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Influence of Repetition Rate on Cytokine Secretion in Peripheral Blood Mononuclear Cells Exposed to Pulsed Infrared Laser Radiation In Vitro

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ABSTRACT

Objective: Immunological effects of infrared laser energy at various exposure parameters have been characterized in previous in vitro studies. The purpose of the present investigation was to assess the influence of pulse repetition rate (PRR) on inflammatory mediator concentrations from peripheral blood mononuclear cells (PBMCs) subjected to pulsed neodymium-doped yttrium aluminum garnet (Nd:YAG) laser energy.

Materials and Methods: Rat PBMCs were cultured then stimulated at lipopolysaccharide concentrations of 0 or 100 ng/ml. Cultures received Nd:YAG laser radiation (1064 nm, 5 W, 30 s) at PRRs of 0 (untreated control), 20, 30, 40, or 60 Hz. Concentrations of six inflammatory mediators were recorded using a magnetic microsphere immunoassay—tumor necrosis factor-α (TNF-α), macrophage inflammatory protein (MIP)-1α, macrophage inflammatory protein (MIP)-2, monocyte chemoattractant protein-1 (MCP-1), interleukin (IL)-6, and IL-10. The main effects of PRR and LPS stimulation on cytokine concentrations, and the interaction between PRR and LPS stimulation, were compared using two-way analysis of variance. Bonferroni post hoc tests were used to identify pairwise differences among groups.

Results: The main effect of PRR was statistically significant for MIP-1α ($P = 0.018$), TNF-α ($P = 0.025$), MCP-1 ($P < 0.001$), MIP-2 ($P = 0.013$), and IL-6 ($P = 0.031$). For each pro-inflammatory cytokine evaluated, at least one PRR resulted in a statistically significant concentration reduction compared with control cultures. However, no statistically significant pairwise differences in cytokine concentrations were observed between laser-irradiated cultures (PRRs of 20, 30, 40, or 60 Hz). The model for the anti-inflammatory cytokine, IL-10, was not statistically significant.

Conclusions: Under the described conditions, statistically significant differences in cytokine secretion were observed between laser-exposed and control cultures, consistent with prior reports. However, PRR exerted no statistically significant effect on the evaluated mediators of inflammation.

KEYWORDS: Lasers; inflammation; monocytes; cytokines; low-level light therapy; lipopolysaccharides

INTRODUCTION

Periodontitis is multifactorial inflammatory disease resulting in periodontal tissue destruction and tooth loss in millions of individuals worldwide. It is clear that pathogenic bacteria are necessary for the initiation and progression of this disease.[1-3] However, despite intensive microbiological investigation through the Human Microbiome Project, researchers have not been able to identify a common core of bacteria consistently associated with health.[4,5] Moreover, it is not one or a few bacterial species but true polymicrobial activity that appears to drive disease progression.[6] Genetic and environmental factors influence the oral microbiota to such an extent that taxonomic profiles among individual patients vary widely.[7] In fact, some sites harboring suspected pathogens show no manifestation of disease, and the microbial community composition at sites of tissue destruction can exhibit marked diversity.[1,6,7]

Regardless of the particular mixture of bacteria present at a periodontitis-affected site, most of the tissue destruction is not directly attributable to microorganisms or their products. Rather, loss of alveolar bone and clinical attachment derives primarily from the host inflammatory response.[8] Dental biofilms result in the emergence a panoply of host-derived pro- and anti-inflammatory cytokines.[8-11] Just as the microbial challenge varies across individuals, so too does the host response to putative periodontal pathogens. For example, Goncalves and colleagues evaluated cytokine secretion from lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells (PBMCs) obtained from healthy patients versus individuals diagnosed with periodontitis.[10] The authors recorded significantly higher tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 levels and significantly lower IL-8 levels from PBMCs isolated from periodontitis patients.[10] In some patients, the immune response to dental

biofilms may be excessive to the need or uncontrolled, and pathways leading to resolution of inflammation may be impaired or inadequate.[8] Chronic inflammation then leads to tissue destruction.

