Granulocyte Macrophage Colony-Stimulating Factor Regulates Dendritic Cell Content of Atherosclerotic Lesions

Zory Shaposhnik, Xuping Wang, Michael Weinstein, Brian J. Bennett, Aldons J. Lusis

Objective—Recent evidence suggests that dendritic cells may play an important role in atherosclerosis. Based primarily on previous in vitro studies, we hypothesized that granulocyte macrophage colony-stimulating factor (GM-CSF)-deficient mice would have decreased dendritic cells in lesions.

Methods and Results—To test this, we characterized gene targeted GM-CSF−/− mice crossed to hypercholesterolemic low-density lipoprotein receptor null mice. Our results provide conclusive evidence that GM-CSF is a major regulator of dendritic cell formation in vivo. Aortic lesion sections in GM-CSF−/− low-density lipoprotein receptor null animals showed a dramatic 60% decrease in the content of dendritic cells as judged by CD11c staining but no change in the overall content of monocyte-derived cells. The GM-CSF−/− mice exhibited a significant 20% to 50% decrease in the size of aortic lesions, depending on the location of the lesions. Other prominent changes in GM-CSF−/− mice were decreased lesional T cell content, decreased autoantibodies to oxidized lipids, and striking disruptions of the elastin fibers adjacent to the lesion.

Conclusion—Given that GM-CSF is dramatically induced by oxidized lipids in endothelial cells, our data suggest that GM-CSF serves to regulate dendritic cell formation in lesions and that this, in turn, influences inflammation, plaque growth and possibly plaque stability. (Arterioscler Thromb Vasc Biol. 2007;27:621-627.)

Key Words: atherosclerosis | dendritic cells | elastin | GM-CSF | macrophages

A large body of evidence now implicates immune functions in the modulation of atherosclerosis.1-3 Advanced lesions contain both T and B lymphocytes as well as antigen-presenting dendritic cells, and experiments with mice deficient in various immune processes exhibit alterations in atherosclerotic lesion development. Recent studies have emphasized a prominent role of dendritic cells in atherosclerosis4-7 and in human lesions, dendritic cells accumulate in rupture-prone areas such as the plaque shoulder, appearing to co-localize with, as well as directly activate, T cells.5,6

To test our hypothesis, we bred GM-CSF deficient mice to hypercholesterolemic low-density lipoprotein receptor-null mice (LDLR−/−), generating double knockout mice. The results revealed a dramatic impact on the content of dendritic cells in lesions, as the double knockout mice had only one-third as many dendritic cells as the LDLR−/− mice. Associated with the decreased lesional dendritic cell content were significant changes in the size and morphology of lesions as well as changes in immune responses to oxidized lipids.

Methods

See cover

In vitro studies have shown that GM-CSF stimulates the differentiation of dendritic cells from bone marrow precursors and monocytes,8 and an in vivo study in mice was also consistent with a role for GM-CSF in dendritic cell maturation.9 We hypothesized that the recruitment and accumulation of dendritic cells in atherosclerotic lesions is controlled in part by granulocyte macrophage colony-stimulating factor (GM-CSF). GM-CSF is induced in cultured aortic endothelial cells by oxidized low-density lipoproteins,10 a primary initiator of inflammation in atherosclerosis.11 Subsequent studies showed that GM-CSF is expressed in human atherosclerotic lesions.12

Methods

Please see the supplemental data section at http://atvb.ahajournals.org for detailed Methods.

GM-CSF null mice backcrossed nine generations to the C57BL/6J background were generously provided by Dr Bruce C. Trapnell (University of Cincinnati College of Medicine). These mice were then crossed to LDLR−/− mice on a C57BL/6J background to produce GM-CSF−/−, LDLR−/−, and LDLR−/− mice on an LDLR-null background. At approximately 10 weeks of age the mice were placed on an ad libitum Western type diet (TD 88137, Teklad) for 12 to 13 weeks before sacrifice. Plasma lipids were determined as described previously.12a Methods for the quantification of atherosclerotic lesions were as previously reported by and Mehrabian et al12b and Tangirala et al.12c Cryosections from the proximal aorta were stained for macrophages (rat anti-mouse MOMA-2, Beckman Coulter),
Results

To determine the effect of a GM-CSF deficiency on dendritic cell content of atherosclerotic lesions, GM-CSF–/– mice were intercrossed with LDLR–/– mice (both on a C57BL/6J background) to generate GM-CSF–/– LDLR–/–, GM-CSF–/– LDLR–/–, and GM-CSF–/– LDLR–/– animals. The mice were fed a high-fat, high-cholesterol “Western” diet for 12 to 13 weeks and then euthanized. Aortic lesions were then examined in both the proximal aorta (by sectioning the aorta) and in the thoracic and abdominal region of the aorta (by en face staining for neutral lipids). Both male and female mice were studied, and plasma lipids, glucose, and insulin levels were determined.

