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An Extraribosomal Function of Ribosomal Protein L13a in Macrophages Resolves Inflammation

Darshana Poddar,* Abhijit Basu,* William M. Baldwin, III,† Roman V. Kondratov,* Sailen Barik,* and Barsanjit Mazumder*

Inflammation is an obligatatory attempt of the immune system to protect the host from infections. However, unregulated synthesis of proinflammatory products can have detrimental effects. Although mechanisms that lead to inflammation are well appreciated, those that restrain it are not adequately understood. Creating macrophage-specific L13a-knockout mice, we report that depletion of ribosomal protein L13a abrogates the endogenous translation control of several chemokines in macrophages. Upon LPS-induced endotoxemia, these animals displayed symptoms of severe inflammation caused by widespread infiltration of macrophages in major organs causing tissue injury and reduced survival rates. Macrophages from these knockout animals show unregulated expression of several chemokines (e.g., CXCL13, CCL22, CCL8, and CCR3). These macrophages failed to show L13a-dependent RNA binding complex formation on target mRNAs. In addition, increased polyribosomal abundance of these mRNAs shows a defect in translation control in the macrophages. Thus, to our knowledge, our studies provide the first evidence of an essential extraribosomal function of ribosomal protein L13a in resolving physiological inflammation in a mammalian host. The Journal of Immunology, 2013, 190: 3600–3612.

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ynthesis of proinflammatory products by animal hosts is an obligatory attempt of the immune system to protect the host from infections. Precise, rapid, and temporal synthesis of inflammatory cytokines and chemokines by monocytes and macrophages serves as an arsenal against the invading microorganisms (1, 2). However, unregulated synthesis of these proinflammatory products can have detrimental effects (3). Thus, the endogenous mechanisms that have evolved to restrict the cytokine storm and permit the resolution of inflammation are prime targets to search for novel anti-inflammatory molecules. Despite its enormous importance, our understanding about inflammation has focused more on the mechanisms that accelerate the process, whereas information about those that restrain it remains limited (4). In this study, we identify one such mechanism in a novel animal model that relies on the abrogation of ribosomal protein L13a-dependent translational silencing by creating viable macrophage-specific L13a-knockout (KO) mice (L13a<sup>fl<sup>ox</sup>/<sup>lox</sup>LysMcRe<sup>+</sup>), in which termination of inflammation is severely compromised. Such unregulated inflammation is consistent with our series of previous studies uncovering an L13a-dependent translational silencing mechanism in IFN-γ-activated monocytic cells (5–10). This silencing is dependent upon the assembly of the gamma-activated inhibitor of translation (GAIT) complex on the GAIT element present in the 3′ untranslated region (UTR) of its target mRNAs upon the phosphorylation-dependent release of L13a from the large ribosomal subunit (10). In addition, our studies showed that P-L13a, as a part of the GAIT complex, can bind the translation initiation factor eIF4G and prevent the formation of the 48S preinitiation complex, thereby blocking the translation initiation of GAIT element–containing mRNAs (8). Our subsequent studies also showed that depletion of L13a by RNA interference in monocytes abrogates GAIT element–mediated translational silencing but, interestingly, overall protein synthesis was not inhibited (11). In addition to our studies, reports from other laboratories provided compelling evidence that controlling the protein synthesis of many inflammatory molecules from the pre-existing mRNAs could be an effective cellular strategy to prevent their accumulation (12).

Monocytes and macrophages can perform pathogenic, as well as protective, functions during innate immune responses against infectious assaults and subsequent tissue homeostasis (13). Priming of these cells with IFN-γ plays a crucial role in the innate immune response, relying in part on increased synthesis of proinflammatory cytokines and mediators to kill the invading organisms (14). Using a genome-wide approach, we showed that, in IFN-γ–induced monocyctic cells, protein synthesis from a novel post-transcriptional operon was severely inhibited; this operon was composed of mRNAs encoding several chemokines and their cognate receptors (i.e., CCL22, CCL8, CCL21, CXCL13, CCR3, CCR4, and CCR6) (9). These studies also identified functional GAIT elements in the 3′UTRs of these mRNAs, which recruited the L13a-containing GAIT complex (9). Emerging evidence suggests a critical role for chemokines and their receptors during the recruitment of mononuclear cells to the sites of inflammation (15). Based on the foregoing, we hypothesize that L13a-dependent
translated silencing could be an endogenous defense mechanism against uncontrolled inflammation. This led us to predict that a deficiency of L13a in macrophages may promote runaway inflammation and associated pathology due to the abrogation of translational silencing of these inflammatory targets. To test this hypothesis, we generated macrophage-specific L13a-KO mice using the Cre-Lox system (16) and studied their response to the inflammatory assaults caused by LPS-induced endotoxemia. The rationale for using the LPS-induced endotoxemia model is based on the fact that LPS stimulates macrophages to produce numerous proinflammatory cytokines and, most importantly, it is also a potent inducer of IFN-γ in the cells of myeloid lineage (17–19). In this study, we show that macrophage-specific L13a-KO mice are highly susceptible to endotoxemia, demonstrating a lower survival rate and invasion of myeloid cells in the peritoneal cavity and in the major organs, with clear signs of organ damage, in comparison with the control mice. Moreover, using macrophages harvested from these KO animals, we observed unregulated expression of several target proteins of the GAIT complex, probably by the abrogation of their translational silencing. To our knowledge, these results provide the first in vivo evidence of an extraribosomal anti-inflammatory function of ribosomal protein L13a and its molecular basis.

