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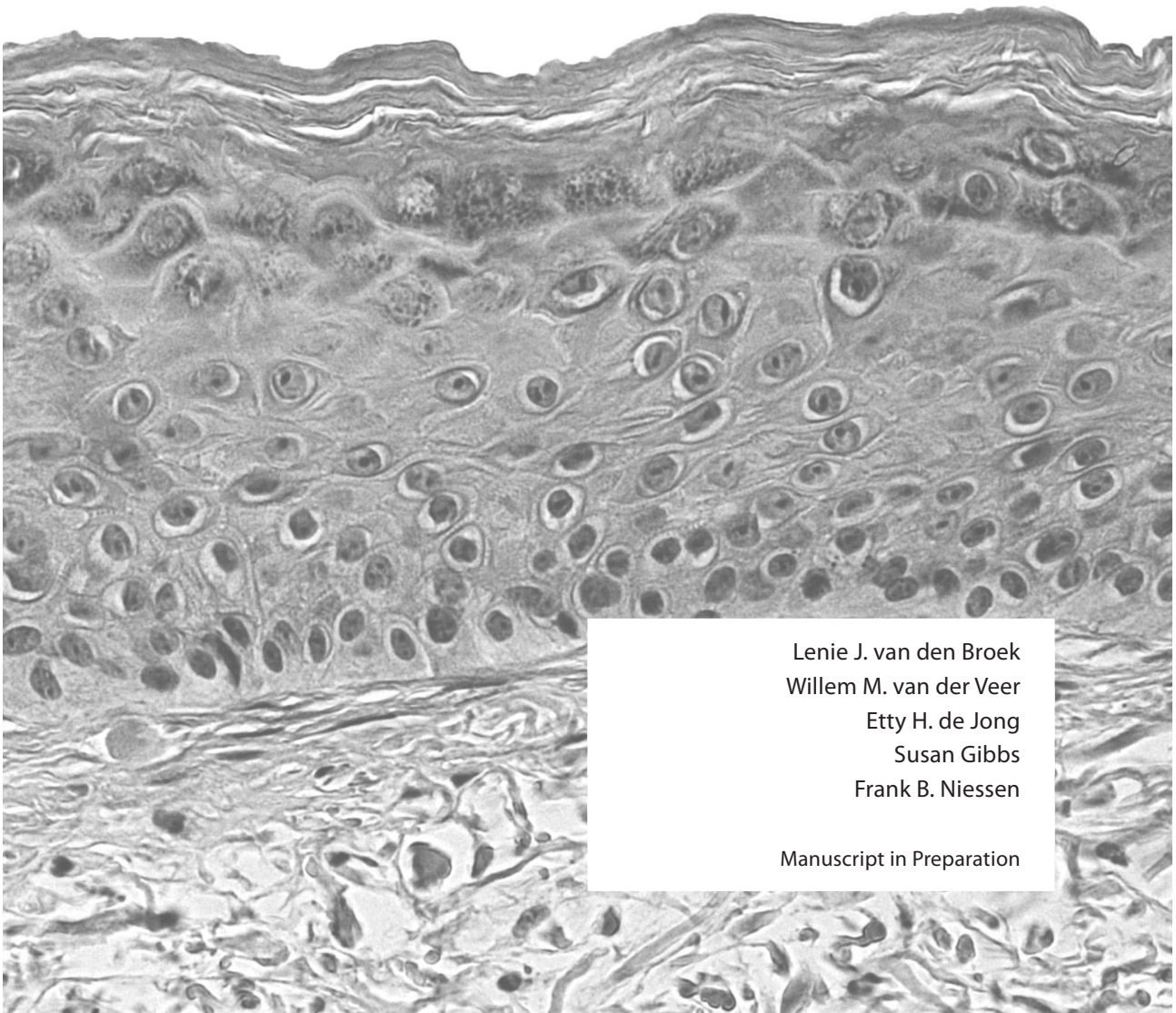
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Chapter 2

Suppressed inflammatory gene expression during human hypertrophic scar compared to normotrophic scar formation



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Manuscript in Preparation

ABSTRACT

Hypertrophic scar formation is a result of abnormal cutaneous wound healing and pathogenesis of hypertrophic scar formation is still poorly understood. A problem next to the lack of suitable animal models, is that often normal skin is compared to hypertrophic scar (HTscar) tissue and not to normotrophic scar (NTscar) tissue. Another drawback is that often only one time period after wounding is studied while scar formation is a dynamic process over a period of several months. In this study, we compared the expression of genes involved in inflammation, angiogenesis and extracellular matrix (ECM) formation in biopsies obtained before and up to 52 weeks after standard surgery in 5 patients who developed HTscar and 6 patients who developed NTscar and furthermore compared this to macrophage infiltration.

It was found that HTscar formation coincided with a prolonged decrease of expression of inflammatory genes (TNF α , IL-1 α , IL-1RN, CCL2, CCL3, CXCL2, CXCR2, C3 and IL-10) and an extended increased expression of ECM related genes (PLAU, Col3A1, TGF β 3). This coincided with a delayed but prolonged infiltration of macrophages (type 2) in HTscar tissue compared to NTscar tissue. Blood vessel protective and stimulating factors (ANGPT1 and FGF2) decreased during HTscar formation whereas vessel disrupting factors (ANGPT2) increased in both groups. These findings were supported by immunohistochemical localization of proteins coding for select genes named above.

Our study emphasizes that human cutaneous wound healing is a dynamic process that needs to be studied over a period of time rather than a single point in time. Taken together our results suggest innate stimulatory therapies may be a better option for improving scar quality than the currently used anti-inflammatory scar therapies.

INTRODUCTION

Cutaneous wound healing is an interactive process initiated immediately upon tissue damage and results in a scar. The most desirable scar is thin, flat and hardly visible and is known as a normotrophic scar (NTscar). Severe trauma (e.g. deep burns) and sometimes even standard surgery can result in wound closure with abnormal scar formation, known as a hypertrophic scar (HTscar)¹. It is not known why some humans form an HTscar after relatively minor injury and others do not. HTscars are red, firm, raised, itchy, sometimes painful and usually develop within 4-8 weeks post injury and may regress over time. Often patients experience diminished quality of life due to loss of joint mobility and disfigurements².

Upon cutaneous injury the wound healing process is initiated and starts with the formation of a blood clot (mainly consisting of fibrin, fibronectin and platelets) to prevent excessive blood loss. Cytokines and growth factors are released by platelets and injured skin resident cells in order to attract immune cells into the wound bed³⁻⁵. These infiltrating immune cells in turn release more cytokines and growth factors, attack invading pathogens and initiate wound repair. The escalating secretion of growth factors and cytokines by these immune cells further amplifies the inflammatory response and activates neighbouring skin residential cells. The skin cells (e.g. keratinocytes, endothelial cells and fibroblasts) start to re-epithelialize the wound, form new microvessels and deposit granulation tissue⁴⁻⁶. In order to further repair and strengthen the dermis, the newly formed collagen is remodelled, reorganized and cross-linked⁴. This process takes about one year and normally results in an NTscar. However the complex cutaneous wound healing process is susceptible to disturbances, which may ultimately result in HTscar rather than NTscar formation.

