CHAPTER 6A

- MODELS TO INVESTIGATE HUMAN WOUND HEALING -

Development of an in vivo mouse model to study human wound healing

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ABSTRACT

Scar formation is a frequently occurring unwanted result of healing of a skin wound. The mechanisms causing scar formation are not fully understood, although many studies have attempted to unravel this issue. Most of these studies were performed in animals or in in vitro models, but both have major drawbacks. In the present study we aimed to develop a representative model in which the human wound healing process can be studied in vivo. Ex vivo human skin was transplanted it on severely immunocompromized mice. After 90 days, human skin was successfully transplanted on the backs of NSG mice. However, most of the transplants still contained a crust, and the transplant that had shed the crust contracted more than 60% of the original size. Because of the crust and contraction, these transplants are not usable for subsequent wound healing studies yet. In conclusion, we have shown that human skin can successfully be transplanted on NSG mice. In order to be useful, parameters such as skin thickness, or transplantation size could be adapted for improvement of this model.
INTRODUCTION

Formation of scars is a frequently occurring unwanted result of healing of a skin wound. Worldwide many people suffer from excessive scars, often causing functional, emotional and psychological problems.\(^1\) The extent of scars can vary greatly. Small superficial wounds result mostly in flat scars. Larger deeper wounds can however give rise to fibrotic or so called hypertrophic scars, which raise above skin level, have a rough texture due to contractures and disorganized collagen accumulation, and cause itching and pain.\(^2\) The mechanisms causing scar formation are not fully understood, although many studies have attempted to unravel this issue. Most of these studies were performed in animals or in \textit{in vitro} models.\(^3\) Regrettably, both have major drawbacks. Animals do not completely reflect the human situation as for example, mice do not develop hypertrophic scars. Moreover, \textit{in vitro} models are usually simplified and lack for instance systemic factors that play a role during \textit{in vivo} repair. In the present study we aimed to develop a representative model in which the human wound healing process can be studied \textit{in vivo}. Therefore, we used \textit{ex vivo} human skin and transplanted it on severely immunocompromized mice.

MATERIALS & METHODS

Animals

\textbf{NOD-scid IL2Rgamma\textsuperscript{null} (NSG; NOD.Cg-Prkdc\textsuperscript{scid} Il2rg\textsuperscript{tm1Wjl/SzJ}) mice were obtained from Jackson Laboratories (Bar Harbor, ME). These mice have a comparable phenotype as severe combined immune deficiency mutation (SCID) mice, but are also deficient for the IL-2 receptor gamma chain. Consequently, these animals are highly immunodeficient and lack mature T cells, B cells, functional NK cells, and are deficient in cytokine (Interleukin-2, -4, -7, -9, -15, and -21) signalling. Neutrophils and monocytes remain in the peripheral blood. Macrophages and dendritic cells are still present in these mice, although they are defective.}\(^4\) Animals were maintained under individually-ventilated-cage (IVC) conditions.

Transplantation experiments

Animal procedures were approved by the ethical committee of the VU University. Six-to-eight-week-old NSG mice (n=5) were anesthetized by isoflurane and depilated.
using a clipper and commercial hair remover (Etos, Beverwijk, The Netherlands). A section of the dorsal skin was resected, leaving the panniculus carnosus intact. This skin was rotated (180°), grafted back on the same animal and sealed by an adhesive foil (Obsite, Smith and Nephew, Hoofddorp, The Netherlands). Subsequently, a gauze (HG compres, Klinion, Oud-Beijerland, The Netherlands) and surgical tape (Microfoam™, 3M, St. Paul, MN) were wrapped around the animals from dorsal to ventral site. Immediately following transplantation, all mice received pain management (burprenofine; 0.05-0.1 mg/kg, and carprofen; 10 mg/kg). Carprofen treatment was applied 24 hours preoperative and repeated at 24 and 48 hours post surgery. After 21 days bandages were removed and the transplants were left to heal. Mice were sacrificed by CO₂ asphyxiation at day 36 post surgery. Transplants were evaluated by macroscopic view.

Subsequently, human skin (12 mm Ø), obtained from patients undergoing abdominoplasty, was grafted on five NSG mice as described above. All skin donors provided informed consent according to institutional and national guidelines. Dressings were replaced 21 days post surgery, and removed after 35 days. Mice were sacrificed by CO₂ asphyxiation, 90 days post-surgery. Transplants were photographed and dissected for further analysis.

