Proresolution Therapy for the Treatment of Delayed Healing of Diabetic Wounds

Yunan Tang,[1] Michael J. Zhang,[1] Jason Hellmann,[1] Madhavi Kosuri,[1] Aruni Bhatnagar,[1,2] and Matthew Spite[1,2,3]

Obesity and type 2 diabetes are emerging global epidemics associated with chronic, low-grade inflammation. A characteristic feature of type 2 diabetes is delayed wound healing, which increases the risk of recurrent infections, tissue necrosis, and limb amputation. In health, inflammation is actively resolved by endogenous mediators, such as the resolvins. D-series resolvins are generated from docosahexaenoic acid (DHA) and promote macrophage-mediated clearance of microbes and apoptotic cells. However, it is not clear how type 2 diabetes affects the resolution of inflammation. Here, we report that resolution of acute peritonitis is delayed in obese diabetic (*db/db*) mice. Altered resolution was associated with decreased apoptotic cell and Fc receptor–mediated macrophage clearance. Treatment with resolvin D1 (RvD1) enhanced resolution of peritonitis, decreased accumulation of apoptotic thymocytes in diabetic mice, and stimulated diabetic macrophage phagocytosis. Conversion of DHA to monohydroxydocosanoids, markers of resolvin biosynthesis, was attenuated in diabetic wounds, and local application of RvD1 accelerated wound closure and decreased accumulation of apoptotic cells and macrophages in the wounds. These findings support the notion that diabetes impairs resolution of wound healing and demonstrate that stimulating resolution with proresolving lipid mediators could be a novel approach to treating chronic, nonhealing wounds in patients with diabetes. ***Diabetes*** **62:618–627, 2013**

Type 2 diabetes is a major health problem reaching epidemic proportions in developed countries, and its incidence is rapidly increasing in developing countries as well (1). It is a significant cause of mortality and is strongly associated with an increase in the risk of cardiovascular disease and cancer (2,3). Extensive research has shown that such clinical complications of type 2 diabetes could be attributed, in part, to low-grade chronic inflammation (4,5). Before the development of type 2 diabetes, changes in the metabolism of glucose and fatty acids activate innate immune responses that give rise to systemic insulin resistance, which in turn perpetuates and establishes a state of chronic inflammation (5). As a result, type 2 diabetes is associated with tissue dysfunction and injury, deficiencies in clearing microbial infections, and impaired wound healing (6).

From the [1]Diabetes and Obesity Center, University of Louisville School of Medicine, Louisville, Kentucky; the [2]Department of Medicine, Division of Cardiovascular Medicine, University of Louisville School of Medicine, Louisville, Kentucky; and the [3]Department of Microbiology and Immunology, University of Louisville School of Medicine, Louisville, Kentucky.
Corresponding author: Matthew Spite, matthew.spite@louisville.edu.
Received 24 May 2012 and accepted 5 August 2012.
DOI: 10.2337/db12-0684
Y.T., M.J.Z., and J.H. contributed equally to this work.

Normally, inflammation protects against infection and injury but must be resolved to prevent inadvertent tissue damage (7,8). The resolution of inflammation is accomplished by time- and site-specific generation of proresolving mediators that control both the magnitude and the duration of the inflammatory response (7,9). Among the mediators of resolution, resolvins have emerged as critical players that exert potent anti-inflammatory actions by blunting excessive polymorphonuclear neutrophil (PMN) infiltration into tissues and decreasing proinflammatory mediator production (7,10). Unlike other anti-inflammatory mediators that suppress inflammation, resolvins also promote macrophage phagocytosis of apoptotic cells and microbes and stimulate the clearance of phagocytes to enable return to homeostasis (7,11,12). These events are critical for resolution of inflammation because lingering apoptotic cells or phagocytes can undergo secondary necrosis causing unwarranted tissue damage (13,14). Hence, disruption of these endogenous pathways of resolution could give rise to chronic inflammation in type 2 diabetes. Nevertheless, diabetes-induced changes in the resolution of inflammation have not been extensively studied. Recent studies from our group and others have shown that proresolving mediators such as resolvins decrease adipose tissue inflammation and improve insulin sensitivity in murine models of type 2 diabetes (15,16). However, it is not known whether type 2 diabetes affects the resolution of inflammation and whether treatment with proresolving mediators would stimulate resolution and ameliorate clinical complications of type 2 diabetes, such as impaired wound healing.

RESEARCH DESIGN AND METHODS

Animals and reagents. Leptin receptor–deficient male mice [B6.BKS(D)-Lepr^{db}/J; *db/db*] and their wild-type controls (C57BL/6J) were purchased from The Jackson Laboratory at 8–12 weeks of age and maintained on normal chow. All procedures were approved by University of Louisville's Institutional Animal Care and Use Committee (no. 11017). Resolvin D1 (RvD1) (7*S*,8*R*,17*S*-trihydroxy-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-docosahexaenoic acid) was purchased from Cayman Chemical (Ann Arbor, MI). The mouse anti-Fpr2 antibody was purchased from Santa Cruz Biotechnology.

Wound healing model and treatment. For studying wound healing, mice were anesthetized by isoflurane inhalation and their dorsal skin was shaved and treated with depilatory cream. The skin was then rinsed, and two circular, full-thickness wounds (skin and panniculus carnosus) were created on the dorsal skin using a 5-mm skin biopsy punch. A donut-shaped silicone splint with an inner diameter of 7 mm was placed so that the wound was centered within the splint (17). The splint was affixed to the skin using Krazy Glue, followed by six interrupted 6-0 nylon sutures. Each wound was covered with a semipermeable polyurethane dressing, which was changed every other day. The splint was fixed around the wound to prevent skin contraction and to allow wounds to heal through reepithelialization and granulation (17). Twenty-four hours after creation of the wound, RvD1 (100 ng/50 μL) was applied to the wound bed (10 μL) and injected intradermally at four points (10 μL/site) every day. Photographic images were analyzed using ImageJ software by tracing the wound margin and calculating the pixel area. For comparing wound healing of wild-type (WT) and *db/db* mice without treatment, the splint was not applied, and the wounds were monitored until day 14.

Histology and immunohistochemistry. Wound tissues were formalin fixed, paraffin embedded, and sectioned. For analysis of apoptotic cells, macrophages, and Fpr2 expression, wounds were harvested at day 5, while granulation tissue formation was assessed at day 27. Deparaffinized sections were stained with hematoxylin-eosin. Wound macrophages were visualized by immunofluorescence microscopy using 5-μm-thick sections. For this, sections were blocked with Rodent Block M Blocking Reagent for 1 h after antigen retrieval and incubated with Alexa-Fluor 647–conjugated anti-CD68 (1:200) for 2 h. Slides were mounted with *SlowFade* Gold antifade reagent with DAPI. Fluorescent photographs were obtained using an EVOS fluorescence microscope. $CD68^+$ cells were quantified in five random fields in all animals. The monitoring of apoptotic cells in wounds was performed using the DeadEnd Colorimetric TUNEL System kit (Promega, Madison, WI). Staining for Fpr2 was performed in wounds using an immunoenzyme method (horseradish peroxidase). Negative staining control experiments were performed by omitting the primary antibody.

