Interleukin-3/Granulocyte Macrophage Colony–Stimulating Factor Receptor Promotes Stem Cell Expansion, Monocytosis, and Atheroma Macrophage Burden in Mice With Hematopoietic ApoE Deficiency

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Objective—Coronary heart disease is associated with monocytosis. Studies using animal models of monocytosis and atherosclerosis such as ApoE−/− mice have shown bone marrow (BM) hematopoietic stem and multipotential progenitor cell (HSPC) expansion, associated with increased cell surface expression of the common β subunit of the granulocyte macrophage colony–stimulating factor/interleukin-3 receptor (CBS) on HSPCs. ApoE−/− mice also display increased granulocyte macrophage colony–stimulating factor–dependent monocyte production in the spleen. We investigated the role of the CBS in cholesterol-driven HSPC expansion, monocytosis, and atherosclerosis.

Approach and Results—Ldlr−/− mice were transplanted with ApoE−/−Cbs−/− or ApoE−/− BM followed by Western-type diet feeding. Compared with ApoE−/− BM–transplanted controls, ApoE−/−Cbs−/− BM–transplanted mice had reduced BM and splenic HSPC proliferation, fewer blood monocytes and neutrophils, and reduced macrophage content and area of early atherosclerotic lesions. More advanced lesions showed diminished macrophage and collagen content; however, lesion size was unchanged, reflecting an increase in necrotic core area, associated with a marked decrease in Abcg1 expression and increased macrophage apoptosis. Compared with wild-type mice, Western-type diet–fed ApoE−/− mice showed increased CBS expression on granulocyte macrophage colony–stimulating factor–producing innate response activator B cells and expansion of this population. ApoE−/−Cbs−/− BM–transplanted Ldlr−/− mice showed a marked decrease in innate response activator B cells compared with ApoE−/− BM–transplanted Ldlr−/− controls.

Conclusions—Increased levels of CBS on HSPCs and splenic innate response activator B cells lead to expansion of these populations in ApoE−/− BM–transplanted Ldlr−/− mice, contributing to monocytosis and increased lesion macrophage content. However, in more advanced lesions, the CBS also has a role in atherosclerotic plaque stabilization. (Arterioscler Thromb Vasc Biol. 2014;34:0000.)

Key Words: atherosclerosis ■ granulocyte-macrophage colony-stimulating factor

Leukocytosis has been associated with higher rates of ischemic vascular disease in numerous prospective and cross-sectional studies.1–3 The monocyte count, in particular, independently predicts risk for coronary artery disease after adjustment for conventional risk factors.4 Monocytosis and neutrophilia have also been observed in animal models of atherosclerosis including pigs and rabbits and seem to contribute to atherogenesis.5,6 Hypercholesterolemia-induced monocytosis has been documented in ApoE−/− mice in association with a marked increase in the Ly-6Csh monocyte subset, a subpopulation that more readily infiltrates lesions.7,8 Hypercholesterolemia-induced neutrophilia has also been reported to contribute to early atherosclerotic lesion formation in ApoE−/− mice.9 Conversely, reduction of monocytes in the circulation through interruption of macrophage colony–stimulating factor (M-CSF) or MCP-1 activity decreased atherosclerosis in ApoE−/− and Ldlr−/− mice.10–12

Recent studies have suggested that hematopoietic stem and multipotential progenitor cell (HSPC) expansion underlies the monocytosis and neutrophilia observed in these models.13,14 Mice deficient in the ATP-binding cassette (ABC) transporters A1 and G1 (ABCA1 and ABCG1), which promote cholesterol efflux from myeloid cells, develop monocytosis, neutrophilia, and expansion of HSPCs in the bone marrow (BM).15 A similar
observation was made in ApoE−/− mice fed a Western-type diet (WTD), where monocytosis and neutrophilia were associated with HSPC expansion.16 Chimeric BM transplantation experiments revealed that the ApoE−/− HSPCs outcompeted wild-type (WT) HSPCs in giving rise to monocytes and neutrophils when transplanted into Ldlr−/− mice and that the AbcamAbcg1−/− HSPCs outcompeted WT HSPCs when transplanted into WT mice, suggesting a cell intrinsic proliferative advantage of ApoE−/− and AbcamAbcg1−/− HSPCs compared with WT HSPCs.16 In both studies, the cell intrinsic proliferative advantage was associated with increased cell surface levels of the common β subunit of the granulocyte M-CSF (GM-CSF)/interleukin-3 (IL-3) receptor (CBS). Another study, using ApoE−/− mice as a model, identified increased numbers of GM-CSF producing cells in the spleen and underscored the importance of extramedullary expansion of HSPCs and myeloid progenitor cells in the spleen as drivers of monocytosis and atherogenesis.17 The same group later described innate response activator (IRA) B cells that protect the hosts from polimicrobial sepsis through LPS-induced production of GM-CSF specifically in the spleen.18 Interestingly, these IRA B cells develop from peritoneal B1a cells in a CBS-dependent manner, suggesting a feed-forward loop in GM-CSF production from IRA B cells under certain stress conditions.

