CD8⁺ T Lymphocytes Regulate the Arteriogenic Response to Ischemia by Infiltrating the Site of Collateral Vessel Development and Recruiting CD4⁺ Mononuclear Cells Through the Expression of Interleukin-16

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Background—Previous studies have demonstrated that macrophages and CD4⁺ T lymphocytes play pivotal roles in collateral development. Indirect evidence suggests that CD8⁺ T cells also play a role. Thus, after acute cerebral ischemia, CD8⁺ T cells infiltrate the perivascular space and secrete interleukin-16 (IL-16), a potent chemoattractant for monocytes and CD4⁺ T cells. We tested whether CD8⁺ T lymphocytes contribute to collateral vessel development and whether the lack of circulating CD8⁺ T cells prevents IL-16 expression, impairs CD4⁺ mononuclear cell recruitment, and reduces collateral vessel growth after femoral artery ligation in CD8⁻/⁻ mice.

Methods and Results—After surgical excision of the femoral artery, laser Doppler perfusion imaging demonstrated reduced blood flow recovery in CD8⁻/⁻ mice compared with C57/BL6 mice (ischemic/nonischemic limb at day 28, 0.66±0.04 versus 0.87±0.04, respectively; P<0.01). This resulted in greater calf muscle atrophy (mean fiber area, 785±68 versus 1067±69 μm², respectively; P<0.01) and increased fibrotic tissue content (10.8±1.2% versus 7±1%, respectively; P<0.01). Moreover, CD8⁻/⁺ mice displayed reduced IL-16 expression and decreased CD4⁺ T-cell recruitment at the site of collateral vessel development. Exogenous CD8⁺ T cells, infused into CD8⁻/⁻ mice immediately after femoral artery ligation, selectively homed to the ischemic hind limb and expressed IL-16. The restoration of IL-16 expression resulted in significant CD4⁺ mononuclear cell infiltration of the ischemic limb, faster blood flow recovery, and reduced hindlimb muscle atrophy/fibrosis. When exogenous CD8⁺ T cells deficient in IL-16 (IL-16⁻/⁻) were infused into CD8⁻/⁻ mice immediately after femoral artery ligation, they selectively homed to the ischemic hind limb but were unable to recruit CD4⁺ mononuclear cells and did not improve blood flow recovery.

Conclusions—These results demonstrate that CD8⁺ T cells importantly contribute to the early phase of collateral development. After femoral artery ligation, CD8⁺ T cells infiltrate the site of collateral vessel growth and recruit CD4⁺ mononuclear cells through the expression of IL-16. Our study provides further evidence of the significant role of the immune system in modulating collateral development in response to peripheral ischemia. (Circulation. 2006;113:118-124.)

Key Words: angiogenesis □ inflammation □ interleukins □ lymphocytes

Atherosclerosis is the leading cause of death in developed countries. One of the compensatory mechanisms involved in the response to impaired blood flow secondary to atherosclerotic plaque development is the formation of collateral blood vessels.¹

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It has recently been shown that cellular components of the immune and inflammatory systems play a pivotal role in modulating collateral vessel development.² In particular, various investigations have demonstrated the importance of monocytes/macrophages in collaterogenesis.³,⁴ A study indicating that nude mice (which are deficient in all components of the T-cell population) exhibit a pronounced impairment in collateral response³ suggested a possible role of CD4⁺ and CD8⁺ subsets in collaterogenesis. The concept that CD4⁺ T lymphocytes play such a role was confirmed by the recent

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demonstration that in the absence of CD4+ T lymphocytes,6 the inflammatory and collateral responses after the induction of ischemia are impaired.

Studies of cerebral infarcts in humans suggest that CD4+ T lymphocytes are not the only T cells involved in collaterogenesis. Thus, within the first few hours of injury, CD8+ T cells infiltrate the periphery of cerebral infarcts.7 Once there, these cells secrete interleukin-16 (IL-16), a potent chemotacticant for several cells of the immune and inflammatory systems, including monocytes and CD4+ T cells.8 Given the role of monocytes and CD4+ T lymphocytes in the process of collaterogenesis,6 the present investigation was designed to test the hypothesis that CD8+ T lymphocytes are critical to ischemia-induced collateral development and that this effect is dependent on the expression of the immune cell chemotactrant IL-16.

