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► To cite this version:

Laure Guenin-Macé, Reid Oldenburg, Fabrice Chrétien, Caroline Demangel. Pathogenesis of skin ulcers: lessons from the *Mycobacterium ulcerans* and *Leishmania* spp. pathogens. Cellular and Molecular Life Sciences, 2014, 71 (13), pp.2443 - 2450. 10.1007/s00018-014-1561-z . pasteur-01387237

HAL Id: pasteur-01387237

<https://pasteur.hal.science/pasteur-01387237>

Submitted on 25 Oct 2016

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Invited review for Cellular and Molecular Life Sciences (CMLS)

Pathogenesis of skin ulcers: lessons from the *Mycobacterium ulcerans* and *Leishmania* spp pathogens

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Running title: Molecular bases of skin ulceration

Abstract

Skin ulcers are most commonly due to circulatory or metabolic disorders and are a major public health concern. In developed countries, chronic wounds affect more than 1% of the population and their incidence is expected to follow those observed for diabetes and obesity. In tropical and subtropical countries, an additional issue is the occurrence of ulcers of infectious origins with diverse aetiologies. While the severity of cutaneous Leishmaniasis correlates with protective immune responses, Buruli ulcers caused by *Mycobacterium ulcerans* develop in the absence of inflammation. Based on these two examples, this review aims to demonstrate how studies on microorganism-provoked wounds can provide insight into the molecular mechanisms controlling skin integrity. We highlight the potential interest of a mouse model of non-inflammatory skin ulceration caused by intradermal injection of mycolactone, an unusual lipid toxin with ulcerative and immunosuppressive properties produced by *M. ulcerans*.

Keywords: Skin, infection, cutaneous Leishmaniasis, *Leishmania* spp, Buruli ulcer, *Mycobacterium ulcerans*, mycolactone.

Introduction

Chronic wounds can result from a variety of causes, such as venous stasis, arterial insufficiency, diabetes, inflammation and cancer. A common complication of their treatment is bacterial colonization, which causes significant morbidity and elevates considerably health care costs. The expenditures for wound care are estimated \$15 billion per year today, and they are expected to rise with the globalization of diabetes ¹. Diabetes affects 285 million patients worldwide, two-thirds of which occur in low- to middle-income countries. It is estimated that 15% of diabetic patients will develop a diabetic foot ulcer, of which 50% (21 million patients) will become infected. In addition to this modern crisis, subtropical and tropical countries have to face the burden imposed by ulcerative pathogens. Both primarily and secondarily infected wounds often result in failure to heal, by mechanisms that are diverse and highly dependent on the infectious agent ². Based on the description of the pathogenic processes employed by *M. ulcerans* and *Leishmania* spp, this paper aims to describe the contribution of infective ulcers to our general understanding of wound formation and repair.

Structure and functions of the skin

Before we review the various conditions leading to skin ulceration, it is important to describe the architecture and functions of this organ. As illustrated (Figure 1), human skin is composed of two superposed layers, the epidermis and the dermis. The epidermis is a stratified squamous epithelium composed of a first layer of proliferating basal keratinocytes

attached to a basement membrane (referred to as stratum basale) covered with suprabasal keratinocyte layers at various stages of differentiation (spinosum, granulosum, lucidum and corneum from deep to superficial). This external layer of the skin is not vascularised and keratinocytes receive oxygen and nutrients by diffusion from the underlying tissues. The dermis is a connective tissue that is essentially composed of an extracellular matrix (ECM) produced by dermal fibroblasts. The ECM is made of fibrous structural proteins including collagen, elastin and laminin, which are embedded in highly hydrated proteoglycans (such as dermatan sulfate and hyaluronan). In contrast to the epidermis, the dermis harbours nerves and sensory organs, hair follicles and various glands (sweat, sebaceous and apocrine). It is perfused by a dense anastomotic network of blood vessels and by draining lymphatics coming from the hypodermis. The hypodermis (also called subcutis) is primarily composed of connective and adipose tissues (accounting for 50% of the body fat) linking the dermis to bone and muscles. The different structure, physico-chemical properties and cellular composition of the tissues composing the skin allow this largest organ of the body to perform numerous functions such as protection against mechanical injuries, thermoregulation, immune surveillance and sensation.

