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SHORT COMMUNICATION

Nuclear Factor Erythroid 2-Related Factor 2 Down-Regulation in Oral Neutrophils Is Associated with Periodontal Oxidative Damage and Severe Chronic Periodontitis

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Corneliu Sima,*[†] Guy M. Aboodi,[‡§] Flavia S. Lakschevitz,[‡§] Chunxiang Sun,[§] Michael B. Goldberg,[‡] and Michael Glogauer[‡§]

From the Department of Applied Oral Sciences,* The Forsyth Institute, Cambridge, Massachusetts; the Department of Oral Medicine, Infection, and Immunity,[†] Harvard School of Dental Medicine, Boston, Massachusetts; and the Department of Periodontology[‡] and the Matrix Dynamics Group,[§] Faculty of Dentistry, University of Toronto, Toronto, Ontario, Canada

The balance between reactive oxygen species and antioxidants plays an important role in periodontal health. We previously demonstrated that high reactive oxygen species production by oral polymorphonuclear neutrophils (oPMNs) in chronic periodontitis (CP) refractory to conventional therapy is associated with severe destruction of periodontium. Herein, we show that inhibition of antioxidant production through down-regulation of nuclear factor erythroid 2-related factor 2 (Nrf2) pathway in oPMN, despite enhanced recruitment in the oral cavity, is associated with severe CP. Twenty-four genes in the Nrf2-mediated oxidative stress response pathway were down-regulated in PMNs of diseased patients. Downstream of Nrf2, levels of oPMN superoxide dismutase 1 and catalase were decreased in severe CP, despite increased recruitment. $Nrf2^{-/-}$ mice had more severe loss of periodontium in response to periodontitis-inducing subgingival ligatures compared with wild-types. Levels of 8-hydroxy-deoxyguanosine were increased in periodontal lesions of $Nrf2^{-/-}$ mice, indicating high oxidative damage. We report, for the first time, Nrf2 pathway down-regulation in oPMNs of patients with severe CP. PMNs of CP patients may be primed for low antioxidant response in the context of high recruitment in the oral cavity, resulting in increased oxidative tissue damage. *(Am J Pathol 2016, 186: 1417—1426; http://dx.doi.org/10.1016/j.ajpath.2016.01.013)*

Periodontal diseases are a group of highly prevalent inflammatory diseases affecting up to 70% of the US population.[1] Chronic periodontitis (CP), the most common form of periodontal disease, is characterized by progressive destruction of tooth-supporting structures as a result of altered host-biofilm interactions in the gingival crevice and unresolved inflammation. Although pathogenic biofilms are required for disease onset, host factors drive inflammation-mediated resorption of alveolar bone and damage to the connective tissue attachment. Neutropenia, altered PMN recruitment into periodontium, and impaired bacterial killing lead to severe forms of periodontitis.[2] On the other hand, hyperresponsiveness and excess reactive oxygen species (ROS) production likely contribute to relapse after CP treatment. Untreated CP is associated with considerable

morbidity and represents a risk factor for numerous systemic conditions with underlying low-grade inflammation. In addition to persistent inflammation, oxidative stress is believed to be a link between CP and metabolic syndrome.[3]

One mechanism suggested to contribute to periodontal tissue breakdown in CP is the increase in endogenous-produced oxidative stress observed in periodontitis lesions.[4,5] We have previously shown that CP patients refractory to conventional therapy present with more severe loss of tooth

attachment when their oral polymorphonuclear neutrophils (oPMNs) produce high levels of ROS, suggesting increased oxidative stress in periodontal tissues through excess PMN-derived ROS in these patients.[6] On the other hand, the protective role of antioxidants (AOs) in CP has been investigated by total antioxidant capacity measurements in whole saliva and gingival crevicular fluid (GCF) samples. Total antioxidant capacity levels were found to be significantly lower in CP patients when compared with healthy controls, although the mechanism responsible for oral AO decrease in CP has yet to be investigated.[7,8] Nonetheless, adjuvant AO therapy for CP has a potential to improve periodontal clinical parameters.[9,10]

Nuclear factor erythroid 2-related factor 2 (Nrf2) regulates gene transcription of a large group of antioxidant and phase 2 detoxifying enzymes playing a central role in cellular defense against oxidative and electrophilic insults.[11,12] In response to oxidative stress, cytoplasmic Nrf2 is released from Kelch-like ECH-associated protein 1 and binds to antioxidant response elements in the promoter region of many antioxidant enzymes, including catalase (CAT), superoxide dismutase (SOD), NAD(P)H:quinone oxidoreductase, glutathione S-transferase, heme oxygenase-1, glutathione peroxidase, glutamate cysteine ligase, and peroxiredoxin I.[12] In addition, because NF-κB−mediated production of cytokines and chemokines, such as tumor necrosis factor-α, IL-6, IL-8 (CXCL8), and chemokine ligand 2, is likely to be reduction-oxidation regulated, Nrf2 may play critical roles in control of inflammatory injury. Nrf2 was shown to play important roles in rheumatoid arthritis, gastrointestinal, renal, brain, skin, and pulmonary inflammation, and atherosclerosis.[13]

Herein, we investigated the oxidative state of local and systemic PMNs from patients with severe CP. It was found that down-regulation of the Nrf2 pathway in oPMNs is associated with increased recruitment in the oral cavity and severe CP. $Nrf2^{-/-}$ mice had increased local oxidative damage and alveolar bone and attachment loss at sites with CP.