The CBS (aka CD131) is the β subunit shared by the receptors of IL-3, GM-CSF, and IL-5. In humans, CD131 is encoded by the gene Csfrb.19 In mice, Csfrb1 encodes IL-3-βc, the ortholog of CD131, whereas Csfrb2 encodes IL-3 receptor class II β chain, a second protein that is homologous to CD131 but can only form a functional receptor with the IL-3α subunit.20 Because of their partial functional redundancy and the fact that a Csfrb1−/−Csfrb2−/− mouse will be used in this study, CBS will be used to denote the protein product of Csfrb1 and Csfrb2 collectively, and Cbs−/− will denote Csfrb1−/−Csfrb2−/−. The CBS plays a central role in hematopoiesis, reflecting the function of GM-CSF and IL-3 in myeloid lineage development.21,22 IL-3 promotes HSPC survival and proliferation, and GM-CSF determines myeloid lineage specification and mediates the development of monocyte and dendritic cells (DCs).23,24 2 cell types that are intimately involved in atherosclerosis.

The hypothesis underlying this study was that increased expression of the CBS in HSPCs and possibly IRA B cells or their progenitors would promote HSPC and IRA B cell expansion, contributing to monocytosis and atherogenesis in ApoE−/− mice.

### Materials and Methods

Materials and Methods are available in the online-only Supplement.

### Results

#### CBS Deficiency Decreased Monocyte Levels Specifically in ApoE−/− BM–Transplanted Mice

To investigate the role of CBS in monocytosis, we generated ApoE−/−Cbs−/− mice by crossbreeding ApoE−/− and Cbs−/− mice (C57BL/6J background). In preliminary studies, we found that CBS deficiency in whole-body ApoE deficiency decreased plasma cholesterol levels (results not shown); therefore, we transplanted ApoE−/− or ApoE−/−Cbs−/− BM into Ldlr−/− mice, which did not change plasma cholesterol levels. At 5 weeks after transplantation, BM reconstitution was >90% (results not shown). We observed a major effect of diet-induced hypercholesterolemia in the Ldlr−/− background on blood monocyte and neutrophil counts as reported.16 ApoE−/−Cbs−/− BM–transplanted Ldlr−/− mice had significantly fewer monocytes than ApoE−/− BM–transplanted Ldlr−/− mice after 5 weeks of WTD feeding, and the difference was even more marked after 8 weeks of WTD feeding (Figure 1A). CBS deficiency also reduced neutrophil counts after WTD feeding (Figure 1B). The changes in monocytes and neutrophil levels were observed in the absence of any significant difference in the cholesterol levels (Figure 1C). We also studied the effect of CBS deficiency on monocyte levels in the presence of apolipoprotein E in the BM. When we transplanted Ldlr−/− mice with WT or Cbs−/− BM and fed them the WTD, no effect on blood monocytes was found (Figure I in the online-only Data Supplement). This was likely because of increased CBS on ApoE−/− but not WT HSPCs, as reported.16 Therefore, for subsequent studies, we used BM donors on the ApoE−/− background.

#### CBS Deficiency Decreased HSPC Expansion in BM and Spleen in ApoE−/− BM–Transplanted Mice

We compared the HSPC levels and their proliferative activity in the BM and the spleen after 9 weeks of WTD feeding (Figure 2; Figure II in the online-only Data Supplement). Compared with ApoE−/− BM–transplanted Ldlr−/− mice, the ApoE−/−Cbs−/− BM–transplanted Ldlr−/− mice had significantly lower numbers of Lin−Sca1+c-Kit+ cells (referred to as HSPCs), common myeloid progenitor cells and granulocyte monocyte progenitor cells in both BM and spleen (Figure 2A and 2B; Figure II in the online-only Data Supplement). ApoE−/− mice are known to develop splenomegaly on WTD feeding,16 and this was also decreased in the ApoE−/−Cbs−/− group (Figure III in the online-only Data Supplement). In BM of the ApoE−/−Cbs−/− group, the decreased HSPC, common myeloid progenitor cell, and granulocyte monocyte progenitor cell percentages were associated with a decrease only in the proliferation of HSPCs (Figure IIA and IIB in the online-only Data Supplement); whereas in the spleen, both HSPCs and granulocyte monocyte progenitor cells showed decreased proliferative activity.
Role of IRA B Cells in Elevated GM-CSF Production in Spleens of ApoE−/− Mice

Given the significant reduction of cell proliferation in the spleens of the ApoE−/−Cbs−/− group, we investigated the underlying mechanisms. In ApoE−/− mice–fed WTD, increased numbers of cells that produce GM-CSF and IL-3 accompanied the increase in monocyte production in the spleen. It has also been reported that peritoneal B1a cells develop into IRA B cells, specifically in the spleen where they produce GM-CSF and IL-3 to protect the host against polymicrobial sepsis. Thus, we examined the possibility that IRA B cells are involved in the enhanced GM-CSF/IL-3 production in the spleen of WTD-fed ApoE−/− mice. Indeed, compared with WT mice, ApoE−/− mice showed elevated numbers of IRA B cells and enhanced IRA B cell proliferation in the spleen (Figure 3A and 3B; Figure IIC in the online-only Data Supplement). These data suggest that CBS facilitates monocytosis in the hematopoietic ApoE−/− background by enhancing HSPC proliferation in the BM and HSPC and granulocyte monocyte progenitor cell production in the spleen.