Materials and Methods

**Generation of macrophage-specific KO mice (L13a<sup>fllox/flox</sup>LysoMCr<sup>Cre<sup>-</sup></sup>)**

Embryonic stem (ES) cells from C57BL/6 mice were transfected with the targeting construct, and the ES cells harboring the loxP allele were screened by genotyping. Cre-dependent depletion was confirmed by infecting Ad-CMV-Cre, followed by recombinase under the control of CMV promoter (Ad-CMV-Cre). Two independent clones of ES cells were injected into blastocysts. The chimeric mouse was generated at the InGenious Targeting Laboratory (Ronkonkoma, NY) by blastocyte injection of rES cells and the resulting chimeric mouse was further crossed with wild type (WT) C57BL/6 females for germline transmission. The neomycin gene from L13a<sup>Cre-lox</sup>-<sup>Cre-lox</sup>mice was removed by crossing with ACTFLPe<sup>+</sup> mice on a C57BL/6 background. The F4 mice (L13a<sup>fllox/flox</sup>LysoMCr<sup>Cre<sup>-</sup></sup>) homozygous for the loxP and Neo deletion allele were confirmed using cell-free in vitro translation systems using rabbit reticulocyte lysates (Promega) in the presence of [35S]methionine. Lysates were made from the peritoneal macrophages of control and L13a<sup>-/-</sup> mice. Immunoblot analysis with anti-L13a Ab was previously raised against a peptide NVEKKIDKY-TEVLKTHG near the C terminus of human L13a (10). This Ab recognizes the chimeric L13a<sup>fllox/flox</sup>LysoMCr<sup>Cre<sup>-</sup></sup>-LysoMCr<sup>Cre<sup>+</sup></sup> homozygous for the loxP and Neo deletion allele were confirmed by genotyping. The Cre-dependent depletion of L13a in the F4 mice was confirmed by infecting the isolated lung fibroblasts from these mice with Ad-CMV-Cre, followed by immunoblot analysis with anti-L13a Ab. The macrophage-specific KO mice (L13a<sup>fllox/flox</sup>LysoMCr<sup>Cre<sup>-</sup></sup>) were generated by crossing the L13a<sup>fllox/flox</sup> mice with LysoMCr<sup>Cre<sup>-</sup></sup> mice (The Jackson Laboratory, Bar Harbor, ME) and identified by genotyping. The macrophage-specific depletion of L13a in L13a<sup>fllox/flox</sup>LysoMCr<sup>Cre<sup>-</sup></sup> mice was confirmed by immunoblot analysis using anti-L13a Ab.

**Abs for immunoblot analysis**

Anti-L13a Ab was previously raised against a peptide NVEKKIDKY-TEVLKTHG near the C terminus of human L13a (10). This Ab recognizes a specific band both for human and mouse L13a between 28 and 21 kDa. Anti-L-28 and anti-actin Abs were from Santa Cruz Biotechnology and Sigma-Aldrich, respectively. Anti-Cre recombinase Ab was from Abcam.

**Animal handling and isolation of leukocytes**

All experiments involving mice were carried out in accordance with National Institutes of Health and Institutional Animal Care and Use Committee guidelines. Age- and sex-matched mice were challenged by i.p. injection of thioglycollate (1.5 ml, 4% solution in distilled water) or LPS (15 mg/kg body weight). The peritoneal lavage was collected with ice-cold PBS after 72 hr for thioglycollate-injected mice and after 24 and 48 hr for LPS-injected mice. The recovered cells were counted with a cell counter. Total leukocytes were isolated from spleen after isolation of a cell pellet from a single-cell suspension and lysing the RBCs from the cell pellet using RBC lysis buffer (eBioscience), following the manufacturer’s suggested method. The mononuclear cells from blood were isolated by layering over Ficoll-Hypaque solution and centrifugation, following established methods (20).

**FACS analysis**

Cells (10<sup>6</sup>/sample) were incubated with rat anti-mouse CD16/CD32 (BD Pharmingen) to block nonspecific binding to FcγRs. Cells were stained with the following Abs: FITC conjugated rat anti-mouse CD11b IgG2b (BD Pharmingen), allophycocyanin-conjugated rat anti-mouse F4/80 IgG2b (Abd Serotec), PE-conjugated rat anti-mouse Ly6G IgG2a (BD Pharmingen), FITC-conjugated rat anti-mouse Mac2 IgG2a (Cedarlane Laboratories), and allophycocyanin-conjugated rat anti-mouse Ly6C (eBioscience). Isotype-control Abs consisted of PE-conjugated IgG2b (eBioscience) and FITC-conjugated IgG1 (eBioscience). For peritoneal cells and blood mononuclear cells, the cells were gated for leukocytes; for splenocytes, the cells were gated for granulocytes. Isotype-control Abs were used to exclude background staining. Analysis of the stained cells was performed with a FACSCanto II flow cytometer (BD Biosciences), and data were analyzed by FACSDiva (BD Biosciences) and FlowJo (TreeStar) software.

**Quantification of cytokine and chemokine expression**

Serum levels of TNF-α and IFN-γ, IFN-γ secreted by total leukocytes, and chemokines secreted by the primary macrophages were determined by ELISA, using commercially available detection kits (R&D Systems). Peritoneal macrophages derived from each mouse were seeded into three replicative wells (5 × 10<sup>5</sup> cells/well). Cells were incubated in RPMI 1640 at 37°C in 5% CO<sub>2</sub> overnight to allow adherence. Nonadherent cells were removed by washing with PBS. The cells were incubated either alone or with murine IFN-γ (500 U/ml; R&D Systems) for different lengths of time. Conditioned medium was collected and subjected to ELISA analysis. Serum levels of IL-1β, IL-6, MCP-1, MIP-1α, RANTES, and KC were determined using cytokine/chemokine ELISA array in a commercial facility (Quansys Biosciences, Logan, UT).

