

Proresolving Nanomedicines Activate Bone Regeneration in Periodontitis

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Abstract

Therapies to reverse tissue damage from osteolytic inflammatory diseases are limited by the inability of current tissue-engineering procedures to restore lost hard and soft tissues. There is a critical need for new therapeutics in regeneration. In addition to scaffolds, cells, and soluble mediators necessary for tissue engineering, control of endogenous inflammation is an absolute requirement for success. Although significant progress has been made in understanding natural resolution of inflammation pathways to limit uncontrolled inflammation in disease, harnessing the biomimetic properties of proresolving lipid mediators has not been demonstrated. Here, we report the use of nano-proresolving medicines (NPRM) containing a novel lipoxin analog (benzo-lipoxin A₄, bLXA₄) to promote regeneration of hard and soft tissues irreversibly lost to periodontitis in the Hanford miniature pig. In this proof-of-principle experiment, NPRM-bLXA₄ dramatically reduced inflammatory cell infiltrate into chronic periodontal disease sites treated surgically and dramatically increased new bone formation and regeneration of the periodontal organ. These findings indicate that NPRM-bLXA₄ is a mimetic of endogenous resolving mechanisms with potent bioactions that offers a new therapeutic tissue-engineering approach for the treatment of chronic osteolytic inflammatory diseases.

Keywords: periodontal disease, cardiovascular disease, bacteremia, resolution of inflammation, lipoxins, resolvins

Introduction

In the face of uncontrolled host responses, tissue engineering, regeneration, and reconstruction of diseased and injured tissues are significantly hampered (Lumelsky 2007). The ideal outcome of acute inflammation is complete resolution (Rock and Kono 2008). Uncontrolled acute inflammation can lead to tissue injury, chronic inflammation, tissue scarring, and fibrosis (Samuelsson et al. 1987; Funk 2001; Serhan et al. 2008). In any contaminated traumatic wound involving hard tissues, an uncontrolled inflammatory response causes neutrophil (PMN)-mediated tissue injury that in turn leads to irreversible bone loss. PMN are essential for protection in microbial host defense, but chronic PMN activation can release noxious materials leading to host injury and loss of organ function (Dalli et al. 2014). In chronic osteolytic inflammatory diseases such as periodontitis (Offenbacher et al. 2008; Williams 2008), sustained microbial challenge and a failure of endogenous resolution pathways result in tissue destruction (Van Dyke 2011). It is now appreciated that resolution of inflammation is an active process: an endogenous anti-inflammatory and proresolving mechanism that activates wound healing with tissue

regeneration instead of fibrosis and scarring (Levy et al. 2012; Ortega-Gomez et al. 2013). Recent results reveal that endogenous control of inflammation directly affects bone healing and regeneration (Hasturk et al. 2006; Hasturk et al. 2007). Mediators of resolution of inflammation also have actions beyond control of PMN, including receptor-mediated control of osteoclast and osteoblast function in wound healing and bone regeneration (Herrera et al. 2008; Zhu et al. 2013).

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Periodontitis is a significant public health problem worldwide. In the United States alone, recent reports estimate that 64.7 million adults, 47% of the adult population, suffer from periodontal disease; 38.5% have moderate to severe disease (Eke et al. 2012). Periodontitis is an inflammatory disease of bacterial etiology that results in host-mediated destruction of the supporting tissues of the teeth, including connective tissue attachment and bone. Clinically, periodontitis is difficult to treat with a high rate of recurrence. Regeneration of hard and soft tissues lost to disease is limited and not predictable (Murphy and Gunsolley 2003; Reynolds et al. 2003; Sculean et al. 2008).

Nonsoluble communication between cells has emerged as an efficient means of long-range communication beyond soluble cytokines and autacoids (Distler and Distler 2010). Membrane-shed vesicles, termed *microparticles*, can be employed to construct nano-proresolving medicines (NPRM) incorporating proresolving lipid mediators (LMs) that can target specific tissues without dilution or inactivation of the mediators (Dalli and Serhan 2012). Incorporation of proresolving mediators into NPRM has proven to be an effective approach for promoting survival in animal models of sepsis (Dalli et al. 2014), reducing inflammation and stimulating resolution (Norling et al. 2011). Here, we report regeneration of soft and hard tissues lost to inflammatory disease using proresolving nanomedicines.

Both lipoxin A₄ and resolvin E1 (RvE1) facilitate bone regeneration in small animal models of periodontitis (Hasturk et al. 2007; Herrera et al. 2008; Van Dyke 2011). We constructed NPRM containing a stable lipoxin analog benzo-lipoxin A₄ (bLXA₄, Sun et al. 2009) to create NPRM-bLXA₄ (Norling et al. 2011) for clinical evaluation in a large animal model of inflammation-induced bone loss. NPRM-bLXA₄ is a mimetic of endogenous resolving mechanisms with potent bioactions that offers a new therapeutic tissue-engineering approach for the treatment of chronic osteolytic inflammatory diseases. Here, we report a proof-of-principle study that documents induction of periodontal regeneration by an endogenous proresolving mediator in a nonhealing large animal model of chronic periodontitis.

