IL-18 Accelerates Atherosclerosis Accompanied by Elevation of IFN-γ and CXCL16 Expression Independently of T Cells

Charlotta Tenger, Anna Sundborger, Jacek Jawien, Xinghua Zhou

Objective—The proatherogenic effect of IL-18 is shown to be dependent on IFN-γ production. It is believed that activated T cells play a proatherogenic role through secretion of IFN-γ. However, recent studies in vitro have shown that macrophages, NK cells, and even vascular smooth muscle cells may also secrete IFN-γ on stimulation by cytokines like IL-18. We therefore investigated whether cells other than activated T cells can play a proatherogenic role via IFN-γ secretion under the stimulation of IL-18 in vivo.

Methods and Results—SCID/apoE knockout mice were injected intraperitoneally with either IL-18 or phosphate-buffered saline 3 times per week for 7 weeks. Our results show that administration of IL-18 leads to 3-fold larger lesions and 1.7-fold higher circulating IFN-γ despite the absence of T cells. In addition, increased IFN-γ, accompanied by elevation of the scavenger receptor/chemokine CXCL16, was observed in both lesions and spleens. Furthermore, our findings revealed that macrophages, NK cells, and vascular cells were the source of IFN-γ under the stimulation of IL-18 in the absence of T cells in vivo.

Conclusion—The current data suggest that the proatherogenic effect of IL-18 can occur in the absence of T cells and that IFN-γ secreted by macrophages, NK cells, and vascular cells is sufficient for the disease progression. (Arterioscler Thromb Vasc Biol. 2005;25:1-6.)

Key Words: atherosclerosis ■ cytokines ■ macrophages ■ NK cells ■ scavenger receptors

Atherosclerosis is a disease of the large and medium arteries, characterized by lipid-laden inflammatory lesions in the vessel wall. It has been found that atherosclerotic lesions of all stages contain significant amounts of macrophages and activated T cells that express cytokines. Interferon (IFN)-γ has been proposed to be a key cytokine in atherogenesis. IFN-γ activates macrophages, which in turn produce proinflammatory cytokines, oxygen radicals, and metalloproteinases. IFN-γ also stimulates endothelial cells to express adhesion molecules and reduces contractility and fibrogenesis in vascular smooth muscle cells (SMCs), as well as inhibits the ATP-binding cassette protein-1, which mediates cholesterol efflux from macrophages. CXCL16, a chemokine of the CXC family that has been found in the atherosclerotic lesion with the function of a scavenger receptor, can also be induced by IFN-γ. Studies have shown that gene deficiency in either IFN-γ receptor or IFN-γ itself results in reduced lesion size, whereas direct injection of IFN-γ in apolipoprotein E knockout (apoE KO) mice accelerates lesion development.

IFN-γ was originally considered to be restricted to T cells and NK cells. IFN-γ secreting CD4+ T cells have been found to be the dominating cell type in the lesions and transfer of CD4+ T cells into an immunodeficient atherosclerotic murine model accelerates the disease. IFN-γ can be induced by a set of cytokines including IL-12 and IL-18. A combination of both cytokines has been found to induce the expression of IFN-γ in vitro, not only in cultured T cells and NK cells but also in macrophages, dendritic cells, and even vascular SMCs. Overexpression of IL-18 binding protein, a natural inhibitor of IL-18, slows the progression of aortic lesions in apoE KO mice. The IL-18/apoE double KO mice show reduced lesion size, whereas direct injection of recombinant IL-18 enhances atherosclerosis.

Materials and Methods
Lyophilized recombinant mouse IL-18 (B002–5; Medical & Biological Laboratories, Nagoya, Japan) was reconstituted in ice-cold saline 3 times per week for 7 weeks. Our results show that administration of IL-18 leads to 3-fold larger lesions and 1.7-fold higher circulating IFN-γ despite the absence of T cells. In addition, increased IFN-γ, accompanied by elevation of the scavenger receptor/chemokine CXCL16, was observed in both lesions and spleens. Furthermore, our findings revealed that macrophages, NK cells, and vascular cells were the source of IFN-γ under the stimulation of IL-18 in the absence of T cells in vivo.

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sterile distilled water to prepare a stock solution of 5 μg/100 μL, as recommended by the manufacturer. The stock solution was further diluted in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA) (PBS-BSA) as a carrier.

