Role of interleukin 12 in hypercholesterolemia-induced inflammation

Department of Molecular and Cellular Physiology, Health Sciences Center, Louisiana State University, Shreveport, Louisiana 71130-3932

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Stokes, Karen Y., E. Chris Clanton, John L. Gehrig, and D. Neil Granger. Role of interleukin 12 in hypercholesterolemia-induced inflammation. Am J Physiol Heart Circ Physiol 285: H2623–H2629, 2003.—We have previously shown that T lymphocytes and interferon-γ are involved in hypercholesterolemia-induced leukocyte adhesion to vascular endothelium. This study assessed the contribution of interleukin 12 (IL-12) to these hypercholesterolemia-induced inflammatory responses. Intravital videomicroscopy was used to quantify leukocyte adhesion and emigration and oxidative stress (dihydrorhodamine oxidation) in unstimulated cremasteric venules (wall shear rate ∼500 s⁻¹) of wild-type (WT) C57Bl/6, lymphocyte-deficient [recombinase-activating gene knockout (RAG1 ‒/‒)] and IL-12-deficient (p35 ‒/‒ and p40 ‒/‒; p35 and p40 are the two subunits of active IL-12) mice for 2 wk. RAG1 ‒/‒HC mice received splenocytes from WT-HC (WT → RAG1 ‒/‒), p35 ‒/‒-HC (p35 ‒/‒ → RAG1 ‒/‒), or p40 ‒/‒-HC (p40 ‒/‒ → RAG1 ‒/‒) mice. Compared with WT-ND mice, WT-HC mice exhibited exaggerated leukocyte adherence and emigration as well as increased dihydrorhodamine oxidation. The enhanced leukocyte recruitment was absent in the RAG1 ‒/‒-ND, p35 ‒/‒-ND, and p40 ‒/‒-ND groups. Hypercholesterolemia-induced leukocyte adherence and emigration were attenuated in RAG1 ‒/‒HC vs. WT-HC mice but were similar to ND mice. Furthermore, compared with WT-HC animals, p35 ‒/‒-HC and p40 ‒/‒-HC mice showed significantly lower leukocyte adherence and tissue oxidative stress responses, but these values were comparable to ND mice. Leukocyte adherence and emigration in WT → RAG1 ‒/‒ mice were similar to responses of WT-HC mice. However, p35 ‒/‒ → RAG1 ‒/‒ mice had lower levels of adherence and emigration vs. the WT → RAG1 ‒/‒ and WT-HC groups. Elevated levels of leukocyte adherence and emigration were restored by ∼50% toward WT-HC levels in p40 ‒/‒ → RAG1 ‒/‒ mice. These findings implicate IL-12 in the inflammatory responses observed in the venules of hypercholesterolemic mice.

HYPERCHOLESTEROLEMIA, a major risk factor for the development of cardiovascular disease, promotes the formation of atherosclerotic lesions through a process that involves multiple cell types and a variety of cytokines (5, 10). T lymphocytes and macrophages have been implicated in the lesion formation that is induced by hypercholesterolemia in both humans and mice (10, 14, 19). The major cytokine products expressed by these cells are interferon-γ (IFN-γ; Refs. 9, 12, 28) and interleukin 12 (IL-12, Refs. 13, 27) in mice. T lymphocyte-derived IFN-γ has also been implicated in the leukocyte-endothelial cell adhesion and oxidative stress that are observed in the microvasculature of hypercholesterolemic mice (21). These microvascular responses to hypercholesterolemia, which are often accompanied by reduced capacity of arterioles to dilate in response to endothelium-dependent dilators (e.g., ACh; Ref. 6) and increased endothelial cell adhesion molecule expression in postcapillary venules, occur long before atherosclerotic lesions are evident in large arteries (1, 17). Although it remains unclear whether these early changes in the microvasculature may contribute to the subsequent induction of inflammation and fatty lesions in large arteries, there is evidence that the hypercholesterolemic microcirculation is more vulnerable to the injurious effects of disease processes that are associated with an acute inflammatory response such as ischemia-reperfusion (23). Therefore, efforts to ablate the actions of IFN-γ and/or other cytokines may negate the deleterious consequences of hypercholesterolemia in both macroscopic and microscopically defined blood vessels.

