T-Cell–Derived Interferon-γ Contributes to Arteriolar Dysfunction During Acute Hypercholesterolemia


Objectives—T-lymphocytes and interferon-γ (IFN-γ) contribute to leukocyte recruitment in postcapillary venules during hypercholesterolemia. Our objectives were to determine whether: (1) T-lymphocytes are the source of this IFN-γ, and (2) whether T-cell–derived IFN-γ also mediates the accompanying arteriolar dysfunction and platelet adhesion.

Methods and Results—Intravital videomicroscopy was used to quantify arteriolar responses to acetylcholine, and leukocyte and platelet adhesion in postcapillary venules of wild-type (WT), immunodeficient (SCID), and IFN-γ−/− mice on a normal (ND) or high-cholesterol (HC) diet. Acetylcholine-induced arteriolar dilation was impaired in WT-HC, compared with WT-ND. This endothelial dysfunction was absent in SCID-HC or IFN-γ−/−HC mice. Vasodilation was impaired by transfer of WT, but not IFN-γ−/−, T-cells to these immunodeficient mice. WT-HC mice exhibited elevated leukocyte and platelet adhesion in venules, versus WT-ND. This blood cell recruitment was attenuated to ND levels in SCID-HC and IFN-γ−/−HC mice, but restored to WT-HC levels by transfer of WT, but not IFN-γ−/− T-lymphocytes.

Conclusions—These data reveal a novel role of T-lymphocyte–derived IFN-γ in the development of endothelial dysfunction in arterioles during hypercholesterolemia and extend our previous observations that IFN-γ mediates both inflammatory and thrombogenic responses to hypercholesterolemia in postcapillary venules. (Arterioscler Thromb Vasc Biol. 2007;27:1998-2004.)

Key Words: T-lymphocytes ■ interferon-γ ■ hypercholesterolemia ■ endothelial dysfunction ■ platelets

Evidence implicating the adaptive immune system in the development of atherosclerosis has grown over the past decade, and it is now recognized that one of the major risk factors for this disease, hypercholesterolemia, can elicit a heightened activation state of T-cells. In acute inflammatory states such as ischemia-reperfusion, T-cells also appear to regulate some of the early inflammatory responses in the microvasculature, such as neutrophil recruitment. Although hypercholesterolemia is well known to induce a chronic inflammatory phenotype in large arteries, recent evidence indicates that this risk factor is also associated with early inflammatory responses throughout the microvasculature, which are manifested as impaired endothelium-dependent vasodilation in arterioles, and enhanced neutrophil and platelet recruitment in postcapillary venules. Lymphocytes, and more specifically CD4+ and CD8+ T-cells, have been shown to contribute to the leukocyte recruitment in postcapillary venules of hypercholesterolemic mice. This appears to be mediated through an interferon-gamma (IFN-γ)–dependent pathway.

In humans with hypercholesterolemia, one of the earliest manifestations of endothelial dysfunction is an impaired endothelium-dependent vasodilation response. This impaired vasodilation response is manifested in both large arteries and in arterioles. We have recently reported evidence indicating that the arteriolar dysfunction caused by hypercholesterolemia is linked to the inflammatory response that occurs in downstream venules. Because T-lymphocytes and IFN-γ appear to modulate the inflammatory responses in venules during hypercholesterolemia, the possibility exists that T-cells, perhaps acting via IFN-γ, also contribute to the impaired arteriolar responses to endothelium-dependent dilators. Although evidence linking T-lymphocytes or IFN-γ to resistance vessel function is limited, it has been reported by de Kimpe et al that arterial rings treated with IFN-γ exhibit an impaired ability to dilate in response to bradykinin, when compared with untreated vessel rings, and IFN-γ can induce/activate a number of factors that can impair endothelial function in arterioles, eg, tumor necrosis factor (TNF)-α. A major objective of this study was to determine whether T-cells, acting via IFN-γ, mediate the endothelium-dependent arteriolar dysfunction that accompanies hypercholesterolemia in mice.