Mice and Experimental Design
Female SCID/apoE KO mice on the C57BL/6 background were obtained from our own breeding and housed in a specific pathogen-free facility. They were fed standard chow and the antibiotics were administered by drinking water (1 mL in 310 mL water 2 times per week). All experiments were approved by the local ethics committee. At 7 weeks of age, the mice were randomly grouped (n = 9 to 10) and injected intraperitoneally with recombinant mouse IL-18 (50 ng/g body weight) diluted in PBS-BSA, or with PBS-BSA only as a control. The mice received 3 injections per week for 7 weeks, were euthanized at 14 weeks of age, and analyzed for atherosclerotic lesion development. To evaluate the role of exogenous IL-18 on the expression of IFN-γ and CXCL16 in lesions and spleens, and to avoid possible side effects caused by long-term cytokine treatment, an additional experiment with short-term injection of IL-18 was applied. Randomly selected groups of female SCID/apoE KO mice (n = 9 to 10) were injected intraperitoneally with either recombinant mouse IL-18 (100 ng/g body weight) diluted in PBS-BSA or PBS-BSA only, and received a second injection after 24 hours. The mice were euthanized 4 hours after the last injection for intracellular fluorescence-activated cell sorter (FACS) staining (n = 4) or after 6 hours for mRNA preparation (lesion n = 5 to 6, spleen n = 6 to 8, aorta n = 9 to 10).

Lesion Quantification, Serum CH, TG, SAA, and IFN-γ Assays
Because of the fact that there is no lesion detectable outside of the aortic root of SCID/apoE KO mice at this age (Zhou et al unpublished findings), the root of aorta was chosen for lesion development assay. Blinded analysis of lesion size in the aortic root was made according to a previously described protocol. To determine the levels of total cholesterol, high-density lipoprotein cholesterol, triglyceride, serum amyloid A protein (SAA), and IFN-γ in the circulation, blood was collected by heart puncture in conjunction with termination. Total serum cholesterol, high-density lipoprotein cholesterol, and serum triglyceride were determined by adding 10 μL of serum onto Vitros II slides and measuring with Vitros DT60II Chemistry System (Ortho-Clinical Diagnostics, Rochester, NY). Serum levels of IFN-γ and SAA were measured with enzyme-linked immunosorbent assay (ELISA) (OptEIA mouse IFN-γ set, #555138; Pharmingen, Calif; and Mouse SAA immunoassay Kit, #KMA0012; Biosource, Nivelles, Belgium). All procedures were performed according to the manufacturers’ instructions. Before diluting the blood for the ELISA assays, the mouse sera were centrifuged at 14,000g for 30 minutes to remove chylomicrons from the sera.

Real-Time Reverse-Transcriptase Polymerase Chain Reaction for Quantification of IFN-γ and CXCL16 Expression
Total RNA was extracted from the lesions in the root of aorta, from the spleens, and from the healthy abdominal aorta after the short-term treatment. The spleens were frozen in dry ice directly after the euthanization. To avoid the possible degradation of the sample RNA, the aorta was perfused with RNA later (#76104; Qiagen, Hilden, Germany) and kept in the same buffer during the isolation of the lesions from the aortic root and the isolation of the healthy abdominal aorta from adventitia. The isolation was performed under a microscope. Total RNA was prepared using an RNeasy Kit (#74104; Qiagen, Hilden, Germany). Good quality of the RNA was confirmed by a 2100 Bioanalyzer (Agilent). The mRNA levels of IFN-γ and CXCL16 were quantified by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) as previously described. The following primers and probes were applied: mouse IFN-γ: FW, 5-AGCAACAGCAAGGCGAAAAA-3; RW, 5-CTGGA-CTGTGGGTGTGTGTA-3; and TM, 5-CCTCAACCTGCGTAATTACTCATGAATGCATTCC-3; mouse CXCL16: FW, 5-TCTCTTCTTCTTGGGTGCGTG-3; RW, 5-CAGCCGACTGCCCTGTTG-3; and TM, 5-TGACCCCTGACGGGATGTC-3. The real-time RT-PCR data are expressed as relative mRNA units normalized to mouse 18S ribosomal mRNA expression. Mouse 18S was quantified by a predescribed TaqMan assay (#4310893E; Applied Biosystems, Warrington, UK).

Immunohistochemistry Staining
To characterize the inflammation of the lesions, we performed immunostaining on sections of the aortic root. The protocol has been described in previous publications. Antibodies used are shown in the Table, and the stainings were visualized by peroxidase substrate kit (SK-4100; Vector Laboratories, Burlingame, Calif). MHC II and vascular cell adhesion molecule-1 were quantified by counting the number of positive cells per mm².

