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RESEARCH ARTICLE

L13a-dependent translational control in macrophages limits the pathogenesis of colitis

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Sustained inflammation from infiltrated immune cells plays a pivotal role in the pathogenesis of ulcerative colitis (UC). Previously, we established the role of ribosomal protein L13a in the regulation of an inflammation-responsive post-transcriptional operon in myeloid cells. However, the role of this protein as a molecular cue to control the severity of colitis is not known. Here, we examined whether L13a-dependent translational control in macrophages could serve as an endogenous defense against colitis. The administration of dextran sodium sulfate induced experimental colitis in myeloid-specific L13a-knockout (KO) and control mice. Pathological scoring and injury to the colon mucosa evaluated the severity of colitis. The steady-state levels of several pro-inflammatory cytokines and chemokines were determined through ELISA and polyribosome profile analysis. Rapid weight loss, severe rectal bleeding, shortening of the colon, and significantly reduced survival rate were observed in the KO mice. Histopathological analysis of the colons of KO mice showed a severe disruption of epithelial crypts with immune cell infiltrates. Elevated levels of several inflammatory cytokines and chemokines and abrogation of their naturally imposed translational silencing were observed in the colons of the KO mice. Higher serum levels of several pro-inflammatory cytokines and the release of gut bacteria and endotoxins into the blood streams of KO mice were detected, suggesting the amplification of the inflammatory response to septicemia. Taken together, these results reveal an essential role for L13a in the endogenous protection against UC and demonstrate the potential for new therapeutic opportunities through the deliberate promotion of this mechanism.
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INTRODUCTION

Ulcerative colitis (UC) is one of the major forms of inflammatory bowel disease (IBD). UC is primarily localized to the colonic mucosa and is pathologically characterized by gastrointestinal inflammation, infiltration of macrophages and other types of leukocytes, and injury to the epithelial crypts.[1,2] The identification of the IBD susceptibility loci that encode cytokines, chemokines, and chemokine receptors suggests a crucial role for cytokine-producing immune cells in the pathogenesis of this disease.[3,4] Indeed, it has been demonstrated that the colonic recruitment of innate and adaptive immune cells and the uncontrolled production of cytokines by these cells leads to defective resolution of inflammation in IBD patients.[1,2] Unresolved chronic inflammation compromises the barrier function of the intestinal mucosa, facilitating the penetration of commensal bacteria from the gut lumen into the intestinal mucosa wall in genetically susceptible hosts.[5,6] IBD is exacerbated through inappropriate immune responses elicited in the host mucosa via commensal populations of bacteria, which disrupt intestinal homeostasis and further damage the mucosal barrier.[7]

Using cultured cells[8–14] and live mouse models,[15,16] we uncovered an endogenous mechanism in macrophages and monocytes that restrains inflammation through the translational silencing of a group of chemokines and chemokine receptors.[13] This process relies on the phosphorylation-dependent release of the L13a protein from the 60S ribosomal subunit[9] and its assembly into a multi-protein RNA-binding complex named the IFN-γ-activated inhibitor of translation (GAIT) complex.[10] The GAIT complex forms under inflam-

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matory conditions and binds GAIT-specific sequence elements in the 3′-untranslated regions (UTR) of target mRNAs[17] that encode several chemokines and chemokine receptors. The binding of GAIT in the 3′-UTR of a target mRNA completely abrogates its translation by preventing translation initiation.[9,12] To determine the physiological significance of this silencing mechanism, we generated myeloid-specific L13a-knockout (KO) mice. In models of lipopolysaccharide-induced endotoxemia[15] and high-fat diet-induced atherosclerosis,[16] these KO mice displayed severe inflammation, unregulated chemokine expression and significantly increased disease susceptibility compared with isogenic control mice that had intact L13a function.

On the basis of these results and the fact that inflammation involving a ''cytokine storm'' that results from infiltrating macrophages plays a pivotal role in the pathogenesis of UC, we examined whether L13a-dependent translational control could serve as an endogenous protection against colitis using myeloid-specific L13a KO mice in a model of experimental colitis induced through dextran sodium sulfate (DSS) provided in the drinking water.[18,19] DSS-induced colitis is a widely accepted experimental model that accurately recapitulates the fundamental clinical and pathological features of UC, including the infiltration of innate immune cells, disruption of the epithelial barrier, and chronic inflammation in the intestine.[20] When DSS was administered in the drinking water, the KO mice with myeloid-specific genetic depletion of L13a showed much more severe colitis than similarly treated control mice with intact L13a. The increased severity of the disease observed in KO mice was associated with a loss of GAIT-dependent translational silencing. These results suggest L13a-mediated translational silencing as an endogenous mechanism that counteracts inflammation and protects mice against experimental colitis.

MATERIALS AND METHODS

Mice

All experiments involving mice were carried out in accordance with the National Institutes of Health and Institutional Animal Care and Use Committee (IACUC) guidelines. The generation of myeloid-specific L13a ''KO'' mice ($L13a^{flox/flox}LysMCre^{+}$) and ''control'' ($L13a^{flox/flox}$) mice on the C57BL/6J background and confirmation of the myeloid-specific depletion of L13a have been described in detail in a previous publication.[15] The conditions for animal housing were in total compliance with the policies of the Laboratory Animal Welfare of National Institute of Health, and the mice were housed in pathogen-free conditions. The Animal Welfare Assurance Number of the facility is A3554-01. Throughout the entire study involving DSS-induced experimental colitis, no signs of infection, abnormal behavior, or unnatural death were observed within the housed colony. All animal studies were conducted using age- and sex-matched control and KO mice aged 8–12 weeks.

Induction of experimental colitis through DSS administration

For pathological scoring, body weight measurements, colon shortening, and septicemia studies, control and KO mice were provided free access to 3% DSS (W/V) (molecular weight: 36 000–50 000, Catalog NO 0216011090, MP Biomedicals, OH, Solon, Ohio, USA) in the drinking water for nine days, after which the treated water was replaced with regular drinking water. For the survival studies, the mice were administered 2.5% DSS in the drinking water for 14 days, followed by regular drinking water for 11 days. For the colonic cytokine analysis, the mice were administered 3% DSS in the drinking water for seven days, followed by regular drinking water for one day. To examine the recovery from DSS-induced colitis, the mice were administered 2.5% DSS in the drinking water for seven days, followed by regular drinking water for four weeks. The same batch of DSS was used for the entire study.

Pathological scoring

The animals were monitored daily for weight loss, gross rectal bleeding, and stool consistency, and these were scored semi-quantitatively on a relative scale of 0–4 as follows: 0 = no blood, normal fecal pellet; 2 = prominent traces of blood, loose fecal pellet; 4 = liquid blood around anus, liquid diarrhea; 1 and 3 were assigned as intermediate scores. To measure colon shortening, the colons were harvested from mice euthanized on day 10 after the initiation of DSS treatment, and the entire colon length from rectum to cecum was measured.

