Interleukin-27 Receptor Limits Atherosclerosis in *Ldlr*−/− Mice

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Abstract

Rationale—Atherosclerosis is a chronic inflammatory disease of the arterial wall. Several proinflammatory cytokines are known to promote atherosclerosis, but less is known about the physiological role of anti-inflammatory cytokines. Interleukin (IL)-27 is a recently discovered member of the IL-6/IL-12 family. The IL-27 receptor is composed of IL-27 receptor A (WSX-1) and gp130 and is required for all established IL-27 signaling pathways. The expression of the IL-27 subunit Ebi3 is elevated in human atheromas, yet its function in atherosclerosis remains unknown.

Objective—The aim of this study was to test the role of IL-27 receptor signaling in immune cells in atherosclerosis development.

Methods and Results—Atherosclerosis-prone *Ldlr*−/− mice transplanted with *Il27ra*−/− bone marrow and fed Western diet for 16 weeks developed significantly larger atherosclerotic lesions in aortic roots, aortic arches, and abdominal aortas. Augmented disease correlated with increased accumulation of CD45+ leukocytes and CD4+ T cells in the aorta, which produced increased amounts of IL-17A and tumor necrosis factor. Several chemokines, including CCL2, were upregulated in the aortas of *Ldlr*−/− mice receiving *Il27ra*−/− bone marrow, resulting in accumulation of CD11b+ and CD11c+ macrophages and dendritic cells in atherosclerotic aortas.

Conclusions—The absence of anti-inflammatory IL-27 signaling skews immune responses toward T-helper 17, resulting in increased production of IL-17A and tumor necrosis factor, which in turn enhances chemokine expression and drives the accumulation of proatherogenic myeloid cells in atherosclerotic aortas. These findings establish a novel antiatherogenic role for IL-27 receptor signaling, which acts to suppress the production of proinflammatory cytokines and chemokines and to curb the recruitment of inflammatory myeloid cells into atherosclerotic aortas.

Keywords

atherosclerosis; chemokines; cytokines; inflammation; leukocyte recruitment; vascular inflammation

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Atherosclerosis is a multifactorial chronic vascular disease perpetuated by inflammation and its mediators, such as cytokines[1] and chemokines.[2] Atherosclerosis is characterized by accumulation of modified low-density lipoproteins in the arterial wall and formation of atherosclerotic plaques. Infiltrating monocytes take up modified low-density lipoprotein and become foam cells.[3] These and other immune cells in turn produce multiple cytokines that promote chronic inflammation and progressive plaque growth.[4]

Previous studies identified various proinflammatory cy-tokines, such as tumor necrosis factor (TNF), interleukin (IL)-17, IL-6, IL-12, and interferon (IFN) γ, as important regulators of atherosclerosis development and progression.[5–13] However, studies of negative regulators of inflammation, such as anti-inflammatory cytokines, are limited.[1] Transgenic mice overexpressing IL-10 showed reduced atherosclerosis,[14] whereas $Apoe^{-/-}$Il10$^{-/-}$ double-deficient mice exhibited an increase in lesion formation.[15] Systemic neutralization of transforming growth factor-β[16] or its genetic deficiency[16,17] resulted in increased lesion development in $Apoe^{-/-}$ mice.

IL-27 is a recently discovered heterodimeric member of the IL-6/IL-12 cytokine superfamily, which consists of 2 subunits: p28 and Ebi3.[18] IL-27 binds to a heterodimeric IL-27 receptor (IL-27R) composed of the common gp130 (*Il6st*) and the unique WSX-1 (*Il27ra*) chains.[19] Gp130 mostly activates STAT3, whereas WSX-1 activates STAT1,[19] which results in a variety of signaling events, such as the T-bet–dependent induction of IL-12 receptor β chain (*Il12rb2*), as well as induction of pro-inflammatory IFNγ and anti-inflammatory IL-10.[20] As a result, IL-27 has been reported to influence the differentiation of CD4 T-cell subtypes, including, in some cases, the stimulation of IL-10–producing type 1 T regulatory (Tr1) cells and Th1 cells, but also the inhibition of T-helper (Th) 17 cells and Th2 cells.[20,21] IL-27 signaling through WSX-1 has been shown to strongly suppress Th17 cell differentiation in an STAT1-dependent fashion.[22–24] IL-27R engagement also blocks STAT3 phosphorylation through a suppressor of cytokine signaling 3–dependent pathway.[25] Although WSX-1 is expressed on B cells, natural killer cells, monocytes, and macrophages, its expression is highest in CD4$^+$ T cells, and most of the in vivo effects of *Il27ra* deficiency are thought to be because of altered T-cell cytokine polarization.[18] In experimental autoimmune encephalitis, a mouse model of multiple sclerosis, the infusion of IL-27 reduces central nervous system inflammation by suppressing Th17 cells.[24] In collagen-induced arthritis, a mouse model of rheumatoid arthritis, IL-27 administration reduced inflammatory cytokines and joint inflammation.[26] Conversely, *Il27ra*$^{-/-}$ mice showed membranous glomerulonephritis and exacerbated disease in the MRL/lpr mouse model of systemic lupus erythematosus.[27]

The role of IL-27 in atherosclerosis has not been addressed. In 2009, Kempe et al[28] reported the enhanced expression of Ebi3 subunit common to IL-27 and IL-35 in human atheromatous plaques. We reasoned that IL-27R signaling may play an important role in the regulation of aortic inflammation and atherosclerosis. Here, we report that *Il27ra* deficiency in hematopoietic cells leads to a significant increase in atherosclerotic lesions in aortic roots, arches, and especially in the abdominal aorta of atherosclerosis-prone *Ldlr*$^{-/-}$ mice. Absence of IL-27R in hematopoietic cells resulted in increased Th17 cells in the aorta and increased production of IL-17 and TNF. Various chemokines, including CCL2, were also upregulated, resulting in the accumulation of CD11b$^+$ and CD11c$^+$ inflammatory macrophages and dendritic cells (DCs) in the arterial wall. Therefore, we propose that IL-27R signaling plays an important anti-inflammatory role in the suppression of atherosclerosis development.

Methods

Mice

C57Bl/6 (B6) (000664) and *Ldlr*^−/− (002207) mice were obtained from Jackson Labs (Bar Harbor, ME)—*Il-27ra*^−/− (WSX-1^−/−) mice were kindly provided by Amgen Inc. *Il-27ra*^−/− mice were subsequently backcrossed into the C57Bl/6 background for 9 generations. Mice were genotyped by standard polymerase chain reaction protocols and used in age- and sex-matched groups. Animal numbers for each specific analysis are given in the Figure legends. Beginning at 8 weeks of age, female and male *Ldlr*^−/−mice were used as bone marrow recipients. Bone marrow was isolated from either *Il27ra*^−/− or C57Bl/6J (wild-type [wt]) mice (6–8 weeks old). After 4 weeks of reconstitution, recipient mice started to receive Western diet for 16 weeks. Littermate controls were used (ie, each cage contained both wt and *Il-27ra*^−/− bone marrow–transplanted mice). Mice were kept in specific pathogen-free conditions in an Accreditation of Laboratory Animal Care–approved barrier facility. Blood counts were measured by an automatic analyzer (Hemavet 950FS, DREW Scientific, Oxford, CT). Alanine aminotransferase in serum was measured by kinetic colorimetric kit (Infinity ALT [GPT] reagent; Thermo Fisher Scientific Inc, Middletown, VA). Animal experiments were approved by the Animal Care Committee at the La Jolla Institute for Allergy and Immunology.

Bone Marrow Transplantation

Recipient mice (*Ldlr*^−/−) were irradiated in 2 doses of 550 rad each (for a total 1100 rad) 3 hours apart. Femurs and tibias of donor mice (wt or *Il27ra*^−/−) were collected, and bone marrow cells were isolated under sterile conditions. Bone marrow cells were resuspended in sterile PBS, and 300 μL of cell suspension containing 5×10^6 cells was intravenously injected (retro-orbitally) into recipient mice. After transplantation, recipient mice were provided with autoclaved food and autoclaved acidified water, containing antibiotic (trimethoprim-sulfamethoxazole) for 2 weeks.

Quantification of Atherosclerosis and Histological Analysis

Whole aortas were excised, fixed, and stained with Sudan IV (counterstain fast green/hematoxylin). Digital images were obtained using Moticam 1000 (Motic, Richmond, Canada) on an Olympus S267 dissection scope (Olympus, Center Valley, PA). For histological lesion size quantification, 5-μm sections were taken starting at the aortic valve plane and covering 300 μm in 50-μm intervals. Sections were stained with Oil red O/hematoxylin/light green stain. Relative collagen contents and composition were analyzed by Picrosirius red staining in polarized light. Photomicrographs were taken with a ×4 objective on a Nikon eclipse 80i microscope. Lesion size was determined using National Institutes of Health Image J and averaged over all sections in each mouse.

