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ORIGINAL RESEARCH

Dual Role of α-MSH in Colitis Progression: Mediating Neutrophil Differentiation via Bone Marrow

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Background: Inflammatory bowel disease (IBD) comprises a group of autoimmune disorders characterized by chronicity and resistance to cure, with an unknown etiology. Recent studies on the brain-gut axis suggest that the central nervous system (CNS), particularly the hypothalamic-pituitary axis (HPA), may play a crucial role in modulating the immune system and influencing disease progression. However, the specific role and mechanism of the HPA in IBD pathogenesis remain unclear. This study aims to investigate the alterations in the HPA and its potential roles during IBD development.

Methods: We utilized a dextran sodium sulfate (DSS)-induced colitis model in mice and employed immunofluorescence, real-time quantitative PCR (RT-qPCR), enzyme-linked immunosorbent assay (ELISA), among other techniques, to evaluate the impact of colitis on the HPA. Additionally, we used flow cytometry, adeno-associated virus-mediated gene silence, parabiosis and single-cell RNA sequencing to uncover the specific roles and mechanisms of the HPA in colitis.

Results: Our results indicate that colitis activates HPA secretion and increases α-MSH. α-MSH acts on the MC5R present on the surface of hematopoietic stem cells (HSCs) in the bone marrow, altering the bone marrow microenvironment and promoting HSCs proliferation and differentiation into neutrophils. This process enhances the clearance of pathogenic microorganisms during the acute phase of colitis, while inducing sustained inflammatory responses during the remission phase.

Conclusion: In summary, our study demonstrates the dual role of HPA activation and α-MSH secretion induced by colitis in the pathogenesis of IBD. These findings offer vital guidance for optimizing personalized treatment of IBD, emphasizing the importance of carefully managing the timing and dosage of α-MSH for its effective clinical application.

Keywords: IBD, α-MSH, hematopoietic stem cells, neutrophils, inflammation

Introduction

Inflammatory bowel disease (IBD), encompassing Crohn's disease and ulcerative colitis, constituted a group of autoimmune disorders with unidentified etiologies, with a rising prevalence annually.[1] The condition was characterized by chronicity, difficulty in achieving a cure, and a frequent lack of response to clinical symptomatic treatments. Furthermore, certain patients exhibited resistance to available therapeutic agents or developed drug resistance over time.[2] Existing literature suggested that genetic predisposition, environmental influences, psychosocial factors, and immune dysregulation were intricately linked to IBD.[3,4] Despite extensive research, the precise pathogenesis and underlying mechanisms remained elusive, and there were no definitive pharmacological interventions capable of reversing the disease process. Therefore, elucidating the regulatory mechanisms governing IBD progression was paramount for advancing our understanding of its etiology and for developing effective treatment strategies.

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Recent studies have increasingly underscored a profound linkage between the brain and gastrointestinal disorders, crystallizing the concept of the "brain-gut axis".[5] This bidirectional communication network integrates signals from the central, autonomic, and enteric nervous system (ENS), as well as from the endocrine and immune systems.[6] As a key hub in the gut-brain axis, the hypothalamic-pituitary-adrenal axis is closely implicated in the pathophysiology of IBD.[7] Under normal conditions, the hypothalamic-pituitary-adrenal axis primarily governs glucocorticoid secretion via corticotropin-releasing hormone (CRH), which can alleviate colitis symptoms by modulating immune responses.[8] However, during psychological stress, chronic glucocorticoid signaling can drive an inflammatory phenotype in ENS glial cells through the cerebrospinal fluid type 1 (CSF1) pathway, thereby exacerbating intestinal inflammation. This process also hinders neuronal transcription in the ENS, reduces the number of mature neurons, and impairs intestinal motility[9]—findings indicative of a dual regulatory role for the HPA that can either mitigate or intensify inflammation depending on physiological context. Furthermore, the HPA may directly interact with immune cells (eg, macrophages, mast cells, T cells, dendritic cells) through neural pathways, thereby exerting significant influence on IBD progression.[10,11] In parallel with these HPA–mediated mechanisms, accumulating evidence points to important neuroimmune interactions in colitis. For instance, studies show that specific brain regions such as the caudal nucleus of the solitary tract (cNST) can markedly influence peripheral inflammatory responses;[12] conversely, activation of brain areas like the insular cortex helps encode immune challenges and can modulate colitis-related inflammation.[13] These observations emphasize the importance of neuroimmune crosstalk in gastrointestinal disorders and underscore the need to elucidate how colitis-induced changes in hypothalamic-pituitary hormone secretion ultimately drive immune dysregulation.

Alpha-melanocyte-stimulating hormone (α-MSH) is an endogenous peptide hormone belonging to the melanocortin family and is primarily encoded by the *POMC* gene in the pituitary gland.[14] Abnormal α-MSH expression has been implicated in various diseases.[15–17] Initially recognized for its role in promoting pigment deposition in melanocytes, recent studies have broadened our understanding of α-MSH to include regulation of cell differentiation and regeneration, immune modulation, and involvement in tissue damage and fibrosis formation.[18] In rheumatoid arthritis, α-MSH exhibits anti-inflammatory effects via the melanocortin 3 receptor (Mc3r).[19] It also participates in inflammatory processes by binding to the melanocortin 5 receptor (Mc5r), which triggers the differentiation of bone marrow hematopoietic stem cells (HSCs) into immune cells implicated in tumor cell regulation.[17] Notably, colitis models have revealed abnormal differentiation of bone marrow HSCs.[20] Through its interactions with melanocortin receptors on various immune cell populations, α-MSH may fine-tune inflammatory pathways in the gut microenvironment,[21,22] making it an attractive candidate for IBD therapy. Yet the precise role of α-MSH in IBD initiation and progression via these bone marrow-derived immune cells remains unclear. This gap underscores the need to clarify how α-MSH–regulated immune pathways contribute to IBD pathogenesis. Unraveling how α-MSH selectively regulates the production, differentiation, and function of immune cells could offer novel insights into disease mechanisms and open new avenues for targeted interventions. However, current understanding remains limited, underscoring the urgent need for comprehensive mechanistic studies that elucidate the precise cellular and molecular pathways of α-MSH in IBD pathophysiology.

In this study, we investigated the activation of the HPA during colitis and found that colitis induced the pituitary to secrete α-MSH. This hormone was then transported to the bone marrow via the peripheral circulation, where it stimulated the differentiation of bone marrow HSCs into neutrophils (Neu). These Neu were subsequently recruited to the site of colitis, exerting their antibacterial effects. During the acute phase of colitis, α-MSH accelerated the clearance of intestinal pathogens by increasing the number of intestinal Neu, thus promoting intestinal health. Conversely, in the remission phase, an excess of Neu contributed to sustained inflammatory responses within the intestine. These results clarified the specific role of α-MSH in the development of colitis, providing important clinical value for optimizing IBD treatment strategies.

Material and Methods

Animals

All animal procedures were approved by the Animal Review Committee of the Army Medical University and conformed to the Chinese National Laboratory Animal Care and Usage Guidelines. Male mice (n = 5-6 per group), aged 6–8 weeks, were housed in a specific pathogen-free environment with ad libitum access to food and water, under a 12-hour light and 12-hour dark cycle. Colitis was induced as previously described.[23] Briefly, the control group received free drinking water, while the

experimental group received 2–3% DSS (36–50 kDa, MP Biomedicals, Canada) in their drinking water. The drinking water in each group was changed every other day. Body weights, the presence of blood stools, and diarrhea were monitored daily.

Disease Severity Index (DAI)

The disease severity of the mice was evaluated using an index that included stool consistency, bleeding, and weight loss. The disease severity index was scored as previously described.[24] Specifically, the following scoring system is used to evaluate rectal bleeding and stool consistency: 0, no observable blood (negative); 1. Trace blood (light blue); 2. Micro blood (blue); 3. Obvious bleeding (dark blue); 4. Meat blood (black) and 0, normal; 1. Loose stool; 2. Pasty semi formed; 3. Liquid feces adhering to the anus; 4. Watery diarrhea. Calculate the percentage change in weight from day 0 to the end of the experiment to evaluate weight loss (0, no weight loss; 1.1–5%; 2.5–10%; 3.10–20%; 4,>20%).

Hematoxylin-Eosin (H&E) Staining

H&E staining was conducted according to previously reported methods.[24] Briefly, colon tissue located approximately 1 cm from the anus was excised, fixed, dehydrated, embedded in paraffin, and sectioned. Subsequently, the sections were stained using an H&E staining kit (Bio Basic Inc., Shanghai, China) following the manufacturer's instructions. Histological changes were observed under an optical microscope. The histopathological scores criteria are as follows: Extent of inflammation: 0, None; 1. Mucosa; 2. From mucosa to submucosal layer; 3. From mucosa to muscle layer; 4, Transmural; Infiltration neutrophils and lympho-histiocytes: 0, None; 1, Focal; 2, Multifocal; 3. Diffuse; Extent of crypt damage: 0, None; 1. Basic one-third; 2. Basic two-thirds; 3. The entire crypt is damaged; 4. Crypt damage + uiceration; Crypt abscess: 0, None; 1, Focal; 2, Multifocal; Submucosal edema: 0, None; 1, Focal; 2, Multifocal; 3. Diffuse; Goblet cell loss 0, None; 1, Focal; 2, Multifocal; 3. Diffuse; Reactive epithelial hyperplasia: 0, None; 1, Focal; 2, Multifocal; 3. Diffuse.

