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Macrophage-Specific Expression of IL-37 in Hyperlipidemic Mice Attenuates Atherosclerosis

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Atherosclerosis, the progressive buildup of plaque within arterial blood vessels, can lead to fatal downstream events, such as heart attack or stroke. A key event contributing to the development of atherosclerosis is the infiltration of monocytes and its associated inflammation, as well as the formation of lipid-laden macrophage foam cells within the vessel wall. IL-37 is recognized as an important anti-inflammatory cytokine expressed especially by immune cells. This study was undertaken to elucidate the role of macrophage-expressed IL-37 in reducing the production and effects of proinflammatory cytokines, preventing foam cell formation, and reducing the development of atherosclerosis. Expression of human IL-37 was achieved with a macrophage-specific overexpression system, using the CD68 promoter in mouse primary bone marrow–derived macrophages via retroviral transduction. Macrophage IL-37 expression in vitro resulted in decreased mRNA (e.g., IL-1B, IL-6, and IL-12) and secreted protein production (e.g., IL-6, M-CSF, and ICAM-1) of key inflammatory mediators. IL-37 expression also inhibited macrophage proliferation, apoptosis, and transmigration, as well as reduced lipid uptake, compared with controls in vitro. The in vivo effects of macrophage-expressed IL-37 were investigated through bone marrow transplantation of transduced hematopoietic stem cells into irradiated atherosclerosis-prone Ldlr−/− mice. After 10 wk on a high-fat/high-cholesterol diet, mice with IL-37–expressing macrophages showed reduced disease pathogenesis, which was demonstrated by significantly less arterial plaque development and systemic inflammation compared with control mice. The athero-protective effect of macrophage-expressed IL-37 has implications for development of future therapies to treat atherosclerosis, as well as other chronic inflammatory diseases. The Journal of Immunology, 2017, 199: 000–000.

The first pathological stage of atherosclerosis begins with the attraction of immune cells, specifically monocytes, to the inflamed endothelial lining of medium and large arteries. Monocytes transmigrate through the endothelium to the intima (1). Chronic inflammation and dysregulation of cholesterol metabolism by macrophages within the plaque are the major driving forces of atherosclerosis progression (2). Macrophages encounter and take up modified lipoproteins (3), which leads to activation of pattern recognition receptors and TLR expression, promoting the production of inflammatory immune mediators (4, 5).

Abbreviations used in this article: AcLDL, acetylated low-density lipoprotein; BMMDD, bone marrow-derived macrophage; BMT, bone marrow transplantation; EdU, 5-ethyl-2′-deoxyuridine; EGF, enhanced GFP; EV, empty vector; HFD, high-fat diet; HSC, hematopoietic stem cell; LDL, low-density lipoprotein; LN, lymph node; OxLDL, oxidative low-density lipoprotein; PFA, paraformaldehyde; Ph-E, Phoenix Ecotropic; RT, room temperature; RT-qPCR, real-time quantitative PCR.

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The online version of this article contains supplemental material.
the potential role of macrophage-expressed IL-37 in the prevention of atherosclerosis.

Materials and Methods

Retroviral vector generation

The CD68s-HA–EGFP retroviral vector was a generous gift from Dr. E. Raines (University of Washington, Seattle, WA). The vector was constructed as described previously (19) and uses a segment of the human CD68 gene promoter to drive macrophage-specific expression of an enhanced GFP (EGFP) reporter gene. CD68s-HA–EGFP or CD68s empty vector (EV) were used as the control for all transduction experiments, as described. The cDNA encoding human IL-37 was obtained from a commercial CMV plasmid purchased from OriGene (Rockville, MD), and the restriction enzymes NotI and HindIII (New England Biolabs) were used to subclone IL-37b–Myc-DDK cDNA in the place of HA-EGFP cDNA.

Transfection of Phoenix Ectropic cells

High-titer retroviral supernatants were generated by transfecting Phoenix Ectropic (Ph-E) packaging cells (American Type Culture Collection) with the retroviral constructs CD68s-EGFP or CD68s-IL-37b using 2× HEPESE-buffered saline and 2 mM CaCl₂, as described previously (23), with the exception that Ph-E cells were not selected using puromycin, and retroviruses-containing cell supernatants were collected at 48 and 72 h for use in HSC transduction. Ph-E cells were grown in DMEM/F12 supplemented with 10% FBS and 1% penicillin/streptomycin.