Histology and Immunofluorescence

For histological analysis, aortic roots were frozen in an optimal cutting temperature compound on dry ice and stored at −80°C. Five-micrometer sections were cut in the aortic valve plane, and immunostaining was performed. Frozen sections were thawed and fixed for 10 minutes in acetone at room temperature, followed by additional fixation for 8 minutes in 1% paraformaldehyde in 100 mmol/L dibasic sodium phosphate containing 60 mmol/L lysine and 7 mmol/L sodium periodate at pH 7.4 on ice. Sections were blocked using the avidin/biotin blocking kit (Vector Labs), followed by 5% normal goat serum (Caltag Laboratories) and 1% BSA (Sigma) in PBS. Sections were stained overnight at 4°C with rabbit anti-mouse smooth muscle actin (polyclonal; Abcam), rat anti-mouse CD11b-fluorescein isothiocyanate (M1/70; BD Biosciences), hamster anti-CD11c (BD Bioscience),

rat anti-mouse IL-27R (provided by Dr Ghilardi, Genentech Inc), rat anti-mouse CD45 Alexa Flour 647 (Biolegend), rat anti-mouse CD4-PE (RM4-5; eBioscience), and rat anti-mouse MOMA-2 (Abcam) followed by staining with secondary antibody: donkey anti-rabbit Alexa Fluor 568 (Molecular Probes), anti-fluorescein isothiocyanate Alexa Fluor 488 (Molecular Probes), goat anti-rat Alexa Fluor 488 (Molecular Probes), and DyLight 649– labeled goat anti-hamster IgG (Jackson Immunoresearch). Images were acquired on a Leica DM6000 upright confocal microscope using HCX PLAPO ×20 and ×40 oil-immersion objectives at 488, 543, and 633 nm excitation wavelengths. National Institutes of Health Image J was used to adjust brightness and 1-step smoothing on all images in parallel.

Aortic Single Cell Preparations and Flow Cytometry Analysis

Single-cell suspensions from aortas were prepared as described previously.[29] Briefly, mice were euthanized by CO_2 inhalation, and aortas were perfused with PBS containing heparin (20 U/mL). Aortas were prepared by surgical removal of all adventitial fat under a dissection microscope, cut into small pieces, and incubated for 55 minutes at 37°C with gentle shaking in a mixture of 450 U/mL collagenase type I, 250 U/mL collagenase type XI, 120 U/mL hyaluronidase, and 120 U/mL DNAseI (all enzymes from Sigma Aldrich, St Louis, MO). Cell suspensions were filtered through 70-μm cell strainers and stained with CD45-PerCP (30-F11; BD Biosciences), T cell receptor-β-AF700 (H57-597; BioLegend), CD4-APC (GK1.5; eBioscience), CD11b-eFluor 450 (M1/70; eBioscience), CD11c-APC (N418; eBioscience), CD8α-Cy7APC (53–6.7; BioLegend), and Aqua LIVE/ DEAD fixable dead cell staining kit (Invitrogen, Carlsbad, CA) for flow cytometry (LSRII; Becton-Dickinson, San Jose, CA). Live CD45^{+} cells were gated and analyzed using FlowJo software (Tree Star Inc, Ashland, OR). For intracellular cytokine staining, cells were restimulated for 6 hours in vitro with phorbol ester and ionomycin in the presence of Brefeldin A, fixed and permeabilized using Cytofix/ Cytoperm kit (BD Biosciences), and stained with IL-17A-PE (BD Biosciences), IFNγ-Cy7PE (XMG 1.2; BD Biosciences), and TNF-APC (MP6-XT22; eBioscience).

Gene Expression

For gene expression analysis, aortas were isolated and snap-frozen in liquid nitrogen. Aortas were homogenized in an RLT buffer (Qiagen), and RNA was isolated using the RNAEasy purification system (Qiagen) and treated with DNase I (Promega) followed by first-strand cDNA synthesis using the Superscript II system, according to the random priming protocol (Invitrogen Life Technologies). Gene expression was analyzed by SYBR green real-time polymerase chain reaction using primers for ribosomal L32, β-actin, T-bet, IFNγ, IL-17, retinoic acid-related orphan receptor-γt, IL-2, IL-10, IL-6, TNF, CCL2, CCL3, CCL5, CCL17, and CCL20. Sequences of the primers were obtained from National Institutes of Health QPrimerDepot (http://mouseprimerdepot.nci.nih.gov). Reactions were performed in triplicate for each gene, and gene expression was normalized to L32 or β-actin expression.

ELISA

Aortas were digested, and cell suspensions were incubated for 48 hours with anti-CD3 and anti-CD28 antibodies in complete RPMI 1640 media containing 10% fetal bovine serum, pen/strep, L-Glu, non-essential amino acids, HEPES, and sodium pyruvate. Supernatants were collected, and cytokine secretion was measured by mouse cytometric bead array (eBioscience). Levels of IL-17A, TNF, IFN-γ, IL-6, and monocyte chemoattractant protein-1 were measured according to the manufacturer's instructions.

Statistical Analysis

Data were analyzed using Prism software (GraphPad). Student 2-tailed *t* test and Mann-Whitney *U* test were used to compare conditions. ANOVA followed by Wilcoxon signed-rank test was used to compare fold induction of gene expression by real-time polymerase chain reaction. Data are expressed as mean±SEM; *P<0.05, ** P<0.01. P<0.05 was considered significant.

Results

IL-27R Deficiency Significantly Increases Atherosclerotic Lesion Development

IL-27R can be expressed by various immune and hematopoietic cells but also can be found in the nonhematopoietic compartment.[18] In aortic roots, IL-27R expression was found in CD4+ T cells, MOMA-2+ macrophages, and endothelial cells as seen by immunofluorescence (Online Figure I). To investigate the role of IL-27R signaling in hematopoietic cells in the pathogenesis of atherosclerosis, we generated bone marrow chimeras using either wt or *Il27ra*−/− bone marrow transferred to irradiated atherosclerosis-prone *Ldlr*−/− recipient mice (*Il27ra*−/−→*Ldlr*−/− and *wt*→*Ldlr*−/− mice). This resulted in the specific ablation of IL-27R on hematopoietic but not on nonhematopoietic cells. Four weeks after bone marrow transplantation, mice were placed on a high-fat western diet for 16 weeks. Reconstitution efficiency was at least 96±2% for myeloid cells and 93±4% for T lymphocytes (Online Figure II). We did not find a difference in body weight or blood leukocyte count between *Il27ra*−/−→*Ldlr*−/− and *wt*→*Ldlr*−/− mice (Online Figure IIIA and IIIB). Remarkably, en face analysis of aortas stained with Sudan IV revealed a significant increase of atherosclerotic plaque size in aortas of *Il27ra*−/−→*Ldlr*−/− mice compared with *wt*→*Ldlr*−/− controls (Figure 1A and 1B). Atherosclerotic plaque size was also increased in aortic roots in the absence of IL-27R (Figure 1C). Interestingly, IL-27R deficiency in hematopoietic cells also led to a dramatic increase in atherosclerotic lesions in the abdominal aorta, which usually is not profoundly affected during atherosclerosis development in *Ldlr*−/− mice but represents an important site of atherosclerosis in human patients (Figure 1B). Both males and females of *Il2 7ra*−/−→*Ldlr*−/− displayed enhanced lesion sizes compared with *wt*→*Ldlr*−/− littermate control mice, suggesting that IL-27R controls atherosclerosis regardless of sex differences. Relative aortic collagen contents and composition were essentially unaltered (Online Figure IVA and IVB). Smooth muscle actin was increased in the aortic arches and abdominal aortas of *Il27ra*−/−→*Ldlr*−/− mice, most likely as a result of outward remodeling (Online Figure IVC–IVF). Plasma lipoprotein and lipid profiles were not different between wt and IL-27R chimeras, suggesting that the lack of IL-27R signaling does not affect atherosclerosis by interfering with lipoprotein metabolism (Online Figure VA). No differences were found in alanine aminotransferase activity in serum (Online Figure VB). Thus, inactivation of IL-27R in hematopoietic cells significantly enhanced atherosclerosis development in a mouse model of atherosclerosis.

IL-27R Deficiency Leads to Increased Accumulation of T Cells in the Aorta of *Ldlr*−/− Mice

Given the relevance of IL-27R signaling in hematopoietic cells for atherosclerosis, we next sought to examine the cellular composition of the leukocyte infiltrates in aortas of *Il 27ra*−/−→*Ldlr* −/− and *wt*→*Ldlr* −/− mice. Flow cytometry revealed a significant increase in CD45+ leukocytes in the aortas of *Il27ra*−/−→*Ldlr*−/− mice (Figure 2A). Among CD45+ cells, we particularly found a significant accumulation of Th cells (TCRβ+ CD4+) cells in the aortic wall of *Il27ra*−/−→*Ldlr*−/−mice (Figure 2B), whereas no significant difference was found in cytotoxic CD8+ T cells. These observations were further supported by immunofluorescence staining for T cells, where significantly higher numbers of T cells were found in both aortic roots (Figure 2C) and abdominal aortas (Figure 2D) of *Il27ra*−/−→*Ldlr*−/− compared with wt controls. We therefore concluded that IL-27R

signaling limits accumulation of CD4 T cells in the aortas of atherosclerotic mice and modulates the CD4 Th response during atherosclerosis development.