Tissue histology and immunostaining

For histological analysis, the colons and spleens were harvested from DSS-treated animals, fixed in acidic methanol (60% methanol, 10% glacial acetic acid, and 30% water) overnight and paraffin embedded. Subsequently, 5 μM sections were cut and deparaffinized using Trilogy (Cell Marque, Austin, TX, USA) in a steamer for 30 minutes. The sections were stained with hematoxylin and eosin (H&E), Gomori Trichrome stain (Richard-Allan Scientific, Kalamazoo, MI, USA) or periodic acid-Schiff (PAS; Poly Scientific R&D Corp., Bay Shore, NY, USA) according to the manufacturer's instructions. Colon sections were also stained for macrophages using purified anti-Mac2 antibody (rat anti-mouse; Cedarlane Laboratories, Burlington, NC, USA) and F4/80 antibody (CI:A3-2) (Thermo Fisher, Waltham, MA, USA) and visualized using the VECTASTAIN Elite ABC Kit (Vector, Burlingame, CA, USA), followed by diaminobenzidine with hematoxylin counterstaining.

Identification of macrophages from isolated intestinal cells

This identification method was carried out according to previously published methods.[21–23] Pieces (1.5 cm) of intestinal tissue were digested from DSS-water-fed and normal drinking water-fed animals and subjected to Type VIII collagenase and DNase I (Sigma-Aldrich) digestion through orbital shaking at 200 rpm for 20 minutes at 37°C. Occasional vortexing was performed to ensure the complete dissociation of the intestinal tissue. The intestinal cells were collected after passing through a

100 μm cell strainer, followed by centrifugation. The cells were incubated with rat anti-mouse CD16/CD32 (BD Pharmingen, San Jose, CA, USA) to block nonspecific binding to FcγRs and were subsequently stained with FITC-conjugated rat anti-mouse CD11b IgG2b (BD Pharmingen) and allophycocyanin-conjugated rat anti-mouse F4/80 IgG2b (Abd Serotec) according to a previously published method.[15]

Quantification of cytokines and chemokines

To prepare colon lysates, the colon samples were transferred to liquid nitrogen for quick-freezing, and the frozen tissues were chopped with razors. Approximately 500 μL of protease inhibitor cocktail (Sigma) was added to each sample and the tissue was further chopped. Subsequently, 1 mL of 1.5% Triton X-100 (Sigma) was added, and the samples were incubated at 4°C for 30 minutes on a shaker. The lysates were collected through centrifugation at 12 000*g* for 10 minutes at 4°C (pellet discarded, supernatant retained as the lysate), and the total protein concentration in the lysate was measured. The levels of IL-1β, IL-6, eotaxin, KC, MIP-1α, and TARC were determined through ELISA using equal amounts of total protein. To prepare colon culture supernatants, proximal colons were chopped into minute pieces using sterile razors and cultured overnight in DMEM media supplemented with 10% fetal bovine serum and antibiotics in a 37°C incubator. The supernatants were collected, and the levels of IL-1β, IL-6, GM-CSF, IL-10, IFN-γ, TNF-α, eotaxin, KC, MDC, MIP-1α, RANTES, and TARC were measured using ELISA. The serum levels of IL-6, eotaxin, MDC, and TARC were also determined using ELISA. All ELISAs were performed using an array at a commercial facility (Quansys Biosciences, Logan, UT, USA). The timing of the collection of the colon and serum samples relative to DSS treatment is indicated in the figure legends.

Polyribosome profiling analysis and determination of GAIT element-mediated translational silencing activity in colon lysates

The polysomal analysis was performed using colon lysates from control and KO mice fed DSS-water for seven days. The colon lysates were prepared by quick freezing the colon tissues in liquid nitrogen, followed by finely chopping the frozen tissues using a sterile scalpel and homogenizing the tissue in a Dounce homogenizer in 1 mL of polysome lysis buffer (10 mM HEPES, pH 7.5, 100 mM KCl, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mg/mL cycloheximide, 50 U of recombinant RNasin (Promega, Madison, WI, USA), 0.1% Igepal-CA630 (Sigma-Aldrich, St. Louis, MO, USA) and 10 mM of sodium orthovanadate (Sigma-Aldrich, St. Louis, MO, USA)). The polysomes were resolved, and the chemokine mRNAs were identified in the fractions using previously established methods.[15,16] GAIT element-mediated translational silencing was determined using a previously reported protocol.[15]

Identification of live bacteria in the circulation of the DSS-treated mice

Blood was isolated from mice fed 3% DSS (w/v) in the drinking water through cardiac puncture into tubes containing 0.1 M EDTA. Plasma was isolated by centrifuging whole blood at 4000 rpm for 5 minutes at room temperature. The plasma from each animal was diluted with sterile PBS in a 1:8 ratio and plated on blood agar plates (Remel Inc., Lenexa, KS, USA). The plates were incubated at 37°C for 24 hours to facilitate colony growth.

Statistical analysis

The log-rank (Mantel–Cox) test was used to determine the statistical significance of differences in the survival rates between DSS-fed control mice and KO mice. The results are represented as the mean ± standard deviation (SD). For all other results, the statistical significance of the differences between groups was determined using a two-tailed Student's *t*-test. p values $\leqslant 0.05$ were considered statistically significant. All statistical analyses were performed using GraphPad Prism 5.0 software.

RESULTS

DSS-induced experimental colitis was more severe in myeloid-specific L13a KO mice

DSS induces experimental colitis in mice, which faithfully recapitulates the majority of the clinical features of UC, including colon shortening, colorectal bleeding with diarrhea, weight loss, and increased mortality.[1,5] The inflammation mediated through innate immune cells such as macrophages play a major role in the pathogenesis of both UC[2] and DSS-induced colitis.[19,24] In the present study, we used a DSS-induced experimental colitis model to determine the role of L13a-dependent translational silencing of a cohort of inflammatory proteins in macrophages as a physiological defense against UC. To this end, multiple aspects of this disease were compared between myeloid-specific L13a KO mice ($L13a^{flox/flox}LysMCre^{+}$, generated and characterized in our previous work[15,16]) versus control animals ($L13a^{flox/flox}$). These studies showed an approximately 99% depletion of L13a protein in peritoneal macrophages but no detectable depletion in other organs, such as liver and kidney. By contrast, we recently observed an approximately 50% depletion of L13a protein in an Ly6G/CD11b double-positive neutrophil-enriched bone marrow fraction from these KO mice (Supplementary Figure 1c and d). However, an approximately 99% depletion in bone marrow-derived macrophages and peritoneal macrophages was observed in the KO mice compared with the controls. However, undifferentiated bone marrow cells showed no depletion of L13a in the KO mice compared with the controls (Supplementary Figure 1a and b). The depletion of L13a expression was quantified (data not shown) by measuring the ratio of the abundance of L13a protein and actin in the same sample using immunoblot analysis. Providing mice with drinking water containing 2.5% or 3% DSS *ad libitum* induces experimental colitis. The loss of body weight following DSS treatment was more severe in KO mice compared with controls beginning on day 5, with the difference between KO and control animals increasing over time (Figure 1a). Kaplan–Meier survival analysis showed the significantly diminished survival

