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CD40/CD40L contributes to hypercholesterolemia-induced microvascular inflammation

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Stokes KY, Calahan L, Hamric CM, Russell JM, Granger DN. CD40/CD40L contributes to hypercholesterolemia-induced microvascular inflammation. *Am J Physiol Heart Circ Physiol* 296: H689–H697, 2009. First published December 26, 2008; doi:10.1152/ajpheart.00962.2008.—Hypercholesterolemia is associated with phenotypic changes in endothelial cell function that lead to a proinflammatory and prothrombotic state in different segments of the microvasculature. CD40 ligand (CD40L) and its receptor CD40 are ubiquitously expressed and mediate inflammatory responses and platelet activation. The objective of this study was to determine whether CD40/CD40L, in particular T-cell CD40L, contributes to microvascular dysfunction induced by hypercholesterolemia. Intravital microscopy was used to quantify blood cell adhesion in cremasteric postcapillary venules, endothelium-dependent vasodilation responses in arterioles, and microvascular oxidative stress in wild-type (WT) C57BL/6, CD40-deficient ($^{-/-}$), CD40L $^{-/-}$, or severe combined immune deficient (SCID) mice placed on a normal (ND) or high-cholesterol (HC) diet for 2 wk. WT-HC mice exhibited an exaggerated leukocyte and platelet recruitment in venules and impaired vasodilation responses in arterioles compared with ND counterparts. A deficiency of CD40, CD40L, or lymphocytes attenuated these responses to HC. The HC phenotype was rescued in CD40L $^{-/-}$ and SCID mice by a transfer of WT T cells. Bone marrow chimeras revealed roles for both vascular- and blood cell-derived CD40 and CD40L in the HC-induced vascular responses. Hypercholesterolemia induced an oxidative stress in both arterioles and venules of WT mice, which was abrogated by either CD40 or CD40L deficiency. The transfer of WT T cells into CD40L $^{-/-}$ mice restored the oxidative stress. These results implicate CD40/CD40L interactions between circulating cells and the vascular wall in both the arteriolar and venular dysfunction elicited by hypercholesterolemia and identify T-cell-associated CD40L as a key mediator of these responses.

T lymphocytes; platelets; oxidative stress; arteriolar vasodilation; leukocyte adhesion

HYPERCHOLESTEROLEMIA IS A well-characterized cardiovascular risk factor that is known to initiate inflammatory and thrombotic responses in the microvasculature (34, 35, 41, 42). These vascular reactions reflect a systemic low-level inflammatory phenotype that appears to predispose the microvasculature to larger injury responses following an exposure to other inflammatory stimuli, such as ischemia-reperfusion (14, 17). Most of the mechanisms that have been implicated in the microvascular and inflammatory responses to acute elevations in blood cholesterol concentration also appear to contribute to the development of large vessel disease that accompanies chronic hypercholesterolemia (3, 9, 12, 35, 36, 40, 43). Immune cell activation and platelet-mediated responses are two

noteworthy examples of mechanisms that appear to underlie both the large vessel and microvascular response to hypercholesterolemia. A signaling system that is linked to inflammation and the activation of both T lymphocytes and platelets is the CD40/CD40 ligand (CD40L) dyad. Whereas this receptor-ligand pair has been implicated in the initiation and progression of atherosclerotic lesions (21, 22, 31), the contribution of CD40/CD40L to the proinflammatory and prothrombotic phenotype assumed by the microvasculature during hypercholesterolemia remains unclear and is the subject of this study.

CD40, a member of the TNF receptor superfamily, and its ligand CD40L are ubiquitously expressed on cell types that have been implicated in the inflammatory and thrombotic processes observed during hypercholesterolemia, including endothelial cells, T lymphocytes, and platelets (30). CD40 is constitutively expressed on these cells and can be upregulated by inflammatory stimuli such as cytokines, whereas CD40L is not normally expressed under control conditions but is induced following cell activation. Endothelial monolayers stimulated with CD40L exhibit an elevated superoxide generation (5, 45) and an upregulation of adhesion molecules (15, 16, 24, 33), and they release chemokines that promote the transmigration of neutrophils and mononuclear cells (44). The ligation of CD40 on endothelial cells (20) or macrophages (23) also leads to the synthesis of interleukin (IL)-12, a potent inducer of IFN- γ . Both IL-12 and IFN- γ have been implicated in hypercholesterolemia-induced microvascular inflammation and oxidative stress (37, 38, 40). It is noteworthy that IFN- γ can be released from T lymphocytes through a CD40-CD40L-dependent mechanism (26) and that IFN- γ can upregulate CD40 on endothelial cells (8), suggesting that CD40/CD40L signaling can lead endothelial cells to assume an inflammatory phenotype through both indirect (through T lymphocytes and IFN- γ) and direct (engagement of endothelial CD40) pathways.

CD40/CD40L is also likely to contribute to the platelet responses elicited by hypercholesterolemia. This possibility is supported by reports describing elevated CD40L expression on platelets and increased plasma levels of soluble CD40L (sCD40L) in hypercholesterolemic patients (7, 29, 32) and mice (19). sCD40L can initiate inflammatory responses by interacting with CD40 on endothelial cells and/or leukocytes. Similarly, the binding of sCD40L to platelet CD40 results in cell activation, the expression of P-selectin on platelets, and the subsequent binding of platelets to leukocytes (13). Such CD40/CD40L-dependent platelet responses may have important implications in the microvascular dysfunction caused by hypercholesterolemia since it has been previously shown that

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the recruitment of adherent platelets in postcapillary venules largely reflects platelet-leukocyte binding (42) and since platelet P-selectin mediates this cell-cell adhesion as well as the arteriolar dysfunction observed in hypercholesterolemic mice (35).

Thus the objectives of this study were to determine whether 1) CD40-CD40L signaling contributes to the inflammatory and thrombogenic responses and impaired vasomotor function in the microvasculature during high cholesterol (HC), 2) platelet-associated CD40 and/or CD40L mediate HC-induced platelet recruitment, 3) HC-induced blood cell recruitment and arteriolar dysfunction are dependent on blood cell and/or vascular wall CD40/CD40L, and 4) these responses and the accompanying oxidative stress are mediated by T-lymphocyte-associated CD40L.