Non-infectious causes of skin ulceration

Acute wounds, due to surgical incision or traumatic injury, typically proceed into a timely reparative process that results in the restoration of anatomic and functional integrity. However in a number of conditions this process is abnormally slow, or interrupted, and wounds become chronic. Venous ulcers are the most common type of chronic wounds,

accounting for up to 80% of all leg ulcers³. Diabetic ulcers represent a growing challenge, as the burden of diabetes mellitus increases and patients continue to age⁴. Less common than venous and diabetic ulcers, ulcers of arterial aetiology are found in older patients who have risk factors of peripheral arterial disease. Pressure ulcers, also known as decubitus ulcers or bedsores, occur when mobility is limited in patients who are post-operative, paralysed or taking sedative medication. They can lead to severe pain, are prone to infection, and associated with high mortality in elderly individuals. Skin ulceration can also result from immune dysfunction. For example *Pyoderma gangrenosum* is thought to result from a neutrophil-instigated destruction of the skin that is frequently associated with other immune-mediated diseases, such as inflammatory bowel disease or rheumatoid arthritis⁵. Finally, malignancies can ulcerate with squamous cell carcinoma commonly having central ulceration at presentation⁶.

Chronic wounds are commonly associated with defective blood flow (ischemia)⁷. Venous insufficiency causes a local rise in blood pressure within superficial venous structures, leading to blood leakage into the interstitial space. Arterial ulcers occur after occlusion of an artery or arteriole, which in the vast majority of cases is a consequence of atherosclerosis or material deposition in small or medium sized arteries. Diabetic ulcers occur in patients with peripheral neuropathy and often-concurrent peripheral vascular diseases. Pressure ulcers usually occur at the muscle-bone interface in patients remaining in the same position for a prolonged period of time. In that case, pathogenesis is believed to result from cycles of ischemia and reperfusion, generating inflammation and necrosis. How defective blood flow leads to skin ulceration nevertheless remains largely unknown, due to the difficulty in establishing satisfactory *in vitro* systems and animal models to study

cutaneous ischemia⁸. In contrast, the comparison of acute versus chronic wounds in human patients, and the development of surgical mouse models of wound healing⁹, have helped improve our understanding of the cellular and molecular mechanisms underpinning skin repair.

Mechanisms governing wound healing

Acute skin wounds typically heal by progressing through four consecutive yet overlapping phases: *(i)* haemostasis, *(ii)* inflammation (with an initial wave of neutrophils that are later replaced by macrophages), *(iii)* a proliferative phase during which the synthesis of new ECM results in scar formation then epithelialisation, and finally *(iv)* a remodelling of the ECM (Figure 1). Although haemostasis and inflammation proceed normally in chronic wounds, they do not translate into the proliferative and remodelling phases of healing. The comparison of acute versus chronic wounds has shown that production of new, fibrin/fibrinogen-rich ECM following clotting is essential in providing a scaffold for cell migration into the wound. Venous hypertension and blood stasis reduces vascular shear stress, resulting in a pro-inflammatory state that promotes leukocyte adhesion and migration¹⁰. The production of pro-inflammatory cytokines such as TNF and IL-1 β by macrophages signal fibroblasts to secrete matrix metalloproteinases (MMPs) that degrade the ECM¹¹. By producing reactive oxygen species (ROS), infiltrating inflammatory cells also generate a pro-oxidant microenvironment in the ulcers^{12,13}. ROS contribute to ECM proteolysis directly by degrading components of the matrix, and by stimulating the synthesis and activity of MMPs. In addition, the attack of ROS on extravasated erythrocytes results in

iron deposition, which in combination with hydrogen peroxide released by activated neutrophils generate highly toxic radicals, further damaging skin tissues and fuelling inflammation¹⁴.