IFN-γ can contribute to an inflammatory response via a variety of mechanisms, such as enhancing the release of other cytokines and the activation of the superoxide-generating enzyme NAD(P)H oxidase. A consequence of these actions by IFN-γ is an increased expression of adhesion molecules on...
vascular endothelium and the subsequent recruitment of inflammatory cells. Previous work in our laboratory implicates T-cells as the major source of this cytokine in hypercholesterolemic mice. An important unresolved issue, however, is whether the inflammatory phenotype that is induced in venules by T-lymphocyte-derived IFN-γ facilitates the recruitment of adherent blood cells other than leukocytes. Although the mechanisms that underlie the recruitment of adherent platelets in venules during hypercholesterolemia remain poorly understood, there is mounting evidence that this process is leukocyte-dependent and involves P-selectin expressed on platelets and on endothelial cells. Hence, another major objective of this study was to determine whether T-cell-derived IFN-γ contributes to the hypercholesterolemia-induced recruitment of platelets in postcapillary venules.

Materials and Methods

Animals
All experimental procedures were performed on male wild-type (WT) C57Bl/6, B6.CB17-Prkdc<sup>−/−</sup>/SjJ mice (SCID), B6.129S-RAG<sup>1</sup>/H<sub>9253</sub> (Rag1<sup>−/−</sup>−/−), or B6.129S7-I<sup>ifng<sup>−/−</sup></sup> mice (IFN-γ<sup>−/−</sup>) (Jackson Laboratory, Bar Harbor, Me; n=5 to 7/group for analysis). Mice (6 to 8 weeks old) were placed either on a normal diet (ND) or high-cholesterol (HC) diet (Teklad 90221 containing 1.25% cholesterol, 0.125% choline chloride, 15.8% fat, Harlan Teklad) for 2 weeks. Hypercholesterolemic SCID, Rag1<sup>−/−</sup>−/−, and IFN-γ<sup>−/−</sup> mice were divided into the following subgroups:

**SCID-HC, Rag1<sup>−/−</sup>−/−-HC, and IFN-γ<sup>−/−</sup>-HC**
SCID, Rag1<sup>−/−</sup>−/−, and IFN-γ<sup>−/−</sup> mice placed on HC for 2 weeks.

**SCID−WT 5d, Rag1<sup>−/−</sup>−/−−WT 5d, SCID−IFN-γ<sup>−/−</sup>−/− 5d**
Lymphocyte-deficient mice reconstituted with T-lymphocytes isolated from either WT-HC or IFN-γ<sup>−/−</sup>-HC mice 5 days before the experiment ie, at day 9 of HC (recipient<sup>−/−</sup>-donor).

**SCID−WT 10d; SCID−IFN-γ<sup>−/−</sup>−/− 10d**
Mice reconstituted with T-lymphocytes isolated from either WT or IFN-γ<sup>−/−</sup>-HC mice 10 days before experiment ie, at day 4 of HC (recipient<sup>−/−</sup>-donor).

A separate group of WT-HC mice received 50 µL anti-asialo GM1 (Wako Chemicals Inc) i.p. at days 11 and 13 of HC. This achieved almost 50% depletion of splenic natural killer (NK) cells as determined by flow cytometry (WT-HC NK depl group). This group was used for measurement of plasma IFN-γ levels only.

Reconstitution Experiments
Splenocytes were isolated as described previously<sup>2</sup>, and T-lymphocytes were isolated from the splenocyte suspension using the MACs system (Miltenyi Biotec Inc) with negative selection for B220<sup>−</sup> and CD11b<sup>−</sup> cells. This procedure yielded a cell population of >95% CD3<sup>+</sup> T-lymphocytes, as verified by flow cytometry. Recipient mice received 5×10<sup>6</sup> T-cells in 200 µL i.p. at 4 or 9 days HC and were allowed to recover for 10 or 5 days, respectively, before intravital microscopy was performed at 2 weeks HC.

Intravital Microscopy
Intravital microscopy of the cremaster microcirculation was performed as previously described. Leukocyte and platelet recruitment were quantified in postcapillary venules, and arteriolar vasodilation responses to acetylcholine were measured. For more details, please see the supplemental materials (available online at http://atvb.ahajournals.org).

Blood Leukocyte Counts
At the end of each experiment, blood was drawn from the heart for manual measurement of circulating blood leukocyte counts with the aid of a hemocytometer.

Serum Cholesterol Levels
Serum was frozen for subsequent measurement of cholesterol levels using a spectrophotometric assay (Sigma Chemicals Co).

Plasma Cytokine Measurements
Separate groups of mice (n=4 to 9 per group) were placed on ND or HC for 2 weeks. Heparinized blood was drawn from an arterial cannula to obtain plasma for cytokine measurements. A cytometric bead array (BD Biosciences) was used to measure plasma IFN-γ concentration (pg IFN-γ per ml plasma), with samples analyzed on a fluorescence-activated-cell sorter (FACS) Caliber.

