

RESEARCH PAPER

Inhibition of inflammasome activation improves the impaired pattern of healing in genetically diabetic mice

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BACKGROUND AND PURPOSE

Type 2 diabetes impairs the healing process because of an exaggerated and persistent inflammatory response, and an altered expression pattern of angiogenic molecules. We investigated the effects of inflammasome blockade in diabetes-related wound-healing defects, in genetically diabetic mice.

EXPERIMENTAL APPROACH

An incisional skin wound model was produced on the back of female diabetic C57BL/KsJ-m +/+ *Lept^{db}* mice (*db⁺/db⁺*) and their normal littermates (*db⁺/m⁺*). Animals were treated daily with two inflammasome blocking agents, BAY 11-7082 (20 mg·kg⁻¹ i.p.), or Brilliant Blue G (BBG, 45.5 mg·kg⁻¹ i.p.), or vehicle. Mice were killed on 3, 6 and 12 days after skin injury to measure expression of the NOD-like receptor NLRP3, caspase-1, VEGF, the inflammasome adapter protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and the chemokine CXCL12. Wound levels of IL-1 β and IL-18 were also measured, along with histological assessments of wound tissue and the time to complete wound closure.

KEY RESULTS

During healing, the diabetic mice exhibited increased activation of NLRP3, caspase-1, ASC, IL-1 β and IL-18. They also showed a reduced expression of VEGF and CXCL12. Treatment with BAY 11-7082 or BBG, to block activation of the inflammasome, decreased the levels of pro-inflammatory molecules. Histological evaluation indicated that inflammasome blockade improved the impaired healing pattern, at day 12 in diabetic mice, along with a decreased time to complete skin healing.

CONCLUSIONS AND IMPLICATIONS

These data strongly suggest that activation of the NLRP3 inflammasome is one of the key contributors to the delayed healing of wounds in diabetic mice.

Abbreviations

DAMPs, damage-associated molecular pattern molecules; NLRP3, NOD-like receptor family, pyrin domain containing 3

Introduction

A disturbed wound-healing process characterizes diabetes and may enhance the overall morbidity and mortality of type 2

diabetic patients (Falanga, 2005; Singh *et al.*, 2005). Generally, skin repair includes a complex programmed sequence of cellular and molecular processes including inflammation, cell migration, angiogenesis, provisional matrix synthesis,

collagen deposition and re-epithelization (Falanga, 2005; Reiber and Raugi, 2005). Angiogenesis plays a central role in wound healing and is associated with expression of several cytokines and angiogenic factors, such as VEGF and the chemokine CXCL12 (SDF-1 α) (Ferrara *et al.*, 2003; Tammela *et al.*, 2005). Healing impairment in diabetics is characterized by an increased and persistent inflammatory response, which in turn delays cellular infiltration and granulation tissue formation, with a consequently decreased collagen organization and reduced angiogenesis (Goodson and Hunt, 1977; Bohlen and Niggli, 1979).

The inflammasomes are multiprotein oligomers that respond to inflammatory stimuli by initiating an intracellular inflammatory cascade, particularly by increasing the cleavage of the inactive precursor of IL-1 β to its active form (Schroder and Tschoop, 2010). One of the largest cytosolic inflammasomes is the NLRP3 inflammasome which comprises the NOD-like receptor NLRP3, the adapter protein termed 'apoptosis-associated speck-like protein containing a caspase recruitment domain' (ASC) and procaspase-1. During type 2 diabetes, an increased accumulation of endogenous damage-associated molecular pattern molecules (DAMPs), such as free cholesterol and membrane complex lipids (i.e. ceramides), serve as activators of the NLRP3 inflammasome. These activators cause inflammasome assembly by interactions in the protein domains of NLRP3, ASC and procaspase-1, with subsequent autolytic cleavage of the inactive pro-caspase into its active form. Active caspase-1 then can cleave the inactive stored forms of IL-1 and IL-18 into the active cytokines, which further cause insulin resistance and organ dysfunction. IL-1 β is predominantly produced by monocytes, macrophages and neutrophils, and is implicated in the pathogenesis of many inflammatory diseases including diabetes (Dinarello, 2011). In addition, activation of the membrane-bound purinergic P2X7 receptor participates in the processing and release of IL-1 β , and this receptor has been proposed as the upstream activator of NLRP3 inflammasome assembly (Ferrari *et al.*, 2006).

Our group has already demonstrated the efficacy of several therapeutic approaches in improving the altered healing and increasing angiogenesis, in the well known *db/db* mouse model (Altavilla *et al.*, 2001; 2011; Galeano *et al.*, 2001; 2003; 2008; 2011). In this study, we evaluated the effects of inflammasome NLRP3 blockade, as a new strategy to ameliorate wound healing in this model.

