GM-CSF Deficiency Reduces Macrophage PPAR-γ Expression and Aggravates Atherosclerosis in ApoE-Deficient Mice

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Objective—Granulocyte-macrophage colony-stimulating factor (GM-CSF) is expressed in atherosclerotic lesions but its significance for lesion development is unknown. Consequently, we investigated the significance of GM-CSF expression for development of atherosclerotic lesions in apolipoprotein E-deficient (apoE−/−) mice.

Methods and Results—We generated apoE−/− mice deficient in GM-CSF (apoE−/−.GM-CSF−/− mice), fed them a high-fat diet, and compared lesion development with apoE−/− mice. We measured lesion size, macrophage, smooth muscle cell, and collagen accumulation at the aortic sinus, and expression of genes that regulate cholesterol transport and inflammation. No differences in serum cholesterol were found between the 2 groups. Lesion size in hyperlipidemic apoE−/−.GM-CSF−/− increased by 30% (P<0.05), macrophage accumulation doubled, and collagen content reduced by 15% (P<0.05); smooth muscle cell accumulation and vascularularity were unaffected. Analysis of PPAR-γ, ABCA1, and CD36 in lesions showed reduced expression (50%, 65%, and 55%, respectively), whereas SR-A doubled. In peritoneal macrophages, PPAR-γ and ABCA1 expression was also reduced by 50% and 70%, respectively, as was cholesterol efflux, by 50%. In lesions, pro-inflammatory MCP-1 and tumor necrosis factor (TNF)-α expression increased 2- and 3.5-fold, respectively, vascular cell adhesion molecule (VCAM)-1 expression enhanced and interleukin (IL)-1 receptor antagonist reduced by 50%.

Conclusions—GM-CSF deficiency increases atherosclerosis under hypercholesterolemic conditions, indicating antiatherogenic role for GM-CSF. We suggest this protective role is mediated by PPAR-γ and ABCA1, molecules that affect cholesterol homeostasis and inflammation. (Arterioscler Thromb Vasc Biol. 2006;26:2337-2344.)

Key Words: apoE−/− mice ■ atherosclerotic lesions ■ GM-CSF deficiency ■ hyperlipidemia ■ inflammatory cytokines ■ PPAR-γ

Atherosclerosis begins early in life and frequently leads to severe complications in later life with high morbidity and mortality. It is much more complex than a simple lipid storage problem, involving inflammatory mechanisms that predominate over anti-inflammatory processes.1,2 Atherosclerotic lesions are characterized by cholesterol accumulation, immune cell infiltrates, which include macrophages, T lymphocytes, and fibrosis.3 Inflammation appears crucial in all stages of atherosclerosis, from the very early stages of lipid accumulation through progression and ultimately complications. Recruitment, activation, survival, and proliferation of inflammatory cells in the vessel wall importantly contribute to atherosclerosis.4 These effects are mediated via adhesion molecules, chemokines, cytokines, and growth factors.2,4–6