Examination of the aortic root by immunostaining, using the GM-CSF–/– mouse as a negative control, clearly revealed the presence of GM-CSF (supplemental Figure I, available online at http://atvb.ahajournals.org). The GM-CSF was present throughout the intimal region, with no clear association with any cell type, as expected for a secreted protein.

GM-CSF is known to promote dendritic cell formation in vitro, and monocytes entering the subendothelial space can differentiate into either dendritic cells or resident macrophages. The factors regulating this transition are not well-characterized but are thought to be endothelial cell derived. We examined the possible effects on dendritic cell content by immunostaining lesions for the dendritic cell marker CD11c and antigen presenting cells (MHC II) is shown in (G) and (H).

A representative example of a leukocyte flow cytometric scattergram for dendritic cells (CD11c) and antigen presenting cells (CD16/CD32) from normal GM-CSF–/– LDLR–/– LDLR–/– mice is shown in (I) and (J).

Three animals per group were examined. Examination of GM-CSF–/– mice revealed a marked decrease in dendritic cell content, which was similar in both male and female mice (Figure 2B). The subset of mature CD86+ dendritic cells comprised ≈16% of the lesion volume in GM-CSF–/– LDLR–/– mice but only 6% of the lesion volume in GM-CSF–/– LDLR–/– mice (P<0.0002) (Figure 2B). The subset of mature CD86+ dendritic cells represented approximately one-third of all lesional dendritic cells and decreased by ≈50% in lesions from GM-CSF–/– LDLR–/– mice (supplemental Figure IX). Overall, approximately half of all macrophages in lesions of GM-CSF–/– LDLR–/– mice were dendritic cells, whereas only ≈12% of macrophages in lesions from GM-CSF–/– LDLR–/– mice on a 12-week western diet were such cells.
circulating levels of dendritic cells or MHC II⁺ cells. Using flow cytometry we measured the total number of CD11c⁺ and CD11c⁺ MHC II⁺ dendritic cells in the circulation. We could detect no differences in either the total number of circulating dendritic cells (represented by CD11c⁺ cells) or of mature dendritic cells (represented by CD11c⁺ MHC II⁺ cells). 5.73±0.52% of blood leukocytes in GM-CSF⁺⁺/LDLR⁻⁻ mice versus 4.43±0.32% of leukocytes from GM-CSF⁺⁺/LDLR⁻⁻ mice stained positive for CD11c. Similarly, we could detect no differences in the total number of circulating MHC II⁺ cells using an antibody against an I-A MHC II alloantigen (Figure 1G and 1H), with 51.81±5.1% of leukocytes from GM-CSF⁺⁺/LDLR⁻⁻ mice and 47.83±2.9% of leukocytes from GM-CSF⁺⁺/LDLR⁻⁻ mice staining positive for that marker.

We sought to determine some of the biological and immunologic ramifications of a lesional dendritic cell deficiency by examining both the T cell content within the lesion and plasma levels of autoantibodies to oxidized lipids. CD4⁺ helper T cell content decreased ~50% within lesions of GM-CSF⁺⁺/LDLR⁻⁻ mice (supplemental Figure IX) but could detect no differences in the total number of circulating CD8⁺ cytotoxic T cells in very small numbers within lesions of GM-CSF⁺⁺/LDLR⁻⁻ mice and were virtually undetectable in lesions from GM-CSF⁺⁺/LDLR⁻⁻ mice (data not shown). This decrease in lesional T cell content, coupled with the decreased dendritic cell content, suggested that there might exist an overall decrease in the inflammatory state of the lesion in GM-CSF⁺⁺/LDLR⁻⁻ mice. However, we could not detect a difference in aortic mRNA levels of either the inflammatory cytokines IL-6 or MCP-1 (supplemental Figure VIII). We also hypothesized that the decreased number of dendritic cells in lesions of GM-CSF⁺⁺/LDLR⁻⁻ mice may result in less activation of B cells responsible for the production of autoantibodies to the immunogenic oxidized lipids present in lesions. Indeed, we observed ~2.5-fold decrease in plasma levels of IgG autoantibodies recognizing malondialdehyde (MDA)-LDL and a 3-fold decrease in plasma levels of IgG autoantibodies recognizing copper oxidized LDL (supplemental Figure V). We also observed a 2-fold increase in the levels of IgM antibodies complexed with ApoB-100 containing lipoproteins (supplemental Figure V). We could detect no change in plasma levels of oxidized lipids bound to ApoB-100 containing lipoproteins as determined with an assay using an anti-E06 antibody or in plasma levels of IgM autoantibodies recognizing MDA-LDL and copper-oxidized LDL (data not shown).