CBS Plays a Central Role in IRA B Cell Expansion in ApoE−/− Mice

Previous studies demonstrated that B1a cells failed to give rise to IRA B cells in the presence of an antibody against CBS, suggesting that CBS is required for the development of IRA B cells. Our laboratory has reported previously that ApoE−/− mice had increased cell surface level of CBS in HSPCs because of defective cholesterol efflux and lipid raft formation. These observations led us to hypothesize that the enhanced IRA B cell formation in spleen could be because of elevated CBS expression on both IRA B cells (Figure 3C; Figure III in the online-only Data Supplement) compared with WT mice. Given that the number of B1a cells did not differ significantly between the 2 groups (Figure IVD in the online-only Data Supplement), these findings suggest that IRA B cell expansion is likely because of enhanced development of IRA B cells from B1a cells and increased IRA B cell proliferation as a result of the increased CBS expression on these 2 cell types. Conversely, lack of CBS decreased the number of IRA B cells in the spleen (Figure 3E) and their proliferative activity (Figure 3F) in Ldlr−/− mice transplanted with ApoE−/− or ApoE−/−Cbs−/− BM, suggesting a
central role for CBS in elevating the number of IRA B cells in ApoE<sup>−/−</sup> mice.

**Decreased Macrophage Content of Atherosclerotic Lesions of ApoE<sup>−/−</sup>Cbs<sup>−/−</sup> Mice:**

Increased Complexity of Advanced Lesions

We next assessed the extent of atherosclerotic plaque burden in ApoE<sup>−/−</sup> and ApoE<sup>−/−</sup>Cbs<sup>−/−</sup> BM–transplanted Ldlr<sup>−/−</sup> mice after 4 weeks or 9 weeks of WTD feeding. After 4 weeks of WTD feeding, ApoE<sup>−/−</sup>Cbs<sup>−/−</sup> BM–transplanted Ldlr<sup>−/−</sup> mice showed a 19% reduction in atherosclerotic plaque size (P<0.05) compared with ApoE<sup>−/−</sup> BM–transplanted Ldlr<sup>−/−</sup> mice (Figure 4A and 4C). This reduced lesion size was associated with a 24% reduction in macrophage-containing area as assessed by staining with an F4/80 antibody (Figure 4B and 4D) while the percentages of macrophages per lesion size were similar in both groups (results not shown). Consistent with these findings, the lesions in these 2 groups were largely cellular with little necrotic core formation (Figure 4A). These findings support our hypothesis that reduced monocyte counts arising through CBS deficiency in ApoE<sup>−/−</sup> hematopoietic tissue leads to decreased macrophage accumulation in early atherogenesis.

After 9 weeks of WTD feeding, CBS deficiency did not reduce the overall atherosclerotic plaque size comparing ApoE<sup>−/−</sup>Cbs<sup>−/−</sup> BM–transplanted Ldlr<sup>−/−</sup> mice and ApoE<sup>−/−</sup> BM–transplanted mice (Figure 5A). However, there was a significant 36% reduction of macrophage content in the ApoE<sup>−/−</sup>Cbs<sup>−/−</sup> BM–transplanted mice (Figure 5B; Figure VA in the online-only Data Supplement), as well as a ~30% reduction in collagen content (Figure 5C; Figure VB in the online-only Data Supplement). On closer characterization of the lesions, we identified a significant increase of necrotic core area in the ApoE<sup>−/−</sup>Cbs<sup>−/−</sup> group (Figure 5D and 5E), suggesting that the potential atheroprotective effect of reduced monocyte and macrophage content of lesions might be counteracted by increased necrotic core formation.

In an effort to understand the mechanisms that might explain the lack of reduction of overall size of advanced lesions, we considered several potential hypotheses. First, because the CBS transduces signals mediated by IL-5 and IL-5 has been implicated in the production of potentially atheroprotective natural antibodies, such as the IgM E06/T15, we measured titers of these antibodies in plasma. The titers of antibodies were not reduced in ApoE<sup>−/−</sup>Cbs<sup>−/−</sup> BM compared with ApoE<sup>−/−</sup> BM–transplanted Ldlr<sup>−/−</sup> mice (Figure VI in the online-only Data Supplement), indicating that this mechanism could not explain the lack of decrease in lesion area in the ApoE<sup>−/−</sup>Cbs<sup>−/−</sup> BM–transplanted Ldlr<sup>−/−</sup> mice. GM-CSF is known to play a role in the development of DCs that activate regulatory T cells (Tregs), an immunosuppressive T-cell population that has been implicated in the production of potentially atheroprotective natural antibodies, such as the IgM E06/T15. We measured titers of these antibodies in plasma. The titers of antibodies were not reduced in ApoE<sup>−/−</sup>Cbs<sup>−/−</sup> BM compared with ApoE<sup>−/−</sup> BM–transplanted Ldlr<sup>−/−</sup> mice (Figure VI in the online-only Data Supplement), indicating that this mechanism could not explain the lack of decrease in lesion area in the ApoE<sup>−/−</sup>Cbs<sup>−/−</sup> BM–transplanted Ldlr<sup>−/−</sup> mice. GM-CSF is known to play a role in the development of DCs that activate regulatory T cells (Tregs), an immunosuppressive T-cell population that has been reported to attenuate atherosclerosis, likely through suppressing both T-cell– and macrophage-mediated inflammation and lesional MCP-1 expression. Therefore, we quantified the level of DCs and Tregs and found that the numbers of both cell types in spleens of ApoE<sup>−/−</sup>Cbs<sup>−/−</sup> BM compared with ApoE<sup>−/−</sup> BM–transplanted Ldlr<sup>−/−</sup> mice (Figure VIIA and VIIB in the online-only Data Supplement), presumably because of the decreased GM-CSF production as a result of reduced IRA B cells. Lymph node populations were unchanged (Figure VIIIC and VIID in the online-only Data Supplement) as expected. In an attempt to assess a possible
causative role of altered Tregs in lesions, we performed gene expression studies using laser capture microdissection analysis of the cellular area of lesions. There was a trend to a reduction of Foxp3 mRNA level in lesions from ApoE−/−Cbs−/− BM compared with ApoE−/− BM–transplanted Ldlr−/− mice (Figure VIIIA in the online-only Data Supplement), with the

