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RESEARCH ARTICLE

Chemerin aggravates DSS-induced colitis by suppressing M2 macrophage polarization

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Chemerin is present in various inflammatory sites and is closely involved in tissue inflammation. Recent studies have demonstrated that chemerin treatment can cause either anti-inflammatory or pro-inflammatory effects according to the disease model being investigated. Elevated circulating chemerin was recently found in patients with inflammatory bowel disease (IBD); however, the role of chemerin in intestinal inflammation remains unknown. In this study, we demonstrated that the administration of exogenous chemerin (aa17–156) aggravated the severity of dextran sulfate sodium (DSS)-induced colitis, which was characterized by higher clinical scores, extensive mucosal damage and significantly increased local and systemic production of pro-inflammatory cytokines, including IL-6, TNF-α and interferon (IFN-γ). Interestingly, chemerin did not appear to influence the magnitudes of inflammatory infiltrates in the colons, but did result in significantly decreased colonic expression of M2 macrophage-associated genes, including Arginase 1 (Arg-1), Ym1, FIZZ1 and IL-10, following DSS exposure, suggesting an impaired M2 macrophage skewing *in vivo*. Furthermore, an *in vitro* experiment showed that the addition of chemerin directly suppressed M2 macrophage-associated gene expression and STAT6 phosphorylation in IL-4-stimulated macrophages. Significantly elevated chemerin levels were found in colons from DSS-exposed mice and from ulcerative colitis (UC) patients and appeared to positively correlate with disease severity. Moreover, the *in vivo* administration of neutralizing anti-chemerin antibody significantly improved intestinal inflammation following DSS exposure. Taken together, our findings reveal a pro-inflammatory role for chemerin in DSS-induced colitis and the ability of chemerin to suppress the anti-inflammatory M2 macrophage response. Our study also suggests that upregulated chemerin in inflamed colons may contribute to the pathogenesis of IBD.
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INTRODUCTION

Chemerin was originally identified as a chemoattractant protein primarily mediating the chemotaxis of plasmacytoid dendritic cells (pDCs) and natural killer (NK) cells.[1,2] Recent studies have suggested that chemerin has other biological functions, such as the regulation of adipocyte differentiation and metabolism as an adipokine.[3,4] Chemerin mRNA is detected at high levels in the skin, lung, colon and liver.[4] Interestingly, chemerin is synthesized as an inactive pro-chemerin, which can be rapidly converted into an active form after the proteolytic removal of 6 or 7 amino acids from its C-terminus by neutrophil-derived serine proteases at sites of inflammation,[5] suggesting a possible role of chemerin in tissue inflammation. Increased chemerin levels are found in the skin lesions of lupus and acute psoriasis and in the kidneys of patients with severe lupus nephritis, which is associated with the tissue infiltration of pDCs, suggesting that chemerin may play a pro-inflammatory role in autoimmune diseases, possibly by mediating pDC chemotaxis.[1,6,7] However, several studies have also demonstrated that the administration of exogenous chemerin exerts potential anti-inflammatory activities in experimental peritonitis and acute lung injury.[8,9] In addition, several synthetic peptides derived from the C-terminal domain of human or mouse pro-chemerin were reported to display significant bioactivity.[8,10,11] For example, the chemerin15 (C15) peptide was shown to ameliorate zymosan-induced peritonitis, which could be due to its capacity to enhance macrophage phagocytosis of microbial particles and apoptotic cells.[8,11] However,

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another study failed to detect any activity of the C15 peptide.[9] It is still unknown whether these peptides are generated *in vivo* due to the lack of a detection assay.

Inflammatory bowel diseases (IBDs), clinically consisting of ulcerative colitis (UC) and Crohn's disease (CD), are chronic inflammatory disorders of the gastrointestinal tract. The incidence of IBD has risen greatly worldwide over the past decades, and extensive attention has been focused on exploring the pathogenesis of IBD. Immunologically, IBD is currently thought to arise from aberrant innate and/or adaptive immune responses to the resident intestinal microflora in genetically predisposed individuals due to the breakdown in diverse regulatory mechanisms that maintain intestinal homeostasis.[12] Multiple immunological factors have been suggested to be major contributors to IBD, including the functional skew of intestinal dendritic cells (DCs) and macrophages from tolerogenic to inflammatory type cells, the colonic recruitment of inflammatory cells such as neutrophils and inflammatory monocytes, and the imbalance between regulatory T cells and pathogenic Th1 and Th17 cells.[13–15] In addition, the role of a defective epithelial barrier has recently been emphasized, as inappropriately activated intestinal epithelial cells are able to initiate and precipitate pathological inflammatory responses in IBD, partly by secreting pro-inflammatory cytokines such as TNF-α and IL-6.[16]

Much of our current understanding of IBD pathogenesis has resulted from studies in various animal models, among which dextran sulfate sodium (DSS)-induced experimental colitis is most commonly used.[17,18] DSS-induced colitis has been considered to be driven by innate immune cells, primarily neutrophils, macrophages and DCs,[19–21] as disease occurs in T and B cell-deficient mice.[22] DSS-induced colitis is triggered by directly disrupting the epithelial barrier, allowing intestinal bacteria to penetrate the injured mucosa and perpetuate mucosal inflammation, which is characterized by increased inflammatory infiltrates and an excessive production of pro-inflammatory cytokines and causes a destructive effect, leading to colitis exacerbation.[23] Macrophages are one of the most abundant leukocytes in the colon and closely involved in IBD pathogenesis.[24] Activated macrophages can be functionally divided into the classically activated or M1 type and the alternatively activated or M2 type in response to the different stimuli in the local microenvironment.[25] It has been reported that M1 and M2 macrophages play opposing roles in DSS-induced colitis.[20] M1 macrophages contribute to the pathogenesis of DSS-induced colitis primarily by secreting pro-inflammatory cytokines and causing tissue damage.[24] In contrast, M2 macrophages contribute to the resolution of DSS-induced colitis primarily by expressing low levels of pro-inflammatory cytokines, but high levels of Arginase 1 (Arg-1), FIZZ1, YM-1 and IL-10.[20,26,27] Recently, it has been reported that M2 macrophages can also antagonize M1 macrophage responses to promote tissue repair.[28] Thus, the factors that modulate the polarization of macrophages could affect the severity of DSS-induced colitis.

A recent clinical study showed elevated circulating levels of chemerin in IBD patients;[29] however, a role for chemerin in IBD has not yet been investigated. In the present study, we investigated the effect of chemerin on the development of DSS-induced colitis by injection of exogenous chemerin or neutralizing anti-chemerin antibody. Then, we evaluate the correlation of the colonic expression of chemerin with disease severity in both mice and humans.

MATERIALS AND METHODS

Animals

C57BL/6 female mice aged 6–8 weeks were purchased from the Chinese Academy of Sciences (Shanghai, China). The animals were kept in a specific pathogen-free environment. All animal experiments were approved by the Animal Care and Use Committee at Fudan University.

