Activating transcription factor 4 protects mice against sepsis-induced intestinal injury by regulating gut-resident macrophages differentiation

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Abstract

Background: Gut-resident macrophages (gMacs) supplemented by monocytes-to-gMacs differentiation play a critical role in maintaining intestinal homeostasis. Activating transcription factor 4 (ATF4) is involved in immune cell differentiation. We therefore set out to investigate the role of ATF4-regulated monocytes-to-gMacs differentiation in sepsis-induced intestinal injury.

Methods: Sepsis was induced in C57BL/6 wild type (WT) mice and *Atf4*-knockdown ($Atf4^{+/-}$) mice by cecal ligation and puncture or administration of lipopolysaccharide (LPS). Colon, peripheral blood mononuclear cells, sera, lung, liver, and mesenteric lymph nodes were collected for flow cytometry, hematoxylin and eosin staining, immunohistochemistry, quantitative reverse transcription polymerase chain reaction, and enzyme-linked immunosorbent assay, respectively.

Results: CD64, CD11b, Ly6C, major histocompatibility complex-II (MHC-II), CX3CR1, Ly6G, and SSC were identified as optimal primary markers for detecting the process of monocytes-to-gMacs differentiation in the colon of WT mice. Monocytes-to-gMacs differentiation was impaired in the colon during sepsis and was associated with decreased expression of ATF4 in P1 ($Ly6C^{hi}$ monocytes), the precursor cells of gMacs. *Atf4* knockdown exacerbated the impairment of monocytes-to-gMacs differentiation in response to LPS, resulting in a significant reduction of gMacs in the colon. Furthermore, compared with WT mice, $Atf4^{+/-}$ mice exhibited higher pathology scores, increased expression of inflammatory factor genes (*TNF-α, IL-1β*), suppressed expression of CD31 and vascular endothelial-cadherin in the colon, and increased translocation of intestinal bacteria to lymph nodes and lungs following exposure to LPS. However, the aggravation of sepsis-induced intestinal injury resulting from *Atf4* knockdown was not caused by the enhanced inflammatory effect of $Ly6C^{hi}$ monocytes and gMacs.

Conclusion: ATF4, as a novel regulator of monocytes-to-gMacs differentiation, plays a critical role in protecting mice against sepsis-induced intestinal injury, suggesting that ATF4 might be a potential therapeutic target for sepsis treatment.

Keywords: Activating transcription factor 4; Mice; Lipopolysaccharides; Monocytes; Leukocytes, mononuclear; Cell differentiation; Macrophages; Sepsis; Homeostasis; gMacs; Intestinal injury

Introduction

Sepsis is a syndrome caused by uncontrolled inflammation in response to infection, which could lead to life-threatening organ damage and dysfunction, and even be fatal.[1-3] Sepsis-induced intestinal injury promotes the translocation of intestinal bacteria and toxins,[4,5] which exacerbates the systemic inflammatory response and accelerates the pathological process of sepsis.[5] Liu *et al*[6] have shown that approximately half of all patients with sepsis develop gastrointestinal dysfunction, and the case fatality rate for patients with sepsis doubles when intestinal dysfunction occurs compared with that for

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patients without intestinal dysfunction.[7] Therefore, the prevention and treatment of sepsis-induced intestinal injury is beneficial for the recovery of patients with sepsis.

Gut-resident macrophages (gMacs) are a heterogeneous group of immune cells that express high levels of the chemokine, CX3C motif, receptor 1 (CX3CR1), with the colon containing the highest abundance of these cells.[8] The gMacs are instrumental in regulating the intestinal inflammatory immune response,[9] supporting submucosal vasculature,[10] and preventing intestinal bacterial translocation.[9,11] A deficiency or severe reduction of gMacs increases intestinal bacterial translocation and vascular leakage in mice.[10-12] Therefore, maintaining gMacs homeostasis is important for the prevention and control of sepsis-induced intestinal injury. Maintenance of a stable gMacs population is achieved *via* the self-proliferation of gMacs and the differentiation of blood-derived Ly6C^{hi} monocytes.[10,13,14] This differentiation process involves a series of stages corresponding to cell populations "P1" (monocytes newly recruited to the intestine), "P2" (mature monocytes), "P3"(immature macrophages), and "P4" (mature macrophages, namely gMacs),[13,15-17] and is regulated by transforming growth factor (TGF)-β signaling.[16]

There are limited reports on the impact of the differentiation of gMacs on immunopathological mechanisms of sepsis-induced intestinal injury owing to the lack of a uniform flow cytometric assay for gMacs. Studies have reported that mouse EGF-like module-containing mucin-like hormone receptor-like 1 (F4/80), CD64, and CD68 are unique markers on the surface of intestinal macrophages;[17-19] however, the most suitable marker for detecting monocytes-to-gMac differentiation is unclear, although there is consensus that lymphocyte antigen 6 complex, locus C (Ly6C), major histocompatibility complex (MHC)-II, and CX3CR1 are essential markers for studying the differentiation of gMacs.[16,18] In addition, some studies have proposed using lymphocyte antigen 6 complex, locus G (Ly6G), Sialic acid-binding immunoglobulin-like lectins-F (Siglec-F), CD11c, CD3e, B220, and killer cell lectin-like receptor subfamily B member 1C (NK1.1) as lineage (Lin) markers of gMacs to exclude interference from other immune cells,[13,15] while other studies have argued against this.[10] Thus, there is currently no consensus on the most suitable marker for detecting monocytes-to-gMacs differentiation.

ATF4 is a member of the basic region-leucine zipper transcription factor superfamily and is expressed in various tissues, including the intestine.[20-22] Hu *et al*[22] demonstrated that ATF4 expression in the intestinal tissues of patients with inflammatory bowel disease (IBD) was significantly lower than that in healthy controls, and that ATF4 affects the development and progression of intestinal inflammation. ATF4 is a key transcription factor in the process through which macrophage colony-stimulating factor regulates the differentiation of bone marrow monocyte/macrophage precursor cells into osteoclasts—a type of specialized macrophage in bone tissue.[20] However, it is not known whether ATF4 regulates the differentiation, proliferation, and effector function of gMacs during sepsis-induced intestine injury. In this study, the cecal ligation and puncture (CLP) model or lipopolysaccharide (LPS) administration was used to induce sepsis in C57BL/6 mice and *Atf4*-knockdown (*Atf4*$^{+/-}$) mice. Subsequently, optimal primary markers for detecting the process of monocytes-to-gMacs differentiation were identified. ATF4 was proven to be a novel regulator of monocytes-to-gMacs differentiation, contributing to the protective effects on sepsis-induced intestinal injury.

