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Research Article

Citrullination of CAMP exacerbating mucosal inflammation in inflammatory bowel disease

Xin Chang,[1,2,3,‡] Haicong Wu,[1,2,3,‡] Yihang Song,[2,3,4,‡] Fenxing Huang,[2,3,4] Yanan Zhu,[2,5] Hongjie Shen,[2,5] Yuntian Ji,[2,3,4] Yu Bai,[2,3,4,*] Zhaoshen Li,[1,2,3,4,*] Shuling Wang,[2,3,4,6,*] Tian Xia[2,3,4,*]

[1]Department of Gastroenterology, The Seventh Affiliated Hospital of Southern Medical University, Foshan 528244, China
[2]Department of Gastroenterology, Changhai Hospital, Naval Medical University, Shanghai 200433, China
[3]National Key Laboratory of Immunity and Inflammation, Naval Medical University, Shanghai 200433, China
[4]National Clinical Research Center for Digestive Diseases, Changhai Hospital, Shanghai 200433, China
[5]Company Eleven of Cadet Battalion Four, College of Basic Medical Sciences, Naval Medical University, Shanghai 200433, China
[6]Institute of Immunology and the CAS Key Laboratory of Innate Immunity and Chronic Disease, Division of Life Sciences and Medicine, University of Science and Technology of China, Hefei 230027, China
*Corresponding authors: Yu Bai, baiyu1998@hotmail.com; Zhaoshen Li, li.zhaoshen@hotmail.com; Shuling Wang, wangshuling0000@126.com; Tian Xia, magicsummer1987@hotmail.com
‡Xin Chang, Haicong Wu, and Yihang Song contributed equally to this work.

Abstract

Background: Cathelicidin (CAMP), plays important roles in pathogen defense, immune regulation, and epithelial barrier maintenance. While previous studies have highlighted its protective function, the post-translational modifications and downstream immune-metabolic effects of CAMP in the pathogenesis of inflammatory bowel disease remain unclear.

Methods: A dextran sodium sulfate (DSS)-induced colitis mouse model was employed to assess the role of CAMP and its citrullination mediated by peptidyl arginine deiminase 4 (PAD4). Proteomic and metaproteomic analyses were performed to investigate microbiota composition and functional shifts. We generated gene-deficient mouse models, CAMP knockout (KO) and PAD4-KO mice, to dissect molecular mechanisms. Epithelial integrity, inflammatory markers, and immune responses have been evaluated at both the protein and mRNA levels. Bone marrow-derived dendritic cells and primary $CD4^+$ T cells were co-cultured to examine the effects of CAMP-related metabolites on antigen presentation and Th17 differentiation. Furthermore, we evaluated the impact of CAMP peptide supplementation and the effects of CAMP-KO mice on DSS-induced colitis.

Results: CAMP citrullination was significantly elevated in DSS-induced colitis mice but restored by PAD4 deletion. Citrullination was found to reduce CAMP protein levels without affecting its transcriptional expression. The absence of CAMP exacerbated intestinal inflammation in DSS-treated mice. Metaproteomic analysis identified 70 differentially expressed proteins and 15 altered microbiota families associated with CAMP deficiency. Elevated levels of arginase-1 and its metabolites, particularly polyamines, enhanced dendritic cell maturation and increased Th17 polarization in CAMP-KO mice.

Conclusions: Our findings highlight that the protein level of CAMP decreased after PAD4-mediated citrullination, thus playing a vital role in regulating taxonomic community structure, restricting arginine metabolism, and regulating dendritic cell–Th17 immune responses in IBD.

Keywords: cathelicidin; peptidyl arginine deiminase 4; citrulliantion; microbiota; inflammatory bowel disease

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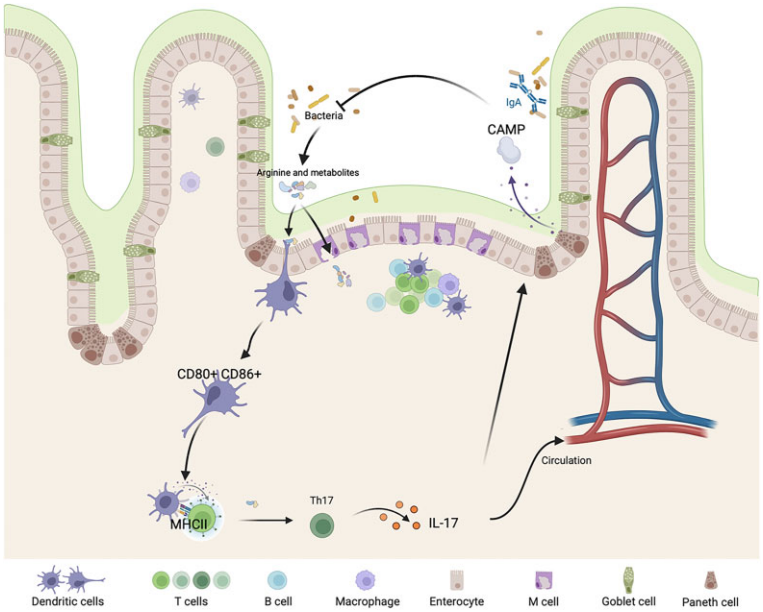
Graphical Abstract



Absence of CAMP contributes to imbalance of intestinal flora with elevated arginase, whose metabolites promote dendritic cell maturation and Th17 polarization.

Introduction

Inflammatory bowel disease (IBD) is a digestive disorder including ulcerative colitis (UC) and Crohn's disease (CD) [1]. In recent years, new biologics and targeted therapies for IBD have continually emerged, such as anti-tumor necrosis factor (TNF) drugs, integrin antagonists, and interleukin (IL) inhibitors [2, 3]. Although these new medications have shown better efficacy than traditional treatments and offer patients more treatment options, the clinical relief is still limited.

While the mechanisms and pathogenesis of IBD remain unclear, it is widely accepted that IBD manifests in genetically susceptible individuals when environmental factors trigger an exaggerated immune response in the intestinal mucosal and immune system, reacting to gut microbial antigens. Gut flora has been recognized as another significant contributor to the maintenance of an intact gut hemostasis which comprises billions of bacteria possessing metabolic, trophic, and immune functions crucial to the body's well-being [4, 5]. During the development of IBD, decreased barrier integrity causes the increased permeability that leads to the overload of microflora [6], which are proposed to provoke abnormal immune responses in the colon and aggravated inflammation [7].

Under such an imbalance of micro-ecology, endogenous antimicrobial peptides (AMPs), including defensins and the cathelicidin family, play crucial roles in regulating the innate immune response. Defensins are small cationic peptides that exert their antimicrobial activity by disrupting the microbial cell membrane, leading to cell lysis. Cathelicidins not only protect against microbiome invasion but also modulate the immune response by interacting with host cells. About 30 cathelicidin family members have been identified in mammalian species. Among them, LL-37 (also known as murine CAMP) is the sole human member, which plays essential roles including antimicrobial activity against pathogens, neutralization of endotoxins, and anti-inflammatory effects [8]. CAMP is stored in immune cells and released upon their activation, or alternatively released by epithelial cells and pancreatic β-cells [9, 10]. Previous research indicates that CAMP not only fights infections caused by bacteria, fungi, and viruses but also regulates immunity, and facilitates tissue repair and angiogenesis [11, 12]. In dextran sodium sulfate (DSS)-induced colitis, administration of CAMP preserved the mucus-secreting layer and significantly reversed the reduction of the mucus layer [13]. Furthermore, CAMP works as a protective mediator in regulating the process of vitamin D in relieving UC [14]. Recent study shows that CAMP can induce neutrophil extracellular traps (NETs) formation and inhibit inflammation in a mouse sepsis model [15]. However, some research found that CAMP contributed to smoking-related airway inflammation by mediating the epithelial–mesenchymal transition [16]. In addition, elevated CAMP forming a complex with DNA was found in plasma and lesions of patients with UC, which revealed that CAMP promoted inflammation by inducing T cell differentiation and activating the toll-like receptor signaling pathway [17]. Considering the dual role of CAMP in inflammation and the immune system, it is important to investigate the mechanisms of action of CAMP thoroughly.

Post-translational modification (PTM) of proteins, involves enzymatic catalysis targeting specific sequences to regulate protein activity, localization, and folding, thus dramatically expanding the functional repertoire of proteins [18]. The immunomodulatory function and activity of CAMP can be influenced by PTM. Lande *et al.* reported that carbamylated-CAMP can maintain both innate and adaptive immune-cells' stimulatory abilities during the development of autoimmune disease, compared with citrullinated-CAMP [19].

