

Interleukin-4 Deficiency Decreases Atherosclerotic Lesion Formation in a Site-Specific Manner in Female LDL Receptor^{-/-} Mice

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Abstract—Activated lymphocytes and mast cells have been detected in human atherosclerotic lesions. Interleukin-4 (IL-4) is a prominent cytokine released during the activation of both these cell types, and its mRNA has been detected in human and mouse atherosclerotic lesions. To define the effects of IL-4 on atherogenesis, bone marrow stem cells from either IL-4^{-/-} or IL-4^{+/+} mice were transplanted into lethally irradiated female low density lipoprotein (LDL) receptor^{-/-} mice. After an interval sufficient to allow engraftment, mice were placed on a diet containing 21% saturated fat, 1.25% cholesterol, and 0.5% cholate. Hematopoietic engraftment was confirmed by the presence of the LDL receptor gene in bone marrow cells. The effect on IL-4 depletion was confirmed by quantifying cytokine release from splenocytes of reconstituted mice. The deficiency of IL-4 in bone marrow-derived cells had no effect on serum cholesterol concentrations or on the distribution of cholesterol among lipoproteins. Atherosclerotic lesion formation was not changed in the aortic root. However, deficiency of IL-4 led to reduced lesion size in the arch ($9.1 \pm 1.1\%$ versus $2.8 \pm 0.8\%$ of intimal area, $P < 0.001$) and the thoracic aorta ($1.2 \pm 0.2\%$ versus $0.4 \pm 0.1\%$, $P < 0.002$). Therefore, IL-4 deficiency reduced atherosclerotic lesion formation in a site-specific manner in female LDL receptor^{-/-} mice fed a high-fat diet. (*Arterioscler Thromb Vasc Biol.* 2002;22:456-461.)

Key Words: atherosclerosis ■ bone marrow transplant ■ interleukin-4 ■ low density lipoprotein receptors ■ T lymphocytes

T lymphocytes are prominent components of human atherosclerotic lesions that are present at all stages of development.¹⁻⁴ Many of these T lymphocytes are activated, as evidenced by the expression of major histocompatibility complex class II and very late activation antigen-1. Evidence of activation of this cell type is also provided by the expression of interleukin-2 receptors (CD25) on a small number of cells^{5,6} and by their proliferation within lesions.⁷ Additional evidence that T lymphocytes are activated within atherosclerotic lesions is implied by the close association of this cell type with macrophages.⁸ T lymphocytes are frequently directly apposed to macrophages in lesions and have been shown to be linked by specialized membrane contacts that are consistent with a functional interaction.⁹ Therefore, these activated T lymphocytes could secrete an array of cytokines that influences the atherogenic process.

Atherosclerotic lesions in apoE^{-/-} and LDL receptor^{-/-} mice also contain T lymphocytes that are predominantly CD4⁺.¹⁰⁻¹² CD4⁺ cells can be further categorized as T helper (Th0, Th1, and Th2) cells, according to the spectrum of their cytokine release.¹³ Currently, there has been no definition of these subtypes within mouse atherosclerotic lesions, although mRNA for the archetype Th1 cytokine,

interferon- γ , has been detected in atherosclerotic lesions from mice, and it appears to function in a proatherogenic manner.^{12,14,15} There is also evidence of the presence of interleukin-4 (IL-4), a Th2 cytokine, in atherosclerotic lesions from mice when the disease is generated under conditions of pronounced hypercholesterolemia.¹⁴

In addition to activated T lymphocytes, IL-4 is also secreted by mast cells and natural killer cells. IL-4 is generally considered to be an anti-inflammatory cytokine. However, in the context of the atherogenic process, there are several processes that are regulated by this cytokine that could hypothetically increase lesion formation through a number of mechanisms, including monocyte recruitment,¹⁶ monocyte adhesion,¹⁷ lipoprotein modification,^{18,19} and macrophage metabolism of modified lipoproteins.^{20,21}

IL-4 is exclusively produced by hematopoietic cells. Therefore, rather than crossbreeding mice to develop atherosclerosis-susceptible mice that are deficient in IL-4, we used bone marrow stem cell transplantation to expedite the generation of mice that are deficient in this cytokine. Furthermore, because IL-4 mRNA is present in mouse atherosclerotic lesions under severe hypercholesterolemic conditions,¹⁴ we selected a diet that promotes a large increase in plasma

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cholesterol concentrations. Under these conditions, we determined that deficiency of IL-4 reduces the extent of atherosclerosis in female LDL receptor^{-/-} mice in the absence of any changes on plasma cholesterol or lipoprotein cholesterol distributions.