METHODS

Animals. Wild-type (WT) C57BL/6J mice, CD45 congenic B6.SJL-PTPRCPEP/BOY mice (which express CD45.1), B6.CB17-*Prkdc^{scid}/SzJ* severe combined immune deficient mice (SCID), B6.129S2-*Tnfrsf5^{tm1mx}* mice (CD40L^{-/-}), and B6.129P2-*Tnfrsf5^{tm1Kik}* mice (CD40^{-/-}) (all on C57BL/6J background) were obtained from Jackson Laboratories (Bar Harbor, ME). Mice (5 to 6 wk old) were placed on either a normal (ND) or HC diet (Harlan Teklad, Madison, WI) for 2 wk. Two separate CD40L^{-/-}-HC groups and two separate SCID-HC groups received splenocytes from donor mice as detailed below (denoted as recipient ← splenocyte donor). Bone marrow transplants were performed between WT and CD40L^{-/-} or CD40^{-/-} mice as described below (denoted as bone marrow donor → recipient). The groups in this study were as follows: 1) WT mice maintained on ND (WT-ND), 2) WT mice placed on a HC diet for 2 wk (WT-HC), 3) CD40^{-/-} mice placed on a HC diet for 2 wk (CD40^{-/-}-HC), 4) CD40L^{-/-} mice placed on a HC diet for 2 wk (CD40L^{-/-}-HC), 5) CD40L^{-/-}-HC reconstituted with splenocytes from WT-HC mice (CD40L^{-/-} ← WT-HC), 6) CD40L^{-/-} mice that received T cells isolated from the WT-HC spleen (CD40L^{-/-} ← WT-HC T cells), 7) lymphocyte-deficient SCID mice placed on a HC diet for 2 wk (SCID-HC), 8) SCID-HC reconstituted with splenocytes from WT-HC mice (SCID ← WT-HC), 9) SCID-HC reconstituted with splenocytes from CD40L^{-/-}-HC mice (SCID ← CD40L^{-/-}-HC), 10) WT mice receiving bone marrow from CD45 congenic WT mice (WT → WT), 11) CD40^{-/-} mice receiving bone marrow from CD45 congenic WT mice (WT → CD40^{-/-}), 12) CD45 congenic WT mice receiving bone marrow from CD40^{-/-} mice (CD40^{-/-} → WT), 13) CD40L^{-/-} mice receiving bone marrow from CD45 congenic WT mice (WT → CD40L^{-/-}), and 14) CD45 congenic WT mice receiving bone marrow from CD40L^{-/-} mice (CD40L^{-/-} → WT).

Splenocyte transfer. The splenocyte isolation procedure and selection of T cells using a MACs system (Miltenyi Biotec, Auburn, CA) have been described previously (36, 40). Recipient mice were injected intraperitoneally with 0.2 ml of the whole splenocyte (50×10^6 cells) or T-cell (5×10^6 cells) suspension at 9 days HC and then allowed to recover for 5 days before intravital microscopy was performed at 2 wk HC.

Bone marrow chimeras. Bone marrow chimeras were generated as described previously (35, 39). Five combinations were used as listed above (denoted as donor → recipient). WT → WT chimeras were WT animals (which express CD45.2 on leukocytes) that received bone marrow cells from CD45 congenic mice, maintaining the expression of the CD40/CD40L dyad. This resulted in a significant increase of circulating leukocytes expressing CD45.1 (of donor origin) from <5% in WT to >90% in the WT → WT chimeras, allowing the verification of a proper chimera reconstitution by flow cytometry, as previously described (35). CD45 congenic bone marrow was transplanted into CD40^{-/-} mice, yielding WT → CD40^{-/-} chimeras with normal blood cell CD40 expression but a CD40-deficient vessel wall.

The CD40^{-/-} → WT chimeras with CD40-deficient blood cells but normal vascular wall expression were produced by transplanting bone marrow from a CD40^{-/-} mouse into a CD45 congenic mouse. WT → CD40L^{-/-} and CD40L^{-/-} → WT chimeras were generated in a similar manner with CD40L absent in either the vascular wall or the circulating cells, respectively.

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 the administration of platelets, and the left carotid artery was cannulated for systemic arterial pressure measurement. Core body temperature was maintained at $35 \pm 0.5^\circ\text{C}$. 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 cremaster muscle was prepared for intravital microscopy ($n = 5-7/\text{group}$) and superfused with bicarbonate-buffered saline at 1 ml/min as described previously (35). Postcapillary venules were selected for observation. A leukocyte was considered adherent if it remained stationary for ≥ 30 s (number/mm²) and was measured throughout the observation period.

Platelets. Approximately 0.9 ml of blood was collected from the donor via the carotid artery into 0.1 ml acid-citrate-dextrose (Sigma, St. Louis, MO). Platelet donors matched the source of the recipient's endogenous circulating platelets, unless otherwise stated. Platelets were isolated by a series of centrifugation steps as described previously (6). We have demonstrated that this technique does not lead to platelet activation, as assessed by P-selectin expression (43). Platelets were considered adherent if they arrested for ≥ 2 s, and the adhesion was expressed as the number of platelets per squared millimeter.

Experimental protocol. Postcapillary venules (20–40 μm diameter) with a wall shear rate (WSR) of $\geq 500/\text{s}$ were chosen for study. WSR was calculated from the velocity of red blood cells [measured using an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University)], and assuming cylindrical shape of the vessel as described previously (27). This threshold was selected based on previous reports describing a propensity for leukocytes to adhere in venules at low WSRs (27). The venule with the least number of adherent and emigrated leukocytes at the end of a 30-min stabilization was chosen for the study. Platelets (in a volume of 120 μl) were infused via the jugular vein over 5 min and allowed to circulate for a further 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 unstimulated vessel, beginning as near to the source of the venule as possible. The mean value of each variable within a single venule was calculated, and comparisons were made between the experimental groups.

Once the venule data were collected, the animals were allowed to stabilize for 20–30 min, and the arterioles with diameters between 15–40 μm and WSR of $\geq 500/\text{s}$ were chosen for study. The diameter and red blood cell velocity were measured in the chosen sections before and after superfusion with 10^{-5} M of the endothelium-dependent vasodilator acetylcholine (ACh) for 5 min. Arteriolar vasorelaxation responses to ACh were expressed as the percent diameter change versus that of baseline.

