



Contents lists available at ScienceDirect

## Vascular Pharmacology

journal homepage: [www.elsevier.com/locate/vph](http://www.elsevier.com/locate/vph)

## 1 Review

Q2 The innate immune system, toll-like receptors and dermal wound  
3 healing: A review ☆Q4 M.J. Portou <sup>a,\*</sup>, D. Baker <sup>a</sup>, D. Abraham <sup>b</sup>, J. Tsui <sup>a</sup><sup>a</sup> Royal Free Vascular, Division of Surgery and Interventional Science, Royal Free Campus, UCL, London, UK<sup>b</sup> Centre for Rheumatology and Connective Tissue Disease, UCL, London, UK

## 7 A R T I C L E I N F O

## 8 Article history:

9 Received 16 November 2014

10 Received in revised form 5 February 2015

11 Accepted 8 February 2015

12 Available online xxxx

## 13 Keywords:

14 Wound healing

15 Innate immune system

16 Diabetes

## A B S T R A C T

Wound healing is a complex physiological process comprised of discrete but inter-related and overlapping stages, requiring exact timing and regulation to successfully progress, yet occurs spontaneously in response to injury. It is characterised by four phases, coagulation, inflammation, proliferation and remodelling. Each phase is predominated by particular cell types, cytokines and chemokines. The innate immune system represents the first line of defence against invading microorganisms. It is entirely encoded with the genome, and comprised of a cellular response with specificity provided by pattern recognition receptors (PRRs) such as toll-like receptors (TLRs). TLRs are activated by exogenous microbial pathogen associated molecular patterns (PAMPs), initiating an immune response through the production of pro-inflammatory cytokines and further specialist immune cell recruitment. TLRs are also activated by endogenous molecular patterns termed damage associated molecular patterns (DAMPs). These ligands, usually shielded from the immune system, act as alarm signals alerting the immune system to damage and facilitate the normal wound healing process. TLRs are expressed by cells essential to wound healing such as keratinocytes and fibroblasts, however the specific role of TLRs in this process remains controversial. This article reviews the current knowledge on the potential role of TLRs in dermal wound healing where inflammation arising from pathogenic activation of these receptors appears to play a role in chronic ulceration associated with diabetes, scar hypertrophy and skin fibrosis.

© 2015 Published by Elsevier Inc. 32

## 38 Contents

39	1. Introduction . . . . .	0
40	2. Normal dermal wound healing . . . . .	0
41	3. The innate immune system and the skin . . . . .	0
42	3.1. The innate immune system . . . . .	0
43	3.2. Toll-like receptors . . . . .	0
44	3.3. Toll-like receptors and the skin . . . . .	0
45	3.4. Toll-like receptors and wound healing . . . . .	0
46	4. Non-healing, hypertrophy and other wound complications . . . . .	0
47	5. Conclusion . . . . .	0
48	References . . . . .	0

## 50 1. Introduction

51 Wound healing is a complex physiological process comprised of discrete but inter-related and overlapping stages, requiring exact timing

53 and regulation to successfully progress, yet occurs spontaneously in response to injury. It is characterised by four phases, coagulation, inflammation, proliferation and remodelling. Each phase is predominated by particular cell types, cytokines and chemokines.

56 Mammals and higher organisms have evolved complex immune defences against pathogenic microbial organisms in the form of the antibody based adaptive immune system and the innate immune system, a primitive evolutionary cellular based system. Pattern recognition receptors (PRRs) on the cell surface of innate immune cells recognise discrete microbial molecular patterns triggering their activation termed

Q3 ☆ This article is based on a presentation at the 8th International Workshop on Cardiovascular Biology & Translational Medicine held in London in September 2013.

\* Corresponding author at: Royal Free Vascular, Division of Surgery and Interventional science, 9th Floor Royal Free Hospital, Pond Street, London NW3 2QG, UK. Tel.: +44 7946619670.

E-mail address: [mjportou@doctors.org.uk](mailto:mjportou@doctors.org.uk) (M.J. Portou).

pathogen associated molecular patterns (PAMPs). A group of highly conserved and prime PRRs are toll-like receptors (TLRs). In addition to PAMPs, TLRs also recognise a range of endogenous self derived molecular patterns released in response to tissue and cellular damage, termed damage associated molecular patterns (DAMPs).

TLRs are expressed by cells comprising the dermis and epidermis of the skin, in addition to the immune cells that reside within the skin or those that are recruited from circulation. The activation and timing of specific TLRs and the presence of conditions affecting TLR expression and activation determine whether TLR activation promotes or inhibits the wound healing process, leading to chronic wounds.

## 2. Normal dermal wound healing

Immediately following trauma to the skin, platelets aggregate at the site of injury with haemostasis achieved following local vasoconstriction and activation of the clotting cascade, resulting in fibrin clot formation [1]. The inflammatory phase of wound healing begins with release of proinflammatory cytokines such as platelet derived growth factor (PDGF), transforming growth factor (TGF- $\beta$ ), fibroblast growth factor (FGF), epidermal growth factor (EGF) and Interleukin 8 (IL-8/CXCL-8) from the newly formed clot and directly from the damaged tissues [2]. These act as potent chemotactic signals to immediately recruit neutrophils to the wound [3]. Circulating polymorphonuclear neutrophils (PMN) begin migration within minutes from the blood into the immature wound bed formed by the clot, peaking within the first 24 h [4]. The neutrophils now present in the wound provide a crucial defence against microbial invasion following disruption to the skin's natural barrier function, clearing both pathogen and tissue debris by phagocytosis [2,5].

