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Impact of Resolvin E1 on Murine Neutrophil Phagocytosis in Type 2 Diabetes

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Diabetic complications involve inflammation-mediated microvascular and macrovascular damage, disruption of lipid metabolism, glycosylation of proteins, and abnormalities of neutrophil-mediated events. Resolution of inflamed tissues to health and homeostasis is an active process mediated by endogenous lipid agonists, including lipoxins and resolvins. This proresolution system appears to be compromised in type 2 diabetes (T2D). The goal of this study was to investigate unresolved inflammation in T2D. Wild-type (WT) and genetically engineered mice, including T2D mice (*db*/*db*), transgenic mice overexpressing the human resolvin E1 (RvE1) receptor (*ERV1*), and a newly bred strain of *db*/*ERV1* mice, were used to determine the impact of RvE1 on the phagocytosis of *Porphyromonas gingivalis* in T2D. Neutrophils were isolated and incubated with fluorescein isothiocyanate-labeled *P. gingivalis*, and phagocytosis was measured in a fluorochrome-based assay by flow cytometry. Mitogen-activated protein kinase (MAPK) (p42 and p44) and Akt (Thr308 and Ser473) phosphorylation was analyzed by Western blotting. The mouse dorsal air pouch model was used to evaluate the *in vivo* impact of RvE1. Results revealed that RvE1 increased the neutrophil phagocytosis of *P. gingivalis* in WT animals but had no impact in *db*/*db* animals. In *ERV1*-transgenic and *ERV1*-transgenic diabetic mice, phagocytosis was significantly increased. RvE1 decreased Akt and MAPK phosphorylation in the transgenic animals. *In vivo* dorsal air pouch studies revealed that RvE1 decreases neutrophil influx into the pouch and increases neutrophil phagocytosis of *P. gingivalis* in the transgenic animals; cutaneous fat deposition was reduced, as was macrophage infiltration. The results suggest that RvE1 rescues impaired neutrophil phagocytosis in obese T2D mice overexpressing *ERV1*.

Excessive inflammation is now recognized as a central component of the most prevalent diseases in developed societies. The complications of diabetes mellitus, particularly type 2, include periodontitis and cardiovascular disease. Fifty percent of the U.S. population has at least some periodontal disease; type 2 diabetes (T2D) doubles the risk of periodontitis (1). A major link between T2D and its complications is inflammation (2, 3). Enhanced inflammation is well characterized in T2D (4), and prolonged inflammation is an important aspect of periodontitis complications (5). Tumor necrosis factor alpha (TNF-α), which has been implicated as a proinflammatory adipokine in T2D and obesity, seems to play an important role. Specific inhibition of TNF-α in diabetic-animal experiments reverses the upregulation of proinflammatory cytokine genes, leukocyte infiltration into the periodontium, and associated bone loss (5).

In periodontitis, after acute infection, the shift to chronicity and persistence of pathogens may be the result of increased inflammation (6–10) and leads to leukocyte-mediated tissue destruction. The increase in inflammation induced by T2D directly contributes to the increased prevalence and severity of periodontitis in T2D (11).

The active endogenous mediators of resolution of inflammation are now known (12–14). It is also well established that sufficient proresolution agonist (lipoxin and resolvin) concentrations in inflammation are necessary to prevent tissue damage (7, 15) and that these pathways are deficient in T2D (16). The actions of these molecules support their potential use in inflammatory diseases. For example, in a sepsis model (cecal ligation and puncture), a resolvin reduced local and systemic bacterial burdens, cytokine production, and polymorphonuclear neutrophil (PMN) accumulation and increased peritoneal mononuclear cell recruitment and macrophage phagocytosis (17). Mouse survival was significantly increased by resolvin treatment. These findings also suggest that excess inflammation impedes bacterial clearance. In T2D, the observed increased susceptibility to infection is associated with impaired phagocytosis and bacterial killing by cells of the innate immune system. This impairment has been shown to be due to the chronic hyperglycemia in poorly controlled type 2 diabetics that primes neutrophils and monocytes, resulting in an exaggerated inflammatory response and tissue damage (18–20).

Genetically engineered animals have been used to study the impact of T2D on the inflammatory response. For example, the leptin receptor-deficient *db*/*db* knockout mouse provides a monogenic model of obesity and T2D (21). The hallmark phenotypic change in *db*/*db* mice is insulin resistance; after 8 weeks of age, *db*/*db* mice are severely obese and hyperglycemic (22). *db*/*db* mice with periodontitis exhibit more aggressive disease with aggravated bone loss (23).

Resolvins, such as resolvin E1 (RvE1), are biosynthesized from the omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid. RvE1, a derivative of EPA, shows remarkable potency in resolving inflammation-related diseases such as asthma (24), retinopathy (25), and periodontal disease (15, 26, 27).

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Emerging evidence suggests that nonresolving inflammation is a critical underlying component of many prevalent chronic diseases such as arthritis, diabetes, and periodontal and cardiovascular diseases (26) and is sustained in part by a deficiency of mediators that normally resolve inflammation (28–30). RvE1 binds to G protein-coupled receptors such as BLT1 (a leukotriene B_4 receptor) and *ERV1* (also known as chemR23, CMKLR1; for a review, see reference 31). It has been demonstrated that activation of the *ERV1* receptor (32, 33) decreases neutrophil migration (34), diminishes inflammatory cytokines, and increases phagocytosis of apoptotic neutrophils by macrophages (35). *ERV1*-overexpressing transgenic mice are protected from *Porphyromonas gingivalis*-induced periodontitis (36).

The purpose of these investigations was to begin to unravel the complexities of deficient resolution of inflammation in T2D and a major comorbidity, periodontitis. We report the engineering of two transgenic animals, one normoglycemic and one type 2 diabetic, that overexpress the receptor for the proresolution agonist RvE1 and the impact of RvE1 on neutrophil phagocytosis by exogenous administration of RvE1. We also report the actions of RvE1 on the modification of neutrophil signaling pathways induced my proinflammatory infections.

MATERIALS AND METHODS

Animals. Male *db*/*db* (homozygous) and *db*/− (heterozygous) mice with a strain FVB [FVB.BKS(D)-*Lepr*^{db}/ChuaJ] background and age-matched nondiabetic wild-type (WT) control mice were obtained from Jackson Laboratories (Bar Harbor, ME).

