Neuroprotectin/protectin D1: endogenous biosynthesis and actions on diabetic macrophages in promoting wound healing and innervation impaired by diabetes

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WOUND HEALING OCCURS IN FOUR separate and overlapping phases: hemostasis, inflammation, proliferation, and remodelling (9, 20). Diabetic wounds are characterized by prolonged chronic inflammation and Mφ inflammatory activities after acute inflammation, and by altered generation of cytokines and lipid mediators (38, 50), which contributes to the impairment of pro-healing functions carried out by macrophages (Mφs) (53, 70). These features contribute to the lack of healing (38, 52, 70) and repair of injured peripheral nerves. The latter results in peripheral neuropathy and insensitivity to injury, and ultimately the poor healing of diabetic wounds such as foot ulcers (18).

Mφs play a critical role in epithelialization and innervation in wound healing, as well as in immune responses. Mφs also produce growth factors, including hepatocyte growth factor (HGF) (7, 73, 80–82), pro-resolving, anti-inflammatory lipid mediators (20, 39, 57, 70), and IL-10 (3, 24, 27, 43). Mφs or monocytes from blood or injury sites convert docosahexaenoic acid (DHA) to pro-resolving lipid mediators such as neuroprotectin/protectin D1 (NPD1/PD1; 10R,17S-dihydroxy-docosa-4Z,7Z,11E,13E,15Z,19Z-hexaenoic acid) (34, 49, 63, 64), maresins (65), and resolvins (64), as well as 14,21-dihydroxy-DHAs (47). NPD1/PD1 stereostructure and potent actions have been thoroughly studied (63). NPD1 promotes resolution of inflammation by regulating leukocyte infiltration, increasing Mφ phagocytosis and evacuation from inflammation site to lymphatic system after efferocytosis (60). NPD1, resolvins D1 (29, 32, 68), and 14,21-diHDHAs (33, 47, 69, 70) accelerate wound healing. In addition, NPD1 also increases corneal nerve recovery and peripheral nerve cell survival (5, 6, 11, 16). Moreover, 14S,21R-diHDHA recovers the reparative functions of diabetic Mφs (70). Therefore, lipid mediators may be partly responsible for the roles of Mφs in wound healing.

These previous findings inspired the three hypotheses in the present study: 1) wounding induces NPD1 biosynthesis in skin, but this process is disturbed by diabetes; 2) Mφs are the key producers of NPD1; and 3) NPD1 promotes Mφs in accelerating diabetic wound healing and nerve regeneration. The putative mechanism involves 1) attenuating inflammation and oxidative stress after acute inflammation (9) in diabetic wounds, and suppressing Mφ inflammatory activities, and 2) increasing the expression of pro-healing molecules. We tested these hypotheses using a combination of a splinted excision wound model on diabetic mice, in vitro assays, and targeted-lipidomic analysis by aqueous reversed-phase chiral liquid chromatogra-
phy with ultraviolet and tandem mass spectrometry (ar chiral LC-UV-MS/MS). The results support our hypotheses.

MATERIALS AND METHODS

Diabetic mouse models. Mice were handled following protocols approved by the IACUC of our institute. We used, Type 2 diabetic db/db (BKS.Cg-m+/+leprdb) and non-diabetic db/+ mice (26 wk old, female). The blood glucose levels were 18–22 mM for db/db mice and 4–6 mM for non-diabetic db/+ mice after 6 h of fasting, as measured in tail blood.

MF isolation and culture. Briefly (28, 70, 83), 3 days after mice were injected with thioglycolate (ip), Mφs were collected by peritoneal lavage and cultured in RPMI 1640 medium containing 25 mM glucose in unprocessed plastic bacterial Petri dishes. More than 95% of adherent cells were F4/80+ Mφs, as our laboratory reported previously (70). NPD1/PD1 was prepared by total organic synthesis previously (70). NPD1/PD1 was prepared by total organic synthesis (Sigma-Aldrich) (5 min at 37°C) (83). The cells were detached by a gentle PBS steam stream formed from precipitate washed with PBS without Ca2+/Mg2+, then resuspended in RPMI 1640, and used as NPD1-treated Mφs; this final Mφ suspension did not contain detectable NPD1. Trypan blue exclusion confirmed that these NPD1-treated Mφs were ≥95% viable.

