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Attenuation of Experimental Atherosclerosis by Interleukin-19

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Abstract

Objective. Interleukin-19 (IL-19) is putative Th2, anti-inflammatory interleukin. Its expression in, and potential role in atherogenesis is unknown. IL-19 is not detected in normal artery, and is expressed to a greater degree in plaque from symptomatic vs. asymptomatic patients, suggesting a compensatory-counter regulatory function. We tested if IL-19 could reduce atherosclerosis in susceptible mice, and identified plausible mechanisms.

Approach and Results. $LDLR^{-/-}$ mice fed an atherogenic diet and injected with either 1.0ng/g/day or 10.0ng/g/day rmIL-19 had significantly less plaque area in the aortic arch compared with controls ($p<0.0001$). Weight gain, cholesterol and triglyceride levels were not significantly different. Gene expression in splenocytes from IL-19 treated mice demonstrated immune cell Th2 polarization, with decreased expression of T-bet, IFNγ, IL-1β and IL-12β, and increased expression of GATA3 and FoxP3 mRNA. A greater percentage of lymphocytes were Th2 polarized in IL-19 treated mice. Cellular characterization of plaque by immunohistochemistry demonstrated IL-19 treated mice have significantly less macrophage infiltrate compared with controls ($p<0.001$). Intravital microscopy revealed significantly less leukocyte adhesion in wild-type mice injected with IL-19 and fed an atherogenic diet compared with controls. Treatment of cultured endothelial cells (EC), vascular smooth muscle cells (VSMC), and bone marrow-derived macrophages (BMDM) with IL-19 resulted in a significant decrease in chemokine mRNA, and in the mRNA-stability protein HuR.

Conclusions. These data suggest IL-19 is a potent inhibitor of experimental atherosclerosis, with diverse mechanisms including immune cell polarization, decrease in macrophage adhesion, and decrease in gene expression. This may identify IL-19 as a novel therapeutic to limit vascular inflammation.

Introduction

Atherosclerosis is a chronic vascular inflammatory disease (1). A series of cytokine-mediated interactions between lymphocytes, macrophage, endothelial, and vascular smooth muscle cells result in local inflammation of the arterial wall (1,2). While a multitude of potential mechanisms have been investigated for the initiation and propagation of atherosclerosis, the role of excess low density lipoprotein in inducing vascular inflammation is a widely acknowledged mechanism in atherogenesis. The Th1 arm of adaptive immunity is characterized by secretion of pro-inflammatory cytokines, and atherosclerosis in particular has been described as a Th1 inflammatory disease (3). Not surprisingly, a large number of studies advocate the importance of Th1 interleukins in the atherosclerotic disease process based on the predominance of Th1 lymphocytes and their cytokines in both human and mouse atherosclerotic lesions (4-6). By comparison, a much smaller number of studies focus on the role of endogenous counter-regulatory mechanisms in atherogenesis. The Th2 arm of immunity elaborate anti-inflammatory interleukins which tend to limit the magnitude of the inflammatory response. Despite their potential for anti-inflammatory effects, uncertainty remains concerning the potential for direct protective effects of Th2 positive cells and interleukins in atherogenesis. The majority of these important studies focus on the archetypical anti-inflammatory cytokine, IL-10. These principal studies suggest that reduction of atherosclerosis attributed to IL-10 is mediated by modulation of immune function by polarizing the T lymphocyte Th2/Th1 ratio toward a more anti-inflammatory phenotype (6-11). However, injection of the other Th2 interleukin, IL-4 into $ApoE^{-/-}$ mice does not reduce development of atherosclerotic lesions, and lesions were in fact reduced in IL-4/ApoE double knock out mice (12,13). Moreover, very little has been published regarding potential protective effects of Th2 interleukins on resident vascular cells (EC and VSMC) in addition to inflammatory cells. Recognition of other Th2 interleukins with anti-atherosclerotic effects, particularly those which may have direct anti-inflammatory effects on resident vascular cells in addition to immune polarization are needed and would have considerable therapeutic potential.

IL-19 was discovered in 2001, and is a member of an IL-10 sub-family which also includes IL-20, IL-22, and IL-24 (14,15). IL-19 is considered to be anti-inflammatory because in T-lymphocytes it promotes the Th2, rather than the Th1 response (16,17). IL-19 signals through the IL-20 heterodimeric receptor complex, a class II cytokine receptor (16,18). Like typical class II receptors, IL-20Rs signal through the JAK-STAT family of signal transducers, and IL-19 activates STAT3 in VSMC, and STAT3 and p44/42 in EC (19,20). Expression of these receptors is tissue-restricted and cytokine inducible (15). Aside from its anti-inflammatory effects, IL-19 is unique among interleukins, including members of its own family. For example, neither IL-10, IL-22, IL-24, nor IL-4 are expressed by EC or VSMC, precluding any autocrine effects of these interleukins on the vasculature (21). IL-19 expression has been associated with some immune-linked diseases, such as rheumatoid arthritis and psoriasis (22). Interestingly, psoriasis has recently been linked with atherosclerosis (23). We have reported a unique role for IL-19 in vascular disease (19,20,24). IL-19 is expressed in EC and VSMC in injured, but not naive arteries, and in stimulated, but not unstimulated cultured EC and VSMC (19,24). This was novel and unexpected because IL-19 expression was previously thought to be restricted to immune cells (14,15,18,21). Expression of IL-19 in atherosclerotic plaque has not been reported, and a role for IL-19 in development of atherosclerosis is currently unknown.

Many investigators have posited that atherosclerosis development is influenced by a balance between pro and anti-inflammatory, or Th1 and Th2 cytokine profile, implying that IL-19 may regulate the development of atherosclerosis (3,5-7). The goals of this study were to determine if IL-19 was expressed in atherosclerotic plaque, to test the hypothesis that IL-19

could reduce atherosclerosis in susceptible mice, and to identify potential mechanisms for these effects.

Materials and Methods

Mice and study design. LDL receptor knock out mice (stock #002207) of both sexes on the C57BL/6 background were purchased from Jackson labs housed in an ALAC-approved facility and maintained on a standard chow diet until study commencement. $LDLR^{-/-}$ were primarily used because these mice do not develop lesion until fed high fat diet, allowing us to synchronize initiation of atherosclerosis with IL-19 administration. Mice entered the study at 3-4 months of age to ensure they were robust enough to endure injections and have enough appetite to consume the atherogenic diet. Normal chow was replaced with an atherogenic diet (42% Fat, 0.2% cholesterol, Harlan atherogenic diet TD.88137) and injected i.p. with 1ng or 10ng/g/day mouse recombinant IL-19 (R&D Inc, Minneapolis, MN) or an equal volume of PBS for 5 days per week for 13 weeks for atherosclerosis, and 12 weeks for intravital microscopy analysis. Wild-type C57BL/6 mice purchased from Jackson labs were used for intravital microscopy and were similarly fed and treated. No mice were excluded from analysis. All animal procedures followed IACUC approved protocols.

Atherosclerotic lesion analysis. Atherosclerotic plaque was determined in the aortic intimal surface by en face staining with Sudan IV and in the aortic root by Oil Red O staining as we described (25). Lesion size in the aortic arch was quantitated by quantitative morphometry using the Image Pro Plus program. Aortic root was frozen in OCT medium and sectioned. Four transverse serial sections spaced 70-100μm apart from the aortic sinus to disappearance of valve cusps per aortic root from mice in each group were stained with Oil Red O, and positive stained areas quantitated as a percentage of total area by quantitative morphometry (26).