Preparation of *ERV1* and *db*/*ERV1* transgenic mice. *ERV1* mice were engineered as previously described (36). FVB mice were bred with *db*/− (heterozygous) mice in order to produce the F_1 generation, *ERV1*$^+$ *db*/−. The F_1 generation mice were bred in, yielding *db*/*ERV1* transgenic mice (*db*$^{-/-}$/*db*$^{-/-}$ with overexpression of *ERV1*). Four experimental groups included WT, *ERV1* transgenic (*ERV1*), *db*/*db*, and *db*/*ERV1* mice. All animal experiments were in conformity with the standards of the Public Health Service policy on the humane care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of The Forsyth Institute.

Genotyping of mice. Genomic DNA was isolated from tail biopsy specimens of mice and screened by PCR with primers directed to mouse *ERV1* (forward primer 5′-CTCGGTCTCCTAGGCAAC-3′) and human *ERV1* (forward primer 5′-GTCTTCCTCCCAATCCAT-3′). The mouse and human *ERV1* amplicons shared the same reverse primer (5′-TAGAA AGCCAGGACCCAG-3′). For the *db*/*db* mice, we used the protocol provided by Jackson Laboratories with restriction enzyme digestion by RsaI and forward primer 5′-AGAACGGACACTCTTTGAAGTCTC-3′ and reverse primer 5′-CATTCAAACCATAGTTTAGGTTTGTGT-3′. The *db*/*db* mice showed double bands (108 and 27 bp), and the WT mice showed a single band (135 bp).

Blood glucose levels. Blood Glucose Test Strips and a Blood Glucose Monitoring System (QSTEPS Biometer Dual Monitoring System; Biomedix, St. Paul, MN) were used to determine the glucose level in a drop of whole blood collected from each mouse.

Resolvin synthesis. RvE1 was prepared by total organic synthesis as described by Arita et al. (32). The structural integrity of RvE1 was monitored by liquid chromatography-UV-tandem mass spectrometry. Immediately before use, RvE1 was diluted in phosphate-buffered saline (PBS) to a final ethanol concentration of <1%.

***P. gingivalis* phagocytosis and killing by neutrophils.** *P. gingivalis* strain A7436 was cultured as previously described (37, 38). After 48 h of anaerobic growth in Wilkins-Chalgren broth in an anaerobic chamber with 85% N_2, 5% H_2, and 10% CO_2, bacteria were harvested by centrifugation; washed three times with sterile, pyrogen-free saline; incubated; and labeled with fluorescein isothiocyanate (FITC; 100 μg/ml of PBS) as previously described (39).

Neutrophils were extracted from peritoneal exudates collected 12 h after the intraperitoneal injection of zymosan-A (1 mg/ml of PBS). The neutrophils were seeded into 24-well plates (1 ml of medium containing 10^6 cells/well), and bacteria were added at a multiplicity of infection of 20. Four different conditions were assigned to each animal strain: (i) control (neutrophils alone), (ii) vehicle (neutrophils plus *P. gingivalis* plus PBS), (iii) neutrophils plus *P. gingivalis* plus RvE1 at 10 ng/ml, and (iv) neutrophils plus *P. gingivalis* plus RvE1 at 100 ng/ml. For the RvE1 groups, the neutrophils were pretreated with RvE1 15 min before a bacterial challenge. After 2 h of incubation, the neutrophils were collected, quenched with 0.2% trypan blue, and analyzed by flow cytometry (FACScan with CellQuest software; BD Bioscience). Phagocytosis was quantified as the percentage of cells containing bacteria (percent fluorescent cells) and the number of bacteria per cell (events per cell).

In separate experiments, the bactericidal activity of neutrophils against phagocytosed *P. gingivalis* was assessed by the CFU method of Amano et al. (40). Data are reported as a killing index as described by Kobayashi et al. (41).

Western blot analysis of Akt and mitogen-activated protein kinase (MAPK) phosphorylation. Protein samples for Western blotting were prepared as previously described (18). Neutrophils were lysed and fractionated by adding 40 μl of 6× SDS sample buffer to 200 μl of the reaction mixture and boiling the samples for 10 min. The final composition of SDS sample buffer after mixing was 2% (wt/vol) SDS, 58.3 mM Tris-HCl (pH 6.8), 6% (vol/vol) glycerol, 5% (vol/vol) 2-mercaptoethanol, 0.002% (wt/vol) bromophenol blue, 1% (vol/vol) protease inhibitors (Sigma, St. Louis, MO), and 1 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO). Aliquots of these samples were separated on 10% (vol/vol) SDS polyacrylamide slab gels, and separated proteins were immediately transferred electrophoretically to polyvinylidene difluoride membranes. The membranes were blocked for 1 h at room temperature with 5% skim milk in Tris-buffered saline (TBS), pH 7.6. The blocking buffer was removed by washing three times with TBS plus Tween 20 (1%), and the membranes were incubated with Akt antibodies (total, phospho-Thr 308, and phospho-Ser473; Cell Signaling Technology, Danvers, MA) and MAPK phosphospecific antibodies (p42 and p44; Cell Signaling Technology, Danvers, MA) overnight. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies (Santa Cruz Biotechnology, Dallas, TX) served as an internal control. The membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibodies for Akt and GAPDH (Santa Cruz Biotechnology, Dallas, TX) and a horseradish peroxidase-conjugated goat anti-rat secondary antibody for MAPK (Santa Cruz Biotechnology, Dallas, TX) in TBS for 1 h at room temperature. Immunoreactive bands detected by chemiluminescence (Immuno-Star; Bio-Rad). Band intensity was determined by densitometric analysis (ChemImager 5500 system; Alpha Innotech Corp.). Protein content was measured by the Bradford method. Akt phosphorylation was normalized to total Akt, and MAPK phosphorylation was normalized to GAPDH.