Methods

Animals and experimental procedure

Animal experimentation was conducted under a protocol approved by the Ethics Committee of Ruijin Hospital Affiliated to Shanghai Jiao Tong University (Shanghai, China), and followed the National Research Council's Guide for the Care and Use of Laboratory Animals. Male C57BL/6 wild-type (WT) mice and *Atf4* knockdown (*Atf4*$^{+/-}$) mice (8–10-week-old, body weight 20–30 g; Shanghai Model Organisms Center, Inc., Shanghai, China; SCXK No. [Shanghai] 2017–0010; SYXK No. [Shanghai] 2017–0012) were used in the study. Animals were housed in plastic cages under standardized conditions (light-dark cycle 12/12 h, temperature 22 ± 2°C, humidity 55 ± 15%). To generate a model of sepsis, WT or *Atf4*$^{+/-}$ mice were treated with either LPS (*Escherichia coli* O111:B4; Sigma-Aldrich, MO, USA), or CLP and sacrificed 24 h later. The LPS model of sepsis was established *via* intra-peritoneal injection (*i.p.*) of LPS at 5 mg/kg body weight, dissolved in 0.1 mL/10 g body weight of 0.9% NaCl (normal saline, NS), as described previously.[23] Control mice received an *i.p.* injection of an equivalent volume of NS. CLP was performed as previously described.[24] Detailed methods are included in the supplementary material and methods, [http://links.lww.com/CM9/B384]. Colon, peripheral blood mononuclear cells (PBMCs), sera, lung, liver, and mesenteric lymph nodes were collected for flow cytometry, hematoxylin and eosin (H&E) staining, immunohistochemistry, quantitative real-time polymerase chain reaction (qRT-PCR), and enzyme-linked immunosorbent assay (ELISA), respectively.

Isolation of PBMCs

Approximately 0.5 mL blood from the internal canthal vein was collected in heparin following orbital enucleation. Erythrocytes in the samples were lysed by adding 3 mL red blood cell lysis buffer (Sangon Biotech, Shanghai, China) for 5 min at room temperature (approximately 25°C). Cells were then washed once and resuspended in fluorescence activated cell sorting (FACS) buffer for flow cytometric analysis.

Isolation of intestinal lamina propria (LP) cells

Colonic LP cells were isolated by following a previously established method, with slight modifications.[25] The detailed method is shown in the supplementary material and methods [http://links.lww.com/CM9/B384].

Flow cytometry

Dead cells were separated from colonic LP cells or PBMCs using the LIVE/DEAD™ Fixable Near-IR Dead Cell Stain kit (Invitrogen, CA, USA) by staining for 20 min and washing in poly butylene succinate (PBS). Cells were then incubated with anti-CD16/CD32 (1:200) for 20 min at 20 to 25°C to block non-specific binding of antibodies to Fcγ receptors. Next, cells were incubated with surface antibodies at recommended dilutions for 30 min at 4°C. To evaluate proliferation, LP cells were then permeabilized with the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, CA, USA) and subjected to intracellular staining with Ki67 monoclonal antibodys (mAbs) (1:100) for 45 min at 4°C. To detect intracellular cytokines, LP cells were incubated with Brefeldin A solution for 3 h to block intracellular protein transport, then after surface staining and permeabilization, cells were stained with intracellular antibodies (interleukin [IL]-10, IL-1β; 1:50–100) for 45 min at 4°C.

Cells were analyzed with a BD FACSAria III flow cytometer (BD, NJ, USA) and data were analyzed using FlowJo software 10.5.3 (Tree Star Inc., OR, USA). The gMacs were gated as indicated in the results or [supplementary figures section, http://links.lww.com/CM9/B384]. The main antibodies and reagents used for flow cytometry are listed in [Supplementary Table 1, http://links.lww.com/CM9/B384].

Histopathology analysis

Histopathology analysis was performed as described previously.[26] Briefly, samples of the colon (~0.5 cm long) near the ileocecal area were collected and flushed with cold NS to remove any fecal content. The tissues then underwent routine histological processing and were embedded in paraffin, sectioned, and stained with H&E. To determine the severity of colitis, H&E-stained sections were evaluated by a pathologist in a blinded fashion using a histological scoring system, as previously described,[26] with the slight modification of adding colonic histological scores for epithelial damage and mononuclear cell infiltration, resulting in a total scoring range of 0 to 8 [Supplementary Table 2, http://links.lww.com/CM9/B384].

Immunohistochemistry

Prepared colon sections were treated according to a standard immunohistochemistry protocol. Anti-CD31 antibody (1:100, Cell Signaling Technology, DC, USA) and anti-vascular endothelial (VE)-cadherin antibody (1:100, Signalway Antibody, CA, USA) were incubated at 4°C overnight. The samples were then incubated with horseradish peroxidase-conjugated secondary antibodies at 37°C for 45 min and developed with 3,3-diaminobenzidine at room temperature in the dark, then washed with PBS. Finally, the nuclei of all sections were counterstained using hematoxylin. Slides were visualized under a digital pathology slice scanner (KF-PRO-120, KFBIO, Yuyao, China) and images were acquired using K-Viewer software (KFBIO). ImageJ software (National Institutes of Health, MD, USA) selected the color of CD31 and VE-cadherin expression on the photo. The average optical density of CD31 and VE-cadherin were calculated, respectively.

Quantitation of gene expression by real-time reverse transcription PCR

Total RNA was extracted from the lung, liver, and colon with TRIzol® Reagent (Invitrogen), according to the manufacturer's instructions, and reversely transcribed using random primer and M-MLV Reverse Transcriptase (Takara, Tokyo, Japan). qRT-PCR was performed with SYBR Green I Master Mix reagent (ThermoFisher, USA) on an ABI 7500 system (Applied Biosystem, MA, USA). Primers (Sangon Biotech, Shanghai, China) used in this study are listed in [Supplementary Table 3, http://links.lww.com/CM9/B384]. Gene expression levels were normalized to that of glyceraldehyde-3-phosphate dehydrogenase. The mean relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, as previously described.[22]

Diamine oxidase (DAO) assay

Serum was separated from blood samples by centrifuging at 3000 r/min for 10 min at 4°C and was stored at −80°C. The serum DAO levels in mice were detected using a DAO enzyme-linked immunosorbent assay kit (CUSABIO, Wuhan, China) according to the manufacturer's instructions. Samples were divided into WT-NS, WT-LPS, *Atf4*$^{+/-}$-NS, and *Atf4*$^{+/-}$-LPS groups. Each sample was tested in duplicate, and a blank control was included, which did not have samples and enzyme-labeled reagents.

Statistical analysis

Statistical analyses were performed using Prism 8 (GraphPad Software, CA, USA). Data are represented as the mean ± standard error. Significant differences were assessed by two-tailed Student *t* test or Mann-Whitney *U* test. Differences were considered statistically significant at $P < 0.05$.