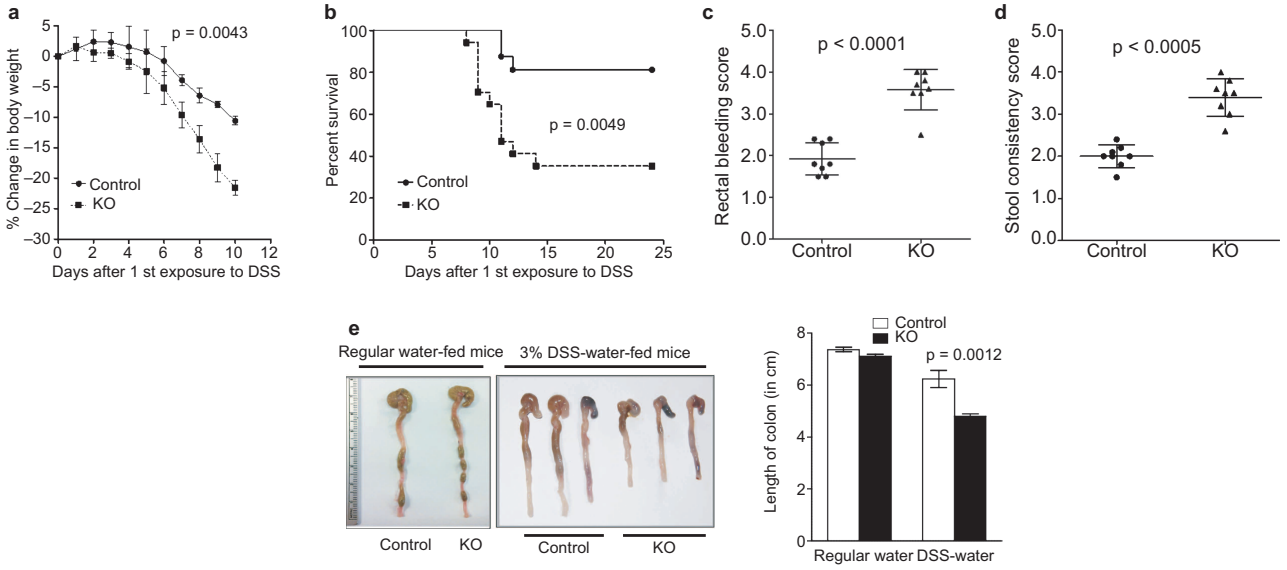

Figure 1 Significantly enhanced susceptibility to DSS-induced experimental colitis in myeloid-specific L13a-KO mice. (a) Changes in body weight during the course of DSS administration. The mice were provided 3% DSS-containing drinking water *ad libitum* for **nine** days followed by **one** day of regular drinking water. The mice were weighed daily, and the % change relative to weight just prior to the start of treatment was determined for each animal. The mean % change ± SD is shown ($n = 10$ for control and 11 for KO, $p = 0.0043$ by paired two-tailed Student's *t*-test). (b) Kaplan–Meier survival plot for mice that w**as** provided with 2.5% DSS-containing drinking water *ad libitum* for 14 days, followed by regular drinking water for 11 days ($n = 16$ per group, $p = 0.0049$ by log-rank test, Mantel–Cox). (c **and** d) Semi-quantitative rectal bleeding and diarrhea scores for mice treated with 3% DSS-containing drinking water *ad libitum* for **nine** days ($n = 8$ per group, $p < 0.0001$ and $p = 0.0005$, respectively, by paired two-tailed Student's *t*-test). See **"**Materials and Methods**" section** for scoring details. (e) Colon shortening in mice fed with 3% DSS-containing water *ad libitum* for **nine** days, followed by **one** day of regular drinking water or mice maintained on regular water (no DSS) for 10 days. On the 10th day the mice were euthanized, and the colons were harvested for length measurements. Representative photos are shown in the left panel. The quantification of the results is shown in the right panel ($n = 4$ per group for regular water-treated animals and $n = 8$ per group for DSS-water-treated animals; $p = 0.0012$ for the comparison of KO and control groups provided with DSS-water using paired two-tailed Student's *t*-test).

of DSS-fed KO mice compared with controls (31% versus 82% on day 25 after treatment initiation, $p = 0.0049$ by log-rank test, Mantel–Cox; Figure 1b). No significant differences in body weight or survival were observed between control mice and KO mice that were provided with regular drinking water, and both groups of animals consumed equivalent amounts of food and DSS-containing water over the course of the entire study (data not shown). Therefore, the effects shown in Figure 1a and b are clearly due to the impact of the myeloid-specific abrogation of L13a on the pathogenesis of DSS-induced colitis.

To further assess the severity of disease in DSS-water-fed KO animals versus control animals, we assigned semi-quantitative pathological scores (see "Materials and Methods" section for details) for two visible signatures of DSS-induced colitis: colorectal bleeding and stool consistency. We observed bloody stool in both groups after day 5 post-treatment initiation; however, colorectal bleeding and bloody diarrhea were significantly more severe in KO mice than in control mice (Figure 1c and d). In addition, colon shortening, which is an essential feature of colitis, was more pronounced in DSS-water-administered KO animals than in similarly treated control animals (Figure 1e). However, no significant difference in the colon length was observed between KO animals and control animals fed regular drinking water.

We also examined whether myeloid-specific L13a KO mice would experience any delay in recovery from disease induced through transient exposure to DSS. The mice were subjected to 2.5% DSS administration for seven days, followed by regular drinking water for four weeks in the recovery phase. Body weights were monitored throughout the study. Both control and KO groups displayed body weight loss until day 11 after the start of treatment and then began to regain weight. However, the control group showed less severe weight loss and recovered significantly more bodyweight than the KO group (Supplementary Figure 2a). Animals surviving to day 35 were euthanized, and sections of the colons were stained with PAS. This showed prominent regeneration of the epithelial crypts in the control group, whereas in the KO group, granulation tissue replaced the ulcerated epithelium (Supplementary Figure 2b). Together, these studies show that the myeloid-specific deficiency of L13a leads to an increased severity of DSS-induced colitis and a substantially delayed recovery from this disease.

Myeloid-specific depletion of L13a increases the disruption of epithelial crypts and widespread infiltration of macrophages in the colon mucosa upon DSS challenge

To verify colon inflammation in the mouse model of DSS-induced colitis, we evaluated the overall morphology of the epithelial crypts through H&E staining (Figure 2a and b),