Despite ongoing research, the pathogenesis of HTscar formation in humans is not well understood. Although studies describe several differences between normal and abnormal scar formation. For example, an increased inflammatory response has been linked to HTscar formation but little is known about the type of immune response and how this differs from that leading to NTscar formation^{7,8}. Furthermore, the development of a T-helper2 response is strongly linked to fibrosis. HTscars are more often seen after delayed wound closure⁹ and the resulting HTscar shows increased numbers of microvessels, increased ECM deposition and decreased apoptosis compared to NTscars⁸. Abnormal expression of proteoglycans, matrix metalloproteinases (MMPs) and their natural inhibitors (TIMPs) (e.g. decreased decorin, MMP1/9 and increased TIMP-1, biglycan and versican) which may lead to increased proliferation, increased matrix production and reduced matrix breakdown, also suggests an altered ECM remodelling in HTscar compared to NTscar formation^{7,8}. However it is not known what the cause is and what the effect of HTscar formation.

Animal models (e.g. mice, pigs, rabbits) are frequently used to investigate HTscar formation. Due to differences in skin physiology, immunology and scar formation between humans and animals the results are difficult to extrapolate¹⁰. Unfortunately, when human clinical studies are done, HTscars are often compared to healthy skin instead of NTscars, enabling only general conclusions to be made on scar formation rather than conclusions on abnormal scar formation¹¹⁻¹³. Another drawback of most studies is that although scar formation is a dynamic process over a period of several months most studies (pre-clinical and clinical) only include one time point and do not study the dynamics of wound healing process¹⁴⁻¹⁶.

In this study, in order to investigate the differences in dynamics of wound healing that lead to an HTscar rather than an NTscar in humans, we compared the expression of genes involved in inflammation, angiogenesis and ECM formation and remodeling in biopsies obtained before and up to 52 weeks after cardiothoracic surgery in 5 patients who developed HTscar and 6 patients who developed NTscar. The findings were supported by immunohistochemical localization of proteins coding for select genes named above in mature scars (>1 year). Our data suggests that HTscar formation when compared to NTscar formation is related to a decreased expression of inflammatory and angiogenic genes and an extended and increased expression of ECM genes related to tissue injury.

MATERIAL AND METHODS

The medical ethics committee of the University Medical Center Groningen approved the study of human cutaneous presternal wound healing after cardiothoracic surgery and all the participants gave written consent.

Human scar tissues were obtained from patients who underwent plastic surgery for scar excision after verbal informed consent according to the Dutch Code for secondary use of anonymous rest material.

Tissue samples for real-time RT-PCR and immunofluorescence staining

Human cutaneous presternal wound healing after cardiothoracic surgery was studied in a standardized manner as described previously¹⁷. In short, a total of 76 patients (> 18 years) undergoing cardiothoracic surgery through a median sternotomy incision (mean length 22 cm) were included in this study from which 42 patients were able to complete the follow up of 52 weeks. All operations were performed at the department of Cardiac Surgery of the University Medical Center Groningen.

Scar evaluations took place under standardized conditions 2, 4, 6, 12, and 52 weeks postoperatively by a plastic surgeon specialized in scar assessments. During all evaluations, the presternal scar was evaluated at 8 cm from the most cranial and caudal border

of the scar and scored as hypertrophic or normotrophic. HTscar was defined as raised above skin level (>1 mm) while remaining within the borders of the original lesion and NTscar was defined as not raised above skin level. After the final evaluation of the last participant, the HTscar group who had developed HTscar in both the cranial and the caudal segment at 12 and 52 weeks, was matched with respect to age, sex, and body mass index to a control group consisting of patients with a complete NTscar at 12 and 52 weeks¹⁸.

During cardiothoracic surgery, from all 76 patients, a skin tissue sample of the caudal part of the incision was collected (control sample, time point 0 postoperatively). During the scar evaluations a 3 mm diameter punch biopsy was taken from the caudal segment of the scar consecutively at 3, 4, 5, 6 and 7 cm from the caudal confine of the scar (patients received local anesthesia with 5 mL of lidocaine hydrochloride (10 mg/ml) combined with epinephrine (0.01 mg/mL)). All collected skin samples/biopsies were frozen immediately in liquid nitrogen and stored at -80°C for real-time RT-PCR.

Scar tissue samples for immunofluorescence staining proteins

Mature scar tissue samples were obtained from patients who underwent plastic surgery for scar excision (HTscar group n = 6; age: 15-50 years; sex: 4 x female, 2 x male; location: abdomen, breast and forearm; age of scar: >1 year < 1.5 year; and NTscar group n = 6; age: 20-50 years; sex: 5 x female, 1 x male; location: abdomen and breast; age of scar: >1 year < 1.5 year).

Real-time RT-PCR

Total RNA was isolated from skin samples and prepared for real-time RT-PCR as described by van der Veer et al¹⁸. Real-time PCRs were performed with exons overlapping primers and minor groove binder probes (Table 1) (Applied Biosystems, Nieuwekerk aan de IJssel, the Netherlands) in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems). All PCR reactions were performed at least in duplicates. For PCR analysis, mean threshold cycle (Ct) values were normalized for the expression of the housekeeping gene GAPDH by calculating the ΔCt ($\text{Ct}_{\text{Gene of interest}} - \text{Ct}_{\text{GAPDH}}$). Relative quantification of the PCR data was calculated by the $\Delta\Delta\text{Ct}$ ($\Delta\text{Ct}_{\text{weekn}} - \Delta\text{Ct}_{\text{week0}}$). The genes are expressed as $2^{-\Delta\text{Ct}}$ to determine the total level of gene expression over time and as $2^{-\Delta\Delta\text{Ct}}$ to determine the relative changes in gene expression in a patient group. Of the 33 genes studied, 32 genes were assessed in a total of 6 and 5 patients forming an NTscar and HTscar, respectively. Due to limited availability of material, FGF2 was only studied in 3 and 4 patients forming an NTscar and HTscar respectively.

Table 1. Exon overlapping primers and minor groove binder probes used for real-time RT-PCR

Gene	Probe	Gene	Probe	Gene	Probe
ANGPT1	Hs00181613_m1	CXCL2	Hs00236966_m1	IL12A	Hs01073447_m1
ANGPT2	Hs00169867_m1	CXCL3	Hs00171061_m1	IL12B	Hs01011518_m1
B2M	Hs00984230_m1	CXCL8	Hs00174103_m1	PECAM1	Hs00169777_m1
C3	Hs00163811_m1	CXCL10	Hs00171042_m1	PLAU	Hs00170182_m1
C5	Hs00156197_m1	CXCR2	Hs00174304_m1	SELE	Hs00174057_m1
CCL2	Hs00234140_m1	FGF2	Hs00266645_m1	TGF- β 1	Hs99999918_m1
CCL3	Hs00234142_m1	GAPDH	Hs99999905_m1	TGF- β 3	Hs00234245_m1
CCL5	Hs00174575_m1	IL1a	Hs00174092_m1	TGF- β R1	Hs00610320_m1
CCL22	Hs01574247_m1	IL1R1	Hs00168392_m1	TNF- α	Hs00174128_m1
COL1A2	Hs00164099_m1	IL1RN	Hs00174099_m1	VEGF	Hs00173626_m1
COL3A1	Hs00164103_m1	IL10	Hs00961622_m1	YWHAZ	Hs00237047_m1

