Regulatory T Cells Modulate Postischemic Neovascularization

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Background—CD4+ and CD8+ T lymphocytes are key regulators of postischemic neovascularization. T-cell activation is promoted by 2 major costimulatory signalings, the B7/CD28 and CD40–CD40 ligand pathways. Interestingly, CD28 interactions with the structurally related ligands B7-1 and B7-2 are also required for the generation and homeostasis of CD4+CD25+ regulatory T cells (Treg cells), which play a critical role in the suppression of immune responses and the control of T-cell homeostasis. We hypothesized that Treg cell activation may modulate the immunoinflammatory response to ischemic injury, leading to alteration of postischemic vessel growth.

Methods and Results—Ischemia was induced by right femoral artery ligation in CD28−/−, B7-1/2−/−, or CD40-deficient mice (n=10 per group). CD40 deficiency led to a significant reduction in the postischemic inflammatory response and vessel growth. In contrast, at day 21 after ischemia, angiographic score, foot perfusion, and capillary density were increased by 2.0-, 1.2-, and 1.8-fold, respectively, in CD28-deficient mice, which showed a profound reduction in the number of Treg cells compared with controls. Similarly, disruption of B7-1/2 signaling or anti-CD25 treatment and subsequent Treg deletion significantly enhanced postischemic neovascularization. These effects were associated with enhanced accumulation of CD3-positive T cells and Mac-3-positive macrophages in the ischemic leg. Conversely, treatment of CD28−/− mice with the nonmitogenic anti-CD3 monoclonal antibody enhanced the number of endogenous Treg cells and led to a significant reduction of the postischemic inflammatory response and neovascularization. Finally, coadministration of Treg cells and CD28−/− splenocytes in Rag1−/− mice with hindlimb ischemia abrogated the CD28−/− splenocyte-induced activation of the inflammatory response and neovascularization.

Conclusion—Treg cell response modulates postischemic neovascularization. (Circulation. 2009;120:1415-1425.)

Key Words: angiogenesis ■ ischemia ■ inflammation ■ cells

Neovascularization, including vasculogenesis, angiogenesis, and collateral growth, characterizes the tissue repair and remodeling that occurs in ischemic vascular diseases. In addition to specific initial triggers, these processes share growth factors, chemokines, and proteases that play different roles in promoting and refining tissue regeneration. It has also been shown that cellular components of the inflammatory system play a critical role in this setting.1,2 Hence, numerous studies have demonstrated the importance of monocyte recruitment in stimulating vessel growth.2–4 Blockade of endogenous monocyte chemotactic protein-1 activity markedly inhibited the ischemia-induced macrophage infiltration that led to impaired collateral vessel and capillary formation in the ischemic hindlimb.5 Furthermore, functional links have been shown between monocyte concentration in the peripheral blood and the enhancement of vessel growth.4 T lymphocytes are also involved, and nude mice, which lack all T-cell subsets, exhibit a pronounced reduction in postischemic vessel growth.6 Furthermore, subsets of T lymphocytes, CD4+ and CD8+ cells, have been suggested to play a role in vascular remodeling.7,8 CD4+ and CD8-deficient mice exhibit a significant reduction in postischemic vessel growth. In addition, CD8-deficient mice display reduced interleukin (IL)-16 expression and decreased CD4+ T-cell recruitment at the site of collateral vessel development. Natural killer cells also display proangiogenic properties. Hence, collateral growth is impaired after natural killer cell depletion and in natural killer cell–deficient mice.9 Leukocytes trigger neovascularization through the release of several angiogenic/arteriogenic factors, including vascular endothelial growth factor (VEGF) and proinflamma-
try cytokines such as tumor necrosis factor-α and IL-1β. Finally, during the inflammatory reaction, antiinflammatory cytokines such as IL-10 and transforming growth factor-β (TGF-β) are also produced and are able to modulate the neovascularization process and orchestrate the tissue response to ischemia.11–13