One approach to treatment of periodontitis involves the use of neodymium-doped yttrium aluminum garnet (Nd:YAG) laser energy (1064 nm). Over fifteen years ago, Yukna and colleagues reported periodontal regeneration—formation of new bone, cementum, and periodontal ligament on previously diseased root surfaces—using this approach.[12] Several years later, another group of investigators confirmed that periodontal treatment using an Nd:YAG laser can in principle result in regeneration, at least under some conditions.[13,14] However, the efficacy of this treatment remains controversial, due in part to high methodological heterogeneity among existing studies.[15,16]

Nd:YAG laser use purportedly influences periodontal treatment through multiple mechanisms. Several authors have shown that Nd:YAG laser output effectively kills putative periodontal pathogens.[17-19] In addition, Nd:YAG laser energy appears to stimulate osteoblasts and has been found to enhance bone regeneration in animal models.[20-23] Nd:YAG laser energy also appears to modulate the host immune response.[24,25] In perspective, reduction in inflammation has been one of the most reproducible effects of laser photobiomodulation (PBM), a term used to describe photophysical and photochemical phenomena unrelated to thermal tissue responses to laser energy.[25]

Wavelengths in the red and near infrared spectral regions (600 through 1200 nm) have shown favorable anti-inflammatory effects.[25] Yamaura and colleagues exposed TNF-α-stimulated synoviocytes isolated from rheumatoid arthritis patients to infrared diode laser

energy (810 nm), with fluence of 5 or 25 J/cm^2.[26] The authors reported dose-dependent reduction in mRNA and protein levels of TNF-α, IL-1β, and IL-8.[26] Similarly, Hwang et al. compared IL-8 and IL-6 expression in cytokine-stimulated macrophages subjected to laser energy (405, 532, or 650 nm) at doses up to 1.6 J/cm^2.[27] All wavelengths significantly reduced IL-8 expression compared with controls; only the 405 nm wavelength produced statistically significant reduction in IL-6 expression.[27] In activated dendritic cells derived from the mouse femur, Chen and colleagues found reduced cell-surface markers of inflammation and IL-12 secretion in response to infrared diode laser irradiation (810 nm, 0.3 or 3 J/cm^2).[28] Dr. Megan Bunting demonstrated a trend for reduced secretion of MIP-1α, IL-6, MCP-1, IP-10, MIP-2, and TNF-α in peripheral blood mononuclear cell (PBMC) cultures receiving Nd:YAG laser irradiation at 5 W.[29] At power levels exceeding 5 W, laser irradiation reduced cytokine levels below detection limits.[29] Dr. Sarah Vargas evaluated the effect of Nd:YAG laser exposure time on PBMC cultures, with average power held constant at 5 W.[30] Nd:YAG laser energy produced statistically significant reduction in secretion of 4 of 27 evaluated inflammatory mediators, and the effect was more pronounced with exposures exceeding 30 s in duration.[30]

Researchers have also assessed the anti-inflammatory effects of lasers emitting in this segment of the electromagnetic spectrum using animal models. Safavi et al. assessed the influence of He-Ne laser irradiation (632.8 nm, 7.5 J/cm^2) on IL-1β, interferon-Y (IFN- Y), and TNF-α expression in wounded gingiva of male Wistar rats.[31] The authors noted statistically significant reduction in IL-1β and IFN- Y expression in the laser-irradiated groups compared with controls.[31] Aimbire and colleagues evaluated the effect of Ga-AsI-Al diode laser irradiation (650 nm, 5.2 J/cm^2) on TNF-α concentrations in diaphragm muscle tissue from male Wistar rats.[32]

The authors recorded statistically significant reduction in TNF-α concentrations in the laser-irradiated group compared with untreated controls.[32] Although existing evidence from in vitro and animal studies suggest a possible clinical role for laser irradiation in limiting inflammation, the ideal wavelength remains unidentified, and parameters such as fluence, irradiance, pulse duration, pulse repetition rate (PRR), exposure time, and number of exposures have not been optimized.

OBJECTIVE

The purpose of this investigation was to assess the influence of PRR on cytokine secretion in stimulated and unstimulated rat PBMC cultures subjected to single applications of Nd:YAG laser output at constant irradiance (W/cm^2) and exposure time.