Immunofluorescence

For immunofluorescence analysis, mice were sequentially perfused through the left ventricle with saline followed by 4% paraformaldehyde (SenBeiJia, China). Subsequently, the brains were excised and post-fixed in 4% paraformaldehyde overnight. The brains were then dehydrated in a gradient sucrose solution at 4°C—initially in 20% sucrose for 24 hours, followed by 30% sucrose for another 24 hours. After dehydration, the brains were sectioned into 3–5 μm slices. For the immunofluorescence staining, the sections were first brought to room temperature, then permeabilized with 0.3% Triton X-100 for 15 minutes, wash three times, and blocked with 3% bovine serum albumin for 1h. The sections were incubated with the c-Fos Rabbit pAb (Invitrogen, USA) overnight at 4°C. The following day, they were washed and incubated with Alexa Fluor 594-conjugated secondary antibodies (Invitrogen, USA) for one hour at room temperature, shielded from light. After additional washes, the sections were mounted with 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) (Beyotime, Wuhan, China). Fluorescence was then observed using a laser confocal microscope (ZEISS, LSM880, Germany).

Real-Time Quantitative PCR (RT-qPCR)

Total RNA was extracted using Trizol Reagent (TaKaRa, Japan) through separation, precipitation, washing, dissolution, following the manufacturer's instructions. RNA quality and concentration were assessed with the NanoDrop One (Thermo Fisher Scientific, USA). Subsequently, 1 μg of RNA was reverse transcribed using the RevertAid RT Reverse Transcription Kit (ABclonal, USA) and following the manufacturer's protocols, to prepare cDNA for RT-qPCR analysis. RT-qPCR was performed on an ABI 7500 RT-PCR system, initiating with a 10-minute denaturation at 95°C, followed by 45 cycles of 15 seconds at 95°C and 1 minute at 60°C, using SYBR Green for detection. The relative expression of the target genes was normalized to the endogenous control gene, β-actin, and analyzed using the $2^{-\Delta\Delta Ct}$ method. At least three biological replicates were used for each qRT-PCR. The specific sequences utilized in the study are listed (Table S1).

Enzyme-Linked Immunosorbent Assay (ELISA)

To quantify the levels of α-MSH, adreno-cortico-tropic-hormone (ACTH), and β-endocrine, serum and bone marrow supernatant from mice were collected and analyzed using ELISA. ELISA kits specifically designed for murine α-MSH (E03M0205)

and β-endocrine (E03E0181) were obtained from Bluegene, China. The ELISA kit for ACTH (ENZ-KIT138-0001) was sourced from ENZO Life Sciences. All assays were conducted in accordance with the manufacturer's protocols.

Flow Cytometry

To assess the population of bone marrow hematopoietic stem and progenitor cells (HSPCs), flow cytometry was carried out. Bone marrow cells were harvested by flushing femurs and tibias of mice with sterile PBS, then filtered through a 70 μm cell strainer. Red blood cells were lysed using red blood cell lysis buffer (Biosharp, Anhui, China). The cells were washed with PBS, resuspended in PBS with 5% FBS, and stained with fluorescence-conjugated antibodies for 45 minutes at 4°C in the dark. Distinct hematopoietic cell populations were identified as follows: LSK (Lin1−c-Kit+Sca1+), slam (Lin1−c-Kit+Sca1+CD48-CD150+), short-term hematopoietic stem cell (STHSC: Lin1−c-Kit+Sca1+CD34+Flk2-), long-term hematopoietic stem cell (LTHSC: Lin1−c-Kit+Sca1+CD34-Flk2-), multipotent progenitors (MPP: Lin1−c-Kit+Sca1+CD34+Flk2+), progenitor (Lin1−c-Kit+Sca1−), common lymphoid progenitor (CLP: Lin1−Il7+c-KitmidSca1mid), common myeloid progenitor (CMP: Lin1−c-Kit+Sca1−CD34+CD16/32mid), granulocyte-macrophage progenitor (GMP: Lin1−c-Kit+Sca1−CD34+CD16/32high). To analyze the cell cycle of HSCs, cells were first stained with surface markers, then fixed and stained with Ki67 (Invitrogen, USA). Following staining, cells were washed, analyzed using a BD Fortessa flow cytometer, and data were processed with FlowJo 10.5.3.

To assess the effect of α-MSH on bone marrow cells, bone marrow cells isolated from femurs and tibias were filtered and lysed with RBC lysis buffer. Cell suspensions were incubated with α-MSH (0.05 μM, TargetMol, USA) alone or combined with an Mc5r inhibitor (3 μM, TargetMol, USA) for 15 minutes, followed by staining with antibodies for Neu (CD45+CD11b+Ly6G+F4/80-) and monocytes (Mon: CD45+CD11b+Ly6G-F4/80-). Samples were assessed on a BD Fortessa flow cytometer.

Flow cytometry of mouse colon was conducted as previously described with minor modifications.[25] Colons were dissected, thoroughly washed with HBSS, cut into 0.5 cm segments, and digested in HBSS containing 10 mm HEPES, 10 mm EDTA, 100 U/mL Penicillin/Streptomycin, 100 μg/mL DNase I, and 2% FBS at 37°C for 40 minutes with intermittent shaking. Post-digestion, tubes were chilled on ice for 10 minutes; supernatants were discarded, and tissues were washed with HBSS. A secondary digestion step involved 100 U/mL Penicillin/Streptomycin, 100 μg/mL DNase I, and 100 μg/mL Liberase TM (Roche, Germany) for 30 minutes at 37°C at 100 rpm. Lamina propria mononuclear cells (LPMCs) were isolated, filtered, and prepared for flow cytometry staining using antibodies listed (Table S2).

Fluorescence-Activated Cell Sorting (FACS)

Bone marrow cells (n=3 per group) were collected and subjected to flow cytometry staining using the same methodology as previously described. The cells were sorted for various populations, including LSK (Lin1−c-Kit+Sca1+), CLP (Lin1−Il7+c-KitmidSca1mid), CMP (Lin1−c-Kit+Sca1−CD34+CD16/32mid), GMP (Lin1−c-Kit+Sca1−CD34+CD16/32high), Neu (CD45+CD11b+Ly6G+F4/80-), Mon (CD45+CD11b+Ly6G-F4/80-), and T cells (CD45+CD3+). Sorting was performed using a MoFlo XDP cell sorter (Beckman Coulter, USA).

Stereotaxic Injection

To specifically interfere with the expression of the POMC gene in the pituitary gland, we conducted stereotaxic injections of virus into mice. Mice were anesthetized with pentobarbital (50 mg/kg, intraperitoneally) and secured in a stereotactic head frame. Throughout the procedure, a heating plate maintained the body temperature at approximately 37°C. A small hole was drilled in the skull using a drill bit, and 500 nl of the virus was injected per mouse using a microinjection needle at a rate of 100 nl/min. The injection site was targeted according to the stereotaxic coordinates from the mouse brain atlas: anteroposterior (AP) −2.5 mm, mediolateral (ML) ± 0.15 to 0.25 mm, dorsoventral (DV) −6.35 to 6.45 mm.[26] The sequence of the shRNA used was CTGCTTCAGACCTCCATAGAT, as previously reported.[17]

Parabiosis

Parabiosis was performed on mice of the same age, sex, and weight, as described previously.[27] Mice were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg). Under sterile conditions, mirror incisions were made along

the adjacent flanks of the paired mice. Nylon monofilament sutures were used to connect the knee and elbow joints of adjacent mice to facilitate coordinated movement. The peritoneal cavities of the two mice were then sutured continuously to ensure they were securely joined. A heating pad was utilized during the surgery to maintain the body temperature of the mice at 37°C. After a recovery period of four weeks, one mouse in each pair was administered 3% DSS orally three times a day for seven consecutive days to induce colitis, while the paired mouse received only water. Following this treatment period, femurs and tibias were collected from the mice for further analysis.

Single Cell RNA Sequencing and Analysis

Bone marrow cells were isolated from the femurs and tibias of mice, then filtered, lysed, and washed. Cells were immediately incubated with α-MSH for 15 minutes, washed twice with PBS, and then directly processed for library preparation (Singleron, China).

Single cell RNA-sequence data were analyzed using the Seurat package (version 4.4.1) in R software (version 4.3.1). Cells with gene counts below 300 or above 7000, or with mitochondrial gene content exceeding 10%, were excluded. The data were first normalized for sequencing depth. Highly variable genes were identified using the FindVariableFeatures function. The dataset was then scaled, and principal component analysis (PCA) was performed using the RunPCA function to reduce dimensionality. Batch effects were corrected using the RunHarmony function from the Harmony package. Cell clustering was conducted using the FindNeighbors function, and visualization of the clusters was achieved using the RunTSNE function. Pseudotime and trajectory analyses were conducted using Monocle 2 software (version 2.14.0) with default settings.

Statistically

All data were presented as means ± standard deviations (SD). Statistical analyses were performed using GraphPad Prism version 7.00 (San Diego, CA, USA). Prior to statistical testing, the normality of the data distribution was assessed using the Shapiro–Wilk test. For comparisons between two independent groups, a two-tailed Student's *t*-test was employed if the data were normally distributed and exhibited homogeneity of variances. In cases involving comparisons among multiple groups, one-way analysis of variance (ANOVA) was conducted, followed by Tukey's post hoc test to adjust for multiple comparisons and control the Type I error rate. A p-value of less than 0.05 was considered statistically significant. All tests were two-tailed, and an alpha level of 0.05 was set for determining statistical significance.

