



HAL
open science

Cutaneous Wound Healing in Diabetic Mice Is Improved by Topical Mineralocorticoid Receptor Blockade

van Tuan Nguyen, Nicolette Farman, Roberto Palacios-Ramirez, Maria Sbeih, Francine Behar-Cohen, Sélim Aractingi, Frederic Jaisser

► To cite this version:

van Tuan Nguyen, Nicolette Farman, Roberto Palacios-Ramirez, Maria Sbeih, Francine Behar-Cohen, et al.. Cutaneous Wound Healing in Diabetic Mice Is Improved by Topical Mineralocorticoid Receptor Blockade. *Journal of Investigative Dermatology*, 2020, 140, pp.223 - 234.e7. 10.1016/j.jid.2019.04.030 . hal-03488459

HAL Id: hal-03488459

<https://hal.science/hal-03488459>

Submitted on 21 Dec 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

Cutaneous wound healing in diabetic mice is improved by topical mineralocorticoid receptor blockade

Van Tuan Nguyen^{1,2,3}, Nicolette Farman¹, Roberto Palacios-Ramirez¹, Maria Sbeih², Francine Behar-Cohen^{1,4}, Sélim Aractingi^{2,4,5}*, Frederic Jaisser^{1,6*}

Short title: Another approach for diabetic wound healing

Affiliations:

¹INSERM, Centre de Recherche des Cordeliers, Sorbonne Université, USPC, Université Paris Descartes, Université Paris Diderot, Paris, France

²Laboratory of progenitors and endothelial cells during and after pregnancy, INSERM UMR 938, Centre de Recherche St Antoine, Sorbonne Université, Paris, France

³Department of Basic Science, Thai Nguyen University of Agriculture and Forestry, Thainguuyen, Vietnam

⁴Faculty of Medicine, Université Paris Descartes, Paris, France

⁵Department of Dermatology, Hôpital Cochin-Tarnier, Paris, France

⁶Inserm, Clinical Investigation Centre 1433, Vandoeuvre-lès-Nancy, France

* contributed equally

Corresponding author:

Frederic Jaisser, MD, Ph.D

UMR 1138, INSERM, Centre de Recherche des Cordeliers,

15 rue de l'Ecole de Médecine, 75006 Paris, France

E-mail: frederic.jaisser@inserm.fr

Phone number: + 33 1 44 27 81 06

ABSTRACT

Skin ulcers resulting from impaired wound healing are a serious complication of diabetes. Unresolved inflammation, associated with the dysregulation of both the phenotype and function of macrophages, is involved in the poor healing of diabetic wounds. Here, we report that topical pharmacological inhibition of the mineralocorticoid receptor (MR) by canrenoate or MR siRNA can resolve inflammation to improve delayed skin wound healing in diabetic mouse models; importantly, wounds from normal mice are unaffected. The beneficial effect of canrenoate is associated with an increased ratio of anti-inflammatory M2 macrophages to pro-inflammatory M1 macrophages in diabetic wounds. Furthermore, we show that MR blockade leads to downregulation of the MR target, lipocalin 2 (Lcn2), which may facilitate macrophage polarization towards the M2 phenotype and improve impaired angiogenesis in diabetic wounds. Indeed, diabetic Lcn2-deficient mice showed improved wound healing, associated with macrophage M2 polarization and angiogenesis. In addition, recombinant Lcn2 protein prevented IL4-induced macrophages switch from M1 to M2 phenotype.

In conclusion, topical MR blockade accelerates skin wound healing in diabetic mice via Lcn2 reduction, M2 macrophage polarization, prevention of inflammation, and induction of angiogenesis.

Key words: Diabetes, Wound healing, Angiogenesis, Mineralocorticoid receptor, Lipocalin 2.

INTRODUCTION

Impaired cutaneous wound healing, responsible for chronic ulcers, represents one of the most important complications of diabetes. Indeed, the incidence of diabetic foot ulcers is increasing because of the high prevalence of diabetes mellitus worldwide and the longer life expectancy of patients (Boulton et al., 2005). The prevalence of global diabetic foot ulcers was recently reported to be 6.3% and up to 13.0% in North America (Zhang et al., 2017). Ulcers remain in a chronic inflammation state that prevents them from healing, resulting in other severe complications, such as pain, infection, and eventually amputation (Greenhalgh, 2003, Noor et al., 2017, Peters and Lipsky, 2013). Previous studies showed that most diabetic amputations are preceded by a foot ulceration, which subsequently results in serious gangrene or infection (Boulton et al., 2005, Lepantalo et al., 2011). Diabetic foot ulcers are thus a major health issue that significantly affects the lives of patients, resulting in a high financial burden in many countries (Boulton et al., 2005). The prevention and appropriate care of diabetic ulcers are thus of major importance.

Several therapies have been proposed for diabetic ulcers but effective treatment is still needed (Clokie et al., 2017, Dinh et al., 2012, Galiano et al., 2004). The lack of therapeutic tools likely results from the complex mechanisms involved in the development of unhealed wounds. Many pathogenic factors, such as vascular defects and neuropathy, are responsible for diabetic ulcers (Ahmed and Antonsen, 2016, Boulton, 2014, Brem and Tomic-Canic, 2007, Dinh et al., 2012). A chronic inflammatory environment is also a common feature observed in unhealed wounds and is mainly associated with the uncontrolled recruitment and activation of inflammatory cells, in particular monocytes/macrophages (Boniakowski et al., 2017, Leal et al., 2015, Okizaki et al., 2015).

During the wound-repair process, macrophages present various phenotypes and functions, depending on the stage of the healing response and how they are activated. During the early phase of wound healing, classically activated macrophages, known as M1 macrophages, are recruited and secrete pro-inflammatory cytokines to kill pathogens and clear away the damaged tissue. In contrast, alternatively activated M2 macrophages secrete anti-inflammatory factors to resolve inflammation and produce factors required for later regenerative phases. The balance between these macrophage subpopulations is pivotal for maintaining a

physiological healing process (Gordon, 2003, Mahdavian Delavary et al., 2011, Mantovani et al., 2004). However, in unhealed wounds, such as diabetic ulcers, macrophages are chronically activated and restrained to the M1 phenotype, heavily contributing to the chronic inflammatory microenvironment observed in these wounds. Moreover, such prolonged inflammation delays the process of tissue regeneration, including re-epithelialization, granulation tissue formation, and vascularization (Boniakowski et al., 2017, He et al., 2017, Maruyama et al., 2007, Okizaki et al., 2015). Enhancing macrophage polarization towards M2 phenotype may help to promote cellular proliferation and angiogenesis and to accelerate diabetic wound closure (He et al., 2017, Leal et al., 2015, Okizaki et al., 2015).

The mineralocorticoid receptor (MR), a steroid receptor that belongs to the nuclear receptor superfamily of transcription factors, plays a key role in various physiological and pathophysiological phenomena, including obesity/diabetes-related complications, such as vascular dysfunction, insulin resistance, metabolic disorders, and chronic inflammation (Guo et al., 2008, Hirata et al., 2009, Jaisser and Farman, 2016). We recently reported the involvement of MR activation in delayed wound closure in type-1 diabetes and the improvement of impaired wound re-epithelialization following local application of a MR antagonist to the wounds (Nguyen et al., 2016). However, we did not explore the integrated impact on either the epidermis or dermis in diabetic wounds or the underlying mechanisms of the benefit of local MR antagonism. It is not known whether MR acts on the inflammatory response and dermal angiogenesis during wound repair in diabetes. Here, we analyzed the positive impact of topical pharmacological MR blockade on impaired wound healing of STZ-treated mice and of db-db mice- a model of type 2 diabetes. We focused on inflammation and angiogenesis, and identified lipocalin 2 (Lcn2), a primary MR target (Buonafine et al., 2018, Martinez-Martinez et al., 2017), as a potential mechanism involved in these beneficial effects.

RESULTS

MR blockade rescues delayed cutaneous wound closure in diabetic mice

We have shown previously that local MR blockade with Canrenoate (Canre) improves the delayed wound re-epithelialization of type-1 diabetic mice (STZ mice) (Nguyen et al., 2016). To exclude MR-independent

effects of Canrenoate, we designed an *in vivo* experimental strategy to interfere specifically with the MR. Topical treatment with MR siRNA blocked MR upregulation in STZ wounds (**Figure S1 a**) and restored the defective re-epithelialization of STZ mice, as compared to those of diabetic mice treated by scrambled siRNA (**Figure S1 c-g**). These results are quite comparable to those previously obtained using Canrenoate, indicating that MR antagonism/silencing is beneficial for wounding in type I diabetic mice.