MATERIALS AND METHODS

Cell Culture

Frozen Rat PBMCs (IQ Biosciences, Berkeley, California, USA) were thawed and suspended in a medium consisting of RPMI, supplemented with 10% FBS penstrap. To remove cryoprotectant, cells were centrifuged for 7 minutes at 700 g, then transferred to 96-well plates (2×10^4 cells per well). Cells were stimulated with 0 or 100 ng/ml of lipopolysaccharide (LPS), and incubated for 24 hours at 37°C.

Irradiation Parameters

An Nd:YAG laser (1064 nm, Lightwalker AT, Fotona, Dallas, Texas, USA) to irradiate cultures. A 320-micron optical fiber directed the laser beam perpendicularly to the plated cells at a distance of 1.8 mm. A standardized support system ensured a reliable and reproducible laser position. For each group, exposure time and power output remained constant, at 30 seconds and 5 W, respectively. PRR was set at 0 (untreated control), 20, 30, 40, or 60 Hz (Figure 1, Table 1). After irradiation, cells returned to the incubator for one hour at 37˚ C.

Evaluation of Cytokine Concentrations

Supernatant was extracted and analyzed using a magnetic microsphere immunoassay (MAGPIX System, Luminex, Austin, Texas, USA) permitting quantification of TNF-α, MIP1- α, MIP-2, MCP-1, IL-6, and IL-10. The sensitivities for these assays were 1.9, 0.08, 9.0, 9.0, 30.7, and 2.7 and pg/ml, respectively (Table 2). The analysis software (MAGPIX System, Luminex) processed the images and determined cytokine/chemokine concentrations in pg/ml using standard curves.

Statistical Analysis

For each evaluated cytokine, a two-way analysis of variance was conducted to compare the main effects of LPS stimulation and PRR, and the interaction between these factors, on cytokine concentration. Bonferroni post hoc tests were used to elucidate statistically significant pairwise differences among PRR levels (0, 20, 30, 40, and 60 Hz). Differences were accepted as significant at an alpha level of 0.05.

RESULTS

A two-way analysis of variance was conducted to assess the influence of two independent variables (PRR, LPS stimulation) on each cytokine concentration. PRR included five levels (0, 20, 30, 40, and 60 Hz) and LPS stimulation consisted of two levels (0 and 100 ng/ml). The ANOVA result for each model is reported in Table 3. The model for IL-10 was not statistically significant ($F(9, 110) = 1.67$, $P = 0.105$). The main effect of PRR was statistically significant for MIP-1α ($F(4,110) = 3.13$, $P = 0.018$), TNF-α ($F(4,110) = 2.90$, $P = 0.025$), MCP-1 ($F(4,110) = 10.51$, $P < 0.001$), MIP-2 ($F(4,110) = 3.35$, $P = 0.013$), and IL-6 ($F(4,110) = 2.76$, $P = 0.031$). The main effect of LPS stimulation was statistically significant for MIP-1α ($F(1,110) = 334.87$, $P < 0.001$), TNF-α ($F(1,110) = 629.16$, $P < 0.001$), MCP-1 ($F(1,110) = 4.33$, $P < 0.04$), and MIP-2 ($F(1,110) = 467.59$, $P < 0.001$). The interaction term between LPS stimulation and PRR was significant for the MIP-1α ($F(4, 110) = 3.39$, $p = .012$) and TNF-α ($F(4, 110) = 2.79$, $p = .030$) models.

The degree to which LPS increased cytokine concentrations varied by cytokine, with the largest increases noted for TNF-α and MIP-2. MCP-1, IL-6, and IL-10 exhibited high baseline concentrations in the unstimulated cultures (Figure 2). In both unstimulated and LPS-stimulated cultures, IL-10 displayed higher variance in concentration levels, compared with the other cytokines evaluated. Among LPS-stimulated cultures, laser irradiation resulted in statistically significant decreases in concentrations of various cytokines, compared with untreated controls: TNF-α at 20 ($P = 0.002$) and 40 ($P = 0.008$) Hz; MCP-1 at 20 ($P = 0.001$), 40 ($P = 0.03$), and 60 ($P = 0.02$) Hz; IL-6 at 30 ($P = 0.04$) and 40 ($P = 0.03$) Hz; and MIP1-α at 20 ($P = 0.02$) and 40 ($P = 0.006$) Hz. All cytokines except for IL-10 exhibited a trend for concentration

reduction in laser-irradiated cultures, with MIP1-α, TNF-α, MCP-1, MIP-2, and IL-6 each exhibiting a statistically significant decrease in concentration at one or more PRRs. Bonferroni post hoc tests identified the PRRs that resulted in statistically significant concentration differences compared with controls (Table 4). No statistically significant differences were noted among laser-irradiated cultures. Thus, PRR had no detectable influence on any cytokine concentration in this investigation.

