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Monocyte derived Colony Forming Unit Endothelial cells (CFU-EC) enhance neovascularization in a validated ischemic hind limb model

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Abstract

Colony-forming unit-endothelial cells (CFU-EC), are formed by (a subset of) monocytes. Moreover, we recently showed that $CD4^+$ T-lymphocytes facilitate this CFU-EC culture by the cytokines produced after activation. The relevance of this interaction is shown by the fact that CFU-EC numbers inversely correlate with the risk for cardiovascular disease. Interestingly, both CD4 positive cells and monocytes have independently been shown to mediate arteriogenesis in *in vivo* models. A specific role for the T-cell-monocyte interaction responsible for the CFU-EC assay in neovascularization, however, was never investigated.

We therefore used $CD4^+$ T-cell stimulated monocytes after short CFU-EC cultures and studied their direct role in neovascularization in an optimized nude mouse ischemic hind limb model. Since mouse strains differ in their revascularization capacity after arterial ligation-mediated hind limb ischemia, we first compared revascularization kinetics from 4 (Balb/C, C57B/6, Swiss, FVB) different nude mouse strains. The intermediate kinetics and small interanimal variance of the nude C57B/6 mice appeared most suitable for neovascularization studies. To enlarge the window in which an effect could be observed, an extended ligation was used.

Mice subjected to femoral artery ligation were intravenously injected with PBS, Monocytes stimulated with activated T-cell supernatant or monocytes stimulated with plain medium. Laser Doppler imaging showed that the mice treated with the monocytes with T-cell supernatant, have a faster recovery of perfusion. In conclusion, the so far only suggested neovascularizing capacity of – in fact T-cell stimulated- CFU-EC monocyte was confirmed in an *in vivo* model. Moreover, our results make it likely that studies by others showing $CD4^+$ T-lymphocytes to enhance ischemic hind limb reperfusion, in fact corroborate our findings that T-cells or better their cytokines, facilitate monocyte dependent neovascularization.

Introduction

Previous studies have shown that endothelial like colonies (CFU-EC) are formed by monocytes in culture[1-3]. In these cultures, the role of activated $CD4^+$ T-cells was found to be of importance. Colony formation was dependent on the presence of $CD4^+$ cells and the cytokine cocktail produced by these cells after activation by an antigen presentation mechanism even greatly induced this colony formation [3]. The relevance of the CFU-EC is shown by clinical studies, in which the number of CFU-EC in PBMC was found to be inversely correlated with the risk for vascular diseases[4]. From these findings it was suggested that these colony forming cells could have a role in vascularization. Although the $CD4^+$ T-lymphocytes were recently shown to be increasing neovascularization in *in vivo* models[5,6], the role of the T-lymphocyte stimulated CFU-EC forming monocyte, however, in this respect is so far unclear.

To test cells for improvement of neovascularization after ischemia, the ischemic hind limb model is often used. This model has been applied in a number of species, like mice, rats and rabbits[7-13]. However, the availability of a large number of genetically modified strains, and their relatively small size makes the mouse the most used animal for this model.

As Shireman *et al* already described, the surgical approach, and methods to measure hind limb perfusion are crucial for the sensitivity of the assay and the interpretation of results derived from this ischemia model. First of all, the levels of femoral artery (FA) ligation or excision are determining the speed and rate of restoration of the hind limb perfusion. Excision of a large part of the FA resulted in a delayed repair and only partial restoration of blood flow, as compared to e.g. a proximal occlusion of the FA [14]. The most important determinant for sensitive and reproducible experiments however is the mouse strain itself. Several groups have shown large differences in the ability to restore perfusion in the ischemic hind limb of the commonly used strains, such as BALB/c, DBA/1J and C57Bl/6J[14-16]. Even within the same mouse strain, interanimal variability can influence the outcome of experiments[17]. The number of pre-existing collaterals, difference in VEGF-A expression and the level of T-cell mediated immune response [14-17] are all implicated in causing these differences within strains.