Accumulating evidence suggests that granulocyte-macrophage colony-stimulating factor (GM-CSF) can play a key role in atherosclerosis. GM-CSF selectively regulates growth and survival of mononuclear phagocytes.7 Atherogenic oxidized low-density lipoproteins induce macrophage expression of GM-CSF.8–11 Atherosclerotic lesions from humans and rabbits exhibit elevated levels of immunoreactive GM-CSF, which is expressed by endothelial cells, smooth muscle cells and macrophages.10,11 Macrophages proliferate within lesions12 and GM-CSF expression frequently colocalizes with proliferating macrophages.13 and is important for their survival.11 GM-CSF appears to regulate type VIII collagen biosynthesis in atherosclerotic lesions,14 stimulates macrophages to produce myeloperoxidase15 and reactive oxygen species,11 increases matrix metalloproteinases,16 and reduces macrophage apoE secretion.17 GM-CSF primed mice produce more proinflammatory cytokines when challenged with lipopolysaccharide (LPS) or tumor necrosis factor (TNF)-α.18 GM-CSF also possesses potential anti-atherosclerotic properties. Pharmacological doses lower plasma cholesterol levels, re-
duce liver cholesterol biosynthesis,\textsuperscript{19} elevate expression of very-low density lipoprotein (VLDL) receptor,\textsuperscript{20} and decrease scavenger receptor expression on cultured human monocytes, leading to reductions in cholesterol ester accumulation.\textsuperscript{21} Monocytes stimulated with GM-CSF produce high levels of IL-1 receptor antagonist protein,\textsuperscript{22,23} increase expression of peroxisome proliferator-activated receptor-\(\gamma\) (PPAR-\(\gamma\)),\textsuperscript{24,25} and suppress interferon (INF)-\(\gamma\) action.\textsuperscript{26} GM-CSF also induces monocytes to secrete soluble VEGF receptor-1, preventing VEGF-A signaling and angiogenesis.\textsuperscript{27} Administration of supraphysiological concentrations of GM-CSF reduces atherosclerosis, smooth muscle cell numbers, and collagen content.\textsuperscript{28} However, despite multiple lines of evidence for a role of GM-CSF in atherosclerosis, the impact and role of physiological levels of endogenous GM-CSF on atherosclerotic lesion formation remain hitherto unknown.

To determine the role of GM-CSF in atherosclerosis, we crossed GM-CSF–deficient mice (GM-CSF\textsuperscript{-/-}) with apoE\textsuperscript{-/-} mice. We found that GM-CSF deficiency resulted in larger lesions with increased macrophage accumulation and reduced collagen content. This was accompanied by reduced expression in lesions of PPAR-\(\gamma\) and ABCA1 and increased expression of proinflammatory TNF-\(\alpha\), MCP-1, and VCAM-1. As PPAR-\(\gamma\) and ABCA1 were also reduced in peritoneal macrophages from GM-CSF–deficient mice, we suggest that reduction of PPAR-\(\gamma\) and ABCA1 in lesional macrophages is the basis for the larger atherosclerotic lesions in these mice. Our data suggest that GM-CSF has a PPAR-\(\gamma\)-dependent protective role in atherogenesis.

\textbf{Methods}

\textbf{Animals}

The generation of GM-CSF\textsuperscript{-/-} mice on a C57BL6 background used in this study has been described previously.\textsuperscript{29} These mice exhibit no perturbation of major hematopoietic populations in marrow or blood. apoE\textsuperscript{-/-} mice on a C57BL6 background were obtained from the Walter and Eliza Hall Institute, Melbourne, Australia. GM-CSF\textsuperscript{-/-} mice were crossed with apoE\textsuperscript{-/-} mice and the apoE\textsuperscript{-/-}.GM-CSF\textsuperscript{-/-} mice backcrossed to produce apoE\textsuperscript{-/-}.GM-CSF\textsuperscript{-/-} mice. Male mice were fed a high-fat diet consisting of 20% butter fat and 0.125% cholesterol from 8 weeks of age for 12 weeks. After pentobarbitone overdose, blood was collected by intracardiac puncture. The aortic sinus was dissected and collected for histological and mRNA analyses. All experiments were approved by AMREP Animal Ethics Committee.

For Materials and Methods used in this article, please see http://atvb.ahajournals.org.

\textbf{Results}

\textbf{GM-CSF Is Upregulated in Aortic Sinus Lesions of ApoE\textsuperscript{-/-} Mice}

GM-CSF was expressed in a temporal manner during development of atherosclerosis in apoE\textsuperscript{-/-} mice (Figure 1). In nonatherosclerotic aortic sinus, no expression of GM-CSF was observed using immunohistochemistry to detect GM-CSF–positive cells or by reverse-transcription polymerase chain reaction (RT-PCR) analysis of mRNA. GM-CSF–expressing cells became detectable in lesions 4 weeks after mice commenced a high-fat diet, peaking at 8 weeks, and then slowly declining by 50% at 12-weeks (Figure 1C).
Atherosclerotic Plaques Are Larger in GM-CSF–Deficient Mice

Cross-sections from lesions in the aortic sinus region were stained with Oil Red-O and lesion area quantified. Plaques from apoE/−/−.GM-CSF/−/− mice were on average 30% larger than plaques from apoE/−/− mice (0.14±0.035 mm² per section and 0.08±0.02 per section, respectively; P<0.05; Figure 2). This increase in lesion size was independent of plasma cholesterol.