Mouse splinted excisional wound-healing model and administration of NPD1-treated db/db-Mφs or NPD1. Briefly (71), two full-thickness wounds (5 mm circular) were made symmetrically across the dorsal midline of db/db mice. A donut-shaped silicone splint was adhered around the wound (25). Two days post-wounding (dpw), wounds were either instilled once with 106 cells/wound of NPD1-treated db/db-Mφs or vehicle treated, or from 2 dpw onward, wounds received a daily injection of NPD1 (50 ng/wound) or RPMI 1640 (vehicle control). Injections were applied to the wound bed (10 μl/bed) and intradermally at four points (10 μl/site) distributed evenly near the wound edge (50 μl total/wound).

Wound healing analysis. Skin wounds rimmed with 3-mm edges were analyzed (70). Serial cryosections (10 μm thick) were made through the center zone or region of the widest wound bed on each wound specimen. The section of each wound representing the widest wound bed or the center of the wound will be chosen for the following analysis. The epithelial gap and granulation tissue area were measured from this section selected after H&E staining. The relative epithelial gap was equal to the advancing epithelial gap (advancing edges of migrated keratinocytes/original epithelial gap × 100%). Granulation tissue was new connective tissue and blood vessel capillaries growing from the base of the wound. Collagen deposition in skin sections was analyzed by a Masson's trichrome stain kit (72), where collagen was stained blue.

Skin nerve-fiber density measurement. Nerve fibers were detected in skin cryosections with a rabbit antibody against the nerve-fiber pan-axonal marker, protein gene product 9.5 (PGP9.5), and a secondary antibody (goat anti-rabbit FITC-conjugated IgG) (Millipore, Billerica, MA) (36). Nuclei were stained with Hoechst 33342, and images were acquired using a Zeiss AxioImager-M1 deconvolution microscope. Nerve-fiber density was measured as % PGP9.5-positive area per field, using ImageJ software.

Targeted lipidomics analysis of skin wounds using ar chiral LC-UV-MS/MS. Lipidomics were performed as reported previously (4, 47, 75). Briefly, wounded skin was extracted three times with cold methanol:butylated hydroxytoluene (1:0.005%). Deuterium-labeled internal standards (prostaglandin-D2-d5, and DHA-d5, 5 ng/each, Cayman Chemical, Ann Arbor, MI) were added to each sample. Part of the pooled supernatants for each sample was cleaned up by C18 solid-phase extraction and then analyzed for the targeted lipidomics study using ar chiral LC-UV-MS/MS with a ChiralPak IA column (150 mm long × 2.1 mm inner diameter × 5 μm; Chiral Technologies, West Chester, PA). The analysis of 8-isoprostane was conducted by hydrolysis of the esterified fatty acids in another portion of the extract (4, 35). The procedures were as follows: 1) the lipid extract was suspended in 50 μl of methanol; 2) 8 μl of 1 M sodium hydroxide and 42 μl of H2O were added; 3) the mixture was then incubated at 42°C for 3 h; 4) the pH was adjusted to 4 with 0.05 M HCl, and the aqueous phase was extracted with 2 ml of hexane:isopropanol (3:2 vol/vol); 5) the tube was centrifuged at 3,000 g for 5 min, the organic phase was removed from the top of the aqueous phase, and the procedure was repeated by washing with 1 ml more of solvent mixture; and 6) the organic extracts were dried and resuspended in methanol for LC-UV-MS/MS analysis.

RESULTS

Injury induced NPD1 biosynthesis in wounds, but this was disrupted by diabetes. We tested the first hypothesis by using ar chiral LC-UV-MS/MS to analyze wounds of non-diabetic db/+ and diabetic db/db mice for NPD1 and its biosynthesis marker 17S-HDHA (Fig. 1A). NPD1 was found in wounds at 1, 3, and 7 dpw but not in the uninjured sham skin (Fig. 1B). 17S-HDHA levels were generally several times higher than those of NPD1, consistent with the upstream position of 17S-HDHA in NPD1 biosynthesis (Fig. 1, A and C). NPD1 and 17S-HDHA reached maximal levels at 3 and 7 dpw, respectively, in db/+ wounds, but their kinetics differed in db/db wounds; NPD1 levels increased continuously from 1 to 7 dpw, whereas 17S-HDHA levels dropped at 3 dpw and then increased again. NPD1 levels were lower in diabetic wounds than in non-diabetic wounds at the same time points. This was also the case for 17S-HDHA. In general, injury induced NPD1 biosynthesis in wounds, whereas diabetes suppressed this biosynthesis, consistent with our first hypothesis.