Methods

Animals

All animal care and experimental procedures were in accordance with the Principles of Laboratory Animal Care (NIH publication no.85-23, revised 1985) and authorized by our Local Institution. They are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 111 animals were used in the experiments described here.

Genetically diabetic female (30–35 g) C57BL/KsJ-m $+/+$ *Lept^{db}* mice (*db⁺/db⁺*) and their normal littermates (22–25 g; *db⁺/m⁺*), were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Animals were 10 weeks old at the start of the

experiments. During the experiments, the animals were kept one per cage, under controlled environmental conditions (12 h light/dark cycle, 23°C), and provided with standard food and water *ad libitum*. After general anaesthesia with sodium pentobarbital (80 mg·kg⁻¹ i.p.), the hair on the back was shaved and two parallel 4 cm incisions were produced with a scalpel (Figure 1A), on the back of all mice as previously described (Altavilla *et al.*, 2001; 2011; Galeano *et al.*, 2001; 2003; 2008; 2011).

Normoglycaemic and diabetic mice, received either BAY 11-7082 (20 mg·kg⁻¹ day i.p.), or Brilliant Blue G (45.5 mg·kg⁻¹ i.p.) every 2 days, or vehicle (a 1:3 solution of DMSO and 0.9% NaCl) for up to 12 days. Ten animals from each strain and treatment arm were killed after 3, 6 and 12 days after wounding and the wounds removed using a scalpel to cut the shape of an ellipse around the lesion (Figure 1A). Of the two wounds, one was used for Western blot, gene expression and ELISA assays, and the other one for histology. Other groups of normoglycaemic and diabetic mice ($n = 21$ in each group) were subjected to wounding and used to measure the time needed to complete wound closure (up to 45 days).

Expression of NLRP3, caspase-1, ASC, and VEGF by Western blot analysis

Expression of NLRP3, caspase-1 and VEGF proteins was evaluated at day 3, 6 and 12 days by Western Blot, as previously described (Galeano *et al.*, 2008; 2011; Altavilla *et al.*, 2011). Primary antibodies for NLRP3 and VEGF were purchased from Abcam (Cambridge, UK), pro-caspase-1 and caspase-1 were obtained from Cell Signaling (Danvers, MA), ASC was purchased from Millipore (Billerica, MA, USA). Secondary peroxidase conjugated antibodies were obtained by Thermo Scientific (Waltham, MA, USA). The protein signals were visualized by chemiluminescence (ECL plus, Thermo Scientific), quantified by scanning densitometry, using a bio-image analysis system (Bio-Profil Celbio, Milan, Italy), and were expressed as integrated intensity, relative to β -actin (Cell Signaling), measured on stripped blots.

Determination of IL-1 β and IL-18 in wound lysates

The wound lysates were prepared, at 3, 6 and 12 days, by homogenizing wound tissue (30 mg) in 1 mL PBS, pH 7.4 using an Ultra Turrax (IKA, Staufen, Germany) homogenizer. The homogenate was centrifuged at 15,000 $\times g$ for 15 min at 4 °C. The supernatant was collected and used for ELISA assays. IL-1 β and IL-18 in these lysates were determined with commercially available mouse-specific ELISA assay kits (from Abcam and eBioscience Inc., San Diego, CA, USA; respectively) and following the manufacturer's instructions. IL-1 β (detection range 2.74–2000 pg·mL⁻¹) and IL-18 (detection range 31.3–2000 pg·mL⁻¹) levels were expressed as pg·mL⁻¹ of wound lysate.

Levels of mRNA for NLRP3, pro-caspase-1, and CXCL12

Total RNA was extracted from wounds by cell lysis with Trizol (Invitrogen, Carlsbad, CA, USA), and quantified as described previously (Galeano *et al.*, 2008). RNA was reverse transcribed to cDNA and used to quantify the amount of NLRP3, pro-