Immunohistochemistry. Human coronary vessels used in this study were graded by a board-certified pathologist and taken from a bank of human vessels excised post-mortem during routine autopsy at the LSU Heath Sciences Center in Shreveport, Louisiana (27). All experiments using human tissue were deemed non-human research by the LSU Institutional Review Board due to the exclusive use of postmortem samples. Immunofluorescence on ten different human coronary artery sections was performed as described (19,23). Briefly, primary antibody incubation was followed by a 30-minute incubation with secondary antibody conjugated to AlexaFluor 568 (red) and AlexaFluor 488 (green) (Molecular probes, Inc., Eugene, OR). Interleukin-19 antibody (Abcam, Inc., Cambridge, MA), GATA3, CD3, T-bet, CD4, FoxP3, CD25, smooth muscle cell αactin, Von Willebrand, and leukocyte common antigen (CD45) (NeoMarkers, Inc, Burlingame, CA), were used at 1.0μg/ml as described (19,23). For mouse aortic root, five-micrometer sections from aortic root fixed in OCT were blocked in 10% goat serum. Sections were incubated with primary antibody at 1μg/ml in 1%BSA/PBS and were applied for 1 hr, followed by incubation with biotinylated secondary antibody (1:200), followed by avidin-biotin peroxidase complex each for 30 min. Non-specific identical isotype control (Neomakers # NC-100-P, and Biolegend #400601) antibodies were used as negative controls. For quantitation of macrophage infiltrate and VCAM1 expression, four transverse serial sections spaced 70-100μm apart from the aortic sinus to disappearance of valve cusps per aortic root from at least 9 mice in each group were immunostained with anti-F4/80, VCAM-1, or CD3 purchased from NeoMarkers, Inc. Quantitative F4/80 and CD3 immunoreactivity was quantitated using the Image Pro Plus program as the percent of each lesion area which stains positive (26). CD4, T-bet, and GATA2 immunoreactivity was quantitated by counting the percentage of dual stained cells per high powered field.

Human carotid plaque sections: Forty human carotid plaques collected at carotid endarterectomies were analyzed and a summary of patient characteristics is found in Table XX. The indications for surgery were plaques associated with ipsilateral symptoms (transient ischemic attacks - TIA, stroke or amaurosis fugax) and stenosis greater than 70%, or plaques not associated with symptoms but stenosis >80%. Patients with ipsilateral carotid artery occlusion or restenosis after previous carotid endarterectomy were excluded from this study. Patients donated a venous blood sample one day before surgery. After surgical removal, plaques were immediately snap-frozen in liquid nitrogen. Plaques were weighed; cross-sectional fragments of one-mm from the most stenotic region were processed for histology as previously described (28). Sections of the one-mm-thick fragment of carotid atherosclerotic plaques were thawed, fixed with ice-cold acetone and permeabilized in 0.5 % Triton-X100 before blocking in 10 % serum. Primary antibody for IL-19 (1.0 µg/mL, Abcam) or IgG control (1.0 µg/mL, Abcam) was used together with biotinylated secondary goat anti-rabbit antibody (1:1000 dilution, Vector Laboratories). The ABC Vectastain Elite kit (Vector Laboratories) was used for visualization of antibody binding. Mounted slides were scanned with an Aperio ScanScope equipped with Console Version 8.2 (Aperio, Vista, CA) and photographed with Aperio ImageScope Version 12.0 (Aperio). The IL-19 positive area of the plaque (% area) was quantified under blind conditions using Biopix iQ Version 2.3.1 (Gothenburg, Sweden). Soluble CD40 ligand (sCD40L) was measured in plasma using Human Cytokine/Chemokine Immunoassay (Millipore Corporation, MA) and analyzed with Luminex 100 IS 2.3 (Austin, TX) according to the manufacturer's instructions and as previously described (29). The study conforms to the principles outlined in the Declaration of Helsinki and was conducted in accordance with approved local ethical guidelines (approval reference no. 472/2005-LUND). All patients gave their informed consent to participate.

Monocyte adhesion assay. Adhesion was assayed as described (30). Briefly, hCaECs were cultured on glass coverslips at a density of 6×10^5 cells/chamber. Confluent ECs were treated with IL-19 (100ng/ml) for 16 hours, then in the presence or absence of oxidized LDL (50µg/ml, Intracell, Inc.) for an additional 6 hours, followed by extensive washing with PBS. THP-1 human monocytes were purchased from the American Type Culture Collection (Cat# TIB-202) and cultured according to vendors instructions. Monocytes (5×10^5 cells/well) were labeled with 2′,7′-bis(2-carboxyethyl)-5(6)-carboxy-fluorescein acetoxymethyl ester (BCECF/AM, 10 µM, Sigma) and incubated with hCaECs for 30 min at 37°C. Unbound THP-1 cells were removed by gently washing twice with PBS, and adherent cells fixed with 4% formalin, photographed by an inverted fluorescent microscope (Eclipse TS-100, Nikon), and counted per high power field. Results are expressed as percentages of the controls and represent mean ± SEM from triplicate experiments.

Intravital microscopy. Leukocyte adhesion was assayed in mesenteric post-capillary venules by intravital microscopy as we described (31). Wild-type C57BL/6 mice were fed an atherogenic diet for 12 weeks, receiving either PBS or 10 ng/g/day 5 days per week IL-19 i.p. Three to four relatively straight, unbranched segments of post-capillary venules with lengths of >100 µm and diameters between 25 and 40 µm were randomly studied in each mouse using a Physiostation Microscope (Nikon Corp.), and the image recorded on A WIN XP Imaging Workstation. All data were analyzed using computerized imaging software. Leukocyte adherence was defined as the number of leukocytes firmly adhered to 100-µm length of endothelium for at least 30 seconds collected from at least 4 mice per group. Blood pressure measured by carotid artery cannulation using a blood pressure monitor (World Precision Instruments, Inc,. Sarasota, FL) was identical for all mice.

Serum lipid analysis. Fasting lipid content in mouse sera was analyzed by Charles River Research Animal Diagnostic Services (Wilmington, MA 01887 USA).

Cells and Culture. Primary human coronary artery vascular endothelial cells, and human coronary artery vascular smooth muscle cells were obtained as cryopreserved secondary culture from Cascade Corporation (Portland, OR) and maintained as we described (22,23). Cells were used from passage 3-5. For BMDM, femurs and tibiae were flushed with sterile DMEM, collected cells washed, resuspended in DMEM+5% FBS, and cultured overnight to remove adherent cells. Non-adherent cells were cultured for 6 days in DMEM+10% FBS in the presence of 100ng/ml M-CSF (Peprotech). The adherent cells were then detached by incubation with Versene solution (GIBCO). For gene expression analysis, cells were pretreated with 100ng/ml IL-19 (R&D, Inc. Minneapolis, MN) for various times, then stimulated with 20ng/ml TNFα (Sigma St. Louis, MO). Some samples remained untreated and used as controls.

RNA extraction and quantitative RT-PCR: RNA from cultured cells or spleen was isolated and reverse transcribed into cDNA as we have described, and target genes amplified using an Eppendorf Realplex4 Mastercycler (20,30). Multiple mRNAs (Ct values) were quantitated simultaneously by the Eppendorf software. Primer pairs were purchased from Integrated DNA Technologies, (Coralville, IA), SYBR green used for detection. The following primer pairs were used:
Human GAPDH: F: CGAGAGTCAGCCGCATCTT, R: CCCCATGGTGTCTGAGCG,
Human MCP-1: F AGCAGAAGTGGGTTCAGGATT, R: TGTGGAGTGAGTGTTCAAGTCT,
Human IL-1β: F: TCCCCAGCCCTT TTGTTG A, R: TTAGAACCAAATGTGGCCGTG,
Human IL-8: F: CCAGGAAGAAACCACGGA, R: GAAATCAGGGCTGCCAAG.
Mouse GAPDH: F: GCAAGGACACTGAGCAAGAG, R: GGGTCTGGGATG GAAATTGT,
Mouse MCP1: F:TTAAAAACCTGGATCGGAACCAA, R:
GCATTAGCTTCAGATTTACGGGT, Mouse IL-1β: F: CTAATAGGCTCATCTGGGATCC, R:
GGTCCGTCAACTTCAAAGAAC.
Mouse FoxP3: F: AAGTACCACAATATGCGACCC, R:TCTGAAGTAGGCGAACATGC
Mouse IFNγ: : CCTAGCTCTGAGACAATGAACG, R: TCAATGACTGTGCCGTGG
Mouse GATA3: F: TACCACCTATCCGCCCTATG, R: CTCGACTTACATCCGAACCC
Mouse T-bet: F: CCTGTTGTGGTCCAAGTTCAAC, R:CACAAACATCCTGTAATGGCTTGT
Mouse IL-19: F: AAATCTCTGGAGCGATGTCAG, R: GGCTAAAAGTATGTTCAGTTCTCC
Mouse IL-12p40: F: GTGAAGCACCAAATTACTCCG, R: AGAGACGCCATTCCACATG
Mouse CXCL2 (human IL-8 homolog): F: CAGAAGTCATAGCCACTCTCAAG, R:
CTCCTTTCCAGGTCAGTTAGC,
Mouse CXCL1 (human IL-8 homolog): F: AGAACATCCAGAGCTTGAAGG, R:
CAATTTTCTGAACCAAGGGAGC,
Mouse RORγ: F: TTTCTGAGGATGAGATTGCCC, R: TTGTCGATGAGTCTTGCAGAG

