Sex-Dependent Attenuation of Plaque Growth After Treatment With Bone Marrow Mononuclear Cells

Wendy D. Nelson,* Andrey G. Zenovich,* Harald C. Ott, Craig Stolen, Gabriel J. Caron, Angela Panoskaltsis-Mortari, Samuel A. Barnes III, Xiangrong Xin, Doris A. Taylor

Abstract—There are clinically relevant differences in symptomatology, risk stratification, and efficacy of therapies between men and women with coronary artery disease. Sex-based differences in plaque attenuation after administration of bone marrow mononuclear cells (BMNCs) are unknown. Forty-five male and 57 female apolipoprotein-E knockout (apoE−/−) mice were fed a high-fat diet. At 14 weeks of age, animals received 4 biweekly intravenous sex-matched (males, n=11; females, n=13) or -mismatched (males, n=12; females, n=14) BMNCs obtained from C57BL/6J mice. The rest of the apoE−/− mice were vehicle treated (males, n=13; females, n=20) or were age-matched untreated controls (males, n=9; females, n=10). Aortic plaque burden, progenitor cell profiles in bone marrow (BM) and 22 circulating cytokines/chemokines were examined 1 week following the final injection. Only female BMNCs infused into male apoE−/− recipients significantly decreased plaque formation (P<0.001). This reparative response univariately correlated with increased CD34+/CD45+ (P=0.02), CD45+ (P=0.0001), and AC133+/CD34+ (P<0.001) cell percentages in the BM of recipients but not with total serum cholesterol or percentage of BM-CD31+/CD45− cells. In a multivariate analysis, BM-AC133+/CD34+ and BM-CD45+ percentage counts correlated with a lower plaque burden (P<0.05). Increased granulocyte colony-stimulating factor levels highly correlated with plaque attenuation (r=−0.86, P=0.0004). In untreated apoE−/− mice of either sex, BM-AC133+/CD34+ cells rose initially and then fell as plaque accumulated; however, BM-AC133+/CD34+ percentages were higher in females at all times (P=0.01). We have demonstrated an atheroprotective effect of female-derived BMNCs administered to male atherosclerotic apoE−/− mice; this reparative response correlated with the upregulation of BM-AC133+/CD34+ and CD45+ cells and of circulating granulocyte colony-stimulating factor. Atherosclerotic female apoE−/− mice did not exhibit atheroprotection after BMNCs of either sex. Our findings may have implications for clinical cell therapy trials for coronary artery disease. Further exploration of sex-based differences in atheroprotection and vascular repair is warranted. (Circ Res. 2007;101:1319-1327.)

Key Words: atherosclerosis ■ bone marrow ■ cytokines ■ gender ■ stem cells

Coronary artery disease (CAD) is the leading cause of death in the industrialized world.1 CAD in men and women represents enormously different clinical challenges attributable to a sex variation in symptomatology, risk stratification, and efficacy of therapies.2 Current treatment targets the existing plaque lesion, via angioplasty, stents, and lipid-lowering agents.2 However, promoting repair to inhibit plaque formation could be more optimal for vascular health. We showed that administration of mononuclear (combined hematopoietic- and stromal-derived) bone marrow (BM) fractions from “young” nonatherosclerotic apolipoprotein-E knockout (apoE−/−) mice or from older C57BL/6 mice attenuated plaque growth in middle-aged apoE−/− mice.3 The BM mononuclear cells (BMNCs) homed to the plaque lesions, suggesting cell-mediated repair of the vessel wall. In apoE−/− mice, a subpopulation of BM CD31+/CD45− vascular progenitor cells (VPCs) diminished with age, which correlated with absence of atheroprotection.3 More recently, higher endothelial progenitor cell (EPC) counts and activity have been inversely correlated with severity of CAD, so that for every increase of 10 EPC colony-forming units, the likelihood of CAD declined by 20%.4 These data suggest that BM EPCs and VPCs may be capable of vascular repair. The impact of donor or recipient sex on BMNC-mediated atheroprotection is unknown. Burke et al showed a higher predominance of plaque erosion in younger women,5 which

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suggests that atheroprotection may have sex-based differences. We investigated the role of sex on vascular repair by examining differences in the capacity of exogenous BMNCs to reduce plaque formation in a sex-matched and -mismatched apoE/H11002/H11002 mice fed a high-fat diet.

**Materials and Methods**

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org. A schematic of the experimental protocol is illustrated in Figure 1.

**Definition of Progenitor Cell Populations**

Because there are variations in the definitions of progenitor cell populations in the literature, in this study, we a priori defined BM-derived EPCs as AC133+/CD34− cells, VPCs as CD31+/CD45low cells, and the BM inflammatory cell–containing fraction as the CD45− subpopulation. BMNCs injected into male and female apoE/H11002/H11002 recipients were derived from wild-type C57BL6/J mice (10−2 weeks of age) and maintained in vitro for 48 hours, as described in the online data supplement.

**Statistical Analysis**

A complete description of statistical analysis procedures appears in the online data supplement. In all analyses, statistical significance was set at P≤0.05.

**Results**

Sex, Aging, Atherosclerosis, and Bone Marrow Progenitor Cells

Atherosclerotic burden (Figure 2A) increased with age in untreated male and female apoE/H11002−/− mice fed a high-fat diet. Male apoE/H11002−/− mice exhibited a significantly greater plaque burden earlier (between 14 and 21 weeks) than female mice (P=0.01). In contrast, in female mice, atherosclerosis accelerated later (ie, between 21 and 32 weeks of age) and resulted in a significantly greater plaque burden versus males (P=0.04) at 32 weeks. BM progenitor cell populations changed over the same time course.