Macrophage Accumulation Is Increased and Collagen Content Decreased in GM-CSF–Deficient Lesions

To evaluate whether the lack of GM-CSF affects inflammatory processes in the lesions, we initially assessed macrophage accumulation. On average, macrophage expression in lesions at the aortic sinus of apoE/−/−.GM-CSF/−/− mice was nearly double the accumulation in apoE/−/− mice (Figure 3A; P<0.05). Deletion of GM-CSF did not affect smooth muscle cell numbers in the lesions, which mostly covered the macrophage-rich lesions but were also apparent to a lesser extent within lesions (Figure 3B; P>0.05). As GM-CSF has been reported to affect vascular collagen content,14,28 we stained sections with picro-sirius red and examined collagen under normal and polarized light. Collagen content in lesions of the apoE/−/−.GM-CSF/−/− mice was 15% lower than in lesions of apoE/−/− mice (P<0.05; Figure 3C). Collagen structure, visually analyzed under polarized light, was different in the apoE/−/−.GM-CSF/−/− lesions with smaller, more disorganized fibrils dominating, indicated by a shift to the violet side of the visible spectrum. The distribution of collagen within lesions appeared to be similar in the 2 groups of mice (Figure 3C).

PPAR-γ and ABCA1 Expression Is Reduced in GM-CSF–Deficient Lesions and in Peritoneal Macrophages

Because PPAR-γ is deficient in alveolar macrophages of patients with alveolar proteinosis, an autoimmune disease with high levels of circulating anti-GM-CSF neutralizing antibodies,30 we examined using real-time PCR whether PPAR-γ was reduced in lesions of apoE/−/−.GM-CSF/−/− mice. We found that PPAR-γ mRNA expression was reduced by 50% in lesions of apoE/−/− mice that were GM-CSF–deficient compared with apoE/−/− mice (P<0.05, n=5 per group; Figure 4A). Because PPAR-γ is a potent transcriptional regulator of genes governing lipid metabolism we next examined whether the reduction in PPAR-γ affected expression of these genes. In lesions of apoE/−/−.GM-CSF/−/− mice, we found that mRNA expression of ABCA1, the ATP-binding cassette protein responsible for cholesterol efflux, was reduced by ~65% (P<0.05), the scavenger receptor SR-A tended to be increased and the scavenger receptor CD36 reduced by 55% (P<0.05; Figure 4A).

To confirm that macrophages are responsible for the decrease in PPAR-γ expression in lesions, we analyzed expression of PPAR-γ, ABCA1, and CD36 in peritoneal macrophages. PPAR-γ mRNA levels in peritoneal macrophages were reduced by 50% and expression of ABCA1 was...
reduced by ≈70% (P<0.05; Figure 4B); CD36 levels also tended to be reduced. To determine whether the reduction in ABCA1 expression was associated with reduced cholesterol efflux, we compared [3H]-cholesterol efflux from thioglycollate-elicited peritoneal macrophages from apoE−/−.GM-CSF−/− and apoE−/− mice. Cholesterol efflux was reduced by 50% in macrophages from the apoE−/−.GM-CSF−/− mice (P<0.05; Figure 4C).

