Interleukin 27 inhibits atherosclerosis via immunoregulation of macrophages in mice

Tetsuaki Hirase,1,3 Hiromitsu Hara,2 Yoshiyuki Miyazaki,3 Noriko Ide,1 Ai Nishimoto-Hazuku,1 Hirokazu Fujimoto,1 Christiaan J. M. Saris,4 Hiroki Yoshida,2* and Koichi Node1*

1Department of Cardiovascular Medicine, Saga University, Saga, Japan; 2Department of Biomedical Sciences, Saga University, Saga, Japan; 3Department of Biosciences and Genetics, National Cerebral and Cardiovascular Center Research Institute, Suita, Japan; and 4Department of Inflammation Research, Amgen, Thousand Oaks, California

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Interleukin 27 (IL-27) is a heterodimeric cytokine consisting of IL-27p28 and Epstein-Barr virus induced gene 3 (EBI3) and belongs to the IL-12 family of cytokines (19). IL-27 binds to a membrane-bound heterodimeric receptor consisting of WSX-1 and gp130 (20) and activates cell signaling predominantly through multiple Janus kinase (Jak) and signal transducer and activator of transcription (STAT) proteins (29). IL-27 receptor complex is expressed not only by T cells but also by innate immune cells, such as macrophages and dendritic cells, and endothelial cells, indicating that multiple cell types are able to respond to IL-27 (23).

Infection of WSX-1-deficient mice that lack specific IL-27 receptors with pathogens resulted in augmented T cell responses and enhanced proinflammatory cytokine production (9, 31). Anti-inflammatory effects of IL-27 signaling that regulates polarization of T cell subsets such as T helper 1 (Th1) cells and T helper 17 (Th17) cells and cytokine production have been demonstrated in animal models of experimental autoimmune encephalitis, allergic asthma, and delayed-type hypersensitivity of skin (2, 15, 16, 21). Therefore, the aim of the present study was to investigate the roles of IL-27 in atherosclerosis. We crossed Ebi3−/− or WSX-1−/− mice with LDLR receptor-deficient (Ldlr−/−) mice that are known to be atherosclerosis-prone when fed a high-cholesterol diet to make IL-27-deficient (Ldlr−/−/Ebi3−/−) and IL-27 receptor-deficient (Ldlr−/−/WSX-1−/−) Ldlr−/− mice. We show an inhibitory role for IL-27 in atherosclerosis in vivo using these mice as well as on administration of recombinant IL-27. We also demonstrate an inhibition of macrophage activation by IL-27 that contributes to the prevention of atherosclerosis. Thus the current study represents a novel immunoregulatory role for IL-27 in atherosclerosis that targets macrophage in mice.

MATERIALS AND METHODS

Animal model. Homozygous Ldlr−/− mice (C57BL/6 strain, Jackson Laboratories) and Ebi3−/− (11) and WSX-1−/− mice (31) (C57BL/6 strain) were crossed to obtain Ldlr+/−/Ebi3+/− and Ldlr+/−/WSX-1+/− mice, respectively. Each heterozygous mouse was crossed to obtain each double-knockout mouse. Genotyping was performed using PCR. Male Ldlr−/−, Ldlr−/−/Ebi3−/−, and Ldlr−/−/WSX-1−/− mice were weaned at 8 wk of age on a high-cholesterol diet (1.25% cholesterol, 7.5% cocoa butter, 7.5% casein; Harlan Laboratories, Indianapolis, IN) and maintained with the diet for 12 or 16 wk. Ldlr−/− and Ldlr−/−/Ebi3−/− mice expressing Ly5.2 (C57BL/6 strain) and congenic Ly5.1 mice (C57BL/6 strain; Sankyo Labo Service, Tsukishima, Ibaraki, Japan) were crossed and each heterozygous mouse was crossed to obtain congenic Ldlr−/− and Ldlr−/−/Ebi3−/− mice expressing Ly5.1. Enzymatic assay for total cholesterol was performed with kits purchased from Wako Pure Chemical Industries (Osaka, Japan). Cholesterol profiles in plasma lipoproteins were analyzed with a dual-detection high-performance liquid chromatography (HPLC) system with 2 tandem...
To detect with FITC-conjugated anti-CD4 antibody (clone GK1.5, eBioscience, blocked with PBS containing 1% BSA. Then sections were incubated with the expression vector using 293fectin (Invitrogen, CA). After 3 days, culture supernatant was collected and 3xFLAG-tagged recombinant single chain IL-27 was purified by affinity chromatography connected TSKgel LipopropakXL columns (300 × 7.8 mm; Tosoh, Japan) by Skylight Biotech (Akita, Japan) (27). All experiments were approved by the institutional animal research committee of Saga University and conformed to the animal care guidelines of the American Physiological Society.

