Conclusions—The predominance of Th1 lymphocytes and their cytokines in the atherosclerotic disease process is acknowledged mechanism in atherogenesis. The Th1 arm of immunity elaborates anti-inflammatory interleukins that tend to limit the magnitude of the inflammatory response. Despite their potential for anti-inflammatory effects, uncertainty remains on the potential for direct protective effects of Th2-positive cells and interleukins in atherogenesis. The majority of these important studies focus on the archetypal anti-inflammatory cytokine, interleukin 10 (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. However, injection of interleukin 4 (IL-4) into apolipoprotein E-deficient (apoE−/−) mice does not reduce the development of atherosclerotic lesions, and lesions were, in fact, reduced in IL-4/apoE–double knockout mice. Furthermore, little has been published on potential protective effects of Th2 interleukins on resident vascular cells (EC and VSMC) in addition to inflammatory cells. Recognition of antiatherosclerotic interleukins, particularly IL-19, as a novel therapeutic to limit vascular inflammation.

Key Words: atherosclerosis • chemokines • interleukin-19 • macrophages

Atherosclerosis is a chronic vascular inflammatory disease. A series of cytokine-mediated interactions among lymphocytes, macrophage, endothelial cells (EC), and vascular smooth muscle cells (VSMC) result in local inflammation of the arterial wall. Although 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 proinflammatory cytokines, and atherosclerosis, in particular, has been described as a Th1 inflammatory disease. 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. By comparison, a much smaller number of studies focus on the role of endogenous counter-regulatory mechanisms in atherogenesis.
those that may have direct anti-inflammatory activity on resident vascular cells in addition to immune polarization, is needed and would have considerable therapeutic potential.

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

Many investigators have postulated that atherosclerosis development is influenced by a balance between proinflammatory and anti-inflammatory cytokines, implying that IL-19 may regulate the development of atherosclerosis. The goals of this study were to determine whether IL-19 was expressed in atherosclerotic plaque to test the hypothesis that IL-19 could reduce atherosclerosis in susceptible mice and to identify the potential mechanisms for these effects.

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

Results
IL-19 Is Expressed in Human Atherosclerotic Plaque
The atherosclerotic plaque microenvironment is biased to Th1 activation in humans and hypercholesterolemic mice. IL-19 content in atherosclerotic plaque has never been reported. Several human coronary arteries obtained postmortem were immunostained with IL-19 antibody to characterize the cellular distribution in atherosclerosis. Overall, 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. In the representative photomicrograph shown in Figure 1A and 1B, abundant IL-19 immunoreactivity localized within plaque from a human patient with type 4 plaque. 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 type 1 or 2. IL-19 cell-specific expression in a representative plaque was established in EC, VSMC, and inflammatory cells by immunoreactive colocalization with the EC marker Von Willebrand, smooth muscle cell α-actin, and leukocyte common antigen CD45 (Figure 1A in the online-only Data Supplement).

Expression of IL-19 was also demonstrated in human carotid endarterectomy sections by immunohistochemistry (Figure 1C and 1G). 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) compared with those from patients without symptoms (P = 0.03; n = 20 in each group; Figure 1I). Clinical characteristics of the individuals included in this study are described in Table I in the online-only Data Supplement. 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 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 interleukin-1 (IL-1) to enhance the density of cell adhesion molecules in the plaque.
vascular EC. As in the atherosclerotic coronary arteries, IL-19 was clearly detected in multiple cell types in these samples (Figure 1E and 1F).