Dihydrorhodamine oxidation. Separate groups of WT-ND, WT-HC, CD40L^{-/-}-HC, CD40^{-/-}-HC, and CD40L^{-/-} ← WT-HC T cells were prepared for intravital microscopy ($n = 5$ to 6/group). Sections of venules and arterioles were chosen as before, and fluorescent images of each section were recorded before [background intensity (I_{Bgrd})] and after superfusion of 1 mM dihydrorhodamine (DHR)-123 for 15 min [DHR intensity (I_{DHR})], using a xenon light source and a fluorescent camera and intensifier (Hamamatsu, Japan). Images were captured onto a 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 (37, 38). The ratio of I_{DHR} to I_{Bgrd}

was calculated for each section, the average ratio for venules and for arterioles in each animal was determined, and comparisons were made between all groups.

Serum cholesterol levels. Serum was frozen for subsequent measurement of cholesterol levels using a spectrophotometric assay (Sigma, St. Louis, MO).

Circulating blood cell counts. At the end of each experiment, blood samples were obtained for platelet and leukocyte counts. Platelets were counted using the unopette system (BD Biosciences), and leukocytes (lymphocytes, monocytes, and polymorphonuclear cells) were counted using crystal violet as a nuclear stain.

Circulating cytokine measurement. The levels of inflammatory cytokines were measured in plasma obtained from separate groups of mice that received no platelets or saline that would dilute the blood ($n = 4-7/\text{group}$). Animals received heparin, which was allowed to circulate for 5 min before blood was drawn for the determination of cytokine levels using a cytometric bead assay as per the manufacturer's instructions (Mouse Inflammation CBA, BD Biosciences). Samples were analyzed on a FACSCaliber. Levels of IL-12, IFN- γ , TNF- α , IL-6, IL-10, and monocyte chemoattractant protein-1 were measured.

Statistical analysis. All values are reported as means \pm SE. ANOVA with Fisher post hoc test was used for statistical comparison of experimental groups, with a statistical significance set at $P < 0.05$.

RESULTS

Serum cholesterol levels increased two- to threefold over normocholesterolemic values in all strains of mice placed on a HC for 2 wk, i.e., WT, SCID, CD40 $^{-/-}$, and CD40L $^{-/-}$ mice, etc. (data not shown). All venules chosen for the study of leukocyte and platelet recruitment demonstrated comparable shear rates, regardless of mouse or diet regimen. There were no significant differences in blood lymphocyte, monocyte, or polymorphonuclear counts between WT and CD40 $^{-/-}$ or CD40L $^{-/-}$ or chimeric mice. SCID-HC mice had significantly fewer circulating lymphocytes when compared with WT or knockout mice. As shown previously (36, 37, 40), the transfer of splenocytes or T cells did not alter circulating leukocyte counts in either SCID or CD40L $^{-/-}$ recipients.

CD40/CD40L contributes to hypercholesterolemia-induced responses in postcapillary venules and arterioles. WT mice placed on a cholesterol-enriched diet for 2 wk exhibited a significantly greater leukocyte adhesion when compared with normocholesterolemic counterparts (Fig. 1A). This cholesterol-induced inflammatory response was attenuated to ND levels in CD40L $^{-/-}$ and CD40 $^{-/-}$ mice placed on HC. Platelets followed a similar pattern of adhesion when matched exogenous platelets were evaluated, i.e., the HC diet elicited an increased platelet adhesion in WT mice that was not detected in either CD40 $^{-/-}$ or CD40L $^{-/-}$ mice placed on the same diet (Fig. 1B). To determine whether the expression of these molecules on platelets was critical to the HC-induced platelet adhesion response, we monitored the adhesion of CD40L $^{-/-}$ and CD40 $^{-/-}$ platelets in WT-HC recipients (Fig. 1B). CD40L $^{-/-}$ platelets exhibited a modest but significant reduction in adhesion compared with WT-HC platelets administered to WT-HC recipients. In contrast, the adhesion of CD40 $^{-/-}$ platelets in WT-HC recipients was comparable with levels found in WT-ND mice, suggesting that platelet-associated CD40, and to a lesser extent CD40L, contributes to platelet adhesion.

Arterioles of mice placed on a HC diet for 2 wk exhibited impaired vasodilatory responses to ACh compared with WT-ND mice (Fig. 2). However, in both CD40 $^{-/-}$ -HC and

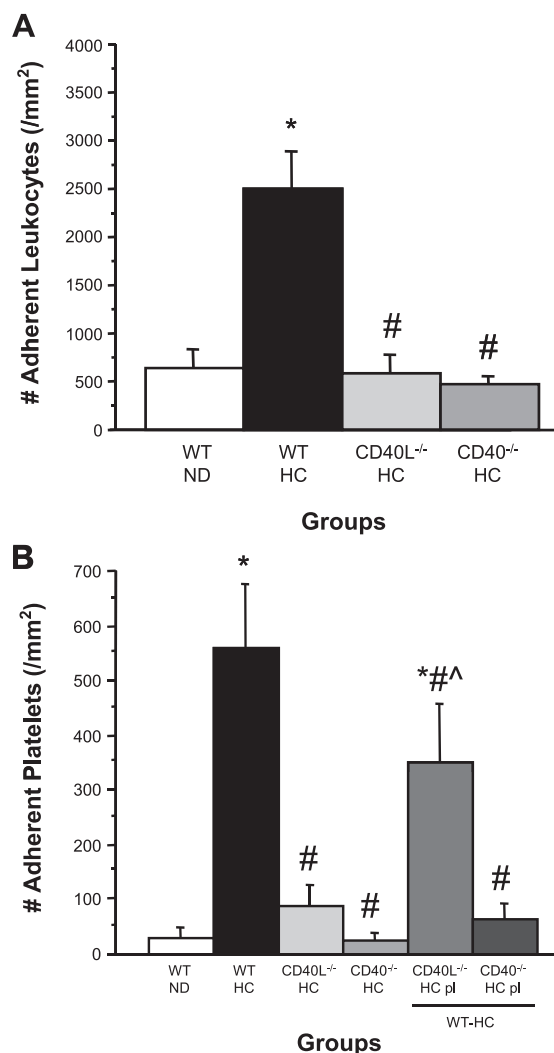


Fig. 1. Leukocyte (A) and platelet (B) adhesion in postcapillary venules of wild-type (WT), CD40L-deficient (CD40L $^{-/-}$), and CD40-deficient (CD40 $^{-/-}$) mice on normal (ND) or high-cholesterol (HC) diet for 2 wk. **B:** platelets were administered to matched recipients in the first 4 groups; the last 2 groups show adhesion of platelets (pl) from CD40L $^{-/-}$ and CD40 $^{-/-}$ donors, respectively monitored in WT-HC recipients. * $P < 0.05$ vs. WT-ND; # $P < 0.05$ vs. WT-HC; ^ $P < 0.05$ vs. CD40L $^{-/-}$.