Ablation of *Il27R* Signaling in Hematopoietic Cells Causes Dysregulation of Th17 and Th1 Responses in *Ldlr⁻/⁻* Atherosclerotic Mice

To gain insights into potential mechanisms by which IL-27R signaling limits atherosclerosis progression, we first performed real-time polymerase chain reaction analysis on aortic arches, abdominal aortas, paraaortic lymph nodes (LNs), and spleens isolated from *Il27ra⁻/⁻→Ldlr⁻/⁻* or wt*→Ldlr⁻/⁻* mice. We found a Th17 signature mRNA expression profile in aortic arches of mice lacking IL-27R signaling, particularly elevated expression of IL-17A and the transcription factor retinoic acid-related orphan receptor-γt mRNAs (Figure 3A). Among other cytokines, mRNA expression of TNF and IL-6 was significantly enhanced in the absence of IL-27R (Figure 3A). Furthermore, we found an upregulation of the Th1 transcription factor T-bet and the Th1 cytokine IFNγ in aortic arches (Figure 3A). TNF and IL-6 expression were also significantly upregulated in the abdominal aortas but not in spleens, suggesting that IL-27R signaling has specific effects at the site of inflammation. We also found a reduction of Foxp3 gene expression in aortic arches and spleens of *Il27ra⁻/⁻→Ldlr⁻/⁻*mice, whereas IL-10 expression remained unchanged.

To further address the question whether inactivation of IL-27R results in dysregulated cytokine production, we measured cytokines in aortic and splenic supernatants. We found a dramatic increase in IL-17A, IL-6, and TNF in the aortic supernatants of *Il27ra⁻/⁻→Ldlr⁻/⁻* mice (Figure 3B). IL-6 and TNF but not IL-17A secretion was also increased in spleens (Figure 3C). Intracellular cytokine staining of CD4+TCRβ+ cells from aortas showed an increased percentage of IL-17A–producing (Th17) T cells in the aortas of *Il27ra⁻/⁻→Ldlr⁻/⁻* mice compared with those of *wt→Ldlr⁻/⁻* (Figure 4A). In addition, we observed enhanced TNF expression in CD4+TCRβ+ T cells (Figure 4A). Interestingly, the numbers of IL17+ TNF+ double-positive cells were also upregulated in the aortas but not in spleens or LNs of *Il27ra⁻/⁻→Ldlr⁻/⁻* mice (Figure 4A–4C). In spleens or LNs of *Il27ra⁻/⁻→Ldlr⁻/⁻* mice, IL-17A- and IFNγ-producing CD4 T cells were reduced or unchanged (Figure 4B and 4C).

To investigate the role of IL-27R signaling in immunosuppressive cells, we analyzed the presence of T regulatory cells (Tregs) and Tr1 cells in the spleen and LN of *Il27ra⁻/⁻→Ldlr⁻/⁻*and *wt→Ldlr⁻/⁻* mice. We found a significant decrease in the percentage of Treg in both spleen and LNs of *Il27ra⁻/⁻→Ld lr⁻/⁻* (Figure 5A). However, Tr1 (Foxp3– IL10+) cells were decreased only in the spleens of *Il27ra⁻/⁻→Ldlr⁻/⁻* (Figure 5B).

Taken together, these findings establish that eliminating IL-27R signaling enhances Th17 immune response and production of IL-17A, IL-6, and TNF in the aortas during atherosclerosis. However, ablation of IL-27R in hematopoietic cells leads to decreased accumulation of immunosuppressive Treg and Tr1 cells in peripheral lymphoid organs, which potentially may result in enhanced inflammation.

Absence of IL-27R Enhances Chemokine Expression

IL-17 signaling has many downstream target genes potentially relevant to atherosclerosis, in particular those encoding various chemokines.[30,31] Several reports suggest that, during inflammation, IL-17 and TNF can act synergistically, particularly in their ability to induce chemokine production.[32] We therefore hypothesized that increased IL-17 production caused by *Il27ra* deficiency could affect the expression of IL-17–dependent chemokines in atherosclerosis. Although we did not find an upregulation of CXCL1 (KC) (Online Figure VI), we observed a significant increase in CCL20, CCL17, CCL5, CCL3, and CCL2 (monocyte chemoattractant pro-tein-1) mRNA levels in aortic arches and abdominal aortas

of $Il27ra^{-/-} \rightarrow Ldlr^{-/-}$ mice (Figure 6A). CCL2 and CCL20 expression was also enhanced in the spleen, and only CCL20 was upregulated in peripheral LNs of $Il27ra$-deficient mice (Figure 6A). Enhanced production of CCL2 protein was detected in the aortic culture supernatant of $Il27ra^{-/-} \rightarrow Ldlr^{-/-}$ mice with or without T-cell stimulation (Figure 6B and 6C). These data suggest that enhanced production of IL-17 and TNF caused by $Il27ra$ deficiency leads to increased expression of various chemokines in mouse aortas, including CCL2.

Absence of *Il27ra* Leads to Enhanced Myeloid Cell Recruitment in Mouse Aortas

CCL2 has previously been shown to increase recruitment of monocytes to atherosclerotic aortas.[33] Enhanced accumulation of these cells in the lesions and arterial wall is associated with acceleration of the disease.[3,29] Indeed, the percentage as well as the number of CD11b^{+} and CD11b^{+}CD11c^{+} myeloid cells was significantly increased in the $Il27ra^{-/-} \rightarrow Ldlr^{-/-}$ mice compared with the control wt$\rightarrow Ldlr^{-/-}$ mice after 16 weeks of Western diet feeding (Figure 7A). To address the localization of these cells in situ, we performed immunofluorescence staining and found enhanced accumulation of CD11b^{+} and CD11c^{+} cells in aortic roots (Figure 7B and 7C, and Online Figure VIIB for isotype controls) and abdominal aortas (Figure 8A–8C), supporting the flow cytometry data. Interestingly, most of the CD11b^{+}CD11c^{+} cells were found in the plaque, whereas the adventitia was dominated by CD11b^{+}CD11c^{−} cells. Most CD11b^{+}CD11c^{+} and CD11b^{+}CD11c^{−} cells also expressed the pan-macrophage marker MOMA-2 (CD169; Figure 7B and 7C). Overall, our data suggest an inhibitory role of IL-27R signaling that limits the accumulation of myeloid cells in the aorta during atherosclerosis progression.

Collectively, our data demonstrate an important inhibitory role of IL-27/IL-27R signaling in hematopoietic cells for controlling Th17 cytokine production and IL-17–dependent chemokine expression. In the absence of IL-27R, IL-17–dependent enhancement of CCL2 expression likely explains increased aortic accumulation of CD11b^{+}CD11c^{+} inflammatory macrophages and DCs.

Discussion

A large body of evidence demonstrates a crucial role of inflammation in the pathogenesis of atherosclerosis.[4] Although the role of various proinflammatory cytokines was extensively studied in atherosclerosis,[1] only 2 antiatherogenic cytokines, IL-10 and transforming growth factor-β, were described. Here, we establish IL-27R signaling as a novel and important atheroprotective cytokine pathway. We show that littermate $Ldlr^{-/-}$ mice receiving $Il27ra^{-/-}$ bone marrow had more severe disease manifestation than those receiving wt bone marrow. This effect was consistently observed both in males and females. Interestingly, IL-27R deficiency in hematopoietic cells also led to a dramatic increase in atherosclerotic lesions in the abdominal aorta, which usually is not profoundly affected during atherosclerosis development in $Ldlr^{-/-}$ mice. In humans, the abdominal aorta represents a clinically important site for atherosclerosis associated with aortic aneurysm formation. In the absence of IL-27R, production of IL-17, IL-6, and TNF was increased in aortas, which correlates with the expansion of CD4^{+} IL-17–producing (Th17) cells and reduction of immunosuppressive Treg and Tr1 cells. Therefore, our data provide collective evidence for an anti-inflammatory and antiatherogenic role of IL-27R signaling in atherosclerosis.

Consistent with the ability of IL-27 to suppress the differentiation of Th17 cells, we found a dramatic increase of IL-17A production in aortas of atherosclerotic mice lacking hematopoietic IL-27R, which positively correlated with enhanced manifestation of the disease. Furthermore, in the absence of IL-27R, we found a significant upregulation of IL-6, which is known to participate in the survival and activation of T cells, in general, and in the

regulation of Th17 lineage differentiation, in particular.[34] The role of IL-6 in atherosclerosis is controversial. Some studies show that IL-6 promotes athero-sclerosis[35]; however old $Il6^{-/-}$ mice have also been reported to show enhanced atherosclerosis.[36] Despite the extensive study of IL-17A in the pathogenesis of atherosclerosis, its role is still debated, although most studies suggest a proatherogenic function of IL-17. In atherosclerosis-susceptible $Apoe^{-/-}$ mice, the proportion of Th17 (and Th1) cells, the expression of IL-17A, and the Th17 signature transcription factor retinoic acid-related orphan receptor-γt were increased compared with nonatherosclerotic wt littermates.[12,13,37–41] Treatment of $Apoe^{-/-}$ mice with neutralizing anti–IL-17A antibody dramatically inhibited the development of atherosclerosis, whereas rIL-17 application significantly promoted the formation of atherosclerotic plaque.[37] Double-deficient $Il17a^{-/-}Apoe^{-/-}$ mice had reduced aortic leukocyte and DC infiltration after a high-fat diet, although the lesion burden was not altered in another study[41] and increased in a third study.[42] Our data support the concept that increased IL-17 production in the absence of IL-27R signaling promotes atherosclerosis.