Immunofluorescence staining

Immunofluorescent stainings were performed exactly as describe previously¹⁹. Primary antibodies directed against CCL2 (MAB679, 25 μ g/ml), FGF2 (MAB233, 25 μ g/ml), TGF- β 3 (DY243, 20 μ g/ml), PLAU (DY1310, 20 μ g/ml) (all R&D System Inn, Minneapolis, Minnesota, USA), CD68 (M0718, 5.3 μ g/ml) (Dako, Glostrup, Denmark), CD163 (MCA1853, 1:100) (AbD Serotec, Dusseldorf, Germany) or corresponding isotype were used. Slides were examined using a fluorescence microscope (Nikon Eclipse 80i, Dusseldorf, Germany) using NIS-Elements AR 2.10 software. The amount of staining of mature scar samples was quantified by measuring the mean (green) fluorescence staining per mm² in three different photographs per samples with a 20-fold magnification area using a computer-assisted fluorescence microscope. The mean fluorescence staining was corrected for mean fluorescence staining of corresponding isotype staining. Staining was quantified in epidermis and dermis.

Statistical analysis

Differences between the NTscar and HTscar forming groups regarding gene expression were tested using by Wilcoxon-Mann-Whitney tests. To adjust for multiple testing when testing for differences in gene expression patterns between groups, the Benjamini-Hochberg method²⁰ was applied to reflect the false discovery rate. Genes with a false discovery rate < 20% and a *p*-value below 0.05 were considered to be significant. Differences between NTscar and HTscar regarding immuno fluorescence staining were tested using a Mann-Withney t-test.

RESULTS

Study group

Human wound healing and scar formation was investigated in patients undergoing cardiothoracic surgery by standardized median sternotomy incision as described previously¹⁷. A total of 76 patients were included of which 42 were able to complete the follow up of 52 weeks. Of these 42 patients a total of 5 patients formed an HTscar at 12 and 52 weeks postoperatively. An NTscar control group consisting of 6 patients who had developed a complete presternal NTscar in both the cranial and the caudal segment at 12 and 52 weeks postoperatively was matched to the HTscar group with respect to age, sex, and body mass index as previously described¹⁸. Figure 1 shows a representative HTscar and NTscar.

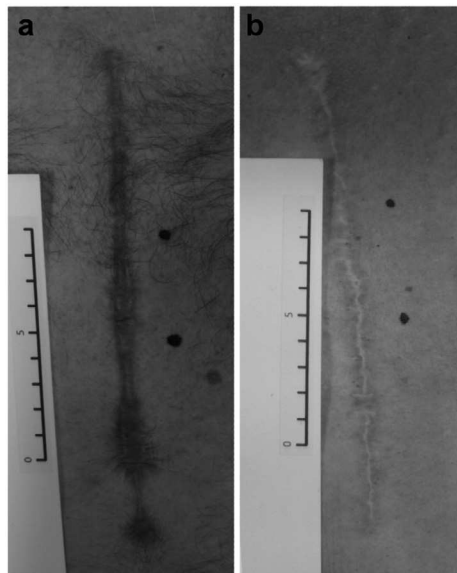


Figure 1. Macroscopic photograph of presternal (a) hypertrophic and (b) normotrophic scar. Bars = 10 cm

Gene expression in HTscar group compared to NTscar group

A total of 33 genes (including GAPDH and B2M) involved in wound healing were analysed for both the HTscar and the NTscar groups (Supplement 1). To distinguish differences between the groups both 1) the normalized Ct values to GAPDH and 2) the relative Ct values to time point 0 were analysed. The two housekeeping genes (GAPDH and B2M) were not differently expressed between the two scar groups or between the different time points. No significant differences were found between NTscar and HTscar

Table 2. Analyse genes

Gene		NTscar in Time	HTscar in Time	Differences NTscar : Htscar (normalized)	Differences NTscar : Htscar (relative)
<i>ANGPT1</i>	Ang	* (↓, ↑)	ns	** (↑)	ns
<i>ANGPT2</i>	Ang	* (↑)	* (↑)	ns	ns
<i>C3</i>	I	ns	ns	** (↑)	0,06 (↑)
<i>C5</i>	I	ns	ns	ns	ns
<i>CCL2</i>	I	ns	ns	* (↑)	ns
<i>CCL3</i>	I	* (↑)	* (↑)	0,07 (↑)	ns
<i>CCL5</i>	I	ns	ns	ns	ns
<i>CCL22</i>	I	ns	ns	ns	ns
<i>Col1A2</i>	ECM	* (↑)	* (↑)	ns	ns
<i>Col3A1</i>	ECM	* (↑)	* (↑)	* (↑)	ns
<i>CXCL2</i>	I	ns	ns	* (↑)	ns
<i>CXCL3</i>	I	ns	ns	ns	ns
<i>CXCL8</i>	I	ns	ns	ns	ns
<i>CXCL10</i>	I	ns	ns	ns	ns
<i>CXCR2</i>	I	ns	ns	** (↑)	ns
<i>IL1a</i>	I	ns	** (↓)	* (↑)	0,06 (↑)
<i>IL1R1</i>	I	ns	ns	ns	ns
<i>IL1RN</i>	I	ns	* (↓)	* (↑)	ns
<i>IL-10</i>	I	* (↑)	ns	** (↑)	ns
<i>IL-12A</i>	I	** (↓)	* (↓)	ns	ns
<i>IL-12B</i>	I	ns	ns	ns	ns
<i>FGF2</i>	Ang	ns	ns	ns	ns
<i>PECAM1</i>	Ang	* (↑)	ns	ns	ns
<i>PLAU</i>	ECM	ns	* (↑)	* (↓)	ns
<i>SELE</i>	I	ns	ns	ns	ns
<i>TGF-beta1</i>	TGF	ns	ns	ns	ns
<i>TGF-beta3</i>	TGF	* (↑)	** (↑)	0,055 (↓)	* (↓)
<i>TGF-betaR1</i>	TGF	* (↑)	* (↑)	ns	ns
<i>TNF-alpha</i>	I	* (↑)	ns	* (↑)	ns
<i>VEGF</i>	Ang	ns	ns	ns	ns
<i>YWHAZ</i>	AP	ns	* (↓)	** (↑)	* (↑)

Ang, angiogenic mediator; AP, adaptor protein; ECM, mediator involved in ECM production/degradation; HKG, housekeeping gene; I, inflammatory mediator; TGF, mediator from TGF- β family. The number of patients forming NTscars and HTscars tested for each gene was 6:5 except for FGF2 which was 3:4. Column 3 and 4: Difference in gene expression in time for NTscar forming group or HTscar forming groups was tested by Kruskal-Wallis test. Column 5: Difference in gene expression between NTscar forming and HTscar forming group of Ct data normalized by GAPDH for area under the curve was tested by Wilcoxon-Mann-Whitney test. Column 6: Difference in gene expression between NTscar forming and HTscar forming group of data relative to time point 0 normalized by GAPDH for area under the curve was tested by Wilcoxon-Mann-Whitney test. ns= not significant; ↑ increase; ↓ decrease; * = $p \leq 0.05$; ** = $p \leq 0.01$

group at time point 0 (time of surgery) for any of the genes studied. However, 15 of the 31 genes normalized to GAPDH showed increased or decreased gene expression during the 52 week study period compared to the before surgery time point 0 in either the HTscar group or the NTscar group or in both groups (Table 2). Furthermore, 14 (including 2 genes showing a trend) of the 31 genes normalized to GAPDH were differently expressed between NTscar and HTscar group (Table 2). Of these 14 genes, 10 were differently expressed in time (overlapping with the 15 genes mentioned above). These 14 genes are described in detail below. The genes were involved in inflammation, angiogenesis or ECM formation. Only 12 genes did not show either a change in gene expression over the 52 week study period or different expression between the HTscar and NTscar groups (Table 2).