Results

Colitis Activated the HPA and Increased α-MSH Secretion

Studies indicated that the HPA, central to brain–gut communication, plays a significant role in IBD. In our research, we utilized a mouse model of colitis induced by DSS to explore the interaction between colitis and the HPA. Our findings indicated that mice began to lose weight on day 5 after DSS administration, with a continued decrease until the study's endpoint (Figure 1A). The DAI was significantly higher in DSS-treated mice compared to vehicle-treated mice (Figure 1B). Correspondingly, colonic length in DSS-treated mice was reduced (Figure 1C), and spleen size showed an increasing trend (Figure 1D). Pathological examinations revealed significant inflammatory cell infiltration, loss of goblet cells, and epithelial cell damage in the colons of DSS-treated mice (Figure 1E). Further analysis focused on the paraventricular nucleus (PVH) of the hypothalamus, a key area involved in regulating pituitary hormone secretion. We observed increased c-Fos expression in the PVH, indicative of neuronal activation (Figure 1F). Gene expression analysis of the pituitary gland showed a significant increase in Pomc transcription, whereas Fshb levels were significantly decreased. No significant changes were observed in Tshb, Prl, and Lhb (Figure 1G). To validate these findings, we measured the levels of the primary peptides derived from the POMC gene product, including α-MSH, β-endorphin, and ACTH, in serum and bone marrow supernatant. β-endorphin levels in serum were significantly elevated, whereas ACTH levels trended downward compared to the vehicle group. In bone marrow, β-endorphin and ACTH levels did not change significantly. Notably, α-MSH levels were significantly elevated in both serum and bone marrow supernatant (Figure 1H). These results indicate that colitis significantly enhanced α-MSH secretion by the pituitary gland in mice.

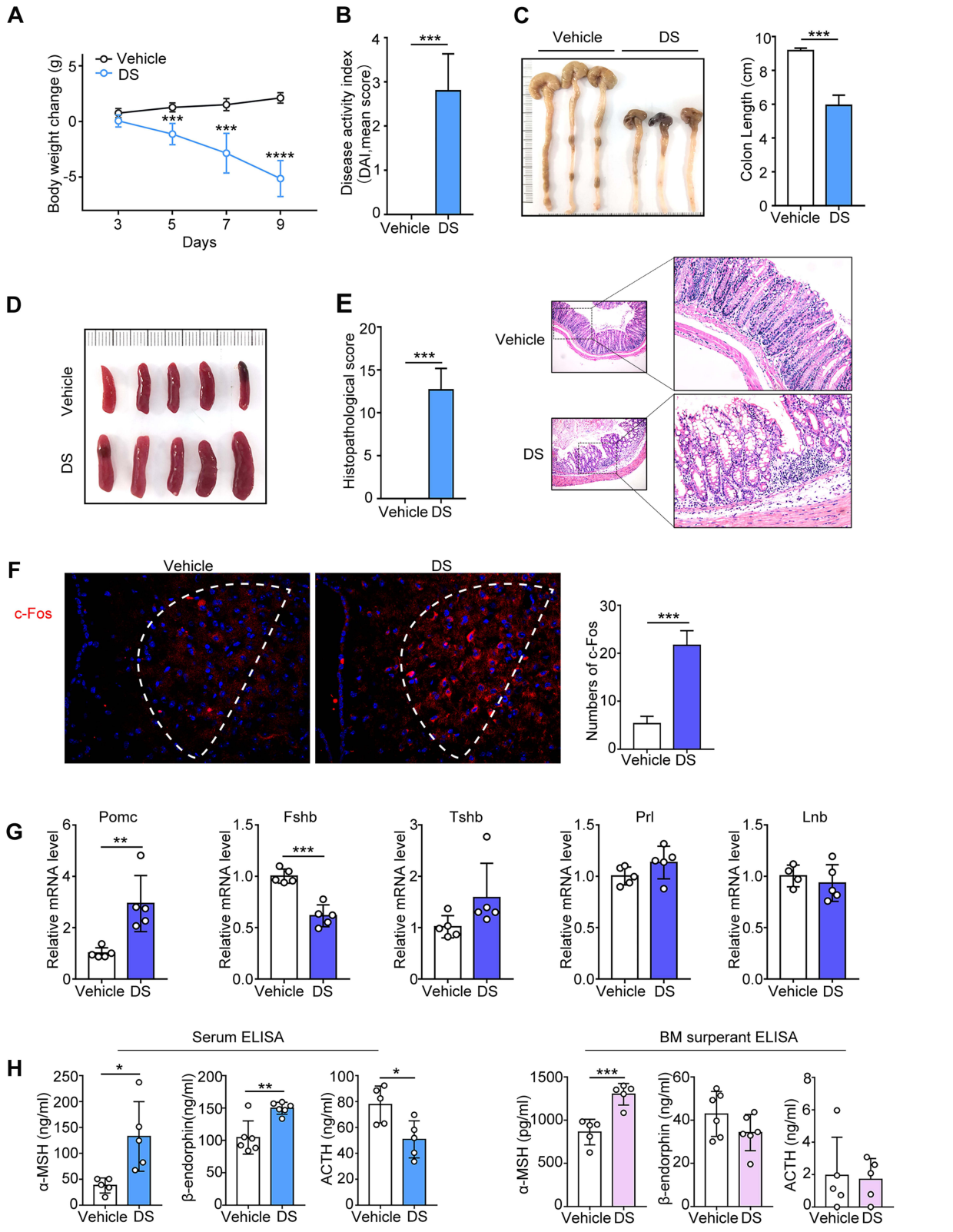

Figure 1 Enteritis Induced α-MSH Secretion Upregulation in the Murine Pituitary Gland. (**A**) The body weight, (**B**) DAI, (**C**) colons, (**D**) spleens, (**E**) H&E staining of mice in Vehicle and DS (2%DSS induced colitis) group were recorded. (**F**) Immunofluorescence analysis was conducted to detect c-Fos expression in the PVH of the hypothalamus in mice, and the positive cells expressing c-Fos were quantified (right). The white dashed line represents the PVH region of the mouse hypothalamus. (**G**) Transcriptional levels of Pomc, Fshb, Tshb, Prl, Lnb in mouse pituitary gland were examined through RT-qPCR. (**H**) The protein levels of α-MSH, β-endorphin and ACTH in mouse serum (left) and bone marrow supernatant were detected by ELISA. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Vehicle: blank control, DS: 2% DSS induced colitis.

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Acute Colitis Induced Profound Disturbances in the Bone Marrow Microenvironment, Impacting HSPCs

The observed increase in α-MSH concentration in the bone marrow supernatant of mice with colitis suggested the potential effect to the bone marrow microenvironment. Previous literature has shown that α-MSH can act on bone marrow HSCs and induce myelopoiesis.[17] We therefore examined the effects of colitis on HSCs and hematopoietic progenitor cells (HPCs) in the bone marrow. Flow cytometry results showed that the frequency of LSK cells was markedly reduced in mice with colitis. Further analysis of HSCs subpopulations revealed significant decreases in Slam, LTHSC, STHSC, and MPP, both in proportion and absolute numbers (Figure 2A and B). This loss of bone marrow HSCs was presumably related to their activation and subsequent differentiation. Additionally, there was an approximate two-fold increase in bone marrow progenitor cells (Figure 2C and D). Among the progenitor cells, the proportion and number of CMPs remained relatively unchanged in both normal and colitis mice. In contrast, the proportion and number of GMPs increased approximately four-fold

