1. Introduction

Immediately after skin injury, multiple physiological responses are initiated, comprising four distinct but overlapping phases of cutaneous wound healing: hemostasis, inflammation, proliferation, and remodeling.\(^{[1,2]}\) Often, impaired wound healing leads to unwanted outcomes like pathological scarring, chronic wounds, or fibrosis. The latter process is characterized by the excessive deposition of extracellular matrix (ECM), resulting in the impairment or even loss of epithelial or organ function.\(^{[3]}\) Wound healing is a multifactorial process involving different cell types and their interactions with the extracellular microenvironment. As a bioactive structure, the ECM controls cell fate and cell–cell communication by its biophysical and biochemical properties, for example, via binding mediators and transducing mechanical forces.\(^{[4–6]}\) The full molecular mechanisms behind this process are insufficiently understood, leading to failure and a lack of therapies particularly in multi-disease situations.

The phases of wound healing are characterized as follows. Initially, platelets trigger hemostasis by the formation of a fibrin clot serving as a scaffold for infiltrating keratinocytes and leukocytes.\(^{[7]}\) Within hours after injury, the inflammatory stage is achieved, characterized by the secretion of platelet-derived growth factor (PDGF), microbial byproducts, and chemoattractants resulting in the mobilization of neutrophils and monocytes.\(^{[8]}\) Next, monocytes differentiate into macrophages of a pro-inflammatory phenotype (M1-Mϕ). This polarization allows the macrophages (Mϕ) to kill microbes, remove neutrophils, and produce several mediators and cytokines like interleukin-1 (IL-1), IL-6, IL-12, and tumor necrosis factor α (TNF-α).\(^{[9–11]}\) In the subsequent proliferative phase, granulation tissue is formed, which is mainly mediated by...
"anti-inflammatory" MΦ (M2-MΦ). This phenotype is characterized by the production of anti-inflammatory growth factors and mediators like transforming growth factor β (TGF-β), vascular endothelial growth factor (VEGF), or IL-10 inducing cellular migration and proliferation at the wound site.\textsuperscript{[12,13]} Especially fibroblasts (FB) attracted by fibroblast growth factor 2 (FGF-2) and TGF-β modulate the initial proliferative phase by their differentiation into myofibroblasts (MyoFB) and their subsequent production of ECM components, for example, collagen type III and fibronectin.\textsuperscript{[14]} The contractile MyoFB is important for wound edge contraction and is characterized by the incorporation of alpha smooth muscle actin (αSMA) into actin stress fibers.\textsuperscript{[15]} Proper wound healing is characterized by the final resolution phase, which is initiated within 2–3 weeks after injury. Mostly FB, MΦ, and endothelial cells are responsible for collagen remodeling including matrix degradation by matrix metalloproteinases (MMPs) leading to a matrix mainly composed of collagen type I (Coll I).\textsuperscript{[16]}

During various phases of wound healing, the cell–cell and cell–ECM interplay is mediated by FB and MΦ. During wound resolution, the anti-inflammatory cytokine IL-10 is predominantly secreted by anti-inflammatory MΦ.\textsuperscript{[16,17]} TGF-β is released by all MΦ phenotypes and is permanently present at the wound site resulting in FB attraction and their differentiation into MyoFB.\textsuperscript{[11,18,19]} In the resolution phase, clearance of MyoFB from the wound site is essential to prevent overshooting scarring and fibrosis. In this context, a controversial influence of TGF-β and IL-10 on FB and MyoFB behavior is discussed.\textsuperscript{[20–22]} Pleiotropic effects of IL-10 on FB and MyoFB are reported in vivo and in vitro. Besides apoptosis or senescence of MyoFB, a potential de-differentiating effect of IL-10 on MyoFB is discussed, too.\textsuperscript{[23]} IL-10 also accounts for the regenerative healing capacity of postnatal tissue.\textsuperscript{[22,24]}

To improve the understanding of molecular mechanisms of wound healing and resolve specific questions in biomedical research and therapy, biomedical engineering tries to come up with new solutions for biomimetic scaffolds for in vitro cell culture. During the last two decades, increased efforts were made to allow for more physiologically relevant results of in vitro cell culture and increased information density. These lead to the development of biomaterial scaffolds, which closely mimic the three-dimensional (3D) microenvironment. Standard in vitro models like a common two-dimensional petri dish cell culture are limited in terms of cellular morphology, actin stress fiber formation, and differentiation as well as mediator presentation.\textsuperscript{[26]} Besides dense polymer-based 3D hydrogels,\textsuperscript{[22–29]} fibrillar 3D networks are widely used as scaffolds for in vitro cell culture studies and display many key characteristics of the natural ECM, for example, low stiffness, micron-sized pores, and a fibrillar character.\textsuperscript{[30]} In this context, Coll I-based matrices exhibit superior properties as Coll I is the most abundant ECM protein in vivo. By its natural origin, Coll I exhibits a biomolecular nano- and microstructure and multifunctional characteristics without the need for further modifications. Native adhesion receptor binding sites,\textsuperscript{[31,32]} biomimetic viscoelastic,\textsuperscript{[33,34]} and tunable mechanical properties\textsuperscript{[35]} as well as degradability by MMPs\textsuperscript{[36]} make Coll I scaffolds a suitable substrate for cellular studies. Moreover, the properties of the Coll I-based networks can be adjusted in a defined manner in terms of modification with other ECM components (e.g., glycosaminoglycans\textsuperscript{[37–39]} and fibronectin).\textsuperscript{[40]} It appears that Coll I-based networks are suitable scaffolds to mimic the 3D ECM microenvironment of cells in wound healing situations.

