Role of TGFβRII in myeloid cell mediated regenerative processes and fibroplasia

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Summary

Tissue repair and fibrosis are controlled by the interaction of different cell lineages, their soluble factors and matrix signals. Recently, macrophages have been found to be crucial for proper tissue repair. In particular, the role of Transforming growth factor-β1 (TGF-β1) has been extensively studied during tissue repair and fibrosis. Fibrosis is characterized by excessive production and deposition of extracellular matrix, as well as immune cell infiltration. Macrophages are one of the main sources of TGF-β1. So far, studies on the mechanisms of tissue repair and fibrosis have mainly focused on macrophages or TGF-β1 individually. However, the specific function of TGF-β1 on macrophages in tissue repair and fibrosis still needs to be elucidated.

To understand the macrophage specific role of TGFβ1-TGFβRII signaling in tissue repair and fibrosis, we generated a mouse model, which lacks TGFβRII in myeloid cells (TGFβRII$^{fl/fl}$/LysMCre). We observed that during mechanical tissue injury TGFβRII signaling in macrophages contributes to wound contraction, possibly by cross—talk between macrophages and fibroblasts. The attenuated wound contraction was accompanied by impaired myofibroblast differentiation and collagen deposition. However, the loss of TGFβRII signaling in macrophages did not lead to reduced expression of TGF-β1, which we proposed as one of the primary mechanisms in wound tissue underlying reduced myofibroblast formation observed in TGFβRII$^{fl/fl}$/LysMCre mice.

Generation of cutaneous fibrosis by bleomycin injection for two and four weeks resulted in reduced fibrosis in TGFβRII$^{fl/fl}$/LysMCre mice, compared to control mice. The mechanisms leading to this phenotype were associated with reduced infiltration of immune cells, reduced deposition of collagen and diminished production of inflammatory mediators such as IL-1β, TNF-α and osteopontin-1 at the early stage of fibrosis formation. At the later stage, the expression of inflammatory mediators in TGFβRII$^{fl/fl}$/LysMCre mice was not altered compared to control mice, possibly due to compensatory mechanisms. Our data leads to the hypothesis that the reduced fibrosis is caused by the reduced expression of inflammatory mediators and accumulation of immune cells at the early stage of fibrosis in TGFβRII$^{fl/fl}$/LysMCre mice.

Our results provide new insights into the crucial role of macrophage specific TGFβRII signaling in tissue repair and fibrosis.

Zusammenfassung

Die Gewebereparatur, als auch die Fibrose sind durch die Interaktion unterschiedlicher Zellen zueinander, löslicher Faktoren und über den Kontakt zur extrazellulären Matrix reguliert. Makrophagen sind wichtige Regulatoren in der physiologischen und pathologischen Wundheilung. TGF-β1 gilt als zentraler Wachstumsfaktor zur Regulation epidermaler und mesenchymaler Reparaturprozesse. Die Entstehung von Fibrose ist maßgeblich durch eine Fehlregulation der Homöostase der extrazellulären Matrix und der Infiltration von Immunzellen charakterisiert, wobei Makrophagen eine entscheidende Rolle zu spielen scheinen. Bisher ist über die Bedeutung von TGF-β1 auf die Makrophagenfunktion in der Wundheilung wenig bekannt.

Um dieser Fragestellung nachzugehen, wurde ein Mausmodell generiert in dem TGFβRII spezifisch in myeloiden Zellen deletiert wird (TGFβRII/LysMCre). Anhand dieses Modells konnten wir zeigen, dass die TGFβRII vermittelte Signalkaskade in Makrophagen maßgeblich zur Wundkontraktion beiträgt. Diese wird möglicherweise durch eine parakrine Interaktion von Makrophagen und Fibroblasten vermittelt. Die veränderte Wundkontraktion in TGFβRII/LysMCre Mäusen geht einher mit einer beeinträchtigten Myofibroblastendifferenzierung und einer verminderten Kollagensynthese. Die Deletion des TGF-β1 Rezeptors in Makrophagen führt nicht zu einer verminderten Expression von TGF-β1 im Wundgewebe von TGFβRII/LysMCre Tieren, was möglicherweise den beschriebenen Phänotypen hätte erklären können.

Die Bleomycin-induzierte Hautfibrose führt in TGFβRII/LysMCre Mäusen zu einer signifikant verminderten Fibrosereaktion. Die Tiere weisen nach zweiwöchiger Injektion ein verringertes Entzündungsfiltrat, eine verminderte Kollagenablagerung, als auch deutlich weniger entzündliche Mediatoren einschließlich IL-1β, TNFα und Osteopontin auf. Wird Bleomycin über einen längeren, vierwöchigen Zeitraum injiziert ist die Entzündungsreaktion mit der der Kontrolltiere vergleichbar. Möglicherweise spielen dabei kompensatorische Mechanismen eine Rolle.

Zusammenfassend liefert unsere Untersuchung einen Hinweis darauf, dass der TGFβ1 Signalweg in Makrophagen während der Wundheilung und in der Fibrosebildung entzündungsstimulierend und profibrotisch wirkt.

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1 Introduction

1.1 Skin: structure and functions

Skin is a compartmentalized multicellular organ of the body, containing several specialized cells and functional structures. It has many functions. The most important function is to form a barrier to the environment, allowing and limiting the inward and outward passage of water, electrolytes and various substances while providing protection against pathogens, UV radiation, toxic agents and mechanical insults. It is composed of two primary layers i.e., epidermis and dermis, which interacts anatomically and functionally. Epidermis and dermis is separated by a lining of basement membrane. Epidermis forms a thin protective layer which is easily regenerated after injury and maintains moisture inside the body. Dermis is the thick layer which gives strength to the skin and contains blood vessels, nerves and adnexal structures like hair follicles, sweat glands, and sebaceous glands.

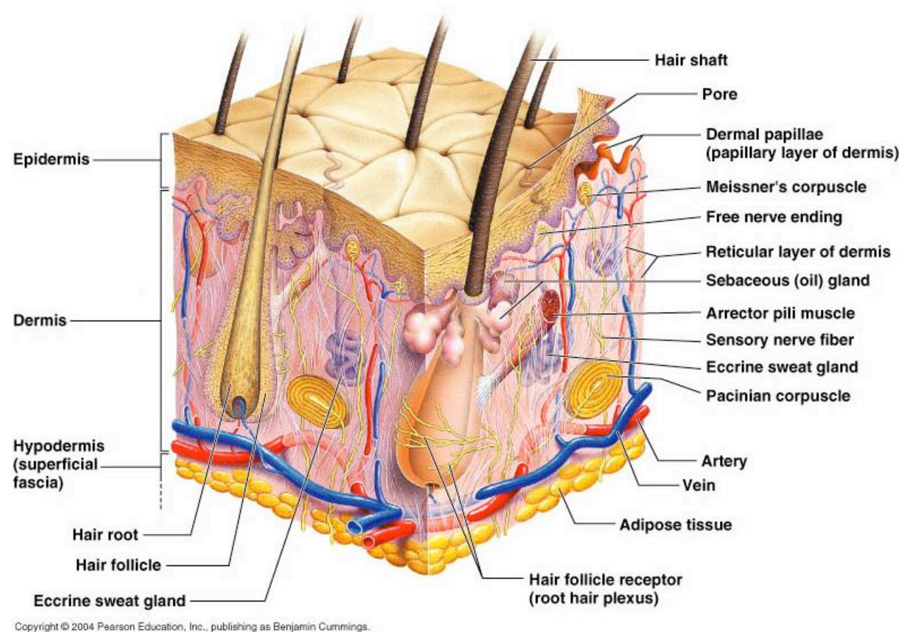


Fig. 1-1: Schematic organization of the skin and its components (Image courtesy Pearson Education Inc.)

Epidermis

The epidermis is the most outermost layer of skin, which provides protective functions and expresses various proteins. Keratinocytes are the building blocks of epidermis and comprise the bulk of this layer (Lee, Jeong et al. 2006). Several inflammatory factors like

prostaglandins, eicosanoids, leukotrienes, histamines and cytokines are synthesized and secreted from the epidermal keratinocytes, which regulates the immune responses (Albanesi, Scarponi et al. 2005).

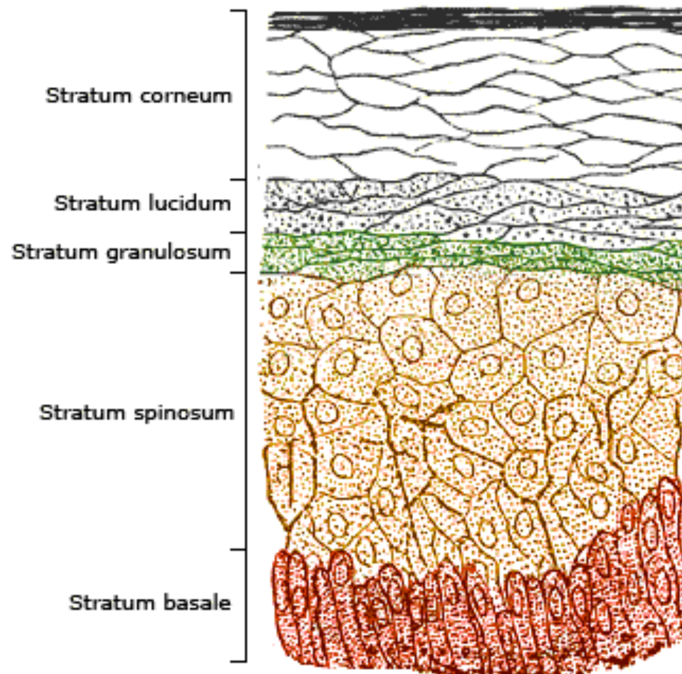


Fig. 1-2: Different layers of the epidermis

The epidermis forms four different layers namely, stratum basale, stratum spinosum, stratum granulosum and stratum corneum (Fig. 1-2). The stratum basale layer of the epidermis is mainly proliferating keratinocytes, which proliferate and then migrate towards outer layer of the skin, which leads to the formation of stratum spinosum. Proliferation and migration of keratinocytes are followed by differentiation by increasing cell-cell adhesion and modulation of cytoskeletal proteins such as keratins (Tseng, Jarvinen et al. 1982; Stoler, Kopan et al. 1988). Immune sentinels like Langerhans cells and other active cells derived from bone marrow are found in all epidermis surfaces but are mainly found in stratum spinosum layer (Brouard and Barrandon 2003). Further, continuing their transition to the cell surface, the cells continue to get flattened and lose their nuclei and their cytoplasm appears granular, hence called stratum granulosum. Continuous movement of the cells results in formation of subsequent terminal differentiated layer called stratum corneum. The stratum corneum consists of hexagonal shaped nonviable cornified cells known as corneocytes. In most of the areas of skin there are 30 to 40 layers of stacked corneocytes, which are more in palms and soles. Each corneocyte is surrounded by the protein envelope and is filled with water retaining keratin proteins. The shape and orientation of the keratin proteins adds strength to the stratum corneum. The cornified

dead cells are continuously shed off and replaced by underlying cells and this whole process contributes to the natural protection of skin against water loss, ultraviolet radiation-induced damage as well as environmental and mechanial insults (Smack, Korge et al. 1994; Proksch, Brandner et al. 2008).

Dermis

The dermis mainly consists of cellular components such as macrophages, mast cells, dendritic cells, lymphocytes, fibroblasts, vascular channels and nerves surrounded by and embedded in an extracellular matrix network consisting of collagen bundles, elastins, fibronectins and proteoglycans. It is mainly 15 to 40 times thicker than epidermis. It consists of three layers namely: Papillary layer, subpapillary layer and reticular layer. The papillary layer projects into the intervals between the epidermal ridges. The fibre components are thin and richly supplied with capillaries, sensory nerve endings and cytoplasm. Subpapillary layer is located underlying epidermis and has same components as papillary layer. Reticular layer is the largest part of the dermis and consists of dense connective tissue composed of fibre components and the lower part of it comes in contact with subcutaneous fat layer. The extracellular matrix (ECM) is the largest component of the dermis, which provides tensile strength and elasticity to the skin. Collagen fibers comprise 70% part of the weight of the dry dermis. The main collagens of the dermis are collagen I and collagen III, which provides tensile strength to skin (Dale, Sherratt et al. 1996).

1.2 Cutaneous wound healing and cutaneous fibrosis

Impaired wound healing, defective regeneration as well as fibrosis of diverse tissues are leading causes of morbidity and mortality in the aged population. A detailed understanding of the complex molecular and cellular events underlying these processes is required to design new therapeutic approaches. Repair of damaged tissues after injury is a fundamental biological process, proceeding in tightly controlled phases, which are significantly affected by age.

1.2.1 Cutaneous wound healing

Cutaneous injury is followed by a physiological healing response aiming at the resolution of the tissue damage. This is a co-ordinated process of cell activation, migration and differentiation, which can be divided into three controlled, regulated and partially

overlapping phases namely: inflammation, proliferation and remodeling. Wound healing kinetics and the cells involved in the process are shown in Fig. 1-4.

1.2.1.1 Inflammatory phase

The inflammatory phase is initiated immediately after tissue damage by local activation of the innate immune system. This phase is characterized by the immediate influx of polymorphonuclear leukocytes (neutrophils), followed by invasion of blood monocytes which differentiate into tissue macrophages. This early response is generated to mount the host response against external injury or microbial invasion. At this stage, tissue macrophages and the recruited pool of blood monocytes get activated and act as phagocytes for dead cells and damaged cellular components such as tissue debris and ECM. The activated cells release several growth factors such as transforming growth factor (TGF-β), vascular endothelial growth factor-A (VEGF-A), platelet derived growth factors (PDGF), basic fibroblast growth factors (bFGF) etc. to promote the recruitment of various cell types, cell proliferation, angiogenesis and production of extracellular matrix (Werner and Grose 2003; Eming, Brachvogel et al. 2007). Thus, inflammation is required for normal wound healing process. However, an excess of inflammation is associated with impaired wound healing (Eming, Krieg et al. 2007), mainly characterized by persistent inflammation, excess deposition of ECM and scar formation and lead to the manifestation of chronic clinical problems such as formation of keloid, scleroderma and non-healing diabetic foot ulcer or venous ulcer (Fig. 1-3).

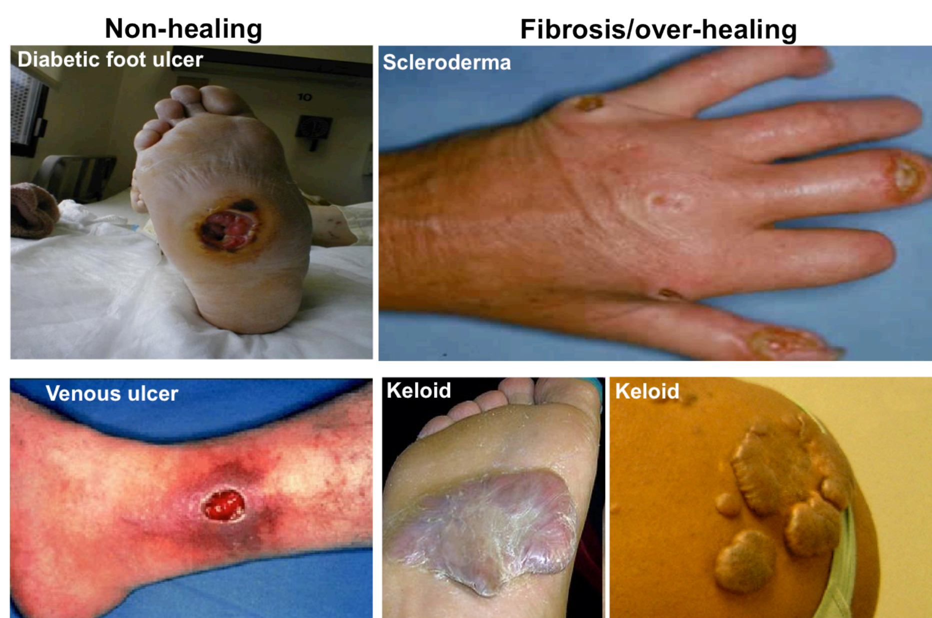


Fig. 1-3: Clinical problems of non-healing and over-healing wounds

1.2.1.2 Tissue regenerative phase/Proliferative phase

The early inflammation response is followed by the subsequent regenerative phase. The regenerative phase is characterized by transient and limited synthesis and deposition of connective tissue, formation of granulation tissue, migration of the keratinocytes (re-epithelialization), angiogenesis, proliferation and influx of fibroblasts, differentiation of fibroblasts into myofibroblasts. The process starts with the accumulation of macrophages to mediate diverse functions such as clearance of the dead cells and pathogens, and the recruitment and activation of other cell types. The recruited macrophages produce chemokines and cytokines, which then activate neighbouring cells such as fibroblasts and recruit other immune cells. The activated fibroblasts then undergo differentiation process and secrete various growth factors such as TGF-β, CTGF, bFGF and PDGF, which regulate proliferation and differentiation of fibroblast as feedback mechanism as well as are pre-requisite for the production of extracellular matrix and contraction of wound (Garrett, Khaw et al. 2004; Grotendorst and Duncan 2005). Deposition of extracellular matrix facilitates cell migration and adhesion. The whole process rebuilds the granulation tissue, which consists of collagens and other ECM into which a new network of blood vessels start to form under the process called neo-angiogenesis. While granulation tissue formation progresses, epithelial cells at the wound margin proliferate to reproduce new epithelial cells and grow to close the wound and restore the epidermal barrier function and the whole process is called re-epithelialization. At the same time, myofibroblasts present at the wound site, which has adhesion properties to ECM, pulls the matrix and minimize the wound area to be healed (Hinz 2006). Contraction of smooth muscle actin in myofibroblasts leads to contraction of the wound edges and finally myofibroblasts undergo apoptosis (Stadelmann, Digenis et al. 1998). This process signals fibroblasts to stop migrating and proliferating and also signals onset of the maturation phase of wound healing (Greenhalgh 1998). The entire process involves a complex epidermal-mesenchymal interaction of cells (Werner, Krieg et al. 2007).

1.2.1.3 Tissue maturation phase

After restoration of the barrier function, the repair process enters into late phase of remodeling. This phase is characterized by scar formation, cell response attenuation and apoptosis. In this stage of wound healing, the tissue matures to its final strength which is never achieved more than 80% of the pre-injury strength. The major cell types which plays role in this phase are macrophages and fibroblasts. The extracellular matrix bound growth factors and MMPs are activated by macrophages and fibroblasts, which results in the degradation of matrix and differentiation of cells (Streuli 1999). The collagen deposition

takes place by active participation of fibroblasts which secretes the collagen in the wound tissue area. Collagen is released in the form of pro-collagen, its precursor form. Pro-collagen is then formed into fibers and arranged in a parallel fashion cross-linked to each other forming thicker and stronger strands. There are substantial differences between the repaired tissue and non-injured skin. In this phase, the remodeling is achieved by the balance between the synthesis of new collagen and the degradation of the old. The deposition of newly formed connective tissue is not well anchored to the underlying connective tissue matrix and is thicker than the normal skin. The collagens at the wounds are reorganized and collagen III gets replaced by collagen I (Braiman-Wiksman, Solomonik et al. 2007). Collagen and extracellular matrix deposition continues in the wound during this stage. Density of cells like macrophages, keratinocytes, fibroblasts, and myofibroblasts is reduced by apoptosis. Firstly, keratinocytes undergo apoptosis followed by myofibroblasts. Finally, as the wound healing process gets switched off after going through remodeling process, the newly formed connective tissue get matured and the colour of the tissue changes from pinkish red to white (Russell 2000).

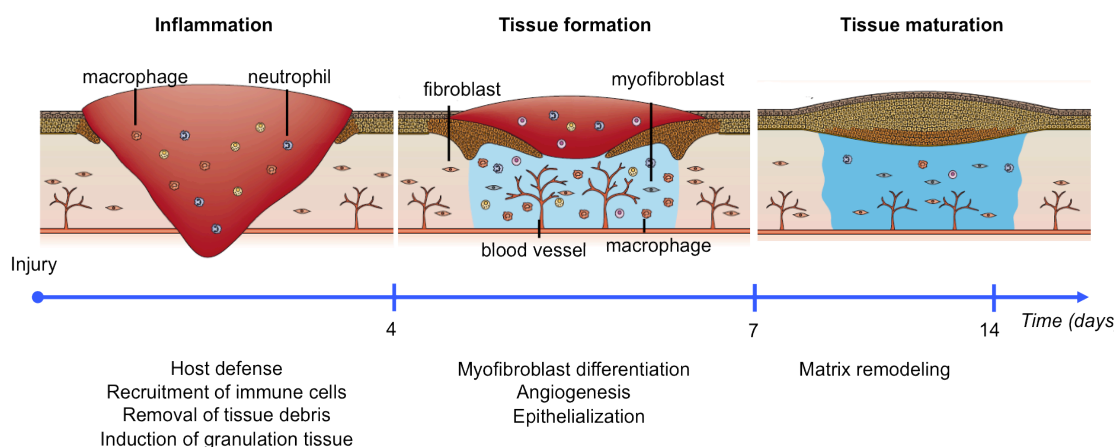


Fig. 1-4: Schematic representation of wound repair kinetics.
Wound healing kinetics starts with external injury leading to clot formation and recruitment of neutrophils and macrophages during inflammatory phase. After 3-4 days of inflammatory phase, keratinocytes proliferate and migrate to close the wound. Tissue formation phase also favors myofibroblast differentiation, blood vessels formation and deposition of extracellular matrices, followed by tissue maturation phase that results in scar formation (Schafer and Werner 2008).

1.2.1.4 Importance of the early pro-inflammatory and late anti-inflammatory phase for normal wound repair

During the process of tissue repair, the inflammatory response is dominated by innate immune cells such as neutrophils and macrophages while the mid proliferative and late remodeling phases are dominated by macrophages and epidermal-mesenchymal cells interaction (Werner, Krieg et al. 2007).

Although, the inflammatory response is the critical importance for the healing process, it has a potential to cause severe tissue damage. Permanent remodeling of the extracellular matrix (ECM) due to imbalanced and uncontrolled inflammation, results in increased function-impairment, fibrosis and scarring (Fig. 1-5). Keloids and hypertrophic scars are the example of skin fibrosis (Bock and Mrowietz 2002; Rahban and Garner 2003). Resolution of the inflammatory response is thus essential to terminate the healing process and to minimize scar formation. Cell-cell interactions among many different tissue resident (e.g. keratinocytes, fibroblasts) and non-resident (hematopoietic) cell types contribute to the physiological repair response and are involved in the pathology of repair and fibrosis. Hematopoietic cells with a prominent role in local innate immunity such as myeloid cells (macrophages and neutrophils) may be of particular importance in controlling the kinetics and the balance of pro- and anti-inflammatory signals regulating tissue repair and fibrosis. Fibroblasts are the central effectors of repair and fibrotic responses in ECM homeostasis.

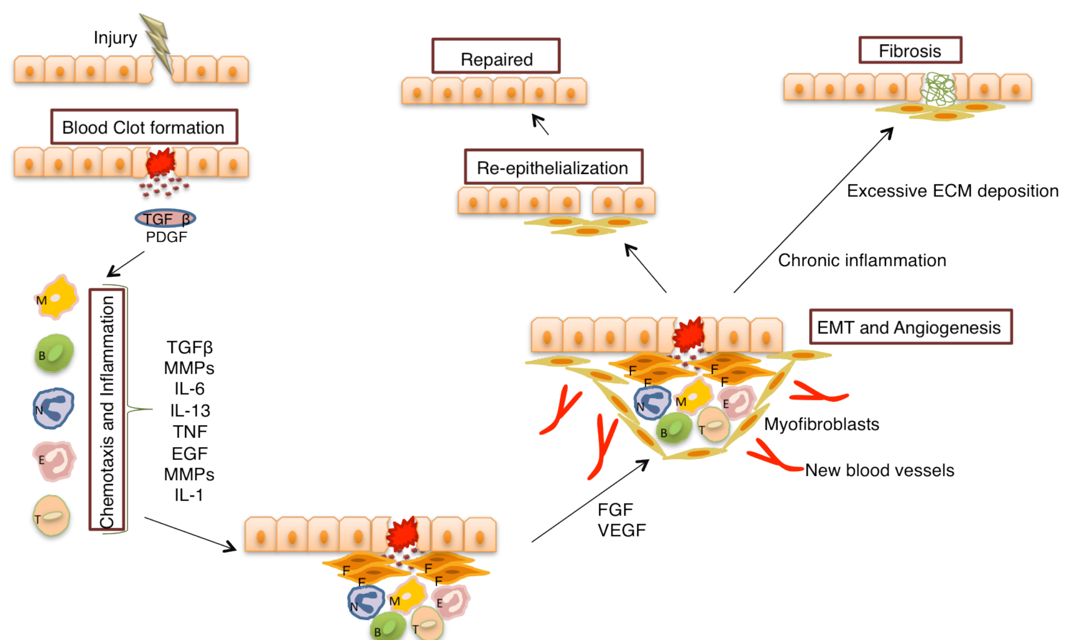


Fig. 1-5: Outcomes of tissue injury: physiological wound healing or fibrosis. Tissue injury is followed by the initiation of blood coagulation cascade which triggers blood clot formation. Platelets, which acts as a reservoir of growth factors and cytokines like PDGF and TGF-$\beta 1$, degranulates and releases these cytokines which act as chemo-attractant for inflammatory cells due to which cells like neutrophils, monocytes and other leukocytes are recruited at the injured site. These inflammatory cells secrete several growth factors and cytokines which help in the formation and restoration of the injured tissue. If the normal healing process gets uncontrolled, it leads to excessive repair process which leads to deposition of excessive ECM in the wound leading to fibrosis (Wynn 2007), modified.

1.2.2 Cutaneous fibrosis

Fibrosis is often viewed as a sustained repair response that has escaped control, resulting in excessive connective tissue deposition. It is characterized by increased accumulation of extracellular matrix, activation of fibroblasts and increased infiltration of immune cells such as monocytes/macrophages, mast cells and T-lymphocytes (Roumm, Whiteside et al. 1984; Kraling, Maul et al. 1995; Yamamoto 2006). The increased amounts of extracellular matrix found in the fibrotic tissues are mainly due to an imbalanced collagen remodeling. This could either be enhanced collagen synthesis and deposition or impaired collagen degradation. Activated and differentiated myofibroblasts are considered as the main cellular source of collagen production and deposition in the fibrotic tissue (Hinz 2007). Collagen synthesis and degradation is controlled by balance between matrix metalloproteinases (MMPs) and their inhibitors, tissue inhibitor of metalloproteinases (TIMPs) (Jinnin 2010).

Fibrotic tissue damage can result from various acute or chronic stimuli, including infection, autoimmune reactions, and mechanical injury. Repetitive injection of bleomycin in mice has also been shown to induce fibrosis (Yamamoto, Takagawa et al. 1999). In mouse models of pulmonary and cutaneous fibrosis, soluble mediators such as chemokines (CCL2 & CCL3) (Smith, Strieter et al. 1994; Smith, Strieter et al. 1995; Ferreira, Takagawa et al. 2006; Ishida, Kimura et al. 2007) and cytokines (IL-13, IL-33 etc.) (Rankin, Mumm et al. 2010) have been shown to contribute fibrosis . Several pro-fibrotic factors such as TGF-β and PDGFb, which are involved in the activation, proliferation and differentiation of fibroblasts into myofibroblasts, also contribute to the fibrosis development. Connective growth factor is another cytokine involved in fibrosis as it activates fibroblasts and extracellular matrix production (Frazier, Williams et al. 1996). It is induced by TGF-β and acts synergistically downstream to TGF-β in fibrosis induction. Moreover, injection of anti-CTGF has been shown to reduce TGF-β1 induced skin fibrosis (Chujo, Shirasaki et al. 2005; Ikawa, Ng et al. 2008). In human scleroderma patients, increased levels of IL-4, IL-6, IL-13 and TNF-α have been reported previously (Needleman, Wigley et al. 1992; Hasegawa, Fujimoto et al. 1997; Hasegawa, Fujimoto et al. 1997; Sato, Hasegawa et al. 2001).

1.3 Role of macrophages and fibroblasts in tissue repair, fibrosis and extracellular matrix production

Macrophages are considered as a master regulator of the tissue repair process. Studies in the 1970s showed that under non-infected conditions, the influx of macrophages was essential for efficient healing of incisional wounds in mice (Leibovich and Ross 1975). Further studies indicated that the reduced numbers of macrophages at the wound site resulted in impaired healing (Werner and Grose 2003; Eming, Krieg et al. 2007; Ishida, Gao et al. 2008). Recently, the role of macrophages during different phases of tissue repair has clearly been investigated by using the *iDTR* model under LysMCre promoter study (Lucas, Waisman et al. 2010). In the *iDTR* model Cre-inducible human diphtheria toxin receptor transgenic mouse line (*iDTR*) in which Cre-mediated excision of a STOP cassette renders, naturally diphtheria toxin (DT)-resistant mouse cells, DT sensitive. However, the exact role of diverse signaling pathway in macrophages during the repair process and its impact on the tissue repair has not been described.

Several chemokines, cytokines, growth factors and fibrotic factors such as CCL2, CCL3, GM-CSF, IL-6, IL-1β, TNF-α, TGF-α, FGF, TGF-β, PDGF and VEGF-α are expressed in wound tissue (Werner and Grose 2003). Considering that macrophages can produce most of the factors, which are modulated during the tissue repair, the role of macrophages during this process could be very diverse and could modulate the repair process in several ways. Macrophages derived chemokines such as CCL2, CCL3 can favor the recruitment of immune cells (Gillitzer and Goebeler 2001) and cytokines such as TNF-α, IL-1β and IL-6 can modulate the inflammatory process. TGFβ1 and PDGF are required for activation and differentiation of fibroblast into myofibroblasts, which is in turn an essential pre-requisite for matrix production and the contraction of collagen matrix (Clark 1993; Heldin and Westermark 1999; Schafer and Werner 2008) (Fig. 1-6). The ability of macrophages to produce pro-fibrotic factors such as TGFβ1 and PDGF, which can be used by fibroblast for activation and production of extracellular matrix, makes them a potential candidate for controlling collagen deposition during the repair process. Macrophages can also directly impact on extracellular matrix deposition and its turn over during tissue repair and fibrosis by secretion of matrix metalloproteinases (MMPs) as well as tissue inhibitor of metalloproteinases (TIMPs), which determines the rate of collagen turn over (Wynn and Barron 2010).