Clinical Perspective on p 1425

Several costimulatory pathways have been described for inflammatory T-cell activation, of which the B7/CD28 and the CD40 ligand/CD40 (CD40L/CD40) pathways play major roles. Interestingly, CD28 interactions with the structurally related ligands B7-1 (CD80) and B7-2 (CD86) are also required for the generation and homeostasis of CD4+CD25FoxP3 regulatory T cells (Treg). Treg cells constitute 10% of peripheral CD4+ T cells; they are specialized in the suppression of pathogenic immune responses against self-antigens and foreign antigens and are required for the maintenance of immune homeostasis.14 A number of critical cytokines affect the survival and function of Treg cells. Production of IL-2 by peripheral pathogenic T cells after CD28 engagement expands the Treg cell population. In addition, production of the immunosuppressive cytokines TGF-β and IL-10 by Treg cells induces deactivation of dendritic cells, which leads to their inability to activate effector T cells and monocytes. Treg cells also suppress the inflammatory reaction through engagement of the cytotoxic T-lymphocyte antigen 4 expressed on Treg cells with B7-1/-2 molecules expressed on effector T cells or on antigen-presenting cells.15 Interestingly, modulation of Treg cell levels through B7/CD28 interaction controls the progression of inflammatory diseases. In this regard, a reduction in Treg cell number and function in both B7-1/-2- and CD28-deficient diabetic mice exacerbates diabetes.16–19 In addition, it has been shown that modulation of the Treg cell response controls the development of atherosclerotic lesions.20 However, the role of the Treg cell repertoire in the control of postischemic neovascularization remains unknown.

Here, we hypothesized that an imbalance between Treg cells and effector immune cells modulates postischemic vessel growth. We assessed the effect of Treg cell response in CD28- or B7-1/-2-deficient mice with surgically induced hindlimb ischemia. To further substantiate the role of endogenous Treg cells in postischemic neovascularization, we also treated CD28-deficient mice with nonmitogenic anti-CD3 monoclonal antibody F(ab′)2 (50 μg per mouse per day) or with 0.1 mL of PBS as a control (intravenous injection for 5 consecutive days), as described previously.21 Ischemia was induced 4 weeks after the onset of treatment to allow the complete recovery of the number of CD3-positive cells, as described previously.21

To analyze the proangiogenic potential of exogenous Treg cells, C57BL/6 CD45.1+ wild-type male mice were also used to isolate spleen Treg cells. A total of 10^6 CD45.1+ wild-type Treg cells and 10^6 splenocytes isolated from CD28-deficient mice were then injected intravenously into Rag1-deficient mice 1 day after the onset of ischemia. All experiments were performed in accordance with the European Community guidelines for the care and use of laboratory animals (No. 07430).

Quantification of Neovascularization

Neovascularization was evaluated 7 and 21 days after ischemia by 3 different methods, as described previously.11–21

Microangiography

Mice were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg), and barium sulfate (3.3 g/mL) was injected into the abdominal aorta. Images were acquired with a high-definition digital x-ray transducer. Vessel density was expressed as a percentage of pixels per image occupied by vessels in the quantification area.

Capillary Density

Frozen tissue sections (7 μm) from calf muscle were incubated with a rabbit polyclonal antibody directed against total fibronectin (1:50; ABCYS, Paris, France). Capillaries were revealed with a fluorescein isothiocyanate (FITC) anti-rabbit antibody (1:10; Amersham, Uppsala, Sweden). Capillary density was evaluated in ischemic and nonischemic legs.

Laser Doppler Perfusion Imaging

Laser Doppler perfusion imaging experiments were performed to assess limb perfusion after ischemic damage. Foot blood flow was expressed as the ratio of ischemic to nonischemic leg.