Besides mimicking the 3D ECM situation, another difficulty in biomedical research is the paucity of proper cellular models. Usage of animal models is controversially discussed. Although this approach is essential, it is not directly transferable to human physiology, for example, since the skin of mice is not attached to the underlying tissue.\textsuperscript{[1]} In the last decade, primary human cells have become a major interest in the field of regeneration, enabling their employment in appropriate models for preclinical studies.\textsuperscript{[41]} Furthermore, commonly used culture of single cell types is of limited complexity and does not address issues concerning communication between different cell types.\textsuperscript{[42]}

Therefore, to address cellular modulation by cell–cell and cell–matrix interactions, it is indispensable to utilize biomimetic in vitro coculture models, which implement the possibility of paracrine signaling. In a recent attempt, paracrine signals were mimicked by a microparticle-based cytokine release system. Ansorge et al. demonstrated that supplementing TGF-β, to cell cultures in a controlled and cell-imitating delivery mode is of major importance for a physiologically relevant outcome.\textsuperscript{[43]}

In sum, there is an obvious need of coculture systems consisting of primary human cells in 3D biomimetic matrices. This enables studying current topics of inter-cellular communication during wound healing, including a better control of the wound resolution phase in order to prevent scarring in pathological situations. To tackle this challenge, we use 3D Coll I-based matrices to model the ECM during wound resolution and investigate a direct coculture of primary human MyoFB and M-CSF differentiated MΦ (M-MΦ). By that, we aim to further clarify the influence of IL-10 release from M-MΦ and its influence on MyoFB dedifferentiation at 3D coculture conditions.

### 2. Results and Discussion

MΦ are the most dominating cell type driving inflammation and controlling tissue repair by secreting a large amount of bioactive mediators.\textsuperscript{[44,45]} As one consequence, FB infiltration into the wound site is regulated and tissue formation is modulated by differentiated FB, so-called MyoFB. Especially during late stages of wound healing, the balance between promotion of tissue formation, scarring and degradation of initially deposited cell debris and preliminary ECM require a locally and timely controlled interaction of both cell types.

In our work, we aimed to establish an in vitro coculture model in a biopolymer-based 3D scaffold. This model should enable the imitation of the in vivo behavior of cells during wound resolution phase and the targeted analysis of the interactions of primary human M-MΦ and MyoFB. In particular, we specifically elucidate the regulatory function of M-MΦ with a focus on the release of the mediator IL-10 in the control of MyoFB dedifferentiation. We analyzed the cellular response in terms of cellular morphology, actin stress fiber formation, MyoFB specific gene expression, and IL-10 release over 6 days inside biomimetic fibrillar 3D Coll I matrices.
2.1. Coculture of Primary Human Macrophages and Myoﬁbroblasts in Biomimetic 3D Matrices over 6 Days

In order to establish a 3D coculture model of primary human M-Mϕ and MyoFB, we used our previously introduced platform of 3D ﬁbrillar Coll I-based matrices with well-deﬁned topology and mechanics. The ﬁbrillar 3D Coll I matrices with their open porous microstructure have been shown to mimic an ECM microstructure comparable to connective tissue and dermis. Choosing a Coll I concentration of 2.5 mg mL−1 in a reconstitution buffer with 250 mM phosphate and a pH of 7.5, we achieved an average pore diameter of 4.1 ± 0.6 µm and a ﬁbril diameter of 0.68 ± 0.06 µm in control Coll I matrices without cells. Elasticity of such 3D Coll I matrices was in the range of 100 Pa as determined by measurements of Young’s modulus by colloidal probe force spectroscopy. In our previous studies, such 3D Coll I matrices were demonstrated to allow for a homogenous cell distribution of FB as well as Mϕ over many days of cell culture. Hence, this type of 3D Coll I-based matrix was considered to be suitable to establish the 3D coculture model. To investigate wound resolution, cell culture experiments were designed as depicted in Figure 1.

Isolated blood monocytes were differentiated to M-Mϕ for 7 days using M-CSF. Mϕ exposed to M-CSF in the absence of inﬂammation are likely to acquire an anti-inﬂammatory “M2-like” phenotype, typically occurring during late wound healing stages. Prior to coculture experiments, M-Mϕ phenotype was veriﬁed by ﬂow cytometry for cell surface receptors CD14, CD86, CD163, and CD206. We found CD14 as well as CD86 and CD206 to be highly expressed, while CD163 is hardly present at the cell surface (see Figure S1, Supporting Information). According to Schilling et al. and other reports, this expression pattern is typical for M-Mϕ.

For coculture experiments, M-Mϕ and MyoFB were seeded into the 3D Coll I matrices during reconstitution of the ﬁbrillar Coll I network and were incubated with and without additional TGF-β1 stimulation for 4 h, 2 days or 6 days. To investigate whether the number of cocultured M-Mϕ exerts inﬂuence on the differentiation state of MyoFB, we mixed several ratios of MyoFB and M-Mϕ, namely 1:1, 1:3, and 1:4 (MyoFB:M-Mϕ).

