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Selective Inhibition of Histone Deacetylase-6 Alters the Composition of Circulating Blood Cells in a Lethal Septic Model

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Abstract

Background—Phagocytes, especially monocytes, macrophages and dendritic cells, play a pivotal role in the innate as well as adaptive immune responses during sepsis. We have shown that inhibition of histone deacetylase (HDAC)-6 improves survival and increases bacterial clearance in a mouse model of cecal ligation and puncture (CLP). The aim of this study was to determine whether this effect was associated with changes in the number and composition of different blood cell types in the circulation.

Methods—C57BL/6J mice were subjected to CLP, and 1 hour later given an intraperitoneal injecton of either Tubastatin A dissolved in dimethyl sulfoxide (DMSO), or DMSO only. Sham-operated animals were treated in an identical fashion but not subjected to CLP. Forty-eight hours later peripheral blood was obtained via cardiac puncture and analyzed using a veterinary Hematrue hematology analyzer.

Results—Tubastatin A administration increased the number of circulating monocytes in the sham-operated as well as the CLP animals. In comparison to the sham, CLP animals displayed an increase in the granulocyte percentage in white blood cells, decrease in the lymphocyte number and percentage, with a resultant increase in the granulocyte to lymphocyte ratio. Treatment of CLP animals with Tubastatin A decreased the granulocyte percentage, and restored the lymphocyte number and percentage, which decreased the granulocyte to lymphocyte ratio. In the sham animals, Tubastatin A increased red blood cell (RBC) number, hematocrit and hemoglobin. This effect was not seen in CLP animals.

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Authors Contributions:
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Conclusions—Tubastatin A treatment has significant impact on the composition of circulating blood cells. It increases the number of circulating monocytes and the RBC cell mass in sham-operated animals. In the CLP animals, it increases the monocyte count, decreases the percentage of granulocytes, restores the lymphocyte population, and decreases the granulocyte to lymphocyte ratio. These results may explain why Tubastatin A treatment improves survival in the septic models.

INTRODUCTION

Severe sepsis causes tremendous burden for health-care systems, with 750,000 new cases and more than 225,000 deaths annually in the United States [1–3]. Severe sepsis and septic shock are among the most elusive syndromes in medicine for which all clinical trials have so far failed to show efficacy [4, 5].

Histone acetylation is an essential epigenetic mechanism that determines the amplitude of cellular and subcelluar signaling, by controlling the chromatin structure, accessibility of transcription factors to the DNA, and the subsequent gene transcription. This process regulated by the opposing actions of two families of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). Histone acetylation relaxes the chromatin structure and promotes gene transcription, whereas histone deacetylation compacts the chromatin structure favoring gene silencing.

The 18 HDAC enzymes are grouped into four classes in humans and mice. Classical HDACs (class I, II and IV) are Zn^{2+} dependent, while the classes III sirtuins act through a NAD^{+}-dependent mechanism [6]. HDAC6 belongs to class IIb HDAC based on domain organization, and is unique among the classical HDAC family in that it is a cytoplasmic microtubule-associated enzyme. HDAC6 deacetylates tubulin, Hsp90 and cortactin, forms complexes with other partner proteins, and involves in a variety of biological processes [7].

Our lab was the first to demonstrate that administration of suberoylanilide hydroxamic acid (SAHA), a histone deacetylase inhibitor (HDACI), improved survival in lethal rodent models of lipopolysaccharide (LPS)-induced endotoxemia and cecal ligation and puncture (CLP)-induced severe sepsis [8–10]. Selective inhibition of HDAC6 with Tubastatin A displays even better survival outcomes in the lethal CLP-sepsis model and increases bacterial clearance in circulation (unpublished data). However, the mechanisms underlying the increased survival outcomes and bacteria clearance after Tubastatin A treatment remain unclear. The current study was therefore designed to determine whether these effects are associated with changes in the number and composition of different blood cell types in the circulation.

MATERIALS AND METHODS

Sepsis Model: Cecal Ligation and Puncture (CLP)

Male C57BL/6J mice (18–26 gm) were purchased from The Jackson Laboratory and housed for 3 days before manipulations. The CLP murine model [11], modified by our laboratory, was used to induce fecal peritonitis. In brief, the peritoneal cavity was opened under inhaled

isoflurane anesthesia. Cecum was eviscerated, ligated below the ileocecal valve using a 5-0 suture, and punctured through and through (2 holes) with a 20 gauge needle. The punctured cecum was squeezed to expel a small amount of fecal material and returned to the peritoneal cavity. The abdominal incision was closed in two layers with 4-0 silk suture. Animals were resuscitated by subcutaneous injection of 1 mL of saline. Sham-operated animals were handled in the same manner, except that the cecum was not ligated or punctured. This protocol was approved by the Animal Review Committee at the Massachusetts General Hospital. All surgery was performed under anesthesia, and all efforts were made to minimize suffering.

Administration of Tubastatin A and Experimental Design

Animals were randomly assigned to the following four groups (n = 5 for sham control, and 12/group for CLP): (a) Sham-operated animals (Sham Control); (b) Sham-operated animals injected with Tubastatin A (Sham + Tub.A); (c) Dimethyl sulfoxide (DMSO) vehicle treated animals after CLP (CLP Control), and (d) Tubastatin A treated animals after CLP (CLP + Tub.A). Mice received intra-peritoneal Tubastatin A dissolved in DMSO (70 mg/kg) or vehicle DMSO 1 h post-procedure. Sham-operated animals were subjected to laparotomy and intestinal manipulation, but the cecum was neither ligated nor punctured.