Next we questioned the effect of Canrenoate on the delayed wound healing of type 2 diabetes using db-db mice, a genetic mouse model of type 2 diabetes. MR mRNA expression was higher in wounded skin and the surrounding normal skin of db-db mice than that of normal db/+ control (CT) mice (**Figure 1a**), raising the question of the role of MR overexpression in abnormal wound repair in type 2 diabetes. Wound healing was strongly impaired in db-db mice relative to that of CT mice (**Figure 1b-c**). Consistent with our observations in STZ mice, local treatment with Canre improved the wound healing delay in db-db mice while it did not modify wound closure in normal CT mice (**Figure 1b-c**). Moreover, keratin-14 staining showed that impaired re-epithelialization of db-db wounds was rescued by local Canre treatment, as illustrated by the longer neo-epidermis at the edges of the wound sections (**Figure 1d-e**) and the shorter residual wound length (the distance between two edges) in Canre-treated db-db mice than in PBS-treated db-db mice (**Figure 1d and f**). This reduction in wound surface is indicative of re-epithelialization rather than wound contraction. The improvement of re-epithelialization was accompanied by a higher number of proliferating Ki67-positive keratinocytes in the neo-epidermis of the wounds of canre-treated than PBS-treated db-db mice (**Figure 1g-h**). These observations suggest that the effects of topical Canre treatment on diabetic wound healing are mediated by MR signaling. Thus, topical MR blockade restored the impaired proliferation of epidermal keratinocytes and blunted the delayed re-epithelialization of wounds in 2 diabetes mouse models. In contrast, these phenomena were not found in wounds of normal CT mice.

Impaired wound angiogenesis in diabetic mice is improved by MR antagonism

Impaired dermal wound angiogenesis is a key contributor to the wound healing defect of diabetes. Herein, we explored the role of MR in the defective wound angiogenesis of diabetic mouse models. The density of CD31⁺ blood vessels was lower in the wounds of STZ-induced (**Figure 2a, in red -b**) and db-db diabetic

mice than those of control mice (**Figure 2e-f**), consistent with previous reports. Decreased vessel density of diabetic wounds was partly rescued by topical treatment of these wounds with Canre (**Figure 2a, b, e, and f**). MR inactivation by local MR siRNA treatment of STZ wounds also showed a beneficial effect on wound angiogenesis (**Figure S1 h-i**). The improvement of vessel density in Canre-treated diabetic wounds was associated with an increased number of CD45⁺CD31⁺ endothelial cells in the wound beds, as quantified by FACS analysis of wound specimens (**Figure 2c, d, g, and h**). Moreover, by quantification of CD31⁺Ki67⁺ double positive cells on wound sections of STZ mice (**Figure 2a**, red and green), we found less proliferating endothelial cells in STZ wounds as compared to those of controls ($2.87\% \pm 1.08$, n=7 vs $7.18\% \pm 0.71$, n=7, respectively), and this decrease was rescued by Canre treatment (STZ + PBS: $2.87\% \pm 1.08$, n=7 vs STZ + Canre: $6.37\% \pm 1.2$, n=7). These observations suggest that the activation of MR in skin of diabetic mice is involved in the impaired wound angiogenesis of these animals and that MR blockade could prevent such defects through increased proliferation of endothelial cells forming novel microvessels in wounds.

MR blockade ameliorates inflammation of diabetic wounds

Chronic inflammation is a common feature of diabetic ulcers. We examined whether MR plays a role in maintaining inflammation in the diabetic wounds by studying the impact of MR blockade on the expression of some pro/anti-inflammatory markers in wounds (Ashcroft et al., 2012, Barrientos et al., 2008, Ramalho et al., 2018). Local treatment with Canre attenuated the overexpression of some pro-inflammatory genes, such as TNF- α , MCP-1, IL-12, and IL-23 in diabetic wounds (**Figure 3a and c**). Consistent with this effect, the repression of anti-inflammatory factors in diabetic wounds was rescued by Canre treatment (**Figure 3b and d**). Thus, MR blockade blunted the inflammation observed in diabetic wounds and resulted in an anti-inflammatory status that could improve the impaired cellular proliferation and poor angiogenesis.

MR antagonism induces the switch of unrestrained M1 towards M2 macrophages in diabetic wounds

A failure to switch from activated M1 macrophages to alternative M2 macrophages leads to chronic inflammation and impaired angiogenesis in various situations of delayed wound healing, including venous leg ulcers and diabetic foot ulcers (Guo et al., 2016, Okizaki et al., 2015, Sindrilaru et al., 2011). We investigated whether the beneficial effect of canrenoate treatment was associated with a switch of activated

M1 macrophages towards the alternative M2 macrophage population. Wounds from both STZ and db-db mice showed more M1 macrophages (Ly6C^{hi}) associated with fewer M2 macrophages (Ly6C^{low}) than those of control mice, whereas the total number of macrophages in diabetic wounds was not different from that in control wounds (**Figure 4**). These observations are consistent with a number of previous reports showing the accumulation and persistence of activated M1 macrophages in diabetic wounds (Guo et al., 2016, Leal et al., 2015, Okizaki et al., 2015). Importantly, the polarization of diabetic-wound macrophages was nearly restored to the levels of control mice by topical canrenoate treatment: Canre blunted the increase in M1 macrophages in STZ and db-db wounds (**Figure 4a, c, e and g**) and the associated decrease in M2 macrophages (**Figure 4a, d, e and h**). MR blockade also blunted over-expression of the pro-inflammatory marker Ly6C by diabetic-wound macrophages (**Figure S2 a-b**). These results suggest that topical MR blockade promotes a shift of pro-inflammatory M1 macrophages towards the anti-inflammatory M2 phenotype to resolve inflammation, promote angiogenesis, and accelerate wound healing.

MR blockade rescues the impaired macrophage expression of angiogenic factors in diabetic wounds

In addition to their pathogen-killing activity, macrophages in wounds also promote cellular proliferation and tissue regeneration, including re-epithelialization and dermal vascularization (Gordon, 2003, Lucas et al., 2010). We tested whether canrenoate treatment improves delayed diabetic wound angiogenesis through the modulation of macrophage polarization by assessing a panel of pro-angiogenic factors in wound tissue. Five days after wounding, gene expression of pro-angiogenic factors was impaired in STZ wounds with a significant decrease of FGF-2, PLGF, Tie-2, and angiopoietin-2 mRNA levels (**Figure 5a**). Importantly, topical canrenoate application restored the expression of these factors in diabetic wounds (**Figure 5a**). We then isolated macrophages (CD11b⁺F4.80⁺Ly6G⁻) from wounded skin by FACS to analyze the expression of pro-angiogenic genes. VEGF-a, FGF-2, PLGF, and Tie2 mRNA levels were significantly lower in macrophages isolated from STZ wounds than those from control wounds, whereas topical application of Canre restored the inadequate expression of these angiogenic factors (**Figure 5b**).

The MR target Lcn2 promotes macrophage phenotypic polarization/switching and angiogenesis in diabetic wounds

We recently showed that Lcn2 is a primary target of aldosterone/mineralocorticoid receptor signaling in many organs and that Lcn2 plays a key role in the action of mineralocorticoids in the cardiovascular system (Buonafine et al., 2018). Lcn2 mRNA and protein expression was much higher in diabetic wounds than in control, non-diabetic wounds (**Figure 6a-b; Figure S3 a-b**). Local canre or MR siRNA treatment lowered the upregulated expression of Lcn2 in diabetic wounds to near the level found in control wounds, indicating that Lcn2 is also an MR target in diabetic skin (**Figure 6a-b; Figure S1 b; Figure S3**). We assessed whether Lcn2 is involved in the impaired healing of diabetic wounds using a global Lcn2-knockout mouse model. Wound closure in diabetic STZ mice was significantly impaired relative to that of CT nondiabetic mice, but Lcn2 inactivation prevented the delay in wound healing in Lcn2-knockout diabetic mice (**Figure 6c-d**). Of note, Lcn2 deficiency did not affect wound healing in normal non-diabetic mice (**Figure 6c-d**). FACS analysis of wound specimens demonstrated that Lcn2 inactivation resulted in M1 to M2 macrophage polarization: the increase of pro-inflammatory M1 macrophages in CT diabetic wounds was blunted in Lcn2-deficient diabetic mice (**Figure 6e**), while the decrease of M2 macrophages in diabetic wounds was prevented (**Figure 6f**). Importantly, Lcn2 deficiency prevented the impaired angiogenesis associated with diabetes, with the presence of more endothelial cells in the wounds of diabetic Lcn2 KO mice than those of CT diabetic mice (**Figure 6g**). Moreover, expression of the pro-angiogenic genes VEGF-a, FGF-2, PLGF and Tie2 was higher in STZ Lcn2 KO macrophages than in STZ control mice (**Figure 6h**).