The process of platelet de-granulation, activation of the complement cascade, and the migration and signalling of PMNs results in the further production of chemotactic factors such as complement component 5 (C5), fibrin by products and TGF- $\beta$ c [6]. These chemokines along with chemokine (C-C motif) ligand 5 (CCL5) produced by keratinocytes, recruit monocytes to the wound, which under the influence of local cytokines undergo differentiation to become mature wound macrophages [6,7]. By days three to five following injury, tissue macrophages become the dominant cell type [8]. Wound macrophages continue the process of wound bed clearance through phagocytosis of apoptotic cells including the early phase PMNs, tissue debris and microbial organisms [8]. In addition, macrophages also directly aid the debridement of injured and devitalised tissue through release of protease and metalloprotease enzymes [8,9]. Over and above their phagocytic role, an important initial function of wound macrophages is the release of cytokines which further aid the recruitment and activation of inflammatory cells [2]. As the inflammatory phase progresses, macrophages produce important growth factors such as KGF, TGF- $\beta$ , VEGF and PDGF which stimulate fibroblast and keratinocyte growth and migration and the process of angiogenesis [1]. It is therefore considered that macrophages are responsible for the transition to the proliferative phase of wound healing [2].

The late inflammatory phase becomes characterised by infiltration of T-lymphocytes under the influence of IL-1, which peak at day 7 after injury. At this stage there is considerable temporal overlap between the late inflammatory, proliferative and early remodelling phases of normal wound healing. [10]. As described, the inflammatory phase involves a well characterised sequence of immune cell infiltration, neutrophils followed by macrophages then finally T-lymphocytes [2].

Like macrophages, T-lymphocytes appear to have a complex yet significant role in the normal process of wound healing, however these processes, functions and pathways remain poorly understood. Studies utilising *In vivo* murine knock-out models have suggested that absent or delayed T-lymphocyte wound infiltration results in an impairment of the healing process [2]. However there appears to be differential roles of CD4 + T helper and CD8 + cytotoxic T cells, with CD4 + cells found to have a positive promoting effect on healing, and CD8 + cells

an inhibitory effect [11]. In addition, T-lymphocytes have a regulatory effect on inflammation and fibrosis and a dermal subgroup of gamma delta T cells produce keratinocyte growth factor (KGF) and insulin-like growth factor 1 which stimulate keratinocyte proliferation, promoting healing [12].

Central to the proliferative phase of wound healing is the formation of granulation tissue. Dermal fibroblast proliferation, migration and differentiation (into contractile myofibroblasts) occurs under the influence of growth factors such as fibronectin, PDGF, FGF, TGF- $\beta$  and C5a, as inflammatory cytokine release diminishes [13]. Fibroblasts are crucial for the production of extracellular matrix comprised of collagen, glycosaminoglycans, proteoglycans, fibronectin and elastin [14]. Angiogenesis occurs as dermal endothelial cells migrate into the newly forming extracellular matrix under the influence of macrophage derived angiogenic factor, forming new capillaries [8].

During the proliferative phase, wound contraction is an important process that occurs through the action of myofibroblasts, differentiated from mesenchymal fibroblast cell lines [15]. Myofibroblasts, unlike fibroblasts express the contractile protein  $\alpha$  smooth muscle actin ( $\alpha$ SMA) and as the wound matures are gradually lost from the granulation tissue [15].

Restoration of the skin's crucial barrier function requires successful epidermal keratinocyte migration, proliferation and differentiation to cover the newly formed granulation tissue and extracellular matrix in a process termed re-epithelialisation [16]. In intact skin, keratinocytes are closely attached to adjacent epithelial cells through desmosomes, and to the extra cellular matrix of the underlying basement membrane by hemidesmosomes [17]. Following injury, keratinocytes become mobilized by undergoing phenotypic changes favouring detachment in a process that remains incompletely understood. However cytokines such as IL-1, IL-6 and TNF- $\alpha$  produced in inflammatory phase seem to help modulate the migratory phenotype of keratinocytes [17]. Migration and proliferation are influenced by growth factors such as IGF1 and epidermal growth factors (EGF) [18]. In addition, EGF, KGF and TGF- $\beta$  have important pro-migratory or pro-proliferative effects on keratinocytes [17]. Essential to the process of keratinocyte migration is production of proteases such as collagenases and matrix metalloproteases [19]. These degrade adhesions between the keratinocyte and the newly formed extracellular matrix to permit cell movement [16]. Disruption of the basement membrane after injury requires migrating keratinocytes to utilize fibronectin, vitronectin and fibrin components of the provisional extracellular matrix for attachment through focal integrin receptors [16]. Closely following migration is rapid proliferation and basement membrane repair through laminin production [20]. Keratinocyte differentiation and keratin production occurs as the epidermal barrier and normal stratified architecture is restored [21].

The remodelling phase is the longest phase of the wound healing process, continuing for weeks to months [8]. This phase is characterised by reduced proliferation and inflammation, active re-organisation of the extracellular matrix and regression of the newly formed capillaries as the nutrient requirements of the wound site reduce [8].

Type III collagen produced by fibroblasts during the proliferative phase is gradually replaced by structural type I collagen, through the action of collagenases and matrix-metalloproteases [16]. During remodelling, collagen becomes more organised and increasingly cross-linked strengthening the scar; fibronectin disappears, and hyaluronic acid and glycosaminoglycans are replaced by proteoglycans. The result is the re-organisation of the extracellular matrix to an architecture more closely resembling normal tissue [2].

## 3. The innate immune system and the skin

### 3.1. The innate immune system

Mammals and other higher vertebrate organisms have evolved complex immune defences against invading pathogenic microorganisms,

comprised of the innate and adaptive immune systems [22]. The innate immune system is in evolutionary terms primitive and unlike the clonal selection antibody-based response of the adaptive immune system, is entirely encoded within the genome [23]. Innate immunity comprises the entire immune response of invertebrate organisms, however in higher species it provides the first line of defence against infectious pathogens and aids adaptive responses through antigen presentation, with the adaptive response concerned with later stages of infection, providing a targeted and specific response and immunological memory [22].