Bone marrow transplantation. Bone marrow cell suspension (2 × 10^5 cells) prepared from the femurs and tibias of male donor mice (8–12 wk old) was intravenously injected into male bone marrow recipient mice irradiated with a single exposure of 1,000 rad at 8 wk of age. Recipient mice were supplemented with the water source containing 2 mg/ml neomycin sulfate for 2 wk following irradiation and then subjected to the assessment of bone marrow chimera ratio using flow cytometry for Ly5.1/Ly5.2 ratio of peripheral blood cells at 12 wk of age. Recipient mice were weaned at 14 wk of age onto the above mentioned high-cholesterol diet and maintained the diet for 16 wk.

Oil red O staining of aorta and quantification of aortic lesion formation. Mice were euthanized after 12 or 16 wk on the high-cholesterol diet, and the atherosclerotic lesions were analyzed as described previously (12, 32). After mice were anesthetized with pentobarbital sodium (80 mg/kg ip; Abbott Laboratories, Abbott Park, IL), the aorta was perfused with normal saline containing 10 U/ml heparin. Then the aortic samples excised from the aortic sinus were dissected free from surrounding tissues, opened longitudinally, and pinned onto a silicon-coated dish. Image analysis was performed on oil red O-stained aortas using the ImageJ Software (NIH, Bethesda, MD). The amount of aortic lesion formation in each animal was measured as the percentage of lesion area per total area of the aorta (12).

Recombinant IL-27. Mouse Ebi3 and IL-27p28 cDNAs were isolated by RT-PCR using total RNA prepared from Con A-activated splenocytes. The fragment encoding Ebi3-(VPVG)2 linker-IL-27p28 was generated by PCR and cloned into p3xFLAG-CMV-14 vector (Sigma-Aldrich). 293F cells were then transiently transfected with the expression vector using 293fectin (Invitrogen, CA). After 3 days, culture supernatant was collected and 3xFLAG-tagged recombinant single chain IL-27 was purified by affinity chromatography using anti-FLAG (M2) affinity gel (Sigma-Aldrich). Protein concentration was determined by titration of immunoblots with anti-FLAG (M2) antibody. In male mice, weaned at 14 wk of age. Recipient mice were weaned at 14 wk of age onto the above mentioned high-cholesterol diet and maintained the diet for 16 wk.

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Immunohistochemistry. Immunohistochemical staining for MOMA-2 in atherosclerotic lesions in the aortic root was performed by the labeled streptavidin biotin method as described previously (18).

Immunofluorescence microscopy. Anti-MCP-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was labeled with Alexa Fluor 647 using Zenon rabbit IgG labeling kit (Molecular Probes, Eugene, OR) following the manufacturer’s protocol.

Frozen sections of aortic root from mice were rinsed with PBS and blocked with PBS containing 1% BSA. Then sections were incubated with FITC-conjugated anti-CD4 antibody (clone GK1.5, eBioscience, San Diego, CA) or anti-MCP-1 antibody labeled with Alexa Fluor 647. To detect α-smooth muscle actin (αSMA), sections were incubated with monoclonal α-smooth muscle actin antibody (Dako, Glostrup, Denmark), followed by the incubation with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (Invitrogen, CA). Following a rinse, sections were mounted. Images were captured using confocal microscopy (LSM5 Pascal, Carl Zeiss, Jena, Germany). Staining intensity of the images was measured using the ImageJ software program (NIH).