Statistical Analysis
All values are reported as mean±SEM. ANOVA with Bonferroni post-hoc test (intravital microscopy data) or Fisher post-hoc test (plasma IFN-γ data) was used for statistical comparison of experimental groups.

Results

Cholesterol Levels, Wall Shear Rate, and Blood Lymphocyte Counts
Placement on a cholesterol-enriched diet for 2 weeks led to a 2- to 3-fold increase in circulating cholesterol levels in all groups (WT or immunodeficient) when compared with mice maintained on a normal diet (supplemental Table I). Blood lymphocyte counts were significantly lower in the SCID and Rag1<sup>−/−</sup>−/− mice versus WT and IFN-γ<sup>−/−</sup> mice, and this remained unchanged after receiving CD3<sup>+</sup> T-lymphocytes from donor mice. Wall shear rate was comparable in all groups.

Hypercholesterolemia Initiates Arteriolar Dysfunction Through a T-Cell–Dependent Pathway
The elevated plasma cholesterol levels in WT-HC mice were associated with a significant impairment of arteriolar relaxation responses to acetylcholine, when compared with normcholesterolemic controls (Figure 1). Lymphocyte deficiency per se in Rag1<sup>−/−</sup>-ND mice did not alter the vasodilatory response to acetylcholine. Furthermore, placement of lymphocyte-deficient SCID or Rag1<sup>−/−</sup>−/− mice on HC did not lead to impaired endothelium-dependent vasodilation; rather, the responses in these mice were similar to the WT-ND group. Administration of T-cells from WT-HC mice to SCID-HC or Rag1<sup>−/−</sup>-HC mice 5 days before observation did not alter the vasodilation responses seen in the immunodeficient-HC mice. The similar data gained from SCID and Rag1<sup>−/−</sup>−/− mice suggest that lymphocyte deficiency per se rather than a SCID-specific phenotype is responsible for the findings, consequently SCID mice were used for the rest of the study. When SCID-HC mice received WT-HC T-lymphocytes 10 days before observation, endothelium-dependent vasodilation was impaired to a level comparable to that detected in WT-HC mice.
The role of T-lymphocytes in hypercholesterolemia-induced impairment of arteriolar dilation responses to acetylcholine. WT and lymphocyte-deficient SCID and Rag1−/− mice were maintained on ND or HC for 2 weeks. Separate SCID-HC or Rag1−/−-HC mice received T-cells from WT mice 5 days (SCID or Rag1−/−-WT 5d) or 10 days (SCID−-WT 10d) before observation. *P<0.005 vs WT-ND; †P<0.005 vs WT-HC; ‡P<0.0005 vs WT-ND; #P<0.0005 vs WT-HC; ^P<0.0001 vs WT-HC.

**Figure 1.** The role of T-lymphocytes in hypercholesterolemia-induced impairment of arteriolar dilation responses to acetylcholine. WT and lymphocyte-deficient SCID and Rag1−/− mice were maintained on ND or HC for 2 weeks. Separate SCID-HC or Rag1−/−-HC mice received T-cells from WT mice 5 days (SCID or Rag1−/−-WT 5d) or 10 days (SCID−-WT 10d) before observation. *P<0.005 vs WT-ND; †P<0.005 vs WT-HC; ‡P<0.0005 vs SCID-HC.

**Figure 2.** The involvement of T-lymphocytes–derived IFN-γ in the altered endothelium-dependent relaxation responses in arterioles of hypercholesterolemic mice. WT and IFN-γ−/− mice were placed on a ND or HC for 2 weeks. Separate IFN-γ−/−-HC mice received T-cells from WT mice 5 days (IFN-γ−/−-WT 5d) before experimentation. Two groups of SCID-HC mice received T-lymphocytes from IFN-γ−/− mice 5 days (SCID−-IFN-γ−/− 5d) or 10 days (SCID−-IFN-γ−/− 10d) before observation. *P<0.0005 vs WT-ND; †P<0.0005 vs WT-HC; ‡P<0.0005 vs IFN-γ−/−-HC; †P<0.0001 vs IFN-γ−/−-WT 5d.