**Histology**

Biopsies were embedded in OCT™ Compound (Sakura Finetek, Alphen aan den Rijn, The Netherlands) and stored at -80°C until sectioning. Cryosections (5 µm) were mounted on Superfrost™ Microscope Slides (Thermo Fisher Scientific, Waltham, MA) and fixed in acetone. Non-specific binding was blocked by incubation with 10% normal mouse serum. Peroxidase was quenched with H₂O₂ and sections were incubated with rat anti human HLA-ABC (Abcam, Cambridge, UK; dilution 1:100) antibody. Subsequently, sections were incubated with a secondary biotin labeled rabbit anti rat antibody, and later with streptavidin-horseradish peroxidase. Detection was performed using diaminobenzidine (DAB) as chromogen substrate (Dako), and sections were counterstained with haematoxylin, dehydrated in ethanol and embedded in entellan (Merck Millipore, Darmstadt, Germany). In addition to HLA-ABC staining, a toluidin blue staining was performed. Sections were micrographed by use of a Zeiss AX10 microscope imager (Carl Zeiss, München, Germany).
RESULTS AND DISCUSSION

To optimize the surgical procedure, we first transplanted autografts on NSG mice. After 36 days, a survival rate of 100% was achieved and the transplants were fully accepted in all animals. In figure 1, an example is depicted, showing a viable skin transplant (Fig. 1A). Underneath the skin, wound edges were highly vascularised (Fig. 1B, arrows). These results indicate that we were able to transplant skin on NSG mice and to keep these transplants viable.

Next, human skin was transplanted on NSG mice. 21 days after transplantation, dressings were removed and the transplants appeared fragile and not completely grown in yet (data not shown). In order to protect the transplants and to allow further repair, new dressings were applied for another 2 weeks, after which transplants were left uncovered in order to heal. Transplants were regularly inspected and after 90 days, a crust on the transplant was still present in 80% of the animals (Fig. 2). In one animal (nr. 5) the crust was completely shed of the transplant and the human skin was reduced in size by >60%, probably due to contraction (from 12 to 4 mm; Fig. 2). Under the crust, the transplant of mouse number 2 had a similar appearance as seen in mouse number 5. Remarkably, in contrast to our observations, Racki et al.5 observed acceptance and fully repair of the human transplant already 5 weeks after surgery. One of the explanations might be that a graft thickness of 300 µm was used, while we transplanted full-thickness human skin (± 3 mm). In contrast to human skin, the autografts healed faster, therefore it seems possible that skin thickness may influence the time of healing.
Since immune cells are involved in repair,\textsuperscript{6,7} complete or partial absence of these cells, as in NSG mice, may have an effect on the rate of transplant acceptance. For instance nude mice that only lack T- and B-cells, accepted human transplants already 30 days after grafting, stressing the importance of immune cells.\textsuperscript{8}

Histological examination revealed that human skin was properly incorporated into the mouse skin (Fig. 3). Human epidermis was thick showing large rete ridges. A clear transition from mouse to human tissue was observed (Fig. 3B, arrows), and confirmed by HLA-ABC staining (Fig. 3C). Furthermore, hair shafts were ubiquitous in the mouse skin. Human cells had clearly migrated into the mouse skin (Fig. 3D). In transplantation models using nude mice, the human skin had similarities with hypertrophic scars, classified by factors such as increased thickness, loss of hair follicles, hypercellularity, and increased mast cell density.\textsuperscript{8} The benefit of NSG mice over nude mice is that less immune mediators are present. These immune factors may be responsible for formation of fibrotic scars as observed in human transplants.\textsuperscript{9-11} When using NSG mice, immune cells can selectively be administrated to wounds, in order to identify which cell could be responsible for hypertrophic scar formation.

In conclusion, we have shown that human skin can successfully be transplanted on NSG mice. For future perspectives the transplantation model must be refined in order to be suitable for subsequent wound healing studies.
Figure 3: Histological structure of human skin transplant on NSG mouse.
A) Toluidin blue staining was used for histological identification, showing inclusion of human skin (H) in mouse skin (M).
B) Magnification, showing a clear border between human and mouse skin, indicated by arrows.
C) Discrimination between human and mouse skin was further detected by HLA-ABC staining.
D) Magnification, showing migrating human cells into mouse skin. Scale bar: 50 µm.
REFERENCES LIST

CHAPTER 6B

- MODELS TO INVESTIGATE HUMAN WOUND HEALING -

M2 macrophages mediate faster wound closure in a new human ex vivo wound healing model

