ORIGINAL REPORT: EPIDEMIOLOGIC RESEARCH

Associations of *P2RX7* Functional Diplotypes with Localized Aggressive Periodontitis

T.H. Harris^{1,2}, M.R. Wallace^{3,4}, H. Huang¹, H. Li³, and L.M. Shaddox^{1,5}

Abstract: Aim: The purpose of this study was to test for the role of the *P2RX7* receptor in localized aggressive periodontitis (LAP).

Methods: Peripheral blood was obtained from 95 subjects with LAP and 76 healthy unrelated controls (HUCs). Three *P2RX7* single-nucleotide polymorphisms (rs1718119, rs2230911, and rs3751143) were genotyped from these subjects, and their peripheral blood samples were stimulated with lipopolysaccharide (LPS) from *Escherichia coli* and tested for inflammatory markers. The 3 *P2RX7* single-nucleotide polymorphisms were found to be in perfect linkage disequilibrium, and a total of 4 haplotypes and 9 diplotypes were identified among all subjects. For both subject populations, the 9 diplotypes were grouped into 4 functional groups and tested for association with subject inflammatory response. To specifically study the effects of extrinsic activation of the *P2RX7* receptor in LAP, peripheral blood samples from were stimulated under

3 treatments: LPS, LPS + ATP, and LPS + ATP + *P2RX7* selective inhibitor. The effects of these treatments on *P2RX7* receptor activity were measured through Luminex protein assay. Last, to test whether receptor stimulation was related to *P2RX7* expression, relative mRNA levels of *P2RX7* were quantified with real-time quantitative polymerase chain reaction.

Results: Several associations between the *P2RX7* diplotypes and LPS-stimulated blood chemokine/cytokine levels were found between the LAP and HUC populations ($P < 0.05$). *P2RX7* activation resulted in statistically significant differences in IL-1 β and IL-12p40 concentrations for both subject populations. The relative *P2RX7* mRNA levels increased significantly after addition of its inhibitor for both LAP and HUC populations.

Conclusions: This study detected an association between *P2RX7* functional diplotypes and *in vitro* immune response of whole blood from subjects with LAP. In addition, we found that inhibition of the activated

P2RX7 receptor leads to increased *P2RX7* mRNA levels, suggesting a feedback loop (ClinicalTrials.gov NCT01330719).

Knowledge Transfer Statement: The results of this study suggest that *P2RX7* functional diplotypes are associated with LAP and their *in vitro* immune response to bacteria. Ongoing studies to uncover the mechanistic link between *P2RX7* and LAP phenotypes could lead to the development of preventive approaches for susceptible subjects.

Keywords: cytokine(s), inflammation, genetics, periodontal diseases, single nucleotide polymorphism, molecular genetics

Introduction

Periodontitis is a complex multifactorial disease that causes damage to tooth-supporting structures such as the periodontal ligament and alveolar bone. One key factor of periodontal disease is the host's abnormal response to microbial communities present in the oral cavity. Under normal conditions,

DOI: 10.1177/2380084419863789. ¹Department of Periodontology, College of Dentistry, University of Florida, Gainesville, FL, USA; ²Department of Oral Biology, College of Dentistry, University of Florida, Gainesville, FL, USA; ³Department of Molecular Genetics and Microbiology, College of Medicine, University of Florida, Gainesville, FL, USA; ⁴University of Florida Genetics Institute, Gainesville, FL, USA; ⁵Current affiliation: Division of Periodontology, Department of Oral Health Practice, College of Dentistry, University of Kentucky, Lexington, KY, USA. Corresponding author: L.M. Shaddox, Division of Periodontology, Department of Oral Health Practice, College of Dentistry, University of Kentucky, 800 Rose St., Room D446, Lexington, KY 40536-0297, USA. Email: lshaddox@uky.edu

A supplemental appendix to this article is available online.

© International & American Associations for Dental Research 2019

the host has a symbiotic relationship with bacteria and biofilms present in the oral cavity. However, in the presence of genetic predisposition and/or environmental factors that have not yet been characterized, the symbiotic relationship becomes abnormal/dysbiotic, resulting in an overly aggressive host response, disrupting the normal balance in bone metabolism and causing tissue destruction (Graves et al. 2011; Fine et al. 2018).

Localized aggressive periodontitis (LAP), previously known as localized juvenile periodontitis and recently termed periodontitis with high risk of progression (grade C), is considered a very uncommon form of periodontitis that primarily affects first molars and incisors and often shows familial aggregation (Marazita et al. 1994; Li et al. 2004; Diehl et al. 2005; Papapanou et al. 2018). It is thought to have both genetic and environmental contributing factors, and its greatest incidence occurs in individuals of African descent (Susin et al. 2014). A previous study showed that whole blood from individuals with LAP and, to a lesser extent, their healthy family members demonstrated a hyperinflammatory immune response after being challenged with periodontopathic bacteria or their by-products (Shaddox et al. 2010; Shaddox et al. 2016). However, specific genetic markers that relate to this inflammatory response have not been yet identified.

Purinergic receptor signaling has been shown to modulate inflammation, and members of this receptor family have been associated with cancer and a number of inflammatory and immune disorders (Burnstock 2017). In particular, the purinergic receptor P2X7 (encoded by gene *P2RX7*) has been extensively studied and found to have a dual activity, as it can either attenuate or exacerbate disease progression in a number of disorders (Burnstock 2017; Savio et al. 2018). Previously, we found associations of haplotypes constructed from 3 nonsynonymous *P2RX7* single-nucleotide polymorphisms

(SNPs) in linkage disequilibrium (rs1718119 [T348A], rs2230911 [T357S], and rs3751143 [E496A]) with the LAP hyperinflammatory response (Harris et al. 2019). These 3 SNPs have been reported in functional assays to cause either a gain (rs1718119, rs2230911) or loss (rs3751143) of function effect in vitro relative to wild type genotypes and are associated with inflammatory, musculoskeletal, and cardiovascular disorders (summarized in Sluyter 2017). To build on our previous observations and further elucidate the role of *P2RX7* in the LAP hyperinflammatory response, in this study we utilized experiments to specifically test P2X7 receptor activity based on the diplotypes in subjects with LAP and periodontally healthy controls. This included measuring *P2RX7* expression and cytokine/chemokine concentrations after adenosine 5'-triphosphate (ATP) stimulation of the receptor and testing for associations between subjects' *P2RX7* functional diplotype group data and in vitro whole blood inflammatory lipopolysaccharide (LPS) response levels.