**Contribution of T-Cell–Derived IFN-γ to Arteriolar Dysfunction During Hypercholesterolemia**

Normocholesterolemic mice deficient in the cytokine IFN-γ exhibited vasodilation responses to acetylcholine that were comparable to their WT counterparts (Figure 2). When these mice were placed on HC for 2 weeks, the absence of IFN-γ conferred protection against the diet-induced attenuation of endothelium-dependent vasodilation. However, hypercholesterolemia-induced impairment of vasodilation was observed after transferring T-cells from WT-HC mice to the IFN-γ−/−-HC mice 5 days before observation. In contrast, in SCID-HC mice receiving IFN-γ−/−-HC T-cells hypercholesterolemia did not promote endothelial dysfunction in arterioles, regardless of whether these cells were transferred 5 or 10 days before the experiment.

**Hypercholesterolemia Promotes an Inflammatory Phenotype in Postcapillary Venules That Is Mediated by T-Cell–Derived IFN-γ**

Mice placed on a high cholesterol diet exhibited elevated leukocyte and platelet recruitment in postcapillary venules when compared with corresponding normocholesterolemic mice (Figure 3). Immunodeficiency, either the SCID mutation (Figure 3) or Rag1 deficiency (not shown), was associated with protection against HC-induced blood cell recruitment, such that levels of leukocyte and platelet adhesion in the immunodeficient mice maintained on HC were comparable to mice on ND. However, administration of T-cells either 5 or 10 days before intravital observation restored the hypercholesterolemia-induced inflammatory and thrombo-
cells may be the source of the elevated plasma IFN-γ in HC mice.

Discussion

Only a mild to moderate elevation of circulating cholesterol levels in humans is required to impair the ability of arteries to respond to endothelium-dependent vasodilators such as methacholine chloride. In fact, endothelial dysfunction is one of the earliest responses to hypercholesterolemia and it is manifested throughout the vascular tree. Recently, we demonstrated that a 2- to 3-fold increase in plasma cholesterol concentration in mice is associated with a reduction in endothelium-dependent vasorelaxation in arterioles, and increased platelet and leukocyte recruitment in venules. The immune system, in particular, T-cells and their cytokines such as IFN-γ, have been implicated in the leukocyte recruit-

![Figure 3](http://atvb.ahajournals.org/)

**Figure 3.** Involvement of T-lymphocytes in leukocyte and platelet adhesion in postcapillary venules of mice maintained on a ND or HC for 2 weeks. WT and lymphocyte-deficient SCID mice were observed. Separate groups of SCID mice received T-cells from WT mice 5 days (SCID→WT-HC 5d) or 10 days (SCID→WT-HC 10d) before experimentation. *P<0.005 vs WT-ND; †P<0.005 vs WT-HC; ^P<0.0001 vs SCID-HC.

![Figure 4](http://atvb.ahajournals.org/)

**Figure 4.** The participation of IFN-γ in hypercholesterolemia-induced recruitment of leukocytes in postcapillary venules was assessed using WT and IFN-γ−/− mice. A separate group of IFN-γ−/− mice received T-cells from WT mice 5 days (IFN-γ−/−→WT-HC 5d) before experimentation, and 2 groups of SCID mice received CD3 splenocytes from IFN-γ−/− mice 5 days (SCID→IFN-γ−/−HC 5d) or 10 days (SCID→IFN-γ−/−HC 10d) before observation. *P<0.0001 vs WT-ND; †P<0.0001 vs WT-HC; ^P<0.0001 vs IFN-γ−/−HC; †P<0.0001 vs IFN-γ−/−→WT 5d.

![Figure 5](http://atvb.ahajournals.org/)

**Figure 5.** The adhesion of exogenous matched platelets in postcapillary venules of WT and IFN-γ−/− mice on ND or HC for 2 weeks. Separate IFN-γ−/− mice received T-cells from WT mice 5 days (IFN-γ−/−→WT-HC 5d) before experimentation. Two groups of SCID mice received T-lymphocytes from IFN-γ−/− mice 5 days (SCID→IFN-γ−/−HC 5d) or 10 days (SCID→IFN-γ−/−HC 10d) before experimentation. *P<0.0005 vs WT-ND; †P<0.0005 vs WT-HC; ^P<0.0001 vs IFN-γ−/−→WT 5d.
Circulating Levels of IFN-γ in the Plasma of Wild-Type and Immunodeficient Mice Placed on a Normal (ND) or Cholesterol-Enriched Diet (HC) for Two Weeks