Isolation and culture of HSCs from bone marrow

Bone marrow was isolated from 8–12-wk-old male C57BL/6 mice. The femurs and tibiae bones were flushed with ice-cold PBS, and the cells were passed through a 40-μm cell strainer. RBCs were lysed using multipurpose RBC lysis buffer (eBioscience), according to the manufacturer’s instructions. After three additional 5-min washes with 0.1% PBST, the membranes were blotted dry and immersed in 10% DMSO (1:500) for 6 h. Cells were then harvested using Cell Stripper and challenged with camptothecin (2 g/ml) or an equivalent volume of DMSO (1:500) for 6 h. Cells were then fixed with 4% paraformaldehyde (PFA) for 10 min and parallelized using 0.1% Triton X-100 in PBS with FBS supplementation at low (2%) and normal (10%) levels, LPS and IFN-γ (10 ng/ml each), or M-CSF (20 ng/ml). Cells were then fixed with 4% paraformaldehyde (PFA) for 10 min and parallelized using 0.1% Triton X-100 in PBS for 10 min, and detection of EdU was performed according to the manufacturer’s instructions using a Click-IT EdU Alexa Fluor 488 Imaging Kit. Cells were washed with PBS, and nuclei were stained with DAPI.

Macrophage proliferation in vitro using 5-ethyl-2′-deoxyuridine

Transduced macrophages (EV versus IL-37b) from C57BL/6 male mice were plated at 2 × 10⁵ cells per well of an eight-well chamber glass slide. Cells were treated with 5-ethyl-2′-deoxyuridine (EdU) for 6 h in parallel with FBS supplementation at low (2%) and normal (10%) levels, LPS and IFN-γ (10 ng/ml each), or M-CSF (20 ng/ml). Cells were then fixed with 4% paraformaldehyde (PFA) for 10 min and parallelized using 0.1% Triton X-100 in PBS for 10 min, and detection of EdU was performed according to the manufacturer’s instructions using a Click-IT EdU Alexa Fluor 488 Imaging Kit. Cells were washed with PBS, and nuclei were stained with DAPI.

Macrophage apoptosis in vitro

BMDMs from C57BL/6 mice were plated in 12-well plates at 7.5 × 10⁵ cells per well. Cells were transfected with EV or IL-37b expression plasmids using Lipofectamine 2000. Twenty-four hours after transfection, cells were challenged with camptothecin (2 μg/ml) or an equivalent volume of DMSO (1:500) for 6 h. Cells were then harvested using Cell Stripper and stained with an Annexin V–FITC Apoptosis Detection Kit (catalog number BMS500FF-100; eBioscience), according to the manufacturer’s directions. Cells were then analyzed for Annexin V–FITC and propidium iodide (PI) fluorescence on an Attune NxT Flow Cytometer (Thermo Fisher) to measure apoptosis.

Cholesterol uptake using Dil-labeled cholesterol

For cholesterol uptake, transduced macrophages (EV versus IL-37b) from C57BL/6 male mice were plated at 2.5 × 10⁵ cells per well in an 8-well chamber glass slide for analysis by microscopy or were plated at 1 × 10⁶ cells per well in a 12-well plate for analysis by flow cytometry. The macrophages were treated or not with LPS to induce IL-37 expression and sonicated using a probe sonicator (Thermo Fisher) and centrifuged at 400 × g for 5 min at 4°C to pellet cell debris. Protein concentrations were determined with a Pierce BCA Protein Assay Kit (Thermo Fisher).

Precast NuPAGE Novex 4–12% Bis-Tris Protein Gels (Invitrogen) were used for SDS-PAGE. SDS loading buffer (4×) was added to 10–20 μg of protein and heated to 95°C for 5 min. Gels were run in 1× NuPAGE MES SDS Running Buffer (Invitrogen). The protein was then transferred to a low-fluorescence polyvinylidene difluoride membrane using Efficient Western Transfer Buffer (catalog number 786-019; G-Biosciences). The membranes were blocked in LI-COR blocking buffer for 1 h. EGFP (Abcam) or IL-37 (R&D Systems) primary Abs were diluted 1:1000 in LI-COR blocking buffer and incubated at room temperature (RT) for 1 h. LI-COR secondary Abs were diluted in LI-COR blocking buffer at RT for 1 h. After three additional 5-min washes with 0.1% PBST, the membranes were visualized on a LI-COR Odyssey infrared scanner, and the images were analyzed using LI-COR Image Studio software (both from LI-COR Biosciences).