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ABSTRACT

Abundant scar formation is an unwanted result of wound healing. Immune cells such as macrophages play a role in formation of scars. Macrophages can exert more pro- (M1) or anti-inflammatory (M2) actions, although there is a continuum of phenotypes between these extremes. Studies that investigated the role of macrophages in wound healing were generally performed in mice. Unfortunately, animals do not completely mirror human conditions. Here, a human ex vivo wound healing model was used in which the activities of M1 and M2 macrophages were investigated. Ex vivo human skin was wounded and macrophages (M1 and M2) were repeatedly applied to the wounds. Wound area was measured over a period of time, and immunohistochemical and gene expression analysis were performed. M2 macrophages induced faster reepithelialization and contributed to fibroblast migration. mRNA encoding telopeptide lysyl hydroxylase (PLOD2) was significantly induced in wounds treated with M2 macrophages. This signifies to involvement of M2 macrophages in fibrosis since PLOD2 is upregulated in fibrotic tissues. In conclusion, M2 macrophages were important for wound closure, but also have the ability to upregulate genes that are involved in fibrotic scar formation, stressing a possible role for M2 macrophages in development of excessive scars.
INTRODUCTION

Dermal wounds are associated with scar formation, while fetal and oral wounds have the capacity to heal without scarring. Understanding the complex processes that lead to scar formation is challenging, and many researchers have attempted to unravel this issue. One of the factors that may be involved in scar formation is the immune system.¹ This is supported by the absence of inflammatory responses in fetal wounds generated during the 1st and 2nd trimester of gestation, which heal without scar formation. In late gestational development wounds when scarring occurs, activated macrophages start to appear.² In addition, PU.1 null mice that genetically lack monocytes/macrophages, and functional neutrophils healed without scars while the healing time was similar to wild-type mice.³, ⁴ These data support that immune responses contribute to scar formation. Nevertheless, specific macrophage depletion had detrimental effects on the repair process, since it reduced wound closure, collagen deposition, and angiogenesis.⁵-⁸ Furthermore in salamanders, that heal by regeneration without scar formation, macrophages were essential for regeneration, as macrophage depletion led to fibrosis and failure to regenerate after wounding.⁹

Macrophages have different phenotypes, which are established by environmental factors. Generally, macrophages mediate more pro- or anti-inflammatory actions, referred to as M1 and M2 respectively, although there is a continuum of phenotypes beyond these extremes.¹⁰ M1 macrophages are necessary to clear the wound from harmful pathogens, but as side effect provoke tissue injury by secretion of pro-inflammatory cytokines.¹⁰ As a consequence of e.g. phagocytosis of dead cells and binding to collagen type I, macrophages start to acquire an M2 phenotype.¹¹, ¹² These M2 macrophages release fibroblast-promoting factors and are therefore assumed to be involved in repair. Furthermore, macrophages in human chronic venous leg ulcers had predominantly an M1 phenotype, which prevented wound closure.¹³ Besides the concept of repair, M2 macrophages are associated with fibrosis. For example, in lung fibrosis alveolar macrophages acquired predominantly an M2 phenotype and fibrosis was reduced when M2 macrophages were diminished.¹⁴, ¹⁵

Studies that investigated the role of macrophages in wound healing have generally been performed in mice, and mostly only the presence of the cells was determined and not their action. From others and our studies however, it was established that mouse and human macrophage features do not correspond
completely (unpublished data). Furthermore, mice heal by contraction, which is different from human wound healing. Therefore it is important to develop wound healing models which better mimic the human repair process. In this study we used an *ex vivo* human wound healing model, and investigated the actions of M1 and M2 macrophages independently during the ongoing repair process.

**MATERIALS & METHODS**

**Human macrophages**

Human macrophages were obtained from healthy donor buffy coats (Sanquin Blood Bank, Amsterdam, The Netherlands), and informed consent was provided by all donors. Peripheral blood mononuclear cells (PBMCs) were isolated by a Ficoll (Lymphoprep™, Axis-Shield, Oslo, Norway) density gradient, and subsequently monocytes were again by density gradient isolated from the PBMC’s using Percoll (GE Healthcare, Uppsala, Sweden). Monocytes were cultured in 100 mm Ø plastic Petri dishes (Greiner Bio-One, Alphen aan de Rijn, The Netherlands) at a concentration of 1 x 10⁶ cells/ml in the presence of DMEM (Invitrogen, Breda, The Netherlands), supplemented 5% (v/v) normal human serum (Bio Whittaker, East Rutherford, NJ, USA) and 1% (v/v) penicillin-streptomycin-glutamine (PSG; Invitrogen). After 5-7 days, monocytes had differentiated towards macrophages.

