Ribosomal Protein L13a Deficiency in Macrophages Promotes Atherosclerosis by Limiting Translation Control-Dependent Retardation of Inflammation

Abhijit Basu, Darshana Poddar, Peggy Robinet, Jonathan D. Smith, Maria Febbraio, William M. Baldwin III, Barsanjit Mazumder

Objective—Unresolved inflammatory response of macrophages plays a pivotal role in the pathogenesis of atherosclerosis. Previously we showed that ribosomal protein L13a-dependent translational silencing suppresses the synthesis of a cohort of inflammatory proteins in monocytes and macrophages. We also found that genetic abrogation of L13a expression in macrophages significantly compromised the resolution of inflammation in a mouse model of lipopolysaccharide-induced endotoxemia. However, its function in the pathogenesis of atherosclerosis is not known. Here, we examine whether L13a in macrophage has a protective role against high-fat diet–induced atherosclerosis.

Approach and Results—We bred the macrophage-specific L13a knockout mice L13a Flox +/+ Cre+/+ onto apolipoprotein E–deficient (apoE−/−) background. L13a Flox +/+ Cre−/− mice on apolipoprotein E–deficient background were used as controls. Control and knockout mice were subjected to high-fat diet for 10 weeks. Evaluation of aortic sinus sections and entire aorta by en face showed significantly higher atherosclerosis in the knockout mice. Severity of atherosclerosis in knockout mice was accompanied by thinning of the smooth muscle cell layer in the media, larger macrophage area in the intimal plaque region and higher plasma levels of inflammatory cytokines. In addition, macrophages isolated from knockout mice had higher polyribosomal abundance of several target mRNAs, thus showing defect in translation control.

Conclusions—Our data demonstrate that loss of L13a in macrophages increases susceptibility to atherosclerosis in apolipoprotein E–deficient mice, revealing an important role of L13a-dependent translational control as an endogenous protection mechanism against atherosclerosis. (Arterioscler Thromb Vasc Biol. 2014;34:533-542.)

Key Words: atherosclerosis ■ chemokines ■ inflammation ■ macrophages ■ ribosomal protein L13a, mouse

Inflammation is central to the pathogenesis of atherosclerosis.1,2 Rapid as well as controlled synthesis of inflammatory cytokines and chemokines by macrophages and monocytes plays an obligatory role in the innate immune system to protect the host from invading microorganisms.3,4 However, unrestricted accumulation of inflammatory products can cause substantial injury to host cells.5 Therefore, endogenous mechanisms have evolved to limit the production of inflammatory molecules and promote resolution of inflammation. These mechanisms are pivotal for the resolution of monocytes. Recently strong evidence has emerged indicating that failure to resolve inflammation by macrophages and monocytes is a major driver of many diseases,6 including atherosclerosis.7 When macrophage inflammation is not resolved, this may initiate endothelial dysfunction triggering acute vascular disease.8,9

A series of previous studies from our laboratory has identified an endogenous mechanism that restrains inflammation. This mechanism involves ribosomal protein L13a-dependent translational silencing of specific mRNAs in interferon (IFN)-γ–activated mononuclear cells.10-12 This translational silencing occurs through phosphorylation-dependent release of L13a from the large ribosomal subunit and assembly of the IFN-γ–activated inhibitor of translation (GAIT) complex on defined GAIT-specific binding elements present in the 3′-untranslated regions of a family of mRNAs encoding inflammatory proteins.11,15 In addition, translational regulation of inflammatory protein synthesis from pre-existing mRNAs as a mechanism to control inflammation has been reported by other laboratories.16 Priming of monocytes and macrophages by IFN-γ is an essential aspect of innate immunity.17 This priming stimulates production of an array of proinflammatory cytokines and mediators to protect the host from infectious agents, but this response is limited in time peaking at ≈4 to 8 hours. In previous studies using a genome-wide approach, we showed that after an 8-hour

Received on: September 19, 2013; final version accepted on: January 3, 2014.
From the Department of Biology, Geology, and Environmental Sciences, Center for Gene Regulation in Health and Disease, Cleveland State University, Cleveland, OH (A.B., D.P., B.M.); Department of Cellular and Molecular Medicine (P.R., J.D.S.), Department of Immunology (W.M.B.), and Department of Molecular Cardiology (M.F), Cleveland Clinic Lerner College of Medicine, Cleveland, OH.
Current address for M.F.: Department of Dentistry, University of Alberta, Edmonton, Canada.
The online-only Data Supplement is available with this article at http://atvb.ahajournals.orglookup suppl doi: 10.1161/ATVBHA.113.302573/-DC1.
Correspondence to Barsanjit Mazumder, PhD, Department of Biology, Geology, and Environmental Sciences, Center for Gene Regulation in Health and Disease, Cleveland State University, 2399 Euclid Ave, SR 261, Cleveland, OH 44115. E-mail b.mazumder@csubio.edu
© 2014 American Heart Association, Inc.
Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org
DOI: 10.1161/ATVBHA.113.302573
533
Cre+/+ Onto apoE−/− Background

The role of L13a-dependent translational silencing as an endogenous mechanism of protection against uncontrolled inflammation was confirmed through the analysis of inflammatory response in macrophage-specific L13a knockout mice. These studies showed that on lipopolysaccharide-induced endotoxemia these animals displayed signatures of severe inflammation. For example, we observed unregulated expression of several chemokines in the primary macrophages harvested from the knockout mice, widespread infiltration of macrophages in organs accompanied by tissue injury and reduced survival rate.

Atherosclerosis is a process that initiates with the activation of the resident cells of the vessel wall triggering the adhesion of leukocytes to the endothelium. The arrested leukocytes migrate through transendothelial space after a chemotactic gradient. These processes are regulated by the orchestrated action of many different chemokines and their cognate receptors. This led us to hypothesize that our newly discovered L13a-dependent mechanism for restricting the synthesis of a cohort of inflammatory proteins could serve as an endogenous protective mechanism against atherosclerosis. To test this hypothesis, we bred the macrophage-specific L13a knockout mice onto an apolipoprotein E–deficient background. The genotypes of all intermediate animals in the breeding scheme that contained the L13a Flox allele were determined with respect to the presence and absence of the Cre and apoE alleles (Figure IA; see Materials and Methods for details in the online-only Data Supplement). Immunoblot analysis using anti-L13a and anti-Cre antibodies confirmed both deletion of L13a protein and expression of the Cre recombinase protein specifically in the macrophages of L13a Flox+/+ Cre+/+ apoE−/− mice but not in L13a Flox+/+ Cre−/− apoE−/− mice (Figure IB, left, upper and middle). In contrast to macrophages, other organs such as liver and kidney showed no depletion of L13a protein (Figure IB, right) and no detectable expression of Cre recombinase (data not shown) in either L13a Flox+/+ Cre−/− apoE−/− mice or L13a Flox−/− Cre−/− apoE−/− mice. These results confirm that the macrophage-specific depletion of L13a in L13a Flox+/+ Cre−/− apoE−/− mice relies on the expression of Cre in the macrophages of these mice and showed that Cre-dependent deletion of L13a was not compromised on breeding the macrophage-specific L13a knockout mice onto an apoE−/− background. Throughout this article we refer to the L13a Flox+/+ Cre+/+ apoE−/− mice with macrophage-specific L13a deletion as knockout mice and to the L13a Flox+/+ Cre−/− apoE−/− mice with normal level of L13a as control mice.

Mendelian distributions of expected genotypes were observed in the births of both control and knockout animals with no incidence of embryonic lethality. During a 6-month observation period under unchallenged conditions, we did not detect any visible sign of pathology in either group of animals such as sporadic death in the colony, retardation of growth, weight loss, infertility, reduced mobility, decreased food intake, etc. These results indicate that animal development is not impeded by the macrophage-specific depletion of L13a on an apoE−/− background.