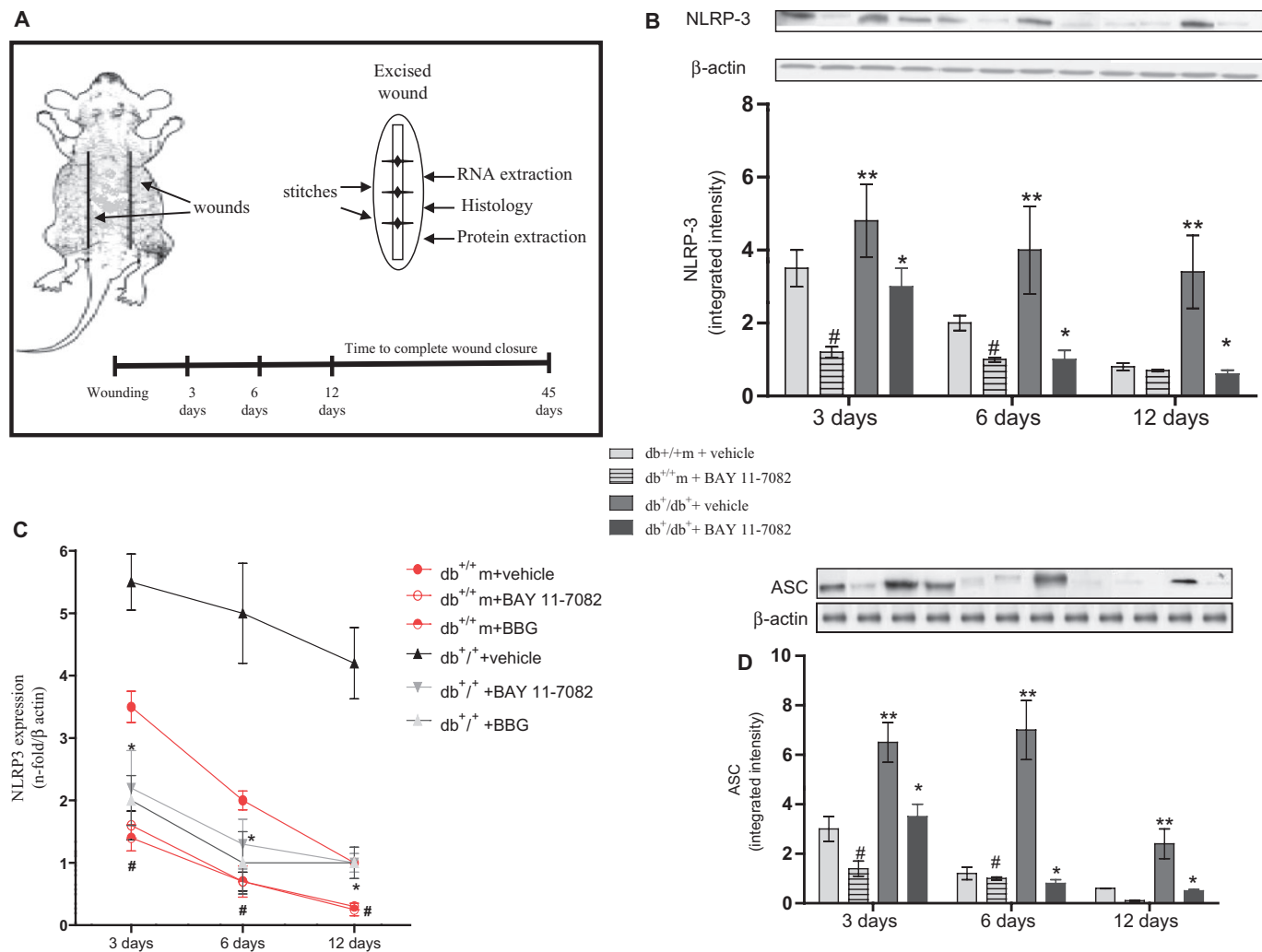


Figure 1

(A) The diagram represents the back of a mouse with the site of the two wounds. The right hand side shows an excised wound (ellipse shape), subdivided to perform the analyses. At the bottom the experimental timeline is shown. (B) NLRP3 protein expression in skin wound samples, collected at 3, 6 and 12 days, from either normoglycaemic ($db^{+/+}/m$) and diabetic ($db^{+/+}/db^{+/+}$) mice, given either vehicle or BAY 11-7082 (20 mg·kg⁻¹ i.p.). Each bar represents the mean \pm SD of seven animals. # $P < 0.01$ versus $db^{+/+}/m$ + vehicle; ** $P < 0.01$ versus $db^{+/+}/m$ + vehicle; * $P < 0.05$ versus $db^{+/+}/db^{+/+}$ + vehicle. (C) NLRP3 mRNA expression in skin wound samples, collected at 3, 6 and 12 days, from either normoglycaemic ($db^{+/+}/m$) and diabetic ($db^{+/+}/db^{+/+}$) mice, given either vehicle or BAY 11-7082 (20 mg·kg⁻¹ i.p.) or BBG (45.5 mg·kg⁻¹ i.p.). Each bar represents the mean \pm SD of seven animals. # $P < 0.01$ versus $db^{+/+}/m$ + vehicle; * $P < 0.05$ versus $db^{+/+}/db^{+/+}$ + vehicle. (D) ASC protein expression in skin wound samples, collected at 3, 6 and 12 days, from either normoglycaemic ($db^{+/+}/m$) and diabetic ($db^{+/+}/db^{+/+}$) mice, given either vehicle or BAY 11-7082 (20 mg·kg⁻¹ i.p.). Each bar represents the mean \pm SD of seven animals. # $P < 0.01$ versus $db^{+/+}/m$ + vehicle; ** $P < 0.005$ versus $db^{+/+}/m$ + vehicle; * $P < 0.05$ versus $db^{+/+}/db^{+/+}$ + vehicle.