No significant differences in the content of smooth muscle cells (supplemental Figure II), macrophages (Figure 1A to 1C), or myeloid cells were observed in GM-CSF⁺⁺/LDLR⁻⁻ mice. We also performed semi-quantitative staining for collagen and elastin content in the lesions. In contrast to the previously reported findings from the normal aorta,¹⁴ we observed no significant differences in lesional collagen staining or aortic gene expression of α₁ (I), α₁ (VIII) procollagen between GM-CSF⁺⁺/LDLR⁻⁻, and GM-CSF⁺⁺/LDLR⁻⁻ female mice (Figure 3A to 3F, and supplemental Figure VIII). Using picrosirius red staining, we observed that the less mature, green stained collagen was the primary form of collagen in the lesions of both groups of mice. We did, however, observe a striking decrease of elastin staining in the GM-CSF⁺⁺/LDLR⁻⁻ male and female mice. In aortic lesions of GM-CSF⁺⁺/LDLR⁻⁻ mice, elastin staining typically encompassed almost the entire circumference of the aortic sections (82%±3%) (Figure 3G to 3H). However, most sections of aortic lesions from GM-CSF⁺⁺/LDLR⁻⁻ mice included large areas of the media devoid of elastin staining (Figure 3I to 3J). In those sections, the percent of circumference staining for elastin varied from 16% to 79%, with an average of 53%±4% (Figure 3K).

Therefore, we examined the gene expression levels of several metalloproteinases (MMPs) able to degrade collagen and the expression of the elastin protein (tropoelastin). We also examined the expression of an inhibitor of MMPs, TIMP1. We reasoned that the decrease we observed in arterial elastin content could be attributed to increased expression of MMPs, decreased expression of tropoelastin, or decreased expression of an inhibitor of MMP activity. No differences were detected between GM-CSF⁺⁺/LDLR⁻⁻ and GM-CSF⁺⁺/LDLR⁻⁻ mice in the aortic expression of the elastases MMP3, MMP12, the gelatinase MMP9, tropoelastin, or TIMP1, as measured by quantitative polymerase chain reaction (supplemental Figure VII).

In both males and females there was ~20% decrease in lesion development in the aortic root of GM-CSF⁺⁺/LDLR⁻⁻ as compared with GM-CSF⁺⁺/LDLR⁻⁻ mice (Figure 4A to 4B) (GM-CSF⁺⁺/LDLR⁻⁻ females, 242,955±13,869 μm² versus GM-CSF⁺⁺/LDLR⁻⁻ females, 196 855±53 238 μm²; and GM-CSF⁺⁺/LDLR⁻⁻ males, 221 316±20 565 μm² versus GM-CSF⁺⁺/LDLR⁻⁻ males, 173 134±13 345 μm²). Heterozygous mice exhibited an intermediate lesion size (GM-CSF⁺⁺/LDLR⁻⁻ females, 212 187±20 262 μm² and GM-CSF⁺⁺/LDLR⁻⁻ males, 204 000±15 881 μm² (Figure 4A, 4B). As compared with the aortic root, there was a larger...
effect of the GM-CSF deficiency on atherosclerotic lesions in the thoracic and abdominal aortae of female mice as measured by en face staining of lipid (Figure 4C). Interestingly, this $\sim 50\%$ decrease in lesions was sex-biased, because males did not show significant evidence of an effect of GM-CSF deficiency on lesion development.

The fasting levels of plasma lipids (total cholesterol, high-density lipoprotein cholesterol, triglycerides, unesterified cholesterol, and free fatty acids) were determined in the mice before euthanization. As shown in supplemental Table I and supplemental Figure X, plasma lipid levels, relative particle and triglyceride distributions were very similar be-

Figure 3. Histochemical analysis of collagen and elastin fibers in GM-CSF $-/-$ deficient mice. Lesions were stained for elastin (black) and collagen (blue). Arrows indicate collagen-stained areas. GM-CSF $-/-$ LDLR $-/-$ (A, G) and GM-CSF $-/-$ LDLR $+/+$ (D, I) sections are shown (magnification 50×). B and E, Magnification of boxed region in (A) and (D). H and J, Magnification of the boxed region in G and I. C and F, Region of a lesion similar to the region shown in (B) and (E) but stained for collagen (red, yellow, green) with picrosirus red. K, Elastin content was shown as the percent of positive stained medial circumference divided by total medial circumference. Four to six female animals per group were examined. Data are presented as means±SEM.

Figure 4. Effect of GM-CSF deficiency on atherosclerotic lesions in the proximal, thoracic, and abdominal aorta. Lesions in GM-CSF $+/+$, GM-CSF $-/-$, and GM-CSF $-/-$ mice were quantitated by lipid staining of the aortic root and proximal aortic sections (A, B) or by en face staining of the thoracic and abdominal aorta (C, D). The data are presented as box plots. Please see the Methods section for an explanation of box plots.
between GM-CSF⁺/⁻LDLR⁻/⁻ and GM-CSF⁻/⁻LDLR⁻/⁻ mice within each sex. After correction for multiple comparisons, no significant differences were observed among the various groups. Consistent with previous observations, there were clear differences in lipid levels between sexes, with females exhibiting somewhat lower levels of all lipid classes. The difference for triglyceride levels between sexes was particularly prominent (supplemental Table I).