The primary structure of LL-37, a member of the CAMP family, contains five arginine residues that are potential targets for citrullination by peptidyl arginine deiminases (PADs), specifically PAD2 and PAD4 [20]. Citrullination refers to the process in which PADs

convert arginine residues in protein peptide chains into citrulline [21]. PADs are calcium-dependent hydrolases that have five different isoforms: PADs 1–4 and PAD6 [22]. Previous findings highlight how PAD2 and PAD4, especially PAD4, as the only isoenzyme of its family that can enter the nucleus, lessen interactions between histones and DNA [23]. Our previous study has revealed the pivotal role of PAD4 in mediating the formation of NETs by citrullination in IBD [24]. Recent study shows that CAMP can take part in NETs formation and inhibit inflammation in a mouse sepsis model [15].

Research conducted in recent years revealed that citrullination impairs LL-37's ability to form complexes with DNA, subsequently damaging its capacity to activate plasmacytoid dendritic cells (DCs) [25]. Furthermore, this modification results in a significant reduction in the peptide's affinity for endotoxins, rendering it unable to prevent endotoxin shock [26]. However, the levels of citrullinated CAMP and its specific role in IBD have not been extensively investigated. Our research utilizes citrullination sequencing and proteomics to investigate the role of PAD4 in the citrullination of CAMP, aiming to elucidate how this modification exacerbates IBD through regulating the microbiota signaling pathway.

Method

Induction of colitis

Male C57BL/6J mice (7–8 weeks old) and Camp$^{-/-}$ mice on C57BL/6J background were purchased from Cyagen. They were allowed free access to standard laboratory chow (Ralston Purina, Chicago, IL, USA) and tap water. All animals were housed in an air-conditioned room with controlled temperature (25°C), humidity (65%–70%), and day/night cycle (12:12 h light : dark). The present study was approved by the University of Hong Kong Committee on the Use of Live Animals for Teaching and Research. Mice were induced with acute colitis by being given 3% DSS (molecular weight, 36–50 kDa; ICN Pharmaceuticals, Costa Mesa, CA, USA) according to the method described by Nakamura *et al.* [8]. The 3% DSS was given in their drinking water for 7 days (from day 0 to day 7). Normal control mice received tap water throughout the experiment.

CAMP treatment

To evaluate the preventive and healing effects of CAMP in UC in mice, CAMP was given either as a parallel treatment, starting together with DSS feeding, or as a posttreatment, starting 7 days after 3% DSS feeding. The full length of the mature CAMP peptide was purchased from Innovagen (Lund, China), and the peptide was dissolved in phosphate-buffered saline (PBS) for rectal administration.

Isolation and induced maturation of bone-marrow-derived dendritic cells

Bone marrow-derived dendritic cells (BMDCs) were isolated from femur and tibia and resuspend with BMDC medium (containing 20 ng/mL GM-CSF and 5–10 ng/mL IL-4) for 5–7 days. To further test the maturation, stimulants such as lipopolysaccharide (LPS, 1 μg/mL) or polyamin (1 μg/mL) can be added on days 6–7, followed by continued culture for 24–48 h. Mature BMDCs will exhibit obvious dendritic protrusions, with increased expression of surface co-stimulatory molecules (CD80, CD86, MHC-II).

T cell isolation from murine spleen and co-culture with BMDCs

Murine splenic T cells were enriched through the use of a commercial magnetic bead-based negative selection kit specifically designed for T cell isolation (EasySep™ Mouse CD4$^+$ T Cell Isolation Kit, STEMCELL Technologies). The selected T cells were seeded in anti-CD3e-coated wells, with the addition of 5 μg/mL anti-CD28 to provide activation and 10 U/mL IL-2 to provide proliferation. CD4$^+$ T cells were identified with CD3 and CD4 antibodies.

The T cells were then carefully pipetted out of wells into pre-warmed T cell medium. The cells were collected and centrifuged at 300 g for 10 min and resuspended in 2 mL of pre-warmed T cell medium and the appropriate concentration was titrated for cell–cell interaction. The stimulation medium was then carefully removed from the BMDCs and they were immediately washed once with serum-free RPMI 1640 complete medium. The T cells were then added to the BMDCs.

Immunohistochemistry of colonic tissues

A Rabbit Immunohistochemistry Application Solutions Kit (Beyotime) was used for immunohistochemistry. To remove the paraffin, paraffin pieces of colon were roasted at 60°C before being submerged in dimethylbenzene, ethyl alcohol concentrations, and ultrapure water. Following blocking with 10% bovine serum albumin, the sections were incubated overnight at a 1 : 200 dilution with rabbit anti-citrullination antibody (1 : 200; Abcam) or rabbit anti-CAMP monoclonal antibody (mAb, 1 : 200; Proteintech), followed by a 1 h incubation with secondary antibody. After being treated with DAB working solution, the tissues were inspected under a microscope at a magnification of 200× . A laser scanning confocal microscope (LSM 800 Zeiss) was used to examine all sample preparations.

Immunofluorescence of colonic tissues

The tight junction protein was tested with the fluorescence system and visualized using immunofluorescence microscopy. Briefly, the colon tissue was stained with rabbit anti-occludin mAb (1 : 200 dilution; Proteintech) and rabbit anti-E-cadherin mAb (1 : 200 dilution; CST), and goat anti-rabbit-FITC and CY3 (1 : 500; Beyotime) were used as secondary Abs for E-cadherin and occludin investigation. After using Hoechest (ThermoFisher) to counterstain the DNA, fluorescence microscopy was used to examine all sample preparations (Zeiss).

Western blots

To get the complete protein, the colons were mashed using a mixer and cleaved by RIPA lysate. Denatured proteins were then isolated using SDS-PAGE. Following that, protein samples were transferred from the polyacrylamide gel to a PVDF membrane at 300 mA for 1.5 h. After 1 h of sealing with 5% nonfat powdered milk, the membranes were incubated overnight at room temperature with primary Abs (1 : 1000 dilution), followed by a 1 h incubation with secondary Abs (1 : 8000 dilution). Finally, using a Millipore ECL Chemiluminescence kit, the blots were created and the integrated density of pixels in each membrane was calculated with Image J software.

Quantitative real-time PCR

RNA was isolated using TRIzol (Life Technologies). RNA was reverse transcribed using iScript (Accurate Biology). Predesigned primers were used to quantify Il6, Tnf, Il12a, and Camp mRNA using SYBR Green SupTaq and a Real-Time PCR System (Applied Biosystems), according to the manufacturer's instructions. Fold-change, normalized to tested cell GAPDH levels relative to the control, was calculated using the ΔΔCT method.

Statistical analysis

All the statistics shown above are expressed as mean ± SD. The statistical analysis was done with the GraphPad Prism 8.0 program. One-way analysis of variance, followed by the Bonferroni *post hoc* test for multiple comparisons multiple comparison tests was utilized. For comparing the differences in protein/mRNA expression level between two independent groups, an unpaired Student's *t*-test was applied. Statistical significance was established by a P value < 0.05.

Results

Citrullination of CAMP participates in the development of IBD

To investigate the mechanisms underlying the occurrence and development of IBD, we constructed a DSS-induced mouse model of enteritis. Through proteomic analysis of intestinal tissues from both control and DSS-treated groups, we identified several differentially expressed candidate proteins, which were categorized based on their functions and associated KEGG pathways (Fig. 1A and B). Excluding proteins with unconfirmed functions, the majority were involved in inorganic ion transport and metabolism, biosynthesis, transport and catabolism of secondary metabolites, and defense mechanisms. Considering that dysbiosis of the gut commensal microbiota contributes to the pathogenesis of IBD, we further analysed the expression of antimicrobial peptide-related families by liquid chromatography mass spectrometery (LC-MS) and found that >50% were up-regulated in DSS-induced inflamed colon. Then, we identified eight differential proteins that were closely related to defense mechanisms, chronic inflammation response and regulation of inflammation response, wound healing, and peptidase activity (Fig. 1C) through enrichment analysis and literature review.

The role of CAMP in the regulation of bacterial flora has aroused our attention. As the sole member of the cathelicidin family in humans, CAMP exhibits protective effects against pathogenic microorganisms and neutralizes endotoxins. It is stored in immune cells and released upon activation or secreted by epithelial cells and pancreatic beta cells. Previous studies have found that high sugar can lead to an increase of mucus-inhibiting bacteria and damage the intestinal barrier. At the same time, it has been proved that CAMP non-specific knockout (KO) can aggravate DSS-induced colitis through microecological changes [27]. However, the effects of PTMs on CAMP's immune function, including changes in activity or protein levels in the context of IBD, remain unexplored. Moreover, prior investigations into CAMP's antibacterial function have primarily focused on *in vitro* validation against single pathogenic strains, and there is a lack of *in vivo* models and broad-spectrum screening relevant to IBD. To address these gaps and further elucidate CAMP's role in maintaining intestinal barriers, we assessed the protein levels of CAMP and occludin, a marker of intestinal epithelial integrity. We observed a significant increase in CAMP levels and a decrease in occludin, E-cadherin, and claudin-1 levels in the intestinal tissues of DSS-treated mice compared to controls (Fig. 1D). Immunofluorescence staining confirmed that this secreted protein is highly expressed in colonic tissue (Fig. 1E). To further elucidate whether CAMP undergoes citrullination during IBD progress, immunohistochemical staining and immunoprecipitation was performed *in vivo*. In colonic tissue, the expression level of citrullination is higher in DSS-induced colitis compared to a healthy control (Fig. 1F). Immunoprecipitation confirmed that DSS-induced colitis significantly increased CAMP citrullination in wild-type (WT) mice but to a lesser extent in the healthy control group (Fig. 1G). Therefore, the level of CAMP as well as its citrullination is increased in the colonic tissue of DSS-induced mice during IBD development.