Measurement of docosahexaenoic acid metabolites in wounds by liquid chromatography–tandem mass spectrometry. Wound tissue was collected from WT and *db/db* mice at day 5 postwounding and immediately homogenized in ice-cold methanol containing deuterated internal standard (1 ng PGE_2-d4), as well as butylated hydroxytoluene to prevent nonenzymatic oxidation during sample preparation. Samples were subjected to solid-phase (C18) extraction (18). Methyl formate fractions were taken to dryness with N_2 gas and resuspended in methanol. Analysis by liquid chromatography–tandem mass spectrometry was performed using a high-performance liquid chromatography system equipped with a Shimadzu C18 column (4.6 mm × 50 mm × 5.0 μm) coupled to a triple quadrupole mass spectrometer (API 2000; Applied Biosystems/Sciex). The instrument was operated in negative ionization mode, and the mobile phase consisted of methanol/water/acetic acid (60:40:0.01, vol/vol/vol) and was ramped to 80:20:0.01 over 3 min and to 95:5:0.01 in the next 14 min at a flow rate of 400 μL/min. The mobile phase was then ramped to 100:0:0.01 over the next 3 min before returning to 60:40:0.01. Free, unesterified docosahexaenoic acid (DHA) and its downstream metabolites were identified by multiple-reaction monitoring (MRM) using established ion pairs for DHA (327 > 283), 17-hydroxyDHA (17-HDHA; 343 > 147), 14-HDHA (343 > 161), and 4-HDHA (343 > 101) (18). The metabolites were quantified by using external calibration curves constructed for each compound using authentic standards (Cayman Chemical). Recoveries were calculated relative to deuterium-labeled internal standard.

Analysis of thymocyte apoptosis. WT and *db/db* mice were treated with dexamethasone (15 mg · kg^{-1} body wt i.p.; in sterile saline) (19). Vehicle (0.1% ethanol in sterile saline) or RvD1 (1 μg) was administered by retroorbital injection initially and 4 h later. Six hours after dexamethasone treatment, the mice were killed and the thymus was collected and formalin fixed. Sections of the tissue were stained using the DeadEnd Colorimetric TUNEL System kit (Promega). The transferase-mediated dUTP nick-end labeling (TUNEL)-positive area was quantified in five random fields per animal using MetaMorph software.

Acute peritonitis. Peritonitis was initiated in WT and *db/db* mice by intraperitoneal administration of zymosan A (0.04 mg · g^{-1} body wt) (12). At 6, 24, or 48 h post-zymosan challenge, mice were killed and the peritoneum was lavaged with Dulbecco's PBS $(DPBS)^{-/-}$. Peritoneal exudates were enumerated by light microscopy, and total leukocyte counts were determined by trypan blue exclusion. Leukocyte populations and apoptotic cells were identified by flow cytometry. For this, peritoneal exudates were suspended in fluorescence-activated cell sorter buffer (1% FBS in PBS) and incubated with Fc Block for 10 min at 4°C. Cells were then stained with fluorescein isothiocyanate (FITC)–conjugated anti-F4/80 and phycoethrin-conjugated anti-Ly6G or appropriate isotype controls for 30 min at 4°C. Flow cytometry analysis was performed using a BD LSRII cytometer equipped with FACSDiva v.6.0. Data were analyzed using the FlowJo V.7.6 software. In selected experiments, FITC–zymosan was instilled in the peritoneum to assess zymosan clearance. Animals were administered zymosan (0.04 mg · g^{-1}) with FITC–zymosan tracer (0.002 mg · g^{-1}) in a 1-mL total volume of sterile saline, and exudates were collected at 72 h post-zymosan challenge. Macrophages were assessed by staining with F4/80 as above, and free FITC–zymosan was determined in parallel by flow cytometry.

Macrophage phagocytosis. Peritoneal macrophages were isolated from WT and *db/db* mice and allowed to adhere to cell culture plates in $DPBS^{+/+}$ (4×10^5 cells/well) for 1 h (20). Macrophages were incubated with FITC-labeled IgG-opsonized zymosan (1×10^6 particles per well) for 60 min at 37°C. Trypan blue (0.025%) was used to quench extracellular fluorescence. Phagocytosis was quantified using a fluorescent microplate reader. To investigate whether RvD1 affects phagocytosis, peritoneal macrophages isolated from *db/db* mice were treated with RvD1 (0.1 nmol/L) for 15 min before the addition of opsonized zymosan. In some experiments, Fpr2 antagonist, WRW4 (10 μmol/L), was added 15 min before RvD1. For testing the involvement of phosphatidylinositol 3-kinase (PI3K) signaling in RvD1-induced changes in macrophage phagocytosis, macrophages were pretreated with wortmannin (200 nmol/L) for 5 min before RvD1 addition.

Measurement of cAMP. Resident peritoneal macrophages were isolated from *db/db* mice and plated onto 24-well plates at a density of 7.5×10^5 cells/well for 1 h in $DPBS^{+/+}$. After washing, cells were stimulated with vehicle (0.1% ethanol in DPBS) or RvD1 (1 nmol/L) for 15 min. Cells were then lysed with 0.1 mol/L HCl for 20 min at room temperature, and cAMP levels were determined by ELISA (Cayman chemical).

Statistical analysis. Data are means ± SEM. Multiple groups were compared using one-way or two-way ANOVA where appropriate, followed by Bonferroni posttests. For direct comparisons, we used an unpaired, two-tailed Student *t* test. In all cases, a P value of <0.05 was considered significant.

RESULTS

To assess the impact of type 2 diabetes on cellular events involved in the development and resolution of inflammation, we examined the time course of leukocyte infiltration in acute peritonitis using WT and *db/db* mice. Despite having elevated resident peritoneal leukocytes (Fig. 1*A*), the extent of leukocyte infiltration in response to zymosan challenge was not different between WT and *db/db* mice after either 6 or 24 h (Fig. 1*B*). However, in WT mice, the number of total leukocytes decreased between 24 and 48 h, whereas leukocyte levels remained elevated in *db/db* mice. Our flow cytometric analyses of the leukocyte differential revealed that PMNs were rapidly cleared between 24 and 48 h in WT but not *db/db* mice (Fig. 1*C* and *D*). However, we found no significant differences in macrophage infiltration during the time course of acute peritonitis (Fig. 1*E*). As macrophage-mediated clearance of apoptotic PMN is a defining feature of active resolution (9), we assessed whether this process was altered in *db/db* mice. As shown in Fig. 1*F*, there was a greater accumulation of annexin V^+ apoptotic PMN 48 h after zymosan challenge in *db/db* mice than in WT mice, indicating that diabetes alters macrophage-mediated clearance of apoptotic PMN. Consistent with defective macrophage phagocytosis, 72 h after zymosan challenge, we found higher levels of FITC-labeled zymosan in the peritoneum of *db/db* mice relative to WT mice, despite no significant differences in total macrophage content (Fig. 1*G*).

Because proresolving mediators such as the resolvins have been shown to stimulate macrophage-mediated clearance of apoptotic PMN and microbes, we next assessed whether these defects could be corrected by stimulating resolution with the prototypic proresolving mediator RvD1. To this end, we challenged *db/db* mice with zymosan and allowed PMN to infiltrate to maximal levels. We then administered RvD1 at 32 h post-zymosan challenge and collected inflammatory exudates 16 h later (48 h after zymosan challenge; see scheme in Fig. 2*A*). We found that RvD1 administration consistently decreased the amount of PMN remaining in the peritoneum relative to vehicle-treated mice, indicating that RvD1 enhanced the clearance of these cells. As an additional test of our hypothesis that stimulating resolution improves macrophage phagocytosis in vivo, we assessed the clearance of apoptotic thymocytes in response to dexamethasone administration (19). As shown in Fig. 2*B*, apoptotic thymocytes accumulated to a significantly higher level in *db/db* mice than WT mice and coadministration of RvD1 markedly reduced the accumulation of apoptotic thymocytes in *db/db* mice. Thus, collectively, these observations suggest that treatment with RvD1 enhances the clearance of apoptotic cells by macrophages and thus restores defective resolution of inflammation.

Previous studies have shown that phagocytosis of apoptotic cells and opsonized microbes is impaired in macrophages isolated from diabetic humans and rodents (21–24).