caspase-1 and CXCL12 mRNA by real-time PCR using TaqMan probes (Applied Biosystems, Foster City, CA, USA); β -actin was used as endogenous control. The results were expressed as the n-fold difference relative to controls (relative expression levels).

Histological examination and time to complete wound closure

Wound samples were fixed in 10% neutral buffered formalin, and processed as previously described (Galeano *et al.*, 2008). All slides were examined by a pathologist, unaware of the treatment, using an eye-piece grid under the microscope from

$\times 20$ to $\times 100$ magnification. The following parameters were evaluated and graded for re-epithelialization, dermal matrix deposition and regeneration, granulation tissue formation and remodelling. The histological specimens were scored as earlier reported (Altavilla *et al.*, 2011; Galeano *et al.*, 2011). The time to complete wound closure, as shown by a closed linear healing ridge, was determined as described previously (Altavilla *et al.*, 2011; Galeano *et al.*, 2011).

Data analysis

All data were expressed as means (\pm SD). Comparisons between different treatment groups were analysed by

one-way ANOVA followed by Tukey's multiple comparison test. In all cases, $P < 0.05$ was selected as the criterion for statistical significance. Graphs were drawn using GraphPad Prism (La Jolla, CA, USA; version 5.0 for Windows).

Materials

The following compounds were supplied as shown: BAY 11-7082 by Adipogene (San Diego, CA); BBG by Sigma Aldrich (Milan, Italy) and sodium pentobarbital by Intervet (Milan, Italy).

Results

Inflammasome blockade and activation of downstream pathways

Diabetic animals exhibited an increased and persistent activation of the NLRP3 inflammasome during the 12 day observation period (Figure 1B; $P < 0.01$ vs. db^{+}/m^{+}). NLRP3 blockade either directly by BAY 11-7082 or via the upstream receptor, by BBG, was confirmed by the blunted protein and mRNA expression in either normoglycaemic and db^{+}/db^{+} mice