Flow Cytometry
To exclude a potential “leakage,” ie, appearance of T and B cells, which has been reported in aged SCID mice, we assessed the frequency of T and B cells by flow cytometry in all experimental mice. Splenocytes were stained on ice for 30 minutes with fluorescein isothiocyanate-conjugated anti-CD3 and PE-anti-CD19 antibodies (Table), and analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Mountainview, Calif). For intracellular cytokine staining, splenocytes from short-term treated mice were first stained on ice for 20 minutes with PE-anti-CD11b or PE-anti-NK1.1 antibodies (Table) in PBS with 5% FCS and 10 μg/mL Brefeldin A, then fixed 20 minutes on ice in 2% formaldehyde, permeabilized 10 minutes at RT in PBS with 5% fetal calf serum and 0.5% saponin, stained for 30 minutes at RT with fluorescein isothiocyanate-anti-IFN-γ antibody (Table) in PBS with 5% fetal calf serum and 0.5% saponin, and finally analyzed with a FACSCalibur.

Statistics
Results are expressed as mean ± SEM. Data were analyzed by the Mann–Whitney significance test. The significance level was set to P<0.05.
Results

IL-18 Accelerates Atherosclerosis in the Absence of T Cells With Elevation of IFN-γ in Circulation

At 14 weeks of age, no T or B cells could be detected by FACS in any of the SCID/apoE KO mice. The mice treated with recombinant IL-18 showed 187% increase in lesion size compared with controls (4338 ± 896 μm² versus 1513 ± 2965 μm²) (Figure 1). The macrophage marker CD68 was expressed throughout the whole lesion, showing that macrophages were the major cell type in the lesions (Figure 2). Vascular cell adhesion molecule-1 can be induced via NFκB, which is activated by proinflammatory cytokines. The stronger expression of vascular cell adhesion molecule-1 therefore implies local inflammation in the lesions after IL-18 treatment (5349 ± 1455 positive cells/mm² versus 1945 ± 107 positive cells/mm² in control) (Figure 2). ELISA assay revealed that lesion development was accompanied by a significant elevation of both IFN-γ (92.13 pg/mL ± 19.61 versus 44.70 pg/mL ± 6.82) (Figure 3E) and SAA (241.58 μg/mL ± 17.76 versus 91.11 μg/mL ± 14.05) (Figure 3F), indicative of a systemic inflammatory response induced by IL-18 treatment. However, IL-18 administration did not affect the serum levels of total cholesterol (7.30 ± 0.40 mmol/L versus 7.81 ± 0.61 mmol/L), triglycerides (0.65 ± 0.03 mmol/L versus 0.71 ± 0.05 mmol/L), or body weight (19.38 ± 0.26 grams versus 20.80 ± 0.58 grams). High-density lipoprotein cholesterol was undetectable in all sera (<0.03 mmol/L).

IFN-γ Is Induced With Elevation of CXCL16 in Both Lesions and Spleens

To further investigate if IFN-γ was induced systemically and to quantify the cytokine production, the mRNA levels of IFN-γ in both lesions and spleens were determined by real-time RT-PCR. In comparison with PBS treatment, IL-18 administration significantly induced IFN-γ expression in both lesions and spleens, suggesting that IL-18 acts systemically (ratio IFN-γ:18S was 7.34 ± 1.67 versus 0.89 ± 0.23 in lesions).
and 1536.10±176.26 versus 103.43±29.40 in spleens) (Figure 3A and 3B). The increased IFN-γ expression was accompanied by a significant elevation of the IFN-γ-inducible scavenger receptor and chemokine CXCL16 mRNA in both lesions and spleens (ratio CXCL16/18S was 27.0±2.75 versus 14.52±2.39 in lesions and 149.11±18.65 versus 83.85±26.49 in spleens) (Figure 3C and 3D).