MCP-1, TNF-α, and VCAM-1 Expression Is Increased in GM-CSF–Deficient Lesions

Because PPAR-γ was reduced in lesions of apoE−/−.GM-CSF−/− mice and has been reported to suppress inflammatory cytokines,31 we next investigated whether expression of inflammatory cytokines in lesions of these mice were increased. Analysis of mRNA from lesions of apoE−/− and apoE−/−.GM-CSF−/− mice indicated increased expression of monocyte chemotactic protein-1 (MCP-1) and TNF-α, ≈2- and 3.5-fold, respectively, in the GM-CSF–deficient mice (P<0.05; Figure 5A), whereas expression of the IL-1 receptor antagonist was reduced by nearly 50% (P<0.05; Figure 5A). VCAM-1 is important in the progression of atheroma and a marker of inflammation.32 Consequently, we also examined its expression in lesions. VCAM-1 expression was also higher in the apoE−/−.GM-CSF−/− mice, expressed in ≈25% of the lesions compared with ≈12% in lesions of apoE−/− mice (P<0.05; supplemental Figure II).

Vascularization of Lesions Is Not Affected by GM-CSF Deficiency

GM-CSF has been reported to induce secretion of soluble VEGF receptor-1, thereby preventing VEGF-A signaling and angiogenesis,27 and angiogenesis inhibitors reduce lesion growth in apoE−/− mice.33 We examined whether such a mechanism could contribute to the increase in lesion size in the GM-CSF–deficient apoE−/− mice. Soluble VEGF receptor-1 (sFlt-1) mRNA expression was reduced by ≈20% and there was a small (≈10%) increase in expression of VEGF receptor-1 mRNA but the differences were not statistically significant (P>0.05; supplemental Figure III). The
number of microvessels in lesions, detected immunohistochemically using anti-CD31 antibodies was also unaffected ($P > 0.05$; supplemental Figure III).

Discussion

Accumulated evidence from in vitro studies suggest that GM-CSF is pro-atherogenic.$^{13,31}$ Here, we found that lesion size and macrophage accumulation is increased in apoE$^{-/-}$ mice deficient in GM-CSF, suggesting that, in vivo, GM-CSF protects against atherosclerosis.

Our observation of reduced PPAR-$\gamma$ expression in atherosclerotic lesions and in peritoneal macrophages of GM-CSF$^{-/-}$ mice deficient in GM-CSF, which regulates cholesterol metabolism and attenuates inflammation.$^{34}$ Our data are consistent with the PPAR-$\gamma$ deficiency in alveolar macrophages of patients with alveolar proteinosis, an autoimmune disease with high circulating anti-GM-CSF neutralizing antibodies that cause GM-CSF deficiency.$^{30}$ PPAR-$\gamma$ is a nuclear transcription factor that is highly expressed in macrophages and macrophage-derived foam cells in atherosclerotic lesions. PPAR-$\gamma$ inhibits macrophage foam cell formation and atherosclerosis$^{35}$ and its deletion in macrophages increases atherosclerosis in low-density lipoprotein receptor-deficient mice.$^{36}$ Whereas PPAR-$\gamma$ promotes monocyte/macrophage differentiation and uptake of oxidized LDL by enhancing CD36 expression,$^{37}$ it attenuates SR-A expression$^{38}$ and induces cholesterol removal from macrophages by promoting ABCA1 expression and function.$^{39}$ Conversely, disruption of the PPAR-$\gamma$ gene lowers expression of ABCA1 in macrophages and reduces cholesterol efflux.$^{40}$ Our finding that ABCA1 expression is also reduced in atherosclerotic lesions and in peritoneal macrophages of GM-CSF and apoE-deficient mice is consistent with GM-CSF being a regulator of PPAR-$\gamma$ expression in macrophages during lesion development. The reduced ABCA1 expression suggests impaired reverse cholesterol transport as one mechanism responsible for the larger lesions in the double knockout mice.$^{40}$ Our suggestion is consistent with the report that increased ABCA1 expression in transgenic mice protects against atherosclerosis while its deletion from leukocytes results in significantly larger and more advanced lesions.$^{41,42}$ Whereas the significance of CD36 and SR-A for lesion development is currently unclear,$^{43}$ our findings that CD36 expression is downregulated and SR-A appeared upregulated provides further support for GM-CSF as a regulator of PPAR-$\gamma$ expression and function in developing atherosclerotic lesions. Our finding that lesion area in GM-CSF$^{-/-}$ mice assessed by macrophage accumulation was greater than that assessed by Oil Red-O staining suggests that GM-CSF might also influence the ratio of free-to-esterified cholesterol deposited in lesions. Both non-esterified and esterified cholesterol are deposited in lesions and Oil Red-O only detects the esterified forms.$^{44,45}$ Also, mice per group. C, Plasma initiated cholesterol efflux from thioglycollate-elicited macrophages of apoE$^{-/-}$, GM-CSF$^{-/-}$ and apoE$^{-/-}$ mice. Bar graphs represent the means $\pm$ SEM of at least 3 mice from each group. $+P < 0.05$ from apoE$^{-/-}$ mice.
oxidized LDL increases free cholesterol accumulation in macrophages.46 Further investigations are warranted to determine whether GM-CSF influences the nature of cholesterol that accumulates in lesions. Together, our data suggest that endogenous GM-CSF plays a major role in regulating cholesterol metabolism in macrophages; one mechanism involves promoting PPAR-γ expression that in turn induces ABCA1 expression.

