Impaired Arteriogenic Response to Acute Hindlimb Ischemia in CD4-Knockout Mice

Eugenio Stabile, MD; Mary Susan Burnett, PhD; Craig Watkins, MD; Timothy Kinnaird, MD; Alessia Bachis, PhD; Andrea la Sala, PhD; Jonathan M. Miller, MS; Matie Shou, MD; Stephen E. Epstein, MD; Shmuel Fuchs, MD

Background—T lymphocytes, components of the immune and inflammatory systems, are involved in such normal processes as wound healing and host defense against infection and in such pathological processes as tumor growth and atherosclerotic plaque development. Angiogenesis is a mechanism common to all of these processes is angiogenesis. Although both CD4+ and CD8+ T cells, main subsets of T lymphocytes, participate in these processes, the CD4+ subset appears more active in secreting multiple cytokines (including a wide range of angiogenic growth factors) and in modulating the trafficking of other cellular components of the immune system (such as monocytes and macrophages). Of note, studies suggest that T cells and macrophages are necessary for the development of collateral blood vessels.

The purpose of the present investigation was to determine definitively whether the immune system is a necessary component of collateral development. In particular, in this investigation we tested the following hypothesis: CD4+ T lymphocytes are critical to ischemia-induced collateral development. To test this hypothesis, we used a mouse model of hindlimb ischemia to compare collateral-mediated recovery of flow after acute induction of ischemia in control wild-type C57BL/6 mice versus that occurring in CD4 knockout mice (CD4−/−), in which the development of CD8+ T cells and myeloid components is unaltered, whereas the number of CD4+ T cells is markedly reduced.

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 CD4−/− mice (Jackson Laboratories, Bar Harbor, Maine) underwent surgical ligation of the left femoral artery to create unilateral hindlimb ischemia (n=14 per group). The study was approved by the Animal Care and Use Committee of the MedStar Research Institute.

Key Words: angiogenesis ■ lymphocytes ■ inflammation
Perfusion Imaging
Repetitive hindlimb blood flow measurements over the region of interest (from the patella to the midfoot) were obtained at baseline, immediately after surgery, and repeatedly over 4 weeks through the use of Laser Doppler Perfusion Imaging (LDPI) (Moor Instruments). Perfusion is expressed as the ratio of left (ischemic) to right (normal) limb.

In Vivo Assessment of Limb Function and Ischemic Damage
Semiquantitative assessment of impaired use of the ischemic limb was performed serially (3=dragging of foot, 2=no dragging but no plantar flexion, 1=plantar flexion, and 0=fLEXing the toes to resist gentle traction on the tail). Semiquantitative measurement of the ischemic damage was also assessed (0=no difference from the right hindlimb, 1=mild discoloration, 2=moderate discoloration, 3=severe discoloration or subcutaneous tissue loss or necrosis, and 4=any amputation).

Tissue Culture
Surgical incision was opened at 7 days after the procedure and a culture swab (Becton Dickinson) was taken under sterile conditions.

Western Blotting and Immunofluorescence Analysis
At day 7 after surgery, muscles were lysed in ice-cold buffer, protein extracted, separated by means of SDS-PAGE, and blotted onto nitrocellulose (Invitrogen). Blots were incubated with primary antibody (1:500 for anti-vascular endothelial growth factor (VEGF) [Chemicon, Temecula, Calif]; 1:1000 for α-tubulin [Santa Cruz, Santa Cruz, Calif]).

At the same time point, adductor muscle cryostat sections were obtained, fixed in methanol, blocked, and incubated first, overnight, with primary antibodies (1:500 for anti-CD4, Santa Cruz; 1:500 for anti-Mac3, Pharmingen [San Diego, Calif]; 1:1000 for anti-VEGF, Santa Cruz) and then at with FITC-anti-goat (Santa Cruz) or Rodamine–anti-rat antibody (Vector Laboratories, Burlingame, Calif) as a detection system. DAPI mounting media (Vector Laboratories) was used to detect viable cells. A microscope ECLIPSE TE300 and Magnafire software (Optronics) was used to analyze cells.

