

Interleukin-18 Enhances Atherosclerosis in Apolipoprotein E^{-/-} Mice Through Release of Interferon- γ

Stewart C. Whitman, Punnaivanam Ravisankar, Alan Daugherty

Abstract—We have previously shown that interferon- γ (IFN- γ) is a potent enhancer of atherogenesis. Interleukin-18 (IL-18) promotes inflammatory responses through release of IFN- γ , although it can also exert direct actions on other inflammatory mediators. In this present study, we determined the effects of IL-18 on atherogenesis and the role of IFN- γ in this response. Male apolipoprotein E^{-/-} mice (*apoe*^{-/-}; aged 16 weeks, n=10/group) were fed a normal diet and injected intraperitoneally for 30 days with either recombinant IL-18 (30 ng/g/day) or saline. Atherosclerotic lesion size was quantified in 2 vascular beds: the ascending aorta and the aortic arch. IL-18 administration did not affect serum cholesterol concentrations or lipoprotein-cholesterol distribution; however, exogenous IL-18 administration increased lesion size 2-fold in both the ascending aorta (50 642±12 515 versus 112 399±13 227 μm^2 , $P=0.004$; saline versus IL-18 groups, respectively) and the aortic arch (3.1±0.3% versus 6.2±0.9% area, $P=0.006$). Exogenous IL-18 promoted a 4-fold increase in the number of lesion-associated T lymphocytes (11±3 versus 50±5 cells; $P<0.0001$) and cells expressing major histocompatibility complex class II (9±3 versus 40±6 cells; $P=0.0002$). To determine the role of IFN- γ production in this response, exogenous IL-18 was administered to *apoe*^{-/-} mice that were IFN- γ deficient. These studies demonstrated that lack of endogenous IFN- γ ablated the effects of IL-18 on atherosclerosis. Therefore, these data strongly implicates IL-18 in the atherogenic process and suggests that IL-18 increases lesion development through enhancement of an inflammatory response involving an IFN- γ -dependent mechanism. The full text of this article is available at <http://www.circresaha.org>. (*Circ Res.* 2002;90:e34-e38.)

Key Words: immunology ■ inflammation ■ cholesterol ■ atherosclerosis ■ apolipoprotein E

Atherosclerosis is an inflammatory process involving the recruitment of specific leukocytes, especially macrophages and T lymphocytes.¹⁻³ A wide array of soluble mediators has been proposed to regulate the inflammatory process.^{1,2} Interferon- γ (IFN- γ) is emerging as a prominent proinflammatory mediator of atherosclerosis. Evidence for its role in the disease process includes the presence of IFN- γ mRNA and protein in atherosclerotic lesions of both humans⁴ and mice.⁵⁻⁷ Deficiency of either the IFN- γ receptor⁸ or the cytokine (S.C. Whitman, P. Ravisankar, P. Veerarahavan, A. Daugherty, unpublished data, 2002) leads to a profound decrease in the severity of atherosclerosis in apolipoprotein E^{-/-} (*apoe*^{-/-}) mice. Conversely, the exogenous administration of recombinant IFN- γ promotes the atherogenic process.¹⁰ Therefore, the role of IFN- γ and its regulator have important consequences for atherogenesis.

One of the primary factors regulating IFN- γ secretion is interleukin-18 (IL-18). This cytokine is a 24-kDa, non-glycosylated polypeptide that lacks a classical signal sequence¹¹⁻¹⁴ and possesses a structure similar to IL-1 β .^{12,15} IL-18 is synthesized as an inactive propeptide that undergoes proteolytic cleavage by the enzyme caspase-1 to generate a

mature, bioactive, 18-kDa molecule.^{12,14,16} In both the mature and propeptide forms, IL-18 shows 64% sequence identity from mouse to human.¹² Cells known to express IL-18 include macrophages/Kupffer cells,^{11,17} keratinocytes,¹⁸ glucocorticoid-secreting adrenal cortex cells,¹⁹ and osteoblasts.²⁰ Because of its ability to stimulate IFN- γ release by both NK cells and T lymphocytes, IL-18 is considered to be a key cytokine in the processes of innate and acquired immunity. IL-18 stimulates IFN- γ production by initiating IFN- γ gene transcription via activation of the IFN- γ promoter in a process that requires *c-jun* binding to the AP-1 site.²¹

A role of IL-18 in atherosclerosis has been inferred following its identification in human atherosclerotic lesions.²² Furthermore, overexpression of the murine IL-18 binding protein, the endogenous inhibitor of IL-18, has been shown to reduce atherosclerotic lesion development in *apoe*^{-/-} mice.²³ Therefore, there is associative evidence to infer a role of IL-18 in the development of atherosclerosis. The present study presents the first direct evidence indicating that IL-18 is proatherogenic, by showing that administration of exogenous IL-18 will enhance the development of atherosclerosis in *apoe*^{-/-} mice and that this

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cytokine increases lesion development through enhancement of an inflammatory response via an IFN- γ -dependent mechanism.