The innate immune system is comprised of numerous different cellular components such as neutrophils, eosinophils, basophils, mast cells, monocytes, macrophages, dendritic cells, NK cells, gamma delta T cells, B-1 cells [24]. Rather than coordinating a non-specific pro-inflammatory or phagocyte response, cell activation, pathogen recognition and a specificity of the innate immunity is conferred by the presence of specific receptors expressed by these immune cells termed pattern recognition receptors (PRRs) [25,26].

### 3.2. Toll-like receptors

Toll-like receptors (TLRs) are key pattern recognition receptors of the innate immune system [27]. Other examples of PRRs include scavenger receptors (SRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs) and B2 integrins [26]. These receptors are highly conserved in evolution and recognise discrete molecular components of invading pathogens termed pathogen associated molecular patterns (PAMPs), such as lipids, lipopeptides, proteins and nucleic acids [22,27]. The recognition of microbial PAMPs by PRRs leads to activation of specific signalling pathways and a variety of cell dependent responses, including pro-inflammatory cytokine release, phagocytosis and antigen presentation [26].

The Toll-like receptor family consists of thirteen identified members of which ten are expressed in humans [24]. TLRs are located either at the cell surface (TLRs 1,2, 4, 5, 6) or in the intracellular compartment (TLRs 3, 7, 8, 9) primarily on exosomes and endoplasmic reticulum [28,29]. TLRs are transmembrane proteins consisting of an ectodomain comprising leucine-rich repeats, a transmembrane domain and an intracellular (TIR) domain [28]. The binding of TLR ligands results in activation through the recruitment of specific adaptor molecules such as myeloid differentiation factor 88 (MyD88), MyD88 adaptor like (MAL), TIR domain-containing adapter-inducing interferon- $\beta$  (TRIF) and TRIF adaptor molecule (TRAM) to the intracellular domain [28,30]. All TLRs except TLR3 utilize one of two signalling pathways, the MyD88 dependent and MyD88 independent (TRIF) pathways, resulting in the activation of nuclear transcription factors such as NF $\kappa$ B, JNK and MAPK [28]. TLR3 signals solely through the TRIF pathway [27]. The result is proinflammatory cytokine and type 1 interferon gene induction [22].

TLRs efficiently recognise distinct components of pathogens that are essential to their metabolism, preventing mutations rendering them undetectable [31]. TLRs 1, 2 and 6 recognise gram positive bacteria cell wall constituents such as lipoproteins, peptidoglycans and lipoteichoic acid [31]. TLR4 is activated by the gram negative bacteria cell wall component lipopolysaccharide (LPS) [32] and TLR5 bacterial flagellin [31]. The intracellular TLRs 3, 7 and 8 recognise double and single stranded viral RNA, and TLR9 non-methylated CpG dinucleotides present in bacterial DNA [31,33].

In addition to exogenous microbial PAMP ligands, TLRs are also activated by a range of endogenous ligands released as a result of tissue and cellular injury termed damage associated molecular patterns (DAMPs). These are usually hidden from recognition, however following injury they are released or revealed, triggering a TLR mediated inflammatory response [31]. It has been suggested DAMPs act as danger signals, released by injured tissues, alerting the immune system of damage [34]. The resulting sterile inflammation is a key stimulator for the recruitment of innate immune inflammatory cells and initiation of

the wound healing process [31]. DAMPs identified as TLR ligands include the extracellular matrix constituent hyaluronic acid, HMGB1 (a nuclear protein), Heat shock proteins (HSPs) 60 and 70, oxidised LDL, fibrinogen and fibronectin [35].

### 3.3. Toll-like receptors and the skin

Intact skin provides an external barrier to the environment, preventing infection by the majority of pathogenic bacteria, viruses and fungi [36]. In addition to this physical defence, cells of the innate immune system present in skin such as dermal mast cells, phagocytes and dendritic cells such as Langerhans cells of the epidermis, and those readily recruited from blood such as neutrophils, macrophages, basophils, eosinophils, NK cells and gamma-delta T cells all express TLRs for pattern recognition [31]. On detection of invading microbial pathogens through recognition of PAMPs, TLR activation results in the initiation of a pro-inflammatory defence response, promoting phagocytosis, immune cell recruitment and antigen presentation [36]. In addition to immune cells, TLRs are also widely expressed by a variety of non-immune cells contained within both the epidermis and dermis which are vital to wound healing [37].

The epidermis is primarily comprised of keratinocytes, which have been demonstrated to express TLRs 1–6 and TLR9 and 10 [38]. Unlike specialist immune cells, keratinocytes and other epithelial cells comprise the boundary and interface with the external environment and are under constant exposure to microbes and PAMPs [31]. They are able to maintain a delicate balance between tolerance of commensal organisms and the detection of infection and injury and subsequent inflammatory response [31]. The relative expression of TLRs by keratinocytes also seems to vary depending on position of the cell, for instance TLR5 is predominantly expressed in the basal layers, whereas TLR9 is expressed to a greater degree by more differentiated cells of the upper epidermal layers [39]. It does appear however all TLRs are functional, and produce distinct immune responses [40]. For instance, activation of keratinocyte TLRs 2, 3, 4, 5 and 9 by their respective ligands resulted in TNF- $\alpha$ , IL-8, CCL2 (basophil chemokine) and CCL20 (macrophage inflammatory protein-3) release [40]. TLR3 and TLR9 activation produced CXCL9 and CXCL10, involved in T-memory cell activation and type 1 interferon production [40].