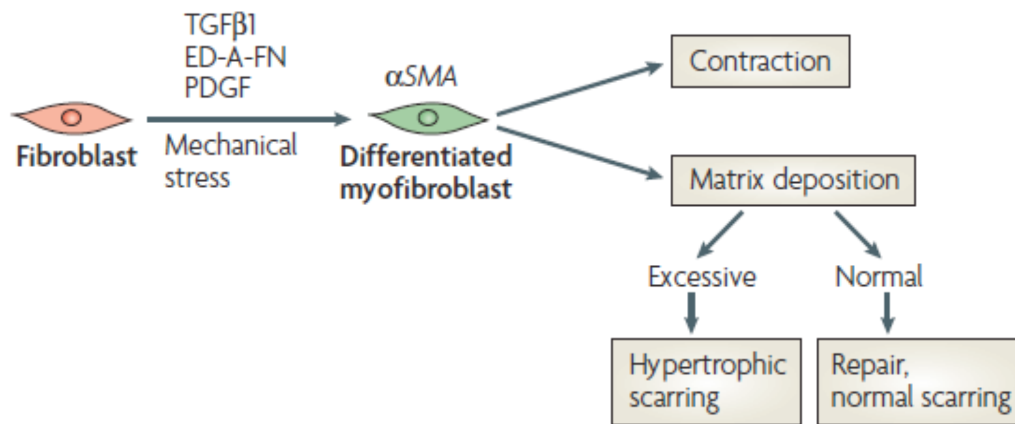


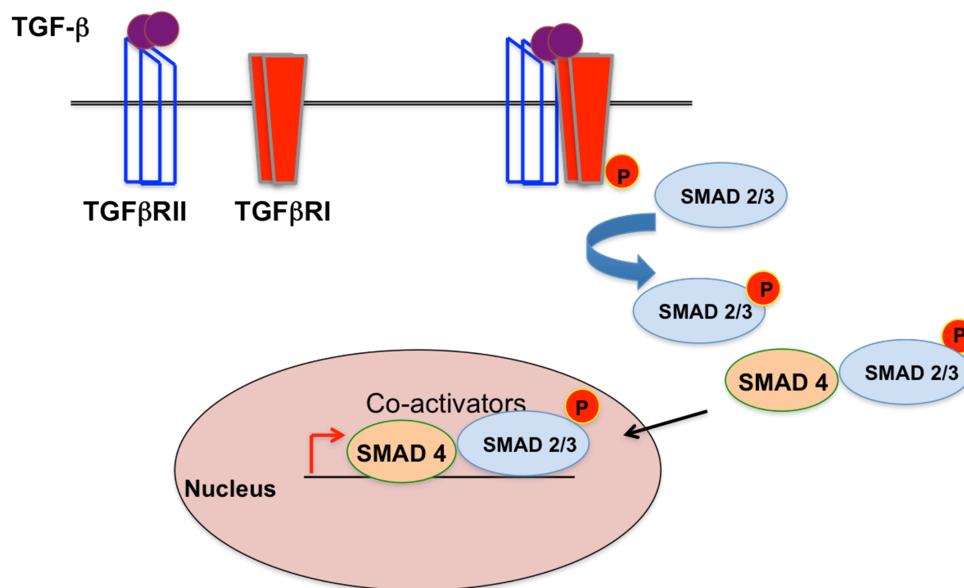
Fig. 1-6: Differentiation of fibroblast into myofibroblast and its function in ECM production and wound contraction (Schafer and Werner 2008).

1.4 TGFβ-TGFβRII signaling

Transforming growth factor-beta (TGF-β) family comprises many related factors with diverse functions such as the regulation of cell growth, differentiation, adhesion, migration and apoptosis in a developmental, context-dependent and cell type specific manner. TGF-β has a profound effect on immune system. It has been implicated in a broad range of pathogenic mechanisms where it imparts a primary effect on immune cells. These include immune defects, which is associated with autoimmune disease, suppression of immune survelliance of tumours, haematopoetic malignancies, susceptibility to parasitic infections and chronic inflammatory diseases leading to fibrotic complications. TGF-β was isolated from blood platelets (Assoian, Komoriya et al. 1983). It is a homodimer protein of 25 kDa.There are three homologous TGF-β isoforms present in mammals denoted as TGF-β1, TGF-β2, TGF-β3 which share 62% to 82% amino acid sequence homology, although each is encoded by a separate gene and controlled by a unique promoter. It has been examined that all these forms of TGF-β are more than 98% conserved between species. TGF-β binds to specific pairs of serine threonine kinase receptor which belongs to a group called type I and type II receptors (Massague 1998). The receptor complexes are heterotetrameric, consist of two type II receptors (75-80 kDa), which bind to the TGF-β ligand and two signal transducing type I receptors (50-60 kDa), which cannot bind the ligand directly and considered to act downstream to type II receptors.

Ligand binding leads to heterodimerization of TGFβRI and TGFβRII. Subsequently, type II receptor activates the type I receptor by phosphorylating the GS domain, which is rich in glycine and serine residues. The phosphorylated type I receptor phosphorylates the

receptor regulated Smads or R-Smads (SMAD-2, SMAD-3), which in turn, phosphorylates SMAD4. The complex then moves into the nucleus and directly regulates gene expression (Massague 2000) (Fig. 1-7). TGF-β1 has also been reported to induce SMAD-independent pathway such as extracellular-signal-regulated kinases (ERK), c-Jun N-terminal kinases (JNK) and Phosphoinositide Kinase-3 (PI3K), however the mechanism and functions of their activation is still unknown (Derynck and Zhang 2003).



Derynck R, *Nature* 2003 (modified)

Fig. 1-7: Schematic representation of TGFβ- TGFβRII signaling.
Dimeric TGFβ binds to TGFβRII that facilitates binding and phosphorylation of TGFβR1. Activated TGFβR1 phosphorylates SMAD 2 and 3 proteins, which bind to SMAD4 and together with this translocate to the nucleus. In the presence of other co-activators, the SMAD complex bind to the target gene and mediate gene expression (Derynck and Zhang 2003).

TGFβRII exists as a homodimer even in the absence of the ligand (Chen and Derynck 1994; Henis, Moustakas et al. 1994), however in the absence of TGFβRI, TGFβRII homodimers are unable to transduce signal through TGF-β (Luo and Lodish 1996). Previous studies have reported that murine TGFβRII has specific binding preference to TGF-β1 over TGF-β2, moreover in the tissue which express low levels of TGFβ-1 such as cartilage and brain, expression of TGFβRII is either very low or absent (Lawler, Candia et al. 1994).

1.4.1 The role of TGF-β1 and TGF-β1 signaling in tissue repair and fibrosis

TGF-β1 is a multifunctional cytokine which plays a central role in wound healing and fibroplasia (Leask and Abraham 2004). It is of prime importance for the induction but also

the resolution of inflammatory responses and stimulates ECM synthesis by fibroblasts. In the skin, it is released by infiltrating cells such as lymphocytes, monocytes/macrophages, fibroblasts, keratinocytes and platelets. After wounding or inflammation all these cells are the potential sources of TGF-β1. The release and action of TGF-β1 stimulates the production of the various ECM proteins and inhibits degradation of matrix proteins under normal situation, synthesis of the matrix proteins is balanced by the degradation of the matrix proteins but, any imbalance in the state of TGF-β1 regulation of matrix deposition contributes to tissue fibrosis. Besides organ fibrosis, conditions like hypertrophic scars and keloids in skin tissue are caused by TGF-β1. Previous studies showed that the amount of TGF-β1 increases in scar deposition and when injected with anti-TGF-β1 and 3 into the wound margins fibrosis gets reduced (Shah, Foreman et al. 1995). Recently, it has been shown that loss of TGF-β1 signaling by targeted deletion of TGFβRII in fibroblasts results in impaired repair process (Denton, Khan et al. 2009).

It is well established that mice with complete deficiency for TGF-β1 die shortly after birth due to multi organ inflammation (Shull, Ormsby et al. 1992). TGF-β1 controls pro-inflammatory and anti-inflammatory responses in immune cells (Letterio and Roberts 1998). It is also a potent chemotactic cytokine for monocytes (Wahl, Hunt et al. 1987). In the context of wound repair and fibroplasia, pro- and anti- inflammatory actions of immune cells are the requisite for the normal repair process. In a mouse model, neutralization of TGF-β by anti-TGF-β antibody or topical application of peptide inhibitor of TGF-β has been shown to improve bleomycin-induced skin fibrosis (Yamamoto, Takagawa et al. 1999; Santiago, Gutierrez-Canas et al. 2005). Mice lacking TGFβRII is embryonic lethal with defective yolk sac vasculogenesis (Oshima, Oshima et al. 1996) and therefore its role in cutaneous wound healing or fibrosis is not well understood. Currently, it is unresolved that whether TGF-β1 directly controls the function of immune cells through TGFβRII during skin repair process and fibrosis.

1.4.2 The role of TGFβ1-TGFβRII mediated functions in macrophages

Macrophages are known for their classical roles in phagocytosis and as antigen presenting cells. In its plethora of functions, gradually its role in maintaining tissue homeostasis and mediating inflammatory and immune responses is gaining recognition from the findings in recent years (Lucas, Waisman et al. 2010). TGF-β1 is one of the cytokines known to secreted by macrophages and in turn known to modulate responses, such as monocyte activation, proliferation, cytokine production, host defense and chemotaxis (Strassmann, Cole et al. 1988; Ashcroft 1999). Studies have implicated that

TGF-β1 plays a bidirectional role on macrophage functions and acts as potent immunosuppressive as well as activating factor (Tsunawaki, Sporn et al. 1988; Ashcroft 1999). The action of TGF-β1 on monocytes/macrophages is dependent on the state of cellular differentiation, cytokine milieu and the concentration gradient of TGF-β1 itself (McCartney-Francis and Wahl 1994; Wahl 1994). Monocytes are known to express TGF-β1 mRNA constitutively but the mRNA and protein levels do not necessarily correlate (Assoian, Fleurdelys et al. 1987; McCartney-Francis, Mizel et al. 1990). Activated monocytes and macrophages secrete increased levels of TGF-β1. Monocytes and macrophages are also known to regulate the expression of TGF-β1 in autocrine and paracrine manner (Wahl, McCartney-Francis et al. 1990). At the site of injury, the platelets degranulate and release a concentrated amount of TGF-β1, which acts as a chemoattractant for the inflammatory cells. TGF-β1 favors recruitment of monocytes *in vitro* in femtomolar concentration (Wahl, Hunt et al. 1987; Wiseman, Polverini et al. 1988). It has been shown that intradermal or intra-articular injection of TGF-β1 stimulates recruitment of monocytes and macrophages and matrix deposition. TGF-β1 mediates monocyte production of cytokines, which acts as additional mononuclear cells chemoattractant (Luger, Charon et al. 1983). Resting monocytes, which are stimulated by picomolar concentration of TGF-β1 responds by up-regulating TGF-β1 transcription and secretion in an autocrine feedback loop (Wahl, McCartney-Francis et al. 1990). After injury, the inflammatory cells, like monocytes have to get recruited at the injury site and for that they need to transverse the endothelial basement membrane. TGF-β enhances the expression of integrins like LFA-1, which binds to ICAM-1 and α3β1 receptors required to mediate the monocyte binding to fibronectin, laminin and collagen (Bauvois, Rouillard et al. 1992; Wahl, Allen et al. 1993). TGF-β1 also enhances the expression of fibronectin receptor α5β1, which enhances the attachment of monocytes to the provisional matrix deposited at the sites of cutaneous wounds (Wahl, Allen et al. 1993). It has been shown that picomolar concentration of TGF-β1 stimulates monocytes to transcribe a variety of cytokines such as TNF-α, PDGF, b-FGF, IL-6, VEGF and IL-1 which in turn modulates cell function (Wiseman, Polverini et al. 1988; McCartney-Francis, Mizel et al. 1990; Jeon, Chae et al. 2007). Therefore, in the cells, which express TGFβRII including macrophages, TGF-β1 can stimulate its own production and the production of the various pro-inflammatory factors, pro-fibrotic and pro-angiogenic factors, ECM proteins, matrix metalloproteinases and tissue inhibitor of metalloproteinases, thus can modulate quality of tissue repair and fibrosis (Fig. 1-8).

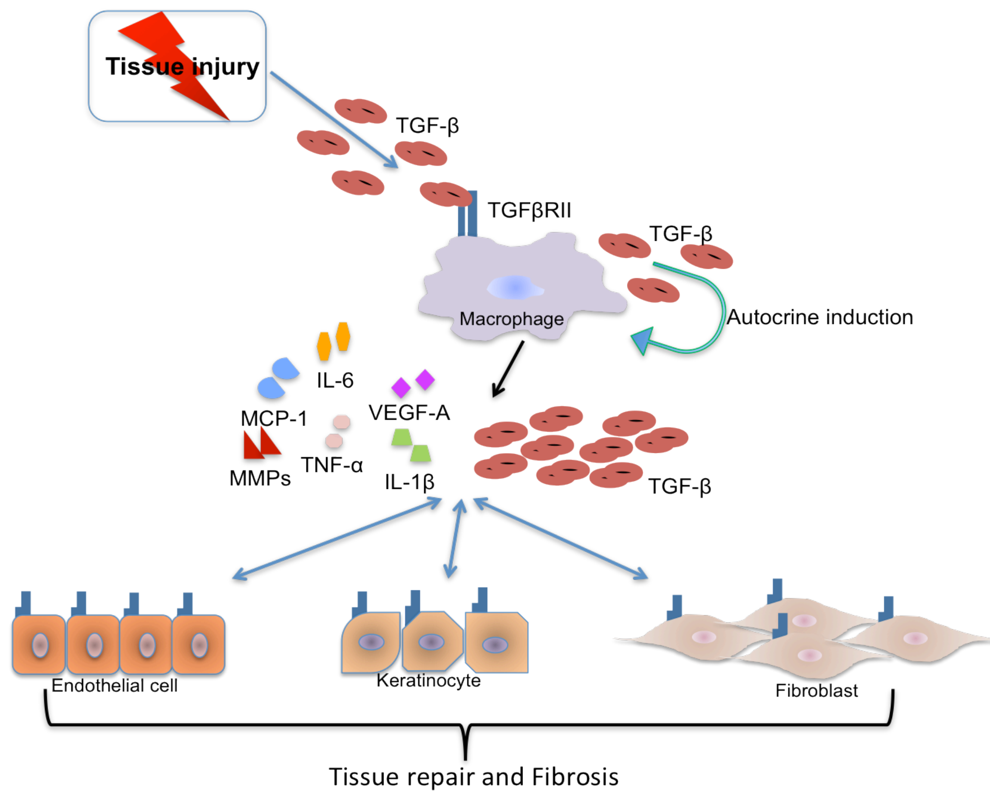


Fig. 1-8: Schematic representation of TGF-β1 mediated functions in macrophages, modulating the outcome of tissue repair and fibrosis.
During tissue repair and fibrosis, TGF-β1 can be secreted from various sources and act on TGFβRII expressing cells. Binding of TGF-β1 to TGFβRII on macrophages induces auto-induction of TGF-β1, which can modulate macrophage function either by autocrine interaction on macrophages or by paracrine interaction on different cell types such as endothelial cells, keratinocytes and fibroblasts. Both autocrine and paracrine interaction of TGF-β1 can lead to the production of other factors such as VEGF, IL-1β, IL-6, TNF-α, MMPs, thus the cells are further modulated to generate tissue repair response or fibrotic response.

TGF-β1 is also known to induce the expression of Fc gamma receptor III (FcγRIII), which in turn enhances the phagocytotic capacity of macrophages (Welch, Wong et al. 1990). The potent proinflammatory effect of TGF-β1 has been shown *in vivo* by using its antagonist, where the local inflammatory effect of TGF-β1 got diminished after treatment. By administration of anti-TGF-β1 antibody locally in the tissue it has been shown that the fibrosis and the influx of inflammatory cells gets reduced which also indicates that inflammatory cells and TGF-β1 expressed by those cells have a profound effect on tissue fibrosis and scar formation.

Besides pro-inflammatory action, TGF-β1 also acts as anti-inflammatory cytokine in modulating macrophage functions. At the site of injury, during the maturation process of monocytes into macrophages, the expression of TGF-β receptor gets down-regulated, which leads to a decline sensitivity of the macrophages towards TGF-β1 response. Furthermore, due to this change the same TGF-β1, which initially acted as a pro-

inflammatory factor, starts acting as anti-inflammatory cytokine and helps in the resolution of the inflammation. *In vitro,* TGF-β inhibits the LPS mediated up-regulation of MIP-1α and MCP-1, which are potent chemoattractant expressed by macrophages (Ashcroft 1999; Li, Wan et al. 2006). Phagocytotic macrophages tend to phagocytose neutrophils and express more TGF-β in the resoluting tissue. This enhanced expression of TGF-β in the local milieu leads to down-regulation of inflammatory cytokines like GM-CSF, TNF-α and IL-8 (Fadok, Bratton et al. 1998). Immunosuppressive effect of TGF-β on macrophages has been reported under *in vivo* condition such as streptococcal cell wall induced arthritis, allergic encephalomyelitis (Brandes, Allen et al. 1991; Kuruvilla, Shah et al. 1991; Racke, Dhib-Jalbut et al. 1991).

1.5 Cre-LoxP system and LysMCre mouse strain

Cyclization recombinase-LoxP (Cre-LoxP) is based on site-specific *in vivo* recombination of LoxP sites and allows successful genetic manipulation of the target genes (Araki, Imaizumi et al. 1997). The system requires insertion of 34 base pair nucleotides named as Lox P site in a way that it flanks the exons to be deleted in the target gene. The sequence consists of two flanking 13 bp inverted repeats and an 8 bp core sequence: ATAACTTCGTATA - GCATACAT –TATACGAAGTTAT.

Cre is the tyrosin recombinase enzyme derived from P1 bacteriophage (Abremski and Hoess 1984; Hamilton and Abremski 1984), which carries out site specific recombination between two loxP sites (Nagy 2000). LysM promoter is expressed in myeloid cell lineage and therefore expression of Cre recombinase under the control of LysM promoter efficiently targets the loxP flanked gene of interests in monocytes, mature macrophages and neutrophils (Clausen, Burkhardt et al. 1999).

Myeloid cell specific TGFβRII mutant mice were generated using LysMCre-loxP DNA recombination system. Mice carrying flanked loxP sites on exon 3 of the TGFβRII alleles (Cazac and Roes 2000) were crossed with the transgenic mice that express Cre recombinase under the control of the endogenous Lysozyme M (LysM) promoter (Clausen, Burkhardt et al. 1999). Myeloid cells, which express Cre recombinase are able to perform recombination of the flanked loxP sites in the alleles and truncation of the TGFβRII protein expression.

2 Aims

The role of TGF-β in wound repair and fibrosis has been well described. TGF-β is secreted by and acts on all cell types. TGF-β produced at the site of injury or fibrosis acts on diverse cell types that expresses its receptor TGFβRII. A complex network of soluble factors and cell-cell interaction controls the wound healing and fibrosis and therefore it is still unclear how a cell type-specific interaction of TGF-β and its receptor, TGFβRII modulates the tissue response.

Macrophages are one of important cell types, which help in the healing process. Additionaly, they are the one of the important sources of TGF-β1. Although, macrophages are actively involved in the repair process, specific contribution of TGFβ1-TGFβRII signaling pathway in macrophages has not been studied so far. Therefore, in this study, we specifically intend to clarify the role of TGFβRII on macrophages in regulating the connective tissue response to mechanical and inflammation-mediated tissue damage in skin.

To address this, the aims are as following:

1. Generation of myeloid cell specific TGFβRII deficient mice

2. Analysis of the consequences of TGFβRII gene deletion in macrophages with respect to the quality and kinetics of skin repair and fibrosis in response to mechanical injury

3. Analysis of the functional impact of TGFβRII in macrophages in regulating skin fibrosis in response to inflammatory tissue damage

4. To study which specific cell functions in tissue repair and fibrosis are controlled by TGFβRII mediated activation of myeloid cells

5. Identification of the mechanisms and mediators, which are responsible for the obtained phenotype

3 Results

3.1 Generation of myeloid cell specific TGFβRII deficient mice

To elucidate the impact of TGF-β1 mediated functions of myeloid cells during skin repair process and fibrosis, mice were generated, in which, TGFβRII can be specifically abrogated in myeloid cell population. The myeloid cell specific TGFβRII deficient mouse was generated by crossing the mouse line carrying the TGFβRII gene, flanked by loxP sites (provided by Jürgen Roes, University College London) (Cazac and Roes 2000) with a mouse line expressing Cre recombinase under the control of the LysM (Lysozyme M) promoter (provided by Irmgard Förster, University Düsseldorf (Clausen, Burkhardt et al. 1999). This system allows the expression of myeloid cell specific Cre recombinase and thereby the deletion of the loxP flanked exon (exon 3) of TGFβRII gene, specifically in the myeloid cells. Excision of exon 3 from the TGFβRII gene resulted in premature termination and truncation of TGFβRII protein synthesis, specifically in myeloid cells.

3.1.1 Myeloid cell specific deletion of TGFβRII

The presence of the floxed allele and transgenic LysMCre promoter was confirmed by genotyping PCR obtained from mouse tail biopsies. The resulting PCR product showed a band of 550 bp for the TGFβRII floxed allele, a band of 500 bp for the TGFβRII wildtype allele, 700 bp for LysMCre and 350 bp for the LysM wildtype allele (Fig. 3-1A). To confirm myeloid cell specific disruption of the TGFβRII gene particularly in macrophages, a Southern blot on genomic DNA and Western blot on the cultured peritoneal macrophages was performed.

Southern blot analysis was performed on the genomic DNA of FACS sorted, F4/80^{+} peritoneal macrophages, isolated from peritoneal cavity of wild type control mice (TGFβRII$^{wt/wt}$), floxed control mice (TGFβRII$^{fl/fl}$) and from mice lacking TGFβRII in macrophages (TGFβRII$^{fl/fl}$LysMCre). NcoI digested genomic DNA resolved on gel was probed with a source specific for exon 2. As revealed on the blot (Fig. 3-1C), the NcoI digested DNA from wild type control macrophage (TGFβRII$^{wt/wt}$) showed a band of 2.7 kb. NcoI digested DNA from TGFβRII floxed control macrophages (TGFβRII$^{fl/fl}$LysMCre) showed a slightly larger band of 2.9 kb. The digested DNA from TGFβRII$^{fl/fl}$LysMCre macrophages also shows a faint band of 2.9 kb and an additional band of 1.8 kb corresponding to the TGFβRII gene with the deleted exon 3. Densitometric analysis of the

1.8 kb band obtained from genomic DNA of TGFβRII$^{fl/fl}$LysMCre macrophages indicated that approximately 80% of TGFβRII were truncated (Fig. 3-1C), thus confirming an efficient deletion at genomic level.

To confirm the deletion of TGFβRII on the protein level, peritoneal macrophages from control and TGFβRII$^{fl/fl}$LysMCre mice were isolated, cultured and analyzed for TGFβRII expression by western blot. In Control macrophages, specific antibody against TGFβRII, detected a band of approximately 70 kDa, whereas TGFβRII$^{fl/fl}$LysMCre macrophages showed loss of this band (Fig. 3-1D), which further confirms an efficient truncation of the TGFβRII expression in macrophages.

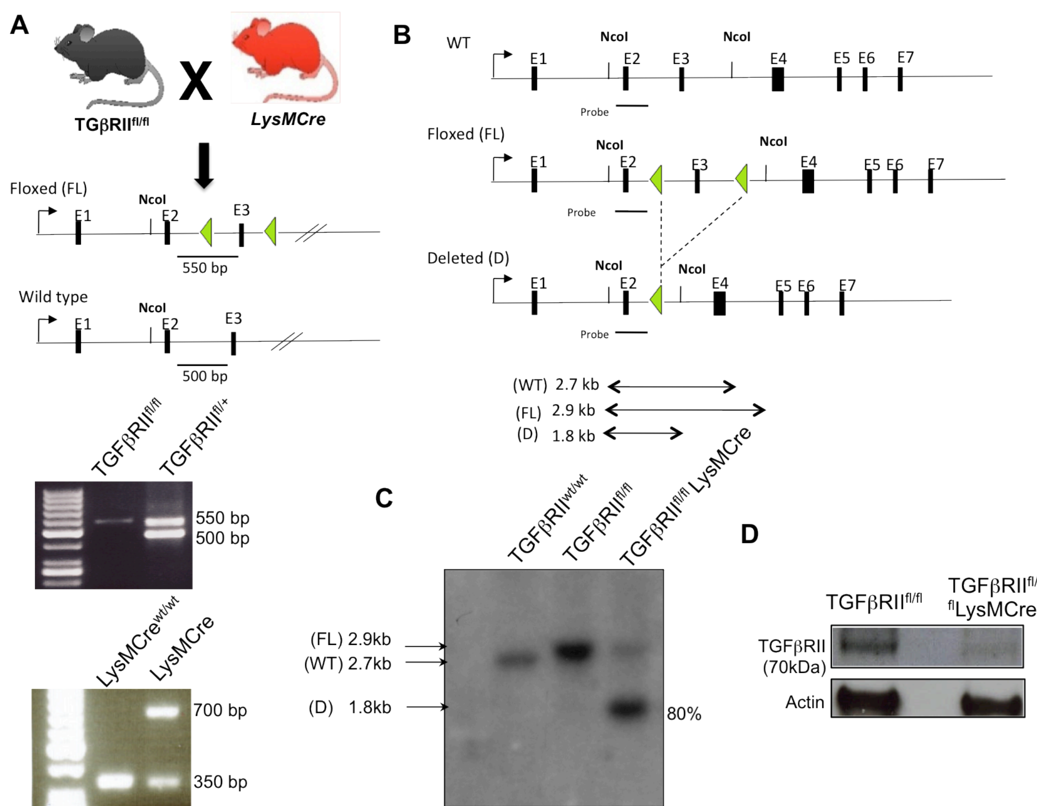


Fig. 3-1: Macrophage specific deletion of TGFβRII
A) Schematic diagram and amplicons of the mouse tail genotyping PCR. Genotyping PCR from mouse tail DNA showed 550 bp band size in TGFβRII$^{fl/fl}$ mouse, whereas TGFβRII$^{fl/+}$ mouse showed 550 bp band of floxed allele as well as 500 bp of wild type allele (upper panel). Genotyping PCR of LysMCre showed a band of 700 bp and a wild type of 350 bp (lower panel). **B)** Schematic diagram of exons of TGFβRII gene, floxed exon and the deleted exon of TGFβRII after Cre recombinase activity detected by southern blot. **C)** Southern blot analysis of genomic DNA obtained from FACS sorted control and TGFβRII$^{fl/fl}$LysMCre peritoneal macrophages. The NcoI digested DNA showed a band of 2.7 kb in TGFβRII$^{wt/wt}$ control, a floxed band of 2.9 kb in floxed TGFβRII$^{fl/fl}$ control without Cre whereas, the digested DNA from TGFβRII$^{fl/fl}$LysMCre showed one band of 2.9 kb (floxed) and another band of 1.8 kb (deleted). Densitometric analysis revealed approximately 80% deletion of the floxed band in TGFβRII$^{fl/fl}$LysMCre mice. **D)** Western blot analysis of TGFβRII expression in cultured peritoneal macrophages showed presence of 70 kDa protein of TGFβRII in control macrophages and abscence in TGFβRII$^{fl/fl}$LysMCre macrophages.

3.1.2 Loss of TGFβ1−TGFβRII signaling in TGFβRII$^{fl/fl}$LysMCre macrophages

TGFβRII binds to TGFβ1, which results in the recruitment of TGFβRI and phosphorylation of cytosolic signal transducers Smad2 and Smad3 (Massague 2000). To confirm the functional impairment of TGFβ1 mediated downstream signaling in TGFβRII$^{fl/fl}$LysMCre macrophages, peritoneal macrophages from control and TGFβRII$^{fl/fl}$LysMCre mice were isolated, cultured and stimulated with 20ng/ml recombinant TGF-β1 (rTGF-β1) for 10 and 30 minutes. The Smad2 phosphorylation was used as a read out and its expression was analyzed by Western blot in stimulated and un-stimulated cells. Macrophages from control mice showed strong phosphorylation of Smad2 after 10 and 30 minutes of rTGF-β1 stimulation, however macrophages from TGFβRII$^{fl/fl}$LysMCre mice did not show phosphorylation of Smad2 at any time point investigated, indicating impaired TGF-β1 mediated downstream signaling in TGFβRII$^{fl/fl}$LysMCre macrophages (Fig. 3-2).