Methods

Animals

LDL receptor^{-/-}, IL-4^{-/-}, and C57BL/6 mice were obtained from Jackson Laboratories, Bar Harbor, Me. LDL receptor^{-/-} and IL-4^{-/-} mice had been backcrossed 10 times into a C57BL/6 background. Mice were housed in a specific pathogen-free room and fed a normal diet (Ralston Purina) before the initiation of the present study. Six weeks after bone marrow transplantation, recipient mice were placed on a modified diet containing 21% saturated fat (wt/wt), 1.25% cholesterol (wt/wt), and 0.5% cholate (wt/wt), from Harlan Teklad, for 4 weeks. Body weight was measured weekly during feeding of the modified diet. All procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Bone Marrow Transplantation

Female LDL receptor^{-/-} recipients (n=10 mice per group), aged 9 months, were provided drinking water containing sulfatrim (4 µg/mL) 1 week before bone marrow transplantation. Recipient mice were lethally irradiated with a total of 9 Gy from a cesium γ source, administered as 2 doses of 4.5 Gy for 2 minutes each, separated by a 3-hour interval. Bone marrow cells were harvested from femurs and tibias of sex- and age-matched IL-4^{+/+} and IL-4^{-/-} (n=2 mice per group) donor mice by flushing with Hanks' buffered saline solution containing 2% (vol/vol) FBS. Lethally irradiated mice received 1×10^7 bone marrow cells by tail vein injection and then were maintained on antibiotic-containing drinking water (sulfatrim) for the first 4 weeks after transplantation.

Genotyping for LDL Receptor and IL-4 in Bone Marrow Cells

Polymerase chain reaction (PCR) analysis of hematopoietic cells was performed to determine the genotype of recipient mice. DNA was isolated from bone marrow by using a Dneasy Kit (Qiagen). Primers specific for either IL-4 (5'-GCACAGAGCTATTGATGGGTC-3', 5'-GCTGTGAGGACGTTTGGC-3', and 5'-TCAGGACATAGCGTTGGC-3') or LDL receptor (5'-AGGTGAGA TGACAGGAGATC-3', 5'-ACCCCAAGACGTGCTCCCAGGATGA-3', and 5'-CGCAGTGCTCCTCATCTGACTTGT-3'), as described by the Jackson Laboratories Web site (www.jax.org), were used to amplify DNA. PCR reactions (20 µL) were performed for 35 cycles with 100 ng DNA, 0.2 mmol/L dNTPs, PCR buffer (for IL-4, 60 mmol/L Tris-HCl, 2.5 mmol/L MgCl₂, and 25 mmol/L ammonium sulfate, pH 10; for LDL receptor, 4.4 mmol/L MgCl₂, pH 8.5; Invitrogen), 100 pmol primers, and 0.3 U *Taq* polymerase (Promega). IL-4 PCR amplification was performed as follows: denaturing at 94°C for 30 seconds, followed by 35 cycles at 94°C (1 minute), 55°C (2 minutes), and 72°C (3 minutes), with a final elongation at 72°C (7 minutes). IL-4 PCR amplification resulted in a 444-bp fragment for a wild-type allele and a 576-bp fragment for the disrupted allele. LDL receptor PCR amplification was performed as follows: denaturing at 94°C for 3 minutes, which was followed by 6 cycles at 94°C (35 seconds), 64°C (45 seconds), dropping 1°C per cycle, and 72°C (45 seconds), which was followed by 25 cycles at 94°C (35 seconds), 58°C (30 seconds), and 72°C (45 seconds), and a final elongation step at 72°C (2 minutes). PCR amplification for the LDL receptor resulted in a 383-bp fragment for the LDL receptor wild-type allele or 800-bp fragment for a disrupted allele. PCR products were analyzed on a 2% agarose gel in Tris borate/EDTA buffer.