Because the primary goal of this study was to test the difference in atherosclerosis susceptibility between control and knockout mice on high-fat diet treatment, we evaluated several closely related parameters of vascular disease such as heart weight, body weight, and total plasma cholesterol levels after 10 weeks of high-fat diet treatment. None of these parameters exhibited any significant difference when compared between control and knockout animals (P=0.75, P=0.85, and P=0.56, respectively; Figure I in the online-only Data Supplement). Therefore, the control and knockout animals did not seem to have any inherent differences in noninflammation-related factors associated with atherosclerosis.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Cre-Dependent Deletion of L13a Is Not Compromised on Breeding the Macrophage-Specific Knockout Mice L13a Flox+/+ Cre−/− Onto apoE−/− Background

We previously reported the generation of mice homozygous for the Floxed L13a gene (L13a Flox+/+)

macrophage-specific L13a deletion (L13a Flox−/− Cre−/−). The macrophage-specific L13a knockout mice were generated by crossing the L13a Flox+/+ mice with LysMCre+ mice (Stock no. 004781, The Jackson Laboratory, Bar Harbor, ME) expressing the Cre recombinase from the endogenous Lysz locus. This resulted in mice in which the Cre recombinase is expressed exclusively in macrophages leading to the deletion of the L13a gene in those cells via the flanking loxP sites (Flox).

To address the role of L13a-dependent translational silencing in high-fat diet–induced atherosclerosis, we bred the macrophage-specific L13a knockout mice onto an apoE−/− background. The genotypes of all intermediate animals in the breeding scheme that contained the L13a Flox allele were determined with respect to the presence and absence of the Cre and apoE alleles (Figure IA; see Materials and Methods for details in the online-only Data Supplement). Immunoblot analysis using anti-L13a and anti-Cre antibodies confirmed both deletion of L13a protein and expression of the Cre recombinase protein specifically in the macrophages of L13a Flox+/+ Cre+/+ apoE−/− mice but not in L13a Flox+/+ Cre−/− apoE−/− mice (Figure IB, left, upper and middle). In contrast to macrophages, other organs such as liver and kidney showed no depletion of L13a protein (Figure IB, right) and no detectable expression of Cre recombinase (data not shown) in either L13a Flox+/+ Cre−/− apoE−/− mice or L13a Flox−/− Cre−/− apoE−/− mice. These results confirm that the macrophage-specific depletion of L13a in L13a Flox+/+ Cre−/− apoE−/− mice relies on the expression of Cre in the macrophages of these mice and showed that Cre-dependent deletion of L13a was not compromised on breeding the macrophage-specific L13a knockout mice onto an apoE−/− background. Throughout this article we refer to the L13a Flox+/+ Cre+/+ apoE−/− mice with macrophage-specific L13a deletion as knockout mice and to the L13a Flox+/+ Cre−/− apoE−/− mice with normal level of L13a as control mice.

Mendelian distributions of expected genotypes were observed in the births of both control and knockout animals with no incidence of embryonic lethality. During a 6-month observation period under unchallenged conditions, we did not detect any visible sign of pathology in either group of animals such as sporadic death in the colony, retardation of growth, weight loss, infertility, reduced mobility, decreased food intake, etc. These results indicate that animal development is not impeded by the macrophage-specific depletion of L13a on an apoE−/− background.

Because the primary goal of this study was to test the difference in atherosclerosis susceptibility between control and knockout mice on high-fat diet treatment, we evaluated several closely related parameters of vascular disease such as heart weight, body weight, and total plasma cholesterol levels after 10 weeks of high-fat diet treatment. None of these parameters exhibited any significant difference when compared between control and knockout animals (P=0.75, P=0.85, and P=0.56, respectively; Figure I in the online-only Data Supplement). Therefore, the control and knockout animals did not seem to have any inherent differences in noninflammation-related factors associated with atherosclerosis.
Macrophage-Specific Deficiency of L13a Promotes High-Fat Diet–Induced Atherosclerosis

Emerging evidence suggests that chemokines and their receptors play a cardinal role in the transendothelial migration of leukocytes. Therefore, deregulation in their synthesis could promote vascular inflammation and the severity of atherosclerosis.\textsuperscript{19,20,22} Our recent studies demonstrated that deficiency of L13a in the cells of myeloid origin abrogates the naturally imposed translational silencing of a cohort of chemokine and chemokine receptor mRNAs in a cellular model\textsuperscript{14} and more importantly the resolution of physiological inflammation in a murine model.\textsuperscript{18} However, the role of this L13a-dependent mechanism in atherosclerosis is not known.

To test the role of L13a-dependent translational silencing in macrophages as a possible mechanism of defense against high-fat diet–induced atherosclerosis, we fed control and knockout mice a high-fat diet for 10 weeks and then evaluated the extent of atherosclerosis in the 2 groups of animals. Mice were euthanized at the end of treatment and their aortas were harvested for immediate analysis using 2 independent procedures, for example, cross-sectional analysis of the aortic sinus and en face analysis of the entire aorta.

Although aortic root lesions can be different in male and female apoE\textsuperscript{−/−} mice fed a chow (low-fat) diet,\textsuperscript{23,24} we did not find a significant difference in aortic root lesions between males and females in our high-fat diet–fed mice for either the control (n=15 for both male and female; \(P=0.48\)) or the knockout cohort (n=10 for both male and female; \(P=0.48\); Figure II in the online-only Data Supplement). Therefore, we combined both sexes in our subsequent analyses. Because aortic root lesion areas were not normally distributed, we used nonparametric statistics to evaluate them. Aortic root lesions detected by Oil red O staining were significantly larger in the knockout mice as compared with control mice (n=20 for knockout, n=30 for control; \(P<0.0001\); Figure 2A and 2B). Median lesion areas in the knockout and control groups were 3.02×10\textsuperscript{5} and 1.57×10\textsuperscript{5} \(\mu\)m\textsuperscript{2}, respectively. In agreement with these findings from cross-sectional analysis of the aortic root, en face analysis of the entire aorta showed that the median en face percentage lesion area was almost 2-fold higher in the knockout mice as compared with control mice (n=16 for knockout, n=30 for control; \(P<0.0001\); Figure 2A and 2B). Median lesion areas in the knockout and control groups were 3.02×10\textsuperscript{5} and 1.57×10\textsuperscript{5} \(\mu\)m\textsuperscript{2}, respectively. In agreement with these findings from cross-sectional analysis of the aortic root, en face analysis of the entire aorta showed that the median en face percentage lesion area was almost 2-fold higher in the knockout mice as compared with control mice (n=16 for knockout, n=30 for control; \(P=0.003\); Figure 3A and 3B). Taken together, these 2 independent measures clearly illustrate that macrophage-specific L13a depletion increases the severity of high-fat diet–induced atherosclerosis.

Uptake of lipids by the macrophages plays a critical role in the pathogenesis of atherosclerosis.\textsuperscript{7} Therefore, we tested whether the observed differences in atherosclerosis between control and knockout mice might be because of an effect of...
L13a deficiency on macrophage lipid uptake. A cholesterol-loading study with acetylated-low-density lipoprotein was performed following a previously published method. This showed that there were no significant differences in cholesterol loading between bone marrow–derived macrophages from the knockout animals and those from control animals (Figure III in the online-only Data Supplement).