While uncontrolled inflammation impairs wound healing, it may not be an absolute requirement for the initial steps of skin ulceration. By studying chronically ischemic but uninjured skin, Dalton *et al.* demonstrated that ECM homeostasis, which relies on the balance between collagen synthesis and degradation by MMPs, is profoundly impacted by defective blood circulation in the absence of inflammation^{15,16}. Compared to controls, ischemic skin tissue samples showed a decreased mechanical resistance. At the molecular level, they were marked by increased expression of the vascular endothelial growth factor VEGF and lactate, indicative of hypoxia. Hypoxic conditions are known to stimulate the production of TGF- β by fibroblasts that in turn promotes fibroblastic collagen synthesis¹⁷ and the concurrent expression of MMP-2 by keratinocytes¹⁸. The increased synthesis of both collagen and MMP-2 collagenase results in an elevated matrix turnover. The hypoxia-inducible factor (HIF)-1 α and TGF- β also stimulate angiogenesis via the production of VEGF^{19,20}. Together, these observations suggested that hypoxia might be sufficient to explain the remodelling of ECM and vessels, weakening the skin and facilitating ulceration.

Infective ulcers

Chronic wounds are always contaminated by microorganisms originating from the surrounding skin (such as *Corynebacteria* and *Propionibacteria* spp) and other endogenous

sources or the external environment (including multi-resistant organisms such as methicillin-resistant *Staphylococcus aureus*). However, wound healing is not compromised unless bacterial proliferation occurs, with generation of host immune responses and tissue injury². The initial bacterial burden, virulence and capacity of invading pathogens to grow within biofilms, combined with the ability of the host to mount protective immune responses both determine the transition to this infectious state. Other risk factors include malnutrition or obesity, diabetes and advanced age.

Chronic wounds can also occur as a result of a primary infection with microorganisms. Amongst the most common ulcerative pathogens are mycobacterium species such as *M. marinum*, *M. tuberculosis* and the causative agent of Buruli ulcer (BU) *M. ulcerans*²¹⁻²⁴. BU has been reported in more than 33 countries with tropical, subtropical and temperate climates and has become a major public health problem in sub-Saharan Africa. In Ghana, the point prevalence of BU can reach 150.8 per 100,000 individuals²⁵. The protozoan parasite *Leishmania* is another major cause of infectious skin ulcers, with an estimated worldwide occurrence of cutaneous leishmaniasis of 1.5 million cases per year²⁶. So-called “tropical” ulcers are common infectious lesions caused by associations of *Fusobacterium ulcerans* and *Borrelia vincenti*, although the role of these bacteria in skin destruction remains poorly understood. Other ulcers of infectious origin include yaws caused by *Treponema pertenuis*, cutaneous diphtheria caused by toxigenic strains of *Corynebacterium diphtheriae* and occasionally by *C. ulcerans*, and mycoses such as sporotrichosis, chromoblastomycosis or eumycetoma. The following paragraphs further describe our current knowledge of the pathogenic processes employed by *M. ulcerans* and *Leishmania* spp.

Mycobacterium ulcerans and Buruli ulcer disease

M. ulcerans is a mycobacterium causing BU in communities associated with an aquatic environment. Outbreaks are commonly associated with changes involving river areas ²⁷. Human to human transmission is rare ²⁸. Infection may occur by exposure of injured skin to contaminated water, although there is evidence for a role of biting insects as vectors of *M. ulcerans*, and aquatic snails and fish as intermediate hosts (reviewed in ²⁹). BU lesions typically start as a non-characteristic, pre-ulcerative stage (nodule, papule, plaque or oedema). After weeks to months, the skin covering the pre-ulcerative lesion eventually opens to form a characteristic ulcer with undermined edges. Histopathological hallmarks of BU are defective inflammatory responses in the presence of acid-fast bacilli, and extensive necrosis of dermal and adipose tissues. Common features also include epidermal hyperplasia in the regions flanking the ulcer, collagen destruction and vascular damage in the underlying dermis and subcutis ³⁰⁻³². In addition, patients with BU display a distinctive profile of immune suppression, marked by the defective capacity of T cells to produce cytokines upon stimulation *ex vivo* ^{33,34}.