Citrullination of CAMP contributes to its upregulation at the protein level

PAD4 plays a significant role in facilitating the citrullination of immunomodulatory proteins, including histones and extracellular proteins, such as NF-κB, p65, and CKMT1, involved in various diseases as highlighted in our research. To explore whether PAD4 is also responsible for the citrullination of CAMP, we generated PAD4 KO mice and found an improvement in intestinal barrier function (supplementary Fig. 1A–G, see online supplementary material) with an alleviation of colonic inflammation in DSS-treated mice (supplementary Fig. 1H–J). Consistently, the citrullination level was decreased in the intestinal tissue of mice after PAD4 KO (Fig. 2A and B). Then, we performed immunofluorescence and found the co-localization of PAD4 and CAMP in colon tissues of DSS-induced mice (Fig. 2C). To further elucidate PAD4 regulatory influence on CAMP, we conducted immunoprecipitation assays, confirming PAD4 binding affinity with CAMP and indicating a notable decrease in CAMP citrullination levels in colitis tissues upon PAD4 KO (Fig. 2D). Given that citrullination has an impact on protein abundance or activity [28–30, 31], we investigated the impact of PAD4-mediated citrullination on CAMP behavior. Interestingly, the protein level of CAMP shows significant upregulation after blocking citrullination modification by PAD4 deficiency (Fig. 2E). Additionally, we also examined the mRNA levels of CAMP in mouse colon tissue and found no differences among the groups (Fig. 2F). Taken together, these findings suggest that citrullination of CAMP, mediated by PAD4, contributes to a decrease in its protein abundance, primarily through the regulation of degradation pathways.

CAMP prevents colitis development in mice

Given that PAD4 mediates the citrullination of CAMP, leading to a decrease in its protein level in colon tissues, we generated mice with CAMP-KO to fully elucidate the role of CAMP in the development of IBD. In line with previous literature descriptions, a low concentration of DSS (2.5%) was sufficient to cause dramatic weight loss in CAMP-KO mice, while it only slightly affected WT mice (Fig. 3A). CAMP-KO mice exhibited more severe colitis than did WT mice, as evidenced by shorter colon length (Fig. 3B), and more severe histopathological damage and loss of mucus to the colon (Fig. 3C). Additionally, we evaluated intestinal barrier function by western blotting and immunofluorescence. The expression of E-cadherin and occludin in inflamed colonic tissue by immunofluorescence is lower in CAMP-KO mice (Fig. 3D and E).

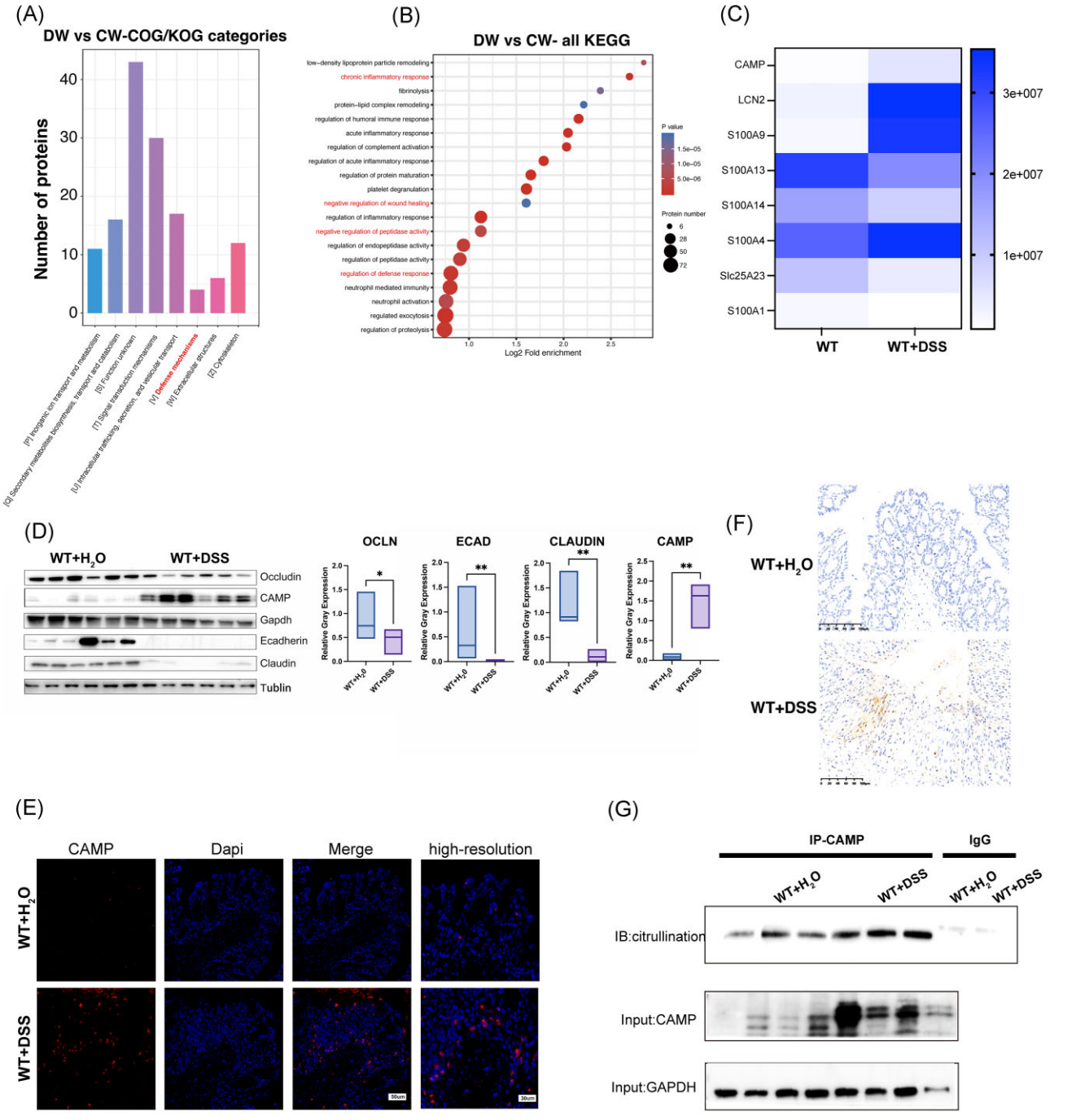


Figure 1. Citrullination of CAMP participates in DSS-induced colitis. (**A**, **B**) GO pathways and KEGG analysis enriched by differentially expressed proteins in LC-MS analysis between DSS-induced colitis and healthy control. (**C**) Expression level of CAMP by LC-MS analysis. (**D**) Representative protein bands of CAMP and occludin in colon tissue ($n = 6$). (**E**) Representative immunofluorescence of CAMP in colon tissue. (**F**) Representative colonic immunohistochemistry of citrullination. (**G**) Immunoprecipitation showing the citrullination of CAMP ($n = 3$). Compared to the healthy control, unpaired Student's *t*-test: * $P < 0.05$; ** $P < 0.01$.

Similarly, following DSS treatment, expression levels of E-cadherin, occludin, and claudin-1 were significantly lower in the colon tissues of CAMP-KO mice compared with WT mice (Fig. 3F). Furthermore, the absence of CAMP led to an increase in proinflammatory cytokine gene expression, indicated by the higher expression levels of IL-6, IL-1B and TNF-a (Fig. 3G). All these data indicate that CAMP deficiency renders mice more susceptible to DSS-induced colitis.