IL-27R engagement has previously been shown to inhibit Th17 lineage commitment through a Stat-1–dependent pathway.[22] IL-27R deficiency in mouse models of multiple sclerosis led to an exacerbated Th17 response, enhanced secretion of IL-17, IL-6, TNF, and GM-CSF, and exacerbated disease.[24,43] This was associated with an increased number of IL-17–producing T cells in the central nervous system. In agreement with this, our work demonstrates that IL-17–producing T cells are more numerous in atherosclerotic aortas of $Ldlr^{-/-}$ mice receiving $Il27ra^{-/-}$ bone marrow. This suggests that, in vivo, the loss of the suppressive effect of IL-27R signaling on Th17 cells dominates disease exacerbation in the $Ldlr^{-/-}$ model of atherosclerosis. In vitro, IL-27 also has been reported to enhance Th1 differentiation.[44] If indeed that was the main effect of IL-27 in vivo, a reduced Th1 signature would be expected in $Il27ra^{-/-}$ mice. However, in a mouse model of infection with $Toxoplasma$ $gondii$, $Il27ra^{-/-}$ mice showed the opposite phenotype with increased accumulation and proliferation of CD4 T cells and increased IFNγ production.[45,46] We also observe enhancement of IFNγ production in aortas of mice receiving $Il27ra^{-/-}$ bone marrow. Overall, our data suggest that in atherosclerosis IL-27R signaling is critically involved in suppression of Th17 and, to some extent, in suppression of the Th1 aortic immune responses. However, IL-27R signaling can be also important for immunosuppressive Treg and Tr1 cell function during atherosclerosis.

Th17 cells in the aortas of $Il27ra^{-/-} \rightarrow Ldlr^{-/-}$ mice also likely contribute to the enhanced TNF production, because the number of IL-17$^+$ TNF$^+$ cells in atherosclerotic aortas is increased. The excess of TNF produced in the absence of IL-27R could potentially synergize with IL-17A to induce downstream proatherogenic events. It was previously demonstrated that TNF and IL-17A can upregulate IL-6 production in osteoblasts by activating the transcription factor C/EBPδ.[47] In endothelial cells, synergistic effects of TNF and IL-17A on expression of adhesion molecules and chemokines have also been demonstrated. Various chemokines, particularly CXCL1, CXCL2, CXCL5, CCL2, and CCL7, have been reported to be induced by both IL-17A and TNF, with a strong synergistic component when both cytokines were present.[32] For example, IL-17A modestly induces expression of CXCL1 in mouse embryonic fibroblasts cells but in combination with TNF has a much stronger effect.[48] In aortas from $Ldlr^{-/-}$ mice receiving $Il27ra^{-/-}$ bone marrow, CCL2, CCL3, CCL5, CCL17, and CCL20 were significantly upregulated. Among them, CCL2, CCL3, and CCL5 are known to promote monocyte recruitment, and CCL2 (monocyte chemoattractant protein-1) potentiates the release of monocytes from the bone marrow and their recruitment to the sites of inflammation.[49,50] Indeed, mice lacking the $Ccl2$ gene are protected from atherosclerosis.[51] CCL3 (macrophage inflammatory protein-1α) is also an important chemoattractant for monocytes,[52] whereas CCL5 (RANTES) is one of the most potent arrest chemokine for monocytes, promoting their transition from rolling to firm adhesion by

inducing rapid integrin activation.[53] Consistent with the known roles of CCL2, CCL3, and CCL5, we found enhanced recruitment of inflammatory monocytes, macrophages, and DCs (CD11b+, CD11b+CD11c+, and CD11c+) to aortas of *Ldlr*−/− mice transplanted with *Il27ra*−/− bone marrow. These myeloid cells have the potential to become foam cells and enhance local inflammation by secreting additional cytokines.[3,54] The increased expression of CCL17 may be secondary to the recruitment of these cells. Indeed, accumulation of CCL17-expressing DCs has been reported to exacerbate atherosclerosis.[55] IL-17A has also been reported to upregulate CCL20,[56] a chemokine that is known to promote recruitment of Th17 cells.[57] In aortas of *Ldlr*−/− mice receiving *Il27ra*−/− bone marrow, we find increased expression of CCL20, which may provide a positive feedback loop to further enhance Th17 recruitment, IL-17 production, and chemokine expression.

Taken together, our data show an important inhibitory role of IL-27R signaling in controlling inflammation during atherosclerosis. Enhanced production of IL-17 and TNF, as well as decreased number of immunosuppressive T cells caused by inactivation of *Il27ra* signaling, leads to increased inflammation in the aortic wall and atherosclerotic plaques. Increased expression of IL-17, TNF, and IL-17–dependent and TNF-dependent chemokines, including CCL2, supports the recruitment of myeloid cells, particularly CD11b+CD11c+ inflammatory macrophages and DC, into the growing atherosclerotic plaque.[50,58] Therefore, our findings may be of potential clinical relevance, suggesting that IL-27 could be used as an anti-inflammatory cytokine in the treatment or prevention of atherosclerosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Non-standard Abbreviations and Acronyms

DC	dendritic cells
IL	interleukin
IL-27R	IL-27 receptor
IFN	interferon;LN, lymph node
Th	T helper
TNF	tumor necrosis factor
Treg	T regulatory cells
Tr1	type 1 T regulatory cells
wt	wild type.

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Novelty and Significance

What Is Known?

- Immune and inflammatory response are critical for atherosclerosis development and progression.

- Interleukin (IL)-27 receptor (IL-27R) signaling is an important regulator of immune responses.

- IL-27R signaling regulates the differentiation of T-helper 1 and T-helper 17 CD4+ T-cell subsets in host defense and mouse models of multiple sclerosis (experimental autoimmune encephalitis) and rheumatoid arthritis.

What New Information Does This Article Contribute?

- This article identifies the role of IL-27R in atherosclerosis.

- This article demonstrates an important inhibitory role of IL-27R signaling in the regulation of inflammation in atherosclerosis.

The role of proinflammatory cytokines in atherosclerosis was extensively addressed in the past decade. However, little is known about anti-inflammatory cytokines and their ability to potentially suppress atherosclerosis. IL-27, a member of IL-6/IL-12 cytokine superfamily, was shown to suppress immune responses, but the role of IL-27R signaling in the pathogenesis of atherosclerosis has been never addressed. Here, we demonstrate that absence of immune-modulating IL-27R signaling during atherosclerosis development results in dramatic acceleration of the disease. The disease manifestation correlated with enhanced activation of T-helper 17 cells and upregulation of IL-17– and tumor necrosis factor–dependent chemokine expression, particularly CCL2, which leads to accumulation of various myeloid cells and progression of atherosclerotic plaque growth. Our data provide new knowledge in the inflammatory and immune aspects of atherosclerosis. It identifies IL-27 receptor as mediating anti-inflammatory effects in the $Ldlr^{-/-}$ mouse model of atherosclerosis. This mechanism controls inflammation and leukocyte recruitment to the aorta. These findings and new insights into the molecular and cellular mechanisms of anti-inflammatory and antiatherogenic effects of IL-27R may provide a rationale for developing new strategies to inhibit inflammation in atherosclerosis.
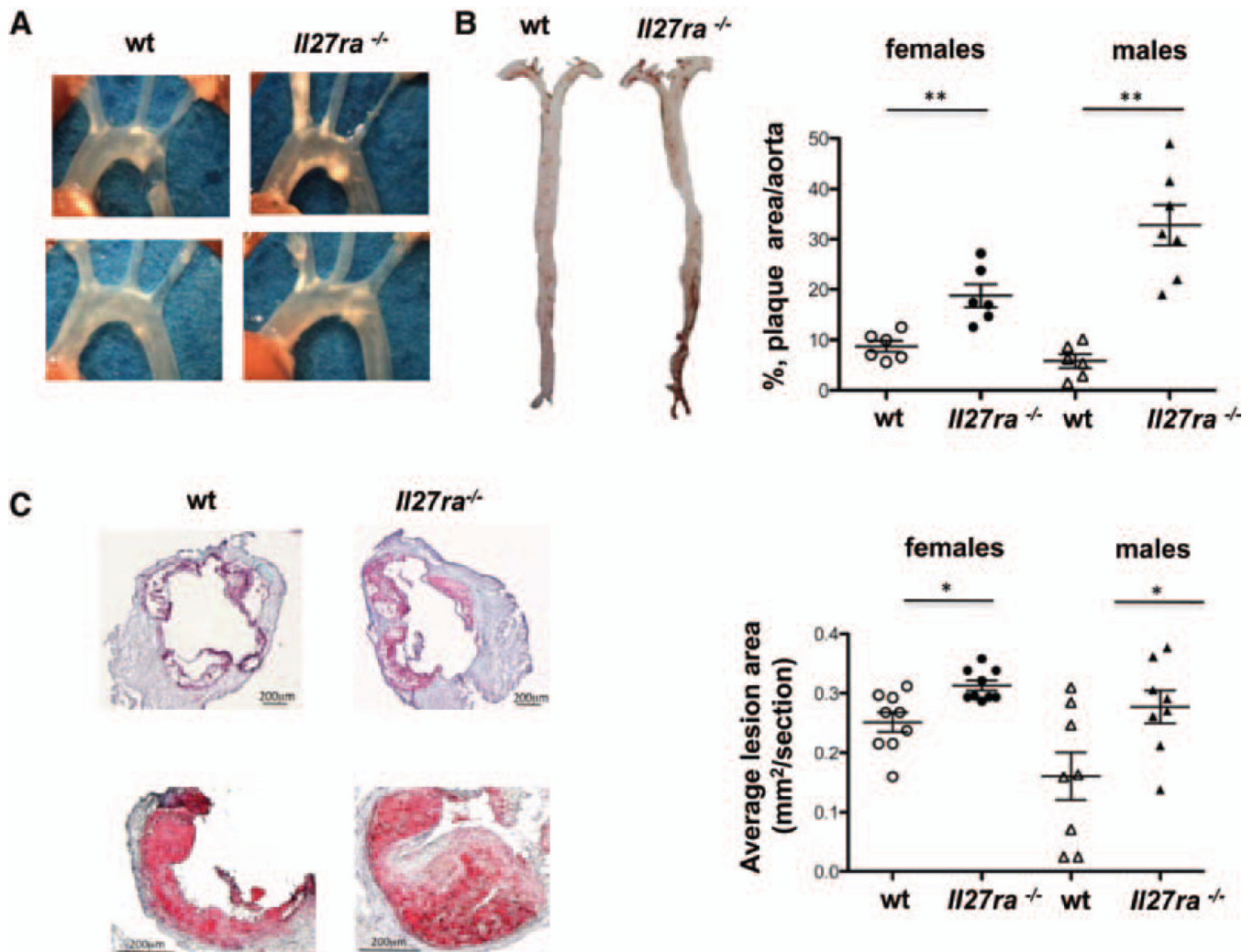
Figure 1. Increased atherosclerotic plaque area in *Il27ra*^{−/−}bone marrow–transplanted mice