Western blotting and protein determination. Western blotting for HuR was performed as we described (20,30). Briefly, cultured EC, VSMC, and BMDM were treated with 100ng IL-19/ml continuously for 4 days, changing the media and adding fresh IL-19 every 24 hours. Extracts were prepared as described (20,30), and lysates frozen until use. Membranes were incubated with a 1:5000 dilution of HuR, GATA3, βactin (Santa Cruz, Inc.), or VCAM-1 (AbCam, Inc) antibody, and a 1:10,000 dilution of secondary antibody. Reactive proteins were visualized using enhanced chemiluminescence (Amersham) according to manufacturer's instructions. Spleens were removed at termination of the experiment and

immediately snap-frozen. Cytokine protein quantitation from spleen was determined by Luminex analysis (Millipore, Inc), according to manufacturer's instructions. Values were normalized for protein concentration of lysates.

Statistical analysis. Results are expressed as mean ± SEM. Differences between groups were evaluated with the use of ANOVA, with the Newman-Keuls method applied to evaluate differences between individual mean values or by paired t tests where appropriate,. Interquartile range determined by the GraphPad Prism statistical analysis program. Differences were considered significant when $p<0.05$.

Results

IL-19 is expressed in human atherosclerotic plaque. The atherosclerotic plaque microenvironment is biased to Th1 activation in humans and hypercholesterolemic mice (32). IL-19 content in atherosclerotic plaque has never been reported. Several human coronary arteries obtained post-mortem were immunostained with IL-19 antibody to characterize the cellular distribution in atherosclerosis. Overall, very little IL-19 immunoreactivity was detected in normal arteries, but surprisingly, we did observe consistent immunodetection in leukocyte, EC, and VSMC in Stary plaque types 4 and 5 (33). In the representative photomicrograph shown in Figure 1A, B, abundant IL-19 immunoreactivity localized within plaque from a human patient with type 4 plaque. Very little to no IL-19 immunoreactivity was detected in medial VSMC in this artery. We did not observe IL-19 expression in Stary plaque classification types 1 or 2. IL-19 cell-specific expression in a representative plaque was established in EC, VSMC, and inflammatory cells by immunoreactive co-localization with the EC marker Von Willebrand, smooth muscle cell α actin, and leukocyte common antigen CD45 (Supplemental Data IA).

Expression of IL-19 was also demonstrated in human carotid endarterectomy sections by immunohistochemistry (Fig. 1C-G). Consistent with a compensatory-counter regulatory role for this cytokine in regulation of atherosclerosis, significantly higher IL-19 levels were found in plaques from patients with symptoms (stroke, transient ischemic attacks, amaurosis fugax) when compared to those from patients without symptoms (P=0.03, n=20 in each group; Fig. 5I). Clinical characteristics of the individuals included in this study are described in Supplemental Data Table 1. Interestingly, a significant negative correlation was observed between the levels of IL-19 (expressed as % positive IL-19 area of the plaque) and levels of soluble CD40 ligand (sCD40L) measured in plasma (r= -0.368; p=0.042). CD40L is a key mediator of cell communication in the immune system and has the ability to trigger the production of inflammatory cytokines such as IL-1 to enhance the density of cell adhesion molecules in vascular endothelial cells (34). As in the atherosclerotic coronary arteries, IL-19 was clearly detected in multiple cell types in these samples (Fig 1E-F).

This is the first description of IL-19 expression in atherosclerotic plaque. IL-19 detection in plaque, but not medial VSMC suggested a compensatory-counter regulatory role for this cytokine in regulation of atherosclerosis.

IL-19 decreases atherosclerotic plaque area in LDLR$^{-/-}$ mice. We hypothesized that systemic administration of IL-19 would be protective and decrease atherosclerosis. Similar to human, little to no IL-19 is detected in normal mouse aorta, but abundant IL-19 is detected in lesions from LDLR$^{-/-}$ mice (Supplemental Data Figure 1B). LDLR$^{-/-}$ mice were utilized for inhibition experiments because they do not develop atherosclerotic lesions until fed a high fat diet, allowing synchronization of initiation of atherosclerosis with IL-19 administration. Mice were injected i.p. with 10ng/g/day murine recombinant IL-19, or an equal volume of PBS for 5 consecutive days per week for 13 weeks. Surface lesion area determined by en face staining of aortic arch and quantitative morphometry show a significant reduction in lesion area between the PBS control and IL-19-treated mice (Figure 2A) (13.9±0.9% vs. 2.9±0.25%, respectively; $p<0.0001$, n=13 in each group). There was no significant difference in lesion area between sexes in either group. Similar results were obtained using ApoE$^{-/-}$ mice fed an atherogenic diet for 12 weeks (26.78±3.41% vs. 10.36±0.94%, $p<0.0001$, for PBS and IL-19-treated mice, respectively). Lesion area assessed by quantitative morphometry in multiple serial transverse sections of Oil Red O stained aortic root was significantly reduced in IL-19 injected mice compared with PBS controls (24.7±1.8% vs. 16.6±1.5% for PBS and IL-19 treated, respectively, $p<0.01$) (Figure 2C). In a second cohort we determined that systemic administration of as little as 1ng/g/day IL-19

could significantly reduce lesion area (17.7±1.7% vs. 5.3±1.2% for PBS and IL-19 treated mice $p<0.0001$, n=11 and 13, respectively) (Figure 2D). Interquartile range values for all studies are presented in Table 1. Lesion area in Oil Red O stained aortic root was significantly lower in 1ng/g/day IL-19-injected mice compared with PBS controls (27.4±1.1% vs. 18.6±1.1% for PBS and IL-19 treated, respectively, $p<0.01$) (Figure 2E.). There was no significant difference in serum lipid profiles (Figure 3A and B). There was no significant difference in weight gain (11.29±0.8 vs. 10.95±0.7 grams for PBS and IL-19 in the 10ng/g/day, or 11.99±1.4 vs. 8.9±0.9 grams for PBS and IL-19 in the 1ng/g/day study, respectively) (Figure 3C) during the course of either dose study. This is the first report demonstrating that systemic administration of IL-19 is anti-atherogenic, and the remainder of the study was directed toward elucidating mechanisms for this effect.

IL-19 polarizes leukocytes to a Th2-like profile. Atherosclerosis is highly influenced by the Th1/Th2 balance (31,32). The global immune status of these mice was determined by quantitation of Th1 and Th2 marker expression in splenocytes immediately removed from mice at the termination of the study. Quantitative RT-PCR demonstrates that splenocytes from IL-19 injected mice had significantly lower mRNA levels of the Th1 markers T-bet (0.82±0.05 vs. 0.66± 0.04 for PBS and IL-19 treated, respectively, $p<0.05$), and IFNγ (0.89±0.05 vs. 0.68±0.02 for PBS and IL-19 treated, respectively, $p<0.05$) (Figure 4). Concordantly, splenocytes from IL-19 injected mice had significantly higher levels of Th2 markers GATA3 (1.06±0.08 vs. 1.62±0.22 for PBS and IL-19 respectively $p<0.001$), and FoxP3 (1.08±0.05 vs. 1.68±0.15 for PBS and IL-19, respectively, $p<0.001$) mRNA. IL-19 treated mice had significantly higher RORγ mRNA compared with controls (3.93±0.27 vs. 6.23±0.46 for PBS and IL-19, respectively, $p<0.01$). Quantitative RT-PCR also shows mRNA for IL-1β and IL-12p40, both potent pro-inflammatory cytokines, are significantly decreased in IL-19 injected mice, (0.78±0.28 vs.0.61±0.5 for IL-1β, $p<0.001$, and 0.88±0.30 vs. 0.72±0.04 $p<0.01$, for PBS and IL-19, respectively). Cytokine protein was assayed by quantitative Luminex analysis and determined significantly less IFNγ and IL-1β protein in IL-19-treated mice compared with controls (16.70±0.8 vs. 9.84±1.3 pg/ml IFNγ for PBS and IL-19, and 33.20±5.5 vs. 21/54±2.4 pg/ml IL-1β for PBS and IL-19, respectively, $p<0.05$). GATA3 protein is also increased in spleen from IL-19 injected mice (Supplemental data IID).