Dorsal air pouch protocol. In order to study neutrophil phagocytosis and killing *in vivo*, air pouches were raised on the dorsa of mice by the subcutaneous injection of 3 ml of sterile air on days 0 and 3, and all experiments were carried out on day 6 (42). Each animal was treated with 50 ng of RvE1 intraperitoneally, followed by 500 μl of sterile PBS or 10^5 cells of *P. gingivalis* strain A7436 in PBS (optical density at 600 nm [OD_{600}], 0.9 to 1.0) 15 min later. Mice were sacrificed 4 h postinjection, and individual air pouches were lavaged three times with sterile PBS (3 ml per lavage) as previously described (42, 43). The exudates were centrifuged at 1,000 × *g* for 5 min, cell pellets were suspended in PBS (200 μl) and counted, and 50 μl of each cell suspension was mixed with 150 μl of 30% bovine serum albumin in PBS, centrifuged onto microscope slides at 500 rpm for 5 min with a cytospin centrifuge, air dried, and Wright-Giemsa stained for identification of individual cell types. An additional sample of 100 μl was incubated in 10 ml of Wilkins-Chalgren broth in an

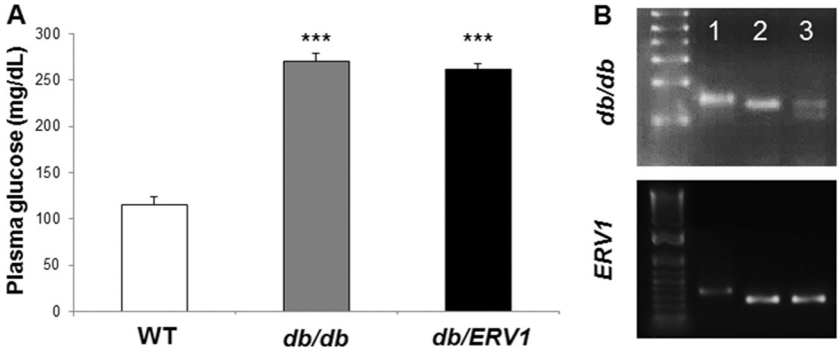

FIG 1 Mouse genotypes and phenotypes. (A) Baseline plasma glucose levels (mg/dl) show that *db*/*db* mouse plasma glucose levels are out of the normal range and that overexpression of *ERV1* does not impact glycemic control. ***: *db*/*db*, $P < 0.001$ compared to WT; *db*/*ERV1*, $P < 0.0001$ compared to WT ($n = 6$). (B) Genotyping results. Lanes: 1, WT, 2, *ERV1* transgenic (*ERV1*), 3, *db*/*db*-*ERV1* transgenic (*db*/*ERV1*).

anaerobic chamber, and after 24 h, the bacterial concentration was measured spectrophotometrically (OD_{600}).

Accumulation of fat containing activated macrophages in *db*/*db* animals is thought to be a source of increased TNF-α and a potential contributor to the enhanced inflammatory phenotype of T2D (44, 45). In order to evaluate the tissue lining, the air pouches were excised, soaked in 10% formalin solution for 72 h, and embedded in paraffin. Sections 5 μm thick were cut, washed, and placed in a water bath. The sections were stained with hematoxylin and eosin and mounted on permanent slides for subsequent analysis with an optical microscope (Zeiss Axiovert). For immunohistochemistry, the sections were blocked with 1.5% goat serum (Vector Laboratories, Burlingame, CA) and then incubated with anti-CD68 antibody (1/2,000 in goat serum; Abcam, Cambridge, MA) overnight at 4°C. The following day, slides were brought to room temperature and biotinylated secondary anti-rabbit IgG (1/200; Vector Laboratories, Burlingame, CA) was applied for 30 min, followed by ABC reagent (Vector Laboratories, Burlingame, CA) for 30 min. Diaminobenzidine solution (Vector Laboratories, Burlingame, CA) was applied and counterstained with hematoxylin. Standardized photos of all groups were taken at a magnification of ×200, and the images were saved on a computer. The thicknesses of the epithelium, connective tissue, and adipose tissue were calculated with ImageJ software (Image Processing and Analysis in Java). In addition, macrophage infiltration, quantified as $CD68^+$ cell counts, was analyzed as total cell counts and as macrophage density (cells/μm^2).

Statistical analysis. Data are expressed as mean values ± the standard errors of the means. The differences between groups in all comparisons were tested by one-way analysis of variance (ANOVA) with Bonferroni corrections for multiple comparisons ($\alpha = 0.05$). All analyses were performed with SPSS statistical software, version 19 (IBM SPSS Statistics; IBM Corporation, Somers, NY).

RESULTS

Glycemic index. Glycemic-index measurements revealed that the WT animals were normoglycemic and *db*/*db* mice were severely hyperglycemic (Fig. 1). The overexpression of *ERV1* in *db*/*db* mice did not change the circulating glucose concentrations (WT, 115.2 ± 8.6 mg/dl; *db*/*db*, 270 ± 8.8 mg/dl; *db*/*ERV1*, 262.2 ± 5.8 mg/dl).

RvE1 increases *P. gingivalis* phagocytosis and killing by neutrophils when ERV1 is overexpressed. The percentage of neutrophils containing bacteria (FITC positive) was lower in *db*/*db* animals (with or without *ERV1* overexpression) than in WT and *ERV1* mutant animals (Fig. 2A). Phagocytosis was also quantified as the number of bacteria (events per cell) as a measure of phagocytosis efficiency. RvE1 ligand, at both concentrations, significantly increased phagocytosis efficiency in the transgenic animals (*ERV1* and *db*/*ERV1*) (Fig. 2B and C). *db*/*db* neutrophil phagocytosis was impaired compared to WT and *ERV1* neutrophil phagocytosis. RvE1 rescued the phagocytosis response in the *db*/*ERV1* animals but not in the *db*/*db* animals.

P. gingivalis killing by neutrophils was significantly increased by RvE1 at both doses in WT animals (Fig. 2D; $P < 0.05$). *ERV1* animals exhibited significantly more killing than the WT without added RvE1 and significantly more killing than the WT at both doses of RvE1. Killing was lower in *db*/*db* mice than in WT animals without added RvE1, and the addition of 10 ng/ml RvE1 did not significantly improve killing. High-dose RvE1 (100 ng/ml) rescued the killing response to a level approaching the WT killing response. Overexpression of *ERV1* in *db*/*ERV1* animals rescued the killing response under all conditions.

