

Arterial colony stimulating factor-1 influences atherosclerotic lesions by regulating monocyte migration and apoptosis^[S]

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Abstract Previous studies have shown that colony stimulating factor-1 (CSF-1) deficiency dramatically reduced atherogenesis in mice. In this report we investigate this mechanism and explore a therapeutic avenue based on inhibition of CSF-1 signaling. Lesions from macrophage colony stimulating factor-1 (*Csf1*)^{+/-} mice showed increased numbers of apoptotic macrophages, decreased overall macrophage content, and inflammation. In vitro studies indicated that CSF-1 is chemotactic for monocytes. Bone marrow transplantation studies suggested that vascular cell-derived, rather than macrophage-derived, CSF-1 is responsible for the effect on atherosclerosis. Consistent with previous studies, CSF-1 affected lesion development in a dose-dependent manner, suggesting that pharmacological inhibition of CSF-1 might achieve similar results. Indeed, we observed that treatment of hyperlipidemic mice with a CSF-1 receptor kinase inhibitor inhibited plaque progression. This observation was accompanied by a reduction in the expression of adhesion factors (ICAM-1), macrophage markers (F4/80), inflammatory cytokines (IL-6, IL-1 β), and macrophage matrix degradation enzymes (MMP-9). We conclude that the M-CSF pathway contributes to monocyte recruitment and macrophage survival and that this pathway is a potential target for therapeutic intervention.—Shaposhnik, Z., X. Wang, and A. J. Lusis. Arterial colony stimulating factor-1 influences atherosclerotic lesions by regulating monocyte migration and apoptosis. *J. Lipid Res.* 2010. 51: 1962–1970.

Supplementary key words inflammation • c-FMS • kinase inhibitor

Colony stimulating factor-1 (CSF-1) regulates monocyte/macrophage survival, differentiation, proliferation (1–4), and migration (5, 6) by activating a signaling cascade medi-

ated by its receptor, the tyrosine kinase proto-oncogene *c-fms* (also known as *Csf1r*). Interleukin-34 (IL-34) has been recently identified as another ligand for CSF1-R; however, its role in atherosclerosis has yet to be elucidated. Inflammatory disorders that display elevated levels of CSF-1 include arthritis, obesity, and atherosclerosis (7). The potential involvement of CSF-1 in atherosclerosis was first suggested by the observation that CSF-1 is induced in endothelial cells by treatment with oxidized lipids (8) and is expressed at high levels in atherosclerotic lesions (8, 9). Subsequently, it was shown that osteopetrotic (*op/op*) mice carrying a *Csf1* null mutation, either on an apolipoprotein E (apoE)^{-/-} or a low density lipoprotein receptor null (LDLR^{-/-}) background, showed a dramatic decrease in the size of atherosclerotic lesions (10–12).

Several significant issues concerning the role of CSF-1 in atherogenesis remain unresolved. First, the mechanism by which the CSF-1 deficiency contributes to lesion development remains unclear. Because CSF-1 influences monocyte/macrophage growth and survival, likely mechanisms include decreased numbers of circulating monocytes; decreased monocyte recruitment to the artery wall; decreased monocyte/macrophage proliferation; decreased macrophage uptake of oxidized lipids; and increased monocyte/macrophage foam cell necrosis/apoptosis. Second, the source of the CSF-1 critical for lesion formation is unclear. Both macrophages and endothelial cells (EC)

Abbreviations: ApoE, apolipoprotein E; B2M, β (2)-microglobulin; CAD, coronary artery disease; CSF-1, colony stimulating factor-1; CSF-1R, colony stimulating factor-1 receptor; cFMS, cellular homolog of the V-FMS oncogene product of the Susan McDonough strain of feline sarcoma virus (equivalent to CSF-1R); EC, endothelial cell; LDLR, low density lipoprotein receptor; PON, paraoxonase; PAF-AH, platelet-activating factor acetyl hydrolase

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appear to be likely candidates as CSF-1 is expressed by both cell types and expression is dramatically induced in EC by oxidized LDL and by proinflammatory molecules, such as bacterial lipopolysaccharide (8). CSF-1 has previously been shown by immunohistochemistry to be abundantly produced in human and rabbit atherosclerotic lesions but not in the normal vessel wall (9, 13).

Because CSF-1 deficiency has such a dramatic impact on atherosclerosis, we have been interested in the possibility that pharmacologic inhibition of CSF-1 signaling could be a useful therapeutic intervention. We have examined the effects of a CSF-1 receptor kinase inhibitor (GW2580) (14) on inflammation and atherosclerosis.

MATERIALS AND METHODS

Animal husbandry

Csf1 heterozygous mice on a C57BL/6JxC3HeB/FeJ mixed genetic background were purchased from the Jackson Laboratory (Bar Harbor, ME) and backcrossed for ten generations to C57BL/6J mice, at which point the mice were crossed to LDLR^{-/-} on the same background. At approximately 10 weeks of age the mice were placed on a Western-type diet (TD 88137, Teklad, Madison, WI) for 13 weeks before sacrifice. The Animal Research Committee of the University of California at Los Angeles approved all animal work.

CSF-1 receptor kinase inhibitor synthesis

GW2580 was synthesized as previously described (14). The compound (or carrier) was administered orally at 80 mg/kg twice a day for 8 weeks to 8-week-old low density lipoprotein receptor (LDLR)^{-/-} female mice.

Quantitation of atherosclerosis, plaque composition, and immunohistochemistry

Atherosclerotic lesions in the proximal aorta and aortic root were measured as previously reported (15). The ascending and descending aorta was pinned out en face and stained with Oil red O as in Tangirala et al. (16). Lesion surface area and total aortic surface area were measured using Image Pro Plus (Media Cybernetics). Frozen sections from the proximal aorta were stained for plaque composition by a Movat pentachrome stain, macrophages (rat anti-mouse MOMA-2, BD Biosciences, San Jose, CA) and proliferating cells (rabbit-anti mouse Ki67, Abcam).