Inflammatory gene expression during hypertrophic and normotrophic scar formation

An inflammatory response involving the release of cytokines is initiated immediately upon tissue damage. A total of 19 inflammatory genes were studied and 9 of these showed a differential expression between the HTscar and NTscar groups (Table 2). Even though no difference was observed between the groups prior to surgery, it was noticeable that in general after surgery the level of inflammatory genes was lower in HTscar compared to NTscar (8 genes showed a significant decrease in mRNA levels; 1 showed a trend to decrease in mRNA levels) (Figure 2). This was observed throughout the study period for HTscar and often became more exaggerated at 52 weeks after postoperatively. For example, pro-inflammatory cytokines (IL-1 α , IL-1RN, TNF α), with exception of IL-1R1 which remained constant, showed a decrease in mRNA levels in HTscar compared to NTscar (Figure 2a). For HTscar, mRNA levels decreased with time and remained low whereas for NTscar, levels decreased to a lesser extent and then normalized or became slightly elevated 52 weeks postoperatively. Inflammatory gene expression also showed decreased expression in HTscar compared to NTscar (significant: CCL2, CXCL2, CXCR2, C3; trend: CCL3) (Figure 2b). For HTscar, CXCR2 showed decreased mRNA levels during the study period whereas for NTscar, mRNA levels decreased to a lesser extent and then normalized 52 weeks postoperatively. For CCL2, CXCL2, C3 and CCL3 the gene expression was less elevated in HTscar compared to NTscar. Ten inflammatory genes were similarly expressed in both HTscar as well as NTscar (e.g. CXCL10, IL12-A)(Table 2). Interestingly, not only (pro)-inflammatory mRNA levels were decreased in HTscar compared to NTscar, also the mRNA level of anti-inflammatory cytokine IL-10 was decreased. The mRNA levels of IL-10 remained constant in HTscar within the 52 week study period (only an increase 2 weeks postoperatively) whereas in NTscar, mRNA levels increased with a net result that again IL-10 mRNA was lower in HTscar compared to NTscar (Figure 2c). Taken together these results suggest a prolonged suppression of inflammatory gene expression in HTscar compared to NTscar.

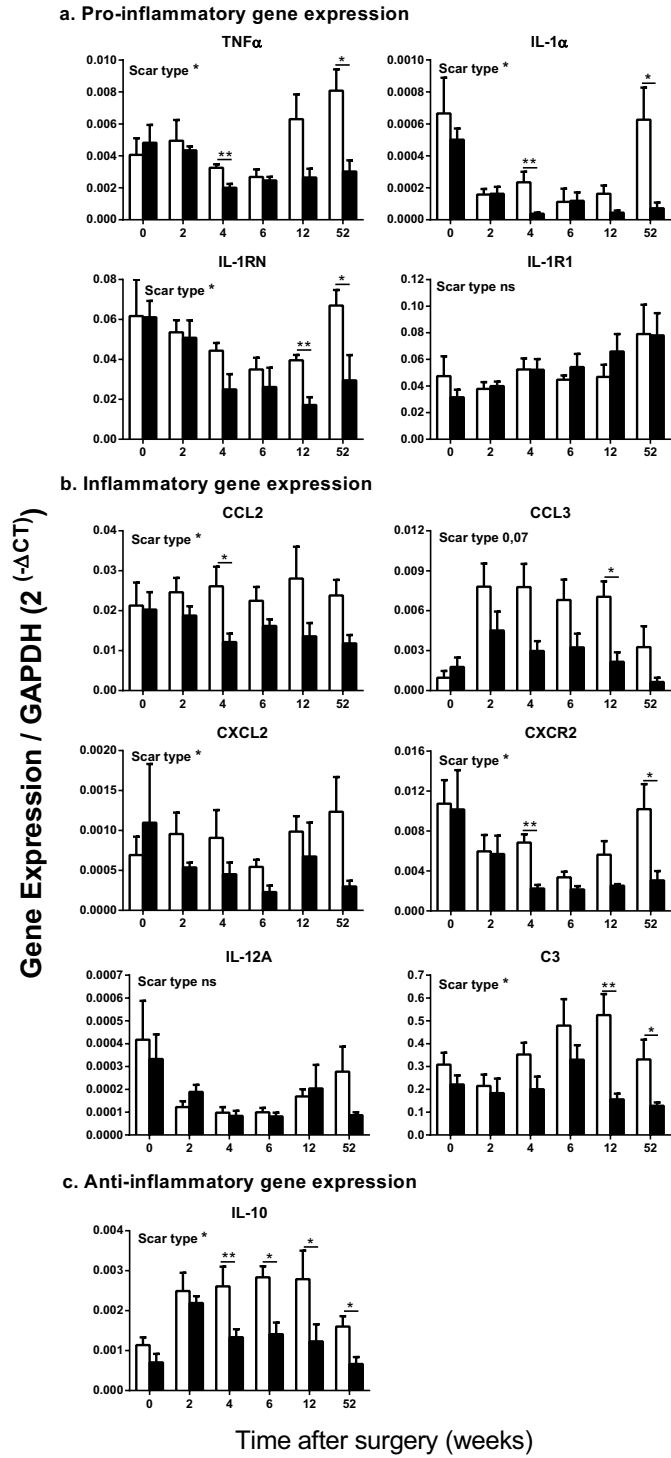


Figure 2. Inflammatory genes are decreased during HTscar formation compared to NTscar formation. Expression of mRNA of (a) TNF α , IL-1 α , IL-1RN, IL-1R1, (b) CCL2, CCL3, CXCL2, CXCR2, IL-12A, C3 and (c) IL-10 normalized to GAPDH expressed as $2^{-\Delta\Delta Ct}$ during surgery (time point 0) and 2, 4, 6, 12, and 52 weeks after surgery. Significance between NTscar (white bars) and HTscar (black bars) groups by Wilcoxon-Mann-Whitney test during entire study period (area under the curve) is shown in top left corner of each graph and Mann-Whitney test for separate time points within the graph is shown (* $p < 0.05$ and ** $p < 0.01$)