The initial homogeneous 3D cell distribution inside the Coll I matrix was analyzed by cell nuclei staining with DAPI, followed by epi-ﬂuorescence microscopy and usage of a home-built image analysis algorithm. As shown in Figure 2A, after 4 h our preparation protocol ensured a homogeneous 3D distribution of MyoFB and M-Mϕ. Additionally, high-resolution cLSM images were taken from samples with stained cell nuclei, f-actin and αSMA (see Figure S2, Supporting Information). Cells exhibited morphologies typically seen in ﬁbrillar 3D Coll I scaffolds. M-Mϕ had a spherical shape and phalloidin staining of f-actin showed the actin cortex at the cell membrane. MyoFB were elongated and stretched between Coll I ﬁbrils. Actin stress ﬁbers in MyoFB were clearly stained by phalloidin, which easily allowed to distinguish M-Mϕ and MyoFB. In addition, actin stress ﬁbers with integrated αSMA were also used to characterize MyoFB.

Coll I matrix topology after cell seeding (Figure 2B) was shown to be comparable to control samples (dotted line = without cells) with an average pore diameter of 3.6 ± 0.3 µm and a mean ﬁbril diameter of 0.9 ± 0.2 µm. Furthermore, the analysis of matrix topology at later time points of cell culture (2 days, 6 days) revealed that our biomimetic ﬁbrillar 3D Coll I matrices can be regarded as a suitable and stable cell culture scaffold, as both pore diameter and ﬁbril diameter did not change essentially during the time course of the coculture experiments.

However, we observed a signiﬁcant decrease in network thickness over the duration of the cell culture. As seen in Figure 2, network thickness decreased from 295 ± 35 µm after 4 h to 130 ± 23 µm after 6 days, correlating with a slight decrease in pore diameter (3.6 ± 0.3 µm [4 h] vs 3.2 ± 0.2 µm [6 days]). Although the changes in pore diameter d pore were small and within the range of data scatter, the decrease in pore diameter together with the decrease in network thickness indicated a contraction of the 3D Coll I matrices without network degradation. As the Coll I network is bound to a ﬂat, stiff sub-strate (coverslip), an increased cell-driven contraction of the 3D network would lead to a unidirectional decrease in network thickness d matrix accompanied by a decrease in pore diameter d pore with (d matrix)1/3 = d pore. The increasing cell number of cell culture period can be expected to cause an increasing network contraction. The observed thickness ratio, more precisely (d matrix)1/3, of (d matrix,4h / d matrix,6d)1/3 = 1.3 ± 0.13 correlated reasonably well with the ratio of pore diameter d pore,4h / d pore,6d = 1.12 ± 0.16. These arguments of a slight matrix contraction are also supported by an increase in matrix stiffness, as an average effective Young’s modulus of 150 ± 50 Pa was determined after the 6 days coculture. The assumption of neglectable matrix degradation is supported by the observed constant ﬁbril diameter of the Coll I matrices. In this context, it has to be mentioned, that we did not observe viscoplastic deformations as reported previously by other groups for Coll I networks. As pointed out in our recent report, we suggest that this property of our
Coll I matrices is caused by the crosslinked character of Coll I fibrils due to their naturally occurring crosslinks via lysyl side chains of acid solubilized Coll I.[33]

2.2. M-Mφ Coculture Controls MyoFB Differentiation Status in Dose-Dependent Manner

Next, we investigated MyoFB differentiation status, as it was hypothesized that it can be controlled by M-Mφ and their secreted mediators in vivo and in vitro.[23,58–60] One major hallmark and often used diagnostic marker of a differentiated MyoFB phenotype is the production of αSMA and its incorporation in actin stress fibers.[15]

We analyzed the MyoFB concerning αSMA presence organized in the cytosol and as actin stress fibers after 6 days of coculture with different numbers of M-Mφ including an optional stimulation with TGF-β1. Figure 3A,B shows characteristic cLSM images of MyoFB with stained f-actin and αSMA with TGF-β1 stimulation in mono- and coculture, respectively. MyoFB and fibroblasts can be easily distinguished by the elongated shape and the staining of actin stress fibers. Cocultured M-Mφ exhibit the typical spherical morphology and staining of the actin cortex. In comparison to MyoFB monoculture, a clearly reduced occurrence of αSMA in MyoFB stress fibers was observed in coculture with $4 \times 10^4$ M-Mφ, pointing toward the impact of M-Mφ on the MyoFB differentiation status. As a control, we additionally stimulated the coculture with recombinant IL-10 (Figure 3Bii) and observed a similar effect. Quantification of the fraction of a SMA-positive MyoFB dependent on the number of cocultured M-Mφ, revealed a dose-dependent decrease of the MyoFB fraction with increasing number of M-Mφ, even under TGF-β1 stimulation (see Figure 3C). Moreover, MyoFB without further TGF-β1 stimulation tended to exhibit a lower fraction of αSMA positive cells but also respond to M-Mφ presence in a dose-dependent manner. However, only at the 1:3 coculture condition this latter effect showed significant differences. This result showed the regulatory influence of M-Mφ and proofed the hypothesis that an increase of regulatory M-Mφ leads to a dedifferentiation of MyoFB. These observations were confirmed by gene expression analysis of αSMA (see Figure 3D). The dose-dependent influence of M-Mφ seen in the quantitative immunofluorescence analysis of αSMA positive cells could be validated.