Peritoneal macrophage and monocyte migration assays

Mice were injected intraperitoneally with 1 ml of 3% brewer's yeast thioglycolate four days before sacrifice. Cells were isolated by flushing the peritoneal cavity with cold PBS. Red blood cells were lysed and peritoneal cells were counted. Monocyte migration was determined in vitro using a human artery wall coculture model (17).

Apoptosis

The ApopTag Fluorescein In Situ Apoptosis Detection Kit (Millipore, Billerica, MA), utilizing a terminal deoxynucleotidyl transferase (TdT)-based technique, was used to quantitate apoptosis within atherosclerotic lesions of the aortic root.

Bone marrow transplantation

Bone marrow from *Csf1*^{+/-} and *Csf1*^{-/-} mice on the C57BL/6J background and *Csf1*^{+/-} and *Csf1*^{-/-} mice on a mixed C57BL/6J

x 129T2/SvEMsJ background was collected. Bone marrow from this mixed background was used because we were unable to obtain *Csf1*^{-/-} mice on an inbred B6 background. The 129 strain was chosen because its haplotype is compatible with B6 mice. Eight-week-old recipients were lethally irradiated and injected via the tail vein with 10⁷ bone marrow cells as previously described (17). Five weeks after transplantation the animals were placed on a Western-type diet for 12 weeks. Leukocyte DNA was isolated to confirm the presence of the *Csf1* null allele or the presence of the *Sry* gene by PCR as appropriate.

Quantitation of inflammatory gene expression

Total RNA was isolated using an RNEASY kit (Qiagen, Valencia, CA). cDNA was synthesized with iScript cDNA Synthesis Kit (Biorad, Hercules, CA). Quantitative PCR was performed on an Applied Biosystems 7700 unit using Platinum SYBR Green qPCR Supermix UDG (Invitrogen, Carlsbad, CA). Samples were analyzed in duplicate and normalized to $\beta(2)$ -microglobulin (B2M) expression. B2M, a component of the MHC I complex, is an ubiquitous and stably expressed factor.

Statistical tests

Data were expressed as mean \pm SEM. Statistical analyses were performed using the nonparametric Mann Whitney test (Statview, SAS Institute, Cary, NC) for all comparisons. Lesion data were displayed as a box plot and whiskers, 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 beyond the 9th and 91st percentiles.

RESULTS

Dose-dependent effects of an CSF-1 deficiency in a defined genetic background

Interaction of Csf1 mutation and genetic background. To study the mechanism by which a CSF-1 deficiency contributes to atherosclerosis, we sought to place the *Csf1* null mutation on an inbred genetic background. For this, we repeatedly crossed the mutation, originally on a mixed C57BL/6JxC3HeB/FeJ background, to inbred C57BL/6J mice. We felt this was important as C3H mice are highly resistant to atherosclerosis and nearly a dozen major loci contribute to the differences in susceptibility between C57BL/6 and C3H mice on an apoE null background (18). After 10 generations of backcrossing, we intercrossed heterozygous mice. Over 120 mice from this cross were genotyped, which resulted in a ratio that was greatly skewed from the expected 1:2:1 ratio. Approximately equal numbers of +/+ and +/- mice were observed; only 2 mice were of the -/- genotype. Thus, both the heterozygous and homozygous null genotypes were associated with dramatically reduced viability. Even on a mixed genetic background, *Csf1*^{-/-} mice had reduced size and weight at birth as well as osteopetrosis and a lack of tooth eruption (19, 20). We chose to use primarily heterozygous mice in this study. While both male and female mice were examined, we present here our analysis on female mice. The male data (supplementary data at www.jlr.org) yielded similar conclusions, although there were some significant differences.

Atherosclerotic lesions. Examination of the aortic root by immunostaining clearly revealed decreased macrophage content and a reduced the rate of plaque formation in female *Csf1*-deficient mice. Intimal lesion macrophage content decreased by 50% (Fig. 1) in female *Csf1*^{+/-} LDLR^{-/-} mice compared with *Csf1*^{+/+} LDLR^{-/-} mice. Movat staining showed that about 45% of the lesion area consisted of matrix components, including collagen and proteoglycans (supplementary Table I). *Csf1*^{+/-} LDLR^{-/-} female mice showed a 40% decrease in proximal aorta atherosclerosis (Fig. 2). This decrease was not observed in the thoracic and abdominal aorta of female *Csf1*^{+/-} mice, as measured by en face staining of lipids (supplementary Fig. I-A). We observed no differences in plasma lipids, circulating monocytes, overall number of cells per unit of lesion area (data not shown), number of proliferating cells (supplementary text and Fig. II-A), fibrous caps, or prominent lipid cores per aortic root section (Table 1, supplementary Tables I, II).

CSF-1 derived from monocyte/macrophages does not influence lesion development. As discussed above, monocytes/macrophages are capable of abundant CSF-1 expression. To test whether monocyte/macrophage-derived CSF-1 contributed to lesion development, we conducted bone marrow transplantation experiments. We transplanted bone marrow from *Csf1*^{+/+} and *Csf1*^{-/-} donors into lethally irradiated LDLR^{-/-} recipients and placed the animals on a 12-week Western diet. No significant differences were detected in

aortic root atherosclerosis (Fig. 2), plasma CSF-1 levels, or circulating monocyte counts (supplementary Table II) between animals receiving *Csf1*^{+/+} or *Csf1*^{-/-} bone marrow.