Male LDLR⁻/⁻ mice maintained on a western diet become insulin-resistant and diabetic, and we examined whether GM-CSF might influence these traits. Male mice tended to have higher plasma insulin levels than female mice, consistent with previous findings but the insulin levels were not significantly altered by the GM-CSF deficiency (data not shown). Interestingly, glucose levels appeared to be decreased in females and not males as a result of the GM-CSF deficiency. The decreased atherosclerotic lesions seen in GM-CSF⁻/⁻LDLR⁻/⁻ females could result, in part, from reduced glucose levels.

Circulating plasma levels of M-CSF were measured by enzyme-linked immunosorbent assay to determine if M-CSF levels may have increased or decreased in response to the GM-CSF deficiency. No change in M-CSF levels was detected between GM-CSF⁺/⁻LDLR⁻/⁻ and GM-CSF⁻/⁻LDLR⁻/⁻ animals (1655±104 pg/mL versus 1561±32 pg/mL).

**Discussion**

We used GM-CSF⁺/⁻LDLR⁻/⁻ mice to examine the regulation and function of dendritic cells in atherosclerotic lesions. GM-CSF is induced in endothelial cells by oxidized lipids and our present studies with GM-CSF⁻/⁻LDLR⁻/⁻ mice have clearly shown that GM-CSF is abundantly present in atherosclerotic lesions. Consistent with previous in vitro studies implicating GM-CSF in dendritic cell differentiation, our results indicate a critical role for GM-CSF specifically in lesional dendritic cell accumulation, because the GM-CSF⁻/⁻LDLR⁻/⁻ mice had only approximately one-third as many dendritic cells and approximately one-half as many mature dendritic cells in atherosclerotic lesions as wild-type mice but had no change in lesional macrophages or myeloid cells. We were able to support our hypothesis that GM-CSF regulates dendritic cell formation within a lesion by showing that the total number of circulating dendritic cells did not change in GM-CSF⁻/⁻LDLR⁻/⁻ mice (Figure 1), suggesting that the decrease of dendritic cells within GM-CSF⁻/⁻LDLR⁻/⁻ lesions was not attributable to decreased numbers of dendritic cells entering the lesion. Recent studies have shown that retention of dendritic cells within a lesion is triggered by their interaction with platelet activating factor or other inflammatory lipids. In the absence of inflammatory lipids, dendritic cells were able to emigrate from the lesion and migrate to lymph nodes, resulting in lesion size reduction. The normal role of dendritic cells is to sample the environment, display antigens and modulate T cell activation, usually after migrating back to the lymph nodes. Thus, the decline in lesional dendritic cell content in GM-CSF⁻/⁻LDLR⁻/⁻ mice could result in decreased activation of T cells, and this could retard lesion growth. The decline in lesional dendritic cells coincided with decreased numbers of CD4⁺ T cells in lesions and with changes in levels of IgM antibodies complexed with ApoB100 containing lipoproteins, suggesting an overall decrease in the inflammatory state of the lesion as well as an enhanced ability of the immune system to eliminate oxidized lipids. It has been shown in humans that dendritic cells accumulate in the rupture-prone shoulders of lesions along with T cells, suggesting that they may contribute to plaque destabilization. Activated dendritic cells express the inflammatory modulator 5-lipoxygenase, which has been associated with both atherogenesis and aortic aneurysm formation.

We observed that hyperlipidemic animals with a GM-CSF deficiency exhibited ~20% to 50% decrease in the size of aortic lesions. The magnitude of the effect of GM-CSF on atherosclerosis is much smaller than that of M-CSF, a growth factor that is also induced by oxidized lipids in endothelial cells and whose function overlaps with that of GM-CSF. The mechanism underlying the very large decline in M-CSF⁻/⁻ mice lesions is thought to relate to decreased monocyte/macrophage recruitment, survival or proliferation within the lesion. The presence of normal levels of M-CSF could be partially compensating for any macrophage survival and growth deficiencies caused by the absence of GM-CSF. Also, the lack of GM-CSF and presence of M-CSF could drive monocytes/macrophages toward a more phagocytic nature that would more efficiently uptake cholesterol in the artery wall, increasing foam cell formation. Diabetes is a risk factor for atherosclerosis in humans and diabetic individuals have increased levels of advanced end products of glycation that interact with receptors on endothelial cells to increase inflammation. To the extent that these effects of hyperlipidemia are mitigated by decreased glucose levels in GM-CSF⁻/⁻ mice, they could be responsible for some component of the decreased atherosclerosis seen in these animals. With respect to the sex specific differences in lesion size, we have observed in this study that female animals exhibited less variable lesion data than male animals. Thus, it is possible we would be able to detect a significant decrease in aortic root lesions in male GM-CSF⁻/⁻LDLR⁻/⁻ animals by increasing out sample size. However, with respect to differences we observed in lesion data measured in the ascending and descending aorta by en face, we hypothesize that the distribution or kinetics of lesion formation in this compartment may differ between male and female mice.