ABCA1 deletion also increases plasma MCP-1 and TNF-α levels, suggesting that it regulates responses to inflammatory stimuli.47 Previous studies suggest that GM-CSF contributes to inflammation through monocyte recruitment, increased cell survival, and/or priming macrophages for activation.7 For example, GM-CSF enhances LPS-induced and TNF-α-induced cytokine production and stimulates IL-1 production by macrophages.18 In contrast, our studies suggest that GM-CSF can also exert anti-inflammatory effects. We found increases in MCP-1 expression in lesions of apoE−/− mice deficient in GM-CSF, consistent with the report that elevated MCP-1 accelerates atherosclerosis, likely by promoting macrophage accumulation.48 Because ABCA1 deficiency in macrophages enhances their responses to inflammatory stimuli,49 it is possible that reduced ABCA1 in macrophages contributed to macrophage accumulation and development of larger lesions in the GM-CSF−/− mice. Moreover, we found that TNF-α expression was elevated in these larger lesions. Because TNF-α is increased in mice deficient in ABCA142 and ABCA1 is reduced in lesions of apoE−/− mice deficient in GM-CSF, it is likely that the increase in TNF-α is also mediated by an ABCA1-dependent mechanism. Further, we found expression of IL-1 receptor antagonist was reduced, consistent with the report that monocytes stimulated with GM-CSF elevated levels of IL-1 receptor antagonist.23 Our finding of enhanced VCAM-1 expression in atherosclerotic lesions of GM-CSF−/− mice provides further support for our suggestion of an anti-inflammatory role for GM-CSF in developing atherosclerotic lesions.

Whereas GM-CSF deficiency did not affect smooth muscle cell content in lesions, it reduced collagen content in lesions, consistent with a stimulatory effect of GM-CSF on collagen production in blood vessels14,49 and contrasting with the effects of administration of supraphysiological amounts of GM-CSF, that resulted in reductions in both smooth muscle cell numbers and collagen content.28 GM-CSF has been reported to increase collagen expression in cultured airway smooth muscle cells by inducing transforming growth factor-β receptors.50 It is possible that GM-CSF acts directly on vascular smooth muscle cells within lesions via a similar mechanism to elevate collagen expression in lesions. Thus, endogenous GM-CSF may also regulate lesion development by promoting more fibrotic and stable atherosclerotic lesions. Although GM-CSF has been reported to inhibit angiogenesis by stimulating expression of soluble VEGF receptor-1 in monocytes,27 vascularity was unaffected in developing lesions in double knockout mice.

In conclusion, we have demonstrated that GM-CSF deficiency led to an increase in size of atherosclerotic lesions in diet-induced hyperlipidemic apoE−/− mice. The increase in lesion size and macrophage accumulation in GM-CSF−/− mice appears due to reductions in PPAR-γ and ABCA1 expression in macrophages. Our results suggest that endogenous GM-CSF modulates lesion development during hyperlipidemia resulting in smaller more stable lesions.

