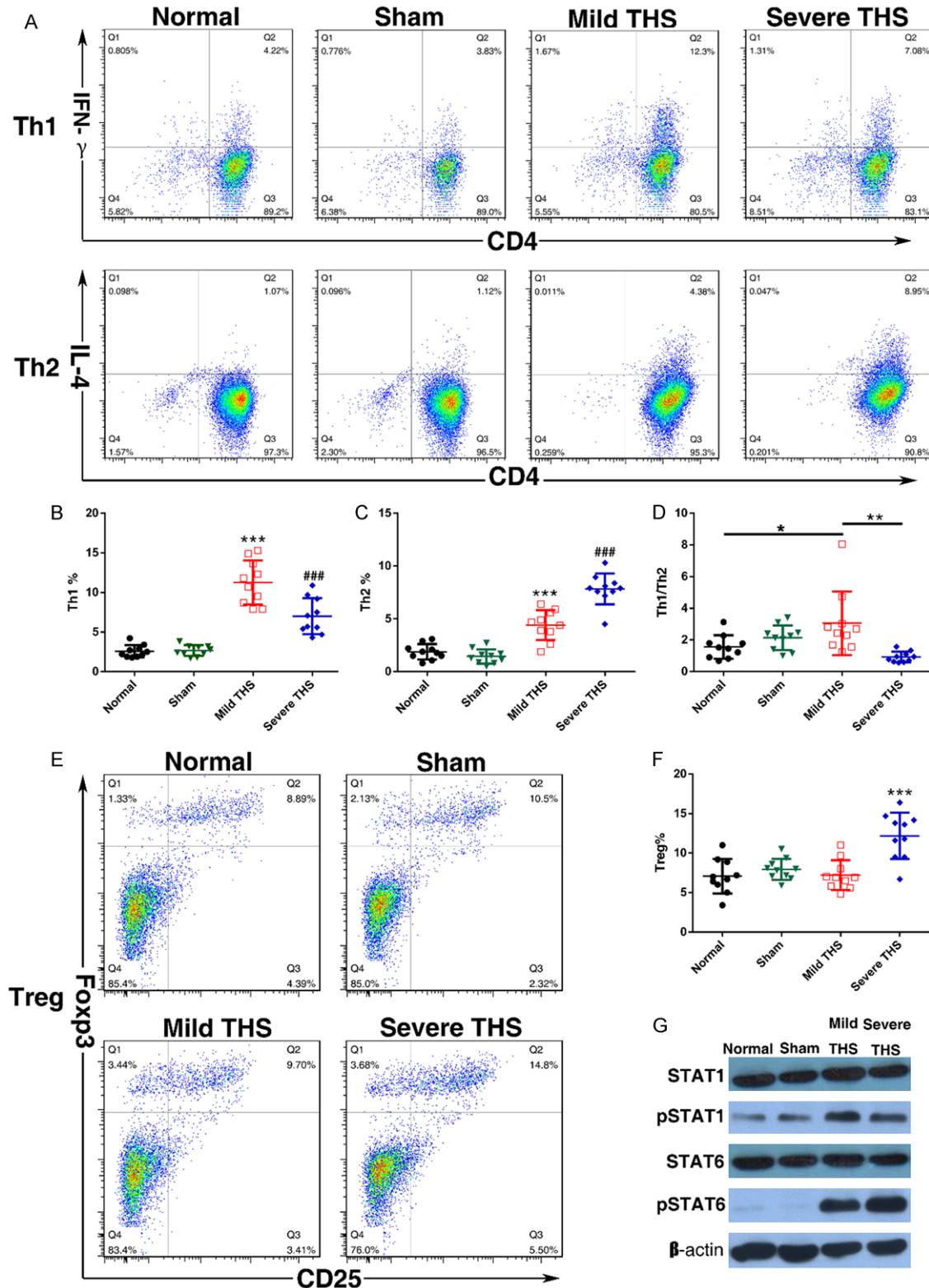
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**Figure 4.** Phenotypic maturation of mesenteric lymph node dendritic cells was impaired after severe THS. Rats from different groups were analyzed by flow cytometry for MHC II (C), CD80 (A), and CD86 (B) expression on mesenteric lymph node dendritic cells. Median fluorescence intensity (MFI) is indicated compared with respec-

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tive isotype controls. Representative data and summary statistics (Fold  $\Delta$  means MFI relative to isotype value) are shown, and in each FCM figure the number in the upper right corner represents the MFI of the maturation marker, while the number below it represents the MFI of the isotype. Data are expressed as mean  $\pm$  SD, and there were  $n = 10$  animals per group. In (A)  $***P < 0.001$  versus other groups; in (B and C)  $*P < 0.05$ ,  $**P < 0.01$ , and  $***P < 0.001$ .