Exogenous supplementation of CAMP reverses colitis

To elucidate the protective effect and efficacy of cathelicidin in IBD, we synthesized a peptide based on our experimental design. Various concentrations of a mouse cathelin-related antimicrobial peptide were administered intrarectally to target tissues in mice with DSS-induced colitis. We assessed both the preventive and healing effects of CAMP, focusing on alterations in gut microbiota

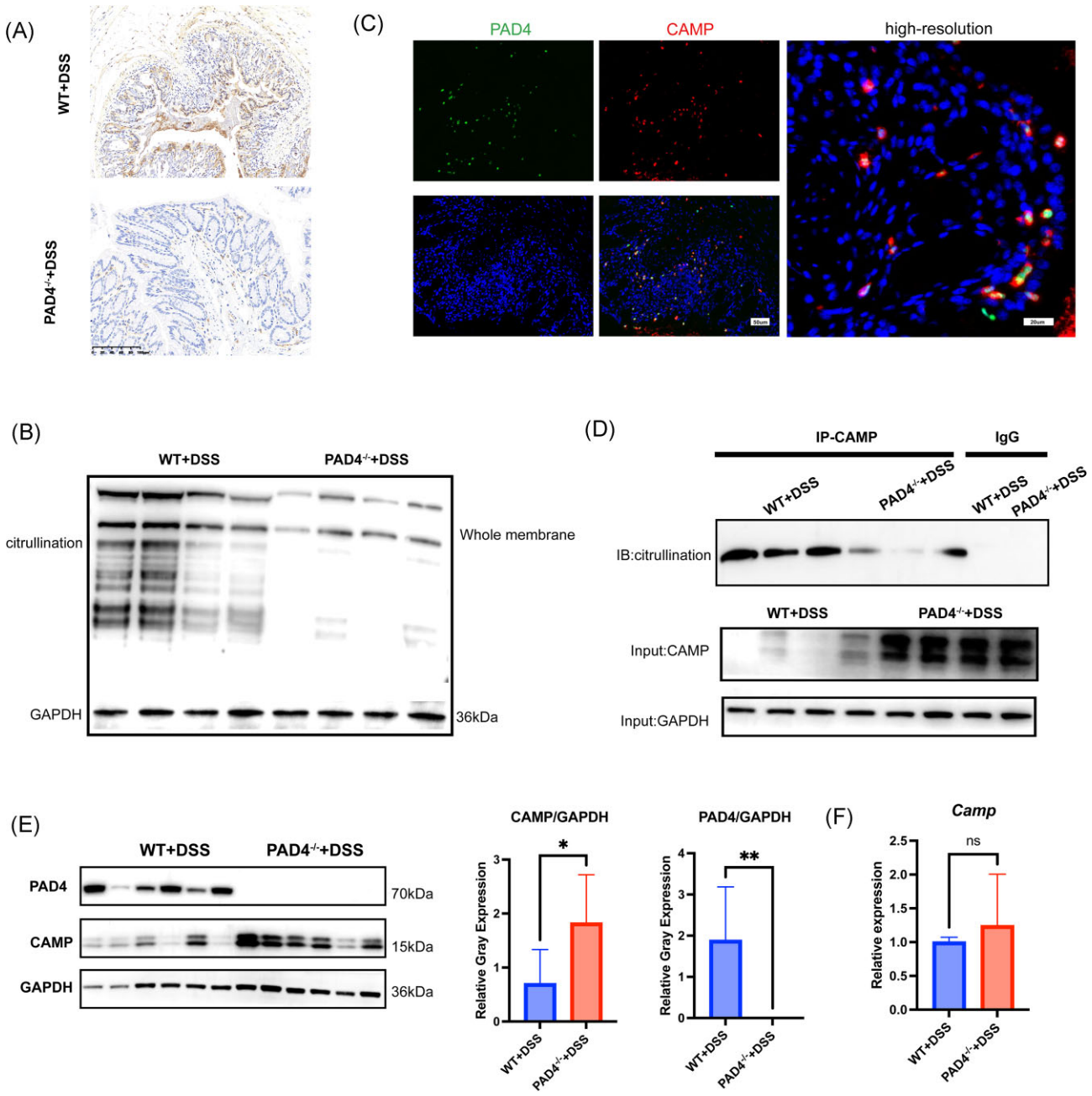


Figure 2. Citrullination of CAMP results in decreased protein level of CAMP. (**A**, **B**) Representative immunohistochemistry and protein bands of citrullination in DSS-induced colitis. (**C**) Representative immunofluorescence of colocalization of PAD4 and CAMP. (**D**) Immunoprecipitation showing the citrullination of CAMP ($n = 3$). (**E**) Representative protein bands of PAD4 and CAMP and their grayscale analysis ($n = 6$). (**F**) Relative mRNA expression of CAMP ($n = 6$). Compared to the WT group, unpaired Student's *t*-test: * $P < 0.05$; ** $P < 0.01$; ns, not significant.

and mucosal mucus during colitis. Mice without CAMP supplementation exhibited more severe colitis, whereas those receiving the synthetic CAMP peptide, particularly at higher doses, showed negligible symptoms. Throughout the modeling period, mice experienced varying degrees of symptoms based on the supplementation conditions. Mice treated with CAMP demonstrated increased body weights, reduced disease symptoms, and less colonic mucosal damage compared to colitis mice treated with PBS. Notably, clinical features indicative of colonic inflammation and tissue destruction were alleviated in a dose-dependent manner with increasing concentrations of CAMP peptide (Fig. 4A and B). These mice displayed accumulation of epithelial erosion, a loss of crypt architecture, and reduced mucus barrier (Fig. 4C).

To further detect the intestinal barrier function, the representative molecules of tight junctions (claudin and occludin family) and adherent junctions (E-cadherin) were further confirmed at the protein level. The colonic tissues were analyzed by western blot, which indicated the remission of barrier function in the CAMP treatment group (Fig. 4D and E). To evaluate the levels of inflammatory factor in colonic tissue, real time-PCR was conducted. Transcriptome analysis indicated decreased levels of inflammatory cytokines with the treatment of CAMP (Fig. 4F). Although there was no statistical difference when comparing the different treatment doses, the expression level of the PBS group is obviously lower than that of the CAMP-treated group, and the expression level is dose dependent. Furthermore, the intestinal