In order to further deepen our understanding of MyoFB dedifferentiation by M-Mφ, we investigated gene expression of other important markers of MyoFB, in particular, matrix protein synthesis. The activation of ECM production and remodeling is of great importance for wound closure and tissue formation. It is known that MyoFB exhibit increased expression of ECM proteins and remodeling activity of the ECM. In our setup, we assumed that MyoFB would dedifferentiate over the culture period with a dynamic change of phenotype (see Figure 3C,D). RNA expression analysis is a suitable method to evaluate the capacity of ECM production after 6 days of coculture. We omitted additional immunofluorescence stainings as
they would only report convoluted effects from an early matrix protein production in all samples as MyoFB is seeded at day 0. Therefore, we have analyzed the expression of Coll I, collagen III (Coll III), and EDA-fibronectin (EDA-FN) as a function of the cocultivated M-Mɸ (Figure 4). In the presence of TGF-β1, gene expression for matrix proteins was four times upregulated in contrast to conditions without TGF-β1 treatment. This induction of gene expression by TGF-β1 decreased with increasing amount of cocultured M-Mɸ. Gene expression of all three genes was at maximum twofold higher for the 1:4 ratio conditions, albeit only slightly different from conditions without TGF-β1. These results showed that the induction of gene expression of the mentioned matrix proteins in MyoFB by TGF-β1 is suppressed by the M-Mɸ. These results support the current perception of processes that appear during later stages of wound healing. Herein, the resolution of preliminary matrix has occurred and contracting, fibrillar ECM producing MyoFB has to be downregulated to prevent overshooting scarring. We herein show, that this process can be modeled in vitro in biometric 3D Coll I matrices in coculture of primary human cells.

Furthermore, the dose-dependence suggested paracrine factors from M-Mɸ to regulate MyoFB dedifferentiation. Additionally, we noticed that this downregulation of MyoFB is not correlated to apoptosis during MyoFB clearance (data not shown). These findings are in line with our earlier results of a FB monocyte system,\textsuperscript{[23]} wherein MyoFB dedifferentiation was stimulated by the presence of IL-10 without apoptosis. Novel data suggest that many of the MyoFB dedifferentiate to adipocytes in vivo not undergoing apoptosis.\textsuperscript{[61]} Hence, our new results suggest a paracrine influence of secreted IL-10 from M-Mɸ in the coculture model.

2.3. Correlation of IL-10 Release and M-Mɸ Number with MyoFB Dedifferentiation

The data shown above are consistent with the idea of a reversible MyoFB activation state and suggest its regulation by IL-10 in a paracrine manner, as it was also reported earlier.\textsuperscript{[23]} Although M-Mɸ phenotype can be quite diverse, IL-10 is known to be highly expressed by M-Mɸ.\textsuperscript{[62]} Accordingly, IL-10 secretion is frequently used as a marker to characterize distinct macrophage phenotypes.\textsuperscript{[63]} That is why we quantified IL-10 concentration in supernatants of our coculture model. After 6 days of coculture, we observed a clear dose-dependent and almost linear increase of IL-10 concentration in cell culture supernatants dependent on the amount of M-Mɸ up to a maximum concentration of 200 ng mL\(^{-1}\).

Figure 3. MyoFB cocultured with M-Mɸ exhibits a dose-dependent dedifferentiation even at TGF-β1 stimulation. A) cLSM image showing typical MyoFB morphology in the Coll I matrix after 6 days of monoculture. Cells were stained for nuclei (blue), αSMA (red), actin (green). Reflection signal of Coll I fibrils is shown in grey. Scale bar: 50 µm. B) cLSM images showing i) typical MyoFB morphology of elongated cells with clear actin stress fibers in the Coll I matrix after 6 days of coculture (MyoFB:M-Mɸ ratio of 1:4 + TGF-β1) and ii) after 6 d of coculture (MyoFB:M-Mɸ ratio of 1:4 + TGF-β1) with additional stimulation with recombinant human IL-10 (10 ng mL\(^{-1}\)) (MyoFB:M-Mɸ ratio 1:4). Arrows indicate position of M-Mɸ with typical spherical morphology of the actin cortex and no signs of actin stress fibers. Cells were stained for nuclei (blue), αSMA (red), and actin (green). Reflection signal of Coll I fibrils is shown in grey. Scale bar: 50 µm. C) Quantitative analysis of fraction of αSMA positive cells, representing MyoFB, after 6 days of coculture. At least 300 cells were manually counted per condition. Data are shown as mean ± SD (n = 3; two-way ANOVA followed by Tukey’s multiple comparisons test; * indicates p ≤ 0.05; # indicates p ≤ 0.05 analyzed by Student’s t-test calculated to unstimulated MyoFB). D) Gene expression of αSMA of MyoFB in coculture analyzed by RT-qPCR. Expression levels were normalized to the reference gene RPS26 and analyzed as ratio to TGF-β1-unstimulated cultures of corresponding composition. Data are shown as mean ± SD (n = 3; Student’s t-test calculated to MyoFB ctrl condition; * indicates p ≤ 0.05).
around 120 pg mL\(^{-1}\) (Figure 5). Hereby, only minor differences between mono- or coculture conditions were observed. This result points toward M-M\(\Phi\) being the only source of secreted IL-10. MyoFB cannot be regarded as relevant source of IL-10, as supernatants from MyoFB without M-M\(\Phi\) coculture exhibited no detectable IL-10 (data not shown) and stimulation of MyoFB by TGF-\(\beta_1\) in neither mono- nor coculture led to any increase of IL-10 secretion. The latter result was expected as TGF-\(\beta_1\) was hypothesized to primarily activate MyoFB differentiation, but not to trigger increased IL-10 secretion by M-M\(\Phi\). These findings imply that paracrine signals from FB do not influence the IL-10-production of M-M\(\Phi\).