In both sexes, reductions in BM-CD34+ cells occurred as atherosclerosis progressed (Figure 2B). In males, percentage cell counts fell in parallel with plaque growth. In females, the reduction of the BM-CD34+ cell percentage occurred 7 weeks prior to the highest plaque burden accumulation; the decline in cell counts was smaller than in male mice of a similar (21 weeks) age (P=0.01). By 32 weeks of age, the BM-CD34+ cell percentages were similar in both sexes.
In male apoE<sup>−/−</sup> mice, BM-VPCs demonstrated a continuous linear fall as plaque accumulated in the vessels (Figure 2C). On the contrary, in females, VPC percentage counts were stable through week 21, even though atherosclerosis progressed, but as plaque burden further increased, BM-VPCs fell significantly.

In both males and females, BM-EPCs exhibited a distinct pattern compared with other BM progenitors (Figure 2D). In contrast to depletion of CD34<sup>+</sup> and VPCs in parallel to plaque buildup, BM-EPCs rose significantly at 21 weeks but then ultimately fell as the atherosclerotic process continued. In female mice, where plaque accumulated significantly slower through week 21 compared with males, BM-EPC percentage counts were higher (P<0.001); conversely, the BM-EPCs declined less at 32 weeks than in male mice (P=0.01), even though female atherosclerotic plaque burden was significantly higher.

**Sex-Dependent Attenuation of Atherosclerotic Plaque Formation by BMNCs**

After donor BMNCs were maintained in vitro for 48 hours, relative EPC number increased, with females exhibiting significantly more EPCs than males (11.1±1.4% [n=3] vs 7.4±1.3% [n=3]; P=0.04). However, VPC percentage fell in both sexes (females: from 19.8±4.9% to 5.8±0.6%, n=3; males: from 18.6±3.5% to 4.1±2.3%, n=3; both P=0.01, paired t test). Similar to EPCs, the VPC percentages were significantly higher in samples derived from female donors (P=0.04).

When BMNCs were administered to apoE<sup>−/−</sup> recipients, a significant reduction in aortic plaque burden was observed in male mice that received female donor BMNCs compared with vehicle-treated and male donor BMNCs (Figure 3A and 3B). No significant changes in plaque burden were observed in female apoE<sup>−/−</sup> recipients irrespective of donor sex (Figure 3B).
Sex and Effects of BMNC Treatment

Exogenous Bone Marrow Progenitors Alter Recipient Bone Marrow

The relative percentages of CD34⁺ cells, EPCs, VPCs, and CD45⁺ cells in the BM of recipients changed after BMNC administration in a sex-dependent fashion. In male recipients, treatment with BMNCs of either sex boosted the percentage of CD34⁺ cells (Figure 3C) and EPCs (Figure 3F) in BM to levels similar to those in treated or untreated females (all \(P\)/NS). These equal increases after BMNC therapy occurred despite the differences in the percentages of EPCs in male and female donor samples. In female recipients, the treatment did not change percentages of BM-CD34⁺ or BM-EPC cells.

Mean levels of BM-VPCs in male and female apoE⁻/⁻ mice were similar after vehicle treatment (Figure 3D). Following BMNC infusions, female recipient mice had higher BM-VPC counts after receiving either male (\(P=0.02\)) or female cells (\(P=0.005\)) versus male recipients.

The inflammatory cell–containing BM-CD45⁺ fraction significantly increased only in male apoE⁻/⁻ mice treated with female BMNCs (\(P=0.04\); Figure 3E): the group of animals that exhibited attenuation of atherosclerotic plaque formation. BM-CD45⁺ counts were unchanged in all other animals.

Cholesterol

Vehicle-treated animals of either sex showed hyperlipidemia appropriate for apoE⁻/⁻ mice fed a high-fat diet (males: 1175±60 mg/dL, \(n=13\); females: 992±93 mg/dL, \(n=20\), \(P=0.1\)). Total serum cholesterol was unchanged in male or female recipients of female BMNCs (males: 1104±74 mg/dL, \(n=12\), \(P=0.45\) versus vehicle; females: 944±72 mg/dL, \(n=13\), \(P=0.3\) versus vehicle). In contrast, total cholesterol concentrations were decreased in males that received male BMNCs (932±40 mg/dL, \(n=11\), \(P=0.01\) versus vehicle) but the lower total cholesterol did not translate into decreased plaque burden. Females treated with male BMNCs showed a
similar trend toward lower cholesterol (853 ± 59 mg/dL, n = 14), although the difference did not reach statistical significance (P = 0.15 versus vehicle).

**Estriol**

After donor BMNCs were maintained in vitro for 48 hours, estriol levels measured in the media from female cells was significantly higher compared with male cells (117.5 ± 64.6 versus 36.3 ± 8.4 pg/mL, P = 0.05; cell-free media control: 13.6 ± 3.5 pg/mL, n = 3 each).

Pooled estriol levels in vehicle-treated male and female apoE−/− mice were similar (2880 and 2565 pg/mL; odds ratio [OR] = 1.12; 95% confidence interval [CI], 0.64 to 2.00; P = 0.4). Administration of BMNCs into male apoE−/− mice increased estriol levels (male BMNCs: 12.397 pg/mL, OR = 4.3, 95% CI, 2.77 to 7.06, P = 0.003 versus vehicle; female BMNCs: 7180 pg/mL, OR = 2.49, 95% CI, 1.51 to 4.27, P = 0.03 versus vehicle). No difference could be detected between males that received male or female BMNCs (OR = 1.40, 95% CI, 0.94 to 2.13, P = 0.35).

Infusions of BMNCs into female apoE−/− mice likewise boosted circulating estriol (male BMNCs: 8840 pg/mL, OR = 3.45, 95% CI, 2.27 to 5.14, P = 0.006 versus vehicle; female BMNCs: 9928 pg/mL, OR = 7.50, 95% CI, 5.31 to 10.71, P = 0.0006 versus vehicle). Significantly more circulating estriol was measured after female BMNCs were administered (OR = 2.18, 95% CI, 1.50 to 3.24, P = 0.04 versus male cells). Recipient estriol levels were not associated with either total cholesterol or plaque burden in any combinations of donor and recipient sex (all G² ≤ 0.03; all P = NS).