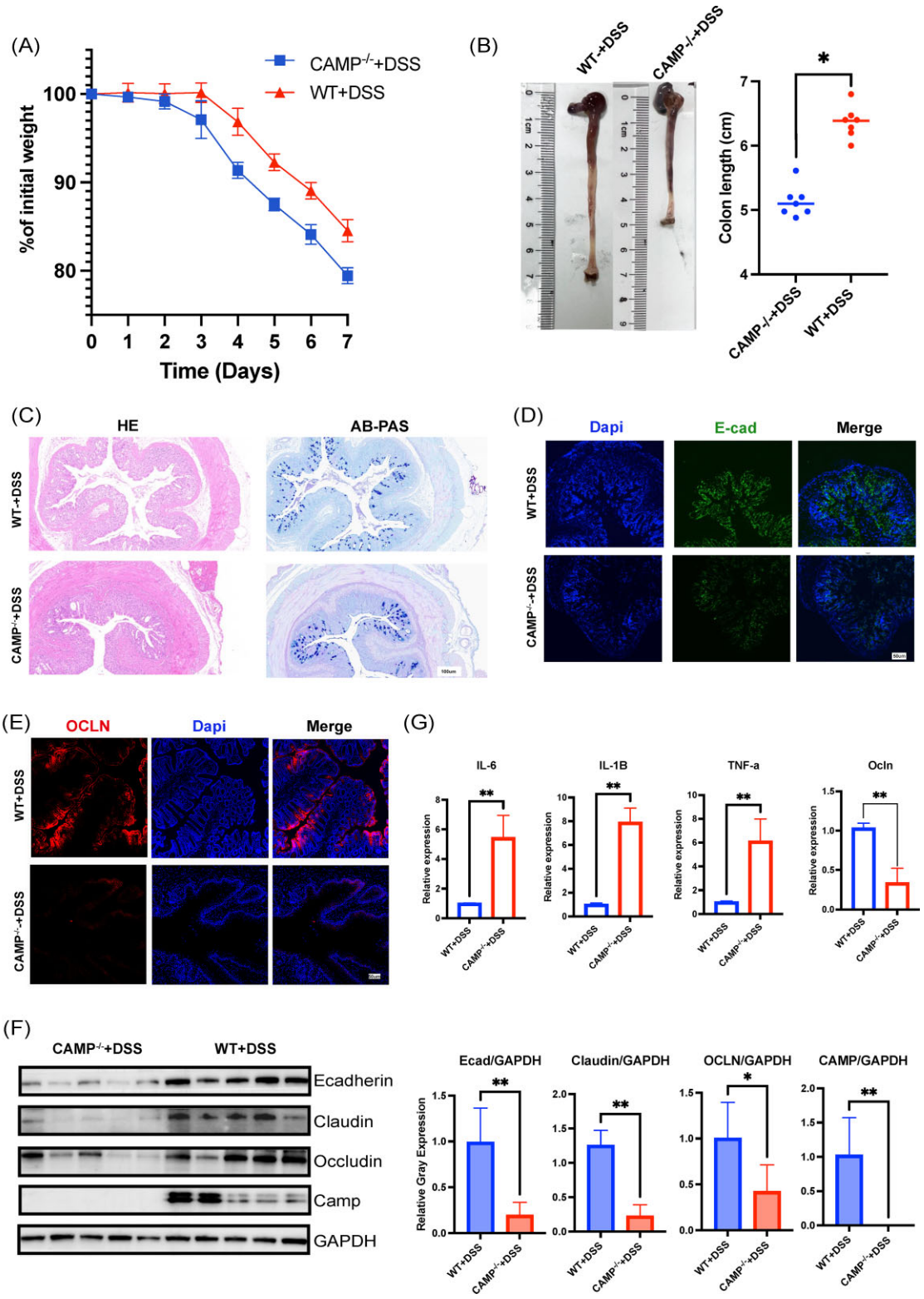


Figure 3. Deletion of CAMP increases the severity of DSS-induced colitis in mice. (**A**) Body weight changes ($n = 6$). (**B**) Representative photographs of the colon and changes in colon length ($n = 6$). (**C**) Representative micrographs of HE and AB-PAS staining. (**D**, **E**) Representative immunofluorescence of E-cadherin and occludin in colon tissue. (**F**) Representative protein bands and grayscale analysis ($n = 5$). (**G**) Relative mRNA expression of pro-inflammation and barrier indicators ($n = 6$). Unpaired Student's *t*-test: * $P < 0.05$; ** $P < 0.01$.

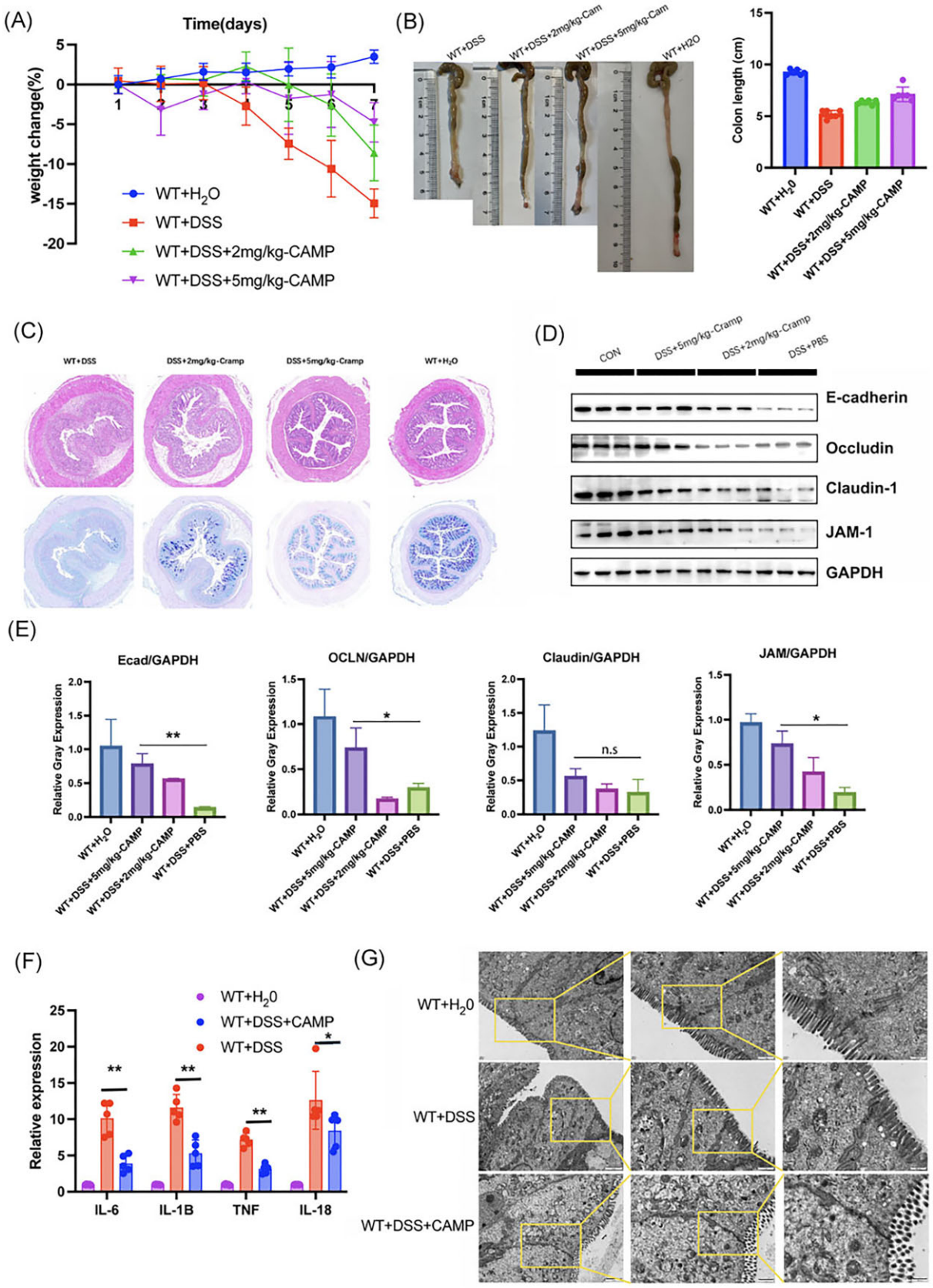


Figure 4. Effect of CAMP administration on the symptoms of DSS-induced colitis in mice. (**A**) Body weight changes ($n = 6$). (**B**) Representative photographs of the colon and changes in colon length ($n = 6$). Colors represent different groups. (**C**) Representative micrographs of HE and AB-PAS staining. (**D**) Representative protein bands of colonic E-cadherin, JAM-1, claudin, and occludin in colon tissue. (**E**) Relative greyscale analysis of the bands ($n = 3$). (**F**) Relative mRNA expression of inflammation indicators. (**G**) Representative micrographs of barrier structure in the intestinal epithelium by TEM. Scale bar: 500 nm. Data are expressed as mean ± SEM. Compared to the control group: * $P < 0.05$; ** $P < 0.01$.

structure morphology of the samples, such as tight junctions and epithelium microvilli arrangements, were observed by SEM. In the CAMP-treated group, the microvilli of the small intestine epithelium were more neatly arranged, tight junction structures appeared more normal, and epithelial cells maintained their integrity compared to the PBS group (Fig. 4G).

Collectively, these data demonstrate that CAMP administration prevents DSS-induced disruption of the intestinal barrier in mice.

CAMP greatly alters the taxonomic community structure and Arg metabolism in the intestine

CAMP acts as a critical human antimicrobial peptide through various mechanisms. We employed MS-based metaproteomics to analyze bacterial proteins present in the colons of both CAMP-KO and littermate WT mice. A total of 2962 bacterial protein peptides from 20 species genera were identified by MS. Metaproteomic data were assessed on multiple taxonomic levels. At the phyla level, Firmicutes and Bacteroidetes were observed as the most dominant phyla, with *Acetatifactor muris*, *Paramuribaculum intestinale*, *Muribaculum intestinale*, *Mucispirillum schaedleri*, *Akkermansia muciniphila*, and *Acutalibacter muris* species making up substantial smaller fractions at the species level (Fig. 5A and B). The relative number of *A. muris* and *Bacteroides caecimuris* protein groups was higher for CAMP-KO than WT mice. The lower LFQ values for the quantifiable protein of *M. intestinale* and *Bifidobacterium pseudolongum* were noted in the colon contents of CAMP-KO mice (Fig. 5B). Notably, three metabolites involved in arginine biosynthesis were identified, generated from *M. intestinale*, *Megasphaera hexanoica*, and *Sporidiobolus salmonicolor*. Furthermore, *M. hexanoica* and *S. salmonicolor* were found to be highly expressed in CAMP-KO colitis mice (Fig. 5C). These altered gut microbiota exacerbate disease progression during IBD. Mice were gavaged with the bacterial solution derived from CAMP-KO mice to mimic the clinical fecal microbiota transplantation (FMT) pattern in germ-free mice, which led to more severe weight loss, shortened intestine, and increased pathological severity (supplementary Fig. 2A–D, see online supplementary material). Fecal microbiota transplantation (FMT) has shown promising therapeutic potential for IBD, particularly in ulcerative colitis. However, in CAMP-deficient mice, fecal microbiota transplantation involving the ten-strain bacterial consortium resulted in exacerbated intestinal inflammation.