The detected levels of IL-10 are in agreement with other in vitro cell culture\[64,65\] and in vivo experiments.\[66\] Schilling et al. measured the IL-10 release of LPS stimulated M-M\(\Phi\) in cell culture supernatants after 16 h and detected similar quantities (5 ng mL\(^{-1}\) for \(1 \times 10^6\) cells) compared to our setup (≈50 pg mL\(^{-1}\) for \(1 \times 10^4\) cells).\[53\]

As elaborated in the section above, MyoFB dedifferentiation was correlated to the amount of cocultured M-M\(\Phi\) and suggested to be regulated in a paracrine manner. A clear reduction of \(\alpha\)SMA positive cells as well as the downregulation of gene expression of \(\alpha\)SMA and relevant matrix proteins can now be discussed in the context of the increased levels of IL-10 dependent on the number of co-cultivated M-M\(\Phi\). Our results allow for a direct correlation of the secretion of IL-10 from M-M\(\Phi\) to a paracrine stimulated dedifferentiation of MyoFB into a FB state. Moreover, measured IL-10 concentrations of around 120 pg mL\(^{-1}\) seem to be sufficient to achieve a fully dedifferentiated state. This concentration is much lower than usually applied cytokine concentrations in such cell culture experiments in ng mL\(^{-1}\) range.\[23\] It points toward the efficacy of a sustained paracrine signal delivery in a coculture system. These results are in good agreement with experiments on biomimetic paracrine delivery systems of TGF-\(\beta_1\), also indicating efficient cell activation in the range of some tens of pg mL\(^{-1}\).\[43\]

2.4. General Discussion

The design and application of materials that support tissue regeneration is permanently of high interest in biomaterials research and engineering. Moreover, multi-disease situations frequently lead to unsatisfactory wound healing processes, which call for...
material-supported intervention. In there, various biomedical questions are unresolved since complex inflammatory processes need to be addressed and underlying mechanisms of cell–cell interaction need to be understood. In this context, strategies to establish and modulate in-vivo-like conditions of physiologically relevant processes are highly requested and there is a need of complex biomaterial in vitro setups with multiple cell types.

In our study on establishing a biomimetic wound resolution model, we used well-defined Coll I matrices for a direct coculture of primary human MyoFB and M-Mφ to mimic the physiological complexity occurring at late stages of wound healing. In this biomimetic scaffold-supported 3D coculture setting, we addressed paracrine cell–cell interactions.

At first, we could demonstrate that cell seeding into the 3D Coll I matrix during Coll I network reconstitution did not significantly alter initial matrix properties in terms of pore and fibril diameter (Figure 2). Additionally, Coll I matrix properties remained stable over the whole cell culture period of 6 days. Only a contraction of the Coll I matrices driven by the increasing cell number was observed over the cell culture period, which had only a minor influence on pore diameter. This observation indicated a constant fibrillar mesh number of the Coll I matrices without matrix degradation or matrix formation.

Next, we investigated cell–cell communication between MyoFB and M-Mφ in our coculture system. In particular, we addressed two very important mediators of cell–cell interaction in wound healing, namely TGF-β1 and IL-10. MyoFB dedifferentiation was specifically considered as a function of the amount of cocultured M-Mφ and TGF-β1. Gene expression analyses revealed a progressive dedifferentiation of MyoFB with higher number of M-Mφ present. Additionally, IL-10 ELISA data displayed that M-Mφ are the main source of supernatant IL-10 and MyoFB does not contribute to the measured IL-10 level in coculture. All experiments demonstrated a counteracting effect of TGF-β1 and IL-10, which was reported previously for a monoculture model of tissue repair.[23] Even under global TGF-β1 presence, IL-10 production is not suppressed. Moreover, with an increasing level of IL-10 in the supernatant it becomes the dominating effect on MyoFB gene expression. Taken together, our results show a dose-dependent effect of M-Mφ on MyoFB dedifferentiation, simulating late wound healing stages. We propose IL-10 to be one of the main paracrine driving force during wound resolution.

In vivo, IL-10 acts on a huge variety of cell types, but has a general suppressing effect on the production of inflammatory cytokines by macrophages and neutrophils mediated by signal transduction via Stat3, the key transcription factor in the JAK/Stat signaling pathway.[62,67,68] Moreover, IL-10 and other members of the IL-10 subfamilies are suppressive cytokines preventing tissue damage caused by infections and inflammation. During the recovery phase of infections, IL-10 regulates and represses the expression of proinflammatory cytokines and stimulates the proliferation of epithelial cells to promote healing.[62,69] However, IL-10 does not only act on FB, but also on Mφ in a self-regulating manner. Hereby, Mφ-derived IL-10 inhibits the differentiation of neighboring cells into classically activated, also named inflammatory, Mφ and thereby allows the population to be self-regulated.[70] Experiments in IL-10 deficient mice have merely shown a contradictory effect of IL-10 on early epithelial wound closure. Whereas wounds of control mice have not been closed at day 7, wounds of IL-10 deficient mice were completely epithelialized.[20] Other reports refer to the importance of IL-10 on FB behavior during early regenerative healing compared to adult FB and an indirect regulation of lymphangiogenesis in the inflamed cornea via the recruitment of Mφ and release of VEGF.[71] In skin, scarring is reduced by the application of IL-10 because excessive ECM deposition and the transition of FB into MyoFB are inhibited.[24] In a recent study on lung fibrosis, locally delivered IL-10 was shown to prevent and reverse pulmonary fibrosis in mice pointing toward the relevance of the presentation form of therapeutically used cytokines and an understanding of the signaling mode.[72] These inconsistent findings of the IL-10 function provide additional support for the compelling need of complex biomimetic in vitro models using primary human cells, as we presented herein.