**Histopathological studies**

Tissues were fixed in 10% neutral buffered formalin (Sigma-Aldrich) for 16 hr and stored in PBS overnight before paraffin embedding and sectioning. For immunostaining, tissues were fixed with a solution of 60% methanol, and 10% H<sub>2</sub>O. Five-micron sections were stained with H&E. Paraffin-embedded tissue sections were deparaffinized with Trilogy (Cell Marque, Austin, TX) in a steamer for 30 min. Tissues were stained for macrophages with purified anti-Mac2 Ab (rat anti-mouse; Cedarlane Laboratories) and anti-Ym1 Ab (anti-rabbit; Wako Chemicals USA), followed by incubation with Biotin-Sp- conjugated appropriate secondary Ab (Jackson ImmunoResearch).

**Determination of in vivo interaction of L13a with different chemokine mRNAs and 60S ribosomal subunit**

The interaction of L13a with cellular mRNAs and 60S subunit was determined following our previously established methods (10). In short, macrophage lysates (500 μg protein) were subjected to immunoprecipitation using 10 μl affinity-purified anti-L13a Ab. L13a-bound RNA was isolated by TRIzol reagent (Invitrogen). For mRNAs, reverse transcription was carried out using oligo-dT primer; random primer was used for the ribosomal subunit. Specific primer pairs were used for PCR amplification (Supplemental Table 1).

**Determination of GAIT element–mediated translational silencing activity of mouse macrophages using cell-free in vitro translation system**

cRNA of the reporter luciferase with a 29-nt GAIt element was generated by in vitro transcription. The cRNA (100 ng) was subjected to in vitro translation using rabbit reticulocyte lysates (Promega) in the presence of [35S]methionine. Lysates were made from the peritoneal macrophages of control and KO mice, and 4 μg these lysates was used to test the translational silencing activity. A 10-μl aliquot of the translation reaction mixture was resolved by SDS-PAGE (7% polyacrylamide), followed by autoradiography.

**Statistical analysis**

The log-rank test (Mantel–Cox) was used to determine the significance of the differences in survival rates. Results are presented as mean ± SD. The statistical significance of the differences between groups was determined by a two-tailed Student t test. All statistical analysis was performed using GraphPad Prism 5.0 software.

**Results**

L13a<sup>fllox/flox</sup>LysoMCr<sup>Cre<sup>-</sup></sup> mice show macrophage-specific depletion of L13a

Macrophages are the principal effectors of inflammatory and innate immune responses. To assess the role of L13a-dependent translational silencing in resolving physiological inflammation, we generated macrophage-specific L13a-KO mice using the Cre-Lox system.
FIGURE 1. Generation of mice homozygous for the loxP allele and macrophage-specific KO mice. (A) Design of the targeting construct and identification of the recombinant mouse ES cells harboring the conditional null allele. The positions of the loxP sites, Neo cassette, long and short homology arms, and the primers used in genotyping are shown (left panel). rES cells were genotyped by PCR using Lox1/SDL2 primers and identified on the basis of the appearance of a doublet band of 325/263 bp (right panel). (B) Confirmation of the presence of conditional null allele in rES cells. rES cells were infected by Ad-CMV-Cre (Vector BioLabs, Philadelphia, PA). The Cre-dependent depletion of L13a was confirmed by immunoblot analysis of the infected cells using anti-L13a Ab. (C) Identification of the F4 mice homozygous for the loxP allele (loxP+/+) and Neo deletion allele. Tail DNA samples of the pups were screened by PCR with the Lox1/SDL2 primer pair for the loxP allele (upper panel) and NDEL3/anti-AT2 primer pair for the Neo deletion allele (lower panel). The appearance of the 280-bp band shows the presence of Neo deletion allele. The stars denote two representative animals in a litter which were genotypically confirmed to be homozygous for the loxP allele and the Neo deletion allele. (D) Identification of F4 mice harboring the conditional null allele. Fibroblasts were isolated from the lung and infected with Ad-CMV-Cre. The Cre-dependent depletion of L13a was confirmed by immunoblot analysis with anti-L13a Ab. (E) The macrophage-specific KO mice were generated by crossing the loxP+/+ mouse with the loxP+/LysM Cre+ mouse and genotyping by PCR with the Lox1/SDL2 primer pair for the loxP allele and Cre-specific primer pair (The Jackson Laboratory) for Cre allele. The star denotes one animal of a representative litter that was successfully genotyped to be homozygous for the loxP allele. (Figure legend continues)
LoxP sites were introduced in the targeting construct after exon 1 and exon 8 of the mouse L13a genomic sequence. The targeting construct also harbors a Neomycin gene as a marker flanked by flippase recognition target sites to facilitate its removal (Fig. 1A, left panel). rES cells harboring the loxP allele were identified by PCR genotyping upon transfection of the ES cells from C57BL/6 mice (Fig. 1A, right panel). Before injecting into the blastocysts, Cre-dependent depletion of L13a protein in the rES cells was confirmed by infecting with Ad-CMV-Cre (Fig. 1B). Germline transmission from the male chimera was carried out by loxP allele with the presence of the Cre transgene. This mouse has been designated as the macrophage-specific L13a knockout mouse. 