Peripheral Blood Analysis

Peripheral blood was obtained by via cardiac puncture 48 h post-procedure using a 1 mL heparinized syringe. Three hundred μl aliquots were then sampled and analyzed within 10 minutes of collection using a veterinary Hematrue hematology bench top analyzer (Heska Corporation, Loveland, CO) [12]. The number and percentage of monocytes, granulocytes and lymphocytes in white blood cell, the number of red blood cells (RBCs) and platelets, hematocrit, and hemoglobin were measured in all animals.

Statistical Analysis

Results were represented as mean ± SEM. Differences between 4 groups were assessed using one way analysis of variance (ANOVA) followed by Bonferroni post hoc testing for multiple comparisons. Student's t-test was used to compare the differences between two groups. All analyses were performed using GraphPad Prism. *P* values of 0.05 or less were considered significant.

RESULTS

Tubastatin A increases circulating monocyte number in sham-operated and CLP animals 48 h post-procedure

HDAC6 inhibitor Tubastatin A increased circulating monocyte number in sham-operated (0.3±0.1 versus $0.6\pm0.1\times10^9$/L, $P = 0.0184$; Figure 1) and CLP animals (0.5±0.1 versus $0.9\pm0.1\times10^9$/L, $P = 0.0076$; Figure 1) 48 h post-procedure. The percent of monocytes in white blood cells was not significantly altered after the inhibition of HDAC6, either in sham-operated (5.4±0.7% versus 6.9±0.4%) or CLP animals (7.6±0.3% versus 8.4±0.5%).

Tubastatin A decreases the granulocyte percentage in blood during severe sepsis

The granulocyte percentage in white blood cells increased 48 h after CLP (15.8±4.4% versus 51.7±2.9%, $P < 0.0001$; Figure 2), which was dramatically decreased after Tubastatin A treatment (51.7±2.9% versus 41.0±2.9%, $P = 0.0224$; Figure 2). In addition, Tubastatin A was not shown to affect the granulocyte percentage in sham-operated animals (15.8±4.4% versus 21.4±4.0%), and the number of granulocytes was not significantly affected by the inhibition of HDAC6 in sham-operated (0.9±0.3 versus 2.0±0.3) and CLP animals (3.1±0.6 versus 4.1±0.5).

Tubastatin A restores the lymphocyte number and percentage in blood during severe sepsis

The number and percentage of lymphocytes decreased 48 h after CLP (lymphocyte number: 5.1±0.5 versus $2.5±0.5×10^9$/L, $P = 0.0051$; lymphocyte percentage: 78.8±5.0% versus 40.7±2.9%, $P < 0.0001$; Figure 3), which were significantly restored by Tubastatin A treatment (lymphocyte number: 2.5±0.5 versus $4.9±0.5×10^9$/L, $P = 0.0039$; lymphocyte percentage: 40.7±2.9% versus 50.6±3.1%, $P = 0.0342$; Figure 3). Tubastatin A did not alter lymphocyte number (5.1±0.5 versus 5.0±0.4) and percentage (78.8±5.0% versus 68.1±3.2%) in sham-operated animals.

Tubastatin A decreases the granulocyte-lymphocyte ratio in a lethal septic model

The granulocyte-lymphocyte ratio increased 48 h after CLP (0.3±0.1 versus 1.4±0.2, $P = 0.0002$; Figure 4), and Tubastatin A attenuated the ratio markedly 1.4±0.2 versus 0.8±0.1, $P = 0.0286$; Figure 4). In sham-operated animals, the granulocyte-lymphocyte ratio was not affected by Tubastatin A treatment (0.3±0.1 versus 0.4±0.1).

Tubastatin A increases RBC number, hematocrit and hemoglobin in sham-operated animals

Tubastatin A increased RBC number (8.5±0.2 versus $8.9±0.1×10^{12}$/L, $P = 0.0192$), hematocrit (36.7±0.7% versus 38.4±0.3% survival, $P = 0.014$) and hemoglobin (12.2±0.2 versus 12.8±0.1 g/dL, $P = 0.0078$) in sham-operated animals, but not in CLP animals (Figure 5 and 6).

Platelet number was not significantly affected by Tubastatin A treatment

Tubastatin A treatment did not affect platelet number significantly in sham-operated (61.0±14.7 versus $55.5±3.4×10^9$/L) and CLP animals (70.5±5.8 versus $84.2±10.9×10^9$/L, Figure 7).

DISCUSSION

We have investigated the effects of HDAC6 inhibitor Tubastatin A on the number and composition of different blood cell types in circulation utilizing a lethal CLP septic model, and discovered some important findings: (1) HDAC6 inhibitor Tubastatin A increases circulating monocyte number in both sham-operated and CLP animals; (2) Tubastatin A decreased the granulocyte percentage, and restored the lymphocyte number and percentage,

which decreased the granulocyte to lymphocyte ratio during severe sepsis; (3) Tubastatin A increases RBC number, hematocrit and hemoglobin in sham-operated animals.

Gene expression is turned on or off by transcriptional regulation and changes in chromatin structure, which are modulated by numerous posttranslational modifications at the amino-terminal tails of nucleosomal histones, such as acetylation/deacetylation, methylation, phosphorylation, and adenosine diphosphate (ADP) ribosylation. The acetylation status of chromatin is balanced by the activities of HATs and HDACs, which can activate or suppress the expression of specific genes [13–15].