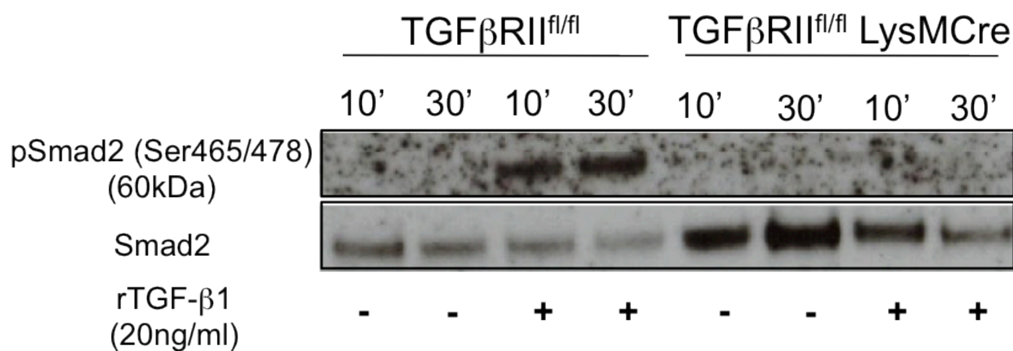


Fig. 3-2: Loss of TGFβ1-TGFβRII signaling in TGFβRII$^{fl/fl}$LysMCre macrophages. Western blot analysis of lysates from peritoneal macrophages isolated from control and TGFβRII$^{fl/fl}$LysMCre mice, stimulated with rTGF-β1 (20ng/ml) for 10' and 30' minutes showed TGF-β1 mediated activation of Smad2 in control macrophages and no phosphorylation of Smad2 in TGFβ RII$^{fl/fl}$LysMCre macrophages. Total Smad2 protein served as a loading control. pSmad2: phospho Smad2.

3.2 Effect of TGFβRII deletion in myeloid cells on thioglycollate induced peritonitis and *in vitro* macrophage chemotaxis

3.2.1 Abolishment of anti-inflammatory effect in TGFβRII$^{fl/fl}$LysMCre mice during an inflammatory model of thioglycollate induced peritonitis

Transforming growth factor beta (TGF-β) is a multifunctional cytokine, whose myriad of functions include the potent suppression of the immune system and the recruitment of monocytes *in vitro* (Li, Wan et al. 2006). After assuring the successful deletion of TGFβRII in myeloid cells, the role of TGFβRII was analyzed in a model of thioglycollate induced peritonitis on infiltration of leukocyes. Thioglycollate was injected into the peritoneum cavity of control and TGFβRII$^{fl/fl}$LysMCre mice and after 4 days, peritoneal cells were harvested and counted under the microscope using haemocytometer chamber slide. This analysis showed an increase of total leukocyte cell numbers in peritoneal lavage of TGFβRII$^{fl/fl}$LysMCre mice compared to control mice, indicating an enhanced inflammatory condition in the peritoneum that might be generated due to the loss of anti-inflammatory effect of TGF-β on myeloid cells (Fig. 3-3).

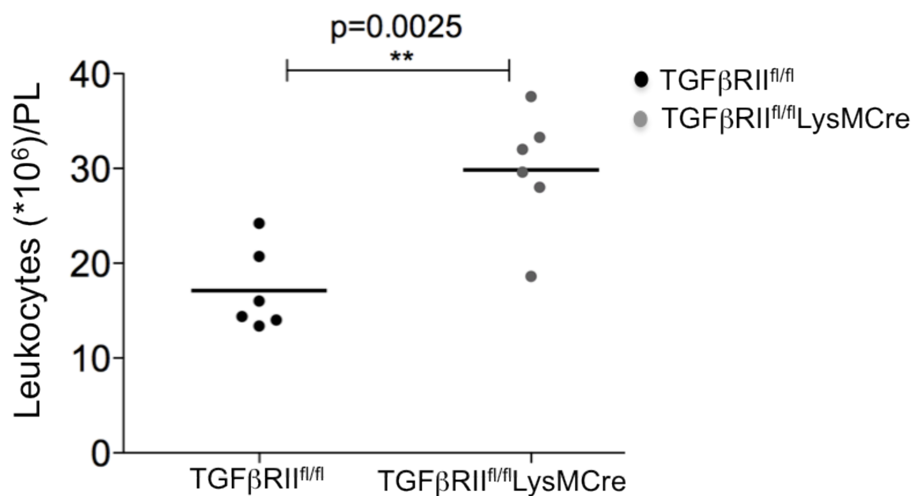


Fig. 3-3: Enhanced leukocyte recruitment in thioglycollate induced peritonitis model due to the loss of TGFβRII in myeloid cells. Peritoneal leukocytes isolated after thioglycollate injection showed an increase in total number in TGFβRII$^{fl/fl}$LysMCre mice compared to control mice. Statistical significance was determined using student's t-test test. PL: Peritoneal lavage.

Enhanced leukocyte recruitment in thioglycollate induced peritonitis in TGFβRII$^{fl/fl}$LysMCre mice raised the question whether the deletion of TGFβRII in myeloid cells directly influences their migration in thioglycollate induced peritonitis.

To address the question, peritoneal cells were isolated from control and TGFβRII$^{fl/fl}$LysMCre mice after four days of thioglycollate injection and analyzed by flow cytometry. However, a similar percentage of CD11b$^+$/F480$^+$ and CD115$^+$/F4/80$^+$ monocytes as well as CD11b$^+$/Gr-1$^+$ neutrophils were observed in both the mice (Fig. 3-4).

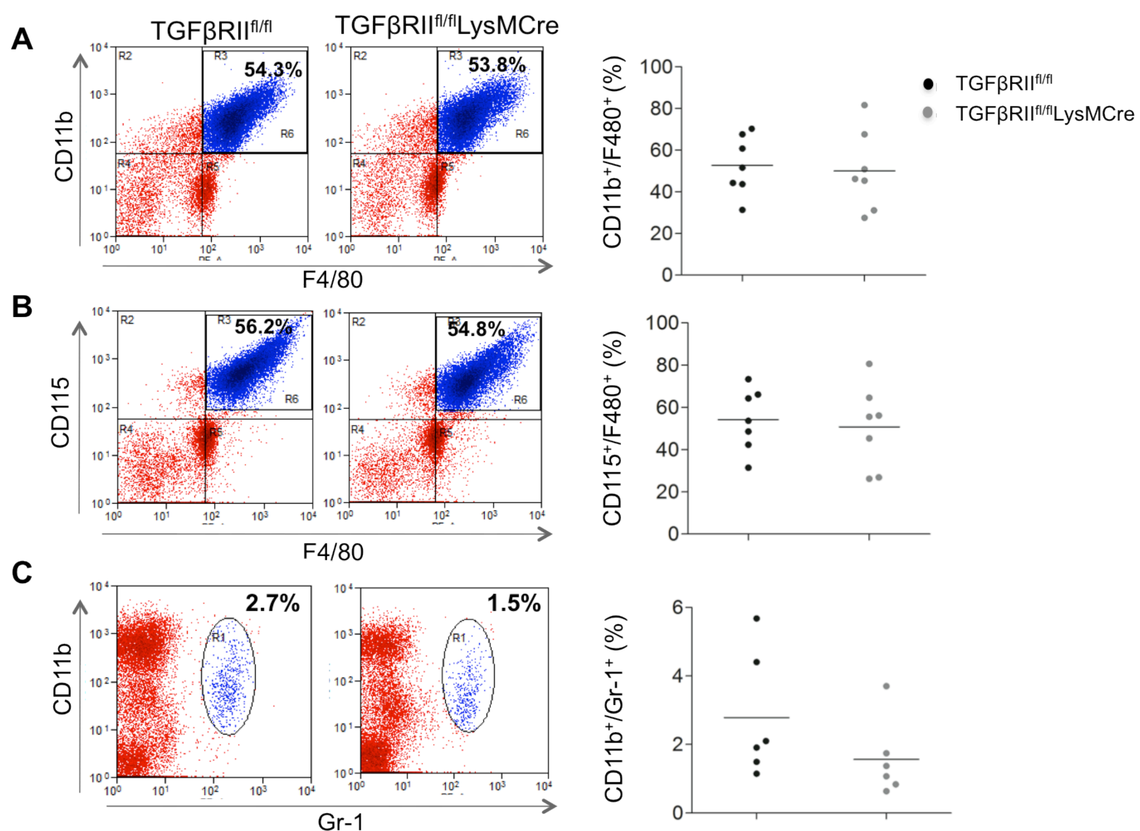


Fig. 3-4: Similar fractions of monocytes/macrophages and neutrophils in thioglycollate induced peritonitis. FACS analysis and quantification of peritoneal cells after 4 days of thioglycollate injection showed similar percentage of CD11b$^+$/F480$^+$ macrophages (A), CD115$^+$/F4/80$^+$ monocytes (B) and CD11b$^+$/Gr-1$^+$ neutrophils (C). Horizontal lines represent the mean of independent experiments. Statistical significance was determined using student's t-test.

3.2.2 Impact of TGFβRII deficiency on *in vitro* random migration of macrophages

To address the question whether the deletion of TGFβRII also impacts *in vitro* migration, peritoneal macrophages were isolated from control and TGFβRII$^{fl/fl}$LysMCre mice, cultured in DMEM medium supplemented with either 1% or 10% of fetal calf serum (FCS). The random migration was determined using Olympus 1X81 microscope under humidified

chamber supplied with 5% CO_2. Interestingly, in the medium supplemented with 1% FCS, migration of TGFβRII$^{fl/fl}$LysMCre macrophages was significantly reduced compared to control macrophages, whereas in the medium supplemented with 10% FCS, similar migration of TGFβRII$^{fl/fl}$LysMCre and control macrophages was observed (Fig. 3-5).

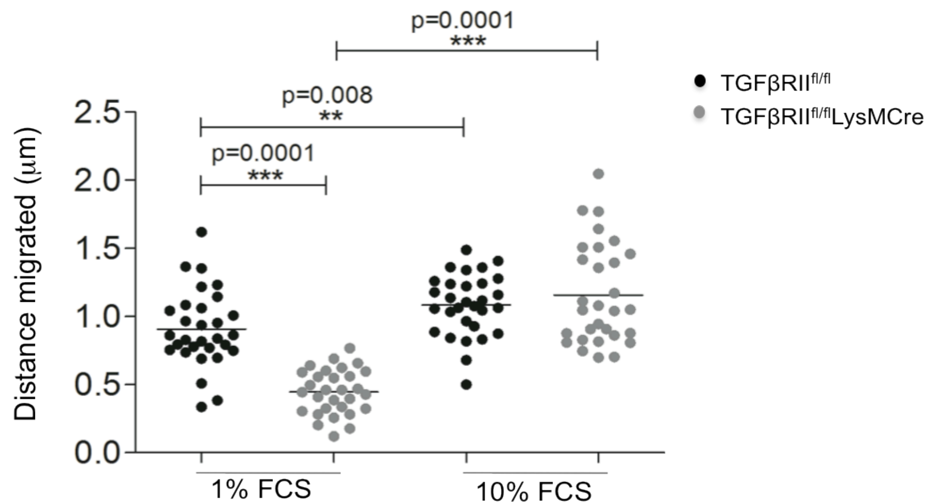


Fig. 3-5: Random migration of peritoneal macrophages.
Random migration of peritoneal macrophages in the medium supplemented with 1% FCS was reduced in TGFβRII$^{fl/fl}$LysMCre macrophages, whereas in 10% FCS supplemented medium, both control and TGFβRII$^{fl/fl}$LysMCre macrophages showed similar random migration. Horizontal lines represent the mean of independent experiments. Statistical significance was determined using student's t-test.

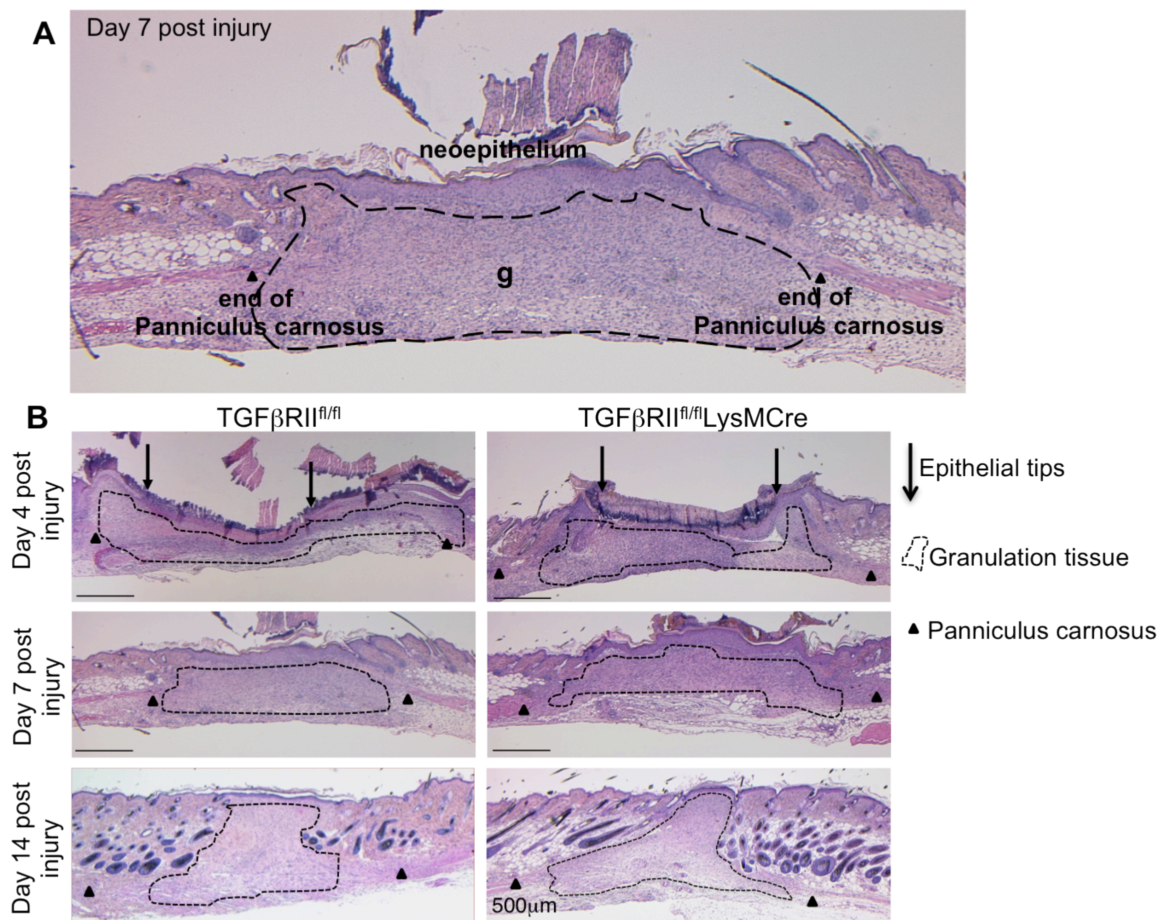
3.3 Impact of TGFβRII deficiency in macrophages on the repair process

3.3.1 TGFβRII deficiency does not impair kinetics of wound closure but delays wound contraction

To investigate the consequences of TGFβRII deficiency in macrophages with respect to the quality and kinetics of skin repair in response to mechanical tissue injury, a 6 mm full thickness wounds were created on the back of control and TGFβRII$^{fl/fl}$LysMCre mice. Healing kinetics and microscopic assessment of various wound healing parameters on day 4, day 7 and day 14 post injury were performed. An overview of wound healing parameters investigated is shown in Fig. 3-6A.

The full thickness wounds on control and TGFβRII$^{fl/fl}$LysMCre mice healed almost with the similar kinetics. Macroscopic observation was further confirmed by histological investigation of H&E stained wound sections, which showed no obvious difference in the

wound closure between control and TGFβRII$^{fl/fl}$LysMCre mice at day 4, day 7 and day 14 post injury (Fig. 3-6B). For histological quantification, the amount of granulation tissue, the distance between the ends of epithelial tips and the length of the epithelial tongue and distance between the ends of the panniculus carnosus were measured on H&E stained paraffin sections. The amount of granulation tissue, the distance between the two ends of the epithelial tips and the epithelial tongue (re-epithelialization) at day 4, day 7 and day 14 post injury were similar in both control and TGFβRII$^{fl/fl}$LysMCre wounds (Fig. 3-6C). By contrast, TGFβRII$^{fl/fl}$LysMCre wounds showed delayed wound contraction, when the distance between the wound edges i.e., panniculus carnosus was measured microscopically. The distance between panniculus carnosus was significantly reduced in TGFβRII$^{fl/fl}$LysMCre wounds compared to control wounds at day 4 post injury and day 7 post injury (Fig. 3-6D). This result indicates that the loss of TGF-β1 mediated response in the TGFβRII$^{fl/fl}$LysMCre macrophages results in delayed wound contraction.



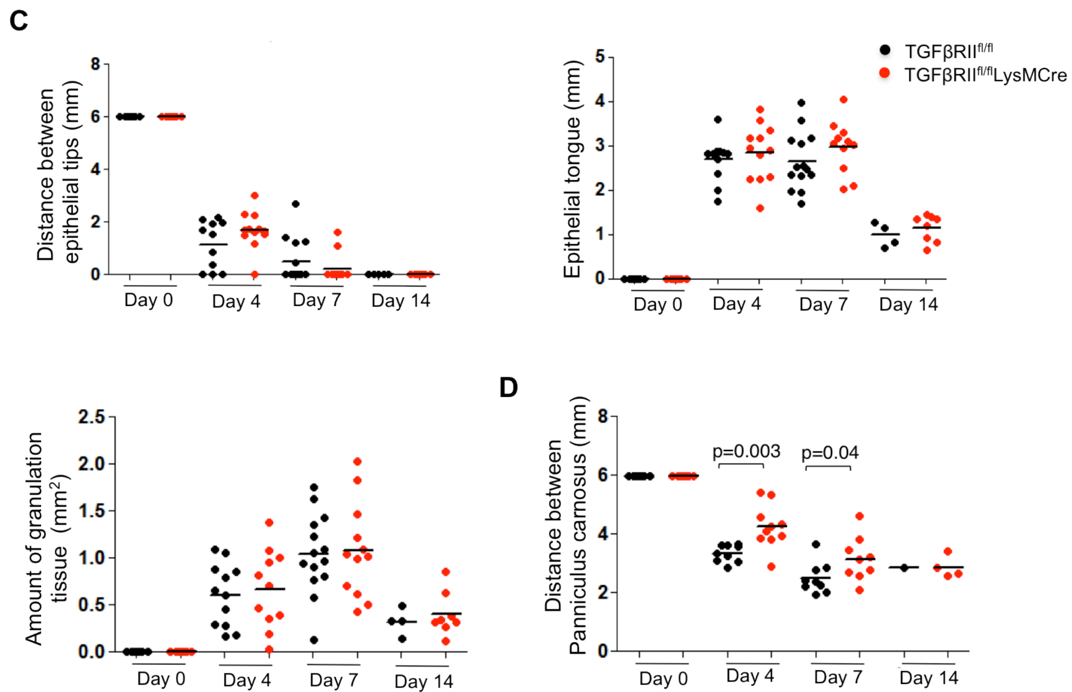


Fig. 3-6: Loss of TGFβRII on macrophages does not impact overall wound repair kinetics, epithelialization, granulation tissue formation but attenuates wound contraction.
A) H&E staining of wound section from day 7 post injury showing general wound parameters (g: granulation tissue) **B)** H&E staining of wound sections from control and TGFβRII$^{fl/fl}$LysMCre mice at day 4, day 7 and day 14 post injury showed no microscopic difference in the healing kinetics. **C)** Microscopic measurement of distance between epithelial tips and epithelial tongue, and the amount of granulation tissue shows no difference at day 4, day 7 and day 14 post injury between control and TGFβRII$^{fl/fl}$LysMCre wounds. **D)** Distance between the edges of the panniculus carnosus at day 4 and day 7 post injury revealed a significantly attenuated wound contraction in TGFβRII$^{fl/fl}$LysMCre wounds compared to control wounds. Horizontal lines represent the mean. Statistical significance was determined using student's t-test.

3.3.2 Impaired myofibroblast differentiation and impaired deposition of collagen at the wound site of TGFβRII$^{fl/fl}$LysMCre mice

Differentiation of fibroblasts into myofibroblast concomitant with the acquisition of contractile features is essential for connective tissue remodelling and wound contraction during normal and pathological wound healing (Hinz 2007). To analyze whether delayed wound contraction in TGFβRII$^{fl/fl}$LysMCre mice was due to impaired myofibroblast differentiation, the presence of myofibroblast in wounds was investigated at day 4, day 7 and day 14 post injury. Wound tissues were stained for alpha smooth muscle actin (α-SMA), which is a marker for the activated fibroblast/myofibroblast (Darby, Skalli et al. 1990). The staining revealed, reduced expression of α-SMA in granulation tissue of TGFβRII$^{fl/fl}$LysMCre wounds compared to control wounds at day 7 post injury (Fig. 3-7A). Quantification of α-SMA positive area in total granulation tissue further confirmed, a significant reduction in TGFβRII$^{fl/fl}$LysMCre wounds compared to control wounds at day 7

post injury, (Fig. 3-7B). This analysis suggested that myofibroblast differentiation in TGFβRII$^{fl/fl}$LysMCre wounds is impaired.

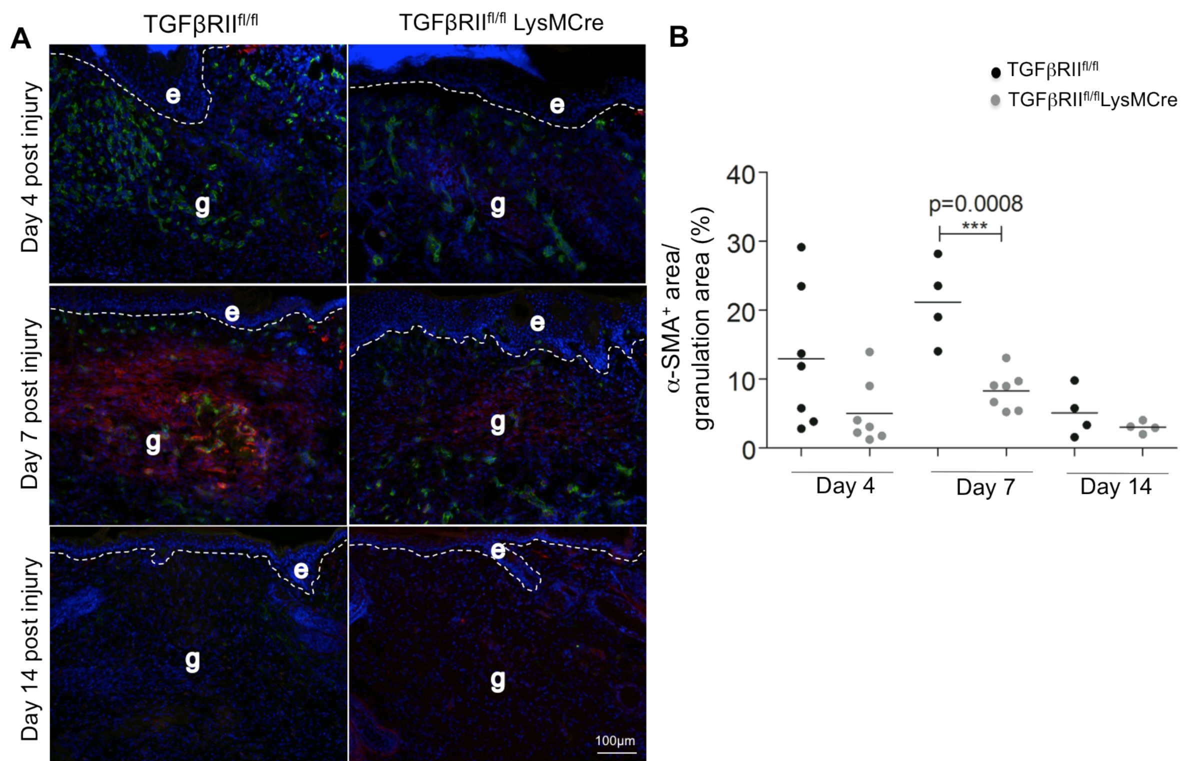


Fig. 3-7: Altered myofibroblast differentiation in TGFβRII$^{fl/fl}$LysMCre wound tissue. A) Immunofluorescense staining and **B)** quantification of the α-SMA positive area, at day 4, day 7 and day 14 post injury within the granulation tissue showed significant reduction of expression in TGFβRII$^{fl/fl}$LysMCre compared to control wounds at day 7 post injury. Statistical significance was determined using student's t-test. Horizontal lines represent the mean. e; epidermis, g; granulation tissue.

One of the essential functions of myofibroblast is the production of collagen (Gabbiani 2003). Since, TGFβRII$^{fl/fl}$LysMCre wounds showed reduced α-SMA positive area, the question was whether this leads to the functional impairment of myofibroblasts. To address this question, collagen bundles were analyzed in the wounds. Wound sections from day 14 post injury were stained with Sirius red, which binds to collagens and can be visualized by polarized microscope. This analysis revealed an attenuated collagen deposition in TGFβRII$^{fl/fl}$LysMCre wounds compared to control wounds (Fig. 3-8).

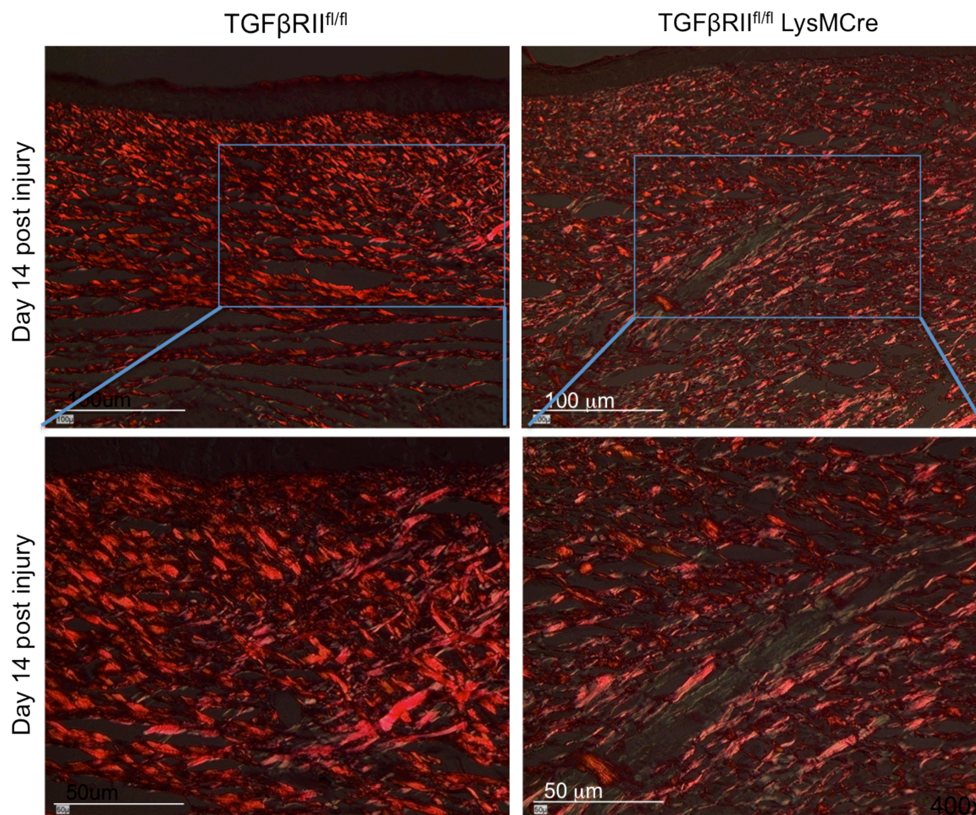


Fig. 3-8: Attenuated collagen deposition in TGFβRII$^{fl/fl}$LysMCre wound. Sirius red staining on control and TGFβRII$^{fl/fl}$LysMCre wound at day 14 post injury showed attenuated collagen deposition in TGFβRII$^{fl/fl}$LysMCre wound compared to control wound (n= 3-4) at 20x and higher 40x magnification.

Connective tissue growth factor (CTGF) is one of the important factors, produced by activated myofibroblast and required for myofibroblast proliferation and differentiation (Grotendorst and Duncan 2005). Moreover, TGF-β1 activated CTGF expression in fibroblast has been shown to be important for myofibroblast differentiation (Garrett, Khaw et al. 2004). To address the question whether myofibroblast function was also affected at the molecular level in TGFβRII$^{fl/fl}$LysMCre wounds, expression of connective tissue growth factor (CTGF) was analyzed on RNA isolated from wound tissue at day 4, day 7 post injury by quantitative real time PCR. Expression of CTGF in TGFβRII$^{fl/fl}$LysMCre wounds at day 4 post injury showed reduction compared to control wounds, however at later stages of the healing, CTGF expression was similar to the level in control wounds (Fig. 3-9). This analysis supports the hypothesis that the deficiency of TGFβRII signaling in the macrophages results in impaired fibroblasts-macrophage interaction, thereby affecting transition of fibroblast to myofibroblast and myofibroblast functions.

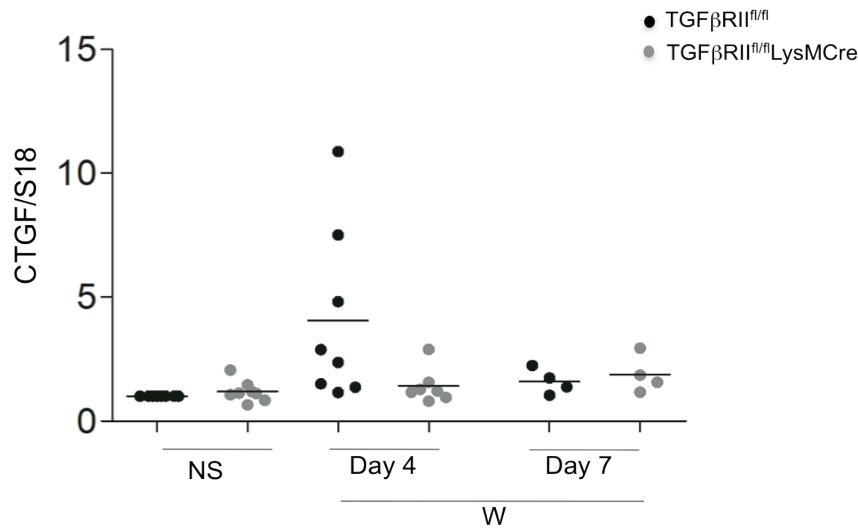


Fig. 3-9: Reduced expression of CTGF in TGFβRII$^{fl/fl}$LysMCre wounds. Real time PCR analysis of total wound tissue at day 4 and day 7 post injury showed no up-regulation of CTGF in TGFβRII$^{fl/fl}$LysMCre wounds at day 4 post injury compared to the up-regulation in control wounds. Expression in wounded skin of control and TGFβRII$^{fl/fl}$LysMCre mice and unwounded skin of TGFβRII$^{fl/fl}$LysMCre mice is normalized over control unwounded skin. **H**orizontal lines represent the mean. Statistical significance was determined using student's t-test. NS: unwounded normal skin, W: wound tissue.