The progression of atherosclerotic lesions involves infiltration and accumulation of macrophages in the intimal plaque region. Disruption of the smooth muscle cell (SMC) layer of the media has been observed in large mouse aortic root lesions, along with the appearance of SMCs forming the fibrous cap. Therefore to gain further insight into the differences in atherosclerosis between knockout and control mice, we analyzed the cellular composition of the atherosclerotic lesion areas using immunohistochemical staining with antibodies against macrophage and SMC markers. Immunohistochemical staining of cross sections of the aortic root region with antibody against macrophage marker Mac2 (Figure 4A) and SMC marker α-actin (Figure 4C) showed significantly increased lesional macrophage-positive (≈2.5-fold; P<0.02; Figure 4B) and SMC-positive areas (≈2.5-fold; P<0.04; Figure 4E) over the entire lesion area in knockout mice as compared with control mice. In addition, lesions in sections from knockout mice had more pronounced fibrous caps than those from control mice (Figure 4C, inset). Immunostaining for α-actin also revealed significant reduction of the α-actin–positive area in the media indicating disruption or phenotypic changes in the aortic medial SMC layer in the knockout mice (Figure 4C and 4D). Infiltration of neutrophils into the area of atherosclerotic lesions plays a significant role in the pathogenesis of atherosclerosis. Therefore, we investigated the presence of neutrophils in the atherosclerotic lesions of knockout and control mice as an additional marker of the severity of disease. Immunohistochemical staining of aortic root cross sections with antibodies against the neutrophil marker Ly6G revealed significantly higher neutrophil infiltration in lesions of knockout mice as compared with control mice (≈2-fold; P<0.02; Figure 4F and 4G).

Taken together these results demonstrate that mice lacking L13a expression in macrophages develop more severe atherosclerosis on high-fat diet treatment than mice with normal L13a expression. This was indicated by increased cross-sectional area of atherosclerotic lesions, increased percentage of the aorta affected by lesions, and increase of macrophage, neutrophil, and SMC content of lesions characteristic of the disease.
Genetic Depletion of L13a in Macrophages
Results in Increased Abundance of Inflammatory Leukocytes in the Circulation and Peritoneum and Elevated Level of Inflammatory Cytokines and Chemokines on High-Fat Diet Treatment

In the murine model of atherosclerosis, a high-fat diet treatment leads to increased release of Ly6C<sup>hi</sup> monocytes from the bone marrow into the circulation. Many of these cells migrate to atherosclerotic plaques and differentiate into foamy macrophages. Therefore, we assessed whether macrophage-specific L13a deficiency affected these aspects of the disease by measuring the abundance of Ly6C<sup>hi</sup> monocytes in the circulation (peripheral blood) and the number of leukocytes into the peritoneal cavity for knockout and control groups of mice at the end of the 10-week high-fat diet treatment period. Fluorescence-activated cell sorting analysis of the peripheral blood leukocytes demonstrated that knockout mice had significantly more Ly6C<sup>hi</sup> cells in the circulation than control mice (≈2-fold; \( P < 0.003 \); Figure 5A). The identity of these cells as circulatory monocytes was further confirmed by staining for CD115 (Figure IV A in the online-only Data Supplement), a well-known marker of murine blood monocytes. We also found significantly enhanced infiltrations of highly inflammatory Gr1-Mac2 (≈2-fold; \( P < 0.006 \); Figure 5B) and F4/80-CD11b (≈2-fold; \( P < 0.013 \); Figure 5C) double-positive leukocytes in the peritoneum. Elicitation with thioglycollate is necessary to harvest leukocytes because without thioglycollate elicitation most of the harvested cells remain unstained with F4/80 and CD11b antibodies showing these cells are not mature myelomonocytic cells (Figure IVB in the online-only Data Supplement). These cellular changes provide further support of the fact that high-fat diet-induced atherosclerosis is more severe in animals lacking macrophage expression of L13a.

Next, we determined whether the increased severity of atherosclerosis observed in the knockout mice was associated with a stronger and systemic inflammatory response. We measured the plasma level of a panel of inflammatory cytokines and chemokines (eg, interleukin [IL]-1\(\alpha\), IL-1\(\beta\), IL-2, IL-4, CCL2, IFN-\(\gamma\), tumor necrosis factor-\(\alpha\), granulocyte macrophage colony-stimulating factor, CCL11, CCL5, CCL3, and keratinocyte-derived chemokine). We found significantly increased plasma levels of all of these cytokines in knockout
mice as compared with control mice (Figure 6). We also investigat
ted whether levels of inflammatory cytokines and chemokines were elevated in the aortic cells of the high-fat diet–fed knockout mice. We performed ELISA analysis of cell lysates prepared from the total cells harvested from collagenase-digested aorta. We found significant increases in the aortic levels of several chemokines (e.g., CCL2, CCL5, CCL11, CCL17, CCL22, and CXCL1; Figure 7). Our cytokine analysis of both plasma and aortic cells identified several previously identified direct targets of the GAIT-mediated translational repression, such as CCL11, CCL3, and CCL22.14,18 Therefore, these results demonstrate that the loss of L13a expression in macrophages led to increased inflammation in high-fat diet–fed knockout mice. Moreover, the increased inflammation in knockout mice seems to be, at least in part, because of elevation of inflammatory factors that are normally subject to L13a-dependent GAIT-mediated translational silencing.

**Disruption of L13a in Macrophages Abrogates Translational Silencing of Several Target mRNAs of GAIT on High-Fat Diet Treatment**

The actively translated pool of heavier polyribosome-bound mRNAs can be readily separated from the pool of lighter translationally repressed nonpolyribosomal mRNAs by sucrose gradient centrifugation, thus allowing an operational distinction between these 2 pools.14,31,32 To directly test whether abrogation of translational silencing of the target mRNAs in the macrophages of high-fat diet–fed knockout mice was associated with the higher inflammatory response in these mice, we investigated the polyribosomal abundance of 3 known target mRNAs of the GAIT complex: CCL22, CXCL13, and CCR3. We found a substantial increase of the polyribosomal abundance of all 3 mRNAs in the macrophages of high-fat diet–fed knockout mice versus control mice (Figure 8A). Comparison of the active (polyribosome bound) and repressed (free and nonpolyribosomal) fractions of these GAIT target mRNAs with those for actin mRNA (as a housekeeping gene control; Figure 8B) clearly demonstrated that genetic depletion of L13a in macrophages specifically impeded the naturally imposed translational repression on GAIT target mRNAs. Together, these findings strongly suggest that GAIT-mediated translational silencing is the mechanism underlying the antiatherogenic role of L13a in murine model of high-fat diet–induced atherosclerosis.

**Discussion**

Inflammatory responses mediated by macrophages play a crucial role in the pathogenesis of atherosclerosis.1,2,8,9 However, there is limited information that directly addressed the role
of endogenous inflammation resolution mechanisms as a physiological defense against atherosclerosis. In this report, we have identified 1 such mechanism. Using a mouse model with macrophage-specific depletion of the gene encoding ribosomal protein L13a, we previously showed that L13a is critical for preventing uncontrolled inflammation in response to lipopolysaccharide-induced endotoxemia. We also identified a macrophage-specific role of L13a in the naturally imposed translational silencing of a cohort of mRNAs encoding several inflammatory proteins, including chemokines and chemokine receptors.

Based on these findings, we hypothesized that L13a-dependent physiological attenuation of inflammation might be important for preventing the development of atherosclerosis. To test this hypothesis, we assessed whether macrophage-specific deficiency of L13a could increase the susceptibility of apoE−/− mice to high-fat diet–induced atherosclerosis. Comparing control apoE−/− mice with normal L13a (control mice) and apoE−/− mice with macrophage-specific L13a knockout (knockout mice), we found that high-fat diet treatment led to significantly more severe atherosclerosis in knockout mice than in control mice. This was revealed by quantitative evaluation of atherosclerotic lesions at 2 independent sites: the aortic sinus and the entire aorta (Figures 2 and 3). It is important to note that this enhanced susceptibility to atherosclerosis was not because of higher plasma cholesterol levels in high-fat diet–fed knockout mice; indeed, plasma cholesterol levels were not significantly different between control and knockout mice (Figure I in the online-only Data Supplement). In addition, we did not find any difference in cholesterol uptake by knockout versus control macrophages in an ex vivo assay (Figure III in the online-only Data Supplement).