DISCUSSION

The aim of this study was to evaluate the influence of Nd:YAG laser PRR on secretion of six cytokines in unstimulated and LPS-stimulated rat PBMC cultures. Prior studies have consistently reported reduction in various inflammatory measures using infrared laser PBM.[25-32] Therefore, reduced proinflammatory cytokine concentrations were anticipated in all laser-treated cultures, and it was hypothesized that PRR would correlate negatively with cytokine concentration. On the contrary, no influence of PRR on the concentration of any cytokine was detected in this investigation. However, all markers of inflammation evaluated in this study except IL-10 exhibited statistically reduced concentrations in laser-treated versus control cultures at one or more PRR, consistent with prior reports.

The sheer number of technical parameters with potential to influence outcome measures represents a major challenge in conducting/interpreting research into biological effects of lasers. Wavelength, pulse duration (pulse width), repetition rate, average power, peak power, irradiance (W/cm^2), fluence (J/cm^2), cumulative dose, timing of laser application, exposure time, and number/frequency of exposures may modify the observed effects. It has

been suggested that fluence (also called energy density) may be the parameter most appropriate for defining the "dose" applied.[25] For multiple outcome measures, biphasic dose responses have been reported. In other words, over a range of PBM does, a maximal response is reached at some value. When the dose in increased beyond that threshold, the observed positive response diminishes or vanishes. At even higher fluence values, the investigator may observe a negative or inhibitory result.[25] Thus, it is suspected that results recorded in this study were highly dependent upon the specific irradiation parameters applied and the target cell type. Although PRR had no apparent effect on cytokine concentrations in the present study, repetition rate could influence cytokine secretion when lower or higher fluence values are applied.

CONCLUSIONS

Under the described conditions, pulsed Nd:YAG laser irradiation produced statistically significant reduction in concentrations for five of six evaluated cytokines at one or more PRR. Findings of the present study do not support a correlation between PRR and pro-inflammatory cytokine concentration. However, these observations may be highly specific for the target cell type and the radiation parameters applied.