Local lymphocyte polarization in plaque was characterized by immunohistochemistry (Supplemental Data IIA,B and Figure 5). A significant increase in the percentage of GATA3 positive T lymphocytes was noted in IL-19 treated mice, compared with PBS injected controls (75.10±4.70% vs. 56.15±6.15 for IL-19 and PBS, respectively, $p<0.05$) (Figure 5A). No difference in T-bet positive T lymphocytes was noted between groups. Collectively, these data suggest one potential mechanism for the IL-19 anti-atherogenic effect is polarization of the immune response to the Th2 and possibly, T regulatory phenotypes.

IL-19 decreases macrophage accumulation in atherosclerotic lesions. To further characterize the cellular content of atherosclerotic lesions, macrophage infiltrate was assessed by immunohistochemistry. Multiple serial sections throughout the aortic root from IL-19 treated and control mice were immunostained using F4/80 antibody to detect macrophage (Supplemental Data IIC). Positively stained areas were quantitated as a percentage of total area by quantitative morphometry. Significantly less macrophage infiltrate in plaque from IL-19 injected mice was observed compared with PBS control mice (20.0±2.7% vs. 36.5±2.2%, respectively, $p<0.001$) (Figure 5C). These data suggest that at least in this experimental model of atherosclerosis, IL-19 can reduce macrophage accumulation.

IL-19 decreases leukocyte-endothelial adhesion in mice fed an atherogenic diet. Decreased macrophage infiltrate in atherosclerotic lesions suggested that IL-19 could reduce leukocyte-EC interaction induced by a chronic atherogenic diet *in vivo*. For these experiments, wild-type C57BL/6 mice were fed an atherogenic diet for 12 weeks, and injected 5 consecutive days per week with 10ng/g/day IL-19 or PBS. Leukocyte-EC interaction was assessed *in vivo* by quantitative intravital microscopy. Figure 5D shows that IL-19 significantly reduced leukocyte adhesion induced by an atherogenic diet (2.1±0.4 for no atherogenic diet, and 3.5±.78 vs. 1.5±0.5 cells/100μm for PBS and IL-19 treated mice, respectively, $p<0.05$). There was no statistical difference in rolling between PBS and IL-19 treated mice. Three additional experiments were performed to characterize a cellular mechanism. First, immunohistochemistry of VCAM1 abundance in plaque was performed. Positively stained areas were quantitated as a percentage of total area and determined significantly less VCAM1 immunoreactivity in IL-19-treated compared with PBS control mice (32.52±4.4% vs. 57.43±6.08% for IL-19 and PBS, respectively, $p<0.01$) (Figure 5E and Supplemental Data III). Second, we used an endothelial cell monolayer adhesion assay and determined that pretreatment of cultured EC with IL-19 could significantly reduce monocyte adhesion to EC oxLDL stimulated EC monolayers (89.9±17.3 vs. 41.1±16.3 monocytes/HPF, $P<0.05$) (Figures 5F and G.) . Third, Human ECs were pre-treated with IL-19 prior to stimulation with oxLDL, and lysates blotted with anti-VCAM1 antibody. Supplemental Data IIIB,C shows that IL-19 pre-treatment significantly decreases VCAM-1 protein abundance. Collectively, these data suggest that a second mechanism for IL-19 anti-atherosclerotic effects is a reduction in leukocyte-EC interactions.

IL-19 decreases expression of chemokines in cultured EC, VSMC, and macrophage. Leukocyte homing to the atherosclerotic lesion is mediated by the local chemokine gradient. We investigated if IL-19 could decrease expression of chemokines in EC, VSMC, and macrophage. Cultured human coronary artery EC, VSMC, and mouse bone marrow-derived macrophages (BMDM) were pre-treated with IL-19 for varying times, then challenged with TNFα. Cytokine mRNA was determined by quantitative RT-PCR. Figures 6A-C demonstrate that in all cell types tested, IL-19 has a potent inhibitory effect on IL-1β, IL-8, and MCP-1 mRNA accumulation, each of which is a potent leukocyte chemoattractant. Interestingly, efficacy of IL-19 inhibition of mRNA varied for different cell types. Differential sensitivity to IL-19 implies complex and perhaps cell type-specific regulatory mechanisms for IL-19 inhibitory effects. Together, this demonstrates that IL-19 can have direct anti-atherogenic effects on resident vascular cells as well as immune cells, suggesting a third mechanism for IL-19 anti-atherosclerotic effects.

We previously reported that IL-19 does not inhibit NF-κB activity, but transiently decreases activation of the mRNA stability factor HuR (20,30). Each of the chemokines tested contains an ARE (AU-rich stability element) in its 3'UTR, which is recognized by HuR. To determine potential molecular mechanisms for IL-19 anti-inflammatory effects, we chronically treated cultured EC, VSMC, and BMDM with IL-19 continuously for 4 days, changing the media and adding fresh IL-19 every 24 hours. Figure 6D shows that chronic stimulation of each of these cell types with IL-19 decreases protein abundance of HuR. This is significant as HuR preferentially stabilizes inflammatory transcripts.

Discussion

The major finding from this manuscript is that systemic administration of IL-19 attenuates atherosclerosis in LDLR$^{-/-}$ mice. Th2 adaptive immune polarization, reduction in macrophage infiltration, and a decrease in macrophage, EC, and VSMC inflammatory cytokine gene expression are likely mechanisms. This is the first description of IL-19 inhibition of atherosclerosis, and is conceptually novel in that IL-19 can have direct atheroprotective effects on non-immune cells. It was unexpected that we were able to detect IL-19 protein in multiple cell types in atherosclerotic plaque from human patients in both coronary and carotid arteries, as current understanding indicates that Th1 cytokine expression dominates, in contrast to Th2 cytokines which are far less prevalent in human atherosclerotic lesions (6,35,36). It was particularly interesting that IL-19 immunoreactivity localized primarily to the plaque, and little to none was detected in medial VSMC, which is similar to expression of IL-20R1 and R2 which was detected in EC and mononuclear cells in human atherosclerotic lesions, but at much lower levels in normal aorta sections (37). IL-19 expression in plaque but not normal media suggested a compensatory, or counter regulatory function for this cytokine. Similarly, elevated levels of IL-19 in plaque from symptomatic patients may occur in an attempt to limit local inflammation. Longitudinal, prospective studies will be required to test this conjecture. Further, IL-19 expression in resident vascular cells (EC and VSMC) also implied a potential novel function for IL-19 independent of lymphocyte Th2 polarization.

Recently, serum IL-19 levels in humans have been reported (38). In patients selected for coronary artery bypass graft surgery (CABG), IL-19 levels were 34.4±17.6 ng/ml prior to surgery. IL-19 rose to 541.3±110.4 ng/ml 24 hours post-surgery, and declined to 77.2±24.9 ng/ml 96 hours after surgery. In our study, systemic administration of 10ng/g/day IL-19 almost completely inhibited plaque formation in the aortic arch, and as little as 1ng/g/day IL-19 decreased plaque area by 70%, suggesting potent anti-atherosclerotic effects of IL-19. This is a straight-forward study design that does not rely on any genetic modification of IL-19, and suggests therapeutic potential for IL-19. IL-19 reduction of plaque size is likely not due to reduction in lipid as there is no significant difference in total serum cholesterol, triglycerides, HDL or LDL in IL-19 treated mice compared with controls, nor is there any difference in weight gain between these two populations. In contrast to IL-19, IL-10 does appear to have lipid-lowering effects, as serum cholesterol is significantly reduced in mice receiving IL-10, which also may have contributed to decreased plaque in that study (10).

