

Depletion of Natural Killer Cell Function Decreases Atherosclerosis in Low-Density Lipoprotein Receptor Null Mice

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Objective—Natural killer (NK) cells are a key component of innate immunity. Despite being identified in human and mouse atherosclerotic lesions, the role of NK cells in the disease process is unknown. To determine this role, we created chimeric atherosclerosis-susceptible low-density lipoprotein (LDL) receptor null (*ldl-r*^{-/-}) mice that were deficient in functional NK cells through expression of a transgene encoding for Ly49A.

Methods and Results—Bone marrow cells from Ly49A transgenic and nontransgenic littermates were used to repopulate the hematopoietic system of lethally-irradiated female *ldl-r*^{-/-} mice. After a recovery period to permit sufficient engraftment, mice were placed on a diet enriched in saturated fat and cholesterol. After 8 weeks, there was no difference in either serum total cholesterol concentrations or lipoprotein cholesterol distribution in mice repopulated with nontransgenic versus Ly49A transgenic marrow cells. Using immunohistochemistry, we detected NK cells in atherosclerotic lesions of both groups of mice. However, deficiency of functional NK cells significantly reduced the size of atherosclerosis by 70% ($P=0.0002$) in cross-sectional analysis of the aortic root and by 38% ($P=0.004$) in *en face* analysis of the intimal surface of the aortic arch.

Conclusion—These studies demonstrate that NK cells infiltrate the vessel wall and promote atherosclerotic lesion development. (*Arterioscler Thromb Vasc Biol.* 2004;24:1049-1054.)

Key Words: atherosclerosis ■ NK cells ■ LDL receptor null mice ■ bone marrow transplantation

Atherosclerotic lesions are characterized by a pronounced infiltration of leukocytes at all stages of disease progression.¹ In humans and animal models of atherosclerosis, the most prominent cells that infiltrate evolving lesions are macrophages and T lymphocytes.²⁻⁴ The ablation of either of these cell types reduces the extent of atherosclerosis in mice that were rendered susceptible to the disease by deficiency of either apolipoprotein E or low-density lipoprotein (LDL) receptors.⁵⁻⁹ Small numbers of B lymphocytes,^{10,11} mast cells,¹² and dendritic cells¹³ have also been demonstrated in lesions, although their function has not been defined.

In addition to the cells mentioned, the presence of natural killer (NK) cell-associated antigen has been noted in human and mouse atherosclerotic lesions, thus providing strong suggestive evidence for the participation of NK cells in the atherogenic process.¹⁴⁻¹⁶ NK cells are derived with lymphocytes from common bone marrow progenitor cells and are characterized by their ability to kill aberrant cells without previous sensitization.^{17,18} These cells represent a critical component of the innate immune system.^{18,19} In humans, NK cells constitute ≈15% of all lymphocytes as defined by expression of CD56 and absence of CD3.¹⁸ However, the lack

of generally accepted markers for this cell type has led to confusion on the specific function of NK cells and hindered the ability to define the presence of this cell type in atherosclerotic lesions.

See page 992

Animal models that combine genetic risks for atherosclerosis with an altered immune system have been invaluable in demonstrating a link between atherosclerosis and immunity.^{2,16} Lack of an animal model that is selectively deficient in NK cells has prevented the creation of a similar animal model aimed at defining the true role of NK cells in atherosclerosis. NK cell function is decreased in mice having the beige mutation,²⁰⁻²² and these mice have been used in 2 separate atherosclerosis studies, yet these studies have yielded different results. Beige mice fed a diet enriched in saturated fat, cholesterol, and cholate do not exhibit any change in atherosclerotic lesions formation.²³ However, when the beige defect was bred into an LDL receptor-deficient background, there was a modest, but statistically significant, increase in lesion size.²⁴ The beige mouse has a very complex phenotype, and although NK cell activity is decreased in these mice, the

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defect is not complete,^{20–22} allowing for residual NK cell activity to persist. Furthermore, given the nature of the mutation in beige mice, which involves a poorly characterized protein required for proper lysosomal trafficking,²⁰ disturbances in cell populations distinct from that of NK cells may ultimately have been responsible for the antiatherogenic effect noted.²⁴