Ldlr^{−/−} mice were lethally irradiated and reconstituted with 5×10^6 unfractioned bone marrow cells from C57BL/6 (wild-type [wt]) or *Il27ra*^{−/−} mice. After 4 weeks of reconstitution, mice were fed with western diet for 16 weeks. **A**, Images of aortic arch of *Ldlr*^{−/−} mice receiving *Il27ra*^{−/−} bone marrow or C57BL/6 (wt) control. **B**, Representative en face images of Sudan IV–stained whole aortas of *Ldlr*^{−/−} mice receiving *Il27ra*^{−/−} or wt bone marrow. **B, right**, Quantification of plaque area as percentage of aortic surface in *Ldlr*^{−/−} mice receiving *Il27ra*^{−/−} or wt bone marrow (n=6–7 females or males per group, respectively). **C, left**, Representative images of aortic roots (**top**) and single valve (**bottom**) of *Ldlr*^{−/−} mice receiving *Il27ra*^{−/−} or C57BL/6 (wt) bone marrow. **C, right**, Aortic root lesions were quantified on frozen sections stained with Oil-red-O in the 300 μm following the aortic valve (wt [n=8–9 males or females] mice and *Il27ra*^{−/−} [n=8–9 males or females] mice from 3 experiments).
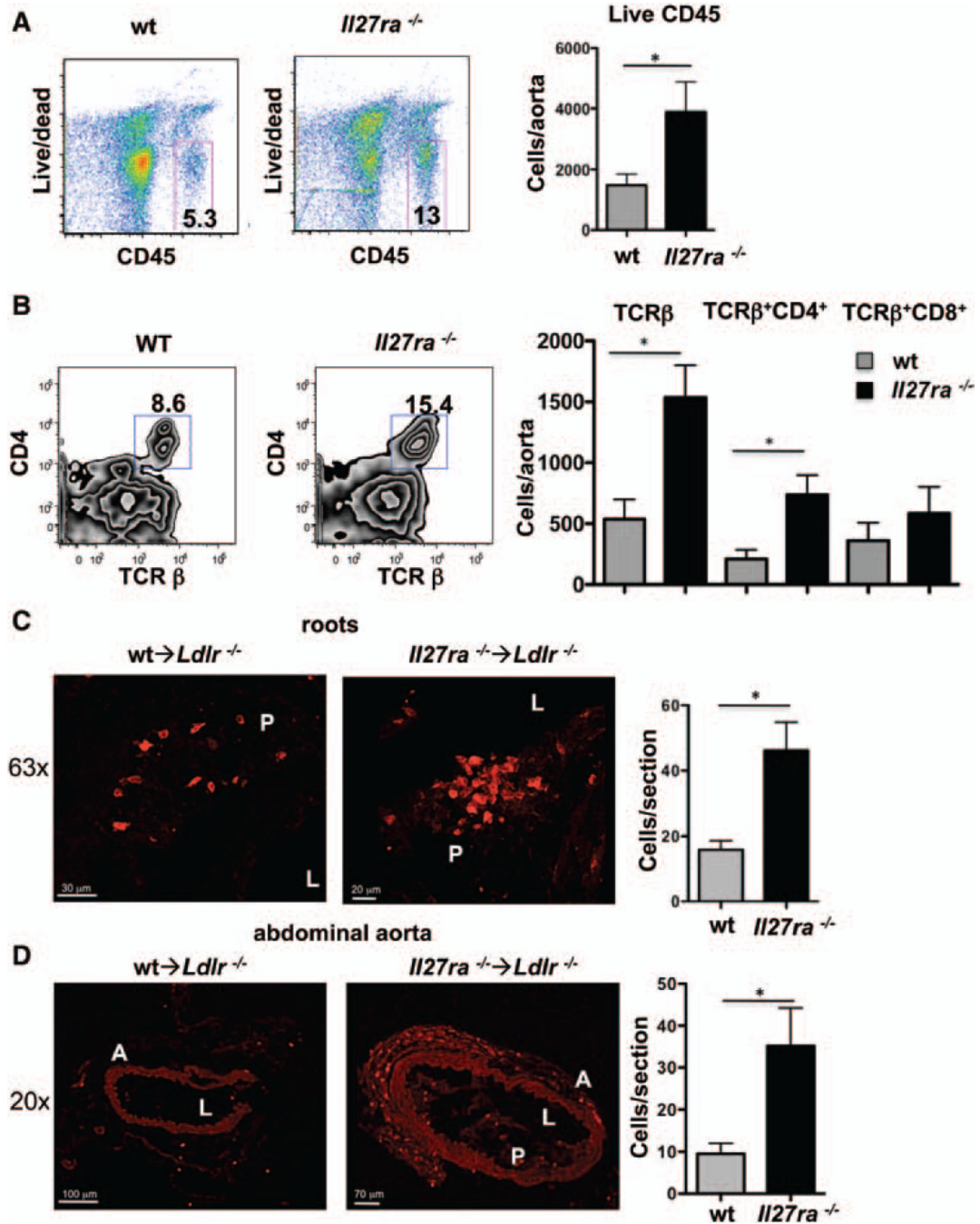
Figure 2. Leukocyte composition of mouse aorta
A, Aortas from bone marrow–transplanted mice were made into single-cell suspensions by enzymatic digestion and stained. Live CD45+ leukocytes were counted in aortas of 16-week Western diet–fed *Ldlr*^{−/−} mice transplanted with *Il27ra*^{−/−} (n=7) or C57BL/6 (wild-type [wt]; n=7) bone marrow. **Left** panels show representative dot plots. **Right** panel shows mean ±SEM, *P<0.05. **B, left,** Live CD45+ cells from aortas were stained for TCRβ and CD4. Numbers indicate percentage in indicated gates. **B, right,** Absolute number of live CD45+, TCRβ+, CD4+TCRβ+, and CD8+TCRβ+ cells in aortas from *Ldlr*^{−/−} mice transplanted with *Il27ra*^{−/−} or wt bone marrow (n=7) each. Mean±SEM *P<0.05. Localization of CD3+ cells