Protein domains are usually independently folded regions of a polypeptide chain and serve specific functions. Functional pathway analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. And 220 different expression bacterial proteins mapped to 32 KEGG Orthology-level pathways (Fig. 5C and D). The complete proteome of the gut microbiota in CAMP-deficient mice with IBD shows high expression of bacterial extracellular solute-binding protein domains (Fig. 5E), which implies that bacterial metabolites play a crucial role in modulating host–microbiota immune interactions through adhesion and antigen presentation. The most abundant functions were lysine degradation, arginine biosynthesis, and nitrogen metabolism (Fig. 5F). In GO analysis, the up-regulated proteins in CAMP-KO mice were mainly enriched in amino acid metabolic processes and extracellular regions with cell surface antigens (Fig. 5G and H). These findings suggest that amino acid metabolism and its enzymes are critical regulators of autoimmune disease, especially Arg metabolism. Moreover, the microbiota metabolism mediated by CAMP may regulate the protein recognition of microbial antigens.

Arginine/polyamine-mediated DCs/Th17 axis in CAMP-KO IBD mice

Recent advances in research have increasingly highlighted the pivotal role of metabolic pathways in modulating epithelial function and immune responses in a range of autoimmune disease. DCs are sentinels of immunity. The expression of a particular set of recognition receptors or the metabolites of a pathogen allows DCs to initiate and trigger T cell-dependent immune responses. Arginine (Arg) has been well investigated as an anti-inflammatory factor related to macrophages and the effect of its metabolites (such as urea and polyamines) on immune cells in psoriasis have been discussed recently. However, relevant studies in the context of IBD are still limited.

To explore the association between Arg1 and CAMP, immunohistochemical analysis was performed to assess Arg1 expression in intestinal tissue sections from WT IBD mice and CAMP-deficient mice (Fig. 5I). Immunofluorescence has been used for testing Arg as the self-antigens presented by DCs initiating the activation of further inflammation (Fig. 5J). These results indicate that CAMP deficiency leads to upregulated Arg expression and increased infiltration of DCs, suggesting that CAMP deficiency may influence the local immune microenvironment by modulating arginine metabolites (such as urea and polyamines) and promoting antigen-presenting cell recruitment, potentially contributing to the exacerbation of intestinal inflammation in the context of IBD.

To investigate the impact of decreasing self-antigens on BMDC maturation, BMDCs were induced with low/mid/high polyamine peptide before the assessment of the relative markers. At the same time, primary T cells isolated from mouse spleens were induced and co-cultured with induced DCs (Fig. 6A). Before co-culturing, primary cells were identified by flow cytometric staining for CD3 and CD4. The high proportion of $CD3^+CD4^+$ cells (≥85%) validated the efficiency and reliability of the magnetic bead-based isolation method (Fig. 6B).

Bright microscopic observation demonstrated an elevated level of cell–cell communication accompanied by an increase in polyamine peptides (Fig. 6C), as well as the maturation markers of DCs. To further evaluate the immunomodulatory potential of the polyamine peptides, we analyzed the expression of maturation markers in BMDCs following stimulation. Compared to the low-stimulatory conditions, this complex elicited a significantly higher expression of key maturation markers, including CD80, CD86, and MHC class II (Fig. 6D–G). Flow cytometry analysis also indicated an increase in $IL17A^+$ $CD4^+$ T cells percentage when co-cultured with BMDCs pre-treated with medium and high levels of polyamine peptide, 2.63% and 4.63%, respectively (Fig. 6H and I). In *in vivo* experiments, the benefits of CAMP supplementation in alleviating IBD were reduced by high concentrations of Arg metabolites (supplementary Fig. 3A and B, see online supplementary material), accompanied by significant expression of $CD4^+$ T cells (supplementary Fig. 3C).

These findings suggest that the polyamine peptide plays a critical role in promoting BMDC maturation, thereby contributing to the modulation of the DCs/Th17 axis in the context of immunological disorders.

Discussion

As the sole cathelicidin gene within the antimicrobial peptides family, CAMP (referred to as LL-37 in humans) constitutes a class of positively charged polypeptides known for their

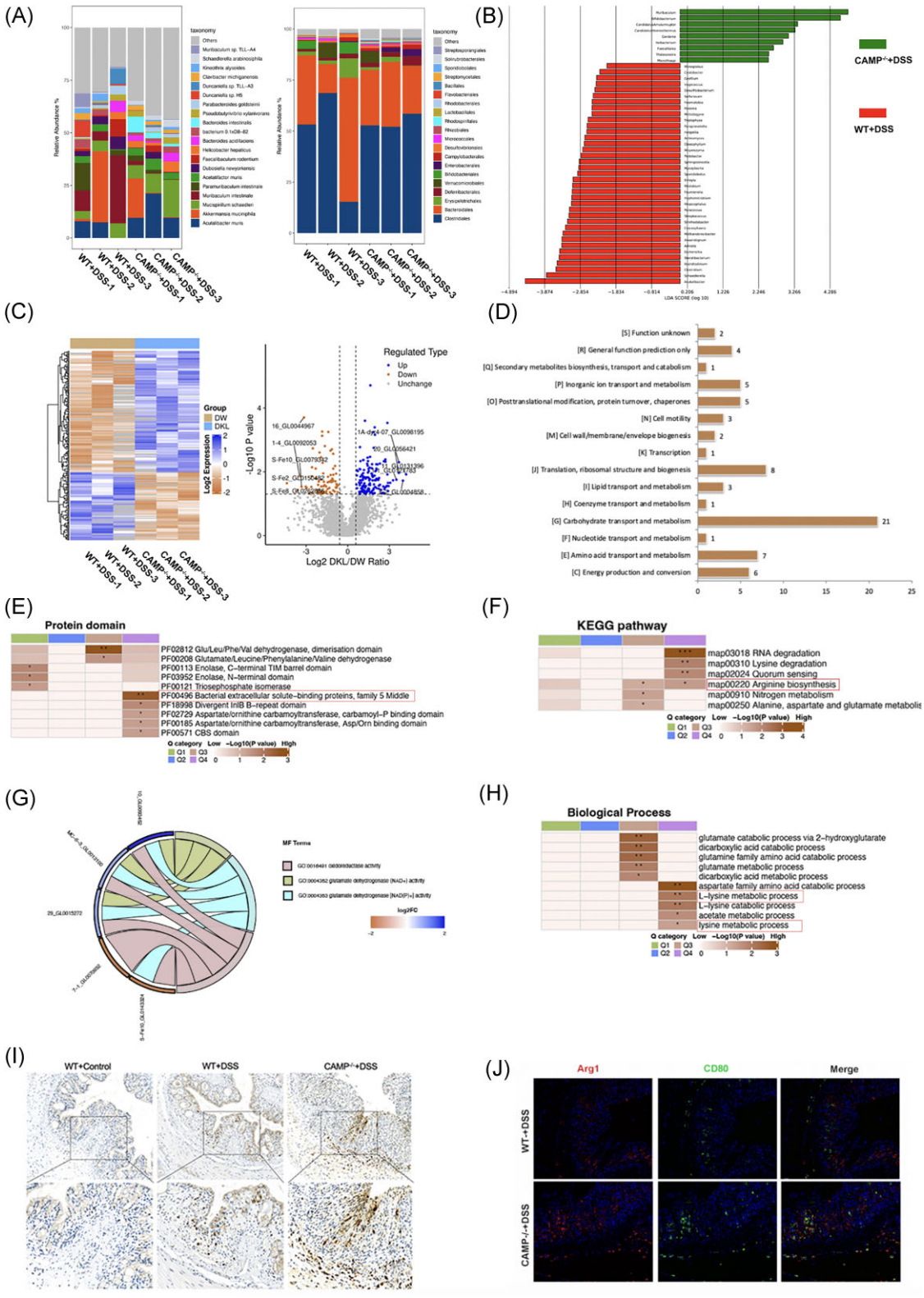


Figure 5. CAMP alters the taxonomic community structure in the intestine. (**A**) Taxonomic distribution of species between WT and CAMP-KO mice. (**B**) Species with the most significant difference by LEfSe analysis. (**C**) Cluster analysis of the relative expression levels of differentially expressed proteins. (**D**) Regulated classification of the different expression proteins. (**E**) Protein domain, (**F**) KGEE pathway analysis, (**G**) molecular functions, and (**H**) biological processes enriched by different proteins between WT and CAMP-KO mice. (**I**) Expression of Arg and (**J**) co-localization analysis in DCs.

