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ORIGINAL ARTICLE

Regulatory T Cells Could Improve Intestinal Barrier Dysfunction in Heatstroke

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Abstract— Intestinal barrier dysfunction plays a pivotal role in multiorgan dysfunction during heatstroke (HS). Neutrophils are involved in intestinal inflammation and thus dampen the mucosal integrity. Regulatory T cells (Tregs) have been shown to orchestrate neutrophils and thus sustain mucosal integrity in miscellaneous inflammation-related diseases. However, whether Tregs are involved in HS-induced intestinal barrier dysfunction remains unknown. Thus, we investigated whether Tregs could alleviate intestinal barrier dysfunction in mice. We found that HS could induce intestinal injury and mucosal barrier dysfunction 0, 24, and 72 h after heat stress. Flow cytometry revealed an increase of neutrophil infiltration and a decrease of Treg frequencies in the small intestinal epithelium 72 h after heat stress. Treg depletion starting 2 days before HS exacerbated intestinal damage and mucosal barrier dysfunction. Adoptive transfer of Tregs at 0 h improved intestinal injury and mucosal barrier dysfunction at 72 h. The manipulation of Tregs affected the neutrophil frequencies in the small intestinal epithelium 72 h after heat stress. Our study demonstrated that Tregs could improve HS-induced intestinal barrier dysfunction, probably *via* modulation of neutrophils in the intestine of mice during HS.

KEY WORDS: heatstroke; regulatory T cells; neutrophils; intestinal barrier dysfunction.

INTRODUCTION

Heatstroke (HS) is a life-threatening injury that is characterized by central nervous system dysfunction and hyperthermia (core temperature > 40 °C). Despite rapid cooling and organ support therapy, many patients still quickly progress to multiple organ dysfunction syndromes (MODS) and experience permanent neurological impairments or death [24].

Gut-derived endotoxin caused by intestinal barrier dysfunction has been considered as the "motor" of MODS in critically ill patients [17]. Emerging evidence has shown that the intestine is highly susceptible to heat injury [21, 30] and gut-derived endotoxin plays a pivotal role in the development of systemic inflammatory response syndrome (SIRS) and MODS in HS patients [12, 18, 26]. The pro-inflammatory cytokines in the intestine are associated with intestinal injury during HS [27]. Neutrophils are involved in intestinal inflammation and mucosal integrity [33]. Meanwhile, inflammatory conditions in the intestinal mucosa further compromise mucosal barrier function [4]. Based on current studies [12, 19], we believe that improvement of intestinal barrier function might be a potential therapeutic target for HS. Recently, immunomodulatory nutrients have been shown to regulate host immune and inflammatory responses and possibly restore the intestinal

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barrier [1]. Unfortunately, severe intestinal injury in early HS might limit the utilization of immunomodulatory nutrients.

Regulatory T cells (Tregs) are a specialized lineage of suppressive $CD4^+$ T cells that have been proven to participate in inflammatory bowel disease [3, 11] and necrotizing enterocolitis [8] *via* inflammation modulation. However, whether Tregs are involved in HS-induced intestinal barrier dysfunction is unknown. Interestingly, studies based on sepsis, which shares a similar mechanism with HS [21], have reported contradictory roles of Tregs in intestinal injury. To our knowledge, whether and how Tregs participate in HS-induced intestinal barrier dysfunction has never been investigated and thus was detected in our study using depletion and transfer techniques to target Tregs in a classic HS model.

METHODS

Animals and Treatments

Male C57BL/6 mice (20–25 g), aged 8–12 weeks, were purchased from the animal center at the Chinese PLA General Hospital. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Chinese PLA General Hospital and Military Medical College.

Mice were divided into HS group and negative control group (NC). The heat stress protocol has been described in detail elsewhere [22]. Briefly, the mice were exposed to an incubator temperature (Ta) of 39 ± 0.2 °C with 50 ± 5% humidity in the absence of food and water, and the rectal temperature was monitored every 10 min using a digital thermometer (ALC-ET06, Shanghai Alcott Biotech Co., Shanghai, China), which was inserted 2 cm into the rectum, until a maximum temperature of 42.7 °C was attained; these selection criteria were formulated to induce moderate HS [23]. The mice were removed from the chamber and allowed to passively recover at room temperature (25 ± 0.5 °C) in a new cage with food and water available *ad libitum*. The control mice underwent the same experimental procedure as the HS mice except that the chamber temperature was maintained at 25 ± 0.5 °C with a relative humidity of 50 ± 5% throughout the experiment.

In vivo depletion of Tregs [14, 29] was achieved by intravenous injection of 100 mg of a monoclonal anti-CD25 antibody (Clone PC61, BioLegend, San Diego, CA) immediately after HS onset. The control mice were injected with rat IgG (BioLegend). The depletion of Tregs was confirmed by staining intestinal cells for the CD4 and Foxp3 markers 72 h after injection of the anti-CD25 antibody.

After HS onset, the mice were injected with 1.2×10^6 $CD4^+CD25^+$ Tregs (or an equal volume of a 0.9% saline solution as a control) *via* the tail vein.

Histopathological Analysis

Mice were anesthetized by intraperitoneal injection of phenobarbital and sacrificed. Samples of the ileal segment 5 cm from the ileocecal valve were harvested immediately after blood collection, sliced into transverse or longitudinal sections, and fixed in 10% neutral-buffered formalin. Then, the tissues were embedded in paraffin blocks, and serial sections were stained with hematoxylin and eosin for microscopic evaluation at × 200 magnification. Morphological changes were assessed and graded in a blinded manner by two certified veterinary pathologists using the intestinal injury score developed by Chiu et al. [7].