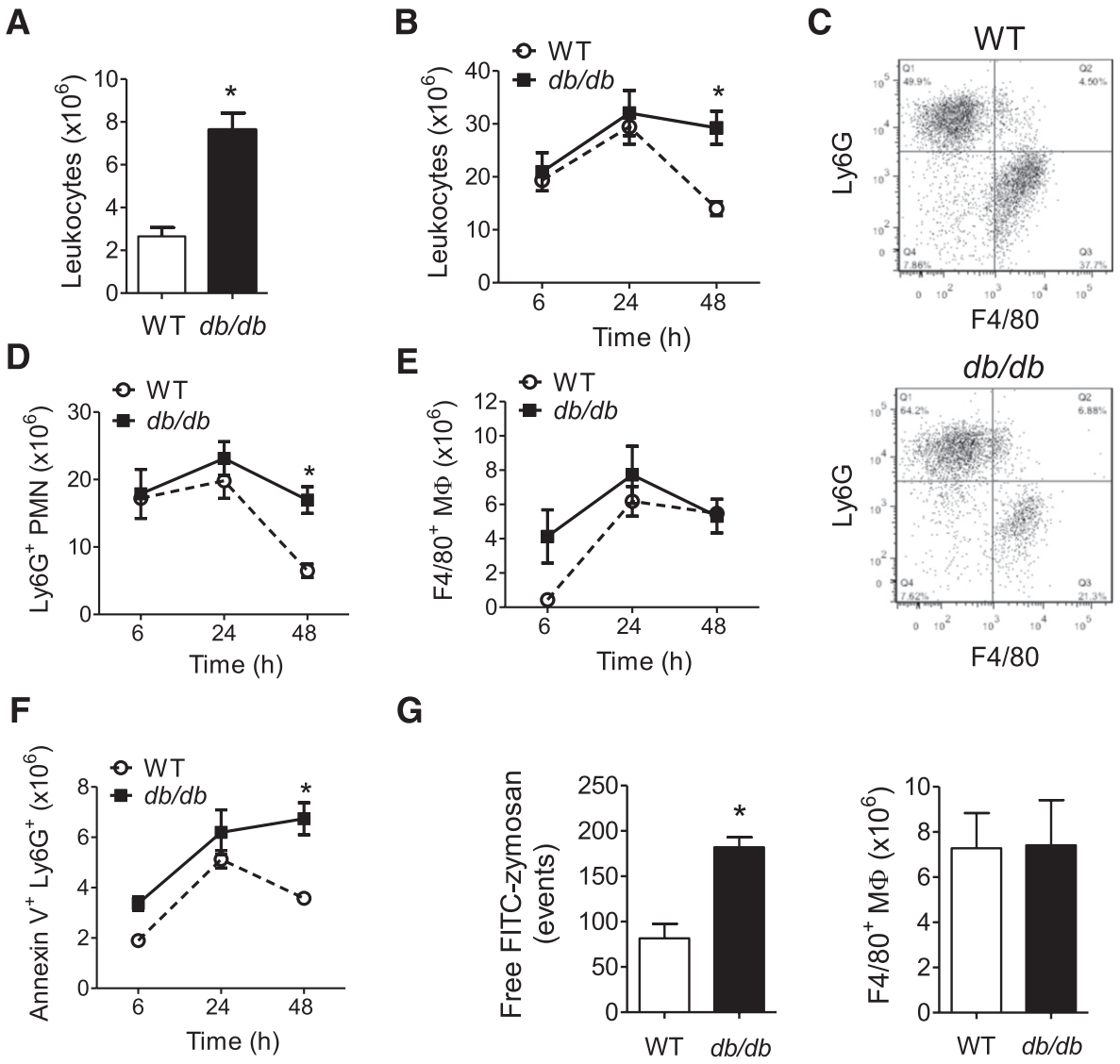

FIG. 1. Resolution of acute inflammation is altered in *db/db* mice. *A*: Resident peritoneal leukocytes in WT and *db/db* mice (n = 4–5). *B*: Time course of total leukocyte accumulation during the course of acute peritonitis stimulated by zymosan (0.04 mg · g^{-1}) challenge (n = 4–7). *C*: Representative flow cytometry dot plots of Ly6G$^+$ cells and F4/80$^+$ cells in peritoneal exudates obtained 48 h post–zymosan challenge in WT and *db/db* mice. *D* and *E*: Time course of Ly6G$^+$ PMNs (*D*) and F4/80$^+$ macrophages (MΦ) (*E*) during acute peritonitis (n = 3–7). *F*: Time course of apoptotic annexin V$^+$ PMN accumulation in the inflamed peritoneum in WT and *db/db* mice (n = 3–4). *G*: Accumulation of FITC–zymosan (*left panel*) and total MΦ numbers (*right panel*) in the peritoneum 72 h post–zymosan challenge (n = 3). Data are means ± SEM. *P < 0.05 by two-way ANOVA (*B* and *D–F*) or Student t test (*A* and *G*).

In addition, we and others have found that resolvins stimulate macrophage phagocytosis (7,12,20). Therefore, to test whether RvD1 rescues defective phagocytosis in diabetic macrophages, we isolated resident peritoneal macrophages from WT and *db/db* mice and assessed their ability to phagocytose IgG-opsonized zymosan. As shown in Fig. 3*A*, the phagocytic ability of macrophages isolated from *db/db* mice was significantly impaired compared with WT mice. We found that pretreatment with RvD1 at a concentration of just 0.1 nmol/L markedly stimulated phagocytosis by macrophages isolated from *db/db* mice (Fig. 3*B*). Because macrophage phagocytosis of both opsonized microbes and apoptotic cells requires PI3K (25) and this pathway is suppressed in diabetic mice (26), we evaluated whether the stimulation of phagocytosis by RvD1 was sensitive to PI3K blockade. Indeed, we found that the enhancement of phagocytosis by RvD1 was completely blocked in the presence of PI3K inhibitor, wortmannin (Fig. 3*B*).

Next, we sought evidence of a direct ligand receptor–dependent relationship between the effects of RvD1 and stimulated phagocytosis because recently the actions of RvD1 on macrophage phagocytosis were shown to involve the activation of G-protein–coupled receptor, formyl peptide receptor 2 (Fpr2) (also known as ALX) (20,27). Our results showed that stimulation of macrophage phagocytosis by RvD1 was blocked by a specific Fpr2 antagonist peptide, Trp-Arg-Trp-Trp-Trp-Trp (WRW4) (Fig. 3*C*) (28). To further examine the involvement of this receptor, we examined signaling events downstream of Fpr2 activation. Previous studies have shown that stimulation of phagocytosis by RvD1 is sensitive to pertussis toxin blockade, implicating coupling of Fpr2 to Gα_i (20). Therefore, we measured the accumulation of a second messenger, cAMP, and found that in macrophages isolated from *db/db* mice, treatment with RvD1 was associated with a significant suppression of cAMP levels (Fig. 3*D*). These observations

