Regulatory T cells influence blood flow recovery in experimental hindlimb ischaemia in an IL-10-dependent manner

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Aims
Ischaemic damage is associated with up-regulation of pro-inflammatory cytokines, as well as invasion of leucocytes and lymphocytes to the injured muscle. Regulatory T cells (Tregs) exert suppressive effects on several immune and non-immune cellular elements. We hypothesized that adoptive Treg cell transfer and depletion will influence re-establishment of flow in the hindlimb ischaemia model, and that this effect would be mediated by the cytokine interleukin (IL)-10.

Methods and results
To study the functional role of Tregs in hindlimb ischaemia, we either adoptively transferred Tregs or functionally blocked Tregs by antibodies to CD25. Initially, we showed that the number and function of Tregs is altered after the induction of ischaemia. Treg ablation resulted in reduced blood flow by laser Doppler at Day 7 that became more robust at Day 14. Adoptive Treg transfer led to a significant improvement of flow in the ligated limb. Treg-mediated improvement in flow was abolished by employing blocking anti-IL-10 antibodies.

Conclusions
These results show that Tregs play an important role in processes that control flow re-establishment after inducible hindlimb ischaemia, and that IL-10 plays a requisite role mediating these effects.

Keywords
Ischaemia • Inflammation • Lymphocyte • Regulatory T cells

1. Introduction
Ischaemia is a restriction of blood supply to tissues, causing a shortage of oxygen and glucose needed for cellular metabolism. Ischaemia results in tissue damage and inflammatory reaction distal to the occlusion. Cytokines are important molecular signals in the inflammatory response to ischaemia and play a potentially dominant role in the complex processes that govern re-establishment of flow to the afflicted organ.

Acute limb ischaemia is defined as a sudden decrease in limb perfusion that threatens the viability of the organ. The incidence of limb ischaemia is ~1.5 cases per 10 000 persons per year. Animal models of hindlimb ischaemia include mice, rats, and rabbits. Surgical procedures range from a single ligation of the femoral artery to a complete excision of the artery.

Previous work relating to the role of the immune system in ischaemia showed that nude mice, which lack all T-cell subsets, exhibit a marked reduction in post-ischaemic vascularity. In addition, CD4⁺ and CD8⁺-deficient mice exhibit a significant reduction in post-ischaemic vessel growth. Moreover, CD8-deficient mice display a reduced interleukin (IL)-16 expression and decreased CD4⁺ T-cell recruitment at the site of collateral vessel development. Natural killer cells also appear to play a role in the hindlimb ischaemia model by exhibiting pro-angiogenic properties. Hence, collateral growth is impaired after natural killer cell depletion and in natural killer cell-deficient mice. Finally, during the inflammatory process, anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (TGF-β) are also produced that potentially modulate the neovascularization process and orchestrate tissue response to ischaemia.

The concept of ‘suppressor’ T cells acting to down-regulate the host’s immune system is known back from the early 1970s. They are involved in the prevention of autoimmune disease, allergies, infection-induced organ pathology, transplant rejection, as well as graft vs. host disease.
suppression of effector T cells (Teffs) and other immune cells. The naturally occurring population of CD4+CD25+ T cells (regulatory T cells; Tregs), both in naive mice and in humans, constitutes 5–10% of the peripheral CD4+ T cells. There are conflicting data regarding the precise mechanism by which Tregs mediate their suppressive effects. 

While cell contact-dependent mechanisms that are independent of cytokines have been demonstrated in vitro, the mechanisms by which Tregs suppress autoimmunity in vivo are more complex, and several suppressor cytokines have been implicated as potential mediators. Moreover, several cellular targets influenced by Tregs have been proposed other than effector T cells and include antigen-presenting cells, endothelial cells (ECs), and cardiomyocytes.

