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ABSTRACT

We have reported a lipopolysaccharide (LPS)-induced hyper-inflammatory response in localized aggressive periodontitis (LAP). It is unknown whether treatment is able to modulate this LPS responsiveness. Fifty-nine individuals with LAP were treated by mechanical debridement and systemic antibiotics. Clinical parameters and cyto/chemokine responsiveness of whole blood stimulated with *Porphyromonas gingivalis* or *Escherichia coli* LPS were monitored at baseline and 3, 6, and 12 months post-treatment. Overall, clinical parameters were improved following treatment. Additionally, *P. gingivalis* LPS induction of eotaxin, IFN γ , IL10, IL12p40, IL1 β , IL6, IP10, MCP1, MIP1 α , GM-CSF, and TNF α was significantly decreased ($p < .05$). Similarly, induction of eotaxin, IFN γ , IL10, IL12p40, GM-CSF, and TNF α by *E. coli* LPS was also reduced post-treatment. These reductions correlated with decreases in clinical parameters. Importantly, these reductions in LPS responsiveness were most robust at 3 months, and some lost significance at 6 to 12 months post-treatment. In conclusion, LPS-induced hyper-inflammatory response in LAP can be partially modulated by periodontal therapy. Conversely, rebound in the hyper-responsiveness of some mediators, in the presence of improved clinical parameters, suggests that this phenotype could be partially influenced by a genetic trait and play a role in future disease recurrence (ClinicalTrials.gov, NCT01330719).

KEY WORDS: immunity, cytokines, chemokines, inflammation, TLR, *Escherichia coli*, *Porphyromonas gingivalis*.

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LPS-induced Inflammatory Response after Therapy of Aggressive Periodontitis

INTRODUCTION

Periodontitis has been acknowledged as the major cause for tooth loss in the adult population, affecting 47% of North Americans (Eke *et al.*, 2012). Aggressive periodontitis (AgP) is a relatively rare (Albandar and Tinoco, 2002) form of disease that progresses rapidly, characterized by an early onset, usually associated with a non-contributory medical history and familial aggregation pattern (Armitage, 1999). When localized, it affects primarily first molars and incisors and rapidly progresses with a higher bone-destruction-to-plaque ratio than observed in chronic periodontitis (Armitage, 1999; Lang *et al.*, 1999).

Localized aggressive periodontitis (LAP) tissue destruction is initiated in part by the interaction of bacterial components with various innate immune receptors, such as Toll-like receptors (TLR), present on host immune cells. Depending on individual host responses and tolerance to bacteria, TLR activation may result in elevated levels of pro-inflammatory mediators, which could in turn exacerbate/accelerate periodontal destruction (Shaddox *et al.*, 2010, 2011; Sun *et al.*, 2010). We have demonstrated that LAP individuals and, to a lesser extent, their unaffected siblings react to certain bacterial lipopolysaccharide (LPS) with an exacerbated inflammatory response (Shaddox *et al.*, 2010). Thus, these hyperactive responses could be the result of a specific bacterial/inflammatory environment of the host and/or a genetic predisposition. The host response is known to play a significant role in LAP (Kinane and Lappin, 2002), and standard treatment aims to reduce offending organisms and consequently the inflammatory burden by mechanical debridement with or without antibiotics. Thus, although treatment may have an impact on the inflammatory environment, it is unknown whether it may affect the LPS hyper-responsiveness observed in LAP.

Thus, the objectives of this study were to evaluate the *ex vivo* LPS-induced inflammatory response following LAP conventional treatment and to correlate this response with clinical parameter reductions. Understanding the effectiveness of conventional therapy on inflammatory response associated with periodontal diseases may lead to novel therapeutic considerations.

MATERIALS & METHODS

Participant Population and Clinical Measurements

Fifty-nine individuals were recruited from the Leon County Health Department, Tallahassee, Florida, from January 2007 to November 2010. All

participants (or their legal representative) signed an informed consent approved by the University of Florida Institutional Review Board (IRB). Participants' complete medical and dental histories were taken. Inclusion criteria were as follows: Participants were between the ages of 5 and 21 yrs, African-Americans, and diagnosed with LAP [presence of ≥ 2 teeth, first molar or incisor, no more than 2 teeth other than molar and incisor (Lang *et al.*, 1999) with PD > 4 mm, in the presence of attachment and bone loss]. Exclusion criteria were as follows: They had been diagnosed with systemic disease or conditions that could influence the progression and/or clinical characteristics of periodontitis, they had used antibiotics within the preceding 3 mos or had taken medications that could influence the characteristics of periodontitis, they were smokers, and (for women) they were pregnant/lactating. At baseline, and 3, 6, and 12 mos after periodontal treatment, peripheral blood was collected, along with measurement of the following periodontal clinical parameters: pocket depth (PD), bleeding on probing (BoP), gingival margin position (GM), clinical attachment level (CAL), and visible plaque (P). All measurements were performed by one calibrated examiner using a UNC-15 periodontal probe at 6 sites *per* tooth, and were recorded with Florida Probe software (Gainesville, FL, USA).