Lcn2 protein induces an unrestrained pro-inflammatory M1 macrophage phenotype *in vitro*

We hypothesized that the MR target Lcn2 participates in the deleterious effect of MR activation in diabetic wound healing by tipping macrophage functional/phenotypic polarization from proangiogenic M2 macrophages towards proinflammatory M1. Macrophages isolated from the peritoneum of wild-type mice were first pretreated with LPS to induce an M1 phenotype and then with IL4 to switch them towards an M2 phenotype (**Figure S4 a**). FACS analysis showed that treatment with recombinant Lcn2 resulted in a higher percentage of Ly6C^{hi} M1 macrophages (**Figure S4 b**), together with fewer Ly6C^{low} M2 macrophages (**Figure S4 c**), indicating that recombinant Lcn2 inhibited the ability of LPS-pretreated macrophages to

switch to the M2 phenotype in response to IL4. This was associated with decreased expression of the angiogenic genes VEGFa and PLGF (**Figure S4 d and e**).

Overall, these data show that MR blockade controls the phenotypic polarization of macrophages towards a repair M2 phenotype and promotes dermal angiogenesis through modulation of the expression and activity of Lcn2, thereby improving the delayed wound healing in diabetes (**Figure S5**).

DISCUSSION

By using two mouse models of diabetes (STZ-induced type 1 and db-db type 2 diabetic mice), we demonstrate that inflammation and impaired healing of diabetic wounds are associated with the activation of MR signaling. Topical inhibition of this pathway provided a clear benefit to improve healing of these pathological wounds. This effect acts via inducing the polarization of macrophages toward the M2 phenotype, helping to resolve inflammation and rescue the angiogenesis defect of diabetic wounds. Moreover, we identified the MR target lipocalin 2 as one of the underlying signaling pathways. In contrast, MR blockade do not modify wound closure from normal mice. We propose that inadequate MR occupancy by exogenous GC or locally produced GC, as well as enhanced MR expression, may explain the benefit of MR blockade in a variety of pathological situations such as dermocorticoid treatments, UV irradiation, diabetic delayed wound healing and perhaps psoriatic skin or glucocorticoid-treated psoriasis (Hannen et al., 2017, Nguyen et al., 2016, Stojadinovic et al., 2016). In addition, local steroidogenesis and modulation of 11 β -Hydroxysteroid dehydrogenase type 1 may interfere with MR/GR signaling balance in skin diseases (Sevilla and Perez, 2018, Slominski et al., 2014, 2015, Tiganescu et al., 2013). Efforts towards better integrations of these factors should help to propose novel therapeutic improvements of delayed wound healing.

During wound healing, macrophages are significantly involved in the repair process (Brancato and Albina, 2011, Lucas et al., 2010, Rahmani et al., 2018). Importantly, they show heterogeneous phenotypes and functions and can switch/polarize between phenotypes to adapt to the wound stage (Boniakowski et al., 2017, Novak and Koh, 2013). The balance of such macrophage populations is pivotal for the progression of

wound healing. However, most diabetic wounds do not progress, but remain in a chronic state of inflammation. This mainly results from a defect in the phenotypic switch of macrophages, leading to the sustained presence of pro-inflammatory M1 macrophages and over-production of pro-inflammatory cytokines in wounds (Boniakowski et al., 2017, Leal et al., 2015, Okizaki et al., 2015). Here, we demonstrate that topical inhibition of the MR promotes the polarization of activated M1 macrophages (CD11b⁺/F4.80⁺/Ly6C^{low}) in diabetic wounds toward an anti-inflammatory M2 phenotype (CD11b⁺/F4.80⁺/Ly6C^{low}). Accordingly, MR antagonism up-regulated the expression of some anti-inflammatory factors, while downregulating the expression of pro-inflammatory cytokines in wound tissues. This is consistent with previous findings that showed the role of MR activation in various diseases involving chronic inflammation, including diabetes (Guo et al., 2008, Jaisser and Farman, 2016, Marzolla et al., 2014). Previous studies reported a central role of MR over-activation in sustained macrophage polarization after renal injury induced by ischemia reperfusion (Barrera-Chimal et al., 2018). In this setting, pharmacological MR antagonism also promoted the polarization of activated M1 macrophages toward an anti-inflammatory M2 phenotype, suggesting that this may be a common mode of action of MR antagonists in wound healing in cardiovascular, renal, or metabolic injury (Barrera-Chimal et al., 2018).

Defective angiogenesis often occurs in diabetic wounds. The defect of wound angiogenesis may be due to inadequate mobilization and abnormal activation of endothelial progenitor cells (Loomans et al., 2004). Several therapies based on promoting the functional activity of endothelial cells and their progenitors have been proposed to improve healing (Liu et al., 2014, Marrotte et al., 2010, Nishimura et al., 2012). Impaired angiogenesis may also be associated with chronic inflammation, inhibiting the production of pro-angiogenic factors and limiting endothelial cell activity. Macrophages are important sources of pro-angiogenic factors. These inflammatory cells have been shown to be involved in many disease settings, including the complications of diabetes (Maruyama et al., 2007, Okizaki et al., 2015). Here, we found that M2 polarized macrophages, induced by the inhibition of MR signaling, expressed higher levels of pro-angiogenic factors than those from non-treated diabetic wounds, contributing to angiogenesis and wound healing improvement.

Overall, our results suggest an important role for MR signaling in the sustained polarization of macrophages and impaired wound angiogenesis in diabetes. These findings provide further insight concerning the impact of MR activation in various skin diseases. Indeed, we previously reported the role of cutaneous MR over-activation in the deleterious effects caused by dermo-glucocorticoid treatment in both mice and humans, including skin atrophy and impaired wound healing: glucocorticoid-induced MR activation impaired the proliferation and activation of epidermal keratinocytes linked to over-activation of epithelial sodium channels. Topical cutaneous treatment with MR antagonists provided significant benefits, limiting atrophy and improving wound healing (Maubec et al., 2015, Nguyen et al., 2016). MR overexpression in keratinocyte was shown to induce epidermal atrophy in mice (Sainte Marie et al., 2007). Boix *et al.* demonstrated that epidermal deletion of MRs leads to increased keratinocyte proliferation and differentiation (Boix et al., 2016). Other studies proposed that epidermal MR cooperates with glucocorticoid receptor acting as an anti-inflammatory factor to counteract skin inflammation and regulate epidermal development in inflamed skin (Bigas et al., 2018, Sevilla and Perez, 2018).

The mechanism underlying the modulation of inflammation and angiogenesis by MR in diabetic wounds may be complex and multifactorial. We identified lipocalin 2, a primary target of aldosterone/mineralocorticoid receptor signaling (Buonafine et al., 2018), as a promising candidate in diabetes-associated delayed wound healing. Lipocalin 2 secretion is increased in the wound lysates of diabetic foot ulcers due to the release of neutrophil extracellular traps (Fadini et al., 2019). Lcn2 activation is also considered to be a biomarker for chronic inflammatory diseases of skin, such as psoriasis and venous ulcers (Serra et al., 2013, Shao et al., 2016). In the present study, wounds from diabetic Lcn2 knockout mice had lower inflammation scores, with a lower ratio of M1/M2 macrophages, with increased angiogenesis, and better wound healing than those from wild-type mice with STZ injection. The role of Lcn2 in the regulation of inflammation is complex. Cheng *et al.* demonstrated that Lcn2 promotes M1 macrophage polarization after cardiac ischemia-reperfusion injury (Cheng et al., 2015). In contrast Guo *et al.* reported that Lcn2-deficient mice displayed up-regulation of M1 macrophage markers and down-regulation of M2 markers in the adipose tissue and liver of mice fed a high-fat diet (Guo et al., 2014). In another setting, Warszawska *et al.* identified Lcn2 as both a marker of deactivated macrophages and a macrophage deactivator in the lungs

of mice with bacterial pneumonia (Warszawska et al., 2013). In the present study, Lcn2 inactivation had a positive effect on diabetic wounds whereas it did not affect the healing of non-diabetic normal mice. Our *in vitro* study on the function/polarization of macrophages showed a direct effect of recombinant Lcn2 protein to prevent the switching of pre-activated M1 macrophages to M2 the phenotype (although a limit of our approach is the use of macrophages isolated from peritoneum rather than those of the skin).

Other factors may influence diabetic wound healing. Abnormalities of hair follicle result in defective hair development and cycling, representing a sign of vascular impairment and organ damage in diabetic patients (Miranda et al., 2016). Efficiency of wound repair is also influenced by hair follicle cycle: wound healing was found to be accelerated when mice are wounded in the late anagen stage, with enhanced re-epithelialization and angiogenesis, and decreased immune cell infiltration (Ansell et al., 2011). It is thus crucial that control and experimental mice are in a comparable stage of hair follicle cycling for adequate comparison. Our diabetic mice and their respective controls are in telogen stage (see Methods), thus providing adequate comparison conditions.