Recently, transgenic mice have been developed that have defective natural cytotoxicity and a selective deficiency in NK1.1⁺ CD3⁻ cells while maintaining functionally normal B and T lymphocytes.²⁵ This phenotype was achieved by expressing the inhibitory major histocompatibility complex (MHC) class I-specific receptor, Ly49A, under the control of the granzyme A promoter. Ly49A is present on all NK cells and is a C-type lectin-like receptor that recognizes the MHC class I ligands, H-2D(d) and D(k). Interactions of these ligands with Ly49A inhibits activation of NK cells, which provides the rationale for the absence of the functional cells in these transgenic mice.²⁵

The development of transgenic mice with selective deficiency in NK activity affords the ability to define the specific role of NK cells in the development of atherosclerosis. Therefore, in the present study, we generated chimeric mice by repopulating the hematopoietic system of lethally irradiated *ldl-r*^{-/-} mice with bone marrow cells obtained from Ly49A transgenic mice or gender-matched nontransgenic littermates. Engrafted mice were placed on a diet enriched in cholesterol and saturated fat, and the extent of atherosclerosis was measured in both the ascending aorta and the aortic arch. Deficiency of NK cell activity decreased the extent of atherosclerosis in both vascular regions without influencing the activation status of lesion-associated cells, as defined by expression of MHC class II.

Methods

Mice

Ly49A transgenic mice were generated directly in a C57BL/6 background as described previously.²⁵ The transgenic mice were bred to C57BL/6 mice and littermates were used for comparing transgenic versus nontransgenic mice. As described previously, Ly49A transgenic mice were healthy, fertile, and had no apparent abnormalities in lymphoid organs.²⁵ The *ldl-r*^{-/-} mice, backcrossed 10 times into a C57BL/6 background, were purchased from the Jackson Laboratory (Bar Harbor). All animal procedures performed were in accordance with our Institute's Animal Care and Use Committee guidelines.

Bone Marrow Transplantation

The technique of bone marrow transplantation was performed using essentially the same procedures described by Boisvert et al²⁶ and by Linton et al,²⁷ with a few minor modifications as noted. Eight-week-old, female *ldl-r*^{-/-} mice (n=24) were maintained on antibiotic-containing water for 1 week before irradiation. Animals were irradiated with a total of 900 rads from a cesium source delivered in 2 equal doses 3 hours apart. Donor bone marrow cells (1×10⁷) were injected into a tail vein of irradiated recipient mice. Four weeks after transplantation, the mice were placed on regular drinking water. Six weeks after transplantation, the mice were placed on a diet enriched in saturated fat (21% of wt/wt) and cholesterol (0.15%; Harlan Teklad diet 88137) and maintained for an additional 8 weeks.

Lipid and Lipoproteins

Serum total cholesterol 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 was resolved by size exclusion chromatography on a Superose 6 column as described previously.²⁸

Polymerase Chain Reaction Analysis

DNA was isolated from bone marrow cells and nonelicited peritoneal macrophages using a commercially available kit (Qiagen). DNA encoding the LDL receptor was detected by polymerase chain reaction (PCR) as described previously,²⁹ and the Ly49A transgene was detected by PCR using primers specific for the Ly49A transgene, (5'-ctc tct ttg cac tgc aga ct-3' and 5'-gct gat tgg ggt ggg aga g-3'), 10X buffer (M190A, Promega) with MgCl₂, and the following reaction conditions: 1 cycle (94°C, 1 minute), 35 cycles (94°C, 1 minute; 54°C, 1.5 minutes; 72°C, 1.5 minutes), and 1 cycle (72°C, 7 minutes).

Spleen Cell Preparation and Flow Cytometry

Immediately after perfusion of the mice, spleens were extracted, and single-cell suspensions were prepared by passage through a tissue strainer. Red blood cells were eliminated by hypotonic lysis in 0.14 mol/L NH₄Cl/0.017 mol/L Tris, pH 7.2, and viable leukocytes were labeled for flow cytometry using fluorochrome-coupled monoclonal antibodies specific for CD3, CD19, and NK1.1 (PharMingen). Cells were analyzed using a FACS Calibur cytometer (Becton Dickinson).

Quantification of Atherosclerotic Lesions on the Intimal Surface of the Aorta

Aortic tissues were prepared as described previously.^{7,30,31} To quantify the extent of intimal surface covered by grossly discernible lesions, aortas were cut and pinned to expose the entire intimal surface. Images of the aorta were captured on a digital camera, and analysis was performed with Image-Pro software (Media Cybernetics).