Measurement of *Ex Vivo* Intestinal Mucosal Permeability

Intestinal permeability was determined by using the everted gut sac method and the fluorescent tracer fluorescein isothiocyanate dextran (MW 4000 Da; FD4), as described elsewhere [31]. Ileal everted gut sacs were prepared in ice-cold modified Krebs–Henseleit bicarbonate buffer (KHBB, pH 7.4). One end of the gut segment was ligated with 4-0 silk. Then, the intestinal segment was everted using a thin metal rod and secured using a 4-0 silk tie to the grooved tip of a 5-mL plastic syringe containing KHBB. The everted gut sac was then distended gently by injecting 0.5 mL of KHBB. During specimen handling, care was taken not to injure the mucous layer based on the appearance of the mucosa. The sac was suspended in a 100-mL beaker containing 80 mL of KHBB with added FD4 (20 mg/mL). The solution in the beaker was maintained at a temperature of 37 °C in a water bath, and a gas mixture containing 95% O_2 and 5% CO_2 was bubbled continuously. A 1.0-mL sample was removed from the beaker before placing the everted gut sac to determine the initial external (mucosal surface) FD4 concentration. The everted gut sacs were incubated for 30 min in the KHBB solution containing FD4. The length and volume of the gut sacs were then measured. The fluid on the serosal side was aspirated into the syringe to determine the FD4 concentration. The serosal and mucosal samples were centrifuged for

10 min at 1000×*g* and 4 °C. Then, 100 μL of the supernatant was diluted with phosphate-buffered saline (PBS) (900 L), and fluorescence was measured using a Perkin-Elmer LS-50 fluorescence spectrophotometer (Palo Alto, CA, USA) at an excitation wavelength of 492 (slit width, 510 nm) and an emission wavelength of 515 nm (slit width, 510 nm). Permeability was expressed as the mucosal-to-serosal clearance of FD4 and calculated using the following equations:

$$M = ([FD4]serosal) \times 0.5$$

$$F = M/30\ min$$

$$C = (F/[FD4]mucosal)/A$$

where M is the mass (in ng) of FD4 in the gut sac at the end of the 30-min incubation period, [FD4]serosal is the FD4 concentration in the serosal fluid aspirated from the sac at the end of the 30-min incubation period, F is the flux of FD4 (in ng/min) across the mucosa, [FD4]mucosal is the FD4 concentration measured in the beaker at the beginning of the 30-min incubation period, A = LD is the calculated area (in cm^2) of the mucosal surface, and C is the clearance of FD4 (in μg cm^{-2} h^{-1}) across the mucosa.

Plasma Endotoxin Analysis

The endotoxin bioassay was performed in duplicate using a GenScript endotoxin detection kit (ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit, L00350) according to the manufacturer's instructions. All micropipette tips, tubes, and other instruments used in this experiment were non-pyrogenic. An endpoint measurement was performed at 545 nm in a microplate spectrophotometer. The results were expressed as endotoxin unit per milliliter.

d-Lactate Detection

Plasma D-lactate levels were measured by a colorimetric D-lactate assay kit (Abcam, ab83429) according to the manufacturer's instructions. The test samples were prepared with assay buffer in a 96-well plate and then mixed with the reaction mixture for 30 min at room temperature. The optical density was measured at 450 nm. A standard curve was generated by serial dilution of a D-lactate standard solution, and the concentrations of the samples were calculated. The results were expressed as millimole per liter.

Diamine Oxidase Detection [6]

Plasma diamine oxidase (DAO) levels were measured by a DAO kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. A total of 80 μL of plasma was added to the test tube, mixed with 800 μL of the reaction mixture, and incubated at 37 °C for 10 min. The optical density was measured at 340 nm. The results were expressed as kilo unit per liter.

Western Blot Analysis [38]

A 5-cm ileal segment was harvested 10 cm from the ileocecal valve, washed in 0.9% saline, and then lysed with ice-cold radioimmunoprecipitation assay (RIPA) Lysis Buffer (Aidlab Biotechnologies Co., Ltd., Beijing, China) supplemented with a protease inhibitor "cocktail." The tissue was homogenized in an ultrasonic cell disruptor (Ningbo Scientz Biotechnology Corporation, Ningbo, China) and then centrifuged at 8000 rpm at 48 °C for 30 min. The protein concentrations were measured using a bicinchoninic acid (BCA) Protein Assay kit (Aidlab Biotechnologies Co., Ltd., Beijing, China). Equal amounts of each extract were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto nitrocellulose membranes. The samples were incubated separately with anti-occludin (1:500, Abcam, ab168986), anti-zonulae occludens (ZO)-1 (1:1000, Abcam, ab96587), and anti-GAPDH (1:5000, Abcam, ab8245) antibodies for 24 h at 48 °C. After washing, the membranes were incubated with the secondary antibodies for 2 h at 48 °C. Full-film scans of the western blotting data were obtained with an Amersham Imager 600 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The band intensity values of the proteins of interest were normalized to that of GAPDH.

Flow Cytometric Analysis

We performed surface staining with the following antibodies according to the manufacturers' instructions: a phycoerythrin (PE) anti-mouse F4/80 antibody (Biolegend, BM8), a PE anti-mouse/human CD11b antibody (Biolegend, M1/70), a fluorescein isothiocyanate (FITC) anti-mouse Ly-6G antibody (Biolegend, 1A8), a FITC-CD4 antibody (553729, BD), and an allophycocyanin (APC)-CD25 antibody (557192, BD). We also performed intracellular staining for Foxp3 with an APC-Foxp3 antibody (17-5773-80, eBioscience) according to the manufacturer's instructions. $CD4^+CD25^+$ Tregs were isolated from the spleens of C57BL/6J mice

using fluorescence-activated cell sorting (FACS). The purity of the population was confirmed by flow cytometry analysis and routinely reached > 90%. Cells were observed using a BD FACS Calibur system (BD Immunocytometry Systems) and analyzed with the FlowJo 7.6 software.