Immunohistochemistry

Inflammatory cell staining was performed at days 7 and 21 after ischemic injury. Briefly, frozen calf sections (7 μm) were incubated with a rabbit polyclonal antibody directed against CD3ε (1:50; DakoCytomation, Glostrup, Denmark) to identify CD3 T lymphocytes, with a rat anti-Mac-3 monoclonal antibody (1:50; BD Pharmingen, San Diego, Calif) to identify macrophages. Immunostainings were visualized with peroxidase-conjugated donkey anti-rabbit, anti-rat, or anti-FITC antibodies (1:100; Jackson ImmunoResearch, West Grove, Pa) with the AEC visualization system (DakoCytomation). Images were acquired, and CD3-, Mac-3-, and CD45.1-positive cells were counted in both the ischemic and nonischemic area with Image J software.

Treg Cell Recovery and Purification

CD4+ cells were negatively selected from CD45.1+ splenocytes with a cocktail of biotin-conjugated antibodies from Miltenyi Biotec (Bergisch Gladbach, Germany; anti-CD8a, anti-CD11b, anti-CD45R, anti-CD5, and anti-ter-119) and anti-biotin microbeads, according to the manufacturer’s instructions. In parallel, cells were labeled with a phycoerythrin-conjugated anti-CD25 antibody (clone 7D4), followed by magnetic cell separation with LD columns. Purified CD4+CD45.1+ cells were subjected to positive selection after incubation with anti-phycoerythrin microbeads and magnetically separated with 2 MS columns, which yielded 85% to 90% CD4+CD25+ cells. CD4+CD25- cells, which did not bind to the

Methods

Mouse Ischemic Hindlimb Model

Ten-week-old C57BL/6 wild-type male mice treated with or without CD25-depleting PC61 antibody (100 μg per mouse at day 0 and day 11) and CD28-, B7-1/-2-, and CD40-deficient mice (Charles River, Orléans, France) underwent surgery to induce unilateral hindlimb ischemia. Animals (10 mice per group) were anesthetized by isoflurane inhalation. The ligature was placed on the proximal part of the right femoral artery, as described previously.11,12

To increase the number of endogenous Treg cells, additional groups of wild-type and CD28-deficient mice were treated with a nonmitogenic anti-CD3 monoclonal antibody F(ab′)2 (50 μg per mouse per day) or with 0.1 mL of PBS as a control (intravenous injection for 5 consecutive days), as described previously.21

Ischemia was induced 4 weeks after the onset of treatment to allow the complete recovery of the number of CD3-positive cells, as described previously.21

To analyze the proangiogenic potential of exogenous Treg cells, C57BL/6 CD45.1+ wild-type male mice were also used to isolate spleen Treg cells. A total of 10^6 CD45.1+ wild-type Treg cells and 10^6 splenocytes isolated from CD28-deficient mice were then injected intravenously into Rag1-deficient mice 1 day after the onset of ischemia. All experiments were performed in accordance with the European Community guidelines for the care and use of laboratory animals (No. 07430).
beads, were harvested from the flow through and contained less than 0.3% of CD4+/CD25+ cells.

**Flow Cytometry**

Blood cells and splenocytes were labeled with FITC-conjugated anti-CD4 (RM4-5 clone; eBioscience, San Diego, Calif) and allophycocyanin-conjugated anti-CD25 (clone PC61.5; eBioscience). An intracellular FoxP3 staining was then performed with phycoerythrin-conjugated anti-mouse/rat FoxP3 (PFJK-16s; eBioscience) according to the manufacturer’s instructions. In Rag1 knockout mice (Rag1KO), phycoerythrin-Cy5 (PE-Cy5)–conjugated anti-CD45.1 (clone A20; eBioscience) was used for Treg cell labeling and then analyzed by flow cytometry on a Canto II device (Becton Dickinson, Franklin Lakes, NJ) with BD FACS DIVA 5 software.

**VEGF Protein Expression**

VEGF-A protein level was determined by Western blot in ischemic and nonischemic legs, as described previously. Antibodies directed against VEGF-A (1:1,000; Santa Cruz Biotechnology, Santa Cruz, Calif) and GAPDH (1:10,000; Sigma, St Louis, Mo) were used for immunoblotting. Results were expressed as a ratio of ischemic to nonischemic leg.