The hair follicle expresses the MR (Jaisser and Farman, 2016), but limited knowledge is available on direct effects of MR signaling (and its blockade) in hair biology. Post-natal over-expression of MR in epidermal mouse skin induced major hair follicle dystrophy leading to alopecia (Farman et al., 2010, Sainte Marie et al., 2007). However, epidermal MR deletion showed no impact on hair follicle number and cycling in normal adult skin (Sevilla and Perez, 2018). Of note, we cannot rule out that in diabetes MR antagonism may affect hair cycle with a subsequent impact on wound healing. Future studies are required to investigate the relationship between MR over-activation, its blockade by canrenoate, and the abnormalities of hair follicle on wound repair and epidermal barrier function in diabetic skin. In the dermis, effect of MR antagonism on elastin and collagen synthesis and tissue remodeling after injuries have been reported (Mitts et al., 2010). Interestingly, it has been highlighted that MR plays a significant role in modulating collagen synthesis in various organs (Jaisser and Farman, 2016). Improvement of the defective dermal remodeling processes in diabetic wounds would represent an important progress.

In summary, we identified a significant role of MR signaling in inflammation and impaired vascular density accompanying delayed diabetic wound healing, using mouse models. This study indicates the efficacy of MR blockade to restore impaired reepithelialization, angiogenesis and to reduce inflammation after an acute wound in diabetic mice; whether these benefits extend to human chronic diabetic wounds as leg ulcers remain to be demonstrated. In a previous report, we showed that application of a topical MR antagonist was safe and effective in healthy volunteers with dexamethasone-associated skin atrophy and delayed wound healing (Maubec et al., 2015, Nguyen et al., 2016). Topical MR antagonism may thus be a simple and useful therapeutic strategy, providing a significant benefit to diabetic patients with delayed wound healing.

MATERIAL AND METHODS

Detailed information is provided in the Supplementary Data.

Wound healing *in vivo*

Two mouse models of diabetes were used for studying wound healing *in vivo*: streptozotocin-induced type I diabetes and type II diabetes (db-db mice), with their appropriate controls. Wounds were generated with 6 mm biopsy punch. For the db-db groups, wounds were done at 10 weeks of age. At that time all the dorsum skin displayed a homogenous pink color. For the streptozotocin groups, diabetes was induced at 10 weeks and we performed wounds at 15 weeks of age. We have given a special attention to select dorsum areas at a telogen stage, featured by pink skin color after hair clipping. The histology of the lateral skin surrounding the healing wounds in both models confirmed our clinical evaluation since we could not find any evidence of anagen hair follicles (**Fig S6**). MR blockade over the wound was achieved by local application of the MR antagonist potassium canrenoate (Canre) or PBS and wounds were photographed to evaluate wound closure. At day 5 (STZ groups) or 7 (db-db groups), mice were euthanized and wound and skin specimens were collected for RNA and protein extraction, immunolabelling, or FACS analysis.

Diabetes was also induced by STZ injections in 10 week-old female Lipocalin 2 knockout mice (Berger et al., 2006) and their appropriate controls before wounding.

Local MR silencing by siRNA in wounds of diabetic type 1 mouse model

Mouse MR Stealth siRNAs (Set of 3, MSS201383, MSS201384, MSS272869) were purchased from ThermoFisher Scientific (Renfrew, UK). Stock solution was made by adding 1ml H₂O to 20 nMol MR siRNA set. The siRNA working solution was prepared by 1:10 dilution of siRNA stock solution with Mirus transfection reagent. After wounding, 100 µl of siRNA solution (0.2 nMol) was locally injected with needle into each wound at day 0 and day 2. The scrambled siRNA (Stealth™ RNAi negative control, medium GC duplex, ThermoFischer Scientific) was prepared according to manufacturer's instruction and used as negative control. Photographs of wounds at day 3 and day 5 were used to evaluate the degree of wound closure. Mice were sacrificed at day 5, and wounded skin samples were collected for histological and molecular analysis.

Animal study approval statement

All animal experiments were approved by the Darwin ethics committee of Pierre et Marie Curie University and the French Ministry of Research (APAFIS#4438-2015092514508030 v7) and in accordance with the INSERM guidelines and European Community directives for the care and use of laboratory animals.

DATA AVAILABILITY STATEMENT

There are no datasets related to this article.

CONFLICT OF INTEREST STATEMENT

The authors have declared that no conflict of interest exists

ACKNOWLEDGEMENTS

We are grateful for the excellent technical assistance of the CEF (animal facility) team and their support with the animal care. We are also grateful to Thorsten Berger and Tak W. Mak (University of Toronto, Canada) for kindly sharing the Lcn2 KO mice. The authors are grateful to the following individuals for providing regular scientific advice and help: Sylvie Dumont and Fatiha Merabtene (Histo-morphology Platform, Saint-Antoine Research Center), H el ene Fohrer-Ting, Estelle Devevre, and Christophe Klein (Cellular Imaging and Cytometry Center, Cordeliers Research Center).

AUTHOR CONTRIBUTIONS

Conceptualization: NF, SA, FJ; Data Curation: VTN, NF, SA, FJ; Formal Analysis: VTN. Funding Acquisition: NF, FBC, SA, FJ; Investigation: VTN, MS, RPR; Methodology: VTN, NF, SA, FJ; Project Administration: NF, SA, FJ, Resources: FBC, SA, FJ; Supervision: NF, SA, FJ; Visualization: VTN, RPR; Writing - Original Draft Preparation: VTN, NF, SA, FJ; Writing - Review and Editing: VTN, NF, RPR, MS, FBC, SA, FJ.

FUNDING

This work was supported by INSERM funding, the Agence Nationale pour la Recherche (ANR-14-CE16-0010-01) and SATT INNOV maturation grant 494. VT Nguyen was a recipient of a PhD grant from the Vietnamese Government and a grant from the Fondation pour la Recherche M edicale (grant FDT20140930932).

REFERENCES

- Ahmed AS, Antonsen EL. Immune and vascular dysfunction in diabetic wound healing. *J Wound Care* 2016;25 Suppl 7:S35-46.
- Ansell DM, Kloepper JE, Thomason HA, Paus R, Hardman MJ. Exploring the "hair growth-wound healing connection": anagen phase promotes wound re-epithelialization. *J Invest Dermatol* 2011;131(2):518-28.
- Ashcroft GS, Jeong MJ, Ashworth JJ, Hardman M, Jin W, Moutsopoulos N, et al. Tumor necrosis factor-alpha (TNF-alpha) is a therapeutic target for impaired cutaneous wound healing. *Wound Repair Regen* 2012;20(1):38-49.
- Barrera-Chimal J, Estrela GR, Lechner SM, Giraud S, El Moghrabi S, Kaaki S, et al. The myeloid mineralocorticoid receptor controls inflammatory and fibrotic responses after renal injury via macrophage interleukin-4 receptor signaling. *Kidney Int* 2018;93(6):1344-55.
- Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. *Wound Repair Regen* 2008;16(5):585-601.
- Berger T, Togawa A, Duncan GS, Elia AJ, You-Ten A, Wakeham A, et al. Lipocalin 2-deficient mice exhibit increased sensitivity to *Escherichia coli* infection but not to ischemia-reperfusion injury. *Proc Natl Acad Sci U S A* 2006;103(6):1834-9.
- Bigas J, Sevilla LM, Carceller E, Boix J, Perez P. Epidermal glucocorticoid and mineralocorticoid receptors act cooperatively to regulate epidermal development and counteract skin inflammation. *Cell death & disease* 2018;9(6):588.
- Boix J, Sevilla LM, Saez Z, Carceller E, Perez P. Epidermal Mineralocorticoid Receptor Plays Beneficial and Adverse Effects in Skin and Mediates Glucocorticoid Responses. *J Invest Dermatol* 2016;136(12):2417-26.
- Boniakowski AE, Kimball AS, Jacobs BN, Kunkel SL, Gallagher KA. Macrophage-Mediated Inflammation in Normal and Diabetic Wound Healing. *J Immunol* 2017;199(1):17-24.