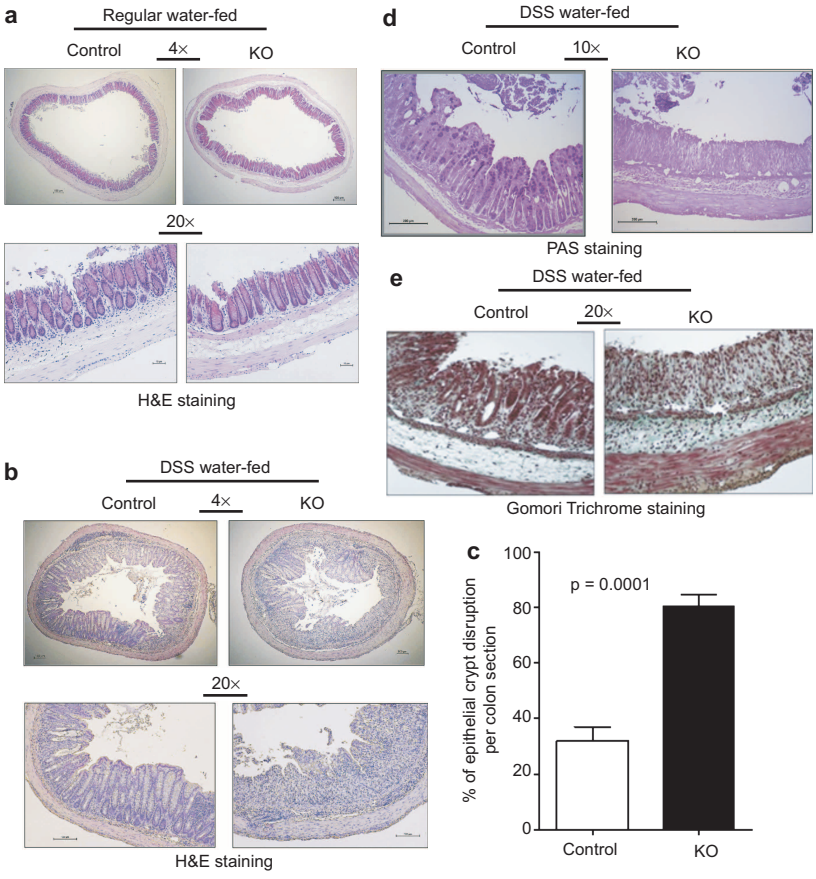

Figure 2 Genetic depletion of L13a in macrophages aggravates the injury to the epithelial lining of the colon in a mouse model of DSS-induced experimental colitis (a) H&E-stained sections of the distal colon from mice fed with regular drinking water for 10 days (no DSS) showed that the epithelial crypt lining is intact and morphologically indistinguishable between KO and control mice. Images taken at original magnification X4 and X20 are shown at 100 μm and 10 μm scales, respectively ($n = 3$ per group, representative images from one animal per group are shown). (b) H&E-stained distal colon sections from mice fed with 3% DSS-containing water for **seven** days followed by **one** day of regular drinking water show greater morphological disruption (mucosal and submucosal inflammation) in KO mice than in controls (pictures taken at 4× and 20× magnifications are shown with 100 μm and 10 μm scales. (c) Quantification of the epithelial crypt disruption in KO versus control mice following DSS treatment. The mice were treated as in b 27 colon sections from **nine** mice per group were analyzed (mean ± SD; $p = 0.0001$ by paired two-tailed Student's *t*-test) (d) PAS staining of the colon sections from DSS-treated mice confirms the increased loss of goblet cells in the colons of KO mice compared with controls. $n = 4$ per group, images from one animal per group are shown at 10× magnification with a 100 μm scale. (e) Gomori Trichrome staining of the colon sections from DSS-treated mice reveals increased connective tissue (green) in the disrupted mucosa and submucosa of KO mice compared with controls.

and the status of mucin-producing epithelial goblet cells was evaluated through PAS staining (Figure 2d). The results showed the extensive replacement of epithelial crypts with granulation tissue (Figure 2b and c) and substantial loss of mucin-producing goblet cells (Figure 2d) in the KO animals that were provided with DSS-containing water, but not in the similarly treated control mice. In addition, Gomori Trichrome staining revealed increased connective tissue in the mucosa and submucosa of colon sections obtained from DSS-fed KO mice relative to controls (Figure 2e). Normal crypt architecture was preserved in both control mice and KO mice that were provided with regular drinking water (Figure 2a).

Next, we investigated whether the extensive damage to the colon lining observed in DSS-challenged KO mice was also accompanied by macrophage infiltration into the colon mucosa, a hallmark of intestinal inflammation. Immunostaining for the macrophage-specific Mac2 marker revealed negligible macrophage infiltration in the colons of both control and KO groups fed with regular drinking water (Figure 3a). By contrast, we observed that there was extensive infiltration of macrophages into the colon mucosa and submucosa of DSS-water-fed KO mice but not in DSS-fed control mice (Figure 3b). The expression of Mac2 (galectin-3) is mostly confined to inflammatory macrophages. Therefore, we also used a more conventional marker for macrophages, such as F4/80, to measure the abundance of F4/80-positive macrophages through immunostaining of the colon sections. The results showed the substantial infiltration of F4/80-positive macrophages into the colon mucosa and submucosa of DSS-water-fed KO mice but not into that of similarly treated control mice (Figure 3d). As expected, negligible F4/80-positive

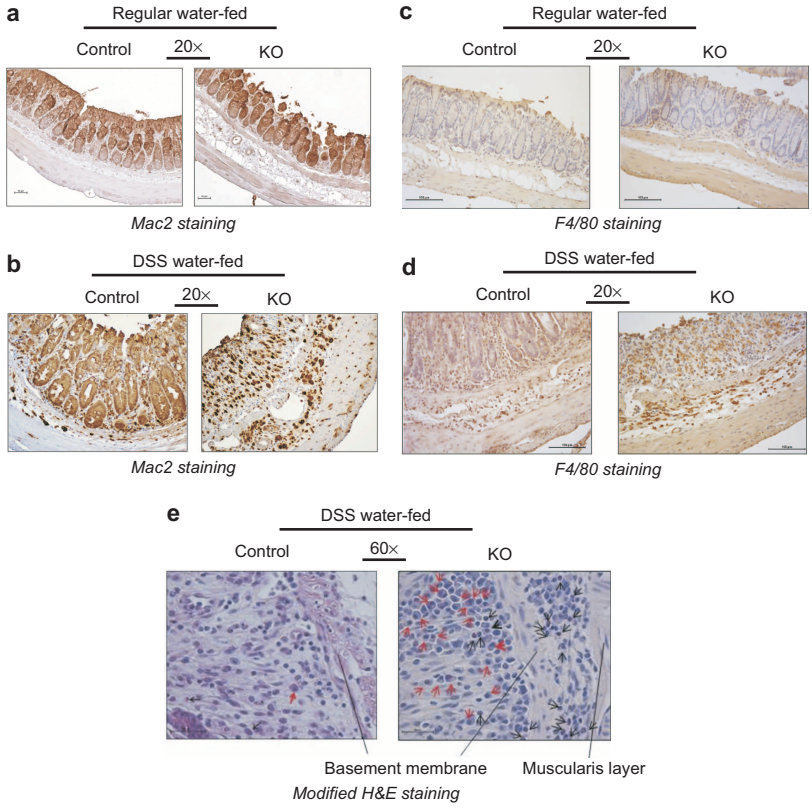

Figure 3 The colonic mucosa of the KO mice shows the widespread infiltration of immune cells. (a) and (c) Colon sections from control and KO mice fed with regular water showed similar distribution of macrophages. The sections were stained with Mac2 and F4/80 antibodies ($n = 4$ per group; images from one representative animal per group are shown at 20× magnification with a 100 μm scale. (b) and (d) Anti-Mac2- and anti-F4/80-stained colon sections from DSS-treated KO mice showing the enhanced infiltration of Mac2- and F4/80-positive macrophages into the lamina propria and submucosa compared with similarly treated control mice. The mice received 3% DSS-containing water for **seven** days, followed by **one** day of regular water ($n = 6$ per group), and the images from one animal per group are shown at 20×magnification with a 100 μm scale. (e) Modified H&E staining shows that colon sections of DSS-treated KO mice contain infiltrates of plasma cells and eosinophils in the lamina propria and submucosa. Plasma cells and eosinophils are indicated with red and black arrows, respectively ($n = 4$ per group; the image for one animal per group is shown at 60× magnification with a 100 μm scale).

