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ORIGINAL ARTICLE

Differential expression of granulocyte, macrophage, and hypoxia markers during early and late wound healing stages following transplantation of tissue-engineered skin substitutes of human origin

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Abstract
Purpose Human pigmented tissue-engineered skin substitutes represent an advanced therapeutic option to treat skin defects. The inflammatory response is one of the major factors determining integration and long-term survival of such a graft in vivo. The aim of the present study was to investigate the spatiotemporal distribution of host-derived macrophage and granulocyte graft infiltration as well as hypoxia-inducible factor 1 alpha (HIF-1-alpha) expression in a (nu/nu) rat model.
Methods Keratinocytes, melanocytes, and fibroblasts derived from human skin biopsies were isolated, cultured, and expanded in vitro. Dermal fibroblasts were seeded into collagen type I hydrogels that were subsequently covered by keratinocytes and melanocytes in 5:1 ratio. These pigmented dermo-epidermal skin substitutes were transplanted onto full-thickness skin wounds on the back of immunoincompetent rats and analyzed at early (1 and 3 weeks) and late (6 and 12 weeks) stages of wound healing. The expression of distinct inflammatory cell markers specific for granulocytes (HIS48) or macrophages (CD11b, CD68), as well as HIF-1-alpha were analyzed and quantified by immunofluorescence microscopy.
Results Our data demonstrate that granulocytes infiltrate the entire graft at 1 week post-transplantation. This was followed by monocyte/macrophage recruitment to the graft at 3–12 weeks. The macrophages were initially restricted to the borders of the graft (early stages), and were then found throughout the entire graft (late stages). We observed a time-dependent decrease of macrophages. Only a few graft-infiltrating granulocytes were found between 6–12 weeks, mostly at the graft borders. A heterogeneous expression of HIF-1-alpha was observed at both early and late wound healing stages.
Conclusions Our findings demonstrate the spatiotemporal distribution of inflammatory cells in our transplants closely resembles the one documented for physiological wound healing.

Keywords Human skin substitute · Tissue engineering · Inflammatory response · Granulocyte infiltration · Monocyte/macrophage recruitment · HIF-1-alpha expression

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Introduction

Autologous split-thickness skin grafts represent the "gold standard" treatment for covering skin defects. However, they become limited in massive burns, i.e., when more than about 60 % of the total body surface is injured [1].

Therefore, in vitro cultured tissue-engineered dermo-epidermal skin analogs have been developed by our group as a potential alternative to conventional techniques [2–6].

Skin wound healing is a vital process for reestablishing tissue integrity and homeostasis following disease or injury. Yet, the role of immune cells in the early and late phases of skin wound repair of tissue-engineered skin analogs after transplantation has so far not been adequately investigated. The healing of a skin wound is a complex biological process that is subdivided into three phases: inflammation, tissue formation, and maturation [7]. The early stage of skin repair is mediated by the inflammatory phase, which is characterized by an immediate influx of polymorphonuclear leukocytes (granulocytes), followed by subsequent invasion of blood monocytes, which then differentiate into tissue macrophages [8–11]. Neutrophil granulocytes combat invading microorganisms, remove cellular debris, and secrete pro-inflammatory cytokines, such as interleukins 1 alpha and beta (IL-α and β) and tumor necrosis factor alpha (TNF-α) [12]. Clearly, neutrophils play an important role in wound healing by activating fibroblasts and keratinocytes [13]. Conversely, neutrophil depletion in the early inflammatory phase has been shown to delay cutaneous wound healing in older animals [14].

Macrophages represent the second major fraction of inflammatory cells recruited into the wound site. They are indispensable for successful wound healing as they secrete important cytokines, chemokines, and growth factors such as platelet-derived growth factor and vascular endothelial growth factor to initiate the formation of granulation tissue [8]. Macrophages are present through all stages of the repair process with an increase during the phase of inflammation, a peak during the phase of tissue formation, and a gradual decline during the maturation phase [15]. Previous findings have demonstrated a crucial role for macrophages to achieve tissue homeostasis by exerting specific functions during the distinct phases of skin repair [16].