**Real-Time Quantitative Reverse-Transcription Polymerase Chain Reaction**

Total RNA was extracted from ischemic and nonischemic tibial anterior muscle according to the Trizol reagent protocol (Invitrogen, Carlsbad, Calif) and from blood with the QIAamp RNA Blood Mini Kit (Qiagen, Valencia, Calif). Total RNA was reverse transcribed with the Quantitect Reverse Transcription Kit (Qiagen). Real-time quantitative polymerase chain reaction was performed on a StepOne Plus (Applied Biosystems, Foster City, Calif). The sequences of primers used were as follows: for IL-1β, forward 5'-AAG-A GCCCATCTCTC-TGTGACTCAT-3', reverse 5'-ACAAAC-CGCTTTTCCATCTTCTTC-3'; for IL-6, forward 5'-CCACTCCTCAACACGTTGTCT-ATA-3', reverse 5'-GGGGGA-GGAAGGACTATT-TTATGT-3'; for tumor necrosis factor-α, forward 5'-GCCCAGACCCTCACACTC-AGA-3', reverse 5'-CGGCAGAGAGGAGGTTGACTT-3'; for IL-10, forward 5'-CAAGCAGAGAGGAGAAGGACTATT-3'; for TGF-β, forward 5'-GGCAAACTGTTTGGAAGTTGACTT-3'; for GAPDH, forward 5'-CTTGATTTCTGGGCCATGCTTCTC-3', reverse 5'-GCCGATGTCAGTGTGACCTTCC-CA-3'. The detection of amplification products was performed with the MESA Green qPCR Master-
Mix (Eurogentec, Brussels, Belgium). Quantification was performed relative to GAPDH. Results were analyzed with StepOne version 2.0 software (Applied Biosystems).

Statistical Analysis

Results were expressed as mean±SEM. Kruskal-Wallis ANOVA was used to compare each parameter. Post hoc Mann–Whitney U tests with Bonferroni correction were then performed to identify which group differences accounted for the significant overall Kruskal-Wallis result.

Results

Downregulation of Endogenous Treg Cell Levels Activates Postischemic Neovascularization

We first examined the effect of Treg cell deficiency on postischemic neovascularization using CD28−, B7−1/2−, and CD40-deficient mice. Numerous studies have indicated that CD28 and B7−1/2 deficiency reduces Treg cell numbers, whereas CD40 deficiency does not result in a major modulation of the number of Treg cells.16–20

Microangiography

At day 7, the angiographic score (Figure 1A) of CD28− and B7−1/2−-deficient mice was increased by 1.7- and 2.3-fold, respectively, compared with control mice (P<0.0167 and P<0.0003, respectively). In contrast, the absence of CD40 slightly decreased ischemic/nonischemic vessel density. At day 21 after the onset of ischemia, the angiographic score was still significantly higher in CD28−/− and B7−1/2−/− mice than in control animals (P<0.0003), whereas the angiographic score in CD40-deficient animals tended to be lower than in wild-type mice.

Capillary Density

Microangiographic results were confirmed by capillary density analysis (Figure 1B). At day 7 of ischemia, the ratio of ischemic to nonischemic capillary density was increased by 1.9- and 1.5-fold in CD28− and B7−1/2−-deficient mice, respectively, compared with controls. CD40 deficiency induced a 2-fold reduction in the number of capillaries...
Similar results were obtained 21 days after surgery.

**Laser Doppler Perfusion Imaging**

Microangiographic and capillary density changes observed in mice deficient for CD28 or B7-1/2 were associated with an increase in blood flow at days 7 and 21 (Figure 1C). In contrast, CD40 deficiency reduced foot perfusion at day 7 compared with control mice. Together, these results suggest that an endogenous Treg cell response inhibits whereas the CD40 costimulatory pathway tends to promote postischemic vessel growth. We next examined the molecular and cellular mechanisms associated with the modulation of postischemic neovascularization in these settings.