The increased severity of high-fat diet–induced atherosclerosis in mice lacking L13a in macrophages was further
illustrated by analysis of the cellular content of atherosclerotic lesions. As compared with control mice, knockout mice displayed higher levels of macrophage infiltration and accumulation in the subintimal region (Figure 4A) and attenuation of the SMC layer in the media (Figure 4C). These results are consistent with the previous findings of others defining breakdown and thinning of SMC layer as a signature of severe atherosclerosis. Recently, a series of reports have implicated matrix-degrading metalloproteinases produced by macrophages in the destruction of SMC in the media and the

Figure 8. L13a deficiency in macrophages abrogates the translational silencing of chemokine (C-C motif) receptor (CCR)3, chemokine (C-C motif) ligand (CCL)22, and chemokine (C-X-C motif) ligand (CXCL)13 mRNAs in high-fat diet–fed mice. A, Macrophages from knockout (KO) mice show increased polyribosomal abundance of the γ-activated inhibitor of translation target mRNAs, such as CCR3, CCL22, and CXCL13. Peritoneal macrophages were harvested from KO and control mice after 10 weeks of high-fat diet treatment. Polyribosome fractions were resolved by sucrose gradient centrifugation. The relative abundance of CCR3, CCL22, CXCL13, and actin mRNAs in the translationally active polyribosomal fractions vs translationally repressed nonpolyribosomal fractions was determined by reverse transcription polymerase chain reaction (RT-PCR) analysis using specific primers (see Materials and Methods for details in the online-only Data Supplement). B, Quantitative representation of the results presented in A. Ratios of the target mRNA/actin mRNA in translationally active and translationally repressed fractions were determined by measuring the band intensities of the corresponding PCR products.
promotion of their migration toward the intima.\textsuperscript{12} However, we do not know whether matrix-degrading metalloproteinases are a direct target of L13a-dependent translational silencing. We previously identified CCL3 as a target of L13a-dependent translational silencing,\textsuperscript{14} and work from others showed that CCL3 regulates neutrophil infiltration in the atherosclerotic lesion.\textsuperscript{39} Consistence with these previous studies, we show here that macrophage-specific depletion of L13a causes both enhanced plasma CCL3 levels (Figure 6) and higher infiltration of neutrophils (Figure 4F) into high-fat diet–induced atherosclerotic lesions.

Previous studies by others have identified Gr1-Ly6C\textsuperscript{hi} monocytes as inflammatory monocytes that show enhanced tissue recruitment in a variety of infectious and noninfectious models.\textsuperscript{36} These monocytes were also reported to be the predecessors of atherosclerotic macrophages.\textsuperscript{28,37,38} Our data showing increased abundance of Gr1-Ly6C\textsuperscript{hi} monocytes in the circulation and highly inflammatory Gr1-Mac2 double-positive macrophages in the peritoneum of knockout mice versus control mice are fully consistent with these previous studies. At the same time it is also important to note that proliferation of the lesional macrophages may also contribute to the enhanced accumulation of macrophages detected in knockout mice. This mechanism has been implicated recently in lesional macrophage accumulation.\textsuperscript{39} Taken together, the results that we obtained by combining macrophage-specific L13a deficiency with the apoE\textsuperscript{-/-} murine model of high-fat diet–induced atherosclerosis model suggest a novel athero-protective role of L13a.

Subsequent analysis of expression of inflammatory proteins in knockout versus control mice indicated that the athero-protective role of L13a is likely because of its translational silencing activity. Using an in vitro cellular model of macrophage and macrophage-specific L13a knockout mice, we previously identified an L13a-dependent translational silencing mechanism targeting a cohort of chemokine and chemokine receptors to resolve lipopolysaccharide-induced endotoxemia. Consistent with this study, we now show increased levels of a group of chemokine and chemokine receptors, including several GAIT target proteins (eg, CCL11, CCL3, and CCL22) in cells of the aorta and in the circulation of knockout mice (Figures 6 and 7). It is interesting to note that we found significantly increased synthesis of other highly inflammatory cytokines (eg, IL-1, IL-4, tumor necrosis factor-\alpha, granulocyte macrophage colony-stimulating factor, etc) in the circulation of high-fat diet–fed knockout mice as well, although they are not the direct targets of GAIT-mediated translational silencing. It is likely that increases in these cytokines could be an indirect or consequential effect of the failure of knockout macrophages to resolve inflammation in the absence of L13a.

Mechanisms involving translation control have been implicated previously in the multifaceted regulation of inflammatory response\textsuperscript{16} and metabolic diseases.\textsuperscript{40,41} However, most of these studies were based on targeting of 1 particular gene (eg, tumor necrosis factor-\alpha,\textsuperscript{12} CD36\textsuperscript{42} Cox-2,\textsuperscript{43} matrix-degrading metalloproteinases-13,\textsuperscript{44} regulated upon activation normal T cell expressed and presumably secreted,\textsuperscript{45} etc). Here, using the macrophages harvested from the high-fat diet–fed knockout and control mice, we demonstrated increased polyribosomal abundance of a group of mRNAs encoding inflammatory proteins in the knockout animals. Therefore, in contrast to previous studies by others based on a monogenic approach, our results identify an endogenous athero-protective mechanism that relies on coordinated translational control of a cohort of proteins mediating inflammation. We think that this is important because responses that lead to the chronic inflammatory diseases, such as atherosclerosis, are orchestrated by the concerted action of many molecules, and therefore, targeting just one may not efficiently achieve a therapeutic outcome. Our study suggests that manipulation of the L13a-dependent translational silencing pathway could lead to effects on numerous inflammatory mediators. In future, it will be fascinating to pursue whether novel and more efficient therapeutic strategies could emerge through manipulation of this pathway.

Acknowledgments

We thank Heather Conger and Nina Dvorina for technical assistance for atherosclerosis and immunohistochemistry assay, respectively. We are grateful to Dr Patricia Stanhope Baker for help with article editing.

Sources of Funding

This work was supported by Public Health Service Grant National Institutes of Health (NIH) HL 79164 and American Heart Association Grant-in-Aid 0855555D (to B. Mazumder), American Heart Association predoctoral Fellowship Grant 11PRE7660008 (to D. Poddard), NIH Grant RO1 HL098193 (to J.D. Smith), and NIH Grant PO1AI087586 (to W.M. Baldwin). B. Mazumder also acknowledges support from the Center for Gene Regulation in Health and Disease and an Ohio Third Frontier Grant.

Disclosures

None.