**FIGURE 2.** Macrophage-specific L13a-KO mice show significantly enhanced susceptibility to endotoxin challenge. (A) Increased mortality of KO mice in response to endotoxin challenge. Age- and sex-matched L13a<sup>lox/lox</sup>, LysMCr<sup>e+</sup>, and L13a<sup>lox/lox</sup>LysMCr<sup>e+</sup> mice were challenged with a sublethal dose of LPS (20 mg/kg) and observed for 120 h for survival. The survival rate was plotted on Kaplan–Meier survival curves (n = 19 in each group), p = 0.042, log-rank test (Mantel–Cox). (B) Increase in breath rate of KO mice upon endotoxin challenge. Control and KO mice were injected with LPS (15 mg/kg) or saline. Breath rate was measured 3 and 24 h postinjection. Twenty-four hours postsaline injection, the breath rates for control mice were set to 1, and the breath rates of all other mice at both time points were plotted on an arbitrary scale. Results are mean ± SD (n = 5). *p = 0.018, two-tailed Student t test. (C) Endotoxin challenge causes a significant reduction in the ambulatory activity of KO mice. After LPS challenge (15 mg/kg), the ambulatory activities of KO and control mice were measured for a period of 25 h using a device equipped to sense the number of sequential laser beam breaks in two dimensions. The ambulatory activities were plotted using an arbitrary scale. Results are mean ± SD (n = 8). *p = 0.049, unpaired two-tailed Student t test. (D) KO mice showed increased serum levels of the markers of inflammation. Serum levels of BUN, AST, and TNF-α in mice were measured after 8 h of LPS challenge (15 mg/kg). Results are mean ± SD (n = 4). *p = 0.011, **p = 0.004, ***p = 0.032, two-tailed Student t test.
crossing with WT C57BL/6 females. The neomycin gene from L13aflx–neo/+ mice was removed by crossing with ACTFLPe+ mice on a C57BL/6 background (The Jackson Laboratory). The F4 mice (L13aflx/flx) homozygous for the loxp and Neo deletion allele were confirmed by PCR genotyping (Fig. 1C). The Cre-dependent depletion of L13a in the F4 mice was confirmed by infecting the isolated lung fibroblasts from these mice with Ad-CMV-Cre, followed by immunoblot analysis with anti-L13a (Fig. 1D). The macrophage-specific KO mice (L13aflx/flxLysMCre+′) were generated by crossing the L13aflx/flx mice with LysMCre+ mice (The Jackson Laboratory), and the mice homozygous for the flx allele and positive for the Cre transgene were identified by PCR genotyping (Fig. 1E). The immunoblot analysis using anti-L13a Ab shows the depletion of L13a in L13aflx/flxLysMCre+ mice only in macrophages and not in other organs, such as liver and kidney (Fig. 1F). Consistent with this result, expression of Cre was observed only in the macrophages of these mice (Fig. 1F, left and middle panels). In contrast, liver and kidney of the same animal showed no detectable expression of Cre (data not shown). This result confirmed the generation of macrophage-specific L13a-KO mice. Throughout this article, we refer to these L13aflx/flxLysMCre+ mice as KO mice and L13aflx/flx mice as control mice.

The KO mice progeny from the mating of L13aflx/flx mice and L13aflx/+/LysMCre+ mice obeyed Mendelian patterns of distribution, with no detected embryonic lethality. The newborn KO animals were indistinguishable from the controls. Under unchallenged conditions, no visible sign of pathology, such as retardation of mobility, growth, or fertility; decreased food intake; weight loss; or any visible change in the major organs (e.g., liver, spleen, lung, kidney) was noted over a 6-mo observation period. Together, these results suggest that macrophage-specific depletion of L13a does not cause any significant defect in animal development.

**Macrophage-specific L13a-KO mice show significantly enhanced susceptibility to endotoxin challenge**

We determined the inflammatory responses of the KO and control group of mice using the LPS-induced endotoxemia model. The relevance of this model is based on the ability of LPS to rapidly induce IFN-γ and a number of other proinflammatory cytokines in cells of myeloid lineage (17–19). IFN-γ–dependent activation of myeloid cells is a critical component of inflammatory responses. We reasoned that if the translational silencing of the cohort of mRNAs encoding inflammatory proteins serves as a defense mechanism against uncontrolled inflammation, then LPS-induced pathological outcomes would be significantly more severe for KO mice compared with the control. To verify this, KO and control mice were subjected to LPS injection with a dose of 20 mg/kg to induce systemic inflammation, and their survival was monitored for 120 h postadministration. The survival of KO mice was decreased significantly compared with the control mice (45 versus 78%), and Kaplan–Meier survival analysis showed the statistical significance of this result (p < 0.05) (Fig. 2A). Tachypnea or a fast breathing rate is also a visible symptom of sepsis (21). These KO animals showed a significantly higher breathing rate even 24 h after LPS treatment (Fig. 2B). Treatment with LPS is known to cause induction of lethargy and reduction in ambulatory activity in mice (22, 23). As a visible response to endotoxin treatment, we quantified the ambulatory activity up to 24 h post-LPS treatment using a device equipped to measure the number of sequential laser beam breaks in two dimensions. A rapid reduction in the ambulatory activity was observed in the KO group in contrast to the control group upon LPS, but not saline, administration (Fig. 2C). To determine other markers of inflammation, we measured the serum levels of blood urea nitrogen (BUN), released liver enzyme aspartate aminotransferase (AST), and TNF-α in the LPS-treated mice; all three were markedly higher in KO mice (Fig. 2D). In addition to these markers of inflammation, we measured the serum level of a panel of other inflammatory cytokines (e.g., IL-1β, IL-6, MCP-1, MIP-1α, RANTES, and KC). Our results showed that the serum levels of all of these cytokines were significantly higher in the LPS-treated KO mice (Fig. 3). Together, these results document that macrophage-specific deficiency in L13a leads to increased susceptibility to endotoxemia.