The overall goal of this study was to determine whether IL-12, which is known to promote the polarization of T cells toward the IFN-γ-producing T helper 1 (Th1) phenotype, contributes to the induction of an inflammatory phenotype in the microcirculation of hypercholesterolemic mice. The specific objectives of the study were 1) to determine whether IL-12 mediates the oxidative stress and leukocyte-endothelial cell adhesion in postcapillary venules of hypercholesterolemic mice, and 2) to define the role of T lymphocytes in IL-12-mediated responses during hypercholesterolemia. Intravital videomicroscopy was used to monitor and quantify the adhesion and emigration of leukocytes [previously determined to be peroxidase-positive cells (20)] and oxidant stress in mouse cremasteric postcapillary venules. The involvement of IL-12 was assessed using hypercholesterolemic IL-12-deficient mice.
(p35−/− and p40−/−) mice (p35 and p40 are the two subunits of active IL-12). The contribution of T lymphocytes to the IL-12-mediated responses was evaluated using hypercholesterolemic, lymphocyte-deficient, recombinase-activating gene knockout (RAG1−/−) mice that were reconstituted with splenocytes derived from either wild-type (WT), p35−/−, or p40−/− mice. Our findings indicate that IL-12 contributes significantly to the leukocyte-endothelial cell adhesion and oxidant stress induced in postcapillary venules by hypercholesterolemia, and that IL-12 acts through a lymphocyte-dependent pathway.

METHODS

Animals. Wild-type C57BL/6J mice, B6.129S-RAG11−/−/Il2rgom (RAG1−/−), B6.129S1-H12a1−/−/Il2rgom (p35−/−), and B6.129-H12b−/−/Il2rgom (p40−/−) mice were obtained from Jackson Laboratory (Bar Harbor, ME). At 5–6 wk of age, mice were placed on either a normal (ND) or high-cholesterol (HC) diet (Teklad 90221, which contains 1.25% cholesterol, 0.125% choline chloride, and 15.8% fat; Harlan Teklad; Madison, WI) for 2 wk (n = 5 or 6 mice/group). RAG1−/− mice on a HC diet were divided into four groups as follows: 1) RAG1−/−/HC: RAG1−/− mice maintained on a HC diet for 2 wk; 2) WT → RAG1−/−/HC diet reconstituted with splenocytes from WT-HC mice; 3) p35−/− → RAG1−/−/HC: RAG1−/−/HC diet reconstituted with splenocytes from p35−/−/HC mice; and 4) p40−/− → RAG1−/−/HC: RAG1−/−/HC diet reconstituted with splenocytes from p40−/−/HC mice.

Reconstitution. The spleen was removed from the donor mouse and placed in cold PBS. The tissue was gently scraped through a screen (E-C Apparatus; St. Petersburg, FL) and suspended in cold PBS. Red blood cells were lysed and the splenocyte pellet was resuspended in cold PBS at a concentration of 2.5 × 10⁷ cells/ml. Recipient mice were injected intraperitoneally at day 9 of a HC diet with 0.2 ml of splenocyte suspension (5 × 10⁷ cells) and were allowed to recover for 5 days.

Surgical protocol. Mice were anesthetized with ketamine hydrochloride (150 mg/kg body wt ip) and xylazine (7.5 mg/kg body wt ip). The right jugular vein was cannulated for administration of heparinized saline, and the left carotid artery was cannulated for systemic arterial pressure measurement using a pressure transducer and monitor (model BP1-B, World Precision Instruments; Sarasota, FL). Core body temperature was maintained by infrared lamp at 35 ± 0.5°C using an intratracheal probe. Animal-handling procedures were approved by the Louisiana State University Health Sciences Center Institutional Animal Care and Use Committee and were in accordance with the guidelines of the American Physiological Society.

Intravital microscopy. The mouse was placed in a supine position on a Plexiglas microscope stage. The right cremaster muscle was isolated, and microcirculation was monitored as previously described (22). Postcapillary venules were selected for observation and data collection. Venular diameter (Dv) was measured online using a video caliper (Microcirculation Research Institute, Texas A&M University; College Station, TX). Centerline red blood cell velocity (Vrbc) was measured online using an optical Doppler velocimeter (Microcirculation Research Institute). Venular blood flow (VBFmean) was calculated as follows: VBFmean = Vrbc/Dv. Leukocyte rolling velocity (Vwbc) and the number of rolling and adherent leukocytes were quantified in the cremaster muscle during playback of videotaped images. Rolling leukocytes were defined as white blood cells that move at a velocity less than that of red blood cells in the same vessel. Rolling leukocyte flux was determined as the number of leukocytes per minute rolling past a specific point within the venule (in no./min), and Vwbc was determined from the average time required for an individual leukocyte to move along 100 μm of the microvessel (in μm/s). A leukocyte was defined as adherent to venular endothelium if it remained stationary for ≥30 s (in no./100 μm) and was measured throughout the observation period. Leukocyte emigration was measured online at the end of each 5-min observation period. Emigrated leukocytes were expressed as the number of interstitial leukocytes per high-powered field of view adjacent to the segment under observation (in no./field).