Materials and Methods

Animals

The Institutional Animal Care Committee (IACUC) at the Forsyth Institute and Pine Acre Rabbitry Farm, Inc. reviewed and approved the animal protocol prior to any study activities. This study conformed to ARRIVE guidelines for pre-clinical animal studies. All procedures used in this study complied with the Association for Assessment and Accreditation of Laboratory Animal Care International-accredited guidelines. Two adult (~50 kg; 18 to 24 mo old) female miniature swine (Hanford) were purchased from

Sinclair Bio Resources (Windham, ME) and acclimatized for 7 d before procedures. The miniature swine, housed in metal pens separately, were maintained at 24 °C ± 2 °C and 55% relative humidity and fed a standard diet (ProLab Mini-Pig Diet; LabDiet, St. Louis, MO) and tap water ad libitum.

Surgical Procedures and Maintenance

Induction of chronic periodontal defects. Animals were pre-medicated with Telazol (4 to 6 mg/kg intramuscularly [IM]) and xylazine (2.2 mg/kg IM) and maintained by isoflurane 3% to 4% for induction and 0.5% to 2% for maintenance (Hasturk et al. 2011). Local anesthesia (2% lidocaine with 1:100,000 epinephrine–0.5 carpule/quadrant) was administered for hemostasis. Mucoperiosteal flaps were elevated with vertical releasing incisions. Interproximal periodontal defects were created using burs (6 mm in depth from the cemento-enamel junction [CEJ] and extending to the mid interproximal buccolingually). Sterile orthodontic wires were positioned below the CEJ and the twisted ends placed in the surgical defects in all 4 quadrants around the second and fourth premolars (Appendix Fig). Flaps were repositioned covering the ligatures using 4-0 Vicryl sutures (Ethicon, Inc., Blue Ash, OH). Animals were fed a soft diet (mixed with applesauce or water) for 6 wk to stimulate plaque accumulation. Oral examinations including digital photographs were repeated at 2 and 4 wk, and ligatures were removed at 6 wk. The sites were left untreated for 38 d to eliminate self-healing as a variable.

Treatments. At day 0 (baseline), animals were anesthetized and blood samples collected via external jugular vein. Mucoperiosteal periodontal flaps were elevated, defects debrided, and root surfaces scaled and root planed. A notch was placed at the defect base, and measurements (from CEJ to crestal bone and to the base of the defect) were recorded. Defects were treated with bLXA₄ alone (10 µg of 2.1 µM solution), NPRM alone, or NPRM containing bLXA₄ (NPRM-bLXA₄). Surgery alone was the negative control. Four defects were treated with each regimen. Randomization was performed by quadrant to prevent spillover to adjacent teeth. The 4-0 Vicryl sutures were placed, and pain medication (buprenorphin) was administered for 2 to 3 d. Sutures were removed at 2 wk.

Maintenance. Animals were monitored monthly to provide oral hygiene and assess healing and inflammation. Periodontal probing was not performed during the healing phase. Probing depth, hard-tissue measurements, histomorphometry, serum lipidomics, and micro computed tomography (µCT) analysis were performed by trained and calibrated examiners (D.N., histology; A.K., clinical, and M.O.F., µCT) who were blinded to treatment allocations.

NPRM with bLXA₄

NPRM were prepared from microparticles from isolated 4×10^8 human neutrophils from a single donor (Norling et al. 2011). Intercalation of active bLXA₄ ((5S, 6R, E)-methyl 5,6-dihydroxy-8-(2-((R,E)-3-hydroxyoct-1-en-1-yl) phenyl) oct-7-enoate (Avanti Polar Lipids, Alabaster, AL) at 0.1 μg per 5×10^5 NPRM with fluorescent phospholipid was performed by aqueous energy dissemination using a sonic dismembrator (output power 15 W, 15 min, 25 °C; Fisher Scientific, Loughborough, UK) as in Norling et al. (2011). NPRM-bLXA₄ was ultracentrifuged at 100,000 g to remove unbound bLXA₄ and resuspended in filtered phosphate-buffered saline. Incorporation of bLXA₄ was confirmed by flow cytometry (BD FACSCanto II; BD Biosciences, San Jose, CA) and liquid chromatography–tandem mass spectrometry (LC-MS-MS) as in Norling et al. (2011). One hundred microliters was delivered topically into the defects with a micropipette (Appendix Fig).