Quantification of Atherosclerotic Lesions in Tissue Sections

Atherosclerotic 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 defined by the region of the aortic root where the aortic cusps disappear and/or the ostia of the coronary arteries are present in the same cross-section as described previously.^{32–34} Atherosclerotic lesion area, defined as intimal tissue within the internal elastic lamina, was determined using Image-Pro software on images that were created using a Spot camera. The mean value of lesion area derived from the 4 sections spaced 100-μm apart in the ascending aorta was taken as the mean lesion size for each animal.

Immunocytochemistry

Immunocytochemistry was performed as described previously,⁴ using sequential sections of the ascending aorta adjacent to the sections stained with Oil Red O. The following reagents were used for immunostaining: a mouse macrophage polyclonal antiserum (1:3,000 dilution; Accurate Chemical Co), an antimouse MHC II monoclonal antibody (LS-004-SN, 1:5 dilution; Biosource International), and the rat anti-mouse monoclonal antibody 4D11 (1 μg/mL; PharMingen) against Ly49G2 (also known as LGL-1). Species-specific biotinylated secondary antibodies and avidin-peroxidase 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). Extracellular elastin and collagen were visualized with a Verhoeff and a Gomori trichrome stain, respectively. Because immunostaining for MHC class II leads to discrete staining of definable cells, lesion-associated MHC class II cells were counted and expressed as the

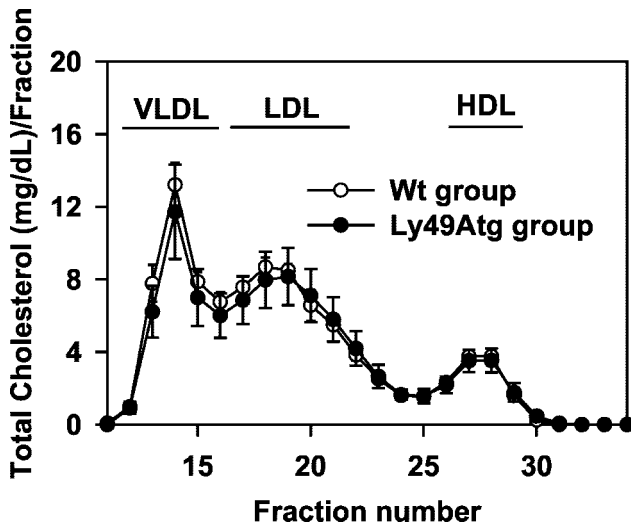


Figure 1. Characterization of serum cholesterol. Serum (50 μ L) from *ldl-r^{-/-}* mice engrafted with either nontransgenic (white circles) or Ly49A transgenic bone marrow cells (black circles) was resolved by size exclusion chromatography using a Superose 6 column. Total cholesterol concentrations were determined in fraction numbers 11 to 40, with each fraction having a total volume of 500 μ L. Symbols represent the means and bars the SEM of values obtained from the serum of 5 mice.

mean number of cells found in the 4 sections analyzed for lesion area, as described previously.^{31–33} However, we have found it difficult to assign a reliable quantitative measure to the content of diffuse staining such as for macrophages, collagen, and elastin. Therefore, we will only note these entities in terms of visually discernible features that are consistently seen in sections from all the mice of a specific group.

Statistics

Data analyses were performed using SigmaStat 2.03 software (SPSS Inc). Statistical differences between groups were determined by Student *t* test after testing that the data complied with the constraints of parametric analysis (Kolmogorov–Smirnov normality test). $P < 0.05$ was considered statistically significant.

Results

All mice survived the bone marrow transplant procedure and appeared healthy throughout the study. There were no overt differences in the appearance or general health of *ldl-r^{-/-}* mice repopulated with marrow from Ly49A transgenic mice compared with marrow from nontransgenic mice. Body, liver, and spleen weights were not significantly different between the groups. Expression of the transgene did not alter the levels of circulating leukocytes, erythrocytes, or platelets (data not shown). Engraftment was verified by PCR detection of the LDL receptor gene and the Ly49A transgene in the bone marrow and in nonelicited peritoneal macrophages of irradiated mice.

Serum cholesterol concentrations were not significantly different between the nontransgenic and transgenic groups (508 \pm 31 versus 605 \pm 47 mg/dL, $P = 0.111$, respectively), with the majority of cholesterol present in the VLDL and LDL subfractions (Figure 1). No changes in serum triglyceride concentrations were found between the nontransgenic and transgenic groups (387 \pm 48 versus 441 \pm 51 mg/dL, $P = 0.463$, respectively).