CD40L $^{-/-}$ -HC mice, arteriolar responses were similar to levels observed in normocholesterolemic mice.

Circulating versus vascular sources of CD40 and CD40L. Due the ubiquitous nature of CD40 and CD40L expression, we used bone marrow chimeras to determine the relative contributions of circulating cell- versus vasculature-associated CD40 and CD40L to the HC-induced microvascular responses. WT mice that received WT bone marrow (WT \rightarrow WT-HC, our control chimera group) exhibited levels of leukocyte (Fig. 3A) and platelet (Fig. 3B) adhesion that were comparable with WT-HC mice. However, in chimeras where CD40L was absent either on the vasculature (WT \rightarrow CD40L $^{-/-}$) or on circulating cells (CD40L $^{-/-}$ \rightarrow WT), the HC-induced leukocyte and platelet adhesion responses were reduced to ND values and to levels seen in CD40L $^{-/-}$ -HC mice where the expression of this molecule is deleted on both circulating and vascular cells. The bone marrow chimeras produced using CD40 $^{-/-}$ mice re-

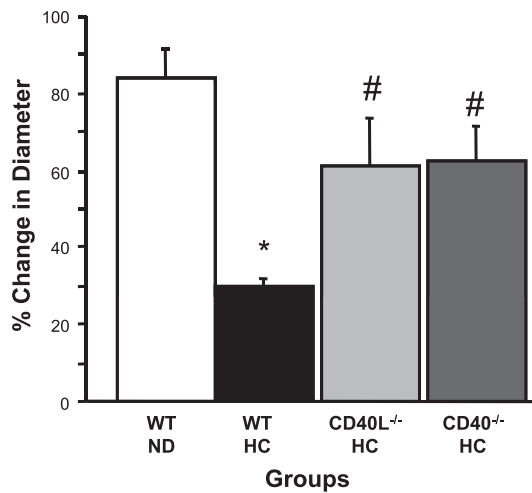


Fig. 2. Arteriolar vasodilation responses to acetylcholine (10^{-5} M) in WT, CD40L^{-/-}, and CD40^{-/-} mice placed on a ND or HC diet for 2 wk. * $P < 0.0005$ vs. WT-ND; # $P < 0.05$ vs. WT-HC.

vealed a quantitatively different pattern, with CD40 deficiency on either vascular or circulating cells yielding a significant (53% and 64%, respectively) but less dramatic (compared with CD40L^{-/-} chimeras) reduction in HC-induced leukocyte adhesion. However, HC-induced platelet adhesion was slightly (37%) reduced by the absence of CD40 on circulating cells but was unaltered in WT→CD40^{-/-} mice.

Arteriolar dysfunction was evident in hypercholesterolemic WT→WT chimeras, with arteriolar vasodilation responses being comparable with nonchimeric WT-HC mice (Fig. 4). The absence of either blood cell or vascular CD40L prevented this arteriolar dysfunction. In contrast to the venular side on which only a partial protection against hypercholesterolemia-induced inflammation was observed, mice deficient in CD40 on either the vascular (WT→CD40^{-/-}) or blood (CD40^{-/-}→WT) cells also showed a complete abrogation of hypercholesterolemia-induced arteriolar dysfunction.

CD40L on T lymphocytes participates in hypercholesterolemia-induced blood cell recruitment and arteriolar dysfunction. As previously reported (36, 37, 40), lymphocyte-deficient SCID mice exhibit significantly blunted HC-induced leukocyte (Fig. 5A) and platelet (Fig. 5B) adhesion responses when compared with immunocompetent WT-HC mice. These adhesion responses were fully restored in SCID-HC mice receiving splenocytes from WT-HC donors but not in SCID-HC mice receiving CD40L-deficient splenocytes, which supports a role for lymphocyte-associated CD40L in the HC-induced recruitment of leukocytes and platelets. The administration of WT-HC splenocytes to CD40L^{-/-} mice also restored the adhesion responses to WT-HC levels. It was determined that the recovery of platelet and leukocyte recruitment was mediated by CD40L on the T-lymphocyte population because CD40L^{-/-} mice that received T cells isolated from WT-HC donors (CD40L^{-/-}←WT-HC T cells) demonstrated blood cell adhesion levels comparable with those of the matched group receiving whole splenocytes and of the WT-HC group.

Lymphocyte-deficient SCID-HC mice showed no evidence of hypercholesterolemia-induced impairment of vasodilation (Fig. 6), despite having elevated serum cholesterol levels. As previously reported, although venular inflammatory responses

were restored in SCID-HC mice receiving splenocytes from WT-HC mice 5 days before observation, this treatment failed to reverse HC-induced arteriolar dysfunction [we have previously shown longer reconstitution in SCID mice is effective in restoring the arteriolar dysfunction (40)]. In contrast, the administration of WT splenocytes to otherwise immunocompetent CD40L-deficient mice led to the restoration of hypercholesterolemia-induced arteriolar dysfunction. Furthermore, CD40L^{-/-}-HC mice that received T cells isolated from WT-HC donors exhibited an impaired arteriolar vasodilation that was comparable with levels observed in WT-HC mice, indicating that CD40L on the T-cell population is critical for the development of endothelial dysfunction in hypercholesterolemic arterioles.