Three-Dimensional μCT Analysis and Quantification of Tissue Regeneration

Samples were placed in a standardized sample holder and scanned using high-resolution μCT (Scanco Medical, Brüttsellen, Sweden) at a spatial resolution of 5.376 μm (voxel dimension) with 1536×1536 pixel matrices. The 2-dimensional image data were stored in Digital Imaging and Communications in Medicine format and transferred to a computer for 3-dimensional reconstruction and analyses. Mandibular and maxillary regions were cropped from consecutive micro-tomographic slice images as volume of interest using Amira 3D software (VSG|FEI Visualization Sciences Group, Burlington, MA; Freire, Sedghizadeh, et al. 2011; Freire, You, et al. 2011). The original spatial resolution was maintained because data were not resampled.

The volume of new bone was measured within a digital box with fixed size (6 high \times 4 diameter at the base) positioned identically for all samples. Tissue type was segmented using global thresholding procedures (bone tissue = 3,741). Newly regenerated bone was determined by applying a cylindrical divider at the base of the previous bone defect. Total and new bone volume is presented as mean and standard deviation of voxel units calculated by Amira. Statistical significance was determined by analysis of variance (ANOVA).

Histology

Bone blocks were fixed in formalin for 3 d; maxillary sites were decalcified using Immunocal for about 6 wk, embedded in paraffin, and 6- μm sections made from each block. To provide quantitative histological measures of local inflammation, the inflammatory cell infiltrate was determined from 4 areas (0.09 mm² in total) of hematoxylin and

eosin–stained slides at 400 \times magnification. Inflammatory cells were clearly distinguishable based on morphologic characteristics and enumerated using Image J software. Data were analyzed by ANOVA with Bonferroni post hoc corrections for multiple comparisons.

Miniature Swine Sera Targeted LC-MS-MS–Based Lipidomics

Blood samples were collected at baseline and at 3 mo, centrifuged at 2,300 rpm, and frozen at -80 °C until analysis. Methanol (4 volumes, 4 °C, 30 min) containing 500 pg of deuterated internal standards d4-LTB₄, d8-5S-HETE, d5-LXA₄, and d4-PGE₂ to facilitate quantification of sample recovery were added to serum. LMs were extracted using C18-silica reverse-phase cartridges as in Dalli and Serhan (2012). Samples were eluted with 6 mL methylformate and dried using Speedvac and suspended in methanol/water for LC-MS-MS. The liquid chromatography–UV coupled with tandem mass spectrometry system includes QTrap 5500 equipped with a Shimadzu SIL-20AC auto-injector and LC-20AD binary pump. An Agilent Eclipse Plus C18 column (100 mm \times 4.6 mm \times 1.8 μm) was used with a gradient of methanol/water/acetic acid of 60:40:0.01 (v/v/v) to 100:0:0.01 at a 0.5-mL/min flow rate. To monitor and quantify the levels of the various LMs, we developed a multiple reaction–monitoring (MRM) method with signature ion fragments for each molecule. Identification was conducted using published criteria (Dalli and Serhan 2012). Calibration curves were obtained using synthetic and authentic LM mixtures, including d8-5S-HETE, d4-LTB₄, d4-PGE₂, LXA₄, LXB₄, LTB₄, PGE₂, PGD₂, PGF_{2 α} , TxB₂, RvE1, RvE2, RvD1, RvD2, RvD3, RvD5, PD1, and MaR1 at 12.5, 25, 50, and 100 pg. Linear calibration curves for each were obtained with r^2 values in the range of 0.98 to 0.99. Quantification was based on peak area of the MRM transition and the linear calibration curve for each compound. Reverse-phase chiral LC-MS-MS was conducted as described (Dalli and Serhan 2012).

Results

There were no complications, including general anesthesia, surgical treatments, or follow-up maintenance. No adverse reactions were noted. During periodontitis induction, the animals developed significant gingival inflammation characterized by gingival edema, bleeding, and redness; however, this did not affect eating and physical activity. A mean monthly weight increase of about 1 kg corroborated this observation.

Bone Evaluation Using μCT

The 3-dimensional region of interest for determination of bone volume changes was defined vertically as the distance from the CEJ of the tooth to the root notch made at the time

of surgery, buccolingually to the limit of the cortical bone and mesiodistally to the root surfaces (Fig. 1). Quantitative bone gain is apparent in Figure 1A, with new bone illustrated in red. NPRM-bLXA₄ treatment led to a statistically significant gain of bone compared with control, bLXA₄ alone, or NPRM alone ($P < 0.05$). Note that the notch in the root placed at the time of surgery at the bone level is no longer visible in the NPRM-bLXA₄-treated samples (Fig. 1C). Quantification of bone volume (Fig. 1B) reveals markedly greater bone gain with NPRM-bLXA₄ treatment. The probing pocket depths of the gingival sulcus at the chronic disease sites ranged from 6 to 8 mm presurgery. All treatments resulted in pocket depth reductions to a final probing pocket depth of 1 to 3 mm.