***P. gingivalis* induces phosphorylation of Akt and MAPK that is reversed by RvE1.** Intracellular signaling through Akt and p38-MAPK was activated by *P. gingivalis* in protein phosphorylation assays, demonstrating that the neutrophils in all groups of mice are responsive to bacterial stimulation. However, phagocytosis by *db*/*db* neutrophils is impaired. In order to determine the impact of RvE1 on activated signaling pathways, Akt and p38-MAPK phosphorylation was assessed after RvE1 treatment in all groups. RvE1 (10 ng/ml) induced a small increase in Akt phosphorylation in *db*/*db* mice, while a significant reduction was observed in *db*/*ERV1* mice. When RvE1 administration was increased to 100 ng/ml, a significant decrease in Akt phosphorylation (threonine 308, serine 473) was observed in both *db*/*ERV1* and *ERV1* mice. Quantification of phosphorylated Akt bands in Western blot assays demonstrated that *P. gingivalis* stimulation induced Akt phosphorylation at threonine 308 and serine 473 in all of the groups. Preincubation with 100 ng/ml RvE1 decreased phosphorylation at both thr308 and ser478 (Fig. 3; $P < 0.05$), while 10 ng/ml RvE1 decreased the phosphorylation of only ser478 in the *db*/*ERV1* group ($P < 0.05$).

A significant decrease in MAPK phosphorylation was also evident when cells were treated with RvE1 (Fig. 4). A decrease in protein phosphorylation occurred in *ERV1* and *db*/*ERV1* mice when cells were treated with RvE1. *db*/*db* and *db*/*ERV1* mice showed greater phosphorylation at both sites prior to incubation with *P. gingivalis*, suggesting preactivation of neutrophils *in situ*. As observed for Akt phosphorylation, preincubation with RvE1 decreased the phosphorylation of ERK at both p42 and p44 when *ERV1* was overexpressed.