Materials and Methods

Subject Population

Patients diagnosed with severe CP (eight subjects, two males and six females, aged 37 to 74 years) and periodontally healthy controls (six subjects, four males and two females, aged 24 to 38 years) were recruited in the periodontics graduate clinic at the Faculty of Dentistry, University of Toronto (Toronto, ON, Canada). Severe CP was defined using the American Association of Periodontology and the Eke et al[1] criteria as the presence of two or more interproximal sites with ≥6-mm attachment loss and one or more interproximal site(s) with ≥5-mm probing depth.[14] All subjects were systemically healthy and nonsmokers. Sample collection was completed before periodontal therapy. Subjects provided a written informed consent to participate, and the study was approved by the Research Ethics Board at the University of Toronto (protocol 24567).

PMN Isolation

Oral rinse and venous blood samples were collected from each subject. Oral rinse samples were collected as previously described.[15] Rinses were collected from subjects who had not eaten or performed oral hygiene for the previous 1 hour to avoid clearance of PMNs before donations.[16] Subjects were asked to rinse with 5 mL 0.9% isotonic sodium chloride solution (Baxter, Toronto, ON, Canada) for 30 seconds (six times with 3-minute breaks) and then asked to expectorate into a sterile 50-mL falcon tube. The same participants provided 10 mL of non-fasting venous blood. PMN isolation procedures from both oral rinse and blood samples were previously described.[15] Isolation procedures were initiated within 2 hours of sample collection. In brief, blood PMNs were isolated using a one-step PMN isolation solution (one-step polymorphs; Axis-Shield PoC, Oslo, Norway) and oPMNs were isolated through a series of filtrations through 40-μm (Fisher Scientific, Loughborough, UK), 20-μm, and 11-μm nylon mesh (EMD Millipore, Billerica, MA). Both isolation procedures resulted in >95% PMNs with >95% viability.

RNA Isolation

Total PMN RNA isolation was completed on the day of sample collection, using mirVana isolation kit (Ambion, Austin, TX) using the manufacturer-suggested protocol, as was previously described by the authors.[15] RNA samples were kept at −80°C until analyzed.

Gene Expression Microarray

Gene expression microarray was completed at the Center for Applied Genomics, Hospital for Sick Children Research Institute (Toronto, ON, Canada). Before gene expression microarray analysis, RNA samples were analyzed on a bioanalyzer using an RNA kit (2100 Bioanalyzer using an RNA 6000 PicoLabChip Kit; Agilent Technologies, Santa Clara, CA). All samples subjected to further analysis presented with an RNA integrity number >8. Microarray analysis was completed for oral and blood PMNs of four CP and four periodontally healthy subjects using the Illumina Human 12WG Expression BeadChip (48,000 gene transcripts; Illumina Inc., San Diego, CA). The microarray data comply with Minimum Information About a Microarray Experiment guidelines.[17] The data set was deposited at Gene Expression Omnibus (*http://www.ncbi.nlm.nih.gov/geo*; accession number GSE435525).

Microarray Data Analysis

The raw microarray data were preprocessed using R loaded with Lumi R package (*http://www.r-project.org*, last accessed January 11, 2016), followed by background correction, completed with BeadStudio Gene Expression Module (Illumina). Data were normalized by quantile normalization implemented with R loaded with Lumi R package. Differentially expressed genes were identified using linear models for

microarray data. Genes were selected controlling for false discovery rate at the level of 0.01 and by a minimum fold change of 2 (at $P < 0.05$). Pathway analyses were performed using the Ingenuity Pathway Analysis software version 01-07 (Ingenuity Systems, Redwood City, CA). Results from analysis of the same data set were previously reported.[18]

RT-qPCR

Quantitative RT-PCR (RT-qPCR) was completed for two subjects from each of the study groups, on the same RNA samples used for microarray analysis. RT-qPCR was performed in triplicate (CFX96 Real-Time System; Bio-Rad, Hercules, CA), as previously described.[15] Total RNA was reverse transcribed into cDNA using murine leukemia virus reverse transcription (Superscript II; Invitrogen, Thermo Fisher Scientific, Waltham, MA), and oligo-dT$_{18}$ VN primer (ACGT, Toronto, ON, Canada) in a 20-mL reaction system. A sample without template RNA, and another without reverse transcription were used as negative controls. *GAPDH* (forward, 5′-GGAGCGAGATCCCTCCAAAAT-3′; reverse, 5′-GGCTGTTGTCATACTTCTCATGG-3′) was used to normalize the expression data of the tested genes (*SOD1*: forward, 5′-GGTGGGCCAAAGGATGAAGAG-3′; reverse, 5′-CCACAAGCCAAACGACTTCC-3′; *CAT*: forward, 5′-TGGAGCTGGTAACCCAGTAGG-3′; reverse, 5′-CCTTT GCCTTGGAGTATTTGGTA-3′).

WB Analysis

SOD1 and CAT protein abundance was investigated through Western blot (WB) analysis. Oral and blood PMNs were isolated from four control subjects (two males and two females, aged 32 to 38 years) and five CP subjects (two males and three females, aged 37 to 74 years). Proteins were resolved by SDS-PAGE, transferred onto nitrocellulose membranes (Amersham-GE, Baie d'Urfe, QC, Canada), and probed with appropriate dilutions of the following antibodies: rabbit anti-human SOD1, anti-human CAT, and anti−β-actin (Cell Signaling Technology, Danvers, MA; CAT: EMD Millipore, Billerica, MA). Immunoreactivity was detected using an electrochemiluminescence system (Perkin Elmer, Akron, OH), and band intensity was quantified using ImageJ software version 1.50e (NIH, Bethesda, MD; *http://imagej.nih.gov/ij*).

Nrf2$^{-/-}$ Mice

Nfe2l2 knockout mice (*Nrf2*$^{-/-}$) are viable and have a normal phenotype under physiological conditions.[19,20] *Nrf2*$^{-/-}$ mice bred on a C57BL/6J background were purchased from Jackson Laboratories (Bar Harbor, ME). C57BL/6J mice were used as wild-type (WT) control. Mice were handled in accordance with the Guide for the Humane Use and Care of Laboratory Animals, and all of the experiments were approved by the Animal Care Committee, Faculty of Dentistry, University of Toronto. The *Nrf2*$^{-/-}$ genotype was verified before breeding using

PCR, following Jackson Laboratories' instructions. Mice of both sexes, aged 6 to 10 weeks, were used.