Several studies documented the role of Tregs at ischaemic environment in stroke, kidney, and pulmonary ischaemia. Others have reported that insufficient recruitment of Tregs results in worsening of ventricular remodelling. A recent study described a role for Tregs in a rat model of myocardial infarction, and in a study in mice it was shown that Tregs become activated after myocardial infarction and facilitate wound healing of the myocardium.25

In a previous study, we have shown that circulating endothelial progenitor cells (EPCs), known to play an important role in the vasculogenic response to ischaemia, associates with peripheral Tregs. This finding prompted us to investigate a possible relation between vasculogenesis and inflammation in an experimental model. In this study, we show for the first time that adoptive Treg cell transfer and depletion influence the re-establishment of flow in the hindlimb ischaemia model, and that this effect is mediated by the cytokine IL-10.

2. Methods

2.1 Animals

All animal studies were approved by The Animal Care and Use Committee of Tel-Aviv Sourasky Medical Center, which conforms to the policies of the American Heart Association and the Guide for the Care and Use of Laboratory Animals. Ten-week-old C57BL/6 wild-type male mice weighing 25 g were purchased from Harlan Laboratories, Israel.

2.2 Hindlimb ischaemia model and laser-Doppler perfusion imaging

Mice were anaesthetized with 2% isoflurane. The femoral artery was exposed by incision of the skin at the middle portion of the left hindlimb. Both the proximal and the distal ends of the femoral artery were ligated and the artery was excised. Control animals were sham operated: skin was incised and ligated. Dypirone (60 mg/kg) was given subcutaneously after incision and the artery was excised. One day after induction of hindlimb ischaemia, mice received 2 × 10^5 murine Tregs (1:1 responder/suppressor). All cells were seeded in a final volume of 200 μL in the presence of soluble anti-mouse CD28 (clone 37.51, eBioscience). After 72 h, [3H] thymidine (1 μCi/well) was added for 18 h, and cell proliferation was then assayed by scintillation counting in a beta counter. Percent inhibition of proliferation was determined with the following formula: 1 – (median [3H] thymidine uptake of 1:1 responder/suppressor co-culture/median [3H] thymidine uptake of Treg only).

2.4 Functional suppression assay for Tregs

For co-culturing of CD4+CD25+ T cells (Teffs) and Treg, 96-well plates were coated with 1 μg/mL of anti-CD3 monoclonal antibody (clone 145-2C11, eBioscience) overnight at 4°C. Teff (responders) and CD4+CD25+ Tregs (suppressors 2 × 10^5 cells/well) were separated from spleens of hindlimb ischaemic mice 14 days after induction of ischaemia or from sham-operated mice. The cells were co-cultured in RPMI medium supplemented with 10% foetal calf serum at different ratios of responders to suppressors (1 : 1 : 1 : 1 and 1 : 1). All cells were seeded in a final volume of 200 μL in the presence of soluble anti-mouse CD28 (clone 37.51, eBioscience). After 72 h, [3H] thymidine (1 μCi/well) was added for 18 h, and cell proliferation was then assayed by scintillation counting in a beta counter. Percent inhibition of proliferation was determined with the following formula: 1 – (median [3H] thymidine uptake of 1:1 responder/suppressor co-culture/median [3H] thymidine uptake of Treg only).

2.5 Study design for in vivo adoptive transfer

One day after induction of hindlimb ischaemia, mice received 2 × 10^5 purified CD4+CD25+ murine Tregs (n = 6) or equal volumes of phosphate-buffered saline (PBS) as control (n = 6), injected once into the tail vein. The mice were then sacrificed on Day 14.

2.6 Treatment with an anti-CD25 monoclonal antibody

One day prior induction of hindlimb ischaemia and 7 days after the procedure, 300 μg of anti-CD25 (clone PC61) was administered intraperitoneally in a volume of 200 μL of sterile saline. Rat IgG1 K isotype control (clone 16-4301, eBioscience) was used as control at the same volume. The extent of Treg depletion was evaluated in a prior study using different concentration of anti-CD25 given via the tail vein.

2.7 Study design for in vivo adoptive transfer with anti-IL-10 treatment

The design is provided in Figure 7A.