Athymic nude mice are well suitable to study human cell transplantation, as they show no immune response against xeno-grafts. Since T-cells play a role in arteriogenesis[5,6], the T-cell lacking nude mice are expected to show a diminished reperfusion capacity. However, studies that compared the nude mouse strain with wild type mice, showed contradictory results. The aim of our study was to identify the most suitable and reproducible nude mouse strain and use this model to test CFU- EC monocytes as neovascularization enhancing therapy after arterial ligation-induced ischemia. In particular, we aimed to characterize if the effect of the $CD4^+$ cytokines on monocytes in the CFU-EC assay was also relevant for the effect on *in vivo* neovascularization of these monocytes.

Materials and Methods

Isolation of cells

Aphaeresis buffy coats, anticoagulated with 0.4% trisodium citrate (pH 7.4) were obtained after informed consent from healthy volunteers of the Sanquin Blood Bank (Amsterdam, The Netherlands). Peripheral blood mononuclear cells (PBMC) were isolated with Ficoll paque density gradient separation. After 2 washing steps with PBS containing citrate and 0.25% plasma protein, PBMC were labelled for 30 minutes with antibody coupled beads against CD4 or CD14 (Miltenyi Biotech GMBH). After a washing step, cells were passed through a magnetic column (LS) for direct selection. Flow cytometric analysis showed over 95% purity.

Culture of cells

Supernatant of activated T-cells was made as follows: isolated $CD4^+$ cells were incubated for 30 minutes with activating anti CD3 (clone CLB.1XE, 1 µg/ml) in combination with activating anti CD28 (clone CLB.15E8, 1 µg/ml)[18]. After a washing step, the activated T-cells were cultured in culture flasks in EndoCult™ medium with supplements (Stem Cell Technologies Inc.) at a density of 1*10^6 cells/ml. After 48 hours of culture at 37 °C, 5% CO_2, the complete culture was centrifuged at a speed of 800g and the supernatant was carefully harvested and stored at -20°C for further use. In all experiments using

activated T-cell supernatant, a pool of supernatants of T-cells of at least 5 donors was used.

Monocytes used for the experiments were cultured at a density of 1*10^6 cells/ml in Endocult medium or in a mixture of Endocult medium 1:1 mixed with pooled supernatant from activated T-cells in fibronectin (FN)-coated 6 wells plates (Biocoat Cellware, BD Biosciences). After 48 hours, non-adherent and loosely adherent cells were harvested by repeatedly pipetting the cell suspension in the plate. Cells were washed twice with PBS infused into the mice within 1 hour.

Ischemic hind limb model

All animal experimental protocols were approved by the animal welfare committee of the Netherlands Cancer Institute (NKI, Amsterdam, The Netherlands) and of the Netherlands Organization for Applied Scientific Research (TNO, Leiden, The Netherlands). In male nude C57B/6 (Taconic), Balb/C (NKI, Amsterdam, The Netherlands), FVB (NKI) and Swiss (Charles River) mice, aged 8-10 weeks, ischemia of the left hind limb was induced in animals by either a single coagulation of the left femoral artery proximal to the bifurcation of the deep and superficial femoral artery (normal), or by removal of a part of the femoral artery from inguinal region until the popliteal artery.

Before surgery (PT), after surgery(T0) and at the mentioned time points after surgery, blood flow was studied using laser doppler perfusion imaging (LDPI) (Moor Instruments)[13]. Mice were placed in a glass bowl connected to a water bath at 37°C to minimize temperature variation. The LDPI system incorporates a 2-mW helium-neon laser to generate a beam of light that sequentially scans tissue surface to a depth of 600 p.m. During the scanning procedure, blood cells moving through the vasculature shift the frequency of incident light. A colour coded image representing the perfusion signal is analysed. This signal is split into six different intervals, and each is displayed as a separate colour. Low or no perfusion was displayed as dark blue, whereas maximal perfusion was displayed as red (fig 1A). Consecutive measurements were obtained over the same region of interest (foot). Colour coded images were recorded, and analyses were performed by calculating the average perfusion for each (ischemic and non-

ischemic) foot. To account for variables, including ambient light and temperature, calculated perfusion was expressed as a ratio of left (ischemic) to right (normal) limb.

Angiography

To study collateral vessel growth, angiography of both hind limbs was performed using polyacrylamide-bismuth contrast (Sigma), as previously described[5].