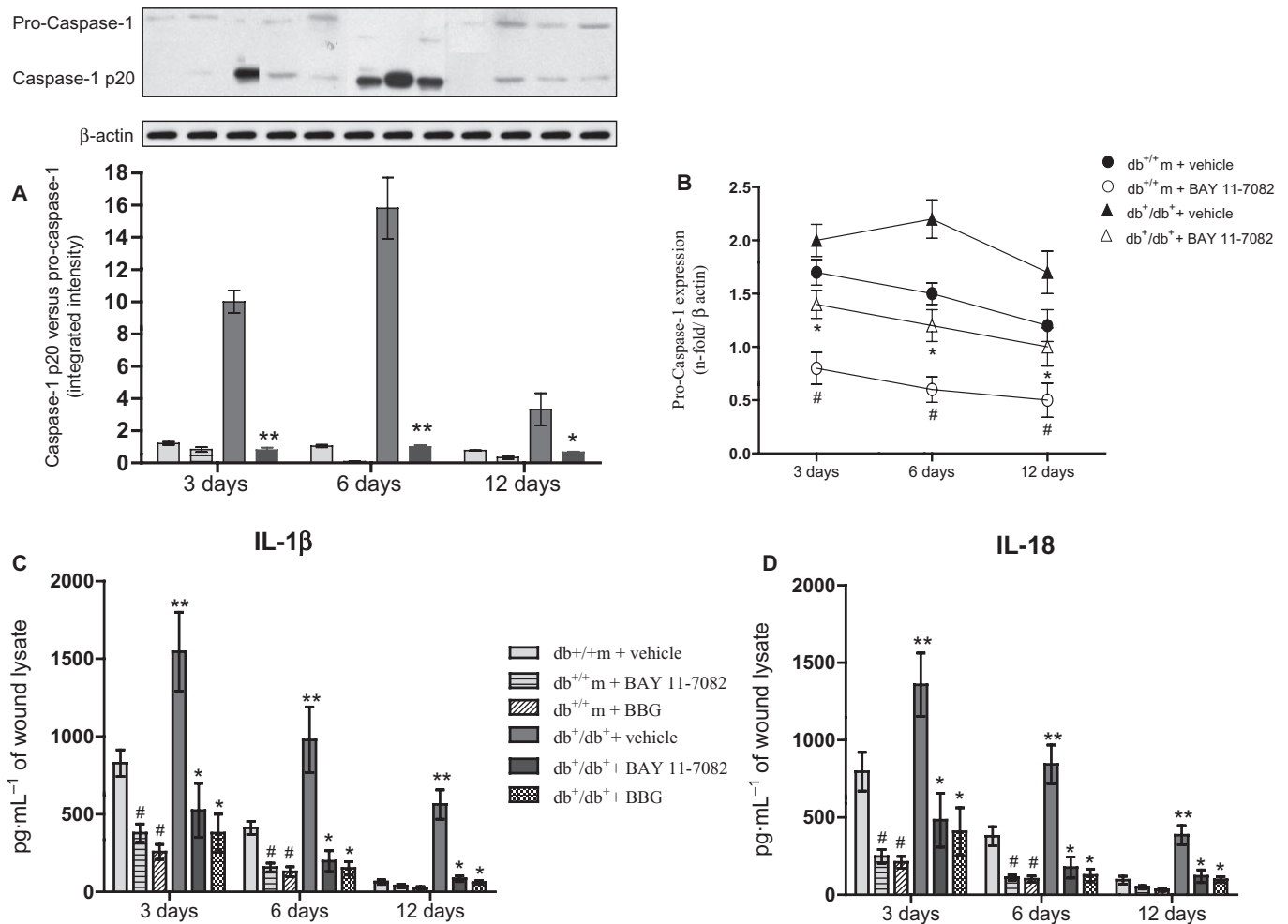


Figure 2

(A) Caspase-1 active protein (p20) versus caspase-1 expression in skin wound samples collected at 3, 6 and 12 days from either normoglycaemic (db^{+}/m^{+}) and diabetic mice (db^{+}/db^{+}) given either vehicle or BAY 11-7082 (20 mg·kg⁻¹ i.p.). Each bar represents the mean \pm SD of seven animals. # $P < 0.01$ versus db^{+}/m^{+} + vehicle; ** $P = 0.0178$ versus db^{+}/db^{+} + vehicle; * $P < 0.05$ versus db^{+}/db^{+} + vehicle. (B) Pro-caspase-1 mRNA expression in skin wound samples collected at 3, 6 and 12 days from either normoglycaemic (db^{+}/m^{+}) and diabetic mice (db^{+}/db^{+}) given either vehicle or BAY 11-7082 (20 mg·kg⁻¹ i.p.). Each bar represents the mean \pm SD of seven animals. # $P < 0.01$ versus db^{+}/m^{+} + vehicle; * $P < 0.05$ versus db^{+}/db^{+} + vehicle. (C) IL-1 β protein levels studied by ELISA in skin wound samples collected at 3, 6 and 12 days from either normoglycaemic (db^{+}/m^{+}) and diabetic mice (db^{+}/db^{+}) given either vehicle, or BAY 11-7082 (20 mg·kg⁻¹ i.p.), or BBG (45.5 mg·kg⁻¹ i.p.). Each bar represents the mean \pm SD of seven animals. # $P < 0.01$ versus db^{+}/m^{+} + vehicle; ** $P < 0.005$ versus db^{+}/m^{+} + vehicle; * $P < 0.05$ versus db^{+}/db^{+} + vehicle. (D) IL-18 protein levels studied by ELISA in skin wound samples collected at 3, 6 and 12 days from either normoglycaemic (db^{+}/m^{+}) and diabetic mice (db^{+}/db^{+}) given either vehicle, or BAY 11-7082 (20 mg·kg⁻¹ i.p.), or BBG (45.5 mg·kg⁻¹ i.p.). Each bar represents the mean \pm SD of seven animals. # $P < 0.01$ versus db^{+}/m^{+} + vehicle; ** $P < 0.005$ versus db^{+}/m^{+} + vehicle; * $P < 0.05$ versus db^{+}/db^{+} + vehicle.