As a deeper skin injury is invariably also associated with the interruption of local blood supply to the tissue, it inevitably results in local hypoxia, which upregulates hypoxia-inducible factor 1 alpha (HIF-1-alpha) signaling. However, limited evidence is available regarding the influence of the local hypoxic microenvironment on chemotactic activity and migratory behavior of inflammatory cells. Previous studies demonstrated the recruitment of monocytes/macrophages and granulocytes from the circulating blood to damaged tissues, where they accumulate and terminally differentiate within ischemic/hypoxic sites with high HIF-1-alpha expression [17–22].

In this experimental study, we wanted to investigate the spatiotemporal distribution of host-derived macrophage and granulocyte infiltration as well as HIF-1-alpha expression in a rat model.

Materials and methods

Human skin samples

The study was conducted according to the "Declaration of Helsinki Principles" and after permission by the Ethic Commission of the Canton Zurich. Parents or adolescent patients gave informed consent to use skin samples. Human foreskins were obtained from patients 1–16 years of age and were used for the isolation of human epidermal keratinocytes, melanocytes, and dermal fibroblasts. Tissue samples for histological examination were embedded in OCT compound (Sakura Finetek, Switzerland) and kept at −20 °C.

Isolation and culturing of primary cells

Keratinocytes and fibroblasts were isolated and cultured as described in Klar et al. [3], melanocytes as specified in Böttcher-Haberzeth et al. [6].

Preparation of tissue-engineered skin analogs

The skin analogs were prepared as previously described [23]. Briefly, 1×10^5 human dermal fibroblasts (passage 1–3) were mixed with rat collagen type I and cultured in transwell cell culture plates in 6-well format containing inserts with 3.0 µm pore-size membranes (BD Falcon, Switzerland). These dermal substitutes were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Switzerland) for five days, followed by a seeding of 7×10^5 keratinocytes and melanocytes (passage 1–3). The skin analogs were cultured for one week in a 1:5 mix of melanocyte (Promocell, Germany) and keratinocyte growth medium (SFM, Invitrogen, Switzerland).

Transplantation of cultured dermo-epidermal skin analogs

The surgical protocol was approved by the local Committee for Experimental Animal Research (permission number 76/2011). Immuno-incompetent female nu/nu rats, eight to ten weeks old (Harlan Laboratories, The Netherlands), were prepared and anesthetized as previously described [23]. Skin analogs were transplanted onto full-thickness skin defects created surgically on the backs of the rats. Custom-made steel rings (diameter 2.6 cm) were sutured into those skin wounds using non-absorbable polyester sutures (Ethibond®, Ethicon, USA) to protect skin analogs from surrounding rat skin. Skin analogs were covered with