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FIGURES

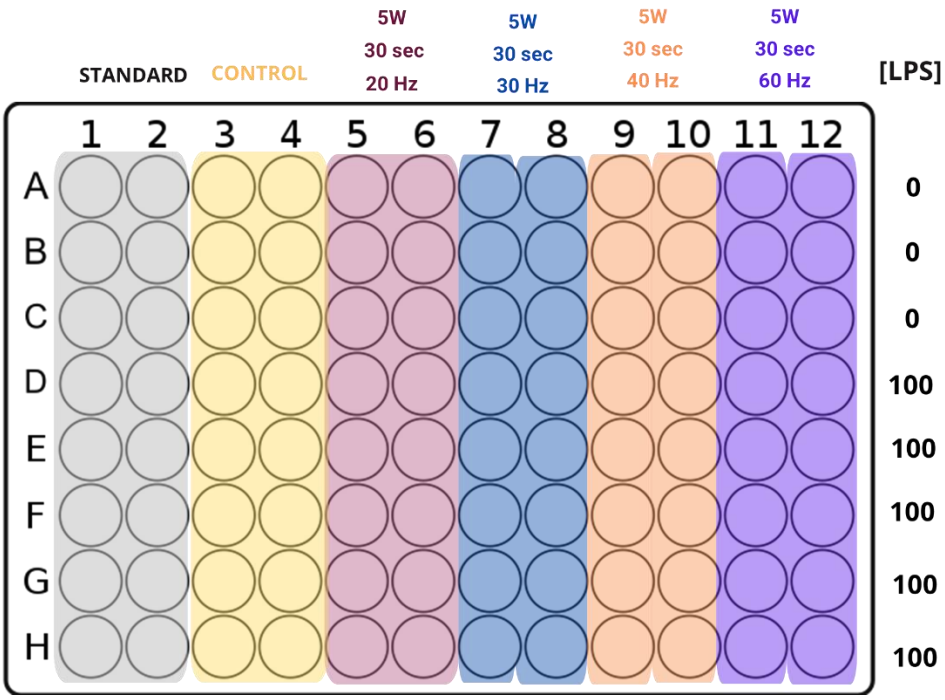

Figure 1. Diagram of 96-well plate depicting experimental design. Exposure time and power output remained constant at 30 sec and 5 W, respectively. For each group, laser parameters differed only in the pulse repetition rate—0 (untreated control), 20, 30, 40, or 60 Hz.

