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Evidence for the in vivo existence and mobilisation of myeloid angiogenic cells and pericyte-like cells in wound patients after skin grafting

Liina Uusitalo-Kylmälä MSc[1] | Katriina Joensuu MD, PhD[2] | Kristiina Hietanen MD, PhD[3] | Juha Paloneva MD, PhD[3,4] | Terhi J. Heino PhD[1]

[1]Institute of Biomedicine, University of Turku, Turku, Finland

[2]Department of Plastic Surgery, Tampere University Hospital, Tampere, Finland

[3]Department of Surgery, Central Finland Central Hospital, Jyväskylä, Finland

[4]Institute of Clinical Medicine, University of Eastern Finland, Kuopio, Finland

Correspondence
Terhi J. Heino, Institute of Biomedicine, University of Turku, Kiinamyllynkatu 10 C4, 20520 Turku, Finland.
Email: terhi.j.heino@utu.fi

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Abstract

Myeloid angiogenic cells (MACs) and pericyte-like cells, derived from peripheral blood mononuclear cells (MNCs) by in vitro culturing, are suggested as relevant cell types for angiogenesis and tissue repair. However, the in vivo existence and relevance of these cells has so far remained unknown. Our aim was thus to study, if MACs and pericyte-like cells exist in circulation during the wound healing of skin graft patients, and to evaluate the cellular features of wound repair. MNCs were isolated from blood samples of healthy controls ($n = 4$) and patients with a traumatic full thickness skin defect ($n = 4$) before skin grafting and on postoperative days 1 and 6. The numbers of circulating $CD14^{+-}$ $CD45^{+}CD31^{+}CD34^{-}$ MACs and $CD14^{+}CD45^{+}NG2^{+}$ pericyte-like cells were assessed by flow cytometry, and gene expression of various pro-angiogenic factors was analysed by qPCR. Wound bed biopsies were taken on postoperative days 6 and 14, and MAC (CD31, CD14 and CD45) and pericyte-related markers (NG2 and PDGFRβ) were histologically studied. MACs and pericyte-like cells were detected in both healthy controls and in patients. Before reconstruction, on average 18% of all circulating MNCs represented MACs and 2% pericyte-like cells in wound patients. Number of MACs significantly increased 1.1−1.7-fold in all patients 1 day after skin grafting ($p < 0.01$). In addition, histological analysis demonstrated effective vascularization of skin grafts, as well as presence of pericytes, and CD14 and CD45 expressing myeloid cells during wound healing. In conclusion, our data shows, for the first time, the presence and mobilisation of MACs and pericyte-like cells in human circulation.

KEYWORDS
angiogenesis, myeloid angiogenic cells, pericytes, skin graft, wound healing

Abbreviations: BSA, bovine serum albumin; DAB, diaminobenzidine; ECFC, endothelial colony forming cell; MAC, myeloid angiogenic cell; MNC, mononuclear cell; MSC, mesenchymal stromal cell; NG2, neural/glial antigen 2; PBS, phosphate-buffered saline; PDGFRβ, platelet derived growth factor receptor β; qPCR, quantitative polymerase chain reaction; RT, room temperature; STSG, split thickness skin graft; TBS, tris-buffered saline; VEGF, vascular endothelial growth factor; VEGFR-1, vascular endothelial growth factor receptor 1; VEGFR-2, vascular endothelial growth factor receptor 2.

1 | INTRODUCTION

Formation of blood vessels via angiogenesis is essential for the normal tissue development and regeneration, for example during wound healing.[1] In certain clinical situations, autologous skin grafting is used for wound coverage, and angiogenesis is known to be crucial for proper adhesion of skin grafts.[2] Revascularization of skin graft involves vascular regression in the graft, ingrowth from the wound bed, and eventually vascular reconnection (inosculation).[2,3] Immediately after skin graft surgery, the vascular networks of the graft and the recipient tissue are separate, but the onset of angiogenesis can be detected in the wound bed as early as 24 h after transplantation and the first signs of revascularization are observed after 72 h.[4] Within 7−14 days, blood vessels from the recipient tissue invade the graft edges, whereas the native graft vasculature begins to regress. Finally, inosculation occurs and restores blood circulation to the graft.[3]

Endothelial progenitors, which promote angiogenesis, were originally identified among the peripheral blood mononuclear cells (MNCs).[5] However, at least two distinct populations of endothelial progenitor cells have been identified to date. Endothelial colony forming cells (ECFCs), also known as late endothelial cells can form tubular structures in vitro and in vivo, whereas myeloid angiogenic cells (MACs), also known as early endothelial progenitor cells increase vascularization through paracrine effects in vitro.[6,7] ECFCs are characterised as being positive for CD31, CD105 and CD146 and negative for CD45 and CD14 surface markers, while MACs are characterised as positive for CD45, CD14 and CD31, and negative for CD146 and CD34.[7] Interestingly, MACs have been suggested to circulate in the bloodstream and thus are also known as circulating angiogenic cells.[7] However, there is currently no in vivo evidence to show that MACs would circulate and contribute to vascularization after tissue damage.