Endogenous NPD1, 17S-HDHA, and 17R-HDHA in wounds were identified based on their ar chiral LC-UV-MS/MS spec-
tra and chromatographic retention times, which matched those of known standards (Fig. 1). For example, the NPD1 from mouse wounds, appearing as peak II in the chromatogram of Fig. 1B, showed MS/MS ions at m/z 359 [M-H-11002]^−, 341 [M-H-11002-H2O]^−, 323 [M-H-11002-H2O2]^−, 315 [M-H-11002-CO2]^−, 297 [M-H-11002-H2O-CO2]^−, and 279 [M-H-11002-H2O2-CO2]^−, consistent with the NPD1 molecular weight (M) of 360 Da (Fig. 1B). The MS/MS ions consistent with the C10 and C17 alcohol-containing positions of NPD1 were observed at m/z 153, 181, 199 [261-CO2-H2O]^−, 217 [261-CO2]^−, 245 [289-CO2]^−, and 261. The triplet bands of the UV spectrum [wavelength of maximum absorbance (λmax) of 271 nm, and two shoulders at 261 and 281 nm] revealed the conjugated-triene structure of NPD1 (Fig. 1B, inset). 17R-HDHA, the epimer of 17S-HDHA, accounted for ~3% of 17S-HDHA (Fig. 1C). Two NPD1 isomers were also found in mouse wounds (Fig. 1B, peak I and III) with LC-UV-MS/MS spectra consistent with those of NPD1 (data not shown).

**Diabetes caused sustained inflammation and oxidative stress after acute inflammation in wounds.** The acute inflammation induced by injury can occur up to 5 dpw. During this phase, the inflammatory cytokines, lipid mediators, neutrophils, and other leukocytes reach maximal levels (9, 20, 48, 58), and then inflammatory factors in the wounds recede to basal levels as the inflammation resolves. However, diabetic wounds show sustained inflammation, which becomes chronic, and resolution of inflammation is impaired (53, 66); this is a major mechanism underlying the observed failure to heal (38, 52, 70). We identified the inflammation marker LTB4 and oxidative stress marker 8-isoprostane by matching their aR chiral LC-UV-MS/MS characteristics to those of known standards. At 7 dpw, after acute wound inflammation (48), higher levels of LTB4 and 8-isoprostane were detected in diabetic than in non-diabetic wounds (Fig. 2, A and B), which was opposite to the trend seen for NPD1 and 17S-HDHA (Fig. 1). The LTB4 extracted from wounds (Fig. 2A) had MS/MS ions m/z 335...
78% in the skin wounds of db/db and 5-hydroxy of LTB4. The LTB4 also had an UV triplet band

A

B

C

D

M̂ reports using similar procedures and doses of CL (23, 40).

no obvious illness or weight loss, consistent with previous

continued consuming food and water normally and showed

some treatment, besides slightly reduced activity, mice

mice with chlondrate liposomes. Following clodronate-lipo-

role of skin-wound M̂

acute inflammation in diabetic wounds.

significantly contributed to inflammation and oxidative stress after

double bands.

typical of a cyclopentyl eicosanoid containing non-conjugated

three hydroxyls, and had a UV singlet with

extracted from wounds (Fig. 2B)


had MS/MS ions

max 270 nm, for its conjugated triene. The 8-isoprostane

with $\lambda_{\text{max}}$ 202 nm, typical of a cyclopentyl eicosanoid containing non-conjugated double bands.

M̂s were the key producers of NPD1 in wounds and significa-

synthesis marker 17S-HDHA also decreased (Fig. 2C). This

finding supported our second hypothesis that M̂s are the key producers of NPD1. Levels of LTB4 and 8-isoprostane at 7 dpw, after acute wound inflammation (9), were also reduced by M̂ depletion in diabetic wounds, suggesting that M̂s were significant contributors to the chronic in-

flammation and oxidative stress occurring after acute in-

flammation in diabetic wounds.

NPD1 accelerated healing and nerve-fiber growth of skin

wounds of diabetic mice. Biosynthesis of NPD1, a lipid medi-

ator known to resolve inflammation and enhance nerve re-

growth in injured corneas (5, 6, 11, 16), was induced by

wounding in skin (Fig. 1). We therefore examined whether

NPD1 could promote wound healing and nerve regrowth in
diabetic mice. After the administration of NPD1 to the wounds

of diabetic mice, HE staining of sections from wounds col-

clected at 7 dpw after depletion by Clodronate liposomes.