macrophage infiltration was observed in KO mice and control mice that were fed with regular drinking water (Figure 3c). As a complementary approach, we also quantified F4/80-CD11b double-positive leukocytes within the total population of isolated cells from the colons of DSS-fed KO and control mice through FACS analysis. Consistent with the results obtained from immunostaining of the tissue sections, DSS-fed KO mice showed a greater abundance of F4/80-CD11b double-positive cells compared with the control (Supplementary Figure 3). In addition, modified H&E staining of the colons of DSS-fed KO mice demonstrated the increased infiltration of plasma cells and eosinophils (Figure 3e). Taken together, these results suggest that absence of L13a expression in macrophages leads to enhanced inflammatory infiltration and tissue injury in the mouse colon following DSS exposure.

Genetic abrogation of L13a in macrophages increases the production of cytokines and chemokines in the mouse colon during experimentally induced colitis

Chemokines and cytokines serve as cues to attract leukocytes and to markedly facilitate the infiltration of these cells into sites of inflammation. To gain further insight into the enhanced recruitment of L13a-depleted macrophages into the colonic mucosa, we measured the steady-state levels of several chemokines and cytokines in colon culture supernatants and colon tissue homogenates. To examine the colon culture supernatants, control and KO mice were treated with 3% DSS-containing water for seven days. The proximal colons were subsequently harvested and cultured overnight in DMEM medium, and the culture supernatant was collected for ELISA array-based assessment of the chemokine/cytokine levels. The supernatants from the colon cultures of DSS-fed KO animals showed significantly higher levels of the 12 tested chemokines and cytokines, including IL1β, IL-6, GM-CSF, IL-10, TNF-α, IFN-γ, eotaxin (CCL11), KC (CXCL1), MDC (CCL22), MIP-1α (CCL3), RANTES (CCL5), and TARC (CCL17), compared with those from DSS-fed control mice (Figure 4). The same assay was used to evaluate the homogenates prepared from the distal colons of the above-mentioned KO and control mice (3% DSS in water for seven days; Supplementary Figure 4). These results showed elevated steady-state levels of IL1β, IL-6,

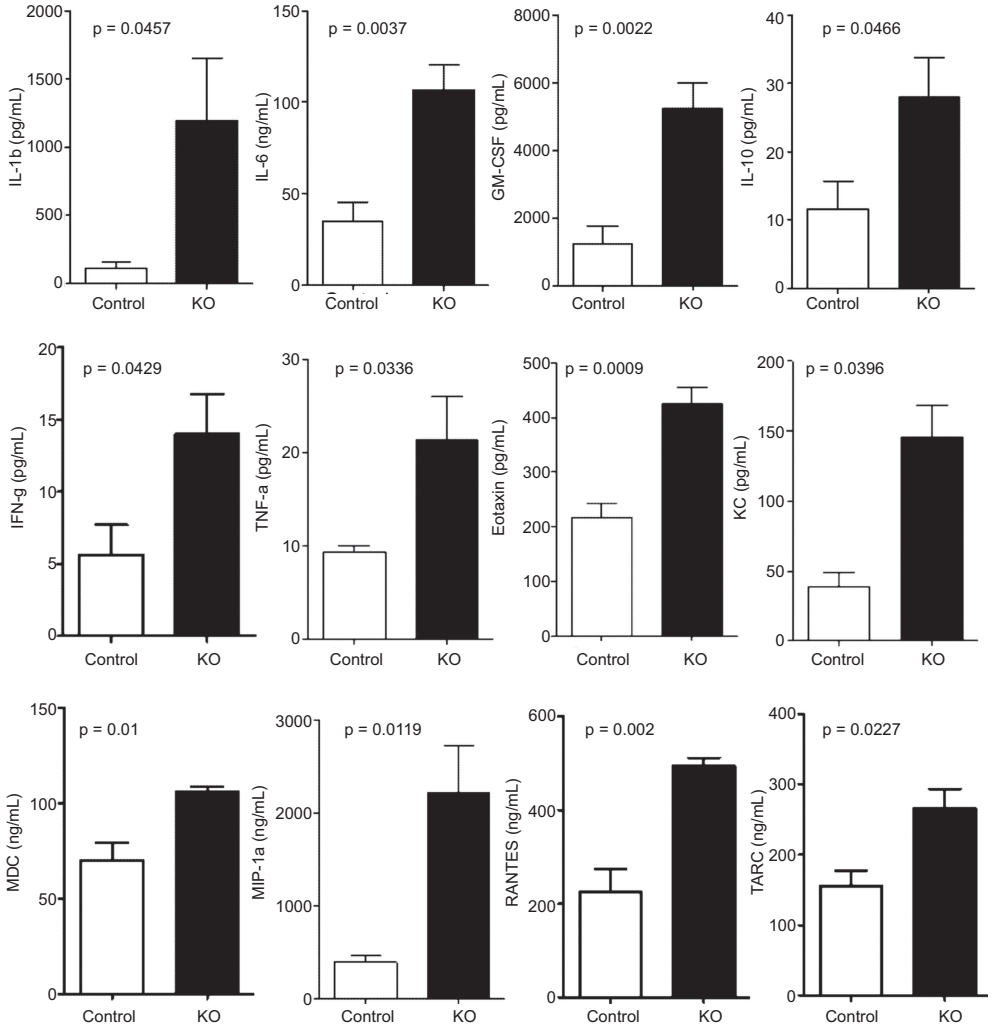

Figure 4 Myeloid-specific depletion of L13a elevates the production of an array of inflammatory cytokines and chemokine ligands in the colons of DSS-treated mice. The colons were harvested from control and KO mice treated with 3% DSS-containing water for seven days. The proximal colon from each mouse was cultured overnight in DMEM, and the culture supernatants were collected to measure the levels of the 12 indicated proteins using ELISA ($n = 5$ per group; mean ± SD is shown; p values were calculated using paired two-tailed Student's t-test).

eotaxin, KC, MIP-1α, and TARC in DSS-fed KO mice compared with DSS-fed controls. Interestingly, several of these molecules, such as CCL3, CCL11, and CCL22, were previously identified as targets of L13a-dependent translational silencing.[13,15] The concentrations of these cytokines and chemokines in colon samples from animals fed regular drinking water (both KOs and controls) were below the lower limit of detection of the assay (data not shown). Collectively, these results demonstrated that the myeloid-specific deficiency of L13a increased the production of pro-inflammatory chemokines and cytokines in this model of DSS-induced experimental colitis.