Figure 4. Common β subunit of the granulocyte macrophage colony–stimulating factor/interleukin-3 receptor (CBS) deficiency reduces lesion size and macrophage-positive area in early atherosclerosis in bone marrow (BM) ApoE deficiency. Ldlr−/− mice were transplanted with ApoE−/− or ApoE−/−Cbs−/− BM and fed the Western-type diet for 4 weeks. Hearts were isolated and fixed, and paraffin sections of the aortic root were made. A, Representative hematoxylin and eosin (H&E) stained sections are shown. B, Sections were stained for macrophages using an F4/80 antibody. Representative F4/80-stained sections are shown. C, Quantification of atherosclerotic lesion area by morphometric analysis on H&E stained sections. D, Quantification of macrophage-positive area on F4/80 stained sections. Each data point represents a single mouse (n=12–16). *P<0.05, as determined by a Mann–Whitney test.

Figure 5. Common β subunit of the granulocyte macrophage colony–stimulating factor/interleukin-3 receptor (CBS) deficiency reduces macrophage-positive area and increases the size of necrotic cores without affecting atherosclerotic lesion size in advanced lesions in bone marrow (BM) ApoE deficiency. Ldlr−/− mice were transplanted with ApoE−/− or ApoE−/−Cbs−/− BM and fed the Western-type diet for 9 weeks. Hearts were isolated and fixed, and paraffin sections of the aortic root were made. A, Sections were stained with hematoxylin and eosin (H&E), and atherosclerotic lesion area was quantified by morphometric analysis (n=24–25). B, Sections were stained for F4/80, and macrophage-positive area was quantified (n=14–16). C, Sections were stained for Masson Trichrome, and collagen-positive area was quantified (n=8). D, Necrotic core area was quantified on H&E stained sections (n=13–16). E, Representative H&E sections containing necrotic cores are shown. Each data point represents a single mouse. *P<0.05, as determined by a Mann–Whitney test.
wide variation probably because of the scarcity of Tregs in lesions. However, this possible decrease in Foxp3 expression was not associated with an expected parallel decrease of Tgf-β and increase in Mcp-1 expression.20 On the contrary, Tgf-β level was significantly increased and Mcp-1 levels decreased in lesions from ApoE−/−Cbs−/− BM compared with ApoE−/− BM–transplanted Ldlr−/− mice (Figure VIIIB and VIIIC in the online-only Data Supplement). These effects are inconsistent with a major effect of lesional Treg content on lesion development in this model.

It has been shown that GM-CSF promotes Abcg1 expression by targeting Pparγ,31 and ABCG1 deficiency has been linked to increased macrophage apoptosis and increased necrotic core formation in lesions.32,33 Although ApoE−/− BM–transplanted Ldlr−/− mice had readily detectable Abcg1 expression, ApoE−/−Cbs−/− BM–transplanted Ldlr−/− mice demonstrated an absence of detectable Abcg1 mRNA in the lesions (Figure 6A), and this was associated with a decrease in Pparγ expression (Figure 6B), a ≈70% increase in the pro-apoptotic protein caspase 3 (Figure 6C and 6E), and a 3-fold increase in the number of apoptotic cells as determined by TUNEL staining (Figure 6D; Figure IX in the online-only Data Supplement).

Discussion

Previous studies have shown increased levels of the CBS associated with increased myeloid proliferative responses to GM-CSF and IL-3 in BM HSPCs of hypercholesterolemic mice with defective cellular cholesterol efflux pathways and also in the widely used ApoE−/− mouse model.15,16 However, the in vivo significance of increased cell surface CBS levels and signaling in monocytosis and atherosclerosis remained...
uncertain. Our study provides direct evidence that the CBS plays a key role in mediating the monocytes that develops in hypercholesterolemic ApoE−/− mice. In the BM and spleen, the CBS is required for the hypercholesterolemia-driven HSPC and myeloid expansion; in the spleen, the CBS also likely increases GM-CSF and IL-3 production by facilitating IRA B cell formation. IRA B cells account for ≈70% of the GM-CSF production in the spleen, suggesting that IRA B cells could contribute to enhanced myeloid proliferation and monocytosis in ApoE−/− BM–transplanted Ldlr−/− mice (Figure 6F). Moreover, CBS deficiency ameliorated plaque burden, including macrophage content during early atherogenesis. Unexpectedly, in more advanced atherosclerotic lesions, CBS deficiency decreased macrophage content but increased necrotic core areas, resulting in no net change of plaque area. This suggests that GM-CSF signaling via the CBS promotes atherosclerotic plaque stabilization in more advanced lesions.

BM-derived HSPCs are known to traffic through blood to extramedullary sites including the spleen where they proliferate and give rise to resident myeloid cells during both steady state conditions and inflammation. This phenomenon also occurs in animal models of atherosclerosis. Abca1−/− Abcg1−/− mice showed infiltration of HSPCs in the lung, spleen, and liver as a result of increased signaling through the IL-23/IL-17/G-CSF axis, and ApoE−/− mice developed splenomegaly with WTD feeding, reflecting enhanced HSPC proliferation and myelopoiesis in ApoE−/− BM–transplanted Ldlr−/− mice (Figure 6F). Moreover, CBS deficiency ameliorated plaque burden, including macrophage content during early atherogenesis. Unexpectedly, in more advanced atherosclerotic lesions, CBS deficiency decreased macrophage content but increased necrotic core areas, resulting in no net change of plaque area. This suggests that GM-CSF signaling via the CBS promotes atherosclerotic plaque stabilization in more advanced lesions.