Other Th2 interleukins have been shown to decrease atherosclerosis in mice; albeit with less potency than IL-19. Systemic IL-10 gene expression mediated by adenovirus injection reduced atherosclerosis by immune cell modulation and reduction in inflammatory cell infiltrate in plaque (10). Transfer of bone marrow from IL-10$^{-/-}$ mice into LDLR$^{-/-}$ mice resulted in increased atherosclerotic plaque formation compared with controls, which was attributed to a decrease in macrophage foam cell apoptosis and monocyte activation (11). Similarly, LDLR$^{-/-}$ mice receiving BM from IL-10 transgenic mice showed a significant decrease in plaque area (8). The synthesis of these studies suggest IL-10 anti-atherogenic effects are mediated by modulation of the adaptive immune response. Intimal area of cross sections of atherosclerotic plaque was reduced in ApoE$^{-/-}$ mice receiving serial injections of 1μg of IL-33; however, lesion area was not measured in this study (39,40). Counter intuitively, IL-4$^{-/-}$ mice do not have increased atherosclerosis, and subcutaneous injection of 1.1/g/day recombinant IL-4 into ApoE$^{-/-}$ mice does not reduce development of atherosclerotic lesions (12,41). Further, lesions were actually reduced in area in IL-4$^{-/-}$/ApoE$^{-/-}$ double knockout mice, and reconstitution of LDLR$^{-/-}$ mice with IL-4$^{-/-}$ bone marrow also reduces lesion formation.

A tip in the Th1/Th2 balance of these seemingly opposing forces toward the Th2 profile has been proposed to have anti-atherosclerotic effects (3-5). Gene expression in splenocytes from IL-19 injected mice indicated that IL-19 appeared to induce Th2 polarization, as Th1 markers T-bet and IFNγ, as well as the pro-inflammatory cytokines IL-1β and IL-12p40 were decreased, and Th2 and T reg markers such as GATA3 and FoxP3 were increased in IL-19-injected mice. This shift in immune system polarization may provide at least one mechanism for IL-19's anti-atherosclerotic effects. Like most studies which utilize animal models, one limitation of this study is that the definitive distinctions in immune polarization observed in mice are often much less clear in humans.

T regulatory cells express the FoxP3 (forkhead/winged helix) transcription factor, and a protective role for this T cell subset has been proposed (41). We were able to detect increased FoxP3mRNA in spleen from IL-19 treated mice, but were not able to detect FoxP3 positive cells in plaque, which could suggest that adaptive immunity may be shifted, but not the local response in the plaque. Alternatively, it has been shown that FoxP3 positive cells are present in very low numbers in plaque, and qRT-PCR from spleen is more sensitive than immunohistochemistry from sections of aortic root (43). Future studies are needed to characterize IL-19 effect on global and local adaptive immunity in the context of atherosclerosis.

Monocyte adhesion constitutes a key cellular event in initiation of atherosclerosis (44). Cellular characterization of plaque determined significantly less macrophage infiltrate in IL-19 injected mice compared with PBS controls. Extending these data, quantitative intravital microscopy determined that leukocyte adhesion is decreased in wild-type mice fed an atherogenic diet One limitation of the intravital study is that endothelium of mesenteric post-capillary venules may not faithfully mirror conditions of the developing atherosclerotic plaque. However, it does allow for direct quantification of leukocyte trafficking in vivo and supports our immunohistochemical determination that IL-19 can reduce leukocyte/EC interactions in an inflammatory environment. The present study also showed that IL-19 can reduce oxLDL-driven VCAM-1 expression in cultured EC, and also reduce monocyte adhesion to oxLDL-stimulated EC monolayers. This is in contrast to IL-4, which has been shown to increase VCAM-1 expression and leukocyte adhesiveness (45). Together, these approaches identify a second mechanism whereby IL-19 may attenuate plaque formation.

IL-19 expression in EC, VSMC, and macrophage in atherosclerotic plaque led us to hypothesize that IL-19 would have anti-inflammatory effects on these cells. Treatment of each these cells with IL-19 prior to TNFα stimulation lead to a significant decrease in mRNA for MCP-1, IL-8, IL-1β, all potent chemoattractants. This may account for the observed decreased macrophage accumulation in plaque, and also decreased adhesion assayed by intravital microscopy. IL-10 is acknowledged to reduce NF-κB activity in VSMC, but did not reduce TNFα or IL-1β-induced expression inflammatory genes (46). In the present study, IL-19 inhibition of mRNA varied for different cell types suggesting complex, cell specific regulatory mechanisms for IL-19 inhibitory effects. Taken in total, our present data are particularly intriguing in their demonstration that a Th2 interleukin can have direct anti-inflammatory effects on cells outside of the T cell lineage, particularly EC and VSMC.

A plausible mechanism for IL-19 induced decrease in these transcripts is its effect on HuR, an inflammation-specific mRNA stability factor (47). We previously reported that in cultured, primary human VSMC, IL-19 inhibitory effects are NF-κB-independent, but did decrease the mRNA stability of inflammatory transcripts by decreasing nuclear to cytoplasmic translocation of HuR (20,30). The present study extends that report, showing that a single addition of IL-19 can rapidly and transiently decrease mRNA abundance of chemokine transcripts in multiple cell types. We then attempted to mirror the *in vivo* scenario in which IL-19 is injected into mice on a daily basis by multiple additions of IL-19 to

these cells and demonstrated HuR protein abundance was reduced. Thus, both reduction in HuR cytoplasmic translocation as well as protein abundance is induced by IL-19. IL-19 regulation of HuR is of note because the modulation of mRNA stability is recently gaining attention as a potential novel therapeutic modality (48). Decreased HuR protein abundance, with subsequent reduction in inflammatory gene mRNA provides a third mechanism for IL-19 attenuation of atherosclerosis.

In summary, IL-19 is expressed in multiple cell types in human atherosclerotic plaque, demonstrated efficacy of IL-19 to reduce atherosclerotic lesion size in mice. The probable mechanisms are a polarization of adaptive immunity to the Th2 phenotype, a decrease in macrophage infiltrate into the plaque, and a decrease in inflammatory gene expression in EC, VSMC, and macrophage. What is particularly important about this study is the ability of EC and VSMC to respond to IL-19. This is potentially paradigm altering as it suggests that resident vascular cells can respond to IL-19 and assume a Th2-like, anti-inflammatory phenotype to promote resolution of the local vascular response to injury. A limitation of the present study is that it can not identify if the protection imparted by IL-19 is mediated primarily by adaptive immune system polarization, a decrease in macrophage infiltrate into the lesion, or by direct anti-inflammatory effects on resident vascular cells, or combinations thereof. Future studies will elucidate the precise cellular mediator(s) of IL-19 effects.

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Disclosures: None

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SIGNIFICANCE

Despite dietary modification and lipid reducing medications, atherosclerotic vascular syndromes account for 50% of all mortality in the United States and is increasing in the developing world. This manuscript describes expression of Interleukin-19, a recently described anti-inflammatory cytokine, in human atheromatous plaque. Administration of IL-19 can significantly decrease severity of atherosclerosis in several murine models by multiple pleiotropic mechanisms, including adaptive immune system polarization, decrease in leukocyte-endothelial interaction, and reduction in inflammatory gene expression in macrophage and resident vascular cells. Together these data support the hypothesis that expression of IL-19 by immune and resident vascular cells is a novel compensatory counter-regulatory mechanism which attenuates atherosclerosis, and suggests that IL-19 itself has therapeutic potential for inhibition of atherosclerosis.

Table 1. Interquartile range (IQR) values for all studies.