- Boulton AJ. Diabetic neuropathy and foot complications. *Handbook of clinical neurology* 2014;126:97-107.
- Boulton AJ, Vileikyte L, Ragnarson-Tennvall G, Apelqvist J. The global burden of diabetic foot disease. *Lancet* 2005;366(9498):1719-24.
- Brancato SK, Albina JE. Wound macrophages as key regulators of repair: origin, phenotype, and function. *Am J Pathol* 2011;178(1):19-25.
- Brem H, Tomic-Canic M. Cellular and molecular basis of wound healing in diabetes. *J Clin Invest* 2007;117(5):1219-22.
- Buonafine M, Martinez-Martinez E, Jaisser F. More than a simple biomarker: the role of NGAL in cardiovascular and renal diseases. *Clin Sci (Lond)* 2018;132(9):909-23.
- Cheng L, Xing H, Mao X, Li L, Li X, Li Q. Lipocalin-2 promotes m1 macrophages polarization in a mouse cardiac ischaemia-reperfusion injury model. *Scand J Immunol* 2015;81(1):31-8.
- Clokie M, Greenway AL, Harding K, Jones NJ, Vedhara K, Game F, et al. New horizons in the understanding of the causes and management of diabetic foot disease: report from the 2017 Diabetes UK Annual Professional Conference Symposium. *Diabet Med* 2017;34(3):305-15.
- Dinh T, Tecilazich F, Kafanas A, Doupis J, Gnardellis C, Leal E, et al. Mechanisms involved in the development and healing of diabetic foot ulceration. *Diabetes* 2012;61(11):2937-47.
- Fadini GP, Solini A, Manca ML, Penno G, Gatti A, Anichini R, et al. Effectiveness of dapagliflozin versus comparators on renal endpoints in the real world: A multicentre retrospective study. *Diabetes Obes Metab* 2019;21(2):252-60.
- Farman N, Maubec E, Poeggeler B, Klatte JE, Jaisser F, Paus R. The mineralocorticoid receptor as a novel player in skin biology: beyond the renal horizon? *Exp Dermatol* 2010;19(2):100-7.
- Galiano RD, Tepper OM, Pelo CR, Bhatt KA, Callaghan M, Bastidas N, et al. Topical vascular endothelial growth factor accelerates diabetic wound healing through increased angiogenesis and by mobilizing and recruiting bone marrow-derived cells. *Am J Pathol* 2004;164(6):1935-47.

- Gordon S. Alternative activation of macrophages. *Nat Rev Immunol* 2003;3(1):23-35.
- Greenhalgh DG. Wound healing and diabetes mellitus. *Clin Plast Surg* 2003;30(1):37-45.
- Guo C, Ricchiuti V, Lian BQ, Yao TM, Coutinho P, Romero JR, et al. Mineralocorticoid receptor blockade reverses obesity-related changes in expression of adiponectin, peroxisome proliferator-activated receptor-gamma, and proinflammatory adipokines. *Circulation* 2008;117(17):2253-61.
- Guo H, Jin D, Chen X. Lipocalin 2 is a regulator of macrophage polarization and NF-kappaB/STAT3 pathway activation. *Mol Endocrinol* 2014;28(10):1616-28.
- Guo Y, Lin C, Xu P, Wu S, Fu X, Xia W, et al. AGEs Induced Autophagy Impairs Cutaneous Wound Healing via Stimulating Macrophage Polarization to M1 in Diabetes. *Sci Rep* 2016;6:36416.
- Hannen R, Udeh-Momoh C, Upton J, Wright M, Michael A, Gulati A, et al. Dysfunctional Skin-Derived Glucocorticoid Synthesis Is a Pathogenic Mechanism of Psoriasis. *J Invest Dermatol* 2017;137(8):1630-7.
- He R, Yin H, Yuan B, Liu T, Luo L, Huang P, et al. IL-33 improves wound healing through enhanced M2 macrophage polarization in diabetic mice. *Mol Immunol* 2017;90:42-9.
- Hirata A, Maeda N, Hiuge A, Hibuse T, Fujita K, Okada T, et al. Blockade of mineralocorticoid receptor reverses adipocyte dysfunction and insulin resistance in obese mice. *Cardiovasc Res* 2009;84(1):164-72.
- Jaisser F, Farman N. Emerging Roles of the Mineralocorticoid Receptor in Pathology: Toward New Paradigms in Clinical Pharmacology. *Pharmacol Rev* 2016;68(1):49-75.
- Leal EC, Carvalho E, Tellechea A, Kafanas A, Tecilazich F, Kearney C, et al. Substance P promotes wound healing in diabetes by modulating inflammation and macrophage phenotype. *Am J Pathol* 2015;185(6):1638-48.
- Lepantalo M, Apelqvist J, Setacci C, Ricco JB, de Donato G, Becker F, et al. Chapter V: Diabetic foot. *European journal of vascular and endovascular surgery : the official journal of the European Society for Vascular Surgery* 2011;42 Suppl 2:S60-74.

- Liu F, Chen DD, Sun X, Xie HH, Yuan H, Jia W, et al. Hydrogen sulfide improves wound healing via restoration of endothelial progenitor cell functions and activation of angiopoietin-1 in type 2 diabetes. *Diabetes* 2014;63(5):1763-78.
- Loomans CJ, de Koning EJ, Staal FJ, Rookmaaker MB, Verseyden C, de Boer HC, et al. Endothelial progenitor cell dysfunction: a novel concept in the pathogenesis of vascular complications of type 1 diabetes. *Diabetes* 2004;53(1):195-9.
- Lucas T, Waisman A, Ranjan R, Roes J, Krieg T, Muller W, et al. Differential roles of macrophages in diverse phases of skin repair. *J Immunol* 2010;184(7):3964-77.
- Mahdavian Delavary B, van der Veer WM, van Egmond M, Niessen FB, Beelen RH. Macrophages in skin injury and repair. *Immunobiology* 2011;216(7):753-62.
- Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol* 2004;25(12):677-86.
- Marrotte EJ, Chen DD, Hakim JS, Chen AF. Manganese superoxide dismutase expression in endothelial progenitor cells accelerates wound healing in diabetic mice. *J Clin Invest* 2010;120(12):4207-19.
- Martinez-Martinez E, Buonafine M, Boukhalfa I, Ibarrola J, Fernandez-Celis A, Kolkhof P, et al. Aldosterone Target NGAL (Neutrophil Gelatinase-Associated Lipocalin) Is Involved in Cardiac Remodeling After Myocardial Infarction Through NFkappaB Pathway. *Hypertension* 2017;70(6):1148-56.
- Maruyama K, Asai J, Ii M, Thorne T, Losordo DW, D'Amore PA. Decreased macrophage number and activation lead to reduced lymphatic vessel formation and contribute to impaired diabetic wound healing. *Am J Pathol* 2007;170(4):1178-91.
- Marzolla V, Armani A, Feraco A, De Martino MU, Fabbri A, Rosano G, et al. Mineralocorticoid receptor in adipocytes and macrophages: a promising target to fight metabolic syndrome. *Steroids* 2014;91:46-53.

- Maubec E, Laouenan C, Deschamps L, Nguyen VT, Scheer-Senyarich I, Wackenheim-Jacobs AC, et al. Topical Mineralocorticoid Receptor Blockade Limits Glucocorticoid-Induced Epidermal Atrophy in Human Skin. *J Invest Dermatol* 2015.
- Miranda JJ, Taype-Rondan A, Tapia JC, Gastanadui-Gonzalez MG, Roman-Carpio R. Hair follicle characteristics as early marker of Type 2 Diabetes. *Med Hypotheses* 2016;95:39-44.
- Mitts TF, Bunda S, Wang Y, Hinek A. Aldosterone and mineralocorticoid receptor antagonists modulate elastin and collagen deposition in human skin. *J Invest Dermatol* 2010;130(10):2396-406.
- Nguyen VT, Farman N, Maubec E, Nassar D, Desposito D, Waeckel L, et al. Re-epithelialization of pathological cutaneous wounds is improved by local mineralocorticoid receptor antagonism. *J Invest Dermatol* 2016.
- Nishimura Y, Ii M, Qin G, Hamada H, Asai J, Takenaka H, et al. CXCR4 antagonist AMD3100 accelerates impaired wound healing in diabetic mice. *J Invest Dermatol* 2012;132(3 Pt 1):711-20.
- Noor S, Khan RU, Ahmad J. Understanding Diabetic Foot Infection and its Management. *Diabetes & metabolic syndrome* 2017;11(2):149-56.
- Novak ML, Koh TJ. Phenotypic transitions of macrophages orchestrate tissue repair. *Am J Pathol* 2013;183(5):1352-63.
- Okizaki S, Ito Y, Hosono K, Oba K, Ohkubo H, Amano H, et al. Suppressed recruitment of alternatively activated macrophages reduces TGF-beta1 and impairs wound healing in streptozotocin-induced diabetic mice. *Biomed Pharmacother* 2015;70:317-25.
- Peters EJ, Lipsky BA. Diagnosis and management of infection in the diabetic foot. *Med Clin North Am* 2013;97(5):911-46.
- Rahmani W, Liu Y, Rosin NL, Kline A, Raharjo E, Yoon J, et al. Macrophages Promote Wound-Induced Hair Follicle Regeneration in a CX3CR1- and TGF-beta1-Dependent Manner. *J Invest Dermatol* 2018;138(10):2111-22.