<table>
<thead>
<tr>
<th>Groups</th>
<th>Plasma IFN-γ Levels (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-ND</td>
<td>12.4±2.06</td>
</tr>
<tr>
<td>WT-HC</td>
<td>49.1±6.83*</td>
</tr>
<tr>
<td>SCID-HC</td>
<td>20.2±1.78†</td>
</tr>
<tr>
<td>SCID→WT 5 days</td>
<td>39.0±8.53*</td>
</tr>
<tr>
<td>SCID→WT 10 days</td>
<td>47.4±11.75*</td>
</tr>
<tr>
<td>SCID→IFN-γ−/− 5 days</td>
<td>62.8±12.95*</td>
</tr>
<tr>
<td>IFN-γ−/−→HC</td>
<td>12.2±0.96†</td>
</tr>
<tr>
<td>IFN-γ−/−→WT 5 days</td>
<td>7.2±1.99†</td>
</tr>
<tr>
<td>WT-HC NK depl</td>
<td>14.3±3.32†</td>
</tr>
</tbody>
</table>

Some mice received T-lymphocytes from donor mice 5 or 10 days before observation. A separate WT-HC group received NK cell-depleting antibody (WT-HC NK depl).

*P<0.05 vs WT-ND, SCID-HC, and IFN-γ−/−→HC groups.
†P<0.05 vs WT-HC group.

ment elicited by hypercholesterolemia7,8 and also contribute to the early stages of plaque development in large vessels of atherosclerosis-prone mice.19–22 Here, we provide the novel evidence that IFN-γ released from T-lymphocytes contributes to the impaired endothelium-dependent dilation of arterioles that accompanies hypercholesterolemia. Furthermore, our findings indicate that T-cell–derived IFN-γ induces the thrombogenic phenotype that is assumed by venules during hypercholesterolemia.

Hypercholesterolemia causes endothelial dysfunction on the arteriolar side of the microvasculature, which is manifested as an impaired vasomotor response to endothelium-dependent dilators such as acetylcholine.3 This is likely to be one of the mechanisms through which hypercholesterolemia exacerbates tissue inflammation and injury in response to other stimuli such as ischemia-reperfusion. Reduced nitric oxide (NO) bioavailability and enhanced NAD(P)H oxidase–derived superoxide generation have both been implicated in the impaired dilation.10,23,24 Because IFN-γ is a potent stimulator of NAD(P)H oxidase,14,15 it is plausible that T-lymphocytes, acting through release of this cytokine, may contribute to this arteriolar dysfunction. Using lymphocyte-deficient mice, we have obtained evidence that strongly implicates T-cells in the impaired endothelium-dependent vasodilation in arterioles of hypercholesterolemic mice. When T-lymphocytes isolated from wild-type mice were transferred into lymphocyte-deficient mice, the wild-type hypercholesterolemic phenotype was reassumed by the arterioles, ie, the impaired dilation response was observed. This rescue of the phenotype occurred despite a failure to replete the circulating T-cell population, suggesting that the transferred T-cells are causing endothelial dysfunction in arterioles through the release of one or more soluble factors, such as cytokines or chemokines.

Although IFN-γ possesses both proatherogenic (eg, promotes adhesion molecule and chemokine expression) and atheroprotective (eg, down-regulation of the LTB4 receptor) properties,25 this cytokine has been implicated in the development of atherosclerosis in large arteries.19 Although IFN-γ has been shown to attenuate the dilation of isolated arterial rings in response to bradykinin,11 and can promote the release of several factors that can impair endothelial function,12 evidence supporting the participation of this cytokine in either the large artery or arteriolar dysfunction associated with hypercholesterolemia or other cardiovascular risk factors is lacking. Hence, we demonstrate for the first time that IFN-γ is a major factor contributing to hypercholesterolemia–induced arteriolar dysfunction in vivo. Using T-cell transfer protocols, we were also able to determine that T-lymphocytes represent the major source of the IFN-γ that mediates the hypercholesterolemia–induced arteriolar dysfunction. Exactly how T-cell–derived IFN-γ induces this endothelial dysfunction in arterioles remains unclear. Because increased reactive oxygen species production and reduced NO bioavailability have both been implicated in the impaired endothelium-dependent responses during hypercholesterolemia,10,23 the possibility that IFN-γ acts on either or both of these factors is worthy of consideration. IFN-γ has been shown to increase BH4 synthesis in endothelial cells through the de novo pathway by stimulating GTP cyclohydrolase-I,26 and there is some evidence that it induces iNOS expression in vascular smooth muscle.27 It has been suggested that the increased NO release in arterial rings exposed to IFN-γ leads to desensitization of the smooth muscle cell to NO, thereby impairing endothelium-dependent vasodilation.11 Alternatively, the ability of IFN-γ to induce NAD(P)H oxidase,14,15 a superoxide-generating enzyme that contributes to hypercholesterolemia–induced arteriolar dysfunction,24 may explain the actions of the cytokine in our model. IFN-γ may also be acting indirectly by inducing the expression of or acting in concert with other cytokines such as TNF-α to alter NO bioavailability or oxygen free radical generation. For example, the combination of TNF-α, interleukin (IL)-1, and IFN-γ is known to reduce eNOS mRNA stability and eNOS catalytic activity in pulmonary artery endothelial cells,28 which could produce an impaired vasodilatory response to endothelium-dependent substances. Additional work is needed to more precisely define the molecular basis for the actions of IFN-γ on arteriolar function.