Statistical Analysis

The analysis was performed using the IBM SPSS Statistics 18.0 software (IBM Corporation, Armonk, NY, USA). Results are presented as mean ± SD. Multiple comparisons of parametric data were performed using one-way analysis of variance (ANOVA), followed by Student–Newman–Keuls (SNK) *post hoc* tests. Student's *t* test was used to compare differences in means. α was corrected by the number of comparisons (α/comparisons) to ensure $\alpha = 0.05$ (*$p < 0.05$, **$0.001 < p < 0.01$, ***$p < 0.001$). Graphs were made *via* GraphPad Prism7 software.

RESULTS

HS Could Induce Intestinal Injury and Barrier Dysfunction

To investigate HS-induced intestinal injury, histological damage and Chiu scores ($n = 5$) were assessed in the NC group and HS groups 0 h, 24 h, and 72 h after HS onset. In the control group and at 0 h after HS, only small blebs were observed at the villus tip, although some pathologists claimed this may be an artifact or even a normal finding (Fig. 1a, b). In contrast, at 24 h, the injury to the ileum was mainly located in the villi, with apparently progressive epithelial necrosis, epithelial loss, and desquamation of the villus epithelium (Fig. 1a, b). The intestinal lesions were further aggravated at 72 h (Fig. 1a, b).

Using the *ex vivo* everted gut sac model in which each time point was measured in a separate group of mice, intestinal permeability to FD4 was first noted to be increased at 24 h after HS and remained elevated throughout the 72-h study period (Fig. 1c, $n = 5$). To further confirm the impairment of the intestinal mucosal barrier in the HS group, we examined serum levels of DAO, D-lactic acid, and endotoxin by enzyme-linked immune sorbent assay (ELISA) kits according to the manufacturer's instructions. The serum DAO (Fig. 1d, $n = 5$), D-lactic acid (Fig. 1e, $n = 5$), and endotoxin (Fig. 1f, $n = 5$) levels in the HS group were significantly increased compared with those in the control group at both 24 h and 72 h.

The tight junction (TJ) proteins occludin and ZO-1 play a key role in maintaining the integrity of the intestinal barrier [38]. The western blotting results showed that occludin and ZO-1 expression was markedly reduced in the intestines of the HS mice at 24 h and 72 h after HS onset compared with the expression levels in the control mice (Fig. 1g).

Intestinal Trafficking of Tregs Was Decreased at 72 h After Heat Stress

Compared with that in the NC group, we found that the proportion of $CD4^+Foxp3^+$ Tregs in the intestine was markedly decreased in the HS intestine at 72 h (Fig. 2a, b). We also characterized other intestine-infiltrating leukocyte populations during HS. FACS demonstrated that neutrophil ($Ly6\text{-}G^+$, Fig. 2c, d) frequency was increased at 72 h after HS.

In Vivo Depletion of Tregs Could Exaggerate Intestinal Injury and Dampen Mucosal Barrier Function 72 h After Heat Stress

After identifying decreased trafficking of Tregs into the intestine, we hypothesized a role for these cells during HS. We initially depleted $CD25^+$ cells with the PC61 (anti-CD25) monoclonal antibody (mAb) *in vivo*. Rat IgG was used as an isotype control. The depletion was started 48 h before heat stress. The mice were killed at 72 h. The efficacy of PC61 mAb treatment was evaluated in the intestine 72 h after HS onset. PC61 mAb treatment led to a significant reduction in $CD4^+Foxp3^+$ Tregs (Fig. 3a, b) in the intestine. After 72 h, the intestine from the PC61 mAb–treated mice showed increased intestinal damage compared with that from the controls (Fig. 3c, d); these changes were associated with increased permeability to FD4 (Fig. 3e); increased serum levels of DAO (Fig. 3f), D-lactate (Fig. 3g), and endotoxin (Fig. 3h); and the downregulation of occludin and ZO-1 (Fig. 3i).

Adoptive Transfer of Tregs Could Improve Intestinal Injury and Mucosal Barrier Function 72 h After HS Onset

Subsequently, 1.2×10^6 $CD4^+Foxp3^+$ Tregs were isolated and purified from the spleens of C57BL/6J mice and then injected intravenously (i.v.) into HS mice immediately after HS onset. An equal volume of PBS was used as a control. At 72 h after HS, transfer of Tregs attenuated intestinal injury (Fig. 4a, b); decreased permeability to FD4 (Fig. 4c); the serum levels of DAO (Fig. 4d), D-lactate (Fig. 4e), and endotoxin (Fig. 4f); and upregulated occludin and ZO-1 expression (Fig. 4g). Moreover, intravenous