2.8 Immunohistochemistry

Identification of ECs and capillaries in ischaemic muscles was performed by immunostaining for CD31 (BD Biosciences) in the gastrocnemius. Arterioles were identified using monoclonal anti-actin, α-smooth muscle (SMA; Sigma, St. Louis, MO, USA) antibodies in the adductor sections. Sections were deparaffinized and washed in distilled water. Sections were incubated with antigen retrieval for 20 min, followed by a PBS wash. Slides were incubated with cas-block for 15 min and with the primary antibody for 60 min. Slides were washed three times in PBS, each time for 2 min. Sections were...
incubated with a secondary antibody goat anti-rat IgG (Jackson Immunoresearch) for 40 min, washed in PBS, and incubated with diaminobenzidine (Invitrogen) for 2 min. Sections were placed in haematoxylin for 5 min, washed in tap water, rinsed in distilled water, and dehydrated. The extent of vascularization was evaluated by blood vessel counts (at least five different fields were counted) under × 200 magnification.

2.9 Quantitative real-time polymerase chain reaction

Total RNA was extracted with a phenol/chloroform EERNA kit (Biological Industries, Beit Ha-Emek, Israel). For cytokine detection, 1 μg of total RNA was transcribed to cDNA using the vero cDNA (Thermo). Quantitative real-time polymerase chain reaction (PCR) was performed with Sybr Green (Applied Biosystems). The sequences of primers used were as follows: for IL-10, forward 5′-CAG-CGGAGGACAA-TAACGT CAC-3′, reverse 5′-CTTGTCTGTGGCCATGCTTCTC-3′; for TGF-β, forward 5′-TGGAGCAACATGTGGAACTC-3′, reverse 5′-GTCAGCA GCGGGTCATTACCA-3′; for TNF-α, forward 5′-CAAGGCTGTAAGAATAACCAA CAAC-3′, reverse 5′-TTTGGAGACATCGGCTGTTG-3′, and for IL-1β, forward 5′-CAGAAGATGAAAGGCTGTTT-3′, reverse 5′-GAAACTG GTATGCTCTCTCA-3′. Using a StepOnePlus instrument (Applied Biosystems), results were derived by the comparative CT (ΔΔCt) method.

2.10 Statistical analysis

Statistical analysis was performed with the GraphPad Prism version 5.00 for windows (GraphPad Software, San Diego, CA, USA). All variables are expressed as mean ± SEM. The Mann–Whitney test (if data were not normally distributed) or unpaired t-test (if data were normally distributed) was used to compare between two groups. To test the hypothesis that changes in measures of % flow between 0, 7, and 14 days varied among the experimental groups, a general linear model two-way repeated-measures ANOVA was used followed by Bonferroni’s multiple comparison test. The model included the effects of treatment, time, and treatment-by-time interaction.

3. Results

3.1 Ischaemia influences Treg number and function

Initially, we wished to determine whether systemic levels of Tregs and their functional suppressive properties are influenced by the hindlimb ischaemia model.

Levels of Tregs were determined in splenocytes at three different time points after the induction of ischaemia: 7, 14, and 21 days. Splenocytes from sham-operated mice served as controls. At Day 7 of ischaemia, levels of Tregs were increased two-fold than in control mice (P < 0.001). At Day 14 after ischaemia, Treg levels were still higher in ischaemic animals (P < 0.001). However, at 21 days, the levels of Tregs were similar in ischaemic mice and sham-operated mice (Figure 1A and B). In addition, we measured the total number of Tregs with no manipulation and compared it with the time course of Treg levels at 7, 14, and 21 days after ischaemia. The results show that once hindlimb ischaemia is performed, there is an increase in Treg levels 14 and 21 days after ischaemia with no change in Treg levels at 7 days (Figure 1A–A).

Functional suppressive activity of Tregs on Teffs from ischaemic mice was significantly reduced when compared with Tregs from non-ischaemic mice at all Treg–Teff ratios (1 : 1, P < 0.01; 1 : 2, P < 0.05; 1 : 4, P < 0.05; Figure 1C). According to previous experiments, some of which were performed in our laboratory (unpublished data), suppression of Treg activity was evident mostly at Day 14 after induction of ischaemia. Moreover, we found that the levels of Tregs were highest at this time point. Thus, it appears that whereas the levels of peripheral Tregs are increased in response to hindlimb ischaemia, their functional suppressive activity is hampered.