HDAC6 is a unique HDAC localized in the cytoplasm, where it associates with non-histone substrates, including heat shock protein 90 (HSP90), α-tubulin and cortactin. It has an ubiquitin binding domain and two catalytic domains [16, 17]. HDAC6 overexpression leads to tubulin deacetylation and increased cell motility [18, 19], whereas specific inhibition of HDAC6 activity or its downregulation by siRNA increases acetylation of α-tubulin and HSP90, reducing cellular motility and inducing degradation of HSP90 client proteins, such as Bcr-Abl, Raf-1, Akt, HER2/Neu, interleukin-1 receptor associated kinase 1 (IRAK1), and hypoxia-inducible factor (HIF)-1α [20–22].

HDAC6 has become a target for drug development to treat cancer, including acute myeloid leukemia, acute lymphocytic leukemia, multiple myeloma, and breast cancer [23–27]. Cardiac HDAC6 catalytic activity is shown to increase in response to chronic hypertension, highlighting the need to determine whether HDAC6 inhibitors are protective in the setting of cardiovascular disease [28]. Meanwhile, inhibition of HDAC6 promotes survival and regeneration of neurons, and has therapeutic potential to ameliorate injury of central nervous system [29]. Notably, Tubastatin A, a selective HDAC6 inhibitor with a drug-like structure, simple synthesis and superior target selectivity, was generated in 2010 [30]. Our laboratory demonstrated that selective inhibition of HDAC6 with Tubastatin A improves long-term survival outcomes significantly in a lethal rodent model of CLP-sepsis, and increases bacteria clearance in circulation (unpublished data).

We discovered that pharmacological inhibition of HDAC6 by Tubastatin A increases circulating monocyte number remarkably in both sham-operated and CLP animals, which may explain the increased bacteria clearance and improved survival outcomes after Tubastatin A treatment in the lethal CLP-sepsis model. Monocytes and macrophages are critical effector cells contributing to the altered innate immune responses against infection, as the most efficient pathogen scavengers and the predominant source of inflammatory cytokines [31–33]. Progressing monocyte and macrophage dysfunction impairs host defenses to invading pathogens, and results in immune dysfunction during severe sepsis and septic shock [34–36]. Tubastatin A treatment replenishes circulating monocyte pool, which may potentially enhance host's overall phagocytosis ability towards foreign pathogens and increases bacteria clearance, rendering significantly better survival outcomes in the lethal CLP-sepsis model.

The relationship between HDAC6 and monocyte development currently remains elusive. It is possible that inhibition of HDAC6 increases the number of monocytes by modulating

epigenetic modifications and activating gene promoters of bone marrow mesenchymal stem cells, facilitating their differentiation into monocytes. Chromatin modifications were reported to be associated with transcriptional activity of monocytes. A genome-wide analysis of histone H3 trimethylation on lysine 4 (H3K4me3) and 27 (H3K27me3), and acetylation of H3 lysines (AcH3) in promoter regions indicated that H3K4me3 and AcH3 correlate with transcriptionally active genes significantly, whereas H3K27me3 is associated with inactive gene promoters [37]. Meanwhile, monocyte acetylated histone H4 levels were significantly elevated in complication-free diabetic subjects, but not in diabetic patients with complications, suggesting that acetylation of monocytes may be a protective mechanism [38].

In addition, we discovered that Tubastatin A decreased the granulocyte percentage, and restored the lymphocyte number and percentage, which decreased the granulocyte to lymphocyte ratio in the lethal CLP model. An elevated preoperative neutrophil-lymphocyte ratio was revealed to be a convenient biomarker to identify patients with a poor prognosis after resection for primary gastric cancer [39]. Preoperative neutrophil-lymphocyte ratio, in combination with CA125, may represent a simple and cost-effective method to identify ovarian cancers, and high neutrophil-lymphocyte ratio may predict adverse outcomes in ovarian cancer [40]. Moreover, critically ill patients with severe sepsis or septic shock had significantly high neutrophil percentage and marked low lymphocyte counts. And the severity of clinical course, according to the Sequential Organ Failure Assessment (SOFA) as well as Acute Physiology And Chronic Health Evaluation II (APACHE II) score, correlated with the divergence of neutrophil and lymphocyte percentages in the white blood picture [41]. Therefore, the decreased granulocyte to lymphocyte ratio after Tubastatin A treatment provides another explanation for the increased survival outcomes in the lethal septic model.

Anti-inflammatory host response during sepsis induces cell apoptosis of the innate and adaptive immune system, and these apoptotic cells result in immunosuppressive effect on surviving immune cells [42–44]. Autopsies of patients with sepsis revealed extensive apoptosis of lymphocytes and gastrointestinal epithelial cells [45], which were similar to animal studies [46, 47]. Our results demonstrated that Tubastatin A is protective against lymphocyte loss in the lethal septic model, resulting in better immune function in Tubastatin A-treated animals. Tubastatin A was also indicated to promote the activity Regulatory T cells (Tregs) in models of inflammation and autoimmunity, including experimental colitis and fully major histocompatibility complex (MHC)-incompatible cardiac allograft rejection, achieved by inhibition of the HDAC6-regulated protein HSP90 [48].