Phenotyping for IL-4 in Spleen Cells

Spleen cells were harvested as described previously.²² IL-4 production was determined in activated and nonactivated spleen cells by using an IL-4 Cytotrap Kit (BioSource).

Lipid and Lipoprotein Analysis

Serum cholesterol and triglyceride concentrations were measured by enzymatic colorimetric assay (Wako Chemical Co). Lipoprotein cholesterol distribution was determined in individual serum samples (50 µL) from 5 mice in each group after fractionation on a single Superose 6 column. Fractions were collected, and cholesterol concentrations were determined by enzymatic colorimetric assay (Wako Chemical Co), as described previously.²³

Removal of Tissue and Blood Samples

Mice were anesthetized by intraperitoneal injection of ketamine (90 mg/kg) and xylazine (10 mg/kg). Terminal blood samples were collected by puncture of the right ventricle. Blood was allowed to clot at room temperature for 1 hour before centrifugation. Mice were perfused with PBS (20 mL) via the left ventricle, while perfusate drained from the severed right atria. The heart and ascending aorta to the iliac bifurcation were removed. The heart was separated from the aorta at the base, embedded in OCT compound, and stored at -20°C. Aortic tissue was placed in freshly prepared paraformaldehyde (4% [wt/vol] in PBS) overnight at room temperature. After tissue fixation, adventitial tissue was removed, and the luminal surface was exposed. Aortas were pinned to a dark surface, and images were captured by using a Spot digital camera (Diagnostic Instruments).^{24,25}

Atherosclerotic Lesion Analysis

The extent of atherosclerotic lesions was quantified in aortas by using Image-Pro computer software (Media Cybernetics), as described previously.^{24,25} Regions of the aorta quantified were defined as follows: (1) arch, from the ascending arch to 3 mm distal to the left subclavian artery; (2) thorax, from the arch to the intercostal artery branch; and (3) abdominal region, from the thorax to the branch of the iliac bifurcation.

Atherosclerotic lesions were quantified in the aortic root as described previously.¹⁵ Sections (8 µm) were collected throughout the aortic sinus, and 9 sections were quantified at 80-µm intervals. Data are represented as lesion area (in square millimeters) in 80-µm intervals from the aortic cusp.

Tissue Sectioning and Immunocytochemistry

Tissue sections were studied in the aortic root and the arch. Sections for the aortic root analysis were acquired as described above for quantification. After en face analysis, aortic arch tissues were placed in OCT compound and frozen. Tissues were sectioned (8 µm) throughout the aortic arch from the apex of the greater curvature to the left subclavian branch.

For immunocytochemistry, tissue sections were blocked in the serum of the secondary antibody host. The primary antibodies used were rabbit anti-mouse macrophage serum (AI-AD31240, Accurate) and rat anti-mouse T lymphocyte (Thy-1.2, PharMingen); these were detected by using an appropriate secondary biotinylated anti-rabbit and anti-rat IgG (1:200, Vector Laboratories), respectively. Subsequently, tissue was incubated with a biotin-avidin-peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories). Immunoreactivity was visualized by using the red chromogen, 3-amino-9-ethylcarbazole (Biomedica Corp), and nuclei were counterstained with aqueous hematoxylin.

Statistical Analysis

All data are represented as mean \pm SEM. Statistical analysis was performed by the Student *t* test. If data were nonparametric, they were analyzed by the Mann-Whitney rank sum test. All data analyses were performed with the use of SigmaStat 2.03 software (SPSS, Inc). Values with $P \leq 0.05$ were considered statistically significant.

Results

Both groups of mice appeared to be in good general health after bone marrow transplantation. There was no significant difference in body weight between the groups during the feeding of the normal or modified diets (data not shown).