Left: percentage of F4/80° M̂s relative to non-depletion in wound

sections. Right: immunohistological images. Data are means ±

SE (n = 5). Significant difference: *P < 0.05; **P < 0.01.
We tested part of our third hypothesis that NPD1 promotes MΦ functions that accelerate diabetic wound healing and nerve-fiber regrowth by treating the db/db-MΦs with or without NPD1 and then injecting these cells into db/db wounds. The injections were performed at 2 dpw, when endogenous MΦs are considerably recruited to wounds (9). The injection suspensions themselves did not contain detectable NPD1, indicating that db/db MΦs metabolized NPD1 during the 24-h incubation. The epithelial gap relative to controls was significantly decreased in the wounds injected with NPD1-treated db/db-MΦs (Fig. 3). Administration of NPD1-treated db/db-MΦs promoted robust increases in granulation tissue area (4.0-fold or 1.2-fold vs. controls or vehicle-treated db/db-MΦs, respectively). Wounds injected with NPD1-treated db/db-MΦs were filled with more collagen, displayed as blue Masson-trichrome stain, and possessed a better organized extracellular matrix compared with control wounds or wounds injected with vehicle-treated db/db-MΦs (Fig. 3). The control wounds showed poor collagen deposition and an immature extracellular matrix. Interestingly, collagen deposition was also increased in the wounds of vehicle-treated db/db-MΦs relative to controls but not in the wounds treated with NPD1 alone. Therefore, better wound healing was obtained by the administration of NPD1-treated

Fig. 3. NPD1 increased MΦ promotion of reepithelialization, granulation tissue formation, and collagen deposition in wounds of diabetic db/db mice. Treatment with NPD1 alone also accelerated the reepithelialization and granulation tissue formation but did not affect collagen level. At 2 dpw of db/db mice, skin excisional wounds were treated with db/db-MΦs, NPD1-treated db/db-MΦs, NPD1, or vehicle control. A: micrographs of HE-stained wound sections. Black bar, epithelial gap; G, granulation tissue. B: relative epithelial gap (top) and granulation tissue area (bottom). C: micrographs of wound sections show Masson-trichrome-stained collagen (blue). D: collagen level (% of granulation tissue area). Original magnification: ×100. Wounds were collected at 7 dpw. Data are means ± SE (n = 8). Significant difference vs. vehicle control: *P < 0.05; **P < 0.01. Significant difference vs. db/db-MΦs: *P < 0.05; **P < 0.01.

NPD1 promoted MΦ functions that accelerate diabetic wound healing and nerve-fiber regrowth. We tested part of our third hypothesis that NPD1 promotes MΦ functions that accelerate diabetic wound healing and nerve-fiber regrowth by treating the db/db-MΦs with or without NPD1 and then injecting these cells into db/db wounds. The injections were performed at 2 dpw, when endogenous MΦs are considerably

Fig. 4. NPD1 treatment of wounds of diabetic mice promoted nerve fiber regrowth. NPD1 also enhanced neurotrophic function of MΦs in diabetic wounds. The treatment of excisional wounds of db/db diabetic mice was the same as described in Fig. 3. A: micrographs of cryosections of wounds collected at 7 and 26 dpw show PGP9.5 nerve fibers (white). B: relative density (%) of nerve fiber area in wounds. Original magnification, ×200; scale bar, 100 μm. Nuclei (dark gray) were stained with Hoechst 33342. Data are means ± SE (n = 8). Significant difference vs. vehicle control: *P < 0.05; **P < 0.01. Significant difference vs. db/db-MΦs: #P < 0.05; ##P < 0.01. ‡Significant difference vs. 7 dpw (P < 0.05).
db/db-MΦs compared with the administration of vehicle-treated db/db-MΦs or vehicle control. Higher densities of PGP9.5+ nerve fibers were found in the wounds of diabetic db/db mice injected with NPD1-treated db/db-MΦs than with db/db-MΦs or vehicle control at 7 dpw (7.7% vs. 5.1% or 3.3%) and at 26 dpw (10.3% vs. 5.7 or 4.8%) (Fig. 4). PGP9.5+ nerve-fiber densities increased for all three groups from 7 to 26 dpw, but, interestingly, a significant increase was only observed for control and NPD1-treated db/db-MΦs. These results indicate that NPD1 augments MΦ neurotrophic functions in diabetic wounds. Together, these data support our third hypothesis proposing that NPD1 promotes MΦ pro-healing activities and neurotrophic functions in diabetic wounds.