Peritoneal macrophage culture, Dil labeled acetyl LDL uptake, and cytokine measurement. Peritoneal-derived peritoneal macrophages were plated on six-well dishes in RPMI 1640 medium supplemented with 10% FCS. After 2 h, nonadherent cells were washed out and adherent cells were starved for 16 h. Then cells were incubated for 4 h in the presence of 10 μg/ml of Dil labeled-acetyl LDL and an excess of unlabeled acetyl LDL (200 μg/ml). Fluorescence intensity in cells was analyzed using fluorescence microscope and quantified using the ImageJ software program. Conditioned media were subjected to ELISA and Luminex microbeads assay to measure IL-10 and other cytokines, respectively. Commercially available mouse IL-10 Quantikine ELISA kit (R&D Systems, Minneapolis, MN) and cytokine mouse 10-plex Panel (Invitrogen, Carlsbad, CA) were used following the manufacturer’s protocol. Experiments were performed in triplicate with six independent cell pools isolated from three mice of each group.

Flow cytometry of Ly6Chigh monocyte subset. Peripheral blood cells were stained with a cocktail of monoclonal antibodies for lineage markers, T cells, CD90-PE, 53-2-1; B cells, B220-PE, RA3-6B2; NK cells, CD49b-PE, DX5 and NK1.1-PE, PK136; granulocytes, Ly6G-PE, IA8; myeloid cells, CD11b-APC, M1/70; and monocyte subsets, Ly6C-FTTC, AL-21 (eBioscience). The cell number of CD11b^hiCD90^loB220^loCD49b^loNK1.1^loLy6G^lo monocytes and the ratio of Ly6C^hi subsets in peripheral blood were measured using flow cytometry using FACSCalibur (BD).

Cell culture of endothelial and vascular smooth muscle cells. Human umbilical vein endothelial cells (HUVECs) purchased from Lonza (Basel, Switzerland) were maintained onto collagen-coated dishes at 37°C under an atmosphere of 5% CO2 in RPMI containing 20% FBS, 20 mg/ml endothelial cell growth supplement, 100 U/ml penicillin, and 100 μg/ml streptomycin. Human aortic smooth muscle cells (Cell Applications, San Diego, CA) were maintained at 37°C under an atmosphere of 5% CO2 in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Flow cytometry for VCAM-1 in HUVECs. HUVECs were treated with recombinant human TNF-α purchased from Peprotech, Rocky Hill, NJ (100 U/ml), or control vehicle for 8 h in the presence or absence of recombinant human IL-27 purchased from R&D Systems (20 ng/ml). Cells scraped with PBS containing EDTA were stained with FITC-conjugated anti-CD106 monoclonal antibody (Serotec, Oxford, UK) and subjected to flow cytometry using FACSCalibur (BD, Franklin Lakes, NJ). The ratio of VCAM-1-positive cells per 1 × 10^5 cells in each group was counted, and the data from three independent experiments performed in triplicate were statistically analyzed.

Cell proliferation assay. PDGF-BB-induced proliferation of human aortic smooth muscle cells were determined by measuring mitochondria-dependent reduction of [3H]-5-tetrazolio]-1,3-benzene disulfonate (WST-1) to formazan (WST-1 as a dye-dependent reduction of 4-[3-(4-lodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) to formazan (WST-1 assay; Roche, Basel, Switzerland). Human aortic smooth muscle cells harvested in a 96-well dish (5 × 10^5 cells/well) were stimulated with PDGF-BB (Peprotech) or control vehicle for 16 h and then incubated in the presence of 10% WST-1 for 1 h at 37°C, and the amount of formazan in each well was estimated by measuring with a multiwell spectrophotometer (SpectraMax 190 Absorbance Microplate Reader; Molecular Devices, Sunnyvale, CA) at a wavelength of 450 nm with a reference wavelength of 650 nm. Data from three independent experiments performed in triplicate were statistically analyzed.