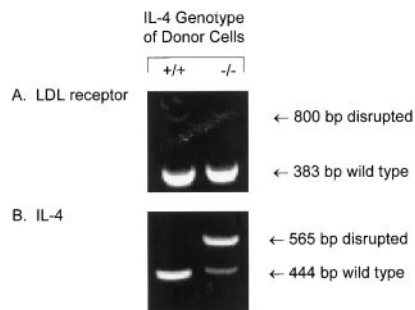


Figure 1. Hematopoietic cells from IL-4^{+/+}→LDL receptor^{-/-} and IL-4^{-/-}→LDL receptor^{-/-} mice exhibit the appropriate genotype after engraftment with either IL-4^{+/+} or IL-4^{-/-} stem cells, as shown in these representative examples. A, PCR amplified a 383-bp wild-type fragment and a 800-bp null fragment in IL-4^{+/+}→LDL receptor^{-/-} and IL-4^{-/-}→LDL receptor^{-/-} bone marrow DNA, indicating the presence of the LDL receptor gene in both groups. B, PCR amplified a 444-bp wild-type fragment in IL-4^{+/+}→LDL receptor^{-/-} bone marrow DNA, indicating the presence of the IL-4 gene and a low level 444-bp wild-type and a 576-bp disrupted fragment in IL-4^{-/-}→LDL receptor^{-/-} bone marrow DNA, indicating that some host stem cells had survived irradiation and contributed to hematopoiesis, albeit at low levels.

Efficacy of Bone Marrow Cell Engraftment

PCR analysis of bone marrow cells at the termination of the study demonstrated the presence of the LDL receptor gene in hematopoietic cells of all recipients in both study groups (Figure 1A). Analysis of IL-4 expression in recipients receiving wild-type bone marrow demonstrated homozygous expression of the IL-4 gene (Figure 1B). In contrast, recipients receiving marrow cells from IL-4^{-/-} donors demonstrated wild-type and disrupted alleles (444- and 576-bp fragments). In agreement with these findings, IL-4 production was decreased in splenocytes of IL-4^{-/-}→LDL receptor^{-/-} mice during basal and LPS-stimulated conditions (Figure 2A and 2B). Taken together, these findings suggest that the hematopoietic system of engrafted mice was chimeric. Nonetheless, there was a marked reduction in IL-4 production in the IL-4^{-/-}→LDL receptor^{-/-} mice.

Serum Cholesterol, Triglyceride, and Lipoprotein Profiles

Ingestion of an atherogenic diet for 4 weeks led to severe hypercholesterolemia in both groups. However, IL-4 deficiency did not alter serum concentrations of either total cholesterol or triglycerides (Table).

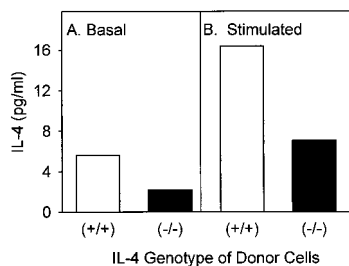


Figure 2. IL-4 production in splenocytes is decreased in IL-4^{-/-}→LDL receptor^{-/-} mice (solid bars) compared with IL-4^{+/+}→LDL receptor^{-/-} (open bars) mice under basal (A) or stimulated (B) conditions. IL-4 elaboration was defined in splenocytes (4×10^5 cells) by using a commercially available kit (Biosource).

IL-4 Deficiency in LDL Receptor^{-/-} Mice Does Not Alter Body Weight or Serum Lipids

	IL-4 ^{+/+} →LDL Receptor ^{-/-}	IL-4 ^{-/-} →LDL Receptor ^{-/-}
Body weight, g	26.4±2.0	27.1±1.1
Cholesterol, mg/dL	982±52	889±41
Triglycerides, mg/dL	180±18	225±27

Values are mean±SEM.

Size exclusion chromatography was performed on serum samples from individual mice to determine whether IL-4 deficiency altered serum lipoprotein distributions. The majority of serum cholesterol was in the VLDL fraction, with no differences in distribution between the 2 groups (Figure 3).

Atherosclerotic Lesion Quantification

Six weeks after bone marrow transplantation, mice were fed an atherogenic diet for 4 weeks to determine the effect of IL-4 deficiency on atherosclerotic lesion formation. The extent of atherosclerotic lesions was quantified in the aortic root and on the intimal surface of the entire aorta. Analysis of the aortic root demonstrated no differences in atherosclerotic lesion formation between the 2 groups (Figure 4). Despite the lack of effect in the aortic root, en face analysis of the aorta demonstrated a 69% reduction in lesion formation in the aortic arch of recipient mice repopulated with IL-4^{-/-} bone marrow cells ($P < 0.001$, Figure 5A). Moreover, there was a 67% reduction in grossly discernible lesions in the thoracic aorta of IL-4^{-/-} recipient mice ($P < 0.002$, Figure 5B). No discernible lesions were present in the abdominal aorta of either group.