In our coculture model of late tissue repair, we clearly demonstrated a paracrine signaling of IL-10 with a regulatory function on wound resolution and MyoFB dedifferentiation. Recently published data also described a similar mode of action for paracrine TGF-β1 signals in a biomimetic in vitro model of early wound healing. Therein, glycosaminoglycan-functionalized agarose microparticles releasing TGF-β1 in a slow and sustained manner were simulating a paracrine cellular source of TGF-β1. This leads to a differentiation of primary human FB into MyoFB in a 3D Coll I matrix at delivered concentration levels two magnitudes lower than standard cell culture conditions of MyoFB differentiation.[43] Here, we now add a model system for late stage wound healing, focusing on TGF-β1 and IL-10 signals in a coculture model. Other paracrine signals are of interest, too, as they are known to regulate wound healing, for example, MCP-1 has long been recognized as an early inflammatory chemokine contributing to sustained inflammation.[74] In chronic wounds, persistent chemotactractant gradients give rise to uncontrolled activation of inflammatory Mφ, owing to a misbalance of early inflammatory cues and thus a chronic inflammation cycle.[74,75] In this context, the investigation of such an early inflammatory response triggered by MCP-1 is also conceivable with our coculture model. It will support a better understanding of paracrine regulation during different stages of cutaneous wound healing.

3. Conclusion

In this study, a direct coculture of primary human cells in a 3D Coll I matrix was successfully established as a tool to mimic processes typically occurring during late stages of wound healing. Specifically, differentiation and paracrine interactions of the involved cell types were of major interest. The previously supposed effect of IL-10 counteracting TGF-β1-driven MyoFB activation was verified. Moreover, IL-10 release of cocultured M-Mφ was identified as a significant paracrine signal in the coculture system. The paracrine influence of regulatory macrophages on MyoFB was characterized by downregulation of the αSMA marker of contractile MyoFB and gene expression of matrix protein synthesis, namely EDA-FN, Coll I, and
Coll III. Hence, our study does not only provide a relevant bio-
mietic ECM-coculture system of wound healing. It also con-
tributes to a deeper understanding of relevant in vivo processes
of late stage wound healing in terms of paracrine cell com-
munication of FB and Mφ. As our ECM model system allows
for additional modifications with other ECM components, for
example, fibronectin[40] and glycosaminoglycans,[39] it will be
worthwhile to further study the modulation of identified parac-
rine cell signaling in dependence on ECM characteristics.

4. Experimental Section

Isolation and Culture of Primary Human Macrophages and Fibroblasts:
Cell isolation and subsequent cell experiments were carried out in
accordance with the approved guidelines of the ethics committee of
the Medical Faculty Leipzig including written informed consent from healthy
donors (ethics committee vote: 384/16-ek).

Primary human dermal FB from foreskin were provided by the
University Hospital Leipzig by an isolation protocol described by
Saalbach et al.[78] Cells were expanded up to 6th passage. Initial cell
seeding during reconstitution of 3D Coll I Matrices: Coll I matrices
were analyzed on a BD LSR II (Becton Dickinson, Franklin Lakes, NJ).
For all cytometry analyses, gates were set according to blank and isotype
controls used in each experiment. Median fluorescence intensity (MFI)
values and percentages were calculated using FlowJo (FlowJo).

Cell Seeding during Reconstitution of 3D Coll I Matrices: 3D Coll I
matrices were prepared similar to protocols described in detail
elsewhere.[10,48] In brief, glass coverslips (diameter 13 mm) were coated with
poly(styrene-alt-maleic anhydride) (PSMA; MW 30 000 g mol−1)
(Sigma-Aldrich). All Coll I preparation steps prior to matrix reconstitution
were performed on ice. Coll I stock solution (4.42 mg mL−1 (rat
tail, Corning, New York)) was prediluted in acetic acid (0.02 M). The
corresponding number of each cell type was suspended in 0.25 mL of
reconstitution buffer (250 mM phosphate buffer (Sigma-Aldrich), pH
adjusted to 7.5 by NaOH (Grüssing, Filsum, Germany) supplement) at
room temperature. 1 mL of prediluted Coll I solution and 0.25 mL of
each cell suspension were thoroughly mixed (final concentrations: Coll I
2.5 mg mL−1, M-Mφ: 0.25 × 10^6 mL−1 to 1.25 × 10^6 mL−1, MyoFB: 0.25
× 10^6 mL−1). A total volume of 40 µL of the Coll I-cell reconstitution
suspension was placed on PSMA coated glass coverslips and Coll I fibroblasts
were added to the M-Mφ monolayer. Each plate was washed with
0.5 mL M-Mφ culture medium.