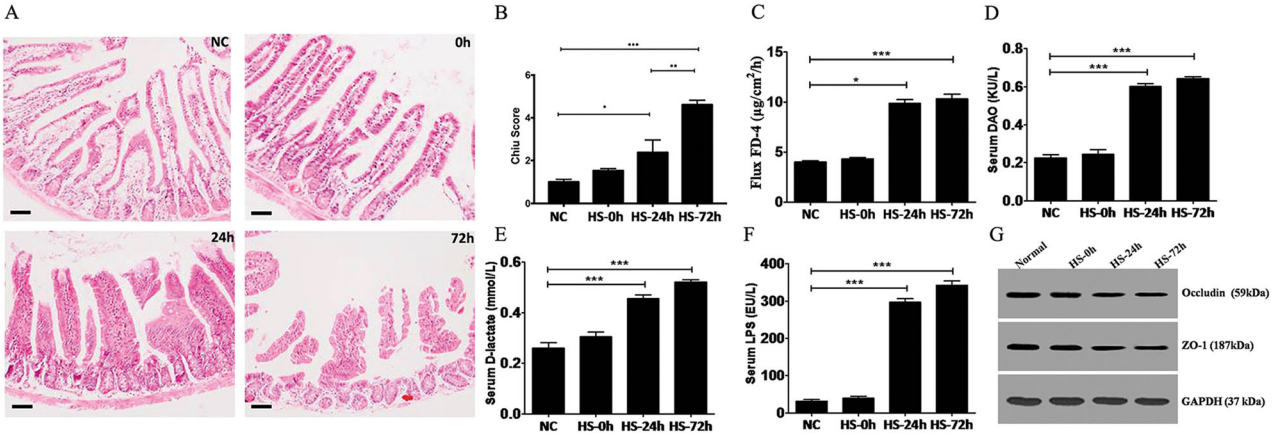

Fig. 1. HS induces intestinal barrier dysfunction. **a** Histological injury to the intestine after heat stress 0 h, 24 h, and 72 h ($n = 5$–7 samples per group). **b** Chiu scores compared with those of NC intestines 0 h, 24 h, and 72 h after HS onset ($n = 5$ samples per group). **c** FD4 levels detected *via* the *ex vivo* everted gut sac model in the HS and NC groups ($n = 5$). Serum DAO (**d**), D-lactic acid (**e**), and endotoxin (**f**) levels detected by ELISA kits in the HS and NC groups ($n = 5$). **g** The tight junction (TJ) proteins occludin and ZO-1 were detected *via* western blotting analysis in the HS and NC groups ($n = 5$). The data are expressed as the mean ± SD; one-way analysis of variance (ANOVA) and Tukey's multiple comparison test were used to do multiple comparisons of Chiu scores in dependent groups. Due to the homogeneity of variance, multiple comparisons of FD-4, DAO, D-lactate, and LPS among dependent groups were performed using one-way analysis of variance (ANOVA). *p* values are adjusted for multiple comparisons. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

injection of Tregs *via* the tail vein resulted in a significant increase in intestinal Tregs at 72 h (Fig. 4h, i).

Treg Manipulation Was Associated with Changes in the Frequency of Neutrophils in the Intestine

Increased neutrophils characterized the HS intestines at 72 h after HS onset (Fig. 2b). Compared with the frequencies in the controls, the PC61 mAb–treated HS intestines showed increases in neutrophil (Fig. 5a, b) frequencies at 72 h after HS. In contrast, HS intestines from mice subjected to adoptive transfer had reduced neutrophil (Fig. 5c, d) frequency at 72 h.

DISCUSSION

In this study, we demonstrated that HS could induce intestinal barrier dysfunction and increase the neutrophil infiltration in the intestine. The depletion of Tregs could exaggerate intestinal injury and intestinal mucosal barrier dysfunction, while the adoptive transfer of Tregs could reverse those effects. Furthermore, the manipulation of Tregs could modulate the frequencies of neutrophils in the intestine. Thus, we demonstrated for the first time that Tregs could improve HS-induced intestinal barrier dysfunction *via* the modulation of neutrophils, which might be a future therapeutic strategy for this life-threatening illness.

DAO is primarily expressed in the small intestine and is rarely detected under normal circumstances [9, 10, 36] but increased significantly in serum when the intestinal injury occurs [5]. D-Lactic acid is a metabolic product of intestinal bacteria, and the change of serum D-lactic acid in serum is a strong indicator of intestinal mucosal barrier damage [28, 34]. Lipopolysaccharide (LPS) is the main pathogenic component of endotoxins, which was released from the damaged intestinal barrier and a key factor for activation of SIRS in HS [25]. TJs are composed of transmembrane proteins (such as occludin) and cytosolic scaffold proteins (ZO-1) [32], which are important components of the mucosal barrier. A destroyed intestinal epithelium, hyperpermeability, and downregulated TJ protein expression have been reported after heat exposure in many studies [30, 37, 38]. In this study, similar results were also found in HS mice, as indicated by intestinal morphological alterations; increased serum levels of LPS, DAO, and D-lactate; hyperpermeability to FD4; and lower expression of the main TJ proteins occludin and ZO-1 in the intestine (Fig. 1).

Tregs are specialized immune cells that play a central role in maintaining immune self-tolerance and are characterized by expression of the forkhead/winged-helix transcription factor Foxp3 in the nucleus [15]. Treg infiltration and its role in intestinal injury have been described in the context of other diseases, such as inflammatory bowel disease [3, 11] and sepsis-induced intestinal injury [13, 39]. However, this effect has never been detected in HS-