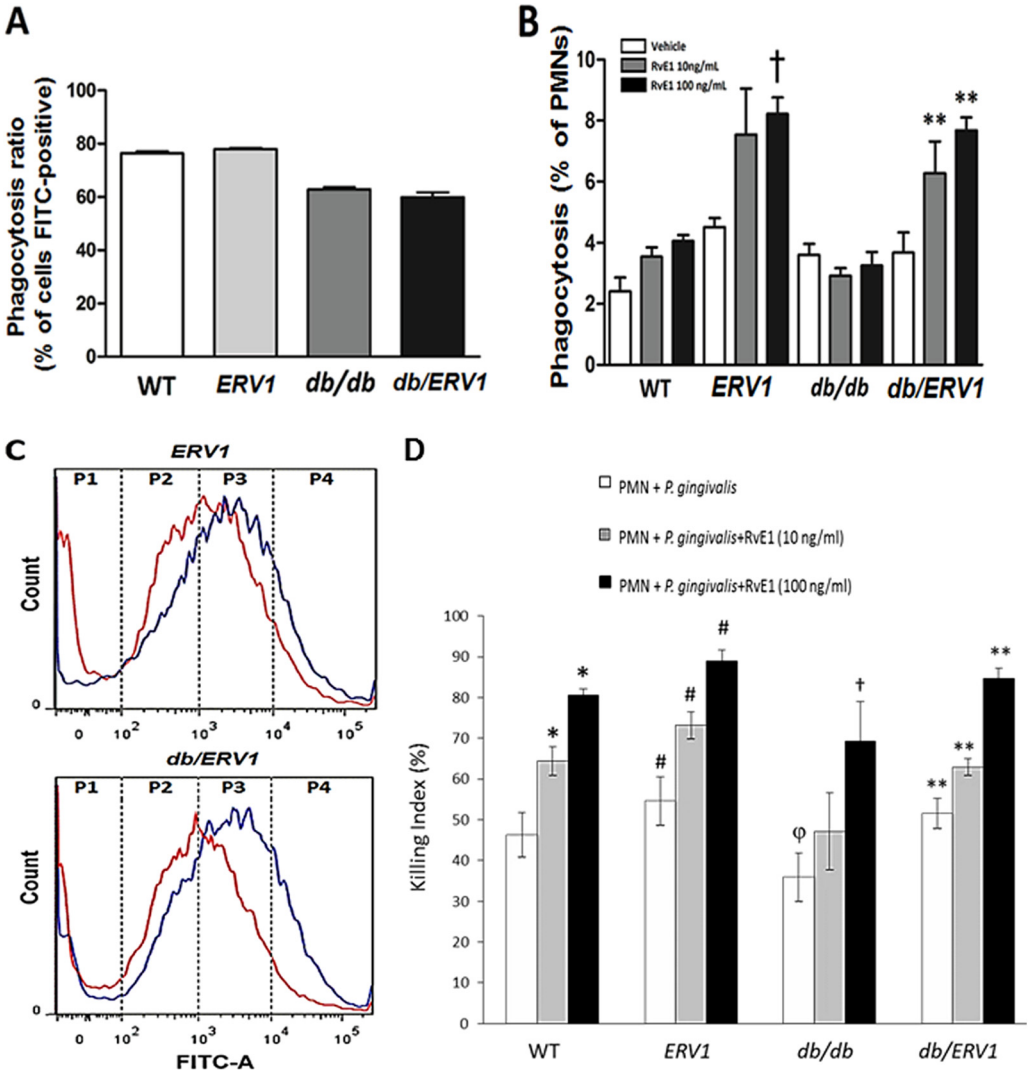

FIG 2 RvE1 increases *P. gingivalis* phagocytosis by neutrophils overexpressing *ERV1*. *P. gingivalis* phagocytosis by neutrophils was quantified by flow cytometry as the percentage of neutrophils containing bacteria (FITC positive) and the number of bacteria per neutrophil. (A) Percentages of FITC-positive neutrophils. Diabetic mice exhibit significantly fewer phagocytizing neutrophils ($n = 9$). (B) Percentages of FITC-positive neutrophils with more than 10,000 events (†, $P < 0.05$ compared to *ERV1* with vehicle; **, $P < 0.01$ compared to *db*/*ERV*1 with vehicle. (C) Distribution of bacteria per neutrophil by quartile. P4 = >10,000 events per cell. Note the shift to more bacteria per neutrophil with RvE1 treatment (red, no treatment; blue, 10 ng/ml RvE1; $n = 8$ nondiabetic and 6 diabetic mice). (D) Neutrophil bactericidal activity is enhanced by RvE1. With a CFU killing assay for *P. gingivalis*, the actions of two doses of RvE1 (10 and 100 ng/ml) were assessed in WT, *ERV1*, *db*/*db*, and *db*/*ERV1* mice. Differences within and between groups were determined by ANOVA with Bonferroni corrections for multiple comparisons. Between-group comparisons revealed that bactericidal activity was lower in *db*/*db* mice than in WT animals (or all other strains) (φ, $P < 0.05$). Treatment of *db*/*db* neutrophils with RvE1 restored the killing response to the level of untreated WT mice at 10 ng/ml, and the effect was significantly greater at 100 ng/ml (†, $P < 0.05$). Overexpression of *ERV1* in diabetic *db*/*ERV1* mice completely normalized the killing response (**, $P < 0.05$). It is interesting that the *db*/*db* killing response was significantly lower than that of the WT at both doses of RvE1 and that this response deficiency was eliminated by the overexpression of *ERV1*. *, $P < 0.05$ compared to PMNs plus *P. gingivalis*; #, $P < 0.05$ compared to the WT under all conditions ($n = 4$).

RvE1 decreases inflammatory cell influx into the air pouch and increases *P. gingivalis* clearance. In order to provide an *in vivo* correlate to the cell-based observations, the impact of RvE1 on cell accumulation, phagocytosis, and killing of *P. gingivalis* by neutrophils was performed in whole-animal experiments using the dorsal air pouch model. In this model, injection of *P. gingivalis* into the lumen of the dorsal air pouches of WT animals is known to induce a significant influx of neutrophils that are gradually cleared. Pretreatment with RvE1 decreased neutrophil influx into the dorsal air pouch after 4 h of stimulation with *P. gingivalis* in all of the groups except *db*/*db* mice (Fig. 5A and B). RvE1 pretreatment increased neutrophil phagocytosis and clearance of *P. gingivalis* in the animals that overexpress *ERV1* (*ERV1* and *db*/*ERV1*), but there was no impact of RvE1 on the *db*/*db* animals, confirming the *in vitro* assay. There was a clear trend to increased *P. gingivalis* clearance in WT animals that did not reach statistical significance ($P = 0.08$).

Accumulation of macrophage-laden fat in cutaneous tissue is a well-known characteristic of diabetic mice (44–46). In order to determine the impact of overexpression of *ERV1*, we compared the dorsal air pouch linings of *db*/*db* and *db*/*ERV1* mice (Fig. 6). Overexpression of *ERV1* clearly reduces adipose tissue deposition and macrophage infiltration into the fat (Fig. 6A). While the thick-

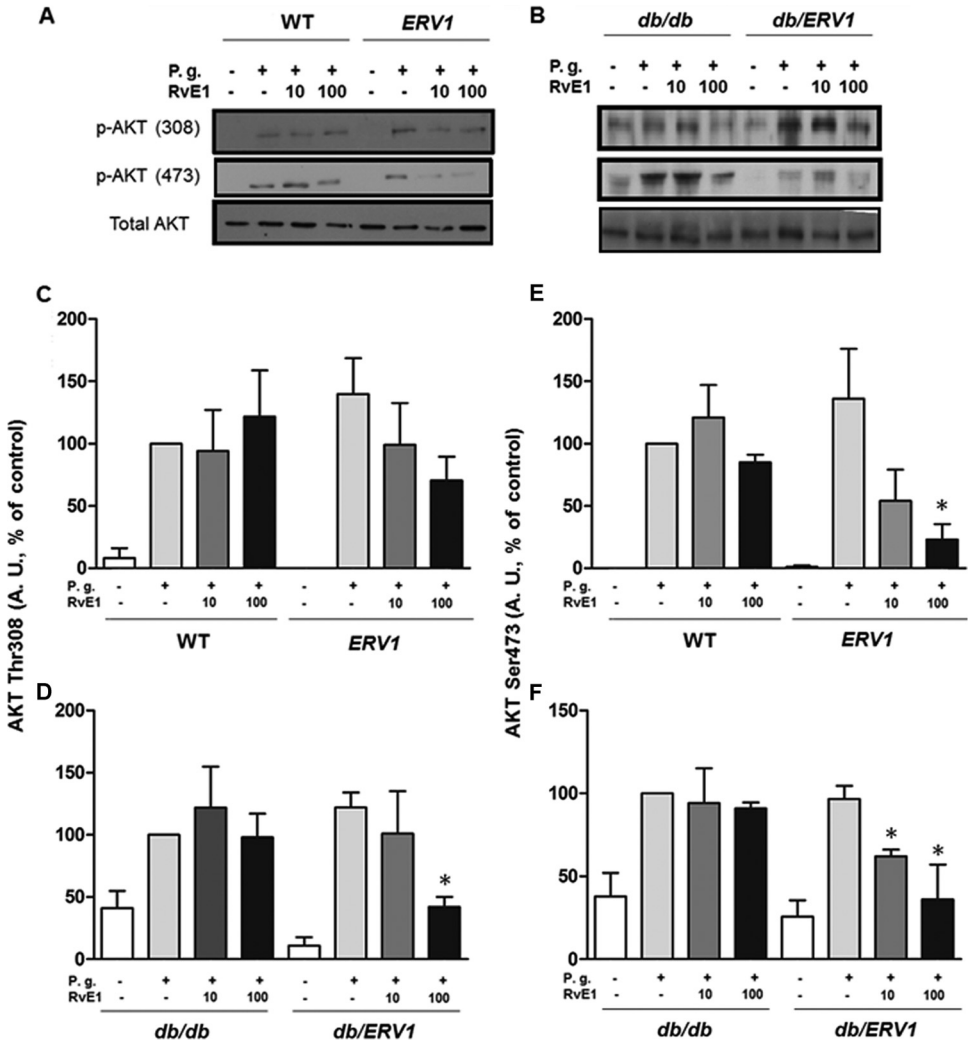

FIG 3 *P. gingivalis* (P.g.) induces phosphorylation of Akt that is reversed by RvE1. (A, B) Representative Western blot images quantified in panels C to F. (C, D) Densitometric quantification of Akt phosphorylation at threonine 308. (E, F) Densitometric quantification of Akt phosphorylation at serine 473. A. U., arbitrary units; *, $P < 0.05$ compared to vehicle control ($n = 4$).

ness of the epithelium and connective tissue is not significantly affected, the thickness of the adipose tissue is reduced by more than half in *db*/*ERV1* animals. The number and density of macrophages (cells/μm^2) in the adipose tissue layer were significantly reduced (Fig. 6B; $P < 0.05$).