Atherosclerotic Lesions Composition

Immunocytochemistry was performed on tissue from the aortic root and arch. All lesions examined in both regions consisted predominantly of lipid-laden macrophages. Small numbers of T lymphocytes were also present. There were no overt differences in the cellular characteristics of lesions from either IL-4^{+/+} or IL-4^{-/-} mice (data not shown).

Discussion

The present study has demonstrated that deficiency of IL-4 in markedly hypercholesterolemic female LDL receptor^{-/-} mice, produced by repopulation with bone marrow stem cells from IL-4^{-/-} mice, led to a reduction in the extent of

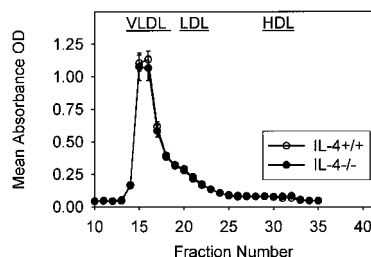


Figure 3. Cholesterol-lipoprotein distributions are not altered by IL-4 deficiency in LDL receptor^{-/-} mice. OD indicates optical density. Lipoprotein-cholesterol distribution was determined in serum from 5 individual mice from each group (IL-4^{+/+}→LDL receptor^{-/-}, open circles; IL-4^{-/-}→LDL receptor^{-/-}, solid circles).

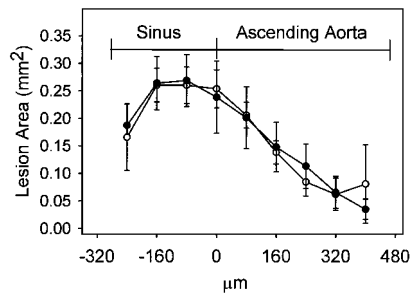


Figure 4. IL-4 deficiency does not alter atherosclerotic lesion formation in the aortic sinus of LDL receptor^{-/-} mice. Lesion size was determined in 8- μ m-thick sections at 80- μ m intervals, covering 720 μ m from the valve cusps. Shown are lesion areas for each section in IL-4^{+/+} \rightarrow LDL receptor^{-/-} (n=10, open circles) and IL-4^{-/-} \rightarrow LDL receptor^{-/-} (n=9, solid circles) mice, aligned to the transitional section as defined by disappearance of the valve cusps.

atherosclerosis in the arch and thoracic regions of the aorta. This reduction occurred in the absence of any observable changes in serum lipid concentrations or distribution of lipoprotein cholesterol.

IL-4 is thought to be secreted only by cells of hematopoietic origin. Therefore, bone marrow transplantation offers the potential to completely deplete IL-4. Bone marrow transplantation has been used to produce a deficient state in atherosclerosis research in many studies ever since its original description in apoE^{-/-} mice.^{26,27} However, this procedure does not commonly lead to complete replacement of the recipient hematopoietic system with donor cells. In accord with the tendency of this procedure to yield chimeric animals, PCR analysis of host bone marrow cells at the termination of the present experiments demonstrated the presence of IL-4 DNA, albeit at a reduced abundance. Furthermore, although IL-4 elaboration from splenocytes was not ablated, IL-4 secretion was significantly reduced in cells derived from mice repopulated with IL-4^{-/-} bone marrow cells under basal and stimulated conditions. Therefore, it is important to emphasize that the effects observed in the present study were in the presence of a reduced ability to synthesize IL-4 and not a complete deficiency, as would be achieved in genetically targeted mice.

IL-4 has been detected in atherosclerotic lesions from mice, but only during the feeding of diets that produced a severe hypercholesterolemic state.¹⁴ Therefore, we designed

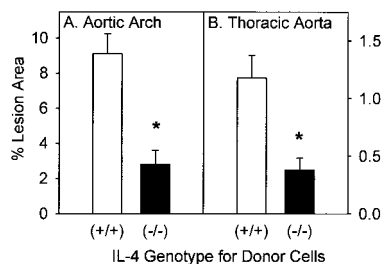


Figure 5. IL-4 deficiency decreases atherosclerotic lesion formation in LDL receptor^{-/-} mice. En face lesion area in IL-4^{+/+} \rightarrow LDL receptor^{-/-} (n=10, open bars) and IL-4^{-/-} \rightarrow LDL receptor^{-/-} (n=9, solid bars) mice in aortic arch (A) and thoracic aorta (B). No discernible lesions were present in the abdominal aorta. *P<0.001 for aortic arch values; *P<0.002 for thoracic aorta values.