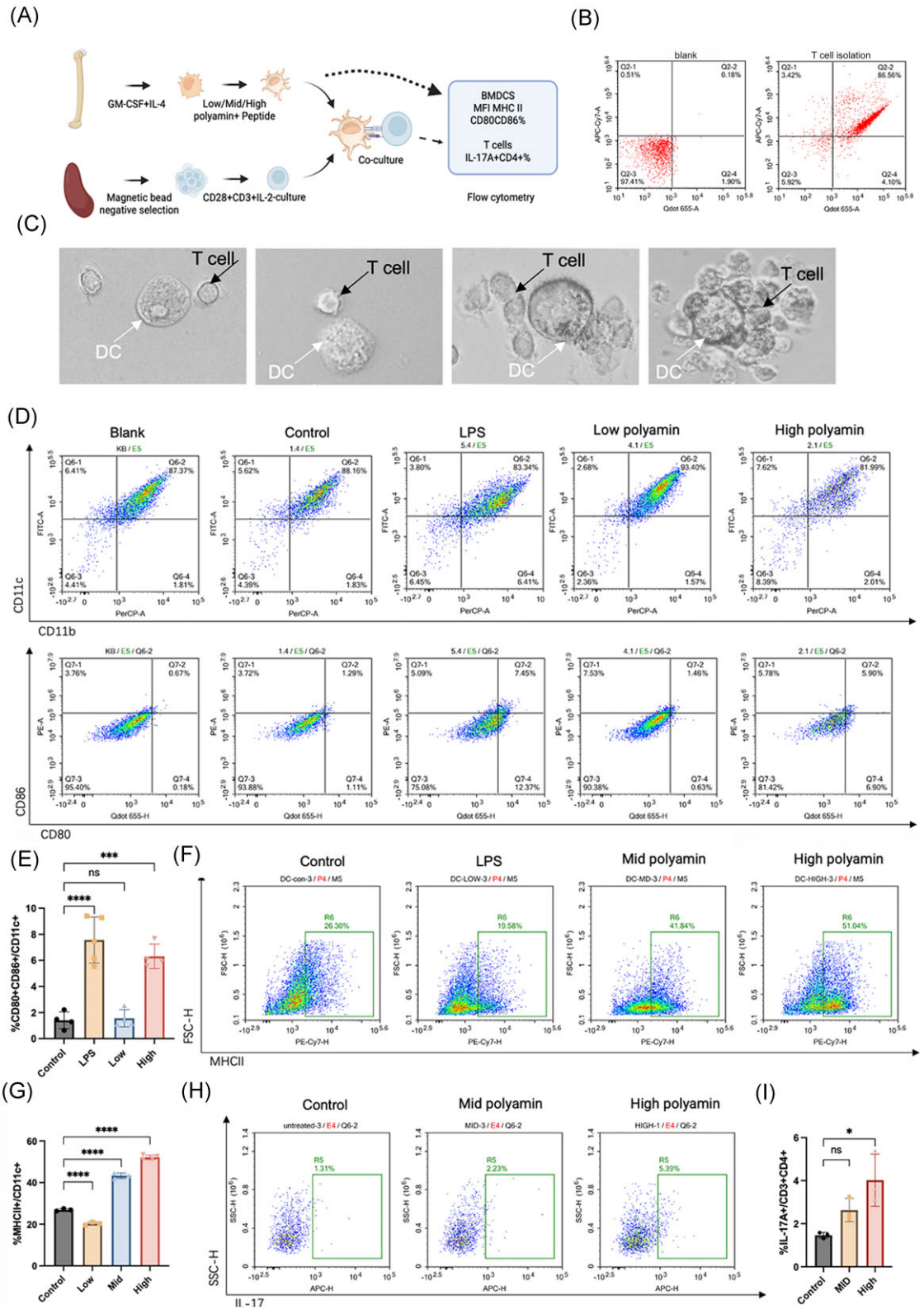


Figure 6. Evaluation of polyamine peptide-induced modulation of BMDC maturation and subsequent CD4$^+$ T cell activation. (**A**) Schematic representation of the experimental workflow. (**B**) Identification of primary CD4$^+$ T cells with cytometry. (**C**) Bright-field microscopy showing enhanced cell–cell interactions with increasing levels of polyamine peptides, suggesting improved immunological synapse formation. (**D**–**G**) Flow cytometry analysis demonstrating upregulated expression of DC maturation markers (CD80, CD86, and MHC class II) in response to increasing concentrations of polyamine peptides. (**H**, **I**) Co-culture with BMDCs pre-treated with medium or high concentrations of polyamine peptides led to a marked increase in IL-17A$^+$ CD4$^+$ T cells. Data are representative of at least three independent experiments.

broad-spectrum antimicrobial activity [32, 33]. These positively charged AMPs adhere to negatively charged bacterial cell membranes, leading to interactions that may alter bacterial cell morphology and, ultimately, result in cell death [34]. Although some clinical trials have shown that CD patients who take oral vitamin D supplements experience an increase in CAMP levels, accompanied by a decrease in the Crohn's Disease Activity Index and intestinal permeability [35], the underlying mechanisms whereby CAMP is upregulated and takes part in IBD have not yet been completely elucidated. This work has shown that in DSS-induced colitis mice, increased production of the antimicrobial peptide CAMP is concomitant with activation of PAD4 in colonic tissue. Furthermore, we observed that citrullination of CAMP mediated by PAD4 leads to a significant decrease in its protein levels, an effect that can be reversed by knocking out PAD4. These findings suggest that PAD4 plays a crucial role in regulating CAMP abundance in the context of IBD. Importantly, our results reveal that elevated levels of CAMP serve a protective role against the progression of IBD. Additionally, we propose that the maintenance of microbiome homeostasis by CAMP may be achieved through the modulation of the taxonomic community structure within the intestine. Our findings provide the first evidence linking CAMP signaling to immune regulation in IBD through a comprehensive analysis of its upstream and downstream pathways in association with gut microbiota and metabolic remodeling.

A growing body of evidence suggests that inflammation is strongly regulated by posttranslational modification of immunomodulators [36], especially by citrullination, that are present at inflammatory foci [37, 38]. Indeed, citrullination modulated by PAD4 has been implicated in various chronic and autoimmune diseases, such as rheumatoid arthritis, interstitial lung diseases, multiple sclerosis, and specific cancer types, among others [38]. In addition, CAMP is highly susceptible to citrullination by PAD4 due to the presence of five arginine residues [20]. However, there is a lack of direct proof to show that the function and expression level of CAMP is connected with the citrullination mediated by PAD4 in IBD. In this study, we demonstrate that citrullination is markedly elevated in mice with DSS-induced colitis. Nonhistone protein modification was regulated by PAD4 and involved CAMP. This post-translational modification substantially reduced protein abundance, leading to alterations in gut microbiota composition and exacerbation of disease progression. Notably, supplementation with CAMP peptides was able to modulate the disease course.

As mentioned previously, LL-37 is the only member of cathelicidin family in humans, and the same protein is named CAMP in mice. It has been reported to exhibit widespread distribution across various tissues and cell types, such as epithelial and immune cells. In the single-cell data we examined, it was found that the expression of CAMP in colitis mice is primarily localized in neutrophils (supplementary Fig. 4, see online supplementary material), which is consistent with previous literature. The role of CAMP in regulating intestinal microecology is significant, with no changes in the digestive tract under the basic state (supplementary Fig. 5, see online supplementary material). Considering the gut microbiota plays a central role in modulating the host immune system, metabolism, and development, CAMP is essential for influencing the onset and progression of several diseases, including IBD. Previous studies focus on the functioning of a single virus or bacterial infection, however metabolic regulation of downstream flora is worth further discussion. Recent studies have demonstrated that metaproteomics, which involves the high-throughput characterization of the complete profile of microbial proteins, offers promising insights into the functional aspects of microbiome research. In this study we used MS-based metaproteomics to detect bacterial proteins present in CAMP-KO and WT mice, both with DSS-induced colitis. Our analysis identified a total of 2 926 unique bacterial proteins from 20 genera. However, although we completed an FMT experiment with CAMP-KO mice with no obvious improvement, human validation, such as with the use of FMT from IBD patients to CAMP-KO mice, will help clarify whether the observed microbial dysbiosis alone is sufficient to exacerbate colitis, independent of other host-related factors mediated by CAMP. This ongoing research will provide further insight into the specific mechanistic roles that FMT plays and the specific bacteria involved in alleviating UC, and may provide a sound scientific basis for future related studies.

Notably, we found that *M. hexanoica* and *S. salmonicolor* were highly expressed in CAMP-KO mice, particularly in relation to the arginine biosynthesis pathway. M.egasphaera hexnaoica is an anaerobic bacterium that has an efficient reverse β-oxidation pathway. In a previous study, the strain showed excellent production of medium chain carboxylic acids using fructose as an electron donor [39]. Some research has reported that *M. hexanoica* exhibited production of various short- and medium-chain carboxylic acids (acetic acid, butyric acid, pentanoic acid, isobutyric acid, isovaleric acid, hexanoic acid, heptanoic acid and octanoic acid) [40, 41]. In IBD, these two strains have not been reported, which deserves further research in the future.