in aortic roots (**C**) and abdominal aortas (**D**) of *Ldlr*$^{-/-}$ mice transplanted with *Il27ra*$^{-/-}$ or C57BL/6 (wt) bone marrow as seen by immunofluorescence. L indicates lumen; P, plaque; A, adventitia. Representative images from 1 of 3 independent experiments. Mean±SEM number of CD3^{+} cells per section in aortic roots (**C, right**) and abdominal aortas (**D, right**) of *Ldlr*$^{-/-}$ mice transplanted with *Il27ra*$^{-/-}$ (n=6) or C57BL/6 (wt; n=6) bone marrow are shown.
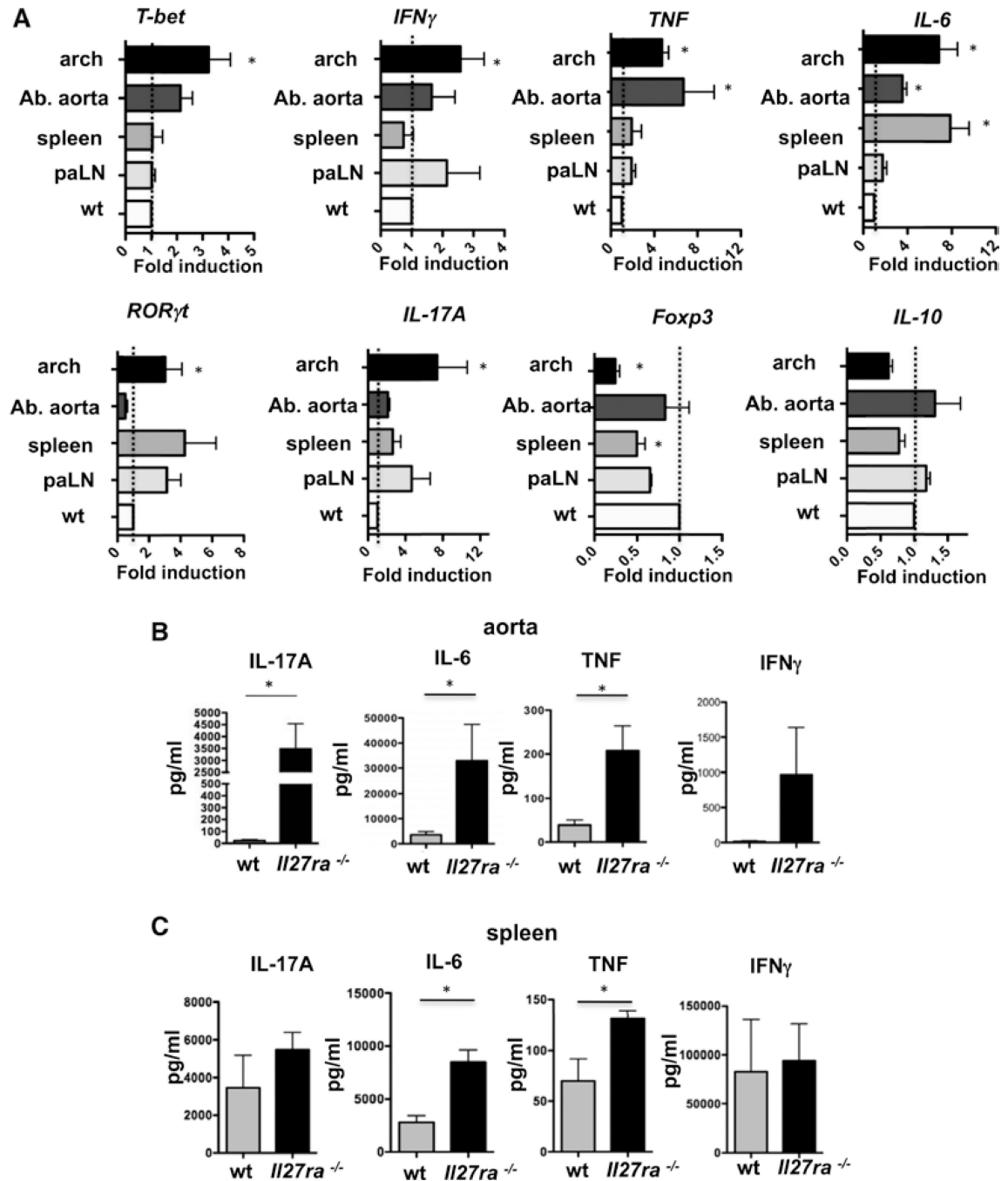
Figure 3. Enhanced production of T-helper 17 cytokines and transcription factors in mice receiving*Il27ra*^{−/−} bone marrow

A, Relative gene expression in *Il27ra*^{−/−}→*Ldlr*^{−/−} (n=6) mice normalized to β-actin and then normalized to gene expression in wild-type (wt)→*Ldlr*^{−/−} (n=6) mice in aortic arches, abdominal aortas (Ab. aorta), paraaortic lymph nodes (paLNs), and spleens after 16 weeks of Western diet. **B** and **C**, Interleukin (IL)-17A, tumor necrosis factor (TNF), IL-6, and interferon (IFN) γ were measured by bead array in supernatants of aortic (n=8; **B**) and splenic (n=8; **C**) cell suspensions, stimulated with anti-CD3/anti-CD28 for 48 h. Mean ±SEM; *P<0.05.
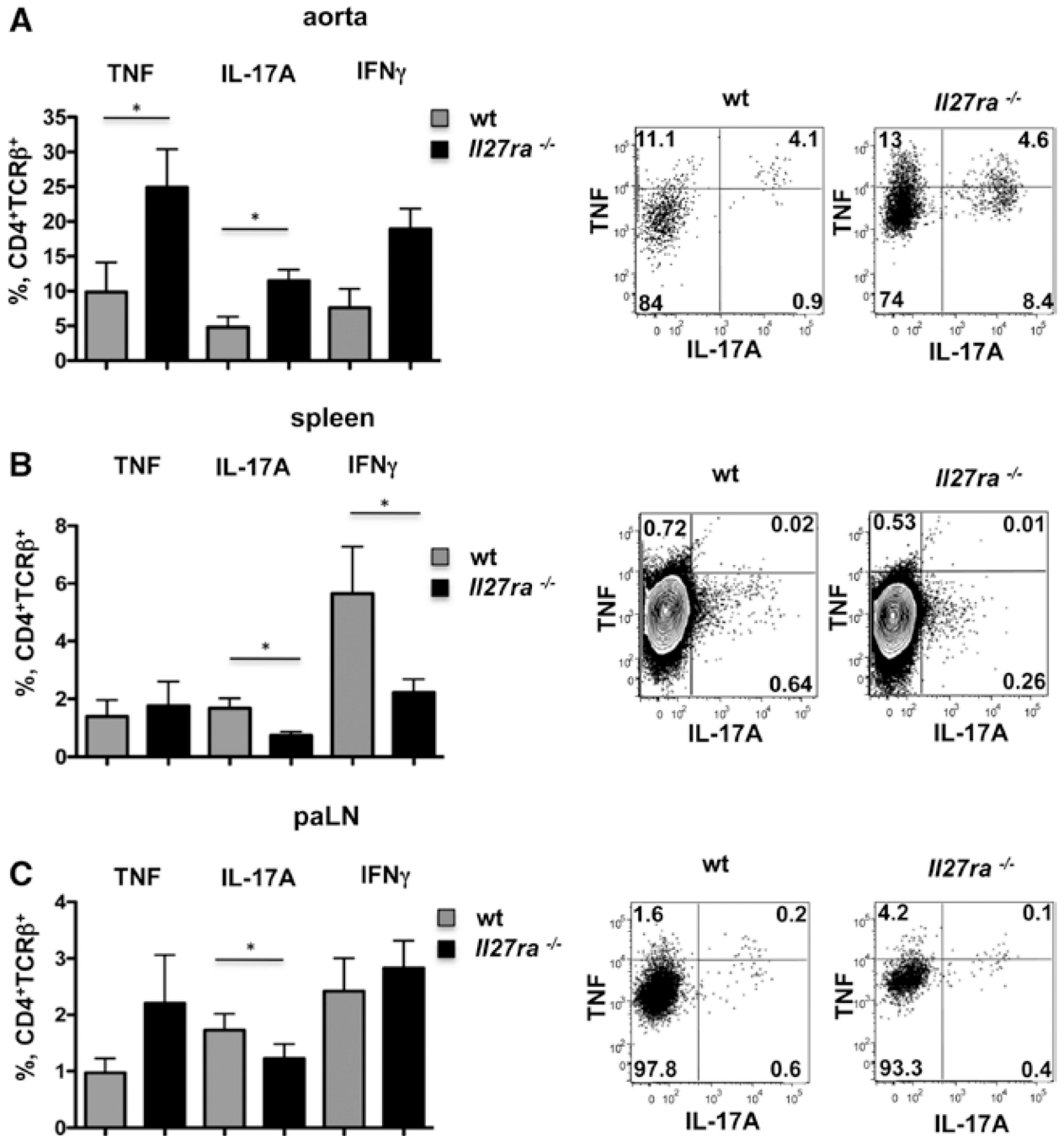
**Figure 4. Enhanced production of T-helper 17 cytokines in aortas of mice receiving *Il27ra*$^{-/-}$ bone marrow**