Another important finding is that inhibition of HDAC6 increases RBC number, hematocrit and hemoglobin in sham-operated animals. Histone modifications have also been implicated in erythrocyte differentiation. Valproic acid (VPA), a non-selective HDACI, was reported to sustains the expression of stemness-related markers in hematopoietic stem/progenitor cells and enhance erythrocyte and megakaryocyte differentiation, by up-regulating genes of growth factor–independent protein 1B (GFI1B) and mixed-lineage leukemia translocated to chromosome 3 protein (MLLT3), mediated by the histone hyperacetylation at their promoter sites [49]. HDACIs FK228 and VPA also enhances the potential of interleukin-3 to stimulate erythropoiesis and megakaryopoiesis [50, 51]. Our results showed that Tubastatin A does not affect platelet number significantly, which indicates that selective inhibition of HDAC6

might have different effect on hematopoietic stem/progenitor cells, compared to non-selective HDACIs.

It is not clear if Tubastatin A has any impact on humoral immunity. We have explored the effects of Tubastatin A on immune organs during severe sepsis (unpublished data), and noted that selective inhibition of HDAC6 was associated with a significant attenuation of thymic and bone marrow atrophy, and splenic apoptosis. These results suggest that Tubastatin A may affect both humoral and cell-mediated immunity.

This study has certain limitation to be acknowledged. It would have been ideal to have cell differential data beyond the 48 hrs time-point. However, the high lethality of the model resulted in very few survivors beyond 48 hrs in the CLP control group. Without the matching data from the control group, any changes in the treated animals would have been difficult to interpret. Even when the animals survived, they were often too sick to permit repeated blood draws safely. Additional molecular mechanisms underlying epigenetic modifications of blood cells need to be investigated. And whether Tubastatin A affects the number and composition of other immune cells, such as bone marrow cells and splenocytes, need to be explored.

CONCLUSION

We have demonstrated that Tubastatin A, a selective inhibitor of HDAC6, has significant impact on the composition of circulating blood cells in a lethal polymicrobial sepsis model. Tubastatin A increases the number of circulating monocytes and the RBC cell mass in sham-operated animals. In addition, it increases the monocyte count, decreases the percentage of granulocytes, restores the lymphocyte population, and decreases the granulocyte to lymphocyte ratio in CLP animals. These results may explain why Tubastatin A treatment improves survival and increases bacteria clearance in the septic models.

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ABBREVIATIONS

AcH3	acetylation of H3 lysines
ADP	adenosine diphosphate
APACHE II	Acute Physiology And Chronic Health Evaluation II
ANOVA	one way analysis of variance
CLP	cecal ligation and puncture
DMSO	dimethyl sulfoxide
GFI1B	growth factor–independent protein 1B
HATs	histone acetyltransferases
HDAC	histone deacetylase
HDACI	HDAC inhibitors
HIF	hypoxia-inducible factor
HSP	heat shock protein
H3K4me3	histone H3 trimethylation on lysine 4
IRAK1	interleukin-1 receptor associated kinase 1
LPS	lipopolysaccharide
MHC	major histocompatibility complex
MLLT3	mixed-lineage leukemia translocated to chromosome 3 protein
RBC	red blood cell
SAHA	suberoylanilide hydroxamic acid
SOFA	Sequential Organ Failure Assessment

Tregs	Regulatory T cells
VPA	Valproic acid

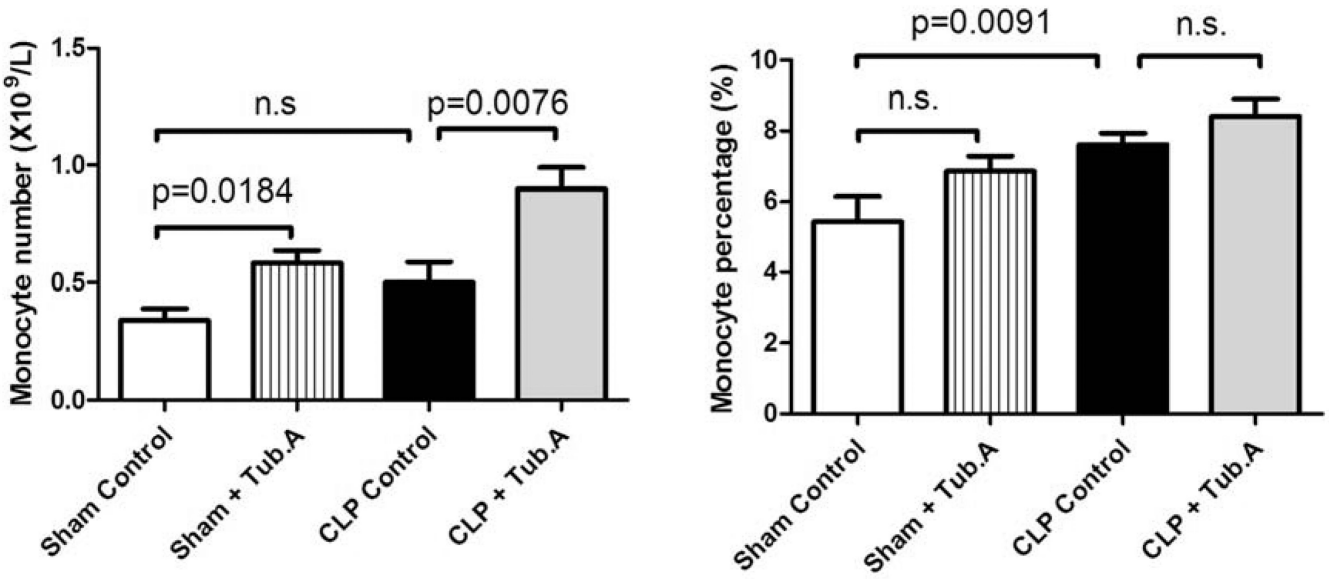

Figure 1. Tubastatin A increases circulating monocyte number in sham-operated and CLP animals 48 h post-procedure

Animals were randomly assigned to four groups as mentioned in Materials and Methods, and peripheral blood was obtained 48 h post-procedure. Three hundred μl aliquots were sampled and analyzed within 10 minutes of collection using a veterinary Hematrue hematology bench top analyzer. The number and percentage of monocytes in white blood cells were measured (means ± SEM, n = 5–12 mice/group). CLP: cecal ligation and puncture; Tub.A: tubastatin A.