The role of CD40/CD40L in hypercholesterolemia-induced oxidative stress. The maintenance of WT mice on a cholesterol-enriched diet for 2 wk promoted an increased DHR oxidation in both venules and arterioles compared with corresponding normocholesterolemic controls (Fig. 7). However, in both CD40L^{-/-} and CD40-deficient mice, HC-induced oxidative

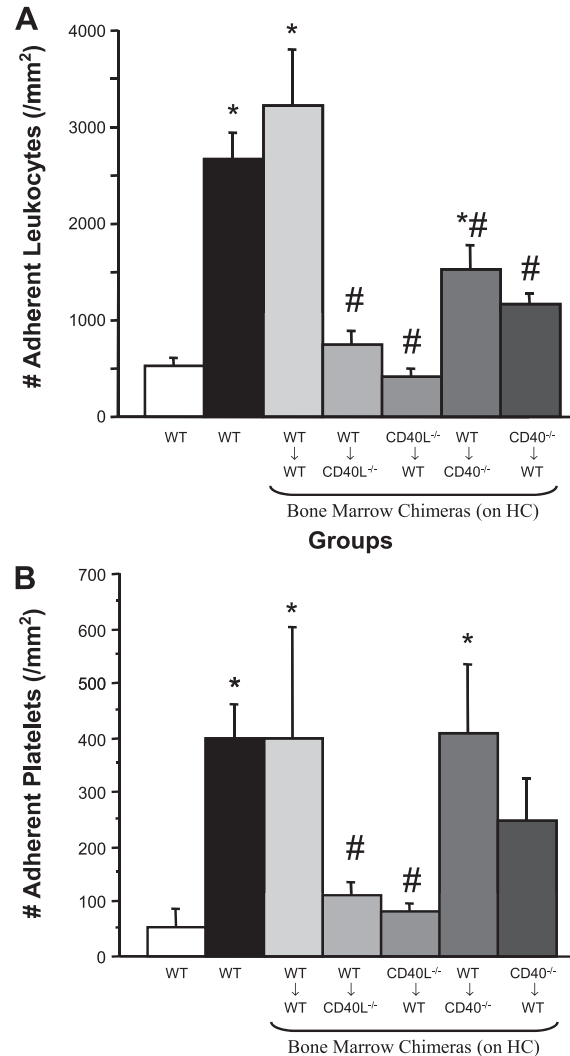


Fig. 3. The adhesion of leukocytes (A) and platelets from matched donors (B) monitored in postcapillary venules of WT mice, and 5 bone marrow chimera groups denoted by bone marrow donor→recipient. WT mice were maintained on a ND or placed on a HC diet for 2 wk; bone marrow chimeras were placed on HC. * $P < 0.05$ vs. WT-ND; # $P < 0.05$ vs. WT-HC and WT→WT-HC.

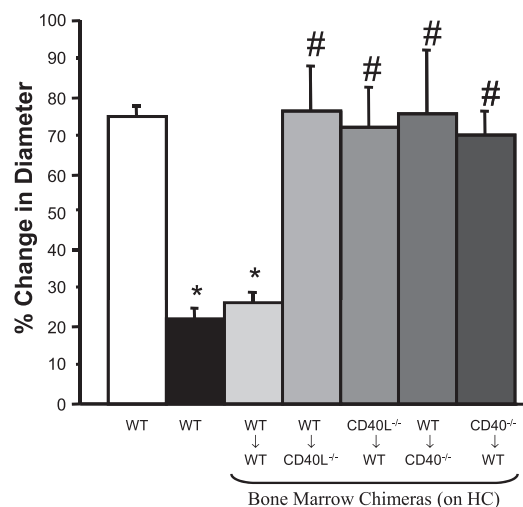


Fig. 4. The percent change in diameter (from baseline) following acetylcholine superfusion in arterioles from WT and chimeric (bone marrow donor→recipient) mice. Mice were placed on either a ND or HC diet for 2 wk. * $P < 0.0005$ vs. WT-ND; # $P < 0.001$ vs. WT-HC and WT→WT-HC.

stress was not detected, i.e., DHR oxidation was no different than that observed in arterioles and venules of WT-ND mice. The HC-induced oxidative stress was restored in CD40L^{-/-}-HC mice that received T cells isolated from WT-HC mice. No differences in the levels of oxidative stress were noted between the arteriolar and venular sides of the microvasculature within any group.

Role of CD40/CD40L in hypercholesterolemia-induced elevation of circulating cytokine levels. Two weeks of cholesterol feeding led to an approximate threefold increase in the circulating levels of IFN- γ (40.9 ± 5.78 pg/ml) and TNF- α (235.9 ± 40.64 pg/ml) compared with WT-ND values [13.7 ± 0.93 ($P < 0.001$) and 82.9 ± 30.65 pg/ml ($P < 0.005$), respectively]. CD40^{-/-}-HC mice demonstrated a significant reduction of these cytokines toward WT-ND levels (IFN- γ , 15.7 ± 1.38 pg/ml, $P < 0.0001$ vs. WT-HC; and TNF- α , 98.2 ± 7.14 pg/ml, $P < 0.01$ vs. WT-HC). However, the hypercholesterolemia-induced elevation of circulating cytokines was not abrogated by a deficiency of CD40L (47.4 ± 4.22 and 228.0 ± 23.24 pg/ml for IFN- γ and TNF- α , respectively).

DISCUSSION

The TNF-like signaling molecule CD40L and its receptor CD40 exhibit an increased expression on circulating cells of mildly hypercholesterolemic patients (7). This ligand-receptor pairing is ubiquitously expressed and has been shown to play a role in the upregulation of adhesion molecules (15, 16, 24, 33), the generation of reactive oxygen species (5, 45), and the release of IFN- γ and other cytokines (20, 23, 26, 30), many of which contribute to the development of an inflammatory and prothrombotic environment in the microcirculation during acute hypercholesterolemia. Here we demonstrate for the first time that the CD40/CD40L dyad is a critical component of the mechanisms that underlie the endothelial dysfunction on both the arteriolar and venular side of the microvasculature during hypercholesterolemia. We also provide evidence that CD40 and CD40L expressed on both the vascular wall and circulating

blood cells participate in these responses, suggesting that this signaling system contributes to HC-induced microvascular dysfunction through a complex, multicellular pathway that includes a central role for T-cell-associated CD40L. Together with our previous findings, these data support a model wherein hypercholesterolemia leads to T-cell-CD40L-dependent IFN- γ generation (40). This in turn promotes arteriolar dysfunction through a pathway involving platelet P-selectin (35), soluble factor(s) (perhaps sCD40L), and NAD(P)H oxidase activation (41). The IFN- γ (40) also contributes to NAD(P)H oxidase-dependent venular blood cell recruitment (41) via platelet CD40 and P-selectin (35), and vessel wall CD40/CD40L (Fig. 8).