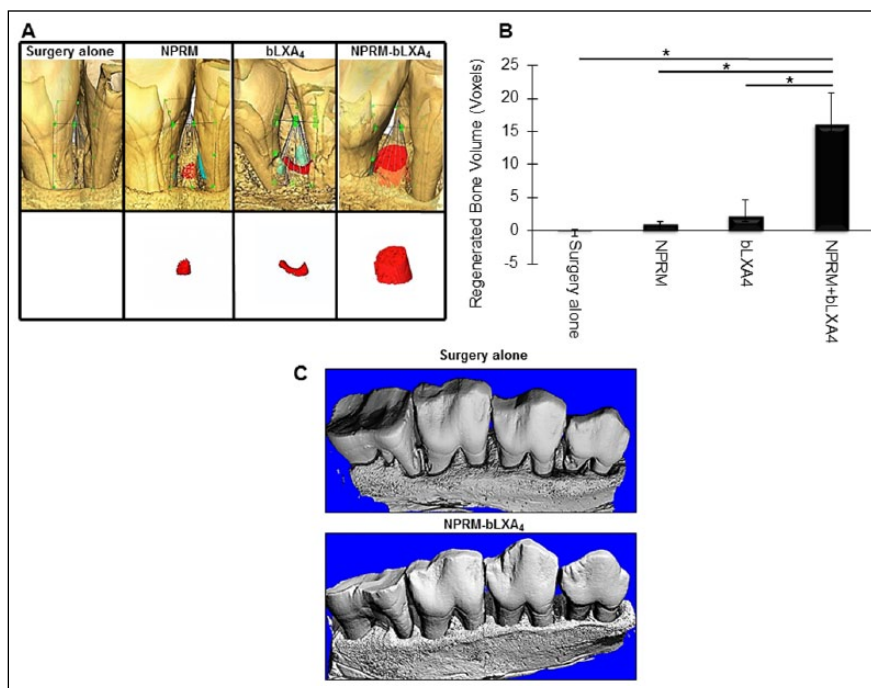


Figure 1. NPRM-bLXA₄ induces bone regeneration. **(A)** Micro-computed tomography (μ CT) images of the 4 treatment conditions: (-) sham surgery, unloaded NPRM, bLXA₄ alone, NPRM-bLXA₄ (red = new bone; $n = 4/\text{group}$). **(B)** NPRM-bLXA₄ induces greater bone formation ($*P < 0.001$, analysis of variance). **(C)** Three-dimensional reconstruction of μ CT of surgery alone and NPRM-bLXA₄-treated sites to provide visual references for changes induced by lipoxin treatment. Note that bone irregularities are resolved and root notches placed at the time of surgery are covered by new bone in the NPRM-bLXA₄-treated specimen.

Histology/Histomorphometry

To differentiate between bone growth and regeneration of the periodontal organ (new root surface cementum, periodontal ligament formation, and new bone), qualitative and quantitative histology results in Figures 2 and 3 reveal that NPRM-bLXA₄-treated sites showed well-organized and newly formed interproximal bone coronal to the root notch placed at the time of surgery with supracrestal connective tissue fibers that are parallel to the newly formed bone crest and anchored to new cementum on the root surface, confirming the μ CT findings and suggesting periodontal regeneration. By contrast, in the surgery alone group, the notch is clearly present coronal to the bone level filled with disorganized granulation tissue with little to no bone regeneration. Linear histomorphometric measurements made using the notch as the reference revealed that sites treated with NPRM-bLXA₄ showed statistically significant ($P = 0.012$) coronal regeneration of alveolar bone without regeneration in the surgery-alone sites. An intermediate response was noted for sites treated with NPRM alone ($P = 0.075$) and bLXA₄ applied in solution phase alone ($P = 0.027$).

Treatment with either the NPRM-bLXA₄ or bLXA₄ alone resulted in histologically healthy connective tissue and vascularization with minimal inflammatory cell infiltration compared with control NPRM or surgery alone (Fig. 3). NPRM-bLXA₄ treatment was specifically associated with increased density of fibroblasts. NPRM without LX analog resulted in an increased inflammatory infiltrate

especially in subepithelial areas, while surgical treatment alone showed dense inflammatory cell infiltration both in the subepithelial/supracrestal and interproximal areas (Fig. 3A). Statistical analysis revealed that NPRM-bLXA₄ and bLXA₄ alone markedly reduced the inflammatory infiltrate; the difference was statistically significant compared with surgery alone (Fig. 3B, $P = 0.012$ and 0.027 , respectively). Control NPRM without bLXA₄ also showed reduced inflammatory infiltrates compared with surgery alone; however, the difference was not statistically significant ($P = 0.067$).