The myeloid-specific depletion of L13a amplifies the DSS-induced inflammatory response to septicemia

To investigate whether the exacerbated DSS-induced colonic inflammation associated with the myeloid-specific depletion of L13a extended to a systemic response, we analyzed the levels of pro-inflammatory cytokines in the serum samples from KO and control mice that were administered either regular or DSS-containing drinking water. Significantly higher levels of IL-6, eotaxin, MDC, and TARC were detected in the serum of DSS-treated KO mice compared with controls (Figure 5a). By contrast, no cytokines or chemokines were detected in the serum of either KO or control mice fed regular drinking water (data not shown).

Because systemic inflammation from DSS-induced colitis is known to lead to splenomegaly,[25,26] we evaluated the size and weight of the spleens collected from KO and control mice after seven days of 3% DSS-water treatment followed by one day with regular water. The results showed an increase in spleen size and weight in DSS-fed KO mice compared with controls (Figure 5b). Moreover, immunostaining of spleen sections for Mac2 revealed an increase in the number and the size of macrophages in the periarteriolar lymphoid sheath for DSS-treated KO mice relative to DSS-treated controls (Figure 5c). Thus, the

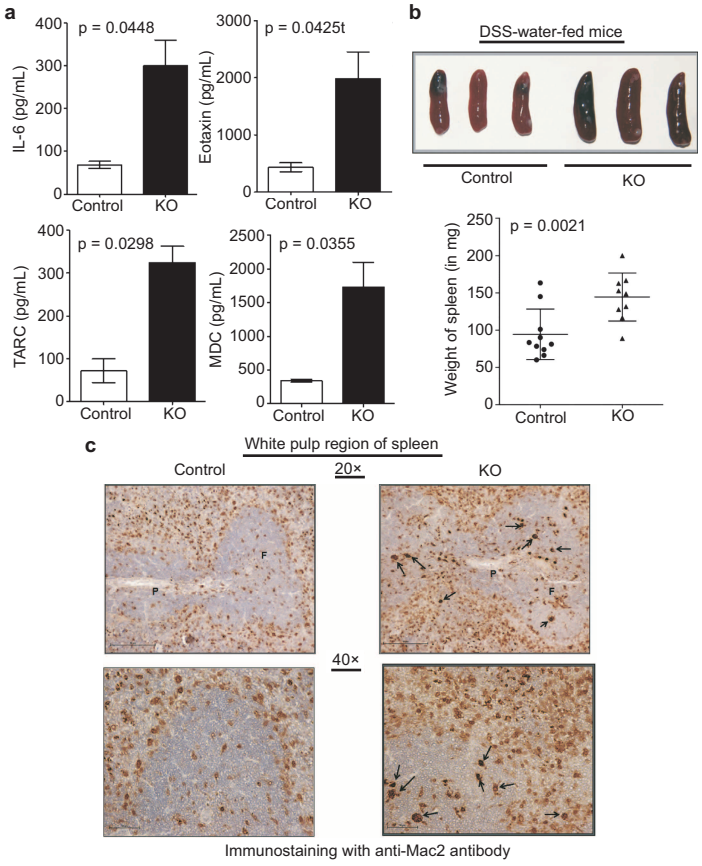

Figure 5 The colonic inflammation in the KO mice is amplified to a systemic response upon DSS treatment. (a) Serum levels of inflammatory cytokines are significantly higher in DSS-water-fed KO mice compared with similarly treated controls. The mice were fed 3% DSS-containing water for **seven** days, followed by **one** day of regular water. Blood was collected through cardiac puncture, and the serum samples were analyzed using ELISA at a commercial facility (Quansys Biosciences) ($n = 8$ per group; mean ± SD is shown; p values were calculated using paired two-tailed Student's t-test). (b) DSS-treated KO mice have larger spleens than DSS-treated controls. The spleens were harvested from the mice described in a and photographed and weighed. Images from **three** representative animals per group are shown. The lower panel shows quantification of the difference in spleen weights in DSS-fed control and KO groups ($n = 8$ per group; mean ±SD is shown; p values were calculated using paired two-tailed Student's t-test). (c) DSS-treated KO mice display increased number and size of macrophages in the white pulp of the spleen compared with DSS-treated control mice. The spleen sections from DSS-treated control and KO mice were stained with Mac2 antibody. Black arrows denote enlarged macrophages. Images at 20× magnification show the white and red pulp areas. P and F indicate the periarteriolar lymphoid sheath and follicles, respectively. Images at 40× magnification show the white pulp region at a 10 μm scale.

results of both these analyses provided further evidence of intensified systemic inflammation upon DSS treatment in the absence of L13a.

A complex interplay between host immune cells and commensal bacteria governs intestinal homeostasis and maintains the integrity of the mucosal barrier. In UC, the penetration of commensal microbes through the damaged epithelial barrier causes inflammation, which markedly contributes to the pathogenesis of UC.[5] To directly test for the presence of live bacteria in DSS-treated KO mice and to determine the association of these microbes with the observed severity of the disease, we tested collected plasma samples in a bacterial colony formation assay. The plasma samples from four out of eight KO mice and one out of nine control mice showed colony formation (Figure 6a). No colonies were detected in the plasma samples obtained from KO and control mice that were fed regular water (Figure 6b). Figure 6b shows a typical colony formation assay, with each plate representing an individual animal. By contrast, we also observed significantly higher endotoxin levels in the plasma of DSS-treated KO mice compared with DSS-treated controls; however, the endotoxin levels were very low and no difference was observed between the two groups that were fed regular drinking water (Figure 6c). Taken together, these results show that the myeloid-specific depletion of L13a magnifies DSS-induced inflammation to a systemic immune response causing septicemia and enhanced severity of this disease.

Genetic depletion of L13a in myeloid cells impedes the translational silencing of GAIT target mRNAs upon DSS-induced experimental colitis

On the basis of the results from a previous study, we hypothesized that the absence of L13a-mediated translational silencing