The DSS-induced colitis model

Mice were given 3% DSS (36 000–50 000 MW; MP Biomedicals, Solon, OH, USA) in their drinking water for 5 days, followed by plain water for 3 or 7 days. Recombinant BSA-free mouse chemerin protein (aa16–157) or PBS as the control was used for the chemerin treatment, and a neutralizing anti-chemerin antibody (ChAb) or isotype control antibody was used for the chemerin blockade experiment (all reagents were purchased from R&D Systems, Minneapolis, MN, USA); chemerin was produced in *E. coli* with less than 0.10 EU per 1 μg of the protein). The chemerin protein or antibodies were injected intraperitoneally (i.p.) every 2 days starting from day 1 until day 8 when the mice were sacrificed. The body weights, stool consistency and rectal bleeding were monitored daily. The colon length was measured from the rectum to the cecum on day 8. The disease activity index (DAI) was scored according to the average of three parameters: stool consistency (0, 2, 4), fecal blood (0, 2, 4) and percentage weight loss (0–4), as previously described.[30]

Histological analysis

The colons were immediately fixed in 10% buffered formalin and stained with hematoxylin and eosin (H&E). The histological scores were calculated as previously described.[31] Briefly, two parameters, epithelium damage and cell infiltration, were measured. The maximum score for each parameter was 4.

Preparation of the colon cell suspension

The colons were excised, placed in calcium/magnesium-free Hank's buffered salt solution with 1% FBS (Gibco, Grand Island, NY, USA) and cleaned of the contents. For acquiring the total colonic cells, pieces were cut into 2 mm and stirred in calcium/magnesium-free Hank's buffered salt solution containing 3 mM EDTA at 37 °C for 15 min twice, and the suspensions were centrifuged at 1500*g* at 4 °C. The supernatants were removed, and the remaining pieces were digested for 1.5 hours with 1 mg/ml type IV collagenase and DNaseI (both from Sigma-Aldrich, St Louis, MO, USA) in Hank's buffered salt solution. The suspensions were passed through 300-mesh sieves to remove the undigested tissues.[32]

Enzyme-linked immunosorbent assay (ELISA)
The colon cells were cultured in 24-well plates (1×10^6 per well) in 1 ml of complete RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin and 10% heat-inactivated FBS (all from Gibco). After a 24-h incubation, the supernatants were collected. The concentrations of chemerin were measured using an ELISA Kit for mouse chemerin (R&D Systems). The concentrations of IL-6, TNF-α and interferon (IFN)-γ were measured using Mouse Ready-SET-Go! ELISA kits from eBioscience (San Diego, CA, USA).

Macrophage polarization *in vitro*
The exudate was collected by washing the peritoneal cavity of mice with 2×5 ml of cold PBS. The harvested cells were then seeded in 48-well tissue culture plates with 4×10^5 cells per well and allowed to adhere for 6 h. The non-adherent cells were removed. The purity of the adherent macrophage population was evaluated by flow cytometry (the purity of isolated macrophages varied between 97% and 99% in three separate experiments). For the polarization of M1 and M2 macrophages, lipopolysaccharide. (LPS, 20 ng/ml) or IL-4 (10 ng/ml) (PeproTech, Rocky Hill, NJ, USA) was added, respectively, in complete RPMI 1640 media for 24 h with or without chemerin (3 nM) (R&D Systems).

Western blotting
Protein extracts were prepared by lysing the macrophages for 5 min in boiling denaturing extraction buffer containing 1% SDS and 10 mM Tris (pH 7.4). The insoluble material was removed by centrifugation for 15 min at 12 000*g* at 4 °C. The total protein concentrations were determined using the BCA assay (Pierce/Thermo Fisher Scientific, Rockford, IL, USA). The cell extracts (standardized to 50 μg of total protein/lane) were separated by 12% denaturing SDS–PAGE and transferred to a PVDF membrane (Amersham Pharmacia Biotech, Freiburg, Germany) by semidry blotting using an electroblotter (Bio-Rad, Hercules, CA, USA) at 0.8 mA/cm^2 for 120 min. The membranes were blocked by 5% milk with PBST and incubated overnight with primary antibodies against total-Stat6, phospho-Stat6 (pSTAT6-Tyr641) and β-Actin (all from Cell Signaling Technology, Beverly, MA, USA) at 4 °C. After being washed in PBST three times, the membranes were incubated for 45 minutes with HRP-conjugated goat anti-rabbit or anti-mouse secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in blocking buffer. The membranes were subsequently washed and incubated with ECL kits (Pierce/Thermo Fisher Scientific, Rockford, IL, USA). The signal intensity was quantified by densitometry using ImageJ software (NIH, Bethesda, MD, USA). The western blotting experiment was repeated at least three times.

Antibodies and flow cytometry
The colon cells and peritoneal macrophages were stained with the following antibodies: fluorochrome-labeled mouse anti-CD45 (30-F11), Gr-1(RB6 8C5), CD11c (N418), F4/80 (BM8), CD11b (M1/70) and CMKLR1 (BZ194) (all from eBioscience). The cells were analyzed on a CyAn, and the data analysis was performed using Summit V5.2.0 (both from Beckman Coulter, Fullerton, CA, USA). CD45$^+$ cells that were F4/80$^+$ CD11b$^+$ were considered as macrophages; CD11c$^+$CD11b$^+$ and Gr-1$^+$CD11b$^+$ gated at F4/80 negative cells were considered as DCs and neutrophils, respectively.

Human biopsies
Biopsy specimens were obtained from the endoscopically inflamed and normal areas (as controls) of each UC patient who signed an informed-consent agreement during a colonoscopy procedure performed at the Huashan Hospital (Shanghai, China). These UC patients underwent a colonoscopy for the diagnosis or assessment of disease activity. The disease severity was calculated according to the Sutherland Disease Activity Index.[33] Briefly, four parameters were calculated: (i) stool frequency (1–3); (ii) rectal bleeding (0–2); (iii) the patient's functional assessment score (0–4); and (iv) endoscopy findings (0–3). A score from 0 to 5 is considered a mild UC patient score, and a score from 6 to 12 is considered a severe UC patient score.

Quantitative RT-PCR
Total RNA from the colons or stimulated macrophages was extracted using the RNA simple total RNA kit (Tiangen, Beijing, China). Then, cDNA was generated using a primescript RT Master Mix (TaKaRa Bio Inc., Otsu, Japan). Real-time PCR was performed using the SYBR green Gene Expression Assay (Applied Biosystems, Foster City, CA, USA) and Applied Biosystems 7500. The fold induction of the target gene expression was calculated using the comparative method by normalization to the internal control β2-actin. The primer sequences of all genes for PCR are as follows: mouse β-actin, 5′-GGCTGTATTCCCCTCCATCG-3′ and 5′-CCAGTTGGTAACAATGCCATGT-3′; mouse Arg-1, 5′-CAGAAGAATGGAAGAGTCAG-3′ and 5′-CAGATATGCAGGGAGTCACC-3′; mouse FIZZ1, 5′-TCCCAGTGAATACTGATGAGA-3′ and 5′-CCACTCTGGATCTCCCAAGA-3′; mouse Ym1, 5′-GGGCATACCTTTATCCTGAG-3′ and 5′-CCACTGAAGTCATCCATGTC-3; mouse IL-10, 5′-CTCTTACTGACTGGCATGAGGAT-3′ and 5′-GAGTCGGTTAGCAGTATGTTGT-3′; human chemerin, 5′-GACAGCCAGCTACTACCAGACATACT-3′ and 5′-CGCATAGGTGGTAACTTGTGTTTC-3′; and human β-actin, 5′-CTACGTCGCCCTGGACTTCGAGC-3′ and 5′-GATGGAGCCGCCGATCCACACGG-3′.