Systemic LMs

To determine the potential impact of local NPRM-bLXA₄ administration on peripheral blood systemic LM levels, whole blood was obtained at the indicated intervals post-treatment. Since both animals received all treatments in this split-jaw design, the changes in LM levels with time were analyzed as an independent variable. LM levels were investigated using LM metabololipidomics and leukotriene B₄, Prostaglandins (PGD₂, PGE₂, PGF_{2 α}), lipoxins (LXA₄, LXB₄, LX pathway markers), and D-series resolvins

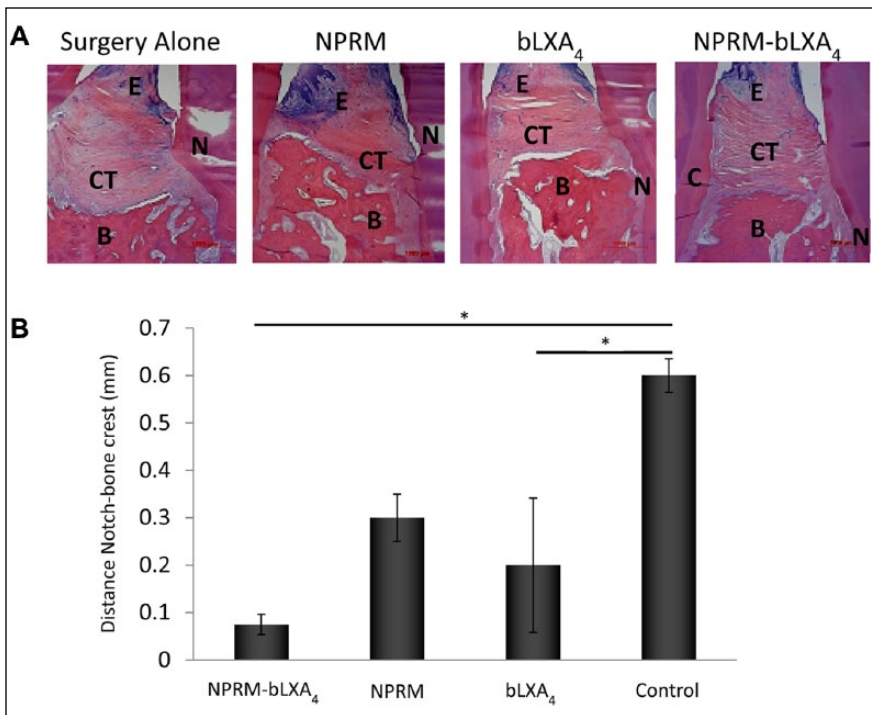


Figure 2. Histology confirms bLXA₄ regeneration of the periodontal organ. **(A)** NPRM-bLXA₄ induces regeneration of new bone, new connective tissue attachment, and new cementum; evidence for regeneration of the entire periodontal organ (N = notch, CT = connective tissue, B = bone, E = epithelium). **(B)** NPRM-bLXA₄ and bLXA₄ alone regenerate periodontium quantified as distance from the bone crest to the root notch created at the time of surgery (**P* < 0.05, analysis of variance; *n* = 4/group).

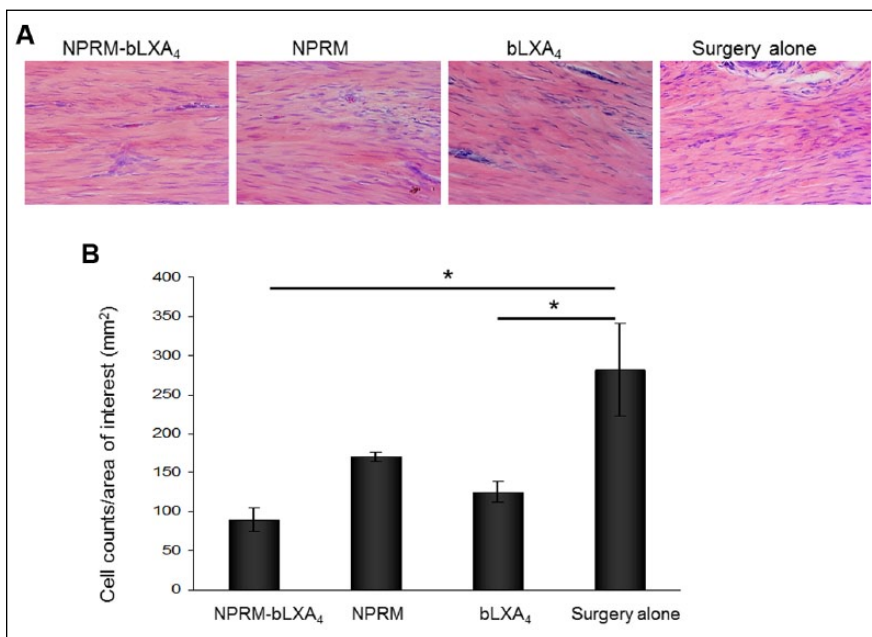


Figure 3. NPRM-bLXA₄ and bLXA₄ alone limit inflammatory cell infiltrate. **(A)** Histological images captured from hematoxylin and eosin-stained slides at 400× magnification at the subepithelial connective tissue at 3 mo. **(B)** Inflammatory cell counts were determined using Image J. (**P* < 0.05, analysis of variance; *n* = 4/group). Three captures were performed for each site, and the averages were used for analysis.