DISCUSSION

We report here that the immunoresolvent RvE1 increases the phagocytosis, killing, and clearance of the periodontal pathogen *P. gingivalis* in healthy (WT) mice but does not rescue deficient phagocytosis and clearance in T2D model *db*/*db* mice and marginally rescues deficient *P. gingivalis* killing. Overexpression of the RvE1 receptor gene (*ERV1*) induced significantly greater phagocytosis and killing in response to RvE1 ($P < 0.01$) *in vitro* in both strains of overexpressing mice, i.e., normoglycemic and diabetic (*ERV1* and *db*/*ERV1*) mice (Fig. 2). Accordingly, *in vivo* experiments measuring *P. gingivalis* clearance in the dorsal air pouch model revealed that RvE1 increased the clearance of bacteria with a concomitant decreased influx of neutrophils, suggesting markedly increased efficiency of phagocytosis with a reduced potential for collateral tissue damage. Phagocytosis, killing, and clearance were impaired both *in vitro* and *in vivo* in neutrophils of *db*/*db* mice. RvE1 had no impact on phagocytosis and a marginal impact on killing by *db*/*db* neutrophils unless *ERV1* was overexpressed (*db*/*ERV1*). We further investigated the actions of RvE1 on signaling pathways in neutrophils preactivated by contact with *P. gingivalis* and observed that preincubation of neutrophils with RvE1 decreased the phosphorylation of Akt at both threonine 308 and serine 478 when *ERV1* was overexpressed; RvE1 stimulation was accompanied by a significant decrease in Akt and MAPK phosphorylation that was more profound in transgenic animals.

Accumulation of cutaneous fat and marked infiltration of the adipose tissue with active macrophages are characteristics of T2D thought to contribute to elevation of systemic inflammation, particularly as a source of the proinflammatory adipokine TNF-α (44–46). Interestingly, overexpression of *ERV1* reduces fat accumulation and markedly reduces macrophages in the adipose tissue of diabetic animals. These data suggest a failure of resolution path-

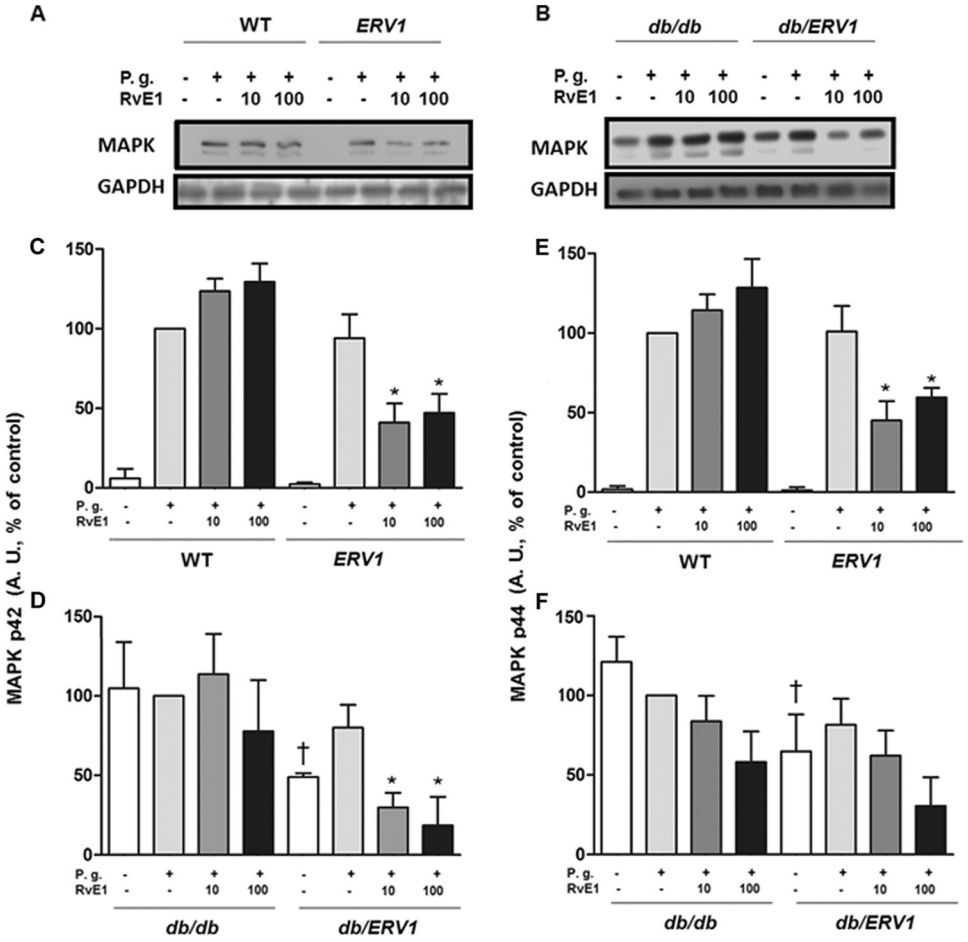

FIG 4 *P. gingivalis* (P.g.) induces phosphorylation of MAPK that is reversed by RvE1. (A, B) Representative Western blot images quantified in panels C to F. (C, D) Densitometric quantification of ERK phosphorylation (p42). (E, F) Densitometric quantification of MAPK phosphorylation (p44). A. U., arbitrary units; *, $P < 0.05$ compared to *ERV1* plus *P. gingivalis* plus vehicle; †, $P < 0.05$ compared to *db*/*db* without *P. gingivalis* plus vehicle; *, $P < 0.05$ compared to *db*/*db* plus *P. gingivalis* plus vehicle; ($n = 4$).

ways in T2D that can be modified by enhancing resolution. The implication is that agonists of resolution of inflammation may be useful as therapeutics for inflammatory complications of T2D in the future.