Fibroblasts located in the dermis produce extra cellular matrix constituents, cytokines, growth factors and have a crucial role in the wound healing process as described above. They have been found to express the full range of human TLRs from 1 to 10 [41]. Studies have demonstrated *in vitro* activation of TLRs 2, 3, 4, 5 and 9 resulted in production of interferon- $\gamma$ , CXCL9, CXCL10 and CXCL11, important in the recruitment of T-cells and NK cells [40]. TLR4 activation in dermal fibroblasts has been demonstrated to result in IL-6, IL-8 and monocyte chemoattractant protein (MCP) [42]. Microvascular cells such as dermal endothelial cells have been shown to highly express TLR4 and to a lesser extent TLR2. *In vitro* treatment with the exogenous TLR4 ligand LPS resulted in NF $\kappa$ B activation. Likewise exposure to the endogenous derived ligand hyaluronan induced IL-8, a potent chemokine, stimulating the recognition of tissue injury and promoting initiation of the early stages of the wound healing process [43].

### 3.4. Toll-like receptors and wound healing

As previously described, recognition of endogenous ligands by TLRs on both immune and non-immune cells of the skin provide alarm signals *via* TLR activation and resulting sterile inflammation alerting to tissue injury. However, the effect of TLR activation on the wound healing process extends beyond the initial recognition of cellular damage, and it appears depending on the location, timing and degree of activation may have a promoting or inhibiting effect on the process of wound healing and tissue regeneration [44] (Table 1).



**Table 1**  
Summary of TLR wound healing studies.

Study	TLR	Model	Wound	Findings
Dasu et al. (2010) [44]	2	<i>In vivo</i> murine, knock out	Diabetic	TLR2 knock out was beneficial for wound healing in diabetes induced animals
Dasu et al. (2013) [45]	4	<i>In vivo</i> murine, knock out	Diabetic	TLR4 knock out improves wound healing and reduces inflammation in diabetic mice
Suga et al. (2013) [7]	2 and 4	<i>In vivo</i> murine, knock out	Non-diabetic	TLR2 and 4 knock out impaired wound healing at days 3 and 7. TLR4 rather than TLR2 regulates healing through TGF- $\beta$ and CCL5
Chen et al. (2013) [43]	4	<i>In vitro</i> , <i>In vivo</i> murine, knock out	Non-diabetic	Injury stimulates TLR4 mRNA expression in keratinocytes. Wound healing is prolonged in TLR4 deficient mice.
Sato et al. (2010) [31]	9	<i>In vivo</i> murine, knock out	Non-diabetic	Wounds treated with TLR9 agonists exhibit accelerated healing. TLR9 deficient animals demonstrate delayed wound healing
Lin et al. (2011) [47]	3	<i>In vivo</i> murine	Non-diabetic	Wound healing is significantly delayed in TLR3 deficient mice compared to wild type
Lin et al. (2012) [48]	3	<i>In vivo</i> murine, human	Non-diabetic	Topical application of TLR3 agonist accelerated wound healing when applied to human and mouse wounds TLR3 deficiency inhibited wound healing

*In vitro* and *in vivo* data has suggested that TLR4 becomes upregulated within the first 12–24 h following injury and slowly decreases to baseline at day 10, and is primarily concentrated in epidermal keratinocytes [45]. The same study demonstrated significantly impaired wound healing in TLR4 deficient mice at days 1–5, with no difference seen from wild type at 10 days [45]. An altered pattern of cytokine release and inflammatory cell infiltration was observed with decreased IL-1 $\beta$  and IL-6, and an increase in neutrophil, macrophage and T-cell infiltrates in the wounds of knockout animals at discreet time points [45]. Another study also observed impairment in wound healing in TLR2 and TLR4 deficient mice at days 3 and 7, but observed a decrease in neutrophil and macrophage infiltration, and reduced TGF- $\beta$  and CCL5 expression [7]. Activation of TLR4 and TLR2 appears therefore to have a beneficial effect on wound healing in the early stages following acute injury, at least in absence of other influences on TLR expression, signalling and activation.

However the story does not end there. Controversy exists as to the exact effect of TLR4 and TLR2 in the wound healing process. Given the seemingly important regulatory role of TLR4 and TLR2 in initiating the early stages of wound healing, it perhaps seems counter-intuitive that wound healing was significantly improved in TLR2 deficient mice with induced diabetes compared to diabetic wild-type animals [46]. The same effect was also observed in diabetic TLR4 deficient mice [47] in apparent contradiction of the studies described above.

In addition to decreased healing time, the wounds from TLR2 deficient mice also demonstrated significantly reduced NF $\kappa$ B activation, IL-6 and TNF- $\alpha$  release [46]. In the same study when comparing wild-type diabetic mice to non-diabetic controls, TLR2 mRNA and protein expression was significantly increased, along with markers of activation such as increased expression of MyD88, IRAK and NF $\kappa$ B [46]. Likewise, TLR4 mRNA and protein expression, IL-6, TNF- $\alpha$  and NF $\kappa$ B activation was increased in wild-type diabetic compared to non-diabetic animals, with a corresponding reduction in IL-6, TNF- $\alpha$  and NF $\kappa$ B activity in the TLR4 deficient diabetic populations [47].

These studies demonstrated significantly increased TLR2, TLR4 and MyD88 expression in diabetic compared to non-diabetic wounds and suggests in diabetes, TLR2 and TLR4 mediated hyperinflammation results in an impairment of wound healing. Persistent activation of TLR2 and TLR4 is also associated with other chronic non-healing wounds such as chronic venous ulceration [48].