Recent studies have highlighted the significance of the spleen as a site of extramedullary myeloproliferation and a source of monocytes in accelerated atherosclerosis after myocardial infarction. Our study shows a critical role of the CBS in facilitating extramedullary monocyte production through the development and expansion of IRA B cells, which likely contribute to myeloid proliferation in the spleen by producing GM-CSF and IL-3. Moreover, a decrease in IRA B cells was associated with reduced monocytosis and lesional macrophage burden, suggesting that these cells may contribute to atherogenesis in the ApoE−/− mouse model.

Recently, Hilgendorf et al also showed an increase in splenic IRA B cells in mouse atherosclerosis models. Based on an elegant chimeric BM transplantation approach that resulted in a specific depletion of GM-CSF–producing B cells, these authors suggested that GM-CSF–producing IRA B cells accelerate atherogenesis by increasing IL12p40-producing CD11b+ DCs in the spleen, leading to activation of CD4+ T cells and production of interferon γ, contributing to macrophage inflammation in lesions. Because we observed a decrease in splenic DCs in ApoE−/− Cbs−/− BM–transplanted Ldlr−/− mice, this mechanism may have also played a role in our study. However, Hilgendorf et al did not observe any effects on monocyte counts. Although this may seem discrepant with our data, Hilgendorf et al used ApoE−/− BM for transplantation, whereas our studies used ApoE−/− BM. IRA B cells are 4-fold expanded in ApoE−/− mice compared with Ldlr−/− mice. When we used ApoE−/− Cbs−/− BM for transplantation into Ldlr−/− mice, we also observed no effect on monocyte counts (Figure I in the online-only Data Supplement). We conclude that the marked expansion of IRA B cells probably contributes to the prominent monocytosis that develops in WTD-fed ApoE−/− BM–transplanted Ldlr−/− mice but not in WTD-fed Ldlr−/− mice where IRA B cell expansion and monocytosis are more subtle. The increase in CBS on HSPCs and IRA B cells may be brought out by defective cellular cholesterol efflux caused by BM ApoE deficiency. Overall, the increased blood monocytes in hypercholesterolemic mice with ApoE−/− hematopoietic tissue is likely caused by both increased CBS on HSPCs and on IRA B cells, driving expansion of both populations.

Studies in the past decade have firmly established a central role of monocyte-derived macrophages in the development of atherosclerotic lesions. Evidence has also emerged to paint a more complex picture of the pathogenesis with differing characteristics between early and advanced atherosclerotic lesions. Strikingly, in our model, the reduction of monocytosis had differential effects on early lesions and advanced lesions. In early lesions, the reduction of monocytes resulted in decreased macrophage content and decreased lesion size, consistent with the largely cellular nature of early lesions. Our findings are consistent with several earlier studies involving GM-CSF injection or deficiency in atherogenesis. With one exception, most of these studies have shown a proatherogenic role of GM-CSF, thought to be related to proliferation of CD11c+ dendritic-like cells in lesions. Many cells that stain for CD11c also stain for macrophage markers, consistent with our findings of decreased lesional macrophages.

In advanced atherosclerotic lesions, CBS deficiency did not decrease lesion size, despite a sustained reduction of blood monocyte counts. This was explained by increased necrotic core formation attributable at least in part to the lack of ABCG1 expression in the lesions. Prior studies have shown that GM-CSF induces macrophage Abcg1 expression mediated by PPARγ. It is known that ABCG1 is critical in sustaining macrophage survival likely by promoting cholesterol and oxysterol efflux, and lack of ABCG1 results in elevated cholesterol and oxysterol levels and enhanced apoptosis. Studies have established that macrophage death may reduce the volume of early atherosclerotic plaques. However, advanced lesions are characterized by defective efferocytosis in which debris from the apoptotic macrophages is not adequately removed, aggravating the inflammatory process in lesions. A recent study found that local macrophage proliferation, rather than monocyte recruitment, plays a major role in maintaining the macrophage content of established atherosclerotic lesions. However, a role for GM-CSF as a proliferative stimulus was specifically excluded in that report, indicating that such a mechanism was not likely involved in our study.

In summary, our study highlights the important role of increased levels of the CBS in mediating cholesterol-driven HSPC expansion in the BM, as well as extramedullary myeloid expansion via IRA B cells in the spleen. These processes contribute to monocytosis and plaque macrophage burden. However, the CBS also seems to play a role in cell survival, at least in part through effects on ABCG1 expression, and thus decreases necrotic core formation and increases collagen content in advanced atherosclerotic lesions. These observations
may have significant implications for the development of new anti-inflammatory therapies for autoimmune diseases such as rheumatoid arthritis or multiple sclerosis based on interruption of GM-CSF signaling. 53

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Disclosures
A.R. Tall is a consultant to Fitzar, Merck, Amgen, and CSL. The other authors report no conflicts.