Results

Optimization and establishment of a flow cytometric assay for detecting monocytes-to-gMacs differentiation in mice

An optimized flow cytometric assay is a prerequisite for detecting monocytes-to-gMacs differentiation in mice. Therefore, the expression of cell-surface markers (Ly6C, MHC-II, CX3CR1, Ly6G, Siglec-F, CD11c, CD3e, B220, NK1.1) was analyzed in F4/80$^+$CD11b$^+$, CD64$^+$CD11b$^+$, and CD68$^+$CD11b$^+$ cells from the colon of WT mice [Supplementary Figure 1A and 2A, http://links.lww.com/CM9/B384]. As shown in the Figure 1, the expression of Ly6C was highest in CD64$^+$CD11b$^+$ cells, and the intensity of MHC-II and CX3CR1 expression in CD64$^+$CD11b$^+$ and CD68$^+$CD11b$^+$ cells was significantly higher than that in F4/80$^+$CD11b$^+$ cells. However, the cell-surface expression of markers, such as CD11c, CD3e, B220, and NK1.1, was similar and consistent among the three cell types, with low to moderate levels [Supplementary Figure 1A, http://links.lww.com/CM9/B384]. The eosinophil marker Siglec-F was

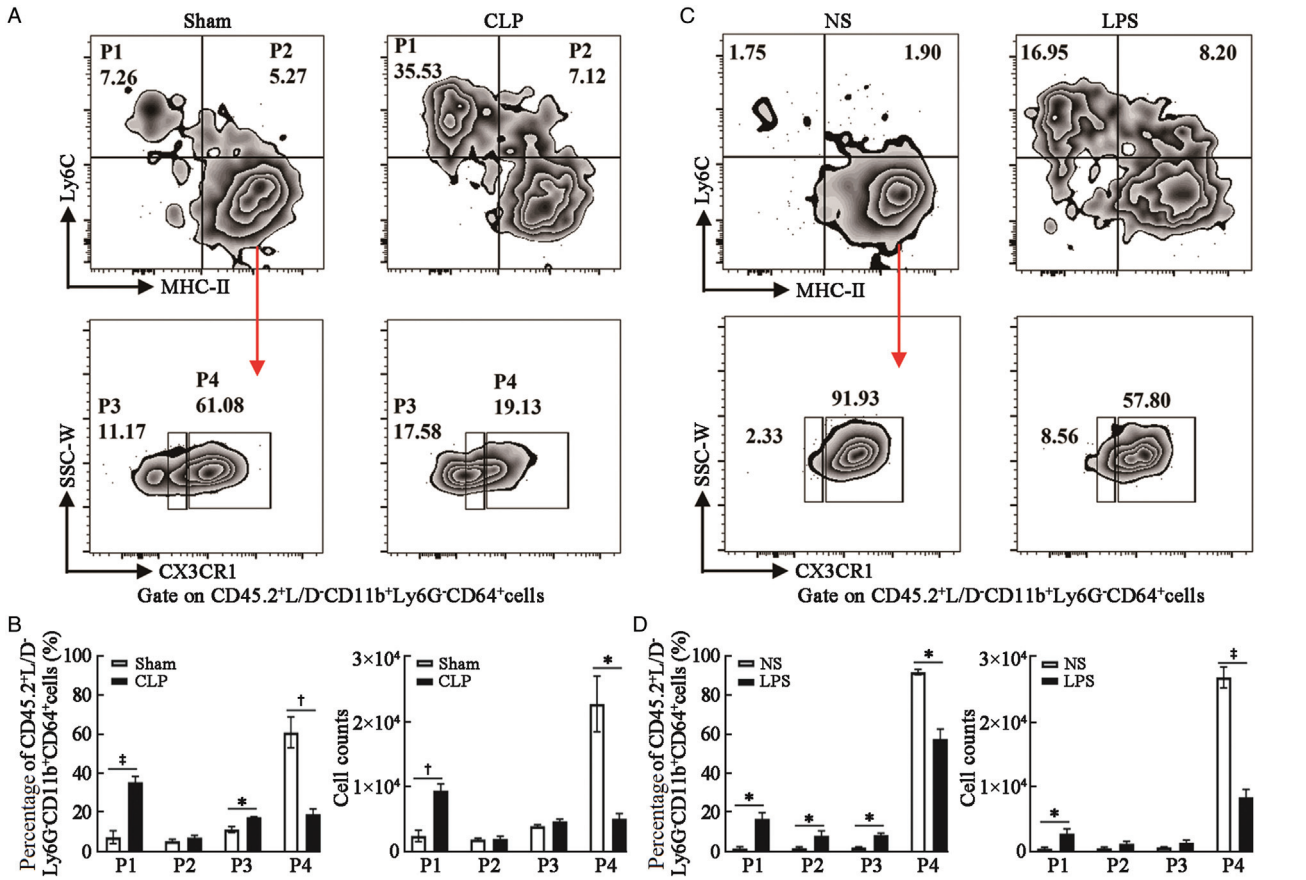

Figure 1: Monocytes-to-gMacs differentiation is impaired in septic mice. (A) Representative flow cytometry plots; (B) the relative abundance of P1 to P4 cells from the CLP of WT mice without (Sham) or with (CLP) sepsis induced by CLP; (C) Representative flow cytometry plots; (D) the relative abundance of P1 to P4 cells in CLP of WT mice without (NS) or with (LPS) sepsis induced by LPS. Values on the flow cytometry plots represent the mean of each group ($n = 4$–5 per group). Data are presented as means ± standard error. $^{*}P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$.CLP: Cecal ligation and puncture; LPS: Lipopolysaccharide; gMacs: Gut-resident macrophages; NS: Normal saline; WT: Wild type.

highly expressed in F4/80^{+}CD11b^{+} cells, while the neutrophil marker Ly6G was highly expressed in CD64^{+}CD11b^{+} cells [Supplementary Figure 1A, http://links.lww.com/CM9/B384]. Furthermore, Siglec-F^{+}CD11b^{+} cells were mostly SSC^{hi}, while CD64^{+}CD11b^{+} cells were mostly SSC^{lo} [Supplementary Figure 1B, http://links.lww.com/CM9/B384]. Therefore, CD64, CD11b, Ly6C, MHC-II, and CX3CR1 constitute an optimal group of primary markers for detecting monocytes-to-gMacs differentiation, without the need for CD11c, CD3e, B220, and NK1.1 as Lin markers to exclude interference from dendritic cells (DCs), T and B cells, and natural killer (NK) cells. However, Ly6G is needed to exclude interference from neutrophils, and side scatter (SSC) parameters are needed to exclude interference from eosinophils [Supplementary Figure 2B, http://links.lww.com/CM9/B384]. Therefore, in subsequent experiments, Live (LIVE/DEAD reagent^{-} [L/D^{-}]) CD45.2^{+}CD11b^{+}Ly6G^{-}CD64^{+}Ly6C^{hi}MHC-II^{-} cells were defined as "P1", Live CD45.2^{+}CD11b^{+}Ly6G^{-}CD64^{+}Ly6C^{+} MHC-II^{+} cells as "P2", Live CD45.2^{+}CD11b^{+}Ly6G^{-}CD64^{+}Ly6C^{-}MHC-II^{+}CX3CR1^{int} cells as "P3", and Live CD45.2^{+}CD11b^{+}Ly6G^{-}CD64^{+}Ly6C^{-}MHC-II^{+}CX3CR1^{hi} as "P4", which are also gMacs [Supplementary Figure 1C, http://links.lww.com/CM9/B384]. The gating strategy of gMacs is shown in [Supplementary Figure 2B, http://links.lww.com/CM9/B384].