**Myeloid-specific depletion of L13a causes enhanced macrophage infiltration in the peritoneum and expansion of leukocyte populations in the spleen**

Infiltration of leukocytes in the peritoneal tissue is a hallmark of inflammation and requires signal generated by chemokines and chemokine receptors (3, 24). We hypothesized that macrophage-specific deficiency in L13a would lead to greater macrophage influx by removing the endogenous translational silencing imposed on this cohort of chemokine and chemokine receptors (9). To measure mononuclear cell influx, we performed FACS analysis of the peritoneal cells isolated from KO and control mice using thioglycollate-induced peritonitis and LPS-induced endotoxemia models. We quantified Gr1-CD11b, F4/80-Gr1, and F4/80-CD11b double-positive cells upon 48 and 24 h of thioglycollate and LPS treatment to test the infiltration of macrophages and neutrophils. Thioglycollate treatment of KO mice for 48 h showed significant enhancement of Gr1-CD11b and F4/80-Gr1 double-positive cell populations relative to control mice (Fig. 4A). In contrast, LPS treatment for 24 h showed significant enhancement of the double-positive cells in all three categories (Fig. 4B). Quantifications of these results from four independent experiments with statistical significance are shown in Supplemental Fig. 1A. Mac2 is a carbohydrate-binding protein and is expressed on the surface of proinflammatory macrophages and monocytes (14, 25). Our studies

**FIGURE 3.** Increased serum level of inflammatory cytokines in KO mice upon endotoxin challenge. Control and KO mice were injected with LPS (15 mg/kg). Blood was collected through cardiac puncture 12 h after injection. Serum was sent to a commercial facility (Quansys Biosciences) and subjected to cytokine/chemokine ELISA array for IL-1β, IL-6, MCP-1, MIP-1α, RANTES, and KC. Results are mean ± SD. The p values were determined using the paired two-tailed Student t test.
show significant enhancement of the proinflammatory F4/80-Mac2 and Gr1-Mac2 double-positive macrophages (Fig. 4C) in KO mice upon endotoxin challenge. Studies by other investigators found that the spleen can serve as a reservoir of leukocytes for rapid deployment of these cells to the sites of inflammation (26). Therefore, we measured populations of splenic leukocytes after RBC lysis of cell suspensions from spleens harvested from KO mice 48 h post-LPS administration. Consistent with our expectation, we found increased abundance of F4/80-Mac2, Gr1-Mac2 (Fig. 4D, Supplemental Fig. 1C), and Ly6Ghi/Ly6Chi (Fig. 5A, 5C) double-positive leukocytes in the LPS-treated KO mice compared with controls. Inflammation causes the release of Ly6C<sup>hi</sup> monocytes from bone marrow into the circulation and subsequent tissue recruitment and differentiation to macrophages (24). Therefore, we also determined the abundance of Ly6C<sup>hi</sup> monocytes in circulation. Our results show that these cells were significantly increased in KO mice (Fig. 5B, 5C).
Results are mean ± SD (n = 4). The p values were determined by the two-tailed Student t test.}

Macrophage-specific depletion of L13a abrogates translational silencing of the GAIT target proteins

Determination of polyribosomal abundance is a widely accepted method to determine the translational efficiencies of mRNAs (9, 29, 30). To directly test the deregulation of the translational silencing of these proteins in the macrophages of KO mice, we investigated the polyribosomal abundance of CCL22, CXCL13, and CCR3 mRNAs. Lysates from peritoneal macrophages from the LPS-treated control and KO mice and from the conditioned medium obtained from the ex vivo cultures of the total leukocytes harvested from KO mice macrophages showed significantly increased levels of L13a targets CCL22, CXCL13, and CCL8 (Fig. 7A, Supplemental Fig. 2). In contrast, no significant difference between the control and L13a-KO macrophages was found following IFN-γ treatment for 8 h. In the mouse model, administration of LPS can rapidly induce IFN-γ in the cells of myeloid lineage (17–19). Therefore, we investigated the ability of the macrophages harvested from LPS-treated KO mice to build up the steady-state level of these target proteins of GAIT (e.g., CCL22, CXCL13, and CCL8) in the conditioned medium of a 24-h ex vivo culture. Indeed, the levels of all three targets were significantly increased in L13a-KO macrophages (Fig. 7B). We then investigated whether these high levels were possibly due to differences in the amounts of IFN-γ produced in response to LPS treatment. However, measurement of IFN-γ in the serum of LPS-treated control and KO mice and from the conditioned medium obtained from the ex vivo cultures of the total leukocytes harvested from these mice showed no significant difference (Fig. 7C). Therefore, these results demonstrate that, in the mouse model, the L13a deficiency in macrophages compromises their ability to control the synthesis of these GAIT target proteins and that the elevated levels of these proteins might contribute to the enhanced inflammatory response of KO mice to endotoxin challenge.

Macrophage-specific depletion of L13a abrogates translational silencing of the GAIT target proteins

Together, these results show that the macrophage-specific deficiency in L13a leads to increased exudation of macrophages and neutrophils in the peritoneum, their increased abundance in the splenic reservoir, and higher levels of inflammatory Ly6Chi mononuclear cells in the circulation in response to inflammatory stimuli.