Cell migration assay. Lower chamber of a Boyden chamber was filled with media containing 50 ng/ml of recombinant human PDGF-BB (Peprotech) or control vehicle. Membrane filter that was precoated with 0.25% gelatin was placed between chambers. Human aortic smooth muscle cells were trypsinized and suspended in DMEM and counted. A total of 2 × 10^4 cells were resuspended and placed in the upper chamber of a Boyden chamber. After 2 h of incubation at 37°C, the cells on the upper side of the membrane were gently scraped off. Cells were fixed with the fixative for 5 s and stained with Diff-Quick Solution (1:1) for 10 min and then stained with Diff-Quick Solution (1:1) for 10 min. Transmigrated cells of three chambers were counted in high-power (×100) microscope fields in three independent experiments.

Statistics. Data are expressed as means ± SE. The significance in each group was evaluated with unpaired Student’s t-test, one-way ANOVA, and Tukey’s test. We considered that P values < 0.05 were statistically significant.
RESULTS

Inhibition of atherosclerosis in vivo by IL-27 in Ldlr−/− mice. We first examined the effect of deleting Ebi3 gene and WSX-1 gene in C57Bl/6 Ldlr−/− mice that are known to be atherosclerosis-prone when fed a high-cholesterol diet. After 16 wk of atherogenic diet, serum cholesterol levels were similar between male Ldlr−/−, Ldlr−/−Ebi3−/−, and Ldlr−/−/WSX-1−/− mice (1,296 ± 104.9 mg/dl, 1,217.6 ± 146.3 mg/dl, 1,176.2 ± 111.8 mg/dl; P = NS). LDL-cholesterol and HDL-cholesterol levels also showed no significant differences between Ldlr−/−, Ldlr−/−Ebi3−/−, and Ldlr−/−/WSX-1−/− mice (402.7 ± 6.2 mg/dl, 421.1 ± 22.7 mg/dl, 396.0 ± 26.6 mg/dl; P = NS., 122.1 ± 2.2 mg/dl, 118.9 ± 15.0 mg/dl, 125.0 ± 2.8 mg/dl; P = NS.). Atherosclerotic lesion size determined by en face lipid staining with oil red O was significantly larger in Ldlr−/−Ebi3−/− and Ldlr−/−/WSX-1−/− mice than in control Ldlr−/− mice (Ldlr−/− mice 23.2 ± 1.5%; Ldlr−/−Ebi3−/− mice 38.2 ± 1.3%; Ldlr−/−/WSX-1−/− mice 28.0 ± 1.3%, P < 0.01) (Fig. 1A). Ldlr−/−Ebi3−/− mice exhibited larger atherosclerotic lesions than Ldlr−/−/WSX-1−/− mice (Fig. 1A). Administration of recombinant IL-27 significantly decreased atherosclerotic lesion size in Ldlr−/− (23.6 ± 1.5% vs. 20.0 ± 3.1%; P < 0.05) and Ldlr−/−Ebi3−/− mice (51.3 ± 6.8% vs. 39.5 ± 4.6%; P < 0.01) fed with a high-cholesterol diet (Fig. 1B). These data indicate an inhibitory role of IL-27 in the development of atherosclerosis in vivo in Ldlr−/− mice.

Since it has been demonstrated that vascular cells as well as immune cells recruited to arterial walls form atherosclerotic lesions (7, 10), we investigated cellular composition of atherosclerotic lesions. Immunohistochemical analysis revealed that MOMA2-positive macrophages were predominantly detected and increased in atherosclerotic lesions by deletion of EBI3 and WSX-1. In contrast, the accumulation of CD4+ positive T cells and α-smooth muscle actin (αSMA)-positive smooth muscle cells showed no significant changes by deletion of EBI3 and WSX-1 (Fig. 1C).