**Table 1. Results of Univariate and Multivariate Regression Analyses of Effects of BM Progenitor Cell Profiles and Total Serum Cholesterol on Atherosclerotic Plaque Burden in Male apoE−/− Mice That Exhibited Plaque Attenuation After BMNC Therapy**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate Regression, R², P</th>
<th>Multivariate Regression, B Coefficient ± SEM, P</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM CD34+ fraction</td>
<td>0.29, 0.02</td>
<td>−0.36 ± 0.24, 0.15</td>
</tr>
<tr>
<td>BM EPCs (AC133+/CD45−)</td>
<td>0.32, 0.001</td>
<td>−0.80 ± 0.35, 0.04</td>
</tr>
<tr>
<td>BM VPCs (CD31+/CD45low)</td>
<td>0.05, 0.19</td>
<td>...</td>
</tr>
<tr>
<td>BM CD45+ fraction</td>
<td>0.43, 0.0001</td>
<td>−0.47 ± 0.19, 0.03</td>
</tr>
<tr>
<td>Total serum cholesterol</td>
<td>0.03, 0.38</td>
<td>...</td>
</tr>
</tbody>
</table>

**Multivariate regression ANOVA: P = 0.02.**

Female mice were not subjected to univariate or multivariate analyses because of the absence of significant differences in plaque burden after BMNC administration (Figure 3B).

**Changes in Cytokines/Chemokines With BMNC Treatment**

After donor BMNCs were maintained in vitro for 48 hours, expression of T-helper (Th)1-type (proinflammatory) chemokine tumor necrosis factor (TNF)-α was similar in the media from cells of either sex, but Th2-type (anti-inflammatory) cytokines interleukin (IL)-4 and IL-5, and the pleiotropic/regulatory cytokine IL-6 were present in 1.9-to 3.0-fold higher concentrations in female cell media versus male cell or cell-free media (data not shown). Granulocyte colony-stimulating factor (G-CSF) and IL-15 were detected only in female BMNC media, whereas male BMNC and cell-free media concentrations were equivalent (G-CSF: 23.9 ± 8.4 pg/mL in female BMNCs and 6.3 ± 5.8 pg/mL in male BMNCs, P = 0.02; IL-15: 34.0 ± 35.2 pg/mL in female BMNCs and undetected in male BMNCs; n = 3 each). None of the other 16 cytokines was detected.

BMNC treatment created an inflammatory response in apoE−/− mice; the type and the magnitude of the effect varied by sex. In male apoE−/− mice, treatment with either male or female BMNCs increased IL-1β, IL-12, TNF-α, and Regulated on Activation, Normal T-Cell Expressed and Secreted, reflecting a Th1-type proinflammatory response (Table I in the online data supplement). Increases of these cytokines/chemokines were numerically larger in male mice treated with female BMNCs: the group of animals that showed attenuation of plaque formation. Along with the increase in Th1-type mediators, Th2-type cytokines (IL-5, IL-10, IL-13, and, to a lesser degree, monocyte chemoattractant protein-1) levels rose as well; larger numerical increases in Th2-type cytokines occurred in male mice with reduced plaque burden. Upregulation of Th1- and Th2-type cytokines paralleled the increase in BM-CD45+ cell fraction in these mice (Figure 3E). In female apoE−/− mice, fewer Th1- and more Th2-related cytokines were upregulated after treatment with either male or female BMNCs (supplemental Table I) but without atheroprotection (Figure 3B).

Of the 22 cytokines/chemokines quantified in male apoE−/− mice treated with female BMNCs, increased G-CSF exhibited the strongest correlation with plaque attenuation; increased Th1- and Th2-type cytokines exhibited correlations with borderline statistical significance (Table 2). As levels of G-CSF increased in mice with attenuated plaque, higher levels of IL-15 and KC (IL-8) were observed (supplemental Figure I). Although median levels of Th2-type cytokines increased after treatment with female BMNCs (supplemental Table I), individual values inversely correlated with G-CSF (supplemental Figure I). The correlations between G-CSF and IL-1β, IL-12, Regulated on Activation, Normal T-Cell Expressed and Secreted, IL-17, and IL-5 were numerically lower in males treated with female (versus male) BMNCs (supplemental Figure I), with the changes approaching statistical significance (data not shown). G-CSF moderately correlated with interferon-γ (but not with interferon-γ inducible...
protein 10) after administration of female BMNCs to male apoE−/− mice (supplemental Figure I).

Median G-CSF levels were higher in vehicle-treated females compared with males and did not increase after either male or female BMNCs (supplemental Table I). G-CSF and IL-15 (but not KC) moderately correlated in vehicle-treated females (but not males) and also in female apoE−/− mice after treatment with female BMNCs (supplemental Figure I).

### Discussion

In this study, we have shown that BMNC-mediated atheroprotection differs in apoE−/− male and female mice fed a high-fat diet and that the difference may be, in part, related to varying degrees of endogenous repair already underway in animals of each sex and supplemented by differences in the response to male- and female-derived BMNC populations. Atherosprotective capabilities of BMNCs were observed only when cells from wild-type female mice were administered to atherosclerotic apoE−/− males. In the context of the natural history of murine atherosclerosis, female BMNCs reduced plaque burden by ≈40%. Of note, a recent large clinical trial of CAD patients demonstrated only a 7% reduction in plaque volume with rosuvastatin. Therefore, atherosoprotection with BMNCs holds an enormous promise, especially considering that 16 million Americans suffer from CAD and that 1 of every 2.7 deaths is attributed to atherosclerosis. A considerable amount of work needs to be done before BMNC treatment of CAD becomes a clinical reality. In addition to discerning the mechanism of atheroprotection, we need to understand why the benefit in male recipients was confined to female BMNCs and why female recipients appeared indifferent to BMNCs.