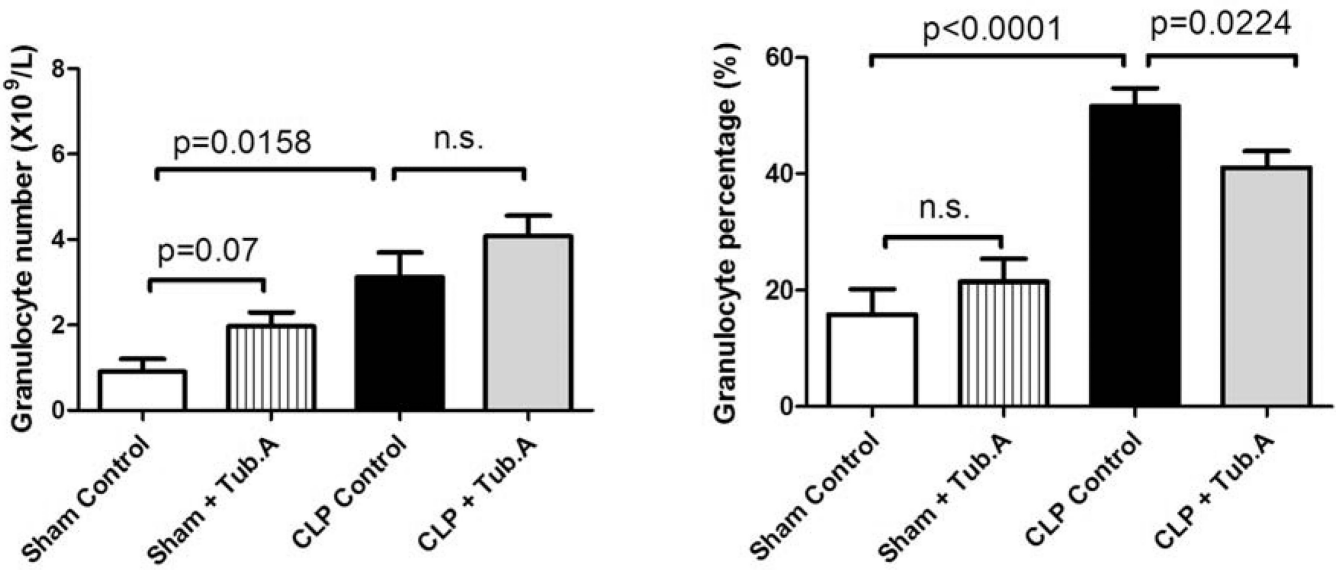

Figure 2. Tubastatin A decreases the granulocyte percentage in blood during severe sepsis

The number and percentage of granulocytes in white blood cells were determined 48 h post-procedure (means ± SEM, n = 5–12 mice/group).