(RvD1-RvD6) serum levels were measured (Fig. 4; Table). These revealed that local treatment with the proresolving mediator analog (bLXA₄) markedly up-regulates systemic expression of other proresolving mediators (Table). Notably, both arachidonic acid and ω-3 polyunsaturated fatty acid-derived mediators were increased, including endogenous lipoxins and resolvins. Concurrently, proinflammatory prostaglandins PGE₂ and PGD₂ were decreased. A spike in systemic prostaglandins at week 12 (Table) appears attributable in large part to an increase in PGF_{2α}, an anabolic mediator associated with bone formation.

Discussion

The current report demonstrates that new NPRM-bLXA₄ activates distinct osteogenic responses in the treatment of chronic inflammatory osteolytic periodontitis when compared with surgery alone and either bLXA₄ or NPRM alone. NPRM-bLXA₄ treatment in this large animal model enhanced hard- and soft-tissue regeneration and reformation of the periodontal organ. NPRM-bLXA₄ treatment was also found to regulate systemic proresolving mediators, where it promoted endogenous biosynthesis of other resolvins and lipoxins and dampened production of proinflammatory mediators. In addition, NPRM-bLXA₄ stimulated production of eicosanoids that are known to favor bone formation systemically (Yao et al. 2009). These findings support the hypothesis that NPRM constructed with bLXA₄ potentiates the anabolic actions of bLXA₄ in regeneration of tissues lost to inflammatory disease by activating distinct proresolving and tissue protective pathways.

Periodontal disease is a bacterial biofilm-induced chronic inflammatory disease resulting in loss of connective tissue attachment to the teeth and osteoclast-mediated alveolar bone resorption (Taubman et al.