Materials and Methods

Materials

Lyophilized recombinant mouse IL-18 (Cat. No. B002-5, R&D Systems, Inc, Minneapolis, Minn) was reconstituted in solution A (sterile phosphate buffered saline [PBS] containing 0.1% bovine serum albumin), to prepare a stock concentration of 50 $\mu\text{g}/\text{mL}$, as recommended by the manufacturer.

Mice and Cytokine Injections

apoe^{-/-} and *ifn- γ* ^{-/-} mice, backcrossed 10 times to the C57BL/6 strain, were obtained from the Jackson Laboratory (Bar Harbor, Maine). The *ifn- γ* ^{-/-} mice were bred to *apoe*^{-/-} mice to generate compound-deficient mice. Polymerase chain reaction (PCR) was used to identify mice homozygous for both apoE and IFN- γ deficiency, as described previously.^{24,25} Breeding pairs of *apoe*^{-/-} and *apoe*^{-/-} \times *ifn- γ* ^{-/-} mice were established and equal numbers of siblings from the resulting litters were divided into 2 groups of 10 male *apoe*^{-/-} mice and 2 groups of 10 male *apoe*^{-/-} \times *ifn- γ* ^{-/-} mice. At 16 weeks old, one group from each strain received daily peritoneal injections of IL-18 (30 ng/g body weight) diluted in PBS, while the second group received daily injections of solution A (to control for the presence of bovine serum albumin), diluted also in PBS. The dose of recombinant cytokine was based on previous studies in which prolonged IL-18 administration via the intraperitoneal route exerted biological affects. Doses of administered recombinant IL-18 have ranged from ≈ 30 ²⁶ to ≈ 400 ²⁷ ng/g body wt/day. Therefore, based on these published findings, we decided to use a dose that was at the lowest value cited in the most current literature. Daily injections were carried out for 30 days, during which time the mice were kept on a normal laboratory diet. Mice were housed in a pathogen-free facility, with all of the animal procedures having the full approval of the University of Kentucky Institutional Animal Care and Use Committee.

Lipid and Lipoproteins

At the end of the study, serum total cholesterol and triglyceride concentrations were determined with enzymatic assay kits (Wako Chemical Co). Lipoprotein cholesterol distributions were evaluated in individual serum samples (50 μL) from 5 mice in each group that were each resolved by size exclusion chromatography on a Superose 6 column as described previously.¹⁰

Acquisition of Tissues

At the end of the injection period, mice were anesthetized by intraperitoneal injection of ketamine plus xylazine (90+10 mg/kg body weight). Terminal blood samples were collected by puncture of the right ventricle, and mice were perfused with PBS. Hearts were separated from the aorta at the base, embedded in OCT, and frozen at -20°C . Aortic tissue was removed from the ascending aorta to the level of the diaphragm and fixed overnight at room temperature in freshly prepared 4% paraformaldehyde. The livers and spleens of each mouse were removed, weighed, and segments of the spleens were frozen in OCT and stored at -20°C .

Quantification of Atherosclerotic Lesions

Atherosclerotic lesion size was determined in 2 vascular beds, the ascending aorta and the aortic arch, using 2 well-validated techniques, as described previously.^{10,28} Lesion size in the ascending aorta was determined from 4 Oil Red O-stained serial sections, cut 10- μm thick, and collected at 100- μm intervals, starting at the region where the aortic sinus becomes the ascending aorta, as described previously.¹⁰ Lesion area, defined as intimal tissue within the internal elastic lamina, was determined using Image-Pro software (Media Cybernetics) on images that were created using a Spot

camera (Diagnostic Instruments). The mean lesion area derived from the 4 serial sections was taken as the mean lesion size for each animal. Lesion size in the aortic arch was measured from en face preparations of this region, and the percentage of intimal surface area covered by atherosclerotic lesions was determined for the aortic arch, as described previously.²⁸