**Inflammatory Cell Infiltration in the Ischemic Area**

First, we assessed the number of CD3 T lymphocytes in the ischemic tissue using CD3 immunostaining (Figure 2A). At day 7 after the onset of ischemia, T-lymphocyte accumulation in the ischemic calf muscle of CD28- and B7-deficient mice was increased by 2.9- and 3.3-fold, respectively (P<0.0003), compared with control mice. Differences were still significant at day 21 (P<0.0003). In contrast, 7 days after ischemia, mice deficient for CD40 presented a 40% reduction in lymphocyte infiltration compared with controls, but this difference did not reach statistical significance.

We next evaluated monocyte/macrophage infiltration (Figure 2B). After 7 days of ischemia, the number of Mac-3–positive monocytes/macrophages was increased by 2- and 1.7-fold, respectively, in mice deficient for CD28 and B7-1/2 compared with control mice (P<0.0003). Monocyte infiltration was still important 21 days after ischemia. In contrast, CD40 deficiency reduced macrophage accumulation in the ischemic tissue (36% reduction), but the difference was no longer significant at day 21. Taken together, these results underscore an important role for the balance between Treg cell and costimulatory pathways in the control of inflammatory cell infiltration into the ischemic muscle.
VEGF-A Protein Levels

A variety of different growth factors that act by stimulating endothelial and smooth muscle cell proliferation and migration are released by inflammatory cells. Among these, VEGF-A is a well-known proangiogenic and proarteriogenic factor. After 7 or 21 days of ischemia, VEGF-A protein content was not affected in mice deficient for CD28, B7-1/2, or CD40 (Figure 3A).

Proinflammatory and Antiinflammatory Cytokine Levels

Infiltrating inflammatory cells also release a vast array of cytokines with proinflammatory, antiinflammatory, and angiogenic potential. We therefore examined cytokine expression using quantitative reverse-transcription polymerase chain reaction (Figure 3B). After 7 days of ischemic damage, messenger RNA (mRNA) levels of the proinflammatory cytokines IL-1β, tumor necrosis factor-α, and IL-6 were markedly increased in CD28- and B7-1/2–deficient mice compared with controls (P<0.0167). In contrast, mRNA levels of the antiinflammatory cytokines IL-10 and TGF-β1 were upregulated in CD40−/− mice. Differences were no longer significant 21 days after ischemia. The present results suggest that the defective Treg cell response induced through CD28/B7 inhibition may promote a switch toward a proinflammatory cytokine profile that leads to activation of postischemic vessel growth, whereas inhibition of the CD40 costimulatory pathway resulted in an antiinflammatory response.

B7 and CD28 are involved in multiple forms of T-cell activation and are not necessarily simply regulators of CD4+CD25+ Treg cells. Therefore, we performed additional experiments using a CD25-specific antibody that depletes Treg cells. After anti-CD25 treatment, the percentage of CD4+CD25hi FoxP3+ Treg cells among CD4+ T cells was dramatically reduced in blood and spleen at day 14 after ischemia (Figure 4A; online-only Data Supplement Figure I). In this setting, the postischemic inflammatory response and neovascularization were increased in anti-CD25–treated animals compared with control mice (Figure 4B and 4C). Taken together, these results demonstrate that the endogenous Treg cell
response controls postischemic inflammatory balance and neovascularization.

Upregulation of Endogenous Treg Cell Levels Inhibits Postischemic Neovascularization

To further substantiate the role of endogenous Treg cells in postischemic neovascularization, we examined vessel growth 21 days after ischemic injury in CD28-deficient mice treated with a CD3ε-specific antibody known to increase the number of Treg cells.