Another important cell type for blood vessel formation and wound healing is the pericyte, which is a multipotent cell present in every vascularized tissue.[8,9] Pericytes of haematopoietic origin are known to express both haematopoietic and monocyte markers, as well as pericyte-markers, such as platelet-derived growth factor receptor-β (PDGFRβ), and neural/glial antigen 2 (NG2), and to contribute to the early stages of angiogenesis.[10] In general, pericytes promote vessel growth and stability, but they have also been suggested to have wider role in wound healing, such as regulation of the inflammatory response and re-epithelialization, as well as in promoting fibrosis.[11,12] Therefore, these cells could be a potential target to accelerate wound healing and to prevent fibrosis, but their origin and contribution to tissue repair is still largely unexplored. Since the mechanisms that underpin both normal and pathological wound healing are not yet fully understood, studying the molecular and cellular features of wound repair and regeneration is of great importance.[13]

In our previous studies, we have shown that interactions between human bone marrow derived mesenchymal stromal cells (MSCs) and peripheral blood derived MNCs induce expression of various signalling molecules and growth factors that are important for angiogenesis.[14] Recently, we reported differentiation of pericyte-like cells and functional MACs in MSC-MNC co-cultures in vitro.[15] In addition, $NG2^+$ pericyte-like cell population derived from cultured MNCs has been demonstrated by us[15] and others.[16] Consequently, the aim of this study was to determine whether MACs and haematopoietic pericytes have a role in neovascularization processes also in vivo. We evaluated the existence and number of these cells in the circulation of human skin graft patients, as well as utilised tissue biopsies to histologically study the expression of various MAC and pericyte related markers during wound healing after skin grafting.

2 | MATERIALS AND METHODS

2.1 | Patients and surgical procedure

Four patients with traumatic full thickness skin defect in the lower limb requiring reconstruction with split thickness skin graft (STSG), as well as four age and gender-matched healthy controls were recruited to the study (Table 1). Tissue injury had occurred on average 23 days before reconstruction (range 18–36 days). Wound bed was revised, after which split thickness skin grafting was performed in local or spinal anaesthesia and STSGs were harvested from patients´ thigh by using a Zimmer dermatome. Thickness of the STSGs were 10/1000 inch each and the grafts were meshed in 1:1.5 manner. After skin grafting, the wounds were covered with traditional wound dressings and the affected limbs were immobilised for 6 days. Blood samples (18 ml) for flow cytometry analysis were drawn before the surgery (day 0), as well as on the first and 6th postoperative days. The 3 mm punch biopsies for histological analysis were taken from the wound bed on the 6th and 14th postoperative days in local anaesthesia. Before participating in the study, patients signed informed consent, under the protocol approved by the Ethical committee of the Tampere University Central Hospital, Finland and collection of samples followed the Declaration of Helsinki ethical principles.

2.2 | MNC isolation

MNCs were isolated by gradient centrifugation (Ficoll-Paque Plus) from peripheral blood samples, which were drawn from the patients ($n = 4$) before the surgery (day 0), and on days 1 and 6 postoperatively, as well as from the healthy controls ($n = 4$). Blood samples were diluted to 1:1 ratio with phosphate buffered saline (PBS), added on top of the Ficoll, and centrifuged 30 min at 300 g without. MNCs were collected from the buffy coat layer and washed twice with PBS. Part of the cells were prepared for flow cytometry analysis and part were lysed for RNA isolation.

2.3 | Flow cytometry analysis

Isolated cells were filtered through 35-μm pore cell-strainer snap cap (Corning Incorporation), calculated, transferred to 96 well plates (100000–200,000 cells / well) and Fc receptors were blocked with

TABLE 1 Characteristics of the patients and healthy controls included in the study.

Patient	Gender	Age (years)	Wound size (cm × cm)	Time from initial injury (days)	Control	Gender	Age (years)
1	M	77	13 × 7	18	1	M	75
2	M	40	3.5 × 7	22	2	M	39
3	M	45	7.5 × 5	19	3	M	42
4	F	60	6 × 4	36	4	F	59

Human BD Fc Block reagent (BD Biosciences). Cells were stained with the following fluorochrome-labelled anti-human monoclonal antibodies (all from BioLegend, unless otherwise stated): CD14 APC/Cy7 (clone 63D3), CD45 PE (clone 2D1), CD31 Brilliant Violet 711 (clone WM59), CD34 Brilliant Violet 421 (clone 561), CD146 APC (clone P1H12) and NG2 Alexa Fluor 488 (clone 9.2.27, Invitrogen, Thermo Fisher Scientific). Cells were fixed with 1% paraformaldehyde and stored at +4°C for 5−7 days, after which flow cytometry analysis was performed using BD LSR Fortessa (BD Biosciences). MACs were characterised as $CD14^+CD45^+CD31^+CD34^-$ cells and haematopoietic pericyte-like cells as $CD14^+CD45^+NG2^+$ cells, and the results were analysed with Flowing Software (Turku Bioscience Centre, Turku, Finland).

2.4 | Real-time quantitative PCR

Total RNA was extracted using GenEluteT Mammalian Genomic DNA Miniprep Kits (Merck) according to the manufacturer's instructions. RNA quality was checked by NanoDrop One spectrophotometer (Thermo Fisher Scientific) and deoxyribonuclease I (DNase I, Merck) was used to purify the RNA samples prior to reverse transcription. cDNA was synthesised from equal amounts of mRNA using SensiFAST cDNA Synthesis Kit (Bioline). Specific primers were purchased from Metabion and primer concentration in qPCR was 25 nM. A list of primers is provided as a Supplementary Table S1. SensiFAST™ SYBR No-ROX Kit was used for real-time qPCR reaction according to the manufacturer's protocol. Beta-actin was used as a housekeeping reference gene and results were analysed by using results from day 0 as the calibrator.