We also performed the reciprocal experiments. We transplanted *Csf1*^{+/+} bone marrow into lethally irradiated *Csf1*^{+/-} LDLR^{-/-} mice and placed them on the same diet regimen as before. Female mice exhibited a 50% decrease in aortic root atherosclerosis (Fig. 2C) and a 35% decrease in plasma CSF-1 levels but no decrease in circulating monocyte counts (supplementary Table III). In this experiment, the male *Csf1*^{+/-} mice showed a trend toward decreased lesion size that did not reach statistical significance (data not shown). We conclude that the levels of artery wall-derived CSF-1 is most important in mediating the effect of CSF-1 on atherosclerosis and that an absence of macrophage-derived CSF-1 has little or no impact on atherosclerosis.

Mechanisms contributing to lesion development

Aortic inflammatory gene expression. *Csf1*^{+/-} LDLR^{-/-} mice demonstrated a 7-fold decrease in aortic *Csf1* expression and a 3-fold decrease in SRA expression (Fig. 3). One would expect a 2-fold decrease in *Csf1* expression when examining heterozygous mice, but as macrophages express this cytokine themselves, the large decrease we observed in aortic *Csf1* expression probably reflects the decreased macrophage content (Fig. 1) and atherosclerosis (Fig. 2) of *Csf1*^{+/-} LDLR^{-/-} mice. SRA is known to

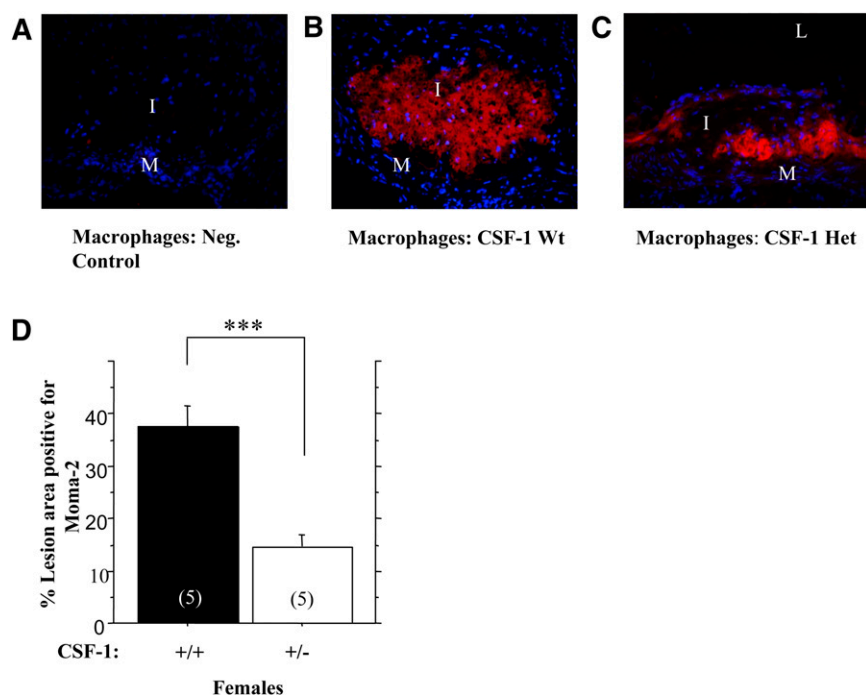


Fig. 1. Histochemical analysis of macrophage content in CSF-1-deficient mice. Aortic root lesion cryosections were stained for macrophage content using a MOMA-2 antibody. (A) The primary antibody was omitted as a negative control. Lesional macrophages in female *Csf1*^{+/+} LDLR^{-/-} animals (B) and *Csf1*^{+/-} LDLR^{-/-} animals (C) were detected and visualized as red-stained regions. Nuclei were stained blue with DAPI. (D) Aortic root atherosclerotic lesion area positive for macrophages was measured and expressed as a percentage of intimal thickening area in each respective section. A total of 3–4 sections from 4–6 animals per group were analyzed. CSF-1, colony stimulating factor-1; I, intima; M, media. (***) $P < 0.001$, magnification 200 \times .