GM-CSF is expressed in smooth muscle cells of normal human arteries and GM-CSF⁻/⁻ mice have been reported to have alterations of the artery wall structure even in the absence of atherosclerosis. These alterations include decreased medial collagen content and decreased, disorganized collagen fibrils. In situ hybridization data showed that the GM-CSF deficiency resulted in decreased numbers of vascular cells expressing collagen and decreased levels of collagen expression, particularly type VIII collagen. We examined collagen and elastin gene expression and distribution in the artery wall and lesions of GM-CSF-deficient animals. Collagen staining was predominantly seen within the atherosclerotic lesions and aortic media using either Masson’s trichrome staining or picrosirus red staining. However, no differences in less mature, loosely packed collagens, tightly
packed collagens, overall collagen content or aortic expression of \( \alpha-1 \) (I) and \( \alpha-1 \) (VIII) procollagen were observed. In our experience, elastic fiber staining intensity and quantity is highly variable within the aortas of LDLR\(^{-/-}\) mice fed a high-fat diet, but the percent of the luminal circumference outlined by elastic fiber is relatively consistent. We used the measure of luminal circumference outlined by elastic fibers to detect changes in elastic fiber staining. Using that standard, GM-CSF\(^{-/-}\) LDLR\(^{-/-}\) mice exhibited a large decline in medial elastic fiber staining. This decline was much greater in magnitude than the decrease in atherosclerosis seen in these animals. Previous studies have indicated that GM-CSF treatment of macrophages induces MMP-1\(^{20}\) (although previously expression of this collagenase could not be detected in the mouse aorta\(^{17}\)) and MMP-12 (macrophage elastase).\(^{21}\) Thus, the absence of GM-CSF would be predicted to decrease MMP expression, not increase it. In any case, we did not detect a difference in the expression of a panel of matrix-degrading molecules consisting of collagenases, elastases, a tissue inhibitor of elastases or tropoelastin gene expression within the aortas of GM-CSF--deficient animals, indicating that other post-transcriptional factors related to GM-CSF are responsible for the abnormal elastin distribution. Based on the expression pattern of GM-CSF and its effect on the artery wall, it is plausible that arterial aneurysms would be more prevalent in GM-CSF\(^{-/-}\) animals; however, we observed no increase in the number of aneurysms within the aortic root (data not shown).

Sugiyama et al\(^{22}\) showed that GM-CSF was expressed in areas of advanced human plaques that were prone to rupture and that GM-CSF was capable of inducing macrophage myeloperoxidase in vitro. Hypochlorous acid, a potent oxidizer produced by myeloperoxidase, can activate matrix metalloproteinase\(^{23,24}\) that degrade the extracellular matrix (ECM) and itself degrade collagen.\(^{25,26}\) However, in contrast to human lesions, myeloperoxidase is not expressed at high levels in murine atherosclerosis\(^{27}\) and is therefore not likely to play a role in the pathology that we have seen in GM-CSF\(^{-/-}\) mice. Exogenous infusion of GM-CSF and M-CSF has been shown to decrease plasma cholesterol. This effect is thought to be mediated by increased macrophage mediated cholesterol uptake.\(^{28}\) Consistent with this, increased cholesterol levels were indeed observed in M-CSF\(^{-/-}\) LDLR\(^{-/-}\) mice.\(^{18}\) However, GM-CSF\(^{-/-}\) LDLR\(^{-/-}\) exhibited no changes in total cholesterol, although female mice showed a small decline in high-density lipoprotein cholesterol. The discrepancy in total cholesterol levels between these two knockout mice could be indicative of an intrinsic change in the character of macrophages that result from a GM-CSF deficiency compared with an M-CSF deficiency. It could also be reflective of the fact that M-CSF null animals have a 50% decline in circulating monocytes, a decrease that may hinder cholesterol clearance, whereas GM-CSF\(^{-/-}\) mice do not have a monocye deficiency.\(^{29,30}\)

In conclusion, our results have revealed a novel regulatory mechanism controlling the composition and inflammatory nature of atherosclerosis. Thus, in response to the accumulation of oxidized lipids in the artery wall, GM-CSF is dramatically induced and regulates the recruitment or differentiation of dendritic cells in the lesion. These dendritic cells within the lesion appear to significantly impact the T cell content of lesions, resulting in a weakened B cell-mediated response against oxidized low-density lipoproteins. Dendritic cell content is likely to be important with respect to the activation of lymphocytes, the formation of foam cells and, perhaps, the stability of the lesion.