It has been established that lymphocytes, in particular both CD4+ and CD8+ T-cells, mediate the leukocyte recruitment in postcapillary venules during hypercholesterolemia.7 Our previous findings suggested that T-lymphocytes release 1 or more soluble factors that induce an inflammatory phenotype in these venules, and we identified IFN-γ as one such factor.8 Here we confirm these findings by isolating CD3+ T-lymphocytes from wild-type or IFN-γ−/− mice for transfer into hypercholesterolemic recipient mice, thereby identifying T-lymphocytes as the source of the IFN-γ. Furthermore, we determined that the T-cells also contribute to the accompanying platelet adhesion that occurs in these venules, and that T-lymphocytes promote this thrombogenic response through the release of IFN-γ. Recent work in our laboratory suggests that platelet recruitment in postcapillary venules of hypercholesterolemic mice is physically supported by leukocytes that are adherent to the vessel wall.3,6 Therefore, a likely scenario to explain IFN-γ–mediated platelet recruitment in hypercho-
lesterolomic venules is that the T-cell–derived cytokine acts directly or indirectly, through the induction of other mediators, on venular endothelium to increase the expression of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), or activates neutrophils to produce chemokines, either of which would promote leukocyte-endothelial cell adhesion. The adherent leukocytes in turn support platelet recruitment through adhesive interactions mediated by constitutively expressed PSGL-1 on leukocytes and the P-selectin that is upregulated on platelets in response to hypercholesterolemia. However, it remains unclear whether IFN-γ specifically enhances the adhesiveness of leukocytes for platelets and whether platelets are being activated through an IFN-γ–dependent or –independent pathway.

We observed that, unlike the inflammatory responses in venules, hypercholesterolemia–induced arteriolar dysfunction was not observed in SCID mice after transfer of T-lymphocytes for 5 days. We confirmed this was not a strain-specific effect by using Rag1−/− mice in which we obtained similar results. When we pursued this in the SCID mice, it was revealed that a longer transfer period (10 days) was required before impaired vasodilatation responses were observed. However, this temporal difference between the arteriolar and venular responses was not observed in IFN-γ–deficient mice receiving WT T-cells. This suggests that T-lymphocytes from IFN-γ−/− mice may be partially activated or somehow initiate the inflammatory process and that T-cell–derived IFN-γ is a required “downstream” mediator. Alternatively, the disparity may simply reflect a difference in the size of the circulating lymphocyte populations between the SCID and IFN-γ−/− mice. Nonetheless, the temporal disparity between the inflammatory processes on the arteriolar and venular sides suggests that, although both require T-cell IFN-γ, either the underlying mechanisms of inflammation are distinct in these microvessels, or that the arteriolar dysfunction is linked to events in occurring in venules. The latter possibility is supported by our previous findings that neutrophil depletion can abrogate both blood cell adhesion in venules and the arteriolar dysfunction. Because the arterioles examined in this study were not paired with venules, the diffusion of soluble factors from venules to arterioles is not a likely explanation for the observed arteriolar dysfunction, as has been demonstrated in arterioles closely paired with venules.