Detecting IL-37 protein by ELISA

A DuoSet ELISA kit for human IL-37 (R&D Systems) was used to analyze cell culture supernatants or mouse serum samples for human IL-37 protein. One hundred microliters of undiluted cell culture supernatant or mouse serum samples diluted 1:4 with 1% BSA in PBS were added to the plate in duplicate, and the assay was performed according to the manufacturer’s instructions.

RNA extraction, cDNA synthesis, and real time–quantitative PCR

Total RNA was recovered from cells or tissues with TRIzol Reagent (Life Technologies). A volume of chloroform equal to 20% of the volume of TRIzol was added, and samples were inverted vigorously to mix. The samples were centrifuged at 12,400 rpm for 15 min in a precooled centrifuge set to 4°C. The aqueous phase was carefully pipetted into a new tube, and an equal volume of nuclease-free 70% ethanol was added. Samples were mixed well, and RNA was isolated using the purification columns provided with the QiAGEN RNeasy RNA extraction kit. An on-column DNA digest was performed for 15 min at RT. RNA concentrations and quality were measured on a NanoDrop 2000. One microgram of total RNA per sample was transcribed to cDNA using the qScript cDNA Synthesis Kit (Quanta Biosciences). Real-time quantitative PCR (RT-qPCR) was performed using SYBR Green (Thermo Fisher) and fluorescent measurements using a Bio-Rad CFX96 Touch Real-Time PCR System.
were also treated with 20 μg/ml DIi-labeled acetylated low-density lipo-protein (AcLDL) or oxidized low-density lipoprotein (OxLDL) for 4 h. The cells in the 12-well plates were detached with Cell Stripper and fixed for 10 min with 4% PFA before being analyzed by flow cytometry for intensity of DIi fluorescence. The cells treated in the glass slides were also fixed for 10 min with 4% PFA and then analyzed with an epifluorescence microscope using a Rhodamine filter.

Cholesterol uptake using AcLDL and BODIPY staining
Differentiated macrophages from the bone marrow of C57BL/6 male mice were transected using Lipofectamine 2000 (EV versus IL-37b) and plated at 2 × 10⁶ cells per well in an 8-well chamber glass slide for analysis by microscopy or were plated at 7.5 × 10⁵ cells per well in a 12-well plate for analysis by flow cytometry. The macrophages were treated or not with LPS and IFN-γ (10 ng/ml each) and were also treated or not with 40 μg/ml AcLDL for 6, 12, or 24 h. The cells in the 12-well plates were detached with Cell Stripper and fixed for 10 min with 4% PFA. Cells were then centrifuged and resuspended in PBS containing the lipid stain BODIPY (1:500, Invitrogen) for 30 min at RT. After washing cells twice with PBS, they were analyzed for green fluorescence intensity, representing lipid content, by flow cytometry using an Attune NxT Flow Cytometer (Thermo Fisher). The cells treated in the glass slides were also fixed for 10 min with 4% PFA and then analyzed with an epifluorescence microscope using an FITC filter.

Transmigration of macrophages
Using a 5-μm-pore filter (Costar), transduced and differentiated BMDMs were plated in the upper chamber of the Transwell filter (100,000 cells per well of a 24-well filter plate) in DMEM/F12 + 0.2% BSA medium. Cells were given 2 h to become adherent before the medium in the lower chamber was replaced with new medium containing the chemotractant MCP-1 (25 ng/ml) to stimulate the transmigration of cells through the filter. After overnight incubation, the transmigrated incubator at 37°C with 5% CO₂, the top filter chamber was carefully and completely swiped with a Q-tip to remove nonmigrated cells. The filter was fixed with 4% PFA for 10 min and stained with the nuclear stain DAPI to visualize and quantify the number of cells that had migrated through the filter. Photographs of the lower side of the filter (three photos per well) were analyzed with ImageJ. Final counts were compared between control and IL-37–expressing macrophages. All conditions were run in triplicate with n = 3 separate experiments.