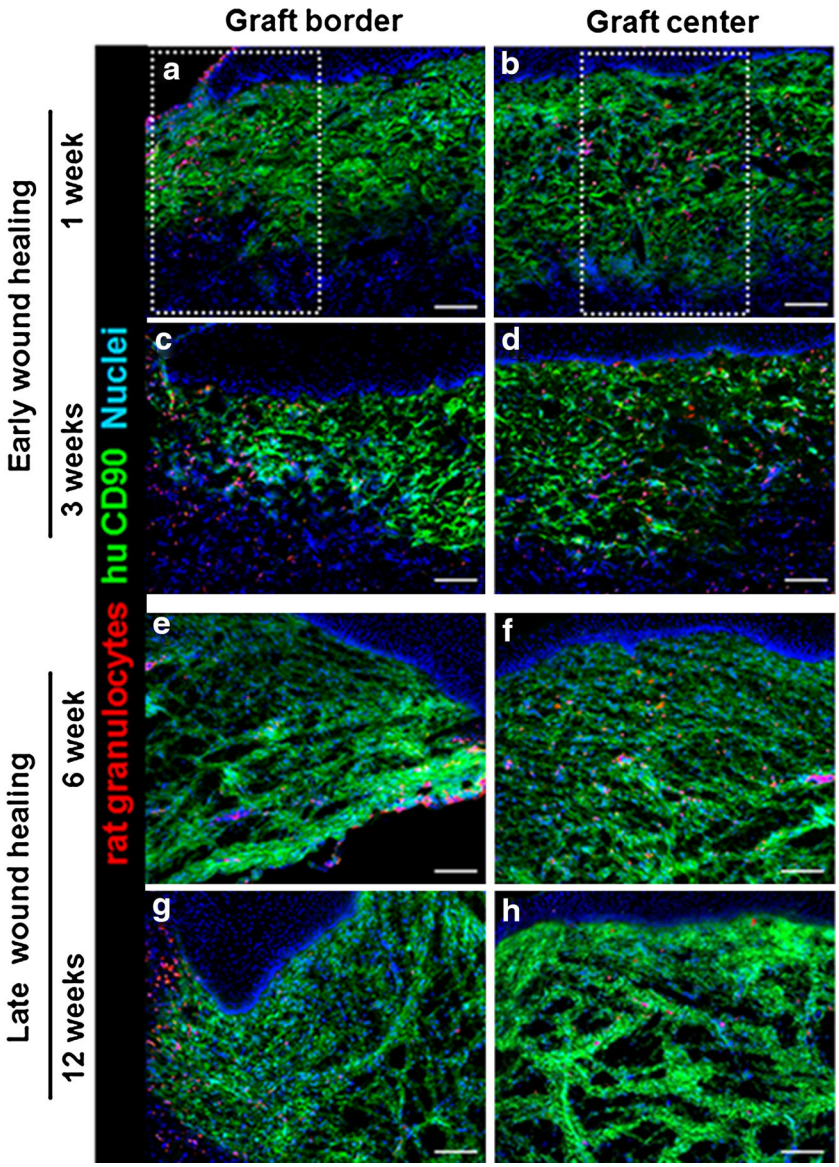

Fig. 1 Infiltration of rat granulocytes into transplanted tissue-engineered dermo-epidermal skin analogs. **a**, **c**, **e**, **g** Staining of rat granulocytes (HIS48; *red*) at the borders and **b**, **d**, **f**, **h** in the central parts of skin analogs. **a**, **b**, **c**, **d** Distribution of rat granulocytes during early (1 and 3 weeks) and **e**, **f**, **g**, **h** late wound healing stages (6 and 12 weeks) post-transplantation. The human dermal compartment is marked by human CD90 staining (*green*). An increased number of infiltrating granulocytes was observed mostly at the graft borders; however, there was also a pronounced increase in HIS48 positive cells in the central parts of the grafts between 3–6 weeks. Cell nuclei are stained with Hoechst (*blue*). *Scale bars* 50 μm

a silicone foil (Silon-SES, BMS, USA), a polyurethane sponge (Ligasano, Ligamed, Austria), and a tape as wound dressing. Dressing changes and photographic documentations were performed weekly. After 1, 3, 6, and 12 ($N = 3$–5) weeks transplanted skin analogs were excised in toto and processed for cryo-sections.

Immunohistochemical staining

Immunofluorescence staining was performed as described in Klar et al. [3]. Rat macrophages (CD11b (anti-Integrin αM), Santa Cruz, Germany) and/or granulocytes (HIS48, Santa Cruz, Germany) were counterstained with human fibroblasts of dermal compartment [CD90 (clone 5E10, 1:50, Dianova, Germany)]. HIF-1-alpha was stained by anti-HIF-1-alpha antibody [(clone EP1215Y, 1:500, abcam, Switzerland)]. Pictures of immunofluorescence staining were taken with a DXM1200F digital camera connected to a Nikon Eclipse TE2000-U inverted microscope. The device is equipped with Hoechst 33342-, FITC-, and TRITC-filter sets (Nikon AG, Switzerland; Software: Nikon ACT-1 version 2.70). Images were processed with Photoshop 7.0 (Adobe Systems Inc., Germany).

Rat macrophage and granulocyte quantification

Numbers of macrophages and granulocytes in skin analogs were determined by counting CD11b and HIS48 positive cells at the graft borders or in the central parts of skin analogs (white dotted insets in Figs. 1, 2). The dermal compartment of skin analogs was delineated by human CD90 staining. Five random high-power fields at 10x or 20x magnification were counted in 3–5 different sections of each skin analog 1, 3, 6, and 12 weeks post-transplantation