Monocytes-to-gMacs differentiation is impaired in septic mice

To understand the impact of sepsis on each stage of cell differentiation (P1–P4), the numbers of P1, P2, P3, and P4 cells (the value represents the average proportion of L/D^{-}CD45.2^{+}CD11b^{+}Ly6G^{-}CD64^{+} cells, and the same thereafter, unless otherwise specified) in the colon of WT mice were analyzed using flow cytometry 24 h after septic model initiation. In both models of sepsis (CLP and LPS), the number of P1 cells in the colon of septic mice increased sharply after 24 h compared with their control mice. However, the number of P4 cells decreased sharply in both models, with P2 and P3 cells exhibiting an increasing trend [Figure 1]. These data indicate that sepsis leads to a decrease in P4 cells and an accumulation of P1 cells in the colon, thus monocytes-to-gMacs differentiation is impaired.

ATF4 is involved in monocytes-to-gMacs differentiation in septic mice

To determine whether ATF4 is associated with impaired monocytes-to-gMacs differentiation in sepsis, the relative abundance and the level of ATF4 protein of P1, P2, P3, and P4 cells in the colon of WT mice were analyzed by flow cytometry at different time points before (0 h) and after (2, 12, and 24 h) LPS treatment. The abundance of P1, P2,

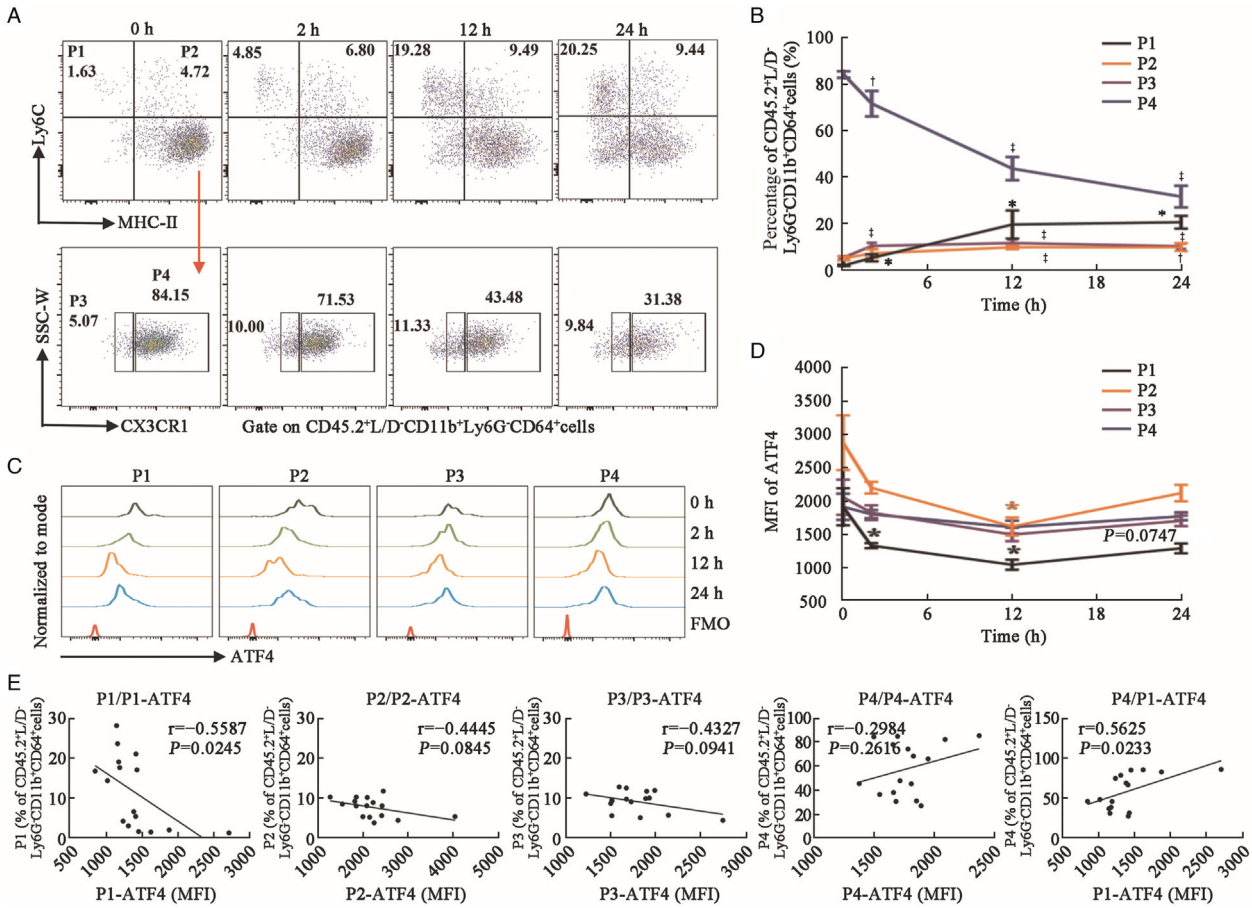

Figure 2: ATF4 is involved in monocytes-to-gMacs differentiation during sepsis. Cells were prepared from the CLP of WT mice at before (0 h), 2, 12, and 24 h after LPS administration. (A) Representative flow cytometry plots; (B) the relative abundance of P1–P4 cells at the indicated time; (C) Histogram of ATF4 expression; (D) Mean fluorescent intensity (MFI) of ATF4 in P1–P4 cells at the indicated time; (E) Pearson correlation analysis for MFI of ATF4 and relative abundance of P1–P4 cells. Values on the flow cytometry plots represent the mean of each group ($n = 4$ at each time point). Data are presented as means ± standard error. $^{*}P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$. ATF4: Activating transcription factor 4; CLP: Cecal ligation and puncture; LPS: Lipopolysaccharide; gMacs: Gut-resident macrophages; WT: Wild type.

and P3 in the untreated WT mice was relatively low (all < 6.00%) while that of P4 cells was relatively high (84.15%). At 2 h after LPS treatment, P1 and P3 cells in WT mice increased significantly, while P2 cells increased slightly, and P4 cells decreased significantly. At 12 h post-LPS, P1 cells increased further, P2 and P3 cells tended to be stable, while the abundance of P4 cells decreased further. At 24 h after LPS treatment, P1, P2, and P3 cells remained stable, while the abundance of P4 cells decreased to less than 50% of the baseline level [Figure 2A, B]. ATF4 expression before LPS challenge was highest in P2 cells, while the expression among P1, P3, and P4 cells was similar. After the LPS challenge, compared with 0 h, the expression of ATF4 in all cells decreased at all subsequent time points, especially at 12 h. The expression of ATF4 in P1 cells decreased significantly at 2 h and 12 h, and almost at 24 h, compared with 0 h. In contrast, the change in ATF4 expression in P4 cells was relatively small. [Figure 2C and 2D]. Pearson correlation analysis showed that the relative abundance of P2 and P3 cells, and especially P1 cells, was negatively correlated with the intracellular level of ATF4, while in P4 cells, there was a positive correlation. Furthermore, ATF4 protein levels in P1 cells were significantly positively correlated with the relative abundance of P4 cells [Figure 2E]. These findings suggest that the impaired monocytes-to-gMacs differentiation is associated with decreased ATF4 protein expression of P1 cells in the colon of mice.