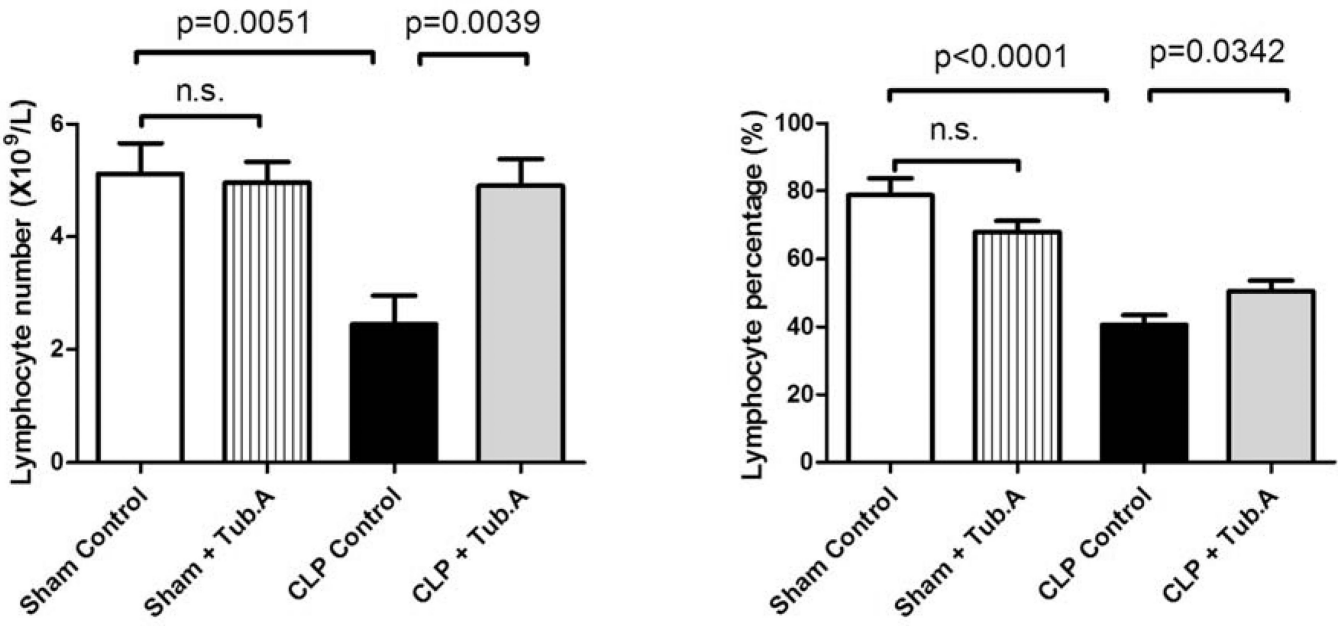

Figure 3. Tubastatin A restores the lymphocyte number and percentage in blood during severe sepsis

Lymphocyte number and percentage in white blood cells were measured 48 h post-procedure (means ± SEM, n = 5–12 mice/group).

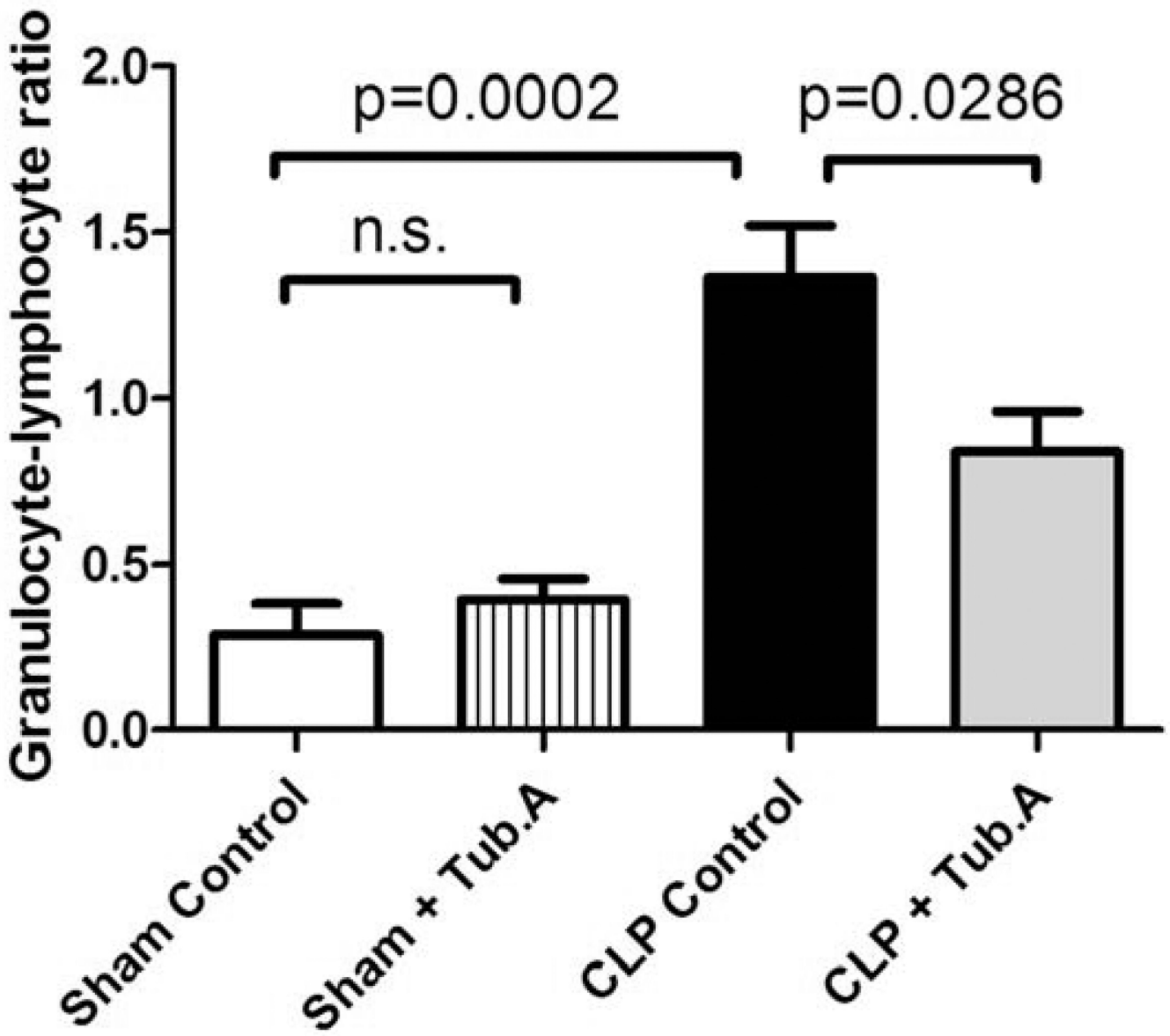

Figure 4. Tubastatin A decreases the granulocyte-lymphocyte ratio in a lethal septic model

The granulocyte-lymphocyte ratio was calculated by dividing the number of granulocytes by the number of lymphocyte 48 h post-procedure (means ± SEM, n = 5–12 mice/group).