Reduced wound contraction and myofibroblast differentiation, accompanied by impaired CTGF and altered collagen deposition at the wound site, posed the question of why this was so. Based on the previous known role of TGF-β1 on macrophages and fibroblasts, two hypotheses can be proposed:

1. TGF-β1 regulates its own production in macrophages in an autocrine manner (Wahl, McCartney-Francis et al. 1990; Lucas, Waisman et al. 2010). Due to the deficiency of TGFβRII, its autocrine regulation might be impaired in macrophages, thereby less TGF-β1 is available to wound fibroblasts to proliferate and differentiate into myofibroblast. Moreover, TGF-β1 is a direct regulator of CTGF production from fibroblast (Garrett, Khaw et al. 2004), thereby the reduced availability of TGF-β1 can also affect CTGF and collagen production from the activated fibroblasts in the wounds of TGFβRII$^{fl/fl}$LysMCre mice. This will lead to the delayed wound contraction.

2. Besides autocrine TGF-β1 regulation, TGFβRII mediated signaling in macrophages also regulate production of fibronectin, osteopontin, platelet derived growth factors, tissue plasminogen activator etc, which are in turn, also required for differentiation of fibroblasts into myofibroblasts and their functions (McCartney-Francis, Mizel et al. 1990; Lenga, Koh et al. 2008; Schafer and Werner 2008). Therefore, macrophage-derived factors under TGFβRII mediated signaling also mediate cross talk between macrophage and fibroblast, which could be disturbed due to the loss of TGFβRII signaling in macrophages.

3.3.3 Similar expression of TGF-β1 in the wounds of control and TGFβRII$^{fl/fl}$LysMCre mice

To address the possibility that TGFβRII$^{fl/fl}$LysMCre wounds may have less availability of TGF-β1 due to an impaired autocrine loop, the expression of TGF-β1 was analyzed on RNA isolated from control and TGFβRII$^{fl/fl}$LysMCre wounds at day 4 and day 7 post injury by quantitative real time PCR. This analysis showed up-regulation of TGF-β1 in both control and TGFβRII$^{fl/fl}$LysMCre wounds at day 7 post injury compared to normal skin, however no significant differential regulation in TGF-β1 expression was observed between control and TGFβRII$^{fl/fl}$LysMCre wounds (Fig. 3-10). This result indicates that possibly a different mechanism is involved to mediate cross talk between macrophages and fibroblasts.

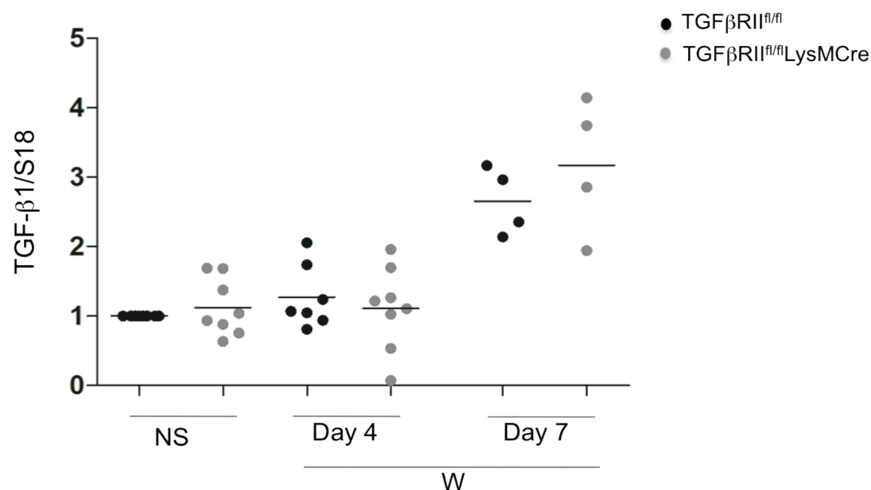


Fig. 3-10: Similar expression of TGF-β1 in control and TGFβRII$^{fl/fl}$LysMCre wound tissues. Real time PCR analysis of total wound tissue at day 4 and day 7 post injury showed no difference in TGF-β1 expression between control and TGFβRII$^{fl/fl}$LysMCre wounds. Expression in wounded skin of control and TGFβRII$^{fl/fl}$LysMCre mice and unwounded skin of TGFβRII$^{fl/fl}$LysMCre is normalized over control unwounded skin**.** Horizontal lines represent the mean. Statistical significance was determined using student's t-test. NS: unwounded normal skin, W: wound tissue

3.3.4 Loss of TGFβRII in macrophages does not impact neo-angiogenesis

TGF-β1 is a regulator of angiogenesis and also induces angiogenic activity in macrophages (Wiseman, Polverini et al. 1988). To assess the impact of TGFβRII deficiency in macrophages on angiogenesis, endothelial cells in the granulation area of wound tissue were quantified. Immunofluorescence staining (Fig. 3-7A; shown in the section 3.3.2) and quantification of CD31 positive cells in control and TGFβRII$^{fl/fl}$LysMCre wounds did not show any significant difference at day 4, day 7 and day 14 post injury (Fig. 3-11A). VEGF−A is a crucial regulator of angiogenesis and its expression in macrophages

is induced by TGF-β1 (Jeon, Chae et al. 2007). To address the question whether expression of VEGF-A is affected due to the deficiency of TGFβRII signaling in macrophages, its expression was analyzed in control and TGFβRII$^{fl/fl}$LysMCre wounds at day 4 and day 7 post injury by real time PCR. However, similar expression levels of VEGF-A was found in control and TGFβRII$^{fl/fl}$LysMCre wounds and correlated with the similar angiogenesis response observed in wounds from both genotypes (Fig. 3-11B).

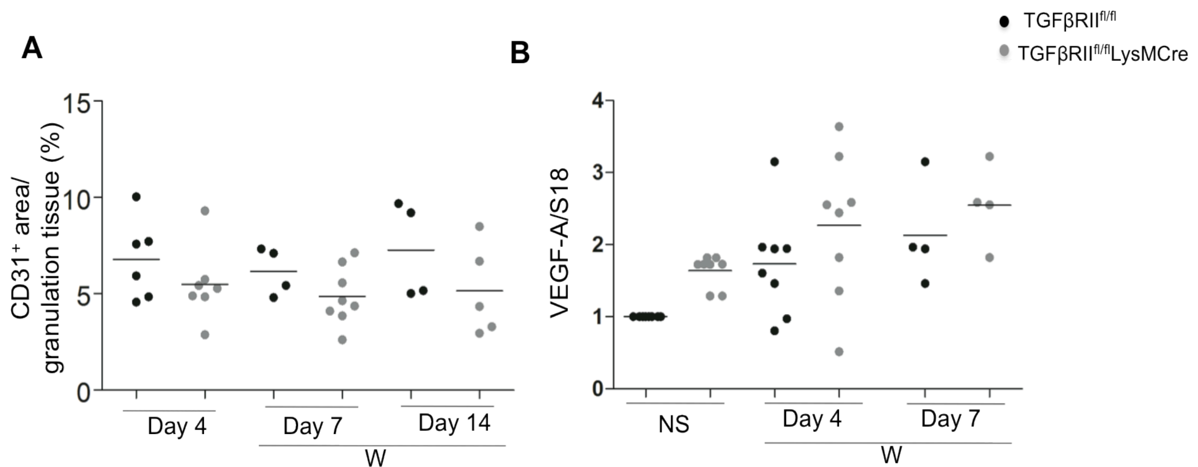


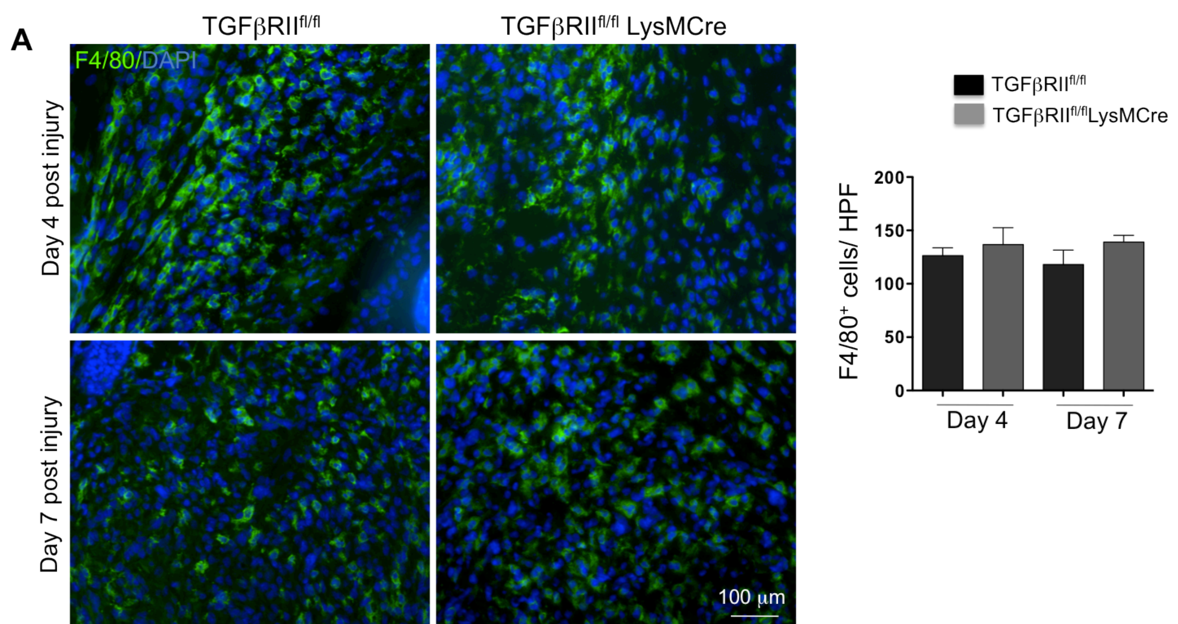
Fig. 3-11: Loss of TGFβRII in macrophages does not impact angiogenesis. A) Quantification of CD31 positive endothelial cells within the granulation tissue at day 4, day 7 and day 14 post injury showed no significant difference between control and TGFβRII$^{fl/fl}$LysMCre wounds. **B)** Real time PCR analysis of VEGF-A expression in wound tissue at day 4 and day 7 post injury showed no difference in regulation between control and TGFβRII$^{fl/fl}$LysMCre mice. Expression in wounded skin of control and TGFβRII$^{fl/fl}$LysMCre mice and unwounded skin of TGFβRII$^{fl/fl}$LysMCre mice was normalized over control unwounded skin**.** Horizontal lines represent the mean. Statistical significance was determined using student's t-test. NS: unwounded normal skin, W: wound tissue.

3.3.5 Loss of TGFβRII in macrophages does not impact infiltration of macrophage into the wound site

TGF-β1 is one of the most potent chemo-attractants for monocytes (Wahl, Hunt et al. 1987) and recruitment of monocytes and macrophages is an essential requirement for the normal healing process as previously shown by depletion of macrophages from the wound site (Lucas, Waisman et al. 2010). However, the role of TGFβ1-TGFβRII signaling in monocyte recruitment during the repair process has not been described very well.

To analyze the impact of TGFβRII deficiency in macrophage on the recruitment to the wound site, cells were analyzed by immunostaining of wound sections and quantified by flow cytometry. Surprisingly, in immunofluorescence stainings, the number of F4/80 positive macrophages seemed to be similar between control and TGFβRII$^{fl/fl}$LysMCre wounds at day 4 and day 7 post injury (Fig. 3-12A). Quantification of CD11b/F4/80

positive cells and CD11b/Gr-1 positive cells was performed by FACS analysis on the cells isolated from control and TGFβRII$^{fl/fl}$LysMCre wound tissue. This analysis revealed two different CD11b^{+}/F4/80^{+} populations with high and low intensity of F4/80 staining, respectively (Fig. 3-12B). However, no significant difference was observed in the percentage of both cell population in control and TGFβRII$^{fl/fl}$LysMCre wounds, which was consistent with the immunostaining (Fig. 3-12B & C). Similar to CD11b/F4/80 positive macophages, no significant difference was observed in CD11b/Gr-1 positive neutrophils between control and TGFβRII$^{fl/fl}$LysMCre wounds (Fig. 3-12D). This result also correlates with the similar recruitment of monocytes to the peritoneal cavity in thioglycollate-induced peritonitis in control and TGFβRII$^{fl/fl}$LysMCre mice (shown in the section 3.2.2). This indicates the involvement of other potent factors in the recruitment of monocytes under *in vivo* inflammation.



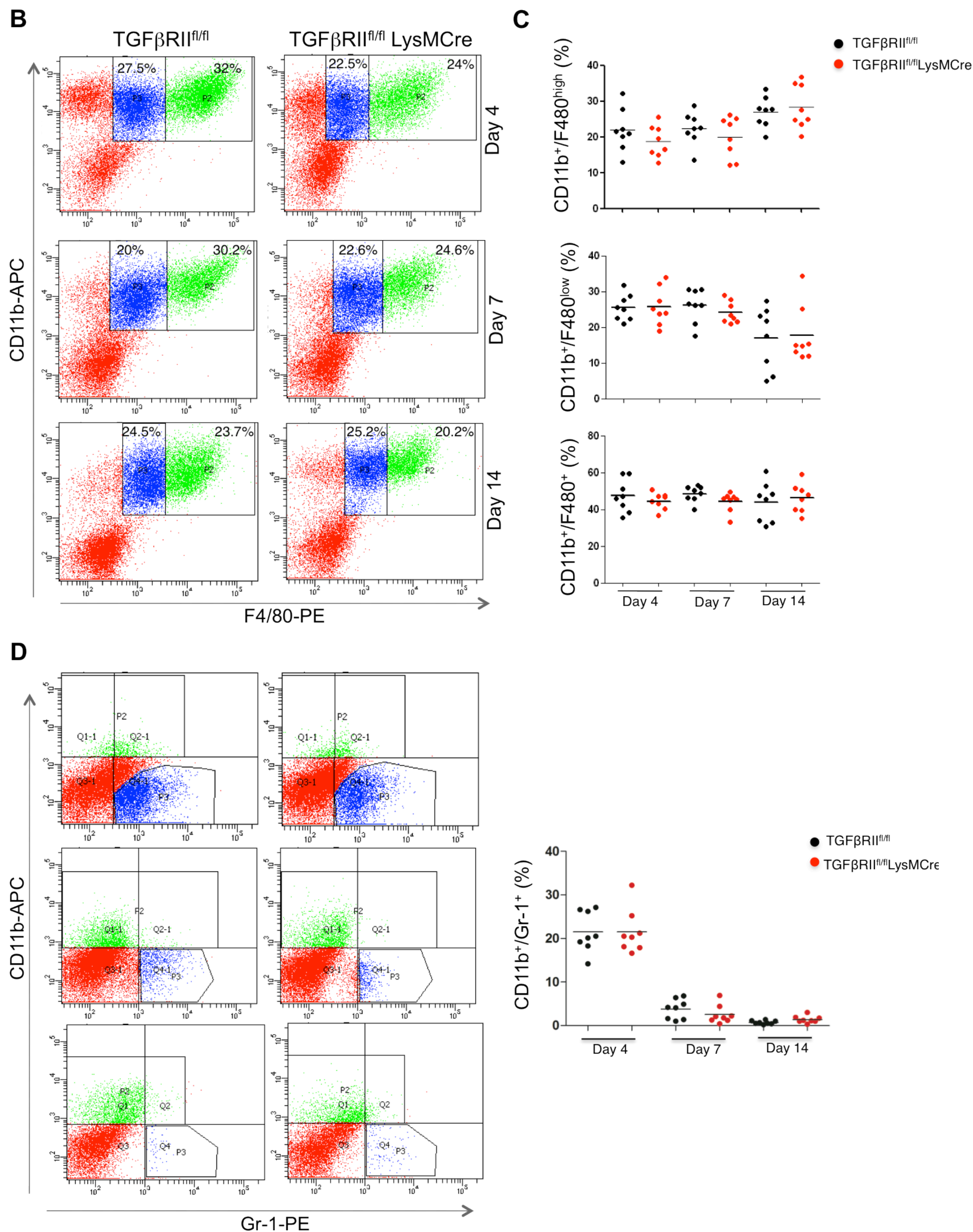


Fig. 3-12: TGFβRII deficiency in macrophages does not alter recruitment of cells to the wound site. A) Immunofluorescence staining and quantification of F4/80 positive cells in wounds showed similar numbers of F4/80 positive macrophages between control (n= 2) and TGFβRII$^{fl/fl}$LysMCre (n=3) mice. **B, C, D)** FACS analysis quantification of macrophages (B, C) and neutrophils (D), isolated from control and TGFβRII$^{fl/fl}$LysMCre wounds at day 4, day 7 and day 14 post injury revealed no difference in the percentage of macrophages and neutrophils population. Horizontal line represents the mean. Statistical significance was determined using student's t-test. NS: unwounded normal skin, W: wound tissue. HPF: high power field. NS: unwounded normal skin, W: wound tissue. HPF: high power field

3.3.6 Loss of TGFβRII in macrophages has no influence on inflammatory cytokine induction

To analyze the impact of loss of TGF-β1 signaling on the regulation of the local inflammatory cytokine milieu in wound tissue, expression of TNF-α and IL-1β was analyzed at day 4 and day 7 post injury by real time PCR. TNF-α and IL-1β are the crucial cytokines, up-regulated during the inflammatory phase of wound healing. As expected, both the cytokines were highly up-regulated in wound tissue at day 4 post injury while their expression declined by day 7 post injury. However, no significant difference in the regulation was observed between control and TGFβRII$^{fl/fl}$LysMCre wounds (Fig. 3-13).

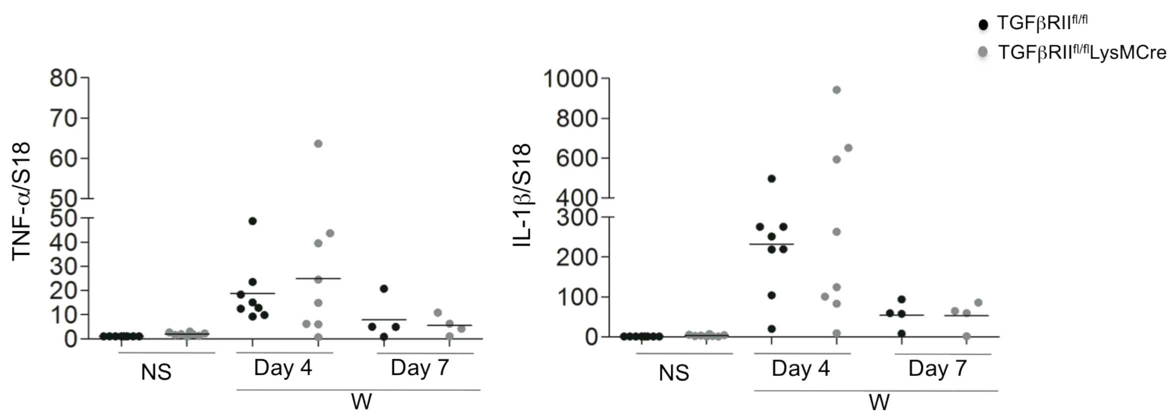


Fig. 3-13: Similar expression of TNF-α and IL-1β in control and TGFβRII$^{fl/fl}$LysMCre wound tissue. Real time PCR analysis of total wound tissue at day 4 and day 7 post injury indicated similar level of up-regulation of TNF-α and IL-1β expression in both control and TGFβRII$^{fl/fl}$LysMCre wounds. Expression in wounded skin of control and TGFβRII$^{fl/fl}$LysMCre mice and unwounded skin of TGFβRII$^{fl/fl}$LysMCre mice was normalized over control unwounded skin. Horizontal lines represent the mean. Statistical significance was determined using student's t-test. NS: normal skin, W: wound tissue.

3.3.7 Loss of TGFβRII in macrophages does not impact pro-fibrotic, pro-inflammatory and pro-angiogenic cytokine regulation in isolated wound macrophages

The wound tissue consists of many cell types and therefore the expression and regulation of fibrotic and pro-inflammatory factors can be a mixed response of all the cell types. To address the specific contribution of control and TGFβRII$^{fl/fl}$LysMCre macrophages in the generation of fibrotic and inflammatory factors at the wound tissue, macrophages were isolated from control and TGFβRII$^{fl/fl}$LysMCre wounds and used to screen various factors. Particularly, the factors which were up-regulated in wound tissues were analyzed in the isolated macrophages. To do this, total cells from control and TGFβRII$^{fl/fl}$LysMCre wounds

at day 4, day 7 post injury were isolated by cell isolation protocol and FACS sorted for F4/80 positive macrophages.

Analysis of TGF-β1 and CTGF on the RNA isolated from wound macrophages at day 4 and day 7 post injury showed a similar expression pattern of both the genes in control and TGFβRII$^{fl/fl}$LysMCre wound macrophages and correlated with their expression regulation pattern in wound tissue at this time point (Fig. 3-14A & see Fig. 3-9, 3-10). This data suggests that similar expression of TGF-β1 in control and TGFβRII$^{fl/fl}$LysMCre wound macrophages might be due to compensatory factors that are involved in TGF-β1 regulation in macrophages. Previously, we showed that the regulation of CTGF was reduced in the wound tissue from TGFβRII$^{fl/fl}$LysMCre mice at day 4 post injury (See Fig. 3-9), however, CTGF regulation in wound macrophages from control and TGFβRII$^{fl/fl}$LysMCre mice showed no difference (Fig. 3-14A) and indicates the involvement of other cell types, contributing to the reduced CTGF expression in TGFβRII$^{fl/fl}$LysMCre wounds, probably, myofibroblasts.

To analyze the influence of TGF-β1 signaling on the regulation of inflammatory cytokines and the expression of angiogenic cytokines in wound macrophages, expression of TNF-α, IL-1β and VEGF-A was analyzed by real time PCR. TNF-α and IL-1β are the crucial cytokines for the inflammatory phase of wound healing. As expected, both the cytokines were highly up-regulated in wound macrophages at day 4 post injury and the expression of IL-6 was similar between control and TGFβRII$^{fl/fl}$LysMCre wound macrophages. By contrast, the TNF-α expression in TGFβRII$^{fl/fl}$LysMCre wound macrophages revealed to be reduced compared to control macrophages, although this difference was not significant (Fig. 3-14B). In contrast to IL-6 and TNF-α, expression of IL-1β was reduced in TGFβRII$^{fl/fl}$LysMCre wound macrophages compared to the control macrophages (Fig. 3-14C). This result indicates differential regulation of some pro-inflammatory functions of macrophages at the wound site due to the loss of TGFβRII. This effect was macrophage specific, as this was not seen in the wound tissue.

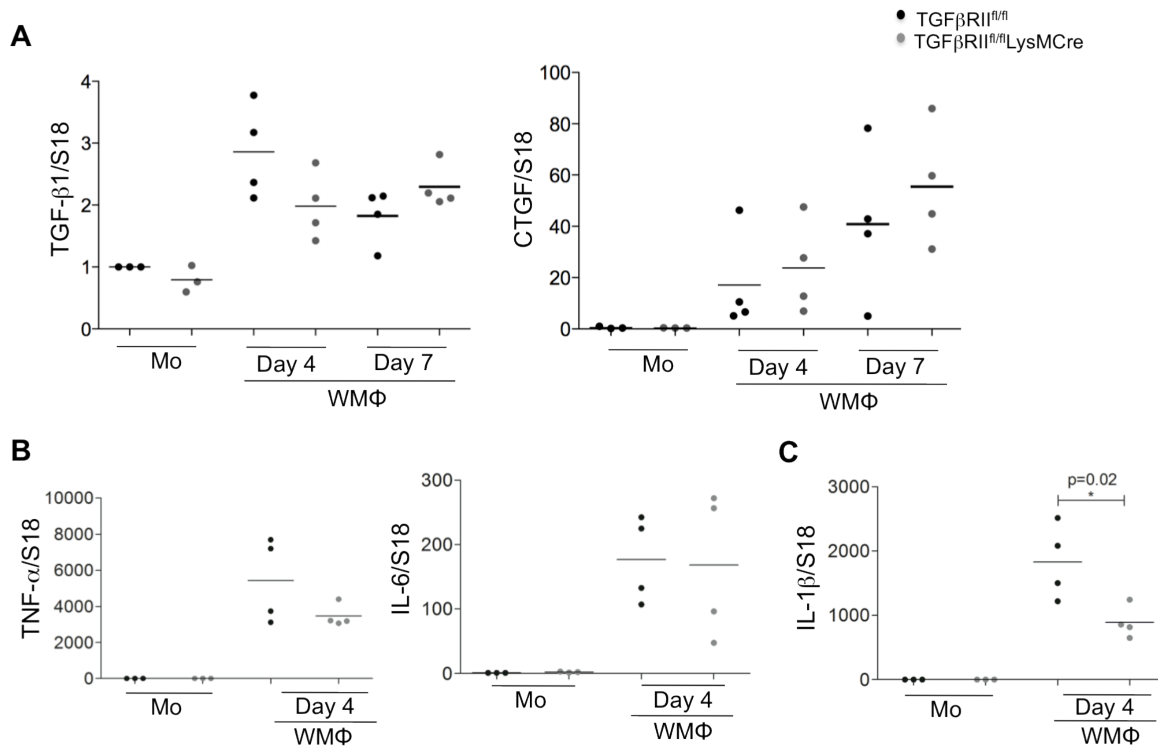


Fig. 3-14: Similar up-regulation of pro-fibrotic and pro-inflammatory cytokines and less up-regulation of IL-1β expression in TGFβRII$^{fl/fl}$LysMCre wound macrophages. A,B,C) Real time PCR analysis of RNA from macrophages isolated from total wound tissue at day 4 and day 7 post injury shows similar level of up-regulation of TGF-β1 and CTGF expression (A), TNF-α and IL-6 (B) and reduced up-regulation of IL-1β in TGFβRII$^{fl/fl}$LysMCre wound macrophages compare to control wound macrophages (B). Expression in wound macrophages from control and TGFβRII$^{fl/fl}$LysMCre mice, and blood monocytes from TGFβRII$^{fl/fl}$LysMCre mice was normalized over control blood monocytes. Horizontal lines represent the mean. Statistical significance was determined using student's t-test. Mo: blood monocytes, WMΦ: wound macrophages.

3.4 Impact of loss of TGFβRII signaling in macrophages on bleomycin induced fibrosis

To elucidate the impact of TGFβRII in the regulation of skin fibrosis in response to inflammatory tissue damage, we used a model of bleomycin-induced skin fibrosis. To induce fibrosis, 100 μg/ml of bleomycin was injected intra-dermal in control and TGFβRII$^{fl/fl}$LysMCre mice. Mice were sacrificed after two weeks and four weeks of treatment for tissue analysis (Fig. 3-15).

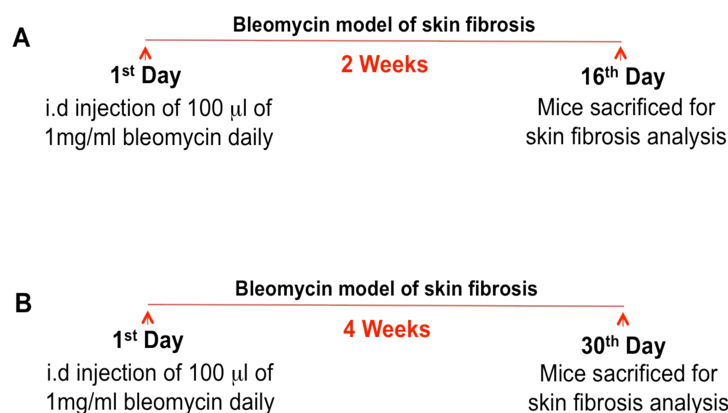


Fig. 3-15: Schematic representation of bleomycin injection to induce cutaneous fibrosis. Mice were injected with 1 mg/ml of bleomycin everyday for 2 weeks (A) or 4 weeks (B) and sacrificed for tissue analysis, real time PCR as well as cell isolation.

3.4.1 Abrogation of TGFβRII in macrophages results in reduced bleomycin induced fibrosis

Macroscopically as well as histopathological analysis of the fibrotic skin revealed reduced fibrosis and reduction of dermal thickness in TGFβRII$^{fl/fl}$LysMCre fibrotic skin compared to control fibrotic skin after two weeks and four weeks of bleomycin injection (Fig. 3-16A). To further confirm the reduced dermal thickness of TGFβRII$^{fl/fl}$LysMCre fibrotic skin, thickness of the dermis was measured. This analysis also showed significant reduction in the dermal thickness of TGFβRII$^{fl/fl}$LysMCre fibrotic skin compared to the control fibrotic skin after two and four weeks of bleomycin injection (Fig. 3-16A), supporting the histological observations. Interestingly, the phenotype observed in TGFβRII$^{fl/fl}$LysMCre mice was similar to the phenotype observed in bleomycin induced skin fibrosis in complete Smad3 knock out (Lakos, Takagawa et al. 2004). Since Smad3 is the downstream signaling target of TGF-β1, the phenotype observed in Smad3 knock out might be partially due to loss of Smad3 in macrophages. Taken together, this result suggests an important role of TGF-β1 signaling in macrophages during fibrosis.