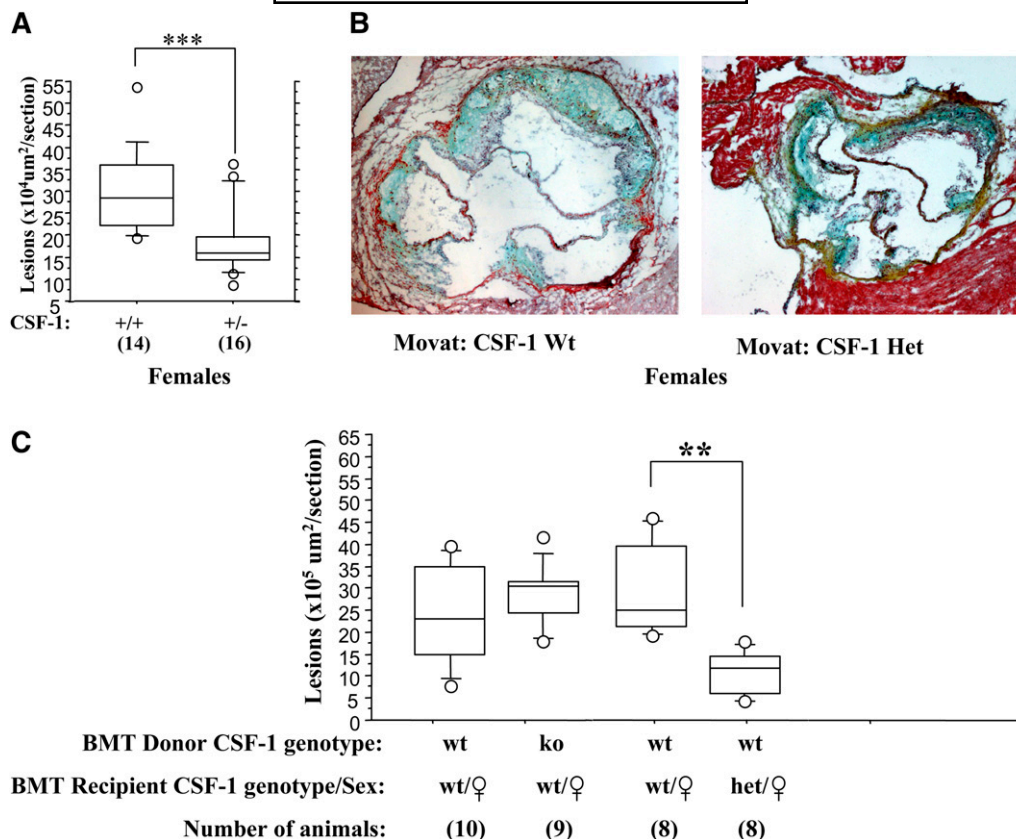


Fig. 2. Effect of a CSF-1 deficiency on atherosclerotic lesions in the proximal aorta. Atherosclerotic lesions in aortic root sections of $Csf1^{+/+}$ and $Csf1^{-/-}$ mice were quantitated by lipid staining with Oil Red O (A) and after bone marrow transplantations (C). Aortic root lesions were stained with Movat Pentachrome to better characterize relative plaque composition. Representative lesion sections are shown in (B) with collagen in yellow, SMCs in red, and proteoglycans in blue. The lesion data are presented as box plots (see “Materials and Methods”). (*** $P < 0.001$, ** $P < 0.01$). CSF-1, colony stimulating factor-1; wt, wild-type at the *Csf1* locus; het, heterozygous at the *Csf1* locus; ko, null at the *Csf1* locus.

be upregulated by *Csf1* (21), but in this case, decreased SRA expression is attributable to decreased macrophage content of the lesion. No significant changes in CD36, KC, or MCP-1 expression were detected (data not shown); however, those cDNAs were detected at levels so low in the control arterial samples that detecting further decreases in expression in *Csf1*-deficient mice would be highly unlikely.

Effect of CSF-1 on monocyte migration. We examined the possible role of CSF-1 in monocyte recruitment using an in vitro monocyte migration assay (17) and an in vivo peritoneal macrophage recruitment assay (supplementary Fig. III-A). Addition of a CSF-1 neutralizing antibody to an in vitro assay consisting of cocultures of endothelial and smooth muscle cells significantly decreased monocyte migration in response to oxidized lipids, whereas control antiserum had no effect. Consistent with this, peritoneal cavity monocyte recruitment in response to thioglycolate exhibited a significant decrease in $Csf1^{+/-}$ LDLR $^{-/-}$ animals. These experiments support the idea that CSF-1 has a chemotactic role in the artery wall and that the haploinsufficiency of *Csf1* in $Csf1^{+/-}$ animals can alter monocyte migration in the context of atherosclerosis.

Circulating monocytes. We did not detect a difference in circulating levels of CD115+ (CSF-1 receptor expressing) monocytes (Table 1, supplementary Fig. III-C).

CSF-1 influences macrophage apoptosis in lesions. The decreased macrophage content we observed in atherosclerotic lesions from *Csf1*-deficient mice raised the possibility of an effect on macrophage apoptosis. We quantitated apoptosis in lesions by measuring DNA fragmentation using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining. The number of apoptotic nuclei varied from 0 to 22 per section in each group and overlapped almost exclusively with lesion areas positive for the

TABLE 1. Circulating monocyte levels measures by flow cytometry

	CSF-1 $^{+/+}$ LDLR $^{-/-}$	CSF-1 $^{+/-}$ LDLR $^{-/-}$
	Females	
Monocytes (CD115+)	1.36 \pm 0.35	2.05 \pm 0.43
% of Leukocytes	n = 6	n = 5

Values are expressed as mean \pm SEM.

Abbreviations: CSF-1, colony stimulating factor-1; LDLR, low density lipoprotein receptor.

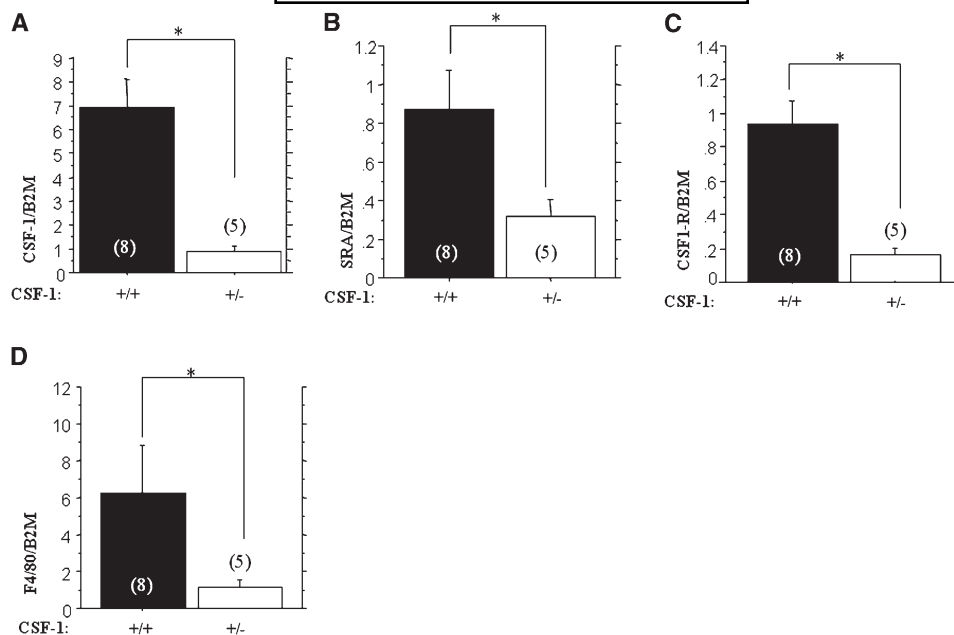


Fig. 3. Inflammatory gene expression is suppressed in atherosclerotic lesions of CSF-1-deficient mice. The ascending and thoracic aorta was dissected at sacrifice after a Western diet to remove adventitial connective tissue before RNA isolation. The expression levels of the inflammatory genes CSF-1, SRA, CSF-1R, and F4/80 were examined by quantitative PCR. Data are presented as means \pm SEM; 5–8 mice of each genotype were tested. (* $P < 0.05$). CSF-1, colony stimulating factor-1; CSF-1R, colony stimulating factor-1 receptor; SRA, scavenger receptor A; B2M, beta(2) microglobulin.