Wound healing studies utilising TLR3 deficient mice resulted in significantly delayed wound healing compared to wild-type controls, led to decreased neutrophil and macrophage recruitment, and reduced CXCL2, CCL2 and CCL3 chemokines [49]. Further to this effect, the TLR3 agonist poly(I:C) significantly accelerated wound healing when applied topically to human and mouse wounds compared to control and resulted in greater neutrophil and macrophage recruitment and upregulated CXCL2 [50].

TLR9 deficient mice demonstrated delayed wound healing compared to wild-type [33]. In addition, topical administration of the TLR9

agonist CpG ODN to wounds resulted in significantly improved healing times, increased macrophage infiltration and increased production of VEGF [33].

#### 4. Non-healing, hypertrophy and other wound complications

Chronic wounds such as foot ulceration are a frequent and challenging complication of diabetes with a life time risk of between 15 and 25% [51,52]. This translates to a 20 $\times$  greater risk of major amputation compared to non-diabetics, and remains the most common cause of hospitalisation amongst diabetic patients [6]. Diabetic foot ulcers are multifactorial in causation, although a predominance for either neuropathy or ischaemia often exists [6]. The result is a wound characterised by poor healing, with the progression of the normal process stalled, or failed to initiate, leading to a chronic, static wound.

The diabetic wound environment differs from the normal acute wound process through a prolonged and persistent inflammatory phase (Fig. 1). There is an exaggerated and sustained neutrophil and macrophage infiltration, which in a db/db mouse model was demonstrated to be associated with deregulated and prolonged chemokine expression, such as macrophage inflammatory protein 2 and macrophage chemoattractant protein 1 [53].

Although the initial infiltration of immune cells is impaired, once activated the result is a hyperinflammatory response with elevated inflammatory cytokine production of TNF- $\alpha$ , IL-1 $\beta$  and IL-6, and increased NF $\kappa$ B regulated matrix metalloprotease (MMP) production leading to excessive extracellular matrix destruction and grossly impaired granulation tissue formation [6]. Neutrophils in particular appear to contribute to these destructive wound conditions through upregulated release of MMP-8 and downregulated release of the MMP inhibitor TIMP1 in chronic wounds [54]. The resulting hostile environment of excess inflammatory cytokine production (TNF- $\alpha$ , IL-6) also impairs other events and processes crucial to healing such as fibroblast migration and proliferation, collagen synthesis and promotes apoptosis in fibroblasts and vascular precursor cells [6].

Diabetic ulceration is an example of chronic inflammation directly leading to a significant impairment in the healing process and the creation of a chronic non-healing wound. As previously described, there is compelling evidence this pathological inflammation is mediated via excessive TLR activation. Another consequence of abnormal TLR mediated inflammation on the wound healing process is in over healing in the form of hypertrophic scar formation [42]. Hypertrophic scars develop following trauma as a result of excessive production of ECM components such as collagen, and although the mechanism remains unclear, are associated with prolonged inflammation and bacterial contamination [42]. Comparison of hypertrophic and normal scar tissue from burns patients demonstrated increased TLR4 staining in hypertrophic tissues and increased TLR4 and MyD88 mRNA in fibroblasts isolated from hypertrophic scars [42]. A corresponding increase in pro-inflammatory cytokines such as PGE2, IL-6, IL-8 and MCP-1 were also