Angiogenic gene expression during hypertrophic and normotrophic scar formation

Since the formation of new blood vessels is essential for wound healing and tissue regeneration we next investigated the expression of genes involved in vessel formation (Figure 3)¹⁸. MRNA levels for ANGPT1 (vessel stabilizing), FGF2 (pro-angiogenic and mitogen, trend) and YWHAZ (cell cycle regulator) were decreased in HTscar compared to NTscar formation whereas the antagonistic ANGPT2 (vessel destabilizing) was increased in both HTscar and NTscar formation during the 52 week study period. MRNA levels for

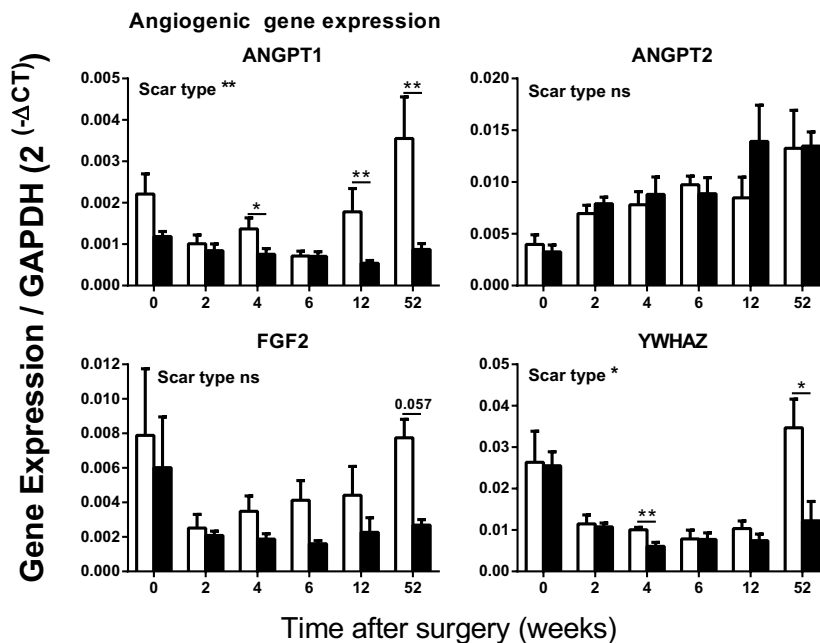


Figure 3. Differential angiogenic gene expression during NTscar formation compared to HTscar formation. Expression of mRNA of Angiopoietin-1, Angiopoietin-2, FGF2 and YWHAZ normalized to GAPDH expressed as $2^{-\Delta\Delta Ct}$ during surgery (time point 0) and 2, 4, 6, 12, and 52 weeks after surgery. Significance between NTscar (white bars) and HTscar (black bars) groups by Wilcoxon-Mann-Whitney test during entire study period (area under the curve) is shown in top left corner of each graph and Mann-Whitney test for separate time points within the graph is shown (* $p < 0.05$ and ** $p < 0.01$). No significant different expression was found for FGF2 between HTscar en NTscar group but probably this was due to that it was only tested in 3 : 4 patients forming an NTscar and HTscar respectively). Data of Angiopoietin-1 and Angiopoietin-2 are previously published in other format¹⁸.

VEGF, a strong pro-angiogenic factor remained unaltered. Taken together, these results indicate that vessel stabilizing and stimulating factors decreased or remained unaltered during HTscar formation whereas vessel disrupting factors increased in both groups.

Genes involved in extracellular matrix formation during hypertrophic and normotrophic scar formation

In contrast to most genes involved in inflammation and angiogenesis, mRNA levels for ECM related genes were clearly increased in both the HTscar group and NTscar group (Figure 4). For Col3A1, TGF β 3 and PLAU the expression remained high in HTscar over the entire 52 weeks period whereas in NTscar mRNA levels began to normalize with time resulting in significantly higher expression in HTscar compared to NTscar 52 weeks postoperatively. TGF β 1, TGF β R1 and Col1A2 remained elevated to a similar extent in both the HTscar and NTscar. Taken together these results indicate extended ECM gene expression in HTscar compared to NTscar formation.

Immunofluorescence staining of HTscar and NTscar

To further support our findings obtained by real time RT-PCR and to gain information on the epidermal and dermal location of proteins expressed in scars, immunofluorescent staining of tissue sections obtained from HTscar and NTscar biopsies was performed for CCL2, FGF2, TGF β 3 and PLAU (Figure 5a&b). By quantifying the amount of fluorescent staining in the tissue sections it was found that CCL2 protein was not significant different expressed in the epidermis of HTscar and NTscar, although a trend was observed. FGF2 protein expression was significantly lower in the epidermis of HTscar compared to NTscar. In further agreement with our RT-PCR data and in contrast to CCL2 and FGF2, TGF β 3 was higher in the epidermis of HTscar compared to NTscar. This expression was particularly located in the basal layer of epidermis. No difference were observed in epidermal PLAU protein expression between HTscar and NTscar. No difference in dermis protein expression was detected between HTscar and NTscar for any of the studied proteins. Taken together, these results support our observations that inflammatory and pro-angiogenic/mitogenic factors are suppressed in HTscar compared to NTscar formation and ECM factors are increased in HTscar compared to NTscar.