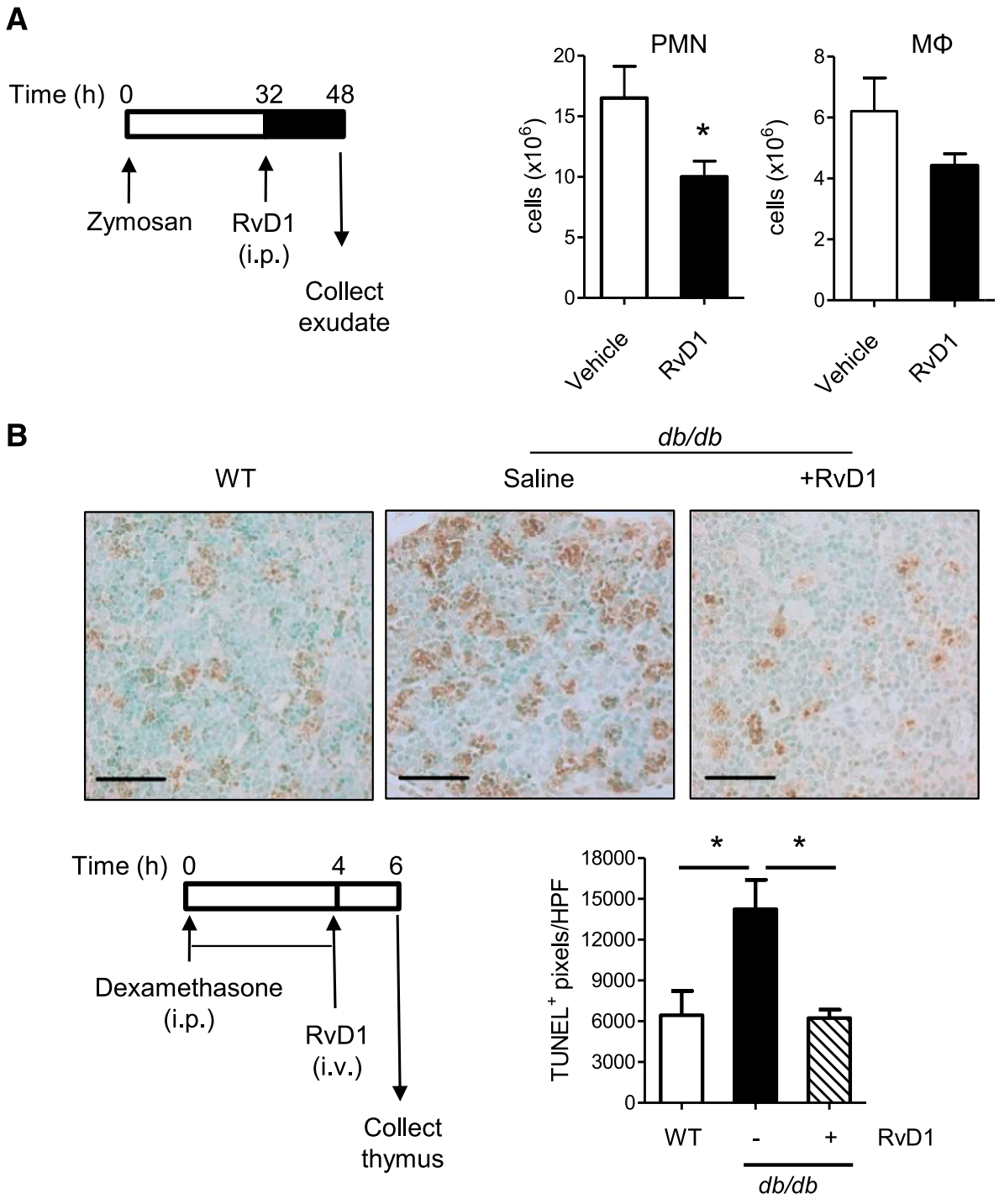

FIG. 2. Resolvin D1 restores resolution of acute inflammation and promotes the clearance of apoptotic cells in obese diabetic mice. *A*: Schematic of treatment protocol of mice challenged with zymosan and then treated with RvD1 and leukocyte differentials (n = 8–9). *B*: Quantification of apoptotic cells in the thymus of WT and *db/db* mice challenged with dexamethasone (15 mg · kg^{-1}) and treated without or with RvD1 (1 μg) (n = 3–4). *Top panel*: Representative images of TUNEL staining per high power field (HPF), scale bars = 50 μm. Sections were counterstained with methyl green. Data are means ± SEM. *P < 0.05 by one-way ANOVA (*B*) or Student t test (*A*). (A high-quality digital representation of this figure is available in the online issue.)

are consistent with and lend further support to the involvement of Fpr2 in mediating the effects of RvD1 on macrophages. Taken together, these data indicate that RvD1 stimulates macrophage phagocytosis and thereby restores diabetic defects in resolution of acute inflammation.

Given that macrophage dysfunction is causally related to delayed wound healing in diabetes (23) and that this dysfunction can be rescued by RvD1, we examined whether stimulating resolution would ameliorate diabetes-induced defects in wound healing. For this, we evaluated the effects of RvD1 on the closure of cutaneous wounds in *db/db* mice. Consistent with previous reports, we found that in comparison with WT mice, wound closure was significantly delayed in *db/db* mice (Fig. 4*A* and *B*) (17). We then determined whether this delayed wound healing is associated with alterations in the resolvin biosynthetic pathway, using

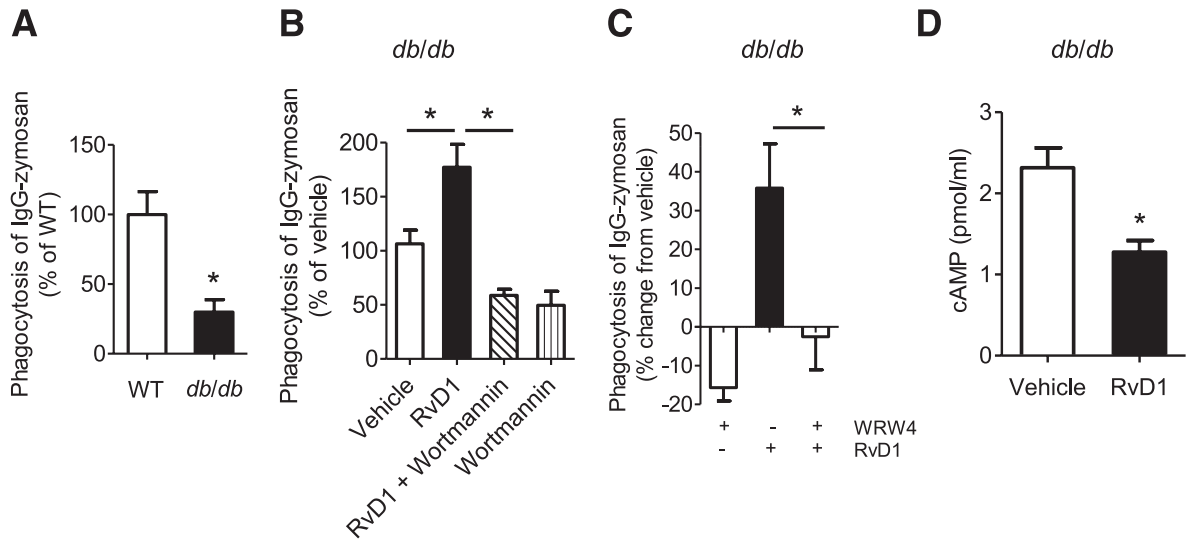

FIG. 3. Resolvin D1 rescues diabetic defects in macrophage phagocytosis. *A*: Phagocytosis of IgG-opsonized FITC–zymosan by peritoneal macrophages isolated from WT and *db/db* mice (n = 7–8). *B*: Phagocytosis in *db/db* macrophages stimulated without or with RvD1 (0.1 nmol/L; 15 min) in the presence or absence of PI3K inhibitor, wortmannin (200 nmol/L) (n = 3–9). *C*: Blockade of RvD1-stimulated macrophage phagocytosis in *db/db* macrophages by Fpr2-antagonist peptide, WRW4 (10 μmol/L) (n = 3–4). *D*: Levels of cAMP in *db/db* peritoneal macrophages stimulated with RvD1 (1 nmol/L, 15 min) (n = 4). Data are means ± SEM. *$P < 0.05$ by one-way ANOVA (*B* and *C*) or Student t test (*A* and *D*).

an LC-MS/MS approach. These measurements showed that despite similar levels of the resolvin biosynthetic precursor DHA (free, unesterified) in the wounds of WT and *db/db* mice, levels of monohydroxydocosanoids, which are markers of proresolving lipid mediator biosynthesis (resolvins, protectins, and maresins) (10), were lower in *db/db* mice (Fig. 4*C*).

To test the therapeutic efficacy of stimulating resolution in treating diabetic wounds, we applied RvD1 to cutaneous wounds of *db/db* mice. In this experiment, silicone splints were used to prevent skin contracture and to more closely monitor tissue growth (17). As shown in Fig. 5*A*, local delivery of RvD1 markedly enhanced the closure of diabetic wounds, and after 8 days of application the wound diameter was significantly smaller in RvD1-treated than in vehicle-treated animals. Consistent with improved wound regeneration, our histological analysis revealed that RvD1-treated wounds had more granulation tissue formation (Fig. 5*B*). Next, we assessed the expression of RvD1 receptor, Fpr2, in nondiabetic and diabetic wounds. Histological analysis of this receptor indicated that while it was abundant in the wounds of both WT and *db/db* mice, the expression of Fpr2 was significantly decreased in the wounds of *db/db* mice compared with WT mice (Fig. 5*C*). Overall, these results suggest that both altered biosynthesis and decreased Fpr2 expression likely underlie diabetes-induced impairment of resolution and wound healing, although these deficiencies can be overcome by exogenous RvD1 treatment.