M1 macrophages were induced by 1 x 10³ U/ml recombinant human IFN-γ (U-Cytech, Utrecht, The Netherlands) and 10 ng/ml LPS-EB Ultrapure (InvivoGen, San Diego, USA). M2 macrophages were generated using 10 ng/ml human IL-4 (ImmunoTools). Both macrophage populations were generated using 10 ng/ml human IL-4 (ImmunoTools). Both macrophage populations were cultured in six-well plates (Greiner Bio-One) for two days.

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**In vitro wound healing model**

Human skin was obtained from healthy donors (n=2) undergoing breast reconstruction, or abdominal dermolipectomy. Donors provided informed consent according to institutional and national guidelines. 10 mm diameter *ex vivo* skin biopsies were collected and (superficial) wounds were generated by means of a 4 mm diameter puncher. This puncher was carefully, with minimal force, pressed on the skin and a 4 mm skin piece was resected. Biopsies were placed on stainless-steel grids and cultured in six-well plates (Greiner-bio-One) at 37°C with 5% CO₂ in DMEM/Ham’s F12 (3 : 1) (Invitrogen, Paisley, UK), supplemented with 2% fetal calf serum (Hyclone, Logan, UT), 1 μM hydrocortisone, 1 μM isoproterenol, 0.1 μM insulin, 1.0 × 10⁻⁵ M L-carnitine, 1.0 × 10⁻² M L-serine, 1 μM DL-α-tocopherol, 130 μg/mL ascorbic acid, a lipid supplement (containing 25 μM palmitic acid, 15 μM linoleic acid, 7 μM arachidonic acid, and 24 μM bovine serum albumin) (all Sigma-Aldrich, St. Louis, MO), and penicillin/streptomycin (100 IU/mL penicillin, 100 μg/mL streptomycin; Invitrogen). Culture medium was refreshed twice a week.

Macrophages (M1 and M2) were harvested by lidocaine (4 mg/ml), washed and suspended in PBS and topically applied to the wounds. Addition of macrophages was repeated every four days. Wounds were photographed at indicated days (Powershot SX220 HS, Canon, ‘s-Hertogenbosch, The Netherlands) and wound area was analysed by Image J software. After 17 days, the skin biopsies were cut in half; one piece was embedded in Tissue Tek® OCT Compound (Sakura Finetek, Alphen aan den Rijn, The Netherlands) for immunohistochemical analysis, and the other part was used for gene expression analysis (wounds and uninjured skin were separated). All skin pieces were frozen until further processing.

**Immunohistochemistry**

Biopsies were cut in 5 µm sections and mounted on collagen coated glass slides. Prior to staining, sections were fixed in acetone and peroxidase was quenched with H₂O₂. Primary antibodies (Table 1) were incubated for 1 hour at room temperature. Next, sections were incubated with Envision (Dako, Glostrup, Denmark) and detection was performed using diaminobenzidine (DAB) as chromogen substrate (Dako). Nuclei were stained using haematoxylin and finally, sections were dehydrated in ethanol and embedded in entellan (Merck Millipore, Darmstadt, Germany). Negative controls were conducted by use of non relevant isotype control antibodies.
RNA isolation
For RNA extraction, biopsies were incubated with Trizol and inserted into a TissueLyser LT (Qiagen, Hilden, Germany) machine, in which biopsies were shaken for 5 minutes at 40 Hz. Lysate was centrifuged and supernatant was replaced to phase lock gel tubes (Qiagen). Chloroform was added and tubes were centrifuged for 10 minutes at 12000xg. The upper water phase was collected and incubated with isopronanol. Next, tubes were centrifuged for 30 minutes at 12000xg. Supernatant was removed and the pellet resuspended in 75% EtOH and subsequently centrifuged for 4 minutes at 12000xg. This latter procedure was repeated three times. Finally, pellets were resuspended in RNase free H$_2$O.