Periodontal Treatment Protocol

Following baseline measurements, participants received full-mouth debridement by means of an ultrasonic device (Cavitron Jet Plus, Dentsply, York, PA, USA) and site-specific scaling and root planing (SRP), under local anesthesia, as required. Oral hygiene instructions and oral care products (Oral B Vitality TM Dual Clean, Procter & Gamble, Cincinnati, OH, USA) were provided to participants. On treatment day, all participants were prescribed 500 mg amoxicillin and 250 mg metronidazole t.i.d. for 7 days (dosage was adjusted for children < 40 Kg). The subjects were re-examined at 3, 6, and 12 months post-treatment, when they received additional mechanical therapy for pockets > 4 mm, as required. Professional plaque removal and reinforcement of home-care procedures were performed at all time points.

In vitro Stimulation of Peripheral Blood and Detection of Cyto/Chemokines

A 4-mL sodium-citrate tube of peripheral blood was collected by venipuncture of each participant at all time-points. No fasting was required prior to collection. Blood was transported in ice to the laboratory, diluted 1:4 in RPMI1640 (Invitrogen, Carlsbad, CA, USA), and stimulated with 1 μ g/mL of ultrapure *Escherichia coli* or *Porphyromonas gingivalis* LPS (InvivoGen, San Diego, CA, USA) or left untreated. Whole blood was stimulated to characterize the patients' overall inflammatory response and not the response of a particular cell type. Culture supernatants were collected 24 hrs post-stimulation and frozen in -80°C until mediator analysis was performed. Supernatants underwent a single thaw cycle. Multiplex cyto/chemokine detection kits (Millipore, St. Charles, MO, USA) were used according to the manufacturer's instructions for the detection and quantitation of 14 cyto/chemokines (eotaxin, TNF α , IFN γ , IL1 β , IL2, IL6, IL8,

IL10, IL12p40, IP10, MCP1, GM-CSF, G-CSF, and MIP1 α). Briefly, culture supernatants and cytokine capture-bead cocktails were incubated for 2 hrs and incubated with biotin-labeled anti-cytokine for 1.5 hrs and in a 1:12.5 dilution of streptavidin-phycoerythrin (SAV-PE) for 30 min. Data were acquired by Luminex[®]200[™] and analyzed with Milliplex Analyst software (Millipore) with standard curves and a five-parameter logistic. Unstimulated levels were subtracted from stimulated levels to normalize the data.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism software. Cyto/chemokine values were log-transformed. Bartlett's test for equal variances was performed, and the Kruskal-Wallis test with Dunn's multiple comparisons was used for comparisons among time points for both clinical parameters and cyto/chemokine levels. We used Backward Stepwise Regression to determine correlations between clinical parameters and cytokine levels. A *p* value < .05 was considered significant and remained in the model. Bonferroni adjustments for multiple comparisons are shown in the Figs. and Table (Rothman, 1990).

RESULTS

Periodontal Treatment Resulted in Improvement of Clinical Parameters

Fifty-nine LAP African-American patients were recruited for this study. Participants' mean age was 14.76 ± 3.72 yrs. Of these, 71% were female (*n* = 34) and 29% male (*n* = 17). In 61%, permanent dentition was affected by LAP (*n* = 36), and 39% had primary dentition affected (*n* = 23). Twenty-seven patients were evaluated at 3 and 6 mos, and 29 at 12 mos. All participants reported compliance with systemic antibiotics.

Periodontal treatment resulted in significant improvement in most clinical parameters (Fig.1). Specifically, at 3 mos there was a significant decrease in plaque index which remained low up to 6 mos post-treatment. There was no difference in BoP sites at any time post-treatment. In contrast, there was a marked reduction in both the percentages and mean pocket depths > 4 mm. Interestingly, prevalence of pockets > 4 mm remained low up to 6 mos, while significant reductions in mean PD of pockets initially > 4 mm remained low up to 12 mos. Similarly, mean CAL > 4 mm was also reduced following treatment up to 12 mos. Analysis of these data indicated that overall clinical parameters were significantly improved by treatment.