**L13a deficiency in macrophages enhances endotoxemia-induced tissue damage**

Extensive tissue damage in the organs, widespread hemorrhage, intravascular congestion, and tissue infiltration of immune cells are the essential features of systemic inflammation caused by LPS-induced endotoxemia (27, 28). Therefore, we investigated whether macrophage-specific deficiency in L13a could cause greater tissue damage and immune cell infiltrates following LPS administration. Histopathological analysis of the lung by H&E staining revealed extensive RBCs in bronchiolar and alveolar spaces of KO mice, whereas the RBCs were mostly intravascular in the control animals (Fig. 6A). Immunohistological studies of the lungs showed significantly more Mac2+ macrophages (Fig. 6B, upper panels) adherent to vascular endothelium. These results are quantitatively expressed in Fig. 6B (lower panel). These data show enhanced adhesion of the macrophages to the vessel wall of KO mice in response to endotoxemia, a hallmark of inflammation. Similarly, analysis of the kidney sections showed conspicuous infiltration of Mac2+ (Fig. 6C) and Ym1+ (Fig. 6D) macrophages in the renal glomeruli of the LPS-treated KO animals. Quantification and statistical significance of these results are shown (Fig. 6C, 6D, right panels). No tissue injury and negligible macrophage infiltrates were found in these organs of the saline-treated animals (data not shown). In summary, these results suggest that the loss of L13a expression in the macrophages leads to the enhanced infiltration of these cells and tissue injury in multiple major organs, consistent with the severe endotoxic shock observed in KO animals.

Disruption of L13a in macrophages causes unregulated synthesis of GAIT target proteins in vivo

To understand the molecular underpinning of the inflammation in L13a-KO animals, we first tested whether the expression of the L13a target molecules indeed increased in the KO group upon endotoxin challenge. Ex vivo cultures of peritoneal, as well as bone marrow–derived, macrophages harvested from KO mice were subjected to 24 h of IFN-γ treatment. The conditioned media of the cultures obtained from KO mice macrophages showed significantly increased levels of L13a targets CCL22, CXCL13, and CCL8 (Fig. 7A, Supplemental Fig. 2). In contrast, no significant difference between the control and L13a-KO macrophages was found following IFN-γ treatment for 8 h. In the mouse model, administration of LPS can rapidly induce IFN-γ in the cells of myeloid lineage (17–19). Therefore, we investigated the ability of the macrophages harvested from LPS-treated KO mice to build up the steady-state level of these target proteins of GAIT (e.g., CCL22, CXCL13, and CCL8) in the conditioned medium of a 24-h ex vivo culture. Indeed, the levels of all three targets were significantly higher in L13a-KO macrophages (Fig. 7B). We then investigated whether these high levels were possibly due to differences in the amounts of IFN-γ produced in response to LPS treatment. However, measurement of IFN-γ in the serum of LPS-treated control and KO mice and from the conditioned medium obtained from the ex vivo cultures of the total leukocytes harvested from these mice showed no significant difference (Fig. 7C). Therefore, these results demonstrate that, in the mouse model, the L13a deficiency in macrophages compromises their ability to control the synthesis of these GAIT target proteins and that the elevated levels of these proteins might contribute to the enhanced inflammatory response of KO mice to endotoxin challenge.

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mRNAs were isolated from the fractions, followed by determination of the GAIT target mRNAs by RT-PCR. Polyribosomal abundance of all three mRNAs was substantially increased in the peritoneal macrophages of KO mice compared with control actin mRNA (Fig. 8A). The translational efficiencies of the target mRNAs were quantitatively expressed by the ratio of the band intensities of the corresponding mRNAs and actin in polysomal and nonpolysomal fractions (Fig. 8B). Essentially similar results were obtained from bone marrow–derived macrophages of KO mice upon treatment with IFN-γ for 24 h in ex vivo cultures (Supplemental Fig. 3). To directly test the abrogation of the GAIT element–mediated translational-silencing activity in the macrophages of KO mice, we reconstituted the translational silencing ex vivo. In this experiment we tested the translational efficiencies of a reporter RNA harboring an active GAIT element in the 3' UTR using the cell-free translation system of rabbit reticulocyte lysates. Results from this ex vivo study showed that treatment of LPS (but not saline) activates the translational-silencing activity of the GAIT element containing mRNA in the macrophage. However, in the macrophage-specific L13a-KO animals, LPS treatment failed
to activate the translational-silencing activity (Fig. 9A). Together, these results demonstrate that macrophage-specific deficiency in L13a may diminish the naturally imposed translational silencing on GAIT element–containing mRNAs in vivo.

The ability of the L13a-containing GAIT complex to silence translation of its target mRNAs relies on its binding to functional GAIT elements in the 3′UTR (9, 10). Therefore, we investigated the in vivo interaction of these target mRNAs with L13a. Comparative RT-PCR analysis of the total RNA isolated from macrophages of the saline- and LPS-treated WT mice showed the presence of CCL22 and CCR3 mRNA only upon LPS treatment (Fig. 9B). To test whether these mRNAs are bound to L13a, peritoneal macrophages from LPS-treated WT mice were subjected to immunoprecipitation using anti-L13a Ab, followed by extraction of RNA. The presence of CCL22 and CCR3 mRNAs was detected by RT-PCR using specific primers; despite its cellular abundance, the control actin mRNA was not detected in the immunoprecipitate. None of the GAIT target mRNAs were detected in the same extracts after immunoprecipitation using Ab against L28, another protein of the large subunit, which demonstrated specificity (Fig. 9C). Using U937 cells, a cellular model of human monocytes, our previous studies showed the regulated release of L13a upon treatment with IFN-γ (10). Because LPS is a potent inducer of IFN-γ in vivo (17–19), we investigated the association of L13a with the 60S ribosomal subunit in the peritoneal macrophages harvested from LPS-treated mice. RT-PCR analysis of the anti-L13a immunoprecipitate using 28S rRNA-specific primers revealed significantly less association of L13a with the 60S subunit in the macrophages isolated from LPS-treated animals compared with those from saline-treated animals (Fig. 9D). This is consistent with the previous report demonstrating the release of L13a from the 60S ribosome in response to IFN-γ treatment (10). Taken together, our results demonstrate that macrophage-specific L13a deficiency may diminish the naturally imposed translational silencing on GAIT target mRNAs and suggest the physiological significance of this mechanism as an endogenous defense against uncontrolled inflammation.