Methods

Subjects

The study was approved by the Institutional Review Board at the University of Florida (201400349), and it followed STROBE guidelines (Strengthening the Reporting of Observational Studies in Epidemiology; Vandembroucke et al. 2014). All participants or their legal representatives signed an approved informed consent form to be included in the study. Participants were recruited between February 2007 and November 2017 from the Leon and Duval county health departments in Tallahassee and Jacksonville, Florida, respectively, and from the dental clinics of the University of Florida, Gainesville and Naples. Subjects with LAP were included if they met the following criteria: self-identified as African American, aged 5 to 25 y, and diagnosed with LAP (following established criteria at the beginning

of this study: characterized by the presentation of periodontal pockets with a depth ≥ 5 mm on the first molar and/or incisor and in no more than 2 other teeth, with concomitant attachment loss ≥ 2 mm and visible radiographic bone loss). Periodontally healthy subjects were defined as having absence of both concomitant attachment loss and radiographic bone loss. Subjects were excluded if they were diagnosed with systemic diseases/conditions (e.g., diabetes, leukemia, neutropenia, lupus, Down's syndrome, rheumatoid arthritis, irritable bowel syndrome, or any inflammatory condition that has shown association with periodontal inflammation), were currently taking medications that could influence the progression and/or clinical characteristics of periodontal diseases (e.g., steroids, cyclosporine, warfarin, chronic use of anti-inflammatory medications), had taken antibiotics within the previous 3 mo, had current use of tobacco, or were pregnant/lactating women. All clinical parameters were collected from certified and calibrated periodontists in the study. The study was conducted according to the Declaration of Helsinki and is registered at ClinicalTrials.gov (NCT01330719).

Genotyping

DNA was extracted and purified from subjects' blood leukocytes with either the standard phenol/chloroform extraction or the PureGene system (Qiagen). The 3 *P2RX7* SNPs (rs1718119 [T348A, exon 10], rs2230911 [T357S, exon 10], and rs3751143 [E496A, exon 13]) were genotyped with TaqMan SNP genotyping assays (ThermoFisher Scientific) at the University of Florida pharmacogenomics core on an Applied Biosystems 7900 HT platform. DNA Sanger sequencing or polymerase chain reaction (PCR)-restriction fragment length polymorphism was used for quality control/validation and determination of genotypes that failed TaqMan genotyping. DNA concentrations were at 10 ng/ μ L for TaqMan genotyping and 50 ng/ μ L for

PCR-based genotyping to fill in missing data. PCR thermal cycling conditions were adapted from manufacturer recommendations: temperature profile included 30 cycles of denaturation at 98 °C for 10 s, annealing between 59 and 62 °C for 30 s, and extension at 72 °C for 30 s. Primers used for *P2RX7* genotyping are listed in Appendix Table 1. Genotypes were tested for Hardy-Weinberg equilibrium. Linkage disequilibrium between SNP pairs was calculated with Cubex (<http://www.oege.org/software/cubex/>) and was found to be complete among all 3 SNPs ($D' = 1.0$). One allele at each SNP was denoted “ancestral” as annotated in its corresponding dbSNP entry and is underlined here (<https://www.ncbi.nlm.nih.gov/projects/SNP/>): rs1718119 (C>T; T348A), rs2230911 (C>G; T357S), and rs3751143 (T>G; E496A). From these 3 SNPs, a total of 4 haplotypes and 9 diplotypes were identified among the study participants, as described in the Results section.

LPS Stimulation of Blood and Quantification of Cytokines/Chemokines

Peripheral blood samples collected by venipuncture from 95 subjects with LAP and 76 healthy unrelated controls (HUCs) at baseline (prior to treatment) were analyzed in vitro for cytokine/chemokine secretion after LPS stimulation as described previously (Shaddox et al. 2010). Briefly, a sample of the whole blood was diluted in RPMI1640 media (Invitrogen) with Antibiotic-Antimycotic (Corning) and stimulated with 1 µg/mL of ultrapure *Escherichia coli* LPS (InvivoGen) or left untreated. After 24 h, culture supernatants were collected and frozen until analysis with multiplex Luminex cytokine/chemokine detection kits (Millipore). Fifteen cytokines and chemokines were quantitated on a Luminex array platform: eotaxin, TNFα, interferon gamma (IFN-γ), interleukin 1 beta (IL-1β), IL-2, IL-7, IL-6, IL-8, IL-10, IL-12p40, IP-10, MCP-1, GM-CSF, G-CSF, and MIP-1α. Data were acquired by Luminex 200 and analyzed with Milliplex

Analyst software (Millipore) with standard curves and a 5-parameter logistic.

P2X7 Receptor Stimulation in Whole Blood and Quantification of Cytokines

To specifically study these outcomes with and without P2X7 receptor activation, peripheral blood samples were collected from 52 subjects (28 LAP and 23 HUC) and stimulated following similar methods to those described here and previously (Spildreorde et al. 2014). Whole blood was diluted in RPMI1640 media (Invitrogen) in 3 treatments: 1) 1 µg/mL of standard *E. coli* LPS (InvivoGen) for 150 min, 2) 1 µg/mL of LPS for 120 min and then an additional 30 min with 6mM ATP (Cayman Chemical), and 3) with 50µM A438079 (P2X7 receptor antagonist; Abcam) added during the final 15 min of the 120-min LPS incubation before ATP addition (Spildreorde et al. 2014). After 2.5 h, the blood cells were separated from the culture supernatants by centrifugation and lysed with TRIzol LS for subsequent RNA extraction, while the culture supernatants were collected and frozen until analysis of specific cytokines (IL-1β, IL-2, IFN-γ, and IL-12p40) with customized multiplex Luminex 200 detection kits (Millipore). These cytokines were chosen for analysis per the results of the full panel in samples that were not subjected to P2X7 stimulation and inhibition. Cytokine data from supernatants were analyzed with Milliplex Analyst software (Millipore) with standard curves and a 5-parameter logistic. LAP and HUC whole blood stimulations were performed with samples from a variety of time points in the study.