Percent of CD4 T cells expressing tumor necrosis factor (TNF), interleukin (IL)-17A, and interferon (IFN) γ by intracellular staining in aorta (**A, left**), spleen (**B, left**), or paraaortic lymph nodes (paLNs) (**C, left**) of *Ldlr*$^{-/-}$ mice transplanted with *Il27ra*$^{-/-}$ (n=6) or wild-type (wt; n=6) bone marrow fed 16 weeks with Western diet. **A–C, right**, Representative fluorescence-activated cell sorter plot of CD4$^+$TCRβ$^+$ T cells stained for IL-17A and TNF in aortas (**A**), spleens (**B**), and paLNs (**C**) of *Ldlr*$^{-/-}$ mice transplanted with *Il27ra*$^{-/-}$ or wt bone marrow fed 16 weeks with Western diet. Mean±SEM; *$P<0.05$.
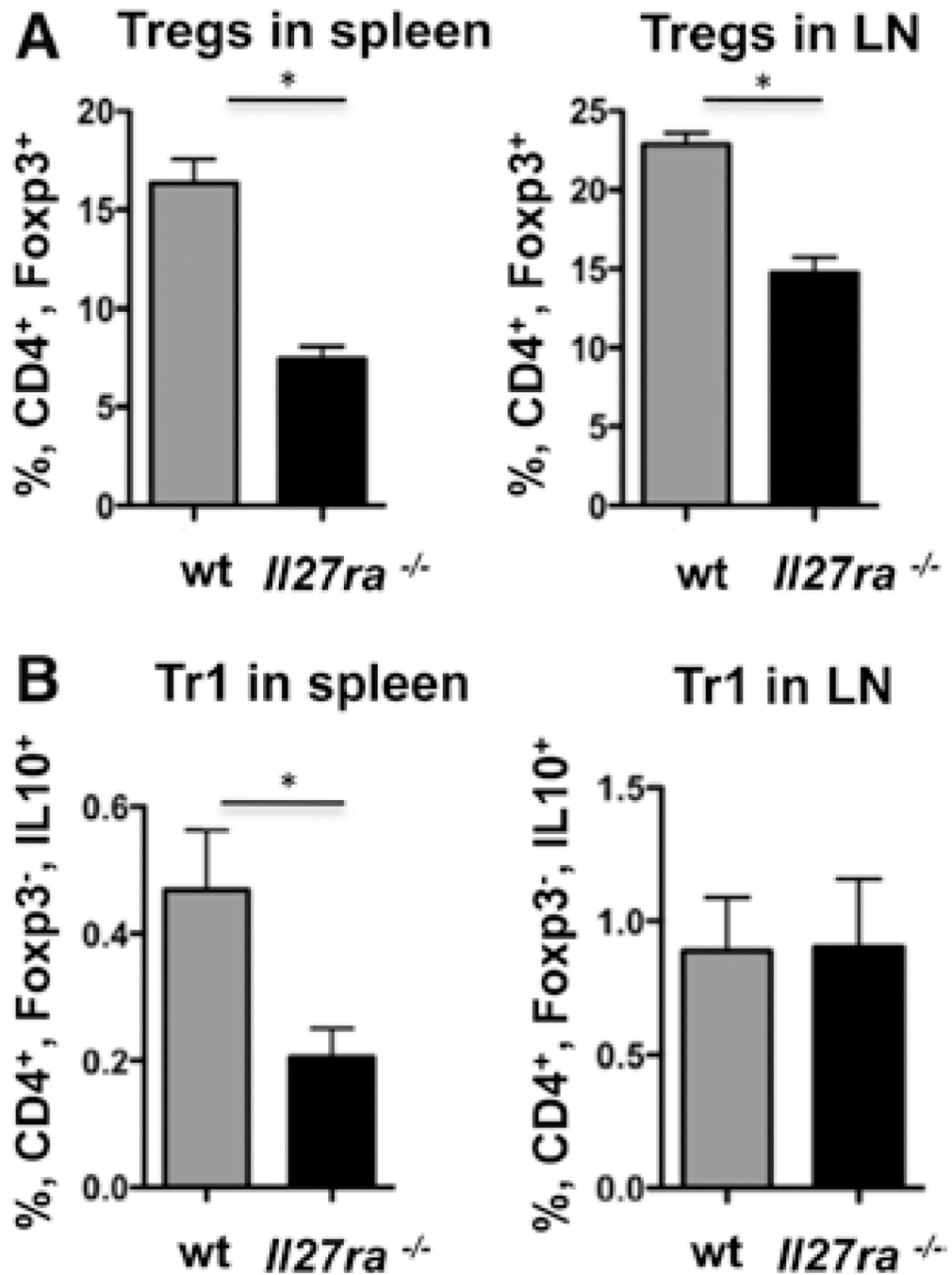
Figure 5. Regulatory T cells in spleen and lymph nodes in*Il27ra*$^{-/-}$ bone marrow–transplanted mice

A, Percentage of live CD4$^+$TCRβ$^+$Foxp3$^+$ T regulatory cells (Treg) in spleens and paraaortic lymph nodes (paLNs) from *Ldlr*$^{-/-}$ mice transplanted with *Il27ra*$^{-/-}$ (n=10) or wild-type (wt) bone marrow (n=10). **B**, Percentage of live CD4$^+$TCRβ$^+$, Foxp3$^-$IL10$^+$ Tr1 cells in spleens and paLNs of *Ldlr*$^{-/-}$ mice transplanted with *Il27ra*$^{-/-}$ (n=10) or wt bone marrow (n=10). Mean±SEM; *P*<0.05. LN indicates lymph node.