Immunocytochemistry

Immunocytochemistry was performed, as described previously,¹⁰ on serial sections of the ascending aorta adjacent to those stained with Oil Red O. The following reagents were used for immunostaining: an anti-mouse Thy1.2 monoclonal antibody (01011D, 7 $\mu\text{g}/\text{mL}$; PharM-ingen) and an anti-mouse major histocompatibility complex class II (MHC II) monoclonal antibody (LS-004-SN, 1:5 dilution; Biosource International). Species-specific biotinylated secondary antibodies and a biotin-avidin-peroxidase complex were subsequently incubated with tissues (Vectastain Elite ABC kit, Vector Laboratories). Immunoreactivity was visualized using the red chromogen, 3-amino-9-ethyl carbazole (Biomedica Corp).

Statistics

Data analyses were performed using SigmaStat 2.03 software (SPSS Inc). Statistical analysis between groups was by Student's *t* test after testing that the data complied with the constraints of parametric analysis. In instances where parametric analysis was not permissible, analysis between groups was conducted using the Mann-Whitney rank sum test. Values with $P \leq 0.05$ were considered statistically significant.

Results

When compared with mice injected with saline, injection of exogenous IL-18 did not affect serum cholesterol concentrations (272 ± 15 versus 253 ± 17 mg/dL, respectively) or lipoprotein-cholesterol distributions (Figure 1). No changes in serum triglyceride concentrations were found between groups (data not shown).

Although serum lipid concentrations did not differ between the 2 groups, injection of exogenous IL-18 caused a 2.2-fold increase in atherosclerotic lesion size in the ascending aorta ($50\,642 \pm 12\,515$ versus $112\,399 \pm 13\,227$ μm^2 , control and IL-18-injected, respectively, $P = 0.004$; Figure 2A). Further analysis also revealed that injection of IL-18 caused a significant increase throughout the ascending aorta ($P \leq 0.01$; Figure 2B). To complement lesion analysis in the ascending aorta, the extent of atherosclerosis between the 2 groups was

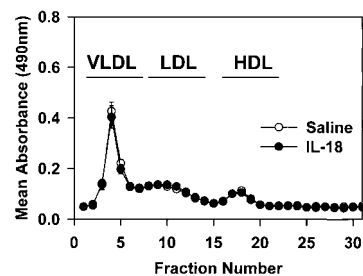


Figure 1. Characterization of serum lipoprotein-cholesterol distributions. Serum (50 μL) from male *apoe*^{-/-} mice receiving either saline (open circles) or recombinant IL-18 (closed circles) injections was resolved by size exclusion chromatography using a Superose 6 column, and the relative concentrations of total cholesterol in each fraction was determined using an enzymatic assay. Symbols represent the means and bars the SEM of values obtained from the serum of 5 mice. VLDL indicates very low-density lipoproteins; LDL, low-density lipoproteins; and HDL, high-density lipoproteins.

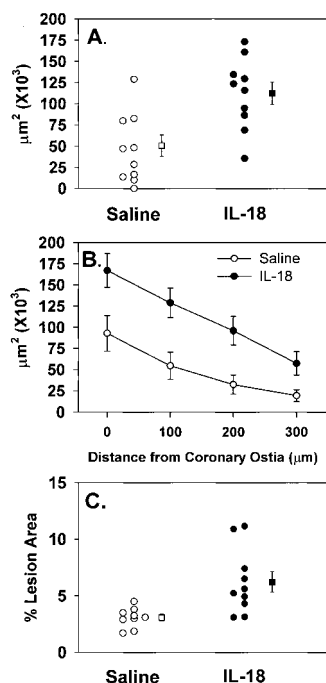


Figure 2. Quantification of atherosclerosis in *apoe*^{-/-} mice. The extent of lesion development in *apoe*^{-/-} mice receiving either saline (open symbols and histobars) or IL-18 (closed symbols and shaded histobars) injections was determined in 2 vascular beds using 2 separate techniques. Atherosclerotic lesion size was determined from cross sections of the ascending aorta and represented as mean lesion size spanning 4 regions of the ascending aorta (A), and as mean lesion size for each of these 4 regions (B). C, Lesion analysis was also determined in a second vascular bed, the aortic arch, by measuring the percentage of lesion area in en face preparations. Values of individual mice are represented as circles, squares are means, and bars are SEM. **P*≤0.01.

also analyzed in the aortic arch, where we found that compared with saline-injected mice, exogenous IL-18 caused a 2-fold increase in atherosclerosis (3.1 ± 0.3 versus $6.2 \pm 0.9\%$ area, *P*=0.006; Figure 2C).