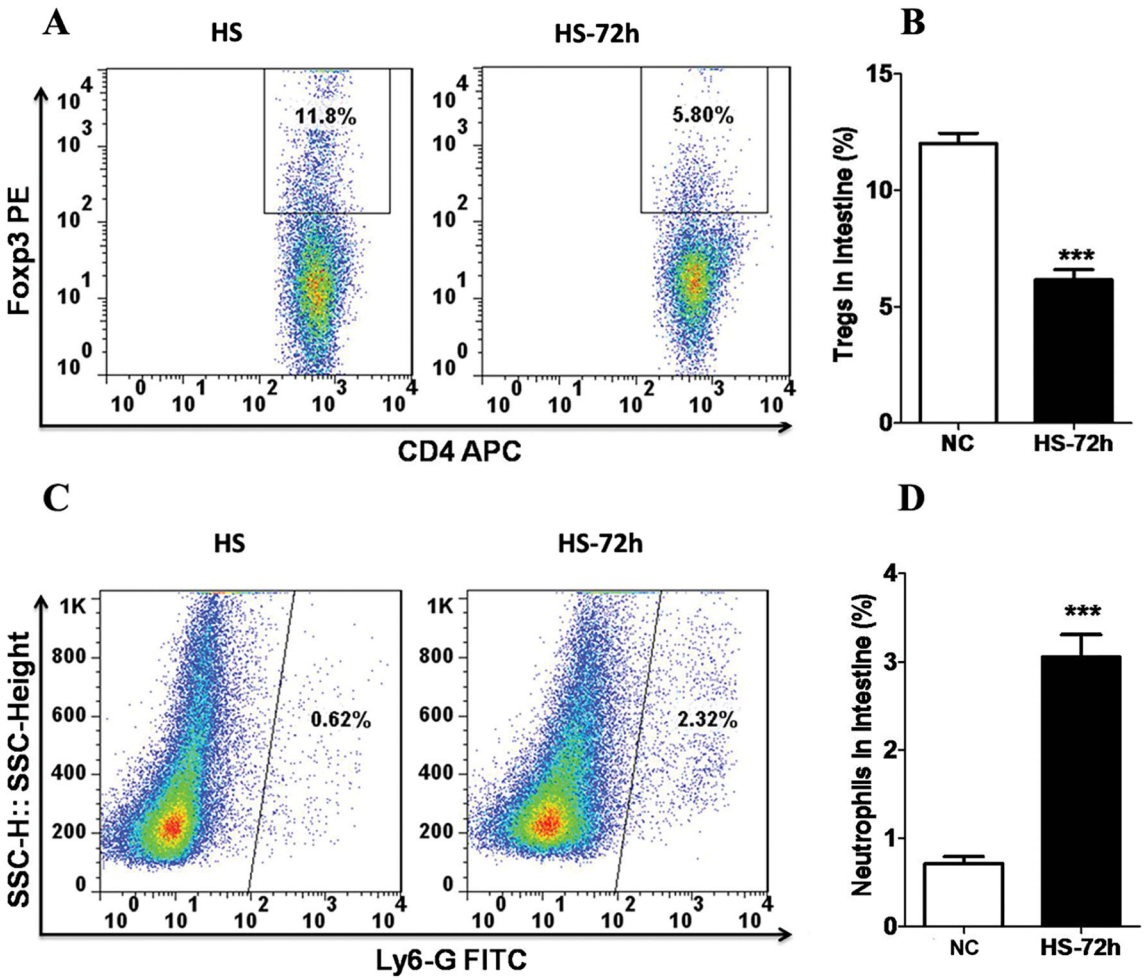

Fig. 2. Intestinal trafficking of Tregs and neutrophils at 72 h after heat stress. **a**, **b** Flow cytometry showing that the proportion of $CD4^+Foxp3^+$ Tregs in the intestine was markedly lower in the HS group than in the NC group at 72 h (12.0 ± 1.0 *vs.* 6.2 ± 1.0, $p < 0.001$, $n = 5$). **c**, **d** Flow cytometry showing that the proportion of neutrophils in the intestine was markedly higher in the HS intestine than in that in the NC group at 72 h (0.71 ± 0.19 *vs.* 3.1 ± 0.6, $p < 0.001$, $n = 5$). The data are expressed as the mean ± SD; Student's *t* test was used to compare differences in means of frequencies of Tregs and neutrophils between dependent groups.***$p < 0.001$.

induced intestinal injury. Our current study demonstrated for the first time that the intestinal infiltration of $CD4^+Foxp3^+$ T lymphocytes significantly decreased 72 h after HS.

Do Tregs participate in HS-induced intestinal injury and mucosal barrier dysfunction? Controversies still exist concerning how Tregs orchestrate local intestinal injury. Some researchers believe that intestinal Tregs exert immunosuppressive effects on other intestinal T lymphocytes and thus favor endotoxin translocation [39], whereas other researchers have demonstrated that Tregs can suppress local inflammation and thus improve intestinal injury and survival in patients with polymicrobial sepsis [13]. To clarify whether and how Tregs participate in catastrophic intestinal injury during HS, we first depleted Tregs by the administration of an anti-CD25 mAb using an established approach [14, 29]. When we investigated the depletion rate of $CD4^+Foxp3^+$ Tregs in the intestine, we observed that Treg depletion was not complete. Unlike natural Tregs that originate in the thymus, CD25 expression on the surface of induced Tregs is variable [2]. Therefore, induced Tregs were not completely depleted in the intestine in our study. Treg depletion before HS led to worse histological damage; hyperpermeability to FD4; increased serum LPS, DAO, and D-lactate levels; and downregulation of TJ proteins. Rat IgG was used as an isotype control to confirm the above effects definitely attributed to the depletion of Tregs other than the immune response to heterogeneous