To this aim, we used an ischemic hindlimb model7 to determine whether collateral-mediated recovery of flow after acute induction of ischemia is impaired in CD8 knockout (CD8−/−) mice compared with control wild-type C57BL/6 mice.

Methods

Mouse Model of Unilateral Hindlimb Ischemia

Under narcosis with intramuscular xylazine (40 mg/kg) and ketamine (100 mg/kg), 12-week-old C57BL/6 and CD8a−/− mice (Jackson Laboratories) underwent surgical ligation of the left femoral artery to create unilateral hindlimb ischemia.9,10 The study was approved by the Animal Care and Use Committee of the MedStar Research Institute.

Perfusion Imaging

Repeated hindlimb blood flow measurements over the region of interest (from the patella to the mid foot) were obtained at baseline, immediately postoperatively, and serially over 4 weeks with laser Doppler perfusion imaging (LDP1) (Moer Instruments). Perfusion is expressed as a ratio of left (ischemic) to right (normal) limb.6 The region of interest did not include the distal foot because this area is more subject to ischemic autospaunation in this animal model.6

In Vivo Assessment of Limb Function and Ischemic Damage

Semiquantitative assessment of impaired use of the ischemic limb and semiquantitative measure of the ischemic damage were performed serially.6,11

Tissue Culture

To ensure the absence of bacterial growth in the immunocompromised CD8−/− animals in both groups, the surgical incision was opened 7 days after the procedure, and a culture swab (Becton Dickinson) was taken under sterile conditions.6

Western Blotting and Immunohistochemical Analysis

At different time points after surgery, adductor muscles were homogenized in ice-cold buffer, and proteins were extracted, separated with SDS-PAGE, and blotted onto nitrocellulose (Invitrogen). Blots were incubated with primary antibody (1:250 for anti–IL-16; 1:1000 for α-tubulin; Santa Cruz).12 At the same time points, adductor muscles were sectioned with a cryostat, fixed in methanol, blocked, and incubated overnight with primary antibodies (1:50 for anti–IL-16; 1:50 for anti-CD4; Santa Cruz). DAPI was used to obtain nuclear staining. An optical microscope (Olympus BX41, Olympus) and Image Pro Plus software (Media Optronics) were used to analyze cellular infiltration.

Histological Analysis

After blood flow assessment over 28 days was completed, hindlimb muscles were removed and fixed in formalin. From the upper thigh, sections were prepared and stained with van Gieson solution. Only arteries were counted. Vessels were identified as arteries if they had a continuous internal elastic laminas, at least a layer of muscle spindles, and a mathematically derived area >300 μm².12,13 The number of arteries present in each thigh was expressed as the ratio of the number of arteries to surface area of muscle analyzed.6

From the gastrocnemius, sections were prepared and then stained with Sirius red, and collagen volume fraction was determined.14 In the same sections, muscle area was calculated and divided by the number of muscle fibers present in the field to obtain the average fiber size for a given field.6

Rescue Experiments by Infusion of CD8+ Cells From C57BL/6 and IL-16−/− Mice

Mononuclear cells were isolated from the spleens of age- and gender-matched wild-type C57BL/6 mice or IL-16−/− mice (on a C57BL/6 background16) and separated into CD8+ and CD8− fractions with positive selection by magnetic labeling (Miltenyi Biotechnology, Inc).

At the induction of ischemia, 3×10⁶ purified CD8+ cells, CD8 depleted, or CD8− IL-16− spleocytes were infused intravenously into CD8−/− mice. To assess homing of injected cells, a subgroup of mice was injected with the same number of CFSE-labeled (Molecular Probes, Inc.) cells.17 Animals were sacrificed serially over 48 hours, and muscles were prepared for fluorescence detection. The percentage of labeled cells in different fields of inflammatory infiltration was calculated.

Statistical Analysis

All results are presented as mean±SEM. A paired Student t test was used to compare raw and normalized values. REMANOVA was used to compare values between groups over time. A value of P<0.05 was considered significant.6

Results

Hindlimb Blood Flow Recovery After Femoral Artery Ligation

In control mice, blood flow fell precipitously after surgery, remained impaired for 3 days, increased to 70% of the nonischemic limb by day 7 (Figure 1a), and ultimately returned to near-normal levels at the end of follow-up. A similar reduction in hindlimb blood flow occurred in CD8−/− mice, but in contrast to control mice, flow recovery was markedly attenuated (Figure 1a). Compared with C57BL/6, flow in CD8−/− mice was significantly less by day 3, a difference persisting at each of the subsequent time points (7, 14, and 28 days); flow never achieved >65% of that measured for the contralateral limb (Figure 1b).