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**Figure 5.** Polarization of naïve T cells induced by mesenteric lymph node dendritic cells. Naïve CD4<sup>+</sup> T cells (1×10<sup>6</sup> cells/well) were co-cultured with OX62<sup>+</sup> MLN-DCs (1×10<sup>5</sup> cells/well) for 3 days at 37 °C with 5% CO<sub>2</sub> in a humidified incubator. Cells were then analyzed by flow cytometry for Th1(CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup>) (A, upper), Th2(CD4<sup>+</sup>IL-4<sup>+</sup>) (A, lower), and Treg (gated on CD4<sup>+</sup>, CD25<sup>+</sup>FOXP3<sup>+</sup>) (E). Summary statistics are shown for Th1 (B), Th2 (C), Th1/Th2 (D), and Treg (F). A Western blot shows the expression of STAT1, STAT6, and their phosphorylated forms in polarized CD4<sup>+</sup> T cells from different groups (G). Data are expressed as mean  $\pm$  SD, and there were n = 10 animals per group. In (B, C and F) \*\*\*P < 0.001 or ####P < 0.001 versus other groups; in (D) \*P < 0.05 and \*\*P < 0.01.

groups. However, the compromised immune barrier function of MLN in the severe THS group could allow *Citrobacter* to break through the MLN and translocate to the visceral organs.

DCs are thought to play a pivotal role in inducing tolerance or mounting protective immune responses. Injury to the epithelial barrier induced by THS may increase the possibility of DCs encountering and being stimulated by certain bacterial antigens. Furthermore, on stimulation with antigens, immature DCs residing in GALT can mature and migrate to MLNs, the place where they direct the polarization of naïve T cells, however, DCs may become dysfunctional after THS due to anoxia and I/R injury. That's why we focused on MLN-DCs in this study. Severe THS downregulated MLN-DCs' MHC II, CD80, and CD86, all of which are involved in executing DCs' antigen presenting capability. And in this study, the intestinal circulation did not recover 48 hours later after sufficient fluid resuscitation in the severe THS group due to low microvascular tissue perfusion, which may suppress the maturation and function of DCs. Many studies have shown that the drift to Th2 or Treg immunosuppressive phenotypes and their associated cytokines, such as IL-4 or IL-10, may explain post-injury T cell immunosuppression [14, 20, 21]. To determine if THS can promote this drift, we analyzed the ability of MLN-DCs to polarize naïve T cells, and interestingly, we found that MLN-DCs from the severe THS group had the ability to cause this drift.

This drift may be related to the ratio change of pSTAT1/pSTAT6. STAT1-deficient mice have been shown to have an impaired expression of IFN- $\gamma$  in splenic T cells [22]. IFN- $\gamma$  is thought to induce the expression of the  $\beta$ 2 chain of IL-12, which may induce an IL-12 response in these cells and an IL-12-driven differentiation into Th1 cells [23, 24]. In addition to partially inhibiting STAT6 signal transduction, which is related to differentiation into Th2 cells [25, 26], IFN- $\gamma$  signaling has also been shown to inhibit IL-4 expression and stabilize the Th1 phenotype

[25, 27]. Treg cell's decreased suppressor function, resulting in a breakdown of immunological tolerance is likely due to augmented expression and activation of STAT1 [28]. We also showed that severe THS increased STAT6 activation and downregulated pSTAT1 expression, which may promote Th2 polarization and inhibit Th1 polarization.

We have shown that THS impairs intestinal mechanical barrier and allows bacteria to reach MLN. However, only severe THS allows the translocation of intestinal bacteria to extraintestinal tissues, which may be correlated with "polarized Th2 and Treg drift" during differentiation of naïve CD4<sup>+</sup> T cells induced by MLN-DCs.

Although we have shown that severe THS impairs the phenotypic maturation of MLN-DCs, the exact mechanism is unclear, which, as well as the role of pSTAT1/6, warrant future investigation.

This study successfully reproduced the clinical phenomenon of severe shock resulting in increased bacterial translocation to extraintestinal tissues, which may be related to the compromised immune barrier function of MLN, as maturation and function of DC's were severely impaired.

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**Disclosure of conflict of interest**

None.

**Address correspondence to:** Dr. Ting-Bo Liang, Department of Hepatobiliary and Pancreatic Surgery, The Second Affiliated Hospital, School of Medicine, Zhejiang University, No. 88 Jiefang Road, Hangzhou 310009, Zhejiang, China. Tel: +86 571 87315006; Fax: +86 571 87315006; E-mail: liangtingbo@zju.edu.cn; liangtingbo2005@gmail.com

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