- Ramalho T, Filgueiras L, Silva-Jr IA, Pessoa AFM, Jancar S. Impaired wound healing in type 1 diabetes is dependent on 5-lipoxygenase products. *Sci Rep* 2018;8(1):14164.
- Sainte Marie Y, Toulon A, Paus R, Maubec E, Cherfa A, Grossin M, et al. Targeted skin overexpression of the mineralocorticoid receptor in mice causes epidermal atrophy, premature skin barrier formation, eye abnormalities, and alopecia. *Am J Pathol* 2007;171(3):846-60.
- Serra R, Buffone G, Falcone D, Molinari V, Scaramuzzino M, Gallelli L, et al. Chronic venous leg ulcers are associated with high levels of metalloproteinases-9 and neutrophil gelatinase-associated lipocalin. *Wound Repair Regen* 2013;21(3):395-401.
- Sevilla LM, Perez P. Roles of the Glucocorticoid and Mineralocorticoid Receptors in Skin Pathophysiology. *Int J Mol Sci* 2018;19(7).
- Shao S, Cao T, Jin L, Li B, Fang H, Zhang J, et al. Increased Lipocalin-2 Contributes to the Pathogenesis of Psoriasis by Modulating Neutrophil Chemotaxis and Cytokine Secretion. *J Invest Dermatol* 2016;136(7):1418-28.
- Sindrilaru A, Peters T, Wieschalka S, Baican C, Baican A, Peter H, et al. An unrestrained proinflammatory M1 macrophage population induced by iron impairs wound healing in humans and mice. *J Clin Invest* 2011;121(3):985-97.
- Slominski AT, Manna PR, Tuckey RC. Cutaneous glucocorticosteroidogenesis: securing local homeostasis and the skin integrity. *Exp Dermatol* 2014;23(6):369-74.
- Slominski AT, Manna PR, Tuckey RC. On the role of skin in the regulation of local and systemic steroidogenic activities. *Steroids* 2015;103:72-88.
- Stojadinovic O, Lindley LE, Jozic I, Tomic-Canic M. Mineralocorticoid Receptor Antagonists-A New Sprinkle of Salt and Youth. *J Invest Dermatol* 2016;136(10):1938-41.
- Tiganescu A, Tahrani AA, Morgan SA, Otranto M, Desmouliere A, Abrahams L, et al. 11beta-Hydroxysteroid dehydrogenase blockade prevents age-induced skin structure and function defects. *J Clin Invest* 2013;123(7):3051-60.

Warszawska JM, Gawish R, Sharif O, Sigel S, Doninger B, Lakovits K, et al. Lipocalin 2 deactivates macrophages and worsens pneumococcal pneumonia outcomes. *J Clin Invest* 2013;123(8):3363-72.

Zhang P, Lu J, Jing Y, Tang S, Zhu D, Bi Y. Global epidemiology of diabetic foot ulceration: a systematic review and meta-analysis (dagger). *Annals of medicine* 2017;49(2):106-16.

FIGURE LEGENDS

Figure 1. Canrenoate improves delayed wound healing in type 2 diabetic mice

(a). Real-time PCR analysis of MR mRNA expression in the skin or wounds (day 7) of db-db mice, relative to that of CT. Photographs (b) and quantification (c) of the wound area of CT and db-db mice treated with Canrenoate (Canre) or PBS at the indicated time post-wounding. (d) Wound sections at day 7 post-wounding labeled with anti-K14 antibody (green) and Dapi (blue). Quantification of the length of the neo-epidermis (e) and diameter of the residual wound (f). Anti-Ki67 (green) staining (g) and quantification of Ki67-positive cells in the neo-epidermis (Epi) and underlying dermis (Der) (h); dotted lines: dermo-epidermis junction. Data represent mean \pm SEM; n= number of mice per group, from 2 experimental series. Statistics: a, e, f, h: Mann-Whitney test; c: two-way ANOVA followed by the Newman-Keuls Multiple Comparison test. * P <0.05; ** P <0.01; *** P <0.001; **** P <0.0001 db-db vs CT; **** P <0.0001 db-db vs db-db + Canre; Scale bar: 500 μ m (d), 100 μ m (g).

Figure 2. Canrenoate improves impaired wound angiogenesis in diabetic mice

(a) Photographs of wound sections doubled-stained for CD31 (red) and Ki67 (green), showing neo-microvessels formed in wound beds of STZ (a) and stained with CD31 only in db-db (e) mice. b and f: Quantification of CD31⁺ microvessels in wound of STZ (b) and db-db (f) mice after treatment with Canre or PBS. c and g: Dot plot of FACS analysis of wound cells for the quantification of CD45⁻CD31⁺ endothelial cells in wound of STZ (d) and of db-db (h) mice after treatment with Canre or PBS. Data represent mean \pm SEM; n= number of mice per group, from 3 (STZ) and 2 (db-db) experimental series. Statistics: One-way ANOVA followed by the Newman-Keuls Multiple Comparison test. *** P <0.001; **** P <0.0001, a and e: Scale bar: 100 μ m.

Figure 3. Canrenoate regulates the wound production of pro/anti-inflammatory factors

Total RNA was extracted from wounded skin of control (CT), STZ (day 5 post-wounding), or db-db (day 7 post-wounding) mice. Cytokines mRNA levels were analyzed by quantitative RT-PCR. Pro-inflammatory cytokine mRNA levels in STZ (a) and db-db (c) wounds treated with Canre or PBS, relative to those of CT wounds treated with PBS. Anti-inflammatory cytokines mRNA levels in STZ (b) and db-db (d) wounds treated with Canre or PBS, relative to those of CT wounds treated with PBS. Data represent mean \pm SEM; n=

number of mice per group, from 2 experimental series. Statistics: One-way ANOVA followed by the Newman-Keuls Multiple Comparison test. * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ STZ or db-db vs CT; § $P < 0.05$, §§ $P < 0.01$; §§§ $P < 0.001$ STZ vs STZ + Canre (**a** and **b**) or db-db vs db-db + Canre (**c** and **d**).

Figure 4. Canrenoate enhances unrestrained polarization of M1 to M2 macrophages

Wounded skin specimens were collected after five (for STZ mice) or seven days (for db-db mice) of treatment with Canre or PBS. Dot plot (**a** and **e**) of FACS analysis of wound cells for the quantification of CD11b⁺F4.80⁺Ly6C^{hi} M1 macrophages (**c** and **g**) and CD11b⁺F4.80⁺Ly6C^{low} M2 macrophages (**d** and **h**) in wound of STZ (**c-d**) and db-db (**g-h**) mice after treatment with Canre or PBS, as the percentage of total CD11b⁺F4.80⁺ macrophages (**b** and **f**). Data represent mean ± SEM; n= number of mice per group, from from 3 (STZ) and 2 (db-db) experimental series. Statistics: One-way ANOVA followed by the Newman-Keuls Multiple Comparison test. * $P < 0.05$, ** $P < 0.01$; **** $P < 0.0001$.

Figure 5. Canrenoate induces macrophage expression of angiogenic factors

Wounded skin specimens from control (CT) or STZ mice were collected five days of treatment with Canre or PBS. Total RNA extracted from whole tissue or sorted macrophages was used to analyze pro-angiogenic factors. (**a**) Pro-angiogenic mRNA levels in whole wounded skin from STZ mice treated with Canre or PBS, relative to those of CT wounds treated with PBS. (**b**) Pro-angiogenic mRNA levels in total macrophages isolated from wounded skin of STZ mice treated with Canre or PBS, relative to those of CT wounds treated with PBS. Data represent mean ± SEM; n= number of mice per group, from 2 experimental series. Statistics: One-way ANOVA followed by the Newman-Keuls Multiple Comparison test. * $P < 0.05$, ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

Figure 6. Lcn2 deficiency prevents the diabetes-induced delay of wound healing

Lcn2mRNA (**a**) and protein (**b**) levels in wounds at day 5 were analyzed by real-time PCR and ELISA. **c-h**: Diabetes was induced in wildtype (CT) and Lcn2 KO mice by STZ injections before wounding. Photographs (**c**) and quantification of the wound area (**d**) from CT and Lcn2 KO mice with or without STZ treatment at the indicated times post-wounding. M1 (**e**) and M2 (**f**) macrophages and endothelial cells (**g**) of wounds at day 5 were quantified by FACS analysis. Total macrophages were sorted from wounded skin at day 5 and angiogenic factors mRNA levels were analyzed by real-time PCR (**h**). Data represent mean ± SEM; n=

number of mice per group, from 2 experimental series. Statistics: **a, e, f, g, h**: one-way ANOVA followed by the Newman-Keuls Multiple Comparison test; **d**: 2-way ANOVA followed by the Newman-Keuls Multiple Comparison test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ CT + STZ vs CT. §§ $P < 0.01$ CT + STZ vs KO Lcn2 + STZ (**d**).