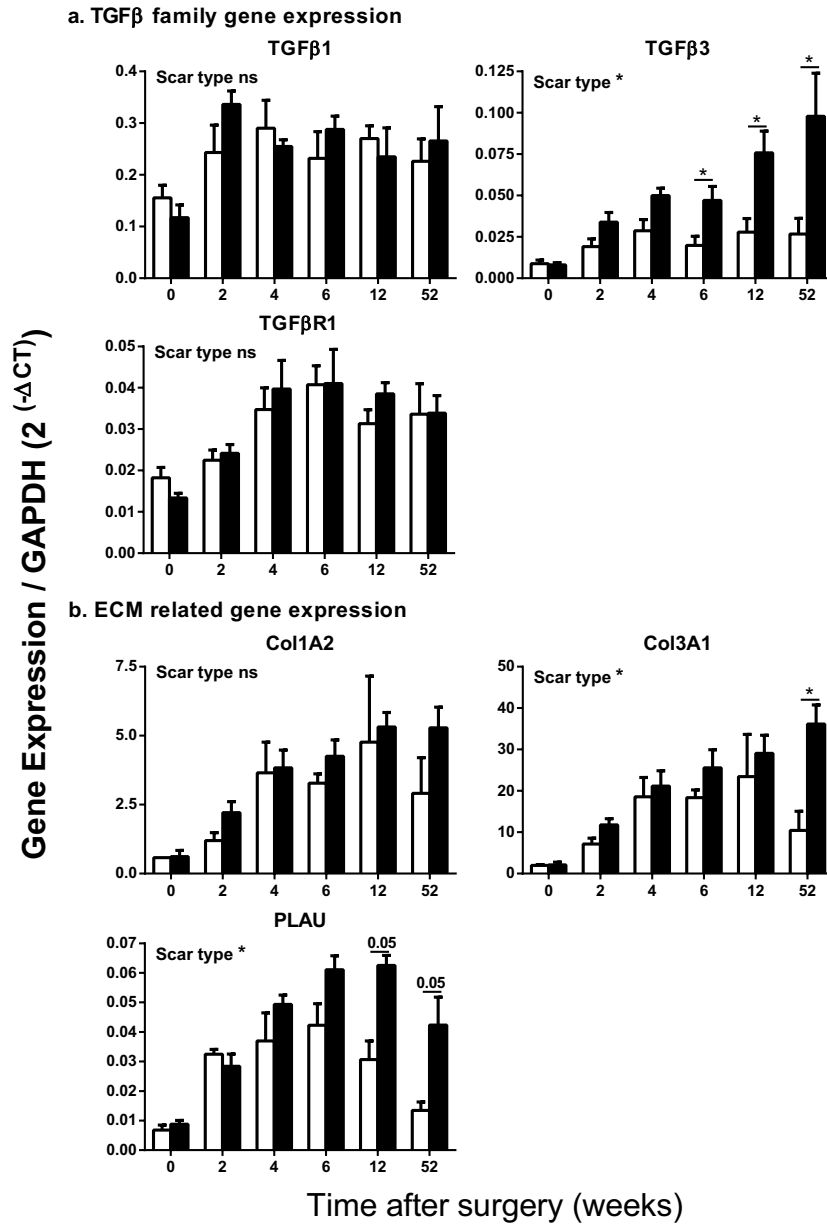


Figure 4. Extracellular matrix related gene expression is increased during HTscar formation compared to NTscar formation. Expression of mRNA of (a) TGF β 1, TGF β 3, TGF β R1, (b) Col1A2, Col3A1 and PLAU normalized to GAPDH expressed as $2^{-\Delta CT}$ during surgery (time point 0) and 2, 4, 6, 12, and 52 weeks after surgery. Significance between NTscar (white bars) and HTscar (black bars) groups by Wilcoxon-Mann-Whitney test during entire study period (area under the curve) is shown in top left corner of each graph and Mann-Whitney test for separate time points within the graph is shown (* $p < 0.05$ and ** $p < 0.01$)

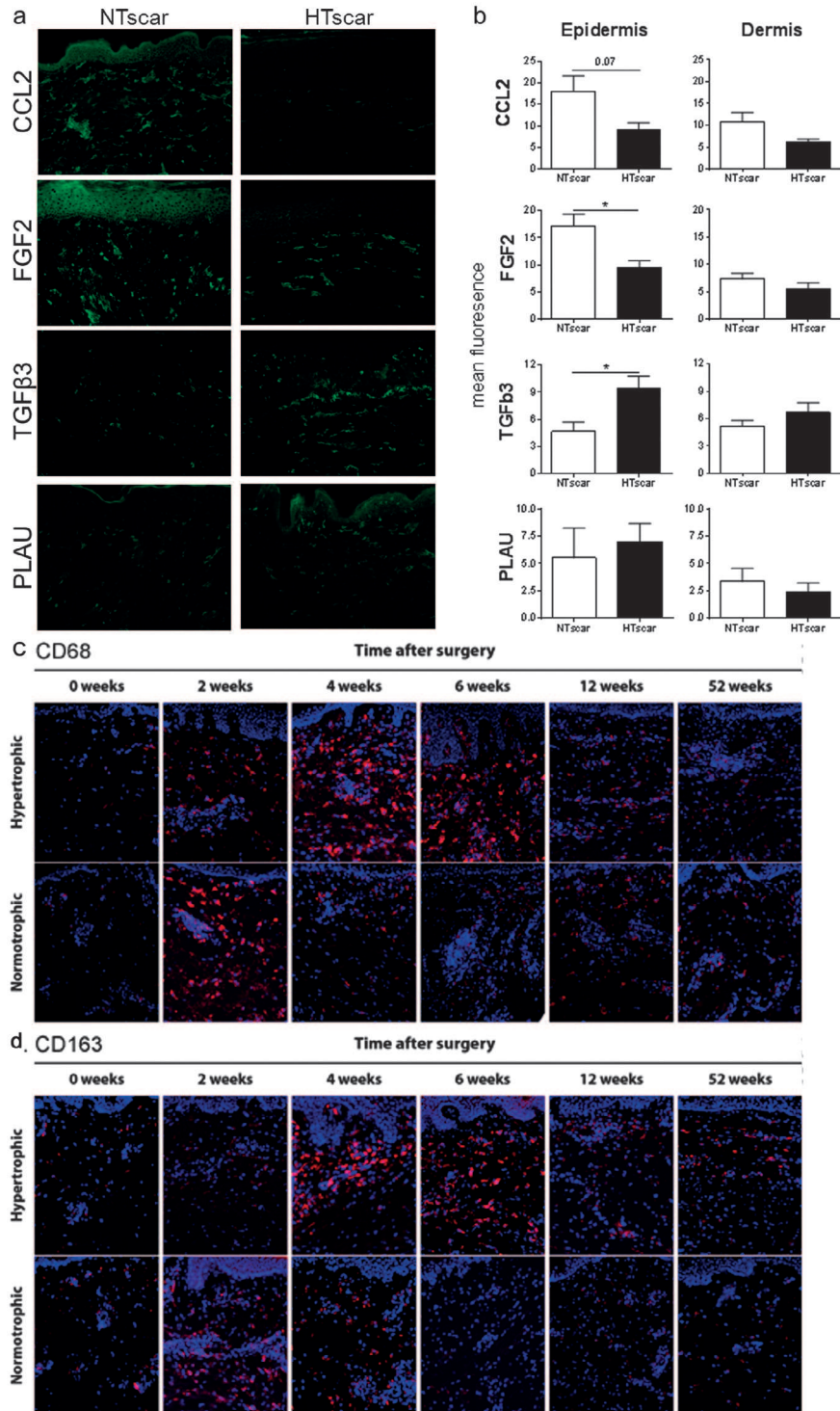


Figure 5. Immunofluorescence staining of NTscar and HTscar tissue samples. (a) CCL2, FGF2, TGF- β 3 and PLAU immunofluorescence stainings of NTscar and HTscar tissue (b) Quantification of CCL2, FGF2, TGF- β 3 and PLAU immunofluorescence stainings of NTscar and HTscar tissue. Data are presented as the mean ($n=6 \pm$ SEM) fluorescence of epidermis and dermis corrected for isotype background staining of corresponding scar sample. Statistical significant differences were calculated using Mann-Whitney t-test ($*p < 0.05$). (c) macrophage CD68 and (d) macrophage type 2 CD163 immunofluorescence staining on tissue collected during surgery (time point 0) and 2, 4, 6, 12, and 52 weeks after surgery of one representative patient of NTscar group and one representative patient of HTscar group (6 NTscar and 5 HTscar forming patients were stained).

Immuno fluorescence staining of macrophage markers during HTscar and NTscar formation

To further determine whether the differences in cytokine and growth factor expression during NTscar and HTscar formation also resulted in a different infiltration rate of immune cells, tissue sections were immunostained with CD68 (pan-macrophage marker) and CD163 (macrophage marker linked to type 2 inflammatory response) (Figure 5c&d). In the NTscar group an increased number of both CD68+ and CD163+ cells was observed 2 weeks after surgery. Four weeks after surgery, the number of CD68+ and CD163+ cells had already decreased to base line levels. In contrast during HTscar formation, the number of CD68+ and CD163+ cells within the tissue did not increase until 4 weeks postoperatively and then remained elevated until at 6 weeks postoperatively. These results show that macrophage infiltration, in particular macrophage type 2, show a delayed and slightly prolonged infiltration in HTscar compared to NTscar.