Bone Marrow PMN Isolation

Bone marrow PMNs were harvested from mice femur and tibia and isolated with Percoll gradient separation, as previously described.[21] Erythrocytes were lysed by addition of 5 mL of Pharm Lysis buffer (BD Biosciences, Mississauga, ON, Canada). Samples were washed and cells were resuspended in phosphate-buffered saline (Sigma-Aldrich, St. Louis, MO) and quantified using a Coulter counter (Beckman Coulter, Brea, CA).

Extracellular ROS Measurements

Extracellular ROS levels were measured by isoluminol-amplified chemiluminescence assay.[22,23] Bone marrow PMNs from six WT and four *Nrf2*$^{-/-}$ mice were incubated in a mixture of 3 mmol/L isoluminol and 6 U of horseradish peroxidase (Sigma-Aldrich) on preblocked 96-well plates, and stimulated with 10^{-6} mol/L phorbol myristate acetate (Sigma-Aldrich). Relative light units (RU) were measured using a fluorescence spectrophotometer (Fisher Scientific, Waltham, MA).

PMN Chemotaxis Assay

PMN chemotaxis was measured using a Zigmond chamber, as previously described.[21] PMN movement for bone marrow PMNs from three WT and three *Nrf2*$^{-/-}$ mice in formylmethionine−leucyl-phenylalanine gradient was recorded using time-lapse video microscopy with differential interference contrast optics (Nikon Eclipse E1000 with Nikon Coolpix 995 camera). Images captured at 15-second intervals for 15 minutes were analyzed with ImageJ software.

Ligature-Induced Periodontitis

As previously described, a split-mouth experimental periodontitis model was used to investigate periodontal tissue destruction in *Nrf2*$^{-/-}$ mice with disease.[24] Mice were anesthetized with ketamine/xylazine, and a 9-0 silk suture was placed in the gingival sulcus around the left second maxillary molar. The suture serves as biofilm retentive medium for subgingival biofilm maturation and induction of periodontitis. The contralateral right second molar served as the control. Mice were euthanized 21 days after ligature placements. Maxillae were harvested and defleshed in a colony of dermestid beetles at Royal Ontario Museum (Toronto, ON, Canada), and then freeze fumigated for 7 days at $-20°C$. Dry skulls were stained with methylene blue (1% in water) for 1 minute. Images of the buccal aspects of second molars (right, healthy; left, periodontitis) were taken in ×5 magnification using a video camera (PixeLINK, Ottawa, ON, Canada) mounted onto a stereomicroscope (Nikon Eclipse E1000). Alveolar bone loss (ABL) was measured by

morphometry from the cemento-enamel junction to the alveolar bone crest in three locations per tooth: midbuccal, mesiobuccal, and distobuccal (using ImageJ software).

Immunohistochemistry

Immunohistochemistry was completed at the Toronto Center for Phenogenomics (Mount Sinai Hospital, Toronto, ON, Canada). Maxillae collected 21 days after induction of periodontitis were fixed in 4% paraformaldehyde, decalcified in 10% formic acid for 7 days, and embedded in paraffin. Coronal sections of healthy and diseased areas were probed with primary goat anti−8-hydroxy-deoxyguanosine (8-OHdG; EMD Millipore, Billerica, MA) and secondary horse anti-goat biotinylated IgG (Vector Labs, Burlington, ON, Canada) and stained using an avidin/biotin tissue staining system (VECTASTAIN Elite; Vector Labs) and hematoxylin and eosin counterstaining. Three regions of interest were defined for each site (ligature and control): alveolar bone crest, sulcular epithelium, and gingival connective tissue. Cells positive for 8-OHdG were quantified using ImageJ software by automated particle counting after setting a uniform intensity threshold across all images analyzed.[25] Numbers of positive particles per area were calculated.

Statistical Analysis

One-way analysis of variance with Tukey-Kramer honestly significant difference post hoc analysis and paired t-tests were used as specified.

Results

Nrf2 Pathway Is Down-Regulated in Oral PMNs of CP Patients

Untreated CP patients and healthy controls were assessed for periodontal clinical parameters before blood and oral PMN collection. Patients with CP had significant loss of clinical attachment and generalized bleeding on probing (Supplemental Table S1). Sequential filtering of oral rinses removed debris and epithelial cells, resulting in 98% purity of oPMN isolates. PMN RNA was further isolated for microarray and RT-qPCR assays and proteins isolated for WB analysis of AO production (Figure 1A). CP patients had significantly higher oPMN numbers compared with healthy controls, despite similar peripheral blood PMN numbers (oPMN: healthy, $1.76 \pm 0.41 \times 10^6$/mL and CP, $3.75 \pm 0.68 \times 10^6$/mL; blood PMN: healthy, $4.90 \pm 0.92 \times 10^6$/mL and CP, $4.06 \pm 0.0.69 \times 10^6$/mL) (Figure 1B).