Given that treatment with RvD1 decreased the accumulation of apoptotic thymocytes and that accumulation of apoptotic cells is a feature of unhealed diabetic wounds (23), we next assessed whether local delivery of RvD1 would modulate the apoptotic cell burden in diabetic wounds. We found that the abundance of apoptotic cells was significantly decreased in RvD1-treated wounds relative to vehicle treatment, as assessed by histological analysis (TUNEL; Fig. 6*A* and *C*). Lastly, we also observed that the number of $CD68^+$ macrophages, which also accumulate in wounds of diabetic mice, was significantly decreased by RvD1 treatment at 5 days postwounding (Fig. 6*B* and *C*) (29). Overall, these results are consistent with our in vitro and acute in vivo studies demonstrating that RvD1 enhances macrophage phagocytosis and clearance of apoptotic cells and suggest that this could in part contribute to the enhanced wound healing observed in RvD1-treated mice.

DISCUSSION

The results of this study demonstrate that type 2 diabetes alters the resolution of inflammation and that these alterations can be acutely corrected by stimulating resolution with proresolving lipid mediator RvD1. In addition, our data indicate that RvD1 also restores diabetic defects in macrophage phagocytosis, which could decrease the accumulation of apoptotic/necrotic cells and microbes in chronically inflamed tissues. We found that stimulating resolution with RvD1 enhanced closure of diabetic wounds and this was also associated with decreased burden of both apoptotic cells and macrophages. Thus, RvD1 could potentially be used as a novel agent to promote wound healing in diabetic patients.

A large body of research demonstrates that inflammatory signaling pathways are chronically activated in obesity and diabetes and that adipose tissue expansion promotes infiltration of mononuclear cells that secrete proinflammatory mediators and sustain insulin resistance (4,5). In healthy tissue, pathogen invasion or tissue injury elicits prompt infiltration of leukocytes that limit the injurious stimulus. Infiltrating PMNs, however, rapidly undergo apoptosis, and they must be cleared by macrophages to prevent inadvertent tissue damage (9). In addition, macrophages also phagocytose microbes and promote their removal. These events are critical for active resolution of inflammation and are regulated by endogenous proresolving mediators (10). Thus, we first examined whether chronic inflammation in obesity and diabetes could result from

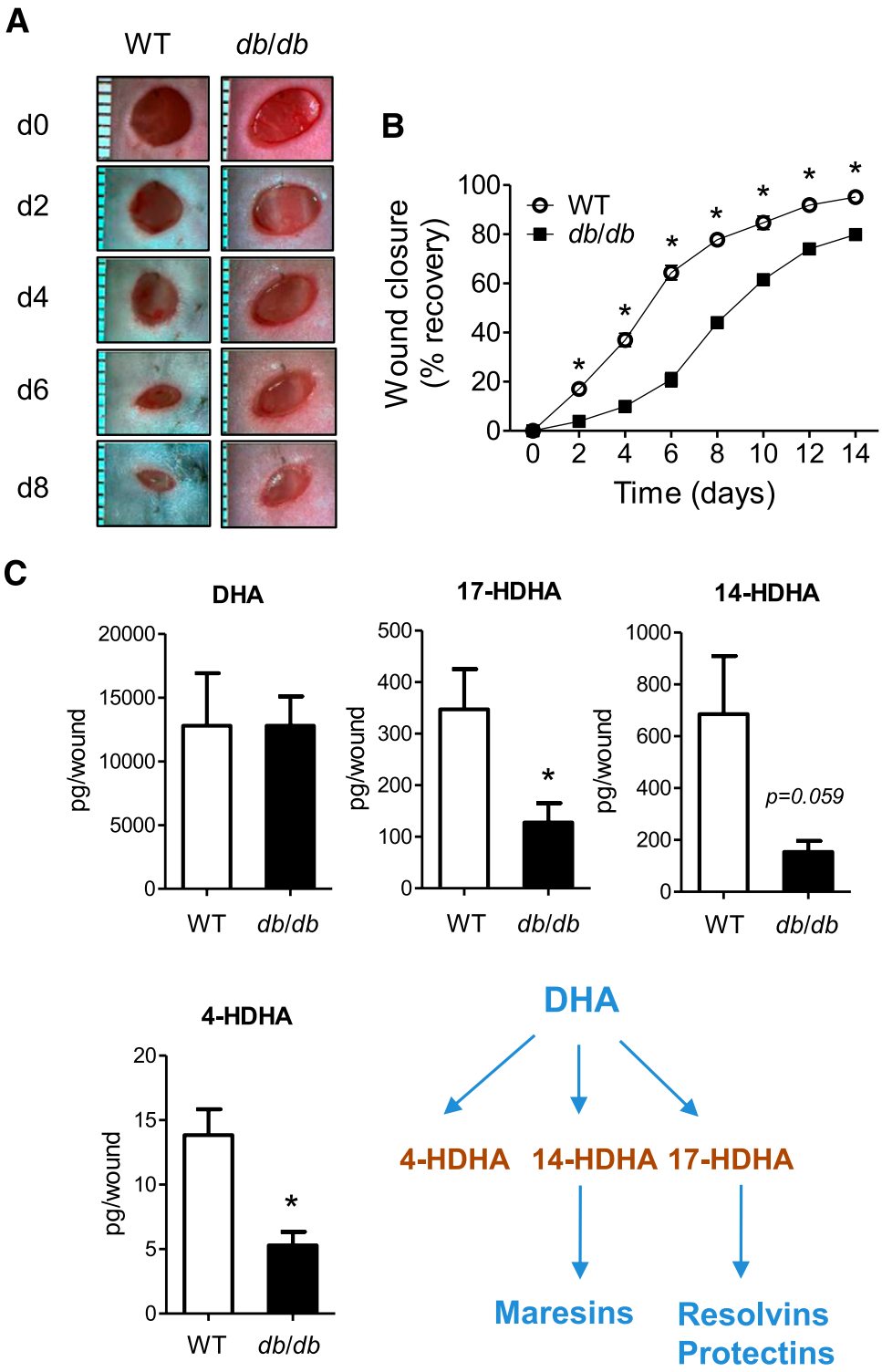

FIG. 4. Diabetes impairs wound healing and metabolism of DHA. *A*: Progressive changes in cutaneous wound closure in WT and *db/db* mice. *B*: Quantitative analysis of wound closure in WT and *db/db* mice ($n = 5$). *C*: Quantification of DHA and downstream metabolites, 17-HDHA, 14-HDHA, and 4-HDHA by liquid chromatography–tandem mass spectrometry analysis using MRM in cutaneous wounds isolated from WT and *db/db* mice at day (d) 5 ($n = 4$). The MRM transitions that were used are as follows: DHA, 327 > 283; 17-HDHA, 343 > 147; 14-HDHA, 343 > 161; and 4-HDHA, 343 > 101. Schematic of downstream mediators generated from DHA is also shown. Data are means ± SEM. *$P < 0.05$ by two-way ANOVA (*B*) or Student *t* test (*C*). (A high-quality digital representation of this figure is available in the online issue.)

failed resolution. For this, we used an acute peritonitis model because it allows for the time-dependent analysis of leukocyte infiltration, apoptosis, and phagocyte clearance. Our results showed that, rather than elevated leukocyte infiltration as might be expected, the macrophage-mediated removal of apoptotic cells and zymosan was impaired in diabetic mice, which further illustrates that specifically targeting these cellular events in resolution could have beneficial effects on chronic inflammation. Importantly, we found that therapeutic administration of proresolving lipid