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 the 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 humans, little to no IL-19 is detected in normal mouse aorta, but abundant IL-19 is detected in lesions from LDLR−/− mice (Figure IB in the online-only Data Supplement). LDLR−/− mice were used 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 intraperitoneally with 10 ng/g per 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 shows a significant reduction in lesion area between the PBS control and IL-19–treated mice (Figure 2A; 13.9±0.9% versus 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% versus 10.36±0.94% for PBS-treated and IL-19–treated mice, respectively; P<0.0001; Figure IC in the online-only Data Supplement). 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% versus 16.6±1.5% for PBS-treated and IL-19–treated mice, respectively; P<0.01; Figure 2C). In a second cohort, we determined that systemic administration of, as little as, 1 ng/g per day IL-19 could significantly reduce lesion area (17.7±1.7% versus 5.3±1.2% for PBS-treated and IL-19–treated mice; P<0.0001; n=11 and 13, respectively; Figure 2D). Interquartile range values for all studies are presented in the Table. Lesion area in Oil Red O–stained aortic root was significantly lower in 1 ng/g per day IL-19–injected mice compared with PBS controls (27.4±1.1% versus 18.6±1.1% for PBS-treated and IL-19–treated mice, respectively; P<0.01; Figure 2E). There was no significant difference in serum lipid profiles (Figure 3A and 3B). There was no significant difference in weight gain.
(11.29±0.8 versus 10.95±0.7 g for PBS and IL-19 in the 10 ng/g per day or 11.99±1.4 versus 8.9±0.9 g for PBS and IL-19 in the 1 ng/g per 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 antiatherogenic, 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.\textsuperscript{28,29} The global immune status of these mice was determined by quantification of Th1 and Th2 marker expression in splenocytes immediately removed from mice at the termination of the study. Quantitative reverse transcription polymerase chain reaction demonstrates that splenocytes from IL-19–injected mice had significantly lower mRNA levels of the Th1 markers T-bet (0.82±0.05 versus 0.66±0.04 for PBS-treated and IL-19–treated mice, respectively; \(P<0.05\)) and interferon-\(\gamma\) (0.89±0.05 versus 0.68±0.02 for PBS-treated and IL-19–treated mice, 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 versus 1.62±0.22 for PBS and IL-19, respectively; \(P<0.001\)) and FoxP3 (1.0±0.2 versus 1.6±0.3 for PBS and IL-19, respectively; \(P<0.001\)).

Figure 3. Interleukin-19 (IL-19) does not modify serum lipids or weight. A, Cholesterol and triglycerides and (B) high-density lipoprotein (HDL) and low-density lipoprotein (LDL) in mice fed atherogenic diet for 13 weeks receiving either PBS or 10 ng/g per day IL-19 at time of euthanasia do not statistically differ between control and IL-19 groups (n=9 each). C, Weight gain does not statistically differ between control and IL-19 groups (n=13 each).

Figure 4. Interleukin-19 (IL-19) polarizes the adaptive immune response to Th2 and T reg. A–G, Quantitative reverse transcription polymerase chain reaction on RNA extracted from spleen of mice receiving either PBS or 10 ng/g per day IL-19. Spleens were removed at the time of euthanasia, RNA extracted and reverse transcribed, and amplified using the primer pairs shown. *\(P<0.05\), 0.01, or **\(P<0.001\) as shown. n=9 spleen each group. H, Cytokine protein concentration from spleen determined by Luminex analysis. **\(P<0.01\) or 0.5, n=4 or 5 spleen per group.
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Protein is also increased in spleen from IL-19–injected mice compared with controls (16.70±0.8 versus 9.84±1.3 pg/mL IL-1β for PBS and IL-19, respectively; P<0.001). No difference in T-bet–positive T lymphocytes was noted between groups. Collectively, these data suggest that one potential mechanism for the IL-19 antiatherogenic 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 (Figure IIC in the online-only Data Supplement). PBS-treated and IL-19–treated mice, respectively (P<0.001) and FoxP3 (1.08±0.05 versus 1.68±0.15 for PBS-treated and IL-19–treated mice, respectively; P<0.001) mRNA. IL-19–treated mice had significantly higher retinoid-related orphan receptor-γ mRNA compared with controls (3.93±0.27 versus 6.23±0.46 for PBS-treated and IL-19–treated mice, respectively; P<0.01). Quantitative reverse transcription polymerase chain reaction also shows that mRNA for IL-1β and interleukin 12 p40, both potent proinflammatory cytokines, is significantly decreased in IL-19–injected mice (0.78±0.28 versus 0.61±0.5 for IL-1β; P<0.001 and 0.88±0.30 versus 0.72±0.04 for PBS and IL-19, respectively; P<0.01). Cytokine protein was assayed by quantitative Luminex analysis and determined significantly less interferon-γ and IL-1β protein in IL-19–treated mice compared with controls (16.70±0.8 versus 9.84±1.3 pg/mL interferon-γ for PBS and IL-19 and 33.20±5.5 versus 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 (Figure IID in the online-only Data Supplement).