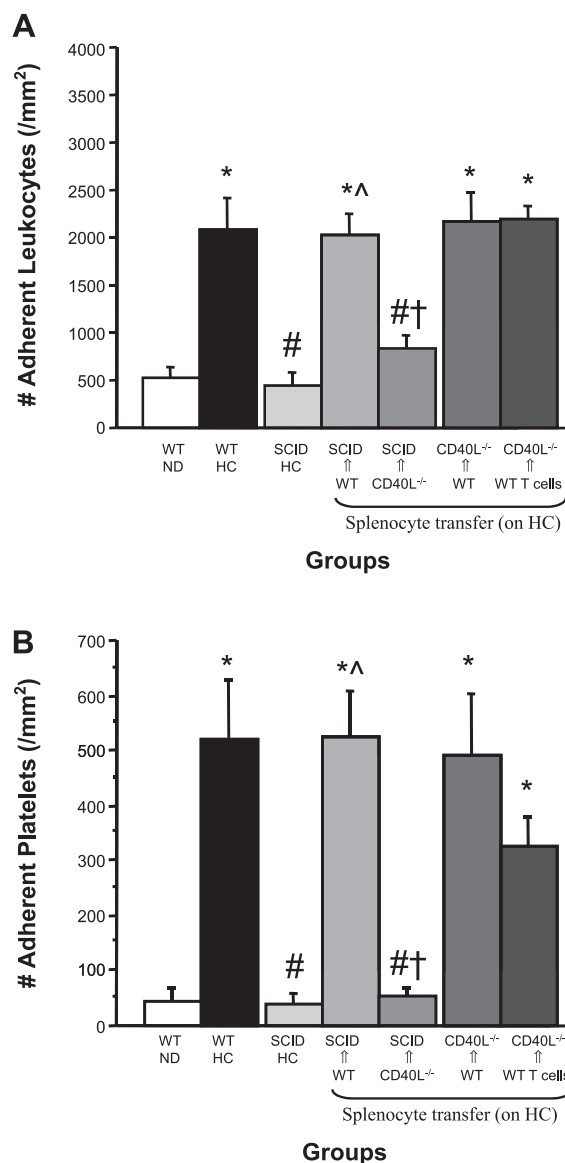


Fig. 5. Leukocyte (A) and platelet (B) adhesion responses in postcapillary venules of WT and severe combined immune deficient (SCID) mice either maintained on a ND or placed on a HC diet for 2 wk. In addition, blood cell recruitment was monitored in SCID and CD40L^{-/-} mice receiving splenocytes or T cells from a donor (recipient←donor) at day 9 of HC. * $P < 0.05$ vs. WT-ND; # $P < 0.0005$ vs. WT-HC; ^ $P < 0.0001$ vs. SCID-HC; † $P < 0.005$ vs. SCID←WT-HC.

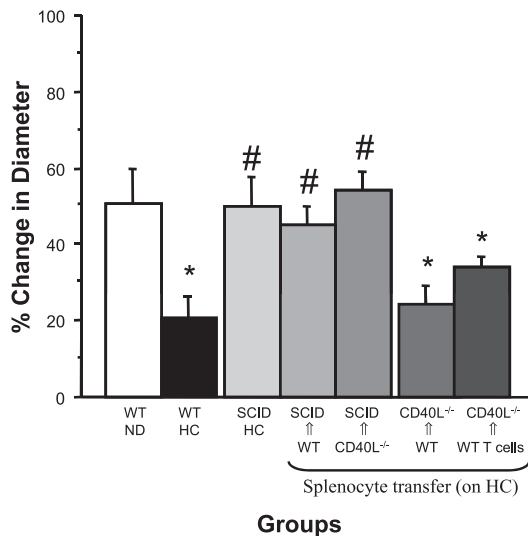


Fig. 6. Arteriolar dilation in response to acetylcholine (10^{-5} M) in WT mice on ND and in WT and lymphocyte-deficient SCID mice on a HC diet for 2 wk. Separate SCID groups and CD40L^{-/-} mice received splenocytes or T cells from a donor (recipient \leftarrow donor) at day 9 of HC. * $P < 0.05$ vs. WT-ND; # $P < 0.005$ vs. WT-HC.

CD40 is basally expressed by endothelial cells, monocytes, and other cell types, and its expression can increase in response to different inflammatory stimuli. CD40L is not expressed under basal conditions, but it is upregulated, particularly on T cells and platelets following stimulation. Since CD40 ligation by CD40L leads to an increased adhesion molecule expression and the release of inflammatory mediators from endothelial cells and leukocytes, it may be expected that both CD40 and CD40L-deficient mice are protected against HC-induced blood cell recruitment in the microvasculature. Although our findings in mutant mice suggest that CD40 and CD40L are equal contributors to the HC-induced microvascular responses, the results derived from the bone marrow chimeras suggest that two components of the dyad exhibit cell target-specific actions wherein one component exerts a more dominant influence over the other. For example, whereas the HC-induced blood cell adhesion responses were completely abrogated in both CD40- and CD40L-deficient mice, the CD40L chimeras exhibited a higher level of protection against blood cell recruitment than the CD40 chimeras. This difference may reflect the variety of alternative receptors for CD40L or CD40 that are differentially expressed on blood and vascular cells. These alternative receptors for CD40L include CD11b (50), GPIIb/IIIa (1), and $\alpha_5\beta_1$ (18), and these alternate ligands for CD40 include C4b binding protein (2) and heat shock protein 70 (48). The recently reported observation that, unlike WT mice, CD40L^{-/-} mice express almost no CD40 under basal conditions (47) raises the additional possibility that the CD40L-deficient mice and chimeras are protected in part due to an associated reduction in CD40 expression. Irrespective of the nature of the differences noted between the CD40/CD40L-deficient mice and their respective bone marrow chimeras, our findings provide strong support for this dyad in mediating the HC-induced blood cell adhesion.