3.2 Depletion of Tregs attenuates revascularization

We next investigated the effect of Tregs on blood flow in mice after in vivo depletion with an anti-CD25-specific antibody. The PC61 mAb (anti-murine CD25 rat IgG1) is widely used for depletion of Tregs. Rat IgG isotype control injection was used as the control treatment in all experiments. Consistent with previous data, we confirmed that injection of PC61 antibody effectively depleted circulating CD25+ cells, 4 days post-injection (P = 0.012; Figure 2A and B) and 7 days post-injection (P = 0.0002; Figure 2C and D).

Next, to characterize the importance of Tregs, we injected PC61 mAb (n = 10) or as control, rat IgG (n = 8) in mice with induced hindlimb ischaemia. LDPI measurements showed (all values are expressed as the percentage of perfusion in the non-ischæmic limb) a significant decrease in blood flow in the PC61 antibody injection group when compared with control IgG mice by measuring the perfusion in the paw area only (P < 0.05; Figure 2E and F). In addition, the number of CD31- and SMA-positive cells in the muscle sections was reduced in the former when compared with the latter group (Figure 3A, D, F, and G).

3.3 The effect of Tregs depletion on vascular progenitor and cytokine expression in skeletal muscle

We tested whether Treg depletion influences systemic levels of progenitor cells that assist in re-establishment of blood flow after hindlimb ischaemia. Tregs depletion did not influence the number of spleen-derived Sca1, flk-1, or Sca1/flk-1 cells (Figure 4A and D).

Infiltrating inflammatory cells also release multiple cytokines with pro-inflammatory, anti-inflammatory, and angiogenic properties. We therefore examined cytokine expression using quantitative real-time PCR (Figure 4E and H). We examined the relative expression of cytokine messenger RNA in the Treg-depleted and ischaemic CD11c-Treg-treated hindlimbs. In Treg-depleted mice, TNF-α expression was substantially up-regulated when compared with controls, 7 days after ischaemia (Figure 4H; P = 0.028). IL-1β mRNA levels showed a similar temporal pattern, but was more pronounced (Figure 4F, P = 0.0053). IL-10 is a key mediator of Treg functions. IL-10 mRNA expression was significantly reduced in Treg-depleted mice when compared with the control group (Figure 4E, P = 0.025).

3.4 The effect of adoptive Treg transfer on post-ischæmic neovascularization

We performed adoptive transfer of 2 × 10⁵ Tregs isolated from C57BL/6 mice to naive C57BL/6 recipients 24 h after induction of hindlimb ischaemia. The freshly isolated Tregs highly (mean purity of 90%) expressed FOXP3, as previously described. LDPI demonstrated a significant increase in blood flow in the Treg-injected group compared with control on Day 14 (Figure 5A and B, P = 0.04). In addition, the number of CD31- and SMA-positive cells was increased in muscle sections from mice adoptively transferred with Tregs compared with PBS-injected mice (Figure 6C and G).
Figure 1  Altered numbers and suppressive properties of Tregs after hindlimb ischaemia. Treg number is increased after hindlimb ischaemia. (A) FACS of Tregs after hindlimb ischaemia when compared with sham-operated animals shows the numbers of CD4^+CD25^+Foxp3^+ Tregs, expressed as a percentage of the total number of CD4 cells, 7, 14, and 21 days after hindlimb ischaemia induction. (A1–A3) FACS of Tregs after hindlimb ischaemia when compared with control animals shows the numbers of CD4^+CD25^+Foxp3^+ Tregs, expressed as a percentage of the total number of CD4 cells, 7, 14, and 21 days after hindlimb ischaemia induction. (B) Representative FACS results showing kinetics of Tregs 7, 14, and 21 days after hindlimb ischaemia induction when compared with sham-operated mice. (C) A functional suppression assay shows a diminished suppressive effect of Tregs from ischaemic hindlimb when compared with sham-operated mice, with significant effects at three ratios of Tregs vs. Teffs, 14 days after the procedure. 1 : 1, 1 : 2, 1 : 4. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 2  Treg depletion employing anti-CD25 antibodies attenuates blood flow recovery. Ischaemia was induced in IgG control-injected mice (n = 8) or PC61 (anti-CD25 antibody)-injected mice (n = 10). Quantitative FACS analysis of C57BL/6J mice treated with IgG isotype control or with an anti-CD25 antibody, (A) 4 days, in blood or (C) 7 days, in the spleen after the injection. (B) Representative FACS results showing kinetics of Tregs 4 days (D) and 7 days after treatment with anti-CD25 or IgG isotype control. (E) Representative laser-Doppler images are shown at each time point, shortly after ischaemia induction, 7 and 14 days after ischaemia. Improvement of blood flow is presented as percentages relative to the non-ischaemic limb in the adductor region to the plantar foot (F) and only in the plantar foot (G). *P < 0.05, **P < 0.01, ***P < 0.0002.
3.5 Anti-IL-10 treatment inhibits post-ischaemic revascularization