2.5 | Histology and immunohistochemistry

Paraffin embedded skin biopsies were cut to 5 μm sections and slides were deparaffinised and rehydrated according to a standard protocol. Before staining, antigen retrieval was performed using either sodium citrate (pH 6) or Tris-EDTA buffer (pH 9) depending on the antibody. Slides were washed with Tris-buffered saline (TBS) plus 0.025% Triton X-100 (TBS-T), and blocked with 10% normal goat serum diluted in TBS plus 1% bovine serum albumin (BSA) for 1 h at room temperature (RT). All antibodies were purchased from Abcam and diluted in TBS plus 1% BSA. Following antibodies were used: mouse monoclonal to CD31 (ab9498, 1:500), mouse monoclonal to CD14 (ab181470, 1:200), mouse monoclonal to CD45 (ab8216, 1:500), rabbit polyclonal to NG2 (ab129051, 1:500) and rabbit monoclonal to PDGFRβ (ab32570, 1:100). Primary antibodies were incubated overnight at +4°C and washed with TBS-T. Blocking of endogenous peroxidases was performed with 0.3% H_2O_2 in TBS for 15 min RT. Secondary antibodies goat anti-mouse (ab205719) or goat anti-rabbit (ab205718) were diluted in TBS plus 1% BSA (1:2000) and incubated for 1 h at room temperature and washed with TBS. The staining was developed with diaminobenzidine (DAB) and Papanicolau's haematoxylin (Millipore) (1:5 dilution) was used for counterstaining. Negative controls were treated similarly, except for exclusion of the primary antibody. Samples were mounted using Pertex (Histolab). Immunohistochemistry with fluorescent detection was otherwise done in similar manner but no blocking of endogenous peroxidase was performed and the dilutions of the antibodies were different: mouse monoclonal to CD31 (ab9498, 1:100), rabbit polyclonal to NG2 (ab129051, 1:100), and rabbit monoclonal to PDGFRβ (ab32570, 1:100). Goat anti-mouse Alexa Fluor 488 (ab150113, 1:1000) and goat anti-rabbit Alexa Fluor 594 (ab150080, 1:1000) were used as secondary antibodies, and mounting medium with DAPI (Vector) was used to mount the samples and stain the nucleus.

2.6 | Image analysis

After staining, samples were scanned with Pannoramic P1000 slide scanner and images were analysed by Fiji. CD31 stained sections were used to quantify the number of blood vessels from both papillary and reticular dermis. Colour deconvolution was performed first, and the threshold levels were then adjusted. Blood vessels larger than 13 μm in diameter were counted and their number was calculated per total dermis area (mm^2). For analysis of CD14 and CD45 stained area, a colour deconvolution was performed, then images were turned into gray-scale and colours were inverted, background was subtracted, and threshold levels were adjusted. Thereafter the stained area (mm^2) was analysed as percentage of the total dermis area.

2.7 | Statistical analysis

Statistical analyses were conducted using GraphPad Prism 8.4.2 software (GraphPad Software, San Diego, California USA). The statistical significance was assessed by using either unpaired t-test or one-way-ANOVA followed by Dunnett's multiple comparison test or

Kruskal-Wallis test followed by Dunn's multiple comparison depending on normality of the data. Two to three technical replicates per sample and four biological repeats were used for qPCR analysis and four technical replicates and four biological repeats for flow cytometry analysis. Data is presented as mean ± SD. Results were considered statistically significant when $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ and $p \leq 0.0001$.

3 | RESULTS

3.1 | MACs and haematopoietic pericyte-like cells exist in the circulation of both healthy controls and skin graft patients and their number increases during the early phases of wound healing

Flow cytometry was used to determine the percentage of $CD14^+CD45^+CD31^+CD34^-$ MACs from the whole MNC fraction. MACs were found in the circulation of all skin graft patients already before reconstruction with the proportion of 16% (patient 1), 15% (patient 2), 12% (patient 3) and 29% (patient 4) of total MNCs, and their proportion significantly increased 1 day after the surgery (27%, 22%, 18% and 34% for patients 1, 2, 3 and 4, respectively) (Figure 1A–D). Number of MACs started to decrease 6 days post-surgery in three patients (17%, 17% and 22% for patients 2, 3 and 4, respectively) (Figure 1B–D), while patient 1 showed even higher numbers of MACs on day 6 (36%) (Figure 1A). We also analysed the number of circulating MACs in healthy controls and the number was significantly lower in controls 1 (age 75 years) (Figure 1A) and 4 (age 59 years) (Figure 1D) compared to the corresponding skin graft patients before surgery. Surprisingly, higher (control 2) (Figure 1B) or similar (control 3) (Figure 1C) proportions of circulating MACs were found in two younger controls when compared to pre-operative samples from corresponding patients. When combining data from all the patients, there was a trend for higher number of circulating MACs in wound patients compared to healthy controls and even for a further increase after the skin graft surgery (Figure 1E) but these differences were not statistically significant. In addition to MACs, circulating $CD14^+CD45^+NG2^+$ haematopoietic pericyte-like cells were also assessed in healthy controls and skin graft patients. Significantly higher cell numbers were observed in healthy controls (11%, 12%, 7%, 15% in controls 1, 2, 3, and 4) when compared to patients before surgery (0.6%, 1%, 4% and 3% in patients 1, 2, 3 and 4, respectively) (Figure 1F–I) and combined data from all four patients confirmed this observation (Figure 1J). The number of circulating haematopoietic pericyte-like cells followed similar trend as circulating MACs in patients 2, 3 and 4, where the number of $CD14^+CD45^+NG2^+$ cells increased 1 day post-surgery (2%, 15% and 5%) and decreased or remained the same on day 6 (1%, 8% and 5%, respectively) (Figure 1G–I). An exception was patient 1, in which the number of haematopoietic pericyte-like cells showed a non-significant trend for decreased cell numbers one day after the surgery (0.5%) but a significant increase on the 6th post-operative day (3%) (Figure 1F).