Real time PCR
RNA was transcribed into cDNA by use of a Reverse Transcription System kit (Promega, Mannheim, Germany), according to the manufacturers’ protocol. Real time PCR was performed using SYBR Green (PE Applied Biosystems, Foster City, CA) as chromogen on a ViiA™ 7 Real-Time PCR System (Applied Biosystems) machine. mRNA expression of α-smooth muscle actin (SMA), biglycan, collagen 1A, decorin, EF1α, fibronectin ED-A, GAPDH, PLOD2, TGF-β1 and VEGF was analyzed (Table 2). The expression levels were normalized to GAPDH and EF1α mRNA expression.

Statistical analysis
Statistical analysis was performed by GraphPad Prism (GraphPad Software Inc., San Diego, USA), using a one-way ANOVA with Bonferroni correction. P values < 0.05 were considered as statistically significant. * P<0.05, ** P<0.01, *** P<0.001

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Table 2: RT-PCR Primers.
RESULTS

M2 macrophages provide faster wound closure and fibroblast migration
First, the effect of M1 and M2 macrophages on reepithelialization was investigated. After wounding, macrophages were applied every four days. Already six days after wounding, a significant difference was observed, as wounds that received M2 macrophages showed faster wound closure, compared to control (PBS) and M1 macrophages. This result was even more evident at the end of the experiment, 17 days after wounding (Fig. 1A, B).

After removal of the stainless-steel grids, an interesting phenomenon was observed. Cells were attached to the bottom of the well (Fig. 2A). Based on morphology, the cells had fibroblast characteristics as indicated by an elongated appearance. After staining for vimentin, indeed fibroblasts were identified (Fig. 2B). Remarkably, the wells in which M2 macrophages were applied to the skin wounds contained large amounts of fibroblasts (Fig. 2). Possibly, fibroblasts had migrated out of the skin under the influence of M2 macrophages.

Figure 1: M2 macrophages provide faster wound closure than M1 and control.
A) Macroscopic appearance of wounds at day 0, 6, 10, and 17 after control (PBS), M1 or M2 macrophage treatment. B) Analysis of wound areas, expressed as percentage of original wound size created at day 0. Scale bar = 0.5 cm.
Effect of M1 and M2 macrophages on microscopic level

Next, effects on microscopic level were evaluated. Collagen fibers were visualized by eosin (Fig. 3). Fibers were visible in control and macrophage conditions, although collagen seemed to be more compact when wounds were treated by M2 macrophages. Fibroblasts start to express α-smooth muscle actin (α-SMA) by factors such as TGF-β or PDGF-CC. Previously, we showed upregulation of α-SMA in fibroblasts by M2 macrophages. However, this effect was not observed in this ex vivo model (Fig. 4). Hardly any α-SMA expressing fibroblasts were present in the wounds regardless of treatment. HSP47 is a collagen specific chaperone protein that is essential for the synthesis and secretion of collagen and used as marker for fibroblasts. In the vicinity of the basement membrane, more fibroblasts were present when M2 macrophages were applied, compared to the other treatments (Fig. 4, inserts).

Administration of M1 and M2 macrophages was clear, since accumulation of macrophages were found at the upper area of the wounds. In control (PBS) treatment skin macrophages were also present, although somewhat deeper in the dermis. Blood vessels were detected in all three conditions, but no differences were noticed (data not shown).
Besides cellular structures, other tissue components were evaluated. Cytokeratin 17 (CK17) is mainly expressed in the basal cells of complex epithelia, and induced after acute skin injury.\textsuperscript{20, 21} Compared to control and M1 macrophage treatment, expression was more widespread throughout the keratin layer upon M2 treatment (Fig. 5). Chondroitin sulphate was previously described to be upregulated after wounding,\textsuperscript{22} but we did not detect this protein in any of the tissues (data not shown). Collagen type IV forms large complexes and is a major component of the basement membrane. Expression of collagen type IV did not differ between the various treatments (data not shown). Finally, cell proliferation was investigated. Proliferating cells were observed at the basement membrane, although no differences between the three treatments were found in unwounded tissue (Fig. 5). In the wound area however, a few proliferating cells were seen but only when M2 macrophages had been administered (Fig. 5, inserts).
Effect of M1 and M2 macrophages on gene expression

RNA was extracted from wounds and unaffected skin to investigate whether macrophages had effect on gene expression level. In control condition (PBS), TGF-β1 expression was clearly lower in wounds than normal skin, and M2 macrophages even decreased TGF-β1 in normal skin (Fig. 6). Possibly the effect of M2 macrophages was spread from wound to unaffected skin area, since the wounds also had lower expression levels of TGF-β1. PLOD2, the gene encoding the enzyme telopeptide lysyl hydroxylase (TLH) that mediates collagen crosslinking, was significantly higher expressed in wounds treated with M2 macrophages, compared to unaffected tissue (Fig. 6). α-SMA and VEGF were not significantly differently expressed by any of the treatments. With respect to extracellular matrix
proteins, macrophages had no influence on its expression. Collagen I, fibronectin (FN) ED-A, biglycan and decorin were equally expressed between wounds and normal tissue, despite addition of macrophages (Fig. 6).