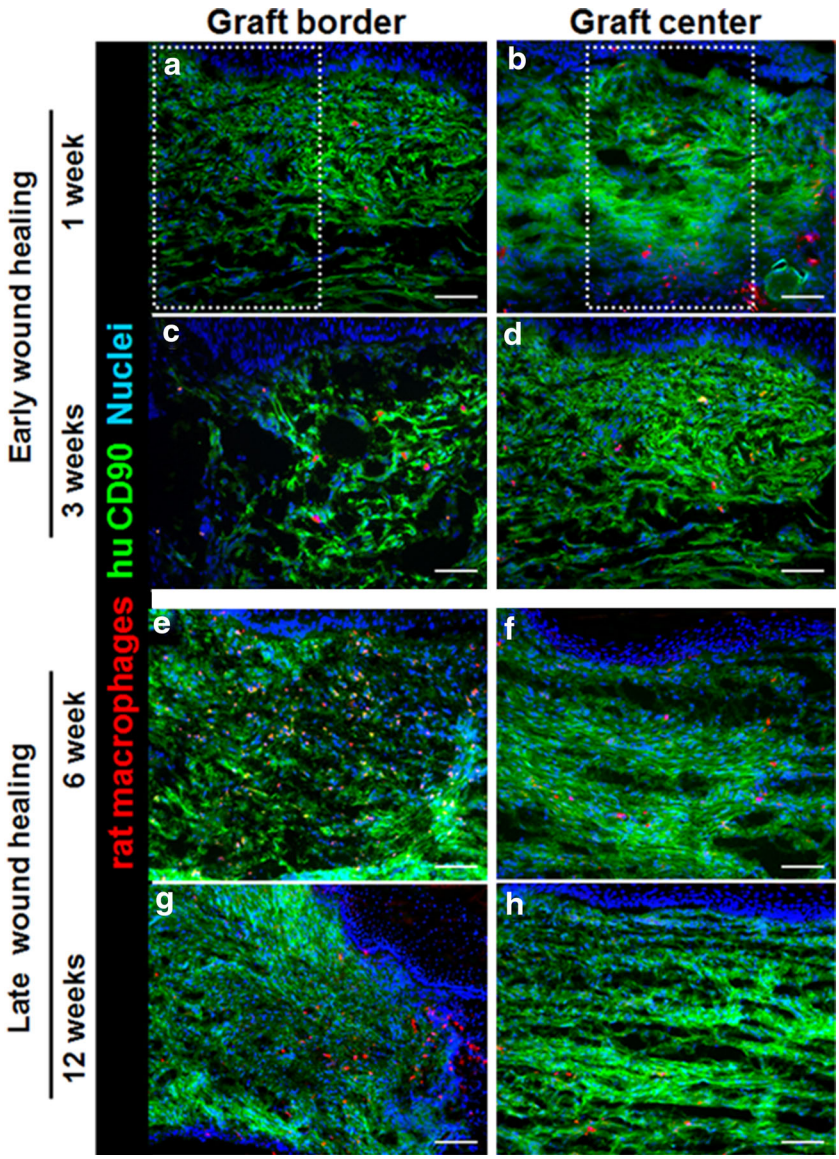

Fig. 2 Infiltration of rat macrophages into transplanted tissue-engineered dermo-epidermal skin analogs. **a**, **c**, **e**, **g** Staining of rat macrophages with CD11b (*red*) at the borders and **b**, **d**, **f**, **h** in the central parts of skin analogs. **a**, **b**, **c**, **d** Distribution of rat macrophages during early (1 and 3 weeks) and **e**, **f**, **g**, **h** late wound healing stages (6 and 12 weeks) post-transplantation. The human dermal compartment is marked by human CD90 staining (*green*). The borders of transplanted skin analogs show markedly enhanced macrophage infiltration as compared to the central parts. Cell nuclei are stained with Hoechst (*blue*). *Scale bars* 100 μm

($N = 3$–5). Immune cell density was expressed as the average total number of rat CD11b or HIS48 positive cells counted in each skin analog per high-power field. All results are reported as mean ± standard deviation (SD). Statistical analysis was performed with GraphPad Prism 4.0 (Graph Pad software, La Jolla, CA, USA).

Results

Rat granulocyte infiltration during early and late wound healing of transplanted skin analogs

To characterize the granulocyte infiltration in transplanted skin analogs, we analyzed the expression of granulocyte marker HIS48 present on basophils, eosinophils, and neutrophils (Figs. 1, 3a). Quantitative evaluation revealed that the number of granulocytes has markedly increased at the border and center of grafts from weeks 1 to 3 after transplantation (early wound healing stage) (Figs. 1a–d, 3a). Granulocyte infiltration decreased slightly at the graft borders 6 post-transplantation, whereas it increased markedly in the graft center (Figs. 1e, f, 3a). The graft borders were still infiltrated by granulocytes 12 weeks after transplantation, but only a few of them were still present in the central parts of grafts (Figs. 1g, h, 3a).