To address whether T-cell–derived IFN-γ may be directly mediating the microvascular responses to hypercholesterolemia, we compared plasma IFN-γ levels between the groups. Our observations that plasma IFN-γ is elevated in hypercholesterolemic WT, but not in immunodeficient, mice is consistent with a direct action of IFN-γ in the microvascular inflammation. The HC-induced elevation of plasma IFN-γ was restored in SCID mice receiving T-lymphocytes from WT donors, which also supports a direct action of IFN-γ and suggests that the donor T-lymphocytes may be a source of elevated IFN-γ. However, the complete restoration of the HC-induced increase in IFN-γ levels in SCID mice receiving IFN-γ−/− T-cells suggests that: (1) although T-lymphocytes are required for the elevated plasma IFN-γ, these cells were not the primary source of the cytokine; (2) high circulating IFN-γ levels are not sufficient to promote the HC-induced microvascular inflammation; (3) other resident cell types, such as NK cells, in the recipient mice produce the IFN-γ in response to a stimulus (other than IFN-γ) from the transferred T-cells.

NK cells have been implicated in the inflammation associated with prolonged hypercholesterolemia during atherosclerosis and represent a major potential source of IFN-γ. Our findings with a NK-depleting antibody in hypercholesterolemic mice support a role for NK cells in generating the elevated plasma IFN-γ. It is possible that T-cells influence another cell type such as monocytes/macrophages to initiate an inflammatory cascade that leads to 2 distinct pathways: (1) NK cell activation and release of IFN-γ into plasma; and (2) further activation of T-cells to produce IFN-γ, this being a key event in the induction of the microvascular inflammation. The fact that the adoptively transferred T-cells were not detected in blood, but were still able to fully restore both the elevated IFN-γ levels and the HC-induced inflammatory phenotype, is somewhat surprising. However it should be noted that the number of transferred cells was approximately equal to the normal circulating T-cell count, consequently the total body (blood and non-blood pools) T-cell population should be sufficient to induce full expression of the inflammatory phenotype.

In conclusion, the results of this study provide the first direct evidence implicating T-cells and the T-cell–derived cytokine IFN-γ as mediators of the endothelium-dependent arteriolar dysfunction caused by hypercholesterolemia. These observations may have important implications in the ongoing research effort to define candidate molecules that link immune dysfunction with cardiovascular disease, and may lead to novel therapeutic strategies (eg, anticytokine agents) for prevention of the arterial vessel dysfunction that precedes the development of atherosclerosis after prolonged elevations in blood cholesterol.

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Disclosures
None.

References


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Platelet Isolation: Platelets were isolated from whole blood by a series of centrifugation steps, and labeled with the fluorochrome carboxyfluorescein diacetate succinimudyl ester (CFSE; Molecular Probes, Eugene OR) as described previously.1

Surgical Protocol: Mice were anesthetized with ketamine hydrochloride (150 mg/kg body weight, i.p.) and xylazine (7.5 mg/kg body weight, i.p.). The right jugular vein of platelet recipient mice was cannulated for infusion of fluorescently labeled platelets. The left carotid artery was cannulated for systemic arterial pressure measurement. Core body temperature was maintained at 35 ± 0.5°C. Animal handling procedures were approved by the LSU Health Sciences Center Institutional Animal Care and Use Committee and were in accordance with guidelines of the American Physiological Society.

Intravital Microscopy: The cremaster muscle was prepared for intravital microscopy as described previously.2,3 Postcapillary venules (20-40 µm diameter) were studied. Venular diameter (Dv) and centerline red blood cell velocity (V_rbc) were measured online using a video caliper and an optical Doppler velocimeter respectively (Microcirculation Research Institute, Texas A&M University). Venular blood flow (VBF_mean) was calculated as follows: VBF_mean = V_rbc/1.6. Venular wall shear rate (WSR) was calculated based on the Newtonian definition WSR = 8(VBF_mean/Dv). In order to minimize inflammation-independent cell recruitment, a threshold WSR of ≥500/s was selected based on previous reports describing a propensity for leukocytes to adhere in venules at low WSRs.4 The microscopic images were recorded on DVD and leukocyte and platelet parameters were analyzed off-line.
**Experimental Protocol:** At the end of 30 min stabilization, if more than one postcapillary venule met the criteria outlined above, the venule with the lowest number of adherent leukocytes was studied in order to minimize bias towards inflammation in HC mice, and to avoid assessing inflammation due to surgical manipulation. If several venules met these criteria, the venule with the most sections available for recording was chosen. 100 x10^6 (120 µl) platelets were infused via the jugular vein over 5 min and allowed to circulate for an additional 5 min. Five minute recordings of the leukocytes (light microscopy) followed by 1 min recordings of the platelets (fluorescent microscopy) were made of the first 100 µm of every 300 µm along the length of the vessel, beginning as near to the source of the venule as possible. Leukocytes or platelets were considered adherent if they remained stationary for ≥30 s or ≥2 s respectively (#/mm²) and were measured throughout the 5 min (leukocytes) or 1 min (platelets) observation period. Leukocyte emigration was measured online at the end of each observation period. Emigrated leukocytes were expressed as the number of interstitial leukocytes per field of view on the television monitor (an area of 0.025 mm²) surrounding the segment under observation (#/field). The mean value of each variable within a single venule was calculated and comparisons were made between groups.