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**Disclosures**

None.

**References**


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Supplementary Material For:

Granulocyte-Macrophage Colony Stimulating Factor Regulates Dendritic Cell Content of Atherosclerotic Lesions

Lusis: GM-CSF controls dendritic cells in atherosclerosis

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Supplementary Methods:

Generation of GM-CSF/LDLR Double Knockout Mice

GM-CSF null mice backcrossed nine generations to the C57BL/6J background were generously provided by Dr. Bruce C. Trapnell (University of Cincinnati College of Medicine). These animals were then crossed to LDLR -/- mice on a C57BL/6J background to produce heterozygous mice that were then crossed to the LDLR -/- mice. GM-CSF +/- LDLR -/- mice were selected and intercrossed to produce GM-CSF +/+, +/-, and -/- mice on an LDLR null background. At approximately 10 weeks of age the mice were placed on an ad libitum Western type diet for 12 to 13 weeks before sacrifice. The University of California, Los Angeles Animal Research Committee approved all animal work.

Plasma Lipids, Glucose, Insulin and M-CSF Measurements

Animals were fasted overnight before being bled from the retroorbital sinus. Plasma was collected and used to determine levels of total cholesterol levels, HDL cholesterol, triglycerides and free fatty acids. Plasma lipids were determined as described previously\(^1\). Plasmas from 4 male GM-CSF +/- LDLR -/- and GM-CSF +/- LDLR -/- mice were pooled and fractionated by gel filtration chromatography using fast protein liquid chromatography (FPLC) to examine the classes and sizes of lipoprotein particles, as previously described\(^1\). Plasma glucose was determined in triplicate using a glucose assay (#315-100; Sigma). Plasma insulin was measured in duplicate using a rat insulin ELISA (ALPCO Diagnostics). Plasma collected at time of sacrifice was used to measure circulating levels of M-CSF by ELISA (R&D Systems).
Measurement of Atherosclerosis and Histological Staining

Methods for the quantification of atherosclerotic lesions in the aortic root were as previously reported by Mehrabian et al.\(^2\). Briefly, the heart and proximal aorta were excised and embedded in OCT compound (Tissue-Tek) before freezing. Serial 10 µm thick cryosections from the middle portion of the ventricle to the aortic arch were collected and mounted on poly-D-lysine-coated slides. In the region from the appearance to the disappearance of the aortic valves, every other section was collected. In all other regions, every fifth section was collected. Sections were stained with oil red O and counterstained with hematoxylin and fast green. Lesion areas were quantitated by light microscopy. Additionally, the ascending and descending aorta (to the diaphragm) was dissected out, cleaned of connective tissue and fixed. The aortas were then pinned out en face and stained with Sudan IV as in Tangirala et al.\(^3\). Lesion surface area and total aortic surface area were measured using Image Pro Plus (Media Cybernetics).

Additional cryosections from the proximal aorta were stained for macrophages (rat anti-mouse MOMA-2, Beckman Coulter), smooth muscle cells (rabbit anti-mouse alpha smooth muscle actin, Spring Bioscience), dendritic cells (hamster anti-mouse CD11c, BD Biosciences), mature dendritic cells (rat anti-mouse CD86, BD Biosciences), GM-CSF (rat anti-mouse GM-CSF, US Biological), and T cells (rat anti-mouse CD4, BD Biosciences; rat anti-mouse CD8, BD Biosciences). Three to four sections from three to five animals per group were analyzed. Sections were dried at room temperature and fixed in acetone (for MOMA-2, actin, CD 86, CD4 and CD11c staining) or 1% PFA (for GM-
CSF staining). Sections were then blocked in 5% serum appropriate for the biotinylated secondary antibody used (rabbit anti-rat, Vector Labs, goat anti-rabbit, Vector Labs or anti-hamster cocktail, BD Biosciences). Staining was detected using the Alkaline Phosphatase Standard ABC Kit (Vector Labs) and Vector Red as a substrate that fluoresces in a spectrum similar to Rhodamine. Sections were counterstained with DAPI and imaged on a Zeiss Axioskop 2 fluorescent microscope. Omission of the primary antibody was included as one control to determine staining specificity. Collagen content was determined using Masson’s trichrome staining and picrosirus red staining. Elastin fiber content was determined using elastica van Gieson staining. Both 3 µm formalin fixed, paraffin embedded sections and 10 µm cryosections were used for Masson’s trichrome, picrosirus red and elastica van Gieson staining. Intimal collagen, macrophage, T cell, dendritic cell and smooth muscle content were normalized to total intimal thickening in each section. Elastic fiber content was expressed as the percent of the circumference of each aortic section positive for the respective marker. Staining for Masson’s trichrome and elastica van Gieson was performed as above. Picrosirus red stained sections were imaged using polarized light microscopy. By this technique, tightly packed collagen fibers appear yellow-red and less mature, while loosely packed collagen fibers appear green. All the histological data presented, other than lesion data, was derived from female animals. Male mice showed results similar to those seen in female mice but for the sake of space and simplicity we focused on characterizing and displaying data from female animals.
Leukocyte Flow Cytometry