Inhibition of bone marrow-derived cell activation in arterial walls by IL-27. We reconstituted bone marrow by bone marrow transplantation after irradiation to investigate the roles of bone marrow-derived cells in atherosclerosis inhibition by IL-27. Bone marrow reconstitution showed no obvious changes in total cholesterol levels of serum (Fig. 2A). Atherosclerotic lesion size in en face aorta stained with oil red O was significantly increased by EBI3 deficiency of bone marrow-derived cells in Ldlr−/− mice (8.1 ± 0.8% vs. 17.1 ± 1.8%; P < 0.01) (Fig. 2, A and B). Bone marrow reconstitution with bone marrow-derived cells from Ldlr−/− mice reduced atherosclerotic lesion size in Ldlr−/−Ebi3−/− mice (10.4 ± 1.1% vs. 12.9 ± 0.6%; P < 0.05) and Ldlr−/−/WSX-1−/− mice (11.8 ± 0.9% vs. 14.4 ± 1.9%; P < 0.05), respectively (Fig. 2, A and B). Bone marrow reconstitution with bone marrow-derived cells from Ldlr−/−Ebi3−/− mice was not sufficient to decrease atherosclerotic lesion size in Ldlr−/−/WSX-1−/− mice (17.8 ± 2.2% vs. 14.4 ± 1.9%; P = NS) (Fig. 2, A and B). Among heterogeneous subsets of monocytes, proatherogenic roles of Ly6C<sup>high</sup> subsets have been demonstrated in atherosclerosis-prone apoE<sup>−/−</sup> mice (24). The cell number of monocytes and the ratio of Ly6C<sup>high</sup> subsets in circulation analyzed by flow cytometry revealed that deficiency of EBI3 and WSX-1 had no obvious effects on the cell number of monocytes and the ratio of Ly6C<sup>high</sup> subsets (Fig. 3A), indicating that IL-27 has a minor role in monocyte recruitment from bone marrow in atherosclerosis. To study the activation of monocytes/macrophages in arterial walls, frozen sections of aorta were stained for monocyte chemoattractant protein 1 (MCP-1), a chemokine that plays key roles in monocyte recruitment to atherosclerotic lesions as well as activation of monocytes/macrophages (3). In mice transplanted with indicated bone marrow, reconstitution with Ldlr−/−Ebi3−/− bone marrow increased MCP-1 expression in atherosclerotic lesions of Ldlr−/− mice compared with reconstitution with Ldlr−/− bone marrow (Fig. 3B). Reconstitution with Ldlr−/− bone marrow decreased MCP-1 expression in atherosclerotic lesions in Ldlr−/−Ebi3−/− and Ldlr−/−/WSX-1−/− mice compared with reconstitution with Ldlr−/−Ebi3−/− and Ldlr−/−/WSX-1−/− bone marrow, respectively (Fig. 3B). Reconstitution with Ldlr−/−Ebi3−/− bone marrow was not sufficient to decrease MCP-1 expression in Ldlr−/−/WSX-1−/− mice (Fig. 3B). These data suggest that deficiency of IL-27 and IL-27 receptor in bone marrow-derived cells promotes the activation of bone marrow-derived cells in arterial walls.

Inhibition of macrophage activation by IL-27. Macrophages activated by various stimuli uptake modified LDL (8) and produce cytokines that modulate function of vascular cells as well as immune cells in arterial walls (26). The effect of IL-27 on macrophage function was assessed using cultured peritoneal-stimulated peritoneal macrophages from the above mice. Macrophages derived from bone marrow lacking EBI3 and WSX-1 showed greater uptake of DiI labeled acetyl-LDL estimated using confocal microscopy than those from control Ldlr−/−/bone marrow (Fig. 4A). Treatment with recombinant IL-27 inhibited uptake of DiI labeled acetyl-LDL in macrophages derived from Ldlr−/− and Ldlr−/−Ebi3−/− bone marrow (Fig. 4B). The inhibition of acetyl-LDL uptake by recombinant IL-27 was abolished in the macrophages from the mice transplanted with bone marrow lacking WSX-1 (Fig. 4B).