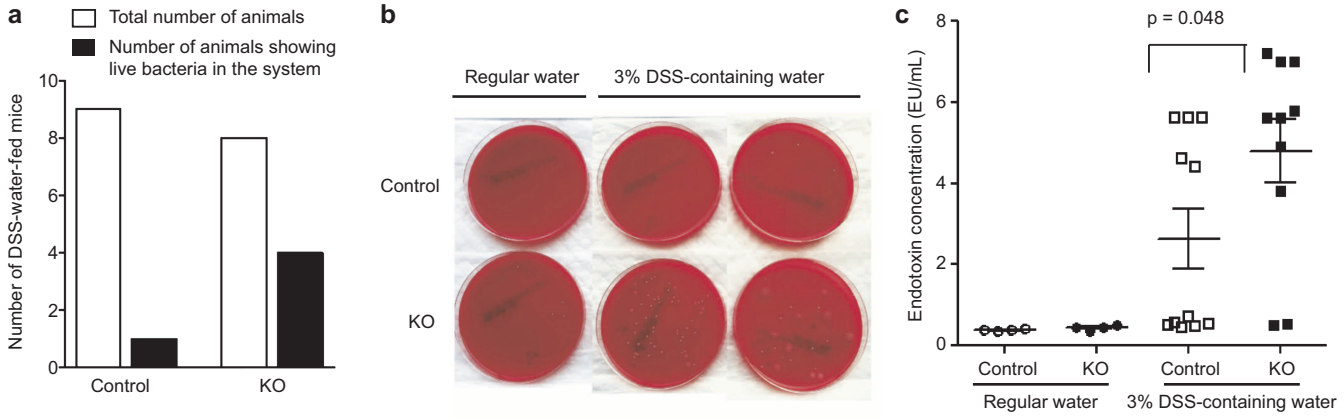

Figure 6 DSS-induced colonic inflammation results in septicemia in mice with myeloid-specific depletion of L13a. The mice were provided with 3% DSS-containing water for nine days followed by **one** day of regular drinking water. Blood was collected through cardiac puncture, and plasma was prepared, diluted 1:8 with sterile PBS and plated onto blood agar plates. Bacterial growth was assessed after incubation for 24 h**ours** at 37°C. (a) The presence of live bacteria was observed in the serum of **one** out of **nine** (11%) DSS-water-fed control mice and **four** out of **eight** (50%) DSS-water-fed KO mice. By contrast, for the group fed with regular drinking water, no live bacteria were detected in the plasma of any of the **eight** tested animals. (b) Representative images of plates with bacterial colonies generated from the plasma of control and KO mice with or without DSS treatment. Each plate represents one animal**.** (c) The plasma samples from control and KO mice were subjected to endotoxin assay using Pierce LAL chromogenic endotoxin quantitation kit. ***n*** = 4 per group for regular drinking water-fed mice and ***n*** = 10 per group for DSS-containing water-fed mice.

of GAIT target mRNAs might be responsible for the increased severity of DSS-induced experimental colitis that is observed in KO mice. To directly test this hypothesis, we reconstituted GAIT-dependent translational silencing *ex vivo* using an *in vitro* translation system of rabbit reticulocyte lysates (RRL) and luciferase reporter RNA with an authentic GAIT element in the 3′-UTR.[17] The lysates prepared from the colons of control and KO mice that were fed either DSS-containing or regular drinking water were added to this *in vitro* translation system to test for the presence of translational silencing activity. This experiment showed that the translational silencing of the GAIT element-containing RNA was activated upon DSS treatment. However, the lysates obtained from the colons of DSS-fed KO mice did not support GAIT element-mediated translational silencing (Figure 7a). These results clearly showed that the myeloid-specific depletion of L13a impedes endogenous translational silencing in response to DSS-induced intestinal inflammation.

To further confirm the deregulation of GAIT-dependent translational silencing as the mechanism responsible for the increased severity of the DSS-induced experimental colitis observed in KO mice, we compared the polyribosomal association of three known GAIT target mRNAs, CXCL13, CCL22, and CCR3, in colon lysates obtained from control mice and KO mice fed with DSS-water for seven days. The results showed a substantial increase in the polyribosomal abundance of all three GAIT target mRNAs in the colons of the DSS-treated KO mice compared with the controls (Figure 7b). Therefore, the loss of L13a expression in macrophages led to the abrogation of translational silencing of GAIT target mRNAs in the colon during DSS-induced experimental colitis.

DISCUSSION

The intestinal lamina propria contains the largest reservoir of macrophages in the body,[27,28] which is maintained by continuous replenishment from circulating monocytes.[29] When the colon is inflamed, these monocytes give rise to highly pro-inflammatory macrophages,[3] and the uncontrolled activation of these innate immune cells plays a critical role in the pathogenesis of UC.[6] However, it was not previously known whether any endogenous mechanism exists in the innate immune cells of the gastrointestinal tract to counteract inflammation and to provide natural protection to the host against UC. The results of the present study suggest that L13a-mediated translational silencing of a cohort of inflammatory proteins in macrophages is one such protective mechanism.

Considering the cardinal role of pro-inflammatory intestinal macrophages in the pathogenesis of UC, we hypothesized that L13a-dependent translational silencing might be important for the maintenance of intestinal homeostasis and prevention of UC. To examine this hypothesis, we used a model of DSS-induced experimental colitis and myeloid-specific L13a KO mice. The evaluation of multiple aspects of this disease showed that DSS treatment led to significantly more severe colitis in KO mice than in control mice. KO mice displayed significantly exacerbated clinical symptoms, including severe weight loss, rectal bleeding, and diarrhea, colon shortening and significantly increased mortality (Figure 1). We further investigated the severity of DSS-induced colitis at the level of the cellular architecture and content of the colon mucosa. Consistent with the disease severity, we observed the increased destruction of the epithelial crypts and infiltration of macrophages and other immune cells in the colonic mucosa (Figures 2 and 3) of DSS-water-fed KO mice compared with control mice. The central