Whole blood from three mice of each genotype was collected from the retroorbital sinus and red blood cells were lysed. The remaining leukocytes were incubated with an anti-mouse CD16/CD32 Fc gamma III/II receptor 2.462 blocking antibody (BD Pharmingen) before double staining with a PE labeled anti-mouse CD11c antibody (BD Pharmingen) and a FITC-labeled anti-mouse I-A/I-E MHC II alloantigen antibody (BD Pharmingen). Unstained and singly stained leukocytes were also included as controls and used to define the appropriate gating parameters. 200,000 to 500,000 cells were counted using a BD FACS scanner running CellQuest software.

Detection of Oxidized Low-Density Lipoprotein Autoantibodies

Plasma levels of EO6, an indicator of oxidized lipids bound to ApoB-100 containing lipoproteins were measured using an anti-EO6 antibody and plasma levels of autoantibodies to both copper and MDA oxidized LDL were determined as were levels of IgG and IgM antibodies complexed with ApoB-100 containing lipoproteins

Quantitation of Gene Expression

Aortas were isolated from animals at the time of sacrifice. Connective tissue and adventitia were removed from the aorta before total RNA isolation using an RNEASY kit (Qiagen). First strand cDNA synthesis was performed using an iScript cDNA Synthesis Kit (Biorad). Real-time quantitative PCR was performed on an Applied Biosystems 7700 unit using Platinum SYBR Green qPCR Supermix UDG (Invitrogen). Samples were analyzed in duplicate and normalized to beta(2)-microglobulin expression.
Statistical Tests

Data were expressed as mean +/- SEM. Statistical analyses were performed using the non-parametric Mann Whitney test (Statview) for atherosclerotic lesion comparisons and measurements of autoantibodies to oxidized lipids. All other comparisons were made using an unpaired t-Test. Lesion data was displayed as a box plot with the whiskers representing the maximum and minimum data points, the central line representing the overall median, the bottom of the box representing the median of the first quartile of the data set and the top of the box representing the median third quartile of the data set. Open circles represent outliers.
Supplementary References:


Online Figure Legends:

Figure I. GM-CSF is expressed in atherosclerotic lesions of LDLR-/- mice

GM-CSF protein was detected in atherosclerotic lesions from GM-CSF +/+ LDLR-/- animals by immunostaining (red) (A) but not detected in lesions from GM-CSF-/- LDLR-/- animals (B). Nuclei are stained by DAPI (blue). L: lumen, I intima, M: media. (magnification 200x)

Figure II. Smooth muscle content within atherosclerotic lesions of GM-CSF deficient animals

Lesion from GM-CSF +/+ LDLR-/- (B) and GM-CSF-/- LDLR-/- (C) animals were stained for actin content (red). In (A) the primary antibody was omitted (negative control). (magnification 200x) Note the fibrous cap covering the lesion in (B) and (C). L: lumen, I: intima, M: media.

Figure III. En face staining of atherosclerotic lesions within the proximal and thoracic aorta of GM-CSF deficient animals

Pictures of lesions (red) from representative aortas are shown.

Figure IV. Fasting glucose levels in GM-CSF deficient animals

Animals were fasted overnight and bled from the retroorbital sinus. Plasma samples were maintained on ice until freezing and glucose measurement as described under methods. Data are presented as means +/-SEM. The number of mice in each group is in parenthesis.
**Figure V. Autoantibody levels to Oxidized Low-Density Lipoproteins**

Titers of IgM antibodies complexed to apoB100 and autoantibodies to MDA modified (MDA-LDL) and copper oxidized LDL (Cu-LDL) were measured as described in Methods. Levels of IgM bound to apoB100 were normalized to the total amount of apoB100 in each sample. Titers of autoantibodies to MDA-LDL and Cu-LDL are expressed as relative light units (RU).

**Figure VI. Quantitation of collagen and smooth muscle content within the proximal aorta of GM-CSF deficient animals**

(A) Collagen content was expressed as a percent of the total lesion in each section. (B) Smooth muscle cell staining was expressed as percent of total lesional area in each section. Data are presented as means +/-SEM.