Rat macrophage infiltration during early and late wound healing of transplanted skin analogs

To assess the influx of macrophages during early (Figs. 2a–d, 3b) and late (Figs. 2e–h, 3b) stages of wound repair, we

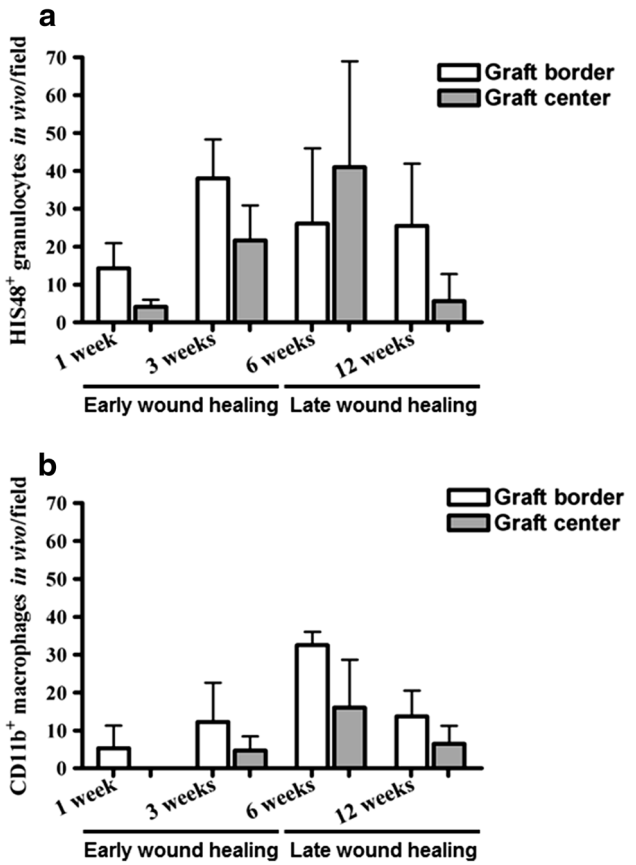

Fig. 3 The quantification of rat granulocyte and macrophage density in transplanted skin analogs. **a** Granulocyte (HIS48) density at the graft borders (*white bars*) and in the central parts (*black bars*). The number of granulocytes increased over time, with the lowest level at 1 and 3 weeks, and the highest between 6–12 weeks post-transplantation. **b** Macrophage (CD11b) density at the graft borders (*white bars*) and in the central parts (*black bars*). An increased number of macrophages were observed at the graft borders as compared to the graft center. Table shows values as the mean (± SD) of total numbers of rat granulocytes (**a**) or macrophages (**b**) counted per high-power field (×10 magnification). $N = 5$ for each group

quantified the expression of macrophage cell markers CD11b (Figs. 2, 3b) and CD68 (data not shown). Host-derived macrophages positive for CD11b marker were observed at the graft borders of skin analogs as early as 1 week after transplantation, but were absent in the graft center (Figs. 2a, b, 3b). By 3 weeks post-transplantation, the number of macrophages almost doubled at the graft borders (Figs. 2c, 3b), but only a few $CD11b^+$ cells were found in the central parts of the graft (Fig. 2d). Six weeks after transplantation, grafts were densely infiltrated by macrophages, which particularly accumulated at graft borders (Figs. 2e, f, 3b). At 12 weeks post-transplantation, only a moderate number of $CD11b^+$ cells were found in the graft (Figs. 2g, h, 3b). The level of macrophages in skin analogs was markedly increased at the graft borders when compared to the graft centers at all time points investigated (Figs. 2, 3b).

Expression of HIF-1-alpha at various wound healing stages of transplanted skin analogs

As ischemia/hypoxia occurs inevitably in wounded tissue, we analyzed HIF-1-alpha expression in skin analogs after transplantation (Fig. 4). As revealed by the staining in Fig. 4a, a‘, the highest level of HIF-1-alpha expression was found at early stages of wound repair, namely at 1 and 3 weeks post-transplantation (Fig. 4a, a‘, data not shown), whereas it decreased after six and again after 12 weeks. Higher magnification in Fig. 4a‘ demonstrates that HIF-1-alpha was strongly expressed by keratinocytes in all epidermal layers and also partially in the dermal compartment delineated by human CD90-staining. Furthermore, the rat tissue surrounding the skin graft also stained positive for HIF-1-alpha (Fig. 4a). The HIF-1-alpha expression level decreased markedly in all skin analogs at later stages post-transplantation from weeks 6 to 12 (data not shown, Fig. 4b, b‘). As revealed in Fig. b‘, the hypoxic regions were restricted to some keratinocytes and only a few cells in the dermis.