Statistical analysis

After testing for normal distribution, statistical differences were assessed using the paired t-test. Significance was assumed at $p < 0.05$. Data are shown as mean±sem, unless mentioned otherwise.

Results

Mouse strains

Four different mouse strains were tested for the kinetics in repair after hind limb ischemia (shown in fig 1B). Data is shown until full recovery or d28. Nude Balb/C mice show a very delayed and only partial repair after the induction of hind limb ischemia. Even after 28 days, only 30% recovery of perfusion was observed. A complete different repair curve is shown by the nude FVB mouse. Already at day 3, a recovery of over 81±10% was observed. Such fast and complete recovery of the blood flow makes this mouse strain unsuitable for studies to identify additional facilitation of reperfusion by cell therapy. The kinetics of the reconstitution of the blood flow in the Swiss mice, with a repair of 80 percent at day 7 makes this mouse strain a possible candidate to test the cells. However, the interanimal variability in this strain was quite large (recovery at d3: 61±25%; range 9-95%) and probably caused by background variation in the pre-existing collaterals in these animals, since the Swiss mice are an outbred strain. As large variation requires large test groups, this strain was also considered less ideal. The C57B/6 mice, show reperfusion kinetics after the induction of hind limb ischemia that can be compared to the Swiss mice, however, the variation between single mice is much

smaller (recovery at d3 66±23%; range 35-95%), making it possible to see a result in smaller groups (Fig 1C).

Surgical approach

The hind limb ischemia can be induced by a single ligation or by an extended ligation, the latter removing a large part of the femoral artery plus branches. Extended ligation results in a bigger window between ischemia and recovery and makes differences between groups better visible (fig 1C). Furthermore, angiography at day 28 showed that the normal ligation induces predominantly the formation of collaterals out of pre-existing arterioles. When extended ligation was applied, this was also observed, however, also newly formed arterioles were observed (fig 1D). It was thus decided to use the C57B/6 mouse with the extended surgery for the experiment, so effects on both angiogenesis and arteriogenesis can be studied.

CFU-EC forming monocytes in the ischemic hind limb model

After choosing the right mouse strain and the extended ligation method, monocytes that were able to perform CFU-EC formation were tested for their ability to accelerate recovery after induction of hind limb ischemia. Monocytes cultured for 48 hours with supernatant from activated T-cells (fig 2A) were compared to monocytes that were cultured without addition of the activated T-cell supernatant (fig 2B). Per mouse, 0.5*10^6 cells were injected intravenously 24 hours after the surgical procedure. Laser Doppler imaging was performed at days 3, 7, 14, 21, and 28. As can be observed from figure 2C, the injection of stimulated monocytes improves perfusion of the ischemic hind limb compared with the groups treated with the control monocytes. This difference is already observed at day 3 and becomes significant at d7 (79.3%±11.3% vs. 48.7%±7.4%; $p<0.05$) and 14 (70.4%±5.3%; $p<0.05$). At day 14 also a significant difference is seen between the PBS treated mice and the stimulated monocytes treated mice (88.4%±9.6% vs. 70.4%±5.3%; $p<0.05$).