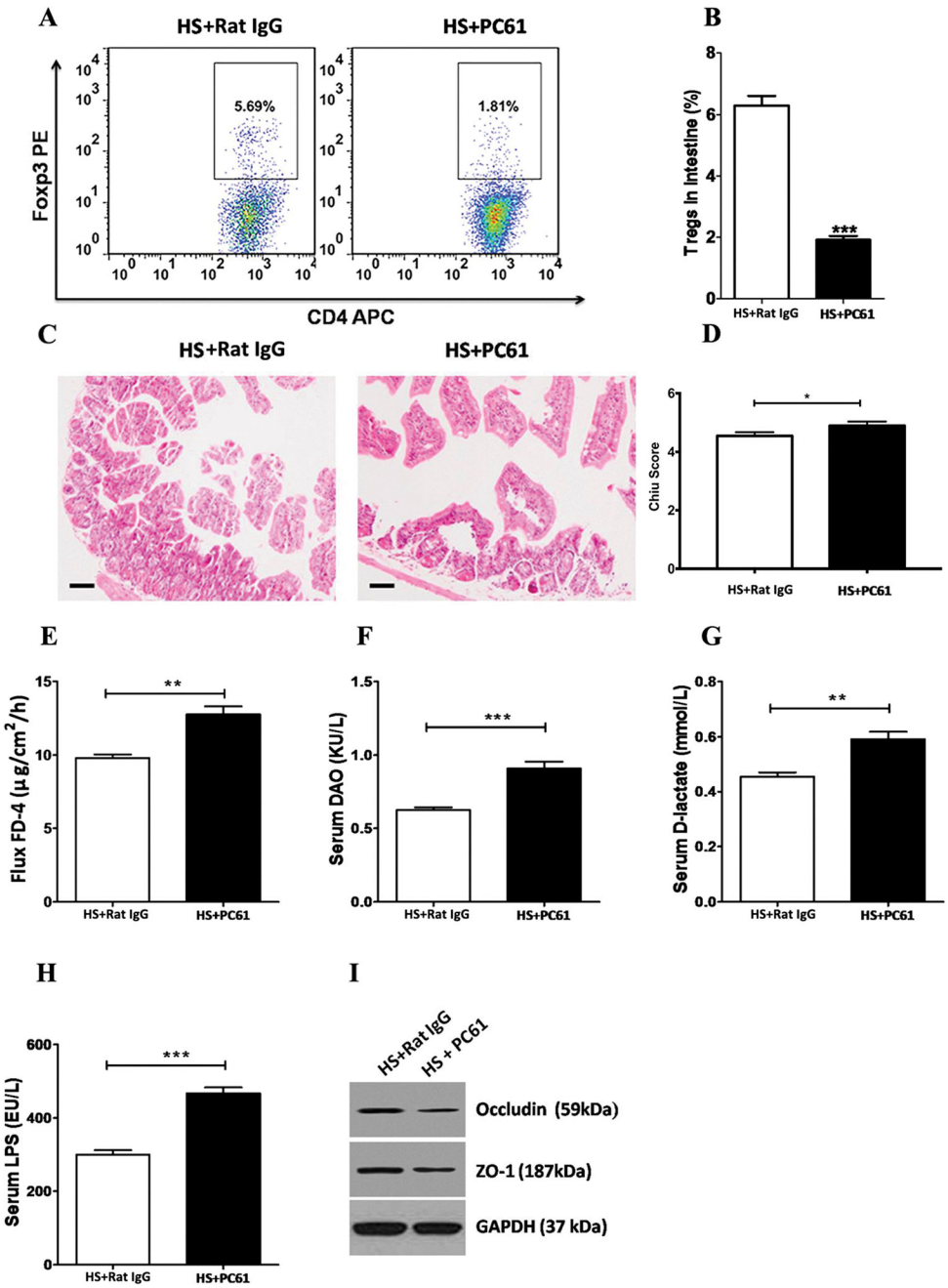

Fig. 3. *In vivo* depletion of Tregs exaggerated intestinal barrier dysfunction at 72 h after heat stress. **a**, **b** Flow cytometry showing that the proportion of $CD4^+Foxp3^+$ Tregs was markedly lower in the intestine in the HS+PC61 mAb group compared with the rat IgG group at 72 h after HS (1.9 ± 0.2 *vs.* 6.3 ± 0.6, $p = 0.0002$, $n = 5$). **c** Histological damage in the intestine at 72 h after heat stress in the PC61 mAb and rat IgG groups ($n = 5$–7). **d** Chiu scores in the intestines treated with the PC61 mAb are much higher compared with rat IgG group at 72 h after HS onset (4.55 ± 0.06 *vs.* 4.9 ± 0.07, $p = 0.0106$, $n = 5$). **e** FD4 levels detected *via* the *ex vivo* everted gut sac model were much higher in the PC61 mAb group compared with rat IgG group (9.8 ± 0.5 *vs.* 12.8 ± 1.2, $p = 0.0011$, $n = 5$). Increased levels of serum DAO (0.625 ± 0.04 *vs.* 0.91 ± 0.1, $p = 0.0005$, $n = 5$; **f**), D-lactic acid (0.45 ± 0.04 *vs.* 0.59 ± 0.06, $p = 0.0025$, $n = 5$; **g**), and endotoxin levels (300.4 ± 26.7 *vs.* 467.4 ± 35.9, $p < 0.001$, $n = 5$; **h**) were observed in the PC61 mAb group compared with rat IgG group. **i** The tight junction (TJ) proteins occludin and ZO-1were detected *via* western blotting in the PC61 mAb and rat IgG groups ($n = 5$). The data are expressed as the mean ± SD; Student's *t* test was used to compare differences in means of Chiu scores, FD4 levels, serum DAO, D-lactate, and LPS between dependent groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