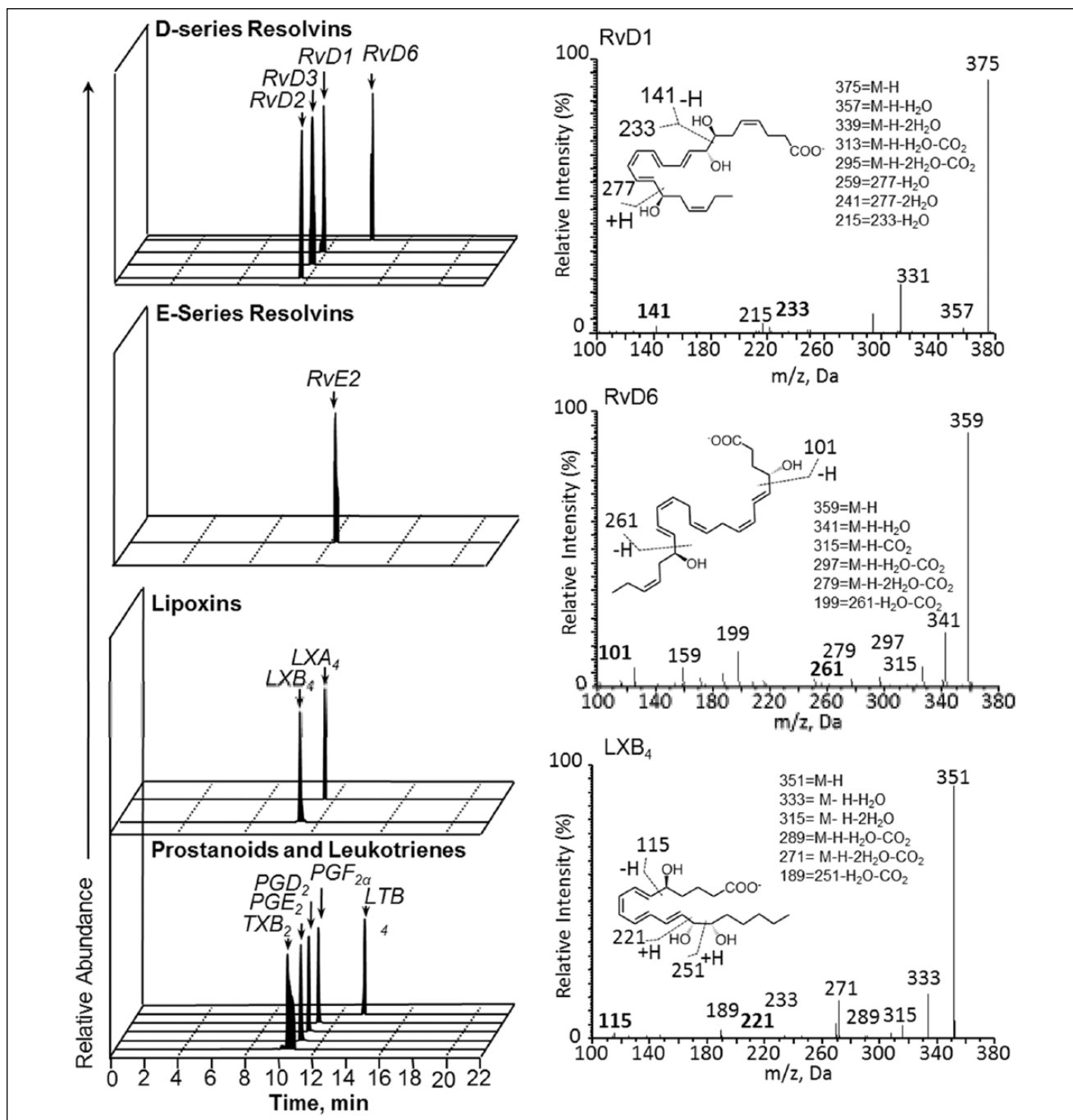


Figure 4. Regulation of peripheral blood lipid mediator profiles by bLXA₄ in periodontal disease. Whole blood was obtained at the indicated intervals post-treatment and serum isolated by centrifugation. Lipid mediator (LM) levels were investigated using LM metabolipidomics (see the Materials and Methods section for details). **(Left)** Representative multiple reaction–monitoring chromatograms of the identified lipid mediators in the peritoneal exudates. **(Right)** Accompanying tandem mass spectrometry spectra employed for identification.

2005). Leukocytes play multiple roles in the progression of periodontal disease, including phagocytosis and killing of bacteria, secretion of inflammatory cytokines, mounting of

specific immune response, and activation of osteoclasts (Graves et al. 2011). Leukocytes are essential in the host defense against oral pathogens; however, in susceptible

Table. Regulation of Peripheral Blood Lipid Mediator Profiles by NPRM-bLXA₄ in Periodontal Disease.^a

	Q1	Q3	Week 0		Week 2		Week 4		Week 8		Week 12	
			Pig 1	Pig 2	Pig 1	Pig 2	Pig 1	Pig 2	Pig 1	Pig 2	Pig 1	Pig 2
DHA bioactive metabolome												
RvD1	375	141	*	2.2	4.4	5.0	5.6	4.8	2.4	8.3	2.1	2.4
RvD2	375	215	*	5.5	10.9	12.2	13.8	11.9	5.8	20.5	5.1	5.8
RvD3	375	147	*	*	*	*	*	2.6	0.5	*	0.9	0.7
RvD5	359	199	*	*	*	*	*	*	*	*	*	*
RvD6	359	159	17.5	20.4	0.4	77.6	17.2	59.8	29.3	21.0	95.6	19.6
DHA	329	285	11,636.5	8,935.0	7,795.2	13,587.3	7,246.8	9,869.2	7,023.3	9,482.8	9,327.7	9,440.0
EPA bioactive metabolome												
RvE1	349	195	*	*	*	*	*	*	*	*	*	*
RvE2	333	199	*	6.4	6.9	7.4	5.9	7.4	7.2	1.9	1.3	17.0
RvE3	333	245	*	*	*	*	*	*	*	*	*	*
EPA	301	257	10,489.0	5,370.3	6,125.2	7,194.1	5,542.4	4,032.6	5,861.9	4,767.2	9,853.8	6,706.3
AA bioactive metabolome												
LXA ₄	351	115	0.5	0.8	0.2	1.1	0.7	3.9	1.4	0.3	1.8	1.3
LXB ₄	351	221	24.4	35.9	100.5	49.8	12.1	84.4	26.7	11.7	26.2	69.5
LTB ₄	335	195	31.2	3.2	102.5	1.6	17.3	0.4	1052.1	82.7	274.0	4.1
PGE ₂	351	189	18.9	7.6	2.8	7.3	5.8	7.2	7.8	3.4	19.2	15.8
PGD ₂	351	233	128.4	62.5	13.9	35.8	24.1	22.1	24.2	9.4	101.8	124.7
PGF _{2α}	353	193	1,646.7	494.0	45.1	327.6	41.1	34.2	93.5	20.4	1,180.0	1,086.1
TxB ₂	369	169	132.1	223.5	46.0	192.4	123.1	56.5	68.1	32.4	620.7	533.9
AA	303	259	152,248.6	68,793.8	79,177.1	72,991.1	70,407.7	63,948.9	68,914.9	49,290.0	102,885.2	73,402.1

^aWhole blood was obtained at the indicated intervals post-treatment and serum isolated by centrifugation. Lipid mediator (LM) levels were investigated using LM metabololipidomics (see the Materials and Methods section for details). Specific bioactive lipid mediator and precursor/pathway markers: Q1, M-H (parent ion); Q3, diagnostic ion in the MS-MS (daughter ion). Results are representative of *n* = 2 pigs and 12 determinations and are expressed as pg/mL of serum. The detection limit was ~1 pg. *Below detection limit.

individuals, unable to resolve the inflammatory lesion, chronic inflammation in the periodontium causes periodontal bone loss (Van Dyke 2011).