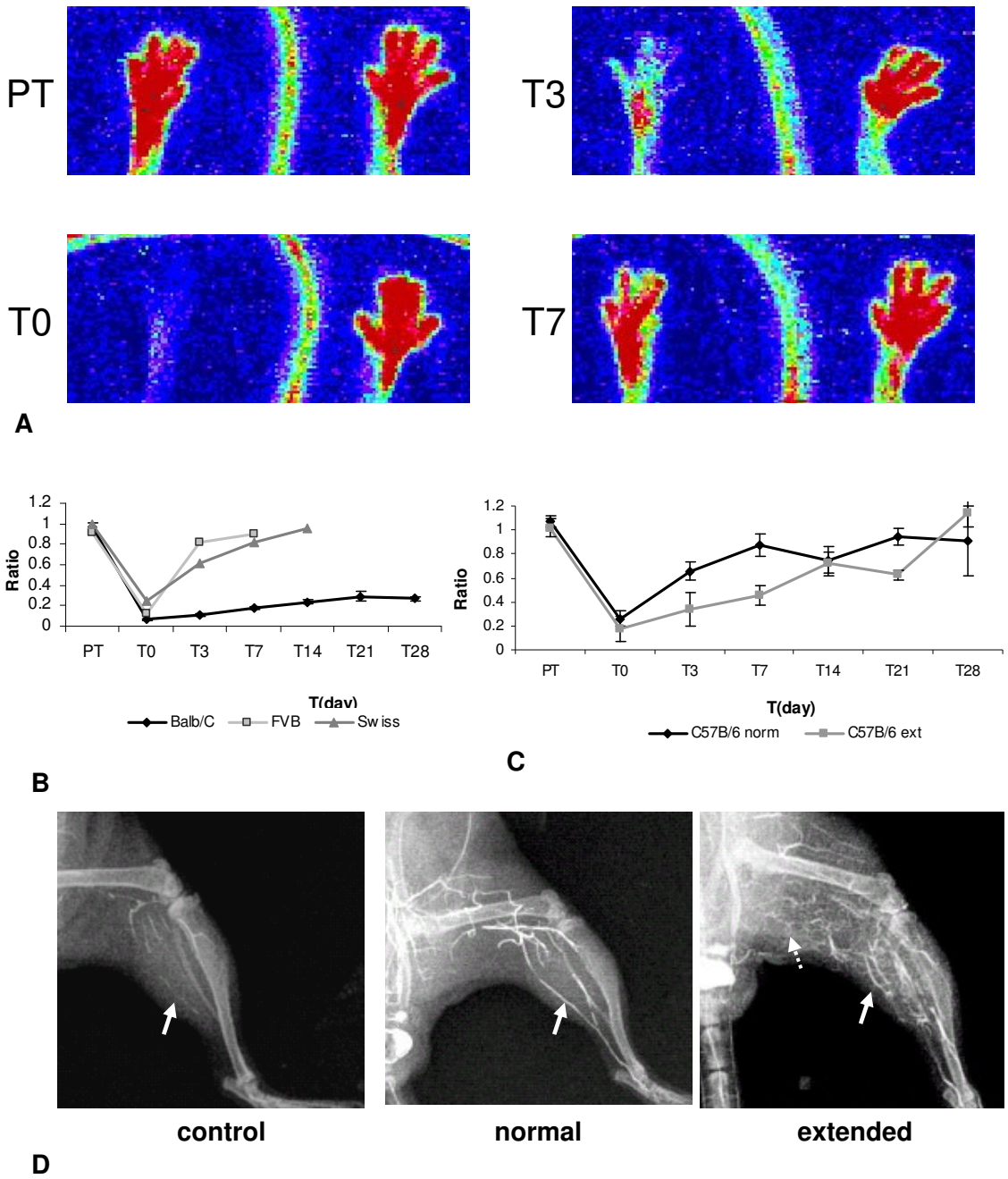
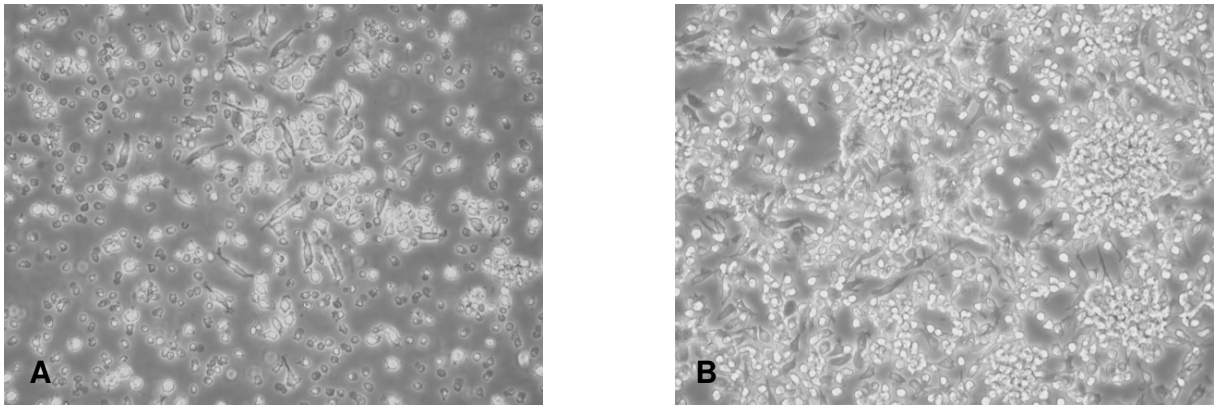