Cytokines produced by cultured peritoneal macrophages were comprehensively measured using ELISA and Luminex microbeads assay. Macrophages derived from bone marrow lacking EBI3 and WSX-1 showed greater production of proinflammatory cytokines, such as MCP-1, IFN-γ, IL-1β, and IL-6, than those from control Ldlr−/−/bone marrow (Fig. 5). Of note, MCP-1 production from macrophages showed a good correlation with MCP-1 expression in atherosclerotic lesions in each group (see Fig. 3B). Treatment with recombinant IL-27 inhibited the production of the cytokines in macrophages derived from Ldlr−/− and Ldlr−/−Ebi3−/− bone marrow, but not in those from Ldlr−/−/WSX-1−/− bone marrow that lack IL-27 receptor (Fig. 5). Although IL-27 has been reported to stimulate production of IL-10, one of the most immunosuppressive cytokines (14), by T cells (1, 4, 22), the production of IL-10 was not changed either by deficiency of EBI3 and WSX-1 or by recombinant IL-27 treatment in cultured macrophages (Fig. 5). These data suggest that IL-27 inhibits modified LDL uptake and production of proinflammatory cytokines by macrophages. Treatment with recombinant IL-27 showed no significant effect in TNFα-induced VCAM-1 expression of cultured human umbilical vein endothelial cells that play pivotal roles in the recruitment of monocytes into arterial walls (Fig. 6A) and PDGF-BB-induced migration and proliferation.
Fig. 1. Inhibition of atherosclerosis in vivo by IL-27 in Ldlr−/− mice and cellular composition of atherosclerotic lesions. A: representative photomicrographs (left) and quantitative analysis (right) of atherosclerotic lesion size in oil red O-stained en face aorta of male Ldlr−/−, Ldlr−/− Ebi3−/−, and Ldlr−/− WSX-1−/− mice fed a high-cholesterol diet (n = 10). Each symbol represents the lesion area measurement from an individual mouse, with the mean per group indicated by a horizontal line. *, **P < 0.01 vs. Ldlr−/− and Ldlr−/− WSX-1−/− mice, respectively. B: effects of IL-27 administration on atherosclerosis in vivo. Quantitative analysis of atherosclerotic lesion size in oil red O-stained en face aorta of male Ldlr−/− and Ldlr−/− Ebi3−/− mice administered with control vehicle or recombinant IL-27 when fed a high-cholesterol diet (n = 10). Each symbol represents the lesion area measurement from an individual mouse, with the mean per group indicated by a horizontal line. *P < 0.05 vs. Ldlr−/− mice administered with control vehicle. **P < 0.01 vs. Ldlr−/− Ebi3−/− mice administered with control vehicle. C: cellular composition of atherosclerotic lesions. Left: representative photomicrographs of atherosclerotic lesions to detect immunohistochemically stained MOMA2-positive macrophages, and overlay of phase-contrast images and CD4-stained or α-smooth muscle actin (αSMA)-stained images by immunofluorescence. Right: staining intensity of three sections each from five mice in each group was expressed as mean ± SE relative to that of control Ldlr−/− mice. *P < 0.01 vs. Ldlr−/− mice.
of cultured human aortic smooth muscle cells that participate in the growth of atherosclerotic lesions (Fig. 6B). These data suggest that the inhibitory role of IL-27 in atherosclerosis is predominantly attributable to bone marrow-derived macrophages, but not to vascular cells.

**DISCUSSION**

We demonstrate in the present study that the deficiency of IL-27 and IL-27 receptor in bone marrow-derived cells accelerates atherosclerosis due to macrophage activation in arterial walls without significant changes in the recruitment of a proinflammatory subset of monocytes in circulation in mice. We also show that recombinant IL-27 treatment significantly inhibits atherogenesis in vivo and macrophage activation in vitro that increases modified LDL uptake and proinflammatory cytokine production. Previous studies have shown that mRNA of EBI3 and p28 that compose IL-27 is expressed highly in monocytes and T cells, suggesting that monocytes and T cells are major sources of IL-27 (19). WSX-1, a component of IL-27 receptor, is reportedly expressed in T cells and myeloid cells (19). Recently, Koltsova et al. showed that targeted depletion of WSX-1 in \(Ldlr^{-/-}\) mice promotes the differentiation and activation of proinflammatory Th17 cells accompanied with the production of proinflammatory IL-17 that contributes to accelerated atherosclerosis (13). Although Koltsova et al. mainly focused on enhanced Th17 responses during atherogenesis in the IL-27 receptor-deficient mice, we revealed excessive activation of macrophages in IL-27/IL-27 receptor-deficient mice.