Flow Cytometry Assessment of Cellular Infiltration
At day 7 after surgery, muscles were removed, weighed, mechanically dissociated and incubated in dissociation buffer (HBSS with 400 U/mL collagenase II and 12 U/mL DNase I). Isolated cells were resuspended and incubated in Fc block solution (PBS–3% FBS), followed by incubation with the primary antibodies (FITC–anti-CD4, FITC–anti-Mac3, PE–anti-CD25; Pharmingen). Analysis was performed with a FACScan (Becton Dickinson), and the data were analyzed through the use of Cell Quest software.

Histological Analysis
After completing blood flow assessment over 28 days, hindlimb muscles were removed and formalin-fixed. From the upper thigh, sections were prepared and stained with van Gieson’s solution. Only arteries identified by the presence of a continuous internal elastic laminae and muscle spindles, present between muscle bundles and fibers and with a mathematically derived area >300 μm² were counted. The number of arteries present in each thigh was expressed as the ratio of the number of arteries to surface area of muscle analyzed.

From the gastrocnemius, sections were prepared and then stained with Sirius red, and collagen volume fraction was determined. 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.

Rescue Experiments by Infusion of CD4+ Cells From C57BL/6 Mice
Mononuclear cells were isolated from age and gender-matched C57BL/6 spleens and separated into CD4-positive and CD4-negative fractions by means of positive selection by magnetic labeling (Miltenyi Biotechnology, Inc.).

Three days after the induction of ischemia, 3×10⁶ purified CD4+ cells or CD4-depleted splenocytes were infused intravenously into CD4⁻/⁻ mice. To assess homing of injected cells, a subgroup of mice were injected with the same number of CFDA-SE labeled (Molecular Probes Inc) cells. Animals were killed after 24 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. An unpaired Student’s t test was used to compare raw and normalized values. Two-way ANOVA was used to compare values between groups over time. A probability value of 0.05 was considered significant.

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, and ultimately returned to near-normal levels by day 28. A similar precipitous reduction in hindlimb blood flow occurred in CD4⁻/⁻ mice, but in contrast to control mice, flow recovery was markedly attenuated (Figure 1a). Compared with C57BL/6, flow in CD4⁻/⁻ mice was significantly less by day 3, a difference persisting at each of the subsequent time points (7, 14, 21, and 28 days); flow never achieved >65% of that measured for the contralateral limb (Figure 1b).

At the end of follow-up, CD4⁻/⁻ animals showed a significant reduction in the density of collateral arteries in the upper thigh of the operated hindlimb when compared with C57BL/6 mice (0.36±0.03 versus 0.46±0.03 arteries >300 μm²/mm² of muscle area; P<0.05). No difference was observed among the nonoperated hindlimbs (Figure 1c).

In control mice, active use of the right foot significantly fell after surgery (Figure 1d), remained impaired for 3 days, improved by day 7, and ultimately returned to near-normal levels by day 14 (ambulatory impairment score: 0.21±0.2). A similar abrupt reduction in hindlimb active use occurred in CD4⁻/⁻ mice, but recovery was markedly attenuated at day 7 (ambulatory impairment score: 1.2±0.2; P<0.01 versus C57BL/6) and remained impaired over follow-up. CD4⁻/⁻ mice also had severe ischemic damage of the limb (Figure 1d) that was still evident by day 14 (tissue damage score: 2.5±0.3 versus 1.5±0.3, P<0.05) and resulted in a 40% incidence of autoamputation by day 28 (data not shown).

Necropsy examination of the calf muscle 28 days after surgery disclosed more pronounced interstitial fibrosis (Figure 2, a and b) and increased muscle fiber atrophy in CD4⁻/⁻ when compared with C57BL/6 mice (Figure 2, a and c).