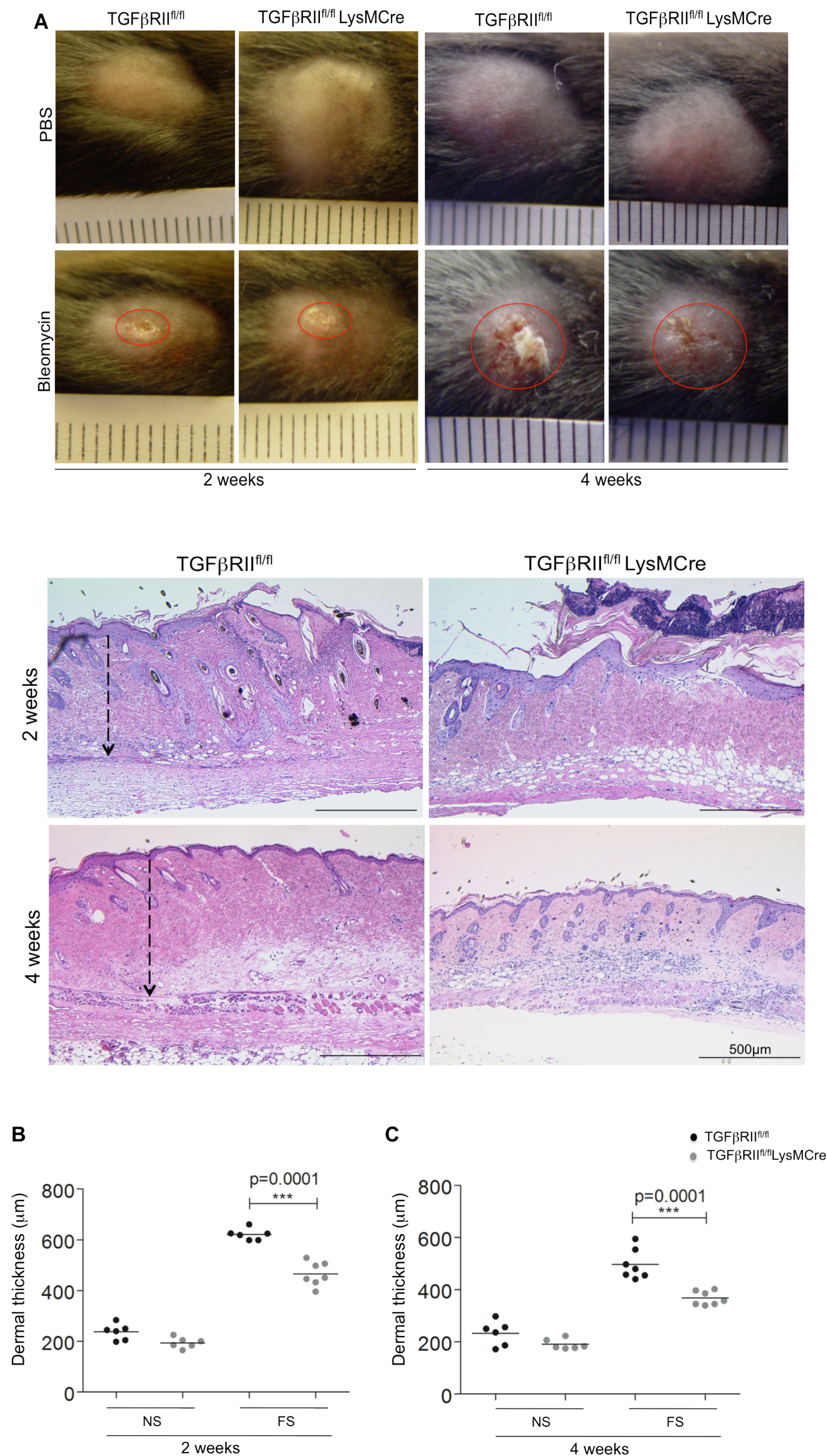
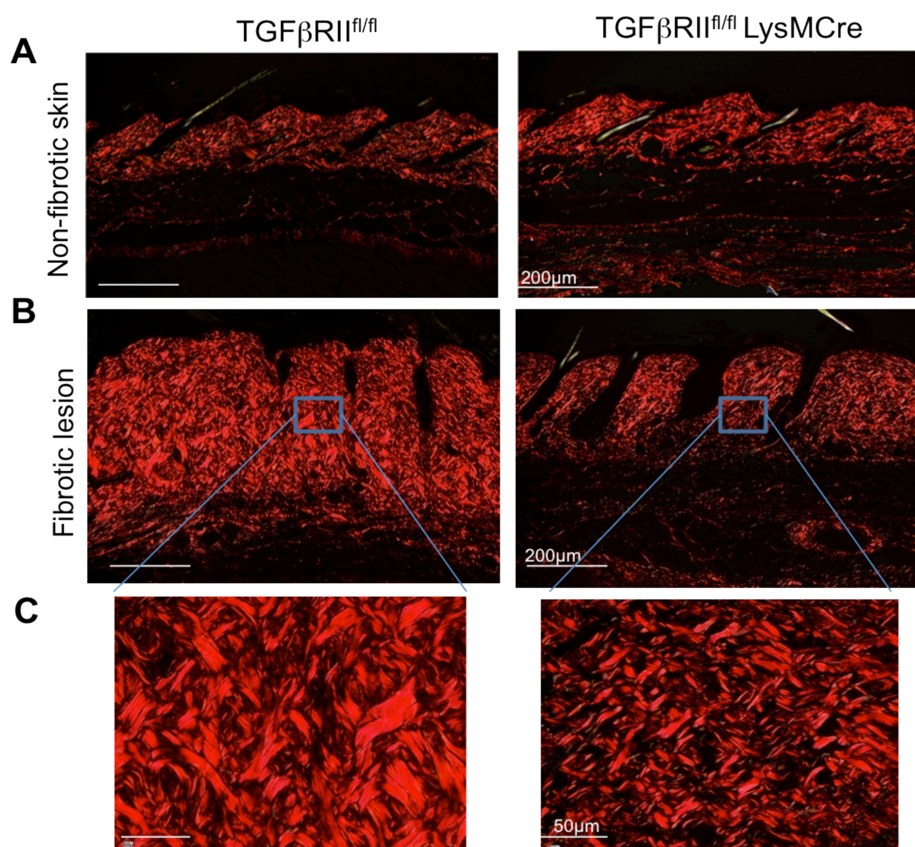


Fig. 3-16: Attenuated bleomycin induced cutaneous fibrosis in TGFβRII$^{fl/fl}$LysMCre mice. A) Macroscopic picture and H&E staining of fibrotic skin after 2 weeks and 4 weeks of bleomycin injection showed reduced fibrosis in TGFβRII$^{fl/fl}$LysMCre fibrotic skin compared to control fibrotic skin. **C, D)** Microscopic measurment of the dermal thickness in the fibrotic skin after 2 weeks (C) and 4 weeks (D) of bleomycin injection showed significant reduction of dermal thickness in TGFβRII$^{fl/fl}$LysMCre fibrotic skin compared to control fibrotic skin. Horizontal lines represent the mean. Statistical significance was determined using student's t-test. NS: normal skin, FS: fibrotic skin.

3.4.2 Abrogation of TGFβRII in macrophages results in impaired collagen deposition in bleomycin induced fibrosis

We next investigated, whether the reduced dermal thickness in bleomycin induced fibrotic skin of TGFβRII$^{fl/fl}$LysMCre was due to reduced collagen deposition in the fibrotic area. To address this question, sections of fibrotic skin were stained with Sirius Red and analyzed under light microscope. The analysis revealed reduced collagen content in TGFβRII$^{fl/fl}$LysMCre fibrotic skin compared to control fibrotic skin. Dermal thickness in the TGFβRII$^{fl/fl}$LysMCre fibrotic skin was hence, accompanied by reduced collagen production (Fig. 3-17A, B &C). Furthermore, in real time analysis of collagen-1α1 and collagen-3α1 in fibrotic dermis, separated from fibrotic skin tended to have less expression of collagen in TGFβRII$^{fl/fl}$LysMCre mice. However, due to variation in samples, this needs further analysis (Fig. 3-17D).



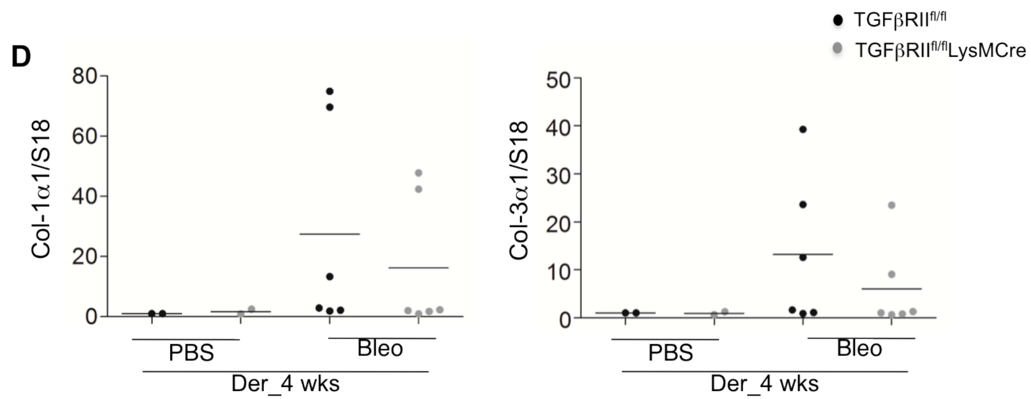


Fig. 3-17: Reduced collagen deposition in TGFβRII$^{fl/fl}$LysMCre fibrotic skin. A, B, C) Sirius red staining of normal skin (A) and of fibrotic skin (B, C) after 4 weeks of bleomycin injection and visualization with polarized light revealed less collagen fibrils in the TGFβRII$^{fl/fl}$LysMCre fibrotic skin compared to control fibrotic skin (B). **C)** Higher magnification shows a clear distinction in collagen bundle structure between control and TGFβRII$^{fl/fl}$LysMCre fibrotic skin. **D)** Real time PCR analysis of fibrotic dermis separated from fibrotic skin after 4 weeks of bleomycin injection tended to have less col-1a1 and col-3a1 expression in TGFβRII$^{fl/fl}$LysMCre mice compared to control mice. Expression in fibrotic dermis from control and TGFβRII$^{fl/fl}$LysMCre mice and PBS dermis from TGFβRII$^{fl/fl}$LysMCre mice was normalized over control PBS dermis. Horizontal lines represent the mean. Statistical significance was determined using student's t-test. Bleo: bleomycin, Der: dermis, wks: weeks.

Myofibroblasts are the primary cell type, required for collagen production and ECM deposition in fibrotic skin. Therefore, we speculated that either a disturbed auto-induction of TGF-β1 synthesis in TGFβRII receptor deficient macrophages or a disturbed communication between macrophages and fibroblasts, might contribute to the reduced fibrosis observed in TGFβRII$^{fl/fl}$LysMCre mice.

3.4.3 TGFβRII deficiency does not affect the number of myofibroblast and endothelial cells in bleomycin induced fibrosis

In order to assess whether reduced collagen deposition correlates with the reduced numbers of myofibroblasts in the fibrotic area, the fibrotic tissue was stained for α-SMA. The quantification of α-SMA positive areas revealed no significant difference between fibrotic area in control and TGFβRII$^{fl/fl}$LysMCre mice (Fig. 3-18A). Additionally, no significant difference in CD31 positive endothelial cellnumbers was observed (Fig. 3-18B).

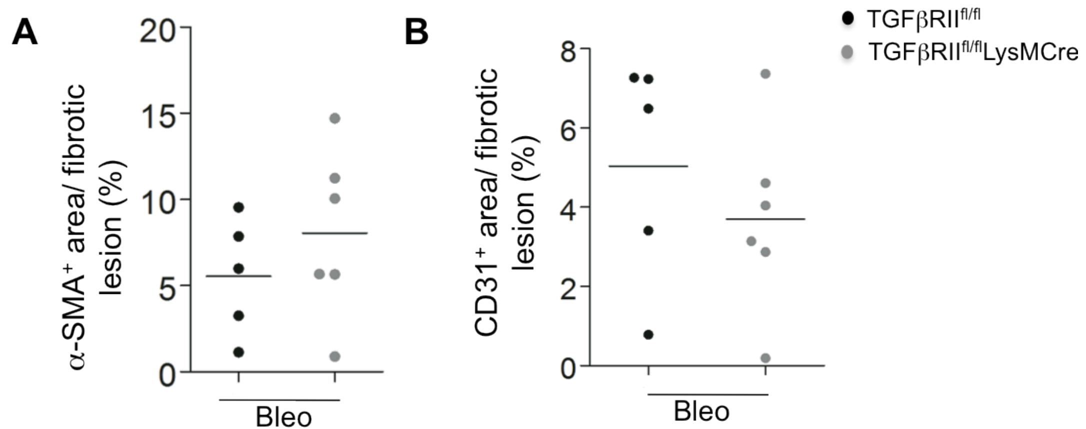


Fig. 3-18: TGFβRII deficiency in macrophages does not impact myofibroblasts differentiation and angiogenesis in bleomycin induced fibrosis. A, B) Quantification in fibrotic area after 4 weeks of bleomycin injection shows similar increase of α-SMA area (A) and CD31 positive area (B) between control and TGFβRII$^{fl/fl}$LysMCre fibrotic skin. Horizontal lines represent the mean. Statistical significance was determined using student's t-test. Bleo: bleomycin.

3.4.4 Possible mechanisms involved in the reduced bleomycin induced fibrosis of TGFβRII$^{fl/fl}$LysMCre mice

In order to find the mechanism behind reduced fibrosis in TGFβRII$^{fl/fl}$LysMCre mice, we set out to investigate different parameters that could potentially contribute to the reduced fibrosis. Inflammation is the prerequisite for fibrosis. Inflammatory mediators, secreted by immune cells, and cell-cell interactions at the local site are of prime importance to generate and sustain fibrotic conditions. Therefore, we analyzed the presence of inflammatory immune cells and their contribution to the local cytokine milieu in bleomycin induced fibrosis of control and TGFβRII$^{fl/fl}$LysMCre mice.

3.4.4.1 Reduced infiltration of myeloid cells in bleomycin induced fibrotic skin of TGFβRII$^{fl/fl}$LysMCre mice

To find out the contribution of inflammatory immune cells, sections from fibrotic skin of control and TGFβRII$^{fl/fl}$LysMCre mice were stained for Gr-1 positive neutrophils and giemsa positive mast cells. Interestingly, a significant reduction in number of Gr-1^{+} neutrophils was observed in the fibrotic area of TGFβRII$^{fl/fl}$LysMCre mice after 4 weeks of bleomycin injection (Fig. 3-19A), however no differences were observed in the number of giemsa positive mast cells (Fig. 3-19B).

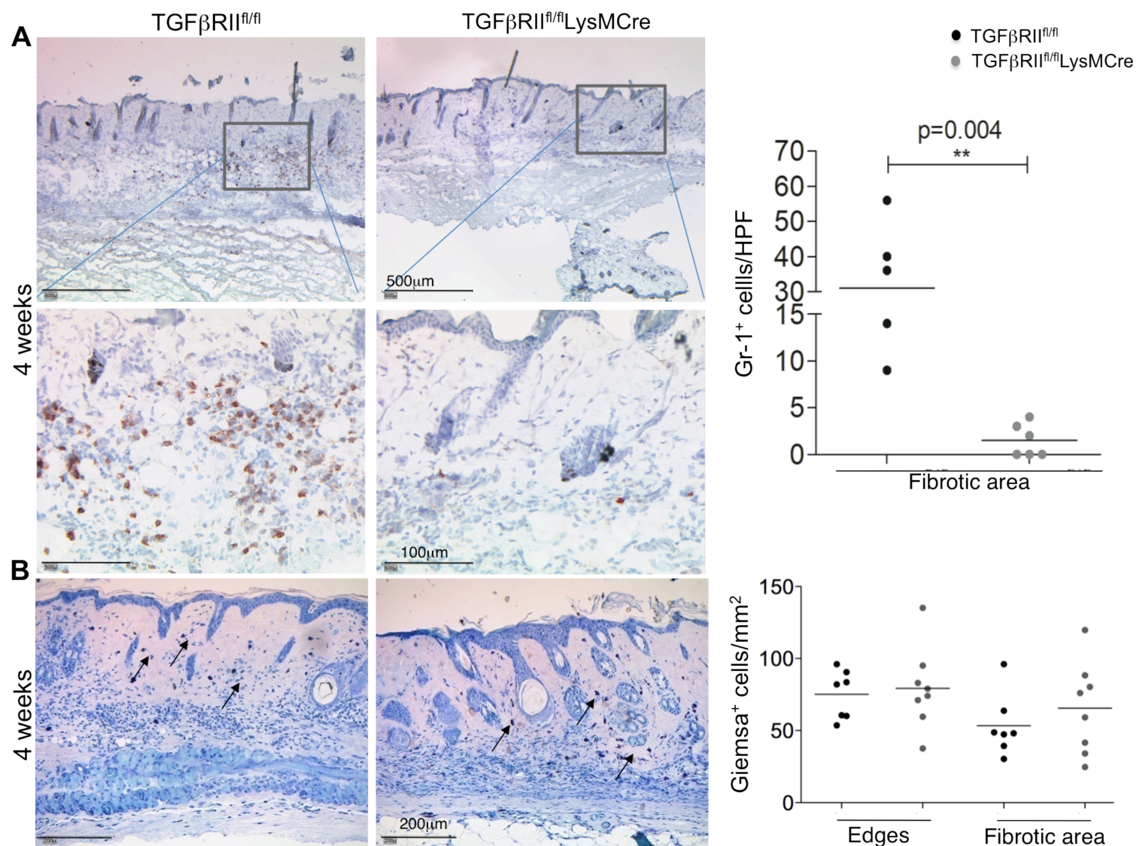


Fig. 3-19: Reduced neutrophils and unaltered mast cells in fibrotic skin after four weeks of bleomycin injection. A, B) Immunohistochemistry and quantification in fibrotic area after four weeks of bleomycin injection shows significantly reduced number of Gr-1^{+} neutrophils in TGFβRII$^{fl/fl}$LysMCre mice compared to control mice (A) and similar number of mast cells in control and TGFβRII$^{fl/fl}$LysMCre mice (B). Horizontal lines represent the mean. Statistical significance was determined using student's t-test. Bleo: bleomycin, HPF: high power field.

To further investigate the altered recruitment of immune cells, cells were isolated from bleomycin induced fibrotic area of control and TGFβRII$^{fl/fl}$LysMCre mice and analyzed by FACS using myeloid cell specific surface markers. Interestingly, after 2 weeks of bleomycin injection the absolute number of total cells, which were normalized to the total tissue weight, was significantly reduced in TGFβRII$^{fl/fl}$LysMCre mice compared to control mice (Fig 3-20A). After 4 weeks, the total number of cells tended to be lower in TGFβRII$^{fl/fl}$LysMCre mice as well, however this needs further investigation (Fig. 3-20A). To address whether the reduced numbers of cells were CD11b^{+} myeloid cells and CD11b^{+}/F4/80^{+} macrophages, the isolated cells were stained for CD11b or double stained for CD11b/F/480 and analyzed by FACS. Interestingly, both, the number of total myeloid cells as well as macrophages were reduced in the bleomycin treated fibrotic skin of TGFβRII$^{fl/fl}$LysMCre mice after 2 weeks compared to control mice (Fig. 3-20B and C).

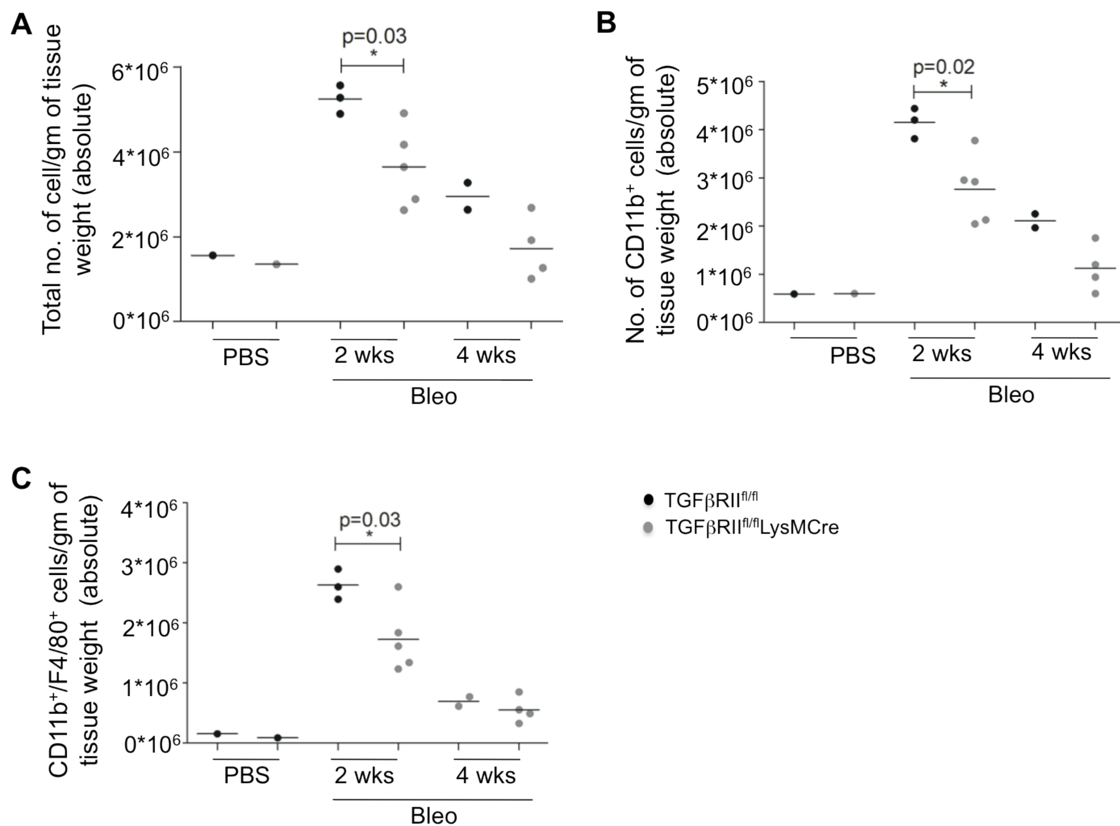


Fig. 3-20: Reduced number of total myeloid cells and macrophages in fibrotic skin of TGFβRII$^{fl/fl}$LysMCre mice. A, B, C) FACS analysis of cells isolated from fibrotic skin indicated reduced number of total cells (A), CD11b^{+} myeloid cells (B) and CD11b^{+}/F4/80^{+} macrophages (C) in TGFβRII$^{fl/fl}$LysMCre mice compared to control mice after two weeks of bleomycin injection. Horizontal lines represent the mean. Statistical significance was determined using students t-test. Bleo: bleomycin.

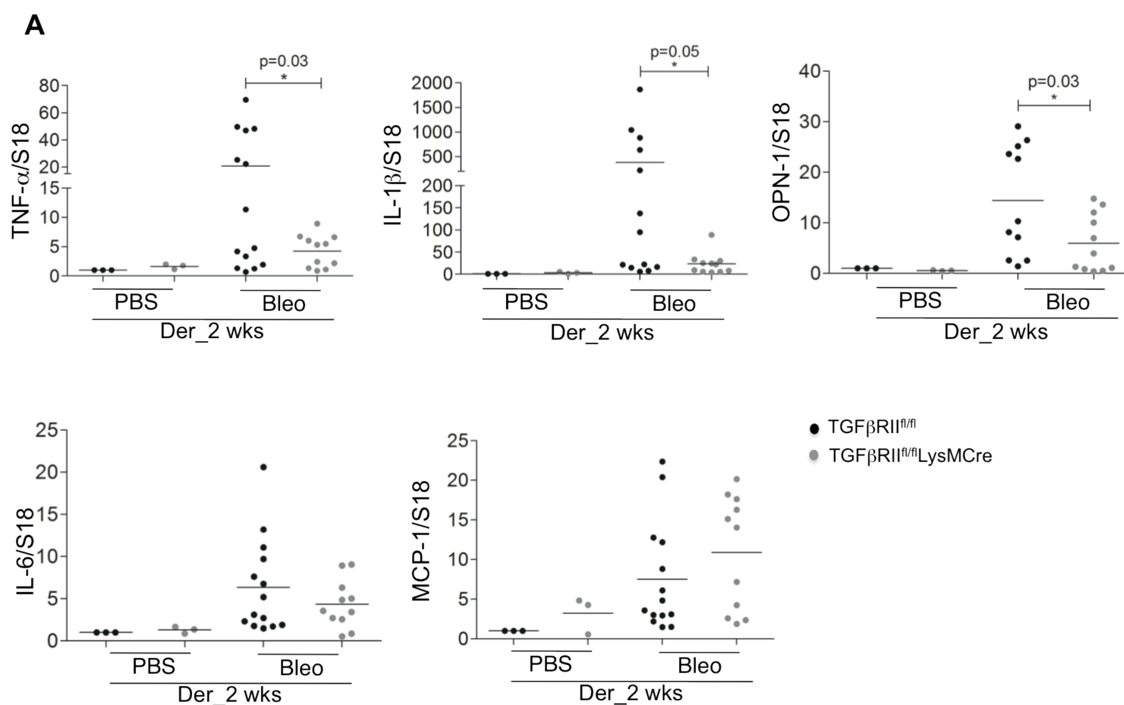
3.4.4.2 Altered dermal inflammatory cytokine profile in bleomycin induced fibrotic skin of TGFβRII$^{fl/fl}$LysMCre mice

Since, TGFβRII$^{fl/fl}$LysMCre mice showed reduced number of infiltrating immune cells in fibrotic skin, we wondered if the local milieu of inflammatory cytokines was also altered. To address the contribution of individual skin compartment in the production of local cytokine milieu, epidermis and dermis were separated from bleomycin induced fibrotic skin by ammonium thiocyanate, followed by RNA isolation and real time PCR of inflammatory cytokines.

Since, the contribution of dermal cells are considered to be of prime importance for the generation of fibrotic response, expression of several pro-inflammatory factors such as IL-1β, TNF-α, osteopontin-1 (OPN-1), IL-6 and MCP-1 was analyzed on dermal RNA from fibrotic skin after 2 week of bleomycin injection. Expression of these factors was significantly up-regulated in bleomycin injected fibrotic dermis compared to PBS injected dermis. However, compared to control fibrotic dermis, in TGFβRII$^{fl/fl}$LysMCre fibrotic dermis, the expression of IL-β, TNF-α and OPN-1 was reduced (Fig. 3-21A), whereas

expression of IL-6 and MCP-1 did not differ significantly (Fig. 3-21A). This analysis points to a reduced inflammatory response generated during fibrosis in TGFβRII$^{fl/fl}$LysMCre mice compared to control mice and correlates with the reduced infiltration of immune cells.

Reduced up-regulation of pro-inflammatory factors and reduced infiltration of immune cells in TGFβRII$^{fl/fl}$LysMCre mice compared to control mice after 2 weeks of bleomycin injection, posed the question that whether this was a transient differential regulation that drives the reduced fibrotic phenotype of TGFβRII$^{fl/fl}$LysMCre mice or a continuous less regulation of these factors are associated with the phenotype. To address this question, dermis was separated from fibrotic skin after 4 weeks of bleomycin injection and analyzed for the expression of pro-inflammatory cytokines by real time PCR. Fibrotic dermis after 4 weeks of bleomycin injection showed a significant differential up-regulation of TNF-α and MCP-1 in TGFβRII$^{fl/fl}$LysMCre mice compared to control mice (Fig. 3-21B), whereas, IL-1β and IL-6 up-regulation was comparable between control and TGFβRII$^{fl/fl}$LysMCre fibrotic dermis (Fig. 3-21B).