Experimental protocol. Postcapillary venules with a WSR of ≥500/s and Dv between 20 and 40 μm were observed. The venule with the least number of adherent and emigrated leukocytes at the end of a 30-min stabilization period was chosen for the study. Five-minute recordings were made of the first 100 μm of every 300 μm along the length of the unstimulated vessel beginning as near to the source of the venule as possible. The mean value of each parameter in a single venule was calculated, and comparisons were made between the experimental groups.

Dihydrorhodamine oxidation. Separate groups of WT-ND, WT-HC, p35−/−-ND, p35−/−-HC, p40−/−-ND, and p40−/−-HC mice were prepared for intravital microscopy, and the cremaster muscle was allowed to stabilize as described. Background fluorescence (Ibg0) of the first 100 μm of every 300 μm was recorded along the length of the selected postcapillary venule using a xenon light source and a fluorescence camera and intensifier (Hamamatsu). Freshly prepared dihydorhodamine (DHR) 123 (1 mM; a nonfluorescent dye that is oxidized to the fluorescent compound rhodamine 123) in bicarbonate-buffered saline was superfused over the cremaster muscle for 15 min. The tissue was then washed with bicarbonate-buffered saline, and the fluorescent image of each section was recorded (IDHR). Images were captured onto computer and an area 100 μm long and twice the vessel width was analyzed for each section using NIH Image 1.62 software as previously described (21). The Ibg0-to-Ibg ratio was calculated for each section, the average ratio for each animal was determined, and comparisons were made between the six experimental groups.

Blood lymphocyte counts. At the end of each experiment, blood was drawn from the heart and 25 μl was mixed with 465 μl of 3% acetic acid and 10 μl of 1% crystal violet. The circulating blood lymphocyte count was performed with the aid of a hemocytometer.

Serum cholesterol levels. Serum was frozen for subsequent measurement of total cholesterol levels in a colorimetric endpoint assay. Briefly, serum samples and standards were mixed with Infinity cholesterol reagent (Sigma Chemicals; St. Louis, MO) and the absorbance measurements were read at a primary wavelength of 500 nm and a secondary wavelength of 660 nm. Serum cholesterol levels of the mice were calculated from a regression plot generated from the standards. All samples were performed in duplicate.

Statistical analysis. All values are reported as means ± SE. ANOVA with a Bonferroni/Dunn post hoc test was used to compare between groups.
RESULTS

Serum cholesterol levels. There was a significant elevation of serum cholesterol levels in all groups of animals placed on a cholesterol-enriched diet for 2 wk when compared with their ND counterparts (Table 1). WT-HC mice exhibited almost a threefold increase in serum cholesterol levels vs. the WT-ND group. Similarly, p35/− and p40/− mice maintained on a HC diet demonstrated a significant elevation of serum cholesterol levels vs. normocholesterolemic groups. Compared with the RAG1/−-ND animals, all lymphocyte-deficient RAG1/−-HC groups showed significantly higher serum cholesterol levels.

Venular WSR. Table 1 shows that no significant differences in WSRs existed between any of the experimental groups, i.e., WT, p35/−, p40/−, ND, or HC.

Peripheral blood lymphocyte count. There was a small but nonsignificant increase in the number of circulating lymphocytes observed in hypercholesterolemic WT animals compared with their normocholesterolemic counterparts (Table 1). A similar pattern was noted in IL-12 knockout animals (both p35/− and p40/− mice). As would be expected, the lymphocyte-deficient RAG1/−-ND mice exhibited a significantly lower number of circulating lymphocytes compared with WT or IL-12 knockout animals (P < 0.005). The lymphocyte deficiency in RAG1/−-ND mice was unaltered by hypercholesterolemia (P < 0.005 vs. WT or IL-12 knockout mice). Administration of splenocytes to RAG1/−-HC mice failed to restore circulating lymphocyte populations in any of the reconstituted groups.