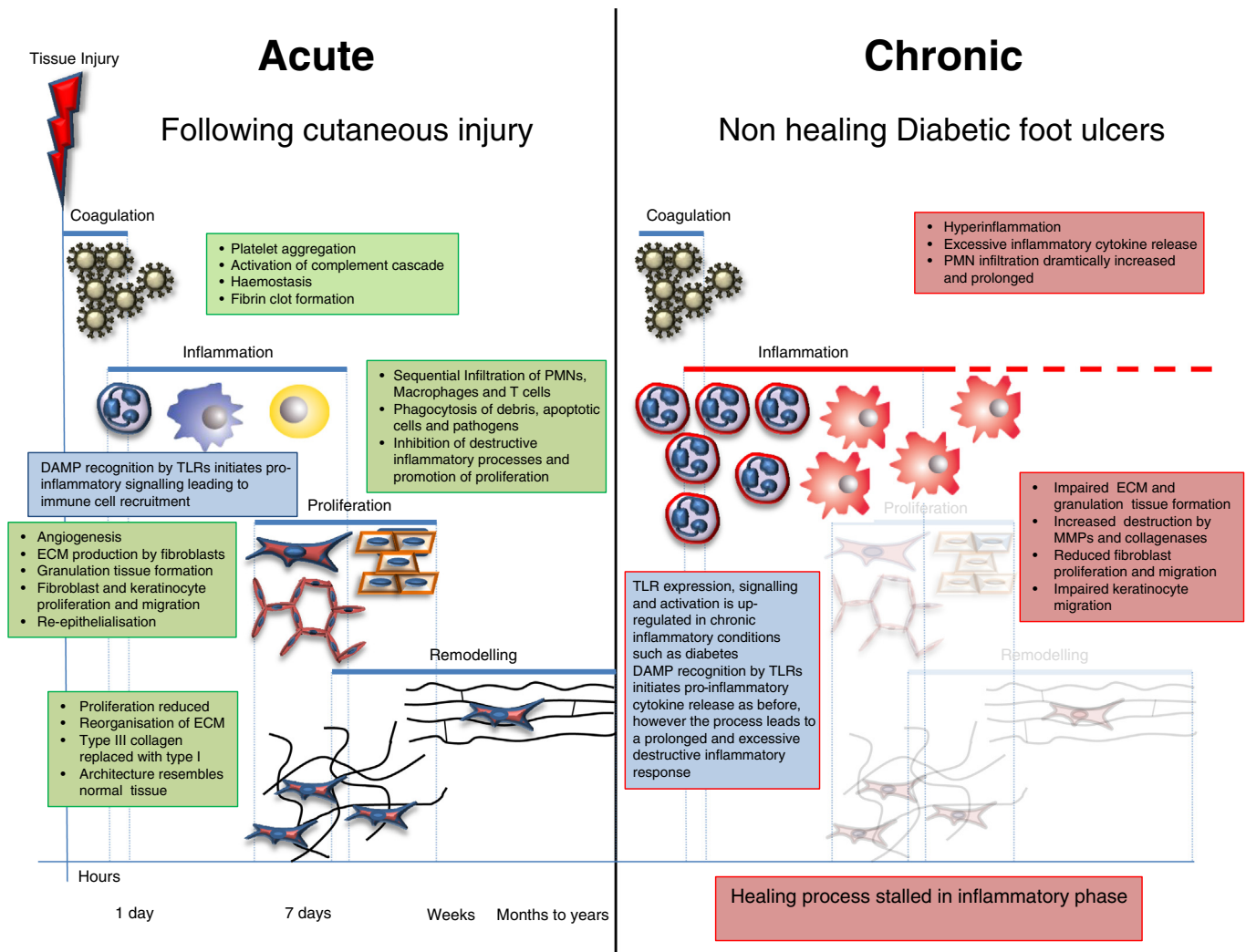


Fig. 1.

Q1

detected. It is therefore suggested that persistent TLR4 activation in dermal fibroblasts leads to hypertrophic scarring, possibly through the increased production of growth factors by supporting cells [42].

Another example of a maladaptive healing response that occurs in response to chronic inflammation is fibrosis. TLR activation is implicated in fibrotic abnormal healing responses in specific organs such as the liver, following repeated tissue injury [31]. TLR4 in particular is thought to contribute to the fibrotic reaction through chronic activation from the continuous translocation of gut bacteria associated with chronic liver diseases, and as demonstrated by the protective effect of TLR4 knock out in animal models of liver injury [31]. There does however appear to be a differential TLR effect on fibrosis depending on the organ involved, with TLR9 implicated in lung fibrosis, and TLRs 2 and 4 likely to have a greater effect in acute renal inflammation rather than chronic kidney fibrotic responses, where evidence is conflicting [31].

The role of TLRs in fibrotic skin reactions has been of particular interest in conditions such as systemic sclerosis. TLR4 activation has been implicated by murine models of skin fibrosis utilising bleomycin, through increased hyaluronan production, a potent TLR4 endogenous ligand [55]. In addition, studies utilising human tissue biopsies from scleroderma patients have demonstrated TLR4 and associated adaptor molecules are overexpressed in affected skin, and correlate with disease progression [56]. *In vitro* studies in ex-planted scleroderma fibroblasts have shown activation of TLR4 resulted in increased collagen production and gene expression of factors associated with ECM production

and remodelling, in addition to an increased susceptibility to the effects of TGF- $\beta$  [57]. Recent work has also identified TLR4 as a crucial mechanism through which in scleroderma, injured keratinocytes interact with fibroblasts through increased production of the protein S100A9, a known ligand of TLR4, leading to increased production of the profibrotic gene CCN2 [58].

It is therefore proposed that in chronic fibrotic skin diseases such as scleroderma, persistent TLR4 activation through endogenous ligand stimulation results in altered response to TGF- $\beta$ , and subsequent dysregulated production and remodelling of the extracellular matrix, leading to profound skin fibrosis.

## 5. Conclusion

Wounds that fail to heal, such as chronic diabetic ulcers, do not progress through the normal stages of the healing process described in detail earlier in this review. It is clear the innate immune system and the pattern recognition receptors that confer specificity such as toll-like receptors have a crucial role in the initiation and regulation of normal wound healing, however the role of the innate immune response in chronic wounds remains controversial.

In addition to the excess morbidity and mortality associated with foot ulceration and subsequent amputation, and with the global burden of diabetes set to reach 350 million people, non-healing wounds of all aetiology are set to remain an enormous economic liability for

healthcare systems around the world. Manipulation of the innate immune response therefore represents a potential novel therapeutic opportunity to reduce the hyperinflammation associated with chronic wounds, and to restart the normal wound healing process.

The impact of dysregulated TLR activation and subsequent chronic inflammation on the wound healing process appears to be significantly more complex however, when the pathological yet intuitively opposed outcomes of non-healing, hypertrophy and fibrosis can all occur in different disease phenotypes within the same tissue, the skin.

## References

[1] Hubner G, Brauchle M, Smola H, Madlener M, Fassler R, Werner S. Differential regulation of pro-inflammatory cytokines during wound healing in normal and glucocorticoid-treated mice. *Cytokine* 1996;8(7):548–56.

[2] Guo S, DiPietro LA. Factors affecting wound healing. *J Dent Res* 2010;89(3):219–29.

[3] Roupe KM, Nybo M, Sjobring U, Alberius P, Schmidtchen A, Sorensen OE. Injury is a major inducer of epidermal innate immune responses during wound healing. *J Invest Dermatol* 2010;130(4):1167–77.

[4] Kim MH, Liu W, Borjesson DL, Curry FR, Miller LS, Cheung AL, Liu FT, Isseroff RR, Simon SI. Dynamics of neutrophil infiltration during cutaneous wound healing and infection using fluorescence imaging. *J Invest Dermatol* 2008;128(7):1812–20.

[5] Segal AW. How neutrophils kill microbes. *Annu Rev Immunol* 2005;23:197–223.