Real-time Quantitative PCR mRNA Analysis

Total RNA was extracted from subjects' whole blood samples from the P2X7 activation experiment ($n = 21$, LAP; $n = 13$, HUC) with TRIzol LS (Invitrogen). Concentrations and purity of total RNA samples were determined via Nanodrop spectrophotometry (ThermoFisher). RNA was reverse transcribed with

iScript cDNA Synthesis Kit (Bio-Rad Laboratories) as described by the manufacturer. Real-time quantitative PCR of *P2RX7*, B-actin (*ACTB*), and β2 microglobulin (*B2M*) to measure mRNA levels was performed with the CFX96-C1000 Touch thermocycler (Bio-Rad Laboratories) with primers detailed in Appendix Table 2. One microliter of the cDNA reaction was amplified in the real-time quantitative PCR experiment with 100 nM of the PCR primers and the SsoAdvanced Universal SYBR Green PCR Supermix (Bio-Rad Laboratories) in a final volume of 20 µL. Data were analyzed with CFX Manager Software (Bio-Rad Laboratories). Relative expression of *P2RX7* was measured with the $2^{-\Delta\Delta C_q}$ calculations (Winer et al. 1999) in the CFX Manager software (Bio-Rad Laboratories), with *ACTB* and *B2M* serving as the reference genes. Samples were run in triplicate and the average used for analysis.

Statistical Analysis

All statistical analyses were performed with GraphPad Prism 7.00 for Windows (GraphPad Software). *P2RX7* diplotype frequencies (combinations of haplotypes) were analyzed for association with LAP and HUC through 2-tailed chi-square analysis. Associations of blood LPS stimulation cytokine/chemokine concentrations with *P2RX7* functional diplotypes in LAP and HUC populations were performed with Mann-Whitney tests or unpaired *t* tests. Associations of IL-1β and IL-12p40 concentrations in LAP and HUC blood poststimulation treatment were performed with the Friedman's test with Dunn's multiple comparisons or repeated measures 1-way analysis of variance with the Bonferroni multiple comparisons correction. Comparisons of the relative levels of *P2RX7* mRNA between subject populations were performed with Mann-Whitney tests.

Results

Study Participants

We recruited 179 subjects with LAP into the overall study, from which subsets

Table 1.

Clinical Parameters for LAP and HUC Populations.

	CAL BL	Affected Sites ^a		Full Mouth ^b		BL	
		PD	BL	PD	CAL	BOP	Plaque
HUC	NA ^c	0.00 ^c	NA ^c	2.3 ± 0.7	NA ^c	19.6 ± 13.3	35 ± 19.9
LAP	3.1 ± 1.2	5.5 ± 0.9	12.7 ± 10.1	2.7 ± 0.7	2.3 ± 0.7	21.1 ± 14.7	39.4 ± 23.3
<i>P</i> value		<0.0001		<0.0001		0.61	0.39
	No HUC	LAP high	No HUC	LAP high	No HUC	LAP high	LAP high

BL, baseline; BOP, bleeding on probing (full mouth parameters); CAL, clinical attachment level; HUC, healthy unrelated control; LAP, localized aggressive periodontitis; NA, not applicable; PD, pocket depth.

Values are presented as mean ± SD.

^aPD >4 mm with CAL >1 mm and bone loss.

^bData based on all sites in the mouth.

^cNo affected sites in healthy controls—thus, no CAL detected.

Table 2.

P2RX7 3-SNP Diplotypes and Frequencies in LAP and HUC Populations.

Diplotype ^a	Subjects, Proportion (<i>n</i>)		Functional Effect	Diplotype Group, <i>n</i>
	LAP	HUC		
11	0.24 (21)	0.20 (15)	Gain of function	78
12	0.25 (22)	0.27 (20)	Gain of function	
13	0.18 (16)	0.09 (7)	Mixed function	23
22	0.09 (8)	0.08 (6)	Wild type	14
14	0.10 (9)	0.09 (7)	Loss of function	49
23	0.07 (6)	0.13 (10)	Loss of function	
24	0.01 (1)	0.09 (7)	Loss of function	
33	0.04 (4)	0.01 (1)	Loss of function	
34	0.02 (2)	0.03 (2)	Loss of function	
44	0	0	Loss of function	

Chi-square analysis showed no significant difference in diplotype frequency distributions between populations, $P > 0.99$.

HUC, healthy unrelated control; LAP, localized aggressive periodontitis.

^aCombination of haplotypes.

had blood samples available for the ATP stimulation and RNA experiments utilized here. Of the 179 subjects in the overall study, 95 were independent and unrelated and thus analyzed in the work presented here. There were also samples available from 76 HUCs, all of whom were also African American. The mean ± SD age of subjects with LAP was 15.09 ± 4.07 years, and 62.64% of the subjects were female.

The cohort of 76 healthy subjects had a mean age of 15.61 ± 5.98 years, and 73.68% of the control population was female. No significant differences were found for both age and sex between the LAP and HUC populations ($P = 0.17$, $P = 0.12$, respectively). Clinical parameters for both populations are shown in Table 1. Diplotype details are presented in Table 2 and described next.