Tissue Inflammatory Responses to Ischemia
We examined histologically the adductor muscle of the operated limb 7 days after femoral artery ligation. Hematoxylin and eosin staining revealed a marked infiltration of
inflammatory leukocytes in the wild-type mice. In contrast, the amount of infiltrated leukocytes was lower in the ischemic tissues of CD4−/− mice (data not shown). Moreover, the number of Mac-3–positive leukocytes was lower in CD4−/− mice than in wild-type mice (Figure 3a). In the same animals, reduced expression of VEGF in the ischemic muscle was observed (Figure 3b). Double staining for Mac-3 and VEGF demonstrated in foci of inflammatory cell infiltration that tissue macrophages express VEGF (Figure 3c).

Animal Health Status After Surgically Induced Hindlimb Ischemia

CD4−/− mice may be more susceptible than wild-type mice to superimposed bacterial infection caused by surgery, which may affect their ability to recover. To rule out this possibility, 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 necrosis and autoamputation in the ischemic limb of CD4−/− mice was not caused by associated septic gangrene. Moreover, CD4−/− mice exhibited, over follow-up, the same weight gain as the C57BL/6 mice, demonstrating absence of any major effect of the surgery on the general health of these mice.

Effect of CD4 Cells Reconstitution in CD4−/− Mice Subjected to Hindlimb Ischemia

At day 3 after surgery, we intravenously infused into CD4−/− mice spleen-derived purified CD4+T cells from control

Figure 1. Deficient collateral development in CD4−/− mice. a, Representative color-coded images reflecting red blood cell (RBC) velocity, an index of blood flow as assessed by laser Doppler imaging. Red is highest velocity, green intermediate, and blue, lowest velocity. b, Cumulative results for C57BL6 mice (n=14) and CD4−/− mice (n=14) are shown graphically as ratio of blood flow in ischemic limb to that in the nonischemic limb at each time point. c, Density of collateral arteries >300 μm² in upper thigh at 28 days after femoral artery ligation. When compared with the reference limb (NI indicates nonischemic limb), C57BL6 mice (C57) showed significantly increased fibrosis in the ischemic limb (I, ischemic leg). This effect was markedly attenuated in CD4−/− mice (CD4) (P<NS). Density indicates the number of arteries present in each thigh, expressed as the ratio of the number of arteries to surface area of muscle analyzed. d, Functional assessment of ischemic muscle over follow-up. Cumulative results for C57BL6 (continuous line) and CD4−/− mice (dotted line) are shown graphically as ambulatory impairment score (left) and ischemic tissue damage score (right) (*P<0.05).

Figure 2. Fibrotic changes in calf muscle after femoral artery ligation. a, Calf muscle: Sirius red–stained histological sections showing increased muscular (yellow) and fibrotic (red) content in C57BL6 (C57) and CD4−/− (CD4) 28 days after femoral artery ligation (NI indicates nonischemic limb; I, ischemic limb). b, When compared with C57BL6, CD4−/− showed significant increase of interstitial fibrosis in the ischemic limb. c, Significant reduction in the average muscle fiber area is observed in the ischemic limb of CD4−/− mice when compared with C57BL6 (*P<0.05).
mice (CD4+ group). Within 24 hours after infusion, exogenous CD4+ cells selectively homed to areas of inflammatory cell infiltration of the ischemic hindlimb (Figure 4a), where they constituted roughly one tenth of the cells in the field (10±3.4%). No exogenous CD4+ cells were found infiltrating muscle fibers of the sham-operated contralateral leg (Figure 4a). No selective homing was observed for CD4-negative cells in the ischemic leg (Figure 4a).

At day 7 after femoral artery ligation, in the ischemic limb of CD4+ mice, more abundant macrophage infiltration and VEGF expression was observed (Figure 4, b, c, and d). Densitometric analysis of the blots showed that CD4+ are able to express 70% of VEGF levels observed in C57BL/6. In addition, the overall number of CD4+ T cells present in the adductor muscle of CD4+ mice was similar to that of C57 BL/6 (122±64 versus 118±27.5 CD25+/CD4+ cells/μg of muscle, P=NS).

Hindlimb blood flow improved in the CD4+ mice when compared with CD4− mice receiving CD4− splenocytes (CD4− group) (Figure 5a). Over follow-up, at all time points, blood flow recovery in the CD4+ group was similar to that of the C57BL/6 mice. These mice had reduced ischemic damage, as observed by improved appearance of the limb (Figure 5b) and by histological evidence of reduced muscle fibrosis/atrophy (Figure 6a), and resulted in a 75% reduction in the incidence of autoamputation (Figure 6b). No difference was detected in all the analyzed end points between the original group of CD4− mice and CD4− group.