Mouse atherosclerosis study: irradiation of donor mice and BMT
Seven- to eight-week-old male recipient Ldlr<sup>−/−</sup> mice (on the C57BL/6 background) were subjected to 1000 rad of whole-body irradiation to destroy their endogenous HSCs. Half of the irradiated mice (n = 15) received 1–2 × 10⁶ HSCs transduced with CD68S-EGFP and the other half received HSCs transduced with CD68S-IL-37b via tail vein injections. Injected mice were allowed a 4-wk recovery period for full reconstitution of their immune populations. Retroviral transduction of HSCs. A macrophage-specific retroviral vector was used to create the CD68S–IL-37b construct (Supplemental Fig. 1A, 1B). Retroviral transduction was performed as described previously (19, 23), with alterations as described in Supplemental Fig. 1A, 1B. The study was conducted with the approval of the institutional animal care committee and was performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Each group consisted of 15 mice except for the control group, which had 20 mice.

Results
Macrophage-expressed IL-37b reduces proinflammatory gene and protein expression
Expression of human IL-37 in mouse BMDMs was achieved via retroviral transduction of HSCs. A macrophage-specific retroviral vector was used to create the CD68S–IL-37b construct (Supplemental Fig. 1A, 1B). Retroviral transduction was performed as described previously (19, 23), with alterations as described in Materials and Methods.
The effect of IL-37 expression on the macrophage inflammatory response was tested by challenging the transduced BMDMs with various inflammatory stimuli relevant to atherosclerosis. EGFP- or IL-37-expressing macrophages were left unstimulated or treated with AcLDL, IFN-γ, or TNF-α overnight. RT-qPCR analysis revealed reduced expression of inflammatory genes, such as IL-1α, IL-1β, IL-6, IL-12, MIP-1β, and TNF-α, compared with EGFP control cells (Fig. 1A). Under conditions of inflammation that would be typical of the plaque microenvironment, macrophage IL-37 expression effectively suppressed inflammatory gene expression.

To determine whether the anti-inflammatory effects of IL-37 on macrophage gene expression were also applicable for the synthesis and secretion of inflammatory cytokines, cell supernatants from EGFP- and IL-37-transduced BMDMs, which were left unstimulated or were treated with AcLDL, were analyzed by Ab array. IL-37 expression resulted in the suppression of inflammatory protein production under basal conditions (Fig. 1B, left panel). Inflammatory mediators, such as ICAM1, IL-6, M-CSF, and MIP-1α, were all downregulated by IL-37 expression in untreated macrophages. A more potent anti-inflammatory effect of IL-37 expression is seen after AcLDL treatment (Fig. 1B, right panel), in which a majority of cytokines tested were downregulated compared with EGFP control, including IL-1α, IL-6, IFN-γ, CXCL9, MIP-1α, and M-CSF. We also wanted to determine whether treating the BMDMs with a more physiologically relevant modified low-density lipoprotein (LDL), OxLDL, would have similar effects as treatment with AcLDL. We were able to confirm that OxLDL had a similar inhibitory effect as AcLDL on the expression of IL-1β and IL-6 (data not shown). This provides new insight into the role of IL-37 in regulating the macrophage inflammatory response to modified LDL uptake.

**IL-37b inhibits macrophage transmigration**

The migratory response of monocytes and macrophages toward chemoattractants is a central feature in the pathogenesis of atherosclerosis. To determine whether IL-37 expression affects the migration of macrophages toward the chemoattractant MCP-1, a transwell filter assay was performed. As shown in Fig. 1C, IL-37 expression significantly reduced macrophage transmigration compared with EV controls. Interestingly, IFN-γ pretreatment increased EV control macrophage transmigration, but it did not have a significant effect on IL-37 macrophages.