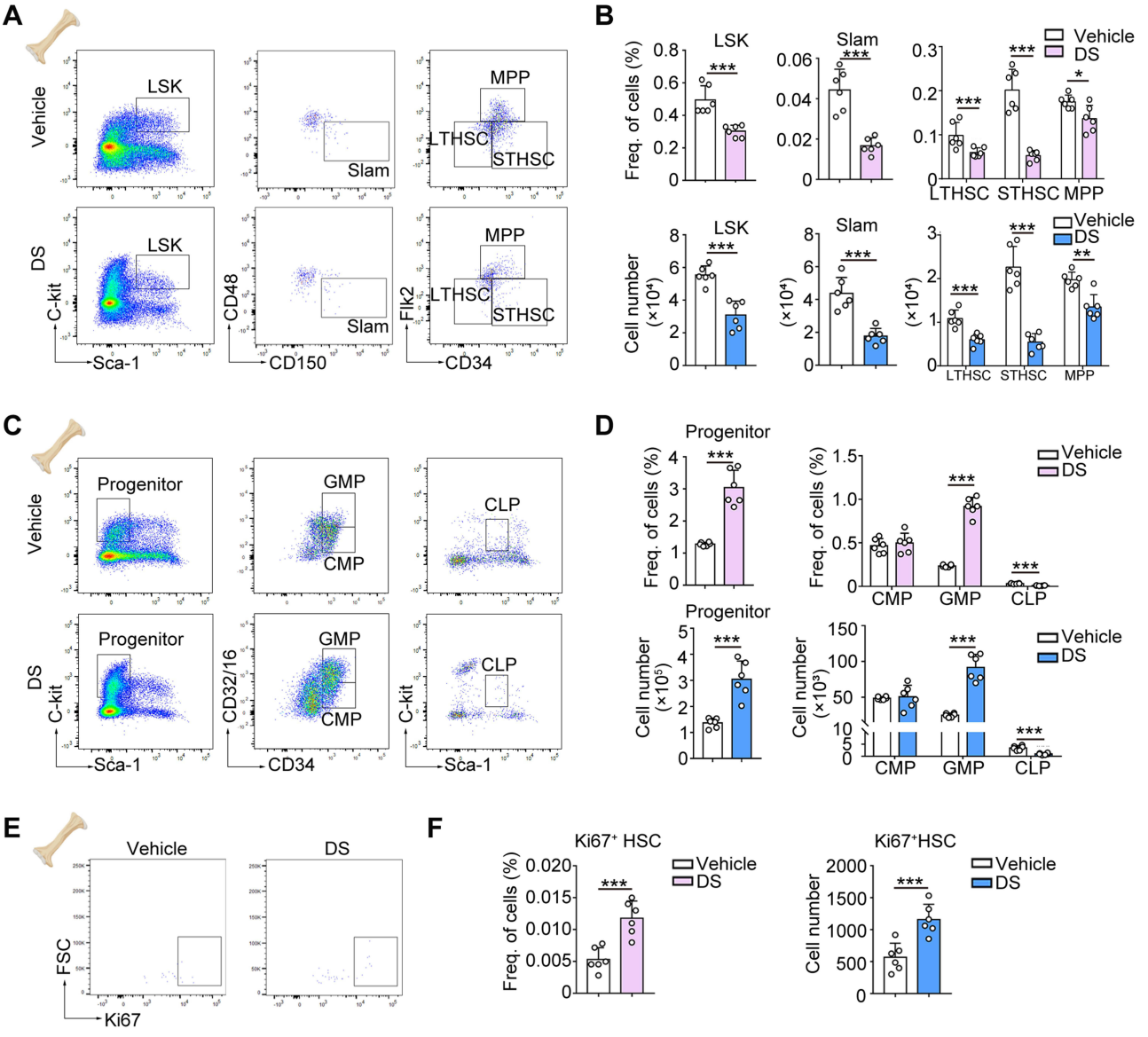

Figure 2 Colitis Induced Disturbances in the Bone Marrow Microenvironment in Mice, Affecting HSPCs Integrity and Balance. (**A** and **B**) The distributions of various HSCs, including LSK, LT-HSC, ST-HSC, MPP, and SLAM cells, were systematically quantified using flow cytometry in mouse bone marrow. (**C** and **D**) Bone marrow progenitor subsets, including CMP, GMP, and CLP, were precisely characterized using flow cytometry. (**E** and **F**) the cell cycle of Slam was assessed and quantified by flow cytometry. *P < 0.05, **P < 0.01, ***P < 0.001.

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in the bone marrow of colitis mice, while CLPs significantly decreased (Figure 2C and D). This pattern indicates that acute colitis induced the activation and differentiation of mouse bone marrow HSCs. Cell cycle analysis of bone marrow HSCs (specifically Slam cells) revealed that the proportion of Ki67-positive HSCs increased by 2.1-fold in mice with colitis (Figure 2E and F). Collectively, these results suggest that DSS-induced colitis triggered the activation and proliferation of HSCs.

Inhibition of α-MSH Secretion Ameliorated Bone Marrow Hematopoietic Disruption Caused by Acute Colitis

Given the observed increase in α-MSH concentration in the bone marrow of colitis-affected mice and the resultant imbalance in HSPCs, we speculated that these changes might be driven by α-MSH. To test this hypothesis, we constructed an adeno-associated virus aimed at suppressing Pomc expression. We then stereotactically injected this virus into the brain to inhibit Pomc expression in the pituitary gland, thereby reducing α-MSH secretion. We subsequently assessed the impact of this intervention on the function and balance of bone marrow HSCs. Our results indicated that after suppressing Pomc expression (DPV), the proportion of LSK cells significantly increased compared to the enteritis control group (DNV). However, replenishing α-MSH in the DPV group led to a significant decrease in the proportion of LSK cells (Figure 3A). Similarly, suppressing Pomc expression significantly rescued the reductions in the proportions of LTHSC, STHSC, MPP, and SLAM cells caused by enteritis. Replenishing α-MSH reversed these effects (Figure 3B and C). Additionally, the proliferation of SLAM cells was significantly reduced after Pomc inhibition compared to the DNV group, with a noticeable increase upon replenishment of α-MSH (Figure 3D). Consistent with these observations, the proportion of GMP cells was significantly decreased following Pomc inhibition, but this decrease was mitigated when α-MSH was reintroduced (Figure 3E and F). In summary, these findings suggest that colitis-induced α-MSH secretion from the HPA disrupts the balance of bone marrow HSPCs, and that modulating α-MSH levels can ameliorate this disruption.

α-MSH Influenced the Ratio of Bone Marrow HSCs and Progenitor Cells via the Bloodstream

We next explored the mechanism by which α-MSH reached the bone marrow and impacted the proportions and proliferation of HSCs. Hormones secreted by the pituitary gland are typically conveyed through the bloodstream to different parts of the body. Based on this, we hypothesized that α-MSH might access the bone marrow via the circulatory system, thereby influencing the ratio and dynamics of bone marrow HSCs. Initially, we established a parabiotic mouse model. After four weeks of parabiosis, we induced enteritis in the right-sided cohabiting mice using DSS for seven consecutive days, and then harvested the bone marrow for further analysis (Figure 4A). The results revealed that the concentration of α-MSH was markedly increased in the DSS-treated mice compared to that in the normal control group (NC-L and NC-R) following DSS induction, consistent with the results shown in Figure 1. Interestingly, the concentration of α-MSH in the DS-L group also exhibited an elevation compared to NC-L and NC-R, implying that α-MSH secreted by the pituitary gland of mice with colitis reached the bone marrow of the parabiotic mice through the bloodstream (Figure 4B). Further flow cytometry analysis confirmed this, showing that the proportions of LSK, SLAM, LTHSC, STHSC, and MPP in mice with enteritis (DS-R) significantly decreased compared to NC-R and NC-L, consistent with our previous findings. More importantly, the mice parabiosed with DS-R, which were not induced by DSS, also exhibited decreased levels of LSK, SLAM, LTHSC, STHSC, and MPP (Figure 4C–E). Overall, these results suggest that enteritis activated the secretion of α-MSH from the pituitary gland, which then traveled to the bone marrow via the bloodstream, inducing an imbalance in the proportions of HSCs.

α-MSH Acted on Bone Marrow HSCs, Leading to an Increase in Neu

We further explored the specific impact of α-MSH on bone marrow HSCs. α-MSH typically exerts its effects by binding to its receptors (Mc1r, Mc2r, Mc3r, Mc4r, and Mc5r). Initially, we sorted LSK cells from mouse bone marrow and assessed the expression of these receptors using qPCR. The results indicated that, compared to the other receptors, Mc5r was most abundantly expressed in LSK cells (Figure 5A). Concurrently, we examined the expression of Mc5r in various bone marrow