[6] Acosta JB, del Barco DG, Vera DC, Savigne W, Lopez-Saura P, Guillen Nieto G, Schultz GS. The pro-inflammatory environment in recalcitrant diabetic foot wounds. *Int Wound J* 2008;5(4):530–9.

[7] Suga H, Sugaya M, Fujita H, Asano Y, Tada Y, Kadono T, Sato S. TLR4, rather than TLR2, regulates wound healing through TGF-beta and CCL5 expression. *J Dermatol Sci* 2014;73(2):117–24.

[8] DiPietro LA. Wound healing: the role of the macrophage and other immune cells. *Shock* 1995;4(4):233–40.

[9] Deodhar AK, Rana RE. Surgical physiology of wound healing: a review. *J Postgrad Med* 1997;43(2):52–6.

[10] Fishel RS, Barbul A, Beschormer WE, Wasserkrug HL, Efron G. Lymphocyte participation in wound healing. Morphologic assessment using monoclonal antibodies. *Ann Surg* 1987;206(1):25–9.

[11] Park JE, Barbul A. Understanding the role of immune regulation in wound healing. *Am J Surg* 2004;187(5A):11S–6S.

[12] Jameson J, Havran WL. Skin gammadelta T-cell functions in homeostasis and wound healing. *Immunol Rev* 2007;215:114–22.

[13] Traversa B, Sussman G. The role of growth factors, cytokines and proteases in wound management. *Prim Intention* 2001;9(4):161–7.

[14] Wild T, Rahbarnia A, Kellner M, Sobotka L, Eberlein T. Basics in nutrition and wound healing. *Nutrition* 2010;26(9):862–6.

[15] Gilbane AJ, Denton CP, Holmes AM. Scleroderma pathogenesis: a pivotal role for fibroblasts as effector cells. *Arthritis Res Ther* 2013;15(3):215.

[16] O'Toole EA. Extracellular matrix and keratinocyte migration. *Clin Exp Dermatol* 2001;26(6):525–30.

[17] Raja Sivamani K, Garcia MS, Isseroff RR. Wound re-epithelialization: modulating keratinocyte migration in wound healing. *Front Biosci* 2007;12:2849–68.

[18] Haase I, Evans R, Pofahl R, Watt FM. Regulation of keratinocyte shape, migration and wound epithelialization by IGF-1- and EGF-dependent signalling pathways. *J Cell Sci* 2003;116(Pt 15):3227–38.

[19] Martins VL, Caley M, O'Toole EA. Matrix metalloproteinases and epidermal wound repair. *Cell Tissue Res* 2013;351(2):255–68.

[20] Amano S, Akutsu N, Ogura Y, Nishiyama T. Increase of laminin 5 synthesis in human keratinocytes by acute wound fluid, inflammatory cytokines and growth factors, and lysophospholipids. *Br J Dermatol* 2004;151(5):961–70.

[21] Usui ML, Mansbridge JN, Carter WG, Fujita M, Olerud JE. Keratinocyte migration, proliferation, and differentiation in chronic ulcers from patients with diabetes and normal wounds. *J Histochem Cytochem* 2008;56(7):687–96.

[22] Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. *Cell* 2006;124(4):783–801.

[23] Staros EB. Innate immunity: new approaches to understanding its clinical significance. *Am J Clin Pathol* 2005;123(2):305–12.

[24] Spirig R, Tsui J, Shaw S. The emerging role of TLR and innate immunity in cardiovascular disease. *Cardiol Res Pract* 2012;2012:181394.

[25] Gordon S. Pattern recognition receptors: doubling up for the innate immune response. *Cell* 2002;111(7):927–30.

[26] Areschoug T, Gordon S. Pattern recognition receptors and their role in innate immunity: focus on microbial protein ligands. *Contrib Microbiol* 2008;15:45–60.

[27] Patel H, Shaw SG, Shi-Wen X, Abraham D, Baker DM, Tsui JC. Toll-like receptors in ischaemia and its potential role in the pathophysiology of muscle damage in critical limb ischaemia. *Cardiol Res Pract* 2012;2012:121237.

[28] Navi A, Patel H, Shaw S, Baker D, Tsui J. Therapeutic role of toll-like receptor modification in cardiovascular dysfunction. *Vascul Pharmacol* 2013;58(3):231–9.

[29] Mann DL. The emerging role of innate immunity in the heart and vascular system: for whom the cell tolls. *Circ Res* 2011;108(9):1133–45.

[30] De Nardo D, De Nardo CM, Nguyen T, Hamilton JA, Scholz GM. Signaling crosstalk during sequential TLR4 and TLR9 activation amplifies the inflammatory response of mouse macrophages. *J Immunol* 2009;183(12):8110–8.

[31] Huebener P, Schwabe RF. Regulation of wound healing and organ fibrosis by toll-like receptors. *Biochim Biophys Acta* 2013;1832(7):1005–17.

[32] Alexander C, Rietschel ET. Bacterial lipopolysaccharides and innate immunity. *J Endotoxin Res* 2001;7(3):167–202.

[33] Sato T, Yamamoto M, Shimosato T, Klinman DM. Accelerated wound healing mediated by activation of toll-like receptor 9. *Wound Repair Regen* 2010;18(6):545–58.

[34] Matzinger P. The danger model: a renewed sense of self. *Science* 2002;296(5566):547–52.

[35] Dasu MR, Ramirez S, Isseroff RR. Toll-like receptors and diabetes: a therapeutic perspective. *Clin Sci (Lond)* 2012;122(5):203–14.

[36] Terhorst D, Kalali BN, Ollert M, Ring J, Mempel M. The role of toll-like receptors in host defenses and their relevance to dermatologic diseases. *Am J Clin Dermatol* 2010;11(1):1–10.