**Expression of IL-37b suppresses proliferation and apoptosis of macrophages**

Because macrophage proliferation and apoptosis are important features of atherosclerosis, we sought to determine whether IL-37b has an impact on these cellular properties. To determine the effect of IL-37 expression or treatment with rIL-37 on macrophage proliferation in vitro, BMDMs were treated with EdU for 6 h in parallel with FBS supplementation at low (2%) and normal (10%) levels, LPS and IFN-γ (10 ng/ml), or M-CSF (20 ng/ml). Under different conditions, including low and normal serum supplementation, inflammatory stimulus (IFN-γ+LPS), and stimulation...
of proliferation (M-CSF), we found that IL-37 suppresses macrophage proliferation (Fig. 2A). An additional experiment was performed using EdU in vivo to determine whether the observed reduction in macrophage proliferation also occurred in the blood, spleen, or aorta of mice treated short-term with rIL-37. After i.p. injection with EdU, with or without rIL-37 (40 ng/g) for 12 h, the mice were sacrificed, and tissues were isolated, digested, and stained for analysis by flow cytometry. We found strong trends of reduced EdU incorporation in CD45+/CD11b+ cells, as well as myeloid cells (using ROSA22/LysM-Cre reporter mice) from the blood and myeloid cells in the aorta, although the differences were not statistically significant (Supplemental Fig. 4).

To check for apoptosis, BMDMs were plated in 12-well plates at 7 × 10^5 cells per well. After transfection with EV or IL-37 expression plasmids, the BMDMs were challenged with DMSO or a 1:500 dilution of 1 mg/ml camptothecin for 6 h to induce apoptosis. Cells were then harvested, stained with Annexin V–FITC and PI, and analyzed by flow cytometry. Compared with controls, IL-37–transfected BMDMs showed reduced apoptosis at baseline, as well as when challenged with DMSO or the apoptosis-inducing chemical camptothecin (Fig. 2B).

**Effect of IL-37b expression on macrophage cholesterol processing**

The uptake of modified lipoprotein within the plaque is central to foam cell formation. Inhibiting cholesterol uptake or increasing cholesterol efflux is beneficial in preventing foam cell formation and protects against plaque growth and development. To investigate whether IL-37 affects macrophage cholesterol uptake, we treated EV- or IL-37–transduced macrophages with Dil-labeled AcLDL and OxLDL and visualized the uptake using fluorescence microscopy (Fig. 3A) and flow cytometry (Fig. 3B, 3C). IL-37 expression had a suppressive effect on the uptake of modified LDL compared with controls, with a significant decrease in AcLDL and OxLDL uptake, implying a protective role for IL-37 in preventing foam cell formation. This was confirmed by performing BODIPY staining of EV- or IL-37–transfected BMDMs incubated with AcLDL for 6, 12, or 24 h and treated or not with LPS/IFN-γ. Although 12 h of treatment with AcLDL+ IFN-γ/LPS was the only condition that resulted in a significant increase in lipid content compared with AcLDL treatment alone, there was a trend toward increased lipid uptake with IFN-γ/LPS at 6 and 24 h (Fig. 4A). The significant decrease in lipid content seen in IL-37–transfected macrophages after 6 h, with or without IFN-γ/LPS stimulation, was no longer apparent after 12 or 24 h, although there was a trend toward less lipid at 12 h (Fig. 4B).

In assessing the effects of IL-37 on lipid efflux, we found that ABCA1 and PPARγ were upregulated with IL-37 overexpression. However, actual cholesterol efflux at multiple time points, whether using ApoA1 or high-density lipoprotein as the cholesterol acceptor, was not influenced by IL-37 (data not shown).

Taken together, the in vitro findings that IL-37 expression reduces macrophage inflammation and migration and inhibits modified LDL uptake support a strong atheroprotective role for macrophage-expressed IL-37. To test whether these in vitro findings hold true in the complex setting of atherosclerosis, an in vivo study using atherosclerosis-prone Ldlr−/− recipient mice was performed.

**BMT of EGFP or IL-37b HSCs into Ldr−/− mice**

Transplantation of EGFP- or IL-37b–transduced HSCs into lethally irradiated Ldr−/− recipient mice was performed as previously described (23). A portion of transduced HSCs was differentiated into macrophages in vitro and analyzed by PCR, as well as by Western blot and flow cytometry, for the presence of IL-37 transcript and protein, respectively (Supplemental Fig. 1C, 1D). Various circulating immune cell populations were analyzed by flow cytometry to confirm successful repopulation with the donor marrow (Supplemental Fig. 2A). Circulating leukocytes were analyzed again after the study mice had been on a high-fat diet (HFD) for 10 wk (Supplemental Fig. 2B), with no differences observed between groups before and after HFD. Serum cholesterol and triglyceride levels, as well as body weight before and after HFD, also were not different between groups (Supplemental Fig. 2C, 2D).