In addition to measuring for differences in lesion size between the 2 groups, immunocytochemical analyses of these same lesions were conducted to examine for any potential changes in the inflammatory status of these lesions. From these analyses, we found that compared with mice injected with saline, IL-18 injections increased the mean number of lesion-associated T lymphocytes by 4.5-fold (11 ± 3 versus 50 ± 5 mean number of cells found in the lesions of the ascending aorta, *P*<0.0001; Figure 3A) and the mean number of cells expressing MHC II by 4.4-fold (9 ± 3 versus 40 ± 6 mean number of cells found in the lesions of the ascending aorta, *P*=0.0002; Figure 3B).

IL-18 is known to stimulate the production of IFN- γ from NK cells, T lymphocytes, and macrophages. Furthermore, we have previously shown that IFN- γ promotes atherosclerosis in *apoe*^{-/-} mice.¹⁰ Therefore, we examined whether administration of exogenous IL-18 to *apoe*^{-/-} mice promoted atherosclerosis via an IFN- γ -mediated process. To accomplish this, we repeated our IL-18 injection studies using *apoe*^{-/-} mice that were deficient in IFN- γ . In this group of

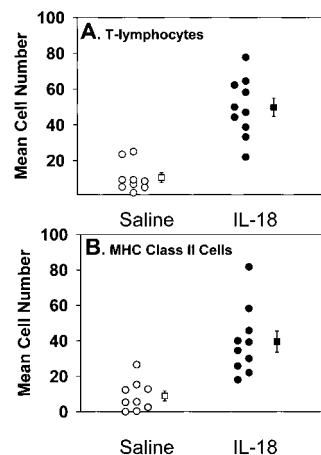


Figure 3. Aortic lesion T lymphocyte and MHC II-positive cell numbers. Numbers of lesion-associated (A) T-lymphocyte and (B) cells expressing MHC II were determined from cross sections of the ascending aorta of male *apoe*^{-/-} mice receiving either saline (open symbols and histobars) or IL-18 (closed symbols and shaded histobars) for 30 days. Segments of heart tissue spanning the aortic sinus and ascending aorta were embedded in OCT, sectioned, and stained with (A) a monoclonal anti-mouse Thy1.2 antibody (7 μ m/mL) or (B) a monoclonal anti-mouse MHC II antibody (1:5 dilution). Values of individual mice are represented as circles, squares are means, and bars are SEM.

mice, serum total cholesterol and triglyceride concentrations or lipoprotein-cholesterol distribution did not differ between groups (data not shown). Furthermore, IL-18 injections did not significantly increase lesion size measured in the ascending aorta ($23\,558 \pm 11\,133$ versus $11\,657 \pm 3860$ μ m², *P*=0.306, controls versus IL-18-injected mice, respectively; Figure 4A) or the aortic arch (1.5 ± 0.4 versus $0.8 \pm 0.3\%$ area of intimal surface, *P*=0.150; Figure 4B). As described previ-

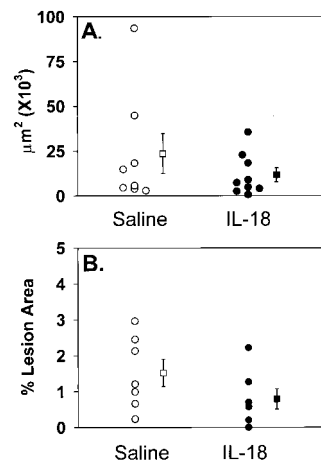


Figure 4. Quantification of atherosclerosis in *ifn- γ* ^{-/-}/*apoe*^{-/-} mice. The extent of lesion development in *apoe*^{-/-} mice receiving either saline (open symbols and histobars) or IL-18 (closed symbols and shaded histobars) injections was determined from cross sections of the ascending aorta and represented as mean lesion size spanning 4 regions of the ascending aorta (A), and lesion analysis was also determined in the aortic arch by measuring the percentage of lesion area in en face preparations (B). Values of individual mice are represented as circles, squares are means, and bars are SEM.

ously (Whitman et al, unpublished data), lesion development in saline-injected *apoe*^{-/-} × *ifn-γ*^{-/-} null mice was reduced by approximately 50% compared with saline-injected, *apoe*^{-/-} × *ifn-γ*^{+/+} mice (Figure 4 versus Figure 2).