Our findings also implicate CD40/CD40L in the vasomotor dysfunction induced by hypercholesterolemia. While blood cell adhesion is not detected in arterioles of HC mice, soluble

factors linked to CD40/CD40L may contribute to the HC-induced arteriolar dysfunction. For example, it is possible that circulating sCD40L, perhaps released from platelets, interacts with endothelial CD40 in arterioles to initiate a cascade of events that lead to impaired endothelium-dependent arteriolar dilation. This possibility is supported by clinical studies showing correlations between sCD40L and vasomotor dysfunction (10, 49) and by a recent report demonstrating that the incubation of porcine aortic rings with a high dose of sCD40L reduced vasomotor responses to bradykinin through endothelial nitric oxide synthase downregulation and oxidative stress (5). However, such a mechanism cannot completely explain our findings in bone marrow chimeras, where comparable roles for vascular and blood cell CD40 and CD40L were demonstrated in the HC-induced arteriolar dysfunction. It is conceivable that CD40-CD40L interactions between circulating cells and/or between blood cells and vascular cells at remote sites (e.g., postcapillary venules) promote the release of one or more soluble factors, including sCD40L, that in turn elicit (via blood transport and/or local diffusion) the arteriolar dysfunction. The different patterns of protection afforded by the bone marrow chimeras on the arteriolar versus venular sides of the microvasculature suggest that the pathway(s) through which CD40 and CD40L result in arteriolar dysfunction differ from the inflammatory mechanisms elicited in venules.

Platelets express CD40 and, upon activation, CD40L (11, 13). Both CD40 and CD40L contributed to the platelet adhesion responses induced by HC. We have previously demonstrated that a majority of the platelets that adhere in postcapillary venules of HC mice are bound to adherent leukocytes (35, 42). While this could indicate that the reduction of leukocyte adhesion in the CD40- and CD40L-deficient mice may be primarily responsible for the corresponding decline in platelet accumulation, we have also shown that platelet P-selectin is upregulated (43) and contributes to the leukocyte adhesion and

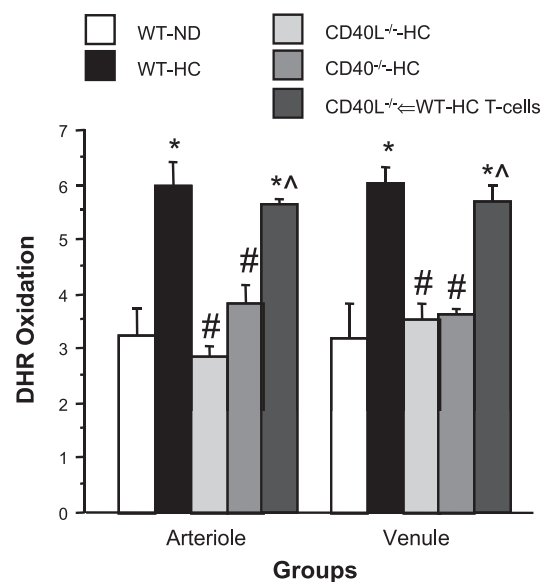


Fig. 7. Dihydrorhodamine (DHR) oxidation in arterioles and venules of WT, CD40L^{-/-}, and CD40^{-/-} mice on ND or HC diet for 2 wk. A second group of CD40L^{-/-} mice received T cells from a WT-HC donor (recipient \leftarrow donor) at day 9 of HC. * $P < 0.0001$ vs. WT-ND; # $P < 0.0005$ vs. WT-HC; ^ $P < 0.0005$ vs. CD40L^{-/-}.

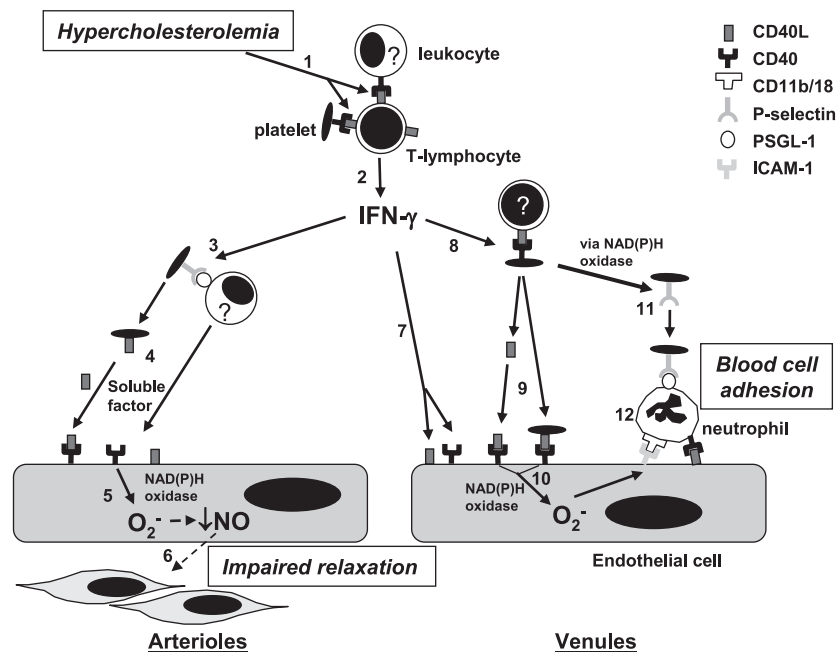


Fig. 8. Based on data from the current and previous studies, we propose the following pathways through which hypercholesterolemia induces vasomotor dysfunction in arterioles and blood cell recruitment in venules. Increased plasma cholesterol, possibly through the generation of antigens presented to T cells via the T-cell receptor, upregulates T-cell CD40L, thereby allowing stimulation of T lymphocytes through the binding of CD40L on the T cell by CD40 on another circulating cell, i.e., either other leukocytes or platelets (1). This results in the release of interferon- γ (IFN- γ) from the T cells (2). IFN- γ directly/indirectly promotes platelet P-selectin-dependent responses (3) that result in the release of soluble CD40L from platelets and perhaps other soluble factors [e.g., regulated upon activation, normal T cell expressed and secreted (RANTES)], which bind their receptors on the arteriolar endothelium (4). Endothelial cell NAD(P)H oxidase is then activated, resulting in an accelerated production of superoxide (5), inactivation of nitric oxide (NO), and an impaired vasodilatory response to acetylcholine (6). In venules, IFN- γ may increase CD40 and CD40L expression on endothelial cells (7) and promote CD40-dependent platelet responses (8) including the activation of endothelial CD40 (9). This results in NAD(P)H oxidase activation, superoxide generation (10), and upregulation of adhesion molecules on the vessel surface, which, along with CD40 and CD40L, support neutrophil adhesion. Occupation of CD40 on the platelet can also stimulate platelet NAD(P)H oxidase and induce platelet P-selectin expression (11). Platelet P-selectin then binds to constitutively expressed P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes, which promotes the accumulation of platelets on the venular wall (12).