Local lymphocyte polarization in plaque was characterized by immunohistochemistry (Figure IIA and IIB in the online-only Data Supplement 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% versus 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 that one potential mechanism for the IL-19 antiatherogenic effect is polarization of the immune response to the Th2 and, possibly, T-regulatory phenotypes.

**Figure 5.** T-lymphocyte and macrophage infiltrate into atherosclerotic lesions. A, Multiple serial sections of the aortic root from the aortic sinus to disappearance of valve cusps per root of 10 ng/g per day interleukin-19 (IL-19)–treated and PBS control mice were sectioned and immunostained using CD3 and GATA3 antibody. The number of CD3–positive cells that also stained positive for GATA3 was quantified by counting individual cells. P<0.001, n=8 mice, using ≥3 sections per aortic root. B, The number of CD3–positive cells that stained positive for T-bet was quantified by counting individual cells. There was no significant difference between groups. C, IL-19 reduces macrophage infiltrate in atherosclerotic lesions. Representative fluorescent photomicrographs of aortic root immunostained with F4/80 antibody. Multiple serial sections of the aortic root from the aortic sinus to disappearance of valve cusps per root of 10 ng/g per day IL-19–treated and PBS control mice were sectioned and immunostained using F4/80 antibody. Positively stained areas were quantified as a percentage of total lesion area by quantitative morphometry. Endothelial cells grown on glass coverslips were pretreated with IL-19 for 16 hours and then with 50 mg/mL oxidized low-density lipoprotein (oxLDL) for 6 hours before addition of THP1 monocytes. Representative photomicrograph shown. G, Results represent means, P<0.05 for 8 high-power fields from triplicate experiments.
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 were injected with 10 ng/g per day IL-19 or PBS for 5 consecutive days per week. 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±0.78 versus 1.5±0.5 cells/100 μm for PBS-treated and IL-19–treated mice, respectively; P<0.05). There was no statistical difference in rolling between PBS-treated and IL-19–treated mice. Three additional experiments were performed to characterize a cellular mechanism. First, immunohistochemistry of vascular cell adhesion molecule-1 (VCAM1) abundance in plaque was performed. Positively stained areas were quantified 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% versus 57.43±6.08% for IL-19 and PBS, respectively; P<0.01; Figure 5E and Figure III in the online-only Data Supplement). Second, we used an EC monolayer adhesion assay and determined that pretreatment of cultured EC with IL-19 could significantly reduce monocyte adhesion to EC oxidized low-density lipoprotein (LDL)–stimulated EC monolayers (89.9±17.3 versus 41.1±16.3 monocytes/high-power field; P<0.05; Figure 5F and S5G). Third, human ECs were pretreated with IL-19 before stimulation with oxidized LDL and lysates blotted with anti-VCAM1 antibody. Figure IIIIB and IIIC in the online-only Data Supplement shows that IL-19 pretreatment significantly decreases VCAM-1 protein abundance. Collectively, these data suggest that a second mechanism for IL-19 antatherosclerotic 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 whether IL-19 could decrease the expression of chemokines in EC, VSMC, and macrophage. Cultured human coronary artery EC, VSMC, and mouse bone marrow–derived macrophages were pretreated with IL-19 for varying times and then challenged with tumor necrosis factor-α. Cytokine mRNA was determined by quantitative reverse transcription polymerase chain reaction. Figure 6A–6C demonstrates that in all cell types tested, IL-19 has a potent inhibitory effect on IL-1β, interleukin-8, and monocyte chemotactic protein-1 mRNA accumulation, each of which is a potent leukocyte chemoattractant. Interestingly, the 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 antithrombogenic effects on resident vascular cells, as well as immune cells, suggesting a third mechanism for IL-19 antitherosclerotic effects.