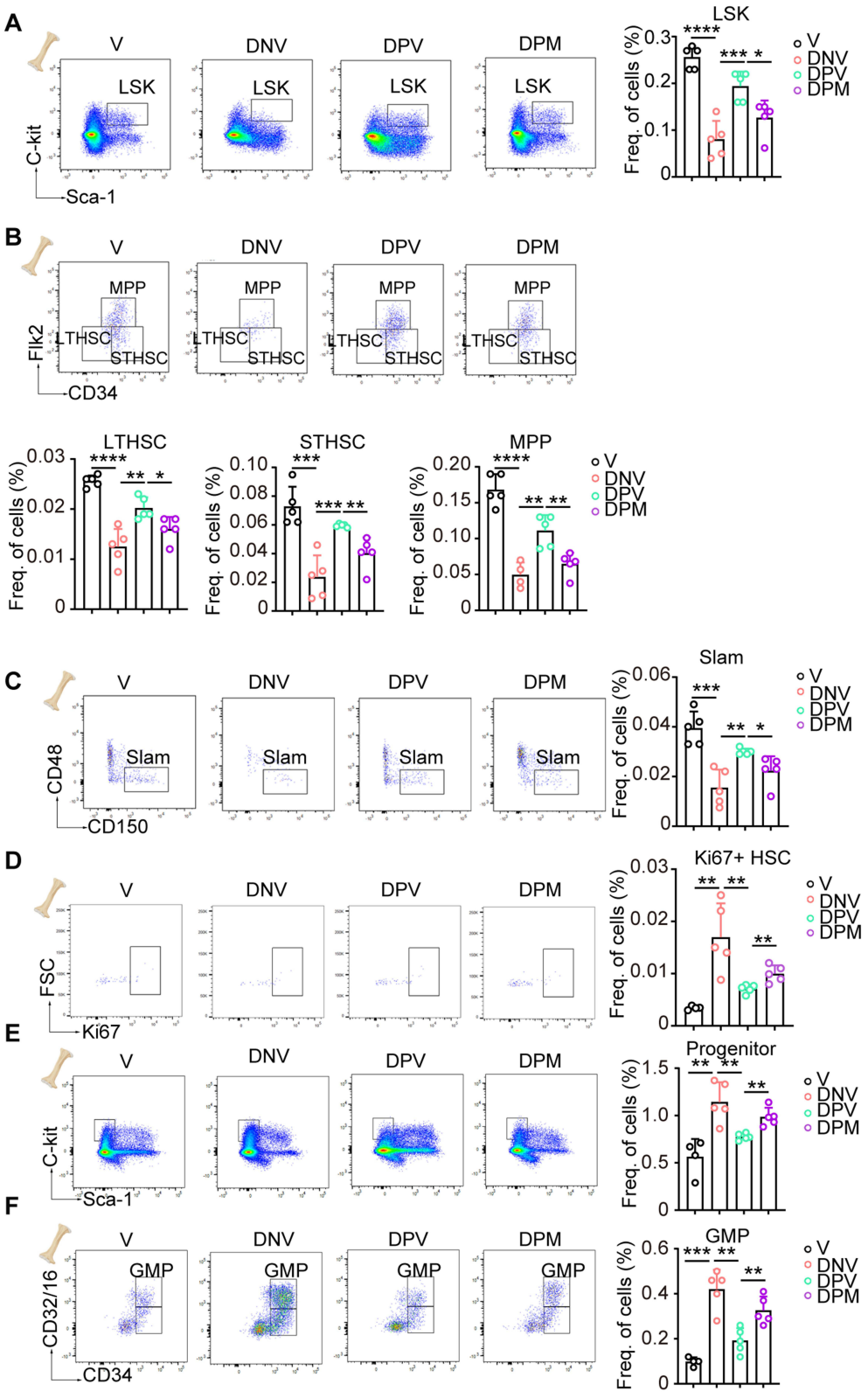

Figure 3 Inhibition of α-MSH Secretion in the Brain Ameliorated Disruption of the Bone Marrow Hematopoietic System Caused by Colitis. (**A**–**C**) The distributions of various HSCs like LSK, LT-HSC, ST-HSC, MPP, and Slam were analyzed after inhibited the expression of α-MSH. (**D**) the cell cycle of Slam after inhibition of α-MSH was assessed by flow cytometry. (**E** and **F**) The populations of progenitors and GMP in mice bone marrow after α-MSH inhibition were quantified through flow cytometry.
Notes: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. V blank control, DNV: 2% DSS+ ShNC, DPV: 2% DSS+ ShPomc, DPM: 2% DSS+ ShPomc+α-MSH.

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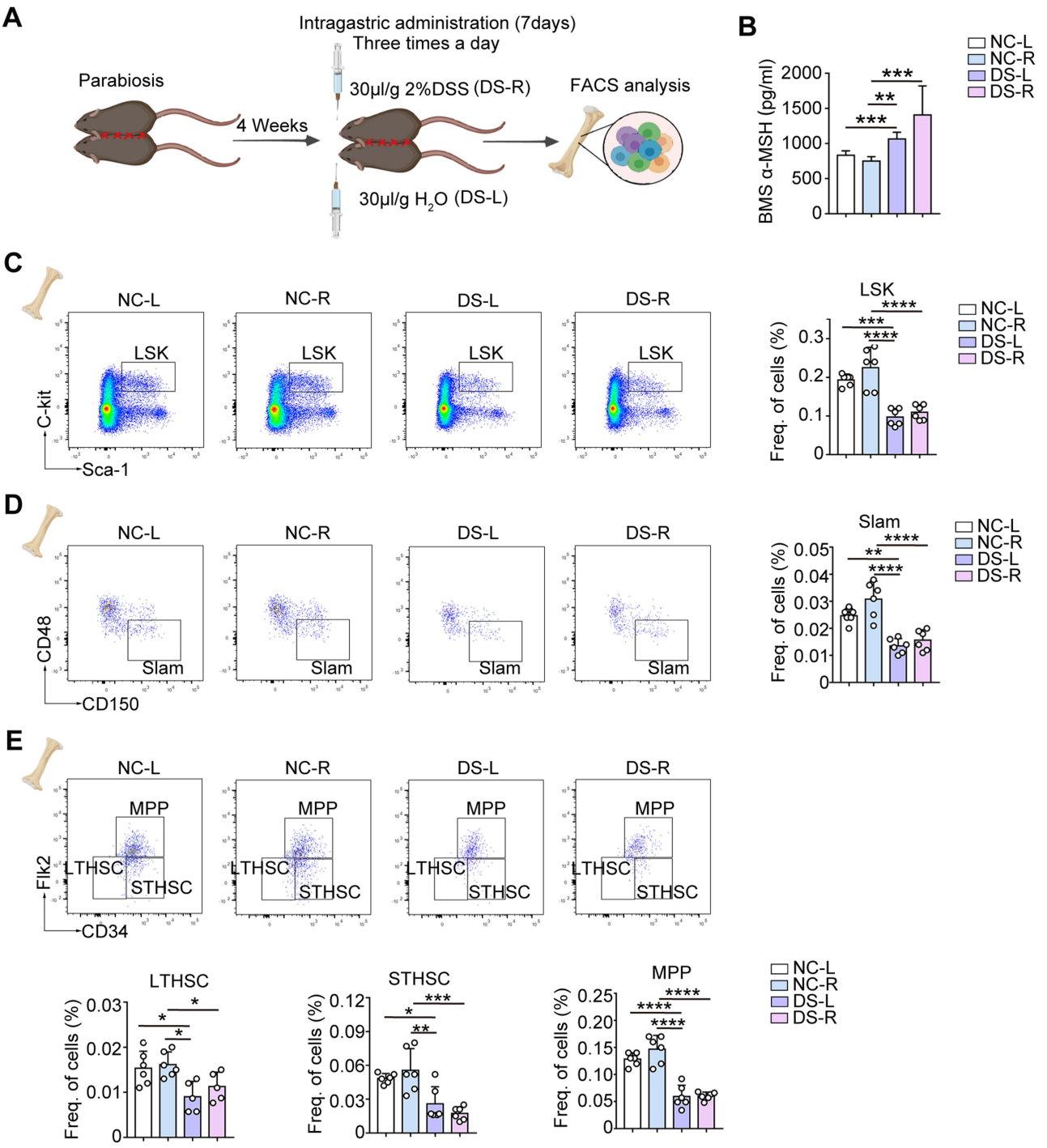

Figure 4 α-MSH Modulates the Ratio of Bone Marrow HSCs and Progenitor Cells via Circulation. (**A**) A parabiotic mouse model was constructed, and after a 4-week period, the right-side mice of DS parabionts were induced with colitis through daily intragastric administration of 3% DSS. Following a continuous 7-day treatment, bone marrow cells were harvested from the mice for flow cytometry analysis. (**B**) the level of α-MSH in bone marrow supernatant was assessed by ELISA. (**C**–**E**) the populations of bone marrow LSK, Slam, LT-HSC, ST-HSC and MPP were assessed and quantified by flow cytometry.

Noted: *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Abbreviations: NC-L, the left parabiont of normal control group; NC-R, the right parabiont of normal control group; DS-L, the left parabiont without DSS administration; DS-R, the left parabiont with DSS administration.

cells, including LSK, CMP, GMP, CLP, Neu, Mon and T cells. The findings suggested that Mc5r was specifically and highly expressed in mouse bone marrow LSK cells (Figure 5B), indicating that α-MSH may influence the proliferation and differentiation capabilities of HSCs by acting on the Mc5r. To directly demonstrate the effects of α-MSH on bone marrow cells, we isolated mouse bone marrow cells and performed single-cell sequencing after stimulation with α-MSH. In total,