We hypothesized that IL-10 secretion from Tregs is a likely mediator of blood flow improvement induced by adoptive Tregs therapy. We thus injected anti-IL-10 blocking antibodies to C57BL/6 mice with inducible hindlimb ischaemia, with or without Tregs (Figure 7A). Seven days after hindlimb ligation, we found that IL-10 blockade resulted in complete abolishment of the favourable effects of Treg delivery on blood flow recorded by LDPI (Figure 7B and F, \( P = 0.0022 \)). In contrast, mice with inducible hindlimb ischaemia treated with anti-IL-10 antibodies did not exhibit a change in limb flow by LDPI when compared with animals that were treated with isotype control (Figure 7C, E, and G, \( P = 0.36 \)). In addition, blood perfusion was improved in control IgG-injected mice with Treg transfer compared with controls (anti-IL-10 with Treg or PBS and IgG isotype control with PBS). Change in blood perfusion between Days 7 and 1 was calculated in four study groups (\( P < 0.001 \)) and showed a significant increase of perfusion in the limbs in control IgG mice transferred with Tregs when compared with the three other groups (Figure 7H). At day 14 after injection difference between the two groups were non-significant (Figure 7I and J).

4. Discussion

In the current study, we investigated the functional role of Tregs in experimental hindlimb ischaemia both by adoptive transfer of Tregs and by in vivo depletion of Tregs.

Hindlimb ischaemia is an acceptable model for testing new therapeutic approaches for patients with peripheral arterial disease, because it provides a reproducible model by which to influence and accurately assess blood flow restoration.

Figure 3  Treg depletion employing anti-CD25 antibodies attenuates vessel growth. Effect of anti-CD25 injection on post-ischaemic blood vessel development was assessed by CD31 in the gastrocnemius and by SMA immunohistochemistry in the adductor (A and C) anti-CD25 antibody-injected mice or (B and D) rat IgG isotype control-injected mice, at Day 14 after surgery. The number of CD31-positive cells (F) was counted in four random images for each mouse and the number of SMA-positive cells (G) was counted in five random images for each mouse. (E) SMA positive cells in wildtype mice. * \( P < 0.02 \), ** \( P < 0.001 \).
Figure 4 The effect of Treg depletion on vasculogenesis and inflammatory markers in hindlimb ischaemic mice. Representative quantitative evaluation of (A) sca-1, (B) flk-1, and (C) sca1/flk1 levels in splenocytes. (D) Representative FACS results of sca1/flk1 numbers, 7 days induction of femoral ligation in hindlimb ischaemic mice. Expression of inflammatory cytokines: (E) IL-10, (G) TGF-β, (H) TNF-α, and (F) IL-1 mRNA levels in ischaemic limb muscle of anti-CD25 antibody-injected mice compared with control IgG-injected mice. *P < 0.05, **P < 0.005.
Adoptive transfer of Tregs increases blood flow recovery after femoral ligation. Ischaemia was induced in C57BL/6 mice by femoral permanent ligation. One day afterwards the mice were injected with PBS (n = 6) or Tregs (n = 5) as outlined in methods. (A) Representative laser-Doppler images are shown at each time point, shortly after ischaemia induction, 7 and 14 days after ischaemia, and are expressed as a percentage of the blood flow in the non-ischaemic limb in the adductor region to the plantar foot (B) and only in the plantar foot (C). *P < 0.05, **P < 0.01.
In a recent interesting and comprehensive study, Zouggari et al. tested the hypothesis that Treg modulation via two major co-stimulatory pathways: CD40-CD40 ligand and B7-CD28 influences flow restoration and inflammatory response in experimental hindlimb ischaemia. They found that whereas CD40-deficient mice exhibited reduced vessel growth, knockout mice for the B7-CD28 pathway demonstrated enhanced vascularization of the occluded limb. They suggested that the endogenous Treg pool is involved in mediating the enhanced vascularization response. However, co-stimulatory signals provide only one regulator of the complex factors that control Treg homeostasis. We thus designed our study to directly approach the isolated role of Treg depletion and transfer in an experimental ischaemia model in wild-type animals.