Discussion

The primary finding of this study is the identification of ribosomal protein L13a in macrophages as a physiological attenuator of endotoxin-induced inflammation. In this study using a new mouse model of macrophage-specific L13a depletion, we identified a protective role for L13a-dependent translational silencing against endotoxic shock caused by uncontrolled inflammation. Targeted disruption of the mouse L13a gene in macrophages resulted in increased mortality during LPS-induced endotoxemia. The increased lethality was associated with widespread tissue damage and infiltration of macrophages in major organs, such as liver, lung, and kidneys. The clinical symptoms of increased inflammation in KO mice included increased lethargy and high breath rate, which were accompanied by significantly higher levels of serum markers of inflammation, such as BUN, AST, and TNF-α, and higher serum levels of a group of other inflammatory cytokines. The increased synthesis of several GAIT target proteins (e.g., CCL22, CXCL13, CCL8, and CCR3) is also consistent with our result that shows the abrogation of GAIT element–mediated translational silencing in the macrophages harvested from LPS-treated KO mice. Most interestingly, our studies also suggest that LPS treatment significantly compromises the association of L13a with the 60S ribosomal subunit in the macrophages of these KO animals. Together, these results demonstrate that macrophage-specific L13a deficiency in an animal model may diminish the naturally imposed translational silencing on GAIT element–containing mRNAs. Thus, we have identified a novel extraribosomal function of L13a as an endogenous defense mechanism against uncontrolled inflammation caused by endotoxin treatment.

Previous studies from our laboratory using in vitro models of monocytes showed the ability of the ribosomal protein L13a-dependent translational silencing to target a cluster of mRNAs encoding different chemokines and chemokine receptors. In ad-

![Figure 7](http://www.jimmunol.org/Downloadedfrom.org/July26,2017)
dition, specific segments present in the 3′UTRs of these mRNAs contained significant folding homologies to the authentic GAIT element, and subsequent experiments showed their role in translational regulation by a single RNA-binding complex (9). These results are consistent with the notion that folding homologies among the elements present in multiple transcripts could offer coregulation of translation by a single RNA-binding complex, demonstrating the existence of a post-transcriptional operon (31, 32). Our results presented using macrophages harvested from KO mice directly showed reduced polysomal abundance of a group of mRNAs encoding inflammatory proteins. To our knowledge, this is the first report of the significance of a ribosomal protein–dependent translational silencing in controlling physiological inflammation at the level of a whole organism. In these studies we tested a few representative targets (e.g., CCL22, CXCL13, CCL8, CCR3) in our animal model, although other targets of L13a identified from the polysome profile analysis of monocytes (9) have yet to be tested in this KO mouse model.

Posttranscriptional control of many mRNAs has been implicated in a variety of cellular events recognized as a signature of inflammation, such as activation and chemotraction of T lymphocytes, influx of neutrophils, NK cell–mediated cytotoxicity, monocyte adhesion, and survival of macrophages in the injured tissue (12, 33). The pathophysiological significance of these control mechanisms has been tested using genetically engineered mouse models with deficiencies in the AU-rich element sequence of TNF-α 3′UTR (34) and RNA-binding proteins, such as TTP (35), TIA-1 (36), and AUF1 (37). All of these studies showed elevated inflammatory responses due to the overexpression of TNF-α caused by the absence of TNF-α mRNA destabilization with a constitutive signature of pathology, even under unchallenged conditions. However, macrophage-specific L13a-KO mice show no sign of any pathology under unchallenged conditions and no developmental defect in the new-
in diverse aspects of inflammation (e.g., controlling the trafficking of activated T lymphocytes (44) and regulatory T cells (45) to inflammatory sites by CCL22, the role of CXCL13 in B cell homing (46) and its correlation with childhood-onset lupus (47), activation of a large cohort of immune cells by CCL8 (48) and its elevated level in intrinsic asthma (49), and promotion of LPS-induced lung inflammation (50) and ulcerative colitis (51) by CCR3. Our data from KO mice showing the elevated levels of these cytokines by macrophage-specific L13a depletion and significant increase in inflammation in response to endotoxin challenge are highly consistent with these previous reports. Another interesting aspect of our results is the presence of higher numbers of both the classically and alternatively activated macrophages that express Mac2- and Ym1, respectively, in the kidney glomeruli of LPS-challenged KO animals (Fig. 4E). Considering the role of Ym1+ macrophages in wound-healing activities (14), our observation could indicate that a fibrotic response is initiated at the onset of acute inflammation.