Periodontal Treatment Temporarily Attenuated LPS-induced Hyper-reactivity

To determine whether periodontal treatment could modulate LPS responsiveness in LAP participants, we evaluated *P. gingivalis* and *E. coli* LPS-induced cyto/chemokine levels 3, 6, and 12 mos post-treatment compared with baseline. Here, unstimulated levels of cyto/chemokines were subtracted from stimulated levels. Following periodontal treatment, there was a reduction in *P. gingivalis* LPS-induced eotaxin, IFN γ , IL10, and TNF α up to 12 mos, and a reduction in IL12p40, IL1 β , IL6, IP10, MCP-1, MIP1 α , and GM-CSF up to 6 mos post-treatment (Fig. 2).

Figure 1. Clinical parameters of LAP participants. BoP = bleeding on probing; PD > 4 = percentage of sites with PD > 4 mm; Mean PD = Mean pocket depth of affected sites; Mean CAL = Mean clinical attachment level of affected sites; Mean PD all sites = mean pocket depth of all probed sites. Values in boxes are represented as means (horizontal line), inter-quartile range (box), and 5th to 95th percentiles (whiskers). *p* values *** < .0001, ** < .001, and * < .05 by the Kruskal-Wallis test with Dunn's multiple comparisons. N = 59 at baseline, n = 27 at 3 and 6 mos, and n = 29 at 12 mos.

Similarly, *E. coli* LPS-induced levels of eotaxin, IFN γ , IL10, IL12p40, GM-CSF, and TNF α were reduced up to 12 mos, while induction of IL12p40 was reduced up to 6 mos post-treatment (Fig. 3). Analysis of these data, together, indicates that periodontal treatment can modulate LPS-induced hyper-inflammatory response, at least in the short term for some mediators.

Attenuated LPS Responsiveness in LAP Correlates with the Improvement in Clinical Parameters

Because clinical parameters and LPS responsiveness were modified by periodontal treatment, we aimed to determine if specific clinical responses correlated with attenuated LPS responses. Both baseline post-treatment reductions of clinical parameters and LPS-induced cyto/chemokine levels were correlated by regression analysis. Significant associations are shown in the Table. Overall, we observed significant moderate correlations between reductions in clinical parameters and inflammatory responses at baseline and mainly at 3 mos post-treatment. Regarding *P. gingivalis* LPS-induced response, we observed associations between

eotaxin, GM-CSF, IFN γ , IL10, IL12p40, and MIP1 α with %BoP as well as between IL1 β with mean PD and CAL at baseline. Additionally, post-therapy, mean CAL and PD reductions were correlated with reductions in eotaxin, GM-CSF, IL6, IL8, MCP-1, and MIP1 α . Upon *E. coli* LPS stimulation, we noted similar correlations at baseline, with the addition of %PD > 4 mm correlating with some markers, and at 3 mos post-treatment, although additional correlations were found at 6 mos between IP10 and plaque% and at 12 mos between IL8 and BoP%.

DISCUSSION

LPS hyper-responsiveness to LPS in an LAP cohort, along with an attenuated hyper-responsiveness in periodontally healthy siblings, has been previously described (Shaddox *et al.*, 2010). Importantly, this hyper-responsiveness was observed in response to LPS from multiple species. Regression analysis demonstrated the hyper-reactivity to be concomitant with the expression of pro-inflammatory cyto/chemokines and the absence of anti-inflammatory mediator expression (Shaddox *et al.*, 2010). Here we sought to determine if non-surgical periodontal treatment would have an effect on this LPS-induced responsiveness.

The clinical outcomes of the present study are consistent with previously reported data on the effects of mechanical debridement along with a combination of amoxicillin and metronidazole

for the treatment of LAP (Tinoco *et al.*, 1998; Haffajee *et al.*, 2003; Guerrero *et al.*, 2005; Beliveau *et al.*, 2012). The lack of significance in BoP following periodontal therapy was expected in our cohort, since the LAP was localized to a few sites in the mouth; thus, percentage bleeding at baseline was very low and correlated little with disease severity. Conversely, this therapeutic approach resulted in not only reduction of deep pockets and clinical attachment gain, but also in stable results over time, up to 12 mos.