Diplotype Analysis of LPS-Only Stimulated Blood

The haplotypes derived from rs1718119, rs2230911, and rs3751143 were as follows, respectively: ACA (designated as 1, predicted to produce a peptide with gain of function), GCA (designated 2, wild type), GGA (designated 3, loss of function), and GCC (designated 4, loss of function). The

functional label of the haplotype was based on multiple lines of functional in vitro evidence from previous literature (Gu et al. 2001; Sluyter et al. 2004; Cabrini et al. 2005; Shemon et al. 2006; Ohlendorff et al. 2007; Roger et al. 2010; Stokes et al. 2010; Sun et al. 2010; Al-Shukaili et al. 2011; Gidlof et al. 2012; Wesselius et al. 2013; Sluyter 2017). Within the subject populations, 9 out of the 10 possible diplotypes (combinations of haplotypes in each subject to represent the full genotype) were identified. As indicated in Table 2, the diplotypes were grouped into 4 functional groups representing the predicted overall P2X7 receptor function for that individual: both haplotypes predicted to have gain of function relative to wild type (or 1 gain haplotype, 1 wild type; diplotypes 11, 12), both with loss of function (or 1 loss, 1 wild type; diplotypes 14, 23, 24, 33, 34, 44), mix function (1 loss, 1 gain; diplotype 13), and wild type (diplotype 22).

In testing for a relationship between the diplotypes and the 15 chemokine/cytokine levels measured from blood stimulated with LPS, we found that LAP-stimulated whole blood with the loss of function diplotype group had significantly lower levels of IL-12p40 and IP-10 as compared with HUCs with the same diplotype group ($P = 0.0465$, 0.0401 , respectively; Fig. 1A, B). We also found that subjects with LAP with gain and mix of function diplotypes separately produced significantly higher levels of IL-2 when compared with the corresponding HUC diplotypes ($P = 0.0276$, 0.0149 ; Fig. 1C, D). Consequently, as literature suggests that the strongest component of the gain effect is derived from the A allele of rs1718119 (also in the mix diplotype), we repeated the analysis by grouping the mix diplotype with the gain diplotypes. This resulted in an analysis with 3 functional diplotype groups (gain + mix, wild type, and loss). In this scenario, the gain + mix function diplotype group was also found to be associated with higher levels of IL-2 in LAP as compared with HUCs in the

same group, with a much stronger P value ($P = 0.0044$; Fig. 1E). None of the other cytokine/chemokine comparisons reached statistical significance for this LPS-only cohort.

Cytokine Levels in LAP and HUC Post-P2X7 Receptor Stimulation

Experiments were then conducted to test for differences after stimulating the P2X7 receptor with ATP (and ATP + P2X7 receptor inhibitor A438079 to show specificity) to test for intrinsic differences in ability of the P2X7 receptor to respond to activation and inhibition in the groups. In both populations, IL-1 β levels were significantly higher after LPS + ATP treatment when compared with either LPS-only or LPS + ATP + inhibitor treatment (for all comparisons, $P < 0.0001$; Fig. 2A). However, there was no difference in IL-1 β levels between the populations for each treatment. Furthermore, LPS + ATP + inhibitor treatment resulted in higher levels of IL-1 β when compared with LPS-only treatment for both the LAP and HUC populations ($P = 0.0099$ and 0.0019 , respectively; Fig. 2A), suggesting slightly incomplete inhibition. In contrast, LPS-only treatment had higher levels of IL-12p40 in LAP and HUC when compared with the other 2 treatments (LAP: $P = 0.0393$, 0.0002 ; HUC: $P = 0.0013$, 0.0013 ; Fig. 2B). No differences were found between the subject populations, and no significant changes in the concentrations of IL-2 and IFN- γ were found after P2X7 receptor stimulation.

Posttreatment *P2RX7* mRNA Expression in LAP and HUC

To test whether P2X7 receptor stimulation (+/- inhibition) was associated with changes in *P2RX7* mRNA levels, the gene's expression was studied after the blood cells were treated with LPS + ATP and LPS + ATP + inhibitor in LAP ($n = 21$) and HUC ($n = 13$) samples. Relative expression of *P2RX7* mRNA, normalized to the average from 2 housekeeping genes, was significantly increased in LPS + ATP +

inhibitor treatment as compared with LPS + ATP treatment for both the HUC and LAP populations ($P = 0.0014$ and 0.0009 , respectively; Fig. 3), although there was no difference between the populations themselves.

Discussion

Our study indicates that *P2RX7* functional diplotypes composed of haplotypes from 3 well-studied SNPs in linkage disequilibrium (rs1718119 [T348A], rs2230911 [T357S], and rs3751143 [E496A]) are associated with greater in vitro inflammatory response changes for 3 cytokines from whole blood from subjects with LAP versus HUCs. In the initial analysis with 4 diplotype groups, the concentration of IL-2 from LPS-stimulated whole blood was higher in subjects with LAP with either the gain or mix of function diplotype when compared with HUCs with the same diplotypes. In a follow-up analysis with 3 functional diplotype groups (combining gain and mix), LAP was also associated with higher levels of IL-2 as compared with HUC samples with gain + mix diplotypes. This supports the conclusion that within the mix diplotype, the gain of function haplotype (gain attributed mostly to the A allele of rs1718119; Roger et al. 2010; Al-Shukaili et al. 2011) had more influence on overall functional activity than the loss of function effect from the other haplotype allele. Similarly, levels of IL-12p40 and IP-10 from LPS-stimulated whole blood were lower in LAP samples with a loss of function diplotype as compared with HUCs with the same diplotype. Thus, for all 3 cytokines, the predicted effect of the *P2RX7* diplotype was related to an abnormal response in LAP versus HUC, possibly due to the already established proinflammatory activity in these subjects. This raises the possibility of a greater effect of this receptor in cytokine/chemokine regulation in subjects with LAP than in healthy controls.