Microarray analysis of PMN gene expression revealed significantly higher *CXCL8*, *CXCR1*, and *TREM1* expression by oPMN in CP compared with blood PMN (6.41-, 1.43-, and 3.1-fold increase, respectively). There was a mild down-regulation (less than twofold) of critical factors involved in ROS production in oPMNs in CP compared with health (Supplemental Table S2). Ingenuity pathway analysis of microarray data predicted

significant down-regulation of AO expression downstream of Nrf2 in CP PMN (blood and oral) compared with health. Down-regulation of AO expression was predicted on the basis of down-regulation found in several upstream and downstream factors in the Nrf2 pathway (Figure 1, C and D). Analysis of oral versus blood PMN samples within each of the study groups revealed that unlike healthy controls, CP patients present a significant down-regulation of the Nrf2-mediated oxidative stress response pathway in oPMNs. Twenty-four and six Nrf2 pathway−associated genes were down-regulated or up-regulated, respectively (more than twofold) in oPMNs of CP patients when compared with blood PMNs (Supplemental Table S3). The same genes had similar expression in blood PMNs of CP and healthy subjects, with the exception of *SOD1* and *AKR1A1* (Supplemental Table S4). SOD1 and CAT, key regulators of superoxide and hydrogen peroxide, were used to verify the gene expression microarray results. RT-qPCR analysis confirmed the reduced expression levels of SOD1 and CAT in CP oPMN (oral/blood ratio: healthy-CAT, 8.70 ± 1.56; SOD1, 3.18 ± 0.90; CP-CAT, 0.15 ± 0.08; SOD1, 0.45 ± 0.22) (Figure 2, A and B). WB analysis of CAT and SOD1 production by oPMN of CP patients revealed significant reduction compared with health (CAT: healthy, 1.88 ± 0.22; CP, 1.09 ± 0.18; SOD1: healthy, 0.8 ± 0.15; CP, 0.41 ± 0.06) (Figure 2, C−F).

$Nrf2^{-/-}$ PMNs Produce Low CAT Levels

$Nrf2^{-/-}$ mice were used as a model of primary AO deficiency to assess the role of PMN AO deficiency on extracellular ROS production and migration toward chemoattractants. Protein extracts of bone marrow PMN assessed by WB showed lower CAT production by $Nrf2^{-/-}$ compared with WT (C57BL/6, 1.52 ± 0.42; $Nrf2^{-/-}$, 0.76 ± 0.09 relative to β-actin) (Figure 3, A and B). Both WT and $Nrf2^{-/-}$ produce high ROS levels in response to phorbol myristate acetate (baseline: C57BL/6, 172.5 ± 28.7 RU; $Nrf2^{-/-}$, 120.75 ± 25.3 RU; stimulated: C57BL/6, 267.3 ± 46.7 RU; $Nrf2^{-/-}$, 230 ± 50.7). Extracellular ROS levels were measured as peak RUs using an isoluminol-amplified chemiluminescence assay. Extracellular ROS production in response to phorbol myristate acetate stimulation was similar in $Nrf2^{-/-}$ and WT PMNs (C57BL/6, 1.53 ± 0.04; $Nrf2^{-/-}$, 1.93 ± 0.18) (Figure 3C). Because ROS were found to regulate migration speed and directionality of PMNs during chemotaxis, we investigated the migratory properties of $Nrf2^{-/-}$ PMNs.[26] $Nrf2^{-/-}$ had higher migration speed along an formylmethionine−leucyl-phenylalanine gradient compared with WT (C57BL6, 4.52 ± 0.34 μm/second; $Nrf2^{-/-}$, 6.86 ± 0.34 μm/second), despite similar overall response to the chemoattractant (migrated cells: C57BL/6, $83.32\% \pm 0.45\%$; $Nrf2^{-/-}$, $84.33\% \pm 4.90\%$) (Figure 3, D and E).

$Nrf2^{-/-}$ Mice Have Increased Periodontal Tissue Breakdown at Sites with Periodontitis