Mycolactone, a diffusible cytotoxin uniquely produced by this pathogen, is believed to cause this intriguing combination of necrosis and immune suppression. *In vitro* experiments have shown that mycolactone is cytotoxic to epithelial cells and fibroblasts ^{35,36}, and blocks the expression of homing receptors and production of cytokines by monocytes, dendritic and T cells at non-cytotoxic concentrations ³⁷⁻⁴². Notably, bacterial killing in BU patients or experimentally infected animals undergoing antibiotic therapy is followed by a massive infiltration of necrotic material with leukocytes and the phagocytosis of dead

mycobacterium⁴³⁻⁴⁷, showing that local and systemic cellular immune responses are suppressed by *M. ulcerans* during ulcer formation.

We reported recently that mycolactone targets and hyper-activates the Neural Wiskott-Aldrich syndrome protein (N-WASP) in epithelial cells⁴⁸. N-WASP is particularly abundant in neurons, but is ubiquitously expressed by mammalian cells⁴⁹. Its best-known function is to regulate actin dynamics by transducing multiple endogenous signals into actin nucleation, via the Arp2/3 complex⁵⁰⁻⁵². By supporting the assembly and homeostasis of E-cadherin junctions⁵³, N-WASP is critical for the maintenance of epithelial cell contacts *in vitro*. *In vivo*, conditional knockdown of N-WASP expression in mouse skin epithelium results in profound defects in hair follicle cycling^{54,55}. It has a more variable impact on skin ulceration and no effect on wound healing. We demonstrated that hyper-activation of N-WASP by mycolactone is also dramatic for the maintenance of skin integrity and functions (Figure 2). *In vitro*, mycolactone-induced activation of N-WASP inhibited the maintenance of cell-cell and cell-matrix adhesive contacts, leading to detachment and death of epithelial cells. In addition, mycolactone triggered N-WASP-dependent defects in cell migration reflected by increased velocity and impaired directionality. Epidermal homeostasis is controlled by the maintenance of such cell-cell contacts and the coordinated migration of keratinocytes (reviewed in⁵⁶). Injection of mycolactone into mouse ears altered the junctional organization and stratification of keratinocytes, leading to a progressive thinning of the external layers with concurrent loss of E-cadherin contacts. This process was efficiently counteracted by co-administration of wiskostatin, a specific inhibitor of N-WASP stabilizing its auto-inhibited conformation⁵⁷. Our study thus suggested that mycolactone-induced activation of N-WASP in epithelial cells and the consequent dynamic

rearrangements of the actin cytoskeleton are the primary cause of epidermal rupture in BU formation.

Leishmania spp and cutaneous Leishmaniasis

A total of about 21 *Leishmania* spp have been identified that are pathogenic to humans. *Leishmania* parasites exist as promastigotes in the sand fly vector and as amastigotes in human hosts. They have developed multiple strategies to survive and multiply within phagolysosomal vacuoles in human phagocytes (macrophages, dendritic cells and neutrophils). The clinical presentation of Leishmaniasis varies significantly according to the species of infection and location⁵⁸. It manifests predominantly as visceral (VL) or cutaneous (CL), with VL being the most severe form and lethal if untreated. CL typically starts as red patches progressing into crusted ulcers with hypertrophied borders that are painless unless there is secondary bacterial infection. Cutaneous lesions are usually solitary and heal spontaneously within a year. However, depending on the infecting species and host-associated risk factors, they may evolve into more generalized forms, like diffuse CL or muco-CL⁵⁹.