Atf4 knockdown deteriorates LPS-induced monocytes-to-gMacs differentiation dysfunction

Atf4 knockdown (*Atf4*$^{+/-}$) mice were generated to clarify the role of ATF4 in the impairment of monocytes-to-gMacs differentiation in sepsis. Compared with WT mice, ATF4 protein expression decreased by approximately 50% in the colonic live CD45.2^{+}, P1, P2, P3, and P4 cells of *Atf4*$^{+/-}$ mice [Supplementary Figure 3A and 3B, http://links.lww.com/CM9/B384], confirming that *Atf4* was successfully knocked down. The *Atf4*$^{+/-}$ mice presented a higher number of P1 cells and a lower number of P4 cells in the colon compared with WT mice. At 24 h after LPS treatment, the number of P1 cells was higher and the number of P4 cells was lower in *Atf4*$^{+/-}$ septic mice compared with WT septic mice [Figure 3A and 3B]. Ki67 is a recognized cell proliferation marker. *Atf4* knockdown led to the enhancement of the proliferation ability of P1 cells but weakened that of P4 cells in both *Atf4*$^{+/-}$ and WT mice [Figure 3C and 3D]. L/D is a dye used to label dead cells in flow cytometry. *Atf4* knockdown significantly

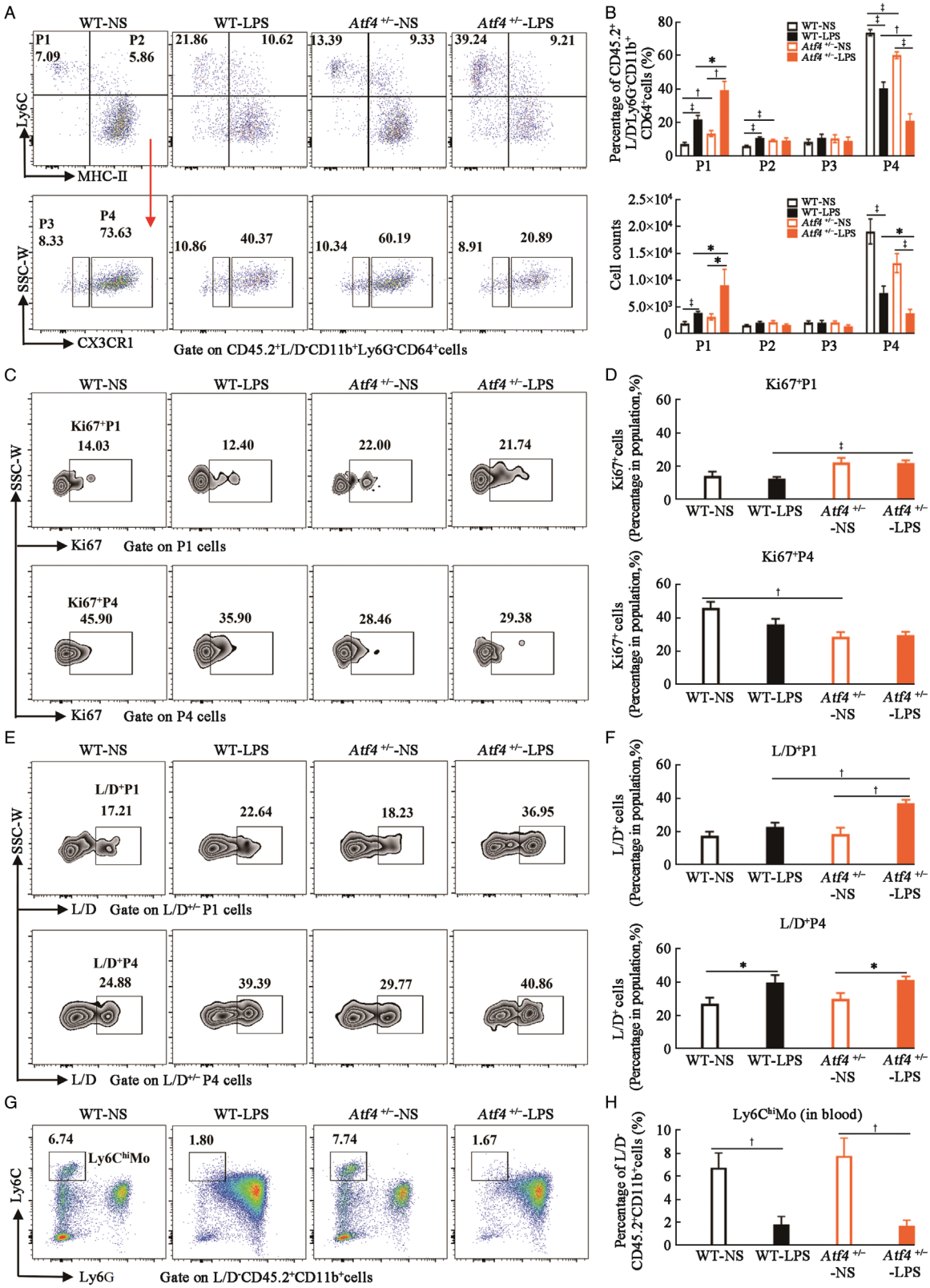

Figure 3: *Atf4* knockdown further deteriorates the impairment of monocytes-to-gMacs differentiation in response to LPS. Cells were prepared from the CLP of WT (black) and *Atf4*$^{+/-}$ (orange) mice without or with sepsis induced by LPS. (A) Representative flow cytometry plots; (B) The relative abundance (top) and the absolute quantity (bottom) of P1–P4 cells; (C) Representative flow cytometry plots of Ki67 expression in P1 (top) and P4 (bottom) cells; (D) The relative abundance of Ki67$^+$ cells in P1 (top) and P4 (bottom) cells; (E) Representative flow cytometry plots of L/D expression in P1 (top) and P4 (bottom) cells; (F) The relative abundance of L/D$^+$ cells in P1 (top) and P4 (bottom) cells. Values on the flow cytometry plots represent the mean of each group. (G) Representative flow cytometry plots of the Ly6ChiMo and (H) The relative abundance of Ly6C^{hi}Mo in L/D$^-$CD45.2$^+$CD11b$^+$cells from blood. (A, B: $n = 7$; C, D: $n = 5$; E, F: $n = 7$; G,H: $n = 7$–8). Data are presented as means ± standard error. $^*P < 0.05$, $^†P < 0.01$, $^‡P < 0.001$. CX3CR1: chemokine, CX3C motif, receptor 1; LPS: Lipopolysaccharide; Ly6G: Lymphocyte antigen 6 complex, locus G; MHC-II: Major histocompatibility complex II; Mo: Monocytes; NS: Normal saline; gMacs: Gut-resident macrophages; WT: Wild type.

increased the ratio of L/D$^+$P1 (the proportion of dead cells in P1) in septic mice, but did not cause a significant change in the ratio of L/D$^+$P4 (the proportion of dead cells in P4) relative to that in the WT mice, neither under normal conditions nor under treatment with LPS [Figure 3E, F]. In addition, *Atf4* knockdown had no significant effect on the LPS-induced reduction in Ly6C^{hi} monocytes of peripheral blood [Figure 3G, 3H and Supplementary Figure 3C, http://links.lww.com/CM9/B384]. Thus, *Atf4* knockdown deteriorates LPS-induced monocytes-to-gMacs differentiation dysfunction, and the exacerbating effect of *Atf4* knockdown on the sepsis-induced impairment of monocytes-to-gMacs differentiation is the predominant factor driving the reduction in P4 cells (gMacs).