Anti-CD3ε Treatment Increases Treg Cell Levels

At day 0 of ischemia, blood levels of Treg cells were 3.4-fold lower in CD28-deficient animals than in wild-type mice ($P<0.0002$). Anti-CD3 therapy increased by 3-fold the number of Treg cells in blood in CD28 knockout mice (CD28KO) compared with untreated mice ($P<0.0002$). At day 7 and 21 after ischemia, Treg cell levels in blood were still lower in CD28-deficient animals than in controls and were upregulated by anti-CD3 treatment. In spleen, Treg cell number was lower in CD28KO animals than in wild-type mice and was upregulated by anti-CD3 antibody (Figures 5A and 5B). We also assessed the expression of the Treg cell marker FoxP3 in blood (Figure 5C) and ischemic calf muscle (Figure 5C) using quantitative reverse-transcription polymerase chain reaction. At day 21, FoxP3 mRNA levels were decreased by 95% and 60% in the blood and ischemic calf muscle, respectively, of CD28-deficient mice compared with control animals. Interestingly, CD3ε-antibody treatment increased FoxP3 mRNA
levels by 19- and 38-fold in blood and ischemic muscle, respectively, compared with untreated CD28-deficient mice. In contrast, no significant differences were observed in FoxP3 mRNA levels in blood and muscle of anti-CD3/H9280 treated wild-type mice.

**Anti-CD3e-Induced Treg Cell Upregulation Inhibits Postischemic Inflammatory Response and Neovascularization**

Twenty-one days after ischemia, anti-CD3e treatment of CD28KO mice reduced angiographic score, capillary number, and foot perfusion by 47%, 50%, and 27%, respectively, compared with CD28-deficient mice given PBS (Figure 6A). Anti-CD3 treatment did not affect vessel growth in wild-type mice compared with untreated wild-type animals. These effects were associated with a significant decrease in the number of CD3-positive T cells (42% reduction) and macrophages (36% reduction) in the ischemic calf muscle of CD28-deficient mice treated with anti-CD3e compared with untreated mice lacking CD28 (Figure 6B). Inflammatory cell infiltration was similar in both anti-CD3–treated and untreated wild-type mice.

Finally, upregulation of endogenous Treg cell levels downregulated IL-1β and tumor necrosis factor-α mRNA levels by 83% and 66%, respectively, and IL-6 mRNA levels were barely detectable in anti-CD3e–treated mice compared with untreated animals. In contrast, TGF-β content was increased by 2.7-fold compared with untreated CD28−/− mice, whereas the IL-10 mRNA level was unaffected (online-only Data Supplement Figure II).

**Exogenous Modulation of Treg Cell Levels Controls Postischemic Neovascularization**

Finally, we attempted to demonstrate a cause-and-effect relationship between the number of Treg cells and the development of vessels. To this purpose, we injected splenocytes isolated from CD28 knockout mice with or without Treg cells in ischemic Rag1KO mice. Twenty-one days after ischemia, the percentage of FoxP3+/CD4+ Treg cells was increased by 6- and 5-fold in blood and spleen, respectively, of Rag1-deficient mice given both CD28KO splenocytes and Treg cells compared with mice treated with CD28KO splenocytes. In addition, we found that 41.7% and 49.5% of blood and spleen CD45.1+ cells, respectively, expressed the FoxP3 marker (Figure 7A). Angiographic score and capillary density were increased by 1.6- and 2.3-fold, respectively, in Rag1KO mice given CD28KO splenocytes compared with PBS-injected CD28KO mice. D21 indicates day 21; Cont, control; Isch, ischemic; and N.Isch, nonischemic.
Discussion

In the present study, we reported that a specific subset of CD4\(^+\) T cells, regulatory T cells, known to be crucial in the maintenance of peripheral immune tolerance are a critical modulator of postischemic neovascularization. B7/CD28 and CD40L/CD40 interactions have been recognized as 2 major costimulatory pathways of T-cell activation. Interestingly, it has been demonstrated that CD28 and B7-1/2 costimulatory molecules also control immunoregulatory T cells, which are known to suppress T-cell responses against self-antigens or foreign antigens. We observed that profound Treg cell number may weaken the effect of anti-CD25 administration postischemic neovascularization compared with CD28-deficient animals.