Protective immunity against Leishmaniasis is mediated by Th1-oriented cellular immune responses, while in contrast Th2 cytokines promote disease progression⁶⁰. Mouse studies using *L. major* have shown that production of IFN- γ by Th1 cells is key to the resolution of CL. Activation of infected macrophages by IFN- γ triggers the production of nitric oxide (NO), resulting in the efficient killing of intracellular parasites. While the targeted

disruption of cytokines promoting the development of Th1/IFN- γ responses (IL-12, TNF) augment mouse susceptibility to leishmanial infection, deficiency for the Th2 cytokines IL-4 and IL-10 leads on the contrary to a better control of the disease. Humans with CL display mixed Th1/Th2 types of immune responses, however the production of IFN- γ is consistently associated with resolution of the disease and treatments based on IFN- γ have a positive impact on wound healing⁶¹. The variability in the intensity of humoral and cellular immune responses in patients with CL is reflected by the diverse pathological presentations. Histological analysis of skin biopsies reveals a wide range of immune profiles, from almost normal to highly inflammatory, with infiltration of macrophages, presence of granulomas and extensive necrosis of dermal tissues⁶². Fas ligand (FasL) and Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) have been reported to contribute to CL pathology by inducing keratinocyte killing and promoting neutrophilic infiltration^{63,64}. Accordingly, the size of CL lesions and healing time correlate with TNF levels in patients with CL⁶⁵. Together, these data thus suggest that skin ulceration in CL results primarily from the Th1 immune responses mounted by the host in response to infection.

Mycolactone injection in mice: a useful model to study skin ulcer pathogenesis?

The examples of CL and BU illustrate the complexity and the diversity of infective ulcer pathogenesis. Exacerbated inflammation is critical in the pathogenesis of CL and in the defective healing of secondarily colonised wounds. In contrast, *M. ulcerans* uses an original mechanism to destroy the skin in the absence of local and systemic inflammation. Mycolactone is a diffusible lipid of 740 Da gaining access to its molecular targets in remote

tissues by passive diffusion⁶⁶. This property, combined with a potent immunosuppressive activity, makes mycolactone a potentially useful tool to study the non-inflammatory components of skin ulceration. Guinea pig and mouse models of infection with *M. ulcerans* have been developed that are based on intradermal, or subcutaneous injection of bacilli^{22,35,67-69}. The resulting ulcers resemble human lesions in many aspects, with comparable epidermal hyperplasia, coagulative necrosis, oedema and vasculopathy. Interestingly, intradermal injection of 5 µg purified mycolactone into mouse ears generates in less than two weeks lesions that are comparable to those observed by infection with *M. ulcerans* in the footpad model⁴⁴ (Figure 3). Furthermore, the histopathological changes occurring upon mycolactone injection mimic the different stages of BU formation. In particular, the epidermal rupture, dermal elastolysis and subcutaneous necrosis that characterizes the transition from pre-ulcerative to ulcerative stage in BU, are adequately reproduced within a ten day period.

We have shown previously that mycolactone mediates epidermal rupture by hyper-activating N-WASP, however the molecular mechanisms leading to the necrosis of the underlying dermis and hypodermis remain to be elucidated. Based on the vascular damages observed in mycolactone-injected or *M. ulcerans*-infected tissues, we can propose that mycolactone also impairs endothelial integrity via the activation of N-WASP, leading to vascular leakage and oedema that pre-dispose skin to ulceration. Recent findings showing that N-WASP regulates endothelial permeability by maintaining adherens junctions⁷⁰ strongly support our hypothesis.

Conclusions

To prevent ulcer formation and improve current wound management practices, it is important to progress in the identification of the different pathways controlling skin integrity. The study of infective ulcers provides important insights into these mechanisms, and in particular into the double-edged contribution of inflammation. The generation of inflammatory responses at the site of infection promotes skin ulceration in CL and delays the healing of secondarily contaminated wounds. Paradoxical reactions with aggravation of the ulcers have been reported in 2-10% of BU patients initiating antibiotic therapy ⁷¹. In these patients, clinical deterioration is efficiently stopped by adjunctive steroid therapies ^{72,73}, suggesting that in case of severe inflammation, immune suppression may improve the treatment of infected ulcers. In spite of the potent immunostimulatory activity and adjuvant potential of mycobacterial cell wall components, *M. ulcerans* induces the formation of BU without triggering inflammation. This original pathogenic process, based on the unique ulcerative and immunosuppressive properties of mycolactone, highlights the importance of epidermal and vascular cell-cell contacts in ulcer pathogenesis. It may provide researchers with a good model to investigate the non-inflammatory processes mediating skin ulceration.