**IFN-γ Is Produced by Macrophages, NK Cells, and Vascular Cells in the Absence of T Cells**

To identify which cell type that, in addition to the T cells, can secrete IFN-γ in vivo and accelerate the disease under the stimulation of IL-18, we performed FACS analysis on splenocytes and real-time PCR on healthy aorta from short-term-treated mice. The FACS analysis revealed that both macrophages and NK cells responded to IL-18 in vivo by producing IFN-γ (30.45±1.63 versus 20.63±2.14, mean IFN-γ fluorescence among all CD11b⁺ cells; and 16.03±0.15 versus 11.96±1.02, mean IFN-γ fluorescence among all NK1.1⁺ cells) (Figure 4A, 4B, and 4C). The FACS analysis was additionally performed on spleen cells from male SCID/apoE KO mice with similar results (data not shown). We also detected a significant increase in IFN-γ mRNA expression in the healthy aorta after IL-18 treatment by real-time RT-PCR (ratio IFN-γ/18S was 6008±3031 in the IL-18 group versus 1635±930 in the PBS group) (Figure 4D), indicating that cells in the vascular wall are also producers of IFN-γ.

**Discussion**

The results of the present study show: (1) administration of IL-18 accelerates atherosclerosis in immunodeficient mice; (2) increased lesion development is accompanied by elevation of IFN-γ in circulation, lesions, and spleen; (3) IL-18 acts systemically; (4) macrophages, NK cells, and vascular cells are sources of IFN-γ under the stimulation of IL-18 in immunodeficient mice; and (5) increased IFN-γ is accompanied by elevation of CXCL16 mRNA in lesions and spleens.

IL-18 has been described as an IFN-γ inducing factor. It is proposed from clinical studies that IL-18 is a strong predictor of cardiovascular death in stable and unstable angina. Recently, Whitman et al have described that exogenous IL-18 enhances atherosclerosis in an IFN-γ-dependent manner in the apoE KO mouse. However, it is still unknown what cell type responds to IL-18 stimulation in vivo. It has long been believed that activated T cells are the major source of IFN-γ in atherosclerosis. Lack of T cells in SCID/apoE KO mice results in a 74% reduction in lesion size and a 85% decrease in circulating IFN-γ levels compared with apoE KO mice, confirming the role of adaptive immunity in atherosclerosis. Nevertheless, lesions containing mainly macrophages still develop in untouched SCID/apoE KO mice. It has been reported that cultured macrophages can be induced to produce IFN-γ. Interestingly, Gerdes et al have found that vascular macrophages and SMCs can secrete IFN-γ under the stimulation of IL-18, and these cells can also express receptors for IL-18. Our current in vivo study confirms this observation. In addition, NK cells have been shown to respond to IL-18 by IFN-γ production in vitro. All these cell types therefore may be of importance in response to IL-18 stimulation and responsible for the IFN-γ secretion in vivo.

The importance of macrophages and NK cells in the progression of atherosclerosis has been emphasized in animal models. In addition to IFN-γ, these cells also produce many other cytokines, including granulocyte-macrophage colony-stimulating factor and IL-10, which can prevent the progression of the disease. It has been reported that IL-18 itself can directly inhibit IL-10 production, and enhance NK cell activity, and exhibit biological function in an IFN-γ-independent manner. These reports imply that IL-18 may affect atherosclerosis via an IFN-γ-independent pathway, even though no such experiment is performed in this perspective. Nevertheless, our study shows that IL-18 executes a proatherogenic role in vivo, with induction of IFN-γ in the absence of T cells. The current data demonstrate that macrophages, NK cells, and vascular cells can be the source of IFN-γ in vivo in response to IL-18, which is sufficient for the disease progression.
genic cytokine, it has been reported that IFN-γ downregulates expression of SR-A and CD36.44,45 Its proatherogenic action has therefore remained unclear. This paradox could now be explained by the findings that IFN-γ upregulates expression of CXCL16 in vivo in lesions and in monocytes and SMCs in vitro.46,47 IFN-γ also enhances the uptake of oxidized low-density lipoprotein in monocytes and SMCs via the CXCL16 receptor.46-48 Our present data have shown that an increased IFN-γ level was paralleled with a significant elevation of CXCL16 mRNA expression in both lesions and spleens after IL-18 administration. This suggests that the proatherogenic role of IL-18 could partly depend on IFN-γ mediated upregulation of CXCL16. Interestingly, two studies have recently shown that IL-18 can induce CXCL16 in SMCs,49-50 however, no experiment shows a direct effect of IL-18 on CXCL16 expression in the absence of IFN-γ in either SMCs or macrophages.

In summary, our findings extend the principle of IL-18 as a proatherogenic cytokine and suggest that the proatherogenic effect of IL-18 can occur in the absence of T cells. IFN-γ secreted by macrophages, NK cells, and vascular cells in vivo is sufficient for disease progression and may involve upregulation of scavenger receptor CXCL16.

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References


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