Study	mouse	condition	median (%)	IQR (%)
10ng/g/day	($LDLR^{-/-}$)	PBS	16.83	14.73-21.72
		IL-19	2.00	1.32- 2.75
1ng/g/day	($LDLR^{-/-}$)	PBS	18.41	11.27-27.81
		IL-19	3.81	2.50- 6.72
10ng/g/day	($ApoE^{-/-}$)	PBS	25.45	18.06-43.92
		IL-19	10.24	8.05-12.06

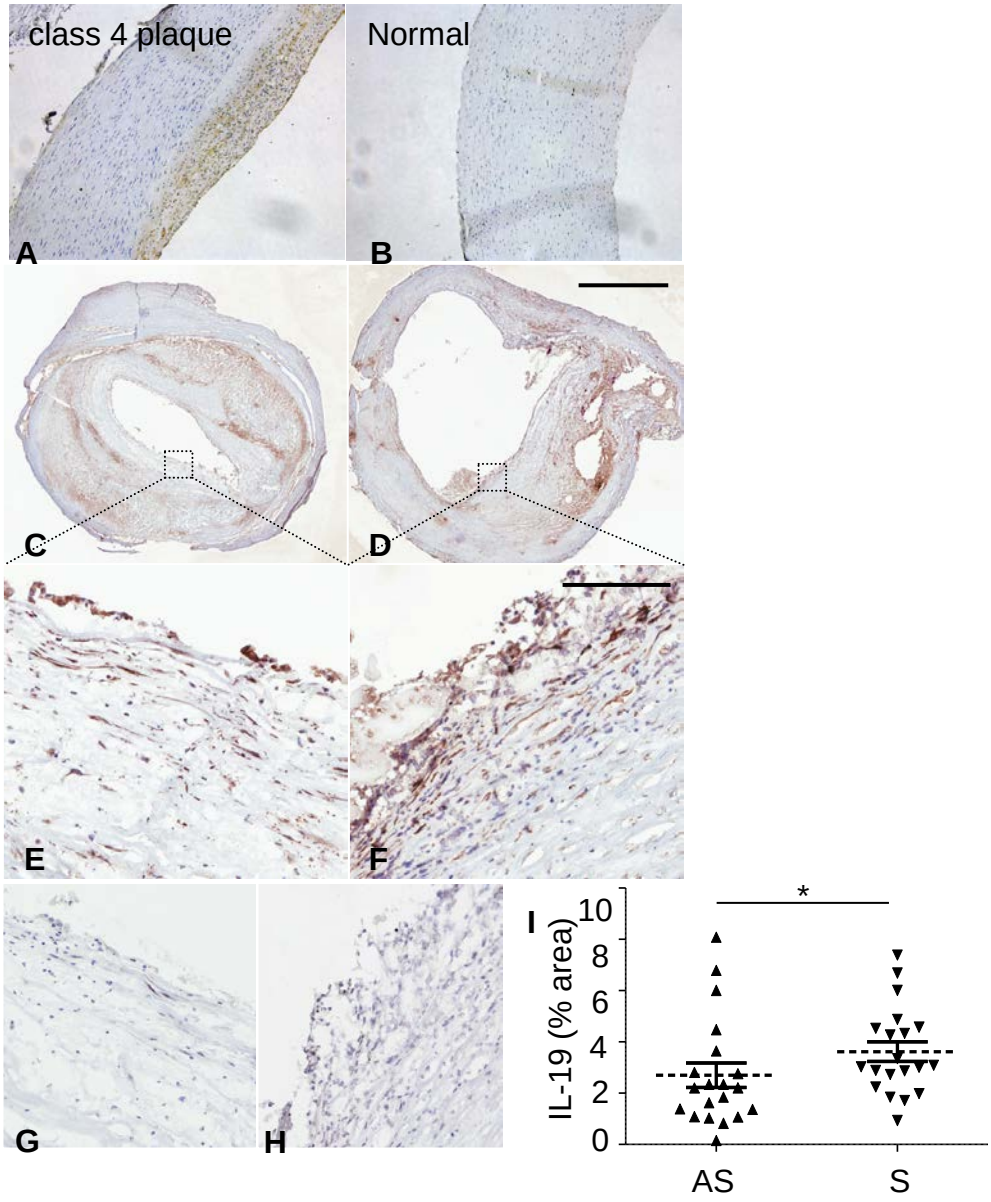

Figure 1

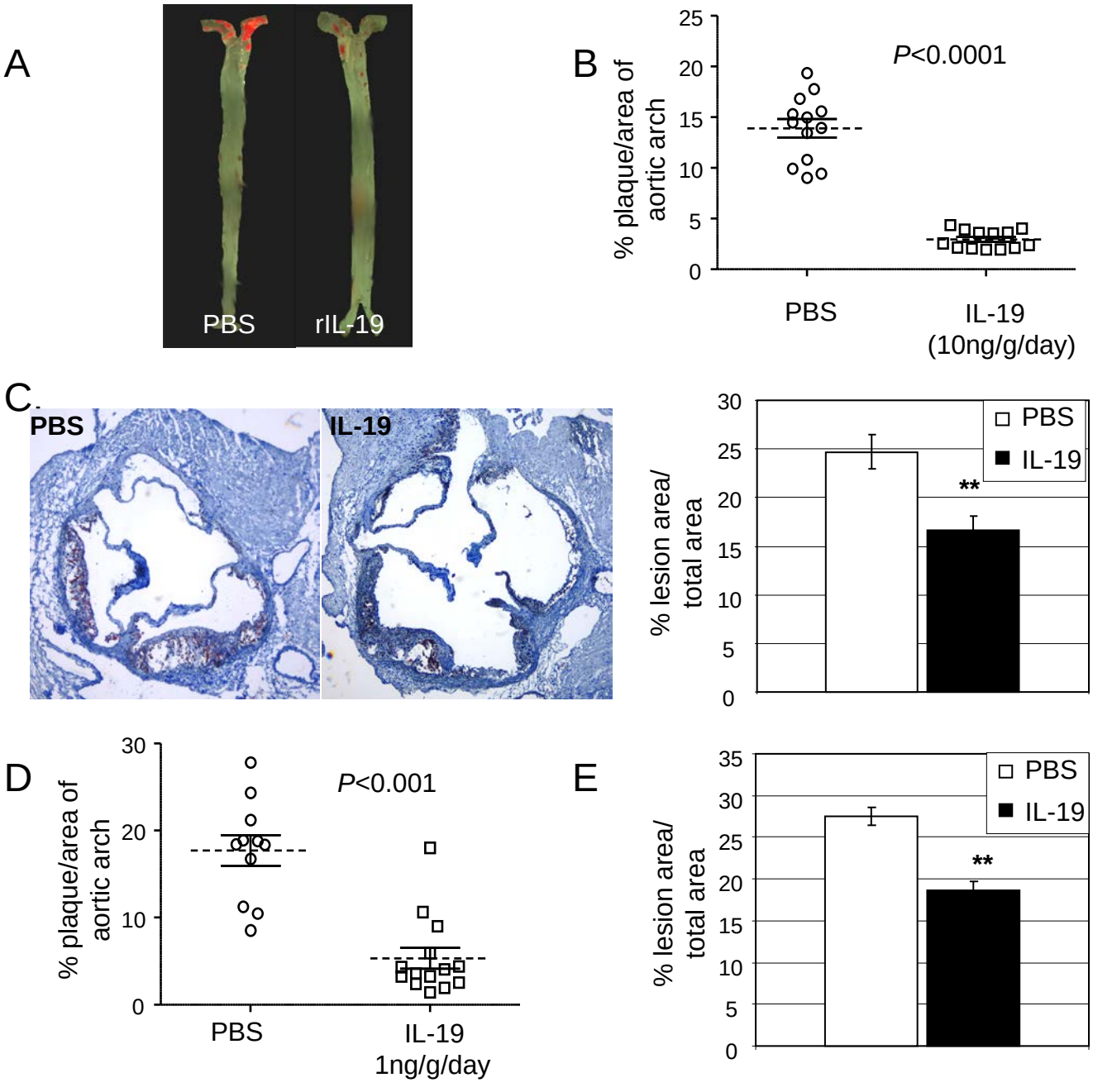
A
PBS
rIL-19
B
% plaque/area of aortic arch
P<0.0001
PBS
IL-19 (10ng/g/day)
C
PBS
IL-19
% lesion area/ total area
PBS
IL-19
**
D
% plaque/area of aortic arch
P<0.001
PBS
IL-19 1ng/g/day
E
% lesion area/ total area
PBS
IL-19
**