Mechanistic insights: actions of NPD1 and NPD1-treated MΦs involve attenuation of chronic inflammation and oxidative stress, and promotion of MΦ production of pro-healing factors. We then tested the part of our third hypothesis. The NPD1-treatment reduced wound levels of TNF-α, LTB4, and 8-isoprostanate at 7 dpw by 48%, 22%, and 70%, respectively, compared with the control (Fig. 5). The NPD1-treated db/db-MΦs also reduced levels of TNF-α, LTB4, and 8-isoprostanate by 74%, 45%, and 69%, respectively, compared with the control. Administration of NPD1 or the NPD1-treated db/db-MΦs increased IL-10 in diabetic wounds by 136% or 243%, respectively, compared with the administration of vehicle-treated db/db-MΦs. These results demonstrate for the first time that NPD1 and activation of MΦs by NPD1 are among these factors. MΦs and injured tissue are known to produce NPD1 (34, 49, 63), and our data show that the NPD1 formation is induced in wounded skin. However, diabetes disturbed NPD1 kinetics, as indicated by lower levels of NPD1 at 1, 3, or 7 dpw. A similar finding was reported in adipose tissue, adjacent to injury sites, of diabetic patients who had lower levels of NPD1 and 17S-HDHA than found in non-diabetic controls (15), suggesting the operation of a similar mechanism involving NPD1 deficiency (53). The investigation of the effects of NPD1 on db/db-MΦs cultured in a simulated diabetic wound microenvironment (hypoxia, LPS-stimulation, and high glucose). NPD1 markedly promoted HGF production by db/db-MΦs in a dose-dependent manner (Fig. 6A). At 20 nM, NPD1 increased the production of anti-inflammatory, pro-healing IL-10 by ~1.3-fold in db/db-MΦs (Fig. 6B). It also reduced production of inflammatory TNF-α by 0.5-fold in db/db-MΦs (Fig. 6C). NPD1 suppressed production of the inflammatory lipid mediators LTB4 and TXA2 (detected as TXB2) in a dose-dependent manner in db/db-MΦs. Treatment with 200 nM NPD1 decreased production of TXB2 by 72% and LTB4 by 54%, as well as 8-isoprostanate by 65%. Therefore, NPD1 treatment reduced the inflammatory and oxidative activities of db/db-MΦs (Fig. 6, D–F). These results demonstrate for the first time that NPD1 and activation of MΦs by NPD1 are among these factors. MΦs and injured tissue are known to produce NPD1 (34, 49, 63), and our data show that the NPD1 formation is induced in wounded skin. However, diabetes disturbed NPD1 kinetics, as indicated by lower levels of NPD1 at 1, 3, or 7 dpw. A similar finding was reported in adipose tissue, adjacent to injury sites, of diabetic patients who had lower levels of NPD1 and 17S-HDHA than found in non-diabetic controls (15), suggesting the operation of a similar mechanism involving NPD1 deficiency (53). The

DISCUSSION

Diabetes impairs the formation or actions of reparative factors, including those involving MΦs, leading to the delay or failure of wound healing (8, 38, 52, 70, 78). This study demonstrates for the first time that NPD1 and activation of MΦs by NPD1 are among these factors. MΦs and injured tissue are known to produce NPD1 (34, 49, 63), and our data show that the NPD1 formation is induced in wounded skin. However, diabetes disturbed NPD1 kinetics, as indicated by lower levels of NPD1 at 1, 3, or 7 dpw. A similar finding was reported in adipose tissue, adjacent to injury sites, of diabetic patients who had lower levels of NPD1 and 17S-HDHA than found in non-diabetic controls (15), suggesting the operation of a similar mechanism involving NPD1 deficiency (53). The
present finding that NPD1 levels in mouse wounds were diminished after MΦs depletion suggests that MΦs are the key cells involved in NPD1 generation in wounds.