As in other osteolytic inflammatory diseases such as arthritis, current tissue-engineering procedures remain limited. There is a critical need for new therapeutics in regeneration. The classic triad of regenerative medicine (scaffold, cells, and soluble mediators) is insufficient because of our inability to control inflammation during regeneration. In rabbit periodontitis, agonists of resolution of inflammation, including lipoxins (LXA₄) and resolvins (RvE1), prevent periodontal bone loss (Hasturk et al. 2006) and in treatment of existing periodontitis permit periodontal regeneration (Hasturk et al. 2007), emphasizing the negative impact of uncontrolled inflammation.

Earlier observations revealed that in addition to the pro-resolving and anti-inflammatory actions of lipoxins, these receptor agonists have direct anabolic actions on bone consistent with the observed increases in bone regeneration. In experiments including transgenic rabbits systems (Serhan et al. 2003), topical application of lipoxin stable analogs dramatically reduced leukocyte infiltration, bone loss, and inflammation. LX-enriched humanized NPRM likewise

reduced PMN influx in murine peritonitis, accelerated healing, and protected against temporomandibular joint inflammation (Norling et al. 2011; Jones et al. 2012), indicating that LX-enriched NPRM offer new therapeutic approaches as mimetics of endogenous resolution of inflammation mechanisms with potent beneficial bioactions in vivo.

The advantage of loading bLXA₄ into NPRM is 2-fold. First, NPRM display anti-inflammatory properties through ALX/FPR2, the receptor for bLXA₄ an Annexin 1, which targets the delivery to inflamed tissues (Dalli et al. 2008). Second, many nanoparticle systems cause nanotoxicity by uptake of nanoparticles and activation of dendritic cells (Hess and Tseng 2007). NPRM provide a new, well-documented means to deliver locally and activate pro-resolving pathways. Thus, NPRM-bLXA₄ nanomedicines exhibit agonist actions in resolution by activating endogenous ALX receptors and releasing precursors for pro-resolving lipids, and they deliver their bLXA₄ cargo at the site of inflammation or surgical intervention (Norling et al. 2011).

Domesticated miniature pigs (miniature swine) are a marked improvement over other animal systems for human translational research because of their smaller size and adaptability to laboratory housing. The pig has more similarities to

humans than other commonly used laboratory animals in anatomy, physiology, immunology, and oral structures and is specifically suitable for regenerative studies (Harding et al. 2013). For these reasons, it is widely used in cardiovascular, digestive tract, and skin studies (Forster et al. 2010). Here, microbe-associated inflammation, leukocyte-mediated bone destruction (periodontitis), and regeneration of hard and soft tissues lost to inflammatory disease were evaluated. In addition, the nonhealing, chronic periodontal lesion in the Hanford miniature swine mimics the human situation including an omnivorous diet and jaw motions during chewing. The miniature swine represent a significant advance in translational research that targets novel approaches to inflammatory diseases (e.g., periodontitis and arthritis, in which inflammation and bone destruction are characteristics of the disease).

This proof-of-principle study does not contain enough animals to be generalizable, but the data are robust and the analysis is valid despite the small *N*. Four teeth were analyzed for each treatment between 2 pigs and statistics corrected for within-pig comparisons. In addition, the development of a large-animal periodontitis model with nonhealing lesions significantly advances the ability to test new treatments in a model significantly closer to the human condition.

In conclusion, the present results provide evidence for a new mechanism in regeneration of bone lost to inflammatory disease, as in periodontitis, using NPRM-bLXA₄ demonstrated here in a large-animal model. NPRM-bLXA₄ is functionally transferred to bone cell serum membrane receptors in addition to leukocytes modulating their responses to promote bone formation. In the complex, ongoing remodeling of bone, the cellular events initiated by bLXA₄ lead to efficient control of the biofilm environment, leading to the elimination of pathogens, up-regulation of endogenous proresolving LMs (e.g., lipoxins and D-series resolvins), as well as direct actions on bone cells that affect osteogenesis, resulting in significant regeneration of bone and connective tissue and reestablishment of the periodontal organ.

Author Contributions

T.E. Van Dyke, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; H. Hasturk, contributed to conception, design, data acquisition, analysis, and interpretation, critically revised the manuscript; A. Kantarci, M.O. Freire, J. Dalli, C.N. Serhan, contributed to data acquisition, analysis, and interpretation, critically revised the manuscript; D. Nguyen, contributed to data acquisition, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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