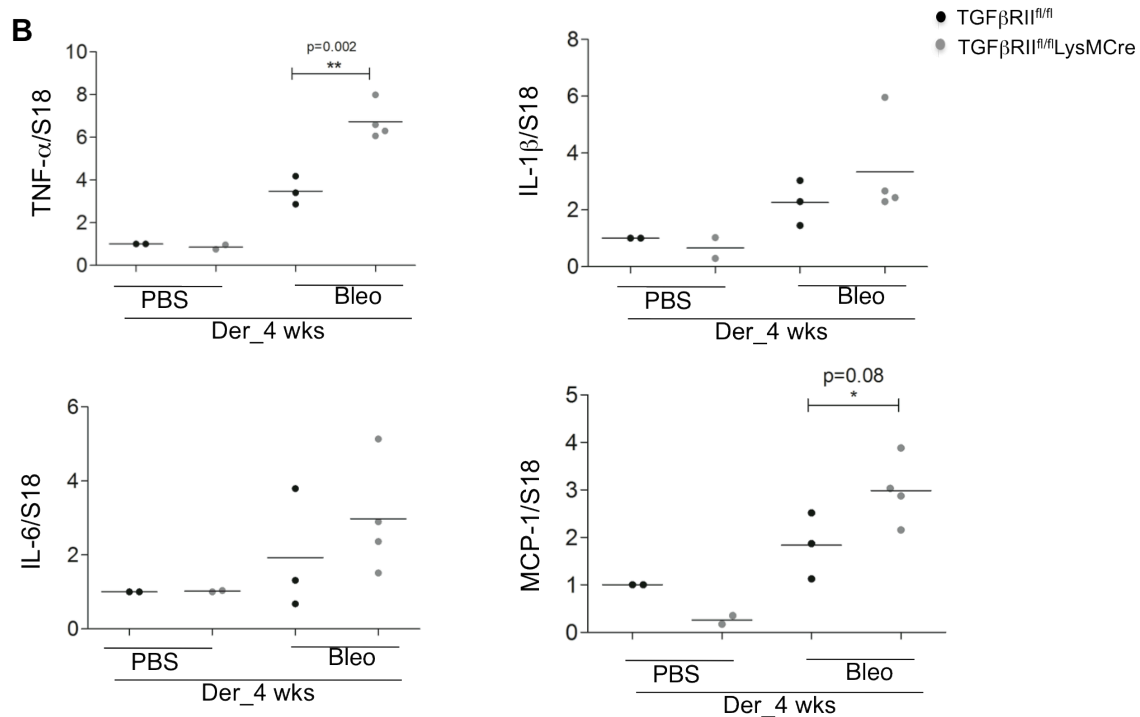


Fig. 3-21: Differential expression of pro-inflammatory cytokines in the dermis of TGFβRII$^{fl/fl}$LysMCre mice during fibrosis. A, B) Real time PCR analysis of dermis, separated from bleomycin injected fibrotic skin after 2 weeks (A) and 4 weeks (B) showed significantly less up-regulation of TNF-α, IL-1β and OPN-1 and comparable expression of MCP-1 and IL-6 in TGFβRII$^{fl/fl}$LysMCre mice after 2 weeks compared to control mice (A). Enhanced up-regulation of TNF-α and MCP-1 in TGFβRII$^{fl/fl}$LysMCre mice compared to control mice and similar up-regulation of IL-1β and IL-6 in both control and TGFβRII$^{fl/fl}$LysMCre mice after 4 weeks of bleomycin injection (B). Expression in fibrotic dermis from control and TGFβRII$^{fl/fl}$LysMCre mice and PBS dermis from TGFβRII$^{fl/fl}$LysMCre mice was normalized over control PBS dermis. Horizontal lines represent the mean. Statistical significance was determined using student's t-test. Bleo: bleomycin, Der: dermis, wks: weeks.

To answer the question whether differential expression of pro-inflammatory cytokines is tissue specific, the expression of TNF-α, IL-1β, IL-6 and MCP-1 was also analyzed in the separated epidermis in the same samples used previously for dermal gene expression analysis. In contrast to the dermal compartment, the expression of TNF-α, IL-1β, IL-6 and OPN-1 in fibrotic epidermis of both control and TGFβRII$^{fl/fl}$LysMCre mice was up-regulated to similar level, thus no differential regulation of these genes was observed between control and TGFβRII$^{fl/fl}$LysMCre mice (Fig. 3-22).

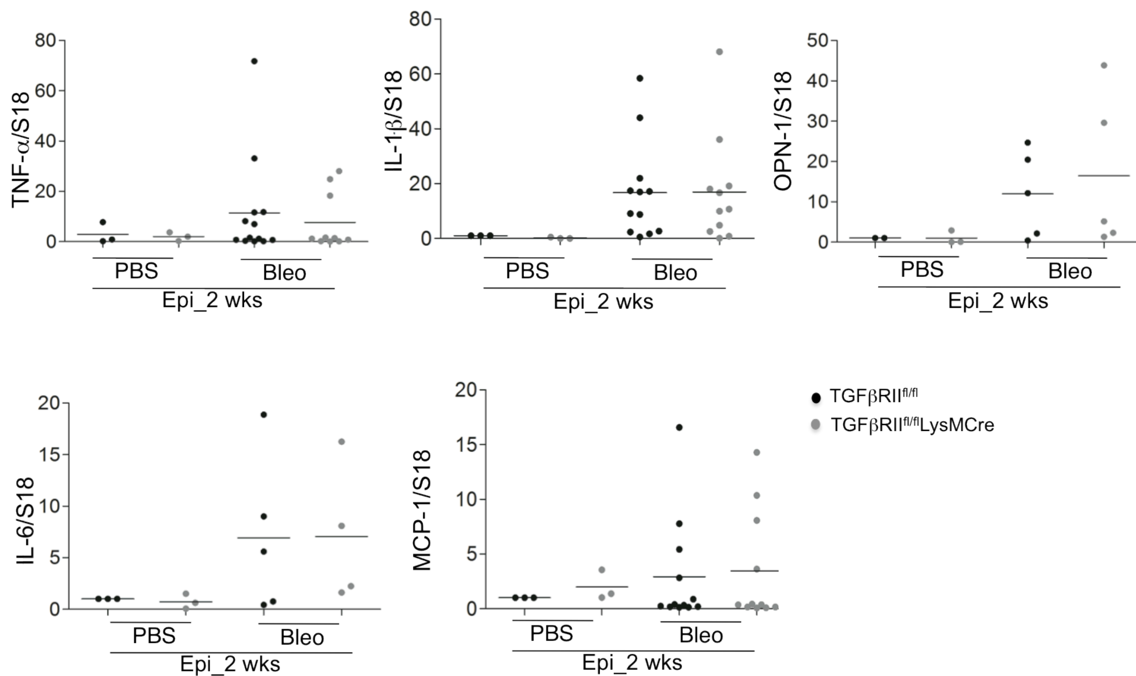


Fig. 3-22: The expression of pro-inflammatory cytokines in fibrotic epidermis of control and TGFβRII$^{fl/fl}$LysMCre mice is not altered. A, B) Real time PCR analysis of fibrotic epidermis separated from bleomycin injected fibrotic skin after 2 weeks showed comparable up-regulation of expression of TNF-α, IL-1β and OPN-1 and unaltered expression of MCP-1 in both control and TGFβRII$^{fl/fl}$LysMCre mice. Expression in fibrotic epidermis from control and TGFβRII$^{fl/fl}$LysMCre mice and PBS epidermis from TGFβRII$^{fl/fl}$LysMCre mice was normalized over control PBS epidermis. Horizontal lines represent the mean. Statistical significance was determined using student's t-test. Bleo: bleomycin, Epi: epidermis, wks: weeks.

3.4.4.3 Similar expression of pro-fibrotic factors in control and TGFβRII$^{fl/fl}$LysMCre fibrotic dermis

Pro-fibrotic growth factors such as TGF-β, CTGF and PDGF-β, directly or indirectly promote accumulation of extracellular matrix as well as proliferation and survival of myofibroblast, which is a prerequisite for the collagen production (Bonner 2004). To further address the underlying mechanism of reduced fibrosis, expression of pro-fibrotic growth factors were determined on the separated dermis from 2 weeks and 4 weeks bleomycin induced skin. However, the expression of TGF-β1, CTGF and PDGF-β isoform was not altered in fibrotic dermis after 2 weeks (3-23A) and 4 weeks (3-23B) of bleomycin injection between control and TGFβRII$^{fl/fl}$LysMCre mice.

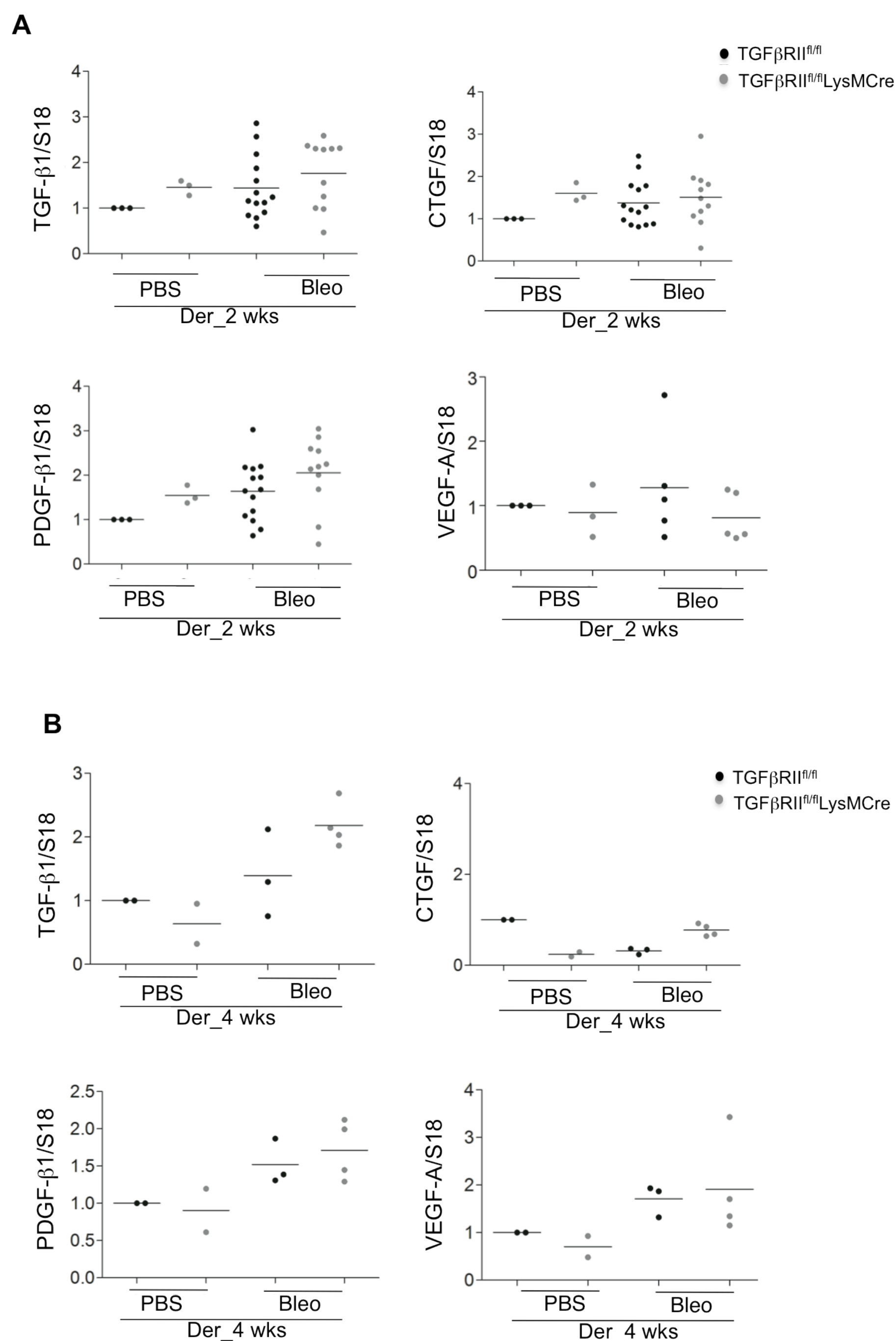


Fig. 3-23: Similar expression of pro-inflammatory cytokines between fibrotic dermis of control and TGFβRII$^{fl/fl}$LysMCre mice. A, B) Real time PCR analysis of fibrotic dermis separated from bleomycin injected fibrotic skin after 2 weeks (A) and 4 weeks (A) showed no difference in expression pattern of TGF-β1, CTGF, PDGF-β or VEGF-A between both control and TGFβRII$^{fl/fl}$LysMCre mice. Expression in fibrotic dermis from control and TGFβRII$^{fl/fl}$LysMCre mice and PBS dermis from TGFβRII$^{fl/fl}$LysMCre mice was normalized over control PBS dermis. Statistical significance was determined using student's t-test. Bleo: bleomycin, Epi: epidermis, wks: weeks.

3.4.4.4 TNF-α expression in fibrotic skin direct correlates with TNF-α expression in TGFβRII deficient macrophage isolated from fibrotic skin

To address the question whether reduced local inflammatory cytokine milieu in fibrotic dermis of TGFβRII$^{fl/fl}$LysMCre mice was directly contributed by macrophages present at the fibrotic lesion, F4/80 positive cells were FACS sorted after isolation from fibrotic skin and the expression of several genes was analyzed by real time PCR. In particular, expression of TNF-α, IL-1β and TGF-β1 was analyzed. Interestingly, the macrophages isolated from 2 weeks of bleomycin injected mice showed significantly reduced expression of TNF-α in TGFβRII$^{fl/fl}$LysMCre mice compared to control mice (Fig. 3-24A). This observation correlates with the previously observed reduced expression of TNF-α in fibrotic dermis at 2 weeks (see Fig. 3-21A). Therefore, this analysis showed a direct contribution of macrophages in producing TNF-α in fibrotic skin, which required TGFβRII expression on macrophages. Unlike expression of TNF-α , expression of IL-1β and TGF-β1 was un-altered in the isolated macrophages from 2 weeks of bleomycin injected control and TGFβRII$^{fl/fl}$LysMCre mice (Fig. 3-24B), indicating source of these factors other than macrophages at the indicate time point.

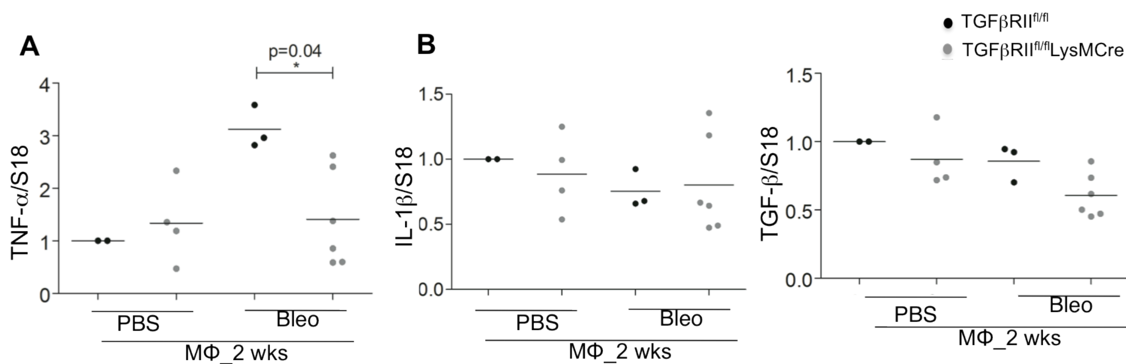
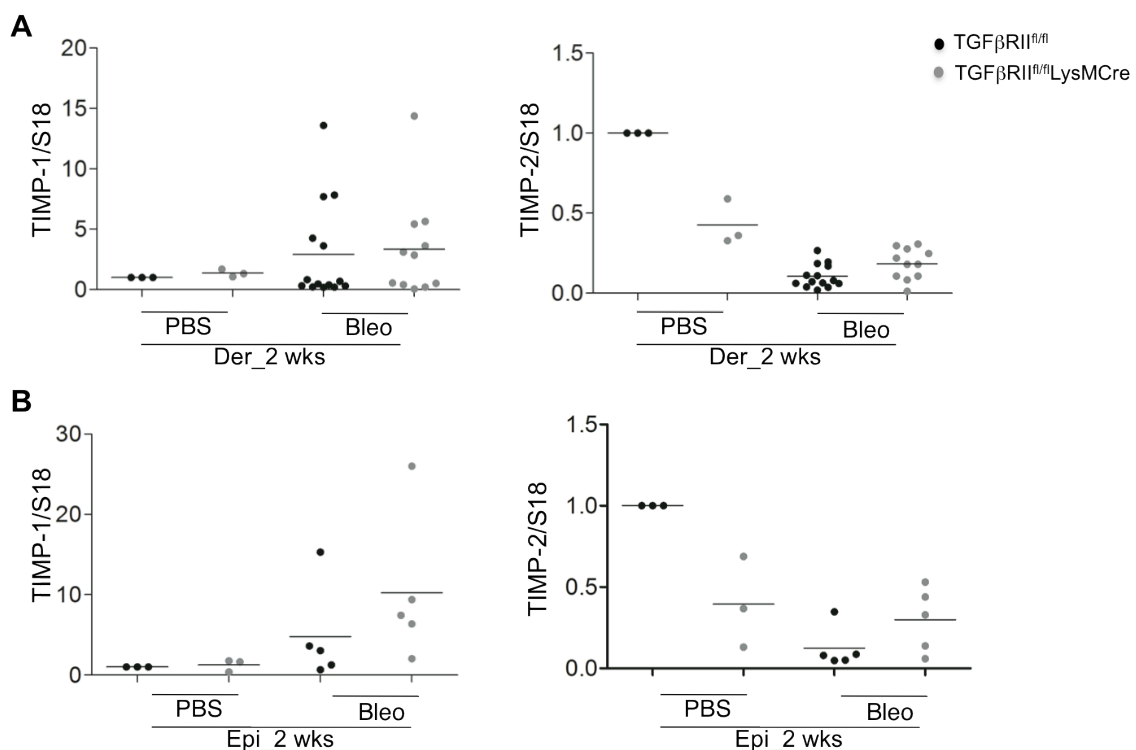


Fig. 3-24: Reduced expression of TNF-α in macrophages isolated from bleomycin injected TGFβRII$^{fl/fl}$LysMCre mice after 2 weeks. A, B,) Real time PCR analysis of TNF-α, IL-1β and TGF-β1 in macrophages isolated from bleomycin induced fibrotic skin after 2 weeks showed less regulation of TNF-α expression in TGFβRII$^{fl/fl}$LysMCre mice compared to control mice (A) and un-altered expression of IL-1β and TGF- β1 in both the mice. Expression in macrophages from fibrotic tissue of control and TGFβRII$^{fl/fl}$LysMCre mice and from PBS injected tissue of TGFβRII$^{fl/fl}$LysMCre mice was normalized over macrophages from PBS injected tissue of control mice. Horizontal lines represent the mean. Statistical significance was determined using student's t-test. Bleo: bleomycin, Epi: epidermis, MΦ: macrophages, wks: weeks.

3.4.4.5 Possible mechanisms of reduced fibrosis and less extracellular matrix deposition in TGFβRII$^{fl/fl}$LysMCre mice

Reduced fibrosis in TGFβRII$^{fl/fl}$LysMCre mice was associated with the reduced collagen deposition, points to a direct mechanism that possibly involves imbalanced turnover of collagen deposition and degradation. The turnover of collagen/extracellular matrix is

mainly controlled by the balanced production of matrix metalloproteinases and of tissue inhibitors of metalloproteinases (TIMPs). TIMPs are known to inhibit matrix metalloproteinases (MMPs), which are mainly associated with degradation of extracellular matrix (Krane 1985). Interestingly, TIMP-1 production from macrophages has recently been speculated to be important for skin fibrosis (Gessner, Hulgens et. al. 2011). Moreover, in fibroblasts, TGF-β1 can up-regulate the expression of TIMP-1 (Mattila, Airola et al. 1998). To address that whether reduced collagen accumulation in fibrotic area was due to imbalanced turnover of collagens and expression of tissue inhibitors of metalloproteinases (TIMPs) at the site, expression of TIMP-1 and TIMP-2 was analyzed in fibrotic epidermis as well as dermis from 2 weeks bleomycin injected mice. Although, expression of TIMP-1 was elevated in dermis (Fig. 3-25A) as well as epidermis (Fig. 3-25B), the expression level was comparable between control and TGFβRII$^{fl/fl}$LysMCre mice. Analysis of the MMP expression would be the further step to look into the mechanism of collagen turn over in fibrotic skin. Although, we have not analyzed the expression of MMPs in the fibrotic skin, MMP activity was analyzed by *in situ* zymography. To do this, frozen fibrotic skin section of control and TGFβRII$^{fl/fl}$LysMCre mice after 4 weeks of fibrosis was incubated with gel containing gelatin substrate and the activity was visualized by coomassie blue staining. As preliminary results the activity of MMPs was observed in control and fibrotic skin (Fig. 3-25C), however whether MMPs are differentially activated in TGFβRII$^{fl/fl}$LysMCre mice need further experiments and analysis.



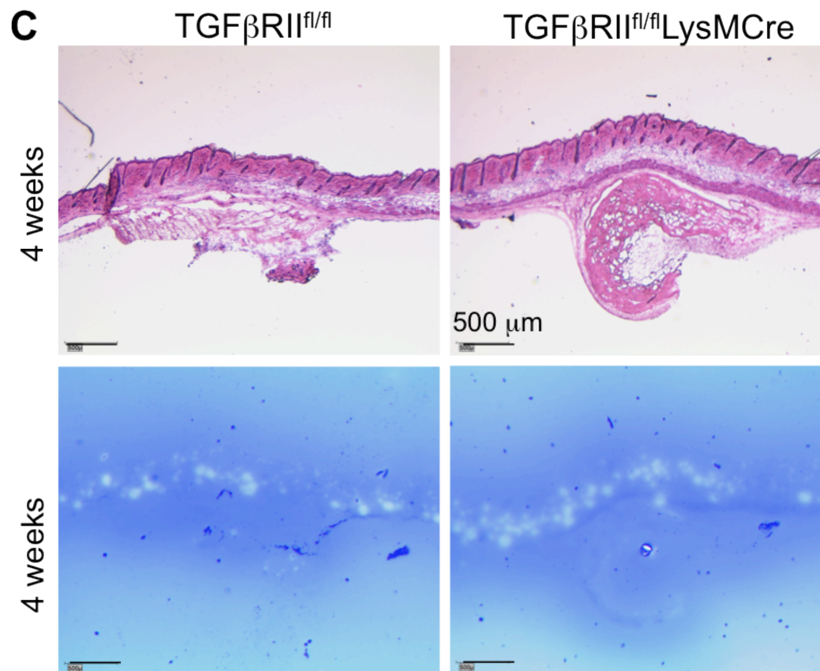


Fig. 3-25: Expression of TIMPs in epidermis and dermis of fibrotic skin after 2 weeks of bleomycin injection. A, B) Real time PCR analysis of fibrotic epidermis (B) and dermis (A) after 2 weeks of bleomycin injection showed comparable up-regulation of TIMP-1 in fibrotic dermis (A) and epidermis (B) of control and TGFβRII$^{fl/fl}$LysMCre mice, whereas TIMP-2 expression was downregulated in both dermis (A) and epidermis (B) but not significantly. **C)** H&E stained sections (upper panel), used for *in situ* zymography of collagenase/MMPs activity on the cryosections, placed and incubated on the gel containing gelatinase as a substrate and visualized by commassie blue staining (lower pannel). Expression in fibrotic epidermis/dermis from control and TGFβRII$^{fl/fl}$LysMCre mice and PBS epidermis/dermis from TGFβRII$^{fl/fl}$LysMCre mice was normalized over control PBS epidermis/dermis. Statistical significance was determined using student's t-test. Epi: epidermis, Der: dermis, Bleo: bleomycin, wks: weeks.

A reduced fibrinolysis and fibrin persistence have been associated with an enhanced accumulation of collagen and the development of skin fibrosis. Plasminogen Activator Inhibitor (PAI) is the inhibitor of fibrinolysis thereby promote fibrosis (Dellas and Loskutoff 2005). A strong correlation between expression of PAI and fibrosis has been noted in bleomycin induced pulmonary fibrosis. Mice lacking PAI gene, show a reduced bleomycin-induced pulmonary fibrosis, whereas, transgenic mice over expressing PAI show a higher collagen deposition in bleomycin-induced pulmonary fibrosis (Eitzman, McCoy et al. 1996; Senoo, Hattori et al. 2010). Based on the previous findings, we expected lower expression level of PAI in blemobycin induced fibrotic skin of TGFβRII$^{fl/fl}$LysMCre mice. However, although, PAI expression was up-regulated in both control and TGFβRII$^{fl/fl}$LysMCre fibrotic epidermis, it was not differentially modulated between control and TGFβRII$^{fl/fl}$LysMCre fibrotic epidermis (Fig. 3-26A). Moreover, in fibrotic dermis expression of PAI was unaltered in both bleomycin injected control and TGFβRII$^{fl/fl}$LysMCre mice compared to PBS injected mice (Fig. 3-26B). Further analysis of PAI-1 expression after 4 weeks of bleomycin injection would provide a better insight into the involvement of PAI in skin

fibrosis. So far, this result shows an association of epidermal PAI expression with skin fibrosis.

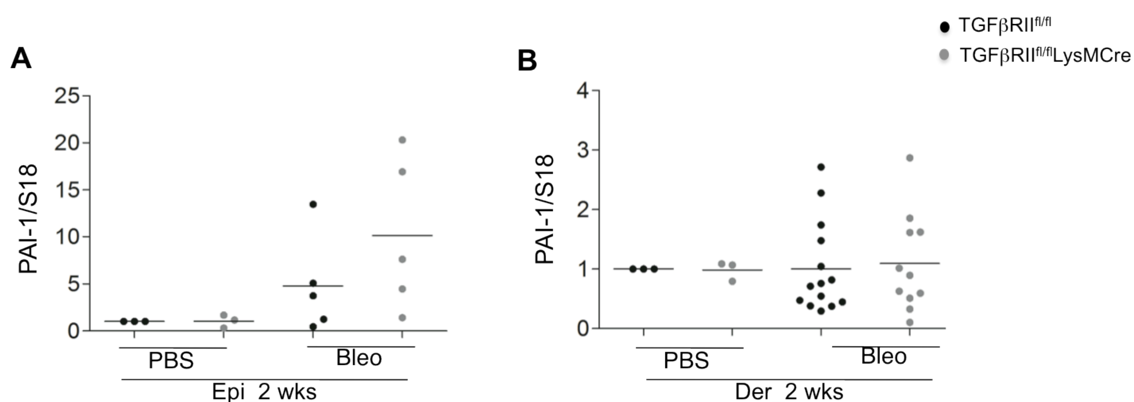


Fig. 3-26: Similar expression of plasminogen activator inhibitor (PAI-1). A, B) Real time PCR analysis of fibrotic epidermis and dermis after 2 weeks of bleomycin injection showed comparable up-regulation of PAI-1 expression in epidermis both control and TGFβRII$^{fl/fl}$LysMCre mice (A) and similar expression of PAI in the fibrotic dermis of control and TGFβRII$^{fl/fl}$LysMCre mice. Expression in fibrotic epidermis/dermis from control and TGFβRII$^{fl/fl}$LysMCre mice and PBS epidermis/dermis from TGFβRII$^{fl/fl}$LysMCre mice was normalized over control PBS epidermis/dermis. Statistical significance was determined using student's t-test. Bleo: bleomycin, Epi: epidermis, Der: dermis, wks: weeks.

4 Discussion

The role of TGF-β and macrophages is well described during different phases of tissue repair process and fibrosis (Leask and Abraham 2004; Lucas, Waisman et al. 2010). However, a cell type specific TGF-β1 mediated function during tissue repair has been poorly studied. Using inducible lineage specific deletion of TGFβRII gene in dermal fibroblasts, fibroblast specific role of TGFβRII signaling has been addressed recently (Denton, Khan et al. 2009). However, the role of TGF-β1 mediated signaling in macrophages during repair process and fibrosis has not been studied so far.

In this project, we have aimed to delineate the function of macrophage specific TGFβRII signaling during mechanical tissue injury and bleomycin induced cutaneous fibrosis. In particular, we addressed the impact of macrophage specific deficiency of TGFβRII signaling on different phases of tissue repair and fibrosis, such as immune cell infiltration, angiogenesis, collagen remodeling and fibroblast differentiation.

Our hypothesis was that TGFβRII signaling in macrophages plays important role in physiological healing process and fibrosis. To address the hypothesis, myeloid cell specific TGFβRII deficient (TGFβRII$^{fl/fl}$LysMCre) mice were generated.

Efficient deletion of TGFβRII and the loss of downstream Smad2 signaling in myeloid cells of TGFβRII$^{fl/fl}$LysMCre mice provided opportunities to explore the functional relevance of TGF-β1 mediated functions of myeloid cells in tissue repair and fibrosis.

4.1 Macrophage specific deficiency of TGFβRII results in context-dependent effect on cell migration under *in vitro* and *in vivo* condition

TGF-β1 is a potent chemoattractant factor for immune cells (Wahl, Hunt et al. 1987; Wiseman, Polverini et al. 1988; Li, Wan et al. 2006) and it also acts as anti-inflammatory factor to resolve the inflammation (Ashcroft 1999). To address the role of TGFβRII mediated signaling in cell migration under *in vivo* inflammatory conditions, control and TGFβRII$^{fl/fl}$LysMCre mice were injected with thioglycollate to induce peritonitis. In the peritoneal lavage after thioglycollate injection, the total fractions of leukocytes were significantly increased in TGFβRII$^{fl/fl}$LysMCre mice compared to control mice. This result indicates that the loss of TGFβRII in macrophages probably potentiate the inflammation in thioglycollate-induced peritonitis model due to the loss of anti-inflammatory effect, leading

to the more recruitment of inflammatory immune cells. We could, however, not observed any significant difference in the migration of CD11b/F4/80 positive macrophages, CD11b/CD115 positive monocytes as well as CD11b/Gr-1 positive neutrophils, indicating that may be the leukocytes other than myeloid cells, are recruited more to the inflammation site.

Serum contains latent/ active TGFβ as well as several other growth factors (Childs, Proper et al. 1982; Masui, Wakefield et al. 1986) and therefore it was examined that whether the deficiency of TGFβRII impaires random migration of macrophages under low and high serum condition. TGFβRII deficiency in macrophage resulted in reduced migration of cells under low serum condition but not under high serum condition. This suggests that TGFβRII indeed impact on the random migration of macrophages under low concentration of TGFβ and other growth factors, whereas in high serum, which contains higher concentration of several growth factors, the migration was not affected, probably due to the compensatory effect of other factors on TGF-β mediated migration.

4.2 Effect of TGFβRII mediated signaling in macrophage on tissue repair

4.2.1 TGFβRII mediated signaling in macrophage is involved in wound contraction

Mechanical tissue injury was induced in control and TGFβRII$^{fl/fl}$LysMCre mice to understand the role of TGFβRII signaling in macrophage in tissue repair and several parameters were analyzed. The loss of TGFβRII signaling in macrophages did not impact the overall re-epithelialization process, formation of granulation tissue and neo-angiogenesis, however significant attenuation of wound contraction was observed in TGFβRII$^{fl/fl}$ LysMCre mice. This result indicates that macrophage specific TGFβRII signaling might play a role during repair process. Previously, we have shown in the publication by Lucas et.al, that during tissue injury, TGFβRII$^{fl/fl}$LysMCre mice show a hemorrhagic phenotype with increase fibrinolysis in the wound tissue at day 7 post injury, suggesting another important aspect of TGF-β1 mediated signaling in macrophages during repair process (Lucas, Waisman et al. 2010).