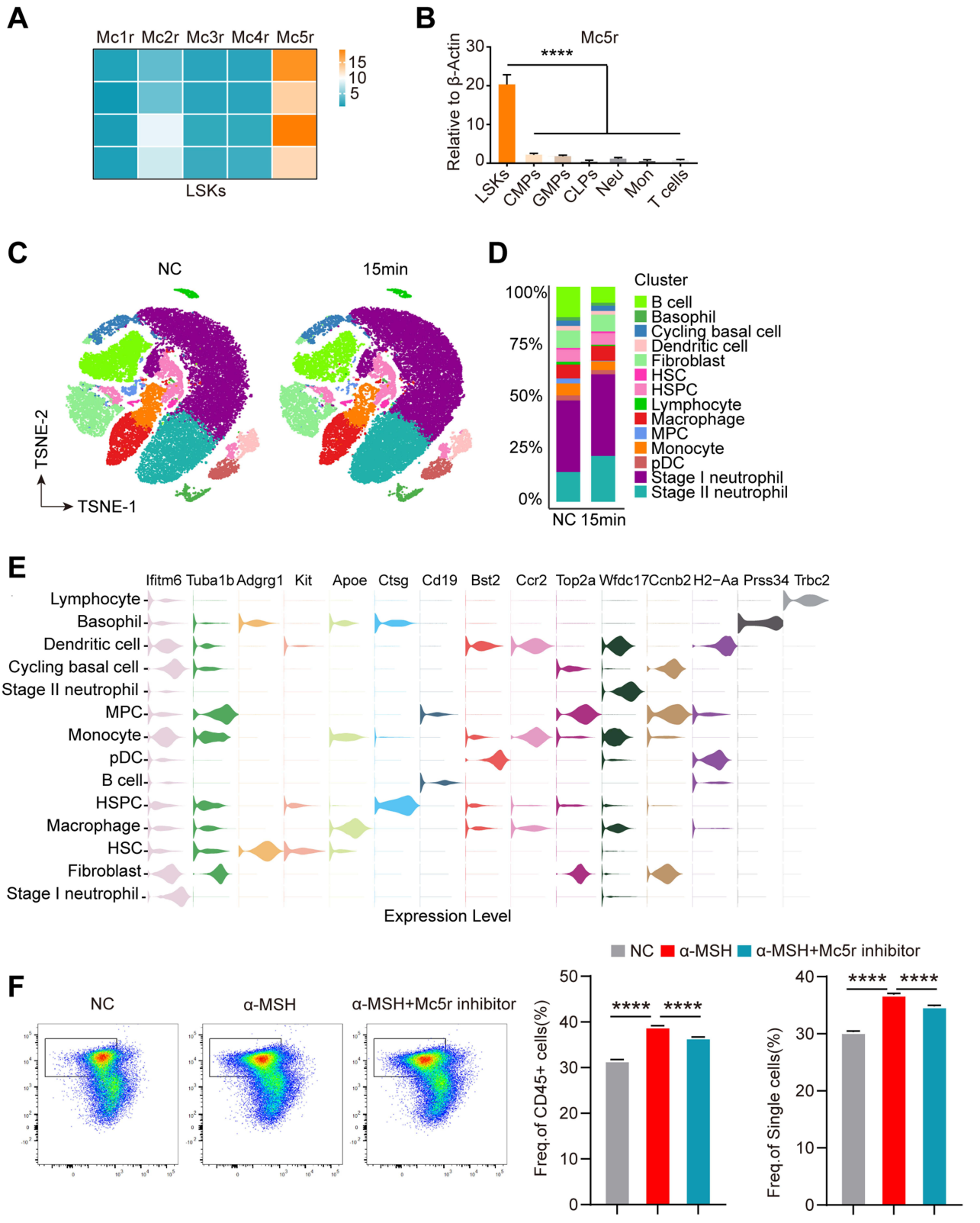

Figure 5 α-MSH Acted on Bone Marrow HSCs, Leading to the Increase of Neu. (**A**) the mRNA levels of Mc1r, Mc2r, Mc3r, Mc4r and Mc5r in LSKs sorted from mouse bone marrow were assayed by RT-qPCR. (**B**) The expression level of Mc5r in LSKs, CMP, GMP, CLP, Neu and Mon were detected using RT-qPCR. (**C**) t-SNE visualization presented single-cell RNA sequencing data from mouse bone marrow cells with and without α-MSH stimulation. (**D**) The populations of different cell types were displayed using bar charts. (**E**) Violin plots illustrated the expression levels of indicated genes within each cluster. (**F**) Flow cytometry analysis quantified the percentage of Neu in bone marrow cells following stimulation with α-MSH alone or in combination with an Mc5r inhibitor for 15 minutes.
Note: ****P < 0.0001.

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27,237 and 28,316 cells in the normal control (NC) group and the α-MSH-stimulated group was detected, respectively. The results, after dimensionality reduction and clustering, were displayed in a t-SNE plot (Figure 5C). The left bar graph represented the relative fraction of each cell type in different groups. Notably, the most pronounced change was observed in Neu; the proportion of Stage I Neu increased from 33% to 38% after α-MSH stimulation, while the proportion of Stage II Neu rose from 13% in the NC group to 21% in the α-MSH-stimulated group (15 minutes) (Figure 5D). Violin plots depicting the distribution of selected marker gene expression levels across various cell types were shown (Figure 5E). Further experiments involving the treatment of bone marrow cells with α-MSH and a Mc5r antagonist confirmed that the proportion of Neu was significantly upregulated following α-MSH treatment compared to the NC group, while treatment with the Mc5r antagonist resulted in a decrease (Figure 5F). In summary, the data collectively suggested that α-MSH led to an increase in the number of Neu by acting on the Mc5r present on LSK cells.

α-MSH Facilitated the Differentiation of Bone Marrow HSCs into Neu

To determine whether α-MSH promotes the differentiation of HSCs in the bone marrow into Neu, we performed dimensionality reduction and clustering on single-cell data of HSCs and HSPCs. We subdivided the bone marrow HSCs into six subpopulations, including CMP, megakaryocyte/erythroid progenitors (MEP), LTHSC, lymphoid-primed multipotent progenitors (LMPP), lymphoid-restricted multipotent progenitors (LYMPP), and multipotent myeloid progenitors (MyMPP). Among these, MyMPP showed a significant increase in proportion after α-MSH stimulation, while the proportions of LYMPP, CMP, MEP, and others exhibited a downward trend (Figure 6A). Bubble plots of the canonical marker genes were used to distinguish cell types (Figure 6B). Density plots from single-cell analysis further confirmed that the population of MyMPP was significantly enhanced after α-MSH stimulation (Figure 6C). To further investigate the impact of α-MSH on gene expression within HSCs, we analyzed the differentially expressed genes among various HSCs subpopulations. We highlighted genes with a fold change greater than 1.5 and a P-value less than 0.05. Genes with a P-value less than 0.01 were defined as significantly differentially expressed, categorized as up_Highly and down_Highly. The top five genes with the highest fold change in differential expression were labeled (Figure 6D). Notably, the expression levels of Egr1, a transcription factor that participated in the proliferation and differentiation of HSCs into Neu, exhibited the highest fold change in LTHSCs. Egr1 expression was also significantly upregulated in both LMPP and MyMPP. Additionally, the transcription factor Fos, which is associated with promoting cell proliferation, showed significant upregulation in LTHSCs, LMPPs, and MyMPPs. This concurrent upregulation of both Egr1 and Fos in these cell populations suggests a coordinated regulatory response to α-MSH that may be driving the differentiation and proliferation of HSCs into Neu (Figure 6D). Consistently with these findings, transcription factors associated with Neu differentiation, such as Egr1, Csf3r, Cebpa, and Cebpb, were significantly upregulated in the α-MSH treatment group. Additionally, transcription factors related to the cell cycle, including Fos, Jun, Nr4a1, Junb, Myc, and Cdkn1a, also showed a marked increase in expression levels after α-MSH treatment (Figure 6E). These results suggest a coordinated regulatory mechanism in response to α-MSH that enhanced the differentiation of HSCs into Neu. Pseudotime analysis results further corroborate this observation (Figure 6F).

α-MSH Played Opposing Roles in the Acute and Remission Phases of Colitis

Although α-MSH was considered a potential treatment for intestinal inflammation, its effects on colitis had been inconsistent and limited. Our findings indicated that colitis induced the differentiation of HSCs into Neu via α-MSH. To understand the impact of α-MSH on the pathology of colitis, we first established a mouse model of colitis induced by DSS. During the acute phase of colitis, we administered α-MSH treatment for two days and then examined the colonic tissue (Figure 7A). The results showed that in the acute phase, α-MSH administration significantly alleviated the colitis-induced shortening of the colon in mice (Figure 7B). The proportion of Neu in the colonic lamina propria was significantly increased after α-MSH treatment in this stage (Figure 7C), and the crypt epithelium, lumina, and cellularity (including goblet cells and colonocytes) were preserved (Figure 7D). This suggests that α-MSH stimulation induced the differentiation of HSCs into Neu, which may promote the clearance of intestinal bacteria during the acute phase of colitis. In contrast, we administered DSS continuously for six days, followed by a switch to normal drinking water starting on

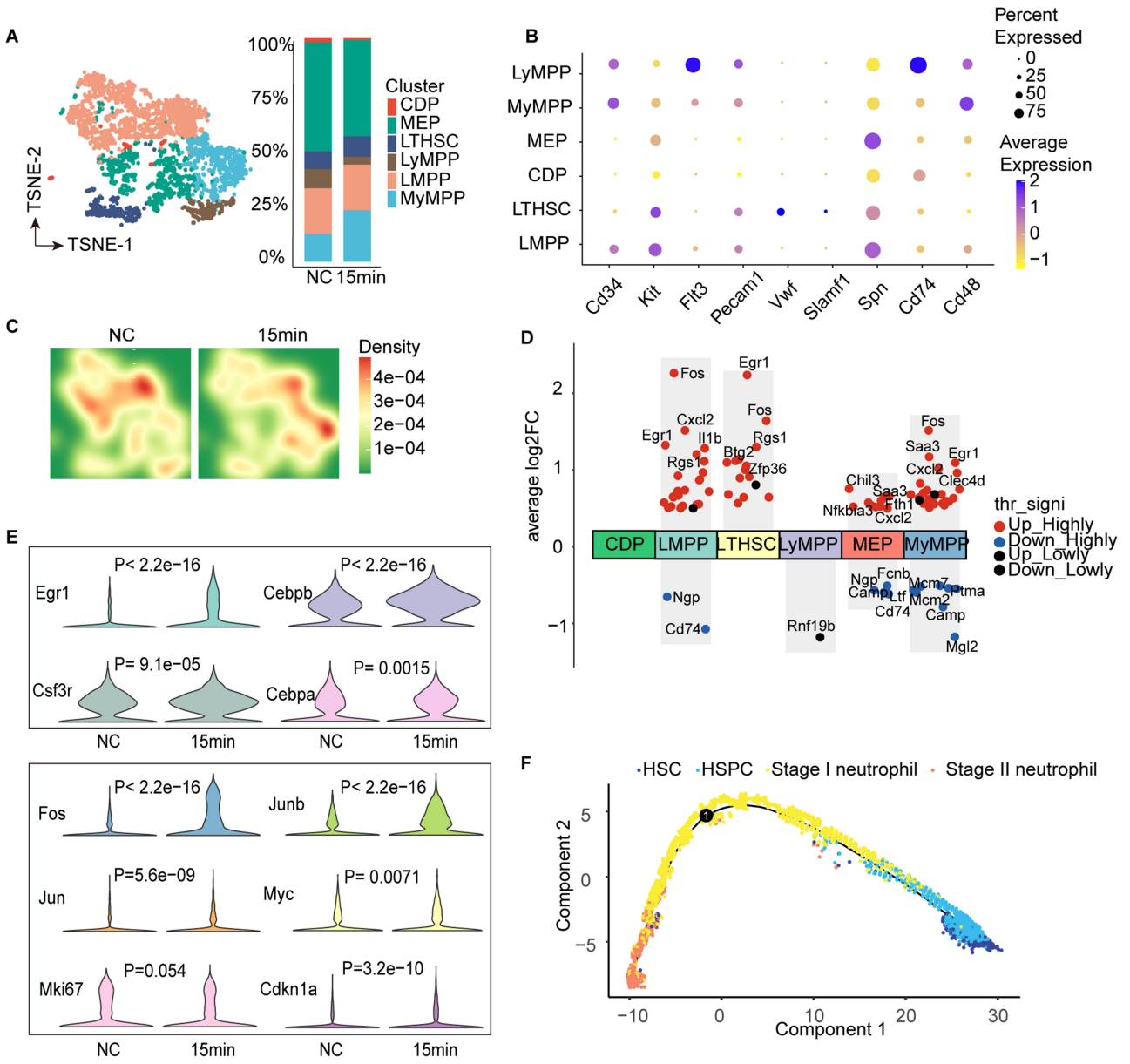

Figure 6 α-MSH Promoted the Differentiation of Bone Marrow HSCs into Neu. (**A**) Single-cell subpopulation classification of HSPCs was visualized via a t-SNE diagram. The proportion of indicated cells was displayed using a bar chart. (**B**) A dot plot illustrated the average expression of specified genes and the percentage of cells expressing these genes within each cluster. (**C**) Cell density across different cell types was shown using density plots. (**D**) Differentially expressed genes (DEGs) across the indicated cell types were calculated. (15min vs NC). (**E**) Violin plots displayed the expression of transcription factors related to the differentiation of HSCs into Neu (upper) and the HSC cell cycle (lower). (**F**) The cell trajectories were visualized by monocle2.