Immunofluorescence Staining and Quantification of Fibroblast
Differentiation: After coculture, cells were washed three times with
PBS and fixed with 4% paraformaldehyde (Roth, Karlsruhe, Germany)
for 15 min. Subsequently, cells were rinsed three times with PBS,
permeabilized with 0.1% Triton X-100 (Roth), and blocked with PBS
containing 2% BSA for 30 min. Afterward, cells were stained with
DAPI (dilution 1:10 000 in PBS; Invitrogen, Vienna, Austria), Rhodamine
phalloidin (dilution 1:200 in PBS; Invitrogen), Alexa Fluor 488 Phalloidin
(dilution 1:200 in PBS; Invitrogen), anti-human αSMA clone 1A4
conjugated with eFluor 570 (dilution 1:200 in PBS; eBioscience) or
conjugated with Alexa Fluor 660 (dilution 1:200 in PBS; eBioscience)
and anti-human CD14 conjugated with Alexa Fluor 488 (dilution 1:100 in PBS;
BioLegend) to room temperature for 1 h. 20 µL FC Receptor Blocking
reagent were added per sample during staining. After staining, Coll I
matrices were kept in PBS at room temperature prior to microscopy
analysis.

Cells were analyzed inside the 3D Coll I matrices using an inverted
cofocal laser scanning microscope (cLSM; LSM700) with a 40×/NA 1.2
water immersion objective (all Carl Zeiss Microscopy, Jena, Germany).
For quantification of MyoFB phenotype, indicated by αSMA staining
of stress fibers, at least 300 cells were manually counted for each
independent experiment and the fraction of MyoFB were calculated.
M-Mφ were distinguished from FB and MyoFB by CD14 cell surface
expression. Experiments were performed for five different donors, each
M-Mφ and MyoFB.

Analysis of Coll I Matrix Parameters and Cell Distribution Inside 3D
Matrices: Coll I matrices were characterized regarding topological
parameters (pore diameter, fibril diameter) using a home-built image
analysis tool, as described previously.[46,48] Mechanical properties of Coll I
matrices by means of determination of Young’s modulus were analyzed
using soft colloidal probe technique, as previously reported.[47,79]

To investigate cell distribution in the 3D Coll I matrices, samples
were rapidly rinsed twice with PBS and cells were fixed with 4% paraformaldehyde for 15 min prior to cell staining with DAPI
(dilution 1:10 000 in PBS) under gentle agitation for 1 h. After washing three times
with PBS, analysis was performed using an epi-fluorescence microscope
(AxioObserver.Z1) equipped with 3D stage and a 10×/NA 0.3 objective
(all Carl Zeiss Microscopy). Image stacks of each sample were taken at
three positions in bright field and fluorescence mode (DAPI signal).
Optical slices (8-bit, 1388 × 1040 pixels (x-y dimension) were taken every
5 µm throughout the whole thickness (z-dimension) of the collagen scaffold.
3D cell distribution in the matrices as well as matrix thickness were
analyzed using a home-built MATLAB (MATLAB R2016b; MathWorks Inc,
USA) script with an automated detection of Coll I matrix top and bottom
surface by bright field scattering signal from the fibrillar Coll I.
Il-10 Analysis in Culture Supernatants: Released IL-10 cytokine concentration was analyzed in cell culture supernatants at day 6 of cell culture using a human IL-10 high sensitivity ELISA kit (eBioscience) according to the manufacturer’s protocol. 1 mL of cell culture supernatants were collected on ice and centrifuged with 362 g at 4 °C for 10 min. The supernatants were stored at −20 °C until analysis. IL-10 positive control, 10 ng mL−1 recombinant human IL-10 (Peptide) were added to the culture media.

Statistical Analysis: Data are presented as arithmetic mean. Error bars indicate standard deviation (SD) of mean. Unless otherwise stated, experiments were performed at least in independent triplicates. Levels of statistical significance were determined by an unpaired Student’s t-test or by one-way or two-way ANOVA followed by Tukey’s post test using GraphPad Prism7 (GraphPad Software, Inc., CA). The significance level was set at p ≤ 0.05.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

Keywords
3D Coll I scaffold, direct coculture, IL-10, primary human cells, wound resolution

Table 1. RT-qPCR primer sequences used in coculture experiments.

| Primer       | Sequence (5’ → 3’)                                    | Accession no. |
|--------------|-------------------------------------------------------|---------------|
| RPS26        | Fwd. CAA TGG TCC TGG CAA AAA G                        | NM_000109.5   |
|              | Rev. TAC AAG AGG GGA AGC                              |               |
| αSMA         | Fwd. ACA CCC TGT TCC AGG CAT C                        | NM_001613.3   |
|              | Rev. TGC TAG GCC CTT GAT CTC                          |               |
| Collagen I   | Fwd. GTG GGT GTG CAC CTT GAG                          | NM_000088.3   |
|              | Rev. GTG CAC CTT GAG                                  |               |
| EDA-FN       | Fwd. CCA TGC CAC AGG TAC TCCTG                        | NM_212482.2   |
|              | Rev. ACA ACC CAG GAT CAC TGC                         |               |
| Collagen III | Fwd. CCA TGG CTG GGA TGG CAG                         | NM_000090.3   |
|              | Rev. CTC CAC CAG TGT TTC CTT GCG                     |               |