The main role of IL-2 (predominantly expressed from T cells) in the immune

Figure 1. Significant associations of HUC (blue dots) and LAP (orange dots) from in vitro LPS-stimulated whole blood cytokine/chemokine data with the P2RX7 functional diplotype groups. Comparisons were made within and between subject populations. Blood from subjects with LAP in the loss of function diplotype group showed significantly lower levels of IP-10 and IL-12p40 when compared with HUCs with the same diplotype (**A, B**). IL-2 levels were significantly higher from blood of subjects with LAP in the gain, mix, and gain + mix of function diplotype groups when compared with the HUCs with the same diplotype groups (**C–E**). Values are presented as log (pg/ml). HUC, healthy unrelated control; LAP, localized aggressive periodontitis; LPS, lipopolysaccharide.

system is to promote proliferation of CD4⁺ and CD8⁺ T cells (Gaffen and Liu 2004). T-cell receptor stimulation in T

cells has been shown to release ATP extracellularly in concentrations sufficient to activate P2X7 receptors (Yip et al.

2009; Rissiek et al. 2015). Such activation results in an influx of Ca²⁺ ions in T cells, leading to upregulation of IL-2, which

Figure 2. Significant cytokine/chemokine results from P2X7 receptor stimulation experiment. IL-1 β and IL-12p40 levels were measured with Luminex assays from LAP and HUC whole blood samples separately treated with LPS only, LPS + ATP, and LPS + ATP + inhibitor. **(A)** Samples from all 3 populations showed higher levels of IL-1 β after LPS + ATP treatment when compared with LPS alone or LPS + ATP + inhibitor ($P < 0.0001$). IL-1 β levels were also significantly higher after LPS + ATP + inhibitor treatment when compared with LPS-only treatment for the LAP and HUC populations ($P = 0.0099$ and 0.0019 , respectively). **(B)** IL-12p40 levels were significantly reduced after LPS + ATP and LPS + ATP + inhibitor treatments as compared with LPS only for both populations (LAP: $P = 0.0393$, 0.0002 ; HUC: $P = 0.0013$, 0.0013). Statistical analyses within populations were preformed with a Friedman's test with Dunn's multiple comparison test or repeated measures 1-way analysis of variance with the Bonferroni correction for multiple comparisons. Significant findings between treatments are indicated by the following: * $P < 0.01$ (LPS vs. LPS + ATP), ** $P < 0.01$ (LPS vs. LPS + ATP + inhibitor), *** $P < 0.01$ (LPS + ATP vs. LPS + ATP + inhibitor). Values are presented as log (pg/ml). ATP, adenosine 5'-triphosphate; HUC, healthy unrelated control; LAP, localized aggressive periodontitis; LPS, lipopolysaccharide.

would in turn increase T-cell proliferation (Yip et al. 2009; Rissiek et al. 2015; Ledderose et al. 2016). The *P2RX7*-mediated Ca^{2+} influx can also be altered by the *P2RX7* genotype. For instance, there is evidence that 1 of the *P2RX7* gain of function SNP alleles studied here increases Ca^{2+} ion flow in lymphocytes of subjects with chronic lymphocytic leukemia (Cabrini et al. 2005). Therefore, it is possible that the gain or mix of function *P2RX7* diplotypes are leading to an increase in *P2RX7*-mediated Ca^{2+} influx within T cells, which in turn produces more IL-2 and increases T-cell proliferation, ultimately contributing to a hyperinflammatory response previously reported in subjects with LAP (Shaddox et al. 2010).

Regarding the other 2 cytokines, other studies have found that P2X7 receptor

activity modulates plasma IL-12p40 levels in *P2rx7* knockout mice, where IL-12p40 levels were significantly lower in *P2rx7*^{-/-} mice after *Toxoplasma gondii* infection than wild type mice (Correa et al. 2017). These results are consistent with the observed decrease of IL-12p40 in our individuals carrying *P2RX7* loss of function diplotypes. This loss of function effect was stronger in subjects with LAP than in HUCs. We can hypothesize that due to an already established hyperinflammatory condition, where IL-12p40 levels are already exacerbated (Shaddox et al. 2010), the loss of function diplotype in these individuals functions to further dampen this inflammatory response, as a protective mechanism.

The significant difference in IP-10 levels between the HUC and LAP groups with loss of function diplotypes was novel

because there is little evidence directly linking the production of IP-10 with LPS/TLR4 stimulation of the P2X7 receptor. However, a recent article reported that the expression and secretion of IP-10 can be induced through UDP-activated P2Y6 receptor activity (Salem et al. 2018), which is also a purinergic G protein-coupled receptor. Another study found that TLR4 stimulation (such as that from the LPS) induces production of not only ATP but also UDP, the primary agonist for the P2Y6 receptor (Dosch et al. 2018). These reports, in conjunction with our results that IP-10 levels are lower in subjects with LAP with *P2RX7* loss of function diplotypes than in healthy controls, suggest that the P2Y6 and P2X7 receptors may both be playing a role in IP-10 synthesis here. This could be further validated in other experiments that attempt to knock

Figure 3. Significant results in real-time quantitative polymerase chain reaction and cytokine/chemokine analyses. Relative *P2RX7* mRNA levels and cytokine (IL-1 β and IL-12p40) concentrations were measured from subjects' whole blood after 3 separate treatments: LPS only, LPS + ATP, and LPS + ATP + inhibitor. The relative *P2RX7* mRNA level from all samples was significantly greater after LPS + ATP + inhibitor treatment than LPS + ATP treatment but not different between the populations. There were no significant differences between the results of the LAP and HUC populations. Values are presented as relative *P2RX7* expression compared to LPS-only treatment.

down this gene in different degrees of inflammatory conditions. A limitation of these experiments is due to the inexact degree of specific P2X7 receptor activation in the assays (i.e., level of ion channel and pore function). Since these 2 functions can have somewhat different downstream effects, the amount and type of activity could be contributing to different results in the literature.