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**Disclosures**
None.
References


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Supplementary Data

Enhanced Materials and Methods

Plasma cholesterol

Plasma cholesterol was analysed enzymatically, using a cholesterol assay kit (Roche/Hitachi) according to manufacturer’s instructions with Cobas Fara II automated clinical chemistry analyser.

Genotyping of GM-CSF-/-.ApoE-/- mice

Genomic DNA isolated from tail biopsies with DNeasy kit (Qiagen) and PCR was used to identify mice doubly deficient for GM-CSF and ApoE genes according to published methods\(^1\) and the web site of the Jackson Laboratories (www.jax.org). Reactions to detect wild type and deletion of GM-CSF shared the same sense primer: 5’ –CCA GGC TCA GAG ACC CAG CTA TCC – 3’; the wild type antisense was: 5’ – GTT AGA GAC TTC TAG CTC TTC – 3’ and knockout antisense 5’ – CGC CAG GGT TTT CCC AGT CAC GAC – 3’. A similar approach was used to detect wild type and deletion of ApoE, sense 5’ – GCC TAG CCG AGG GAG AGC CG – 3’, antisense for wild type 5’ – TGT GAC TTG GGA GCT CTG CAG C – 3’, and knockout antisense was 5’ – GCC GCC CCG ACT GCA TCT – 3’.

Tissue Preparation, Histology and Immunohistochemistry

Dissection of the heart and aorta was performed under a microscope. For subsequent histological/immunohistochemical analysis the heart and proximal aorta were collected, washed in ice cold PBS, gently blotted and embedded in Tissue Tek OCT Compound (optimum cutting temperature, Sakura Finetek, CA), frozen in liquid nitrogen cooled isopentane and stored in liquid nitrogen. The heart containing the aortic root and ascending aorta were cut and serial sections (6\(\mu\)m) discarded until a section showed the 3-valve cusps and round aorta. Sections were cut and collected until the cusps were no
longer visible\(^2\). Sections were stained with Oil Red O for lipid content, with picro-sirius red to determine collagen associated with lesions and also used for immunohistochemistry. For immunohistochemistry sections were stained as previously described\(^3\), with rat anti-mouse macrophage (CD68) antibody 1:100 dilution (BD Pharmingen), rabbit anti-mouse \(\alpha\)-SMC actin (Abcam) 1:500 dilution, rat anti-mouse VCAM-1 (BD Pharmingen) 1:50 dilution and rat anti mouse endothelial cells (CD31) antibody (BD Pharmingen) 1:100 dilution for an hour. Anti-rat (BD Pharmingen) and anti-rabbit (Vector laboratories) biotinylated secondary antibodies at 1:200 dilutions were detected with the Vectastain Elite ABC kit (Vector Laboratories) and 3'3'-diaminobenzidine substrate (Sigma). Sections were counterstained with hematoxylin.

**Quantification of Atherosclerotic Lesions**

The lesion area was measured using Optimus Image Analysis Software v5.2. Values reported represent mean lesion area from 3 sections for each animal. Quantitation of macrophage and smooth muscle cells in lesions was determined by calculating total area for CD68, the percentage of alpha-SM actin and VCAM-1 of cross-sectional area of the lesion, respectively. Collagen levels were similarly determined under normal light and its characteristics assessed under polarized light. CD31 expression was determined as percentage of positive cells associated with lesions.