A
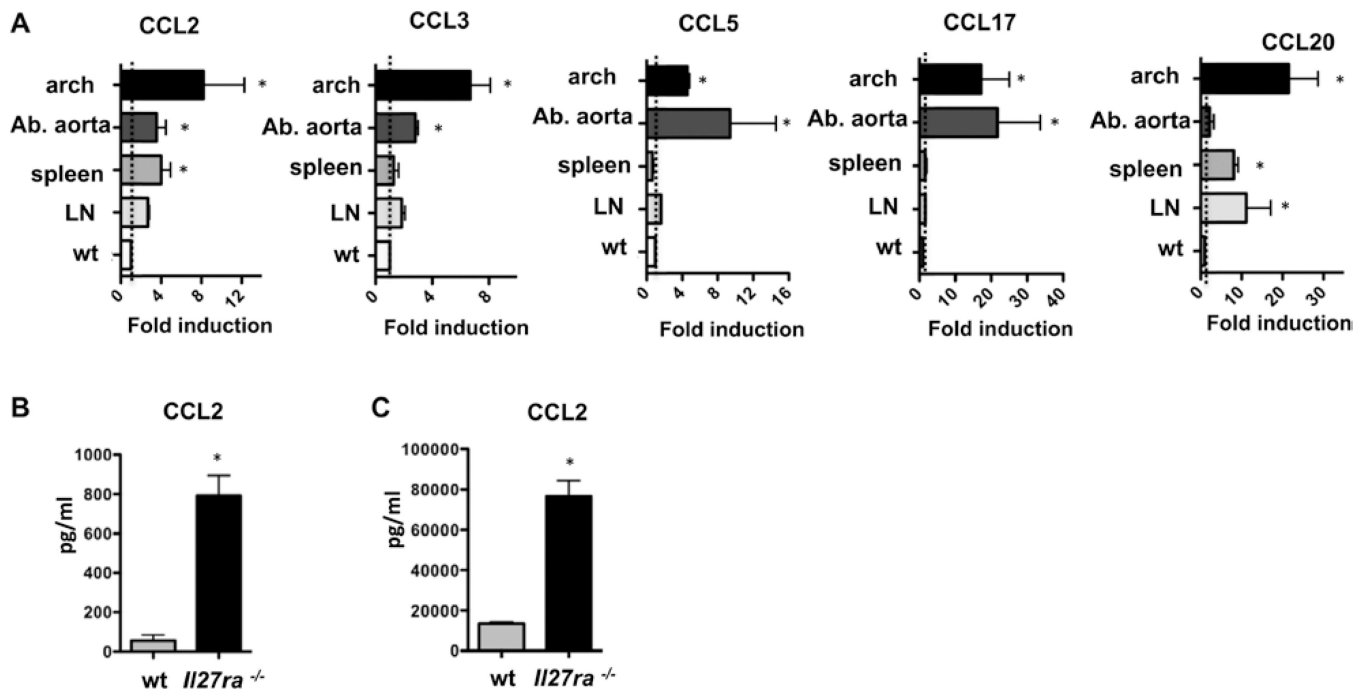
Figure 6. Analysis of interleukin (IL)-17–dependent chemokine expression in mouse aortas and lymphoid organs
A, Relative chemokine gene expression in *Il27ra*$^{-/-}$→*Ldlr*$^{-/-}$ (n=6) normalized to β-actin and then normalized to gene expression in wild-type (wt)→*Ldlr*$^{-/-}$ (n=6) mice in aortic arches, abdominal aortas (Ab. aortas), paraaortic lymph nodes (LNs), and spleens after 16 weeks of Western diet. CCL2 protein in supernatants of aortic cell suspensions unstimulated (n=6; **B**) or stimulated with anti-CD3/anti-CD28 for 48 h (n=8; **C**). Mean±SEM; *P<0.05.
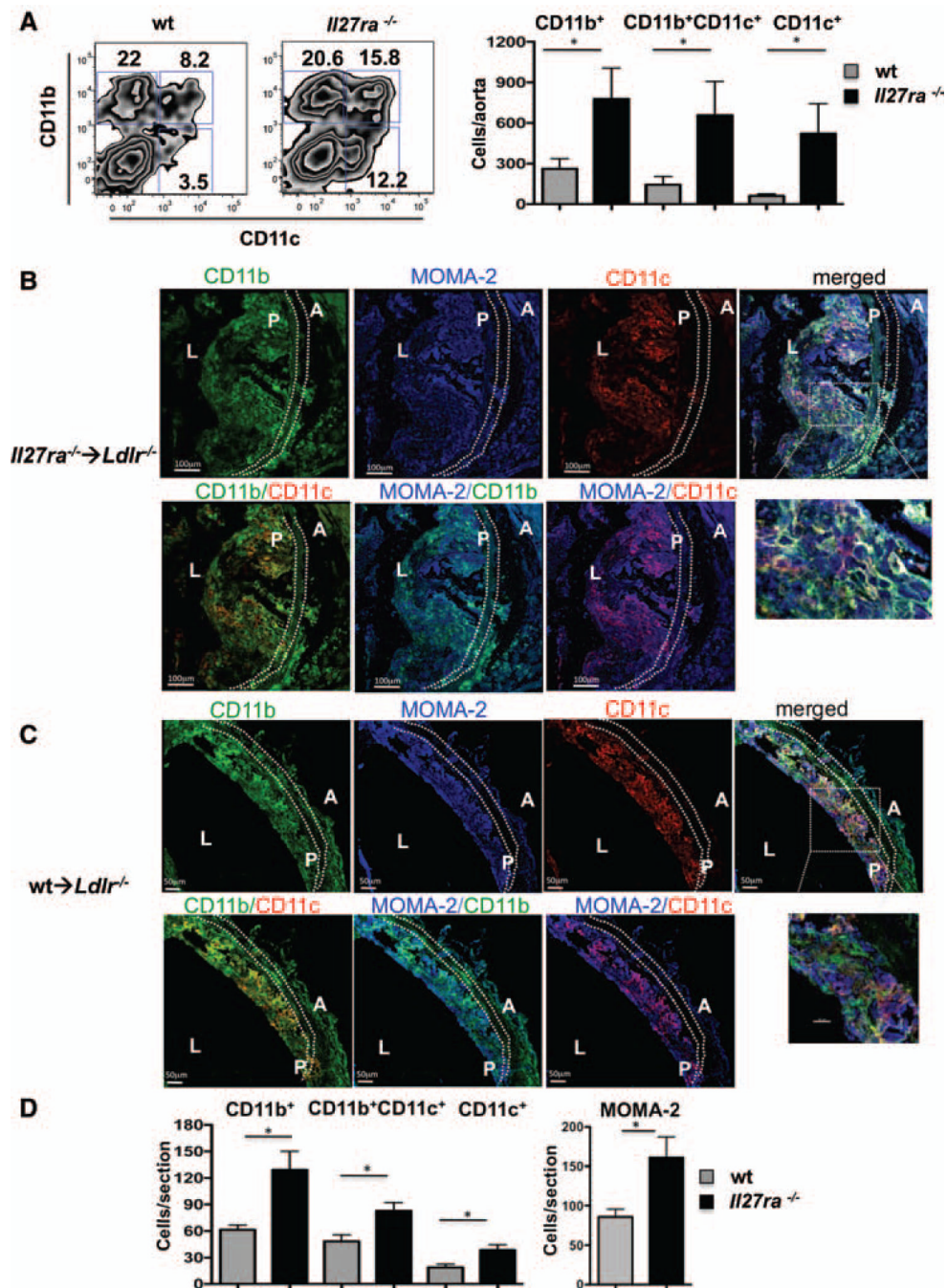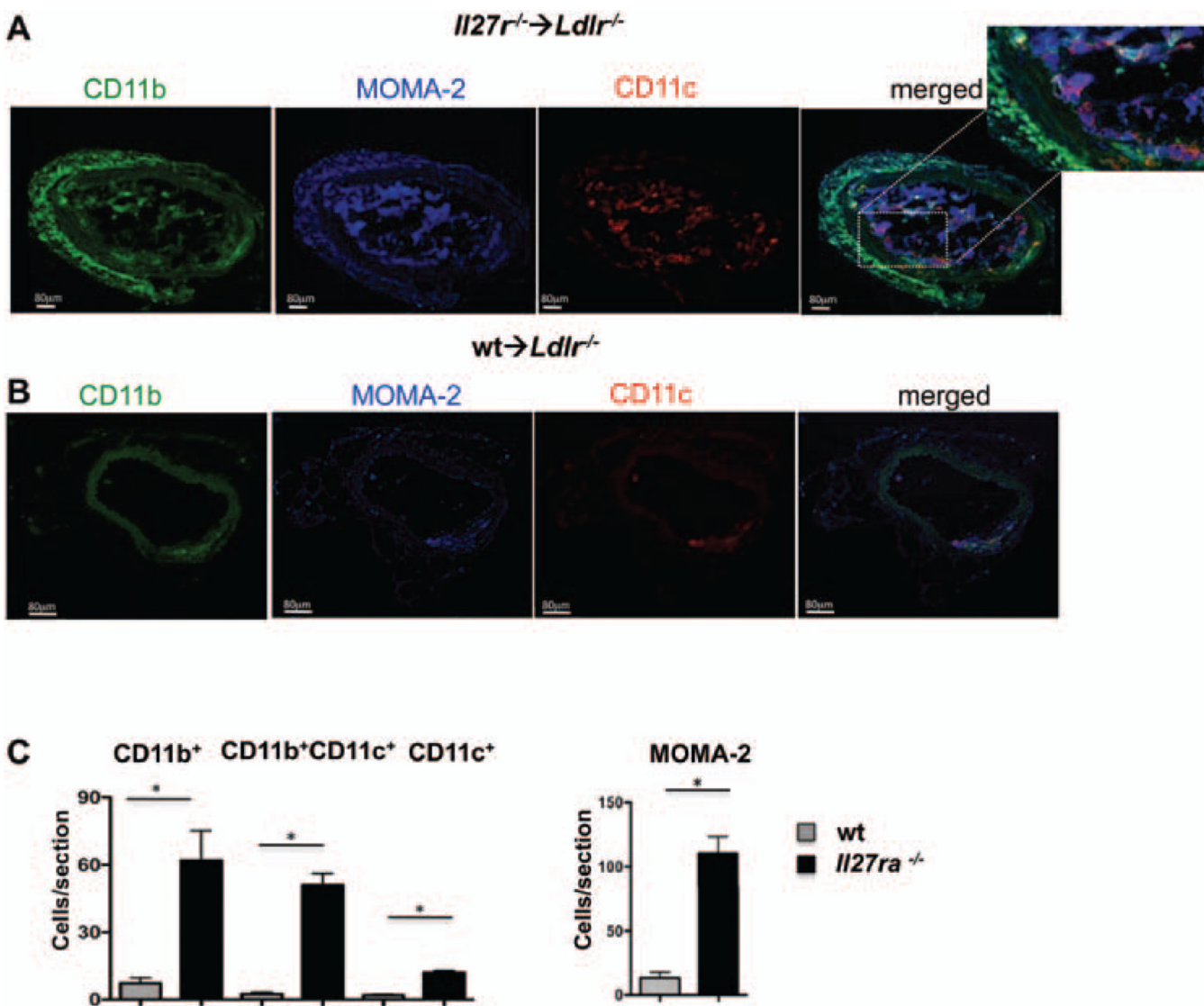
Figure 7. Interleukin-27 receptor (IL-27R) controls the recruitment of myeloid cells to atherosclerotic aortas

A, left, Live CD45^{+} cells from aortas of $Ldlr^{-/-}$ mice transplanted with bone marrow from $Il27ra^{-/-}$ or wild-type (wt) mice fed with Western diet for 16 weeks were stained for CD11b and CD11c. Numbers indicate percentage in each quadrant. **A, right**, Absolute number of live CD45^{+}, CD11b^{+}CD11c^{−}, CD11b^{+}CD11c^{+}, and CD11b^{−}CD11c^{+} cells per aorta of $Ldlr^{-/-}$ transplanted with $Il27ra^{-/-}$ (n=8) or wt (n=7) bone marrow was determined based on flow cytometry data. Mean±SEM; *$P<0.05$. **B** and **C**, Localization and abundance of CD11b^{+}CD11c[−](green), CD11b^{+}CD11c^{+} (yellow), CD11b^{−}CD11c[+](red), and MOMA-2^{+} (blue) cells in aortic roots of $Ldlr^{-/-}$ mice transplanted with $Il27ra^{-/-}$ (**B**) or C57BL/6 (wt;

C) bone marrow characterized by immunofluorescence. Dotted white lines indicate border of lamina muscularis. L indicates lumen; P, plaque; A, adventitia. Representative images from 1 of 6 independent experiments. **D**, Quantification of CD11b$^+$CD11c$^-$, CD11b$^+$CD11c$^+$, CD11b$^-$CD11c$^+$, and MOMA-2$^+$ cells in the aortic roots of *Ldlr*$^{-/-}$ mice transplanted with *Il27ra*$^{-/-}$ (n=6) or C57BL/6 (wt) (n=6) bone marrow as in **B** and **C**. Mean ±SEM; *P<0.05.

Figure 8. Interleukin-27 receptor (IL-27R) controls the recruitment of myeloid cells to abdominal aortas of atherosclerotic mice

Localization and abundance of CD11b$^+$CD11c$^-$(green), CD11b$^+$CD11c$^+$ (yellow), CD11b$^-$CD11c$^+$(**red**), and MOMA-2$^+$ (**blue**) cells in abdominal aortas of $Ldlr^{-/-}$ mice transplanted with $Il27ra^{-/-}$ (**A**) or C57BL/6 (wild-type [wt]) (**B**) bone marrow characterized by immunofluorescence. Dotted white lines indicate border of lamina muscularis. L indicates lumen; P, plaque; A, adventitia. Representative images from 1 of 6 independent experiments. **C**, Quantification of immunofluorescent staining for CD11b$^+$CD11c$^-$, CD11b$^+$CD11c$^+$, CD11b$^-$CD11c$^+$, and MOMA-2$^+$ cells in the abdominal aortas of $Ldlr^{-/-}$ mice transplanted with $Il27ra^{-/-}$ (n=6) or C57BL/6 (wt; n=6) bone marrow as shown in **A** and **B**. Mean±SEM; *P<0.05.