Experiments stimulating subjects' whole blood with LPS alone did not test the P2X7 receptor in isolation or in full activity. Therefore, we stimulated subjects' whole blood with LPS-only, LPS + ATP (to activate the P2X7 receptor), and LPS + ATP + P2X7 selective inhibitor to amplify and isolate the P2X7 receptor activity as measured by concentrations of 2 cytokines (IL-1 β and IL-12p40; Fig. 2). We found that the addition of ATP resulted in higher levels of IL-1 β for the 2 subject populations (LAP, HUC). Furthermore, the addition of the inhibitor to the ATP stimulations led to a significant decrease in IL-1 β concentrations for both populations (LAP, HUC) relative to LPS + ATP. The

changes observed in IL-1 β concentrations in our stimulations are consistent with the accepted strong activity of the P2X7 receptor on IL-1 β production in the literature, indicating that the stimulation and inhibition were successfully detecting predominantly P2X7 receptor activity. However, there was no difference in LPS-only stimulated IL-1 β concentrations between the LAP and HUC groups, in contrast to a previous study (Shaddox et al. 2010). This may be due to heterogeneity in the subject populations and the stimulation times, as the current study utilized different stimulation methodology due to the specific function of the P2X7 receptor.

In contrast to the IL-1 β findings, the addition of ATP to whole blood stimulations led to a decrease in IL-12p40 concentrations for the LAP and HUC populations (Fig. 2B). Moreover, the lack of significant difference in IL-12p40 concentrations with or without the inhibitor suggests that the decrease in this cytokine may not be due to the activity of the P2X7 receptor but rather another ATP activated receptor, such as

P2Y11, or even the presence of the loss of function diplotype in a few subjects, as discussed earlier. Several studies have reported that an inhibitory effect of ATP on IL-12p40 and IL-12p70 production in human monocyte-derived dendritic cells and blood monocytes is mediated by the P2Y11 receptor (Schnurr et al. 2005; Gabel 2007; Idzko et al. 2014). Therefore, it is possible that both the P2X7 receptor (as evidenced from diplotype data and from literature) and the P2Y11 receptor participate in the control of IL-12p40 expression.

In the study of *P2RX7* mRNA in LAP and HUC populations after P2X7 receptor stimulation (+/- inhibition; Fig. 3), we found that relative *P2RX7* mRNA expression rose after inhibitor treatment, suggesting a feedback loop stimulating greater expression of *P2RX7* to respond to the ATP challenge. If this observation is reflecting a real phenomenon, whereby inhibition of the receptor in the face of strong ATP stimulation triggers an increase in *P2RX7* expression (relative to LPS only), this could be attributed to a possible compensatory effect (Chang et al. 2011). No differences were found between the LAP and HUC groups, which could suggest that receptor levels per se are not playing a key role in the inflammatory response in LAP. Alternatively, there could be underlying heterogeneity within blood cell populations, which could mask a significant result from 1 cell type.

Although this study did not investigate the effects of intact oral bacteria on P2X7 activity, only purified LPS to trigger a TLR4 response, a recent study found evidence that the JP2 clone of *Aggregatibacter actinomycetemcomitans* (Aa), which is also known to ligate to TLR4, secretes eATP and is able to trigger a chemokine response from periodontal fibroblasts in an P2X7-dependent manner (Ding et al. 2016). This response to an invading pathogen recruits immune cells to the site of infection. This immune response is not able to eliminate Aa, as 1 of its main virulence factors, leukotoxin, induces cell death in leukocytes (Kachlany 2010). Therefore,

the ultimate effect of the secreted eATP is as an additional virulence factor. The increase in cell death due to *Aa* leukotoxin leads to a rise in localized ATP concentrations and further amplifies P2X7-mediated inflammation. This inflammatory response could be further exacerbated in the presence of *P2RX7* gain of function variants, and the effects of specific pathogens, such as *Aa*, need to be further investigated in this LAP population, since *Aa* and specially JP2 clone are highly associated with LAP (Burgess et al. 2017). Moreover, other periodontal pathogens could be investigated, such as *Porphyromonas gingivalis*, as done by other investigators to identify the role of this receptor or other purinergic receptors in other forms of periodontitis (Kim et al. 2018).

In this study, we have exclusively investigated the activity of the P2X7 receptor on immune cells; however, the P2X7 receptor is almost ubiquitously expressed in humans and is of particular interest in bone. There is significant evidence in the literature indicating that the P2X7 receptor plays a role in bone metabolism, and its genetic variants have been associated with osteoporosis and other bone resorption phenotypes (Burnstock 2017). Theoretically, the previously proposed hyperinflammatory response to bacteria seen in subjects with LAP could upregulate local extracellular ATP levels, which could then activate P2X7 receptors present on nearby bone cells, increasing osteoclastic activity and leading to tooth loss. Now that we have established a link between P2X7 activity and LAP, there is rationale to further investigate the activity of P2X7 receptors in osteoclasts and within periodontal tissues in subjects with LAP. Such direct investigation at the site of inflammation, rather than in peripheral blood, will provide a more robust test of the role of the P2X7 receptor.

While there is some evidence that P2X7 may play a role in chronic periodontitis, there has been little work evaluating the effects of purinergic receptors in LAP disease (Ramos-Junior et al. 2015). Here we present evidence that *P2RX7*

functional diplotypes are associated with in vitro inflammatory response of whole blood from subjects with LAP. However, as we have limited this study to 3 of the most common *P2RX7* SNPs, in full linkage disequilibrium, it is possible that less common variants at other sites in the gene might contribute to a somewhat different protein function than that expected in some patients, which could be explored in future work to investigate such heterogeneity. Some other variants will not be in linkage disequilibrium with those used here. Such future, more extensive studies should analyze linkage disequilibrium haploblocks across the gene as well as test for sites of high-frequency recombination to ensure that the linkage disequilibrium interpretation is correct. Gene expression studies may be useful as well to correlate with haploblock information. Similarly, future work should investigate whether there are any expressed *P2RX7* pseudogenes, which could affect gene expression (thus function) by sequestering miRNAs targeting *P2RX7*. Furthermore, other purinergic receptors (e.g., P2Y6 and P2Y11) may be involved in the hyperinflammatory phenotype of this disease; thus, further studies are also warranted to investigate specific roles of purinergic receptors in the hyperinflammatory response in LAP.