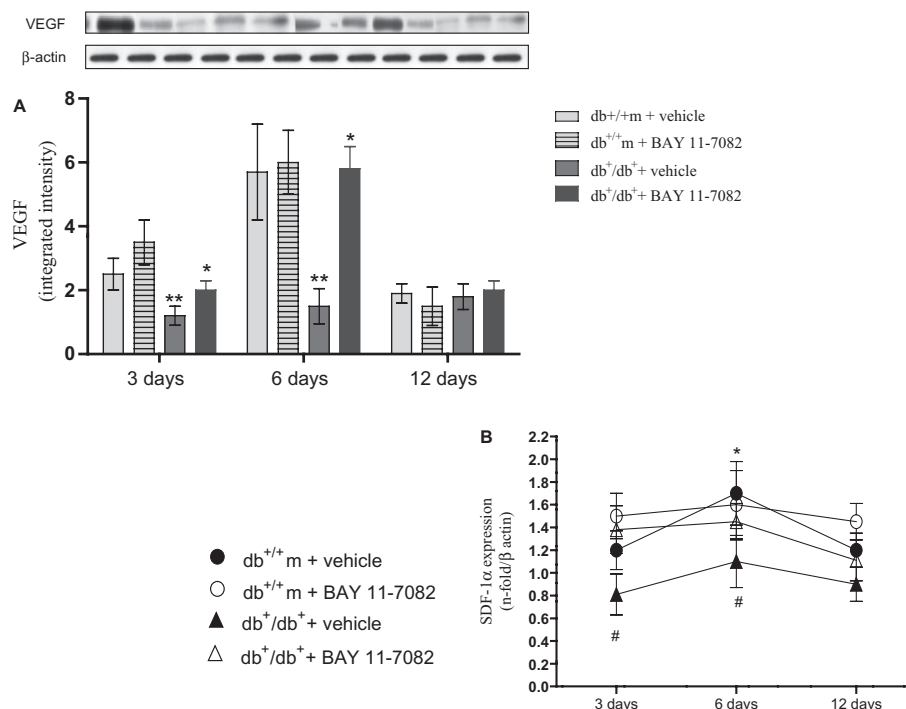


Figure 3

(A) VEGF expression in skin wound samples collected at 3, 6 and 12 days from either normoglycaemic ($db^{+/+}m$) and diabetic mice ($db^{+/+}db^{+/+}$) given either vehicle or BAY 11-7082 (20 mg·kg⁻¹ i.p.). Each bar represents the mean ± SD of seven animals. ** $P < 0.01$ versus $db^{+/+}m$ + vehicle; * $P < 0.05$ versus $db^{+/+}db^{+/+}$ + vehicle. (B) CXCL12 mRNA expression in skin wound samples collected at 3, 6 and 12 days from either normoglycaemic ($db^{+/+}m$) and diabetic mice ($db^{+/+}db^{+/+}$) given either vehicle or BAY 11-7082 (20 mg·kg⁻¹ i.p.). Each bar represents the mean ± SD of seven animals. # $P < 0.01$ versus $db^{+/+}m$ + vehicle; * $P < 0.05$ versus $db^{+/+}db^{+/+}$ + vehicle.

(Figure 1B,C; $P < 0.05$), at all time points. As a direct consequence of NLRP3 blockade, a reduction in ASC activation (Figure 1D) was observed, at all time points. Also p20 caspase-1, which is proteolytically activated from a pro-enzyme, was also significantly reduced in BAY 11-7082-treated diabetic and normoglycaemic animals (Figure 2A, $P < 0.05$). Pro-caspase-1 mRNA expression was also significantly reduced in BAY 11-7082-treated diabetic and normoglycaemic animals (Figure 2B). Raised levels of the active forms of IL-1 β and IL-18 in wound lysates were sustained at 3, 6 and 12 days in $db^{+/+}db^{+/+}$ animals, compared with controls (Figure 2C,D; $P < 0.01$). NLRP3 blockade either by BAY 11-7082 or by BBG markedly reduced both cytokines in wound lysates, at each time point (Figure 2C,D; $P < 0.05$).

VEGF and CXCL12 expression in wounds

In the wounds of non-diabetic animals, high levels of VEGF protein and CXCL12 mRNA (Figure 3A, B respectively) were found, especially at days 3 and 6, with CXCL12 expression remaining high at day 12.

Wounds from diabetic mice showed a markedly reduced expression of CXCL12 mRNA, and protein levels of VEGF, compared with values from non-diabetic mice at days 3 and 6 following wounding. Treatment of diabetic mice with BAY 11-7082 enhanced expression of CXCL12 mRNA and of VEGF protein at days 3 and 6. (Figure 3A, B).

Histology

Figure 4 shows the histological scores (Figure 4A) of all groups, and representative histological pictures (Figure 4B–D) of diabetic animals treated with vehicle, or BAY 11-7082, or BBG at day 12. In normoglycaemic animals, the dermis remodelling and wound closure process were complete (Figure not shown). However, the administration of BAY 11-7082 or BBG further improved wound healing (Figure not shown).