Over the past decade, intervention studies have been conducted to determine if the treatment of periodontal infections could result in decreased systemic inflammation as measured by circulating levels of pro-inflammatory mediators. However, contradictory results have been described. One rationale for this is that different clinical profiles between aggressive (AgP) and chronic periodontitis (CP) were not considered (Fokkema *et al.*, 2003; Ide *et al.*, 2003; Yun *et al.*, 2007; Ikezawa-Suzuki *et al.*, 2008; Behle *et al.*, 2009). A recent study showed that individuals with generalized AgP presented higher levels of TNF α and IL17 than those with CP and healthy individuals, prior to treatment,

Figure 2. *P. gingivalis*-induced cyto/chemokine levels. Values in boxes represent cyto/chemokine levels (log pg/ml) as means (horizontal line), inter-quartile range (box), 5th to 95th percentiles (whiskers), at baseline (BL), 3 mos (3 mos), 6 mos (6 mos), and 12 mos (12 mos) after treatment. *p* values *** < .0001, ** < .001, and * < .05 by the Kruskal-Wallis test with Dunn's multiple comparisons. For Bonferroni multiple comparisons adjustment, consider *p* ≤ .004.

Figure 3. *E. coli*-induced cyto/chemokine levels. Values in boxes represent cyto/chemokine levels (log pg/mL) as means (horizontal line), interquartile range (box), and 5th to 95th percentiles (whiskers), at baseline (BL), 3 mos (3 mos), 6 mos (6 mos), and 12 mos (12 mos) after treatment. p values *** $< .0001$, ** $< .001$, and * $< .05$ by the Kruskal-Wallis test with Dunn's multiple comparisons. For Bonferroni multiple comparisons adjustments, consider $p \leq .004$.

and although serum levels of some cytokines improved significantly as a result of periodontal therapy, TNF α levels remained higher in individuals with AgP compared with healthy individuals (Duarte *et al.*, 2010).

It is important to note that these studies are a ‘snapshot’ of the inflammatory status at the time of sample collection. In the current study, we examined the LPS-induced cytokine responsiveness rather than systemic levels of circulating cytokines. While we evaluated the effect of non-surgical treatment on whole-blood LPS stimulation, a previous study (Radvar *et al.*, 2008) evaluated the effect of surgical periodontal treatment on *E. coli* LPS-induced IL6 production of peripheral blood monocytes in individuals with AgP and CP. Although periodontal treatment was clinically successful, it did not result in a change of unstimulated or LPS-stimulated IL6 production by monocytes in CP or AgP. The authors concluded that AgP peripheral monocytes might be hyper-responsive for IL6 production. Similarly, in our study, non-surgical periodontal treatment did not affect *E. coli* LPS-induced IL-6 production, although *P. gingivalis* LPS-induced IL-6 production was reduced in the short term (3 mos) following periodontal treatment. In fact, our results show a change in multiple *P. gingivalis* LPS-induced cyto/chemokine levels in the short term following periodontal treatment. Interestingly, we observed a much less robust reduction in *E. coli* LPS-induced cytokines. The fact that we used a different treatment approach and whole-blood stimulations may account for the differences found between the 2 studies. Additionally, the differences in inflammatory response patterns induced by *P. gingivalis* and *E. coli* LPS described here could be explained by the receptors ligated by these specific LPS. Specifically *P. gingivalis* LPS has been shown to preferentially ligate TLR2 (Hirschfeld *et al.*, 2001) and, to a lesser extent, TLR4 (Darveau *et al.*, 2004). *E. coli* was utilized in this and previous studies in periodontal diseases because of its ability to ligate robustly to TLR-4 (Tapping *et al.*, 2000), the same receptor *Aggregatibacter actinomycetemcomitans* LPS preferentially utilizes (Mochizuki *et al.*, 2004). Because of the strong association of *A. actinomycetemcomitans* to LAP in this population (Shaddox *et al.*, 2012) and the less robust reduction in *E. coli* LPS-induced inflammatory responses, one could hypothesize a strong role for TLR4 ligation in the continued hyper-inflammatory response in LAP. However, the role of TLR-specific mechanisms in LAP needs to be further evaluated.