References


Significance

Uncontrolled synthesis of inflammatory molecules by macrophages and monocytes contributes significantly to the pathogenesis of atherosclerosis. Endogenous mechanisms that regulate the resolution of inflammation have been identified, and their roles in providing physiological protection against atherosclerosis have not been established. In particular, our previous work revealed ribosomal protein L13a-dependent translational silencing of a cohort of chemokine and chemokine receptors in interferon-γ-stimulated macrophages and showed that this mechanism is critical for protection against lipopolysaccharide-induced endotoxemia. Using a mouse model of macrophage-specific L13a depletion in an apolipoprotein E−deficient background, we now demonstrate that L13a-dependent translational silencing in macrophages serves as a key endogenous defense against high-fat diet–induced atherosclerosis. These findings suggest that pharmacological agents capable of promoting L13a-dependent translational silencing could provide a new therapeutic opportunity for prevention and treatment of atherosclerosis.
Ribosomal Protein L13a Deficiency in Macrophages Promotes Atherosclerosis by Limiting Translation Control-Dependent Retardation of Inflammation
Abhijit Basu, Darshana Poddar, Peggy Robinet, Jonathan D. Smith, Maria Febbraio, William M. Baldwin III and Barsanjit Mazumder

Arterioscler Thromb Vasc Biol. 2014;34:533-542; originally published online January 16, 2014; doi: 10.1161/ATVBAHA.113.302573
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/34/3/533

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2014/01/16/ATVBAHA.113.302573.DC1
http://atvb.ahajournals.org/content/suppl/2014/01/16/ATVBAHA.113.302573.DC2

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Quantification of the lesion area in the aortic root
**Basu_Supplemental Fig. III**

- **Unloaded**
  - Control: [Cholesterol level]
  - KO: [Cholesterol level]
  - *P* = 0.6557

- **Loaded**
  - Control: [Cholesterol level]
  - KO: [Cholesterol level]
  - *P* = 0.3461
Supplementary Figure Legends

Supplementary Fig. I. Body weight, heart weight and serum total cholesterol levels were not significantly different between control and KO mice after 10 weeks of high-fat diet feeding. Total cholesterol levels were determined by an enzymatic method using Cholesterol Liquicolor (Stanbio). Results shown are mean +/- SD, N=13 mice/group, P values for comparison of body weight, heart weight and cholesterol levels between KO and control groups were 0.8508, 0.7463 and 0.5636 respectively, using paired two-tailed Student’s t-tests.

Supplementary Fig. II. The extent of high-fat diet-induced atherosclerosis is not influenced by gender within either the control or KO groups of mice. Atherosclerosis values (area of atherosclerotic lesions on cross sections of the aortic root) were not normally distributed as determined by the Kolmogorov-Smirnov test; therefore, the non-parametric Mann-Whitney test was used to determine the statistical significance of differences between the two genders in each group. Values for individual animals are plotted and the horizontal lines in the figure denote the median value. The P values for comparison of males and females in the control group and in the KO group were 0.4807 and 0.48143 respectively. In the control group N=15 for both Male and Female and in the KO group, N=10 for both Male and Female.

Supplementary Fig. III. The Cholesterol loading efficiency of the macrophages is not affected by L13a deficiency. Bone marrow-derived macrophages from control and KO mice were incubated in the absence (“unloaded”, left panel) or presence (“loaded” right panel) of acetylated human LDL for 24 hours. The amount of total cholesterol in lipids extracted from the cells is shown as µg of cholesterol per mg of cellular protein (average value for 2 mice with each sample run in triplicate, error bars indicate standard deviations; see Materials and Methods for assay details). There was no significant difference in the cholesterol content of control and L13a-deficient (KO) macrophages under either “unloaded” or “loaded” conditions (P=0.6557 and 0.3461 respectively).

Supplementary Fig. IV. (a) The identity of the mononuclear cells isolated from blood as monocytes was confirmed by staining with rat anti-mouse CD115 IgG2a (eBioscience). FACS analysis of the mononuclear cells from control and KO mice showed positive staining for CD115. (b) The number of peritoneal macrophages that can be isolated from non-thioglycollate injected mice is extremely low. Peritoneal macrophages were harvested 72 hours after saline injection and stained with rat ant-mouse F4/80 IgG2b (Abd Serotec) and rat anti-mouse CD11b IgG2b (Cedarlane Laboratories). The majority of the cells remained unstained. Only 1% and 6% double positive cells were observed for the control and KO mice, respectively. Since the number of cells harvested after saline injection was too low to provide useful information, we used thioglycollate elicitation for isolation of peritoneal macrophages (see Materials and Methods).
Materials and Methods

Generation of macrophage-specific KO mice on an apoE-/ background (L13a Flox+/ Cre+/ apoE-/- mice):

In our previous publication we reported generation of mice homozygous for the Floxed L13a gene (L13a Flox+/+) and macrophage-specific L13a KO mice (L13a Flox+/ Cre+/)\(^1\). In the current study, the L13a Flox+/+ mice were mated with the mice homozygous for the Apolipoprotein E (apoE) null allele [(apoE-/-), The Jackson Laboratory, Bar Harbor, ME] and L13a Flox+/ apoE-/- mice were identified by PCR-based genotyping of the offspring. The control mice (L13a Flox+/ Cre+/ apoE-/-) mice were identified by genotyping the offspring generated by brother-sister mating of L13a Flox+/ apoE-/- mice. Subsequently, Flox+/ apoE-/- mice were mated with the macrophage-specific KO mice (L13a Flox+/ Cre+/) in order to generate the L13a Flox+/ Cre+/ apoE-/- mice. The experimental mice i.e. the macrophage-specific L13a KO mice on an apoE null background (L13a Flox+/ Cre+/ apoE-/-) were generated by intercrossing L13a Flox+/ Cre+/ apoE-/- mice.

PCR-based genotyping was performed using genomic DNA isolated from tail snips and the allele-specific primer pairs listed below. The sequences of the primers used to identify the presence and/or absences of the Fllox and Cre alleles are based on our previous publication\(^1\). The primers used to identify the presence and/or absence of the apoE allele are based on the genotyping instructions provided by The Jackson Laboratory.

**Primer pair to detect the loxP allele:**
- loxP (forward): 5'-AGGTTCTGCTTGAGCATCTGAG-3'
- loxP (reverse): 5'-CCGTCAGGATGCCTACTACCCAG-3'

**Primer pairs to detect the presence or absence of the apoE allele:**
- apoE (forward, common for presence or absence): 5'-GCCTAGCCGAGGGAGAGCCG-3'
- apoE (reverse, for the presence of apoE): 5'-TGTTGACTTGGGAGCTCTGCAGC-3'
- apoE (reverse, for the absence of apoE): 5'-GCCGCCCCGACTGCATCT-3'

**Primer pairs to detect the presence or absence of the Cre allele:**
- Cre (forward, common for presence or absence): 5'-CTTGGGCTGCCAGAATTTCCTC-3'
- Cre (reverse, for the presence of Cre): 5'-TTACAGTCGGCCAGGCTGCAC-3'
- Cre (reverse, for the absence of Cre): 5'-CCCAGAAATGCAGATTAC-3'

**Atherosclerotic lesion analysis:**

Atherosclerosis was induced in 4-week-old mice by feeding the animals a Western-type “high fat” diet containing 0.2% cholesterol and providing 42% calories as fat (TD88137, Harlan Teklad) for 10 weeks. Anesthetized mice were euthanized by cervical dislocation. The hearts of euthanized mice were perfused with cold 4% paraformaldehyde in PBS followed by fixing in 10% phosphate-buffered formalin for 48 hours and then embedded in Optimal Cutting Temperature (OCT) medium. For aortic root analysis serial cryosections of 10 µm thickness were taken from the region of the proximal aorta through the aortic sinuses and stained with Oil red O as previously described\(^2\). Images were captured using a Nikon Eclipse 55i microscope and a CCD camera (Nikon DS-U2). The lesion areas were quantified using Image Pro Plus software (Lakewood, CO). En face analysis was performed as described by Febbraio et al\(^3\). The entire aorta from the heart extending 5-10 mm after bifurcation of the iliac arteries and including the subclavian right and left common carotid arteries was removed from euthanized mice.
The aorta was dissected longitudinally and stained with Oil red O. Stained aortas were mounted on glass slides with PBS and digital images were captured using a scanner. Aortic lesions and the total aortic area were manually selected and analyzed using Adobe Photoshop CS5. The lesion size was quantified as a percentage of total aortic area.