The promotion of diabetic wound healing by NPD1 suggests that the wound-induced formation of NPD1 is a reparative response of the skin. NPD1 is potently anti-inflammatory and pro-resolving (63), and clearly reduced chronic inflammation in diabetic wounds after acute inflammation (Fig. 5). There are multiple types of intracellular mechanisms that could underlie NPD1 promotion of reparative functions of diabetic MΦs in addition to the involvement of the expression of IL-10 and HGF and the suppression of chronic inflammation and oxidative stress we observed in this report. MΦ efferocytosis of apoptotic cells may be involved. NPD1 enhances the MΦ phagocytosis capability (60, 62), and the MΦ engulfment of apoptotic cells leads to the production of HGF with concomitant activation of PI3K, MAPK, and Rho A pathways (54, 55). MΦ efferocytosis also induces microRNA-21 by silencing PTEN and GSK3β, triggers release of IL-10, and suppresses LPS-induced TNF-α expression (17). NPD1 actions on diabetic MΦs may include the activation of peroxisome proliferator-activated receptor (PPAR) γ. NPD1 upregulates PPARγ transcriptional activity in primary human neuronal-glial cells (84). It is likely that such activation could also take place in MΦs. The PPARγ pathway regulates MΦ increased expression and secretion of both IL-10 (1, 14, 59) and HGF (19, 44). Alternatively, NPD1 could have restored diabetic MΦ pro-healing functions by activating/phosphorylating the mTOR, AKT, and/or p70S6K, similar to the mechanism described in retinal pigment epithelial cells and neutrophils under oxidative stress (21, 26, 30, 31). The mTOR regulates cell growth and cell proliferation (26, 31), AKT signaling is essential in shifting inflammatory MΦ phenotype to anti-inflammatory phenotype (10), and p70S6K are associated with cell movement in the phagocytic process (41). These pathways may underlie the NPD1 rescue of the pro-healing functions of diabetic MΦs and should be systematically investigated in the future.

In this report, we found that NPD1 accelerates wound healing and promotes the pro-healing function of MΦs by enhancing wound epithelialization and granulation tissue formation similar to our previous reports utilizing 145,21R-diHDHA (69, 70), and by promoting collagen deposition and regrowth of nerve fibers. Interestingly, NPD1 appears to be more potent than 145,21R-diHDHA in inducing the increase of granulation area by db/db MΦs (70). DHA-derived pro-resolving lipid mediators are likely to act in concert to regulate wound healing when they are generated together in the body or administered together. For example, both NPD1 and 145,21R-diHDHA are induced in wounds (69), and they could act together in promoting the healing. We should comprehensively assay the efficacies of various prohealing lipid mediators, including NPD1 and 145,21R-diHDHA, under the same experimental conditions in the future for therapeutic intervention.

The promotion of innervation in diabetic wounds by NPD1 treatment, as shown here, demonstrates a further neurotrophic property of NPD1 in rescuing diabetes-damaged peripheral nerve fibers in skin. This is consistent with previously observed enhancement of innervation in corneal injury by NPD1 (16). The functional outcome of NPD1-promoted innervation in diabetic wounds warrants further study.

We observed that the presence of diabetes also corresponded to a greater degree of chronic inflammation and oxidative stress in wounds after acute inflammation, as indicated by higher levels of inflammatory TNF-α and LTB4 (13, 56, 67), and of the oxidative stress marker 8-isoprostane (22, 46, 79) at 7 dpw (Figs. 2, A and B, and 5). Depletion of MΦs in diabetic wounds suppressed the levels of these molecules after acute inflammation (Fig. 2C), confirming previous reports that endogenous diabetic MΦs contribute significantly to chronic inflammation and oxidative stress in diabetic wounds (39). Thus MΦs are a “double-edge sword”; they can show impaired pro-healing functions or can even deteriorate healing if not well regulated, as happens in diabetic wounds. In diabetic mice, MΦs display dysregulated production of growth factors and lipid mediators, which contributes to impairment of diabetic wound healing (22, 70). Therefore, the treatment of diabetic wounds should aim at restoring MΦ pro-healing functions as a potential strategy for achieving adequate healing, as suggested by the findings reported here.