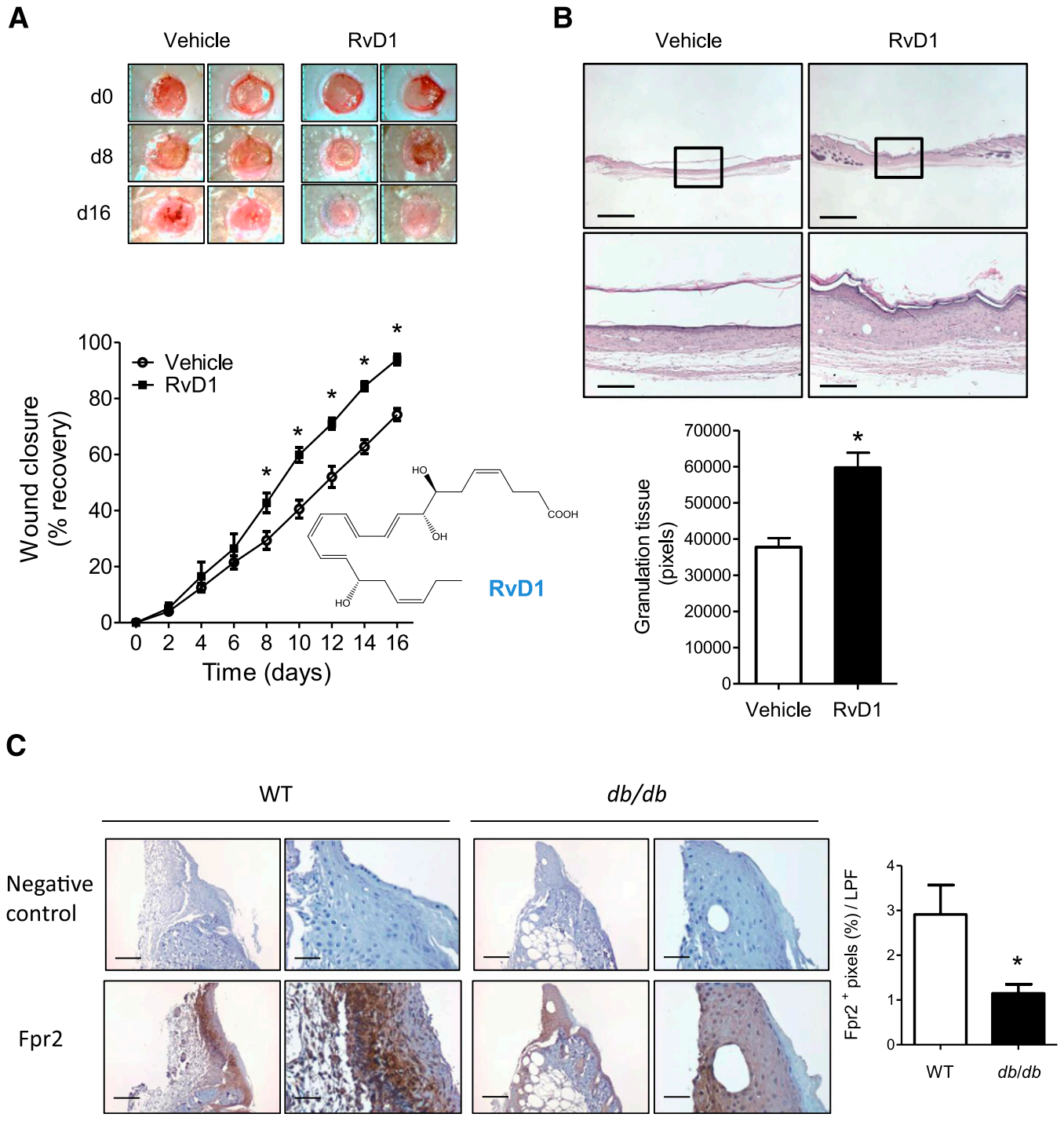

FIG. 5. RvD1 accelerates wound closure and granulation tissue formation in *db/db* mice. *A*: Representative images of vehicle (0.1% ethanol in saline) and RvD1-treated (100 ng per day [d] per wound) splinted wounds in *db/db* mice. *Lower panel*: Quantitative analysis of wound closure in *db/db* mice treated without or with RvD1, with the structure of RvD1 shown in the inset (n = 5–9). *B*: Assessment of granulation tissue area in vehicle or RvD1-treated wounds in *db/db* mice. Representative histological sections of hematoxylin-eosin staining (*top panels*, scale bar = 1,000 μm; *lower panels*, scale bar = 200 μm). *Lower panel*: Quantification of granulation tissue area (day 27; n = 5). *C*: Histological analysis of RvD1 receptor, Fpr2, in wounds isolated from WT and *db/db* mice at day 5 (n = 5). Sections were counterstained with hematoxylin-eosin; scale bars = 200 μm (*left two panels*) and 50 μm (*right two panels*) for each group. Quantification of Fpr2 receptor density is shown as pixels per low power field (LPF). Data are means ± SEM. *P < 0.05 by two-way ANOVA (*A*) or Student t test (*B* and *C*). (A high-quality digital representation of this figure is available in the online issue.)

mediator, RvD1, decreased PMN accumulation in the peritoneum. Although we administered RvD1 at the time of maximal PMN infiltration, we cannot rule out the possibility that decreased PMN accumulation in response to RvD1 treatment could be due to decreased infiltration. Thus, we next turned to a distinct model of phagocyte-mediated clearance of apoptotic cells that does not depend on infiltrating cells but, rather, on the apoptosis of resident cells (19). The results of these studies demonstrated that RvD1 decreases the accumulation of apoptotic thymocytes in