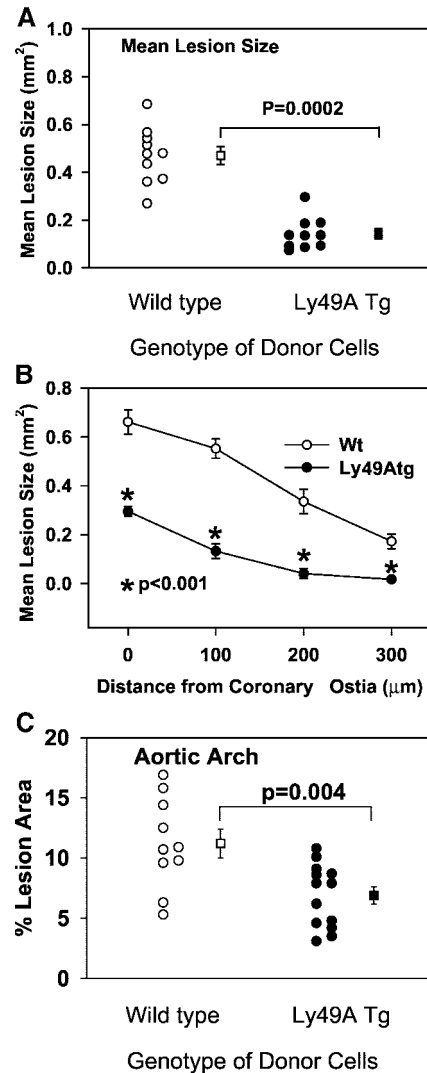


Figure 2. Quantification of atherosclerosis. The extent of atherosclerotic lesion development in *ldl-r^{-/-}* mice undergoing bone marrow transplantation from either nontransgenic (white symbols) or Ly49A transgenic (black symbols) mice was determined. Atherosclerotic size was determined from cross-sections of the ascending aorta and is represented as (A) mean lesion size in 4 regions of the ascending aorta and (B) as mean for each of these four regions. Lesion analysis was also determined in a second vascular bed, the aortic arch, by measuring (C) the percent intimal area covered by grossly discernible lesions. Values of individual mice are represented as circles, squares are means, and bars are SEM. * $P = 0.0002$; $P < 0.001$ and $P = 0.004$ for graphs A, B, and C, respectively.

Flow cytometric analysis of spleen cell preparations revealed that expression of the Ly49A transgene did not significantly reduce the presence of NK1.1⁺ CD3⁻ (2.2% \pm 0.7% versus 1.1% \pm 0.7% of viable cells; $P = 0.105$). No change in the population of spleen-associated T lymphocytes (CD3⁺) was noted between groups (30.7% \pm 2.2% versus 28.7% \pm 1.0% of viable cells; $P = 0.194$).

The extent of atherosclerosis was quantified using 2 different techniques in 2 separate vascular beds: sequential cross-sectioning of the aortic root and *en face* analysis of percent lesion area of the aortic arch. Both measurements demonstrated significantly less atherosclerosis in vascular

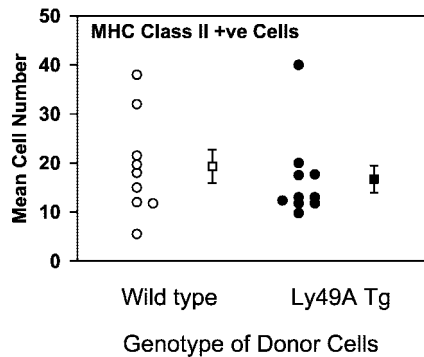


Figure 3. Quantification of MHC class II-positive cells in atherosclerotic lesions. The number of lesion-associated cells expressing MHC class II antigen as determined from sections of the ascending aorta of lethally irradiated female *ldl-r^{-/-}* mice that had been engrafted with bone marrow cells from wild-type (white symbols) or Ly49A transgenic (black symbols) donors after 8 weeks on an atherosclerotic diet. Segments of heart tissue spanning the aortic sinus and ascending aorta were embedded in optimal cutting temperature (OCT) compound, sectioned and stained with a monoclonal anti-mouse MHC class II-antibody (1:5 dilution). The tissue used to measure the number of lesion-associated MHC class II expressing cells was obtained from serial sections collected adjacent to those used in Figure 2A. Values of individual mice are represented as circles, squares are means, and bars are SEM.