Recently, a study by Hellingman et al. reported apparently opposing results, showing that Treg-depleted mice exhibited suppressed restoration of blood flow. However, in this model, surgical manipulation included electrocoagulation of the femoral artery. This procedure evokes considerably more robust tissue damage, with resultant extensive local inflammation that is less likely to mirror ischaemia in humans. This heightened local immune response is likely to significantly alter the effect of immunomodulating agents, and thus this model is not comparable with femoral ligation inducing hindlimb ischaemia.

Here, we show for the first time that Treg levels are increased 7 and 14 days after femoral ligation and apparently normalize at 21 days. In contrast, it appears that induction of limb ischaemia evokes a functional defect in the ability of Tregs to suppress effector cell proliferation, a finding that has not been described so far. The increase in Tregs in response to ischaemia can either be explained by a stimulated release of Tregs from haematopoietic pools or by peripheral conversion of effectors as a result of ischaemia-derived humoral factors. The reduced restoration of blood flow. However, in this model, surgical manipulation included electrocoagulation of the femoral artery. This procedure evokes considerably more robust tissue damage, with resultant extensive local inflammation that is less likely to mirror ischaemia in humans. This heightened local immune response is likely to significantly alter the effect of immunomodulating agents, and thus this model is not comparable with femoral ligation inducing hindlimb ischaemia.

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Figure 7 IL-10 blockade in mice adoptively transferred with Tregs. (A) Experimental plan outline: 14 mice were injected with anti-IL-10-blocking antibody (LEAF™ purified anti-mouse IL-10 mice, clone 505012, BLG) and 14 mice were injected with rat IgG K isotype control (functional grade purified, clone 16-4301, eBioscience). Hindlimb ischaemia was induced and 1 day afterwards, seven mice from each group were injected iv Tregs or PBS. Representative laser-Doppler images are shown at 7 days post-ischaemia induction, (B) anti-IL-10 antibodies with Tregs treatment, (C) anti-IL-10 antibodies with PBS injection, (D) IgG isotype control with Tregs treatment, and (E) IgG isotype control with PBS. Percentage of change in blood flow ischaemic vs. non-ischaemic limb at 7 days (F–H). (I) Tregs-injected mice and (J) PBS-injected mice shortly after ischaemia, and 7 days and 14 days after ischaemia.

**P, 0.002, ***P, 0.001.
function of Tregs can be explained by factors associated with oxidative stress that are induced by ischaemia and likely influence their ability to suppress effector T-cell function. We have previously shown that oxidized LDL, a product of oxidative modification of this lipoprotein, hampers Treg functions more potently that it does so to other T-cell populations. 38

Owing to the apparent discrepancy between behaviour of the number and function of Tregs following induction of hindlimb ischaemia, we went on to study the effect of either depletion or provision of Tregs in this model. Indeed, mice treated with anti-CD25 antibodies exhibited a reduced blood flow pattern by LDPI in comparison with IgG-treated animals at Day 7 that became more robust at Day 14. One limitation that should be acknowledged with regard to the LDPI measurements is that it cannot accurately differentiate between skin perfusion and deeper muscular perfusion. To partially overcome this limitation, we have also analysed perfusion in the paws that is more likely to represent superficial perfusion and correlate with previous measurements. Perfusion results were supported by an attenuated vessel growth evident in immunohistochemistry, employing CD31 and SMA antibodies.