The process of wound contraction is primarily governed by differentiated fibroblasts i.e, myofibroblast, which acquires a contractile phenotype during repair process and helps in the contraction of wounds during the healing process. Our result primarily indicates an

additive role of TGFβRII signaling in macrophage during this process, however this could either be mediated by macrophage or by fibroblasts to which TGFβRII mediated signaling in macrophage is an important pre-requisite. To address whether the reduced contraction in TGFβRII$^{fl/fl}$LysMCre wound is associated with altered myofibroblast differentiation and function, expression of smooth muscle actin and production of CTGF was analyzed in the wound tissue. This result showed an impaired myofibroblast differentiation associated with reduced CTGF expression in TGFβRII$^{fl/fl}$LysMCre wounds. Collagen production from activated fibroblasts is the hallmark of myofibroblast function (Hinz 2007) and therefore the accumulation of collagen was analyzed in the wound tissue, which showed reduced deposition of collagen in TGFβRII$^{fl/fl}$LysMCre wounds compared to control wounds.

Interestingly, mice deficient in TGFβRII in dermal fibroblast show phenotype of delayed wound healing, delayed wound contraction, reduced collagen deposition and impaired myofibroblast differentiation (Denton, Khan et al. 2009), which resembles to the phenotype observed in TGFβRII$^{fl/fl}$LysMCre mice. Therefore, our data add onto the previously known role of TGFβ1 in mediating fibroblast differentiation and collagen production. However, how can we explain, that the lack of TGFβRII signaling in fibroblast or macrophage, leads to the similar phenotype? At this stage, our data suggests that impaired fibroblast differentiation could be responsible for the phenotype. We therefore hypothesized that the modulation of TGFβRII signaling in macrophage results in impaired cross talk between macrophage and fibroblast, which is required for myofibroblast differentiation and function. Although, a cross talk between fibroblasts and macrophages mediated by paracrine factors in pathological wound repair process has not been studied, it has been reported under *in vitro* condition in different context. Previously it has been shown that co-culture of macrophage and fibroblast in hydrogel affects the fibroblast proliferation and migration (Zeng and Chen 2010). Moreover, paracrine soluble factors like IL-1β , TNF-α and IL-6 from macrophages were found to be important for fibroblast mediated cartilage degradation in a mouse model of rheumatoid arthritis (Scott, Weisbrot et al. 1997). . Macrophages isolated from control and TGFβRII$^{fl/fl}$LysMCre mice and its co-culture with fibroblast under *in vitro* condition would be a crucial step to look further, if the loss of TGFβRII signaling on macrophages affects the fibroblast differentiation by secretion of paracrine factors.

In order to find the paracrine mediators, downstream to macrophage specific TGFβRII signaling in TGFβRII$^{fl/fl}$LysMCre wounds, which might be essential for myofibroblast differentiation, expression of several cytokines and growth factors were analyzed in wound tissue from control and TGFβRII$^{fl/fl}$ LysMCre mice at day 4, day 7 and day 14 post

injury. At this stage, expression of none of the potential growth factors such as TGF-β1, IL-1β or IL-6 was impaired in the TGFβRII$^{fl/fl}$ LysMCre wound tissue.

In the wound tissue, several cell types can contribute to the local cytokine milieu and therefore the expression of inflammatory factors was analyzed on macrophages isolated from wound tissue of control and TGFβRII$^{fl/fl}$ LysMCre mice. However, expression of all the analyzed cytokine was similar between control and TGFβRII$^{fl/fl}$ LysMCre mice except IL-1β. Interestingly, it has been shown in previous studies that in macrophages, TGF-β1 controls regulation of IL-1β (Allen, Manthey et al. 1990; McCartney-Francis, Mizel et al. 1990). It has also been shown that *in vitro,* under serum free conditions, IL-1β controls collagen production by lung fibroblasts (Elias, Freundlich et al. 1990), and induces collagen synthesis by different cell types such as hepatic stellate cells (Zhang and Yao 2012). Therefore, we proposed that the loss of TGFβRII signaling in macrophages results in impaired autocrine interaction of TGF-β1 on macrophages leading to the reduced IL-1β expression. However, whether the reduced IL-1β expression was involved in the macrophage-fibroblast cross talks and contributed to the impaired wound contraction and reduced collagen accumulation in TGFβRII$^{fl/fl}$ LysMCre mice, requires further investigation.

Although, TGF-β1 is known to auto-induce its own production (Wahl, McCartney-Francis et al. 1990) and this could be one of the primary factors mediating mechanisms of impaired cross talk of macrophage and fibroblast, both, in wound tissue and isolated macrophages, expression of TGF-β1 was similar between TGFβRII$^{fl/fl}$ LysMCre mice compared to control mice. This result could either indicate compensatory expression of TGF-β1 from different cell types such as epithelial cells, neutrophils, endothelial cells, fibroblasts (Li, Wan et al. 2006), which are known to produce TGF-β1. Since, mRNA expression of TGF-β1 does not necessarily correlates with the protein expression (Assoian, Fleurdelys et al. 1987; McCartney-Francis, Mizel et al. 1990), alternatively, expression of TGF-β1 may be altered at the protein level and activation state in TGFβRII$^{fl/fl}$ LysMCre wounds, which we did not addressed so far.

4.2.2 TGFβRII mediated signaling in macrophage does not impact cell recruitment in wound tissue

TGF-β1 is known to mediate monocyte recruitment (Wiseman, Polverini et al. 1988), therefore it was investigated that whether the deficiency of TGFβRII in macrophages impacts on cell migration *in vivo* during tissue repair. We found that under *in vivo* situation, loss of TGFβRII in macrophages did not alter the overall recruitment of CD11b/F4/80

positive macrophages or CD11b/Gr-1 positive neutrophils. This result can be explained by the fact that *in vivo* chemotaxis of monocytes can be mediated by many other chemokines produced at the wound site, such as CCL2and MIP-1α (DiPietro, Polverini et al. 1995; DiPietro, Burdick et al. 1998). Hence, the overall kinetics of monocyte recruitment remained unaltered between control and TGFβRII$^{fl/fl}$LysMCre wounds.

Previous studies showed that TGF-β1 induces angiogenesis under *in vivo* conditions (Roberts, Sporn et al. 1986; Ferrari, Cook et al. 2009). Moreover, TGF-β1 can induce VEGF-A in macrophages, which is an essential mediator of angiogenesis (Wiseman, Polverini et al. 1988; Jeon, Chae et al. 2007). Due to the loss of TGFβRII mediated signaling, it was assumed that VEGF-A production and angiogenesis will be impaired in TGFβRII$^{fl/fl}$LysMCre wounds. However, in contrast to this, expression of VEGF-A and CD31 positive cells remained unaltered between control and TGFβRII$^{fl/fl}$LysMCre wounds. This could be possibly explained by the previous known fact that several cell types such as endothelial cells and epithelial cells can also produce VEGF-A (Brown, Yeo et al. 1992; Frank, Hubner et al. 1995; Lee, Chen et al. 2007) and thereby sufficiently contribute to the overall VEGF-A expression during wound healing.

4.3 Impact of TGFβRII mediated signaling in macrophage on fibrosis

4.3.1 TGFβRII mediated signaling in macrophage generates fibrotic response

It is well studied that increased ECM deposition is a hallmark of fibrosis. Fibroblasts as a cell type and TGF-β1 as a soluble mediator are of prime importance in contributing collagen production in fibrosis (Eckes, Mauch et al. 1996; Jelaska, Arakawa et al. 1996; Kawakami, Ihn et al. 1998). However, macrophage specific role of TGF-β1 signaling in generating fibrosis is still unknown. To this direction, our data show an essential role of macrophage specific TGFβRII signaling in fibrosis.

Here we show, that the macrophage specific TGFβRII signaling is essential to generate inflammation-induced early and late fibrotic response in mice. In the absence of TGFβRII signaling in macrophages, TGFβRII$^{fl/fl}$LysMCre mice exhibit reduced fibrosis, accompanied by the reduced dermal thickness and the reduced collagen deposition. Interestingly, the fibrotic response observed in the TGFβRII$^{fl/fl}$LysMCre mice was similar to the phenotype observed in mice lacking Smad3, which is a downstream signaling molecule of TGFβRII

(Lakos, Takagawa et al. 2004). In the study by Lakos et.al, mice lacking Smad3 also showed a reduced bleomycin induced fibrotic response, however complete lack of Smad3 in this mouse model limits the study to find the cell type specific role of Smad3. At this point, our data suggests that macrophage-specific TGFβRII signaling axis might be an important contributor to the fibrosis.

4.3.2 TGFβRII mediated signaling in macrophages results in reduced expression of pro-inflammatory factors

To find out the possible mechanisms involved in the reduced fibrotic response due to macrophage specific loss of TGFβRII signaling, the early and late inflammatory response was further investigated in control and $TGF\beta RII^{fl/fl}$LysMCre mice. Interestingly, after two weeks of bleomycin injection, $TGF\beta RII^{fl/fl}$LysMCre mice showed reduced number of myeloid cells/macrophages compared to control mice. This data shows that macrophages specific TGFβRII signaling is associated with recruitment of these cells in fibrosis. Since under normal conditions, the recruitment of myeloid cells in the blood of control and $TGF\beta RII^{fl/fl}$LysMCre mice were similar as studied by FACS analysis of blood cells for myeloid cell markers (data not shown), we proposed that the difference observed in the recruitment of cells in $TGF\beta RII^{fl/fl}$LysMCre mice was bleomycin induced and mediated by TGFβRII signaling in macrophages during fibrosis.

Since, inflammation is the pre-requisite to induce fibrosis and $TGF\beta RII^{fl/fl}$LysMCre mice showed reduced number of inflammatory cells, this might be associated with reduced inflammatory response generated in $TGF\beta RII^{fl/fl}$LysMCre mice leading to reduced fibrosis.

Therefore, the next question was why does a $TGF\beta RII^{fl/fl}$LysMCre mice show less immune cell infiltration and what makes the inflammatory response in $TGF\beta RII^{fl/fl}$LysMCre mice different from control mice? A most likely explanation would be that $TGF\beta RII^{fl/fl}$LysMCre mice are unable to recruit sufficient macrophages or myeloid cells at the fibrotic site and therefore as a consequence, less inflammatory cytokines are produced, which are probably required for the paracrine interaction with fibroblast, and collagen deposition.

To address this hypothesis, expression of several inflammatory mediators was analyzed in bleomycin injected fibrotic skin. Interestingly, $TGF\beta RII^{fl/fl}$LysMCre mice showed a differential down-regulation of inflammatory mediators, such as TNF-α, IL-1β and osteopontin-1 (OPN-1) in the early fibrotic phase. This result suggests that these factors might be important for the reduced inflammation and reduced collagen production in

TGFβRII$^{fl/fl}$LysMCre fibrotic tissue, either by direct involvement or by mediating paracrine interaction between macrophage and fibroblast. Since these are less produced in TGFβRII$^{fl/fl}$LysMCre fibrotic tissue, the fibrotic response is attenuated.

Seemingly, all of the less regulated cytokines in TGFβRII$^{fl/fl}$LysMCre mice have previously been associated with the generation of fibrotic response and collagen production *in vitro* and *in vivo*. OPN-1 is a glycoprotein, which has cytokine-like properties. It is a chemotactic and activating factor for several cell types such as macrophages and T-cells (Weber, Zawaideh et al. 2002; Buback, Renkl et al. 2009). It is also a pro-fibrotic factor (Pardo, Gibson et al. 2005). It is secreted by many immune cells such as macrophages, and has been shown to be involved in myofibroblast differentiation and repair process (Lenga, Koh et al. 2008; Mori, Shaw et al. 2008; Buback, Renkl et al. 2009). Interestingly, mice lacking OPN-1 show reduced bleomycin-induced lung fibrosis with reduced production of active TGF-β1 (Berman, Serlin et al. 2004). Moreover, knock down of OPN-1 reduces the immune cell recruitment and type I collagen production *in vivo,* during repair process (Mori, Shaw et al. 2008). Although, in macrophages, relation between OPN-1 and TGF-β1 has not been established, in various other cells such as osteoblasts and HeLa cells, TGF-β1 has been shown to up-regulate the expression of OPN-1 (Fagenholz, Warren et al. 2001; Piva, Gavassini et al. 2012). In a co-culture of macrophage and fibroblasts, macrophage secreted factors have been found to regulate OPN-1 expression from fibroblast and therefore OPN-1 expression is indeed regulated during fibroblast-macrophage interaction If TGF-β1 mediates expression of OPN-1 in macrophages as well, it can be well explained that due to loss of TGFβRII in macrophages, expression of OPN-1 has been impaired. Moreover, OPN-1 expression from macrophages has recently been speculated to be important for skin fibrosis (Gessner, 2011). TGF-β1 mediated signaling in macrophage directs fibroblast to secrete OPN-1 and since in TGFβRII$^{fl/fl}$LysMCre mice this signal is lacking, less OPN-1 was produced from fibroblasts for proper function, which leads to the observed phenotype. This possibility is based on the observation made by Mori et.al. (Mori, Shaw et al. 2008), who showed that macrophages produce inductive signals for OPN-1 regulation from fibroblasts, however the pathway, which provides this signal is unknown. Taken together, based on the previous known role of OPN-1, it is likely that the reduced expression of OPN-1 may have contributed to the reduced immune cell infiltration, reduced collagen deposition and reduced fibrosis in TGFβRII$^{fl/fl}$LysMCre mice. However, *in vitro* stimulation of macrophages with recombinant TGF-β1 and expression analysis of OPN-1 will address the point that whether OPN-1 expression in macrophages is regulated by TGF-β1 and further experiment would identify that whether OPN-1 expression is linked to the observed phenotype in TGFβRII$^{fl/fl}$LysMCre mice.

Similar to OPN-1, TGFβRII$^{fl/fl}$LysMCre mice showed down-regulation of IL-1β expression in fibrotic dermis compared to control mice. IL-1β has been variably reported to either up-regulate or down-regulate collagen production from fibroblasts. Under *in vitro* condition, recombinant IL-1 has been found to up-regulate collagen synthesis in lung fibroblasts (Elias, Freundlich et al. 1990), moreover in rheumatoid synovial cells/fibroblasts, mononuclear cells derived IL-1β have been shown to up-regulate collagen mRNA (Krane, Dayer et al. 1985). In another study, IL-1 homologue from monocyte culture supernatant has been shown to down-regulate collagen production from normal dermal fibroblast and scleroderma fibroblasts (Whiteside, Buckingham et al. 1984). However, these studies have been done *in vitro*, and an *in vivo* situation can be dependent on various other factors and could either be two of them, which has not been studied so far. Therefore, the reduced expression of IL1β in fibrosis of TGFβRII$^{fl/fl}$LysMCre mice can either directly or indirectly involved in the observed reduced fibrosis and reduced collagen production, which needs further investigation

4.3.3 Loss of TGFβRII mediated signaling in macrophage results in reduced expression of TNF-α

Another pro-inflammatory factor, TNF-α was consistently down regulated in the fibrotic dermis (2 weeks) as well as in the isolated macrophages from the fibrotic tissue (2 weeks) of TGFβRII$^{fl/fl}$LysMCre mice as compared to control mice. However, after 4 weeks of bleomycin injection, TNF-α regulation was significantly up-regulated more in the fibrotic dermis of TGFβRII$^{fl/fl}$LysMCre mice compared to control mice, indicating a dual role of TNF-α, which involves transient down-regulation of TNF-α, followed by up-regulation at the later stage of fibrosis in TGFβRII$^{fl/fl}$LysMCre mice.

Under *in vivo* condition, the role of TNF-α has been described as suppressor as well as activator of pulmonary fibrosis. It has been shown that after 2 weeks of bleomycin injection, TNF-α is up-regulated and if treated with anti-TNF-α IgG antibody, fibrosis and collagen production can be restored to the normal level with reduction in macrophages and other cells (Piguet, Collart et al. 1989; Zhang, Gharaee-Kermani et al. 1997). In contrast to these reports, which showed pro-inflammatory role of TNF-α, anti-inflammatory action of TNF-α has also been reported in \ pulmonary fibrosis. Mice lacking TNFR p55, which is one of the receptors for TNF-α has been shown to generate strong bleomycin induced fibrosis, accompanied by reduced expression of MMP-1, leading to reduced collagen degradation (Murota, Hamasaki et al. 2003). In another study, mice lacking TNF-α showed an intense pulmonary fibrosis due to the persistent inflammation and the

reduced apoptosis of inflammatory cells (Kuroki, Noguchi et al. 2003). On the other hand, transgenic mice overexpressing TNF-α showed abrogated bleomycin induced fibrosis and reduced collagen production (Fujita, Shannon et al. 2003), which also show an anti-inflammatory and anti-fibrotic role of TNF-α during pulmonary fibrosis. Reduced fibrosis in TGFβRII$^{fl/fl}$LysMCre mice, accompanied by the reduced TNF-α expression at the early stage and enhanced TNF-α regulation at the later stage suggest that in the early stage, reduced inflammation might be the consequence of reduced pro-inflammatory action of TNF-α, leading to reduced fibrosis, reduced immune cell infiltration, accompanied by reduction in other pro-inflammatory cytokines such as IL-1β and OPN-1. Whereas, at the later stage, over production of TNF-α led to its anti-inflammatory function resulted in reduced fibrosis and reduced collagen deposition. Our data also show that this effect of TNF-α requires TGFβRII mediated signaling in macrophages.

In vivo dual role of TNF-α also correlates with *in vitro* studies, where the role of TNF-α on collagen production by fibroblasts has been variably reported. Whereas, in several reports, TNF-α has been shown to down-regulate ECM deposition either by inducing production of collagenase or by inhibition of collagen synthesis (Scharffetter, Heckmann et al. 1989; Mauviel, Lapiere et al. 1994; Greenwel, Tanaka et al. 2000; Verrecchia, Wagner et al. 2002), several other reports showed that TNF-α up-regulates expression of type I and type III collagen in fibroblasts (Elias, Freundlich et al. 1990; Sarkar, Vellaichamy et al. 2004). However, *in vitro* conditions can be very different from *in vivo* situation, where a complex network of cells and soluble factors contribute to a cumulative phenotype of fibrosis. Moreover, all the reports, which describe TNF-α and collagen are context dependent and involve activation of other pathways such as JNK and NF-κB.

In conclusion, this study reveals previously unknown function of macrophage specific TGFβRII signaling during repair process and fibrosis. The study further supports the previous known *in vitro* role of TGF-β1 mediated signaling such as collagen production, induction of pro-inflammatory factors etc under *in vivo* situation. The novel insight from the study, which we obtained are: First, macrophage specific TGFβRII signaling mediates fibrosis. Second, macrophage specific TGFβRII signaling is involved in the regulation of pro-inflammatory cytokines such as TNF-α, IL-1β and PN-1 and accumulation of immune cells. Third, macrophage specific TGFβRII signaling facilitates wound contraction, probably through paracrine interaction between macrophages and fibroblasts during repair process.

4.4 Hypothetical model for fibrosis mediated by TGFβRII signaling in macrophages

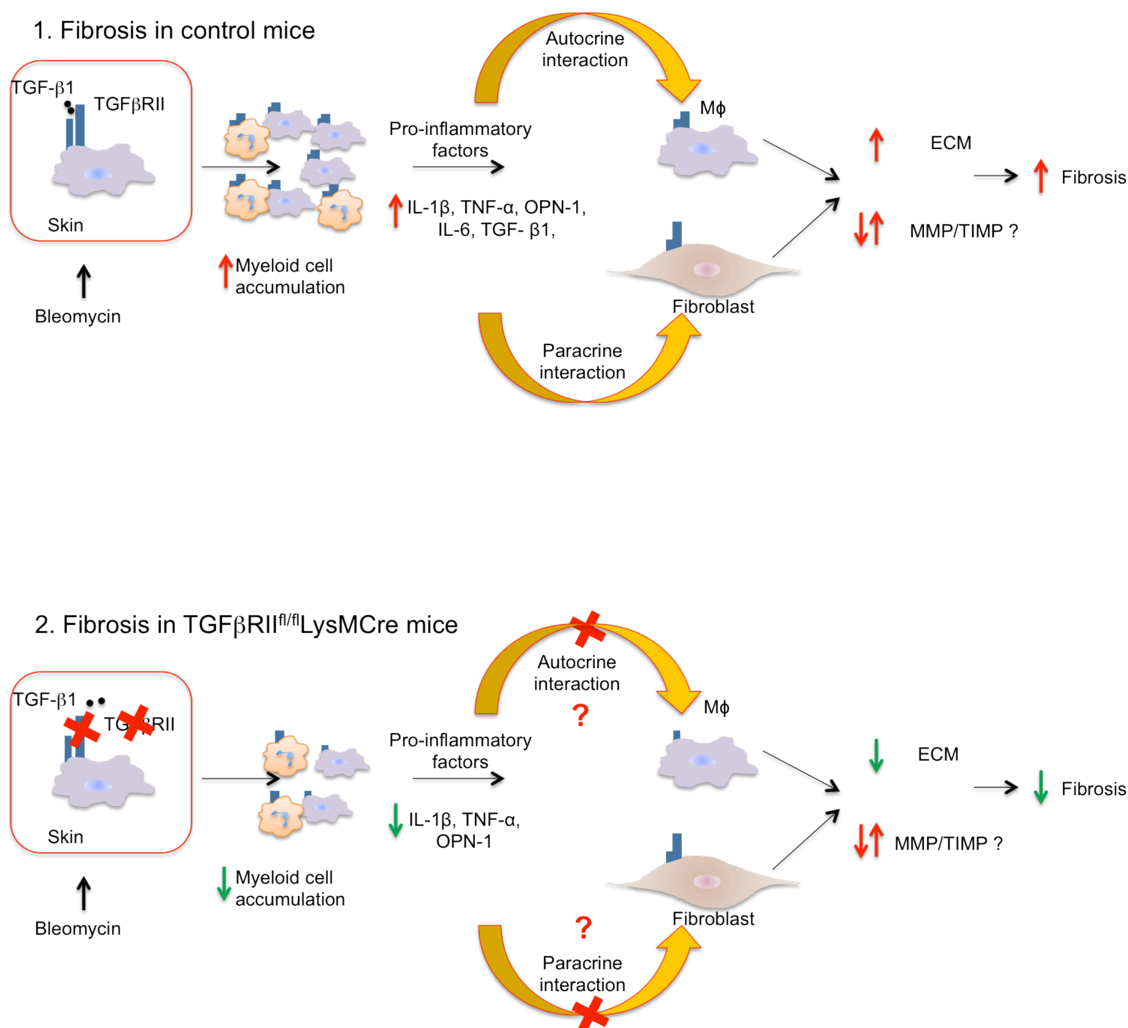


Fig. 4-1: Model for TGFβRII mediated function in macrophages during fibrosis. During fibrosis, early recruitment of myeloid cells in the skin produce inflammatory cytokines such as IL-1β, TNF-α, IL-6, MCP-1, leading to either autocrine interaction of the secreted factors on the same cells such as macrophage or on the other cells such as fibroblast. The interaction between cells and soluble mediators then activate ECM deposition, leading to severe fibrosis. However, deficiency of TGFβRII on macrophages results in the reduced accumulation of inflammatory cells and the reduced production of inflammatory mediators, leading to impaired autocrine or paracrine interaction mediated by these factors. The impaired interaction then leads to reduced activation of cells for ECM deposition and reduced fibrosis.

5 Materials and Methods

5.1 Materials

5.1.1 Buffers used

Ammoniumthiocynate (pH 6.8): 0.1 M $NaKHPO_4$, 5 mM NH_4SCN,

Anticoagulation buffer: 1 mM EDTA in PBS supplemented with Heparin (1:4000, Heparin-Natrium-25,000-ratiopharm®, Ratiopharm GmbH, Ulm, Germany)

ACK lysis buffer: 150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA

FACS buffer: 1% BSA, 2 mM EDTA in PBS

PBS: 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4; 2.6 mM KCl, 136 mM NaCl, pH 7.4

TAE buffer (pH 8.0): 40 mM TrisHCl 20 mM NaAc, 1 mM EDTA

TE buffer (pH 8.0): 10 mM TrisHCl, 1 mM EDTA

5x loading dye: 40% Glycerol, 0.04% bromphenol blue, 0.2% orange G, 1 mM EDTA

Tail lysis buffer (pH 8.0): 100 mM Tris-HCl, 5 mM EDTA, 0.2% (w/v) SDS, 0.2 M NaCl, 500 mg/ml proteinase K

Endogenous peroxidase blocking solution: 0.03% H2O2 and 0.15 mol/L NaN_3 in PBS

RIPA buffer: 50 mM Tris/HCl (pH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA supplemented with mammalian protease inhibitors (Sigma-Aldrich).and phosphatase inhibitor (Roche).

MES buffer (pH 7.3): 2.5 mM MES, 2.5 mM Tris base, 0.005% SDS, 0.005 mM EDTA

Transfer buffer (pH 7.2): 25 mM Bicine, 25 mM Bis-Tris (base free), 1 mM EDTA

Tris-buffered saline (TBS) (pH 7.6): 0.5 M Tris base, 1.5 M NaCl

Membrane blocking solution: 5% dried milk powder in Tris-buffered-saline, supplemented with Tween20 (TBS-T)

Membrane Stripping buffer (pH 2.2): 0.2 M Glycin, 0.1% SDS, 1% Tween 20

Transfer buffer: 5 M NaOH, 5M NaCl**,** distilled water

Denaturation buffer: 0.25 N HCl, 37%HCl, distilled water

SSC buffer (20x, pH 7): 0.3 M Na-Citrate, 3M NaCl

Tris-HCl buffer (pH 7.4): 50 mM Tris-HCl, 5 mM $CaCl_2$

Coomassie blue: 0.01% Coomassie blue R-250, 50% MeOH, 5% acetic acid

Destaining solution: 10% MeOH, 10% acetic acid

5.1.2 Chemicals and reagents

Name	Company
Bleomycin (Bleomedac)	Medac Gesellschaft, Germany
DMEM medium	Invitrogen, Darmstadt, Germany
Penicillin/Streptomycin	Biochrom, Berlin, Germany
Fetal Calf Serum (FCS)	PAA, Pasching, Austria
Normal goat serum	DakoCytomation Inc., Carpinteria, USA
Bovine serum albumin	Carl Roth GmbH, Germany
EDTA	Biochrom AG, Germany
AEC substrate	DakoCytomation Inc., Carpinteria, USA
Antibody diluent	DakoCytomation Inc., Carpinteria, USA
β-mercaptoethanol	Sigma, USA
NuPAGE 4-12% Bis-Tris Gel	Invitrogen, USA
NuPAGE MES SDS running buffer	Invitrogen, USA
Mammalian protease inhibitor cocktail	Sigma-Aldrich, USA
PhosStop phosphatase inhibitor	Roche, F. Hoffmann-La Roche Ltd, Basel, Switzerland
LDS-sample loading buffer	Invitrogen, USA
Western Lightning Chemiluminsecence Reagent	Perkin Elmer, USA
Page Ruler pre-stained	Fermentas GmbH, St. Leon-Rot, Germany
peqGOLD 50 & 1 kb bp ladder	Peqlab, Erlangen, Germany
Agarose	Bio-Budget, Germany
DNA Salmon sperm	Sigma-Aldrich, USA
10X Tango buffer	Fermentas, Germany
Quick Hyb Hybridization solution	Stratagene, Germany
Ketanest S (25 mg/ml)	Park Davis GmbH, Karlsruhe, Germany
Rompun (2%)	Bayer HealthCare AG, Germany
Isofluoran	Abott, Germany
Ammonium thiocyanate (NH_4SCN)	Sigma, USA
Thioglycollate (4%)	Sigma-Aldrich, St. Louis, USA
Tissue Tek O.C.T. Compound	Miles, Elkhart, IN, USA
Tween 20	Merck, Darmstadt, Germany

5.1.3 Commercial Kits

Kits	Company
BCA Protein Assay Reagent Kit	Pierce Protein Research Products, Thermo Scientific
RNeasy Micro Kit	Qiagen, Hilden, Germany
RNeasy Plus Mini Kit	Qiagen, Hilden, Germany
RNeasy Fibrous Tissue Kit	Qiagen, Hilden, Germany
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems, Carlsbad, CA, USA
QIAquick Gel Extraction kit	Qiagen, Hilden, Germany
Ladderman probe labeling unit	Takara, Japan
QuickZyme Total Collagen Assay	QuickZyme biosciences, Leiden, Netherlands

5.1.4 Recombinant proteins and enzymes

Protein	Concentration	Company
rTGF-β1	20 ng/ml	eBioSciences Inc, San Diego, USA
Taq DNA Polymerase	100 ng/ml	Bio Budget, Krefeld, Germany
DNase I	30 K unit	Qiagen, Hilden, Germany
Liberase	0.15 U/ml	Roche Applied Science, Germany
Proteinase K	500 mg/ml	Sigma-Aldrich, Germany
NCoI	2 U/10 μg DNA	Fermentas, Germany

5.1.5 Technical equipments and softwares

Medimachine	BD Biosciences, Germany
7300 Real Time PCR system	Applied Biosystems, Carlsbad, CA, USA
T3000 Thermocycler	Biometra, Göttingen, Germany
Flow Cytometers (FACSDiva software)	FACS Canto^{TM} (BD, Heidelberg, Germany)
Graph Pad Prism and Software	GraphPad Software, Inc., San Diego, CA, USA
Light microscope; Leica DM 4000B (Diskus 4.50 Software)	Carl H. Hilgers, Königswinter, Germany
Nikon eclipse E800 (NIS-Elements AR 2.30 Software)	Nikon, Melville, NY, USA
Olympus 1X81 microscope system	Tokyo, Japan
Image J	Bethesda, MD, USA