tissues from *ldl-r^{-/-}* mice repopulated with Ly49A transgenic bone marrow cells (Figure 2A through 2C). The reduction was particularly striking in the case of the aortic root, where the extent of atherosclerotic lesions was decreased by 70% (0.471 ± 0.04 versus 0.143 ± 0.02 mm², $P < 0.001$; Figure 2A) in all 4 regions of the ascending aorta that were measured (Figure 2B). Analysis of a second vascular bed, the aortic arch, showed that *en face* lesion area was significantly decreased by 38% in the NK cell-deficient mice ($11.2\% \pm 1.2\%$ versus $6.9\% \pm 0.7\%$ of intimal area covered by lesion, $P = 0.004$; Figure 2C).

In addition to defining lesion size for the 2 groups, immunocytochemical analysis of the lesions was conducted to determine if there was any change in cellular composition or inflammatory status. Based on visual examination by 2 independent observers, there were no overt differences in the gross characteristics of lesions from both groups of mice, with all lesions composed predominantly of macrophages, and we found no visible difference in the extracellular distribution of either elastin or collagen (data not shown). In addition, quantitative analysis of cells expressing MHC class II, a marker of immunological activation, showed that depletion of NK function did not affect the mean number of cells expressing MHC class II (19 ± 3 versus 16 ± 3 cells in the lesions of the ascending aorta, $P = 0.6$; Figure 3). The anti-mouse Ly49G2 antibody, 4D11, was used to detect NK cells by immunohistochemistry. Although 4D11 will detect NK cells, it also will stain positive DX5-positive T lymphocytes (NK-T cells) and also a population of memory CD8-positive T lymphocytes in C57BL/6 mice. However, 4D11 will not cross-react with macrophages, which is the largest impediment to the use of the traditional NK cell immunohistochemical antibody asialo-GM-1. Staining atherosclerotic tissue from both groups with the monoclonal antibody 4D11

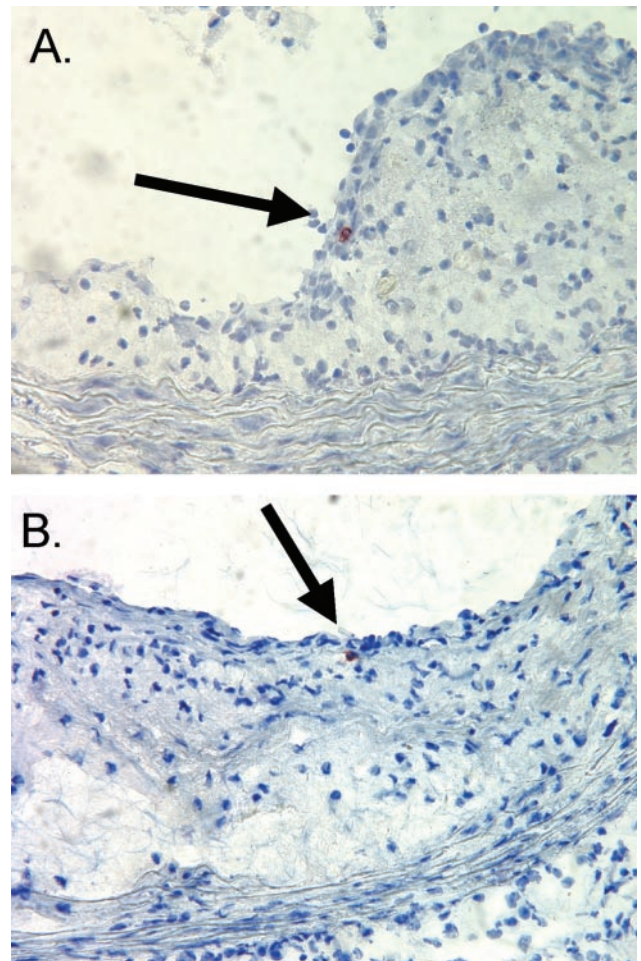


Figure 4. Detection of NK cells in atherosclerotic lesions by immunohistochemistry. NK cells (identified by the arrows) in atherosclerotic lesions were determined in sections cut from snap-frozen tissue of the ascending aorta of lethally irradiated female *ldl-r^{-/-}* mice that had been engrafted with bone marrow cells from wild-type (A) or Ly49A transgenic (B) donors after 8 weeks on an atherosclerotic diet. The segments of heart tissue spanning the aortic sinus and ascending aorta were embedded in OCT, sectioned and stained with a rat anti-mouse monoclonal antibody, 4D11 (1 μ g/mL; PharMingen), against Ly49G2. The ascending aortic tissue corresponds to serial sections used in Figure 2A. Magnification is $\times 400$.

showed positive staining of the same intensity with the number of cells per lesion being relatively small, ≈ 1 to 2 cells per lesion examined (Figure 4).