the present study to recapitulate this condition because according to the observation of Zhou et al,¹⁴ it was assumed that IL-4 deficiency would have the most impact on lesion formation during severe hypercholesterolemia. The origin of IL-4 within atherosclerotic lesions has not been defined but could be attributable to activated T lymphocytes or natural killer cells, both of which have been detected in lesions from atherosclerosis-susceptible mice.^{10-12,28} Mast cells are also a rich source of IL-4, but although they are present in human lesions,^{29,30} their presence has not been described in atherosclerotic mouse tissue.

The procedure used in the present study resulted in the expression of LDL receptor by bone marrow cells of LDL receptor^{-/-} mice. The presence of LDL receptors has been shown to influence the development of lesions in female fat-fed C57BL/6 mice. In contrast, leukocyte-specific LDL receptors have no effect on the development of atherosclerosis in male LDL receptor^{-/-} mice.^{31,32} The effect of LDL receptors on leukocytes has not been defined in female mice, although the effects of LDL receptors in these cell types appear to be abrogated by the pronounced hypercholesterolemia that occurs in LDL receptor^{-/-} mice fed a high-fat diet.³¹

Relatively few studies have quantified atherosclerotic lesions in the aortic root and also throughout the aorta. Most have not reported equivalent changes in lesion size in these 2 regions in response to an intervention. Rather, most report either a lesser effect^{33,34} or no effect³⁵⁻³⁷ in the root, compared with the aortic intima. Region-specific differences have also been noted in lesion formation during immune deficiency in the aortic root and the brachiocephalic trunk.³⁸ Therefore, our finding of region-specific effects of an intervention on lesion formation has been observed by others, although the reason for these disparities are unknown.

One other study has evaluated the effects of IL-4 deficiency on the development of atherosclerosis.³⁹ These studies were performed in C57BL/6 mice fed a high-fat diet and did not demonstrate any effect of IL-4 deficiency in the development of atherosclerotic lesions. This lack of effect may be due to the paucity of lymphocytes in lesions from this model. However, administration of recombinant HSP65 or *Mycobacterium tuberculosis* accelerates fatty streak formation, and this enhanced lesion formation was ablated by IL-4 deficiency. Therefore, the result of this previous study is in agreement with our own data in defining IL-4 as a proatherogenic molecule.

Interleukin-10 (IL-10) can facilitate Th2 CD4⁺ cell responses, and its mRNA has been detected in atherosclerotic lesions.⁴⁰ Previous studies in which the effects of IL-10 on lesion formation have been studied in C57BL/6 mice that overexpress or are deficient in this cytokine demonstrate an antiatherogenic effect.^{41,42} These data are in contrast to our findings, in which IL-4 exerts proatherogenic effects. Differences in our findings may be accounted for by IL-10 being highly pleiotropic and being secreted by T lymphocytes and macrophages. In contrast, secretion of IL-4 by macrophages has not been demonstrated. Moreover, IL-10 has potent deactivating effects on macrophages, suggesting an autocrine role involving feedback and inhibition of the synthesis of cytokines by activated macrophages.^{43,44} Furthermore, studies investigating the role of IL-10 in atherosclerosis were per-

formed in C57BL/6 mice, which are an inbred atherosclerosis-susceptible mouse strain.⁴⁵ Lesions in these mice are composed of macrophage foam cells and contain no T lymphocytes. In contrast, immunostaining for T lymphocytes and macrophages was observed in lesions of both groups in the present study, as described previously.¹⁰

There are several potential mechanisms by which IL-4 may exert its effects on atherosclerosis, including several effects on lipoprotein metabolism through the upregulation of 15-lipoxygenase,^{18,38} CD36,²¹ and class A scavenger receptors.^{20,33,39,46} IL-4 can also exert effects on endothelial cells,^{17,47} smooth muscle cells, and macrophages,⁴⁸ which could impact the disease process. Further studies will define whether any of these multiple potential mechanisms are responsible for the effect of IL-4 on atherosclerosis.

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