In control mice, active use of the right foot fell significantly after surgery (Figure 1c), remained impaired for 7 days, and gradually returned to near-normal levels by day 28 (ambulatory impairment score, 0.23±0.16). A similar abrupt reduction in hindlimb blood flow occurred in CD8−/− mice, but recovery was markedly attenuated at day 7 (ambulatory impairment score, 1.7±0.18; P<0.05 versus C57BL/6) and remained impaired during the rest of the study. CD8−/− mice also displayed severe ischemic damage of the limb (Figure 1c) that was still evident by day 28 (tissue damage score,
2.5±0.38 versus 0.8±0.2; P<0.05) and resulted in an increased incidence of minor autoamputations by day 28.

At the end of the study, CD8−/− animals showed a significant reduction in the density of collateral arteries in the upper thigh of the operated hind limb compared with C57BL/6 mice (0.33±0.02 versus 0.44±0.04 arteries >300 μm²/mm² muscle area; P<0.05) (Figure 1c). No difference was observed among the nonoperated hind limbs.

Animal Health Status After Surgically Induced Hindlimb Ischemia

CD8−/− mice may be more susceptible than wild-type mice to superimposed infection resulting from surgery, which may affect their ability to recover. To rule out this possibility, at least as it relates to bacterial infection, we cultured specimens of the ischemic muscle at day 7 after surgery. No bacterial growth was detected in either group. Thus, the increased incidence of tissue necrosis in the ischemic limb of CD8−/− mice cannot be attributed to septic gangrene. Moreover, CD8−/− mice exhibited over follow-up the same weight gain as the C57BL/6 mice, demonstrating an absence of any major effect of surgery on the general health of these mice.

Effect of CD8+ Cell Reconstitution in CD8−/− Mice Subjected to Hindlimb Ischemia

Immediately after surgery, we intravenously infused into CD8−/− mice spleen-derived purified CD8+ T cells from control mice (CD8+ group). Exogenous CD8+ cells selectively homed to areas of inflammatory cell infiltration of the ischemic hind limb. No exogenous CD8- cells were found infiltrating muscle fibers of the contralateral hind limb (Figure 2a).

Distal blood flow improved in the CD8−/− mice receiving infusion of purified CD8+ cells (CD8+‑treated group) compared with CD8−/− mice receiving CD8− splenocytes (CD8−‑treated group) (Figure 2b). Over follow-up, at all time points, blood flow recovery in the CD8−‑treated group was similar to that of the wild-type C57BL/6 mice. These mice exhibited reduced ischemic damage, as observed by improved appearance of the limb (Figure 2c) and reduced ambulatory impairment (Figure 2c).

Extent of Ischemic Damage in the Distal Limb

To confirm the results derived from the LDPI data and of the pathological assessment of collateral vessel development, we performed an additional independent assessment of the ischemic damage of the operated hind limbs by analyzing the extent of gastrocnemius muscle fibrosis/atrophy (Figure 3). Necropsy examination of the calf muscle 28 days after surgery revealed more pronounced fibrotic tissue content (10.8±1.2% versus 6.6±1.3%; P<0.01) (Figure 3a and 3b) and increased muscle fiber atrophy in CD8−/− compared with wild-type C57BL/6 mice (mean fiber area, 785±68 versus 1067±69 μm²; P<0.01). Moreover, the improved hindlimb blood flow recovery observed in the CD8−‑treated group was associated with reduced muscular fibrosis and atrophy (Figure 3a and 3b). No difference was detected in the extent of muscle fibrosis/atrophy between the original group of CD8−/− mice and the CD8−‑treated group.