macrophage marker MOMA-2 (**Fig. 4A–B**, supplementary Fig. VI-A, B). Female *Csf1*^{+/-} lesion sections did not show an increase in apoptotic cell content relative to lesion. However, after normalizing for the number of apoptotic cells by the percentage of MOMA-2-positive lesion area, we observed approximately 2-fold increased apoptosis in *Csf1*^{+/-} LDLR^{-/-} mouse lesions ($P < 0.05$) (**Fig. 4D**).

Additional studies were conducted to determine cell survival using mouse bone marrow derived macrophages from either *Csf1*^{+/+} or *Csf1*^{+/-} mice. Cells were cultured for six days in regular media or media supplemented with recombinant CSF-1. We observed that both groups of cells treated with CSF-1 continued to proliferate over the six days and displayed a spread out morphology typified by various cellular projections. In contrast to that, cells starved of CSF-1 displayed a much more rounded morphology and appeared not to proliferate at all. Their numbers declined precipitously after six days in culture compared with cells not starved of CSF-1 (data not shown).

Pharmacologic inhibition of CSF-1 signaling decreases atherosclerosis

On the basis of the clear dose-dependent inhibitory effect of a CSF-1 reduction on lesion formation, we tested the effect of blocking CSF-1 signaling by inhibiting the intrinsic kinase activity of the CSF-1 receptor with a highly selective, orally bioavailable, small-molecule kinase inhibitor GW2580 (14). We initially detected the ability of this compound to inhibit LDL- or MCP-1-mediated monocyte migration in a dose-dependent manner using an artery wall coculture model (supplementary Fig. IV-A). This suggested there was

potential for the compound to be effective in inhibiting atherogenesis in vivo. The inhibitor was dosed orally twice a day at 80 mg/kg into LDLR^{-/-} female mice for 8 weeks. This dose was chosen based on previous work (14) indicating that free drug levels in the plasma would remain within the IC₅₀ range for suppression of monocyte proliferation while maintaining 100-fold selectivity for kinase inhibition. Lesion size was reduced ~40% ($P < 0.05$), and relative plasma PON activity was elevated 28% ($P < 0.05$) (**Fig. 5**). Using the same coculture model mentioned previously, we observed that HDL from the group treated with GW2580 retained the protective capacity to inhibit LDL-mediated monocyte migration while HDL from control mice lost this ability (supplementary Fig. IV-C). No changes in plasma lipid levels were detected (supplementary Fig. V).

GW250 was added to cultured peritoneal macrophages to determine if phosphorylation of Akt, an event known to occur downstream of CSF-1R signaling and an important factor in cell survival/proliferation, had been altered. Thioglycolate-elicited peritoneal macrophages isolated from C57BL6/J mice were cultured for one week in regular media, media supplemented with 10 ng/ml of recombinant CSF-1 (as a positive control), or with 1 μ M of the CSF-1R kinase inhibitor GW250. This concentration of inhibitor should selectively inhibit all endogenous signaling only through this receptor. Western blots of cell lysates were probed for phospho-Akt and total Akt levels. It was observed that phospho-Akt levels decreased 89% using GW2580 after normalization to total Akt levels while increasing 155% upon addition of recombinant CSF-1 (supplementary Fig. VII).

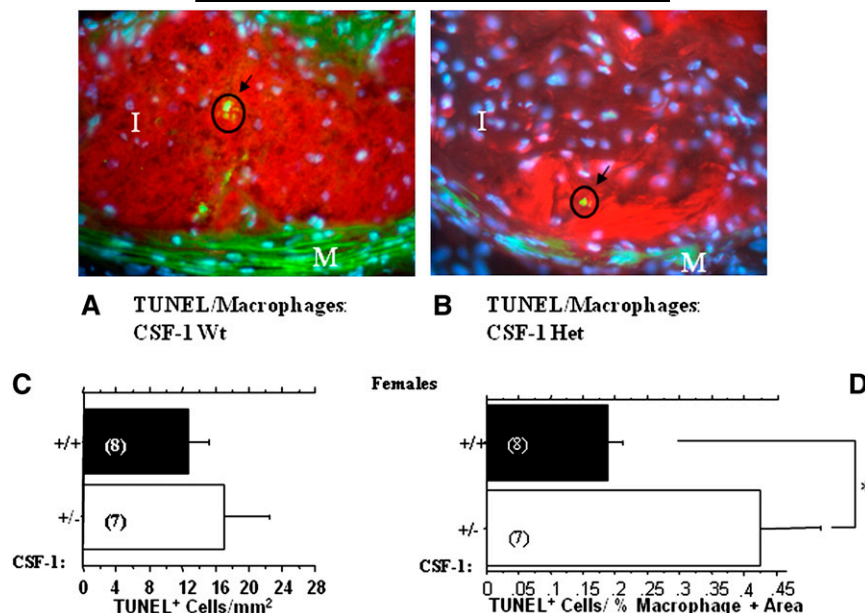


Fig. 4. Quantitation of apoptosis in the aortic root of CSF-1-deficient mice. Aortic root plaque cryosections were stained for apoptotic cells (green) using a TUNEL assay and counterstained for macrophages with a MOMA-2 antibody in red. Nuclei were stained blue with DAPI. Apoptotic nuclei (yellow areas indicated with arrows) were detected within *Csf1*^{+/+} LDLR^{-/-} lesions (A) and *Csf1*^{+/-} LDLR^{-/-} lesions (B). Apoptotic cells were within areas of macrophage infiltration (black circles). Quantitation of lesional apoptosis (C–F). (**P* < 0.05, magnification 400×). CSF-1, colony stimulating factor-1; I, intima; LDLR, low density lipoprotein receptor; M, media; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; wt, wild-type at the *Csf1* locus; het, heterozygous at the *Csf1* locus.