Gel documentation	Peqlab, Germany
Mixer mill MM 400	Retsch, Germany
Victor Multilabel reader	Perkin Elmer, USA
Biophotometer	Eppendorf, Germany
Hybridization roller bottle GL45	Witeg, Germany
Illustra ProbeQuant G-50 microcolumns	GE Healthcare. USA
Metallblock thermostat (Heating block)	Bachhofer GmbH, Reutingen, Germany
Hybridization oven OV-10	Biometra, Goettingen, Germany
Scintillation counter LC-6500	Beckmann, USA
Contamat FHT 111M	Medizin-GmbH, Duesseldorf, Germany

5.1.6 Other materials

Name	Company
Optical adhesive film	Applied Biosystems, UK
96 well plates	Applied Biosystems, Singapore
40 μm nylon filter	BD Biosciences, Germany
70 μm nylon filter	BD Biosciences, Germany
Centrifuge tubes (15/50 ml)	BD Falcon™, Germany
Polylysine glass slides	Menzel GmbH, Germany
Medicons	BD Biosciences, Germany
Thermo mixer	Eppendorf, Hamburg, germany
PVDF membrane (8 μm pore size)	Millipore Corporation, Billerica, USA
Biopsies Punch (5-8 mm)	Stiefel, Offenbach, Germany
Nitrocellulose membrane	GE healthcare, USA

5.1.7 Primary antibodies

Table 5-1

Antibodies	Host/Isotype	Clone	Dilution	Company
β-Actin	Mouse	C4	WB: 1/1000	Santa Cruz biotechnology, Germany
α-SMA-Cy3	Mouse IgG2a	1A4	IF: 1:250	Sigma-Aldrich, St. Louis, MO, USA
CD11b-PE/APC	Rat IgG2b	FC: 1:100	FC: 1/100	BD Pharmingen, Germany
CD19-APC	Rat IgG2a	1D3	FC: 1/100	Miltenyi, Bergisch Gladbach, Germany
CD31 (Pecam-1)	Rat IgG2a	MEC 13.3	IF: 1/1000	BD Pharmingen, Heidelberg, Germany
CD115-APC	Rat IgG2a	AFS98	FC: 1/250	eBioscience Inc., San Diego, USA
CD16/32	Rat IgG2a	93	FC: 1:60	eBioscience Inc., San Diego, USA
ERK1/2	Rabbit	Polyclonal	WB: 1/1000	Cell Signaling Technology
F4/80	Rat IgG2a	BM8	IF: 1/200	Dianova BMA AG, Augst, Switzerland
F4/80-PE/FITC	Rat IgG2a	BM8	FC: 1:200	AbD Serotec, Düsseldorf, Germany Gladbach, Germany
Gr-1	Rat IgG2b	RB6-8C5	IHC: 1/150	BD Pharmingen, Heidelberg, Germany
Gr-1-PE	Rat IgG2b	RB6-8C5	FC: 1/10	Miltenyi, Bergisch Gladbach, Germany
P-Smad-2 (Ser465/478)	Rabbit	Polyclonal	WB: 1000	Cell Signaling Technology
Smad-2	Rabbit	L16D3	WB: 1000	Cell Signaling Technology
TGFβRII	Rabbit	Polyclonal	WB: 1/1000	SantaCruz Biotechnology, Germany

5.1.8 Secondary antibodies

Table 5-2:

Antibodies	**Dilution**	**Company**
Goat anti rat (H+L)- Alexa 488	1:500	Invitrogen, Paisley, UK
Swine anti-rabbit-HRP	1:1000	Dako, Glostrup, Denmark
Rabbit anti-mouse-HRP	1:1000	Dako, Glostrup, Denmark
Goat anti-rat-IgG+M (H+L)-HRP	1:250	Southern Biotech, USA

5.1.9 Oligonucleotides

Table 5-3: Quantitative real time PCR primers for murine genes (5´→ 3´)

mS18	FP: GATCCCAGACTGGTTCCTGA
	RP: GTCTAGACCGTTGGCCAGAA
mIL-1β	FP: GGACCCCAAAAGATGAAGGGCTGC
	RP: GCTCTTGTTGATGTGCTGCTGCG
mIL-6	FP: ACACATGTTCTCTGGGAAATC
	RP: AAGTGCATCATCGTTGTTCATACA
mCTGF	FP: CCTCCGTCGCAGGTCCCATCA
	RP: CCATAGCAGGCCGGGTGCAG
TGF-β1	FP: TGGAGCAACATGTGGAACTC
	RP: GTCAGCAGCCGGTTACCA
mTIMP-1	FP: GCATTGAGCTTTCTCAAAGACC
	RP: AGGGATAGATAAACAGGGAAACACT
mTIMP-2	FP: AGGTACCAGATGGGCTGTGA
	RP: GTCCATCCAGAGGCACTCAT
mCol1-α1	FP: AGCTTTGTGGACCTCCGGCT
	RP: ACACAGCCGTGCCATTGTGG
mCol3-α	FP: GTTTTATGACGGGCCCGGTGC
	RP: GACCAAGGTGGCTGCATCCCA
mTNF-α	FP: GACCCTCACACTCAGATCATCTTCT
	RP: CCTCCACTTGGTGGTTTGCT
mPAI-1	FP: CTCCTCATCCTGCCTAAGTT RP: GCCAGGGTTGCACTAAACAT
mOPN-1	FP: GCCTCCTCCCTCCCGGTGAAA
	RP: TCCTCGCTCTCTGCATGGTCT
mPDGF-b	FP: AGTCGGCATGAATCGCTGCTG
	RP: TCCGGAGTGTGCTCGGGTCAT

miNOS	FP: CCACCTTGGTGAAGGGACTGAGCT
	RP: AGGGGCAAGCCATGTCTGAGACT
mVEGF-A	FP: TGTACCTCCACCATGCCAAGT
	RP: TCGCTGGTAGACGTCCATGAA

Table 5-4: Genotyping primer sequence and amplicons (5´→ 3´)

Mouse	Primer Sequence	Product size (bp)
LysMCre	FP: 5´- CTTGGGCTGCCAGAATTTCTC -3´ RP: 5´- TTACAGTCGGCCAGGCTGAC -3´	LysMCre: 750 WT: 350
TGFβRII	FP: 5´-GCACAGGTACACATCTCTGCAC-3´ RP: 5´-TGTAATCGTTGCACTCTTCCATGT -3´	WT: 500 FL: 550

Table 5-5: Exon 2 specific TGFβRII southern blot probe

Exon 2	Primer Sequence	Product size (bp)
TGFβRII	FP: 5´-CCTCAGTTAACAGTGATGTCATGG -3´ RP: 5´-GAAAGCCAGAGGAGGCCACAG -3´	737 bp

5.2 Methods

5.2.1 Flow cytometry

5.2.1.1 Single cell suspension

5.2.1.1.1 Isolation of peritoneal cells

Mice were sacrificed and peritoneal cavity was flushed with approximately 8 ml of ice-cold FACS buffer. The obtained cells were centrifuged at 300 g for 8 minutes at 4°C and the pellets were re-suspended in 1 ml FACS buffer. The cells were then counted in Neubauer counting chamber and used for cell surface staining and FACS analysis.

5.2.1.1.2 Isolation of skin cells from wound tissue and bleomycin fibrotic tissue

The cells were isolated from wound and fibrotic tissue by combination of enzymatic and mechanical digestion. Wound tissue at day 4, day 7 and day 14 post injury or bleomycin induced fibrotic tissue after 2 weeks and 4 weeks of injection was excised, minced and digested in liberase blendzyme (0.15 U/ml) in DMEM (Dulbecco's modified eagle medium) for 90 minutes at 1000 rpm and 37°C on a thermomixer. Subsequently, the mechanical digestion was accomplished using BD™ Medimachine for 5 minutes in PBS containing 10% FCS at room temperature and filtered through 70 μm cell strainer. The cell suspension was centrifuged at 300 g for 8 minutes at 4°C and mixed with PBS/2mM EDTA followed by additional filtration through 40 μm cell strainer. The suspension was centrifuged and the pellets were re-suspended in 1 ml FACS buffer followed by counting and staining for FACS analysis.

5.2.1.1.3 Blood leukocyte isolation

Mice were sacrificed and blood was taken from the heart in 5 ml of anticoagulation buffer and centrifuged at 300 g for 8 minutes at 4°C. Erythrocytes were lysed by adding 5 ml of ACK lysis buffer two times for 8 minutes at room temperature. The reaction was stopped by addition of 45 ml of ice-cold FACS buffer and subsequently, the filtered through a 70 μm cell strainer. The cell suspension was centrifuged and the cells were re-suspended in 1 ml FACS buffer before counting.

5.2.1.2 FACS staining and analysis

The final cell suspension in FACS buffer was taken in 2 ml of FACS tube, centrifuged at 300 g for 8 minute at 4°C and the pellets were re-suspended in 60 μl of FACS buffer. Subsequently, the cells were incubated with Fcγ receptor blocking antibody CD16/32 for

10 minute on ice, followed by incubation with cell surface marker antibody at the dilution specified in the table 5-1. Minimum 20,000-50,000 cells were counted using BD FACSCanto™ machine and analyzed using the FACSDiva software.

5.2.2 Isolation of genomic DNA

Mouse tail biopsies or isolated cells from peritoneal cavity were incubated in tail lysis buffer on a thermo mixer at 56°C over night followed by centrifugation at 13,000 rpm and precipitation by adding an equivalent of isopropanol and centrifugation at 13,000 rpm for 10 minutes. The obtained DNA pellets were washed with 500 μl of 70% ethanol at 13,000 rpm, air dried at room temperature for approximately 2 h and re-suspended in 125 μl (in case of tail biopsies) or 30 μl (in case of cultured cells) 1x TE buffer.

5.2.3 Polymerase chain reaction (PCR)

For genotyping PCR, 10-100 ng template DNA was amplified in a reaction volume of 25 μl containing 2 mM $MgCl_2$, 200 nM each primer, 200 μM dNTPs and 0.05 U/μl Taq-Polymerase in 1x reaction buffer. The optimal annealing temperature of the specific primers was estimated with the following formula: TA=59.9+0.41(GC%)-600/L (GC%, GC content in percent, L, total number of base pairs). All primers used for genotyping are listed in table. 5-4. PCR amplified DNA fragments were analyzed following addition of 5x loading dye and gel electrophoresis on 1.5% (w/v) agarose gels (1x TAE, 0.5 mg/ml ethidium bromide) at 120 V. The bands were visualized under UV-light (peqlab Gel Documentation system)

5.2.4 RNA extraction, reverse transcription PCR and quantitative real time PCR

For isolation of total RNA from wound tissue or fibrotic tissue, the tissue was homogenized in a mixer mill, and RNA was extracted using the RNeasy Fibrous Tissue Kit. Total RNA from monocytes/macrophages was isolated using the RNeasy Mini Kit as per manufacturer’s instructions. The RNA concentration was determined by spectrophotometric measurement and calculated by standard equation: RNA (μg/ml) = OD (260) * 40 * dilution factor. 500-1000 ng of each RNA sample was reversely transcribed using the High Capacity cDNA RT Kit according to the manufacturer’s protocols.

Quantitative real time PCR was performed with Power SYBR Green PCR mix in triplicates

using 7300 Real Time PCR system. S18 (18S rRNA subunit; ribosomal protein S18) as a housekeeping gene and efficiency matched primers of the target genes were used for the RT-PCR. The comparative method (ΔΔCT) of relative quantification was used to determine the expression levels of target genes normalized to the housekeeping gene S18. Data were expressed as fold change over control wounds or PBS injected fibrotic skin or blood monocytes. Wherever possible, exon spanning primers were designed with an annealing temperature of 60°C ± 1°C using either NCBI primer BLAST or Roche Universal primer design software. All the used oligonucleotides are listed in table 5-3.

Master mix for quantitative real time PCR

SYBR Green mix	10 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
Double distilled H_2O	8.0 µl
cDNA	1.0 µl

Quantitative real time PCR program

50°C	2 min
95°C	10 min
95°C 60°C	15 s 60 s 30 x
72°C	10 min
4°C	Pause

Default dissociation curve program was used for melting curve.

5.2.5 Separation of epidermis and dermis

Bleomycin injected fibrotic skin and PBS injected skin were used for the separation of epidermis and dermis. The skin was incubated with 0.5 M ammonium thiocyanate, pH 6.8 for 35 minutes on ice and subsequently, the epidermis was scrapped off. The separated epidermis or dermis was kept in either RNAlater® solution for RNA isolation or the dermis was used immediately for the analysis of the total collagen content.

5.2.6 Culture and maintenance of peritoneal macrophages

Isolation of peritoneal macrophages for culture and stimulation was performed under sterile conditions as described in the section 5.2.1.1.1. The cells were re-suspended in

DMEM medium supplemented with 10% FCS and penicillin-streptomycin (100 U/ml penicillin and 0.1 mg/ml streptomycin). A minimum $1*10^6$ cells per ml was seeded into the 6 well plate dishes and incubated for 4 h in an incubator supplied with 5% CO_2 at 37°C. Non-adherent cells were removed by washing two times with fresh DMEM medium. Macrophages were starved overnight in DMEM supplemented with 1% FCS before stimulation with recombinant human TGF-β1 for 10 minutes, 20 minutes and 30 minutes and lyzed for western blot analysis.

5.2.7 *In vitro* random migration and analysis

Peritoneal cells were harvested and cultured as described in the section 4.2.7. To analyze random migration macrophages were cultured in 24 well plate at a cell density $1*10^6$ per well in DMEM medium supplemented with 1% FCS and 10% FCS. The cells were kept under Olympus 1X81 microscope under humidified chamber supplemented with 5% C02 at 37^0C for 48 h. The trajectory length travelled by macrophages was analyzed using manual tracking plug-in from ImageJ software.

5.2.8 Southern blot

5.2.8.1 DNA digestion

5-10 μg isolated DNA from peritoneal macrophages were digested in a reaction volume of 50 μl containing 1X Tango buffer, 2 U of the restriction enzyme Nco1 overnight at 38°C. The digested DNA was resolved by agarose gel electrophoresis on 0.7% agarose gel at 50 V. The resolved gel was kept in denaturation buffer, washed in distilled water and transferred onto a nitrocellulose membrane.

5.2.8.2 Transfer of DNA on nitrocellulose membrane and pre-hybridization

To transfer the gel on nitrocellulose membrane, the transfer apparatus was set in the following order:

3 sheets of gel-size whatmann paper pre-soaked in transfer buffer

Pre-run gel (upside down over the whatmann paper)

Nitrocellulose membrane (gel size)

3 sheets of whatmann paper

Paper towels (as many so that it do not get wet)

A weight for efficient transfer

The whole assembly was kept overnight at room temperature for the transfer of DNA on nitrocellulose membrane followed by baking in an oven at 80^0C for 2 h. Subsequently, the blot was washed with distilled water, inserted in a hybridization roller bottle with 10 ml of QuickHyb hybridization solution and incubated inside the hybridization oven at 60^0C for 1 h. Meanwhile, Radioactive labeled probed was prepared as described in the next section.

5.2.8.3 Generation of TGFβRII specific probe and purification

To generate the probe and labeled with radioactive (P32)-α-dCTP, genomic DNA was amplified using TGFβRII exon 2 specific primer for probe labeling.

Master mix for exon 2 amplification

10 X buffer	2.5 μl
dNTP mix	0.5 μl
Forward primer	0.5 μl
Reverse primer	0.5 μl
MgCl2	2 μl
Taq DNA polymerase	0.25 μl
Distilled water	17.75 μl
Genomic DNA	1 μl

PCR program for exon 2 amplification

95°C	5 min
95°C 56°C 72°C	45 s 60 s 33 x 60 s
72°C	7 min
10°C	Pause

After amplification PCR, 10 μl of PCR product was loaded and run on 1.5% agarose gel at 120 V. The amplified product was cut out of the gel with sharp scalpel and purification steps were performed using QIAquick gel extraction kit according to the manufacturer's protocol. The obtained purified DNA was measured and used for radioactive probe labeling.

5.2.8.4 Radioactive probe labeling and purification

100 ng of the purified exon 2 amplicon (section 5.2.8.3) was mixed with 2 µl of random primer (provided with the labeling kit) denatured to 95^{0}C for 5 minutes followed by cooling on ice for 5 minutes. To label the probe Takara labeling kit was used as detailed in the manufacture's protocol.

Master mix for probe labeling

10 X buffer	5 µl
dNTP mix (0.2 mM each, without dCTP)	2.5 µl
Bca DNA polymerase (2U/µl)	1 µl
(P32)-α-dCTP (radioactive labelled)	2.5 µl
DNA with incubated random primer	15-17 µl

The radioactive labeled probe was purified on Illustra ProbeQuant G-50 microcolumns column (supplied with the kit).

5.2.8.5 Hybridization and detection

100 µl of salmon sperm DNA was added to the labeled probe, heated to 95^{0}C for 5 minutes, cooled on ice and then added to the hybridization bottle containing nitrocellulose membrane with transferred digested DNA and incubated overnight at 63^{0}C inside the hybridization oven. Subsequently, the membrane was washed with 2X SSC containing 0.1% SDS at room temperature and the radioactivity was measured with the scintillation counter followed by additional washing with 0.1x SSC containing 0.1% SDS at 65^{0}C until the radioactivity value drops to 0.6 bq/cm^2 as measured by Contamat FHT 111M. The membrane was then exposed to X-ray film. The expected size of the floxed band was 2.9 kb, wild type band was 2.7 kb and deleted band was 1.8 kb on the film.

5.2.9 Western blot

5.2.9.1 Sample preparation and SDS-PAGE (Sodiumdodecyl-sulphate-polyacrylamide-gel electrophoresis

For Western blot, cultured macrophages stimulated with rTGF-β1 were lysed in RIPA buffer supplemented with protease inhibitor cocktail and phosphatase inhibitor and incubated for 1 h at 4°C. The sample was pre-cleared by centrifugation at 13,000 rpm and 4°C and supernatant was used to determine protein concentration by BCA assay to ensure equal protein loading. The samples (30-50 µg) were reduced in sample loading buffer containing 20% β-mercaptoethanol by heating for 10 minutes at 95°C. SDS-PAGE

was performed following the modified protocol of Laemmli (Laemmli 1970) using 4-12% Bis-Tris SDS-PAGE gels (NuPAGE® Novex®, Invitrogen). Electrophoresis was performed at 200 V for 45 minutes in the Xcell SureLock® Mini-Cell system using MES running buffer.

5.2.9.2 Protein-transfer and detection

Proteins resolved by SDS-PAGE were transferred to a PVDF membrane using a tank blot system (SureLock System and Tank blot module, Invitrogen). Before use, the PVDF membrane was activated with methanol. The blotting module with no air bubbles. The blotting module contained cathode- 2 transfer sponge- 4 pieces of Whatman filter paper- SDS-PAGE gel- PVDF membrane- 4 pieces of Whatman filter papers- 2 transfer sponges- anode. All components were soaked with transfer-buffer before use. The transfer was performed for 1.25 h at 30 V.

Immunodetection was performed by blocking the PVDF membrane with 5% dry milk in TBS supplemented with 0.05% Tween-20 (TBS-T) for 45 minutes, followed by overnight incubation with appropriately diluted primary antibody at 4°C (table 5-1). Equal protein loading was detected using actin as a loading control. The membrane was stripped in stripping solution for 5-10 minutes, blocked and re-probed with primary antibody against actin and incubated for 1.5 h at room temperature.

After incubation, membrane was washed with TBST (15*3) and probed with HRP-conjugated secondary antibody for 1 h at room temperature, washed and protein bands were visualized by western lightning chemiluminescence. The chemiluminescence signal was detected on X-ray film.

5.2.10 Histology

5.2.10.1 Histochemistry, Sirius red and Giemsa staining

Skin samples were fixed in 4% formalin over night and embedded in paraffin. 6 μm sections were stained with hematoxylin/eosin (H&E), Giemsa stain, and Sirius Red stain following standard protocols in a routine histology laboratory of University hospital of Cologne.

H&E staining was used for an overview and quantification of common wound healing parameters and bleomycin induced fibrotic skin. In this case Hematoxylin stains basophilic structures such as nucleic acids purple, whereas eosin stains eosinophilic structures like the cytoplasm and protein pink, and erythrocytes red. To analyze collagen fibers, Sirius

Red staining was performed and visualized under polarized light. Giemsa staining was used to score mast cells infiltration, which obtained dark purple cytoplasmic granule staining.

5.2.10.2 F4/80 staining on cryosections.

10 μm cryosections were fixed in ice-cold acetone for 2 min, washed with PBS (3*5') and blocked with 10% normal goat serum in PBS containing 5% BSA for 1 h at room temperature. Sections were then incubated for 2 hours at room temperature with primary antibody F4/80, diluted in Dako antibody diluent, washed with PBS (4*15) and incubated with Alexa-488 conjugated Goat-α-Rat secondary antibody dilute in Dako antibody diluent for 2 h at room temperature. Nuclei were visualized using 1 μg/ml DAPI solution. The sections were mounted with Gelvatol.

5.2.10.3 α-SMA/CD31 staining

Cryosections were fixed in ice-cold acetone for 2 minutes, air dried for 20-30 minutes, washed with PBS for 10 minutes and blocked with 10% fetal calf serum for 30 minutes at room temperature. The sections were then probed with primary antibody Rat-α-CD31 diluted in 10% FCS/PBS, washed with PBS (4*10') and incubated with Alexa-488 conjugated secondary antibody Goat-α-Rat for 2 h at room temperature. To double stain, the sections were simultaneously incubated with Cy3 conjugated mouse-α-SMA primary antibody and 1 μg/ml DAPI in 10% FBS/PBS to visualize the nuclei. Sections were then washed with PBS (4*10') and mounted with gelvatol.

5.2.10.4 Gr-1 staining

Cryosections were fixed in ice-cold acetone for 3 minutes, washed with PBS (3*5'), incubated with endogenous peroxidase blocking solution, blocked with 10% FCS for 30 minutes and probed with primary antibody Rat-α-Gr-1 diluted in 1% BSA/PBS overnight at 4°C. The sections were then washed with PBS (4*15'), incubated with HRP-conjugated secondary antibody Goat-α-HRP diluted in 1% BSA/PBS for 1 h at room temperature, washed with PBS (4*15') and incubated with few drops of aminoethyl carbazole (AEC) substrate solution for 5-20 minutes at room temperature. The sections were then washed with tap water for 5 minutes and counterstained with Hematoxylin.

5.2.11 Morphometric analysis

5.2.11.1 Quantification of wound healing parameters and bleomycin induced fibrosis

The microscopic wound area and fibrotic dermal thickness were quantified on H&E stained paraffin sections using Leica DM4000B light microscope (Leica Microsystems, Wetzlar, Germany; Diskus 4.50 Software, Diskus, Königswinter, Germany). The measurement of the distance between the two epithelial tips was used to determine the extent of epithelialization. The measurement of the distance between the ends of the panniculus carnosus was used as a measure of wound contraction. In bleomycin induced fibrotic skin, the dermal thickness was determined to quantify the fibrotic response.

5.2.11.2 Quantification of histochemical stainings

Organization and maturation of collagen bundles was assessed on paraffin sections of day 14 wounds and fibrotic skin (2 weeks and 4 weeks of bleomycin injection) stained with Sirius Red and visualized by polarized light (Leica DM4000B, Leica Microsystems, Wetzlar, Germany). To determine the number of mast cells and Gr-1 positive neutrophils, the stained cells were counted in the entire area of fibrotic tissues.

5.2.11.3 Quantification of immunohistochemical stainings

Immunofluorescence microscopy was conducted using Eclipse 800E Microscope; Nikon, Melville, NY, USA). Number of macrophages was determined by counting cells in the total granulation tissue and at the wound edges. For quantitative analysis of CD31 and α-SMA, the area in the granulation tissue, which stained positive were calculated using analysis ImageJ software (Wayne Rasband, National Institute of Mental Health, Bethesda, MD, USA).

5.2.12 *In situ* zymography

In situ Zymography was performed on the cryosections of fibrotic skin. Gel containing 30% polyacrylamid, 5% of bovine gelatine, 10% APS and TEMED in Tris-HCl buffer was polymerized on the slides and kept in humid chamber. 8 to 10 μm thick cryosections were cut and placed on the gelatine gel and incubated overnight in a humid chamber at 37^0C. Subsequently, the sections were stained with methylene blue for 1 minute and washed with distilled water. Pictures were taken under the microscope and coordinates are measured. The sections were removed from the gel using soft tissue and 5% SDS at 50^0C and the tissue incubated gelatine slides were washed in distilled water, stained with

Coomassie blue for 10-20 minutes, destained in distilled water. The white spots against the dark blue gelatine background were used as an indication of enzyme activity.

5.2.13 *In vivo* experiments

5.2.13.1 Mouse strains

Mouse strains were maintained and bred under standard pathogen-free conditions as per institutional guidelines. To generate TGFβRII$^{fl/fl}$LysMCre mice, TGFβRII floxed mice (TGFβRII$^{fl/fl}$) (Cazac and Roes 2000) were crossed with a transgenic mouse line expressing Cre recombinase under myeloid cell specific lysozyme M (LysMCre) promoter (Clausen, Burkhardt et al. 1999). TGFβRII floxed mouse strain was maintained on a mixed C57/Bl6 & SV129 background, and LysMCre was maintained on a C57/Bl6 background.

5.2.13.2 Genotyping

Isolated Genomic DNA from three weeks old mice was used for genotyping PCR as described in the section 5.2.3. The PCR program used for genotyping is listed below and the primer sequences are listed in the table 5-4.

Genotyping master mix

Mix	TGFβRII & LysMCre
10X buffer	2.5 μl
dNTP´s	0.5 μl
FP1	0.5 μl
RP1	0.5 μl
$MgCl_2$	2 μl
Taq-Polymerase	0.25 μl
Double distilled H_2O	18.25 μl
cDNA	1 μl

Genotyping PCR program

Temperature	TGFβRII & LysMCre
95°C	5 min
95°C 56°C 72°C	45 s 60 s 12 x 60 s
95°C 52°C 72°C	45 s 60 s 18 x 60 s
72°C	7 min
10°C	Pause

5.2.13.3 Thioglycollate-induced peritonitis and peritoneal cell harvest

To induce peritonitis, 4% of thioglycollate in PBS was prepared, autoclaved and matured in the absence of light in a dark bottle for at least six months before use. Mice were injected with 1 ml of 4% thioglycollate and peritoneal cells were harvested from peritoneal cavity four days after injection (section 5.2.1.1.1).

5.2.13.4 Wounding

Mice were anesthetized by intra-peritoneal injection of ketanest/2%Rompun. Four full thickness wound of 6 mm diameter were generated on the shaved back of mice using a standard biopsy punch. At day 4, day 7 or day 14 post injury and tissues were collected. For histological analysis, the tissue were bisected in caudocranial direction and was either fixed overnight in 4% paraformaldehyde or embedded in OCT compound for immunohistochemical staining. Serial sections from the central portion of wounds were used for histological analysis. For RNA isolation, tissues were excised and stored in RNAlater® solution at -20°C. For cell isolation, wound tissues were excised and processed immediately.

5.2.13.5 Bleomycin induced fibrosis

Cutaneous fibrosis was induced by intra-dermal injection of 100 μl of 1 mg/ml bleomycin everyday for five days per week until 2 weeks and 4 weeks on the shaved back. Tissue was excised and used for histological studies, RNA isolation and cell isolation.

5.2.14 Statistical analysis

Statistical analysis was performed using GraphPad Prism5 (GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was analyzed using unpaired student's t-test. $P \leq 0.05$ was considered significant.

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7 Abbreviations

7-AAD	Aminoactinomycin D
APC	Allophycocyanin
APS	Ammonium persulfate
α-SMA	Alpha-smooth muscle actin
bFGF	Basic fibroblast growth factor
Bleo	Bleomycin
bp	Base pairs
BSA	Bovine serum albumin
°C	Degree Celsius
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CSF-1	Colony stimulating factor
cDNA	Copy deoxyribonucleic acid
CTGF	Connective tissue growth factor
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
der	Dermis
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
DT	Diphtheria toxin
Epi	Epidermis
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluoresceinisothiocyanat
fl	Floxed
g	Granulation tissue
H&E	Hematoxylin and eosin stain
HPF	High power field
ICAM	Intercellular adhesion molecule
IFN-γ	Interferon-γ
i.p	Intraperitoneal
iDTR	Inducible diphteria toxin receptor
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JNK	c-Jun N-terminal protein kinase
kb	Kilo base pairs

kDa	Kilo Daldon
LysM	Lysozyme M
MΦ	Macrophage
MCP-1	Monocyte chemotactic protein-1
MES	2-(*N*-morpholino) ethanesulfonic acid
mg	Milligram
μl	Microlitre
μm	Micrometer
MIP-1α	Macrophage inflammatory protein
min	Minute
MMP	Matrix metalloprotease
NK cells	Natural killer cells
p-value	Probability-value
PAGE	Polyacrylamide gel electrophoresis
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffer saline
PC	Panniculus carnosus
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PFA	Paraformaldehyde
PMSF	Phenylmethylsulphonylfluorid
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RPM	Rounds per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
S	Second
S18	ribosomal protein S18
SDS	Sodium dodecyl sulphate
Ser	Serine
Tm	Annealing temperature
TAE	Tris base acetic acid EDTA
TE	Tris base EDTA
TEMED	Tetramethylethylenediamine
TGF-β1	Transforming growth factor-beta
TGFβRII	Transforming growth factor-beta receptor II
TIMP	Tissue inhibitor of metalloproteinases
TNF-α	Tumor necrosis factor-alpha
VEGF	Vascular endothelial growth factor
V	Volt

W	Wound tissue
WMΦ	Wound macrophages

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9 Erklärung

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