In line with these results, treatment of CD28\(^{-}\/) mice with the nonmitogenic anti-CD3 monoclonal antibody enhanced regulatory T-cell levels and led to a reduction in postischemic neovascularization of CD28\(^{-}\/) mice. Similarly, restoration of self-tolerance induced in anti-CD3–treated nonobese diabetic (NOD) mice by upregulation of Treg cell levels led to diabetes remission. These immunosuppressive properties of anti-CD3 antibodies have also been exploited in a mouse model of atherosclerosis and were revealed to reduce plaque development. Finally, we also demonstrated a cause-and-effect relationship between the number of Treg cells and the development of vessels. For this purpose, we injected 1 million Treg cells and 10 million splenocytes, which led to a physiological ratio of Treg cells and splenocytes in vivo. The number of Treg cells was similar in the spleen of Rag1\(^{-}\/) mice cotransferred with Treg cells and CD28\(^{-}\/) splenocytes compared with C57Bl/6 wild-type mice. Furthermore, the number of Treg cells in blood was only 2-fold higher in CD28\(^{-}\/) mice than in mice treated with anti-CD25 antibody. Ten days after ischemia, we observed a complete recovery in the number of Treg cells cotransferred with CD28KO mice. Subsequently, we made an additional intraperitoneal injection of CD25-specific antibody at day 11 to reduce Treg levels again. Therefore, it is likely that the transient increase of Treg number may weaken the effect of anti-CD25 administration postischemic neovascularization compared with CD28-deficient animals.

Figure 7. A, Flow cytometry analysis of FoxP3\(^+\)/CD4\(^+\) and FoxP3/CD45.1\(^+\) cells in blood and spleen of ischemic Rag1KO mice treated with PBS, splenocytes isolated from CD28KO mice (spleenocytes CD28KO), and both splenocytes CD28KO and CD45.1\(^+\) Treg cells 21 days after ischemia. B, Quantitative evaluation of microangiography, capillary density, and foot perfusion in Rag1KO mice treated with PBS or splenocytes derived from CD28KO mice with or without CD45.1\(^+\) Treg cells. C, Quantitative evaluation of C3- and Mac-3–positive cells in calf muscle of Rag1KO mice treated with PBS or splenocytes derived from CD28KO mice with or without CD45.1\(^+\) Treg cells. Values are mean±SEM (n=10); 3 pairwise comparisons. *P<0.0167, **P<0.0003 vs PBS-injected Rag1KO mice and #P<0.0167, ##P<0.0033 ###P<0.0003 vs Rag1KO mice receiving CD28KO splenocytes. ND indicates not detected; Isch, ischemic; N, nonischemic; D7, day 7; and D21, day 21.
In contrast, the CD40L/CD40 costimulatory pathway only marginally reduces the Treg compartment, whereas T-cell activation is profoundly affected. Accordingly, the present results showed that CD40 deficiency impaired the postischemic inflammatory reaction and blood vessel growth. Thus, the balance between Treg cell and costimulatory functions greatly determines the issue of the immunoinflammatory response, which significantly impacts postischemic vessel growth.

To investigate potential mechanisms involved in the angiostatic effects related to Treg cells, we first examined the effect of Treg cell downregulation on inflammatory cell infiltration. The increase in neovascularization observed in CD28−/− mice, B7-1/2−/− mice, or anti-CD25−/−treated wildtype mice was associated with upregulation of T lymphocyte and monocyte recruitment into the ischemic area. In support of our findings, previous studies have shown that abrogation of the Treg compartment by complete disruption of CD28 signals induces T-cell activation and increases the severity of inflammatory disease in experimental models of diabetes and atherosclerosis. In contrast, anti-CD3 treatment of CD28−/− mice, which negatively regulates inflammatory cell infiltration, leads to inhibition of vessel development.