DISCUSSION

In this study we investigated the differences in dynamics of wound healing that led to the formation of HTscar rather than NTscar in a group of patients undergoing standard surgical procedures. This gave us the opportunity to study scar formation in a standardized manner (type of trauma and location). It was found that HTscar formation coincided with a prolonged decrease in expression of inflammatory and angiogenic genes and an extended increased expression of ECM related genes. This coincided with a delayed but prolonged infiltration of macrophage (type 2 cells) in HTscar tissue compared to NTscar tissue.

Directly upon cutaneous injury the wound healing process is initiated by an inflammatory response. Inflammatory chemokines and cytokines are produced by platelets, injured skin residential cells and infiltrating immune cells resulting in an amplification of the response^{3,4}. Here we show that inflammatory mediators are clearly lower during HTscar formation compared to NTscar formation. This might influence the rate of infiltration of immune cells. Indeed we found positive cells for pan-macrophage marker CD68

and macrophage scavenger receptor CD163 (linked to M2) only at 4-6 weeks postoperatively in HTscar forming patients compared to 2 weeks post operatively in NTscar forming patients. This suggests that the time of arrival of the macrophages is important in scar formation.

When looking in more detail, it was found that both pro-inflammatory and anti-inflammatory cytokine mRNA levels are reduced during HTscar formation compared to NTscar formation. Pro-inflammatory cytokines IL-1 α and TNF α , which are released by keratinocytes and several types of immune cells directly upon skin trauma, have been described to play a central role in initiating inflammation^{4,21}. Since these cytokines are reduced during HTscar it suggests that the inflammatory response at the start of HTscar formation is suppressed compared to that in NTscar formation. These findings are in line with previously reported decreased levels of IL-1 α and TNF α in HTscars compared to NTscars^{22,23}. C3, in the skin mainly produced by keratinocytes²⁴, is described to be important in regulating skin inflammation by attracting immune cells and is found to be increased in inflammatory skin diseases (e.g. psoriasis and atopic dermatitis)²⁴. Inflammatory mediators CCL2 and CCL3 are also described to be important in attracting monocytes⁵. Our finding that these chemokines are suppressed in HTscar compared to NTscar is in line with our observation that macrophage infiltration is delayed in HTscar. Anti-inflammatory cytokine IL10 is produced by monocytes (especially M2) and lymphocytes during a T helper-2 (TH2) response, which is linked to fibrosis⁷. In contrast, we found decreased IL-10 during HTscar compared to NTscar formation, which is in line with the findings of Henderson et al²⁵ and Prevascar (use of hr-IL10) clinical trial phase two for reduction of scarring in the skin (<http://www.renovo.com/fr/news/prevascar-phase-ii-results>). The decreased IL-10 expression during HTscar formation is in line with our delayed infiltration of CD163 positive cells (likely M2 macrophages) during HTscar formation.

The failure of immunosuppressive therapies to improve scar formation can be partly explained by our results that both pro-inflammatory and anti-inflammatory genes were decreased during HTscar compared to NTscar formation. Taken together our results may imply that reduced initial inflammation results in a locally suppressed but prolonged inflammation in HTscar compared to NTscar. A similar phenomenon is also seen in HTscars formed after deep burn injury. These burn patients are described to have a suppressed immune system directly after burn injury^{26,27} and to have HTscar frequency of up to 91% after deep burn injury²⁸.

Blood vessel protective and stimulating factors (ANGPT1 and FGF2) decreased during HTscar formation whereas the vessel disrupting factor ANGPT2 increased in both groups. Also PLAU, involved in ECM degradation and thereby promoting endothelial migration, was elevated in HTscars at 12 and 52 weeks post operatively, which may cause more microvessel formation. In line with this, we showed previously that during HTscar forma-

tion a significant increase of the number of microvessels occurred at 12 and 52 weeks post-surgery compared to NTscar formation¹⁸. However potent angiogenic factors VEGF was not differently expressed. The decreased expression of FGF2 is in line with findings of Eto et al, who showed that FGF2 may have therapeutic potential for the treatment of HTscars by up regulation of MMP-1 and HGF¹².

Interestingly YWHAZ, an adapter protein which activates, stabilizes or inactivates proteins involved in cell cycle/apoptosis^{29,30} and which is used as a housekeeping gene (e.g. wound healing)³¹, is differently expressed during HTscar and NTscar formation. Due to the multitude of binding partners for YWHAZ, a specific definition of its cellular role or function is difficult to define, but the differential expression observed between NTscar and HTscar group deserves further attention.

The TGF- β family plays a critical role in regulating multiple cellular responses that occur during cutaneous wound healing^{32,33}. In particular, the role of TGF β 1 in the induction of ECM production by fibroblasts and the differentiation of fibroblasts into myofibroblasts is linked to abnormal scarring^{7,34}. Surprisingly TGF β 1 was not differently expressed during HTscar formation compared to NTscar formation in our study group. This discrepancy with the literature could be due to the use of animals in other studies, since it has been shown to be difficult to extrapolate results between animals and humans with respect to scar formation¹⁰. In agreement with our results, Hakvoort et al showed in human skin biopsies that no difference in protein expression of TGF β 1 in HTscars occurred compared to NTscars³⁵. Interestingly, TGF β 3 (thought to be anti-fibrotic^{36,37}) was increased in the HTscar group compared to the NTscar group. In agreement with Hakvoort et al we found an increased immune-staining of TGF β 3 in the epidermis of HTscars³⁵. Our results explain the failure of phase 3 clinical trial of Juvista for scar reduction using hr-TGF β 3 (<http://www.renovo.com/en/news/juvista-eu-phase-3-trial-results>). We found that TGF β 3 is mainly expressed in the epidermis of NTscar compared to HTscar whereas Honardoust et al suggest that reduced TGF β 3 in deep dermis leads to hypertrophic scarring³⁷. This suggest that the location of TGF β 3 (which cells types are in the vicinity) is crucial for its pro- and anti-fibrotic function.

Taking in account the number of genes differentially expressed in HTscar compared to NTscar formation it is not surprising that a single therapeutic is not effective in reduction of scar formation³⁸. Our data suggests that HTscar when compared to NTscar formation seems to be related to a prolonged decreased expression of inflammatory and angiogenic genes and an extended prolonged increased expression of ECM related genes. Therefore instead of the currently used anti-inflammatory scar therapies, our results suggest that possibly innate immune stimulatory therapies may prove more beneficial for improving scar quality. Importantly, human cutaneous wound healing is a dynamic process that needs to be studied over a period of time if we want to identify the mechanisms involved in abnormal hypertrophic scar formation.