To get further insight into the impact of AO deficiency on periodontal tissue breakdown in periodontitis, ABL at sites
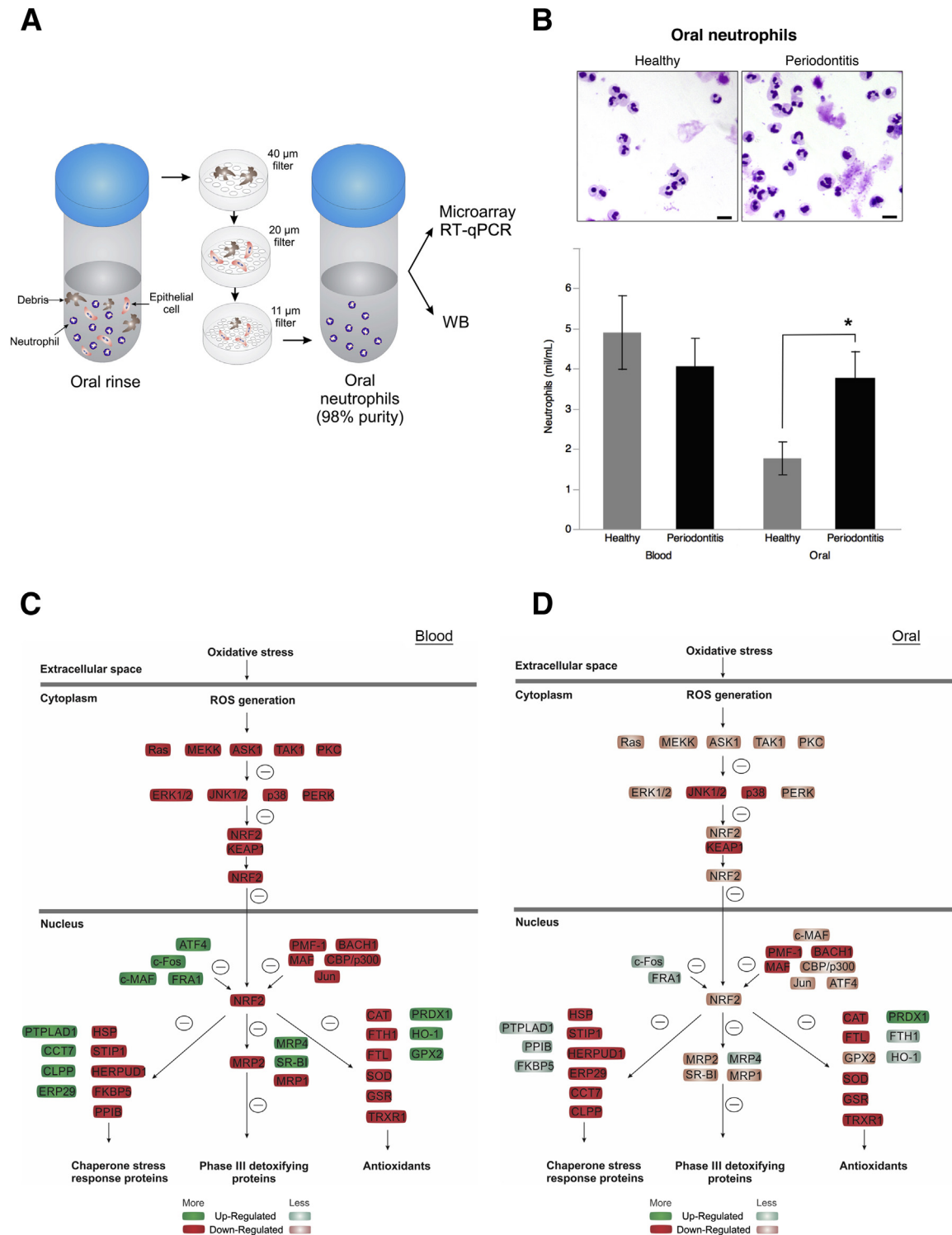
Figure 1 Nuclear factor erythroid 2-related factor 2 (Nrf2) antioxidant (AO) pathway is down-regulated in polymorphonuclear neutrophils (PMNs) of chronic periodontitis (CP) patients despite high oral recruitment rate. **A:** Oral rinses with 0.9% saline solution were used to isolate oral PMNs (oPMNs). Sequential filtering through 40-, 20-, and 11-μm filters removed debris and epithelial cells from the rinse, which resulted in 98% purity of oPMN samples. PMN RNA was further isolated for microarray and RT-qPCR assays and proteins isolated for Western blot analysis of AO production. **B:** Representative micrographs of Diff-Quick stained cytospins of oPMNs illustrating increased recruitment in CP patients compared with healthy controls. Oral and blood PMNs from eight CP patients and six healthy controls were quantified with a Coulter counter. **C** and **D:** PMN RNA was isolated from oPMNs of healthy and CP patients, as described in *Materials and Methods*. Blood PMNs were isolated using a one-step PMN isolation protocol. Regulation of Nrf2 antioxidant stress response pathway was predicted on the basis of microarray gene expression data using the Ingenuity Pathway Analysis software for blood and oPMN in CP compared with health. Data are given as means ± SD (**B**). *P < 0.05 (paired *t*-test). Scale bar = 10 μm (**B**). ROS, reactive oxygen species; RT-qPCR, quantitative RT-PCR.
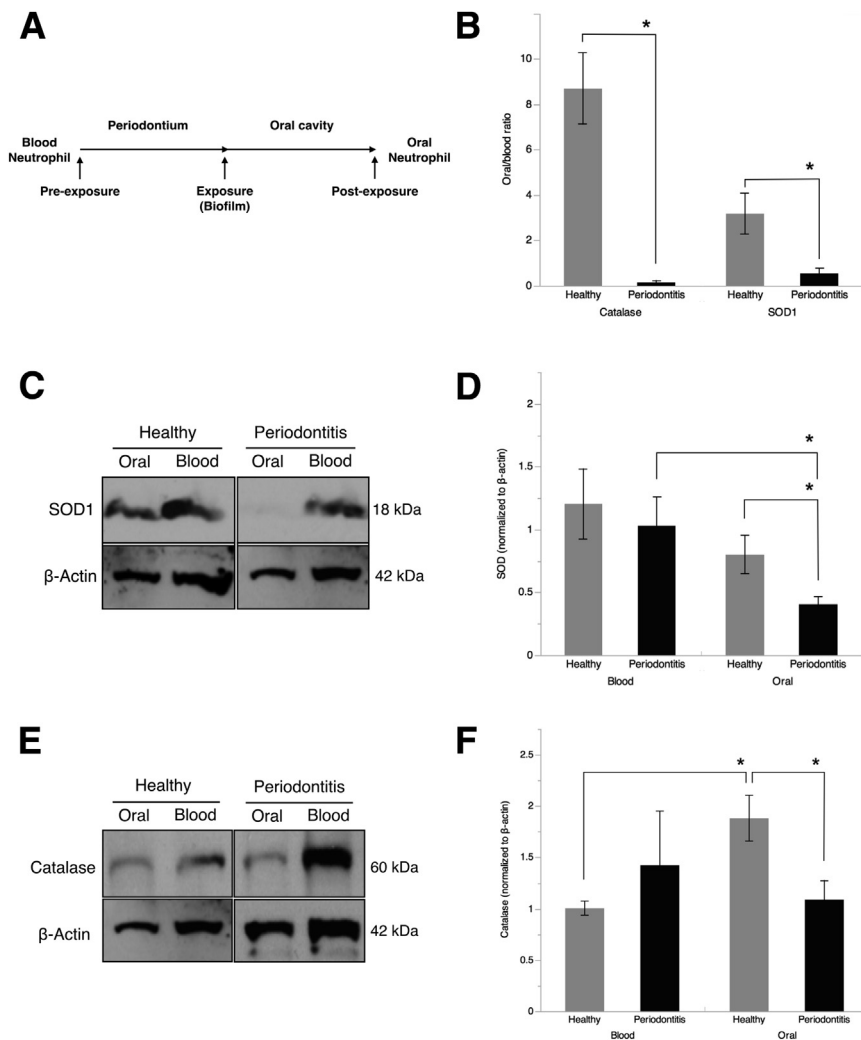
Figure 2 Oral polymorphonuclear neutrophils (oPMNs) of chronic periodontitis (CP) patients produce low levels of catalase (CAT) and superoxide dismutase (SOD1). **A:** We investigated the PMN antioxidant (AO) response to subgingival biofilms by considering blood PMNs as preexposure and oPMNs as postexposure in the context of PMNs' migratory path from vasculature to the oral cavity through the gingival crevice, where periodontitis-inducing subgingival biofilms reside and induce the inflammatory response. PMN RNA was isolated from oral and blood PMN, as described in *Materials and Methods*, and quantitative RT-PCR performed for *CAT* and *SOD1*. **B:** Oral versus blood ratios for *CAT* and *SOD1* normalized to *GAPDH* gene expression were compared in health and disease. **C–F:** CAT and SOD1 production by oral and blood PMNs was analyzed by Western blot analysis and normalized to β-actin. The oral/blood band intensity ratio was then calculated for each patient. All data were expressed as means ± SEM (**C–F**). $n = 4$ (**B–F**, controls); $n = 5$ (**C–F**, patients). *$P < 0.05$ [paired *t*-test (**B**) or one-way analysis of variance (**C–F**)].