Exogenous injection of Treg cells in Rag1KO mice also downregulates inflammatory cell accumulation in the setting of ischemia. Macrophage and T-cell accumulation into ischemic tissue promotes neovascularization through production of angiogenic growth factors. Inflammatory cell infiltration is also associated with the expression of proinflammatory and proangiogenic cytokines. Regulatory T cells inhibit the activation of effector immune cells at least in part by producing or promoting the production of suppressive cytokines such as IL-10 and TGF-β. Hence, it is likely that a deficit in Treg cells promotes vessel development by its stimulatory effects on proinflammatory mediators. In contrast, anti-CD3–induced upregulation of Treg cell levels increased expression of TGF-β. Accordingly, anti-CD3–induced long-term remission in overtly diabetic NOD mice and atherosclerotic mice is TGF-β dependent.

In conclusion, the present study reports for the first time that CD4+CD25+FoxP3+ regulatory T cells play a key role in postischemic neovascularization through control of the effector immune cell response. Our findings also suggest that modulation of the regulatory arm of the immune response could provide novel angiogenic strategies for the treatment of ischemic diseases.

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

A specific component of the immune system known as the CD4+CD25+ regulatory T cell (Treg) repertoire is specialized for the suppression of both T helper 1 and T helper 2 pathogenic immune responses against self-antigens or foreign antigens and controls T-cell homeostasis. Interestingly, modulation of Treg cell levels controls the progression of inflammatory diseases. In this regard, the reduction of Treg cell number and function in both B7-1/2− and CD28-deficient diabetic mice exacerbates diabetes mellitus. In addition, it has been shown that modulation of the Treg cell response controls the development of atherosclerotic lesions. However, the role of Treg cells in the control of posts ischemic neovascularization remains unknown. In the present study, we report that Treg cells are a critical modulator of vessel growth. We observed that profound Treg cell reduction caused by B7/CD28 deficiency or anti-CD25 treatment increased posts ischemic revascularization, and we showed that exogenous administration of Treg cells reduced vessel growth in this setting. These results provide new insights into the immune regulation of vessel growth and may lead to new therapeutic approaches that involve immune modulation with Treg cells. In particular, our findings suggest that modulation of the regulatory arm of the immune response could provide novel strategies for the treatment of ischemic diseases that target angiogenesis.
Regulatory T Cells Modulate Postischemic Neovascularization
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In the article “Regulatory T Cells Modulate Postischemic Neovascularization” by Zouggari et al, which appeared in the October 6, 2009 issue of the journal (Circulation. 2009;120;1415–1425), one of the author’s names was spelled incorrectly. The name should have read “Esther Lutgens, MD, PhD.”

In addition, the author affiliations on page 1415 should read, “From Paris-Cardiovascular Research Center (Y.Z., L.W., J.V., C.L., C.C., A.R., M.D., B.I.L., Z.M., J.-S.S.), INSERM U970, Hôpital Européen Georges Pompidou, Université Paris 5, Paris, France; Service de Réanimation médicale (H.A.-O.), Hôpital Saint-Antoine, Paris, France; and Institute for Molecular Cardiovascular Research, RWTH Aachen University, Aachen, Germany (E.L.).”

The online version of the article has been corrected. The authors regret the errors.

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**Supplemental Material**

**Figure I.** Time course of CD4+CD25+Foxp3+ Treg levels in blood of wild-type C57BL/6 mice treated with or without anti-CD25 antibody. Evaluation by flow cytometry. 1 pair-wise comparison. *** P<0.001 versus PBS-injected wild-type mice. ND indicates not detected.

**Figure II.** Quantitative evaluation of IL-1β, TNF-α, IL-6, IL-10 and TGF-β mRNA levels in ischemic calf muscle of wild-type C57BL/6 mice, untreated CD28KO mice, and anti-CD3ε treated CD28KO mice, 21 days after ischemia (n=6). Values are mean±SEM. 2 pair wise comparison * P<0.025, ** P<0.005, *** P<0.0005 versus control mice and # P<0.025, ## P<0.025 versus PBS-injected CD28KO mice.
Figure I
Figure II