Atf4 knockdown did not enhance the inflammatory effector function of Ly6C$^+$ monocytes and gMacs in septic mice

Next, the effect of ATF4 on the function of P1, P2, P3, and P4 cells was explored. Under steady-state conditions in WT mice, only 5.13% of P4 cells expressed IL-10 and 23.45% of P4 cells expressed IL-1β, but 24.10% of P1 cells and 39.98% of P2 cells expressed IL-10, and more than 30.00% of P1 cells and P2 cells expressed IL-1β [Figure 4A–4D]. Furthermore, P1 cells hardly expressed MHC-II, while P4 cells expressed the highest level of MHC-II [Figure 4E and 4F], indicating that P1 cells and P2 cells (Ly6C$^+$ monocytes) are the main inflammatory effector cells, and P4 cells (gMacs) are the predominant immune surveillance cells. *Atf4* knockdown significantly reduced IL-10 expression and slightly increased IL-1β expression of P1 and P2 cells, and significantly reduced MHC-II expression of P2 and P4 cells [Figure 4A–4F]. Following LPS treatment in WT mice, there was a significant decrease in IL-10 expression of P1 cells and an almost significant reduction in that of P2 cells, with a significant decrease in MHC-II expression of P4 cells [Figure 4A, 4B, 4E, 4F]. However, LPS induction of sepsis in *Atf4* knockdown mice did not further enhance IL-1β expression in P1 cells and P2 cells. On the contrary, it led to a decreasing trend in IL-1β expression of P1, P2, P3, and P4 cells and in MHC-II expression of P4 cells [Figure 4C–4F]. This indicates that *Atf4* knockdown did not enhance the inflammatory effector function of Ly6C$^+$ monocytes and gMacs in septic mice.

Atf4 knockdown aggravates sepsis-induced intestinal injury

Since *Atf4* knockdown affects the numbers and functions of the colonic immune cells, the role of ATF4 in sepsis-induced intestinal injury was subsequently explored. At 24 h after LPS treatment, *Atf4*$^{+/-}$ and WT mice presented with colonic epithelial cell atrophy and inflammatory cell infiltration [Figure 5A]. Compared with WT septic mice, *Atf4*$^{+/-}$ septic mice exhibited higher intestinal pathology scores [Figure 5B], significantly higher expression of inflammatory genes (*TNF-α, IL-1β*) [Figure 5C], and a higher trend in the serum levels of DAO (a marker of intestinal barrier damage) [Figure 5D]. The *Atf4*$^{+/-}$ septic mice also had significantly lower expression of CD31 (a marker of microvascular density) [Figure 5E and 5F], and lower expression of VE-cadherin (a marker of vascular integrity[10]) [Supplementary Figure 4A and 4B, http://links.lww.com/CM9/B384], and increased translocation of intestinal bacteria to extra-intestinal sites (lymph nodes, lungs) compared with the WT septic mice[Figure 5G and 5H]. These observations suggest that *Atf4* knockdown aggravates sepsis-induced intestinal injury.

Discussion

In this study, CD64, CD11b, Ly6C, MHC-II, CX3CR1, Ly6G, and SSC were identified as optimal primary markers for detecting the process of monocytes-to-gMacs differentiation in the colon of mice by flow cytometry. By utilizing this optimized assay, impaired monocytes-to-gMacs differentiation was revealed during sepsis and was associated with decreased ATF4 protein expression of P1 cells in the colon of mice. Moreover, *Atf4* knockdown promoted the aggregation of Ly6C^{hi} monocytes (P1 cells) and significantly reduced gMacs (P4 cells) in the colon of septic mice, owing to more serious impairment of monocytes-to-gMacs differentiation. Furthermore, *Atf4*$^{+/-}$ mice displayed severe intestinal injury, higher expression of inflammatory factors and lower expression of CD31 and VE-cadherin in the colon, and increased translocation of intestinal bacteria to extra-intestinal sites in response to LPS. Moreover, the aggravation of sepsis-induced intestinal injury by *Atf4* knockdown is not caused by the enhanced inflammatory effect of Ly6C$^+$ monocytes (P1 cells and P2 cells) and gMacs.

The flow cytometric strategies adopted in previous studies on monocytes-to-gMacs differentiation have generally been based on CX3CR1-green fluorescent protein-reporter mice.[16,17,27] In this study, a flow cytometric array that was suitable for detecting monocytes-to-gMacs differentiation in WT mice was optimized. CD64, CD11b, Ly6C, MHC-II, and CX3CR1 were identified as the optimal group of primary markers for detecting monocytes-to-gMacs differentiation. Ly6G is needed to exclude interference from neutrophils and SSC parameters are utilized to exclude interference from eosinophils, but CD11c, CD3e, B220, and NK1.1 as Lin markers were not required to exclude interference from DCs, T, B, and NK cells. This strategy reduces the cost of experimental procedures and avoids excluding gMacs that have low expression of CD11c and CD3e, thus providing a reliable technical solution for detecting monocytes-to-gMacs differentiation in WT mice.

Accumulating evidence has indicated that monocytes-to-gMacs differentiation is impaired in experimental models of colitis.[17,18,28] In our study, both the CLP model and the LPS-induced sepsis model proved that impaired monocytes-to-gMacs differentiation contributed to a reduction in P4 cells and an accumulation of P1 cells in the intestine during sepsis. Following a rapid decrease in P4 cells upon induction of sepsis, circulating Ly6C^{hi} monocytes (P1) are recruited to the colon as early as 2 h after LPS treatment. Thus, impaired monocytes-to-gMacs differentiation is likely to be involved in the early pathological stage of sepsis-induced intestinal injury. To clarify the reasons underlying the reduction in gMacs in sepsis, the proliferation, death, and origin of gMacs were analyzed. Using *Atf4*$^{+/-}$ mice, it was confirmed that *Atf4*