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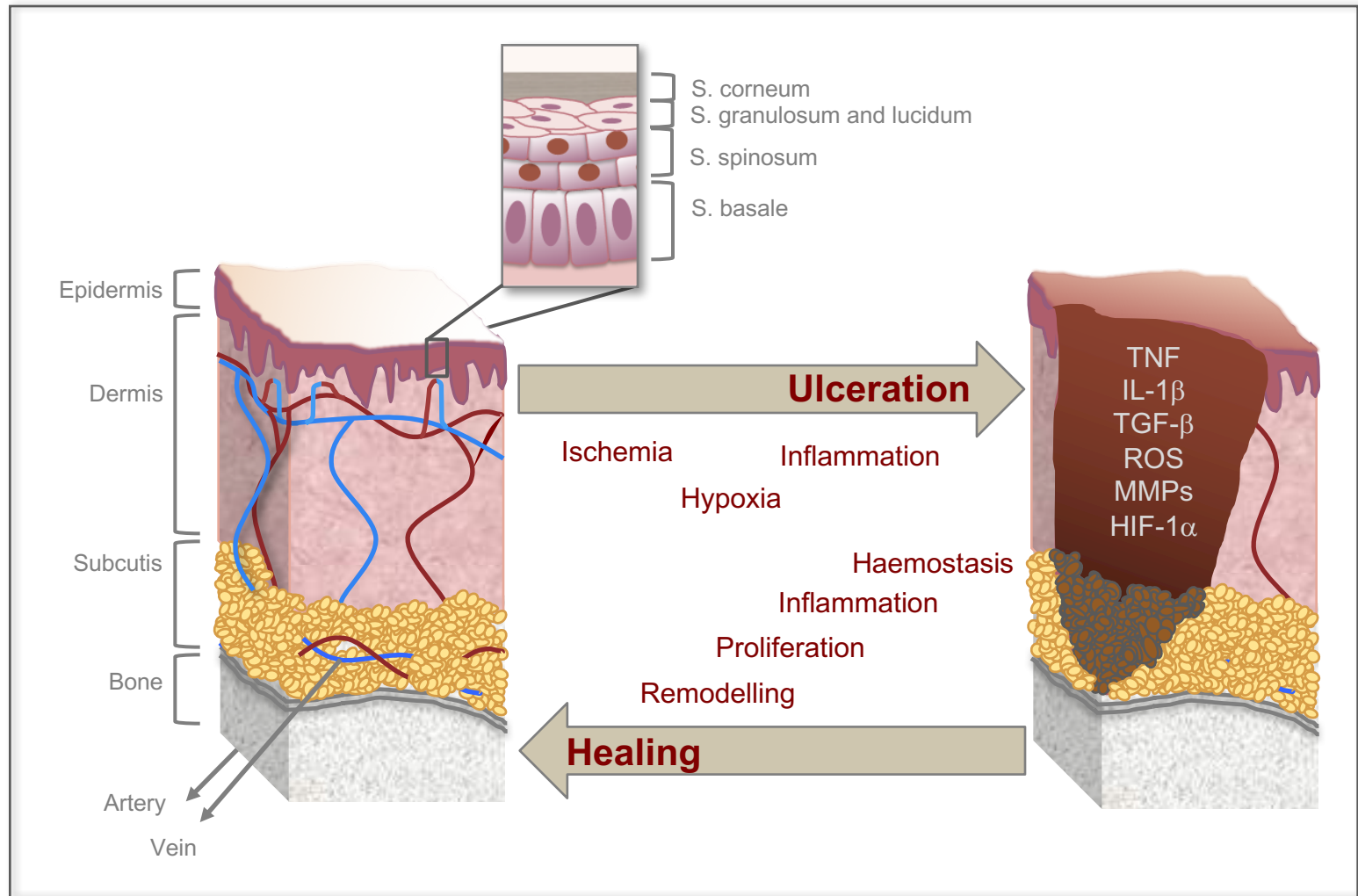


Fig. 1 Structure of human skin. The different tissues and cell layers composing this organ are depicted, together with the physiological mechanisms and molecular species varying during skin ulceration and healing.