References


**Significance**

Monocytosis has been associated with an increased cardiovascular risk in humans and increased atherosclerosis in mice. The common β subunit of the granulocyte macrophage colony-stimulating factor (GM-CSF)/interleukin-3 receptor is increased on hematopoietic stem and multipotential progenitor cells of several mouse models exhibiting monocytosis and accelerated atherosclerosis. We investigated the role of the common β subunit of the GM-CSF/interleukin-3 receptor in monocytosis and atherosclerosis in hypercholesterolemic mice containing *ApoE*⁻⁻ bone marrow. The common β subunit of the granulocyte GM-CSF/interleukin-3 receptor deficiency decreased monocytosis, bone marrow and splenic hematopoietic stem and multipotential progenitor cell proliferation, and splenic innate response activator B cells, which are the main source of splenic GM-CSF, resulting in decreased macrophage accumulation and lesion size in early atherosclerosis. In advanced atherosclerosis, common β subunit of the GM-CSF/interleukin-3 receptor deficiency decreased macrophage accumulation, but did not affect lesion size, associated with increased apoptosis, decreased collagen, and increased necrotic cores, suggesting decreased plaque stability. This reflected a decrease in macrophage ATP-binding cassette G1 expression, which is known to be increased by GM-CSF. These findings could have important implications for therapies aimed at disrupting the GM-CSF pathway, which are currently being developed for various autoimmune disorders in humans.
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Detailed Methods

Animals. Ldlr<sup>−/−</sup>(B6.129S7-Ldlr<sup>bm1Her</sup>), Apoe<sup>−/−</sup>(B6.129P2-Apoel<sup>tm1Unc</sup>), WT (C57BL/6J) and Cbs<sup>−/−</sup> (B6.129S1-Csf2rb<sup>2m1Cgb</sup> Csf2rb<sup>2m1Cisc/J</sup>) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Apoe<sup>−/−</sup> and Cbs<sup>−/−</sup> mice were intercrossed to obtain Apoe<sup>−/−</sup>Cbs<sup>−/−</sup> and Apoe<sup>−/−</sup> littermates. Apoe<sup>−/−</sup> or Apoe<sup>−/−</sup>Cbs<sup>−/−</sup> BM was transplanted into female Ldlr<sup>−/−</sup> recipient mice per genotype using a protocol described in previous studies. Five weeks after BM transplantation, recipient mice were fed a WTD (21% milk fat, 0.2% cholesterol; TD88137, Harlan Teklad) for up to 9 weeks. Animals had ad libitum access to food and water. All protocols were approved by the Institutional Animal Care and Use Committee of Columbia University.

White Blood Cell Counts. Blood was drawn freshly via tail bleeding and white blood cell (WBC) as well as monocyte and neutrophil cell counts were determined using the FORCYTE Veterinary Analyzer (Oxford Science, Inc).

Total Plasma Cholesterol levels. Blood was collected through tail vein bleeding followed by centrifugation for 10 minutes at 10,000 x g using a refrigerated centrifuge. Plasma cholesterol levels were measured using the Cholesterol E kit (Wako Diagnostics) as per the manufacturer's instructions.

Flow Cytometry – Bone marrow and spleen HSPCs. Bone marrow (BM) was harvested by flushing the femurs and tibias with ice-cold PBS. Spleens were crushed on a cell strainer on a 50 ml Falcon tube to release the cells into ice-cold PBS. BM or spleen cells were then incubated with BD Pharm Lyse on ice for 5 min, followed by centrifugation to remove lysed red blood cells (RBCs). The remaining cells were then resuspended in HBSS (0.1% BSA w/v, 5mM EDTA) and incubated with a cocktail of antibodies to lineage committed cells (CD45R, CD19, CD11b, CD3e, TER-119, CD2, CD8, CD4, Ly-6G: All FITC, eBioscience), Sca1-Pacific Blue, and cKit-APC Cy7. For the further identification of progenitor cells, antibodies to CD16/CD32 (FcyRII/III) and CD34 were used. HSPCs were identified as Lin<sup>−</sup>Sca1<sup>−</sup>cKit<sup>−</sup>, CMPs were identified as Lin<sup>−</sup>Sca1<sup>−</sup>cKit<sup>−</sup>CD34<sup>int</sup>FcyRII/III<sup>int</sup>, and GMPs were identified as Lin<sup>−</sup>Sca1<sup>−</sup>cKit<sup>−</sup>CD34<sup>hi</sup>FcyRII/III<sup>hi</sup>. LSK HSPCs were identified as Lin<sup>−</sup>, Sca1<sup>−</sup> and cKit<sup>+</sup>.<sup>2,4</sup> To assess the proliferation activity of HSPCs and progenitor cells, we permeabilized the cells by incubating them with BD CytoFix/CytoPerm (554714) buffer followed by washing with ice-cold Perm/Wash buffer. The cells were then suspended in HBSS containing 3µM of DAPI (4’,6-Diamidino-2-Phenylindole, Dihydrochloride, Invitrogen). Stained cells were analyzed using BD LSRII running FACSDiVa software and results were analyzed using FlowJo software.

Flow Cytometry – BM and spleen IRA B cells. BM and spleen leukocytes were collected as described in the section above. These cells were incubated with the following antibodies for 30 minutes on ice: IgM-APC, CD23-PE Cy7, and CD19-APC Cy7. Cells were washed and permeabilized as described above and incubated in Perm/Wash buffer containing GM-CSF-PE antibody for 30 minutes on ice, followed by resuspension in HBSS containing 3µM of DAPI. Stained cells were analyzed using BD LSRII running FACSDiVa software and IRA B cells were identified as IgM<sup>hi</sup>CD19<sup>−</sup>CD23<sup>low</sup>GM-CSF<sup>+</sup>. In the settings where cells surface CBS was measured, CBS-PE antibody was included in the incubation before permeabilization of the cells, and a GM-CSF-FITC antibody was used after the permeabilization. Results were analyzed using FlowJo software.