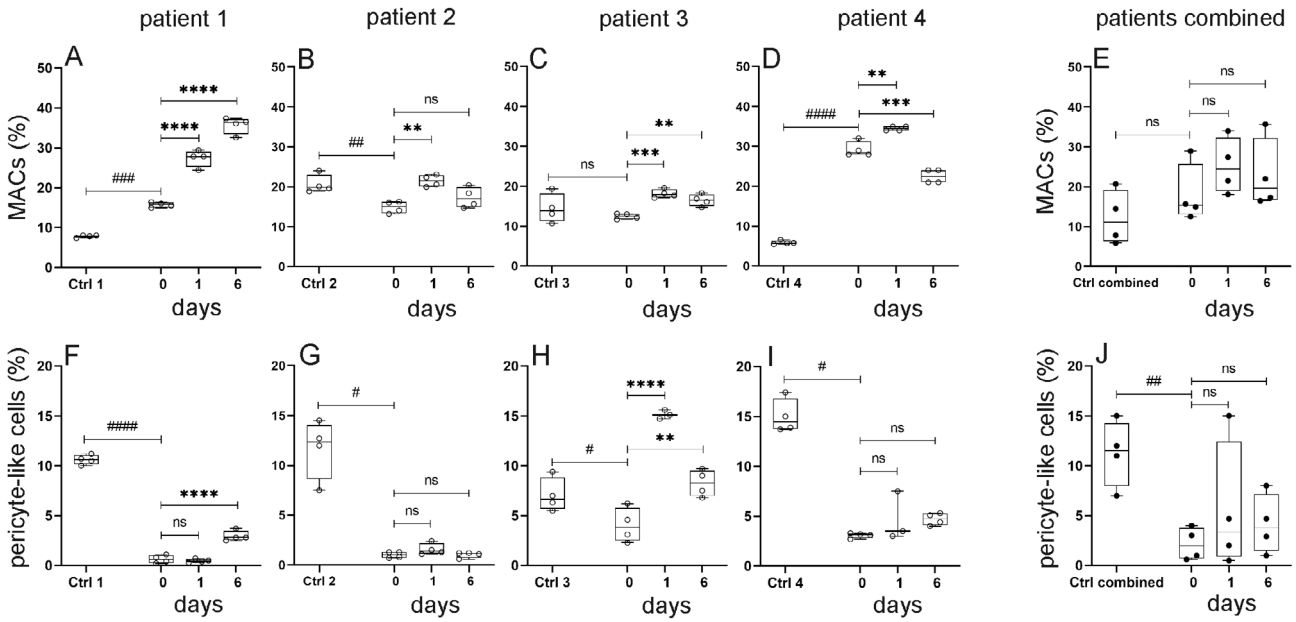

FIGURE 1 Percentages of $CD14^+CD45^+CD31^+CD34^-$ myeloid angiogenic cells (MACs) (A–E) and $CD14^+CD45^+NG2^+$ haematopoietic pericyte-like cells (F–J) among peripheral blood MNCs were determined by flow cytometry on pre-operative day 0 and on post-operative days 1 and 6. Three to four technical repeats per sample (shown as hollow circle) were used for the individual patient analysis (A–D and F–I). Combined data of MACs (E) and pericyte-like cells (J) from all four patients (shown as black circle) was also analysed. Statistical significance between healthy controls and corresponding patient on pre-operative day 0 was assessed by using unpaired t-test and is denoted as $^{\#}p \leq 0.05$, $^{\#\#}p \leq 0.01$, $^{\#\#\#}p \leq 0.001$, and $^{\#\#\#\#}p \leq 0.0001$. Statistical significance between the pre-operative day 0 and other time points was assessed by one-way-ANOVA followed by Dunnett's multiple comparison test or Kruskal-Wallis test followed by Dunn's multiple comparison and is denoted as $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ and $^{****}p \leq 0.0001$. ns, non-significant

3.2 | Gene expression of pro-angiogenic factors in circulating MNCs differs between the patients

To study the gene expression of different molecular markers related to angiogenesis, mRNA expression levels of *PECAM-1* (CD31), *KDR* (VEGFR-2), *FLT-1* (VEGFR-1), *VEGF-A*, *PDGFRβ* and *CSPG4* (NG2) in total MNC fraction were studied by qPCR (Figure 2A–L). Expression levels were analysed from the whole MNC fraction both before skin graft surgery and on days 1 and 6 post-surgery in all four patients. No distinct differences were observed in the expression of these factors that would apply to all patients except for *PDGFRβ*, which was upregulated on day 6. This change between days 1 and 6 did not however reach statistical significance in patient 4 (Figure 2I), or when data from all four patients was combined (Figure 2J). The only statistically significant difference, which was observed in the combined patient data, was the downregulation of *CSPG4* (NG2) on post-operative day 6 (Figure 2L).