To better understand the anti-inflammatory effects of GW2580, inflammatory gene expression was determined in the atherosclerotic aorta and the liver. As in the *Csf1*^{+/-} mouse lesions, 70%–80% reductions were observed in the tissue macrophage-specific marker F4/80 (*P* < 0.01), the pan-monocyte/macrophage marker CSF1-R (the CSF-1 receptor) (*P* < 0.001), and the scavenger receptor SRA (*P* < 0.01) (Fig. 6A). Matrix metalloproteinase-9 (MMP-9) expression, an enzyme secreted by macrophages that can degrade the matrix and fibrous cap causing lesion destabilization, declined ~90% (*P* < 0.001). ICAM-1 and E-Selectin, both factors expressed by the endothelium early in plaque formation and involved in the firm adhesion of monocytes to the artery wall prior to their entry, showed 70%–80% reduced expression in the CSF-1R inhibitor-treated group. Urokinase plasminogen activator receptor (uPAR), a direct target of CSF-1 signaling, was significantly downregulated as well (Fig. 6B). In contrast to the aorta, hepatic CSF-1R expression tripled (*P* < 0.05) while F4/80 levels declined 50% (Fig. 6C), suggesting that while the overall macrophage content of liver decreased, there appears to be a feedback loop linking the inhibition of CSF-1 signaling with increased expression of CSF-1 receptor in the remaining tissue macrophages. The expression of other key inflammatory mediators and markers of inflammatory macrophages, such as IL-6 and IL-1 β , decreased by 50%–80% in the liver. Hepatic CSF-1 expression itself declined 50% (*P* < 0.05).

Genetic deficiencies of CSF-1 or its receptor are known to increase bone density and decreasing adiposity. We studied adiposity using NMR and bone density using DEXA in response to GW2580 treatment. Except for a

small change in adiposity (from 19.1% to 22.5%, *P* < 0.05), no differences were observed. We also found no changes in food intake or blood monocyte counts in mice treated with the inhibitor (data not shown).

DISCUSSION

Our studies with *Csf1*^{+/-} LDLR^{-/-} mice showed that the effect of CSF-1 on atherosclerosis was dependent on decreased artery wall-derived CSF-1, rather than monocyte/macrophage-derived CSF-1. It resulted in decreased lesional macrophage content and increased lesional macrophage apoptosis. Also, our studies with a specific CSF-1 receptor kinase inhibitor (GW2580) suggest that pharmacologic inhibition of CSF-1 signaling may be a promising therapy for atherosclerosis.

Studies of *Csf1*-deficient mice on ApoE^{-/-} (12) and LDLR^{-/-} backgrounds (11) have revealed dramatic decreases in aortic atherosclerosis. These studies were conducted using mice on a mixed background (12) where the *Csf1*^{-/-} mice had significant decreases in circulating monocytes and were generally unhealthy, with striking osteopetrosis. In the current study we used *Csf1*^{+/-} LDLR^{-/-} animals backcrossed for ten generations to the C57BL/6J background to demonstrate an approximate 2-fold decrease in female aortic root lesions. Although not reaching significance, aortic root lesions in male *Csf1*^{+/-} mice trended strongly toward a similar decrease in lesion size while showing a significant decrease in lesions when measured by an en face approach. Sex-specific and region-specific differences in lesion susceptibility are frequently

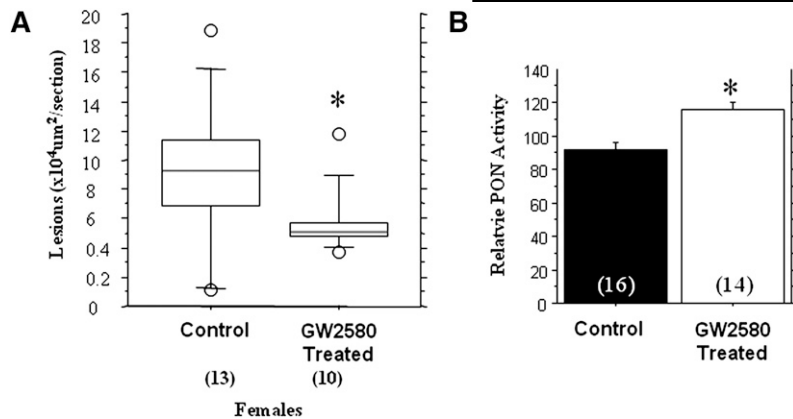


Fig. 5. Effect of the CSF-1 receptor kinase inhibitor on atherosclerotic lesions in the proximal aorta and on plasma PON activity. LDLR^{-/-} female mice on a Western diet treated twice a day by oral gavage for 8 weeks with 80 mg/kg of the CSF-1 receptor kinase inhibitor. Aortic root atherosclerotic plaques were quantitated by lipid staining with Oil Red O (A). The lesion data are presented as box plots (see “Materials and Methods”). Plasma was collected at sacrifice in heparin-coated tubes, and relative plasma PON activity after the 8-week treatment was determined (B). (**P* < 0.05). CSF-1, colony stimulating factor-1; LDLR, low density lipoprotein receptor; PON, paraoxonase.

observed in mouse atherosclerotic lesion studies. We believe the data demonstrates that decreasing CSF-1 levels resulted in decreased atherosclerosis but that the interplay of complex factors outlined above are responsible for the variations observed between males and females.