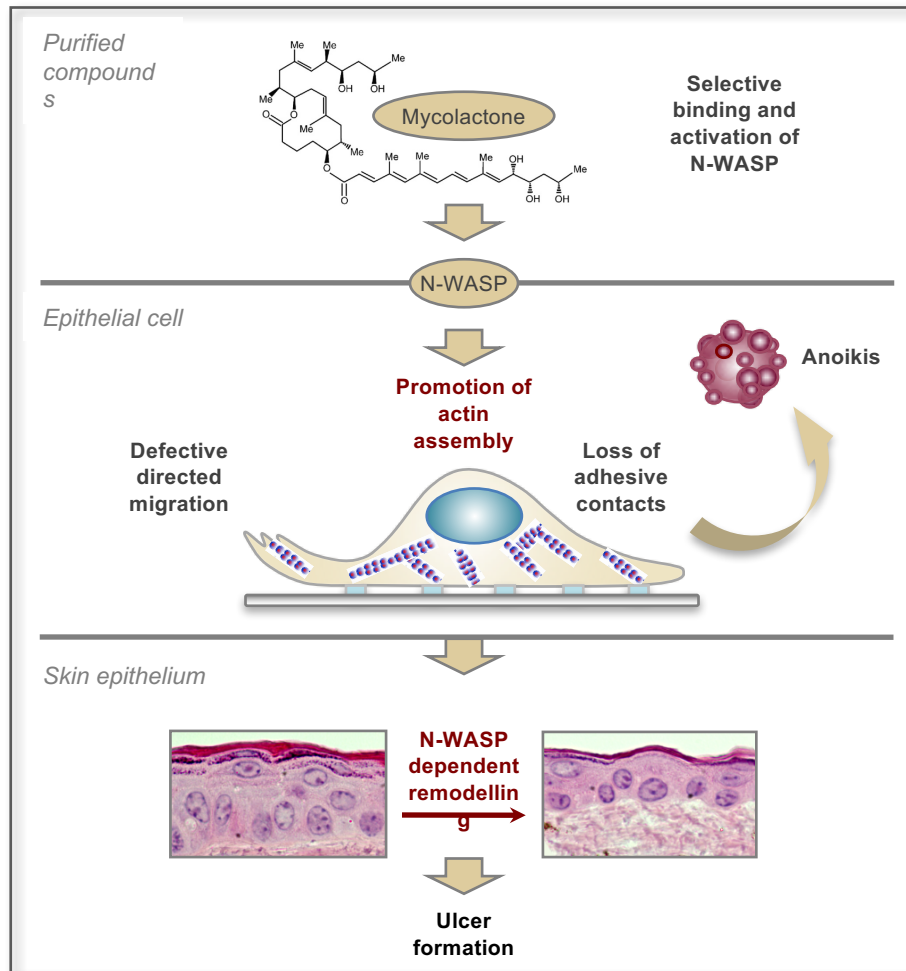


Fig. 2: Remodelling and rupture of the epidermis upon activation of N-WASP by the bacterial factor mycolactone. The impact of mycolactone on the biological functions of N-WASP *in vitro*, in cell models and *in vivo* are illustrated.