In addition, when compared with mice injected with saline, the injection of IL-18 into IFN-γ-deficient mice did not affect the mean number of lesion-associated T lymphocytes (2 ± 1 versus 2 ± 1 mean number of cells found in the lesions of the ascending aorta; *P* = 0.784) or the mean number of cells immunostaining positive for MHC II (5 ± 3 versus 3 ± 1 mean number of cells found in the lesions of the ascending aorta; *P* = 0.381).

Discussion

Recently, Mallat et al²² demonstrated that IL-18 (mRNA and protein) is expressed in human carotid atherosclerotic plaques retrieved by endarterectomy. Furthermore, this group defined that exogenous expression of the murine IL-18 binding protein (the endogenous inhibitor of IL-18) reduced atherosclerotic lesion development in *apoe*^{-/-} mice.²³ Both of these studies associatively imply that IL-18 is a proatherogenic cytokine. However, as important as these 2 studies are at bringing awareness of the potential contribution of IL-18 to the disease process, they fail to provide direct evidence that IL-18 is in fact enhancing lesion progression. The present study provides this needed proof that IL-18 will promote lesion development and goes on to show that IL-18 enhances atherosclerosis via the promotion of an inflammatory process at the site of lesion development and that this proinflammatory response requires the production of IFN-γ.

IL-18 by itself is generally a weak stimulator of IFN-γ release. However, it functions synergistically with other co-stimulators, particularly IL-12, to induce pronounced secretion of IFN-γ production by T cells,^{29,30} NK cells,³¹ and macrophages.³² Therefore, as it relates to this study, in order for exogenous IL-18 to have promoted atherosclerosis via an IFN-γ-mediated mechanism, it would require that at least one co-stimulus to have been present within the early developing lesions. As it turns out, one such co-stimulus has been identified by at least 2 laboratories. Recently, work by Uyemura et al³³ and Lee et al,⁶ have identified IL-12 in atherosclerotic lesions from both humans and mice, respectively. Furthermore, Lee et al⁶ have shown that administration of exogenous IL-12 to *apoe*^{-/-} mice will promote lesion development.

Direct evidence exists for the secretion of IFN-γ within atherosclerotic lesions of humans^{33–36} and *apoe*^{-/-} mice.^{5,6} This cytokine can influence the development of atherosclerosis through a number of mechanisms that have been demonstrated in cultured cells. These include effects on 15-lipoxygenase,³⁷ lipoprotein-metabolism,³⁸ lipoprotein-modification,³⁹ lipoprotein-cell recognition,^{36,40,41} apoptosis,⁴² extracellular matrix synthesis,⁴³ and macrophage ABCA1 expression.⁴⁴ However, these diverse effects of IFN-γ on cultured cells render it difficult to predict whether IFN-γ would promote or retard atherogenesis. Recently we have demonstrated that IFN-γ promotes atherosclerosis in *apoe*^{-/-} mice. We have shown that intraperitoneal administration of exogenous IFN-γ into *apoe*^{-/-} mice promoted

atherosclerosis.¹⁰ Conversely, deficiency of endogenous IFN-γ production in *apoe*^{-/-} mice, via targeted deletion of the *ifn-γ* gene, decreased atherosclerosis (Whitman et al, unpublished data). In this latter study, compound-deficient mice were generated by crossing strain-matched *ifn-γ*^{-/-} and *apoe*^{-/-} mice and comparing them to *apoe*^{-/-} × *ifn-γ*^{+/+} mice. IFN-γ deficiency did not affect serum cholesterol concentrations or lipoprotein-cholesterol distributions in any groups. Furthermore, IFN-γ deficiency had no effect on the extent of atherosclerosis in female mice in either the aortic root or aortic arch. In contrast, IFN-γ deficiency markedly decreased lesions size in male *apoe*^{-/-} mice, highlighting a pronounced gender-specific effect with regard to this proinflammatory cytokine. In the present study, only male *apoe*^{-/-} mice were used and so the effect of IL-18 on lesion development in female *apoe*^{-/-} mice is not known. This gender-specific effect of IFN-γ on *apoe*^{-/-} mice warrants future studies to determine whether endogenous and exogenous IL-18 exerts effects in female mice.

Thus, IFN-γ is a potent proatherogenic cytokine, and having shown its role in promoting lesion development, we have now started to turn our attention toward defining the cellular sources of IFN-γ and the signaling responsible for its release. In summary, the present study provides strong evidence that IL-18 is a proatherogenic cytokine. Furthermore, its potent proinflammatory effect during atherosclerotic lesions formation is dependent on the presence of IFN-γ.

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