Figure 1: The ischemic hind limb model in different nude mouse strains
A/B: Laser Doppler technique shows the increase in perfusion in colour coded images, before and after normal ligation and during repair. Highly perfused tissue is red, while tissue with low of no perfusion appears blue (A). Laser Doppler measurements in different mouse strains show large variety within mouse strains (B). **C:** The extended ligation in the C57B/6 mice enlarges the window to measure improvement of vascularization, compared to the normal ligation. **D:** Angiography shows that after the normal ligation, collaterals are formed ((arteriogenesis, white arrow), while the extended ligation induced both collateral formation and formation of newly formed cells (dashed arrow): angiogenesis.



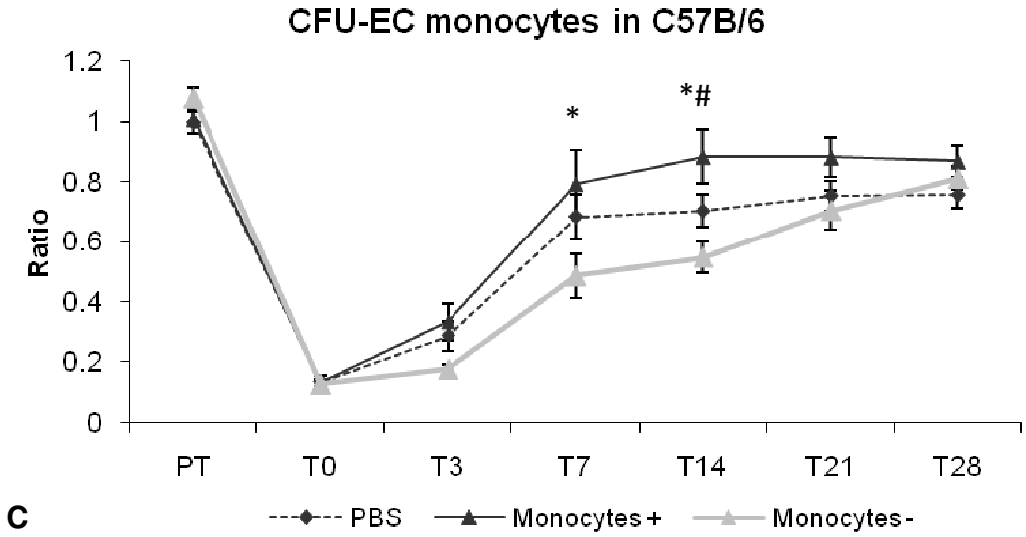


Figure 2: Monocytes increase neovascularization *in vivo*

A: monocytes were cultured in Endocult medium with and without 1:1 T-cell supernatant. Stimulated monocytes form colonies consisting of round cells in the middle, surrounded by spindle shaped cells (**B)**. **C:** Nude mice were injected intravenously with PBS, monocytes cultured in Endocult medium or monocytes stimulated with supernatant from activated T-cells. Monocytes stimulated with T-cell cytokines significantly improve hind limb perfusion compared to injection with control monocytes (*, $P<0.05$) and PBS injection (#$P<0.05$).

Discussion

In this study it was shown that the CFU-EC forming monocyte can accelerate and improve perfusion after ischemia in the ischemic hind limb model.