Scoring of the presence and severity of atherosclerotic lesions by both assays (Oil red O staining of aorta cross sections and en face analysis) was performed in a blinded manner (group identities/genotypes were not known at the time of scoring).

**Immunohistochemical analysis:**

Mouse hearts were fixed in 10% neutral buffered formalin (Sigma-Aldrich) for 24 hours and stored in PBS overnight before paraffin embedding. Five μm thick sections were prepared and stained for macrophages and smooth muscle cells using purified anti-mouse monoclonal antibody against Mac2, 1:1500 dilution (CL8942AP, Cedarlane Laboratories) and anti-alpha smooth muscle Actin antibody, 1:250 dilution (ab5694, Abcam), respectively, followed by incubation with Biotin-SP-conjugated appropriate secondary antibodies (Jackson ImmunoResearch). Staining for neutrophils was performed using anti-Ly-6G, 1:50 dilution (RB6-8C5, BioXCell), followed by the appropriate secondary antibodies. For each antibody, we analyzed 5 different hearts from each study group (genotype) and 2 to 3 sections from each heart. The images were captured using a Nikon Eclipse 55i microscope and a CCD camera (Nikon DS-U2) and stained areas were quantified using Image J software (National Institute of Health).

**Preparation of extracts from aortic cells:**

Digestion of the aorta and subsequent preparation of a single cell suspension of aortic cells was done according to previously published procedures. Briefly, aortas were dissected and minced then incubated with collagenase A (1 mg/ml) (Roche) for 2 hours at 37°C. The cells were then spun down, washed twice with ice-cold PBS and lysed in buffer (25mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP-40 and 5% glycerol) using sonication. After sonication the lysates were centrifuged and the supernatant recovered. The total protein concentration in the supernatant ("the extract") was measured before ELISA analysis was performed.

**Cytokine and chemokine assays:**

Blood was collected from anesthetized mice by cardiac puncture. Plasma was prepared from the blood samples and aortic cell extracts were prepared as described above. Levels of cytokines and chemokines were quantified in plasma and aortic cell extract samples using an ELISA-based array in a commercial facility (Quansys Biosciences, Logan UT).

**Antibodies for Immunoblot analysis:**

The peptide sequence NVEKKIDKYTEVLKTHG from the C terminus of human L13a was used to raise a rabbit polyclonal anti-L13a antibody in rabbits. This antibody was used previously to detect human5 and mouse1 L13a appearing as a specific band between 28 and 21 kDa on SDS PAGE. The anti-actin antibody and anti-Cre recombinase antibodies used in the study were from Sigma-Aldrich and Abcam, respectively.
Animal handling and isolation of peritoneal macrophages and peripheral blood mononuclear cells:

All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) and National Institutes of Health (NIH). For peritoneal macrophage isolation, age- and sex-matched mice were challenged by i.p injection of thioglycollate (1.5 ml, 4% solution in distilled water). 72-hours after thioglycollate injection, macrophages were collected by peritoneal lavage with ice-cold PBS. The collected cells were counted before use in downstream applications. Peripheral blood mononuclear cells were isolated by layering whole blood collected by cardiac puncture over Ficoll-Hypaque solution and performing centrifugation as described⁶.

FACS analysis:

To block nonspecific binding of antibodies to FcγRs, cells (10⁶/sample) were incubated with rat anti-mouse CD16/CD32 (BD Pharmingen). The cells were then washed and stained with PE-conjugated rat anti-mouse Ly6G IgG2a κ (BD Pharmingen), FITC-conjugated rat anti-mouse Ly6C (BD Pharmingen), FITC-conjugated rat anti-mouse Mac2 IgG2a (Cedarlane Laboratories), APC-conjugated rat anti-mouse F4/80 IgG2b (Abd Serotec), FITC-conjugated rat anti-mouse CD11b IgG2b (BD Pharmingen) and PE-conjugated rat anti-mouse CD115 IgG2a (eBioscience). FITC-conjugated rat IgM κ, IgG2a and IgG2b (BD Pharmingen) were used as isotype controls to exclude background staining. After antibody staining and washing, the cells were fixed with 4% paraformaldehyde and analyzed using a FACSCanto II flow cytometer (BD Biosciences). Data was collected from cells within gates set for leukocytes and was analyzed using FACSDiva (BD Biosciences) software.

Polyribosome profiling analysis in mouse peritoneal macrophages:

Peritoneal macrophages were harvested from mice by peritoneal lavage after thioglycollate elicitation as described above. Cell lysates were prepared from the collected macrophages in polyribosome lysis buffer [10mM HEPES (pH 7.5), 100 mM KCl, 2.5mM MgCl₂, 1mM DTT, 50U recombinant RNasin and 0.1% NP-40]. 9.0 O.D units of lysate were loaded on top of a 5-50% sucrose density gradient and ultracentrifuged for 17,000 rpm in a Beckman SW32.1 Ti rotor for 18 hours at 4⁰C. Fractions were collected using an ISCO gradient fractionation system equipped with a UA-6 detector following an upward displacement method. The presence of ribosomal subunits and heavy polyribosomes in collected fractions was monitored via continuous UV absorption profiles at A₂₆₄. Total RNA from the these fractions were isolated by extraction with Trizol (Invitrogen) and used for Reverse Transcription (RT)-PCR. RT-PCR reactions were carried out using Superscript RT-PCR system (Invitrogen) followed by PCR amplification using specific primer pairs for detection of CCR3, CCL22, CXCL13 and Actin mRNA (see below). The PCR products were visualized on 1.2% agarose gels.

Primer sequences:

Mouse β-Actin (NM_007393):  
Forward: 5’ GTCCCTCACCCCTCCCAAAGC 3’  
Reverse: 5’ AGGTAAGGTGTGCACTTTTAT 3’
Mouse CCL22 (NM_009137):
Forward: 5' TTCTTGCTGTGGCAATTCAGACCT 3'
Reverse: 5' CAGGTCCTCCTCCCTAGGACAGTT 3'

Mouse CXCL13 (NM_018866):
Forward: 5' CTTGTAAAACGCAGGCTTCCACA 3'
Reverse: 5' GGGTCACAGTGCAAAGGAATATA 3'

Mouse CCR3 (NM_009914):
Forward: 5' TTCTACCGGCCCTCACATAC 3'
Reverse: 5' ATCCAGAGGCACCTCCTGA 3'

Cholesterol uptake analysis:

Bone marrow-derived macrophages were obtained from control and KO mice. Bone marrow cells were flushed from mouse femurs and resuspended in macrophage growth medium consisting of DMEM supplemented with 10% FBS and 20% L-cell-conditioned medium as a source of macrophage colony stimulating factor as previously described. Cells were seeded in 12-well plates and allowed to differentiate for 15 days (with the medium renewed twice per week) before their use in cholesterol uptake assays. Human low-density lipoprotein (LDL) was prepared by ultracentrifugation and acetylated according to the published procedure. The cells were incubated for 24 hours at 37°C in macrophage growth medium alone (unloaded) or containing acetylated LDL (50µg/ml). At the end of the incubation period lipids were extracted from cells and total cholesterol was quantified as described. Proteins were dissolved in 0.2N NaOH containing 0.2% SDS and quantified by BCA assay. Amounts of cholesterol were expressed as µg of cholesterol per mg of cellular protein.