A large body of work has addressed the role of CSF-1 in a variety of macrophage functions (22). We sought to distinguish the effects of a macrophage-specific CSF-1 deficiency on atherosclerosis, as opposed to an artery wall-specific de-

ficiency on atherosclerosis, using bone marrow transplantations. These experiments suggested that artery wall-derived CSF-1 is the primary source of circulating CSF-1 and is the key source of CSF-1 as it relates to atherosclerosis. This could be due to differences in the half-life, stability, activity, and cell signaling properties among the three major isoforms of CSF-1: cell surface, secreted glycoprotein, and secreted proteoglycans. For example, cell-surface CSF-1 has a longer half-life than the secreted forms but only works

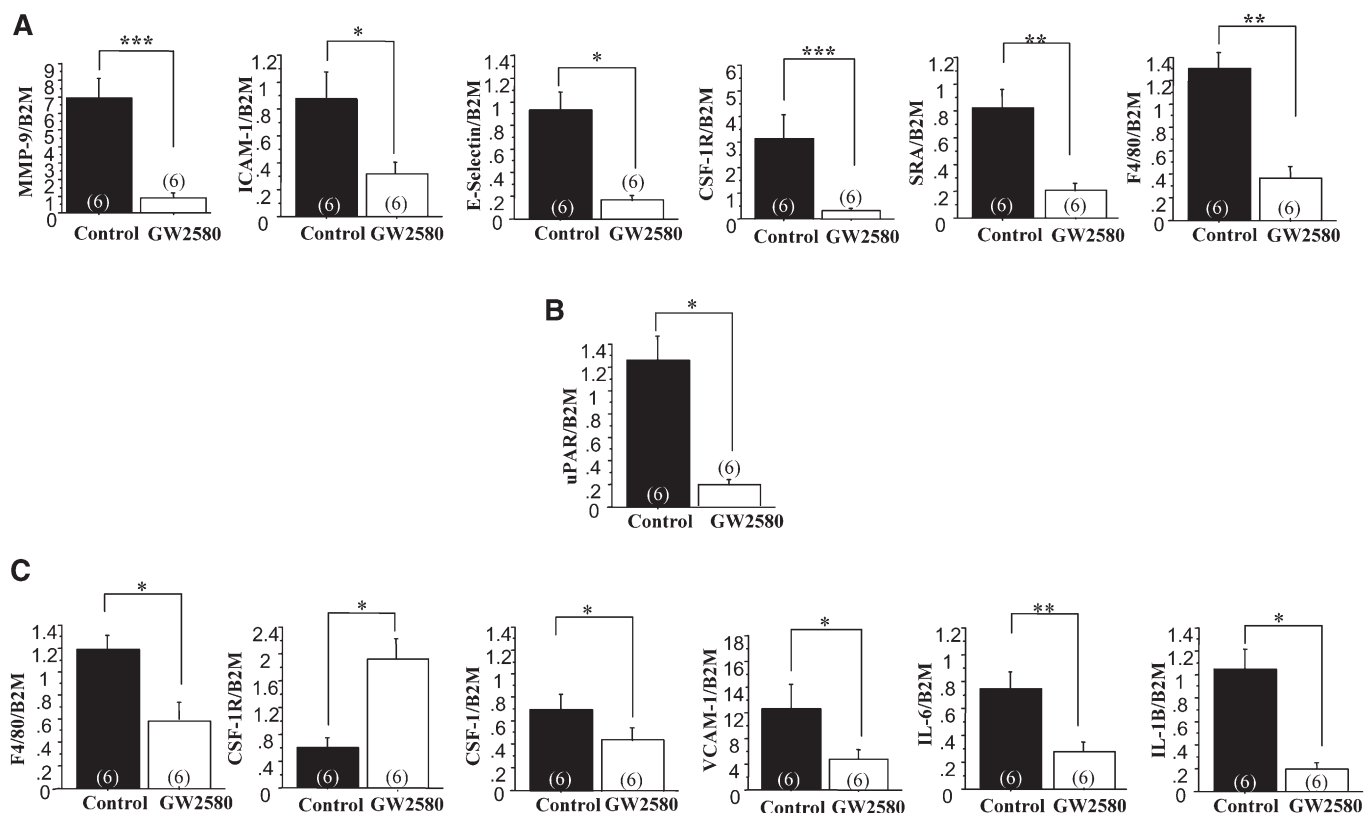


Fig. 6. Aortic and hepatic inflammatory gene expression is suppressed in LDLR^{-/-} mice treated with the CSF-1 receptor kinase inhibitor GW2580. The relative expression levels of the macrophage and endothelial cell derived inflammatory genes MMP-9, ICAM-1, E-Selectin, CSF-1R, SRA, and F4/80 were examined by quantitative PCR of aortic RNA from LDLR^{-/-} female mice on a Western diet treated with GW2580 (A). Aortic uPAR expression was examined (B), and expression of the hepatic inflammatory genes F4/80, CSF-1R, CSF-1, VCAM-1, IL-6, and IL-1β was examined (C). Data are presented as means ± SEM. From each category 6 mice were tested. (****P* < 0.001, ***P* < 0.01, **P* < 0.05). CSF-1, colony stimulating factor-1; CSF-1R, colony stimulating factor-1 receptor; IL, interleukin; LDLR, low density lipoprotein receptor; MMP-9, matrix metalloproteinase-9; uPAR, urokinase plasminogen activator receptor; ICAM-1, intercellular cell adhesion marker-1; SRA, scavenger receptor A; VCAM-1, vascular cell adhesion molecule-1.

when in direct contact with the macrophage. In an inflammatory kidney model, (23) the secreted proteoglycan form of CSF-1 enhances macrophage accumulation beyond what is observed with cell-surface CSF-1. Therefore, differences between the isoforms of CSF-1 produced by macrophages and the artery wall cells could have significant effects on plaque formation and macrophage function within the artery wall or even prior to entering the vessel. Infusion of a neutralizing antibody to the CSF-1 receptor in ApoE^{-/-} mice resulted in significantly decreased atherosclerotic lesions despite no significant differences in circulating monocytes (24). That report and our work indicate that reducing signaling through the CSF-1 receptor (primarily expressed on mononuclear phagocytes) by decreasing artery wall-derived CSF-1 or blocking CSF-1 receptor ligand binding results in decreased atherosclerosis.