First, 4 nude mouse strains were tested for their kinetics in repair after induction of unilateral hind limb ischemia. The nude Balb/C mice, showed a very slow and incomplete repair. Although this makes the window to see cell therapy induced neovascularization rather broad, at the same time, this strain probably will allow only studies on the development of newly formed vessels (angiogenesis) and much less of the fast maturation of collaterals (arteriogenesis). The most likely explanation for this may be an underdeveloped pre-existent collateral bed, as was shown by Chilton *et al* and Van Weel *et al* [5,16]. Since arteriogenesis indeed is fast and probably the most relevant process to compensate acute ischemic disease, and while monocytes or monocytic cells are explicitly implemented in mediating arteriogenesis, this mouse strain was regarded as less suitable [16,19]. A mouse strain with a complete other kinetics of repair is the FVB mouse. Already within 3 days after surgery an 81±10% recovery was observed. This extremely fast spontaneous perfusion recovery, however, will make any therapy-induced improvement in neovascularization undetectable. Another available nude mouse strain is the Swiss nude mouse. Although the kinetics of repair of this mouse is suitable, the variability within the separate mice was too large, so a large number of mice will be needed to see any significant effect of cell therapy. This is probably caused by the fact that this mouse strain is outbred. The inbred C57B/6 mouse strain showed reperfusion kinetics that was comparable with the Swiss nude mice; however, the interanimal variation was much smaller. To enlarge the time for recovery, an extended surgery was applied; enlarging the window to study the effect on neovascularization that is created.

Three groups were compared: i.v. injection with PBS, monocytes that were cultured for 48 hours in fresh medium and monocytes cultured for 48 hours in fresh medium 1:1 with T-cell conditioned medium. The monocytes that were cultured with T-cell derived cytokines showed a significant improvement of repair compared to PBS injection and to injection with monocytes that were cultured without the cytokines. Since this effect was already observed at day 3 (although not yet significant), the effect is most likely mainly

dependent on arteriogenesis, since arteriogenesis is most dominant in the first reperfusion phase after induction of hind limb ischemia. Immunohistochemistry for capillaries and SMC containing arterioles will tell more about which effect was induced by the monocytes. Moreover, detection of human CD45mRNA by RQ-PCR in the tissue will also unravel if the effect of the human monocytes is also dependent on more durable engraftment in this model.

The monocytes that were used in this study were stimulated with a partly unknown cocktail of stimulated T-cell released cytokines. In a recent study from our group, this cytokine cocktail was shown to strongly induce quantitative colony growth of monocytic cells with a clear pro-angiogenic and arteriogenic phenotype as reflected protein and gene analysis[3]. The CFU-EC directed monocytes that were used for the animal studies were cultured 3 days shorter than the commercial CFU-EC assay itself. Earlier experiments showed that the monocytes stimulated for 48 hours with T-cell supernatant showed colony formation in plain medium (data not shown). This indicated that a 48 hour stimulus is enough to induce the CFU-EC phenotype. The advantage of this shorter culture period is that it allows better detachment and resuspending the monocytic cells for intravenous therapy. The use of activated T-cell supernatant in essence not only speeds up but also augments the monocytic differentiation and colony growth. We thus envision this approach to be a first step towards increasing the effectivity of monocyte dependent revascularizing cell therapy and solving the many GMP issues that have to be tackled to get these mechanisms into clinical therapy. Future studies in this respect should examine which cytokine(s) induce this pro-arteriogenic phenotype of monocytes.

Other groups have also shown that shortly cultured monocytes of endothelial like cells have a regenerative effect in the hind limb model. However, these cultures involved 7 days and contained addition of angiogenic cytokines, like VEGF[20]. Especially, VEGF did not stimulate the CFU-EC and was not present in the supernatant of the activated T-cells we used. We have showed that TNF is present in the T-cell supernatant and Rohde *et al* have also showed a role for this cytokine. Blocking TNF in preliminary experiments indeed showed an inhibition of colony formation. However, contrasting experiments with addition of TNF did not increase colony formation by monocytes, indicating that other factors in the T-cell supernatants are involved. As factor in optimization of this

therapy, we envision that the T-cell induced arteriogenic phenotype should be as stable as possible. Moreover, it must be ensured that pro-arteriogenic monocytes have lost its ability to participate as e.g. foam cell in plaque formation in atherosclerosis.

In conclusion, we established that in an *in vivo* model the CFU-EC phenotype of monocytes truly represents a neovascularizing capacity. Moreover, our results make it likely that studies by others showing $CD4^+$ T-lymphocytes to enhance ischemic hind limb reperfusion, in fact agree with our findings that T-cells or better their cytokines, facilitate a monocyte dependent neovascularization.

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