**EGFP and IL-37 expression in BMT mice**

ELISA analysis of serum samples for the presence of IL-37 protein revealed a range of concentrations in the IL-37 BMT mice, with no expression detected in the EGFP control mice (Fig. 5A). IL-37
serum levels were measured following 10 wk on an HFD, with IL-37 BMT sera concentrations in a similar range as pre-HFD, whereas EGFP mice did not show detectable IL-37 protein (Fig. 5A).

At sacrifice, the femurs from seven mice per group were harvested, and the bone marrow was cultured for analysis of BMDMs. As seen in Fig. 5B, clear expression of EGFP or IL-37 was detectable by Western blot in each group.

**FIGURE 3.** Macrophage-expressed IL-37 reduces lipid uptake in vitro. Transduced and differentiated IL-37b-expressing or EV control macrophages were treated with DiI-labeled AcLDL or Dil-labeled OxLDL (20 μg/ml), with or without parallel IFN-γ (20 ng/ml) treatment. After 4 h, lipid uptake was visualized with fluorescence microscopy (original magnification ×100) (A) (DiI in red, nuclei stained with DAPI in blue) and quantified by flow cytometry (B and C) for DiI-labeled AcLDL and Dil-labeled OxLDL, respectively (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 4.** Macrophage expression of IL-37b reduces neutral lipid content in BMDMs. EV- or IL-37–transfected BMDMs were treated with 25 μg/ml AcLDL, with or without IFN-γ/LPS for 6, 12, and 24 h. BMDMs were plated at 7 × 10⁵ cells per well in a 12-well plate for analysis by flow cytometry or at 2 × 10⁵ cells per well in an 8-well chamber slide for analysis by fluorescent microscopy. Following treatment, cells were fixed in 2% PFA for 10 min and stained with BODIPY at 1:500 dilution of 1 mg/ml in PBS for 30 min. (A) AcLDL uptake is shown in green with DAPI-stained nuclei in blue (original magnification ×100). (B) Quantification of BODIPY-stained lipids by flow cytometry. Mean fluorescence intensity is shown for green fluorescence in the FITC channel (n = 3). *p < 0.05, **p < 0.01.
Finally, aortic root sections were analyzed for the presence of EGFP or IL-37 in the plaque areas using immunohistochemistry (Fig. 5E). The staining of EGFP and IL-37 proteins within the respective BMT mice confirmed the presence of transduced macrophages within the atherosclerotic plaque.

Systemic inflammation is reduced by macrophage IL-37 expression in vivo

To gain a general view of the inflammatory state of the BMT mice, lumbar LNs collected at sacrifice were analyzed for inflammatory gene expression by RT-qPCR. LN transcripts from IL-37 mice showed a significant reduction in IL-6 and IL-1β expression compared with LNs from EGFP mice (Fig. 5C), indicating that IL-37 expression had a systemic anti-inflammatory effect in vivo.

Additionally, serum samples from EGFP and IL-37 BMT mice were analyzed with a Luminex magnetic bead assay. The absolute quantities of circulating inflammatory cytokine levels measured were not significantly different between IL-37 and EGFP BMT mice (Supplemental Fig. 3). However, linear regression analysis revealed a significant negative correlation between IL-37 protein abundance and M-CSF levels in the serum of IL-37 BMT mice ($R = -0.7071, p = 0.0047$) (Fig. 5D), implying a systemic anti-inflammatory role for IL-37 in vivo. There was no significant correlation for each of the other cytokines analyzed, although there was a positive trend between IL-37 and IL-4 serum concentrations (Supplemental Fig. 3B).

Macrophage-expressed IL-37 reduces total plaque area in Ldlr−/− mice

To analyze the atherosclerotic plaque development in EGFP and IL-37 BMT mice, the entire aortas were opened and stained en face with Oil Red O to visualize atherosclerotic plaque. Aortas from IL-37 BMT mice, but not EGFP mice, clearly demonstrated the athero-protective effect of macrophage-expressed IL-37 in lesion-prone Ldlr−/− mice. Aortic root sections from EGFP or IL-37 BMT mice were also analyzed for plaque area following staining with Oil Red O. As seen in Fig. 6B, aortic root plaque area was significantly reduced in IL-37 BMT mice compared with EGFP BMT mice, further supporting the athero-protective role of IL-37 in vivo.