Wounds of diabetic mice treated with vehicle showed poor to mild re-epithelialization at day 12, with only partly organized granulation tissue (Figure 4B). In contrast, moderate to complete re-epithelialization and well-formed granulation tissue were observed in the wounds of diabetic mice following NLRP3 blockade either with BAY 11-7082 or BBG (Figure 4C, D).

Wound closure

Complete wound closure was indicated by a closed linear ridge, with no open areas between stitches. Mice were observed for up to 45 days and complete wound closure was observed at day 22 ± 2 in vehicle-treated normoglycaemic animals, while wounds in diabetic mice closed completely only after 39 ± 3 days (Figure 5). Treatment of diabetic mice with BAY 11-7082 or BBG accelerated wound closure, reducing the time to complete closure to almost normoglycaemic values (Figure 5, $P < 0.001$ vs. vehicle).

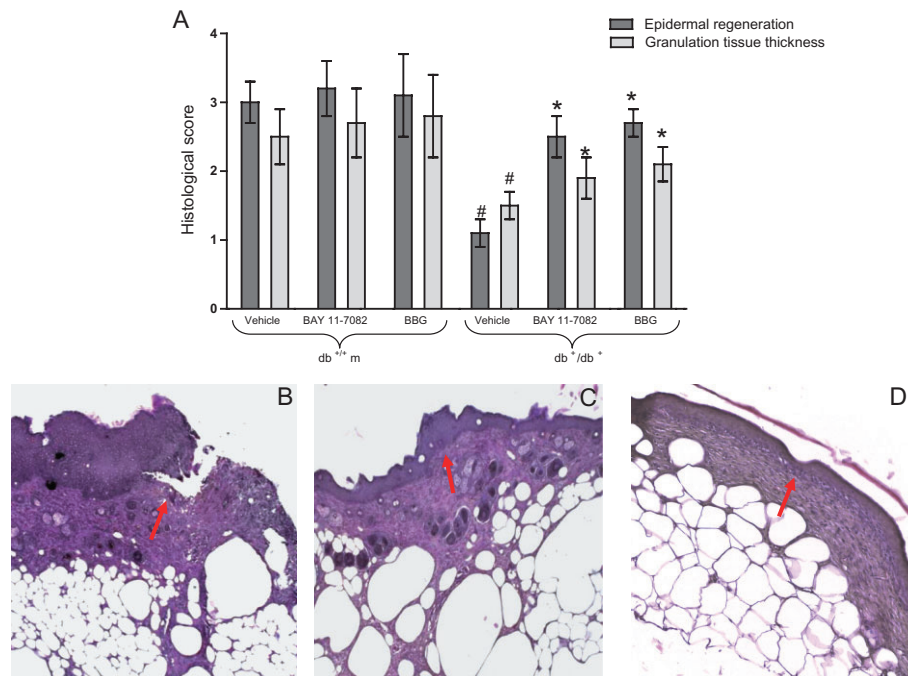


Figure 4

(A) Histological scores in wound samples, collected at 12 days, from either normoglycaemic (db^{+}/m^{+}) and diabetic mice (db^{+}/db^{+}) given either vehicle, or BAY 11-7082 (20 mg·kg⁻¹ i.p.), or BBG (45.5 mg·kg⁻¹ i.p.). Each bar represents the mean \pm SD of seven animals. [#] $P < 0.01$ versus db^{+}/m^{+} ; ^{*} $P < 0.05$ versus db^{+}/db^{+} + vehicle. (B) Histological photomicrographs, haematoxylin-eosin stain, vehicle-treated diabetic (db^{+}/db^{+}) wounds. Incomplete re-epithelialization (see arrow), and poorly formed granulation tissue. (C) Histological photomicrographs, haematoxylin-eosin stain, BAY 11-7082 treated diabetic (db^{+}/db^{+}) wounds. Re-epithelialization is almost complete (see arrow), well-organized granulation tissue and no evidence of inflammation or oedema. (D) Histological photomicrographs, haematoxylin-eosin stain, BBG-treated diabetic (db^{+}/db^{+}) wounds. Re-epithelialization is complete (see arrow), the granulation tissue is well-organized and inflammation is absent.