**Discussion**

In the present investigation, we used a mouse hindlimb model of ischemia to determine whether the immune system is a neces-

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**Figure 3.** Macrophage infiltration and VEGF expression at 7 days after femoral artery excision. Immunofluorescence staining (a, left and middle) showing macrophages (stained in red, pointed by arrows) infiltrating the interstitial space between muscle fibers of the adductor muscle of the ischemic limb (nuclei are stained in blue). Number of muscle-infiltrating macrophages is consistently reduced in CD4−/− mice (a, right) (*P<0.05). b, VEGF expression from ischemic hindlimbs in CD4−/− and C57BL/6 mice. c, Immunofluorescence staining for VEGF (stained in green, left) and macrophages (expressing Mac-3, stained in red, middle). Macrophages are able to express VEGF (colocalization of antigenity for Mac-3 and VEGF, right) when they infiltrate the interstitial space between muscle fibers of the ischemic limb.

**Figure 4.** CD4-positive T-cell selective homing and macrophage recruitment into ischemic hindlimbs in CD4+ mice after intravenous infusion of spleen derived-purified CD4+ cells. a, Fluorescence staining shows (top) exogenous CD4+ T cell (nuclei are stained in blue, fluorescent marker stains cytosol in green) present in interstitium between muscle fibers of the adductor muscle of the ischemic limb (CD4+ group). No exogenous CD4+ T-cell infiltration was evident in nonischemic sham-operated limb (middle). CD4-depleted splenocytes do not show any homing in the muscle of ischemic hindlimbs when infused in CD4−/− mice (CD4−) (bottom). NI indicates nonischemic limb; I, ischemic limb. b, Fluorescence staining at sites of inflammation in the ischemic leg. Mice from the CD4+ group show CD4+ T cells (upper, stained in green, pointed by arrows) and macrophage infiltration (bottom, stained in red, pointed by arrows) in the ischemic limb at 7 days after surgery. c, Number of muscle-infiltrating macrophages is consistently increased in CD4+ when compared with CD4− (*P<0.05). d, Western blot showing VEGF expression in muscle of the operated limb of C57BL/6, CD4+, and CD4−.
sary component of collateral development. In particular, we tested the validity of the hypothesis that CD4+ T lymphocytes are critical to ischemia-induced collateral development. The major findings of our study are (1) the inflammatory response and collateral development in response to ischemia are impaired in CD4−/− versus C57BL6 wild-type mice; (2) such changes have important biological consequences to limb function; (3) spleen-derived purified CD4-positive T cells, when infused into CD4−/− mice, selectively localize to the ischemic limb, increase macrophage recruitment, and result in blood flow recovery, limb salvage, and reduced muscle atrophy and interstitial fibrosis.

One of the important end points of our study is based on the assessment of limb flow by means of laser Doppler perfusion imaging. LDPI-assessed flow correlates well with microsphere-assessed perfusion and the number and size of second- and third-generation collateral branch arteries; we found the number of collateral arteries significantly increased in the operated limb of wild-type mice, changes that were markedly attenuated in the CD4−/− mice (Figure 1c). These results complement the flow-based findings derived from LDPI measurements.

The impaired flow and reduced number of collateral vessels present in the CD4−/− mice correlated with more prolonged 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 CD4−/− mice, we assessed calf muscle histology at 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 (Figure 2).