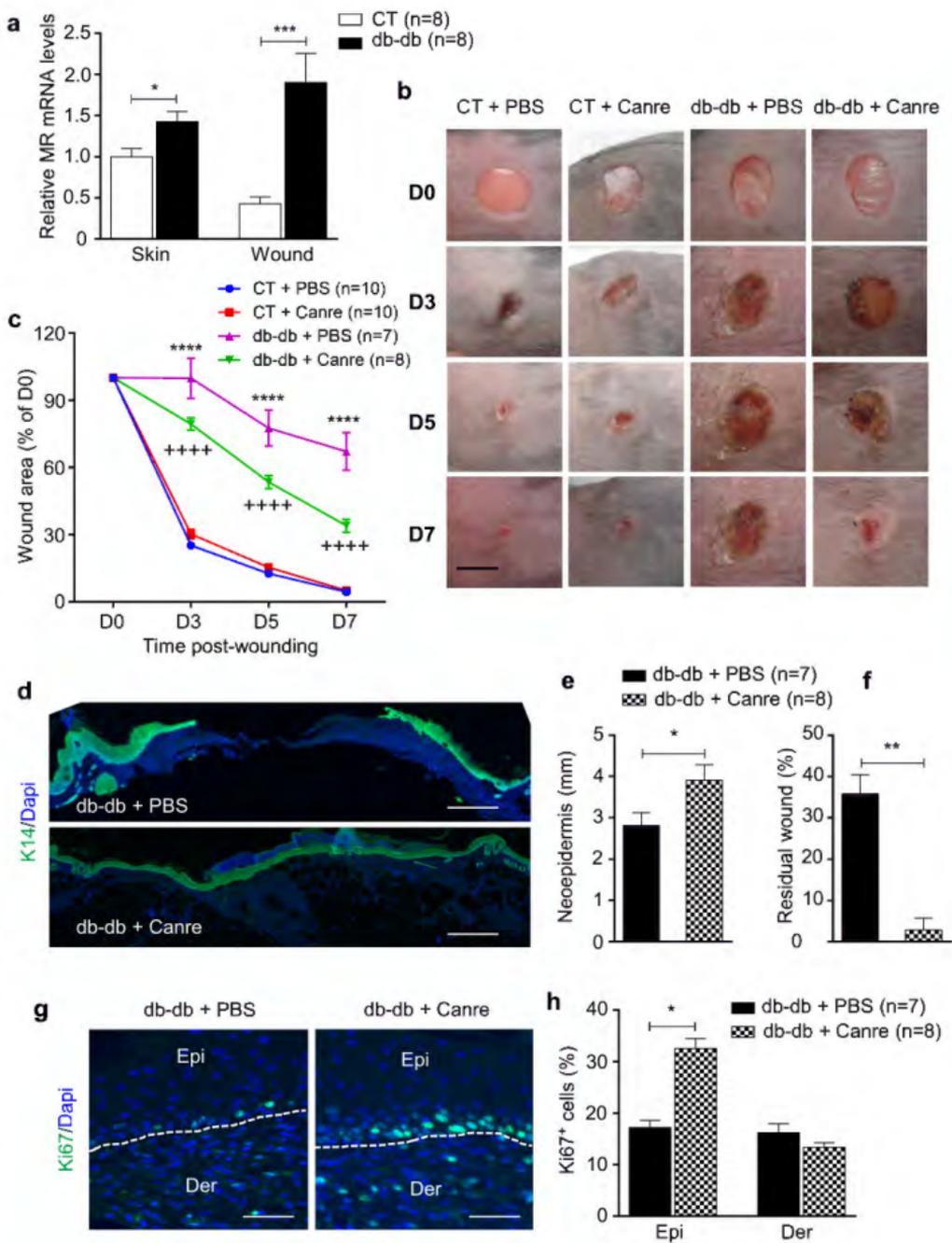
Figure 1

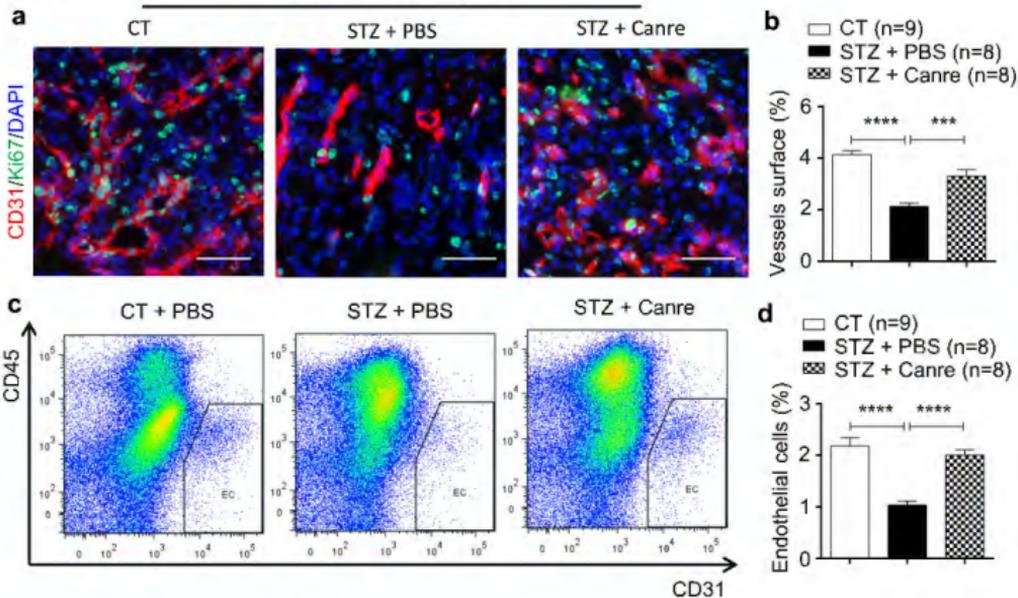
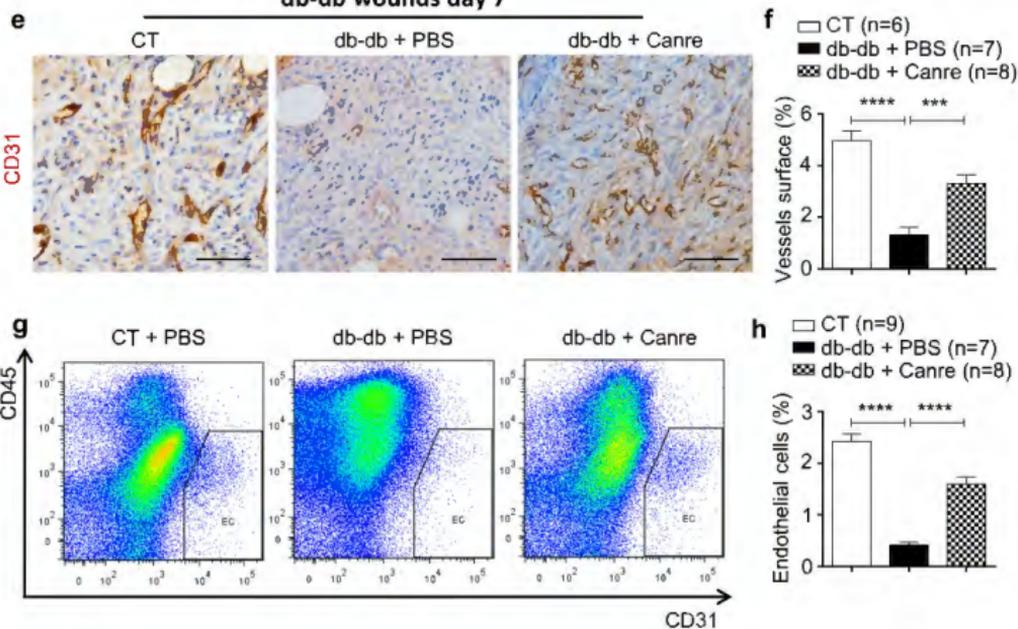
Figure 2**STZ wounds day 5****db-db wounds day 7**