Figure 2

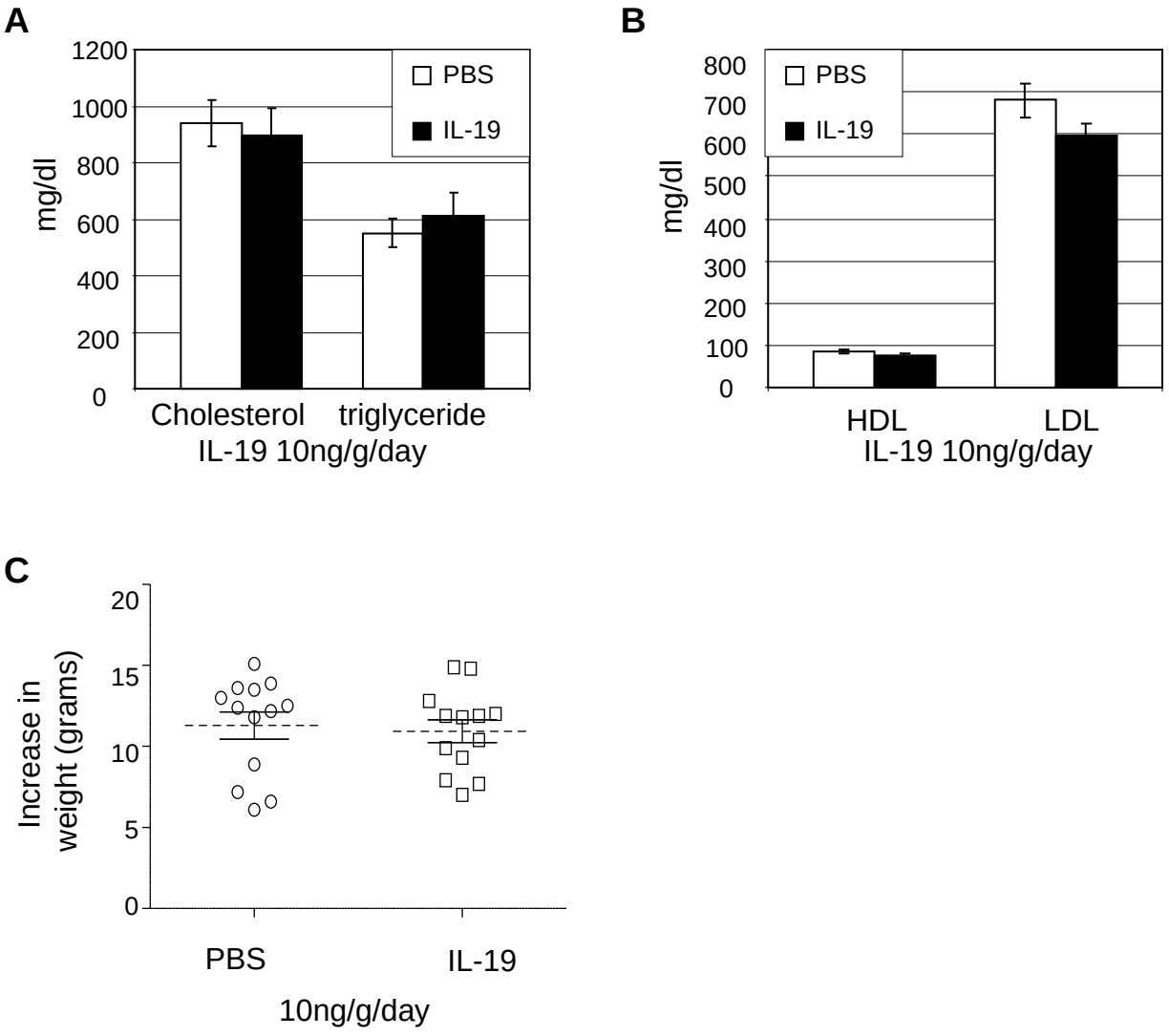
A
1200
1000
800
600
400
200
0
mg/dl
PBS
IL-19
Cholesterol
triglyceride
IL-19 10ng/g/day
B
800
700
600
500
400
300
200
100
0
mg/dl
PBS
IL-19
HDL
LDL
IL-19 10ng/g/day
C
20
15
10
5
0
Increase in weight (grams)
PBS
IL-19
10ng/g/day

Figure 3

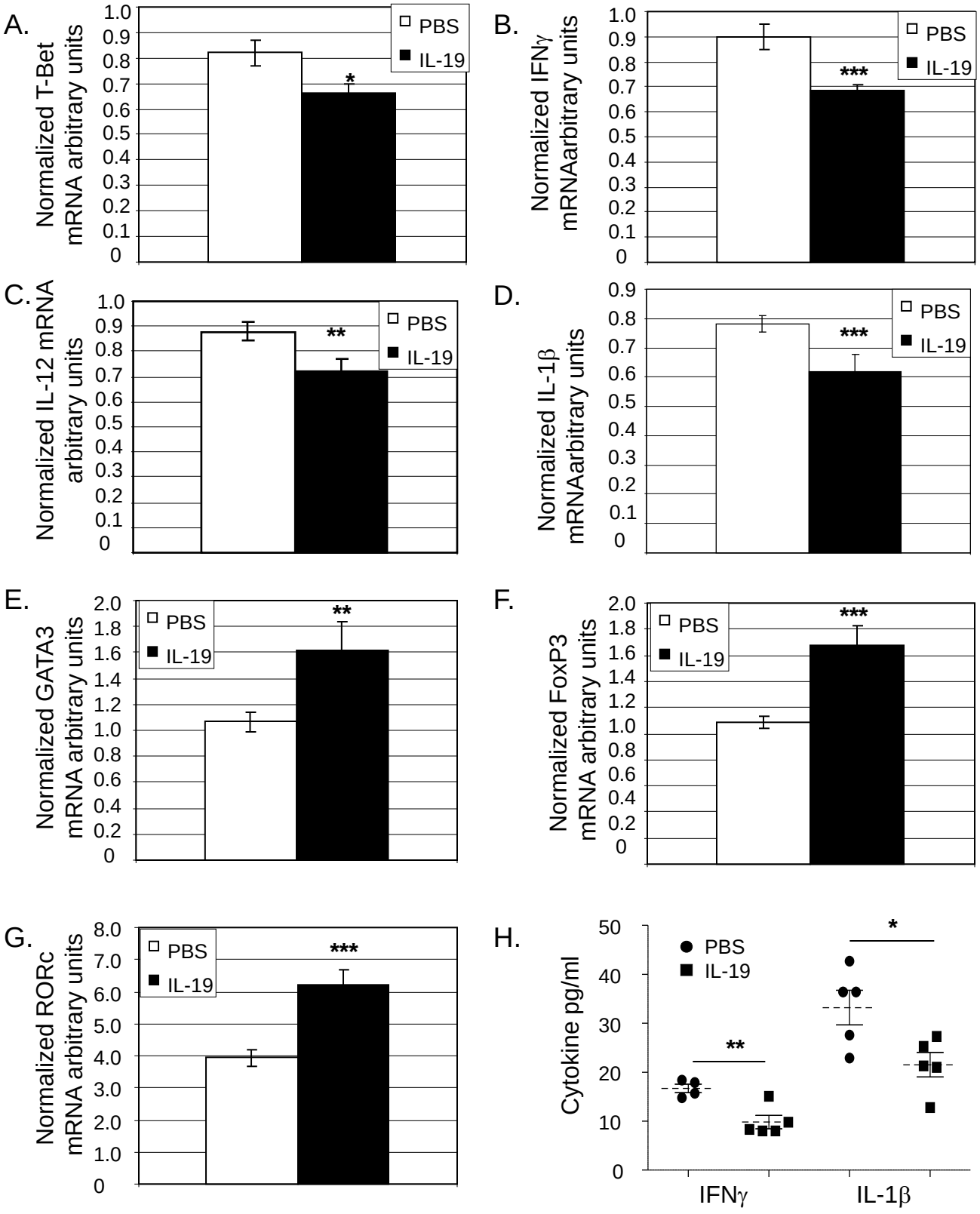
A.
Normalized T-Bet mRNA arbitrary units
PBS
IL-19
*
B.
Normalized IFNγ mRNAarbitrary units
***
C.
Normalized IL-12 mRNA arbitrary units
**
D.
Normalized IL-1β mRNAarbitrary units
E.
Normalized GATA3 mRNA arbitrary units
F.
Normalized FoxP3 mRNA arbitrary units
G.
Normalized RORc mRNA arbitrary units
H.
Cytokine pg/ml
IFNγ
IL-1β