3.3 | Skin graft is successfully vascularized and NG2 and PDGFRβ expressing cells are observed at graft site during wound healing

As successful vascularization plays an important role in skin graft attachment and wound healing, the formation of blood vessels was demonstrated by CD31 staining in patients' skin biopsies on 6 (Figure 3A) and 14 days post-surgery (Figure 3B). Formation of blood vessels was observed in all patients (Figure 3C), thus verifying

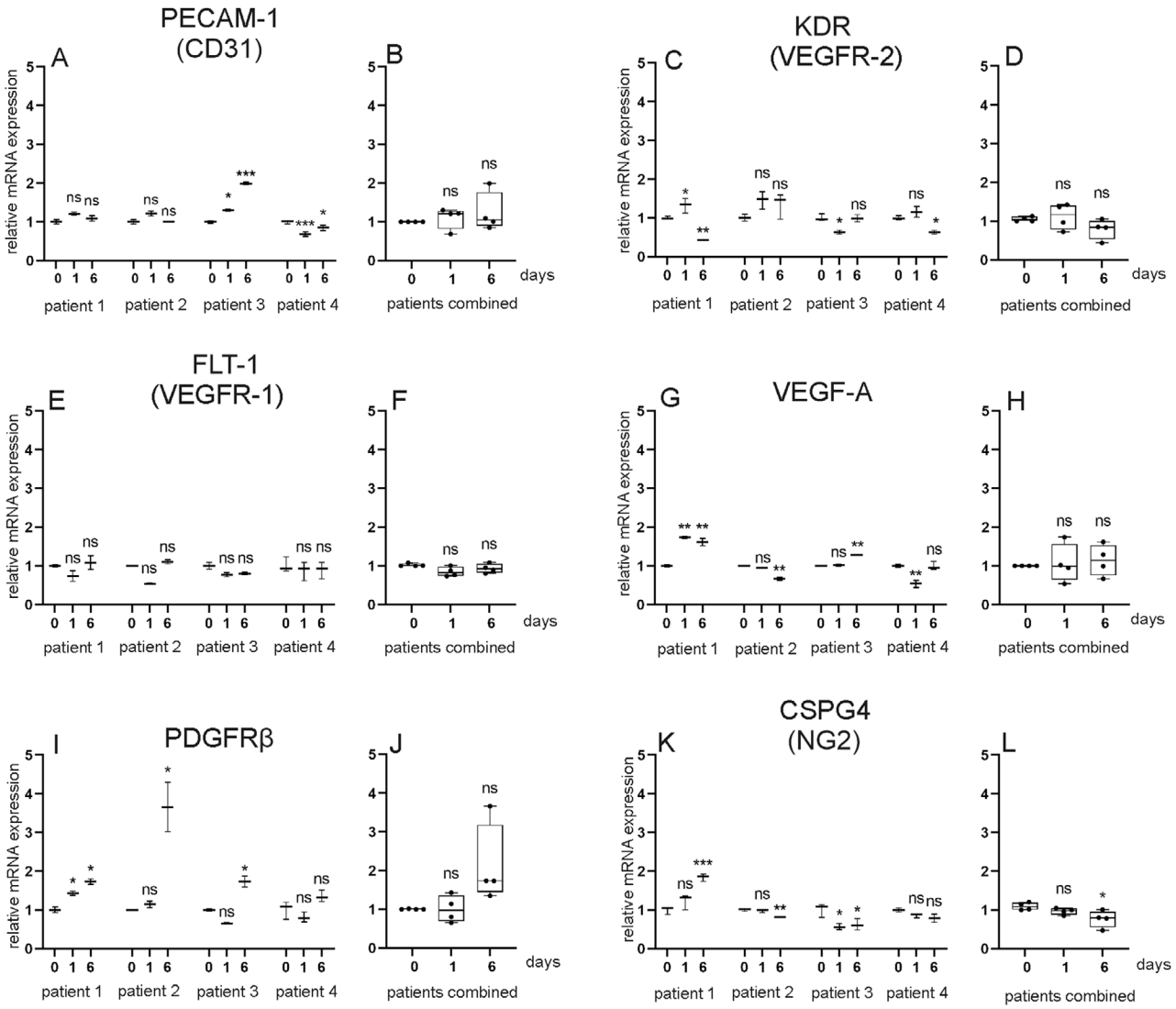

FIGURE 2 Gene expression of *PECAM-1* (A), *KDR* (C), *FLT-1* (E), *VEGF-A* (G), *PDGFRβ* (I) and *CSPG4* (K) was studied by quantitative PCR from four skin graft patients before surgery (day 0) and after the surgery on days 1 and 6. Two to three technical repeats per sample were used for the analysis. Expression of the genes was also studied by combining the data from all four patients (shown as black circles) (B, D, F, H, J, and L). Statistical significance was assessed by one-way-ANOVA followed by Dunnett's multiple comparison test or Kruskal-Wallis test followed by Dunn's multiple comparison depending on normality of the data and is denoted as $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$. ns, non-significant

the effective vascularization of skin grafts. Presence of NG2$^+$ and PDGFRβ$^+$ cells was analysed with immunohistochemistry and skin biopsies exhibited extensive non-vascular expression of NG2 (Figure 3D,E) and PDGFRβ (Figure 3G,H). Both markers were also associated with blood vessels, since as NG2 (Figure 3D–F) and PDGFRβ (Figure 3G–I) expressing cells were found around small vessels, suggesting that these cells represent pericytes.