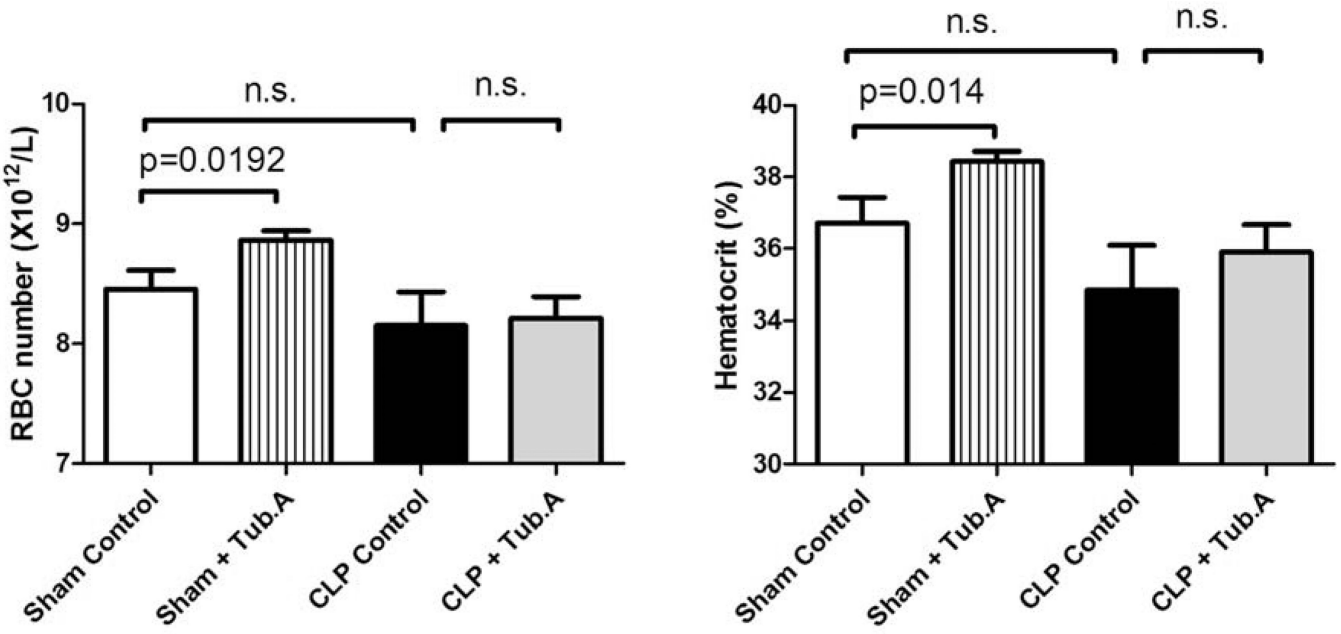

Figure 5. Tubastatin A increases RBC number and hematocrit in sham-operated animals

The RBC number and hematocrit were examined 48 h post-procedure (means ± SEM, n = 5–12 mice/group). RBC: red blood cell.