It is well-known that periodontal treatment is able to reduce local bacterial load (Haffajee *et al.*, 2006) as well as lower systemic LPS levels (Pussinen *et al.*, 2004). Thus, it could be hypothesized that the reduction in overall local and systemic inflammatory burden as a result of periodontal treatment may have attenuated the LPS-induced host-responsiveness we observed, although this was not directly evaluated/correlated here. Conversely, the exposure of innate immune cells to low (rather than high) doses of LPS results in what is known as ‘LPS tolerance’ (Nahid *et al.*, 2011). Thus, it is also plausible that the removal of excessive bacterial burden by periodontal treatment, resulting in lower levels of systemic LPS (Pussinen *et al.*, 2004), induced LPS tolerance and the decreased LPS-responsiveness observed *in vitro*. Importantly, LPS-induced cross-tolerance has been described, whereby LPS from one organism (*i.e.*, *P. gingivalis*)

Table. Correlations between Clinical Parameters and *P. gingivalis*- and *E. coli*-induced Cyto/Chemokines

<i>P. gingivalis</i>		Correlations*	<i>p</i> value	Adj. R ²
Eotaxin	BL	BOP%	.002*	0.206
	3 mos	Mean PD > 4	.022	0.333
GM-CSF	BL	BOP%	.025	0.100
	3 mos	Mean PD > 4	.019	0.317
IFN-γ	BL	BOP%	.014	0.104
IL-10	BL	BOP%	.006	0.189
	3 mos	Plaque%	.005	0.409
IL-12p40	BL	BOP%	.010	0.126
IL1β	BL	Mean PD > 4	.045	0.158
		Mean CAL > 4	.035	
IL-6	3 mos	Mean CAL > 4	.025	0.584
		Plaque	.003*	
IL-8	3 mos	Mean CAL > 4	.026	0.290
MCP1	3 mos	Mean CAL > 4	.019	0.524
	6 mos	BOP%	.016	0.431
		Plaque%	.038	
MIP1α	BL	BOP%	.004*	0.143
	3 mos	Mean PD > 4	.016	
		Mean CAL > 4	.035	
		Plaque%	.027	
<i>E. coli</i>		Correlations*	<i>p</i> value	Coefficient <i>r</i> ²
Eotaxin	BL	BOP%	.001*	0.137
		% PD > 4	.034	
GM-CSF	3 mos	Mean PD > 4	.026	0.203
	3 mos	BOP%	.013	
		Mean PD > 4	.044	0.534
IFN-γ	BL	BOP%	< .001*	0.242
		% PD > 4	.001*	
	3 mos	Mean PD > 4	.010*	
	Plaque%	.040		
IL-10	BL	BOP%	.001*	0.211
		% PD > 4	.004*	
	3 mos	Mean PD > 4	.015	0.320
		BOP%	.015	
IL-12p40	BL	BOP%	< .001*	0.218
		% PD > 4	< .001*	
IL-1β	3 mos	Mean PD > 4	.040	0.214
IP-10	BL	BOP%	.047	0.073
	6 mos	Plaque%	.048	
IL-8	12 mos	BOP%	.039	0.464
TNF-α	BL	BOP%	.043	0.094
		PD > 4%	.015	

*Only $p \leq .05$ by backward stepwise regression is provided here (Adj R² = overall adjusted R² in each model). Mean PD > 4 = Mean pocket depth of affected sites. Mean CAL > 4 = Mean pocket depth of affected sites. BOP% = percentage of bleeding on probing. %PD > 4 = percentage of sites with PD > 4. Plaque% = percentage of sites with plaque. BL = baseline; 3 mos = reductions at 3 mos; 6 mos = reductions at 6 mos; 12 mos = reductions at 12 mos. *Significant after Bonferroni multiple comparison adjustments ($p \leq .004$).

can confer tolerance to that of another (*i.e.*, *E. coli*) (Nahid *et al.*, 2013). This could explain the similar reductions in *E. coli* and *P. gingivalis* LPS-induced inflammatory mediators evaluated here.

It should be noted that although periodontal treatment resulted in an overall attenuation of LPS responsiveness, we observed a rebound of LPS-induced responsiveness for a subset of inflammatory markers at 12 mos following treatment. Interestingly, an overall less effective modulation of *E. coli* LPS responsiveness (such as IL-6 and IL1 β) was observed when compared with *P. gingivalis* LPS stimulation (such as IL-12, IL1 β , and IL-6). Analysis of these data, along with our previous evaluation of unaffected sibling LPS-responsiveness, prevents us from ruling out a genetic predisposition of hyper-reactivity to LPS in these patients (e.g., polymorphisms of the genes coding for the synthesis of inflammatory mediators and for the hyper-reactivity of inflammatory cells or even *via* epigenetic regulation). Therefore, because the periodontal treatment provided was able to maintain clinical results stable for up to 12 mos, even in the presence of a rebound in LPS hyper-reactivity, it is imperative to evaluate the long-term stability of these clinical results to determine the role this overactive inflammatory response plays in the host-susceptibility for disease recurrence.

Treatment of LAP with SRP and systemic antibiotics was effective in partially modulating LPS responsiveness, which positively correlated with reductions in clinical parameters.

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