An uncontrolled innate immune response is central to chronic inflammatory diseases, including T2D and its complications, including periodontitis. Physiologic changes induced by hyperglycemia and metabolic dysregulation in T2D result in chronic inflammation. The prevailing hypothesis suggests that the hyperglycemic state drives the formation of excess advanced glycation end products, known as AGE, and the expression of its cognate receptor, RAGE (47). This, in turn, induces a proinflammatory cytokine profile contributing to elevation of inflammation and cellular dysfunction systemically. In both metabolic syndrome and T2D, chronic inflammation is associated with insulin resistance, impaired metabolism, and impaired wound healing. In health, an injury induced by pathogens or trauma induces acute inflammation that is characterized on a cellular level by rapid neutrophil infiltration that peaks within hours and is thereafter rapidly cleared to be replaced by monocyte/macrophage infiltration to clear the wound. These events are mediated to a great extent by specialized lipid mediators of resolution of inflammation that induce neutrophil apoptosis and nonphlogistic macrophage activation. Specialized proresolving mediators, such as resolvin E1, greatly influence the fate of innate immune cells. Our group and others have previously demonstrated that RvE1 enhances macrophage phagocytosis and efferocytosis (48).

An important concept that is supported by these data is that excess inflammation actually inhibits bacterial clearance and promotes bacterial growth. This was first demonstrated in murine colitis. In these experiments, neutrophil infiltration was inhibited, but phagocyte clearance and zymosan phagocytosis were enhanced *in vitro* and *in vivo* by RvE1 (49, 50). Later work with another member of the resolvin family, resolvin D2 (RvD2), in a murine sepsis model confirmed this concept, showing that in a cecal ligature and puncture model, animals receiving RvD2 exhibited greater survival and enhanced clearance of bacteremia (17). We also reported that treatment of periodontitis with RvE1 induced spontaneous clearance of periodontal pathogens (7). We questioned whether the resolution agonist RvE1 impacts the neutrophil response *in vitro* and *in vivo* with induced peritoneal neutrophils and an air pouch model, respectively, in diabetic and normoglycemic *ERV1* transgenic mice. After RvE1 treatment, phagocytosis was greater in *db*/*ERV1* and *ERV1* animals than in

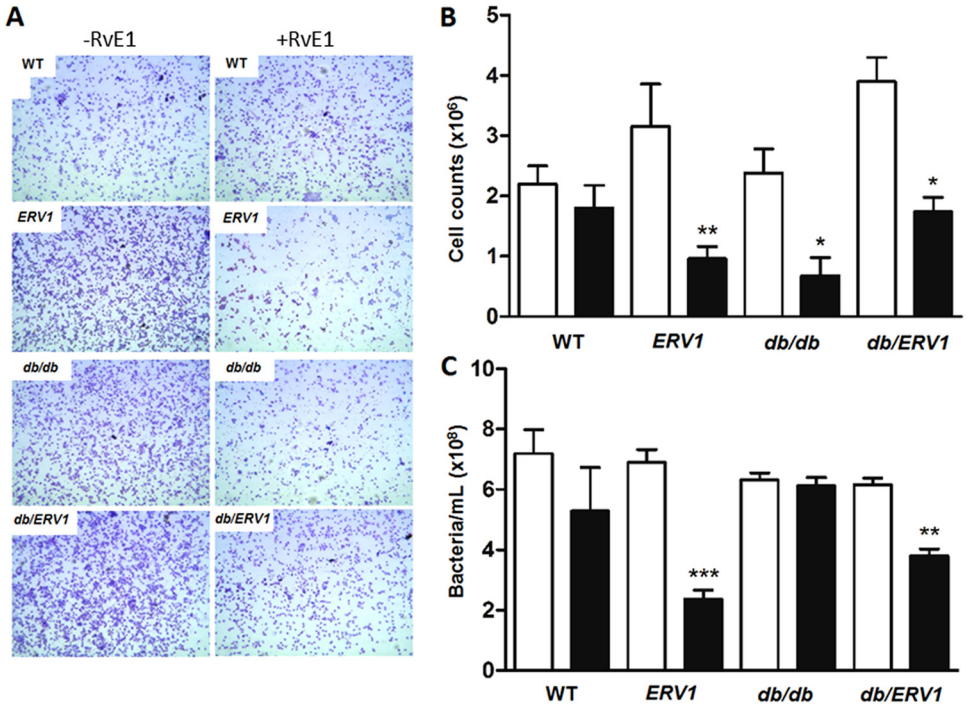

FIG 5 RvE1 decreases cell influx and increases *P. gingivalis* clearance. Neutrophils and bacteria were harvested from the air pouch after 4 h by lavage. (A) Representative images of the cellular infiltrate (Wright-Giemsa, <95% neutrophils) with or without RvE1 treatment in all four strains of mice. (B) Total number of neutrophils in the pouch after 4 h ($n = 4$). (C) Bacteria in the air pouch after 4 h. Bacterial numbers were quantified densitometrically (OD_{600}) after 24 h of growth in Wilkins-Chalgren broth and compared to a standard curve. Bars: black, RvE1; white, control. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ ($n = 6$).

db/*db* and WT animals. Thus, RvE1 was more efficient in modulating the phagocytic phenotype in *ERV1* positive genotype mice (*db*/*ERV1*, *ERV1*) that resulted in damped accumulation of neutrophils with more efficient bacterial clearance.

Previous observations have showed that ω-3 polyunsaturated fatty acids (PUFAs) enhance metabolism in both diabetic and obese mice. Improved whole-body insulin sensitivity and increased activity of resolvin pathways were observed upon dietary supplementation of ω-3 PUFA (51, 52). Consistent with the actions of ω-3 PUFAs, RvD1 and RvD2 each rescued impaired expression and secretion of adiponectin in a time- and concentration-dependent manner. ω-3 PUFA supplementation also modulated proinflammatory signals (TNF-α, interleukin-6 [IL-6], and IL-1β) and cellular function in lean and obese mice. Treatment of animals with exogenous RvD1 prevented hepatic steatosis and reversed insulin resistance, upregulating insulin response genes and increasing insulin tolerance (53). It is interesting that neutrophil functional abnormalities were not likewise corrected by RvE1 treatment. We did not follow blood glucose longitudinally in our experiments, but *ERV1* transgenic animals did not show improvements in glycemic control cross-sectionally. However, it is clear that overexpression of *ERV1* was necessary to improve the neutrophil response in *db*/*db* mice. There are at least four possible explanations for this. (i) The leptin mutation in *db*/*db* mice directly affects *ERV1* expression and function. (ii) There may be a threshold effect in which resolvin administration is sufficient to upregulate insulin response genes but insufficient to reverse neutrophil functional abnormalities. (iii) RvE1 treatment in our model was acute. Longer treatment regimens may be necessary to affect change. (iv) Overexpression of *ERV1* in myeloid cells replaces nonfunctional BLT-1 on *db*/*db* neutrophils.