The presence of macrophages and smooth muscle cells in the atheroma is not affected by IL-37

Aortic root sections were subjected to immunofluorescent staining to detect macrophages or smooth muscle cells within the plaque. Each aortic root section was imaged at 20× magnification and analyzed with ImageJ to determine macrophage-positive areas. MOMA-2 staining is shown for EGFP and IL-37 sections in Fig. 6C, along with the macrophage-positive area, expressed as a percentage of total plaque for quantification. Although the total plaque area was reduced in IL-37 mice, there was no difference observed in macrophage content per plaque area between the EGFP and IL-37 groups, indicating that the composition of the plaque was very similar in the two groups.

Discussion

The experiments detailed in this study demonstrate that macrophage-specific IL-37 expression leads to suppression of inflammation, cholesterol uptake, cell proliferation, and apoptosis, as well as macrophage transmigration in vitro. Furthermore, BMT of IL-37-transduced HSCs resulted in reduced plaque development and decreased systemic inflammation in atherosclerosis-prone Ldlr−/− mice compared with controls. To our knowledge, this is the first study elucidating the role of macrophage-expressed IL-37 in the context of atherosclerosis, and it provides key evidence for future investigation into its potential therapeutic value in prevention of atherosclerosis.

**FIGURE 5.** Macrophage-expressed IL-37 in vivo quells inflammation. EGFP- or IL-37b–transduced HSCs were transplanted into lethally irradiated Ldlr−/− recipient mice, followed by a 4-wk recovery and a 10-wk HFD to induce atherosclerosis. (A) IL-37 protein was measured by ELISA in the sera of all study mice before and after HFD. (B) BMDMs cultured from the femurs of study mice at sacrifice were analyzed by Western blot for IL-37 or EGFP protein expression. (C) LNs collected at sacrifice were analyzed by RT-qPCR for inflammatory gene expression ($n = 14$). (D) Serum M-CSF concentration in EGFP or IL-37 study mice was measured by Luminex assay (left panel). Linear regression analysis with IL-37 serum concentration reveals a significant negative correlation (right panel). (E) Five-micrometer-thick aortic sinus sections were stained using primary Abs against EGFP or IL-37 and detected with an HRP secondary Ab, followed by staining with an AEC kit. EGFP and IL-37 protein (both shown in red) were detected in the plaques of EGFP and IL-37 BMT study mice, respectively. Background control staining is shown in the insets (original magnification ×200) ($n = 14$). *$p < 0.05$. ND, not detected.
the treatment and prevention of human atherosclerosis, as well as other chronic inflammatory diseases.

Expression of various inflammatory cytokines known to be detrimental in the context of atherosclerosis (24) was reduced by macrophage IL-37 expression in vitro. This protective effect of IL-37 was observed after classical inflammatory stimuli, such as IFN-γ and TNF-α, as well as AcLDL treatment, indicating that IL-37 also protects against a type of inflammation caused by modified LDL that has not previously been reported. IL-37 retains its anti-inflammatory properties, which is crucial for effective function in a state of hyperlipidemia.

Inflammatory mediators implicated in atherogenesis that were reduced by IL-37 in vitro and in vivo include IL-1β, IL-6, and M-CSF. IL-6 is associated with unstable angina in humans (25) and is known to stimulate production of matrix-degrading enzymes by macrophages (26). IL-37 expression in macrophages consistently reduced IL-6 transcript and protein expression in vitro and in vivo, as evidenced by reduced IL-6 transcripts in LNs isolated from IL-37 mice compared with controls. Serum IL-6 levels were undetectable by Luminex analysis (data not shown); thus, it remains unclear whether IL-37 had an effect on circulating levels of IL-6 protein.

Macrophage expression of IL-37 led to reduced IL-1α and IL-1β gene and protein expression in vitro, as well as decreased IL-1β transcripts in the LNs of IL-37 BMT mice compared with controls. It has been shown that, within minutes of macrophage inflammatory activation, IL-37 expression closely follows that of IL-1α and IL-1β (9), with the gene regulatory regions of all three genes found in the IL-1 locus coming into close proximity following LPS stimulation (27). This programmed expression of IL-37 during the inflammatory response likely acts to modulate excessive inflammation.