Leukocyte-endothelial interactions. Both leukocyte rolling flux and \( V_{\text{rbc}} \) values remained unchanged by hypercholesterolemia in WT mice (data not shown). IL-12 knockout animals (p35/− or p40/−) similarly showed no change in rolling parameters (data not shown). In hypercholesterolemic IL-12-deficient groups revealed that p35/−-HC mice exhibited significantly reduced levels of leukocyte adhesion compared with WT-HC animals (P < 0.0001). Similarly, in the p40/−-HC group, leukocyte adhesion was significantly decreased to ND levels (P < 0.0001 vs. WT-HC; Fig. 1A). Leukocyte emigration showed a similar pattern of responses to leukocyte adhesion (Fig. 1B) when compared with the normocholesterolemic counterparts (P < 0.0001). Examination of the IL-12-deficient mouse strains revealed that p35/−-HC mice exhibit significantly reduced levels of leukocyte adhesion compared with WT-HC animals (P < 0.0001). Similarly, in the p40/−-HC group, leukocyte adhesion was significantly decreased to ND levels (P < 0.0001 vs. WT-HC; Fig. 1A). Leukocyte emigration showed a similar pattern of responses to leukocyte adhesion (Fig. 1B). Leukocyte emigration in both the p35/−-HC and p40/−-HC groups was significantly attenuated compared with WT-HC mice (P < 0.0001). Indeed, leukocyte emigration in both hypercholesterolemic IL-12-deficient groups remained at levels comparable to the corresponding ND animals.

Table 1. Serum cholesterol concentration, venular wall shear rate, and circulating blood lymphocyte counts in wild-type, recombinase-activating gene-knockout, interleukin 12-knockout mice maintained on a normal or high-cholesterol diet for 2 wk

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum Cholesterol (mg/dl)</th>
<th>Wall Shear Rate (s⁻¹)</th>
<th>Blood Leukocyte Count (no./µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-ND</td>
<td>69.3 ± 5.32</td>
<td>803 ± 104.2</td>
<td>5,030 ± 268.6</td>
</tr>
<tr>
<td>WT-HC</td>
<td>179.6 ± 7.95</td>
<td>624 ± 62.3</td>
<td>5,530 ± 1,062.1</td>
</tr>
<tr>
<td>RAG1/−-ND</td>
<td>79.5 ± 3.12</td>
<td>831 ± 91.3</td>
<td>100 ± 28.9†</td>
</tr>
<tr>
<td>RAG1/−-HC</td>
<td>190.0 ± 13.26</td>
<td>800 ± 109.2</td>
<td>333 ± 185.6†</td>
</tr>
<tr>
<td>WT → RAG1/−</td>
<td>158.1 ± 11.99</td>
<td>749 ± 96.3</td>
<td>150 ± 57.7†</td>
</tr>
<tr>
<td>p35/−-ND</td>
<td>59.2 ± 9.39</td>
<td>835 ± 76.7</td>
<td>4,325 ± 371.2</td>
</tr>
<tr>
<td>p35/−-HC</td>
<td>152.0 ± 11.62</td>
<td>974 ± 207.6</td>
<td>5,730 ± 1,040.6</td>
</tr>
<tr>
<td>p40/−-ND</td>
<td>70.3 ± 3.15</td>
<td>775 ± 107.4</td>
<td>5,251 ± 437.2</td>
</tr>
<tr>
<td>p40/−-HC</td>
<td>159.2 ± 18.22</td>
<td>813 ± 126.3</td>
<td>4,585 ± 497.5</td>
</tr>
<tr>
<td>p35/− → RAG1/−</td>
<td>214.1 ± 15.64</td>
<td>681 ± 69.8</td>
<td>75 ± 106.1†</td>
</tr>
<tr>
<td>p40/− → RAG1/−</td>
<td>165.9 ± 25.36</td>
<td>674 ± 61.5</td>
<td>130 ± 36.7†</td>
</tr>
</tbody>
</table>

Values are means ± SE. Several groups of mice received splenocytes from donor WT, p35/−, or p40/− mice 5 days before experimentation (donor → recipient); animals were HC unless otherwise stated. WT, wild type; ND, normal diet; RAG, recombinase-activating gene; HC, high cholesterol. *P < 0.005 vs. respective ND groups; †P < 0.005 vs. non-RAG1/− groups.