with ligature-induced periodontitis was measured by morphometry in $Nrf2^{-/-}$ mice. Statistically significant ABL was observed in $Nrf2^{-/-}$ mice at sites with periodontitis compared with C57BL/6 (WT) mice (239.7 ± 16.4 μm versus 162.4 ± 7.6 μm, respectively). Both groups presented significant ABL at periodontitis sites compared with contralateral healthy molars, and no differences were found between control sites (Figure 4, A and B). Loss of attachment was measured on histological sections of diseased molars stained with Masson's trichrome. $Nrf2^{-/-}$ mice had more epithelial and connective tissue attachment loss compared with WT mice (172.6 ± 8.1 μm versus 166 ± 2 μm, respectively) (Figure 4, C and D). These results demonstrate a more severe tissue breakdown in $Nrf2^{-/-}$ mice, indicating a cytoprotective role of Nrf2 in periodontitis.

$Nrf2^{-/-}$ Mice Have High 8-OHdG Levels at Sites with Periodontitis

To assess ROS damage at sites with periodontitis, we quantified 8-OHdG−positive cells and found increased numbers around ligated molars of $Nrf2^{-/-}$ mice compared with healthy molars and with WT control mice (control: C57BL/6, 1.97 ± 0.39; $Nrf2^{-/-}$, 1.49 ± 0.19; ligature: C57BL/6, 2.08 ± 0.25; $Nrf2^{-/-}$, 6.23 ± 1.05 cells/square pixels) (Figure 4, E and F). Positive cells identified at the control sites are considered as physiological background level of DNA oxidation.[27] Analysis of 8-OHdG−positive cell levels in different regions of interest in the soft tissues and along the alveolar bone surface measured cells/area found significant ROS-mediated damage in the epithelium and connective tissue compared with bone.

Discussion

The current study demonstrates, for the first time, that the Nrf2 pathway is down-regulated in oPMNs of patients with severe CP compared with periodontally healthy controls. Twenty-four upstream and downstream Nrf2 pathway factors were down-regulated twofold to fivefold in oral compared with blood PMNs of patients with CP who also recruited oPMNs in higher numbers. In addition, we showed
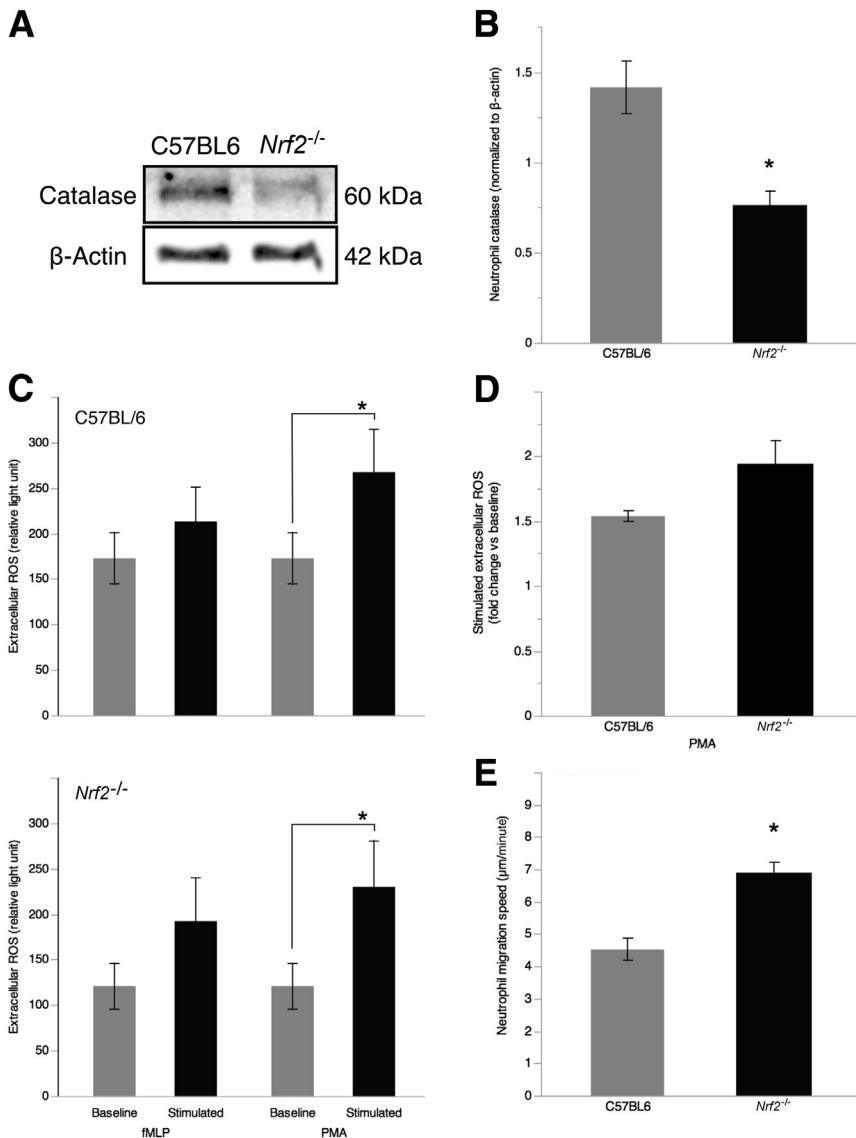
Figure 3 Polymorphonuclear neutrophils (PMNs) of nuclear factor erythroid 2-related factor 2 ($Nrf2^{-/-}$) mice produce low levels of catalase (CAT) and have increased migration speed. **A:** CAT production by bone marrow PMNs from $Nrf2^{-/-}$ mice was assessed by Western blot analysis and compared with wild-type C57BL/6 mice. **B:** CAT levels normalized to β-actin were compared between genotypes. **C:** Extracellular reactive oxygen species (ROS) production by $Nrf2^{-/-}$ PMN in response to fMLP and 10^{-6} mol/L PMA (stimulation, 30 minutes) was measured by isoluminol-amplified chemiluminescence. **D:** Chemotaxis was measured in Zigmond chambers along an fMLP gradient (10^{-6} mol/L). PMN movement was recorded using time-lapse video microscopy. **E:** Migration speed and efficiency (percentage migrated cells) were measured by playback analysis of 15-minute recordings. All data were expressed as means ± SEM (**E**). $n = 4$ mice per group (**B** and **E**). *$P < 0.05$ (t-test). fMLP, formylmethionine—leucyl-phenylalanine; PMA, phorbol myristate acetate.