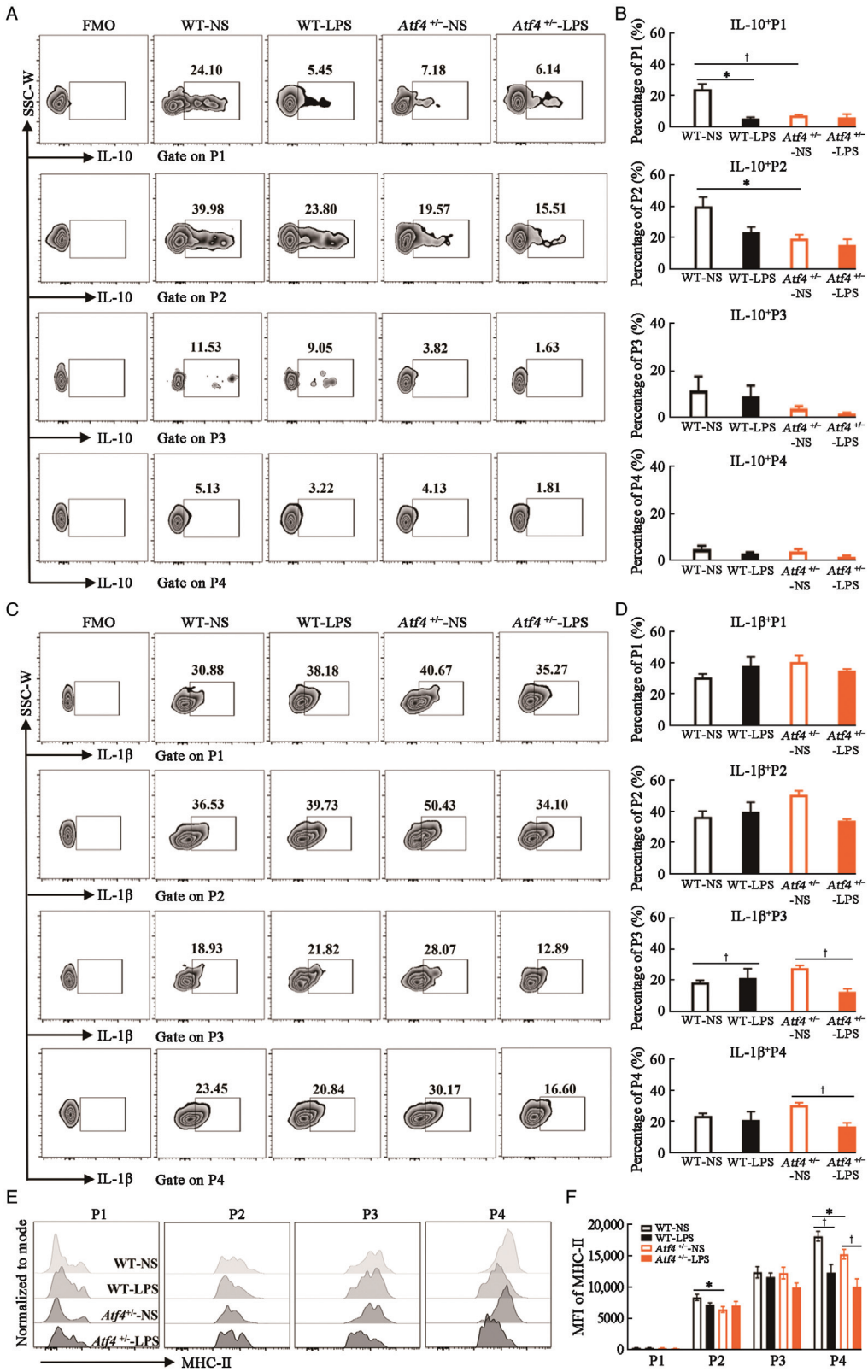

Figure 4: *Atf4* knockdown did not enhance the inflammatory effector function of Ly6C$^+$ monocytes and gMacs in septic mice. Cells were prepared from the CLP of WT (black) and *Atf4*$^{+/-}$ (orange) mice without or with sepsis induced by LPS. Representative flow cytometry plots of (A) expression of IL-10 and (B) the proportion of IL-10$^+$ cells in P1–P4 cells. Representative flow cytometry plots of (C) expression of IL-1β and (D) the proportion of IL-1β$^+$cells in P1–P4 cells. Histogram of (E) MHC-II expression and (F) the MFI of MHC-II in P1–P4 cells. Values on the flow cytometry plots represent the mean of each group (A–D: $n = 3$–4; E, F: $n = 7$ per group). Data are presented as means ± standard error. $^*P < 0.05$, $^\dagger P < 0.01$, $^\ddagger P < 0.001$. FMO: Fluorescence minus one; IL: Interleukin; gMacs: Gut-resident macrophages; LPS: Lipopolysaccharide; MFI: Mean fluorescent intensity; MHC-II: Major histocompatibility complex II; NS: Normal saline; SSC: Side scatter; WT: Wild type.