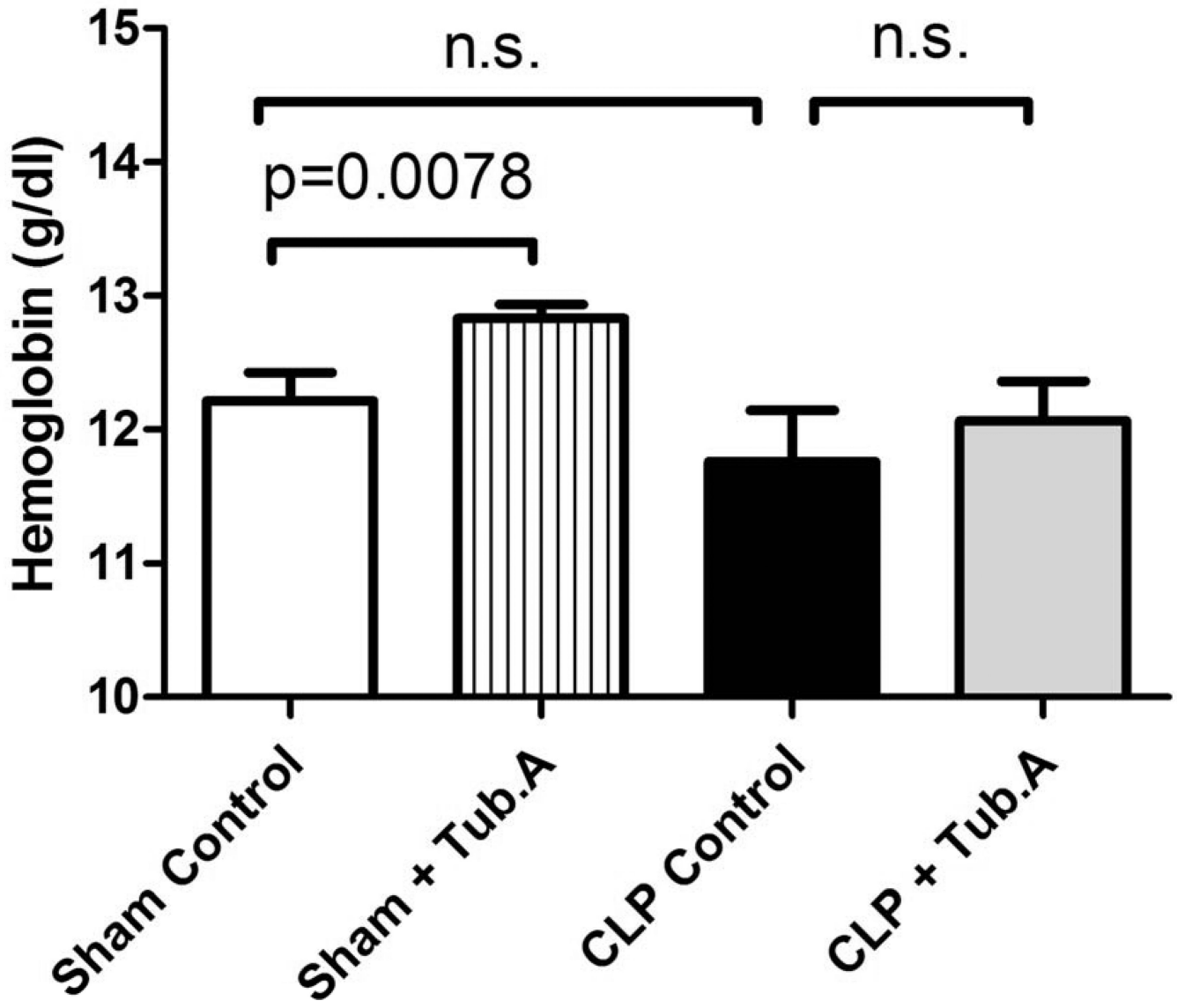

Figure 6. Tubastatin A increases hemoglobin in sham-operated animals

Hemoglobin was measured 48 h post-procedure (means ± SEM, n = 5–12 mice/group).

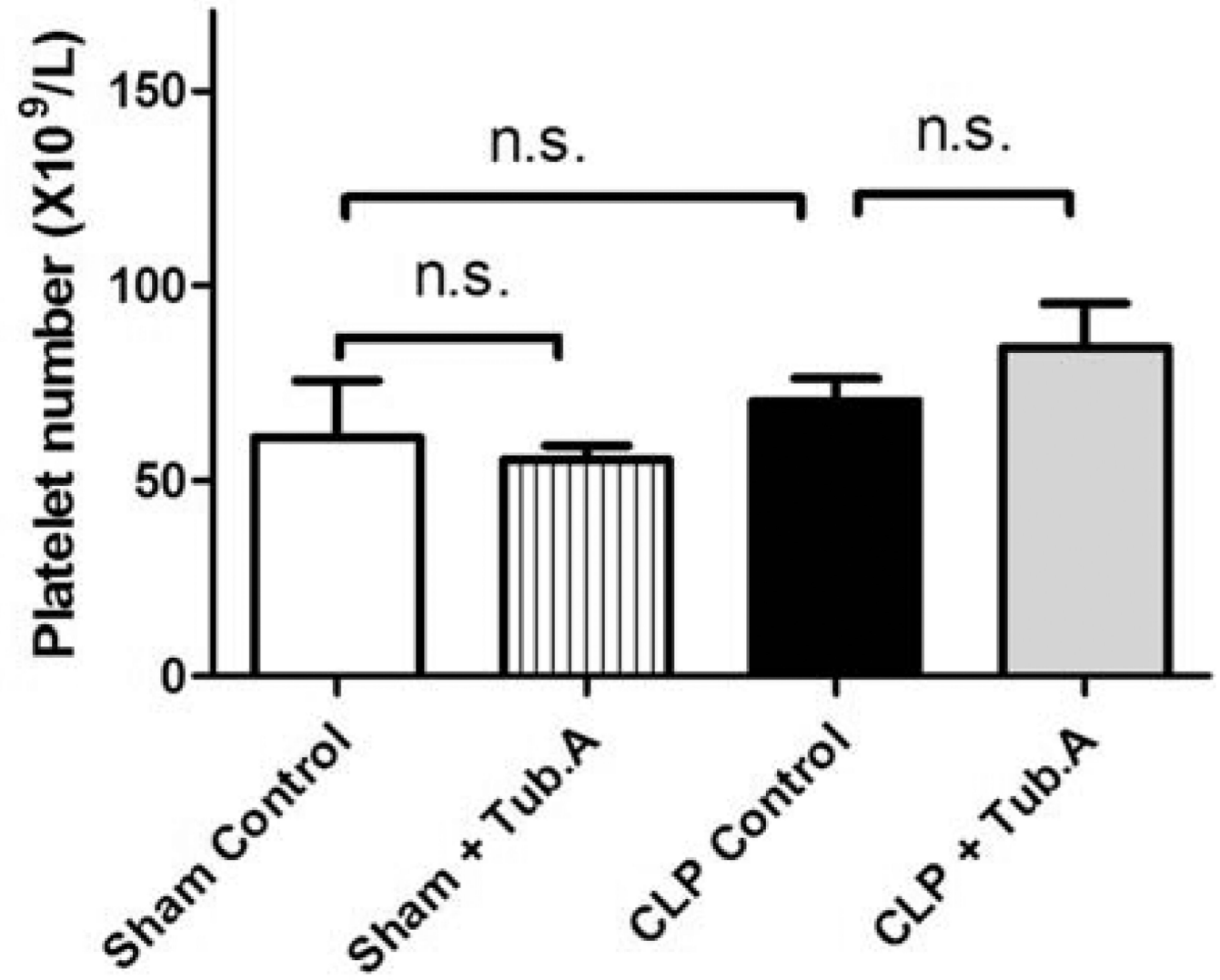

Figure 7. Tubastatin A does not affect platelet number significantly

The platelet number was measured 48 h post-procedure (means ± SEM, n = 5–12 mice/group).