that oxidative damage as a result of *Nrf2* knockout results in more severe loss of periodontal tissues in an animal model of periodontitis. The Nrf2 pathway controls the expression of a wide array of genes essential for production of cytoprotective factors, its activation leading to cell survival and tissue protection during oxidative stress conditions.[11,12] It has also been shown that osteoclastic bone resorption is substantially promoted in Nrf2-deficient osteoclast precursor cells compared with WT cells.[28] This must be kept in mind when interpreting our results because the lack of AO also affects the osteoclasts in addition to the PMNs. The role of CAT in maintaining periodontal health was previously demonstrated in acatalasemia patients who are systemically healthy, but have an increased incidence of CP.[29] Similarly, SOD1 levels were decreased in whole saliva and serum samples from CP patients.[30—32] Therefore, our results indicate that PMN *Nrf2* down-regulation in the context of increased periodontal recruitment aggravates tissue destruction. ROS neutralization may play a role in

preventing a continuous PMN influx to allow for resolution of inflammation to proceed because PMN chemotaxis is a ROS-dependent process.[33,34]

Nrf2 down-regulation in oPMNs of CP patients as cause for reduced PMN ability to neutralize ROS and withstand oxidative stress supports previous reports of increased oxidative damage in CP.[4,10] Interestingly, ingenuity pathway analysis predicted a more significant Nrf2 pathway down-regulation in blood PMNs of patients with CP compared with healthy controls. This seems to suggest that constitutive *Nrf2* expression may be required for adequate PMN responses to subgingival biofilms, which is associated with periodontal health. The difference in AO production we observed may also be a reflection of age and sex differences between CP patients and healthy controls in our study. Several studies demonstrated a significant increase in intracellular ROS production by peripheral blood PMNs in CP patients within a similar age range compared with sex- and age-matched healthy controls.[21,35] However, the
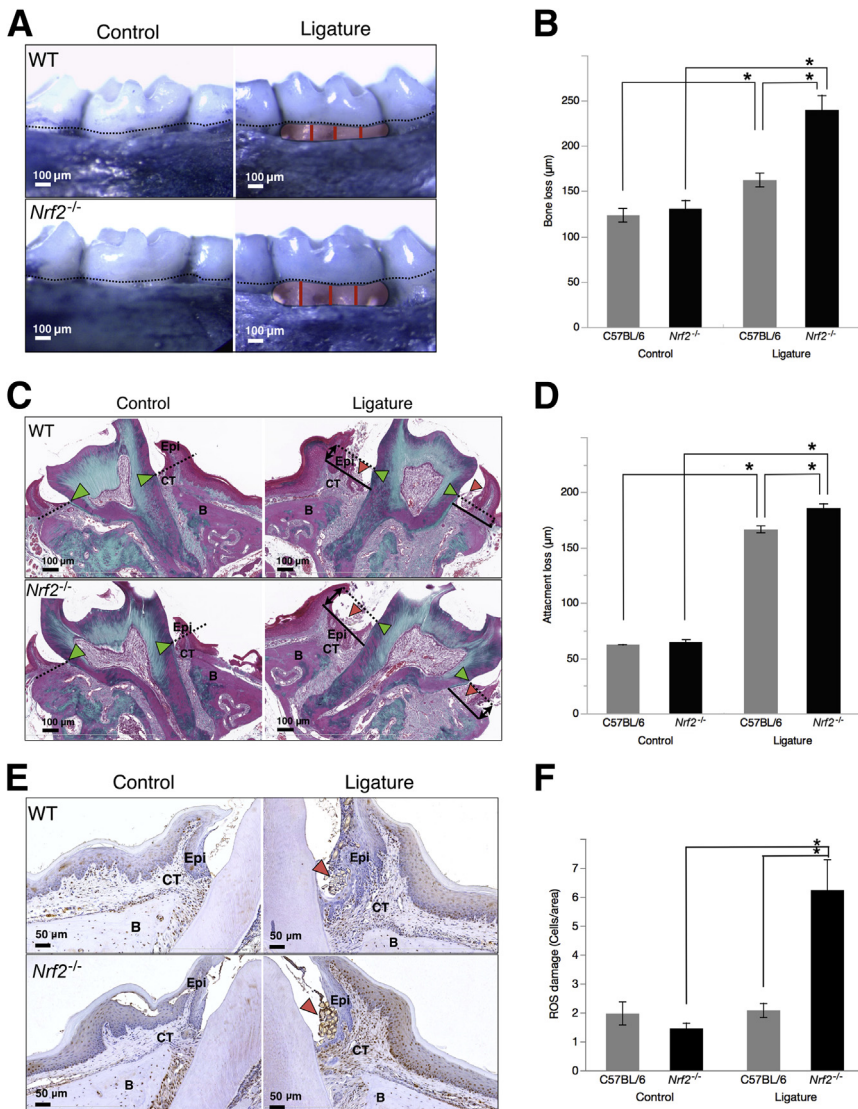
Figure 4 In response to ligature-biofilms, nuclear factor erythroid 2-related factor 2 ($Nrf2^{-/-}$) mice develop severe bone and attachment loss associated with high oxidative stress. **A:** Periodontitis was induced in $Nrf2^{-/-}$ mice using a ligature-biofilm model. Representative micrographs of buccal aspects of ligated and contralateral healthy control molars demonstrate the bone loss 21 days after induction of periodontitis. **Dotted lines** represent the cement-enamel junction. **Red lines** represent the measurement of alveolar bone loss along the exposed roots. **B:** Alveolar bone loss was measured by morphometry from the cement-enamel junction (**dotted lines** in **A**) to the bone crest along the exposed roots (**red lines** in **A**) on defleshed methynene blue—stained maxillae. **C** and **D:** Loss of connective tissue attachment was measured on Masson trichrome—stained sections from the cement-enamel junction as reference for baseline base of gingival sulcus (**green arrowheads**, **dotted lines**) to the base of the periodontal pocket (**solid line**) on buccal and palatal surfaces of ligated molars. **Red arrowheads** indicate the ligature. **E:** Reactive oxygen species damage in periodontitis lesions surrounding the ligature (**red arrowheads**) was assessed by immunohistochemistry for 8-hydroxydeoxyguanosine (8-OHdG), a specific marker for oxidative damage to DNA. **F:** Oxidative damage was quantified in epithelium (Epi), connective tissue (CT), and at bone level (B) by counting 8-OHdG$^+$ cells per surface area. All data were expressed as means $\pm$ SEM (**F**). $n = 12$ (**B**, WT); $n = 11$ (**B**, $Nrf2^{-/-}$); $n = 3$ mice per group (**C**, **D**, and **F**). $*P < 0.05$ (one-way analysis of variance). WT, wild type.