Flow Cytometry – Peritoneal B1a cells. Peritoneal leukocytes were collected by lavage of the peritoneal cavity with ice-cold PBS. After centrifugation, the cells were resuspended in BD PharmLyse buffer to remove RBCs. The cells were then stained in HBSS buffer with the
following antibodies: B220-FITC, IgM-APC, CD5-Per CP, CD11b-APC Cy7, and CBS-PE. Peritoneal B1a cells were identified as B220^IgM^CD5^CD11b^.

**Flow cytometry – Tregs and DCs in the spleen and lymph nodes.** Tregs and DCs were isolated from the iliac lymph nodes and the spleen. Briefly, lymph nodes or spleens were diced and digested in 30% Collagenase (Sigma C2674) in PBS at 37°C. The digested tissue was then passed through a syringe for several times and filtered through a 40 µm cell strainer. The cells were collected by centrifugation followed by removal of RBCs with BD Pharm Lyse. WBCs were then collected, washed, and resuspended in HBSS containing the appropriate antibodies. The antibodies used for Treg surface staining were: CD4-FITC and CD25-AP. After staining, cells were permeabilized as described above, followed by staining with FoxP3-PE antibody. Tregs were identified as CD4^+CD25^+FoxP3^+. In a separate preparation, WBCs from the spleen and lymph nodes were stained using the following antibodies: CD45-APC Cy7, MHC-II-PE, CD11c-A700. DCs were identified as CD45^+MHC-II^+CD11c^+. The samples were examined on a BD LSR II flow cytometer and data were analyzed using FlowJo software.

**Immunohistochemistry of atherosclerotic lesions.** Following euthanasia, the heart of each mouse was perfused with ice-cold PBS and fixed in formalin. Hearts were embedded in paraffin and the aortic root was sectioned at 5-µm intervals, and 6 sections of the same order from each mouse were then stained with H&E to assess overall tissue morphology.

H&E stained sections were also used for assessment of the necrotic cores as described previously.  Briefly, necrotic core area was determined as acellular area under the fibrous cap of lesions. Area was determined as the acellular area, lacking nuclei and cytoplasm, from H&E-stained sections. Necrotic core area was differentiated from regions of dense fibrous scars by the presence of macrophage debris.

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For the quantification of macrophage content within the lesions, sections were stained for F4/80, followed by Harris Hematoxylin counterstain. To determine collagen content, a Masson Trichrome Staining was performed. For caspase 3 staining, sections were stained for cleaved caspase 3 using an antibody from Cell Signaling.

Apoptotic cells in atherosclerotic lesions were detected by the TUNEL (TdT-mediated dUTP nick end labeling) technique using the in situ cell death detection kit, TMR red (Roche). For this staining, frozen sections were used that were collected after the heart was embedded in OCT. Sections of the aortic root were washed with buffer (PBS containing 0.2% Tween-20), incubated in 0.3% H_2O_2 for 10 min, and rinsed. For the TdT reaction, the sections were incubated in TdT reaction mixture containing TMR red dUTP for 2 hr at 37°C in a humidified chamber. After washing, genomic DNA was stained with DAPI for 1 min at room temperature and then the slides were mounted with coverslips. Images were captured and quantified using Olympus IX 70 fluorescence microscope.

**Measurement of E06 antibody titers.** Chemiluminescent enzyme immunoassay was used as previously described.  In brief, plates were coated with monoclonal anti-T15/E06–idiotypic antibody AB1-2 in PBS (5 µg/ml), overnight at 4°C. After washing and blocking steps, 50µl serially diluted antisera from pooled plasma (n=5-6) were added, and incubated for 1.5h at room temperature. Bound E06 antibodies levels were detected using AP-conjugated goat anti-mouse IgM (Sigma) and a 50% aqueous solution of Lumiphos 530 (Lumigen, USA). Data are expressed as relative light units counted per 100 milliseconds (RLU/100 ms). A standard curve was constructed with purified E06 (Avanti).

**Laser Capture Microdissection and Q-PCR.** After euthanizing each mouse, the heart and the arterial tree were perfused with 10mL of ice-cold PBS. The hearts were embedded in OCT and flash-frozen on dry ice. Using a cryomicrotome, sections were cut serially at 8-µm intervals,
generating 25 sections that spanned the entire aortic root, and mounted on slides. For comparison of lesional mRNA expression between the groups, the sections of the same order were used for each mouse. Atherosclerotic lesions were visualized and selectively captured using a PALM laser capture microdissection (LCM) machine. RNA was isolated using the RNeasy Micro Kit (Qiagen). Linear amplification of the RNA was performed using the MessageAmp II aRNA Kit (Ambion). The purity of the obtained RNA was determined using a NanoDrop machine (Thermo Scientific). RNA with an A260/280 of >1.8 was used for cDNA synthesis. cDNA conversion of the amplified RNA was performed using the SuperScript VILO cDNA Synthesis Kit (Invitrogen). qPCR was performed in an 7500 Real-Time PCR system (Applied Biosystems) using SYBR Green.

**Statistical Analysis.** Statistical analysis was performed using 2-tailed parametric Student’s t-test. For atherosclerosis studies, the Mann Whitney test was used. Values are expressed as mean ± SEM. A P value of less than 0.05 was considered significant.