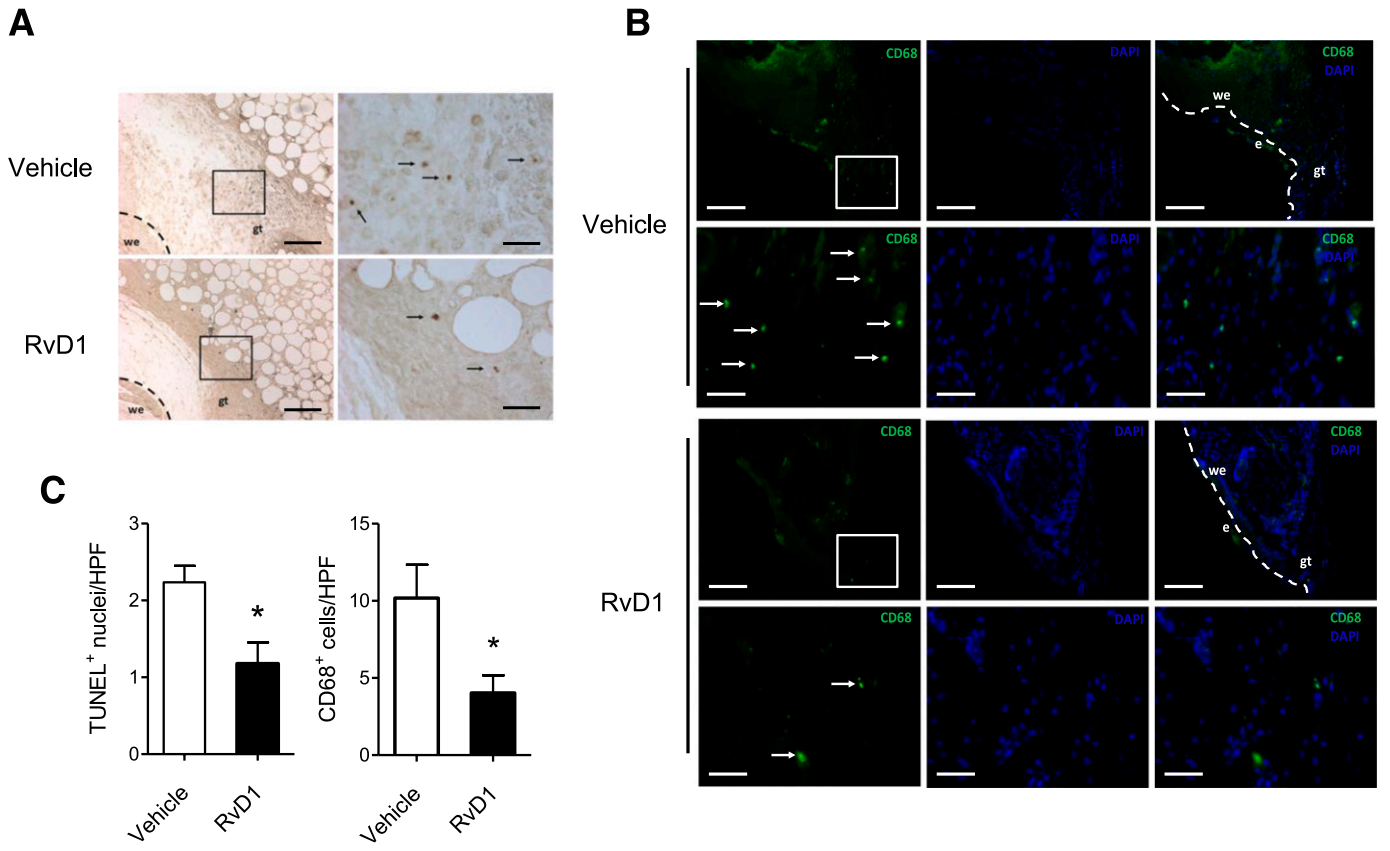

FIG. 6. Apoptotic cell and macrophage levels are decreased in diabetic wounds treated with resolvin D1. *A*: Representative images of TUNEL staining in *db/db* wounds treated without or with RvD1 (day 5; *n* = 4/group). Scale bars = 200 μm (*left panel*) and 50 μm (*right panel*). *B*: Immunofluorescence imaging of CD68$^+$ macrophages in *db/db* wounds treated without or with RvD1 and harvested at day 5. Scale bars = 200 μm (*upper panel*) and 50 μm (*lower panel*) for each group. *C*: Quantification of TUNEL$^+$ cells and CD68$^+$ cells in *db/db* wounds treated without or with RvD1 (*n* = 4) per high power field (HPF). Data are means ± SEM. **P* < 0.05 by Student *t* test. (A high-quality digital representation of this figure is available in the online issue.)

response to dexamethasone administration. These in vivo data strongly suggest that RvD1 improves macrophage-mediated clearance in diabetes and highlight the importance of the metabolic environment in diabetes in altering innate immune responses required for resolution.

An important biological action of resolvins is that they stimulate macrophage phagocytosis and emigration from tissues. We and others have previously demonstrated that resolvins, such as RvD1 and RvD2, enhance the phagocytosis of apoptotic cells, opsonized zymosan, and live bacteria (11,12,30). Importantly, stimulation of macrophage phagocytosis by RvD1 is mediated by Fpr2 (20,27). It has also been shown before that macrophages isolated from both diabetic humans and rodents have impairments in their ability to undergo phagocytosis (21,24). This defect extends to uptake of both apoptotic cells and microbes and has been demonstrated in several distinct rodent models of both type 1 and type 2 diabetes, including *db/db*, *ob/ob*, NOD, and streptozotocin-induced diabetes in mice (22,24,26,31). Thus, as a direct test of the hypothesis that RvD1 enhanced phagocyte-mediated clearance, we assessed its actions on primary macrophages isolated from *db/db* mice. We found that RvD1 enhanced diabetic macrophage phagocytosis in a receptor-dependent manner, suggesting that the proresolving role of RvD1 in vivo could be attributed in part to its targeted actions on macrophages.

Delayed wound healing is one of the most prominent clinical manifestations of type 2 diabetes. Current management of diabetic wounds is focused primarily on debridement, offloading, antibiotic therapy, and in some cases, surgical revascularization (e.g., angioplasty and bypass) (6). Even with therapeutic management, diabetes still accounts for >60% of nontraumatic amputations of the lower limb, and nearly one-third of diabetic foot ulcers require surgery (1,32). Thus, novel therapeutics aimed at controlling inflammation, reducing infection, and improving wound closure are urgently needed. Deregulated inflammation impairs the normal wound-healing response in diabetes, which is associated with increased susceptibility to soft-tissue infection despite accumulation of leukocytes. In fact, diabetic wounds have elevated levels of both PMN and macrophages and express higher levels of proinflammatory cytokines (29). Macrophage dysfunction is central to altered healing of diabetic wounds. This leads to failed clearance of apoptotic cells and microbes and gives rise to tissue necrosis (23). The results of the current study demonstrate that RvD1 enhances wound closure in diabetic mice and that this is associated with decreased accumulation of apoptotic cells and macrophages in the wounds. The beneficial effects at RvD1 on wound healing observed here are likely to be of high clinical importance because reducing wound-healing time would reduce susceptibility to infection. Moreover, the enhancement of diabetic macrophage phagocytosis by RvD1 suggests that local delivery of resolvins could also combat existing wound infection as an adjunctive therapy to antibiotics.

Indeed, recent studies have demonstrated that resolvins lower the threshold for antibiotic therapy in live infection, and we have previously shown that resolvins enhance leukocyte-mediated bacterial killing (11,12). Moreover, even though the current study suggests that macrophages may be the primary targets of RvD1, we have previously demonstrated that RvD1 also has direct actions on human epidermal keratinocytes, and thus RvD1 could have multiple cellular targets within wounds (33). Further studies will be required to interrogate fully the prohealing roles for resolvins.

Previous studies have documented that n-3 polyunsaturated fatty acids (PUFAs) improve metabolic parameters in obese and diabetic mice. In particular, either dietary supplementation or genetic manipulation (*fat-1* transgenic) to increase omega-3 PUFA levels has been shown to be associated with improved whole-body insulin sensitivity and increased flux through proresolving lipid mediator biosynthetic pathways (16,34,35). Moreover, we have recently shown that RvD1 enhances glucose tolerance and decreases macrophage accumulation in hypertrophied adipose tissue in *db/db* mice (15). The results of the present studies extend these findings by illustrating that proresolving lipid mediators improve macrophage function and clinically relevant end points (i.e., wound healing). Interestingly, we found that levels of free, unesterified DHA were similar in wounds isolated from nondiabetic and diabetic mice but that biosynthetic intermediates in proresolving lipid mediator pathways were markedly decreased. This result is consistent with recent findings by another group, which also reported deficient levels of 14-HDHA and another DHA-derived mediator, 14*S*,21*R*-dihydroxyDHA, in diabetic wounds (36). Given that clinical trials with n-3 PUFA have not yielded conclusive beneficial effects in humans with diabetes (37), the findings of the current study suggest that alterations in downstream metabolism of DHA might contribute to the discrepancies between rodent and human studies and that more targeted therapeutics with proresolving lipid mediators such as resolvins may be more efficacious than dietary supplementation with n-3 PUFAs.

In summary, the results of the current study demonstrate that resolution of inflammation is altered in type 2 diabetes and that defective macrophage-mediated resolution could be restored by proresolving lipid mediator RvD1. Notably, local delivery of RvD1 enhanced wound closure in diabetic mice, suggesting that stimulating resolution has beneficial functional outcomes. As resolvins are currently in phase III clinical trials for the treatment of other inflammatory pathologies (i.e., dry eye), the results of this study could be readily translated into clinical therapy for accelerating wound healing in patients with diabetes. The findings of the current study also have wide implications for developing future strategies for the treatment of other diabetes complications and several chronic autoimmune and cardiovascular diseases associated with unresolved chronic inflammation (8).

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants HL106173 (to M.S.) and P20RR024489 (Diabetes and Obesity Center to A.B. and M.S.). The study was also supported by U.S. Department of Defense Grant BAA10-1 (to A.B. and M.S.).

No potential conflicts of interest relevant to this article were reported.