investigation of peripheral blood PMNs may not provide a full understanding of the mechanisms underlying PMNs' role in CP pathogenesis.

Recently, our group demonstrated specific gene expression in oPMNs obtained from healthy subjects, when compared with peripheral blood PMNs.[15] We further found that in a subset of patients with CP, who are refractory to conventional therapy, oPMNs are hyperactive and produce high ROS levels associated with more severe loss of periodontal tissues.[6] One study showed increased stimulated levels of ROS in GCF of CP patients compared with healthy controls but no difference in superoxide generation by blood PMNs or in AO capacity of cell-free GCF in disease versus health.[36] Malodialdehyde (MDA), a thiobarbituric acid reactive substance used as a marker of lipid peroxidation to assess oxidative damage, was found increased in GCF and saliva of patients with CP.[10,37,38] Tsai et al[37] reported 200- to 400-fold higher MDA levels in GCF compared with saliva but similar salivary AO capacity. However, salivary

AO capacity may have a lower prediction value for CP activity than oPMN AO capacity, considering that most oPMNs reach the oral cavity through the gingival crevices.[39–41] To our knowledge, the current study is the first to report an association between reduced oPMN AO production and severe CP, and opens the question of whether blood PMNs in patients with CP are primed to a low response to specific subgingival bacteria. Whether low AO production by oPMNs in severe CP is because of systemic priming or because of local inflammatory milieu remains unknown.

The microarray data found that blood PMNs of CP patients have a more pronounced Nrf2 pathway downregulation, indicating systemic priming. One study reported high 8-OHdG levels in whole saliva and GCF of patients with CP in a Japanese population, but systemic levels of 8-OHdG were not measured.[42] However, GCF levels of 8-OHdG were suggested as a biomarker for DNA damage caused by oxidative stress and an indicator for CP severity.[43] Konopka et al[44] showed that 8-OHdG levels in

gingival blood of patients with aggressive periodontitis were higher than those with CP, suggesting a correlation between ROS damage and disease severity. D'Aiuto et al[45] have shown that severe CP patients have higher Diacron-reactive oxygen metabolites in serum and reduced total serum AO capacity compared with healthy controls. In search for salivary biomarkers for oxidative stress and alveolar bone loss in CP, a recent study has found increased salivary 8-OHdG and MDA levels, and reduced salivary total anti-oxidant capacity in CP patients. A correlation between salivary MDA levels and C-terminal telopeptide of type I collagen, a biomarker for bone loss, was also reported.[46] These findings point to a possible systemic low AO expression in CP, but the relative contribution of oPMNs to low oral AO capacity may be critical for disease progression.

In summary, our present study indicates that *Nrf2* down-regulation in blood and oPMNs of CP patients may predispose to severe periodontal tissue breakdown, likely through increased local oxidative damage. Furthermore, PMNs of CP patients may be primed for low AO response in the context of high recruitment in the oral cavity.

Acknowledgments

Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2016.01.013*.

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