To definitively prove that the impaired development of collateral flow we observed in the CD4−/− mice was caused by a deficient supply of CD4-positive T cells, we performed a rescue experiment in which we infused into CD4−/− mice spleen-derived purified CD4-positive T cells. Exogenous CD4-positive T cellsselectively homed to the ischemic tissue within 24 hours after infusion, recruited macrophages, and restored arteriogenic cytokine production. The overall benefit of CD4-positive T-cell infusion was evident by restoration of recovery of flow to the levels observed in the wild-type

**Figure 5.** Hindlimb blood flow and ischemic tissue damage in CD4−/− mice after infusion of spleen derived-purified CD4 positive cells. a, Hindlimb perfusion ratios recorded by laser Doppler imaging in ischemic hindlimb of CD4−/− mice after infusion of CD4-positive (CD4+) or of splenocyte CD4-depleted cells (CD4−). b, Ischemic tissue damage score of operated hindlimb over follow-up in CD4−/− mice after intravenous infusion of spleen-derived, purified CD4-positive cells (continuous line) or CD4-depleted splenocytes (dotted line) (P<0.05).

**Figure 6.** Fibrotic changes and autoamputation rate in CD4−/− mice after infusion of spleen-derived, purified CD4-positive cells. a, Ischemic limb calf muscle Sirius red-stained histological sections showing increased muscular (yellow) and fibrotic (red) content in CD4+ and CD4−, 28 days after femoral artery ligation. b, CD4+ shows significant reduction of interstitial fibrosis (top) and a bigger muscle fiber area (bottom) (P<0.05 vs CD4−). c, Administration of CD4+ positive T cells reduced the incidence of autoamputation (bottom) by 75% (P<0.05). Top, Representative macroscopic photographs of CD4− mouse that underwent necrotic autoamputation of the forefoot.
parental strain and by preserved muscular structure, as demonstrated by a reduction in fibrosis and atrophy.

We conclude from our results that one of the most likely mechanisms contributing to the collateral-enhancing effects of CD4+ T cells appears to reside in the classic immune response activity of CD4+ T cells1; They induce monocyte-macrophage accumulation in the ischemic muscle. The accumulating macrophages then secrete a broad array of cytokines and growth factors, including VEGF, which facilitate collateral development.

Macrophages appear to be central to arterial remodeling in tissues undergoing arteriogenesis, accumulating at sites of collateral artery growth and angiogenesis. They are an important source of VEGF, TNFα, and bFGF, which are evident during collateral growth. Moreover, increased monocyte accumulation induced by LPS promotes angiogenesis and collateral growth; in addition, absence of macrophages is associated with a deficient arteriogenic response. A specific role of T cells in arteriogenesis was suggested by a prior study, which demonstrated that nude mice that lack all T cells and are severely immunocompromised exhibit an impaired collateral response to hindlimb ischemia. Finally, in addition to the effects on monocytes/macrophages, it is likely that CD4+ T cells have other proarteriogenic influences.

Importantly, ischemia per se may not be the critical stimulus for induction of the cellular response that is necessary for arteriogenesis. Collaterals originate from preexisting vessels located proximal to the obstructed major artery and therefore proximal to the ischemic tissue. Although little or no flow normally occurs in these small high-resistance vessels, flow becomes established after occlusion of their companion major conductive vessel. This increase in flow then leads to vascular remodeling and to the development of large functioning collaterals, changes presumably induced by shear-related endothelial responses. The same responses may be involved in recruiting CD4+ T lymphocytes, just as they have been demonstrated to recruit monocytes, after they are recruited to the target region, may help orchestrate and enhance the recruitment of monocytes and other players involved in arteriogenesis. An interesting question for future studies is whether ischemia is a trigger for a specific type of inflammatory response—one that leads to arteriogenesis rather than just to angiogenesis—or if it induces the same inflammatory response as other noxious stimuli, but in the context of the increased shear stress this response enhances vessel remodeling and collateral development.

Based on the findings of this investigation and the growing body of work exploring the mechanisms involved in arteriogenesis, we propose a novel concept. We assign an important role to CD4+ cells in collateral development and suggest that the effects of such cells are causally related, in part, to their capacity to attract the monocyte/macrophage to the region of collateral growth, which in turn triggers the development of collaterals through the synthesis of arteriogenic cytokines. The mechanisms by which CD4+ cells recruit monocytes in this model are as yet unknown. Although we cannot provide an answer to this question at present, our study’s importance derives from the proof of concept it provides demonstrating that cells of the immune and inflammatory systems are of critical importance to the development of collateral vessels.

References

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