the seventh day. α-MSH was administered on the seventh and eighth days, with colon samples collected on the ninth day for analysis (Figure 7E). This design allowed us to evaluate the effects of α-MSH treatment during the recovery phase of DSS-induced colitis, following an initial period of inflammation. The data illustrated that administration of α-MSH during the remission phase notably promoted colonic atrophy observed in the murine model (Figure 7F). The proportion of Neu within the colonic lamina propria was markedly elevated following α-MSH treatment during the remission phase (Figure 7G). Histopathological examination of the colonic tissue revealed significant mucosal damage (Figure 7H). Collectively, these data suggest that α-MSH stimulation may have induced the differentiation of HSCs into Neu, potentially leading to a sustained inflammatory response within the colon, thereby hindering the natural reparative mechanisms of the tissue.

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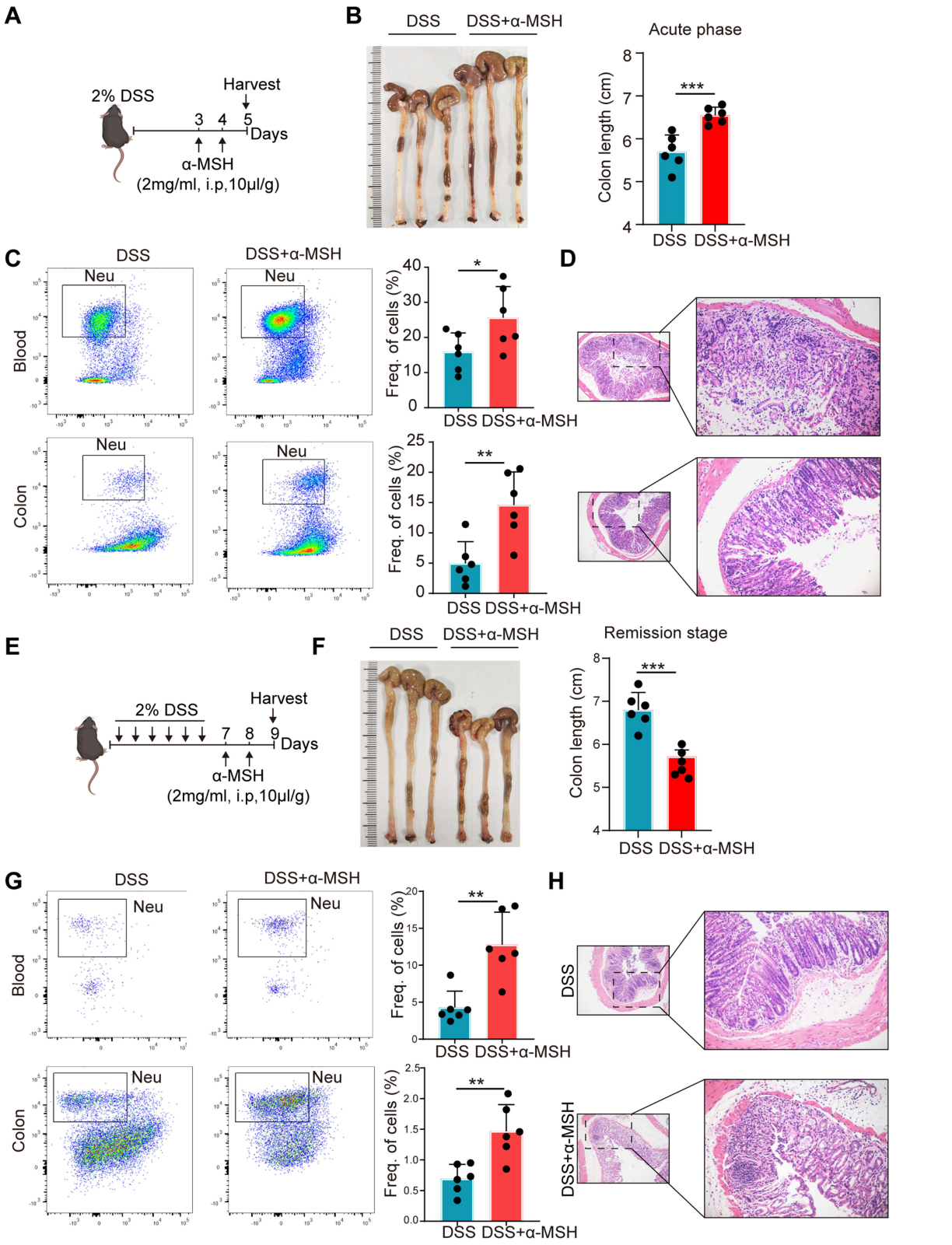

Figure 7 α-MSH Plays an Opposite Role in the Acute and Remission Phases of Colitis. (**A**) Diagram illustrating the administration of α-MSH during the acute phase of DSS-induced colitis in mice. (**B–D**) In acute mice, representative images of the colon and quantification of colon length, flow cytometry images and quantification of Neu in the lamina propria, and histopathological examination of colon H&E. (**E**) Diagram depicting the administration of α-MSH during the remission phase of DSS-induced colitis in mice. (**F–H**) In remission mice, representative images of the colon and quantification of colon length, flow cytometry images and quantification of Neu in the lamina propria, and histopathological examination of colon H&E.

Notes: *P < 0.05, **P < 0.01, ***P < 0.001.

Discussion

Under pathological conditions, organ systems such as the immune system can transmit signals to the CNS via neuroendocrine pathways.[28,29] Upon receiving these signals, the CNS may modulate the body's immune response or the state of disease by releasing hormones or other signaling molecules. This regulatory mechanism plays a critical role in various diseases, including autoimmune disorders like IBD.[29,30] However, dysregulation of this mechanism may exacerbate the disease. For example, an overactive inflammatory response can activate specific brain regions, which may then exacerbate the inflammatory response through neural pathways, creating a vicious cycle that accelerates disease progression.[12] This phenomenon is commonly observed in chronic pain and depression.[31,32] In this study, we demonstrated that colitis activates the hypothalamus and pituitary to overexpress α-MSH (Figure 1), which, in the early stages of colitis, promotes the generation of Neu and accelerates the clearance of pathogenic microorganisms from the intestine. Conversely, during the remission phase of colitis, α-MSH stimulation leads to persistent inflammatory responses in the intestine, detrimental to tissue repair (Figure 7). Mechanistically, we found that suppressing the expression of pituitary α-MSH using an adeno-associated virus could rescue the imbalance of bone marrow HSCs and HSPCs caused by colitis (Figure 2 and 3). Furthermore, in vitro treatment of bone marrow cells with α-MSH confirmed that α-MSH acts on the Mc5r on the surface of bone marrow HSCs to promote their differentiation into Neu, thereby participating in the progression of colitis (Figure 5 and 6). These findings deepen our understanding of the pathogenesis of colitis and provide potential therapeutic targets for IBD. The dual role of α-MSH, depending on the phase of colitis, highlights the complexity of targeting neuroendocrine factors in therapeutic strategies and underscores the importance of precise timing and contextual considerations in treatment approaches.