Tissue Inflammatory Responses to Ischemia

We examined the adductor muscle of the operated limb 48 hours after femoral artery ligation. Hematoxylin and eosin staining revealed differing numbers of inflammatory leukocytes in the wild-type versus CD8−/− mice. In particular, the number of infiltrating CD4+ leukocytes was lower in CD8−/− mice than in wild-type mice (Figure 4a). Moreover, in control animals, IL-16 was induced after femoral artery ligation and was expressed at the site of mononuclear cell infiltration (Figure 4b). In contrast, IL-16 expression after ischemia was reduced in the CD8−/− mice (Figure 4b).
IL-16 Deficiency Impairs the Ability of Exogenous CD8⁺ Cells to Restore Hindlimb Blood Flow Recovery in CD8⁺/⁻ Mice

In CD8⁺/⁻ mice, expression of IL-16 in the ischemic muscle was observed at the site of exogenous cell infiltration 2 days after femoral artery ligation and infusion of CD8⁺ cells (Figure 5a). Colocalization of CFSE and IL-16 fluorescent signal demonstrated that in reconstituted CD8⁺/⁻ mice, exogenous CD8⁺ T cells express IL-16 (Figure 5a). At 48 hours after femoral artery ligation, in the ischemic limb of CD8⁺-treated CD8⁺/⁻ mice, IL-16 expression was almost equal to that observed in wild-type C57BL/6 mice (Figure 5b). The presence of this cytokine is associated with an increased number of infiltrating leukocytes in the ischemic limbs of CD8⁺-treated CD8⁺/⁻ mice compared with CD8⁺-treated mice (Figure 5c). Finally, when IL-16-deficient CD8⁺ T cells were infused into CD8⁺/⁻ mice after femoral artery ligation, they homed to the ischemic hind limb (data not shown), but we did not observe an increased recruitment of CD4⁺ mononuclear cells to the ischemic hind limbs. This inhibition in CD4⁺ cell recruitment is associated with the lack of ability to rescue blood flow recovery in CD8⁺/⁻ mice (Figure 5c and 5d) and more pronounced muscle atrophy and fibrosis of the ischemic limb (data not shown).

Discussion

The major findings of our study are that (1) collateral development in response to ischemia is impaired in CD8⁺/⁻ compared with C57BL/6 wild-type control mice; (2) CD8⁺/⁻ mice have reduced IL-16 expression after femoral artery ligation and an impaired inflammatory response at the site of collateral vessel growth; (3) spleen-derived purified CD8⁺ T cells, when infused into CD8⁺/⁻ mice, selectively localize to the ischemic limb, recruit CD4⁺ mononuclear cells, and increase blood flow recovery; and (4) the ability of CD8⁺ T
cells to regulate inflammatory responses and blood flow recovery after ischemia is dependent on IL-16 expression. One of the important end points of our study is based on the assessment of limb flow by means of LDPI. LDPI-assessed flow correlates well with microsphere-assessed perfusion and the number and size of second- and third-generation collateral branch arteries. In accordance with the LDPI-based data, we found that the number of collateral arteries significantly increased in the operated limb of wild-type mice, changes that were markedly attenuated in the CD8\(^{-/-}\) mice.

The impaired collateral vessel development observed in the CD8\(^{-/-}\) mice correlated with a more prolonged and severe...
functional impairment of the limb, an increased incidence of ischemic necrosis, and an increased incidence of autoamputation. As an independent parameter of the severity of ischemic injury of CD8−/− mice, we assessed calf muscle histology 4 weeks after femoral artery ligation. In these animals, single muscle fiber size was markedly reduced compared with wild-type mice, a change associated with increased tissue fibrosis.

The differences in flow between the wild-type and knockout mice probably cross a flow threshold for tissue viability (below which cell death will occur). Thus, the decrease in flow in the CD8−/− mice, even though relatively small, probably accounts for the large changes we observed in muscle atrophy and fibrosis.

To prove definitively that the impaired development of collateral flow we observed in the CD8−/− mice was caused by a deficient supply of CD8+ T cells, we performed a rescue experiment in which we infused spleen-derived purified CD8+ T cells into CD8−/− mice. Exogenous CD8+ T cells selectively homed to the site of active collateral development, restored flow-recovery to the levels observed in the wild-type parental strain, and preserved calf muscular structure, as demonstrated by a reduction in fibrosis and atrophy.

When we assessed the pattern of the inflammatory response in the ischemic limbs of the CD8−/− animals, we observed that the overall number of mononuclear cells infiltrating the interstitium among muscle fibers was lower than that seen in wild-type C57BL/6J mice. In particular, the number of infiltrating CD4+ cells was reduced by 50%.