M-CSF gene expression has long been known to be linked to atherosclerosis development (28), and its expression within the plaque leads to differentiation of macrophages and expression of scavenger receptors (29). M-CSF deficiency in ApoE-knockout mice results in reduced plaque formation (30), emphasizing the importance of limiting its expression to prevent the disease. M-CSF protein production was reduced by IL-37 expression in unstimulated and AcLDL-stimulated macrophages in vitro. In addition, circulating levels of M-CSF in IL-37–transduced BMT mice were negatively correlated with serum IL-37 protein concentration.

The observation that IL-37 expression reduces macrophage transmigration toward MCP-1 corresponds with the reduction in inflammatory cytokines and chemoattractant molecules seen in IL-37–expressing macrophages. MCP-1 secretion from IL-37–expressing macrophages was reduced in vitro, although the serum levels were not detectable. Thus, it is unclear whether they differed between study groups in vivo. Although not explored in this study, a possible mechanism for IL-37 in reducing macrophage migration could involve decreased phosphorylation of various kinases and transcription factors, as shown by Nold-Petry et al. (31) to physically decrease the chemotactic response.

In addition, the reduced proliferation of macrophages by IL-37 could be a major contributing factor to the attenuated plaque size found in IL-37 BMT mice compared with controls, because the vast majority of the plaque is composed of macrophages at this early stage. Recently, Robbins et al. (32), using a symbiosis model of atherosclerosis-prone CD45.1 and CD45.2 mice put on an HFD, showed that the majority of BrdU+ proliferating macrophages within developed lesions were from the host parabiont rather than from the donor, indicating that proliferation is a significant contributor to the macrophage content of lesions. Because rIL-37 also strongly inhibited macrophage proliferation, it is very likely that the IL-37–expressing macrophages within the plaque microenvironment secrete IL-37 that could act to reduce the proliferation of nearby macrophages, even if they did not express IL-37. The observation that macrophage apoptosis is decreased by IL-37 indicates that necrotic core formation may be attenuated in IL-37–expressing mice, because macrophage death is associated with the presence of a necrotic core.

Of note, the macrophage content of the aortic root plaque did not differ between EGFP and IL-37 BMT mice, indicating that the
composition of the plaques was similar. However, the total plaque area was reduced in IL-37 BMT mice, reflecting an overall reduction in macrophage infiltration. There was noticeably less IL-37 staining in IL-37 mice than EGFP staining in EGFP BMT mice, possibly as a result of reduced infiltration of IL-37–expressing macrophages. Taken together, the data described above support the anti-inflammatory function of IL-37 that was likely a significant contributing factor in the reduction in atherosclerosis development in vivo.

In terms of lipid metabolism, IL-37 expression in macrophages led to a reduction in modified LDL uptake, as shown by the reduced Dil-labeled AcLDL and OxLDL content of the cells after 4 h of incubation, as well as by reduced BODIPY staining in IL-37–expressing macrophages, indicating lower neutral lipid content. Aside from the obvious benefit that this would have in reducing foam cell formation, it may have anti-inflammatory benefits, because activated plaque macrophages become especially pathogenic when they take up modified LDL and become lipid-laden foam cells (2, 33). Although we observed decreased uptake of modified LDL in macrophages as a result of IL-37 expression, we did not observe changes in cholesterol efflux to the acceptors ApoA1 or high-density lipoprotein at multiple time points (data not shown). Taken together, the reduction in modified lipid uptake by IL-37 expression, coupled with its well-established role in preventing inflammation, are likely the two key components of its protective mechanism against the development of atherosclerosis in vivo.

Although the goal of expressing IL-37 specifically in macrophages to deliver the anti-inflammatory cytokine to the plaque microenvironment was successful, the relatively high concentration of IL-37 protein in the sera of IL-37 mice at the 4- and 14-wk timepoints implicates a possible systemic role for IL-37 in reducing inflammation, are likely the two key components of its protective mechanism against the development of atherosclerosis in vivo.

The separate mechanisms of action were not investigated in this study, and the particular contribution of each in preventing atherosclerosis development remains to be determined. The results of this study warrant further investigation of IL-37 as a potential agent for therapy against atherosclerosis, as well as various other inflammatory diseases.

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Disclosures
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