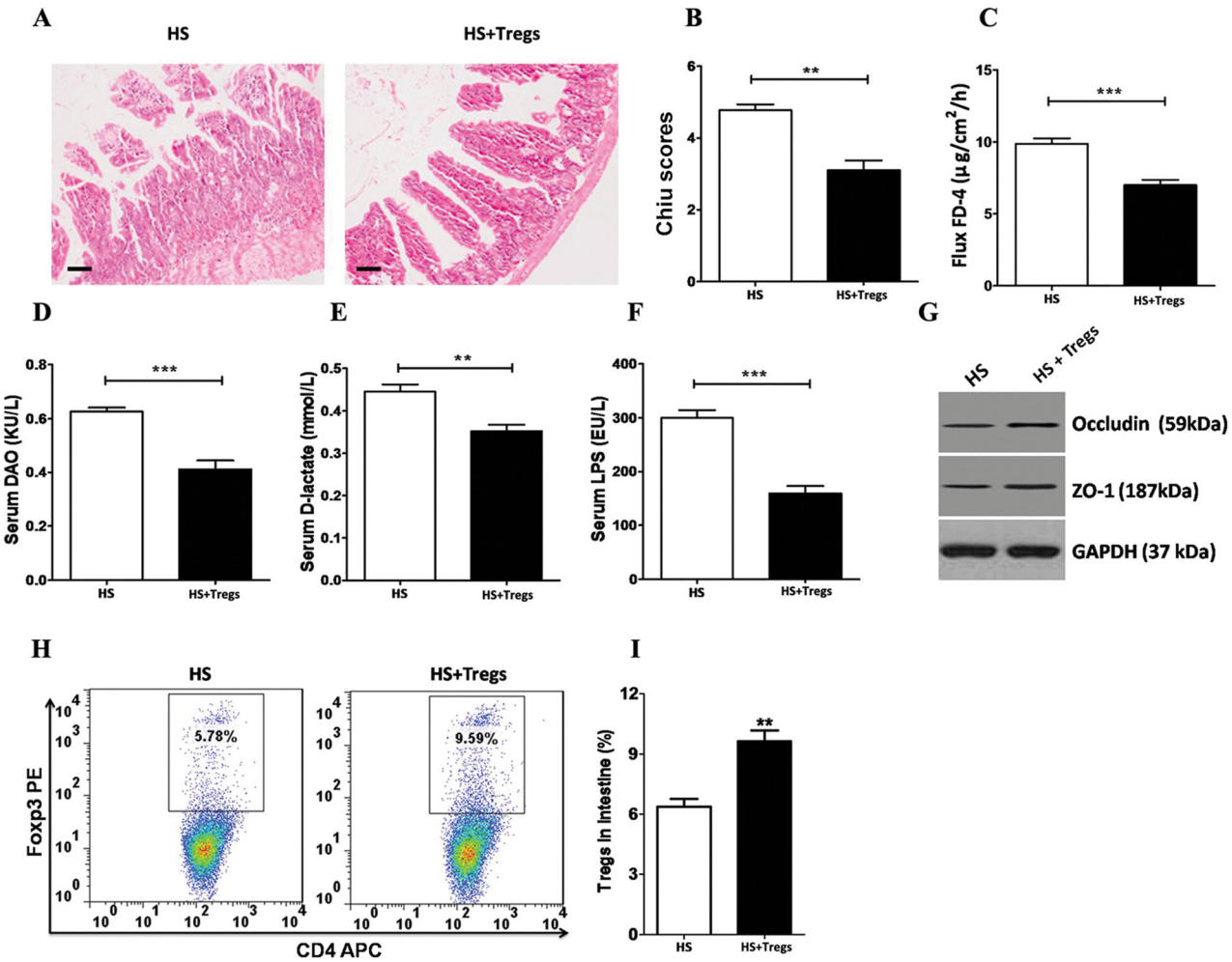

Fig. 4. Adoptive transfer of Tregs improved intestinal barrier function at 72 h after HS onset. **a** Histological damage in the intestine after heat stress at 72 h in the HS and HS+Tregs groups ($n = 5$–7 per group). **b** Chiu scores in the intestines treated with or without Tregs at 72 h after HS onset (4.76 ± 0.15 *vs.* 3.10 ± 0.27, $p = 0.0017$, $n = 5$). **c** FD4 levels detected *via* the *ex vivo* everted gut sac model were much lower in the HS+Tregs group compared with the HS group (9.88 ± 0.87 *vs.* 7.0 ± 0.84, $p = 0.0007$, $n = 5$). Serum DAO (0.63 ± 0.03 *vs.* 0.41 ± 0.07, $p = 0.0003$, $n = 5$; **d**), D-lactic acid (0.44 ± 0.04 *vs.* 0.35 ± 0.03, $p = 0.004$, $n = 5$; **e**), and endotoxin (300.0 ± 32.33 *vs.* 159.5 ± 30.27, $p = 0.0001$, $n = 5$; **f**) levels detected by ELISA kits were significantly lower in the HS+Tregs compared with HS groups. **g** The tight junction (TJ) proteins occludin and ZO-1 were detected *via* western blotting analysis in the HS+Tregs and HS groups ($n = 5$). **h**, **i** Flow cytometry showing that the proportion of $CD4^+Foxp3^+$ Tregs in the intestine was significantly higher in the HS+Tregs group at 72 h than in the HS group (6.38 ± 0.37 *vs.* 9.63 ± 0.55, $p = 0.008$, $n = 5$). The data are expressed as the mean ± SD; Student's *t* test was used to compare differences in means of Chiu scores, FD4 levels, serum DAO, D-lactate, and LPS between dependent groups. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

proteins or non-specific binding to other membrane molecules. Then, we utilized a complementary approach to determine whether Tregs played a beneficial role in HS-induced intestinal injury and barrier dysfunction. We transferred $CD4^+CD25^+$ Tregs into mice *via* the tail vein immediately after HS. Treg transfer not only affected intestinal histology and the Treg frequency at 72 h but also correlated with reduced serum LPS, DAO, and D-lactate levels and permeability to FD4. Moreover, the upregulation of TJ proteins was observed at 72 h. Collectively, these data demonstrated that Tregs could protect against HS-induced intestinal injury and barrier dysfunction.