Author Contributions

T.H. Harris, L.M. Shaddox, M.R. Wallace, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; H. Huang, H. Li, contributed to data acquisition and interpretation, critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Acknowledgments

The authors thank all the collaborating sites, doctors, and staff for assisting with subject recruitment. We also thank the subjects for their participation. The authors thank the National Institute of Dental and Craniofacial Research,

National Institutes of Health, for the financial support (grant R01DE019456). This study was supported by a grant from the National Institute of Dental and Craniofacial Research to L.M.S. (R01DE019456). The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

References

- Al-Shukaili A, Al-Kaabi J, Hassan B, Al-Araimi T, Al-Tobi M, Al-Kindi M, Al-Maniri A, Al-Gheilani A, Al-Ansari A. 2011. P2x7 receptor gene polymorphism analysis in rheumatoid arthritis. *Int J Immunogenet*. 38(5):389–396.
- Burgess D, Huang H, Harrison P, Aukhil I, Shaddox L. 2017. *Aggregatibacter actinomycetemcomitans* in African Americans with localized aggressive periodontitis. *JDR Clin Trans Res*. 2(3):249–257.
- Burnstock G. 2017. Purinergic signalling: therapeutic developments. *Front Pharmacol*. 8:661.
- Cabrini G, Falzoni S, Forchap SL, Pellegatti P, Balboni A, Agostini P, Cuneo A, Castoldi G, Baricordi OR, Di Virgilio F. 2005. A His-155 to Tyr polymorphism confers gain-of-function to the human P2X7 receptor of human leukemic lymphocytes. *J Immunol*. 175(1):82–89.
- Chang MY, Lu JK, Tian YC, Chen YC, Hung CC, Huang YH, Chen YH, Wu MS, Yang CW, Cheng YC. 2011. Inhibition of the P2X7 receptor reduces cystogenesis in PKD. *J Am Soc Nephrol*. 22(9):1696–1706.
- Correa G, Almeida Lindenberg C, Moreira-Souza AC, Savio LE, Takiya CM, Marques-da-Silva C, Vommaro RC, Coutinho-Silva R. 2017. Inflammatory early events associated to the role of P2X7 receptor in acute murine toxoplasmosis. *Immunobiology*. 222(4):676–683.
- Diehl SR, Wu T, Michalowicz BS, Brooks CN, Califano JV, Burmeister JA, Schenkein HA. 2005. Quantitative measures of aggressive periodontitis show substantial heritability and consistency with traditional diagnoses. *J Periodontol*. 76(2):279–288.
- Ding Q, Quah SY, Tan KS. 2016. Secreted adenosine triphosphate from *Aggregatibacter actinomycetemcomitans* triggers chemokine response. *Mol Oral Microbiol*. 31(5):423–434.
- Dosch M, Gerber J, Jebbawi F, Beldi G. 2018. Mechanisms of ATP release by inflammatory cells. *Int J Mol Sci*. 19(4):E1222.