We attempted to dissect the sex-based differences in the progression of atherosclerosis and the changes in the relative numbers of endogenous BM progenitor cells. In addition, we sought to begin to understand the mechanistic impact of exogenous BMNC administration after repair by assessing 22 circulating cytokine/chemokines. Our data offered some understanding of the interactions that mediate vascular repair while uncovering notable sex differences. Specifically, the speed of progression of atherosclerosis and the amount of plaque accumulated differed in male and female apoE−/− mice. Changes in BM progenitors accompanied disease progression in a sex-dependent fashion, which suggested differences in endogenous repair.

Male and female animals responded differently to BMNCs at the level of the BM and the vessel. BMNCs administered to male and female apoE−/− mice boosted BM-EPC counts, ≈2-fold, to levels indistinguishable from either treated or untreated females. Yet female donor BMNCs contained significantly more EPCs, and their delivery attenuated plaque formation. This result suggests that rather than simply traveling to the BM, the “excess” EPCs participated in vascular repair. Our multivariate analysis, which showed the correlation of reduced plaque burden with the percentages of EPCs delivered, supports this supposition. Combined with our previous observations that exogenous cells travel to the sites of injury,3 the data from this study suggest that BMNCs act locally as well as at the level of BM. However, the outcome of treating male apoE−/− mice with male BMNCs, where BM-EPCs increased but plaque formation was not attenuated, suggests that the BM effect alone is insufficient for repair. Therefore, the dose of EPCs delivered may be a critical component of success of atheroprotection. If so, reducing plaque with male BMNCs may be as simple as administering a higher percentage of EPCs. However, male animals that received female cells exhibited attenuation of plaque formation. Because the sex-based difference in the in vitro and in vivo hormonal and inflammatory milieu may be responsible for the differences in the reparative capacity of exogenous cells, infusing higher numerical counts of EPCs may not suffice. Dose–response studies combined with in vitro functional assays of male and female cells are required to clarify whether female EPCs are truly more “reparative” or whether the number of EPCs delivered determines atheroprotection.

In treated animals, an increase in “inflammation” was registered, together with a lower plaque burden. Specifically, upregulation of CD45+ cell–containing BM fraction (which contains precursors of granulocytes, monocytes, B cells, etc) correlated with repair. Even though inflammation is observed with progression of atherosclerosis,3 in our study, it was associated with repair. As indicated previously,3 we propose that initial inflammation is a “positive” signal to trigger recruitment of endogenous BM progenitors. When cells capable of repair are recruited in sufficient numbers, repair ensues and inflammation decreases. If insufficient numbers of and/or cells functionally incapable of repair are recruited, the repair process stops and inflammation increases and becomes a “negative” signal to the detriment of the tissue. Our data, as well as that of 2 recent studies, support this hypothesis. Veillard et al11 demonstrate that inflammation surges between weeks 4 and 10 of high-fat diet in apoE−/− mice, but then acute phase response transforms into chronic inflammation as atherosclerosis progresses. Inoue et al12 provide evidence that local arterial inflammation serves as a signal for the release of...
BM progenitors after stent deployment to promote the healing cascade within the vessel.

Increased G-CSF levels correlated with the reparative response. In addition, hematopoietic cytokines IL-15 and KC (IL-8) correlated with the G-CSF increase. IL-8 has been shown to mediate progenitor cell migration. IL-15 stimulates proliferation of dendritic T cells, and there is evidence of regulatory CD4+/CD25– T-cell engagement by IL-15. Caux et al. have shown that dendritic T cells are derived from CD34+ progenitors in presence of TNF-α. Our animals exhibited increased BM-CD34+ cells and circulating TNF-α, together with reduced plaque burden. Therefore, it is tempting to hypothesize that 2 processes are required for attenuation of plaque: (1) inflammation, to mobilize the necessary BM progenitors and to trigger digestion of lesions by macrophages; and (2) vessel repair, which includes engraftment of EPCs to renew the activated endothelium, and a decrease in smooth muscle activation and in attachment of lipid particles. Both processes have been shown to involve G-CSF. Higher G-CSF levels in vehicle-treated female mice, where plaque growth was stable, support its role in endogenous repair. Whether G-CSF is the conductor of the repair process and other cytokines/chemokines are the orchestra remains to be confirmed. Although G-CSF reduced left ventricular remodeling after acute myocardial infarction, clinical data of filgrastim (G-CSF) administration in CAD patients have been mixed, and untoward effects, including restenosis and de novo lesions, have been reported. Exacerbation of atherosclerosis in apoE−/− mice fed a high-fat diet after G-CSF treatment (10 μg/kg per day for 5 days, every other week for 4 weeks) puts forward the question of an optimal regimen to produce comparable (with BMNCs) vascular repair.

The significantly higher G-CSF and exclusive IL-15 production by female (versus male) BMNC fraction in vitro reaffirms our idea that attenuation of plaque is the result of actions of the infused BMNCs, but the secondary activation of endogenous repair via mobilization of BM progenitors and concomitant Th1-Th2 shift is also necessary for repair. The cytokines/chemokines measured in the media of cultured BMNCs differed from the published data on those produced by CD34+ cells. Specifically, TNF-α, IL-1β, Regulated on Activation, Normal T-Cell Expressed and Secreted, and IL-8 were secreted by CD34+ cells. These differences in the inflammatory milieu suggest that administration of CD34+ cells versus BMNCs may activate different pathways, which, in turn, may beget dissimilar clinical outcomes. However, age (ie, functional capacity and available number of cells) may influence the reparative capacity of BMNCs, CD34+ cells, and EPCs. We have shown previously atheroprotection with BM mononuclear fractions derived from young but not older atherosclerotic apoE−/− mice.