Fig. 1. Effects of hypercholesterolemia on the number of adherent (A) and emigrated (B) leukocytes in normal wild-type (WT) and interleukin 12 (IL-12) knockout (p35/− and p40/−) mice placed on normal (ND) or high-cholesterol (HC) diet for 2 wk. Adherence and emigration of leukocytes were significantly higher in the WT-HC group compared with the WT-ND group (*P < 0.0001). These increases were prevented in HC mice that lacked either p35 (p35/−-HC) or p40 (p40/−-HC; †P < 0.0001 vs. WT-HC).
In RAG1−/− mice, neither rolling flux nor V_ebc values were changed after placement on HC for 2 wk; i.e., these leukocyte rolling parameters were comparable to those observed in WT mice (data not shown). RAG1−/−-ND animals exhibited similar levels of leukocyte adhesion as those found in the WT-ND group. However, in RAG1−/−-HC mice, the increment in leukocyte adhesion normally produced by HC was not observed, and the level of adhesion was comparable to that found in WT-ND and RAG1−/−-ND groups (Fig. 2A; P < 0.0001 vs. WT-HC). The hypercholesterolemia-induced elevation of leukocyte adhesion was fully restored to WT-HC levels in RAG1−/−-HC mice that received splenocytes from WT-HC mice 5 days before observation (WT → RAG1−/− group; P < 0.0001 vs. WT-ND and RAG1−/−-ND). The findings were similar for leukocyte emigration (Fig. 2B). RAG1−/−-ND mice exhibited low levels of leukocyte emigration comparable to normocholesterolemic wild types. In the RAG1−/−-HC group, leukocyte emigration was reduced to ND levels (P < 0.0001 vs. WT-HC). The normal leukocyte emigration responses to 2 wk of HC feeding were restored in the RAG1−/− mice that received splenocytes from WT-HC mice 5 days before the experiment (WT → RAG1−/− group; Fig. 2B; P < 0.0001 vs. RAG1−/−-HC).

When RAG1−/−-HC mice were reconstituted with splenocytes from p35−/−-HC animals, there was no restoration of the leukocyte adhesion response to hypercholesterolemia (Fig. 3A; P < 0.001 vs. WT-HC and WT → RAG1−/− groups). In fact, leukocyte adhesion remained at levels comparable to RAG1−/−-HC, p35−/−-HC, and WT-ND mice. The transfer of p40−/−-HC splenocytes into RAG1−/−-HC mice led to an ~50% restoration of the leukocyte adhesion response, which was significantly greater than adhesion in p40−/−-HC mice (P < 0.005) but still lower than the adhesion levels seen in WT-HC or WT → RAG1−/− mice (P < 0.005). A similar pattern of responses was noted for leukocyte emigration (Fig. 3B). When p35−/−-HC splenocytes were transferred into RAG1−/−- mice, leukocyte emigration remained at ND levels comparable to RAG1−/−-HC and p35−/−-HC mice (P < 0.0001 vs. WT-HC and WT → RAG1−/− groups). RAG1−/−-HC mice receiving splenocytes from p40−/−-HC mice (p40−/− → RAG1−/− group) exhibited significantly more leukocyte emigration than was observed in the p40−/−-HC or p35−/− → RAG1−/− groups (P < 0.0005; Fig. 3B). However, this restoration of leukocyte emigration was incomplete, with emigration in the p40−/− → RAG1−/− group remaining significantly lower than that observed in either WT-HC or WT → RAG1−/− mice (P < 0.005).

Oxidative stress. WT-HC mice, when compared with their normocholesterolemic counterparts, exhibited significantly enhanced oxidative stress as indicated by DHR oxidation (P < 0.0005; Fig. 4). Oxidative stress in p35−/− mice maintained on a ND was similar to levels seen in WT-ND animals. However, when p35−/− mice were placed on a HC diet for 2 wk, an HC-induced oxidative stress was not observed (P < 0.0005 vs. WT-HC) and DHR oxidation was comparable to that observed in WT-ND mice. Similarly, p40−/−-ND mice exhibited DHR oxidation values that were equivalent to other normocholesterolemic groups. However, p40−/−-HC mice showed an attenuated DHR oxidation response to hypercholesterolemia (P < 0.0005 vs. WT-HC) similar to that seen in the p35−/−-HC mice. These findings indicate that deletion of either the p35 or p40 components of IL-12 blunts the hypercholesterolemia-induced oxidative stress in postcapillary venules.