Statistical analyses:

Statistical analyses were performed using GraphPad Prism 5.0 software. Differences between two groups with normal distributions (determined by the Kolmogorov-Smirnov test) were evaluated using 2-tailed Student's t-tests. Two-group comparisons with non-normal distributions were analyzed using the Mann-Whitney rank sum test. Values shown on graphs represent the per-group mean +/- the standard deviation (SD) for data with normal distribution and the per-group median for data with non-normal distribution. P values less than or equal to 0.05 were considered to be statistically significant.
References

Baseline Supplementation Fig. 1

Body Weight (gm)

Control       KO

Total Cholesterol (mg/dL)

Control       KO

Heart Weight (gm)

Control       KO
Quantification of the lesion area in the aortic root

Lesion area (µm²)

Control

P = 0.4807

KO

P = 0.4813

Male  Female

Male  Female
Basu_Supplemental Fig. III

**Unloaded**

- Control: 10 ± 2 µg/mg of cellular protein
- KO: 20 ± 2 µg/mg of cellular protein
- P = 0.6557

**Loaded**

- Control: 20 ± 2 µg/mg of cellular protein
- KO: 30 ± 2 µg/mg of cellular protein
- P = 0.3461
Supplementary Figure Legends

**Supplementary Fig. I.** Body weight, heart weight and serum total cholesterol levels were not significantly different between control and KO mice after 10 weeks of high-fat diet feeding. Total cholesterol levels were determined by an enzymatic method using Cholesterol Liquicolor (Stanbio). Results shown are mean +/- SD, N=13 mice/group, P values for comparison of body weight, heart weight and cholesterol levels between KO and control groups were 0.8508, 0.7463 and 0.5636 respectively, using paired two-tailed Student’s t-tests.

**Supplementary Fig. II.** The extent of high-fat diet-induced atherosclerosis is not influenced by gender within either the control or KO groups of mice. Atherosclerosis values (area of atherosclerotic lesions on cross sections of the aortic root) were not normally distributed as determined by the Kolmogorov-Smirnov test; therefore, the non-parametric Mann-Whitney test was used to determine the statistical significance of differences between the two genders in each group. Values for individual animals are plotted and the horizontal lines in the figure denote the median value. The P values for comparison of males and females in the control group and in the KO group were 0.4807 and 0.48143 respectively. In the control group N=15 for both Male and Female and in the KO group, N=10 for both Male and Female.

**Supplementary Fig. III.** The Cholesterol loading efficiency of the macrophages is not affected by L13a deficiency. Bone marrow-derived macrophages from control and KO mice were incubated in the absence (“unloaded”, left panel) or presence (“loaded” right panel) of acetylated human LDL for 24 hours. The amount of total cholesterol in lipids extracted from the cells is shown as µg of cholesterol per mg of cellular protein (average value for 2 mice with each sample run in triplicate, error bars indicate standard deviations; see Materials and Methods for assay details). There was no significant difference in the cholesterol content of control and L13a-deficient (KO) macrophages under either “unloaded” or “loaded” conditions (P=0.6557 and 0.3461 respectively).

**Supplementary Fig. IV.** (a) The identity of the mononuclear cells isolated from blood as monocytes was confirmed by staining with rat anti-mouse CD115 IgG2a (eBioscience). FACS analysis of the mononuclear cells from control and KO mice showed positive staining for CD115. (b) The number of peritoneal macrophages that can be isolated from non-thioglycollate injected mice is extremely low. Peritoneal macrophages were harvested 72 hours after saline injection and stained with rat anti-mouse F4/80 IgG2b (Abd Serotec) and rat anti-mouse CD11b IgG2b (Cedarlane Laboratories). The majority of the cells remained unstained. Only 1% and 6% double positive cells were observed for the control and KO mice, respectively. Since the number of cells harvested after saline injection was too low to provide useful information, we used thioglycollate elicitation for isolation of peritoneal macrophages (see Materials and Methods).
Materials and Methods

Generation of macrophage-specific KO mice on an apoE−/− background (L13a Flox+/* Cre+/* apoE−/− mice):

In our previous publication we reported generation of mice homozygous for the Floxed L13a gene (L13a Flox+/*) and macrophage-specific L13a KO mice (L13a Flox+/* Cre+/*)\(^1\). In the current study, the L13a Flox+/* mice were mated with the mice homozygous for the Apolipoprotein E (apoE) null allele [(apoE−/−), The Jackson Laboratory, Bar Harbor, ME] and L13a Flox−/* apoE−/− mice were identified by PCR-based genotyping of the offspring. The control mice (L13a Flox−/* apoE−/−) mice were identified by genotyping the offspring generated by brother-sister mating of L13a Flox−/* apoE−/− mice. Subsequently, Flox−/* apoE−/− mice were mated with the macrophage-specific KO mice (L13a Flox+/* Cre+/*) in order to generate the L13a Flox+/* Cre+/* apoE−/− mice. The experimental mice i.e. the macrophage-specific L13a KO mice on an apoE null background (L13a Flox+/* Cre+/* apoE−/−) were generated by intercrossing L13a Flox+/* Cre+/* apoE−/− mice.

PCR-based genotyping was performed using genomic DNA isolated from tail snips and the allele-specific primer pairs listed below. The sequences of the primers used to identify the presence and/or absences of the Flox and Cre alleles are based on our previous publication\(^1\). The primers used to identify the presence and/or absence of the apoE allele are based on the genotyping instructions provided by The Jackson Laboratory.

Primer pair to detect the loxP allele:

\[\text{loxP (forward): 5'}-\text{AGGTTCTGCTTGAGCATCTGAG} - 3']
\[\text{loxP (reverse): 5'}-\text{CCGTCAGGATGCCTACTACCCAG} - 3']

Primer pairs to detect the presence or absence of the apoE allele:

\[\text{apoE (forward, common for presence or absence): 5'}-\text{GCCTAGCGAGGGAGAGCCG} - 3']
\[\text{apoE (reverse, for the presence of apoE): 5'}-\text{TGTTGACTTGGGAGCTCTGAC} - 3']
\[\text{apoE (reverse, for the absence of apoE): 5'}-\text{GCCGCCCCGACTGCATCT} - 3']

Primer pairs to detect the presence or absence of the Cre allele:

\[\text{Cre (forward, common for presence or absence): 5'}-\text{CTTGAGCTTGAGAGGGCGGAG} - 3']
\[\text{Cre (reverse, for the presence of Cre): 5'}-\text{TACAGTGGGCCGGCTGAG} - 3']
\[\text{Cre (reverse, for the absence of Cre): 5'}-\text{CCAGAAATGCGCCAGATTAC} - 3']

Atherosclerotic lesion analysis:

Atherosclerosis was induced in 4-week-old mice by feeding the animals a Western-type “high fat” diet containing 0.2% cholesterol and providing 42% calories as fat (TD88137, Harlan Teklad) for 10 weeks. Anesthetized mice were euthanized by cervical dislocation. The hearts of euthanized mice were perfused with cold 4% paraformaldehyde in PBS followed by fixing in 10% phosphate-buffered formalin for 48 hours and then embedded in Optimal Cutting Temperature (OCT) medium. For aortic root analysis serial cryosections of 10 µm thickness were taken from the region of the proximal aorta through the aortic sinuses and stained with Oil red O as previously described\(^2\). Images were captured using a Nikon Eclipse 55i microscope and a CCD camera (Nikon DS-U2). The lesion areas were quantified using Image Pro Plus software (Lakewood, CO). En face analysis was performed as described by Febbraio et al\(^3\). The entire aorta from the heart extending 5-10 mm after bifurcation of the iliac arteries and including the subclavian right and left common carotid arteries was removed from euthanized mice.
The aorta was dissected longitudinally and stained with Oil red O. Stained aortas were mounted on glass slides with PBS and digital images were captured using a scanner. Aortic lesions and the total aortic area were manually selected and analyzed using Adobe Photoshop CS5. The lesion size was quantified as a percentage of total aortic area.