3.4 | CD14 and CD45 expressing cells participate in wound healing

Since CD14$^+$ monocytes and macrophages have a major role during the inflammatory phase of the wound healing, the presence of CD14$^+$ cells at graft site were also analysed (Figure 4A–D). More CD14$^+$ cells were observed on day 6 than on day 14 (Figure 4E), except for patient 4, in whom the number of CD14 expressing cells appeared to slightly increase over time. The presence of CD45$^+$ cells, representing differentiated haematopoietic cells, was also evaluated (Figure 4F-I). In patients 1 and 2, there was an increase in the number of CD45$^+$ cells on postoperative day 14, while in patients 3 and 4 the cell numbers were decreasing (Figure 4J). Interestingly, CD14, CD45 and CD31 expressing cells were found to be co-localised within the same regions in the regenerating skin on days 6 (Figure 4A,B,F,G,K,L) and 14 (Figure 4C,D,H,I,M,N).

4 | DISCUSSION

Previous studies have suggested that MACs are generated only in vitro due to cell culture conditions not existing in vivo,[7] and the significance of these cells, along with proposed circulating haematopoietic pericyte-like cells[16] has so far remained obscure. Here we show, for the first time, that MACs and pericyte-like cells exist in human circulation. We detected MACs and pericyte-like cells of monocytic origin in both healthy controls and in patients with a traumatic tissue defect and furthermore show here that the number of MACs increases after skin grafting, suggesting that these cells could contribute to wound healing in vivo.

We quantified circulating MACs in four patients with a traumatic skin defect and observed that the percentage of MACs was increased 1 day after the skin graft surgery in all patients, suggesting that these cells might contribute to wound healing. A previous study demonstrated a rapid rise of another type of endothelial progenitor cells, that is, CD45$^-$ ECFCs in the circulation of burn patients within 24h.[17] Together with our data, this indicates that the circulating cells

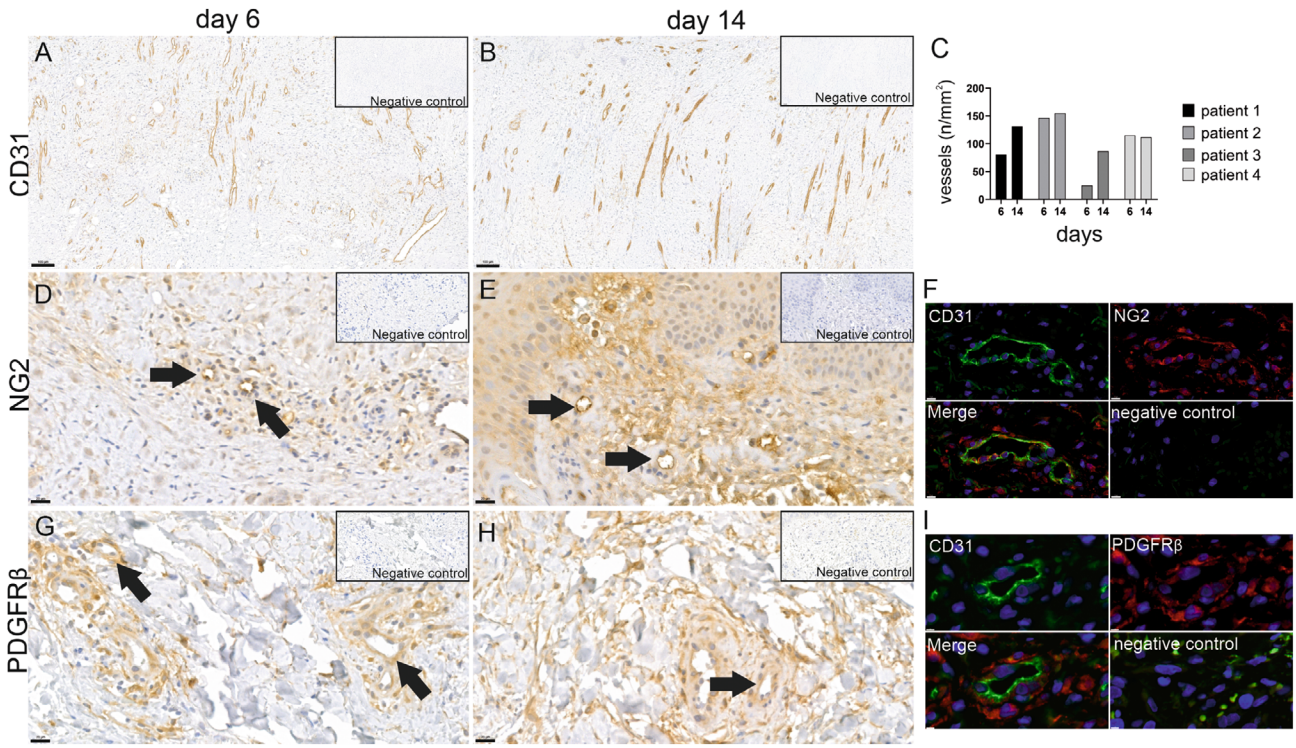

FIGURE 3 Immunohistochemical analysis was used to determine the vascularization of the skin graft and to evaluate the NG2 and PDGFRβ expressing cells during wound healing. Formation of blood vessels was demonstrated by CD31 staining on day 6 (A) and 14 (B) (scale bar 100 μm) and the blood vessel density was calculated with Fiji (C). Skin displayed wide non-vascular expression of NG2 (D, E) and PDGFRβ (F, G), but positive cells were observed also around small vessels (arrows) (scale bar 20 μm). Immunofluorescence staining demonstrated that NG2 (F, red label) (scale bar 10 μm) and PDGFRβ (I red label) (scale bar 5 μm) positive cells wrap around CD31 positive endothelial cells (green label) lining the inner surface of small vessels (day 6) [Color figure can be viewed at wileyonlinelibrary.com]