Statistical analysis
The comparisons between two groups were performed using unpaired one-tailed Student's *t*-tests. Multiple-group comparisons were performed using ANOVA followed by a Bonferroni correction to compare each group. The statistical analysis was performed with GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). A *P* value less than 0.05 was considered statistically significant.

RESULTS

Chemerin aggravates DSS-induced colitis

Previous studies showed that the administration of chemerin could play opposing roles, either pro-inflammatory or anti-inflammatory, according to different inflammatory disease models.[1,2,6,8,9,34] In this study, we aimed to determine the effect of exogenous chemerin on the development of DSS-induced colitis. First, we examined whether chemerin itself was able to cause systemic and/or local inflammation. The i.p. injection of recombinant chemerin (500 ng per mouse) to naïve C57BL/6 mice resulted in increased serum levels of chemerin but with no effect on the circulating numbers of neutrophils or the serum levels of pro-inflammatory cytokines such as IL-6 and TNF-α (Supplementary Figure 1a–c). Furthermore, i.p. injection of chemerin had no effect on the percentage of macrophages with undetectable infiltrating neutrophils (data not shown) in the peritoneal cavity of naive C57BL/6 mice (Supplementary Figure 1d). These data suggest that i.p. injection of chemerin is unable to cause systemic or local inflammation in naïve C57BL/6 mice. We next i.p. injected exogenous chemerin or PBS as a control to DSS-exposed C57BL/6 mice, according to the methods shown in Figure 1a. The clinical signs of colitis, including body weight, stool consistency and rectal bleeding, were monitored daily, and the length and the histology of the colons were examined on day 8. DSS-exposed mice showed a significant increase in the circulating levels of chemerin, which further increased following chemerin treatment (Supplementary Figure 2). Interestingly, we observed higher mortality in chemerin-treated mice than in control mice following DSS exposure on day 8 (1 of 18 for the DSS group *vs.* 3 of 16 for the chemerin-treatment group). The DSS exposure caused significantly greater weight loss starting from day 5 throughout the experiment, as well as a significantly decreased colon length on day 8 (Figure 1b and c). Chemerin-treated

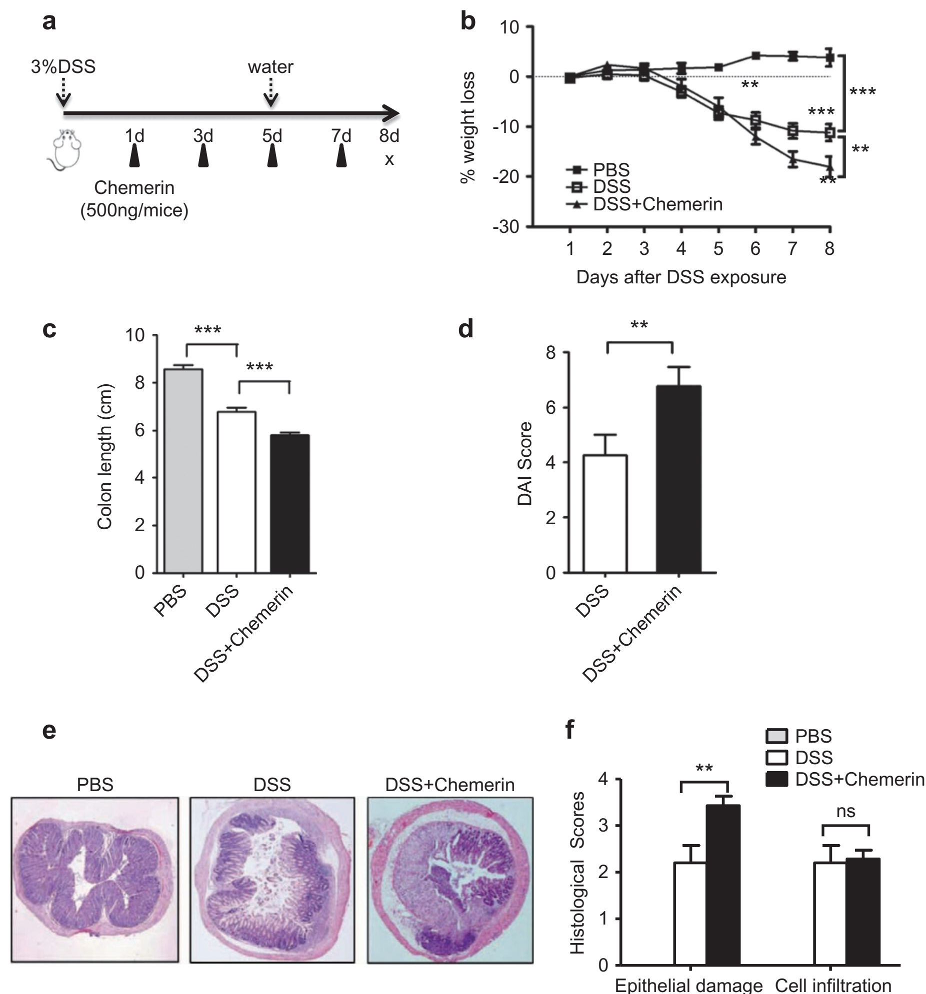

Figure 1 Exacerbated colitis in chemerin-treated mice following DSS exposure. (**a**) The methods for DSS-induced colitis and chemerin administration. (**b**) Weight loss was measured every day and expressed as the percentage change from day 0. (**c**, **d**) Colon length (**c**) and clinical DAI (**d**) were assessed at necropsy on day 8 following DSS exposure. (**e**) Representative photomicrographs of colon sections stained with H&E were examined at ×40 magnification. (**f**) Histological scores were determined as described in the section on 'Materials and methods'. The data are pooled from three independent experiments and expressed as the mean±s.e.m. (n=15–18 per group). *P<0.05, **P<0.01, ***P<0.001. DAI, disease activity index; DSS, dextran sulfate sodium; H&E, hematoxylin and eosin.

mice showed significantly greater weight loss and colon shortening, as well as significantly higher DAI scores, compared to control mice on day 8 following DSS exposure (Figure 1b–d). Consistent with the exacerbated clinical signs, chemerin-treated mice showed a greater severity in histological damage as evidenced by a widely disrupted tissue architecture, the disappearance of intestinal crypts and goblet cells, marked mucosal hypertrophy and edema compared to control mice following DSS exposure (Figure 1e). However, it's worth noting that there was no obvious increase in inflammatory infiltrates observed in the H&E colon sections of chemerin-treated mice. This result was further confirmed by an evaluation of the histological scores (Figure 1f). Collectively, these results suggest that chemerin exacerbates the clinical parameters and histological damage of DSS-induced colitis.