Interestingly, the arginine biosynthesis pathway in the microbiota was altered, as indicated by the changes in the abundance of upstream metabolites and enzymes, resulting in lower levels of arginine and higher levels of aspartate in the colon after CAMP deletion. Many researchers demonstrated that arginine metabolism plays a crucial role in the pathophysiology of IBD [42]. Its components, the arginine–creatine and arginine–polyamine axes, primarily exert protective effects against inflammation, in contrast to the pro-inflammatory arginine–nitric oxide (iNOS) axis [43]. Arginine biosynthesis and metabolism serves as an essential substrate in bacterial metabolism and host–pathogen interactions. In *Escherichia coli* and *Salmonella*, arginine metabolism contributes to energy production, acid resistance, and the regulation of virulence gene expression [44, 45]. Many other bacteria and fungi use the more widespread and energetically more favorable non-linear or cyclic pathway for arginine biosynthesis. Meanwhile, targeting the microbiota–arginine metabolism axis has been revealed as a cure to alleviate DSS-induced IBD in mice [46]. Arginine is a semi-essential amino acid essential for protein synthesis and closely linked to gut pathophysiology [47]. Much IBD work regarding Arg metabolic pathways has been summarized [43, 46]. The roles of the Arg–nitric oxide and Arg–urea pathways in IBD remain controversial, while the Arg–polyamine and Arg–creatine pathways appear to be protective [43]. Supplementation with Arg shows promise as a therapeutic approach for IBD; however, the optimal dosage may vary among individuals and disease stages [43, 48].

Furthermore, exploring the inhibitors of Arg metabolic pathways and other treatment modalities warrants further investigation. In various immunopathological conditions, the upregulation of arginase and its downstream metabolites has been implicated in the amplification of immune responses as antigen presentation [49, 50]. Accumulating evidence has highlighted the pivotal role of antigen presentation in the initiation and progression of IBD [51–52]. In particular, peptides derived from microbial or host sources can serve as self-antigens when presented by antigen-presenting

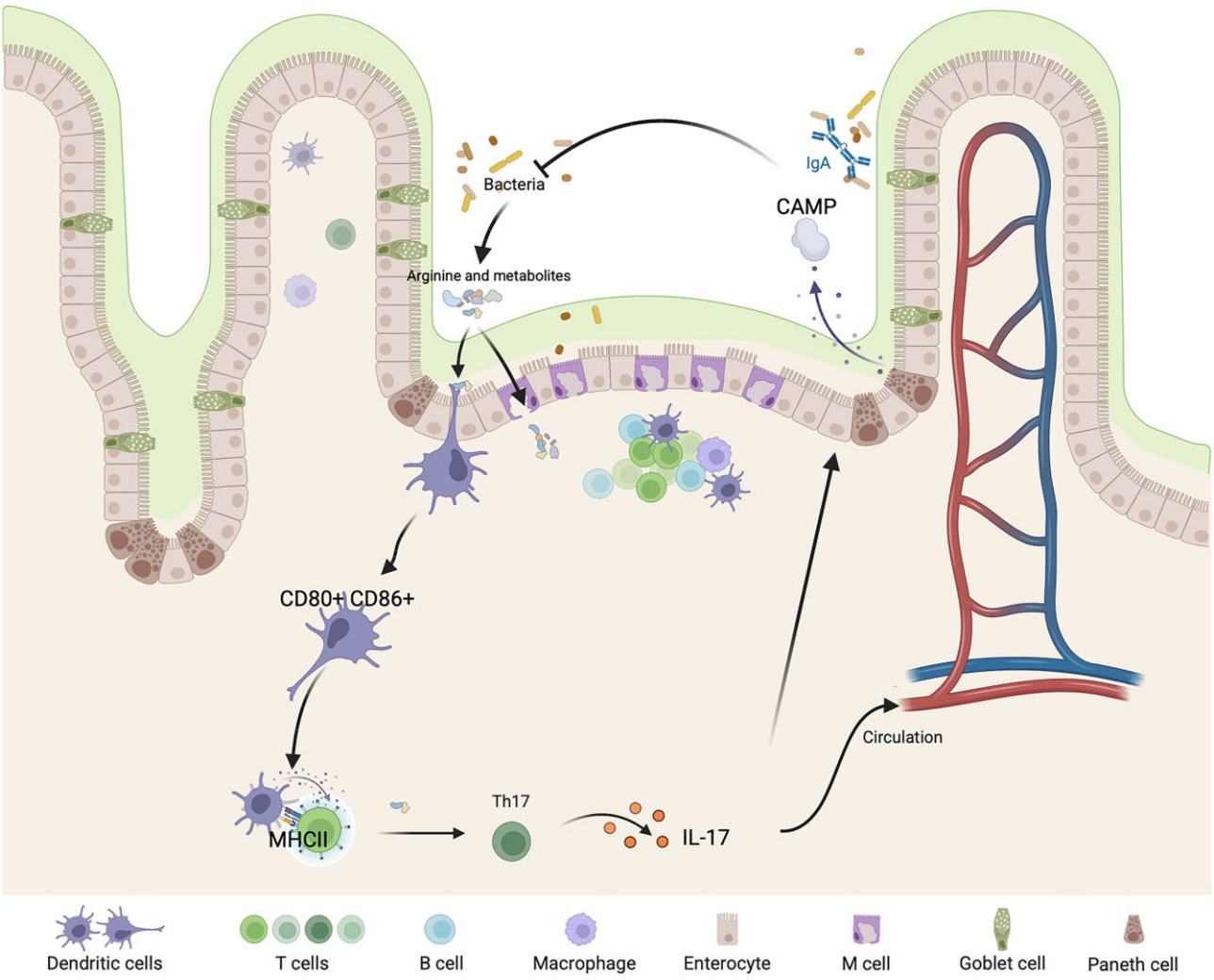


Figure 7. Schematic model illustrating the protective role of CAMP in regulating the DC–Th17 axis during intestinal inflammation.

cells (APCs), triggering aberrant T cell activation [53, 54]. Lou *et al*. revealed that the RNA + polyamine + Hnrnpa1226-237 complex is considered as a self-antigen [49], with aberrant polyamine metabolism driven by the overexpression of arginase-1 in psoriatic keratinocytes, enhancing the sensing of this complex by DCs, and uncovering that arginase1 (Arg1)/polyamine is overexpressed in psoriasis patients and mice, inducing the *in situ* accumulation of self-antigens. Targeting the reduction of peptide levels and their associated antigen presentation in the intestinal environment has increasingly been recognized as a promising therapeutic strategy for autoimmune disorders [55].

This study highlights upstream modification and regulation of CAMP as well as the downstream influence of microbiota. Furthermore, in conjunction with analysis of intestinal metabolic alterations, we examined the influence of arginine-derived metabolites (polyamines) on DC maturation and their antigen presentation capacity. The results indicate that arginine metabolism plays a pivotal role in shaping the DC/Th17 axis, thereby contributing to immune modulation in the gut microenvironment. Within the intestinal microenvironment, the abundance of such peptides and their presentation via MHC class II molecules have been linked to heightened immune responses [56]. Recent studies have demonstrated that reducing peptide levels and limiting their antigen presentation can attenuate immune activation, especially along the DC–Th17 axis [55]. Consequently, modulation of peptide abundance and presentation in the gut has emerged as a promising therapeutic target for the treatment of autoimmune diseases.

In summary, in addition to our strong suggestion that CAMP is a protective mediator by regulating the DC/Th17 axis in IBD (Fig. 7), we demonstrate that CAMP can be citrullinated by PAD4 which results in a degradation in protein level. However, the lack of validation for other antimicrobial peptides regulated by PAD4 limits the ability to fully establish the unique role of CAMP in citrullination during IBD development. Moreover, due to a lack of immunoprecipitating antibody, the specific site of citrullination in CAMP has not been well documented in this study. As we faced challenges in identifying specific bacterial species within the flora, additional macroprotein samples are necessary to achieve high-precision screening and verification. Our data provide substantial evidence for the novel hypothesis that CAMP, beyond being affected by PAD4-mediated citrullination, can also regulate the microbiota and influence downstream metabolic processes in immune cells of IBD.

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Author contributions

X.C., S.W., Y.B., and Z.L. conceived and designed the research and supervised the studies. X.C., H.W., Y.S., F.H., Y.Z., H.S., and Y.J. performed the experiments, conducted the statistical analyses and interpreted the data. X.C. and H.W. wrote the manuscript. S.W., T.X., and Y.B. revised the manuscript. All authors read and approved the final manuscript.

Supplementary data

Supplementary data is available at *PCMEDI Journal* online.

Conflict of interest

All the authors declare that they have no conflict of interest.

Ethics approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Changhai Hospital (Approval No. CHEC [A.E]2025-064). Animal experiment protocols complied with the Guide for the Care and Use of Laboratory Animals. Written informed consent to participate was obtained from all participants prior to inclusion in the study.

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