Y.T., M.J.Z., and J.H. designed and carried out experiments, analyzed data, and contributed to the writing of the manuscript. M.K. carried out experiments and analyzed data. A.B. designed experiments and contributed to the writing of the manuscript. M.S. planned the project, designed experiments, analyzed data, and wrote the manuscript. M.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

The authors thank Dr. Lucy Norling and Ms. Stefania Bena (William Harvey Research Institute, London, U.K.) for assistance with Fpr2 staining.

REFERENCES

1. *CDC Fact Sheet: National Diabetes Fact Sheet: National Estimates and General Information on Diabetes and Prediabetes in the United States*. Atlanta, GA, Centers for Disease Control and Prevention, 2011
2. Sarwar N, Gao P, Seshasai SR, et al.; Emerging Risk Factors Collaboration. Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies. Lancet 2010;375:2215–2222
3. Gallagher EJ, LeRoith D. Diabetes, cancer, and metformin: connections of metabolism and cell proliferation. Ann N Y Acad Sci 2011;1243:54–68
4. Hotamisligil GS, Erbay E. Nutrient sensing and inflammation in metabolic diseases. Nat Rev Immunol 2008;8:923–934
5. Chawla A, Nguyen KD, Goh YP. Macrophage-mediated inflammation in metabolic disease. Nat Rev Immunol 2011;11:738–749
6. Jeffcoate WJ, Harding KG. Diabetic foot ulcers. Lancet 2003;361:1545–1551
7. Serhan CN, Chiang N, Van Dyke TE. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. Nat Rev Immunol 2008;8:349–361
8. Nathan C, Ding A. Nonresolving inflammation. Cell 2010;140:871–882
9. Serhan CN, Brain SD, Buckley CD, et al. Resolution of inflammation: state of the art, definitions and terms. FASEB J 2007;21:325–332
10. Zhang MJ, Spite M. Resolvins: anti-inflammatory and proresolving mediators derived from omega-3 polyunsaturated fatty acids. Annu Rev Nutr 2012;32:203–227
11. Chiang N, Fredman G, Bäckhed F, et al. Infection regulates pro-resolving mediators that lower antibiotic requirements. Nature 2012;484:524–528
12. Spite M, Norling LV, Summers L, et al. Resolvin D2 is a potent regulator of leukocytes and controls microbial sepsis. Nature 2009;461:1287–1291
13. Ravichandran KS. Beginnings of a good apoptotic meal: the find-me and eat-me signaling pathways. Immunity 2011;35:445–455
14. Vandivier RW, Henson PM, Douglas IS. Burying the dead: the impact of failed apoptotic cell removal (efferocytosis) on chronic inflammatory lung disease. Chest 2006;129:1673–1682
15. Hellmann J, Tang Y, Kosuri M, Bhatnagar A, Spite M. Resolvin D1 decreases adipose tissue macrophage accumulation and improves insulin sensitivity in obese-diabetic mice. FASEB J 2011;25:2399–2407
16. González-Périz A, Horrillo R, Ferré N, et al. Obesity-induced insulin resistance and hepatic steatosis are alleviated by omega-3 fatty acids: a role for resolvins and protectins. FASEB J 2009;23:1946–1957
17. Galiano RD, Michaels J 5th, Dobryansky M, Levine JP, Gurtner GC. Quantitative and reproducible murine model of excisional wound healing. Wound Repair Regen 2004;12:485–492
18. Yang R, Chiang N, Oh SF, Serhan CN. Metabolomics-lipidomics of eicosanoids and docosanoids generated by phagocytes. Curr Protoc Immunol 2011;Chapter 14:Unit 14.26
19. Park D, Han CZ, Elliott MR, et al. Continued clearance of apoptotic cells critically depends on the phagocyte Ucp2 protein. Nature 2011;477:220–224
20. Krishnamoorthy S, Recchiuti A, Chiang N, et al. Resolvin D1 binds human phagocytes with evidence for proresolving receptors. Proc Natl Acad Sci USA 2010;107:1660–1665
21. Lecube A, Pachón G, Petriz J, Hernández C, Simó R. Phagocytic activity is impaired in type 2 diabetes mellitus and increases after metabolic improvement. PLoS ONE 2011;6:e23366
22. Ferracini M, Martins JO, Campos MR, Anger DB, Jancar S. Impaired phagocytosis by alveolar macrophages from diabetic rats is related to the deficient coupling of LTs to the Fc gamma R signaling cascade. Mol Immunol 2010;47:1974–1980

23. Khanna S, Biswas S, Shang Y, et al. Macrophage dysfunction impairs resolution of inflammation in the wounds of diabetic mice. PLoS ONE 2010;5:e9539
24. O'Brien BA, Huang Y, Geng X, Dutz JP, Finegood DT. Phagocytosis of apoptotic cells by macrophages from NOD mice is reduced. Diabetes 2002;51:2481–2488
25. Leverrier Y, Okkenhaug K, Sawyer C, Bilancio A, Vanhaesebroeck B, Ridley AJ. Class I phosphoinositide 3-kinase p110beta is required for apoptotic cell and Fcgamma receptor-mediated phagocytosis by macrophages. J Biol Chem 2003;278:38437–38442
26. Li S, Sun Y, Liang CP, et al. Defective phagocytosis of apoptotic cells by macrophages in atherosclerotic lesions of ob/ob mice and reversal by a fish oil diet. Circ Res 2009;105:1072–1082
27. Norling LV, Dalli J, Flower RJ, Serhan CN, Perretti M. Resolvin d1 limits polymorphonuclear leukocyte recruitment to inflammatory Loci: receptor-dependent actions. Arterioscler Thromb Vasc Biol 2012;32:1970–1978
28. Bae YS, Lee HY, Jo EJ, et al. Identification of peptides that antagonize formyl peptide receptor-like 1-mediated signaling. J Immunol 2004;173:607–614
29. Wetzler C, Kämpfer H, Stallmeyer B, Pfeilschifter J, Frank S. Large and sustained induction of chemokines during impaired wound healing in the genetically diabetic mouse: prolonged persistence of neutrophils and macrophages during the late phase of repair. J Invest Dermatol 2000;115:245–253
30. Palmer CD, Mancuso CJ, Weiss JP, Serhan CN, Guinan EC, Levy O. 17(R)-Resolvin D1 differentially regulates TLR4-mediated responses of primary human macrophages to purified LPS and live E. coli. J Leukoc Biol 2011;90:459–470
31. Liu BF, Miyata S, Kojima H, et al. Low phagocytic activity of resident peritoneal macrophages in diabetic mice: relevance to the formation of advanced glycation end products. Diabetes 1999;48:2074–2082
32. Jeffcoate WJ, Lipsky BA, Berendt AR, et al.; International Working Group on the Diabetic Foot. Unresolved issues in the management of ulcers of the foot in diabetes. Diabet Med 2008;25:1380–1389
33. Norling LV, Spite M, Yang R, Flower RJ, Perretti M, Serhan CN. Cutting edge: Humanized nano-proresolving medicines mimic inflammation-resolution and enhance wound healing. J Immunol 2011;186:5543–5547
34. Titos E, Rius B, González-Périz A, et al. Resolvin D1 and its precursor docosahexaenoic acid promote resolution of adipose tissue inflammation by eliciting macrophage polarization toward an M2-like phenotype. J Immunol 2011;187:5408–5418
35. White PJ, Arita M, Taguchi R, Kang JX, Marette A. Transgenic restoration of long-chain n-3 fatty acids in insulin target tissues improves resolution capacity and alleviates obesity-linked inflammation and insulin resistance in high-fat-fed mice. Diabetes 2010;59:3066–3073
36. Tian H, Lu Y, Shah SP, Hong S. 14S,21R-dihydroxydocosahexaenoic acid remedies impaired healing and mesenchymal stem cell functions in diabetic wounds. J Biol Chem 2011;286:4443–4453
37. De Caterina R, Madonna R, Bertolotto A, Schmidt EB. n-3 fatty acids in the treatment of diabetic patients: biological rationale and clinical data. Diabetes Care 2007;30:1012–1026