Discussion

Here, we have examined the spatial and temporal distribution of host-derived monocytes/macrophages and granulocytes in relation to oxygen gradients in transplanted pigmented dermo-epidermal skin analogs of human origin. Our findings provide evidence that granulocytes appear faster and in higher numbers than monocytes/macrophages during early wound healing of skin analogs. However, the number of both inflammatory cell types decreased slowly at later wound healing stages. A number of aspects call for a detailed comment.

First, our study demonstrates that the early inflammatory phase of skin graft healing in vivo is dominated by the activation of the immune system and influx of polymorphonuclear neutrophils. These findings are similar to the ones of earlier studies investigating the chain of events following a trivial skin injury [24, 25]. This early inflammatory phase is essential for the clearance of bacteria and cellular debris at the wound site [7, 8]. Second, we observed a progressive increase in the number of granulocytes which peaked at 6 weeks post-transplantation and decreased thereafter. However, most other studies looking at skin healing, investigated wounds only up to 14 [26] or 21 days post-injury. [27]. Both of these studies found a progressive decrease of granulocytes with time. However, it is not possible to directly compare those results with our

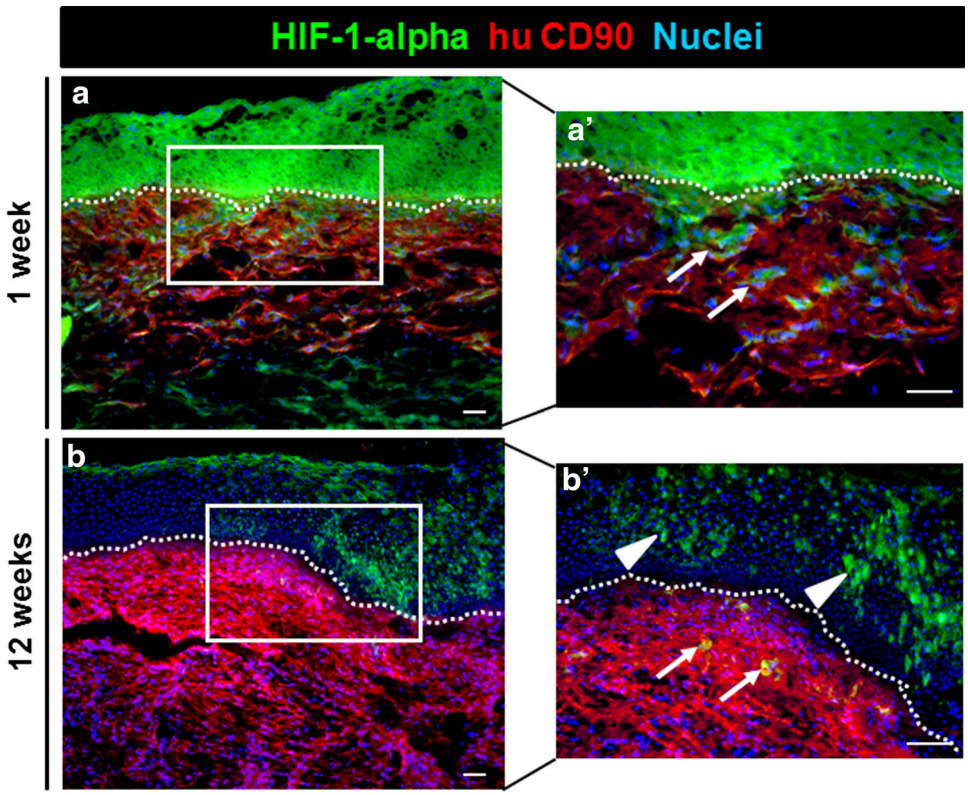

Fig. 4 HIF-1-alpha expression in transplanted skin analogs. **a**, **a'**, **b**, **b'** Cells expressing HIF-1-alpha (*green*) are counterstained with human CD90 (*red*) to mark the human dermal compartment of skin analogs. **a'**, **b'** Insets show a higher magnification of **a** and **b** images, respectively. Note the high expression of HIF-1-alpha in all layers of the epidermis, as wells as partially in the dermis early after transplantation (1 week) (*white arrows*). In contrast, only moderate numbers of HIF-1-alpha expressing cells were found in the epidermis (*white arrowheads*) and dermis (*white arrows*) at later stages (12 weeks) post-transplantation. Cell nuclei are stained with Hoechst (*blue*). *White dotted lines* indicate the dermo-epidermal junction. *Scale bars* 50 μm