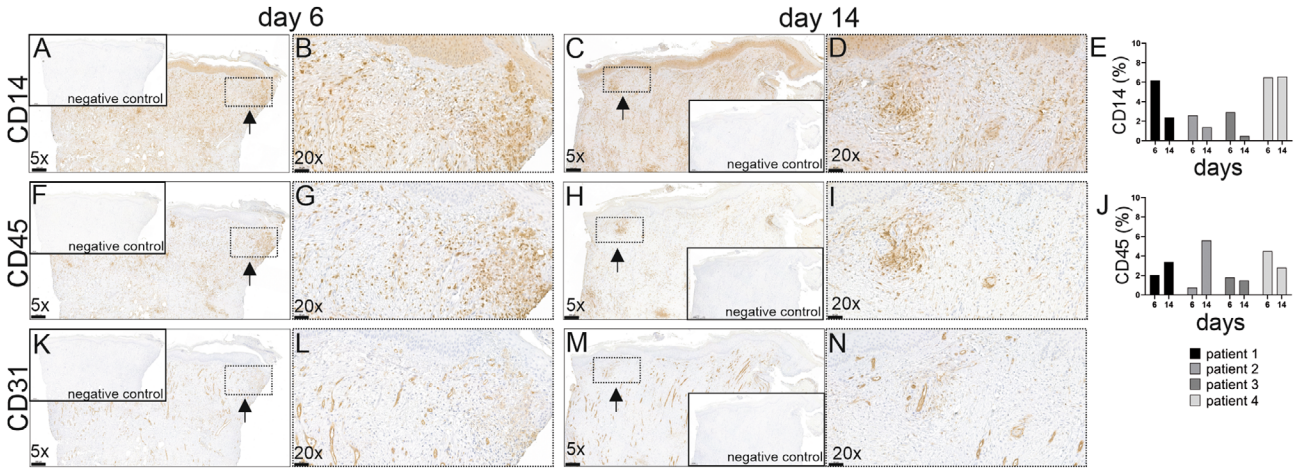

FIGURE 4 Immunohistochemical analysis was used to determine the presence of CD14$^+$ (A–D) and CD45$^+$ (F–I) cells in the regenerating skin and their numbers were analysed with ImageJ program by quantifying the percentage of stained area (E, J). CD14, CD45 and CD31 expressing cells were found to be co-localised within the same tissue areas (indicated by arrows) both on days 6 (A,B, F,G, and K,L) and 14 (C,D, H,I, and M,N). Larger magnifications of the areas of interest (arrows) are presented in adjacent panels (boxes with dashed line). Scale bar for images are 200 μm (5× magnification) and 50 μm (20× magnification) [Color figure can be viewed at wileyonlinelibrary.com]

promoting angiogenesis, whether they are ECFCs or MACs, could be quickly mobilised in response to tissue trauma. However, to our surprise, we found similar or even higher levels of MACs in younger controls compared to skin graft patients at pre-operation, whereas the number of circulating MACs was significantly lower in older controls. These results suggest that besides stimulating blood vessel formation after trauma, MACs could have other age-related functions. In fact, the number of circulating endothelial progenitor cells is known to reflect age-related vascular health and remodelling.[18] For example, a previous study has shown that higher age is associated with lower preoperative levels of circulating CD34$^+$CD133$^+$ ECFCs in patients undergoing coronary artery bypass grafting.[19] Thus, age might be a significant factor contributing to the number of circulating MACs as well. Even though in vitro generated MACs are known to enhance angiogenesis in a paracrine manner in vitro[6,15] and in vivo[6,20] it has not yet been demonstrated if MACs actually exist in vivo. As we observed high percentages of circulating MACs both in healthy controls and during the early phases of wound healing and quantitative analysis of blood vessels verified effective angiogenesis by day 14 in the same patients, it can be hypothesized that MACs are not only generated in vitro but they do exist in human circulation in vivo.

To investigate the possible mechanisms through which angiogenesis occur, we analysed the gene expression of endothelial markers CD31 (*PECAM-1*), vascular endothelial growth factor-A *(VEGF-A)* and VEGF receptors VEGFR-1 (*FLT-1*) and VEGFR-2 (*KDR*). CD31 is known to be involved in endothelial cell migration and angiogenesis,[21] while VEGF-A is the most important stimulator of angiogenesis, functioning through its receptors VEGFR-1 and VEGFR-2.[22] However, changes in gene expression of these factors after skin graft surgery were not consistent in all patients and no statistically significant differences were observed when the data from all patients was combined. These results suggest that MACs could contribute to vascularization through some other signalling pathways, such as stromal cell-derived factor 1 (SDF-1)[15] or interleukin-8.[6] In addition, since gene expression analyses were made for the whole MNC fraction and not solely for isolated MACs, potential changes in MAC gene expression might be covered by the presence of other major cell types, such as T-cells among the MNCs. Ruling out this possibility would require another study with more patients and larger blood sample sizes to obtain enough MACs for detailed qPCR analyses.