Aiming to test whether Treg ablation was associated with reduced vasculogenesis, we studied systemic EPC numbers. EPCs are well described in the context of ischaemic models and play a role in supporting vessel growth. Moreover, we have previously shown that peripheral Treg numbers are associated with circulating EPCs in healthy subjects. 26

The level of Flk1+/Sca1- cells is an acceptable index of endothelial progenitor numbers and its correlate in humans is inversely associated with cardiovascular disease risk. 27 Among the various surface markers of circulating EPCs, Sca-1/Flk-1 is one of the most common acceptable markers in rodents. 28 We and others assessed circulating EPCs by flow cytometry and correlated cell numbers with cardiovascular outcomes in humans. 41–43 A brief view may add a new perspective to this ongoing debate of EPC subsets. 44–46 EPC definition may not be restricted to a single cellular lineage or the combination of surface markers, but incorporate a family of functionally associated subsets of endothelial-regenerating cells. T-cell capacity for de novo vessel formation and their contribution to restoration of damaged endothelium through replacement of diverse EC subsets seems predilected to evaluate and define EPCs. 19

Interestingly, in this study, there was no difference in the circulating EPC levels measured by expression of Sca1 and flk1 markers, between the Treg-ablated mice and IgG isotype control group. Inflammatory cell infiltration is followed by secretion of pro-inflammatory cytokines. 10 Treg inhibition of effector immune cells, at least in part, by producing or promoting the production of suppressive cytokines such as IL-10. 47 IL-10 has been shown to have anti-angiogenic properties in models of ischaemia. 48 Accordingly, we have found that mice with inducible hindlimb ischaemia that underwent Treg ablation by anti-CD25 antibodies exhibited lower expression of IL-10 when compared with elevated levels of the pro-angiogenic cytokines TNF-α and IL-1.

Treg depletion-mediated compromise in hindlimb blood flow does not necessarily suggest that transfer of Tregs will improve flow. For this purpose, we carried out the adoptive transfer studies. Indeed, we have found repeatedly that adoptive transfer of Tregs from healthy age-matched syngenic mice improved blood flow after femoral ligation in recipient animals.

These LDPI observations were reinforced by immunohistochemistry studies, showing that CD31- and SMA-positive vessels were significantly more abundant in hindlimbs of animals adoptively transferred with Tregs. The PBS and IgG groups appear non-identical between Figures 3B and 6B. However, we should bear in mind that collateral vessel formation following ischaemia is a very variable process that relies many factors including the age and weight of the mice, as well as technical procedural issues. Therefore, we only compared experimental groups that underwent the procedure on the same day.

As IL-10 appeared to be a potential candidate secreted by Tregs and reduced upon their ablation in our initial experiments, we designed a study whereby Treg-transferred animals were treated with either blocking IL-10 or irrelevant antibodies. Indeed, our findings clearly show that IL-10 blockade was capable of completely abolishing the protective effects conferred by Treg adoptive transfer. It can thus be assumed that Tregs’ favourable effect on re-establishment of blood flow in the hindlimb ischaemia model is IL-10-dependent.

In conclusion, we show here for the first time that Tregs are increased yet functionally defective in mice undergoing femoral ligation. Depletion of Tregs results in diminished re-establishment of flow in this experimental model, whereas adoptive intravenous provision of Tregs improves blood flow via the occluded hindlimb in an IL-10-dependent manner. If these findings are further supported by complimentary experimental work, they may pave the way for developing novel means of adoptive cell therapy in patients with peripheral vascular disease.

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