As research into the brain-gut axis advances, it is increasingly recognized as a pivotal bridge in modulating intestinal immunity in patients with IBD, where the CNS and immune system are intricately interconnected. Studies have documented abnormal activation of neurons in brain regions such as the insular cortex, thalamus, and amygdala in mice with colitis.[13] However, the mechanisms by which these brain regions modulate the intestinal immune response in IBD remain poorly understood and warrant further in-depth exploration. Our study demonstrated that colitis significantly activates the PVH of the hypothalamus in mice (Figure 1), leading to the secretion of α-MSH by the pituitary gland. This hormone plays a crucial role in the progression of colitis by promoting the differentiation of bone marrow HSCs into Neu. Consistent with our findings, the DSS-induced colitis model has shown that activation of neurons in the insular cortex of the brain via the autonomic nervous system enables these neurons to store and encode specific peripheral immune response information. Inhibition of insular cortex neurons can improve the intestinal immune microenvironment and, to some extent, alleviate the symptoms of colitis in mice.[13] Furthermore, the hepatic vagus nerve has been found to sense the intestinal microenvironment in IBD mice and transmit information to the nucleus of the solitary tract in the brainstem. This signaling promotes the differentiation of intestinal regulatory T cells, thereby maintaining intestinal immune homeostasis.[33]

The bone marrow serves as a crucial immune organ, producing various immune cells essential for initiating and maintaining the body's immune responses.[34,35] It has been established that bone marrow receives signals from the brain primarily through two mechanisms: on the one hand, it senses signals such as chemokines, hormones, and cytokines in the peripheral circulation system;[34,35] on the other hand, it is directly innervated by the sympathetic nervous system, transmitting information on the differentiation of immune cells.[36] Bone marrow HSCs are intimately linked to inflammatory processes,[37] playing a significant role in the inflammatory pathology of IBD,[6] although the underlying mechanisms remain to be fully elucidated. In this work, we observed that α-MSH, induced by colitis, can reach the bone marrow via the peripheral circulation, altering the bone marrow microenvironment. Single-cell sequencing revealed that under the influence of α-MSH, bone marrow LTHSC, LMPPs, and MyMPPs highly express transcription factors such as Egr1, Cebpb, Cebpa, and Csf3r, which significantly promote the generation of Neu.[38–40] Transcription factors related to cell proliferation, including Jun and Fos, were also significantly upregulated,[41,42] confirming the regulatory axis of pituitary-α-MSH-bone marrow HSC-Neu in colitis. As research into the brain-bone marrow axis deepens, studies demonstrate the brain's crucial role in regulating the differentiation and function of bone marrow cells. When the brain is

affected by disease, it can release signals to bone marrow cells, contributing to the disease's onset and progression. For instance, acute brain injury releases norepinephrine through the sympathetic nervous system, which acts on the β3 adrenergic receptor (Adrb3) on HSCs, promoting their proliferation and the generation of Mon with immune-regulatory functions.[36] These Mon migrate to the brain's damaged areas, secreting regulatory factors like IL-10 to reduce neuroinflammation. Similarly, in multiple sclerosis, autoimmune T cells home to the bone marrow via CXCL12 and highly express CCL5, promoting a myeloid differentiation bias in bone marrow HSCs. This results in a surge of Mon and Neu that migrate to the CNS, exacerbating inflammatory damage.[35] Moreover, different brain regions regulate distinct responses in bone marrow cells. For example, the motor cortex, via spinal neuronal signals, regulates the production of CXCL1 in skeletal muscle, inducing Neu mobilization to peripheral tissues to participate in immune responses.[34] The hypothalamus, through the HPA, releases glucocorticoids that mediate Mon migration from blood and lymph nodes to the bone marrow, and also acts on the Nr3c1 receptor on HSCs to promote their mobilization.[43]

α-MSH is widely recognized for its role in regulating inflammatory immune responses. In experimental Autoimmune Encephalomyelitis (EAE) and experimental autoimmune uveitis (EAU), α-MSH alleviates symptoms by modulating T cells.[44,45] Similarly, in systemic lupus erythematosus (SLE) and rheumatoid arthritis, it reduces inflammatory cell infiltration and mediator release.[46,47] However, the therapeutic potential of α-MSH in IBD remains controversial. Some studies indicate that inhibiting α-MSH worsens colitis,[48] while in TNBS-induced colitis models, α-MSH injections alleviate intestinal pathology.[49] Conversely, in DSS-induced models, α-MSH does not significantly affect TNF-α levels and may slightly increase them.[50] These mixed results suggest that α-MSH's promotion of myeloid differentiation in HSCs adds complexity to its role in disease progression.[17,51] Our research reveals that α-MSH exerts differential effects during the acute and remission phases of IBD. In the acute phase, α-MSH enhances the proliferation and differentiation of neutrophils, facilitating the clearance of pathogenic microorganisms from the intestine and thereby controlling the inflammatory response. During the remission phase, α-MSH may intensify inflammation and impede tissue repair processes. The contradictory effects of α-MSH in the acute versus remission phases of colitis may be attributed to several factors: First, the disease stage specificity, acute phase characterized by intense inflammation and tissue damage, α-MSH supports the generation and migration of neutrophils to eliminate pathogens and reduce inflammation, while in remission phase, the focus shifts to tissue repair and regeneration, excessive neutrophil may hinder the recruitment of repair-associated macrophages and fibroblasts, thereby obstructing the tissue healing process. Second, the dual functional roles of α-MSH, beyond promoting HSCs differentiation, α-MSH may directly modulate the anti-inflammatory functions of immune cells.[52,53] This dual role may contribute to the ambiguous therapeutic outcomes observed in IBD treatment with α-MSH. Most existing studies on α-MSH primarily investigate its direct effects on immune cells, with limited exploration of its broader impact on the host system. To fully elucidate the complex immunoregulatory functions of α-MSH, future research should examine its effects on the host hematopoietic system and intrinsic immune cell functions.

Conclusion

In this study, we discovered for the first time that the body activates the secretion of α-MSH by the pituitary gland during colitis (Figure 8A and B). This hormone travels through the peripheral circulation to the bone marrow, where it acts on HSCs, inducing their differentiation into Neu that participate in the progression of colitis (Figure 8C and D). In the early stages, this brain-α-MSH-HSC-Neu regulatory axis aids in accelerating the clearance of pathogenic microorganisms. However, in the later stages, it fosters a persistent inflammatory response that hinders tissue repair (Figure 8A and D). Our findings implying α-MSH may exert a dual regulatory role in colitis, which enhancing our understanding of the complex pathogenesis of this condition. This insight into the divergent roles of α-MSH depending on the disease phase highlights the intricate interactions between the neuroendocrine and immune systems in the context of inflammatory diseases.

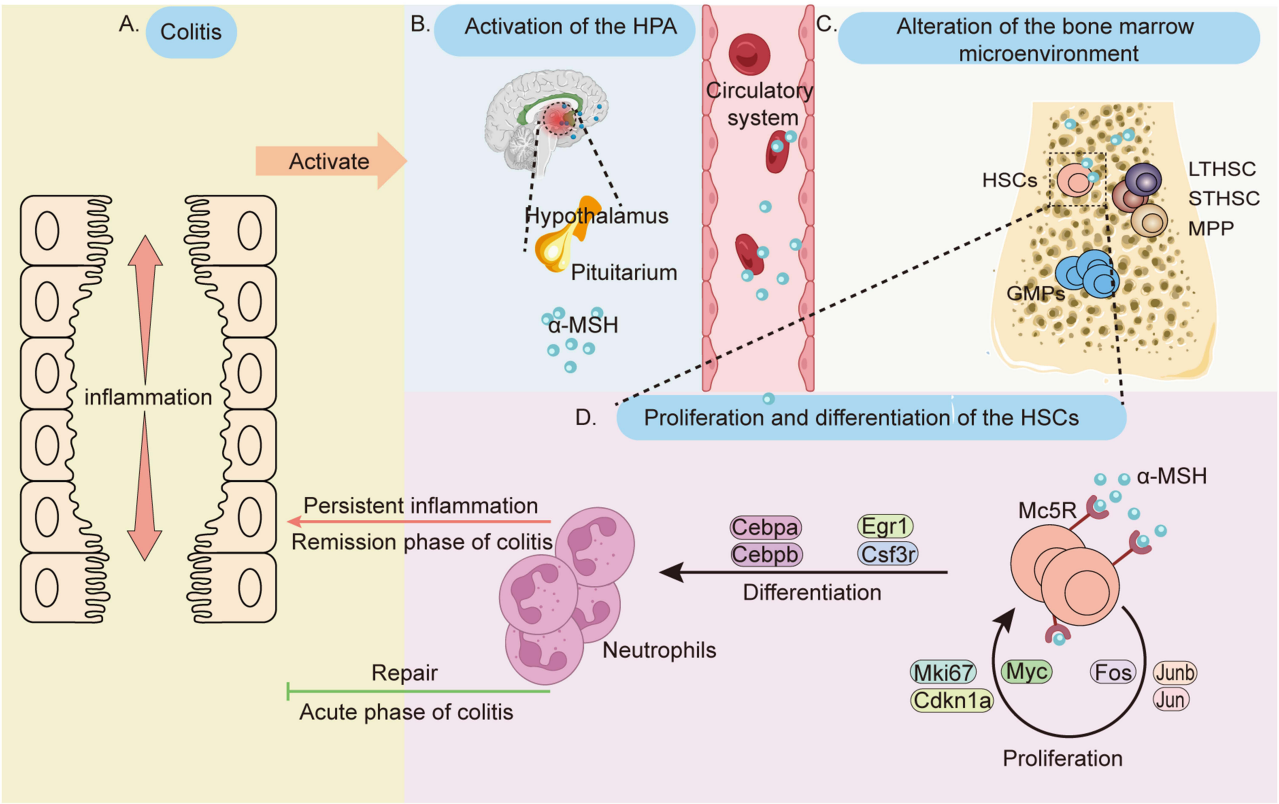

Figure 8 Work model. (A) During colitis, inflammation activates the hypothalamic-pituitary axis, leading to the secretion of α-MSH. (B) This hormone then enters the bone marrow via the circulatory system, (C and D) where it influences HSCs to differentiate into Neu, which play a role in the progression of colitis. In the early stages, this brain-α-MSH-HSC-Neu regulatory axis enhances the clearance of pathogenic microorganisms. However, in the later stages, it contributes to a sustained inflammatory response, thereby impeding tissue repair.

Data Sharing Statement

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval and Consent to Participate

All animal experiments were in accordance with the Army Medical University and Chinese national laboratory animal care and usage guidelines. Animal trial registration number: SYXK (Yu) 2022-0018.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The author(s) report no conflicts of interest in this work.

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