**DISCUSSION**

Predominantly, animal models are used to investigate wound healing processes. Subsequently this information is translated to humans, although animals regrettably do not completely mirror human conditions. For instance, mice heal by contraction while human repair is based on reepithelialization and formation of granulation tissue. In addition, anatomical conditions such as skin thickness, presence of rete ridges and sweat glands differ between the two species. Here, we used a new *ex vivo* human wound healing model and studied the effect of M1 and M2 macrophages on repair.
Figure 6: Gene expression in normal and wounded skin.
After 17 days of culture, normal skin (grey bars) and wounds (black bars) were separated and mRNA levels of TGF-β1, PLOD2, α-SMA, VEGF, Collagen I, FN ED-A, Biglycan, and Decorin were measured.
M2 macrophages induced faster wound closure than M1 macrophages or control treatment. Previously, we showed that M2 macrophages secrete more platelet derived growth factor-CC than M1 macrophages. This growth factor is chemotactic for fibroblasts which may explain the enhanced repair after addition of M2 macrophages. Moreover, M2 macrophages mediated fibroblast migration and/or proliferation since large quantities of these cells were found at the bottom of the culture plate. Earlier, human mesenchymal stem cells were shown to induce an M2 phenotype in macrophages, which led to improved dermal wound healing. Moreover, enhanced reepithelialization of mouse wounds was correlated with increased numbers of M2 macrophages, stressing the importance of M2 macrophages during repair.

Based on immunohistochemical analysis, HSP47 positive fibroblasts were predominantly present at the dermal-epidermal junction when M2 macrophages were administered. This indicates that more fibroblasts were attracted to the wound, and thereby likely contribute to repair. Conversely, in contrast to adult wounds HSP47 was not increased in fetal wounds, which may suggest that HSP47 is involved in scar formation. In addition, enhanced HSP47 expression, together with excessive collagen accumulation is regularly observed in fibrotic diseases. Animal models revealed that downregulation of HSP47 suppressed fibrosis by reduction of collagen type I, III and α-SMA. CK17 was more present in epithelia treated with M2 macrophages. CK17 is rapidly upregulated during wounding, and delayed healing was observed in CK17 knock-out mice.

After control (PBS) treatment, TGF-β1 was lower in wounds than normal skin. In general, TGF-β1 is higher expressed in wounds than normal skin, and promote scar formation. In our ex vivo setting factors that induce TGF-β1 expression are possibly lacking. Keratinocytes are an important source of TGF-β1 and absent in the wound area which may explain the reduced TGF-β1 expression compared to normal skin. Stressed keratinocytes adjacent to the wound borders (normal skin) secrete more TGF-β1. Next to that, influx of systemic components such as platelets, growth factors or immune cells can be responsible for increased TGF-β1 levels in wounds, but are absent in our ex vivo model. Upon M1 or M2 treatment however, TGF-β1 expression was equal between wounds and normal skin. Interestingly, upon application of M2 macrophages, TGF-β1 was reduced in normal skin compared to control (PBS) treatment. Wound closure was enhanced
by M2 macrophages and hypothetically skin around the wound might be less stressed compared to control treatment, thereby producing less TGF-β1.

Finally, PLOD2 was significantly increased in wounds treated with M2 macrophages. TLH, the enzyme PLOD2 encodes for, was highly expressed in fibroblasts derived from fibrotic skin.\textsuperscript{36} This points to a relation between fibrosis and M2 macrophages.

In conclusion, we present a new \textit{ex vivo} human wound healing model in which M2 macrophages appear to be important for wound closure, but also have the ability to upregulate gene expression of molecules involved in fibrotic scar formation. In order to prevent such scars a balance for the presence and persistence of M2 macrophages in wounds must be maintained. To further evaluate the role of M2 macrophages in fibrosis formation, it is recommended to investigate their effects on the long term in this \textit{ex vivo} model.
REFERENCE LIST