IL-16 is a potent chemoattractant for several immune cells such as monocytes, eosinophils, and dendritic cells.8,17–19 Similarly, IL-16 has been characterized as a natural ligand for CD4, through which it induces a migratory response in CD4+ T cells.17 IL-16 is also likely to regulate TH1-cell recruitment and activation at sites of inflammation such as those of active collateral vessel growth. When we followed up the expression of IL-16 after femoral artery ligation in the adductor muscles, we observed a lack of induction of this cytokine in CD8−/− mice. Of relevance, within the first hours of ischemia, CD8+ cells infiltrate the periphery of cerebral infarcts and secrete IL-16.7

In our experiments, CD8+ T cells from IL-16−/− mice did not improve blood flow recovery in CD8−/− mice, indicating that IL-16 is a likely effector in this process. The lack of effect on blood flow was also associated with a reduced recruitment of CD4+ mononuclear cells at the site of collateral vessel development, thus demonstrating the important role of this cytokine in the initial phases of the inflammatory response to peripheral ischemia.

Collaterals originate, at least in part, from preexisting vessels located proximal to the site of arterial obstruction and therefore proximal to the ischemic tissue.20,21 Although little or no flow occurs in these vessels under normal conditions, pressure gradients created by the distal obstruction promote their opening and the establishment of flow. The increase in blood flow and shear force activates endothelial cells, with subsequent upregulation of cell adhesion molecules and chemokines and resultant recruitment of inflammatory cells.22

The present investigation, in conjunction with the data available in the literature,6 demonstrates that lymphocytes contribute importantly to the process of arterial remodeling that leads to collaterogenesis. After ischemia induction, the inflammatory response and collateral vessel development are compromised in CD4+ T-cell–deficient mice. At the site of active collateral vessel growth, in the absence of CD4+ T cells, there is impaired recruitment of macrophages, which are pivotal cellular determinants for optimal collateral vessel growth.6 These cells localize around developing collaterals and secrete several proangiogenic factors.6 Our results suggest that CD8+ T cells are one of the first responders to the local stimuli involved in collateral growth. When these cells infiltrate the region of developing collaterals, they express IL-16, which contributes to the recruitment of CD4+ mononuclear cells (T cells and macrophages). These cells, in turn, secrete many of the numerous cytokines that play a critical role in collaterogenesis.

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Disclosures
None.

References
Surgical and catheterization-based techniques to improve blood flow to the heart and legs of patients with atherosclerotic obstructive disease are highly successful. However, it is generally recognized that if interventions could be developed that augment collateral development (collaterogenesis), they would play a critical role in treating many patients. Therefore, it has been discouraging that no pivotal “angiogenesis” trial has shown robustly positive results. This suggests that more insight into basic mechanisms responsible for collaterogenesis is necessary to design better therapeutic strategies. The present study was derived from observations suggesting that cellular components of the immune and inflammatory systems play pivotal roles in modulating collaterogenesis. Thus, in the absence of CD4+ T lymphocytes or monocytes, not only are ischemia-induced inflammatory responses impaired, but collateral development also is impaired. The present investigation demonstrates that CD8+ T lymphocytes are important to ischemia-induced collateral development and that this effect is dependent on the expression of the immune cell chemoattractant IL-16, which specifically attracts monocytes and CD4+ T cells. The relevance of these results to the design of new therapies is particularly interesting because traditional risk factors (advanced age, male sex, diabetes mellitus, cigarette smoking, hypertension, and increased lipid levels) correlate with deficient T-cell function. Thus, given the critical role of T cells in collaterogenesis, such interactions may contribute to impaired collateral development, a conclusion that could lead to the development of novel interventions to enhance collaterals.
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In the recently published paper by Stabile et al, “CD8⁺ T Lymphocytes Regulate the Arteriogenic Response to Ischemia by Infiltrating the Site of Collateral Vessel Development and Recruiting CD4⁺ Mononuclear Cells Through the Expression of Interleukin-16,” which appeared in the January 3/10, 2006, issue of the journal (Circulation. 2006;113:118–124), the affiliation for Dr Andrea la Sala has been mistakenly reported as both the IRCCS San Raffaele, Rome, Italy, and the National Institutes of Health, Bethesda, Md. Instead, Dr la Sala is affiliated exclusively to the Laboratory of Molecular and Cellular Biology, IRCCS San Raffaele, Rome, Italy.

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