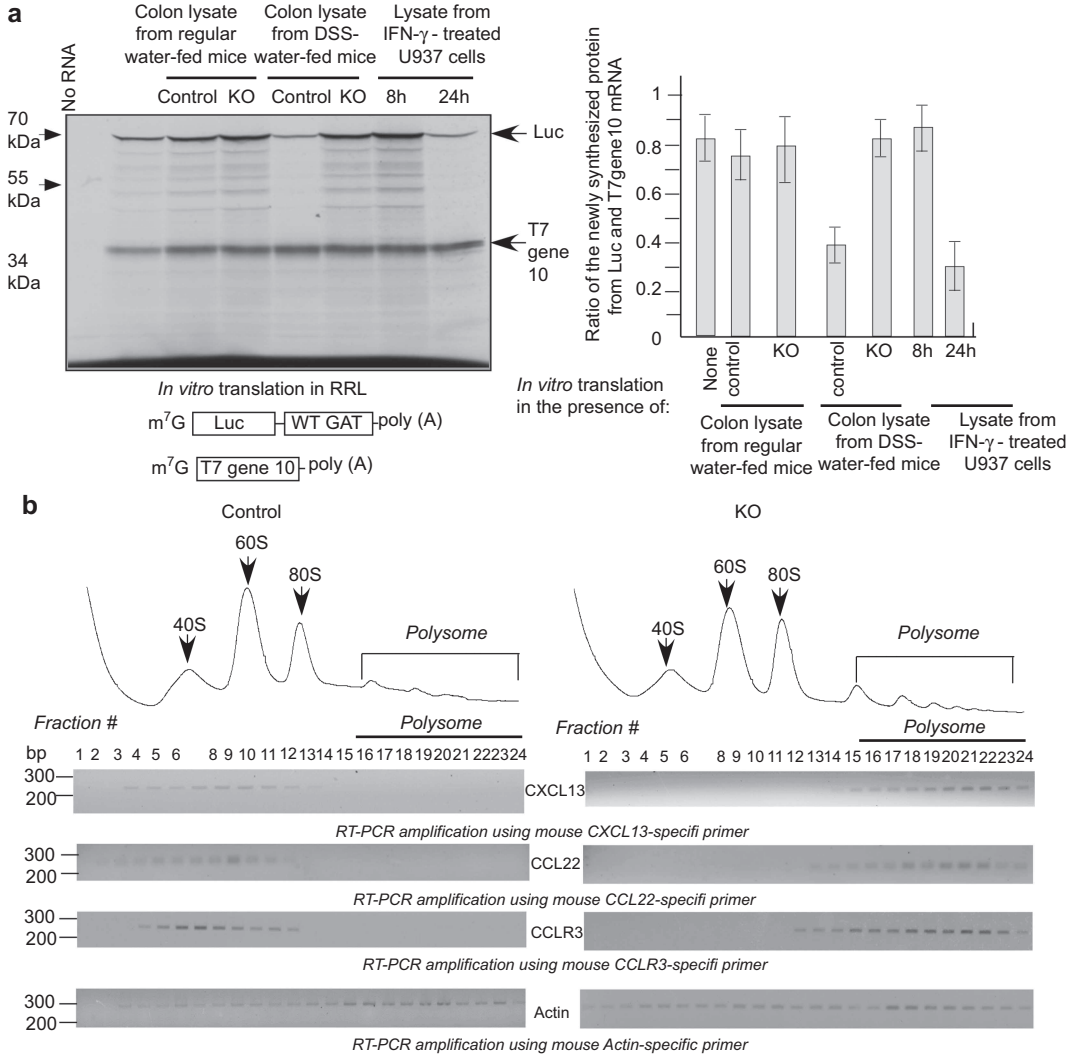

Figure 7 L13a-dependent translational silencing of GAIT element-containing mRNAs is induced through DSS-treatment in control mice but not in KO animals. (a) DSS-induced colonic inflammation activates the silencing of a GAIT element-containing reporter RNA in an L13a-dependent manner. A GAIT element-containing reporter luciferase RNA and control T7 gene 10 RNA (illustrated schematically at the bottom of the panel in a) were *in vitro* translated using rabbit reticulocyte lysates (RRL). The lysates of the colons harvested from DSS-water-fed control or KO mice were added into the *in vitro* translation system to test for the presence of silencing activity. The lysates from U937 cells treated with IFN-γ for 8 h**ours** and 24 h**ours** were used as positive controls. The translation products were visualized through SDS-PAGE, followed by autoradiography. The right panel shows the quantification of the results from three independent experiments after measuring the band intensities of the newly translated proteins from the Luc and T7 gene10 mRNA using a RRL *in vitro* translation system**.** (b) Myeloid-specific depletion of L13a abrogates translational silencing of several endogenous GAIT-regulated mRNAs (CXCL13, CCL22**,** and CCR3) in the colons of DSS-water-fed mice. The lysates were prepared from the colons of DSS-treated control or KO mice and subjected to polyribosome fractionation through a sucrose gradient. RT-PCR analysis of the fractions was conducted using primer pairs specific for each target mRNA mentioned compared with the controls. KO mice showed the increased abundance of CXCL13, CCL22**,** and CCR3 (but not actin) mRNAs in translationally active heavy polyribosome fractions.

role of inflammatory cytokines[6] and chemokines[30,31] in the pathogenesis of UC has recently been well appreciated. The chemokine-mediated aberrant chemoattraction of leukocytes in the inflamed intestinal epithelium promotes the destruction of epithelial crypts. Using both colonic culture supernatants and colonic lysates, we observed significantly higher levels of chemokines and cytokines in the colons of DSS-treated KO animals compared with DSS-treated control animals. These differentially produced molecules included several known targets of L13a-dependent and GAIT-mediated translational silencing, namely, MIP-1α (CCL3), MDC (CCL22), and eotaxin (CCL11) (Figure 4 and Supplementary Figure 4). Moreover, all of these chemokines have been previously implicated in the pathogenesis of UC.[32–34]

One striking feature of the present study is the finding that DSS-induced intestinal inflammation could be amplified to a systemic inflammatory response and ultimately to sepsis through the genetic depletion of L13a in macrophages, as evidenced by the findings of the significantly elevated serum levels of several inflammatory cytokines (Figure 5a), splenomegaly

(Figure 5b), and presence of live bacteria and endotoxins in the plasma of DSS-treated KO mice but not of control mice (Figure 6). The immunostaining of spleen sections showed an increase in both the number and size of Mac2-positive macrophages in the white pulp of DSS-fed KO mice compared with controls (Figure 5c). One potential interpretation of this finding is that the splenic macrophages of the KO mice are highly phagocytic. It is important to note that the physical and biochemical barrier formed by the intestinal epithelial cells separates the luminal microbiota from the mucosal immune system.[35] The luminal goblet cells, which secrete mucin and antimicrobial peptides, are essential for this barrier function[36,37] and for the organization of the mucous layer at the epithelial surface.[38] These results showed the severe damage of epithelial crypts (Figure 2b and c), loss of mucin-producing goblet cells (Figure 2d), and the appearance of live bacteria in the serum upon induction of experimental colitis in animals with myeloid-specific L13a deficiency. Taken together, the results of the present study suggest that the absence of L13a in macrophages impairs intestinal homeostasis, thereby promoting a systemic response.

We previously used a cellular model of human monocytes[13] and an animal model of myeloid-specific L13a KO mice[15] to show the ability of L13a-dependent translational silencing to block the translation of different chemokine and chemokine receptors harboring GAIT elements in the 3'UTRs of the mRNA. Using animal models of LPS-induced endotoxemia[15] and high-fat diet-induced atherosclerosis,[16] we also showed the potential of this mechanism to resolve physiological inflammation. These studies are consistent with emerging evidence from other groups regarding the importance of translational control in innate immunity and inflammation.[14,39] Here, we recapitulated the translational silencing of reporter mRNA harboring the GAIT element in the 3′-UTR using an *in vitro* translation system. This experiment clearly showed that the L13a deficiency of the macrophages infiltrating the colon abrogates GAIT element-mediated translational silencing (Figure 7a). L13a-dependent and GAIT element-mediated translational silencing is activated in response to DSS-induced colonic inflammation in control mice but not in KO mice (Figure 7a), and interestingly this mechanism also targets DSS-induced mRNAs encoding inflammatory proteins. Indeed, polyribosomal profiling of the colon lysates showed a higher translational status of several chemokines (e.g., CCR3, CCL22, CXCL13, etc.) in the colons of DSS-fed KO mice compared with DSS-fed controls (Figure 7b). These chemokines harbor GAIT elements in the 3′-UTRs.[13] Clearly, these findings are highly consistent with the notion that the termination of inflammation is a self-limiting response.

Clinical trials to treat IBD using recombinant anti-inflammatory cytokines, such as IFN-β, IL-10, and IL-11, or antibodies targeting specific inflammatory cytokines, such as TNF-α, IFN-γ, and IL-17A, have received considerable attention. Unfortunately, except for treatment with neutralizing antibodies to TNF-α, these interventions produced disappointing outcomes and in some cases, even aggravated the disease,[6,40] strongly suggesting that targeting only a single molecule might not achieve efficient therapeutic outcomes for complex diseases such as IBD, UC, or Crohn's disease, which result from the concerted actions of many inflammatory molecules. In the present study, we identified L13a-dependent translational silencing as a novel inflammation-resolving mechanism that impacts the synthesis of multiple molecules relevant to these diseases. Therefore, the pharmacological manipulation of this mechanism could offer significant promise as a therapeutic strategy against IBD, UC, and/or Crohn's disease.

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