In humans, haploinsufficiency of many ribosomal proteins, such as S19, S24, S17, S15, S7 L35A, L5, L11, and L36, causes genetic disorders, such as Diamond-Blackfan anemia (52); in mice, deficiencies lead to various abnormalities in ribosome biogenesis (52). In sharp contrast, our previous in vitro studies of human monocytes showed that depletion of L13a caused no defect in translation of the target mRNA harboring the GAIT element in the 3'UTR using a cell-free in vitro translation system of rabbit reticulocyte lysates by adding only purified recombinant L13a. An aliquot of the immunoprecipitate was subjected to SDS-PAGE, followed by autoradiography to see the translated product of the reporter RNA. (B and C) Macrophages from LPS-treated WT mice show in vivo association of CCL22 and CCR3 mRNA with L13a. (B) RT-PCR of the total RNA isolated from macrophages of LPS-treated mice shows induction of CCL22 and CCR3 mRNA. Aliquots of these lysates were subjected to immunoprecipitation with anti-L13a or anti-L28 Abs using a Seize X immunoprecipitation kit (Pierce) to avoid contamination from L and H chain. (C) RNA was extracted from the immunoprecipitates, followed by RT-PCR with specific primers (upper panel). The efficiency of the immunoprecipitation was confirmed by immunoblot analysis (lower panels). (D) Reduced association of L13a with the 60S ribosomal subunit in the macrophages from LPS-treated mice. Macrophages isolated from either LPS- or saline-injected mice were subjected to immunoprecipitation using anti-L13a Ab. An aliquot of the immunoprecipitate was subjected to RT-PCR with mouse 28S rRNA-specific primer (upper panel). The other aliquot was subjected to immunoblot analysis using anti-L13a Ab (lower panel).
upregulating p53 translation by the depleted ribosome. However, to our knowledge, our results provide the first evidence for a novel extraribosomal function of ribosomal protein L13a in limiting inflammation. In macrophages, this mechanism is driven by the phosphorylation-dependent release of L13a from the 60S ribosomal subunit (10, 56) to efficiently block the expression of a cohort of inflammatory proteins (e.g., a group of chemokine and chemokine receptors) directly at the level of translation (9). In addition, previous work from other investigators identified the intermediate signaling molecules responsible for L13a phosphorylation and release from ribosome (57). Our studies show the ability of the L13a-dependent silencing mechanism to control physiological inflammation caused by endotoxia. Together, our results suggest that a genetic defect in this pathway may contribute to the progression of inflammatory diseases and that manipulations of this pathway by small molecules may offer novel therapeutic strategies.

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Disclosures
The authors have no financial conflicts of interest.

References

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Poddar Fig. S3

(a) Western blot analysis showing protein levels of CCR3, CXCL13, CCL22, and Actin in fraction 1-12. Polysome and non-polysome were separated and analyzed for each fraction.

RT-PCR amplification using mouse CCR3 specific primer
RT-PCR amplification using mouse CXCL13 specific primer
RT-PCR amplification using mouse CCL22 specific primer
RT-PCR amplification using mouse Actin specific primer

(b) Bar graph showing the ratio of the abundance of target mRNA and actin in polysomal and non-polysomal fractions for CCL22, CXCL13, and CCR3 in Control and KO conditions.

Control   | KO
---------|---------
CCL22    |        |        |
CXCL13   |        |        |
CCR3     |        |        |
Supplementary Figure Legends

Figure S1 (related to Fig. 4) Enhanced infiltrates of the leukocytes in the peritoneum of the KO mice. a, Statistical analysis of the data obtained from 4 animals in each group. Control and KO mice were injected with saline, thioglycollate and LPS (15 mg/kg). 24 and 48 hr post injection total leukocytes were harvested from peritoneal cavity. These cells were subjected to FACS analysis with appropriate antibodies in order to identify the percentage of Gr1/CD11b, F4/80/Gr1 and F4/80/CD11b double positive cells. Results are mean ± s.d, n=4, the corresponding P values were shown at the top of the bar, paired two-tailed Student’s t-test.

Figure S2 (related to Fig. 7a) ex vivo studies using bone marrow derived macrophages of the KO mice show high accumulation of chemokine ligands in response to 24 hr of IFN-γ treatment. Mouse bone marrow cells were differentiated into macrophage by culturing in presence of L929 cell conditioned media. The bone marrow derived macrophages were treated with IFN-γ (500 U/ml). The chemokine ligands CXCL13, CCL22 and CCL8 were measured by ELISA from the conditioned medium after different periods of IFN-γ treatment. Results are mean ± s.d, n=4, the corresponding P values were shown at the top of the bar, same P values were obtained using paired two-tailed Student’s t-test and repeated measures 2 way ANOVA.

Figure S3 (related to Fig. 8) Bone marrow derived macrophages from KO mice show increased polyribosomal abundance of CCL22, CXCL13 and CCR3 mRNAs. a, Bone marrow derived macrophages from control and KO mice were prepared from ex-vivo differentiation of bone marrow cells in the presence of L929 cell conditioned media. These cells were treated with IFN-γ for 24 hr and subjected to polyribosome fractionation
by sucrose gradient. Fractions were subjected to RT-PCR analysis using specific primers. 

b, Quantifications of the results from a. Ratio of the abundance of the target mRNA and actin in polysomal and non-polysomal fractions were determined by measuring the intensities of the corresponding bands.
Supplemental Table S1

The following primers were used:

Lox1: 5’ AGGTTCTGCTTGAGCATCTGAG 3’
SDL2: 5’ CCGTCAGGATGCCTACTACCCAG 3’
NDEL3: 5’ GGGTGAATCCTTGCTCACGGGAT 3’
Anti AT2: 5’ GCTCTTGATCTTGTGTGTGCTGACTT 3’
Mouse 28S (X00525)
Forward: 5’ GTTGTTGCCATGGTAATCCTGCTCA 3’
Reverse: 5’ CAGAAGCAGGTGGTCTGACGATGT 3’
Mouse β-Actin (NM_007393)
Forward: 5’ GTCCCTCAACCTCCTCAAAAAGC 3’
Reverse: 5’ AGGTAAGGTGTGCACCTTTAT 3’
Mouse CCL22 (NM_009137)
Forward: 5’ TTCTTGCTGTGGCAATTCAGACCT 3’
Reverse: 5’ CAGGTCTCCTCCTCCCTAGGAGACGT 3’
Mouse CXCL13 (NM_018866)
Forward: 5’ CTGTAAAACGCAGGCTTCCACA 3’
Reverse: 5’ GGTCACAGTGCAAGGAATATA 3’
Mouse CCR3 (NM_009914)
Forward: 5’ TTCTACGGGCCCTCAACATAC 3’
Reverse: 5’ ATCCAGAGACACCTCCTGTA 3’