arteriolar dysfunction observed in hypercholesterolemic mice (35). It is known that the ligation of platelet CD40 by CD40L leads to the upregulation of P-selectin on the cell surface (13); therefore, it is conceivable that the binding of CD40L (possibly platelet associated/derived) to platelet CD40 initiates platelet activation and the translocation of P-selectin to the platelet surface. This P-selectin may mediate platelet interactions with other cell types, resulting in their activation and culminating in the inflammatory responses observed in our study, as implicated using this model in a previous study by our group (35). While this scenario is supported by the attenuation of leukocyte adhesion and arteriolar dysfunction in CD40- and CD40L-deficient mice, and in bone marrow chimeras where either CD40 or CD40L was absent on the blood cells, it is difficult to separate the platelet versus leukocyte-specific contributions of either molecule using bone marrow chimeras. Hence, we used the strategy of administering platelets from CD40- or CD40L-deficient mice into WT recipients and monitoring their adhesion. This approach previously enabled us to identify roles for platelet P-selectin and NAD(P)H oxidase in the HC-induced platelet adhesion response (41, 43). The current study reveals that platelet CD40 is required for HC-induced platelet adhesion, with platelet-associated CD40L playing a lesser role. It is plausible that sCD40L or cell-associated CD40L in the WT-HC recipients normally engages CD40 on WT platelets, which leads to cell activation, P-selectin expression, and platelet adhesion. However, these responses are not elicited by

sCD40L or cell-associated CD40L in CD40^{-/-} platelets. A somewhat unexpected finding in this study was the observation that CD40^{-/-} platelets adhered, albeit at a significantly reduced level, in venules of CD40^{-/-}→WT bone marrow chimeras. Whether these mice used an alternative pathway to mediate this adhesion response is unclear. For example, it is possible that the blunted leukocyte adhesion response in these chimeras may have allowed the CD40^{-/-} platelets to adhere directly to endothelium via a platelet-CD40-independent pathway.

Based on our previous findings that T lymphocytes play a major role in mediating the microvascular dysfunction associated with hypercholesterolemia (36–38, 40) and our new observation that blood cell-associated CD40L is also major contributor to this microvascular dysfunction, we tested whether T lymphocytes use CD40L-dependent pathways to mediate their deleterious effects in the microvasculature of HC mice. Using splenocyte or T-cell transfer from WT or CD40L^{-/-} donors into immunodeficient mice, and from WT donors into CD40L^{-/-} mice, we revealed a role for T-cell-associated CD40L in eliciting the HC-induced dysfunction in both venules and arterioles. CD40L likely exerts its effects on T lymphocytes by mediating cell activation and the release of cytokines such as IFN- γ , which was identified as a key mediator of the HC-induced responses using the same T-cell transfer strategies employed in the present study. While we have previously demonstrated that T-cell-derived IFN- γ does appear

to contribute to the HC-induced microvascular responses and that circulating IFN- γ levels are elevated in response to HC, we found that NK cells, rather than T lymphocytes, are likely to be the source of this circulating IFN- γ , and these NK cells do not contribute significantly to this model of inflammation (40). Here we observed that IFN- γ levels are not reduced in CD40L-deficient mice, whereas CD40^{-/-} mice did exhibit a reduction in circulating IFN- γ levels. Hence, our results suggest that the interaction of CD40 with an unidentified ligand (other than CD40L) may be responsible for inducing IFN- γ secretion from NK cells during HC.

The enhanced oxidative stress induced by HC was comparable in arterioles and venules. Although the similarity between the two vessel types was somewhat unexpected, it is likely that different cell types were involved in arterioles (endothelial cells and smooth muscle cells) versus venules (adherent leukocytes and platelets, and endothelial cells). While it is also conceivable that these vessels differ somewhat with regard to the relevant roles of oxidant-generating enzymes, the superoxide-generating enzyme NAD(P)H oxidase has been implicated in both the blood cell recruitment and arteriolar dysfunction associated with HC (39, 41). It has recently been demonstrated in several cell types that CD40-CD40L interactions can activate NAD(P)H oxidase (e.g., endothelial cells and platelets) and enhance superoxide production (e.g., endothelial cells, platelets, and neutrophils) (4, 5, 46). The converse may also be true in that CD40L may be upregulated on platelets via a gp91^{phox}-dependent pathway (25), and neutrophils from patients deficient in NAD(P)H oxidase express lower levels of CD40 following activation (28). Our findings that HC-induced oxidative stress was abrogated in both CD40- and CD40L-deficient mice support the CD40/CD40L-mediated activation of NAD(P)H oxidase. However, we have also previously shown that T-cell-derived IFN- γ , an inducer of NAD(P)H oxidase, contributes to HC-induced oxidative stress in both arterioles and postcapillary venules (40). Taken together with our findings here that T-cell CD40L participates in the arteriolar dysfunction and blood cell adhesion in our model, it is plausible that CD40-CD40L signaling plays a more indirect role in mediating the HC-induced oxidative stress response. This possibility is supported by our observation that HC-induced oxidative stress was restored in CD40L^{-/-} mice following the administration of WT-HC T cells, suggesting that the engagement of T-cell CD40L leads to IFN- γ release, which results in the activation of other cell types, ultimately leading to enhanced superoxide production, inflammation, and impaired endothelium-dependent vasodilation.

In conclusion, our findings provide evidence that CD40/CD40L signaling plays a major role in the endothelium-dependent responses (blood cell adhesion, impaired vasodilation, and oxidative stress) that are elicited in the microcirculation during the early stages of hypercholesterolemia. Taken together with our previous findings, our results also reveal a complex multicellular involvement of CD40 and CD40L in the HC-induced microvascular responses, with T-lymphocyte-associated CD40L serving a critical role in modulating leukocyte recruitment, oxidative stress, and vasomotor dysfunction, whereas CD40 on platelets is critically important for the HC-induced recruitment of this blood cell population in the microvasculature, as depicted in Fig. 8. Therapeutic strategies directed at CD40 and/or CD40L may prove beneficial in

reducing the deleterious vascular responses to hypercholesterolemia and other risk factors for cardiovascular disease.

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