Female recipient mice responded completely differently to exogenous BMNCs from males. Even though circulating cytokines/chemokines were elevated and BVMPCs increased (versus males), BM-CD34+ cells and BM-EPCs remained stable and so did plaque burden. Although the exact mechanism(s) of lack of plaque attenuation are unknown, our data let us propose several possibilities. First, the females were treated earlier in the course of atherosclerosis (prior to the maximal increase in plaque burden) compared with males. Secondly, at the time of treatment, BM-VPCs counts did not decline proportionately to plaque (as they did in males), which together with higher BM-CD34+ counts and upregulated G-CSF in vehicle-treated mice suggests a better ongoing endogenous repair than in males. In addition, females exhibited a stronger Th2-type response following BMNCs than males. Of note, Pinderski et al. showed inhibition of atherosclerosis in LDL receptor–deficient mice that had IL-10–overexpressing T cells. Finally, failure to increase BM-EPCs despite receiving a relatively high dose of EPCs (in BMNC samples) implies a regulatory mechanism. Therefore, exogenous BMNCs were placed in an environment in which endogenous repair was more efficient (than in males) and were thus extraneous, so that no additional atheroprotection could be seen. This finding itself may have clinical implications and suggests that cell delivery should be timed with need. In other words, females may benefit from BMNC administration significantly later than males.

Sex-mismatched hematopoietic stem cell transplantation in humans may cause acute graft-versus-host reaction (GVHD), in which donor T-cells attack the minor histocompatibility (Y-chromosome) antigens of the host, causing interferon-γ- and IL-2–driven cytokine storm, along with antibody production by the host to reject transplanted cells. If GVHD were to occur, atherosclerosis would be exacerbated. On the contrary, atheroprotection occurred in male apoE−/− mice that received female BMNCs. Similarly, a lack of significant increase in plaque growth in females that received male cells favors the absence of GVHD.

Plaque attenuation occurred without reduction of hypercholesterolemia. In recent years, the core understanding of atherosclerosis has shifted away from the exclusive engagement with hyperlipidemia to a long-term relationship with inflammation. In fact, male BMNCs reduced total cholesterol in recipients of both sexes but without atheroprotection. Although this finding was unanticipated, recent evidence suggests the existence of cholesteryl ester transfer protein in BM, which could partially explain the reduction in total cholesterol but does not account for failure to lessen plaque burden.

Lastly, estrogen has been shown to influence BM progenitor cell–based myocardial repair after acute myocardial infarction. In our study, even though administration of sex-matched or -mismatched BMNCs increased estriol levels, there was no direct relationship of estriol levels and atheroprotection. Although we acknowledge the limitation of the pooled data, we also recognize that more insight into the role of estrogen could have been obtained if estrus cycle data were collected and related to atheroprotection. Estrus cycle may modulate reparative activity of BMNCs, as recently shown by Masuda et al. In summary, the present study provides a first step toward defining and deciphering sex differences in vascular repair. Although apoE−/− mice have a significantly greater hyperlipidemia than do CAD patients, it is evident that atherosclerosis begets different inflammatory milieus in males and females. Likewise, endogenous repair and response to cell
therapy differs between the sexes. These differences may account for some of the sex disparities in plaque morphology seen by pathologists and for differences in symptomatology and efficacy of therapies between men and women seen by clinical cardiologists.

Conclusion
We have shown a sex difference in response to BMNC therapy for atheroprotection. Administration of BMNCs derived from wild-type female donors attenuated plaque formation in only male atherosclerotic apoE−/− mice. The percentages of EPCs and CD45+ cells in the BM of recipients, as well as G-CSF levels, significantly correlated with a lower plaque burden. Male BMNCs administered to males and BMNCs from donors of either sex infused into females showed no reduction in plaque. In males, Th1- and Th2-type cytokines increased with BMNC therapy and increases in these cytokines correlated with plaque attenuation. In contrast, females exhibited a stronger Th2-type response following administration of BMNC of either sex. Endogenous BM progenitor cell populations changed with atherosclerosis: CD34+ cells and VPCs decreased, but EPCs rose and then fell as plaque accumulated.

Overall, our findings may have implications for clinical cell therapy trials for CAD, because men and women may exhibit differential responses to exogenous BMNCs. Further exploration of sex differences in vessel repair is warranted.

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Disclosures
None.

References
and regulate normal hematopoiesis in an autocrine/paracrine manner. 


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ONLINE SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

The study conformed to the Guide to Care and Use of Laboratory Animals (National Academy of Sciences, 1996). The study protocol was approved by the University of Minnesota Institutional Animal Care and Use Committee (IACUC) prior to initiation of any experiments.

Animals

All male and female mice were purchased from Jackson Laboratory (Bar Harbor, Maine), housed specific pathogen-free and handled according to the University of Minnesota IACUC guidelines. Recipient mice were apolipoprotein E null mice (ApoE\textsuperscript{-/-}) (stock # 2052) on a C57BL6/J background, and donor mice were wild type C57BL6/J (stock #0664).