One of the functions of CSF-1 is its ability to act directly as a monocyte chemotactic factor (5, 25). We showed a specific role for CSF-1 in monocyte migration in the context of a model of the artery wall by neutralizing oxidized LDL-stimulated endothelial and smooth muscle cell-derived CSF-1. In this model, MCP-1 induced in response to oxidized LDL is thought to be primarily responsible for monocyte migration into the artery wall. Our results show that CSF-1 also has a significant role in this process, and it is possible that CSF-1 and MCP-1 signaling pathways interact in the monocyte or that CSF-1 itself can induce endothelial cell-derived MCP-1 (26). We also showed that *Csf1*^{-/-} LDLR^{-/-} animals have significantly fewer thioglycolate-elicited peritoneal cells, a population of cells rich in macrophages, which suggests an *in vivo* role for CSF-1 in macrophage migration. This *in vivo* role of CSF-1 was substantiated in other studies where a single treatment of GW2580 blocked the migration of intravenously injected GFP-expressing macrophages into subcutaneous mouse 3LL lung cancer tumors 24 h post injection (27).

The peripheral leukocyte surface markers CD115 (the CSF-1 receptor) has been used to define populations of monocytes. We observed no change the overall frequency of CD115+ monocytes (Table 1) in *Csf1*^{+/-} LDLR^{-/-} mice as compared with *Csf1*^{+/+} LDLR^{-/-} mice, suggesting that CSF-1 does not alone strongly regulate these aspects of monocyte development. The data also suggest that the decreased atherosclerosis observed in CSF-1 deficient mice is not attributable to fewer circulating monocytes.

CSF-1 signaling through its receptor, the kinase c-fms, likely mediates macrophage survival via activation of phosphatidylinositol 3-kinase and Akt kinase activity (28, 29). CSF-1R inhibition in peritoneal macrophages with GW250 reduced endogenous phospho-Akt levels 89% (supplementary Fig. VII). Oxidized LDL is known to be toxic to macrophages (30) and other vascular cells, and we hypothesized that CSF-1-deficient macrophages within lesions would be more prone to undergo apoptosis. Indeed, we observed about a 2-fold increase in the number of TUNEL-positive cells that coincided with MOMA-2-positive regions of atherosclerotic lesions in *Csf1*^{+/-} LDLR^{-/-} mice (Fig. 4). Despite this, the exact nature of the causal relationship between decreased CSF-1 expression and apoptosis is unclear as

it could be due to the decreased plaque size in CSF-1-deficient mice. As previously reported, we also observed that a lack of CSF-1 supplementation severely impaired cultured bone marrow macrophage survival.

On the basis of our work and previous studies, we explored the potential of therapeutic inhibition of CSF-1 action for treatment of atherosclerosis. We tested GW2580, a compound that has been demonstrated to have at least 100-fold greater inhibitory capacity for the CSF-1R kinase when tested against an array of related kinases, such as the PDGF and VEGF receptor tyrosine kinases (14). GW2580 was administered at a dose required to maintain free-plasma levels (unbound to albumin) of the inhibitor in the range of 100 nM, near the IC₅₀ for inhibiting monocyte growth, with the assumption that the albumin-associated compound has very limited bioactivity. At the dose used, GW2580 suppressed atherogenesis to about the same extent observed in CSF-1 heterozygous mice (Fig. 2A). Although other kinase inhibitors, including imatinib (31) and SU11248 (32), can inhibit CSF-1 receptor kinase activity, they also inhibit several other kinases and are not selective for this single target.

We also observed that GW2580 could suppress monocyte migration in an artery wall coculture model and that HDL from treated LDLR^{-/-} mice retained the ability to block LDL-mediated monocyte migration while HDL from control LDLR^{-/-} mice lost this protective capacity (supplementary Fig. IV-C). This phenomenon presumably occurs as a result of the elevated inflammatory state engendered by a high-fat diet in atherosclerotic mice (17) and is associated with the displacement of PON1 and platelet-activating factor acetyl hydrolase (PAF-AH) enzymes from HDL particles. Both PON1 and PAF-AH have the ability to inactivate oxidized lipids. The ability to suppress lesion formation, improve the anti-oxidant capacity of HDL, and reduce the overall inflammatory state while not altering lipid levels, body composition, or bone density (data not shown) suggests that, in principal, pharmacologic inhibition of CSF-1 signaling can selectively suppress inflammation and lesion formation, properties that are desirable when considering the use of a compound in human coronary artery disease (CAD).

By analyzing a panel of inflammatory gene expression profiles derived from the aorta and liver of mice treated with GW2580, we are able to generate an improved understanding of the molecular changes associated the decreased lesion formation. It is known that the early steps of atherosclerosis involve oxidized lipid-mediated expression of monocyte adhesion molecules and that deficiencies in VCAM-1 and ICAM-1 have significant effects on lesion size (33). Further development of atherosclerosis occurs via the conversion of migrating monocytes into activated inflammatory macrophages that are characterized by the production of cytokines, such as IL-1 β , IL-6, and others. The rupture of an unstable plaque represents another manifestation of activated macrophages in light of the evidence that several matrix metalloproteinases are found in lesions. Expression of the activated form of MMP-9 resulted in the disruption of plaques within the innominate artery of ApoE^{-/-} mice (34). GW2580 treatment substantially decreased the expression of all these genes as well as general markers of mature

macrophages (Fig. 6). These data suggest that inhibition of CSF-1 signaling suppresses lesion formation at early, intermediate, and late stages.

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