**Fig. 2.** Promotion of atherosclerosis by bone marrow-derived cells lacking IL-27 or IL-27 receptor. A: representative photomicrographs of atherosclerotic lesions in oil red O-stained en face aorta and total cholesterol levels in serum of \(Ldlr^{-/-}\), \(Ldlr^{-/-}Eb3^{-/-}\), and \(Ldlr^{-/-}WSX-1^{-/-}\) mice fed with a high-cholesterol diet after transplantation of the indicated bone marrow. B: quantitative analysis of atherosclerotic lesion size in each group is shown. Each symbol represents the lesion area measurement from an individual mouse, with the mean per group indicated by a horizontal line. *\(P < 0.01\) vs. \(Ldlr^{-/-}\) mice transplanted with \(Ldlr^{-/-}\) bone marrow. **, #\(P < 0.05\) vs. \(Ldlr^{-/-}Eb3^{-/-}\) mice transplanted with \(Ldlr^{-/-}Eb3^{-/-}\) bone marrow and \(Ldlr^{-/-}WSX-1^{-/-}\) mice transplanted with \(Ldlr^{-/-}WSX-1^{-/-}\) bone marrow, respectively.
Fig. 3. IL-27 suppresses bone marrow-derived cell activation in arterial walls, but not the recruitment of proinflammatory Ly6Chigh monocytes from bone marrow. A: the cell number of CD11b<sup>hi</sup>CD90<sup>lo</sup>CD49b<sup>lo</sup>NK1.1<sup>lo</sup>Ly6G<sup>lo</sup> monocytes (top) and the ratio of Ly6Chigh subset (bottom) in peripheral blood from Ldlr<sup>−/−</sup>, Ldlr<sup>−/−</sup>Ebi3<sup>−/−</sup>, and Ldlr<sup>−/−</sup>WSX-1<sup>−/−</sup> mice fed with a high-cholesterol diet for 16 wk after transplantation of the indicated bone marrow. No significant differences in monocyte number and the ratio of Ly6Chigh subset between each group were detected. B: deficiency of IL-27 and IL-27 receptor in bone marrow-derived cells increases MCP-1 expression in atherosclerotic lesions. Left: representative photomicrographs of overlay of phase-contrast images and Alexa Fluor 647-conjugated anti-MCP-1 antibody-stained images of sections of aortic root from Ldlr<sup>−/−</sup>, Ldlr<sup>−/−</sup>Ebi3<sup>−/−</sup>, and Ldlr<sup>−/−</sup>WSX-1<sup>−/−</sup> mice transplanted with the indicated bone marrow. Transplantation of Ldlr<sup>−/−</sup>bone marrow cells into Ldlr<sup>−/−</sup> and Ldlr<sup>−/−</sup>Ebi3<sup>−/−</sup> mice was not determined (ND). Right: quantitative analysis of MCP-1 expression. Fluorescence intensity of three sections each from five mice in each group was expressed as mean ± SE relative to that of Ldlr<sup>−/−</sup> mice transplanted with Ldlr<sup>−/−</sup> bone marrow. *, **, ***P < 0.01 vs. Ldlr<sup>−/−</sup>, Ldlr<sup>−/−</sup>Ebi3<sup>−/−</sup>, and Ldlr<sup>−/−</sup>WSX-1<sup>−/−</sup> mice transplanted with Ldlr<sup>−/−</sup>, Ldlr<sup>−/−</sup>Ebi3<sup>−/−</sup>, and Ldlr<sup>−/−</sup>WSX-1<sup>−/−</sup> bone marrow, respectively.
settings. Currently the reason for this possible discrepancy is not known; difference in housing condition or timing of sampling may have resulted in the difference. Together with the preceding report, IL-27 affects macrophage functions as well as CD4-positive T cell functions during development of atherosclerosis. As such, IL-27 expressed in arterial walls seems not only to modulate macrophage function, for instance, phagocytosis and subsequent cytokine production, but to affect activities of various types of immune and nonimmune cells via modulating cytokine production by macrophages and other immune cells involved. Compared with the preceding report by Koltsova et al. (13), we clearly showed that EBI-3-deficient mice, mice deficient for a subunit of IL-27 cytokine, demonstrated essentially similar phenotypes with the IL-27 receptor-deficient mice, which further substantiated the role of IL-27 in atherosclerosis. The phenotypes between the two strains of mice, however, were not identical. Differential expression levels and/or differential inducibility upon cell activation of IL-27 and IL-27 receptor in different types of cells that participate in atherosclerosis may contribute to differences in atherosclerotic lesion size between \(Ldlr^{-/-}\)-\(Ebi3^{-/-}\) and \(Ldlr^{-/-}\)-\(WSX-1^{-/-}\) mice (Fig. 1A).

