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T-cell-pre-stimulated Monocytes Promote Neovascularisation in a Murine Hind Limb Ischaemia Model

A.A. Hellingman [a,f], J.J. Zwaginga [b,c,f], R.T. van Beem [d], TeRM/Smart Mix Consortium[g], J.F. Hamming [a], W.E. Fibbe [b], P.H.A. Quax [a,e], S.B. Geutskens [b,e,*]

[a] *Department of Vascular Surgery, Leiden University Medical Center, Leiden, The Netherlands*
[b] *Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands*
[c] *Jon J van Rood Center for Clinical Transfusion Medicine Research, Leiden, The Netherlands*
[d] *Department of Experimental Immunohematology, Sanquin Research and Landsteiner Laboratory, Amsterdam, The Netherlands*
[e] *Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Leiden, The Netherlands*

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Abstract *Aim:* Monocytes play a significant role in neovascularisation. The stimuli that differentiate monocytes along a pro-angio-/arteriogenic-supporting pathway are currently unclear. We investigated whether pre-stimulation of human monocytes with soluble T-cell-derived factors improves revascularisation in murine hind limb ischaemia as a new option for therapeutic angio- and arteriogenesis.
Design: Human monocytes were cultured with or without soluble T-cell-derived factors. Unstimulated and pre-stimulated monocytes were transfused after induction of hind limb ischaemia in nude mice.

* Corresponding author. Department of Immunohematology & Blood Transfusion and Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Building 1, C2-144, PO Box 9600, 2300 RC Leiden, The Netherlands. Tel.: +31 715263872; fax: +31 715266755.
E-mail address: s.b.geutskens@lumc.nl (S.B. Geutskens).
[f] Authors contributed equally.
[g] Collaborators Translational Excellence in Regenerative Medicine (TeRM)/Smart Mix consortium: M.A. Lijkwan (Department of Vascular Surgery, Leiden University Medical Center, Leiden, The Netherlands), L. Seghers (Department of Vascular Surgery, Leiden University Medical Center, Leiden, The Netherlands), M.R. de Vries (Department of Vascular Surgery, Leiden University Medical Center, Leiden, The Netherlands), P.J. van den Elsen (Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands; Department of Pathology, VU University Medical Center, Amsterdam, The Netherlands), A.J. van Zonneveld(Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Leiden, The Netherlands; Department of Nephrology, Leiden University Medical Center, Leiden, The Netherlands), C.E. van der Schoot (Department of Experimental Immunohematology, Sanquin Research and Landsteiner Laboratory, Amsterdam, The Netherlands).

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Methods: Blood flow was measured with laser Doppler perfusion imaging. Collaterals were visualised by immunohistochemistry and angiography. Monocytes were characterised by flowcytometry and Bio-Plex assays.
Results: Transfusion of T-cell-pre-stimulated monocytes significantly improved blood flow recovery after hind limb ischaemia and increased collateral size and collateral and capillary number in the post-ischaemic paw. Pre-stimulated monocytes produced a wide variety of factors that support neovascularisation such as platelet-derived growth factor-BB, vascular-endothelial growth factor, interleukin-4 and tumour necrosis factor-α. Few transfused human cells were detected in the muscle tissue, suggesting that paracrine rather than direct effects appear responsible for the enhanced recovery of blood flow observed.
Conclusion: These results show a beneficial role for T-cell-pre-stimulated monocytes in neovascularisation, rendering the monocyte a potential candidate for regenerative cell therapy that promotes revascularisation in peripheral arterial disease patients.

Collateral artery formation (arteriogenesis) may prevent tissue damage caused by arterial stenosis or occlusion, and is crucially important to limit the consequences of peripheral arterial disease (PAD). Despite improvements of vascular and endovascular techniques, major amputation is still described in one-third of patients with critical limb ischaemia, underscoring the urgent need for new therapeutic alternatives.[1]

Cell therapy is a promising novel therapeutic approach for the treatment of patients with PAD. Selection of the most efficient cell type for therapeutic purposes is a central issue. Much attention has been given to the use of endothelial progenitor cells (EPCs) that may augment revascularisation by direct incorporation into the vessel wall. Transfusion of EPCs isolated from circulating mononuclear cells or the increase of circulating EPC numbers via bone marrow (BM) mobilisation have been described to promote neovascularisation in different animal models.[2,3] However, outcomes of clinical studies in which BM-derived cells (including EPCs) were transplanted into ischaemic limbs of patients showed only minor improvements.[4–8] Moreover, the controversy about the nature of human blood EPC complicated the isolation procedure and, therefore, the development of a cellular therapy.

The monocyte was highlighted as an alternative candidate for cell therapy that may facilitate vascular regeneration. Monocytes are pluripotent progenitor cells that, depending on the stimulus encountered, may differentiate into immune effectors such as macrophages or dendritic cells (DCs). Notably, a still growing number of studies additionally emphasises the involvement of the monocyte or its mature progeny in angio- and arteriogenic processes. The beneficial effects of monocyte transfusion were underscored by several recent studies. Herold et al.[9] demonstrated increased blood flow recovery after ligation-induced ischaemia by transfusion of autologous monocytes that were engineered by adenoviral transduction to express granulocyte/macrophage-colony-stimulating factor (GM-CSF), possibly by stimulating the mobilisation of EPCs from the BM. Interestingly transfusion of untransduced autologous monocytes stimulated *ex vivo* with GM-CSF was ineffective. The importance of a stimulus to mature monocytes along a certain pathway was emphasised by a study of Urbich et al.[10] Here, human blood-derived mononuclear cells, $CD14^-$ or $CD14^+$-monocyte-enriched populations that were stimulated in endothelial medium with vascular-endothelial growth factor (VEGF) to generate EPCs improved blood flow recovery in a hind limb ischaemia model, whereas $CD14^-$- or $CD14^+$-populations that were not stimulated before infusion did not.

Besides monocytes, T-lymphocytes play an eminent role in adult vascular repair as was exemplified by the hampered blood flow recovery and reduced collateral density upon hind limb ischaemia in various murine models that lack $CD4^+$-T-helper lymphocytes.[11,12] Moreover, significantly lower numbers of monocytes/macrophages accumulated in the ischaemic muscles of $CD4^+$-deficient mice,[11] suggesting that T-cell/monocyte interactions are involved in arteriogenesis. We previously reported that co-culture of monocytes with $CD4^+$-T-cells was indispensable for the formation of colony-forming-unit (CFU)-Hill colonies.[13] CFU—Hill colonies are correlated to vascular reactivity and although they were initially thought to represent colony growth of EPCs,[14] it is now widely accepted that these colonies are of haematopoietic origin.[15,16] Instead of EPC-derived colony growth, this *in vitro* culture actually represents the efficiency of cluster formation between autologous T-lymphocytes and naïve $CD14^+$-monocytes. Enhanced cluster formation was observed upon prior activation of $CD4^+$-T-cells, and replacement of T-cells by the addition of activated $CD4^+$-T-cell-derived soluble factors similarly stimulated cluster formation.[13]

The aim of this study was to further investigate the phenotype of the T-cell-pre-stimulated monocytes and to examine whether pre-stimulation of naïve human $CD14^+$-monocytes with T-cell-derived soluble factors promotes their capacity to improve vascular regeneration in a murine hind limb ischaemia model for PAD.

Materials and Methods

Isolation and culture of human primary cells

Human $CD14^+$-monocytes or $CD4^+$-T-cells-enriched fresh apheresis mononuclear cell preparations from healthy donors were obtained at Sanquin Bloodbank NW Amsterdam after informed consent and with the approval of the