We previously reported that IL-19 does not inhibit nuclear factor-κB activity but transiently decreases activation of the mRNA stability factor human antigen R (HuR). Each of the chemokines tested contains an AU-rich stability element in its 3′-untranslated region, which is recognized by HuR. To determine potential molecular mechanisms for IL-19 anti-inflammatory effects, we chronically treated cultured EC, VSMC, and bone marrow–derived macrophage 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 because HuR preferentially stabilizes inflammatory transcripts.

Discussion

The major finding from this article is that systemic administration of IL-19 attenuates atherosclerosis in LDLR−/− mice. Adaptive immune polarization, reduction in macrophage infiltration, and inflammatory cytokine gene expression in immune and resident vascular cells 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 nonimmune 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 because current understanding indicates that Th1 cytokine expression dominates, which is in contrast to Th2 cytokines that are far less prevalent in human atherosclerotic lesions. 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 the expression of interleukin-20 receptor 1 and receptor 2 that was detected in EC and mononuclear cells in human atherosclerotic lesions, but at much lower levels in normal aorta sections. IL-19 expression in plaque but not normal media suggested a compensatory 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. 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. In patients selected for coronary artery bypass graft surgery, IL-19 levels were 34.4±17.6 ng/mL before surgery. IL-19 increased to 541.3±110.4 ng/mL 24 hours after surgery and declined to 77.2±24.9 ng/mL 96 hours after surgery. In our study, systemic administration of 10 ng/g per day IL-19 almost completely inhibited plaque formation in the aortic arch and as little as 1 ng/g per day IL-19 decreased plaque area by 70%, suggesting...
potent antiatherosclerotic effects of IL-19. This is a straightforward 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 caused by reduction in lipid because there is no significant difference in total serum cholesterol, triglycerides, high-density lipoprotein, or LDL in IL-19–treated mice compared with controls, nor is there any difference in weight gain between these 2 populations. In contrast to IL-19, IL-10 has lipid-lowering effects because serum cholesterol is significantly reduced in mice receiving IL-10, which also may have contributed to decreased plaque in that study.

Some 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 of plaque inflammatory cell infiltrate. Transfer of bone marrow from IL-10−/− 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. Similarly, LDLR−/− mice receiving bone marrow from IL-10−/− transgenic mice showed a significant decrease in plaque area. Together, these studies suggest that IL-10 antiatherogenic 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 1 μg of interleukin-33; however, lesion area was not measured in this study. Counterintuitively, IL-4−/− mice do not have increased atherosclerosis, and subcutaneous injection of 1.1/μg per day recombinant IL-4 into apoE−/− mice does not reduce the development of atherosclerotic lesions. Furthermore, 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.

Figure 6. Interleukin-19 (IL-19) decreases mRNA abundance of chemotactic cytokines in resident vascular and immune cells. A–C, Cultured vascular smooth muscle cells (VSMC) or endothelial cells (EC) were serum-starved 24 hours. Bone marrow–derived macrophages (BMDM) were isolated from C57B6 mice. All cells were pretreated with IL-19 for the times shown and then stimulated with 10 ng/mL tumor necrosis factor-α for 4 hours. Total RNA was reverse transcribed and target mRNA quantified by quantitative reverse transcription polymerase chain reaction. mRNA was normalized to GAPDH. Differences in IL-19 pretreated vs untreated controls are significant where indicated (P≤0.05 or 0.01, n=3 experiments), and asterisks are for all targets at that time, unless otherwise noted. D, Chronic treatment with IL-19 reduces human antigen R (HuR) protein. Cultured VSMC or EC were serum-starved 24 hours. BMDM were isolated from C57B6 mice. Cells were stimulated with IL-19 continuously for 4 days, changing the media and adding fresh IL-19 every 24 hours. At the indicated times, lysates were immunoblotted with HuR and GAPDH antibody. Western blots shown are representative of ≥3 independent experiments, with identical results. MCP indicates monocyte chemotactic protein.
immunohistochemistry from sections of aortic root.40 Like most studies that use 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. Future studies are needed to characterize the effect of IL-19 on global and local adaptive immunity in the context of atherosclerosis.