Scoring of the presence and severity of atherosclerotic lesions by both assays (Oil red O staining of aorta cross sections and en face analysis) was performed in a blinded manner (group identities/genotypes were not known at the time of scoring).

**Immunohistochemical analysis:**

Mouse hearts were fixed in 10% neutral buffered formalin (Sigma-Aldrich) for 24 hours and stored in PBS overnight before paraffin embedding. Five μm thick sections were prepared and stained for macrophages and smooth muscle cells using purified anti-mouse monoclonal antibody against Mac2, 1:1500 dilution (CL8942AP, Cedarlane Laboratories) and anti-alpha smooth muscle Actin antibody, 1:250 dilution (ab5694, Abcam), respectively, followed by incubation with Biotin-SP-conjugated appropriate secondary antibodies (Jackson ImmunoResearch). Staining for neutrophils was performed using anti-Ly-6G, 1:50 dilution (RB6-8C5, BioXCell), followed by the appropriate secondary antibodies. For each antibody, we analyzed 5 different hearts from each study group (genotype) and 2 to 3 sections from each heart. The images were captured using a Nikon Eclipse 55i microscope and a CCD camera (Nikon DS-U2) and stained areas were quantified using Image J software (National Institute of Health).

**Preparation of extracts from aortic cells:**

Digestion of the aorta and subsequent preparation of a single cell suspension of aortic cells was done according to previously published procedures4. Briefly, aortas were dissected and minced then incubated with collagenase A (1 mg/ml) (Roche) for 2 hours at 37°C. The cells were then spun down, washed twice with ice-cold PBS and lysed in buffer (25mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP-40 and 5% glycerol) using sonication. After sonication the lysates were centrifuged and the supernatant recovered. The total protein concentration in the supernatant (“the extract”) was measured before ELISA analysis was performed.

**Cytokine and chemokine assays:**

Blood was collected from anesthetized mice by cardiac puncture. Plasma was prepared from the blood samples and aortic cell extracts were prepared as described above. Levels of cytokines and chemokines were quantified in plasma and aortic cell extract samples using an ELISA-based array in a commercial facility (Quansys Biosciences, Logan UT).

**Antibodies for Immunoblot analysis:**

The peptide sequence NVEKKIDKYTEVLKTHG from the C terminus of human L13a was used to raise a rabbit polyclonal anti-L13a antibody in rabbits. This antibody was used previously to detect human5 and mouse1 L13a appearing as a specific band between 28 and 21 kDa on SDS PAGE. The anti-actin antibody and anti-Cre recombinase antibodies used in the study were from Sigma-Aldrich and Abcam, respectively.
Animal handling and isolation of peritoneal macrophages and peripheral blood mononuclear cells:

All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) and National Institutes of Health (NIH). For peritoneal macrophage isolation, age- and sex-matched mice were challenged by i.p injection of thioglycollate (1.5 ml, 4% solution in distilled water). 72-hours after thioglycollate injection, macrophages were collected by peritoneal lavage with ice-cold PBS. The collected cells were counted before use in downstream applications. Peripheral blood mononuclear cells were isolated by layering whole blood collected by cardiac puncture over Ficoll-Hypaque solution and performing centrifugation as described.

FACS analysis:

To block nonspecific binding of antibodies to FcγRs, cells (10^6/sample) were incubated with rat anti-mouse CD16/CD32 (BD Pharmingen). The cells were then washed and stained with PE-conjugated rat anti-mouse Ly6G IgG2a κ (BD Pharmingen), FITC conjugated rat anti-mouse Ly6C (BD Pharmingen), FITC conjugated rat anti-mouse Mac2 IgG2a (Cedarlane Laboratories), APC conjugated rat anti-mouse F4/80 IgG2b (Abd Serotec), FITC conjugated rat anti-mouse CD11b IgG2b (BD Pharmingen) and PE conjugated rat anti-mouse CD115 IgG2a (eBioscience). FITC conjugated rat IgM κ, IgG2a and IgG2b (BD Pharmingen) were used as isotype controls to exclude background staining. After antibody staining and washing, the cells were fixed with 4% paraformaldehyde and analyzed using a FACSCanto II flow cytometer (BD Biosciences). Data was collected from cells within gates set for leukocytes and was analyzed using FACSDiva (BD Biosciences) software.

Polyribosome profiling analysis in mouse peritoneal macrophages:

Peritoneal macrophages were harvested from mice by peritoneal lavage after thioglycollate elicitation as described above. Cell lysates were prepared from the collected macrophages in polyribosome lysis buffer [10mM HEPES (pH 7.5), 100 mM KCl, 2.5mM MgCl₂, 1mM DTT, 50U recombinant RNasin and 0.1% NP-40]. 9.0 O.D units of lysate were loaded on top of a 5-50% sucrose density gradient and ultracentrifuged for 17,000 rpm in a Beckman SW32.1 Ti rotor for 18 hours at 4°C. Fractions were collected using an ISCO gradient fractionation system equipped with a UA-6 detector following an upward displacement method. The presence of ribosomal subunits and heavy polyribosomes in collected fractions was monitored via continuous UV absorption profiles at A_{264}. Total RNA from these fractions were isolated by extraction with Trizol (Invitrogen) and used for Reverse Transcription (RT)-PCR. RT-PCR reactions were carried out using Superscript RT-PCR system (Invitrogen) followed by PCR amplification using specific primer pairs for detection of CCR3, CCL22, CXCL13 and Actin mRNA (see below). The PCR products were visualized on 1.2% agarose gels.

Primer sequences:

Mouse β-Actin (NM_007393):
Forward: 5’ GTCCCTCACCTCCCAAAGC 3’
Reverse: 5’ AGGTAAGGTGTGCACTTTTAT 3’
Mouse CCL22 (NM_009137):
Forward: 5' TTCTTGCTGTGGCAATTCAGACCT 3'
Reverse: 5' CAGGTCCTCCTCCCTAGGACAGTT 3'

Mouse CXCL13 (NM_018866):
Forward: 5' CTTGTAAAACGCAGGCTTCCACA 3'
Reverse: 5' GGGTCACAGTGCAAAGGAATATA 3'

Mouse CCR3 (NM_009914):
Forward: 5' TTCTACCGGCCCTCACATAC 3'
Reverse: 5' ATCCAGAGAGCACCTCCTGA 3'

Cholesterol uptake analysis:

Bone marrow-derived macrophages were obtained from control and KO mice. Bone marrow cells were flushed from mouse femurs and resuspended in macrophage growth medium consisting of DMEM supplemented with 10% FBS and 20% L-cell-conditioned medium as a source of macrophage colony stimulating factor as previously described. Cells were seeded in 12-well plates and allowed to differentiate for 15 days (with the medium renewed twice per week) before their use in cholesterol uptake assays. Human low-density lipoprotein (LDL) was prepared by ultracentrifugation and acetylated according to the published procedure. The cells were incubated for 24 hours at 37°C in macrophage growth medium alone (unloaded) or containing acetylated LDL (50µg/ml). At the end of the incubation period lipids were extracted from cells and total cholesterol was quantified as described. Proteins were dissolved in 0.2N NaOH containing 0.2% SDS and quantified by BCA assay. Amounts of cholesterol were expressed as µg of cholesterol per mg of cellular protein.

Statistical analyses:

Statistical analyses were performed using GraphPad Prism 5.0 software. Differences between two groups with normal distributions (determined by the Kolmogorov-Smirnov test) were evaluated using 2-tailed Student's t-tests. Two-group comparisons with non-normal distributions were analyzed using the Mann-Whitney rank sum test. Values shown on graphs represent the per-group mean +/- the standard deviation (SD) for data with normal distribution and the per-group median for data with non-normal distribution. P values less than or equal to 0.05 were considered to be statistically significant.
References