Figure 4

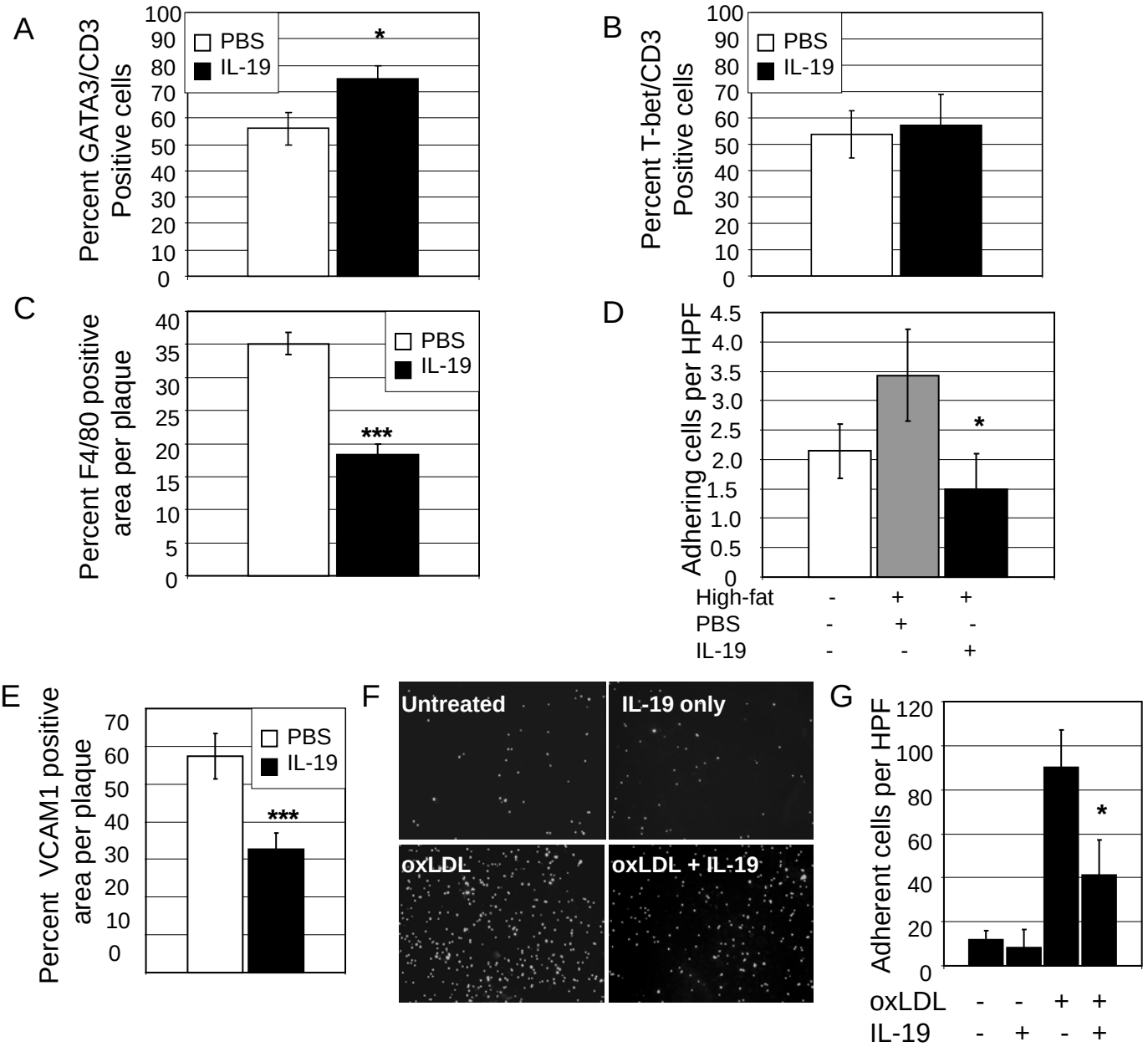
A
Percent GATA3/CD3 Positive cells
PBS
IL-19
*
B
Percent T-bet/CD3 Positive cells
C
Percent F4/80 positive area per plaque
***
D
Adhering cells per HPF
High-fat - + +
PBS - + -
IL-19 - - +
E
Percent VCAM1 positive area per plaque
F
Untreated
IL-19 only
oxLDL
oxLDL + IL-19
G
Adherent cells per HPF
oxLDL - - + +
IL-19 - + - +

Figure 5

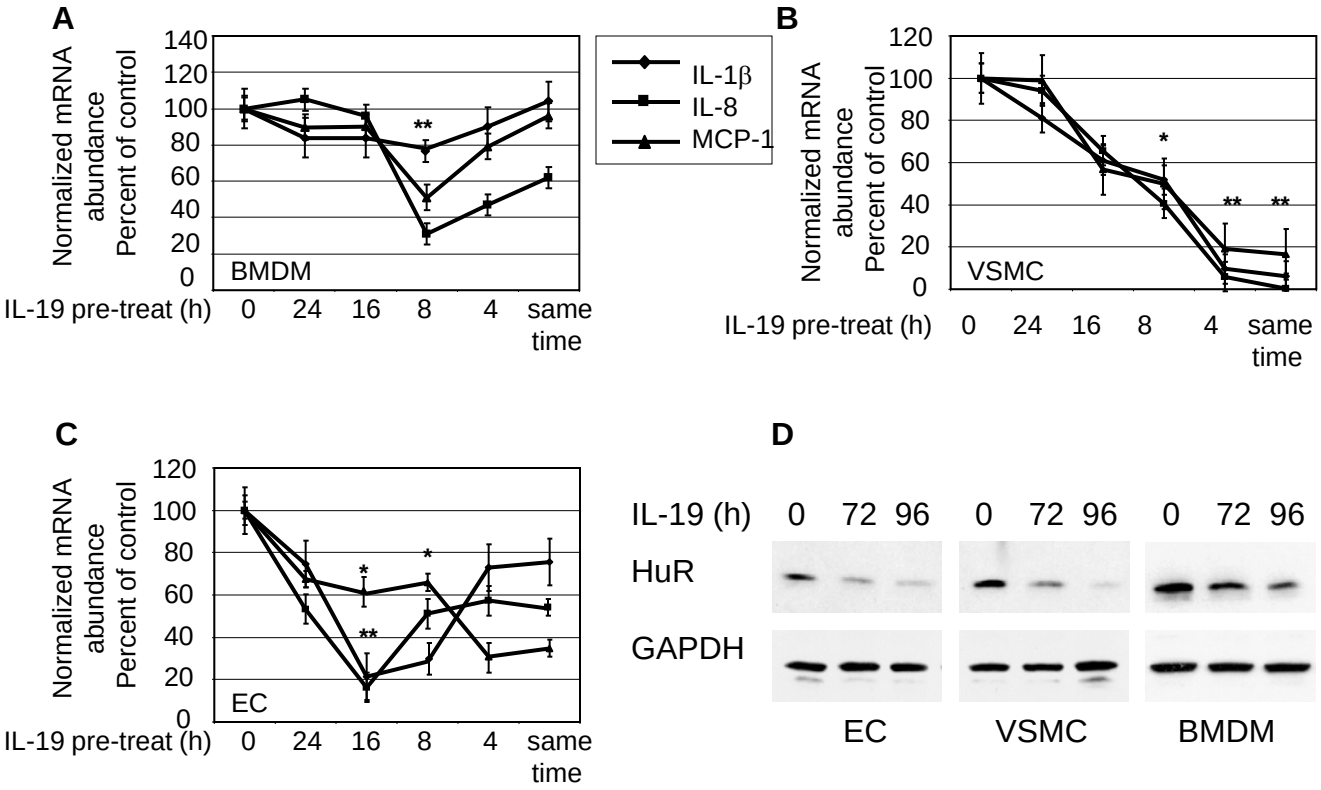
A
Normalized mRNA abundance
Percent of control
140
120
100
80
60
40
20
0
BMDM
IL-1β
IL-8
MCP-1
IL-19 pre-treat (h) 0 24 16 8 4 same time
B
Normalized mRNA abundance
Percent of control
120
100
80
60
40
20
0
VSMC
IL-19 pre-treat (h) 0 24 16 8 4 same time
C
Normalized mRNA abundance
Percent of control
120
100
80
60
40
20
0
EC
IL-19 pre-treat (h) 0 24 16 8 4 same time
D
IL-19 (h) 0 72 96 0 72 96 0 72 96
HuR
GAPDH
EC
VSMC
BMDM

Figure 6