[37] Pasparakis M, Haase I, Nestle FO. Mechanisms regulating skin immunity and inflammation. *Nat Rev Immunol* 2014;14(5):289–301.

[38] Miller LS, Modlin RL. Human keratinocyte toll-like receptors promote distinct immune responses. *J Invest Dermatol* 2007;127(2):262–3.

[39] Miller LS, Sorensen OE, Liu PT, Jalian HR, Eshtiahpour D, Behmanesh BE, Chung W, Stamer TD, Kim J, Sieling PA, et al. TGF-alpha regulates TLR expression and function on epidermal keratinocytes. *J Immunol* 2005;174(10):6137–43.

[40] Miller LS, Modlin RL. Toll-like receptors in the skin. *Semin Immunopathol* 2007;29(1):15–26.

[41] Jang S, Park JS, Won YH, Yun SJ, Kim SJ. The expression of toll-like receptors (TLRs) in cultured human skin fibroblast is modulated by histamine. *Chonnam Med J* 2012;48(1):7–14.

[42] Wang J, Hori K, Ding J, Huang Y, Kwan P, Ladak A, Tredget EE. Toll-like receptors expressed by dermal fibroblasts contribute to hypertrophic scarring. *J Cell Physiol* 2011;226(5):1265–73.

[43] Taylor KR, Trowbridge JM, Rudisill JA, Termeer CC, Simon JC, Gallo RL. Hyaluronan fragments stimulate endothelial recognition of injury through TLR4. *J Biol Chem* 2004;279(17):17079–84.

[44] Dasu MR, Isseroff RR. Toll-like receptors in wound healing: location, accessibility, and timing. *J Invest Dermatol* 2012;132(8):1955–8.

[45] Chen L, Guo S, Ranzer MJ, DiPietro LA. Toll-like receptor 4 has an essential role in early skin wound healing. *J Invest Dermatol* 2013;133(1):258–67.

[46] Dasu MR, Thangappan RK, Bourgette A, DiPietro LA, Isseroff R, Jialal I. TLR2 expression and signaling-dependent inflammation impair wound healing in diabetic mice. *Lab Invest* 2010;90(11):1628–36.

[47] Dasu MR, Jialal I. Amelioration in wound healing in diabetic toll-like receptor-4 knockout mice. *J Diabetes Complications* 2013;27(5):417–21.

[48] Pukstad BS, Ryan L, Flo TH, Stenvik J, Moseley R, Harding K, Thomas DW, Espevik T. Non-healing is associated with persistent stimulation of the innate immune response in chronic venous leg ulcers. *J Dermatol Sci* 2010;59(2):115–22.

[49] Lin Q, Fang D, Fang J, Ren X, Yang X, Wen F, Su SB. Impaired wound healing with defective expression of chemokines and recruitment of myeloid cells in TLR3-deficient mice. *J Immunol* 2011;186(6):3710–7.

[50] Lin Q, Wang L, Lin Y, Liu X, Ren X, Wen S, Du X, Lu T, Su SY, Yang X, et al. Toll-like receptor 3 ligand polyinosinic:polycytidylic acid promotes wound healing in human and murine skin. *J Invest Dermatol* 2012;132(8):2085–92.

[51] Fadini GP, Albiero M, Menegazzo L, Boscaro E, Pagnin E, Iori E, Cosma C, Lapolla A, Pengo V, Stendardo M, et al. The redox enzyme p66Shc contributes to diabetes and ischemia-induced delay in cutaneous wound healing. *Diabetes* 2010;59(9):2306–14.

[52] O'Loughlin A, McIntosh C, Dinneen SF, O'Brien T. Review paper: basic concepts to novel therapies: a review of the diabetic foot. *Int J Low Extrem Wounds* 2010;9(2):90–102.

[53] Wetzler C, Kampfer H, Stallmeyer B, Pfeilschifter J, Frank S. Large and sustained induction of chemokines during impaired wound healing in the genetically diabetic mouse: prolonged persistence of neutrophils and macrophages during the late phase of repair. *J Invest Dermatol* 2000;115(2):245–53.

[54] Nwomeh BC, Liang HX, Cohen IK, Yager DR. MMP-8 is the predominant collagenase in healing wounds and nonhealing ulcers. *J Surg Res* 1999;81(2):189–95.

[55] Yoshizaki A, Iwata Y, Komura K, Ogawa F, Hara T, Muroi E, Takenaka M, Shimizu K, Hasegawa M, Fujimoto M, et al. CD19 regulates skin and lung fibrosis via toll-like receptor signaling in a model of bleomycin-induced scleroderma. *Am J Pathol* 2008;172(6):1650–63.

[56] Stifano G, Affandi AJ, Mathes AL, Rice LM, Nakerakanti S, Nazari B, Lee J, Christmann RB, Lafyatis R. Chronic toll-like receptor 4 stimulation in skin induces inflammation, macrophage activation, transforming growth factor beta signature gene expression, and fibrosis. *Arthritis Res Ther* 2014;16(4):R136.

[57] Bhattacharyya S, Kelley K, Melichian DS, Tamaki S, Fang F, Su Y, Feng G, Pope RM, Budinger GR, Mutlu GM, et al. Toll-like receptor 4 signaling augments transforming growth factor-beta responses: a novel mechanism for maintaining and amplifying fibrosis in scleroderma. *Am J Pathol* 2013;182(1):192–205.

[58] Nikitorowicz-Buniak J, Shiwen X, Denton CP, Abraham D, Stratton R. Abnormally differentiating keratinocytes in the epidermis of systemic sclerosis patients show enhanced secretion of CCN2 and S100A9. *J Invest Dermatol* 2014.