Study Design

Recipient animals (ApoE\textsuperscript{-/-}) were fed on a high fat diet #88137 (Harlan-Teklad, Madison, Wisc.; composition: 42% fat, 1.25% cholesterol), beginning at 3 weeks of age, to facilitate formation of atherosclerotic plaque lesions. At 14 ± 1 weeks of age, these mice were administered a total of 4 bi-weekly injections of bone marrow mononuclear cells (BMNC) derived from 10 ± 2 week old C57BL6/J donor mice and cultured as described below. Each ApoE\textsuperscript{-/-} animal received approximately 1 x 10\(^6\) BMNCs/300\(\mu\)l intravenously (IV) through the tail vein in a recipient-donor matched and mismatched fashion. The number of mice in each treatment group is detailed in each figure. The recipients were harvested 1 week post final injection (21 weeks of age). Aortic plaque, bone marrow progenitor cell populations (BMPC), circulating total cholesterol, estriol and plasma cytokine/chemokine levels were analyzed in each group to determine effects of BMNC treatment.
Preparation of Injected Cells

Bone marrow from the tibia and femoral hind legs was isolated from C57BL/6/J mice at 10 ± 2 weeks of age. Cells were maintained in vitro for 2 days in LTC media containing: alpha-MEM (Invitrogen, Carlsbad, Calif.), 12.5% horse serum (Invitrogen), 12.5% fetal bovine serum [FBS] (Hyclone, Logan, Utah), 1 μM hydrocortisone (Sigma, St. Louis, Missouri), 5 μM 2-mercaptoethanol (Invitrogen), and 50 U/μg penicillin/streptomycin (Invitrogen) as previously detailed by our group.(2) Hematopoietic-enriched (non-adherent) cells were harvested, washed twice and suspended in 0.9% saline for IV injection into the ApoE−/− recipients. To maintain blinding of the investigators, BMNCs injections were performed by appropriately trained specialists of Research Animal Resources of the University of Minnesota.

Cholesterol, Estriol and Cytokine Measurements

Blood, harvested from the recipient mice via cardiac puncture with an EDTA-coated syringe immediately after euthanasia, was analyzed using CardioCheck (PTS, Indianapolis, Indiana) Cholesterol Strips for quantification of hyperlipidemia.

Estriol measurements on pooled samples (n = 3 - 6 per group) were made by ELISA using a non-species-specific kit (Assay Designs, Ann Arbor, MI) per manufacturer’s instructions.

Plasma was obtained by spinning approximately 100 μL of harvested blood at 2.8 x g for 10 minutes. Cytokine/chemokine levels were evaluated by using the multiplex method (Luminex platform, Luminex, Austin, Tex.) and utilizing mouse-specific bead sets (Linco, St. Charles, Missouri). Values were interpolated from recombinant protein standards supplied by the manufacturer (Linco). The panel included: IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, TNF-α, IFN-γ, G-CSF, GM-CSF, MCP-1 (CCL2), MIP-1α (CCL3), RANTES (CCL5), KC (CXCL8), and IP-10 (CXCL10).

Animals for both estriol and cytokine measurements were randomly selected from treatment groups by the investigator (W.D.N.) blinded to results of aortic plaque burden, EPCs or VPCs. Levels of aortic plaque, EPCs and VPCs were not different in animals selected for
Histological Evaluation of Atherosclerotic Plaque Lesions

Aortas were harvested, cleaned, split lengthwise for an en face preparation (Figure 1), and stained using Oil red O (1-8-[4-dimethylphenylazo]-2-naphthalenol) to examine plaque deposition. Images were obtained using a digital Nikon (Enfield, New Jers.) E990 camera attached to a Leica (Wetzlar, Germany) S6D microscope. Plaque burden was derived (Oil red O-positive area divided by the total vessel area and expressed as percentage) using ImageProPlus (Media Cybermetics, Silver Springs, Maryland) software.

Fluorescence Activated Cell Sorting (FACS)

Composition of the injectate. To examine progenitor cell composition of the donor cells actually injected, FACS analysis was performed in exact similarity to the assessment of bone marrow progenitor cells from BMNC-treated recipients (detailed below). A sample of the processed bone marrow was obtained both before and after (non-adherent fraction) the 48 hour culture period to determine the changes occurred during this process.

BMNC-Treated ApoE−/− animals. Bone marrow from recipient mice at 21 weeks (one week following the final injection) was harvested and processed to remove red blood cells using ACK lysis buffer. The bone marrow cells were not subjected to culturing. The cells were suspended in 500 μL PBS buffer containing 2.5% FBS and 0.2% sodium azide (FACS buffer). The following antibodies were used with the corresponding isotype controls: CD31-APC (clone MEC 13.3); CD45-PerCP (clone 30-F11); CD34-PE (clone RAM34 – all from BD Biosciences, San Jose, Calif.); and AC133-FITC (clone 13A4 – eBiosciences, San Diego, Calif.). Cells were incubated for 20 minutes, washed twice, and resuspended in 1 mL of FACS buffer. Labeled cells were analyzed on FACSCalibur (BD Biosciences) and BMPCs were gated and evaluated using FlowJo (TreeStar, Ashland, Oreg.) software.
Statistical Analysis.

Results are expressed as mean ± SEM. ANOVA with post-hoc Tukey HSD (honestly significant difference) testing was used for multiple pairwise comparisons within groups of similar recipient sex. Equal variance and normality were determined prior to ANOVA. Where appropriate, differences between two groups (males versus females) were established by two-sided Student t-test. Univariate and multivariate regression analyses were applied to aortic plaque burden, total cholesterol, and BM cell populations. Univariate regression curve fit for each independent variable was derived from the most appropriate model (linear, power, exponential, polynomial or logarithmic) selected based on an initial dot-plot, and Durbin-Watson test was applied. Univariate curve fits and multivariate regression were tested by ANOVA.

Logarithmic transformation \([\log_e(x)]\) was applied to cytokine/chemokine concentration values, and the data were presented as median and range. Kruskal-Wallis ANOVA determined significant differences after treatment in sex-matched recipients, and Wilcoxon/Mann-Whitney U-test was then employed for two-group comparisons (males versus females). Binomial Pearson product-moment coefficients \((r)\) were derived to uncover relationships of cytokine/chemokine values to aortic plaque burden. Pearson’s \((r)\) correlation matrix was produced for the cytokine/chemokine with the highest \(r\) value (against plaque burden) to evaluate its relationship to patterns of immune responses in each treatment group, and results were tabulated using a color-coded matrix.