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SUPPLEMENT CHAPTER 2

Supplement 1: Function of genes in cutaneous wound healing

Abbrev.	Full Name	Function	Secreted by or expressed on	Target
ANGPT1	Angiopoietin 1	Ang (vasoprotective); anti-Inflam ^{1,2}	Perivascular cells (pericytes)	EC
ANGPT2	Angiopoietin 2	Antagonist ANGPT1; Ang (vessel destabilizing) ¹	EC (mainly tip cells)	EC
B2M	β -2 microglobulin	Component of MHC class1 molecule; Housekeeping gene	Nearly all nucleated cells	Component MHC-I (T cell)
C3	Complement component 3	Inflam (innate immune system); chem. for E, MC, Mo, leuk. ³⁻⁶	Hepacytes (main) and nearly all cells	C3a binds to C3Ra on N, Ba, E, MC, T, B, Mo, Ma, DC, EC, KC
C5	Complement component 5	Inflam (innate immune system); Chem. for Mo. +N. ³⁻⁵	Hepacytes, MC, Fb	N, MC, Mo, DC
CCL2	Monocyte chemotactic protein-1	Inflam; Chem.; pro-Ang; Epith; ⁷⁻⁹	PI, MC, T, Mo, KC, EC, Fb, SMC	Ba, NK, T, Mo, DC, EC (CCR2,4)
CCL3	Macrophage inflammatory protein-1 α	(acute-)inflam ¹⁰	N, NK, MC, T, B, Mo, Ma, DC, KC, Fb	N, Ba, E, NK, T, Mo, DC, Fb (CCR1,4,5)
CCL5	Rantes	Inflam; Ang; Mit; Chem. for Stem cells ^{11,12}	PI, N, MC, Mo, DC, KC, EC, Fb	N, Ba, E, NK, T, Mo, DC, KC, Fb (CCR1,3,5)
CCL22	Macrophage-derived chemokine	Inflam ¹³	Ma, DC, KC	NK, T, Mo, DC, KC, Fb (CCR4)
Col1A2	Collagen alpha-2(I)	Component ECM	Fb, SMC	Component ECM
Col3A1	Collagen alpha-1(III)	Component ECM	Fb	Component ECM
CXCL2	Macrophage inflammatory protein-2 α	Inflam; pro-Ang ^{9,14,15}	Mo, Ma, KC, Fb	N, Ba, E, Me, EC (CXCR2)
CXCL3	Macrophage inflammatory protein-2 β	Inflam; pro-Ang ^{9,14,15}	Mo, Ma, KC, EC, Fb	N, Ba, T, Mo, Me, KC, EC, Fb, (CXCR2)
CXCL8	Interleukin-8	Inflam; pro-Ang; Epith; Fib; Gran ^{8,9,14}	PI, N, MC, Mo, Ma, KC, Fb	N, Ba, T, KC, EC, Fb (CXCR1,2)
CXCL10	Interferon gamma-induced protein 10	Inflam; anti-Ang ^{8,9,14}	T, Mo, Ma, DC, KC, EC, Fb	NK, T, EC, Fb (CXCR3)
CXCR2	Interleukin 8 receptor, beta	Inflam; pro-Ang ^{8,9,14}	N, Ba, E, T, Mo, KC, EC, Fb	Binds CXCL1, 2, 3, 5, 6, 7, 8
FGF2	Basic fibroblast growth factor	pro-Ang; Epith; Mit; TR; Gran ¹⁶	Mo, KC, EC, Fb, adi	KC, EC, Fb, SMC (FGFR2b)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Glycolysis; Housekeeping gene	Nearly all cells	Converts glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate

Abbrev.	Full Name	Function	Secreted by or expressed on	Target
IL1 α	Interleukin 1 α	Pro-Inflam; Mit; Hematopoeises ¹⁶	PI, N, Ma, KC, EC, SMC	N, T, B, Fb, KC, EC, SMC (IL1R1&2)
IL1R1	Interleukin 1 receptor, type I (CD121a)	Inflam ¹⁶	N, T, B, KC, EC, Fb, SMC	Binds IL1 α , IL1 β , IL1RN
IL1RN	Interleukin-1 receptor antagonist	Inflam ¹⁷	N, Ma, Mo, KC, EC, adi	IL1R1&2 (prevent binding IL1 α and IL1 β)
IL-10	Interleukin-10	Anti-Inflam; anti-fibrotic ^{18,19}	T, B, Mo, Ma, KC	NK, B, T, Mo, Ma, DC, KC, EC, Fb
IL-12A	Interleukin-12 subunit α	Inflam ²⁰	N, B, Mo, Ma, DC, KC	NK, T, KC
IL-12B	Interleukin-12 subunit β	Inflam ²⁰	N, B, Mo, Ma, DC, KC	NK, T, KC
PECAM1	Platelet endothelial cell adhesion molecule / CD31	Inflam; Ang (cell adhesion, activation, migration) ²¹	PI, N, NK, T, B, Mo, Ma, EC	CD38, GAGs
PLAU	Urokinase-type plasminogen activator	Degradation ECM ²²	N, NK, Mo, KC, EC, Fb	Converts plasminogen to plasmin (Act. by binding to uPAR)
SELE	E-selectin / CD62 antigen-like family member E	Inflam; Neovasc ^{23,24}	EC	Ligands are expressed by E, N, NK, T, Mo
TGF-beta1	Transforming growth factor- β 1	(anti-)Inflam; MyoFibs; ECM; Gran; TR ²⁵⁻²⁷	PI, T, Mo, Ma, KC, EC, Fb	N, NK, MC, T, B, Ma, Mo, DC, KC, EC, Fb
TGF-beta3	Transforming growth factor- β 3	ECM; Gran; TR ^{27,28}	PI, Mo, Ma, KC, Fb	KC, EC, Fb
TGF-betaR1	Transforming growth factor- β receptor I	Inflam; ECM; Gran; TR (receptor TGF β 1-3) ²⁵⁻²⁸	N, NK, MC, T, B, Ma, Mo, DC, KC, EC, Fb	Binds TGF β 1, 2 and 3
TNF-alpha	Tumor necrosis factors alpha	Pro-Inflam; Mit ²⁹	N, MC, NK, T, B, Ma, Mo, Fb, KC, EC	N, E, T, B, Ma, Mo, DC, KC, EC, Fb
VEGF	Vascular endothelial growth factors	Ang; Mit for EC ³⁰	PI, N, Mo, Ma, KC, EC, Fb, SMC	N, E, Ba, Mo, Ma, EC, SMC, Fb
YWHAZ	14-3-3 protein zeta/delta	Adapter protein: mediates signal transduction (cell cycle/apoptosis) ³¹	Nearly all cells	Modulates activity of the binding partner

Abbrev, abbreviation; Act., activated; Adi, adipocytes; Ang, angiogenesis; Ba, basophil; B, B-lymphocytes; BMDSC, bone marrow derived stem cell; Chem, chemotactic; Collagen, collagen production; DC, dendritic cell; E, eosinophil; EC, endothelial cell; ECM, extracellular matrix; Epi, epithelial cell; Epith, epithelization; GAG, Glycosaminoglycan; GF, growth factor; Gran, stimulates granulation tissue formation; Inflam, inflammatory mediator; KC, keratinocytes; Leuk., Leukocytes; Ma, Macrophage; MC, mast cell; Me, melanocyte; Mit, mitogen; Mo, monocyte; MSC, mesenchymal stromal derived cells; MyoFibs, involved conversion fibroblasts to myofibroblasts; N, neutrophil; Neovasc., Neovascularization; NK, natural killer cell; PI, platelets; SMC, smooth muscle cell; T, T-lymphocyte; TR, tissue remodelling

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