Monocyte adhesion constitutes a key cellular event in the initiation of atherosclerosis.41 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 postcapillary 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 shows that IL-19 can reduce oxidized LDL–driven VCAM-1 expression in cultured EC and also reduce monocyte adhesion to oxidized LDL–stimulated EC monolayers. This is in contrast to IL-4, which has been shown to increase VCAM-1 expression and leukocyte adhesiveness.42 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 of these cells with IL-19 before tumor necrosis factor-α stimulation led to a significant decrease in mRNA for monocyte chemotactic protein-1, interleukin-8, and IL-1β, all potent chemotactants. 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 nuclear factor-κB activity in VSMC but did not reduce tumor necrosis factor-α– or IL-1β–induced expression of inflammatory genes.43 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 presumed Th2 interleukin can have direct anti-inflammatory effects on cells outside 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.44 We previously reported that in cultured, primary human VSMC, IL-19 inhibitory effects are nuclear factor-κB independent but did decrease the mRNA stability of inflammatory transcripts by decreasing nuclear to cytoplasmic translocation of HuR.20,28 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 that HuR protein abundance was reduced. Thus, both reduction in HuR cytoplasmic translocation and reduction in protein abundance are induced by IL-19. 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, which demonstrated the 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 a potentially paradigm-altering procedure because it suggests that resident vascular cells can respond to IL-19 to 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 cannot identify whether 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 mediators of IL-19 effects.

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Disclosures

None.

References


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 article describes expression of interleukin-19, a recently described anti-inflammatory cytokine, in human atheromatous plaque. Administration of interleukin-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 interleukin-19 by immune and resident vascular cells is a novel compensatory counter-regulatory mechanism that attenuates atherosclerosis and suggest that interleukin-19 itself has therapeutic potential for inhibition of atherosclerosis.
Attenuation of Experimental Atherosclerosis by Interleukin-19

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IL-19 expression in atherosclerotic plaque. A. IL-19 is expressed in multiple cell types in human atherosclerotic plaque. For cell-specific co-staining, immunofluorescence immunohistochemistry of an artery from a human patient with atherosclerosis was co-stained with anti-IL-19 (red) and: pan-leukocyte antigen CD45 (green), SMCα actin (green), or Von Willebrand (green). Arrows define merged localization. B. 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. Magnification 400X for wild type, 600X for plaque and immunofluorescence. C. Graphic depiction of atherosclerotic lesion size quantitated from en face stained aortic arches from 3 month old ApoE-/- mice after consuming atherogenic diet for 12 weeks, injected with either PBS or 10ng/g/day IL-19. n=7 (PBS) and 9 (IL-19), p<0.0001.
T lymphocyte and macrophage infiltrate into atherosclerotic lesions. A. Representative photomicrograph showing a significant increase in GATA3 positive T lymphocyte infiltrate in aortic root from IL-19 treated mice compared with PBS-injected controls. B. Representative photomicrograph showing no significant difference in T-bet positive lymphocyte infiltrate in IL-19 treated mice. Magnification 600X for A. and B. C. Representative fluorescent photomicrographs of aortic root immunostained with F4/80 antibody and oil Red O. D. GATA3 protein expression in spleen. Representative immunoblot from lysates used for Luminex analysis blotted with anti-GATA3 antibody.
IL-19 reduces VCAM1 expression in vivo and in cultured EC. A. Immunohistochemistry of VCAM1 abundance in aortic root. 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. Quantitation is shown in Figure 5E. B. Human coronary artery EC were treated with 100ng IL-19 for the times shown, then with 50μg/ml oxLDL for 24 hours. Lysates were immunoblotted with anti-VCAM1 and GAPDH antibody. Representative blot is shown. C. IL-19 significantly reduces VCAM-1 protein abundance. Western blot was quantitated by densitometry from at least 3 experiments, P<0.05 for times indicated compared with no IL-19 treatment.
Supplemental Data Table I