study due to different experimental time points, inflammatory cell markers, and animal models used for the analysis. Interestingly, Baskaran et al. [28] describe decreased granulocyte recruitment in remote organs following a burn injury, but a sustained granulocyte homing into the wound site (rat model). These effects were independent of the burn size. Our observation that we still detect granulocytes in our skin analogs at later stages post-transplantation, especially at the graft borders, seems to be an analogous phenomenon.

Influx of granulocytes after wounding is typically followed by a subsequent infiltration of monocytes/macrophages [29, 30]. We detected only low numbers of macrophages during the early wound healing phase (1–3 weeks), with a peak at 6 weeks, and a marked decrease at 12 weeks post-transplantation. Overall, the number of macrophages was lower than the one of granulocytes. In addition, the majority of infiltrating macrophages were concentrated at the graft borders. These findings are perfectly consistent with the sequence of events observed during normal skin wound healing [15]. It has been shown, that macrophages require a longer time to become active in the inflamed tissue area than neutrophils do. However, after several days to several weeks, the macrophages are the dominating phagocytic cells within the healing area and, in parallel, there is an increased bone marrow production of new monocytes [31, 32]. This points at the specific functions of macrophages recruited during the different phases of skin repair after an injury as demonstrated by Lucas et al. [16]. Their study shows that the depletion of macrophages restricted to the early stage of tissue repair (inflammatory phase) significantly reduced the formation of vascularized granulation tissue and impaired epithelialization [16]. In contrast, depletion of macrophages in the consecutive mid-stage phase (tissue formation) resulted in severe hemorrhage and delayed or absent wound closure of the injured tissue [16].

Further, our study provides new insights into the relationship between macrophages/granulocyte infiltration and HIF-1-alpha expression in a wound healing setting, i.e., engraftment of our tissue-engineered skin substitutes. As to be expected, during the initial healing phase (1–3 weeks), there was a high HIF-1-alpha expression as a reflection of abnormally low tissue oxygenation. This ischemic/hypoxic microenvironment is essential to stimulate angiogenesis and regulate the early inflammatory cell recruitment as myeloid cells sense low oxygen gradients and move along them to migrate toward the areas of inflammation [33, 34].

We have previously demonstrated that the upper dermal compartment of our skin analogs remains avascular for up to 2 weeks after grafting with host vessel ingrowth starting only at 1 week in the lower dermal compartment [3, 4]. This timeline corresponds well with the enhanced expression of HIF-1-alpha observed in our skin analogs between

1–3 weeks and its downregulation to reach normoxic levels 6–12 weeks post-transplantation, and it is paralleled by the onset of the maturation phase that leads to complete skin restoration in vivo. Our findings are in line with those of several other studies: The group of Ninikoski et al. [35] has also demonstrated extremely low oxygen tensions in a rodent wound chamber model using a subcutaneously implanted viscose cellulose sponge at the first and second day post-injury, which steadily decreased to reach a minimum by days 5–7. Other investigators showed a sustained hypoxia up to 14 days post-injury using subcutaneous sponge-induced granulation tissue model in rats [36]. In a study on anastomosis healing (rabbit aorta), normoxia was reestablished 6 weeks post-injury, which is consistent with our results [37].

In summary, this is, to the best of our knowledge, the first study investigating the spatiotemporal distribution of inflammatory cells and HIF-1-alpha expression as tissue hypoxia parameter in a wound healing setting consisting of transplanting tissue-engineered human skin substitutes. This long-term in vivo assay reveals that, basically, wound healing occurs in the classical and well described way. This confirmatory knowledge has significant clinical implications with regard to the planned application of such laboratory grown skin substitutes in patients.

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Conflict of interest The authors declare that they have no conflict of interest.

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