Comparisons between pooled samples (estriol) were carried out with z-test, and 95% confidence intervals (CI) were estimated. Log-linear Chi-square analysis \((G^2)\) of 3-way contingency tables was used to evaluate first- and second-order interactions between total cholesterol, estriol and sex. SPSS software (version 15.0, SSPS Inc., Chicago, Ill.) was used for all calculations, and graphs were generated using SigmaPlot (version 10.0, Systat Software, San Jose, Calif.).
Online Table 1. Median Cytokine Levels [\log_e(x)] in ApoE\textsuperscript{−/−} Mice Treated with Gender-Matched and Mismatched BMNCs.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Males, Vehicle-Treated, median (range), n = 4</th>
<th>Males, Male-BMNC Treated, median (range), n = 4</th>
<th>Males, Female-BMNC Treated, median (range), n = 5</th>
<th>ANOVA p-value</th>
<th>Females, Vehicle-Treated, median (range), n = 5</th>
<th>Females, Male-BMNC Treated, median (range), n = 5</th>
<th>Females, Female-BMNC Treated, median (range), n = 5</th>
<th>ANOVA p-value</th>
</tr>
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<tbody>
<tr>
<td><strong>Th1-type pro-inflammatory cytokines:</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>4.26 (1.09)</td>
<td>5.90 (2.05)</td>
<td>6.26 (1.84)</td>
<td>0.02</td>
<td>5.52 (2.40)*</td>
<td>6.13 (3.00)</td>
<td>7.84 (3.66)</td>
<td>0.18</td>
</tr>
<tr>
<td>IL-12</td>
<td>4.63 (7.59)</td>
<td>5.54 (0.85)</td>
<td>6.07 (1.54)</td>
<td>0.01</td>
<td>5.10 (1.22)</td>
<td>6.23 (1.37)</td>
<td>6.72 (4.78)</td>
<td>0.05</td>
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<tr>
<td>TNF-α</td>
<td>4.26 (0.93)</td>
<td>5.90 (0.84)</td>
<td>6.26 (1.48)</td>
<td>0.02</td>
<td>3.45 (0.89)</td>
<td>4.29 (1.51)</td>
<td>4.80 (1.48)</td>
<td>0.01</td>
</tr>
<tr>
<td>RANTES</td>
<td>3.82 (0.78)</td>
<td>4.33 (1.21)</td>
<td>4.55 (1.50)</td>
<td>0.02</td>
<td>4.35 (1.28)*</td>
<td>4.31 (2.38)</td>
<td>5.71 (2.39)</td>
<td>0.28</td>
</tr>
<tr>
<td>INF-γ</td>
<td>4.39 (1.61)</td>
<td>4.60 (0.54)</td>
<td>4.31 (1.28)</td>
<td>0.71</td>
<td>4.17 (2.18)</td>
<td>4.31 (1.08)</td>
<td>4.72 (7.51)</td>
<td>0.73</td>
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<tr>
<td>IP-10</td>
<td>7.97 (0.65)</td>
<td>8.96 (1.69)</td>
<td>8.27 (1.23)</td>
<td>0.25</td>
<td>7.68 (0.39)</td>
<td>8.78 (1.41)</td>
<td>7.70 (0.94)</td>
<td>0.08</td>
</tr>
<tr>
<td>IL-1α</td>
<td>5.68 (1.50)</td>
<td>5.78 (1.09)</td>
<td>6.12 (0.72)</td>
<td>0.10</td>
<td>5.06 (1.45)</td>
<td>5.88 (0.98)</td>
<td>5.75 (1.85)</td>
<td>0.18</td>
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<td>MIP-1α</td>
<td>6.96 (2.66)</td>
<td>7.34 (0.56)</td>
<td>7.44 (0.55)</td>
<td>0.34</td>
<td>6.94 (2.68)</td>
<td>7.19 (1.97)</td>
<td>7.25 (2.89)</td>
<td>0.73</td>
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<td><strong>Th17 cytokine:</strong></td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>IL-17</td>
<td>3.81 (1.59)</td>
<td>4.76 (1.57)</td>
<td>4.64 (2.66)</td>
<td>0.17</td>
<td>4.84 (0.63)*</td>
<td>4.35 (1.82)</td>
<td>6.13 (4.24)</td>
<td>0.45</td>
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<td><strong>Th2-type anti-inflammatory cytokines:</strong></td>
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<tr>
<td>IL-4</td>
<td>1.53 (5.15)</td>
<td>2.20 (0.95)</td>
<td>2.23 (1.56)</td>
<td>0.3</td>
<td>0.57 (0.62)</td>
<td>1.90 (1.70)</td>
<td>2.15 (1.44)</td>
<td>0.09</td>
</tr>
<tr>
<td>IL-13</td>
<td>5.56 (1.52)</td>
<td>6.57 (0.84)</td>
<td>7.16 (1.28)</td>
<td>0.03</td>
<td>5.88 (1.46)</td>
<td>7.00 (1.14)</td>
<td>7.31 (2.79)</td>
<td>0.04</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.78 (6.98)</td>
<td>5.34 (2.29)</td>
<td>5.69 (1.67)</td>
<td>0.03</td>
<td>4.78 (7.30)</td>
<td>5.28 (1.91)</td>
<td>6.67 (2.28)</td>
<td>0.04</td>
</tr>
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<td>IL-9</td>
<td>4.76 (3.34)</td>
<td>4.80 (1.94)</td>
<td>5.10 (2.06)</td>
<td>0.9</td>
<td>3.66 (0.46)</td>
<td>4.91 (3.57)</td>
<td>4.54 (2.04)</td>
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<tr>
<td>IL-5</td>
<td>3.51 (0.93)</td>
<td>4.53 (1.20)</td>
<td>4.90 (0.97)</td>
<td>0.02</td>
<td>4.86 (0.71)*</td>
<td>5.06 (1.40)</td>
<td>5.78 (3.69)</td>
<td>0.73</td>
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<tr>
<td>MCP-1</td>
<td>5.07 (1.49)</td>
<td>6.16 (0.44)</td>
<td>6.01 (1.00)</td>
<td>0.06</td>
<td>5.61 (0.62)</td>
<td>6.46 (0.87)</td>
<td>6.76 (0.96)</td>
<td>0.008</td>
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<td><strong>Hematopoietic and regulatory cytokines:</strong></td>
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<td>IL-2</td>
<td>4.33 (0.68)</td>
<td>5.06 (0.76)</td>
<td>4.67 (1.23)</td>
<td>0.18</td>
<td>4.73 (2.69)</td>
<td>4.41 (1.14)*</td>
<td>4.93 (3.34)</td>
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<tr>
<td>IL-6</td>
<td>4.05 (0.58)</td>
<td>4.83 (3.64)</td>
<td>5.39 (2.52)</td>
<td>0.02</td>
<td>4.81 (2.09)</td>
<td>4.40 (2.02)</td>
<td>6.08 (3.02)</td>
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<tr>
<td>IL-7</td>
<td>3.84 (5.03)</td>
<td>5.70 (1.91)</td>
<td>6.26 (3.52)</td>
<td>0.16</td>
<td>5.72 (2.61)</td>
<td>7.32 (3.06)*</td>
<td>5.73 (1.17)</td>
<td>0.04</td>
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<tr>
<td>GM-CSF</td>
<td>5.51 (8.73)</td>
<td>5.78 (1.23)</td>
<td>6.13 (0.64)</td>
<td>0.26</td>
<td>5.69 (1.14)</td>
<td>5.78 (1.11)</td>
<td>6.08 (8.85)</td>
<td>0.40</td>
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<tr>
<td>KC</td>
<td>5.00 (7.68)</td>
<td>5.03 (0.65)</td>
<td>4.50 (1.30)</td>
<td>0.27</td>
<td>4.70 (1.14)</td>
<td>5.07 (0.69)</td>
<td>4.99 (7.88)</td>
<td>0.43</td>
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<tr>
<td>IL-15</td>
<td>5.02 (4.30)</td>
<td>7.19 (1.92)</td>
<td>7.08 (5.65)</td>
<td>0.43</td>
<td>6.83 (2.83)</td>
<td>8.39 (2.55)</td>
<td>7.22 (0.97)</td>
<td>0.18</td>
</tr>
<tr>
<td>G-CSF</td>
<td>6.97 (0.79)</td>
<td>7.07 (1.06)</td>
<td>8.15 (0.80)</td>
<td>0.02</td>
<td>7.60 (1.36)*</td>
<td>7.47 (1.24)</td>
<td>7.00 (2.23)</td>
<td>0.68</td>
</tr>
</tbody>
</table>