**References**

Supplemental Figure I. CBS bone marrow deficiency in \textit{Ldlr}^- mice did not affect blood monocyte levels. \textit{Ldlr}^- mice were transplanted with \textit{Cbs}^- BM, and fed the WTD. At 5 weeks post-BM transplantation (presented as baseline), mice were fed the WTD. Blood monocytes were monitored at baseline, 5 and 8 weeks of WTD feeding. N=6.
ApoE<sup>−/−</sup> bone marrow → Ldlr<sup>−/−</sup> mice

ApoE<sup>−/−</sup>/Cbs<sup>−/−</sup> bone marrow → Ldlr<sup>−/−</sup> mice
Supplemental Figure II. Role of CBS in the proliferation of BM and spleen HSPC, CMP, and GMP. Ldlr−/− mice were transplanted with Apoe−/− or Apoe−/−Cbs−/− BM, and fed the WTD for 9 weeks. BM and spleen cells were isolated. HSPC, CMP, and GMP were identified using flow cytometry, and DAPI staining was performed to assess their proliferative activity. A. Representative flow cytometry results for the identification of proliferating HSPCs and GMPs in the BM. B and C. Percentages of cells in the S/G2M phase in the BM (B) and spleen (C). N=9-10. *P<0.05, **P<0.01.
Supplemental Figure III. CBS deficiency reduced spleen weight in Apoe<sup>+/−</sup> BM transplanted Ldlr<sup>−/−</sup> mice. Ldlr<sup>−/−</sup> mice were transplanted with Apoe<sup>+/−</sup> or Apoe<sup>+/−</sup>Cbs<sup>−/−</sup> BM, and fed the WTD for 9 weeks. Spleens were isolated and weighed. N=9-10. *P<0.05.
Supplemental Figure IV

A  IRA B cells in the spleen

WT

ApoE−/−

B  IRA B cells in the bone marrow

WT

ApoE−/−
Supplemental Figure IV. Characterization of spleen and BM IRA B cell content and peritoneal B1a cells in Apoe<sup>-/-</sup> mice. WT or Apoe<sup>-/-</sup> mice were fed WTD for 5 weeks and spleen and BM IRA B cell content, and peritoneal B1a cell content, proliferation and CBS surface expression were assessed using flow cytometry. A-B. Gating of the IRA B cell population is shown for spleen (A) and BM (B) cells. C. Percentage of BM IRA B cells. D. BM IRA B cell proliferation as assessed after DAPI staining. E-F. Representative flow cytometry plots of cell surface CBS expression in spleen IRA B cells (E) and peritoneal B1a cells (F). G. Percentage of peritoneal B1a cells. Results are shown as mean±SEM. N=5.
Supplemental Figure V. CBS deficiency reduced macrophage-positive lesion area and increased necrotic cores in advanced atherosclerotic lesions. *Ldlr<sup>−/−</sup>* mice were transplanted with *Apoe<sup>−/−</sup>* or *Apoe<sup>−/−</sup>Cbs<sup>−/−</sup> BM and fed the WTD for 9 weeks. Hearts were isolated and fixed, and paraffin sections of the aortic root were made. **A.** Sections were stained for macrophages using an F4/80 antibody. Representative F4/80-stained sections are shown. **B.** Sections were stained for Masson-Trichrome to assess collagen positive area (blue). Only the collagen area in the plaque was measured. Representative Masson-Trichrome stained sections are shown.
Supplemental Figure VI. CBS deficiency does not reduce atheroprotective IgM production in Apoe<sup>−/−</sup> BM transplanted Ldlr<sup>−/−</sup> mice. Ldlr<sup>−/−</sup> mice were transplanted with BM of specified genotype followed by 9 weeks of WTD feeding. Dilution curves of serum IgM binding to the monoclonal anti-T15/EO6 antibody were produced by pooling 5-6 mice from each group.
Supplemental Figure VII. BM CBS deficiency causes moderate decreases in DCs and Tregs in spleen but not lymph nodes. Ldlr\(^{-}\) mice were transplanted with BM of specified genotype followed by 9 weeks of WTD feeding. DCs in spleen (A) and lymph nodes (C) were identified as CD45\(^{+}\)MHC-II\(^{+}\)CD11c\(^{+}\). Tregs (CD4\(^{+}\)CD25\(^{+}\)FoxP3\(^{+}\)) from spleen (B) and lymph nodes (D) are expressed as a percentage of total CD4\(^{+}\) T cells. N=5. *P<0.01.
Supplemental Figure VIII. BM CBS deficiency on the Apoe<sup>-/-</sup> background reduced Foxp3 and Mcp-1 mRNA, while it increased Tgf-β mRNA in atherosclerotic lesions. Ldlr<sup>-/-</sup> mice transplanted with BM of specified genotype were fed with WTD for 9 weeks, and frozen sections of aortic root were prepared. RNA was extracted from laser-captured macrophages from atherosclerotic lesions, and Foxp3 (A), Mcp-1 (B), and Tgf-β mRNA (C) were assessed. A.U., arbitrary units. N=10. *P<0.05.
Supplemental Figure IX. BM CBS deficiency increased apoptosis in advanced atherosclerotic lesions. *Ldlr*−/− mice transplanted with BM of specified genotype were fed WTD for 9 weeks, and paraffin and frozen sections of aortic roots were prepared. TUNEL staining was performed on frozen sections to identify apoptotic cells. Representative images are shown.