**Analysis of Gene Expression by Real-Time and RT-PCR**

Total RNA was extracted from aortic arches and peritoneal macrophages snap frozen in liquid nitrogen using RNeasy kit (Qiagen) and quantitated by measuring absorbance at 260nm. Reverse-transcription was performed using Taq man reverse transcription reagent kit (Applied Biosystems). Quantitative gene expression analysis was performed on an ABI PRISM 7500 fast real-time PCR system (Applied Biosystems) using SYBR Green technology (Applied Biosystems). Primers were designed using Primer Express (Applied

RT-PCR was performed as previously described, using oligonucleotide primers to detect GM-CSF and GAPDH (GM-CSF: sense: 5’ – CCA CCC GCT CAC CCA TCA CTG TC-3’, antisense 5-GCT GGC CTG GGC TTC CTC ATT TTT – 3’; GAPDH: sense 5’ – ATG TTT GTG ATG GGT GGC TTC CTC ATT TTT – 3’, antisense 5’ – TAG CCA TAT TCA TTG TCA TAC CAG G – 3’) and a one step RT-PCR kit (SuperScript RT-PCR system (Life Technologies Invitrogen)).
Isolation of Peritoneal Macrophages

Mice were injected with 1ml of sterile 4% Brewer thioglycolate medium (Sigma). Four days later the mice were culled and peritoneal cavity flushed with ice-cold Dulbecco’s phosphate buffer pH7.4. Cells in the buffer were recovered by centrifugation and plated in DMEM supplemented with 10% FBS. After 3 hours non-adherent cells were washed off with ice cold PBS and the macrophages were collected by scraping.

Cholesterol efflux assay

Cholesterol efflux assay was modified from those previously described. Briefly, thioglycolate elicited peritoneal macrophages from ApoE-/- GM-CSF-/- and ApoE-/- mice were seeded onto a 12 well plate at 5 X 10^5 cells per well and grown in DMEM supplemented with 10% mouse serum from either ApoE-/- GM-CSF-/- or ApoE-/- mice. After 24 hours [^3H]-cholesterol was added and cells cultured for a further 48 hours. Then, following 6 washes with PBS, the cells were incubated for 18 hours in DMEM alone before adding plasma to treatment wells for another 2 hours. Then the incubation media and cell lysate were collected and total cell and media [3H]-cholesterol determined by liquid scintillation counting. Cholesterol efflux was calculated as the ratio of[^3H] label in the medium to[^3H] label in the medium +[^3H] label in the cells.

Statistical Analysis

Data are presented as mean ± SEM. When the data followed a normal distribution, an unpaired t test was used to assess statistical significance of differences between means. Mann-Whitney rank sum test was used in other cases. Difference between means were considered statistically significant when p<0.05.
References
Figure I.

Mouse characteristics (A) Detection of 1000 and 246bp PCR products representing GM-CSF and ApoE knockout alleles in the ApoE-/-,GM-CSF-/- mice. PCR products of wild type ApoE and GM-CSF alleles are 155 and 1246 bp respectively. Numbers represent different mice genotypes using tail derived DNA. (B) Body weights and (C) plasma cholesterol levels of the ApoE-/- and ApoE-/-,GM-CSF-/- mice after feeding a high fat diet. Results are the means ± SEM of at least 20 mice in each group. +P < 0.05 from ApoE-/- mice.
Figure II.

Immunohistochemical staining with an anti-VCAM-1 antibody in the tunica intima of the aortic sinus in ApoE-/-,GM-CSF-/- mice (A) and ApoE-/- mice (B). (C) Bar graphs represent VCAM-1 protein expression as % atheroma in ApoE-/-,GM-CSF-/- and ApoE-/- mice; results are means ± SEM of at least 13 mice from each group. † P < 0.05 from ApoE-/- mice.
Figure III.

GM-CSF and lesion vascularity. (A) There is no increase in the number of microvessels per unit area of atheroma in ApoE-/-,GM-CSF/- mice. Results are means ± SEM of at least 13 mice in each group (B) mRNAs encoding soluble VEGF receptor-1 (sFlt-1) determined by real-time PCR tended to be reduced whilst mRNA encoding VEGF receptor-1 (Flt-1) tended to be induced in lesions of ApoE-/-,GM-CSF/- mice, but changes were not statistically significant (p>0.05). Results are means ± SEM of 5 mice in each group.