Chemerin increases the secretion of pro-inflammatory cytokines in DSS-induced colitis

Excessive secretion of pro-inflammatory cytokines is closely linked to exacerbated intestinal inflammation. We therefore investigated whether chemerin treatment influenced the secretion of pro-inflammatory cytokines in DSS-induced colitis. For this purpose, we measured the concentrations of IL-6, TNF-α and IFN-γ in the culture of colonic cells from mice on day 8. As shown in Figure 2, there was abundant secretion of IL-6, TNF-α and IFN-γ in the culture of colon cells from DSS-exposed mice. Furthermore, significantly increased concentrations of all three cytokines were detected in the culture of colon cells from chemerin-treated mice compared to the controls (Figure 2a). Chemerin treatment also significantly increased the serum levels of IL-6 and TNF-α in DSS-exposed mice (Figure 2b), although IFN-γ was not detectable in the sera of the mice from all three groups. Thus, these results indicate that chemerin promotes systemic and local production of pro-inflammatory cytokines, which is consistent with the exacerbated DSS-induced colitis observed in chemerin-treated mice.

Chemerin has no effect on the proportion of inflammatory cells in DSS-exposed colons

Although histological analysis showed no obvious increased inflammatory infiltrates in DSS-exposed colons after chemerin treatment, given the previous studies showing that chemerin

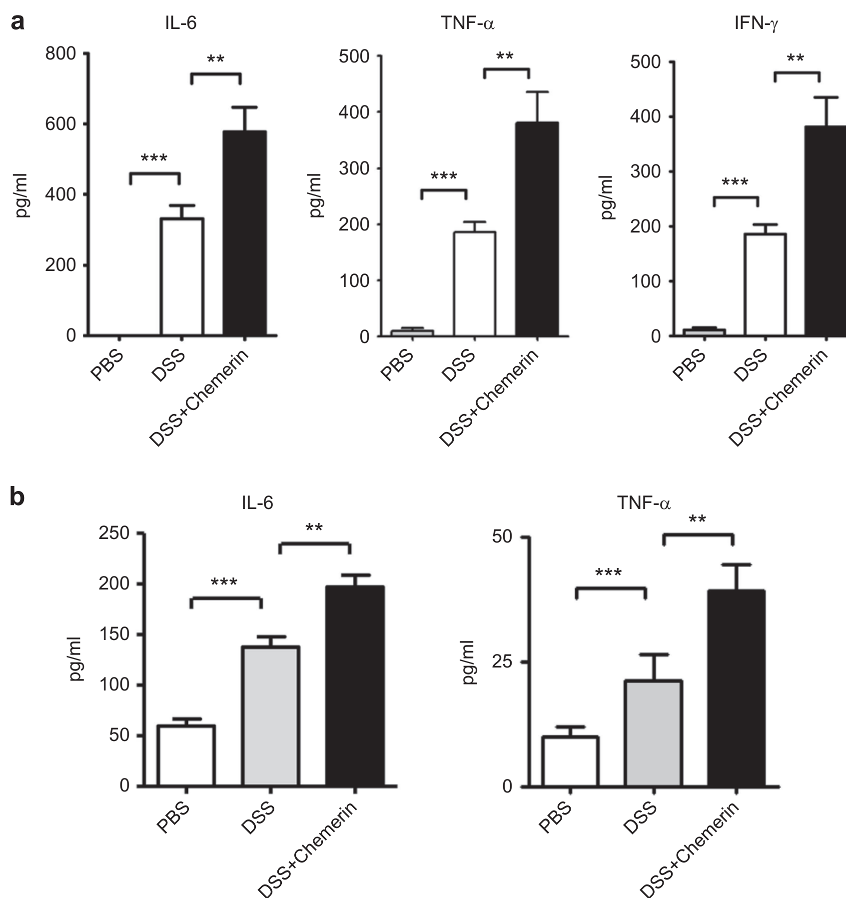

Figure 2 Increased production of pro-inflammatory cytokines in chemerin-treated mice following DSS exposure. (**a**) The concentrations of the pro-inflammatory cytokines IL-6, TNF-α and IFN-γ in the supernatant of colon cells after 24 h of culture were measured by ELISA. (**b**) The serum levels of the pro-inflammatory cytokines IL-6 and TNF-α were measured by ELISA. **P*<0.05, ***P*<0.01, ****P*<0.001. The data are expressed as the mean±s.e.m. (*n*=4–6 per group). Similar results were obtained in three independent experiments with 4–6 mice per group. DSS, dextran sulfate sodium; ELISA, enzyme-linked immunosorbent assay; IFN, interferon.

regulates the tissue trafficking of immune cells, we next investigated whether chemerin treatment influenced the mucosal infiltration of neutrophils, macrophages and DCs by flow cytometric analysis of the digested colon tissues. As expected, there were significantly increased percentages of $CD45^+$ leukocytes in the colons of DSS-exposed mice; however, chemerin treatment did not increase the percentages of $CD45^+$ leukocytes, which even slightly decreased but did not reach statistical significance (Figure 3a). Comparable proportions of neutrophils, DCs and macrophages were found among the $CD45^+$ leukocytes from the colons of DSS-exposed mice regardless of chemerin treatment (Figure 3b and c). We next examined the percentages of infiltrating NK cells and pDCs, both of which have been shown to respond to the chemotactic activity of chemerin by expressing its receptor, CMKLR1. Flow cytometric analysis revealed that DSS exposure had no effect on the percentages of $CD11c^+PDCA\text{-}1^+$ pDCs, but caused increased percentages of $NK1.1^+CD3^-$ NK cells in the colons. No differences were found in the percentages of colonic pDCs and NK cells in chemerin-treated mice compared with controls (Supplementary Figure 3). Collectively, these data suggest that chemerin has no effect on the colonic infiltration of inflammatory cells in DSS-induced colitis. Thus, the upregulated secretion of pro-inflammatory cytokines in the colons from chemerin-treated mice could not be due to increased inflammatory infiltrates.

Chemerin reduces the expression of M2 macrophage-associated genes in DSS-induced colitis

It is known that macrophages can change their function from the inflammatory M1 type that is the major source for pro-inflammatory cytokines to the healing M2 type during tissue injury or infection including DSS-induced colitis.[25,35] We next investigated whether chemerin was able to alter the shift of M1 to M2 macrophage function, which could cause persistently upregulated levels of pro-inflammatory cytokines. To this end, we examined the mRNA expression levels of M2 macrophage-associated genes including Ym1, FIZZ1, Arg-1 and IL-10