Clinical characteristics of the endarterectomy Patients (N=40)

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<tr>
<td>Body mass index (kg/m²)</td>
<td>26.7±4.2</td>
</tr>
<tr>
<td>Hypertension</td>
<td>30 (75.0)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>14 (35.0)</td>
</tr>
<tr>
<td>Symptomatic</td>
<td>20 (50.0)</td>
</tr>
<tr>
<td>Stroke</td>
<td>10 (25.0)</td>
</tr>
<tr>
<td>Amaurosis fugax</td>
<td>4 (10.0)</td>
</tr>
<tr>
<td>Transient ischemic attacks (TIA)</td>
<td>10 (25)</td>
</tr>
</tbody>
</table>

Values are expressed as mean± standard deviation or N (%)
Materials and Methods

Mice and study design. LDL receptor knock out mice (stock #002207) and ApoE knock out mice (Stock #002052) 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 the primary platform for these studies because they do not develop lesion until fed atherogenic chow, 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 (1). 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 (2).

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 Health Sciences Center in Shreveport, Louisiana (3). 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 (4,5). 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 α actin, Von Willebrand, and leukocyte common antigen (CD45) (NeoMarkers, Inc, Burlingame, CA), were used at 1.0µg/ml as described (4,5). 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 (2). 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 Supplemental Data Table 1. 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 (6). 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 (7). 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 (8). 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 (9). 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 (5,8). 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 (5,8). 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: CGAGAGTCAGCCGCATCTTT, R: CCCCATGGTGTTCTGAGCCG,
Human MCP-1: F AGCAGAAGTGGGTTCAGGATT, R: TGTGGAGTGAGTGTTCAAGTCT,
Human IL-1β: F: TCCCCAGCCCTTTGTTGA, R: TTAGAACCATTGTGGCCGTG,
Human IL-8: F: CCAGGAAGAAACCACGGA, R: GAAATCGGATGCAAAG.
Mouse GAPDH: F: GCAAGGACACTGAGCAAGAG, R: GGGTCTGGAGTGAATTGTG,
Mouse MCP1: F: TTAAAAACCTGGATCGGAACCAA, R: GCATTAGCTTCAGATTTACGGGT,
Mouse IL-1β: F: CTAATAGGCTCATCTGGGATCC, R: GGTCCTGGGATGAAATTGTG,
Mouse FoxP3: F: AAGTACCACAATATGCACCC, R: TCTGAAGTAGGCGAACATGC
Mouse IFNγ: F: CCTACTGCTGACAAATGAACG, R: TCAATGACTGTGGCCGTG
Mouse GATA3: F: TACCCTATTCCGCTATGG, R: CTCGACTTACATCCGACCCTG
Mouse T-bet: F: CCTGTTTGGGTCCAAAGTTACAA, R: CCACAAAATCCTGTAATGGCTTGT
Mouse IL-19: F: AAATCTTGAGGGATGTCGAG, R: GGGTAAAGTAGTGGTCTCC
Mouse IL-12p40: F: GTGAAGCAGCAATATTACTCAG, R: AGAGGCCATTCCCCAT
Mouse CXCL2 (human IL-8 homolog): F: CAGAAGCTCATAGCCACTCTCAAG, R: CTCCCTTCCAGGTCTTAGGC
Mouse CXCL1 (human IL-8 homolog): F: AGAACATCCAGAGCTTGAGGG, R: CAATTTTCTGAACCAAGGAC
Mouse RORγ: F: TTTCTGAGGATGAGATTGCCC, R: TTGTCGATGAGTCTTGCAGAG

Western blotting and protein determination. Western blotting for HuR was performed as we described (5,8). 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 (5,8), 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 $p<0.05$.

References