Myeloid lineage cells are known to be essential during the inflammatory phase of wound healing, for example, by secreting inflammatory cytokines and growth factors, thus further regulating tissue repair and remodelling.[23] Abnormalities in their myeloid cell function can lead to deficient wound healing and contribute to development of chronic wounds. Because CD14 and CD45 are considered as common immune cell markers, which can provide important information about the progress of skin healing, we histologically evaluated the presence of CD14$^+$ and CD45$^+$ cells at the wound site in our patient samples. Dynamic changes were found, as decreasing numbers of CD14$^+$ cells were seen in three out of four patients and increasing numbers of CD45$^+$ cells in two out of four patients over time. CD14 expressing monocytes are known to normally arrive at the wound site after neutrophils and have a major role during the inflammation phase by differentiating into macrophages.[13] After the macrophage-predominant inflammatory phase, wound healing progresses towards proliferation and remodelling and the number of these myeloid cells decreases. We also observed fewer CD14$^+$ cells at the wound site by 14 days after skin grafting, indicating that the healing had progressed along successful vascularization. Nevertheless, it should be noted that monocytes can also differentiate into fibrocytes,[24,25] which are a unique leukocyte subpopulation implicated in wound healing. They express the haematopoietic marker CD45[26] and contribute to the remodelling phase of wound repair, which could explain our observation on the

higher incidence of $CD45^+$ cells on day 14. However, since $CD14^+$ monocytes and macrophages also express CD45[26] it cannot be concluded whether the cells at wound site represent monocyte/macrophages or fibrocytes. However, cell clusters co-expressing CD14, CD45 and CD31 were found at the same locations within the regenerating tissue, suggesting that part of these cells might be $CD14^+CD45^+CD31^+$ MACs, which could have migrated to injury site to promote healing. Further studies are nevertheless still needed to verify the potential presence of MACs at the tissue site. In addition, the interpretation of results is confounded by a variety of factors known to affect wound healing, such as patient's age, gender, and chronic diseases,[27] which is also reflected in our data with substantial inter-individual differences in cell and gene expression parameters.

Another important cell type involved in angiogenesis is the pericyte, which are considered to stabilise the vessel wall, control endothelial proliferation and thereby the growth of new capillaries.[8] It has also been suggested that pericytes play a key role in wound healing by interacting with platelets, inflammatory cells, and connective tissue cells, in addition to endothelial cells.[11,12] However, the origin of skin pericytes is not fully known. Mouse studies have shown that pericytes originate from fibroblasts during wound healing[28] and that myeloid progenitors differentiate into pericytes during development of the embryonic skin vasculature.[29] Our data shows that $CD14^+CD45^+NG2^+$ haematopoietic pericyte-like cell population exists also in the circulation of healthy controls and patients after skin grafting. Gene expression analysis of MNCs showed a consistent upregulation of pericyte-marker *PDGFRβ* after skin graft surgery, although this did not reach statistical significance in patient 4 nor in combined patient data. PDGF/PDGFRβ axis is one of the key pathways between pericytes and endothelial cells,[30] suggesting that $PDGFR\beta^+$ circulating pericytes could be attracted to the wound site via the PDGF-B secreted by endothelial cells during angiogenesis. Data on NG2 is more difficult to interpret, since even though a trend for increased numbers of circulating $CD14^+CD45^+NG2^+$ haematopoietic pericyte-like cells was observed on post-operative day 6, gene expression analysis surprisingly showed downregulation of *CSPG4* (NG2) in MNCs at the same time point. This data is however confounded by presence of other cell types among MNCs and future experiments should thus be performed with more specific cell populations. Nonetheless, in our immunohistochemical analysis, NG2 and PDGFRβ expressing pericytes were found wrapped around endothelial cells, indicating a proper cellular organisation of the newly formed blood vessels at the wound site. In addition, a wide non-vascular NG2 staining was observed possibly representing keratinocytes,[31,32] epidermal stem cells[33] or dermal stromal cells, such as hair follicle stem cells or adipocytes,[32] which have also been reported to express NG2, at least in mice. Diffuse PDGFRβ staining was also seen in dermal connective tissue, presumably representing dermal fibroblasts. Data from mouse studies has indicated that PDGFRβ function in dermal fibroblasts is critical for wound healing in vivo.[34,35]

In summary, the present study demonstrates for the first time the presence of circulating MACs and haematopoietic pericyte-like cells in both healthy donors, as well as in traumatic wound patients after skin grafting. Interestingly, the number of these cells increased in circulation within the first 24 h and was followed by a successful vascularization at the wound site by day 14. However, it cannot be definitely concluded, whether the mobilisation of MACs and pericyte-like cells in patients is caused by donor site defect, wound bed preparation or skin grafting, or whether it could also be influenced by anaesthesia and/or medications. Furthermore, circulating MACs and haematopoietic pericyte-like cells were also detected in healthy controls, suggesting that these cells could function in other processes as well. Thus, further studies with larger patient groups are needed to verify our observations. Moreover, it would be of great interest to assess the functionality of MACs and pericyte-like cells isolated from circulation and to include additional time points for blood and tissue sampling. In the future, comprehensive cellular and molecular analyses on isolated MACs should be performed to fully understand their role in tissue repair. In conclusion, our data however supports the hypothesis that pro-angiogenic cells exist in circulation and could potentially contribute to wound repair and regeneration.

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CONFLICT OF INTEREST

The authors have no financial conflicts of interest.

DATA AVAILABILITY STATEMENT

Data available on request due to privacy/ethical restrictions.

ORCID

Liina Uusitalo-Kylmälä https://orcid.org/0000-0003-0215-0339
Terhi J. Heino https://orcid.org/0000-0003-2274-6834

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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