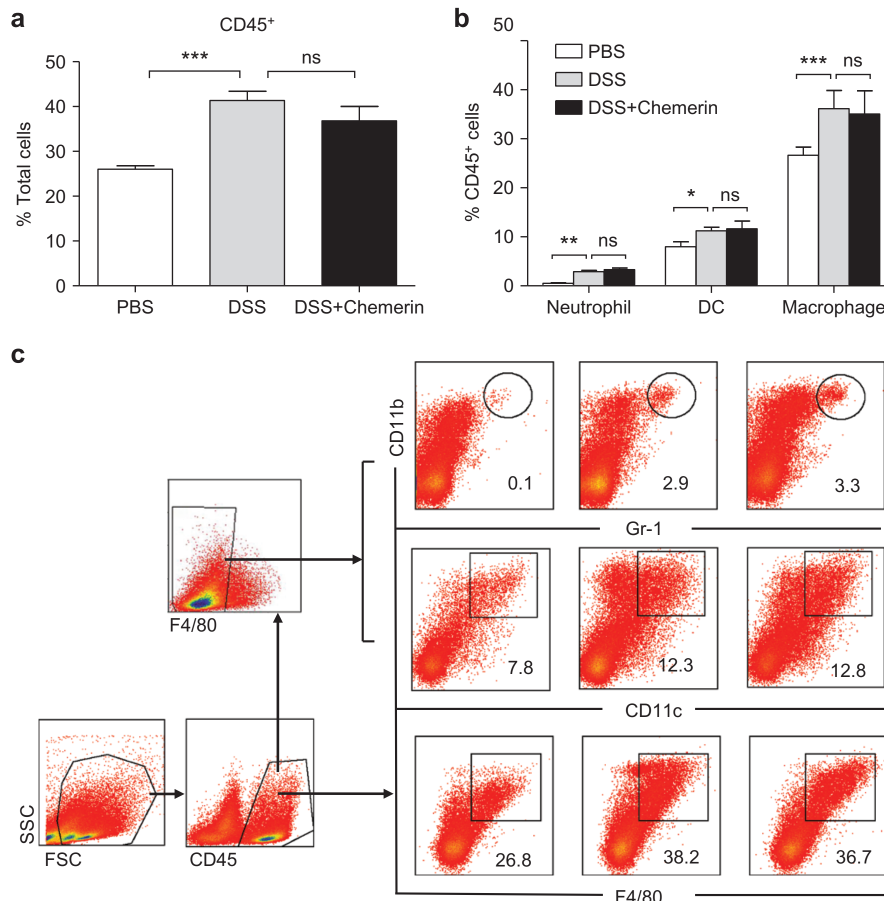

Figure 3 Chemerin has no effect on the colonic recruitment of inflammatory cells following DSS exposure. Colon cells were prepared from enzymatically digested colon tissue, then were stained with Gr-1, CD11b, CD11c and F4/80 and analyzed by flow cytometry. $F4/80^+CD11b^+$ were considered as macrophages, $CD11c^+CD11b^+$ and $Gr\text{-}1^+CD11b^+$ gated at F4/80 negative cells as DCs and neutrophils, respectively. (**a**, **b**) The percentages of $CD45^+$ cells (**a**) and neutrophils, DCs and macrophages (**b**) in a single-cell suspension prepared from enzymatically digested colon tissue. Data are expressed as the mean±s.e.m. (n=4–6 per group). (**c**) Representative flow cytometric analysis of colonic cells. The numbers indicated are the percentage of neutrophils, DCs and macrophages. Similar results were obtained in three independent experiments with 4–6 mice per group. *P<0.05,, **P<0.01, ***P<0.001, ns=not significant. DC, dendritic cell; DSS, dextran sulfate sodium.

by RT-PCR analysis of the colon tissues obtained on days 5 and 8 following DSS exposure. DSS exposure led to significant increases in the colonic mRNA expression of Arg-1, Ym1, FIZZ1 and IL-10 (Figure 4), suggesting that M2 macrophages accumulate in the colon of DSS-induced colitis. Notably, there was significantly decreased colonic expression of all the M2 genes in chemerin-treated mice compared to the controls (Figure 4). Collectively, these data suggest that chemerin treatment impairs the skewing of macrophages towards the anti-inflammatory M2 type in DSS-exposed colons, which may contribute to the exacerbated colitis.

Chemerin suppresses IL-4-induced M2 macrophage polarization *in vitro*

We next investigated whether chemerin was able to directly modulate macrophage polarization. To this end, we isolated peritoneal macrophages from C57BL/6 mice for macrophage polarization under different stimuli combined with chemerin. The majority of the peritoneal macrophages were found to express CMKLR1, as a previous study reported.[36] We confirmed the findings of Bondue *et al.*[37] in which chemerin had no effect on LPS-induced M1 macrophage activation and the secretion of pro-inflammatory cytokines (data not shown), suggesting the upregulated levels of pro-inflammatory cytokines in chemerin-treated mice was not due to an enhanced M1 macrophage response. To determine whether chemerin directly influenced the M2 polarization *in vitro*, peritoneal macrophages were stimulated with chemerin or IL-4 alone or with IL-4 and chemerin together, and the mRNA expression levels of the M2 macrophage-associated genes were examined by RT-PCR. As expected, IL-4 stimulation greatly induced the mRNA expression of Arg-1, Ym1, FIZZ1 and IL-10, whereas chemerin alone had no effect on the expression of these M2 genes (Figure 5a). Furthermore, we found that the addition of chemerin significantly reduced the mRNA expression of Arg-1, Ym1, FIZZ1 and IL-10 in IL-4-stimulated macrophages (Figure 5a). It is known that IL-4-induced M2 macrophage polarization is dependent on STAT6, a master regulator of M2 genes.[38] Therefore, we further examined whether the addition of chemerin influenced the total STAT6 and STAT6 phosphorylation in IL-4-stimulated macrophages (30 min) by western blot. STAT6 phosphorylation was undetected in unstimulated macrophages (data not shown). As expected, obvious STAT6 phosphorylation was observed in IL-4-stimulated macrophages (Figure 5b). The addition of chemerin reduced the intensity of phosphorylated STAT6, but not total STAT6, in IL-4-stimulated macrophages (Figure 5b). This result was further confirmed by a significant reduction in pSTAT6 when normalized to total STAT6 (Figure 5b). Interestingly, we found that IL-4 significantly upregulated the expression level of CMKLR1 in macrophages (Figure 5c and d), which may enhance the responsiveness of macrophages to chemerin. Collectively, these results suggest that chemerin could directly suppress IL-4-induced M2 macrophage polarization partly by inhibiting STAT6 phosphorylation.

Chemerin levels are elevated in the inflamed colons from DSS-induced colitis and UC patients

Circulating levels of chemerin were reported to be elevated in IBD patients.[29] We next investigated whether chemerin was