**Endothelium-Dependent Arteriolar Vasodilation Responses:** In all groups, once the venule data were collected, the bicarbonate-buffered saline (BBS) superfusion was increased to 2 ml/min and the animals were allowed to stabilize for 20-30 min. Arterioles with diameters between 15-40 µm and WSR of ≥500/s were chosen for study. After baseline measurements of diameter and Vrbc were taken in a single section from each arteriole of interest, the preparation was superfused with 10^-5 mol/L of the endothelium-dependent vasodilator, acetylcholine (ACh), for 5
min (this dose was based on a previous dose response study in this model\textsuperscript{5}). Diameter and \(V_{\text{rbc}}\) were measured in the vessel regions as before. The preparation was then superfused with BBS and allowed to return to baseline values. Any preparation that contained arterioles that were unresponsive to ACh was then superfused with the endothelium-independent vasodilator, papaverine (10\textsuperscript{-3} mol/L) to determine if the lack of response was a feature of the smooth muscle rather than the endothelium. Data from arterioles that also failed to respond to papaverine were excluded from the study. There was no difference between arteriolar responses to papaverine in the ND and HC groups. Arteriolar vasorelaxation responses to ACh were expressed as the percentage diameter change versus baseline.
References


Table I: Cholesterol levels, wall shear rate and circulating lymphocyte counts in wildtype and immunodeficient mice placed on a normal (ND) or cholesterol-enriched diet (HC) for two weeks. Some mice received T-lymphocytes from donor mice 5 or 10 days prior to observation.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cholesterol (mg/dL)</th>
<th>WSR (s⁻¹)</th>
<th>Lymphocytes (#/µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-ND</td>
<td>68.2±4.76</td>
<td>734±50.6</td>
<td>7204±726.1</td>
</tr>
<tr>
<td>WT-HC</td>
<td>160.6±6.84*</td>
<td>586±23.9</td>
<td>5623±372.9</td>
</tr>
<tr>
<td>SCID-HC</td>
<td>154.4±13.03*</td>
<td>851±144.7</td>
<td>21±14.9†</td>
</tr>
<tr>
<td>SCID←WT 5d</td>
<td>163.9±13.81*</td>
<td>632±30.9</td>
<td>130±80.0†</td>
</tr>
<tr>
<td>SCID←WT 10d</td>
<td>152.4±21.69*</td>
<td>716±83.7</td>
<td>113±51.5†</td>
</tr>
<tr>
<td>RAG1⁻⁻-ND</td>
<td>54.0±14.15</td>
<td>645±40.5</td>
<td>33±16.7†</td>
</tr>
<tr>
<td>RAG1⁻⁻-HC</td>
<td>152.4±12.84*</td>
<td>640±41.2</td>
<td>50±25.8†</td>
</tr>
<tr>
<td>RAG1⁻⁻←WT 5d</td>
<td>176.1±23.36*</td>
<td>732±61.7</td>
<td>25±17.1†</td>
</tr>
<tr>
<td>IFN-γ⁻⁻-ND</td>
<td>50.2±10.92</td>
<td>752±87.0</td>
<td>4413±997.4</td>
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<tr>
<td>IFN-γ⁻⁻-HC</td>
<td>142.6±18.27*</td>
<td>867±105.3</td>
<td>5817±276.8</td>
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<tr>
<td>IFN-γ⁻⁻←WT 5d</td>
<td>147.0±11.83*</td>
<td>605±62.2</td>
<td>6717±1010.3</td>
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<tr>
<td>SCID←IFN-γ⁻⁻ 5d</td>
<td>132.6±19.95*</td>
<td>744±87.3</td>
<td>100±50.0†</td>
</tr>
<tr>
<td>SCID←IFN-γ⁻⁻ 10d</td>
<td>161.2±6.20*</td>
<td>817±126.8</td>
<td>240±48.5†</td>
</tr>
</tbody>
</table>

* P<0.05 versus all ND groups
† P<0.05 versus WT and IFN-γ⁻⁻ groups