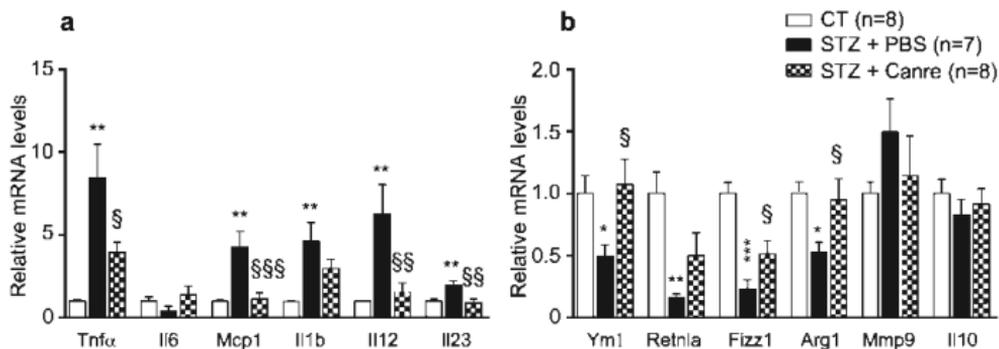
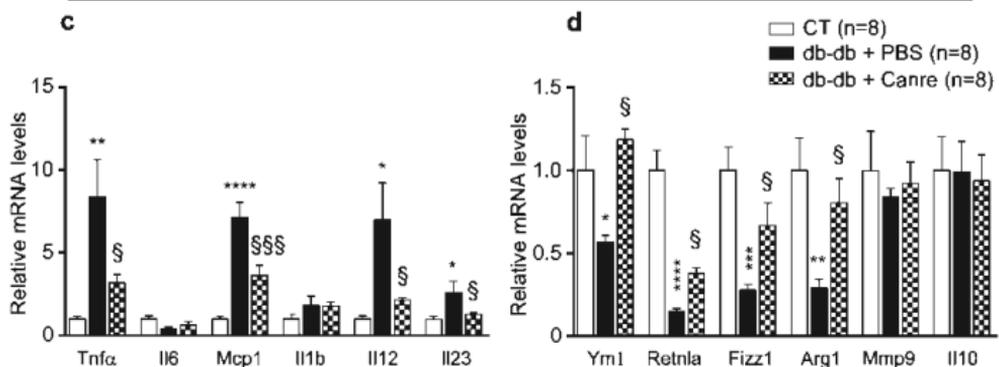
Figure 3**STZ wounds at day 5****db-db wounds at day 7**

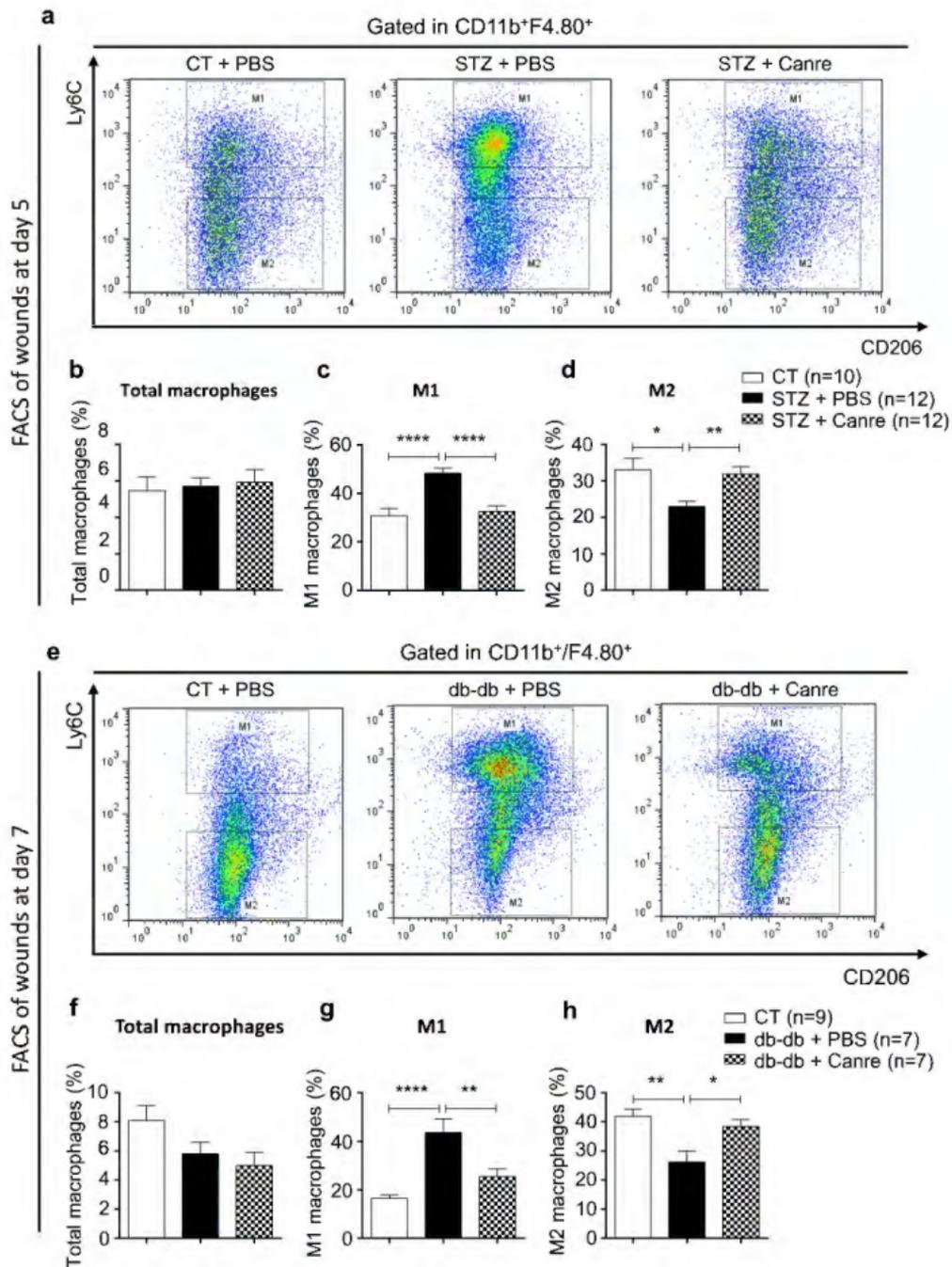
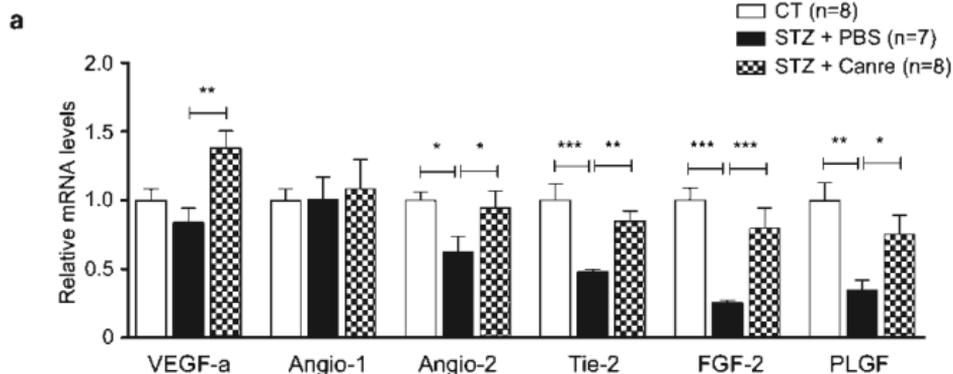
Figure 4.

Figure 5**Whole wounds at day 5**

b **Sorted wound macrophages at day 5**

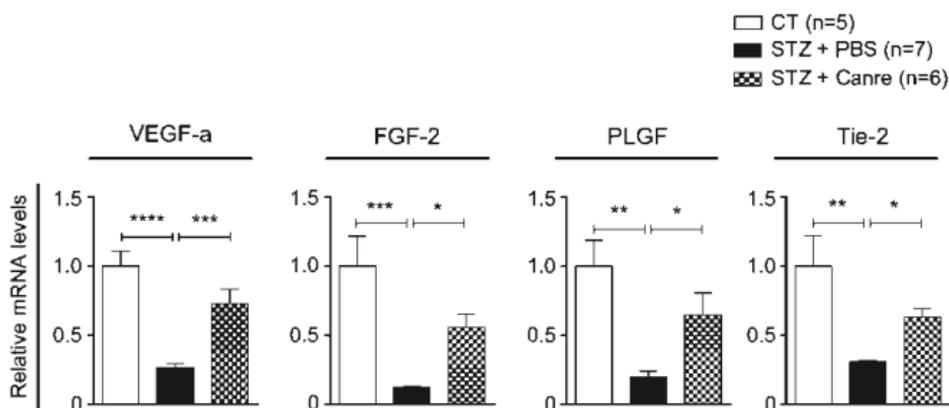


Figure 6