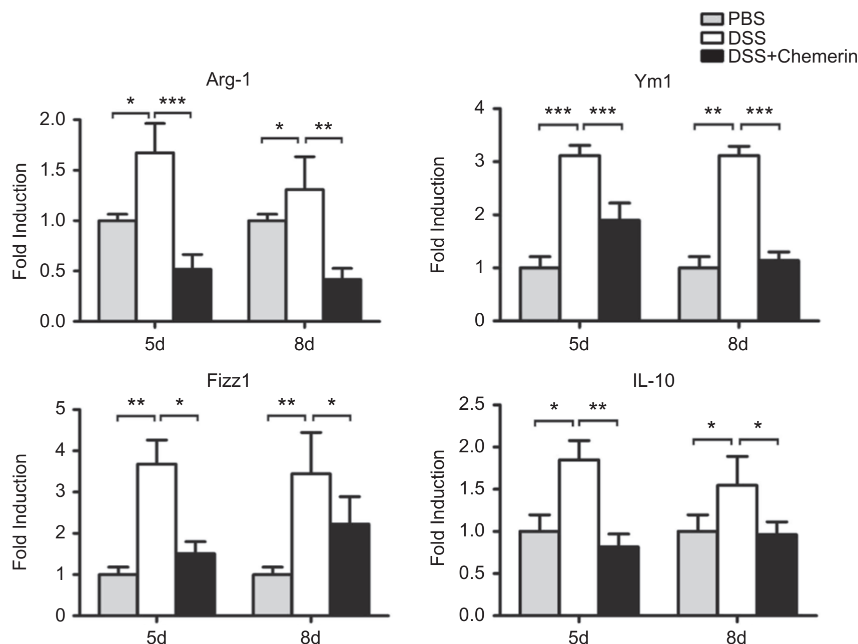

Figure 4 Decreased expression of M2 macrophage associated genes in chemerin-treated mice following DSS exposure. The colonic expression of M2 macrophage-associated genes Arg1, Ym1, FIZZ1 and IL-10 was examined by RT-PCR analysis. $*P<0.05$, $**P<0.01$, $***P<0.001$. The data are expressed as the mean±s.e.m. (n=4–6 per group). Similar results were obtained in three independent experiments with 4–6 mice per group. Arg-1, Arginase 1; DSS, dextran sulfate sodium.

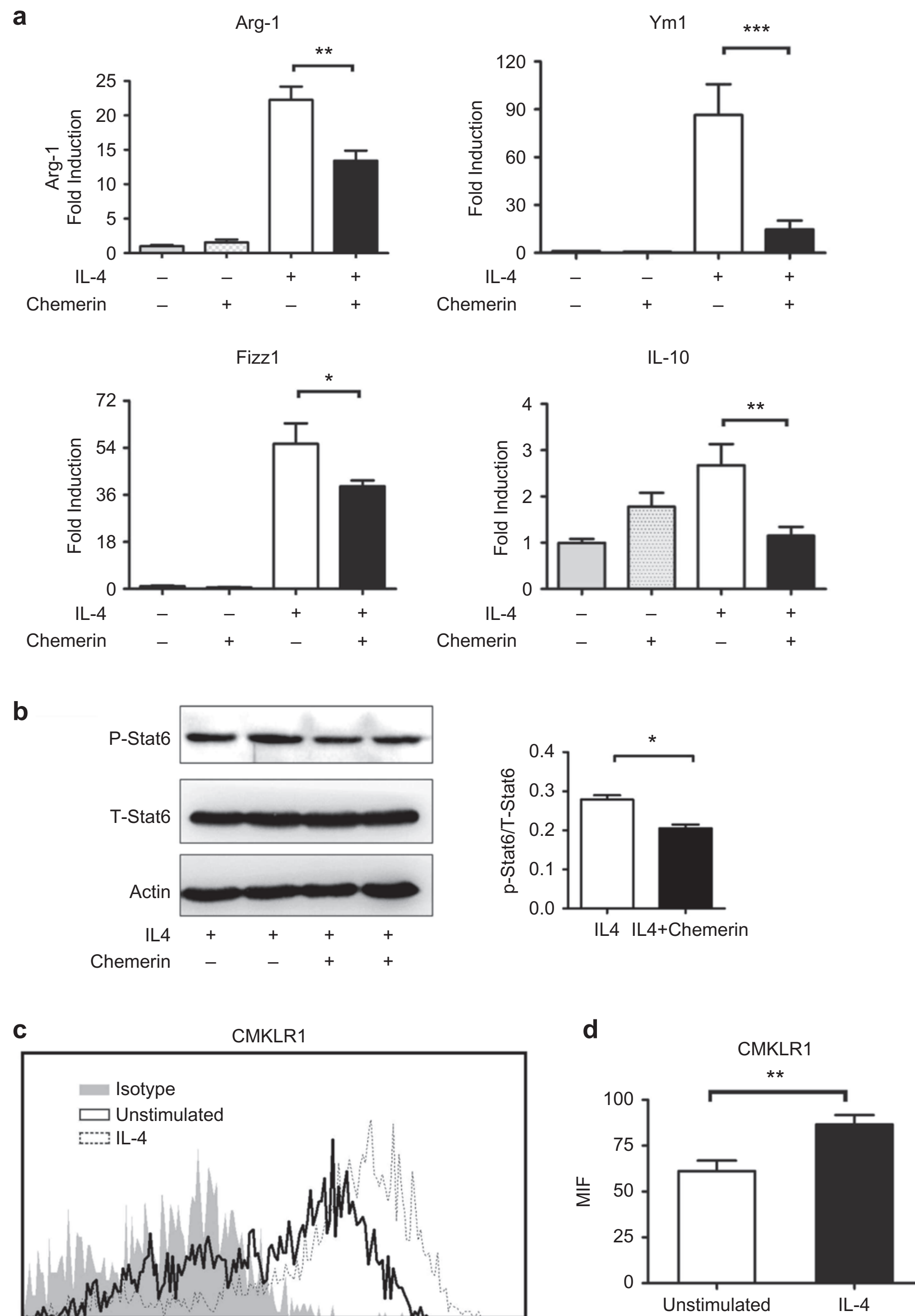

Figure 5 Chemerin suppresses IL-4-induced M2 macrophage polarization *in vitro*. Peritoneal macrophages were isolated and stimulated with chemerin or IL-4 alone or together. (**a**) The mRNA expression of Arg1, Ym1, FIZZ1 and IL-10 by macrophages was examined by RT-PCR analysis. (**b**) STAT6 phosphorylation was measured in stimulated macrophages by western blot. (**c**) A representative histogram of CMKLR1 expression on macrophages. (**d**) MIF of CMKLR1 expression in freshly isolated macrophages and IL-4-stimulated macrophages. *$P<0.05$, **$P<0.01$, ***$P<0.001$. The data are pooled from three independent experiments and expressed as the mean±s.e.m. ($n=6$ per group). Arg-1, Arginase 1; Mean fluorescent intensity.

upregulated locally in a DSS-induced mouse model of IBD. To this end, we measured the concentrations of chemerin in the culture of colon cells from DSS-exposed mice at different time points by ELISA. The production of chemerin was significantly upregulated on days 5 and 8 following DSS exposure (Figure 6a). Moreover, the upregulated colonic chemerin production paralleled the severity of DSS-induced colitis, both of which reached peak on day 8 (Figure 6a and 1b) and returned to the baseline levels on day 12 when the colitis completely resolved (Figure 6a and data not shown). We further compared the chemerin mRNA expression in the colon biopsies of endoscopically normal and inflamed areas collected from UC patients and found that chemerin mRNA expression was significantly upregulated in the inflamed tissues compared to the