DISCUSSION

There is a growing body of evidence that the deleterious actions of hypercholesterolemia in the cardiovascular system are not limited to the arterial tree. Postcapillary venules respond to hypercholesterolemia with endothelial cell dysfunction that is manifested as
cytes (WT-3) prepared with the WT-HC and WT showed significantly lower adherence and emigration responses compared with their ND counterparts (*P < 0.0005 vs. WT-ND). This response was abolished in mice deficiency accelerated formation of atherosclerotic lesions both IFN-γ and IL-12 in human atherosclerotic lesions and in the aortas of hypercholesterolemic apoE-deficient mice (8, 13); 2) presence of T cells in human atherosclerotic lesions that are situated in close proximity to macrophages that stain positive for IL-12 (27); and 3) accelerated formation of atherosclerotic lesions with enhanced lymphocyte infiltration and expression of IFN-γ in young apoE-deficient mice receiving recombinant IL-12 daily for a month (13). IL-12 plays a key role in the modulation of both the innate and adaptive immune responses. It is primarily produced by phagocytic cells, particularly monocytes, although it may also be synthesized by neutrophils (3) and endothelial cells (15). A chemotactic role for IL-12 has been identified for neutrophils, and this chemotactic property is dependent on superoxide release from the neutrophil (2). In addition, IL-12 promotes a Th1-type phenotype in T lymphocytes, which stimulates the release of IFN-γ from these cells (25). IFN-γ in turn is capable of stimulating the release of IL-12 from monocytes (16), thereby allowing for positive-feedback regulation between IL-12 and IFN-γ during inflammation.

In the present study, the influence of IL-12 deficiency on hypercholesterolemia-induced inflammation was evaluated using mice that are genetically deficient in either the p35 or p40 subunit of IL-12. The active form an increased adhesion of neutrophils and platelets to the vessel wall, and an enhanced production of reactive oxygen species including superoxide anion (21, 24). The recruitment of adherent and extravasating neutrophils in venules that occurs within 2 wk of the onset of hypercholesterolemia appears to be mediated by a mechanism that involves circulating T lymphocytes (both CD4+ and CD8+ T cells; Ref. 20), IFN-γ (largely derived from T cells), and oxidative stress (21). The existing evidence in the literature suggests that T-cell-derived IFN-γ is an important stimulus for enhanced NADPH oxidase-derived superoxide (11), which ultimately promotes neutrophil adhesion. In the present study, we further explored this immune mechanism of hypercholesterolemia-induced microvascular dysfunction by assessing the potential contribution of IL-12, a cytokine that has been closely linked to IFN-γ in atherogenesis (13).

There are several lines of evidence in the literature that suggest a possible role for IL-12 in the inflammation and microvascular dysfunction associated with hypercholesterolemia. These include 1) detection of both IFN-γ and IL-12 in human atherosclerotic lesions (5, 27) and in the aortas of hypercholesterolemic apoE-deficient mice (8, 13); 2) presence of T cells in human atherosclerotic lesions that are situated in close proximity to macrophages that stain positive for IL-12 (27); and 3) accelerated formation of atherosclerotic lesions with enhanced lymphocyte infiltration and expression of IFN-γ in young apoE-deficient mice receiving recombinant IL-12 daily for a month (13). IL-12 plays a key role in the modulation of both the innate and adaptive immune responses. It is primarily produced by phagocytic cells, particularly monocytes, although it may also be synthesized by neutrophils (3) and endothelial cells (15). A chemotactic role for IL-12 has been identified for neutrophils, and this chemotactic property is dependent on superoxide release from the neutrophil (2). In addition, IL-12 promotes a Th1-type phenotype in T lymphocytes, which stimulates the release of IFN-γ from these cells (25). IFN-γ in turn is capable of stimulating the release of IL-12 from monocytes (16), thereby allowing for positive-feedback regulation between IL-12 and IFN-γ during inflammation.