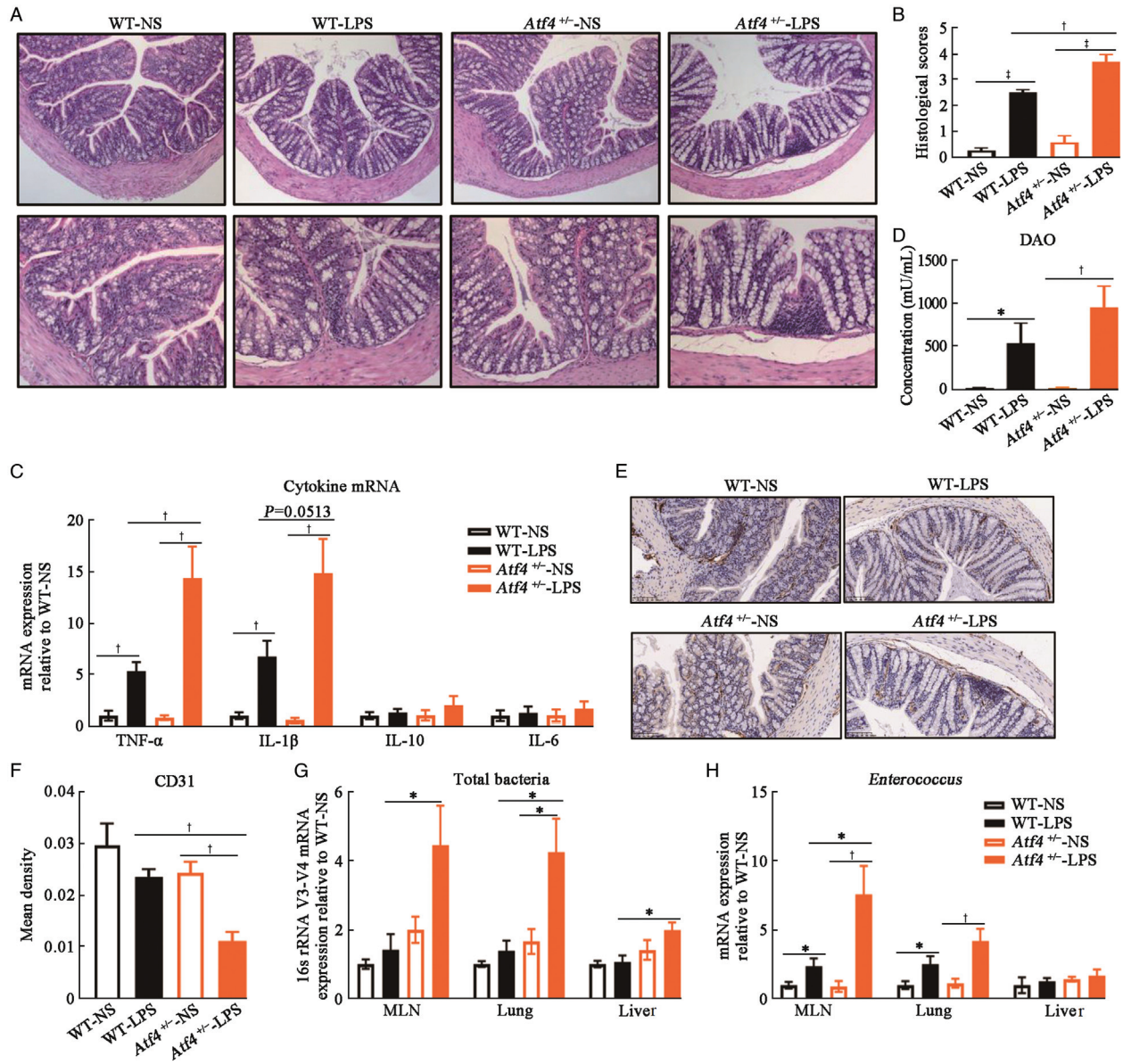

Figure 5: *Atf4* knockdown aggravates LPS-induced intestinal injury. Colon, sera, MLN, lung, and liver were prepared from WT (black) and *Atf4*$^{+/-}$ (orange) mice without or with sepsis induced by LPS. (A) H&E staining of colonic tissue (top, Original magnification × 100; bottom, Original magnification × 200) and (B) histology score. (C) Relative expression of indicated cytokine genes of colonic tissue was analyzed by real-time PCR. (D) Sera levels of DAO determined by ELISA. (E) CD31 staining of colon by immunohistochemistry (Original magnification × 200) and (F) AOD statistics. (G, H) Real-time PCR analysis of bacterial translocation in MLN, lung, and liver (G: Total bacteria, H: *Enterococcus*). For A, B: $n = 4$; C: $n = 5$–6; D: $n = 4$–5; E, F: $n = 3$; G, H: $n = 5$ to 6 per group. Data are presented as means ± standard error. $^{*}P < 0.05$, $^{\dagger}P < 0.01$, $^{\ddagger}P < 0.001$. AOD: Average optical density; DAO: Diamine oxidase; ELISA: Enzyme-linked immunosorbent assay; H&E: Hematoxylin and eosin; IL: Interleukin; LPS: Lipopolysaccharide; MLN: mesenteric lymph nodes; PCR: Polymerase chain reaction; NS: Normal saline; rRNA: Ribosomal RNA; WT: Wild type.

knockdown led to a further reduction in gMacs, primarily by exacerbating the impaired monocytes-to-gMacs differentiation induced by sepsis, rather than by promoting the death of gMacs or decreasing their proliferative capacity, or by affecting the abundance of Ly6C^{hi} monocytes in peripheral blood. Consistently, Bain *et al*[18] also showed that reduced levels of gMacs under experimental colitis were partly due to impaired monocytes-to-gMacs differentiation.

Hu *et al*[22] reported an important role for ATF4 in the development and progression of IBD and confirmed through basic research that intestinal epithelial cell-specific deletion of *Atf4* induces spontaneous colitis. The results of our research revealed that *Atf4* deficiency exacerbated sepsis-induced intestinal injury. *Bain* et al[18] showed that owing to impaired Ly6C^{hi} monocytes-to-gMacs differentiation, large amounts of monocytes and pro-inflammatory macrophages infiltrate the colon and secrete inflammatory factors, which is the pathological basis for experimental colitis. In our study, although Ly6C^{hi} monocytes also accumulated in the colon following LPS treatment, there was no change in pro-inflammatory macrophages (P3 cells) and a significant decrease in gMacs. Intestinal macrophages have been reported to either suppress excessive inflammation in the intestine by producing IL-10,[15] or to maintain immune tolerance by expressing MHC-II and IL-10.[29] In contrast,

IL-1β secretion by monocytes/macrophages in intestinal tissues is closely associated with intestinal inflammation and pathological injury.[30] Our data indicated that neither Ly6C$^+$ monocytes nor gMacs secreted large amounts of inflammatory factors in septic conditions, but instead showed a reduction or no change in IL-1β, or downregulated IL-10 in Ly6C$^+$ monocytes and MHC-II molecules in gMacs. Therefore, we propose that sepsis-induced intestinal injury is governed by a different immunopathological mechanism to that associated with experimental colitis; that is, impaired monocytes-to-gMacs differentiation in sepsis, and hypofunction of Ly6C^{hi} monocytes and gMacs may play a more important role in sepsis-induced intestinal injury than in experimental colitis. This hypothesis is supported by colonic pathologic findings showing that infiltration of mononuclear cells is lower in sepsis-induced intestinal injury compared with that in experimental colitis.[22] Further research is needed to clarify the role of gMacs in the development, progression, and prognosis of sepsis-induced intestinal injury and to identify possible therapeutic targets.

TGF-βR signaling regulates Ly6C^{hi} monocytes-to-gMacs differentiation, and *Tgfbr1* knockdown leads to accumulation of P1 cells in the mouse colon, and thus prevents differentiation toward gMacs.[16] Therefore, we further investigated the genetic mechanism through which sepsis reduced gMacs and found that TGF-βR1 expression of P1 cells was upregulated after *Atf4* knockdown and further upregulated in LPS models of sepsis (our unpublished data). These observations, combined with the fact that *Atf4* is a transcription factor downstream of TGF-βR signaling,[31] suggest that the exacerbating effect of *Atf4* knockdown on the sepsis-induced impairment of Ly6C^{hi} monocytes-to-gMacs differentiation may be related to TGF-βR signaling. However, the underlying mechanism requires further research.

To our knowledge, this research on the role of ATF4 in the regulation of monocytes-to-gMacs differentiation in sepsis-induced intestinal injury is rare but valuable [Supplementary Figure 5, http://links.lww.com/CM9/B384]. However, several limitations of the study need to be addressed. Firstly, there is no direct evidence on where ATF4 over expression effectively attended sepsis-induced intestinal injury owing to technical limitations. Secondly, the specific mechanism of ATF4 in regulating the differentiation of gMacs requires further detailed investigation. Nevertheless, those limitations do not affect the new insights that this study brings to the immune-related mechanism of sepsis-induced intestinal injury.

Conclusions

In summary, sepsis induces monocytes-to-gMacs differentiation dysfunction associated with decreased expression of ATF4 in P1 cells. *Atf4* knockdown aggravates monocytes-to-gMacs differentiation dysfunction in response to LPS, and finally exacerbates sepsis-induced intestinal injury. Thus, ATF4-mediated monocytes-to-gMacs differentiation might represent a potential therapeutic target for sepsis-induced intestinal injury.

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Conflicts of interest

None.

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