Discussion

The innate and acquired immune systems have been implicated in the atherogenic process. Among the components of innate immunity, monocyte-derived macrophages are present in abundance at all stages of the disease process.¹ Their pivotal role in atherogenesis has been demonstrated by the attenuation of lesion formation in monocyte deficiency in both apolipoprotein E^{-/-} mice³⁵ and *ldl-r^{-/-}* mice.³⁶ Two other cellular components of innate immunity for which there is a paucity of information regarding their atherogenic role are neutrophils and NK cells. In the case of the latter cell type, we designed this study to use the first mouse model of

functionally deficient NK cells²⁵ to define the role of these cells in the development of atherosclerosis in *ldl-r^{-/-}* mice fed a cholesterol and saturated fat-enriched diet. Development of *ldl-r^{-/-}* mice that are deficient of functional NK cells substantiated the participatory role of NK cells in atherosclerosis. Furthermore, our work indicates that the proatherogenic role of NK cells does not involve an alteration in the dyslipidemic state of the mouse, given that the noted reduction in atherosclerosis in mice devoid of functional NK cells occurred in the absence of any effect on serum lipid (cholesterol and triglyceride) concentrations or changes in the lipoprotein cholesterol distribution.

The presence of NK cells in atherosclerotic lesions has been suggested by immunohistochemistry, although the reagents used were not specific for this cell type.^{14,15} Despite the ambiguity regarding their identification, there exist chemoattractants that would promote recruitment of NK cells in lesions. In particular, MCP-1, which is present in lesions³⁷ and is previously shown to have a prominent role in atherogenesis, is a chemoattractant for NK cells.³⁸ Therefore, lack of NK cell recruitment may contribute to the reduced atherosclerosis observed in mice deficient in MCP-1,^{39,40} or its major receptor CCR-2.^{41,42} A major factor influencing NK cell activity is IL-15, which has recently been detected in atherosclerosis.^{43,44} Detection of IL-15 provides further evidence that NK cells could be regulated in the local milieu of atherosclerotic lesions.

The mechanism of NK cell deficiency influencing atherosclerosis is not clear. One of the prominent cytokines released by activated NK cells is interferon gamma. This cytokine has been implicated in the atherosclerotic process via direct effects^{31,32,45,46} and indirectly via IL-12 and IL-18.^{33,47,48} Also, a link between interferon-gamma release from NK cells after macrophage activation has been demonstrated by the ability of severe combined immunodeficient (*scid*) mice to respond to infection by *Listeria monocytogenes*.⁴⁹ However, in our current study, there are indications that changes in interferon-gamma may not be responsible for the differences seen in Ly49A transgenic versus littermate controls. This is primarily based on the lack of change of MHC class II expression in macrophages, which has been used as a marker of macrophage activation. Changes in this macrophage marker have been noted previously in other atherosclerosis studies in which interferon-gamma has been manipulated.^{31–33}

In addition to interferon-gamma, NK cells possess the potential to elaborate many other cytokines that may influence the formation of atherosclerotic lesions. These include granulocyte macrophage colony-stimulating factor, tumor necrosis factor-beta, IL-13, and IL-10, which exert a spectrum of effects on the development of atherosclerotic lesions.^{50–53} Therefore, the effect of NK cells on lesion formation may depend on the local environment of the cells, which will define the elaboration of specific cytokines. Another secretory product of NK cells is perforin. However, this does not appear to account for the changes in the extent of atherosclerosis, because Schiller et al²⁴ demonstrated that perforin deficiency did not change the extent of lesion formation in hyperlipidemic *ldl-r^{-/-}* mice.

In conclusion, we have demonstrated that deficiency of functional NK cells leads to reduced size of atherosclerotic lesions in diet induced hyperlipidemic *ldl-r^{-/-}* mice. The relatively limited information of NK cell biology and reagents has confined our ability and that of others²⁴ to fully define the role of this cell type in the atherogenic process. However, with the evolution of specific reagents, it will be possible to define the role of NK cells under a range of atherosclerotic conditions and determine the specific mediators that are responsible for the effects on the disease process.

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