qPCR Analysis: Prior to perform quantitative real-time polymerase chain reaction (RT-qPCR) day 6 of cell culture, total RNA was isolated using TRIzol reagent (LifeTechnologies) and the ReliaPrep RNA Tissue Miniprep System (Promega, Mannheim, Germany). The RNA amount was determined by UV/vis spectrophotometry with a µ-Cuvette (BioSpectrometer; Eppendorf AG, Hamburg, Germany). Both, cDNA synthesis and RT-qPCR were performed using a LineGene 9600 (Bioer, Hangzhou, China). cDNA synthesis was performed according to the supplier’s protocol (GoScript ReverseTranscription, Promega). RT-qPCR was performed using GoTag qPCR Master Mix (Promega). 

Table 1 provides an overview about the primers used for the αSMA, EDA-FN, Collagen I (α1), Collagen III, and RPS26 (reference genes) (all from Eurofins genomics, Ebersberg, Germany). RT-qPCR protocol was set as follows: 5 min denaturation at 95 °C, 45 amplifications of denaturation (95 °C, 15 s), annealing under primer-specific conditions (20 s) and target gene-specific extension (20 s at 72 °C). Subsequent fluorescence measurement was conducted for 20 s at 72 °C. Melting curve analysis at the end of each run confirmed the specificity of the PCR products. Genes were normalized to the non-regulated reference gene RPS26. Results are expressed as fold induction with respect to conditions without TGF-β, treatment.

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[1] S. A. Eming, P. Martin, M. Tomic-Canic, *Sci. Transl. Med.* 2014, 6, 265sr16.
[2] G. C. Gurtner, S. Werner, Y. Barrandon, M. T. Longaker, *Nature* 2008, 453, 314.
[3] M. Walraven, B. Hinz, *Matrix Biol.* 2018, 71–72, 205.
[4] R. O. Hynes, *Science* 2009, 326, 1216.
[5] G. Charras, E. Sahai, *Nat. Rev. Mol. Cell Biol.* 2014, 15, 813.
[6] S. W. Volk, S. A. Iqbal, A. Bayat, *Adv. Wound Care* 2013, 2, 261.
[7] R. A. F. Clark, *Ann. N. Y. Acad. Sci.* 2001, 936, 355.
[8] B. K. Sun, Z. Sipplashili, P. A. Khavari, *Science* 2014, 346, 941.
[9] T. J. Koh, L. A. DiPietro, *Expert Rev. Mol. Med.* 2011, 13, e23.
[10] R. F. Diegelmann, M. C. Evans, *Front. Biosci.* 2004, 9, 283.
[11] S. Barrientos, O. Stojadivonic, M. S. Golkin, H. Brem, M. Tomic-Canic, *Wound Repair Regener.* 2008, 16, 585.
[12] D. M. Mosser, J. P. Edwards, *Nat. Rev. Immunol.* 2008, 8, 958.
[13] S. Werner, R. Grose, *Physiol. Rev.* 2003, 83, 835.
[14] J. J. Tomasek, G. Gabbiani, B. Hinz, C. Chapronnier, R. A. Brown, *Nat. Rev. Mol. Cell Biol.* 2002, 3, 349.
[15] B. Hinz, *Exp. Eye Res.* 2016, 142, 56.
[16] T. A. Wynn, A. Chawla, J. W. Pollard, *Nature* 2013, 496, 445.
[17] T. A. Wynn, K. M. Vannella, *Immunity* 2016, 44, 450.
[18] B. Hinz, *J. Invest. Dermatol.* 2007, 127, 526.
[19] J. J. Tomasek, G. Gabbiani, B. Hinz, C. Chapronnier, R. A. Brown, *Nat. Rev. Mol. Cell Biol.* 2002, 3, 349.
[20] S. A. Eming, S. Werner, P. Bugnon, C. Wickenhauser, L. Siewe, O. Utermöhlen, J. M. Davidson, T. Krieg, A. Roers, *Am. J. Pathol.* 2007, 170, 188.
[21] D. Hos, F. Bucher, B. Regenfuss, M.-L. Dreisow, F. Bock, L. M. Heindl, S. A. Eming, C. Cursiefen, *Am. J. Pathol.* 2016, 186, 159.
[22] A. King, S. Balaji, L. D. Le, T. M. Crombleholme, S. G. Keswani, *Adv. Wound Care* 2014, 3, 315.
[23] J. Sapudom, X. Wu, M. Chkolnikov, M. Ansorge, U. Anderegg, T. Pompe, *Biomater. Sci.* 2017, 5, 1858.
[24] J.-H. Shi, H. Guan, S. Shi, X.-Z. Bai, X.-L. Hu, X.-B. Fang, J.-Q. Liu, K. Tao, X.-X. Zhu, C.-W. Tang, D.-H. Hu, *Arch. Dermatol. Res.* 2013, 305, 341.
[25] B. M. Baker, C. S. Chen, *J. Cell Sci.* 2012, 125, 3015.
[26] D. W. Infanger, M. E. Lynch, C. Fischbach, *Annu. Rev. Biomed. Eng.* 2013, 15, 29.
[27] M. V. Tsurkan, K. Chwalek, K. R. Levental, U. Freudenberg, C. Werner, *Macromol. Rapid Commun.* 2010, 31, 1529.