Data presented in log\_e-units. ANOVA p-values reflect the statistical significance of inter-group comparisons among the animals of the same recipient sex that received different treatments (i.e., vehicle-, or male BMNCs, or female BMNCs). * indicates comparison of similarly-treated male and female ApoE\textsuperscript{−/−} mice, p values (0.01 < p \leq 0.05), Wilcoxon/Mann-Whitney U-test. Abbreviation: BMNCs, bone marrow mononuclear cells.
Online Figure 1

Male ApoE-/- Mice

Female ApoE-/- Mice

**Th1 and pro-inflammatory cytokines**
- IL-1β
- IL-12
- TNF-α
- RANTES
- INF-γ
- IP-10
- IL-1α
- MIP-1α

**Th17 cytokine**
- IL-17

**Th2 and anti-inflammatory cytokines**
- IL-4
- IL-13
- IL-10
- IL-9
- IL-5
- MCP-1

**Hematopoietic and regulatory cytokines**
- IL-2
- IL-6
- IL-7
- GM-CSF
- KC
- IL-15

Legend:
- $r \geq 0.80$
- $0.50 \leq r \leq 0.79$
- $-0.49 \leq r \leq 0.49$
- $-0.79 \leq r \leq -0.50$
- $r \leq -0.80$
ONLINE SUPPLEMENTARY MATERIAL

FIGURE LEGEND

Online Figure 1.

Color-coded representation of correlations between G-CSF and individual cytokine levels in male and female ApoE⁻/⁻ mice fed on a high-fat diet and treated with vehicle (V, n=4 for male and n=5 for female recipients); or male donor BMNCs (M, n=4 for male and n=5 for female recipients); or female donor BMNCs (F, n=5 for both male and female recipients). Pearson’s r for each pair (G-CSF and a cytokine/chemokine) were derived and then color-coded according to strength of the correlation reflected by the r value. Specifically, when both G-CSF and a cytokine/chemokine concentration increased, the box received red color; when G-CSF rose but the cytokine fell, the box received a green color. When r ≥ 0.80 (e.g., IL-15 in male ApoE⁻/⁻ mice treated with F BMNCs the entire box was colored red, and this was considered a strongly positive correlation. When r ranged between 0.50 to 0.79 (e.g., KC (IL-8) in male ApoE⁻/⁻ mice treated with F BMNCs), only half a box was colored in red, and this was considered a moderately positive correlation. Similarly, when r ≤ -0.80 (e.g. IL-1α in male ApoE⁻/⁻ mice treated with F BMNCs), the correlation was considered strongly negative, and the entire box was colored green; but when r ranged between -0.79 to -0.50, only half a box was colored in green (e.g., GM-CSF in male ApoE⁻/⁻ mice treated with F BMNCs) and deemed a moderately negative correlation. When any r ranged between -0.49 and 0.49, we considered that a weak or no relationship existed between G-CSF and a cytokine/chemokine, and those boxes were colored in black. Abbreviation: BMNCs, bone marrow mononuclear cells.