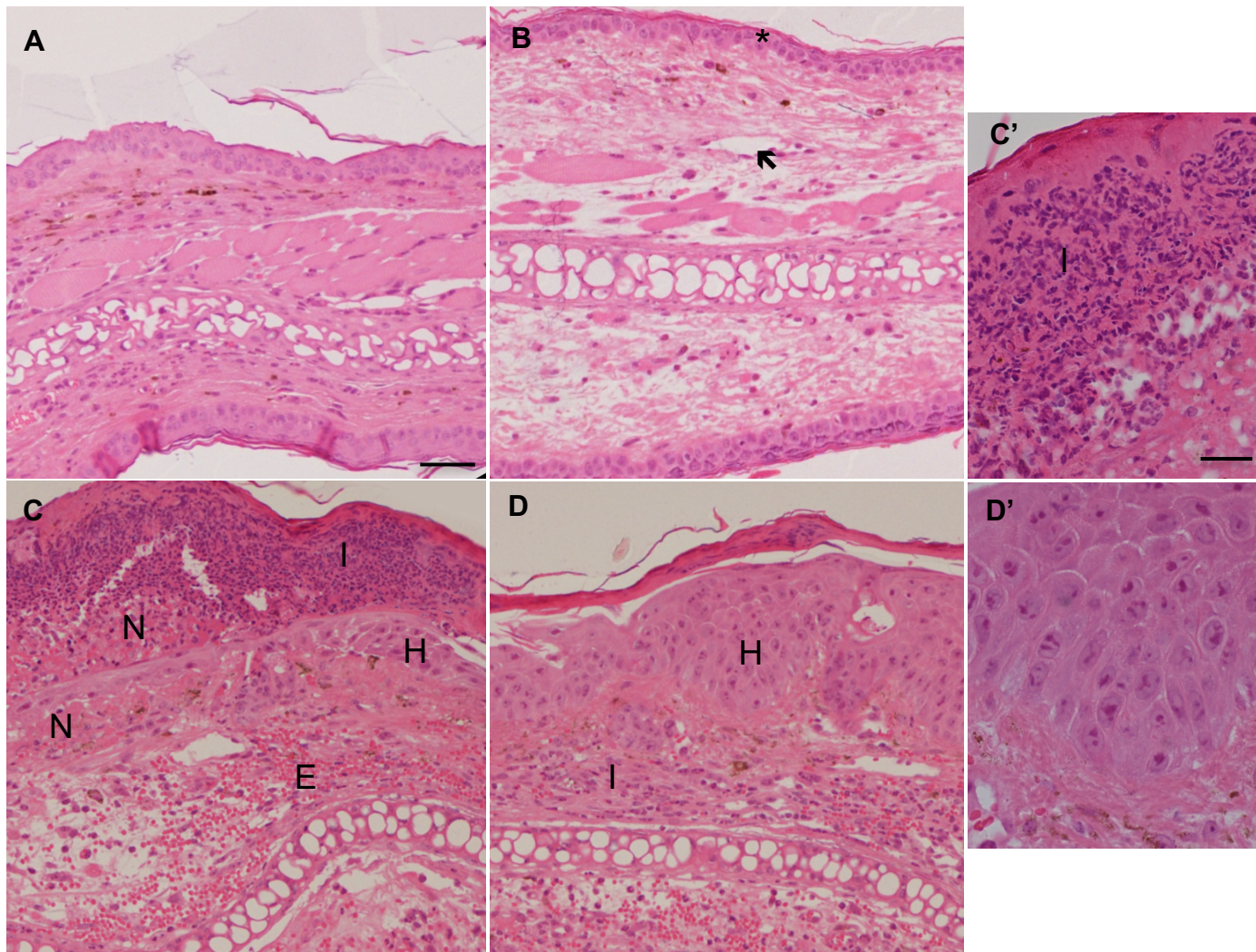


Fig. 3 Histopathological features of mycolactone-injected skin. Hematoxylin and eosin staining of mouse ears, injected with vehicle (A) or mycolactone after 2 (B) or 9 days (C, C', D, D') in the dermis of the external side of the ear. Two days post-injection, mycolactone injection induces a marked oedema and a profound disorganization of the dermal architecture and a progressive thinning of the epidermis (*) near the site of injection (B). Notice the loose aspect of blood vessels (↗). Nine days after mycolactone injection, hyperplasia (H) is observed in the regions flanking epidermal destruction, with inflammation characterized by neutrophilic infiltration (I), necrosis (N) of subcutaneous tissue (C,C') and hypertrophy of keratinocytes (D,D'). In the subcutaneous tissue, extravasation of erythrocytes (E) without hemolysis suggests an increased vascular permeability due to reduced endothelial integrity. (A-D, 10x, C'-D', 40x) Scale bars: 50 μ and 20 μ for 10x and 40x magnifications, respectively.