Intracellular signaling after stimulation with RvE1 has been shown to be through extracellular binding to at least two receptors (*ERV1* and BLT-1). RvE1 has been shown to positively activate Akt phosphorylation in HeLa cells and human macrophages through *ERV1* (54). In our previous studies of RvE1 and osteoclasts, we observed that RvE1 attenuates the nuclear translocation of NF-κB, as well as the phosphorylation of Akt (55). Interestingly, the data suggest that when neutrophils, where the major receptor for RvE1 has been reported to be BLT-1 (33), are stressed, functional *ERV1* is expressed, which seems to dominate the response to anti-inflammatory mediators. This was demonstrated for chemerin (an anti-inflammatory protein ligand for *ERV1*) in myocardial infarction (56). The nature of *ERV1* expression and function by neutrophils from *db*/*db* animals remains to be elucidated, but it is clear that the phagocytosis and killing response to RvE1 is impaired. Overexpression of *ERV1* by *db*/*ERV1* transgenic animals rescues the response, implicating *ERV1* and also suggesting the BLT-1 is not functioning on *db*/*db* neutrophils.

The mechanism of the interactions between T2D and its complications, such as periodontitis, is unclear. It is, however, well documented that type 2 diabetic (*db*/*db*) mice exhibit more periodontal destruction than the WT (23) and that *ERV1* transgenic mice are resistant to periodontitis (36). Our work and the work of others provide further insights revealing that phagocyte interactions are dysregulated in people with diabetes and likewise with periodontitis (26, 57). Thus, mechanisms related to inflammation in periodontitis may be linked with the primary cause of T2D, inflammation. Without a clear understanding of these pathways, we cannot identify targets for the development of small molecules for pharmacologic intervention to predictably harness natural pathways to treat these important noncommunicable diseases.

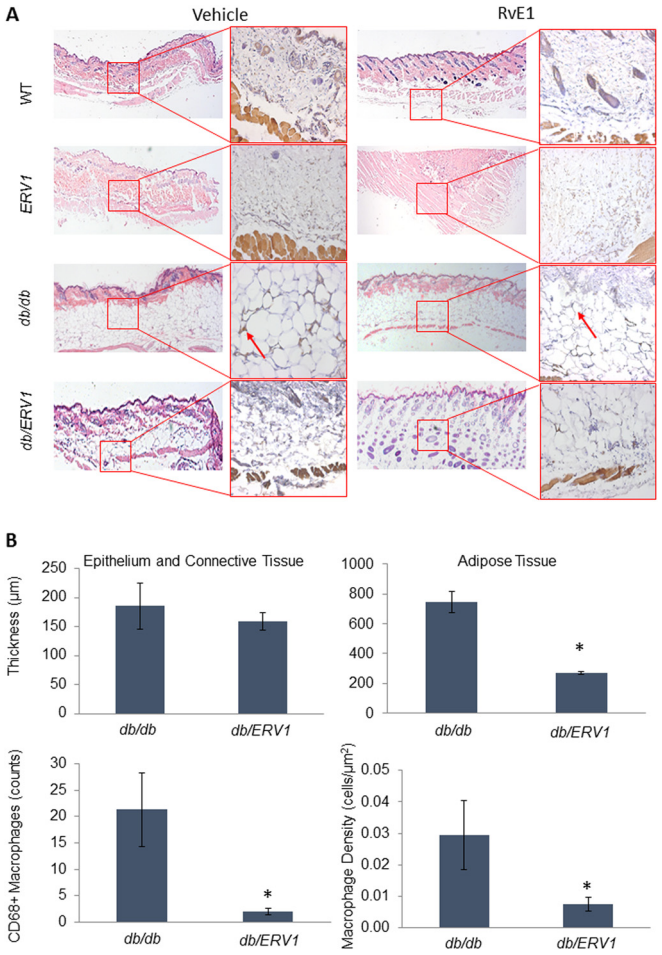

FIG 6 *ERV1* overexpression reduces cutaneous fat accumulation and macrophage accumulation in adipose tissue of diabetic mice. Dorsal air pouches were raised on the backs of all strains of mice. Tissues were stained with hematoxylin and eosin. Tissue macrophages were identified by immunohistochemical detection of CD68 expression. (A) Epithelium, connective tissue, and adipose tissue distribution is demonstrated in each group (*ERV1*, transgenic mice overexpressing ERV1; *db*/*db*, diabetic mice; *db*/*ERV1*, diabetic mice overexpressing *ERV1*) with or without RvE1 treatment. Larger panels demonstrate hematoxylin-and-eosin staining (magnification, ×2.5). $CD68^+$ macrophages in the adipose tissue layer are shown in inserts (arrows; magnification, ×20). Histological analysis of the air pouch lining reveals significant cutaneous adipose tissue accumulation with significant accumulation of $CD68^+$ macrophages in *db*/*db* mice that is not evident in diabetic *ERV1*-overexpressing (*db*/*ERV1*) mice. (B) Epithelium, connective tissue, and adipose tissue thicknesses were measured, $CD68^+$ macrophages were counted, and macrophage density in adipose tissue was calculated. Quantification of tissue thickness and macrophage numbers in the adipose tissue reveals significant reductions of both in *db*/*ERV1* animals. *, $P < 0.01$ [ANOVA] compared to the *db*/*db* group.

The characterization of the impact of RvE1 on the complete cycle of inflammation resolution at the phagocyte level will address major clinical complications of T2D. The relationship between diabetes and periodontal disease is reciprocal. Infections, including periodontal infections, have a significant impact on diabetic control, and diabetes is a significant risk factor for the development and severity of periodontal disease (58). New data clearly demonstrate that type 2 diabetics are refractory to standard periodontal therapy, which further emphasizes this interrelationship (59, 60).

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