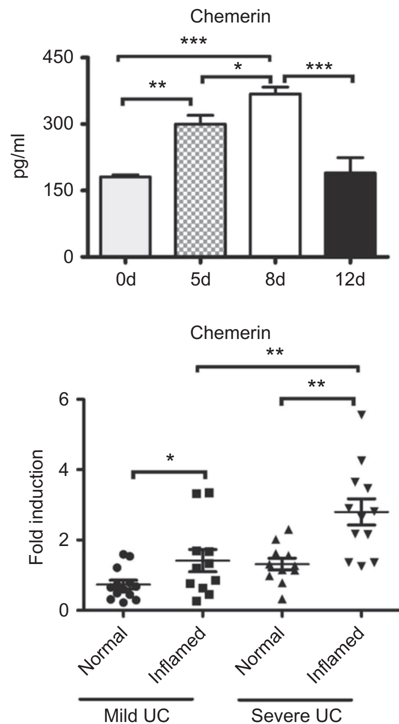

Figure 6 Elevated colonic chemerin levels in DSS-induced colitis and UC patients. The concentrations of chemerin in the supernatants of colon cells collected from DSS-exposed mice at different time points after 24 h of culture. (**b**) The mRNA expression levels of chemerin in the colon biopsies of endoscopically normal and inflamed areas collected from UC patients were determined by RT-PCR analysis. $*P<0.05$, $**P<0.01$, $***P<0.001$. The data in **a** are expressed as the mean± s.e.m. (n=4–6 per group), and similar results were obtained in three independent experiments with 4–6 mice per group. DSS, dextran sulfate sodium; UC, ulcerative colitis.

normal-appearing tissues (Figure 6b). More importantly, the inflamed tissues from patients with severe UC expressed significantly higher chemerin levels than those tissues from patients with mild UC (Figure 6b). Collectively, these results suggest that the colonic chemerin expression levels positively correlate with the severity of the DSS-induced IBD mouse model and human UC.

The blockade of endogenous chemerin attenuates the intestinal inflammation in DSS-exposed mice

We next investigated the role of endogenous chemerin in DSS-induced colitis by taking advantage of the available neutralizing anti-ChAb. ChAb or an isotype control Ab was i.p. injected to DSS-exposed mice according to the methods shown in Figure 7a. Although the administration of ChAb failed to improve the clinical signs such as weight loss and the length of the colon of DSS-exposed mice (data not shown), a great improvement in the histological damage was observed in the H&E colon sections from ChAb-treated mice compared to the isotype controls (Figure 7b), which was further confirmed by the histological scores (Figure 7c). ChAb treatment also had no effect on the proportions of neutrophils, DCs and macrophages in the DSS-exposed colons (Figure 7d); however, significantly decreased concentrations of TNF-α and IFN-γ, but not IL-6, were found in the culture of colon cells from ChAb-treated mice compared to the isotype controls (Figure 7e). An increased expression of M2 macrophage-associated genes was also found in ChAb-treated mice compared to the isotype controls, although only increased Arg-1 expression reached statistical significance (Figure 7f). Collectively, these results suggest that upregulated chemerin in DSS-induced colitis could contribute to intestinal inflammation.

DISCUSSION

Chemerin appears to play opposing roles, either pro- or anti-inflammatory, depending on the context of tissue inflammation.[39] Chemerin expression was detected in the colon, and elevated circulating levels of chemerin were found in IBD patients;[29] however, the role of chemerin in intestinal inflammation has not yet been investigated. We herein demonstrate that chemerin aggravates intestinal inflammation in DSS-induced colitis, which could be due to its ability to suppress M2 macrophage polarization. Furthermore, we demonstrate that locally upregulated chemerin levels positively correlate with the severity of DSS-induced colitis and human UC.

Several studies demonstrated the anti-inflammatory effects of chemerin and CMKLR1 in lung inflammation, which are associated with significant decreases in the lung recruitment of neutrophils and macrophages and the production of pro-inflammatory cytokines and chemokines.[9,34] In contrast, our study showed that chemerin treatment resulted in a greater severity of DSS-induced colitis. Interestingly, this severity was not associated with the magnitude of the colonic infiltration of inflammatory leukocytes, according to histological analysis as well as flow cytometry, which showed comparable proportions of $CD45^+$ leukocytes in the DSS-exposed colons regardless of chemerin treatment. Innate leukocytes including neutrophils, macrophages and DCs are central to both the induction and regulation of DSS-induced colitis.[19–21] We further found that the proportions of neutrophils, macrophages and DCs in the DSS-exposed colons were not affected by chemerin treatment. The proportions of pDCs and NK cells, for which chemerin was originally identified as a chemoattractant, were also not affected. Thus, the ability of chemerin to regulate the tissue recruitment of immune cells could be tissue context-dependent. In DSS-induced intestinal inflammation, the higher inflammatory state and the higher production of other chemokines could affect cell recruitment independently of chemerin.

We speculated that the exacerbation of DSS-induced colitis could be due to the ability of chemerin to modulate the functions of inflammatory innate cells instead of their colonic recruitment. CMKLR1 has been detected in macrophages, but not neutrophils and inflammatory DCs,[36] which was also confirmed by our findings that infiltrating macrophages, but not infiltrating neutrophils and inflammatory DCs, express CMKLR1 in the colon (data not shown). This result suggests that the intestinal macrophage is directly responsive to