- Fine DH, Patil AG, Loos BG. 2018. Classification and diagnosis of aggressive periodontitis. *J Clin Periodontol.* 45 Suppl 20:S95–S111.
- Gabel CA. 2007. P2 purinergic receptor modulation of cytokine production. *Purinergic Signal.* 3(1–2):27–38.
- Gidlöf O, Smith JG, Melander O, Lovkvist H, Hedblad B, Engström G, Nilsson P, Carlson J, Berglund G, Olsson S, et al. 2012. A common missense variant in the ATP receptor P2X7 is associated with reduced risk of cardiovascular events. *PLoS One.* 7(5):e37491.
- Graves DT, Oates T, Garlet GP. 2011. Review of osteoimmunology and the host response in endodontic and periodontal lesions. *J Oral Microbiol.* 3. doi:10.3402/jom.v3i0.5304.
- Gu BJ, Zhang W, Worthington RA, Sluyter R, Dao-Ung P, Petrou S, Barden JA, Wiley JS. 2001. A Glu-496 to Ala polymorphism leads to loss of function of the human P2X7 receptor. *J Biol Chem.* 276(14):11135–11142.
- Harris TH, Wallace M, Huang H, Li H, Mohiuddeen A, Gong Y, Kompotiati T, Harrison P, Aukhil I, Shaddox LM. 2019. Association of P2RX7 functional variants with localized aggressive periodontitis. *J Periodontol Res* [epub ahead of print 10 July 2019] in press. doi:10.1111/jre.12682
- Idzko M, Ferrari D, Eltzschig HK. 2014. Nucleotide signalling during inflammation. *Nature.* 509(7500):310–317.
- Kachlany SC. 2010. *Aggregatibacter actinomycetemcomitans* leukotoxin: from threat to therapy. *J Dent Res.* 89(6):561–570.
- Kim H, Kajikawa T, Walsh MC, Takegahara N, Jeong YH, Hajishengallis G, Choi Y. 2018. The purinergic receptor P2X5 contributes to bone loss in experimental periodontitis. *BMB Rep.* 51(9):468–473.
- Ledderose C, Bao Y, Ledderose S, Woehrle T, Heinisch M, Yip L, Zhang J, Robson SC, Shapiro NI, Junger WG. 2016. Mitochondrial dysfunction, depleted purinergic signaling, and defective T cell vigilance and immune defense. *J Infect Dis.* 213(3):456–464.
- Li Y, Xu L, Hasturk H, Kantarci A, DePalma SR, Van Dyke TE. 2004. Localized aggressive periodontitis is linked to human chromosome 1q25. *Hum Genet.* 114(3):291–297.
- Marazita ML, Burmeister JA, Gunsolley JC, Koertge TE, Lake K, Schenkein HA. 1994. Evidence for autosomal dominant inheritance and race-specific heterogeneity in early-onset periodontitis. *J Periodontol.* 65(6):623–630.
- Ohlendorff SD, Tofteng CL, Jensen JE, Petersen S, Civitelli R, Fengler M, Abrahamsen B, Hermann AP, Eiken P, Jorgensen NR. 2007. Single nucleotide polymorphisms in the P2X7 gene are associated to fracture risk and to effect of estrogen treatment. *Pharmacogenet Genomics.* 17(7):555–567.
- Papapanou PN, Sanz M, Buduneli N, Dietrich T, Feres M, Fine DH, Flemmig TF, Garcia R, Giannobile WV, Graziani F, et al. 2018. Periodontitis: consensus report of Workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-implant Diseases and Conditions. *J Clin Periodontol.* 45 Suppl 20:S162–S170.
- Ramos-Junior ES, Morandini AC, Almeida-da-Silva CL, Franco EJ, Potempa J, Nguyen KA, Oliveira AC, Zamboni DS, Ojcius DM, Scharfstein J, et al. 2015. A dual role for P2X7 receptor during *Porphyromonas gingivalis* infection. *J Dent Res.* 94(9):1233–1242.
- Rissiek B, Haag F, Boyer O, Koch-Nolte F, Adriouch S. 2015. P2X7 on mouse T cells: one channel, many functions. *Front Immunol.* 6:204.
- Roger S, Mei ZZ, Baldwin JM, Dong L, Bradley H, Baldwin SA, Surprenant A, Jiang LH. 2010. Single nucleotide polymorphisms that were identified in affective mood disorders affect ATP-activated P2X7 receptor functions. *J Psychiatr Res.* 44(6):347–355.
- Salem M, Tremblay A, Pelletier J, Robaye B, Sévigny J. 2018. P2Y6 receptors regulate CXCL10 expression and secretion in mouse intestinal epithelial cells. *Front Pharmacol.* 9:149.
- Savio LEB, de Andrade Mello P, da Silva CG, Coutinho-Silva R. 2018. The P2X7 receptor in inflammatory diseases: angel or demon? *Front Pharmacol.* 9:52.
- Schnurr M, Toy T, Shin A, Wagner M, Cebon J, Maraskovsky E. 2005. Extracellular nucleotide signaling by p2 receptors inhibits IL-12 and enhances IL-23 expression in human dendritic cells: a novel role for the camp pathway. *Blood.* 105(4):1582–1589.
- Shaddox L, Wiedey J, Bimstein E, Magnuson I, Clare-Salzler M, Aukhil I, Wallet SM. 2010. Hyper-responsive phenotype in localized aggressive periodontitis. *J Dent Res.* 89(2):143–148.
- Shaddox LM, Spencer WP, Velsko IM, Al-Kassab H, Huang H, Calderon N, Aukhil I, Wallet SM. 2016. Localized aggressive periodontitis immune response to healthy and diseased subgingival plaque. *J Clin Periodontol.* 43(9):746–753.
- Shemon AN, Sluyter R, Fernando SL, Clarke AL, Dao-Ung LP, Skarratt KK, Saunders BM, Tan KS, Gu BJ, Fuller SJ, et al. 2006. A Thr357 to Ser polymorphism in homozygous and compound heterozygous subjects causes absent or reduced P2X7 function and impairs ATP-induced mycobacterial killing by macrophages. *J Biol Chem.* 281(4):2079–2086.
- Sluyter R. 2017. The P2X7 receptor. *Adv Exp Med Biol.* 1051:17–53.
- Sluyter R, Shemon AN, Wiley JS. 2004. Glu496 to Ala polymorphism in the P2X7 receptor impairs ATP-induced IL-1 beta release from human monocytes. *J Immunol.* 172(6):3399–3405.
- Spildreorde M, Curtis SJ, Curtis BL, Sluyter R. 2014. Extracellular adenosine 5'-triphosphate and lipopolysaccharide induce interleukin-1beta release in canine blood. *Vet Immunol Immunopathol.* 157(1–2):105–110.
- Stokes L, Fuller SJ, Sluyter R, Skarratt KK, Gu BJ, Wiley JS. 2010. Two haplotypes of the P2X(7) receptor containing the Ala-348 to Thr polymorphism exhibit a gain-of-function effect and enhanced interleukin-1beta secretion. *FASEB J.* 24(8):2916–2927.
- Sun C, Chu J, Singh S, Salter RD. 2010. Identification and characterization of a novel variant of the human P2X(7) receptor resulting in gain of function. *Purinergic Signal.* 6(1):31–45.
- Susin C, Haas AN, Albandar JM. 2014. Epidemiology and demographics of aggressive periodontitis. *Periodontol 2000.* 65(1):27–45.
- Vandenbroucke JP, von Elm E, Altman DG, Gotzsche PC, Mulrow CD, Pocock SJ, Poole C, Schlesselman JJ, Egger M, STROBE Initiative. 2014. Strengthening the reporting of observational studies in epidemiology (STROBE): explanation and elaboration. *Int J Surg.* 12(12):1500–1524.
- Wesselius A, Bours MJ, Henriksen Z, Syberg S, Petersen S, Schwarz P, Jorgensen NR, van Helden S, Dagnelie PC. 2013. Association of P2X7 receptor polymorphisms with bone mineral density and osteoporosis risk in a cohort of dutch fracture patients. *Osteoporos Int.* 24(4):1235–1246.
- Winer J, Jung CK, Shackel I, Williams PM. 1999. Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. *Anal Biochem.* 270(1):41–49.
- Yip L, Woehrle T, Corriden R, Hirsh M, Chen Y, Inoue Y, Ferrari V, Insel PA, Junger WG. 2009. Autocrine regulation of T-cell activation by ATP release and P2X7 receptors. *FASEB J.* 23(6):1685–1693.