**Figure VII. Quantitation of macrophage and matrix catabolic gene expression in the artery wall**

The gene expression of the matrix catabolic enzymes MMP 3, MMP 9 and MMP 12 as well as TIMP 1, an inhibitor of this catabolic process was examined by quantitative PCR. Also examined was the relative arterial gene expression of the macrophage markers CD68 and CSF-1. Data are presented as means +/-SEM and 6 mice of each genotype were tested. We could detect no difference in the expression of any of these genes between GM-CSF +/+ LDLR-/- or GM-CSF -/- LDLR-/- mice.

**Figure VIII. Quantitation of Collagen, Elastin and Inflammatory gene expression in the artery wall**

The gene expression of the vascular collagens alpha-1 (I) (Col1A1), alpha-1 (VIII) (COL8A1) procollagen, elastin (tropoelastin) were examined by quantitative PCR as were the inflammatory cytokines IL-6 and MCP-1. Data are presented as means +/-SEM and 6 mice of each genotype were tested. We could detect no difference in the expression of any of these genes between GM-CSF +/+ LDLR-/- or GM-CSF -/- LDLR-/- mice.
Figure IX. T cell and Mature Dendritic cell content within atherosclerotic lesions of GM-CSF deficient animals

Lesions from GM-CSF +/- LDLR-/-(A, C) and GM-CSF-/- LDLR-/-(B, D) animals were stained for T cell and mature dendritic cell content (red). (magnification 200x)

L: lumen, I: intima, M: media. (E) and (F) display the percentage of the aortic root atherosclerotic lesion area positive for CD4+ T cells (E) and CD86+ mature dendritic cells (F) expressed as a percentage of the area of intimal thickening in each respective section.

Figure X. Plasma Lipoprotein Fractionation by Size Exclusion Chromatography

Plasma lipids

(A) Pooled plasma samples from four male GM-CSF +/- LDLR-/-(black diamonds) and GM-CSF -/- LDLR-/-(white squares) mice were fractionated by fast protein liquid chromatography (FPLC). Individual 0.5 mL fractions were collected and used to determine the relative cholesterol and triglyceride content as described in Methods. (B) Triglyceride levels from each plasma sample fraction are plotted. Note that the vast majority of triglycerides found between fractions 1 and 20, coinciding with the presence of VLDL and LDL particles.

Figure XI. A model of the effect of GM-CSF on the artery wall in atherosclerosis

Circulating LDL becomes trapped within the arterial extracellular matrix and becomes oxidized. This oxidized LDL induces arterial monocyte recruitment through the induction of chemokines such as monocyte chemotactic protein (MCP-1) and monocyte survival/differentiation factors M-CSF and GM-CSF. M-CSF mediated signaling nudges the monocyte towards a phagocytic macrophage fate while GM-CSF mediated signaling
nudges the monocyte toward a dendritic cell fate. In the presence of hyperlipidemia and oxidized lipids, these dendritic cells accumulate within the growing lesion and interact with lymphocytes, increasing local inflammation and resulting in plaque rupture.
Figure I.

A

GM-CSF: GM-CSF KO

B

GM-CSF: GM-CSF WT
Figure II

A. SMC: Negative Control

B. SMC: GM-CSF WT

C. SMC: GM-CSF KO
Figure III

GM-CSF:  

Females:  
+/-  
-/-  

Males:  
+/-  
-/-
Figure IV

Plasma Glucose (mg/dl)

GM-CSF: (+/+) (+/-) (-/-) (+/+) (+/-) (-/-)

Females

Males

P < 0.008

(15) (20) (16) (17) (24) (12)
Figure VI

A

GM-CSF: ++/++/---

% Collagen Staining

(6) (6)

B

GM-CSF: ++/++/---

% Smooth Muscle Staining

(4) (5)
Figure VII
Figure VIII

Figure IX

A

CD86+ Dendritic Cells: GM-CSF WT

T cells: GM-CSF WT

B

CD86+ Dendritic Cells: GM-CSF KO

T cells: GM-CSF KO

C

D
Figure IX

E

% CD4 Staining

GM-CSF: +/- (-)

P<0.003

F

% CD86 Staining

GM-CSF: +/- (-)

P<0.02
Figure X

The figure illustrates the distribution of total cholesterol (µg/150 µL) across different fractions. The x-axis represents the fraction number, while the y-axis shows the total cholesterol concentration. Two conditions are compared:

- GM-CSF+/+ LDLR -/-
- GM-CSF -/- LDLR -/-

The peaks indicate the presence of three main lipoprotein fractions: VLDL, LDL, and HDL. The graph shows a clear difference in cholesterol distribution between the two conditions.
Figure X

B

![Graph showing Triglycerides (ug/150uL) per Fraction number for GM-CSF +/+ LDLR -/- and GM-CSF -/- LDLR -/-]
Figure XI

Monocytes

MCP-1

Matrix

GM-CSF

M-CSF

ox-LDL

T cell

Inflammation and Rupture

DC

SMC
Table I. Plasma Lipid Levels

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Values were expressed as mean +/- SEM