In the present study, the influence of IL-12 deficiency on hypercholesterolemia-induced inflammation was evaluated using mice that are genetically deficient in either the p35 or p40 subunit of IL-12. The active form of post-dihydrorhodamine (DHR) fluorescence intensity to background fluorescence intensity (I_{DHR}/I_{Rgd}) in normal (WT) and IL-12 knockout (p35−/− and p40−/−) mice on ND or HC diet for 2 wk. Adherence and emigration responses to hypercholesterolemia were comparable to WT-HC levels in RAG1−/−HC mice that were reconstituted with WT-HC splenocytes (WT → RAG1−/− group). However, RAG1−/−HC mice reconstituted with p35−/−HC splenocytes (p35−/− → RAG1−/− group) showed significantly lower adherence and emigration responses compared with the WT-HC and WT → RAG1−/−HC groups (#P < 0.001) but were similar to p35−/−HC mice. Conversely, RAG1−/−HC mice reconstituted with p40−/−HC splenocytes (p40−/− → RAG1−/− group) exhibited 50% restoration of adhesion and emigration responses (P < 0.005 vs. WT-HC and WT → RAG1−/− groups and P < 0.005 vs. p40−/−HC group).

Fig. 3. Effects of hypercholesterolemia on the number of adherent (A) and emigrated (B) leukocytes in WT, IL-12 knockout (p35−/− and p40−/−), and reconstituted lymphocyte-deficient (RAG1−/−) mice placed on ND or high-HC diet for 2 wk. Adherence and emigration responses to hypercholesterolemia were comparable to WT-HC levels in RAG1−/−HC mice that were reconstituted with WT-HC splenocytes (WT → RAG1−/− group). However, RAG1−/−HC mice reconstituted with p35−/−HC splenocytes (p35−/− → RAG1−/− group) showed significantly lower adherence and emigration responses compared with the WT-HC and WT → RAG1−/−HC groups (#P < 0.001) but were similar to p35−/−HC mice. Conversely, RAG1−/−HC mice reconstituted with p40−/−HC splenocytes (p40−/− → RAG1−/− group) exhibited 50% restoration of adhesion and emigration responses (P < 0.005 vs. WT-HC and WT → RAG1−/− groups and P < 0.005 vs. p40−/−HC group).

Fig. 4. Effects of hypercholesterolemia on tissue oxidative stress (ratio of post-dihydrorhodamine (DHR) fluorescence intensity to background fluorescence intensity (I_{DHR}/I_{Rgd}) in normal (WT) and IL-12 knockout (p35−/− and p40−/−) mice on ND or HC diet for 2 wk. Tissue oxidative stress was significantly elevated in WT-HC mice compared with their ND counterparts (*P < 0.0005 vs. WT-ND). This response was abolished in mice deficient in either IL-12 subunit (p35−/−HC or p40−/−HC; #P < 0.0005 vs. WT-HC).
of IL-12 is the heterodimer p70, which is made of two covalently linked subunits, p35 and p40. Mice deficient in either subunit cannot secrete active IL-12. Our finding that a deficiency in either p35 or p40 results in a profound attenuation of hypercholesterolemia-induced leukocyte-endothelial cell adhesion and oxidative stress in postcapillary venules strongly implicates a role for lymphocyte-derived IFN-γ. We previously reported that hypercholesterolemic SCID mice exhibit an attenuated recruitment of adherent and emigrating neutrophils in cremasteric venules, compared with their WT counterparts (20). Furthermore, we showed that reconstitution of severe combined immunodeficiency mice with splenocytes harvested from WT but not IFN-γ−/− mice restored the inflammatory phenotype that is normally seen with hypercholesterolemia (21). In the present study, we demonstrate a similar attenuation of the inflammatory responses to hypercholesterolemia in lymphocyte-deficient RAG1−/− and full restoration of the responses after reconstitution with splenocytes from WT mice. The reduced inflammation found in RAG1−/−/HC mice was independent of changes in total cholesterol levels. Indeed, although one could argue that alterations in individual lipoprotein levels, in particular low-density lipoprotein, may explain the protection in these mice, this is unlikely, because Song et al. (19) did not find significant alterations in low-density lipoprotein cholesterol of lymphocyte-deficient mice vs. immunocompetent mice in a model of atherosclerosis. Thus these findings add further support to a role for lymphocytes in microvascular inflammatory responses to hypercholesterolemia. When the possibility of a link between IL-12-dependent and lymphocyte-dependent pathways was investigated using RAG1−/−/HC mice that received splenocytes from p35−/−/HC or p40−/−/HC mice, our findings were consistent with a role for IL-12 in the lymphocyte-mediated inflammatory responses to hypercholesterolemia. Because our previous work also implicates a role for lymphocyte-derived IFN-γ in this model system, it appears likely that a major action of IL-12 in hypercholesterolemia is to induce IFN-γ production by T lymphocytes. We previously demonstrated (21) that IFN-γ levels were elevated in the serum of WT-HC mice when compared with WT-ND animals. However, the changes in serum IFN-γ levels approached the resolution of the assay; therefore, although it would be relevant data for this study, we are reluctant to draw conclusions based on that approach here.