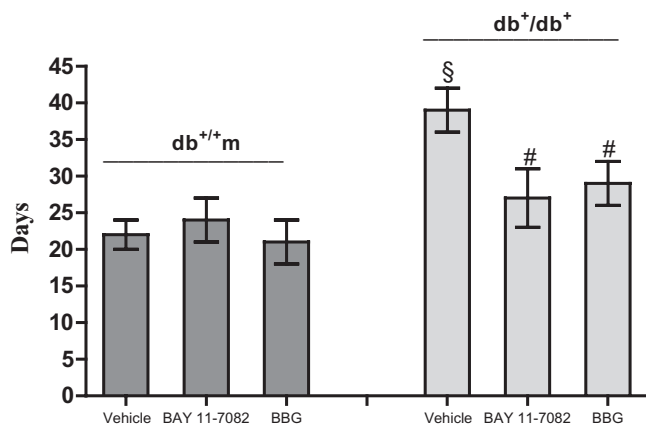


Figure 5

Time to complete the wound closure in normoglycaemic (db^{+}/m^{+}) and diabetic mice (db^{+}/db^{+}) given either vehicle, or BAY 11-7082 (20 mg·kg⁻¹ i.p.), or BBG (45.5 mg·kg⁻¹ i.p.). Each bar represents the mean \pm SD of seven animals. [§] $P < 0.001$ versus db^{+}/m^{+} ; [#] $P < 0.001$ versus db^{+}/db^{+} + vehicle.

Discussion

The structure and function of the skin are maintained at the epidermal surface by an equilibrium between keratinocyte

survival and death, which requires continual regeneration of this tissue. Among the early phases of a wound-healing response is infiltration of immune cells at the wound site (Martin and Leibovich, 2005). The accumulation of DAMPs during chronic inflammatory diseases is thought to contribute to systemic inflammation and disease pathogenesis. During the pathogenesis of type 2 diabetes, the NLRP3 inflammasome has been proposed to sense and mediate downstream inflammatory events of 'glucotoxicity' (Zhou *et al.*, 2010), and thus be responsible for a constant pro-inflammatory status.

Our work clearly demonstrates the efficacy of NLRP3 inflammasome blockade on healing of wounds, via reduced activation of the inflammatory cascade. The I- κ B kinase- β inhibitor, BAY 11-7082, selectively inhibited NLRP3 inflammasome activity in macrophages, independently of its inhibitory effect on NF- κ B activity (Juliana *et al.*, 2010). In our experiments, this inhibitor decreased the inflammatory pathways downstream of the NLRP3 inflammasome and, consequently, improved angiogenesis. However in a complex biological process such as wound healing, it is difficult to completely separate the role of NF- κ B blockade from inflammasome blockade. In order to clarify this point, we used another way of blocking activation of NLRP3 inflammasomes, by blocking its upstream activator, the purinergic P2X7 receptor (Ferrari *et al.*, 2006; Juliana *et al.*, 2010; Zhao *et al.*, 2013). This approach also resulted in inhibition of NLRP3 inflammasomes and in normalisation of the healing in diabetic mice.

In diabetes, the deficiency in neovascularization arises from inadequate VEGF production and release in wounds. Consequently, the normal angiogenic process is delayed and poorly functional. Disturbance of the mechanisms underlying angiogenesis and vasculogenesis, including altered VEGF and CXCL12 expression, has been previously described in *db/db* animals (Nguyen *et al.*, 2010; Bitto *et al.*, 2013). The present findings further strengthen these observations, suggesting that reduced inflammation at the wound site improves both angiogenesis and vasculogenesis, improving, in turn, the healing of wounds in the diabetic mice.

To our knowledge this is the first report that actually demonstrates a clear involvement of the NLRP3 inflammasome and its downstream cascade, in the impaired wound healing in obese-diabetic animals. Increased NLRP3 expression in adipose tissue has been correlated with obesity-associated insulin-resistance in type 2 diabetic patients and in obese mice, with strong caspase-1 auto activation and consequent IL-1 β production in adipose tissue (Vandanmagsar *et al.*, 2011). In agreement with these findings, we found that mice treated with BAY 11-7082 demonstrated a marked reduction in active caspase-1 expression and reduced IL-1 β and IL-18 production, at the wound site. These cytokines were also markedly reduced when the upstream P2X7 receptor was pharmacologically blocked, emphasizing that activation of the NLRP3 inflammasome is one of the mechanisms involved in impaired healing during glucotoxicity.

All these effects normalised skin remodelling, as suggested by the histological data and reduced the time to complete closure, which represents the main outcome for any therapeutic intervention aimed at improving the healing of wounds in diabetics.

In conclusion, results from this study demonstrate for the first time, that direct inflammasome blockade can ameliorate the altered wound healing in diabetic mice, not only by blunting the inflammatory cascade, but also by increasing angiogenesis-related molecules, at least in obese-diabetic animals.

Funding

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Conflict of interest

None of the authors have any conflict of interest.

Significance of the work

The healing of wounds during diabetes is hardly impaired by an exaggerated inflammatory reaction as well as by a reduced expression of angiogenic molecules. The pharmacological blockade of the inflammasome could be a strategy to halt inflammation and increase angiogenesis, thus restoring the healing pattern.

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