Recent studies have revealed that macrophages differentiate into multiple subsets including classic macrophages activated by IFN-\(\gamma\)-mediated Th1 type response (M1 macrophage phenotype) and the subsets activated alternatively by T helper 2 (Th2) response (M2 macrophage phenotype) (17). Each subset represents diversified roles in the regulation of inflammation including atherogenesis (30). M1 macrophages play proinflammatory roles through the promotion of Th1 response and higher production of proinflammatory IL-12 with lower production of anti-inflammatory IL-10. In contrast, M2 macrophages play immunoregulatory roles through phagocytic activity, the promotion of Th2 response, and higher production of anti-inflammatory IL-10. We demonstrate augmented production of IFN-\(\gamma\) by IL-27- and IL-27 receptor-deficient macrophages, suggesting that IL-27 inhibits macrophage cell-autonomous activation and M1 polarization by regulating IFN-\(\gamma\) secretion in a paracrine and an autocrine fashion. Although it is reported that IL-27 controls IL-10 production in T cells that contribute to an anti-inflammatory role of IL-27 (1, 4, 22), the present study indicates that IL-10 production in macrophages is not significantly changed by IL-27. The contribution of IL-10 produced by macrophages may be minor in the inhibition of
Atherosclerosis by IL-27. Further studies are necessary to elucidate roles of IL-27 in the macrophage polarization and the regulation of each subset, while IL-27 may modify pathological microenvironment in arterial walls that controls atherogenesis through the regulation of cytokine production by macrophages. It has been established that macrophage activation in arterial walls is a key process of the rupture of atherosclerotic lesions that causes thrombotic occlusion of coronary arteries and resultant acute coronary syndrome (6). Immunoregulation by immunosuppressive cytokines in local lesions of arterial walls is considered to be beneficial to prevent atherosclerosis and related diseases. We also reported in C57BL/6 mice that IL-27 inhibits pancreatic β cell injury by macrophage-mediated islet inflammation that contributes to the development of diabetes (5). Accordingly, we suggest that anti-inflammatory IL-27-IL-27 receptor could be a favorable therapeutic target for vascular and metabolic diseases whereby chronic inflammation driven at least partly by macrophages plays important roles.

It has been shown that IL-27 activates Jak/STAT signaling pathways through phosphorylation upon ligation of IL-27 re-
Fig. 6. IL-27 has a minor role in the regulation of activated vascular cells. A: effects of recombinant human IL-27 on TNF-α-induced VCAM-1 expression in HUVECs. The ratio of VCAM-1-positive cells determined using flow cytometry from three independent experiments performed in triplicate in each group is shown as mean ± SE. *P < 0.01 vs. control. Recombinant human TNF-α increased VCAM-1 expression in HUVECs, while the treatment with recombinant human IL-27 demonstrated no significant effects. B: effects of recombinant human IL-27 on PDGF-BB-induced proliferation and migration of cultured human aortic smooth muscle cells. Left: cell proliferation of human aortic smooth muscle cells determined by WST-1 assay. The data shown are values for the intensity of formazan that were measured in triplicate in three independent experiments and normalized to control as mean ± SE. *P < 0.05 vs. control. PDGF-BB-induced proliferation was not blocked by recombinant human IL-27. Right: the numbers of migrated human aortic smooth muscle cells evaluated using a Boyden chamber method in triplicate in three independent experiments are shown as mean ± SE. *P < 0.01 vs. control. Treatment with recombinant human IL-27 was not able to inhibit cell migration in response to PDGF-BB in human aortic smooth muscle cells.
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