An interesting and potentially important observation in the present study is that reconstitution of RAG1−/−/HC mice with p40−/−/HC splenocytes (p40−/− → RAG1−/− group) resulted in a 50% restoration of the inflammatory phenotype (leukocyte adhesion/emigration), whereas reconstitution with p35−/−/HC splenocytes (p35−/− → RAG1−/− group) showed no such effect (Fig. 3). It is unlikely that the discrepancy in the inflammatory responses between the p35−/− → RAG1−/− and p40−/− → RAG1−/− groups can be simply explained by the fact that T cells from IL-12-deficient animals become unresponsive to IL-12 and differentiate toward a Th2 phenotype from which they cannot be “rescued” by subsequent stimulation with IL-12. If this were the case, both groups should have yielded a similar response. A more likely explanation of our findings with the p35−/− → RAG1−/− and p40−/− → RAG1−/− experiments relates to the facts that the production of p35 and p40 are independently regulated and the synthesis of active IL-12 (a p70 heterodimer) is more dependent on the transcription of p40 (26). Indeed, it has been demonstrated that the expression of p40 is correlated with the secretion of the active form (p70) of IL-12 (18). RAG1−/− mice should be able to produce IL-12 in response to hypercholesterolemia because they still possess monocytes and neutrophils. In the absence of T lymphocytes (RAG1−/− mice), IL-12 cannot bind to T cells and stimulate the production of IFN-γ. However, upon injection of splenocytes from WT mice, the transferred T lymphocytes would be exposed to IL-12 in the recipient and consequently respond by producing and secreting IFN-γ, thereby enabling the hypercholesterolemic RAG1−/− mouse to exhibit the inflammatory phenotype. Furthermore, splenocytes harvested from WT hypercholesterolemic mice have most likely existed in a proinflammatory environment (including exposure to IL-12 and IFN-γ) and may already be partially or fully activated. Hence, their transfer into RAG1−/−/HC recipients fully restores the inflammatory phenotype. Conversely, T cells harvested from the hypercholesterolemic IL-12-deficient mice likely experience a less-intense inflammatory environment before transfer into RAG1−/− animals and thereby elicit a diminished inflammatory response in the recipient mice.

The question then arises as to why splenocytes from p40−/− mice partially restore the inflammatory response in RAG1−/− mice, whereas the p35−/− splenocytes do not. The answer may relate to the fact that p40 is capable of forming homodimers that may act as an antagonist to functional IL-12 (p70) (7). If hypercholesterolemia promotes p40 production in p35−/− mice as it does in WT mice (13), then the resulting (p40)2 homodimers may bind to T cells and occupy the receptors without stimulation. Because p35 is not secreted in the absence of p40, T cells from p40−/− mice do not secrete p35 (4). Consequently, splenocytes from p40−/− mice should be responsive to IL-12 formed in the recipient RAG1−/− mice and could initiate at least a partial response to hypercholesterolemia.

We have previously demonstrated that hypercholesterolemia is associated with oxidative stress in postcapillary venules (21) and that NADPH oxidase-derived superoxide mediates the leukocyte recruitment in this model (22). Furthermore, venules in mice that are genetically deficient in IFN-γ, which is a cytokine known to stimulate NADPH oxidase in leukocytes (11), exhibit a significantly attenuated oxidative stress during hypercholesterolemia (21). In the present study, we extend our earlier observations by demonstrating that IL-12-deficient animals also respond to hypercholester-
cholerasteraemia with an attenuated oxidative stress. Collectively, these findings support an intimate interaction between IFN-γ and IL-12 in mediating the inflammatory phenotype in hypercholesterolemia and are consistent with the possibility that IL-12 is critical for the induction of a Th1-type phenotype in T lymphocytes, which results in enhanced production and release of IFN-γ and ultimately elicits the oxidative stress and leukocyte-endothelial cell interactions observed in the hypercholesterolemia microvasculature.

DISCLOSURE

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REFERENCES