As observed by other researchers (50) and our laboratory previously (70), administration of exogenous db/db-MΦs to wounds at 2 dpw also improved wound healing (Fig. 3). The MΦ density in wounds of db/db mice at 2 dpw is lower compared with non-diabetic db/+ mice as reported previously (76, 77). Adding exogenous db/db-MΦs supplemented the MΦ deficit in db/db wounds, which might contribute to the improvement of db/db wound healing. The db/db-MΦs administrated to the wounds at 2 dpw, a time point of the acute inflammatory phase of healing, are likely to directly act on non-inflammatory aspects of the early phase of wound healing. They did not significantly increase the levels of TNF-α or oxidative stress marker 8-isoprostane but increased the level of inflammation marker LTB4 in db/db wounds after the acute inflammatory phase (at 7 dpw) (Fig. 5). It is reported that MΦs derived from bone marrow cells of db/db mice cross-bred with C57BL/6 GFP-transgenic mice increased TNF-α level in wounds at 5 dpw when these MΦs were transplanted to wounds at 3 dpw (2). Different animal strains, organs, timing of MΦ transplantation, and/or healing phases might contribute to this macrophase difference across the pro-inflammatory marker expression. The db/db MΦs used in this manuscript are likely to consist of diversified populations, including M1-like and M2-like phenotypes (38, 45). The db/db MΦs without incubation with NPD1 can still produce reparative HGF and IL-10 (Fig. 6) (38) as well as VEGF (50, 70), although at lower levels compared with db/db MΦs incubated with NPD1, which might contribute to the db/db-MΦ improvement of wound healing.

NPD1 reduced inflammatory and oxidative activities, as manifested by dampened production of TNF-α, LTB4, TXB2, and 8-isoprostane in db/db-MΦs treated in vitro with NPD1 (Fig. 6). Endogenous diabetic MΦs also contributed substantially to chronic inflammation and oxidative stress in diabetic wounds (Fig. 2C) (39). Therefore, the reduction of chronic inflammation and oxidative stress in diabetic wounds by NPD1 or NPD1-treated MΦs at 7 dpw after acute wound inflammation could largely result from the attenuation of these inflammatory, oxidative activities of MΦs. NPD1 promoted healing, at least in part, by reducing chronic inflammation due to a MΦ-associated mechanism.

Treatment of db/db-MΦs with NPD1 increased the secretion of HGF, a factor that regulates the major cellular processes
responsible for wound repair, including reepithelialization and collagen deposition (20, 39, 57, 70). It also increased secretion of anti-inflammatory, pro-healing cytokine IL-10 from db/db-MΦs (38). These observations suggest that the mechanism by which NPD1 promotes MΦ reparative functions in diabetic wounds also includes the promotion of MΦ paracrine/autocrine functions, such as the production of HGF and IL-10, which can promote the pro-healing functions of neighboring cells or of the MΦs themselves (39). NPD1 alone did not affect collagen deposition; therefore, it is possible that NPD1 alone, in our experimental conditions, was insufficient in activating endogenous MΦs for collagen deposition, whereas in vitro pretreatment with NPD1 did. This implies that, in regard to collagen deposition, exogenous MΦs respond better than endogenous MΦs to activation by NPD1, suggesting that the microenvironment during the activation might affect the outcome, since there are many other cells and extracellular molecules surrounding MΦs in vivo, whereas there are only MΦs and limited molecular species in the medium in vitro. These results could indicate that, in diabetic skin wounds, there is a potential therapeutic advantage to using NPD1-treated MΦs over using NPD1 directly. The findings of NPD-treated db/db MΦs in this report are of translational significance for therapeutic intervention on diabetic wound treatment. However, their implication for endogenous wound db/db-MΦs needs to be systematically confirmed in the future.

The bioactions and mechanisms for NPD1 in diabetic wound healing were not studied previously. Our new data advances the field as follows over what was already published. First, they unbiasedly show, with full-scan MS/MS and UV spectra and chiral LC chromatograms, the existence of endogenous NPD1 in mouse wounds and the diabetic diminishment of NPD1 in the wounds. The NPD1 deficiency in diabetic wounds could be, at least partly, responsible for the diabetic impairment of skin wound healing. Second, they provide the time courses of NPD1 in diabetic and non-diabetic wounds, which are important to the field in studying NPD1-related mechanisms and therapeutic intervention in diabetic wound healing. These results are consistent with the recent discovery by Claira et al. (15) that NPD1 is a chiral molecule, the existence of endogenous NPD1 in vivo, whereas there are only NPD1 and limited molecular species in the medium in vitro. These results could indicate that, in diabetic skin wounds, there is a potential therapeutic advantage to using NPD1-treated MΦs over using NPD1 directly. The findings of NPD-treated db/db MΦs in this report are of translational significance for therapeutic intervention on diabetic wound treatment. However, their implication for endogenous wound db/db-MΦs needs to be systematically confirmed in the future.

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