How do Tregs participate in HS-induced intestinal injury and barrier dysfunction? In our study, intestine samples were harvested 0 h, 24 h, and 72 h after the Tc reached 42.7 °C, and the histopathological analysis confirmed that the lesions and barrier dysfunction were further aggravated in the absence of continuous heat stress, which was consistent with a former study [27]. These data indicated that

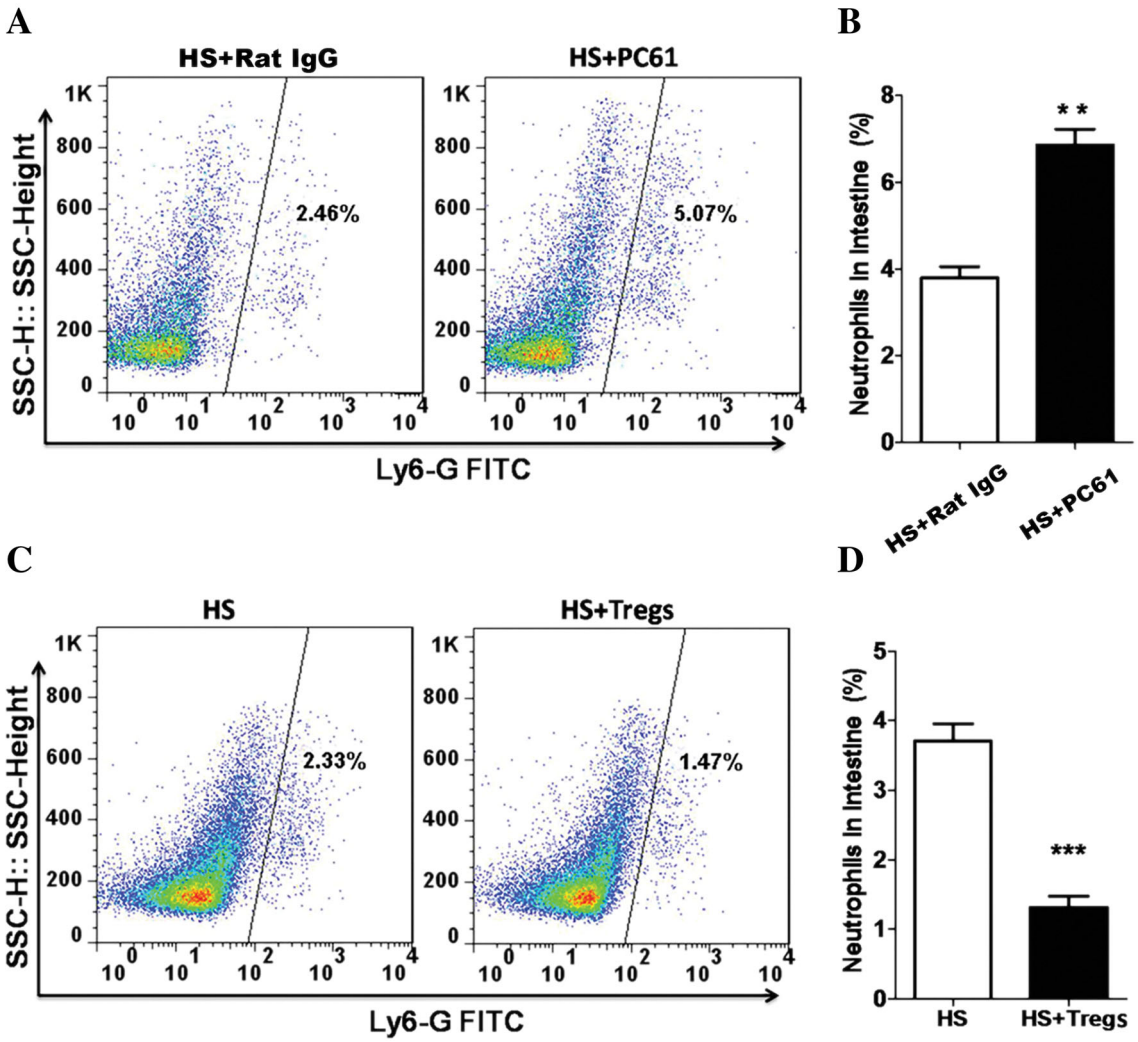

Fig. 5. Treg manipulation was associated with a change in the neutrophil frequency in the intestine. Flow cytometry showing that the proportion of neutrophils (**a**, **b**) in the intestine was significantly higher in the HS+PC61 mAb group at 72 h than in the HS+Rat IgG group (3.78 ± 0.57 *vs.* 6.86 ± 0.81, $p = 0.001$, $n = 5$), whereas the proportion of neutrophils (**c**, **d**) in the intestine was markedly lower in the HS+Tregs group at 72 h than in the HS group (3.71 ± 0.54 *vs.* 1.31 ± 0.36, $p < 0.001$, $n = 5$). The data are expressed as the mean ± SD; Student's *t* test was used to compare differences in means of frequencies of neutrophils in the intestine between dependent groups. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

there must be potential mechanisms that can induce small intestine injury and barrier dysfunction other than the direct thermal effect. Accumulating evidence has indicated that inflammatory pathogenesis may contribute to this phenomenon. Serum levels of IL-1β, IL-10, and IL-12p40 were significantly correlated with intestinal injury scores [27]. However, the neutrophil, which is proved to be a key factor in intestinal inflammation [33], is rarely detected in HS. In our study, we found a significant increase in the frequency of neutrophils in the intestinal mucosa, which might be the potential targets of HS-induced intestinal injury and barrier dysfunction.

Tregs have been shown to maintain gut homeostasis by preventing inappropriate innate (such as neutrophils) immune responses to commensal bacteria in inflammatory bowel disease [16, 20]. Neutrophil depletion or treatment with an inhibitor of neutrophil activation could ameliorate experimental colitis [35]. Thus, we further clarified whether Tregs could orchestrate neutrophil infiltration in our experiment. Manipulation of Tregs affected the neutrophil frequency in the intestine, suggesting that these cells were potential targets of Tregs in our model.

In summary, our findings reveal a novel role for Tregs as protector of HS-induced intestinal barrier function after

HS. As there is no specific therapy available to attenuate intestinal barrier dysfunction during HS, these data highlight the exciting potential to harness Tregs for the future therapeutic strategy.

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COMPLIANCE WITH ETHICAL STANDARDS

All animal procedures were approved by the Institutional Animal Care and Use Committee of the Chinese PLA General Hospital and Military Medical College.

Conflict of Interest. The authors declare that they have no conflicts of interest.

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