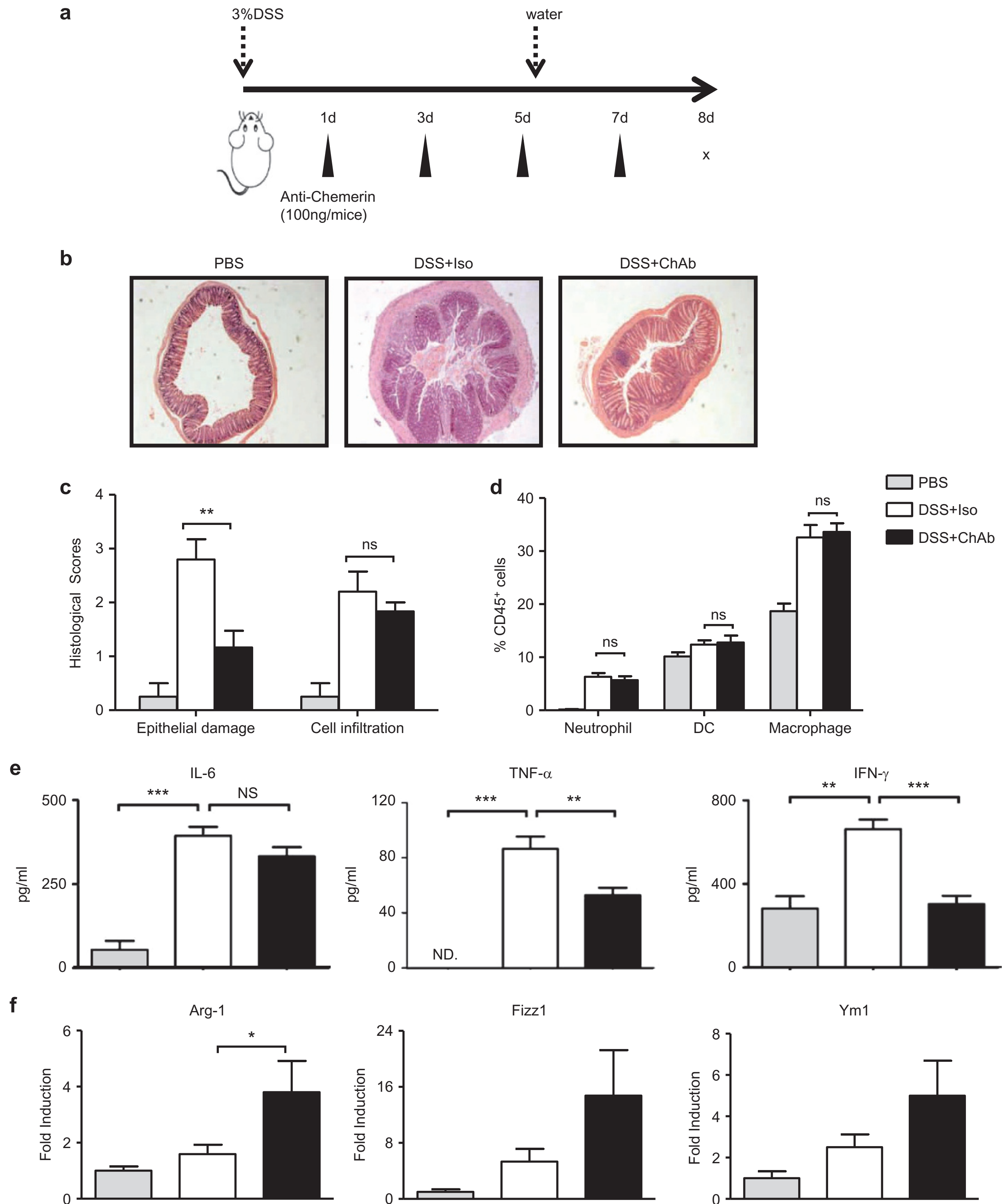

Figure 7 Attenuated intestinal inflammation in neutralizing anti-chemerin (ChAb)-treated mice following DSS exposure. (**a**) The methods for DSS-induced colitis and ChAb administration. (**b**) Representative photomicrographs of colon sections stained with H&E were examined at ×40 magnification. (**c**) Histological scores were determined as described in the section on 'Materials and methods'. (**d**) The percentages of neutrophils, DCs and macrophages in the colons were determined by flow cytometry as described above. (**e**) The concentrations of the pro-inflammatory cytokines IL-6, TNF-α and IFN-γ in the supernatant of colon cells after 24 h of culture were measured by ELISA. (**f**) The colonic expression of the M2 macrophage-associated genes Arg1, Ym1, FIZZ1 and IL-10 was examined by RT-PCR analysis. The data are expressed as the mean±s.e.m. (n=5 per group). Similar results were obtained in three independent experiments with five mice per group. ns=not significant, *P<0.05, **P<0.01, ***P<0.001. ChAb, chemerin antibody; DC, dendritic cell; DSS, dextran sulfate sodium; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; UC, ulcerative colitis.

chemerin. Accumulating studies show that macrophages are functionally heterogeneous and closely involved in the initiation and the resolution of intestinal inflammation by being activated into either pro-inflammatory M1 or anti-inflammatory M2 macrophages.[20,25] Several studies have suggested that the defect in M2 polarization or the activity of M2-associated effector molecules leads to increased severity in mouse models of colitis,[20,40] whereas adoptive transfer of *in vitro*-derived M2 macrophages attenuates colonic inflammation in mice.[26] A clinical study also has suggested that the numbers of M2-like macrophages inversely correlate with the active status of patients with CD.[26] We therefore hypothesized that chemerin may regulate the macrophage skewing in DSS-exposed colons. We first ruled out the possibility that chemerin enhances the M1 macrophage response because published data and ours have demonstrated that chemerin has no effect on the secretion of pro-inflammatory cytokines by LPS-induced M1 macrophages (Supplementary Figure 4). DSS-induced colitis is believed to be driven largely by innate immune responses involving pro-inflammatory cytokine production not only from hematopoietic but from non-hematopoietic cells such as epithelial cells. Thus, it is likely that the increased levels of pro-inflammatory cytokines are, at least in part, due to persistent epithelial damage because of impaired anti-inflammatory M2 macrophage skewing in chemerin-treated mice, as a greater severity of epithelial damage was observed in chemerin-treated mice following DSS exposure. Indeed, we found that chemerin treatment resulted in significantly decreased M2-associated genes in DSS-exposed colons, suggesting locally impaired M2 macrophage responses. Furthermore, we found that chemerin was able to directly suppress the polarization of M2 macrophage *in vitro*, as the addition of chemerin greatly reduced the expression of M2-associated genes by IL-4-stimulated macrophages, as well as the phosphorylation of STAT6, which is critical for M2 gene expression.[41] It is also possible that chemerin could influence the conversion of M1 macrophages into M2 macrophages in the presence of IL-4, as a recent study suggested that the repolarization of macrophages through IL-4 may occur in joint inflammation.[42] Intriguingly, the upregulated CMKLR1 expression in macrophages was observed following IL-4 stimulation, which is consistent with a previous study showing that M2-inducing TGF-β upregulates, whereas M1-inducing factors downregulates, CMKLR1 expression in macrophages.[36] Thus, it is possible that upregulated CMKLR1 expression by IL-4 enhances the responsiveness of macrophages to chemerin, which in turn suppresses IL-4-inducing STAT6 phosphorylation and subsequent M2 macrophage polarization. However, the exact molecular mechanism needs to be further investigated.

Significant increases in the systemic and local secretion of chemerin were detected following DSS exposure. Interestingly, the colonic expression of chemerin positively correlated with the severity of DSS-induced colitis, as the chemerin expression peaked on day 8 when the highest scores of DAI and intestinal histopathology were observed and returned to the baseline levels on day 12 when the colitis completely resolved. More importantly, elevated chemerin levels in the inflamed colonic tissues from UC patients positively correlated with the disease severity. These results suggest that locally upregulated chemerin levels may be involved in intestinal inflammation. Finally, the pro-inflammatory role of chemerin in DSS-induced colitis is also confirmed by the experiment of *in vivo* blockade of chemerin. ChAb administration significantly improved the intestinal histopathology associated with a significantly decreased colonic secretion of pro-inflammatory cytokines. The proportions of innate inflammatory leukocytes in DSS-exposed colons still remained unaffected by ChAb treatment, strongly supporting the idea of chemerin regulating the function, but not the recruitment, of inflammatory cells, particularly macrophages, in intestinal inflammation. This idea is also supported by the finding of enhanced colonic expression of M2-associated genes following ChAb treatment. Unfortunately, we failed to observe significantly improved clinical manifestations in ChAb-treated mice following DSS exposure. This result may be due to the insufficient blockade of endogenous chemerin, as ChAb administration only partly decreased the upregulated chemerin production in DSS-exposed colons, which was still higher than the baseline levels in control mice (data not shown). The exact role of endogenous chemerin needs to be further investigated by using chemerin-deficient mice or developing a more effective neutralizing anti-chemerin antibody.

In conclusion, our study reveals a pro-inflammatory role of chemerin in DSS-induced colitis, which could be mediated by its ability to suppress the polarization of anti-inflammatory M2 macrophages and suggests that locally elevated chemerin in IBD patients may contribute to the pathogenesis of IBD. These results further highlight the complex function of chemerin in regulating tissue inflammation and suggest a potential therapeutic target for chemerin in the treatment of intestinal inflammation.

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