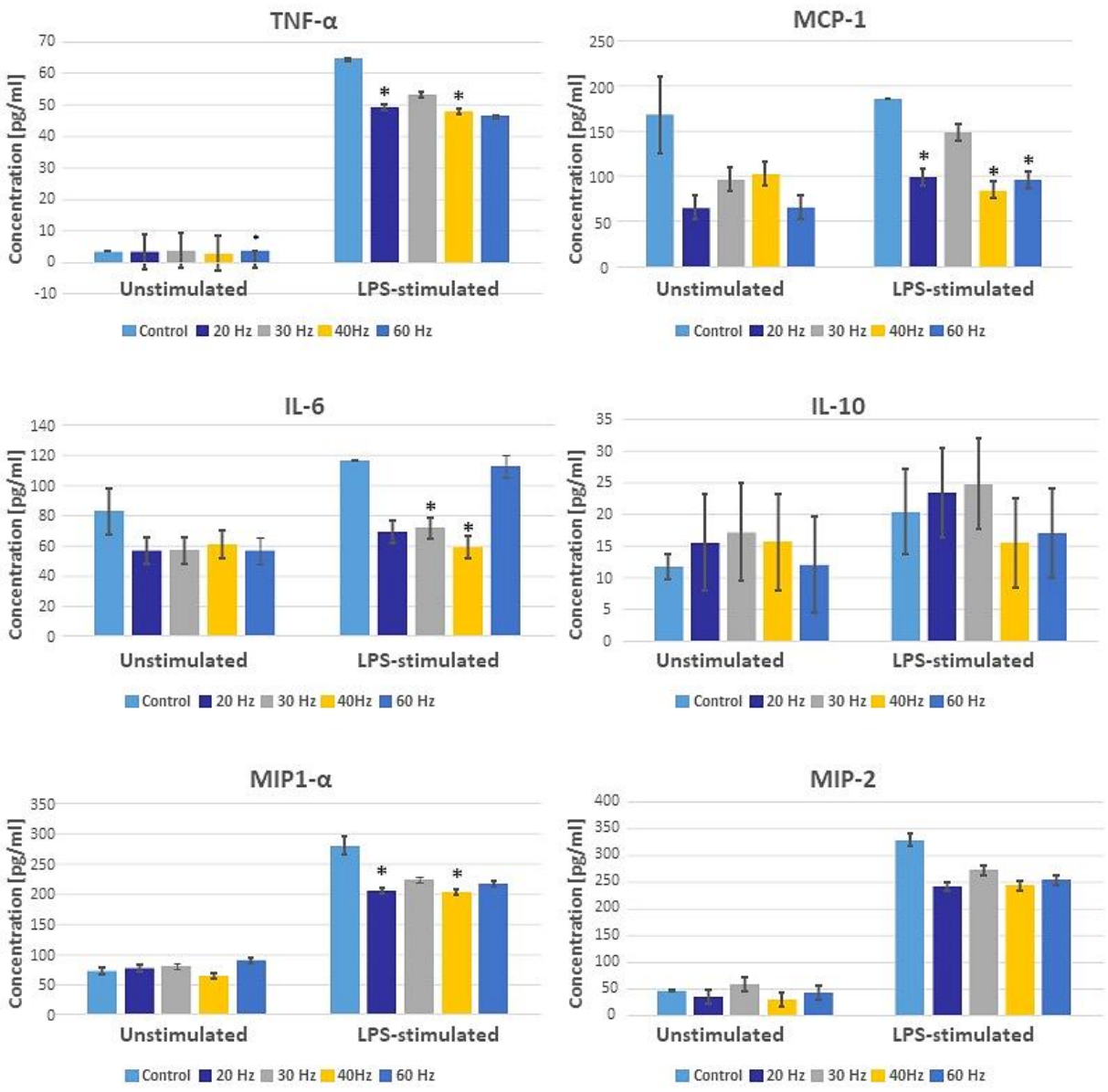

Figure 2. Cytokine secretion in response to pulsed neodymium-doped yttrium aluminum garnet laser irradiation at various repetition rates in lipopolysaccharide (LPS)-stimulated and unstimulated peripheral blood mononuclear cell cultures. Statistically significant decreases in concentration compared with control (no laser irradiation) are identified by asterisks.

TABLES

Table 1. Nd:YAG laser irradiation parameters by treatment group.

Group	Average Power (W)	Peak Power (W)	Pulse Energy (mJ)	Pulse Duration (μs)	Repetition Rate (Hz)	Fiber Diameter (μm)	Irradiance at tip (W/cm^2)	Fluence at tip* (J/cm^2)	Distance to target (mm)	Irradiation time (s)
Control	0	0	0	0	NA	NA	0	0	NA	0
1	5	2500	250	100	20	320	6217	311	1.8	30
2	5	1667	167	100	30	320	6217	207	1.8	30
3	5	1250	125	100	40	320	6217	155	1.8	30
4	5	833	83	100	60	320	6217	103	1.8	30

*Per pulse

Table 2. Inflammatory mediators evaluated by magnetic microsphere immunoassay and the associated minimum detectable concentrations (MDCs)

Analyte		2-Hour Protocol	
		MDC (pg/mL)	MDC + 2 SD (pg/mL)
1	Interleukin-6 (IL-6)	30.7	86.2
2	Interleukin-10 (IL-10)	2.7	6.9
3	Macrophage inflammatory protein-1α (MIP-1α)	0.8	2.1
4	Macrophage inflammatory protein-2 (MIP-2)	9.0	21.8
5	Monocyte chemoattractant protein-1 (MCP-1)	9.0	21.8
6	Tumor Necrosis Factor-α (TNF-α)	1.9	7.2

Table 3. Results of two-way analysis of variance for each cytokine evaluated

MIP-1α						
Source	Type III Sum of	df	Mean Square	F	Sig.	Partial Eta
Corrected Model	686106.37[a]	9	76234.04	40.93	<.001	.770
Intercept	2534268.15	1	2534268.15	1360.71	<.001	.925
LPS	623689.25	1	623689.25	334.87	<.001	.753
PRR	23326.62	4	5831.66	3.13	.018	.102
LPS * PRR	25245.42	4	6311.36	3.39	.012	.110
Error	204870.87	110	1862.46			
Total	4306291.87	120				
Corrected Total	890977.24	119				
a. R Squared = .770 (Adjusted R Squared = .751)						
TNF-α						
Source	Type III Sum of	df	Mean Square	F	Sig.	Partial Eta
Corrected Model	69762.81[a]	9	7751.42	73.27	<.001	.857
Intercept	85882.54	1	85882.54	811.81	<.001	.881
LPS	66559.77	1	66559.77	629.16	<.001	.851
PRR	1225.05	4	306.26	2.90	.025	.095
LPS * PRR	1180.24	4	295.06	2.79	.030	.092
Error	11637.04	110	105.79			
Total	217768.59	120				
Corrected Total	81399.85	119				
a. R Squared = .857 (Adjusted R Squared = .845)						
MCP-1						
Source	Type III Sum of	df	Mean Square	F	Sig.	Partial Eta
Corrected Model	188278.65[a]	9	20919.850	6.02	<.001	.330
Intercept	1385150.30	1	1385150.297	398.78	<.001	.784
LPS	15047.14	1	15047.135	4.33	.040	.038
PRR	146017.24	4	36504.311	10.51	<.001	.276
LPS * PRR	15607.98	4	3901.995	1.12	.349	.039
Error	382083.34	110	3473.485			
Total	2125855.83	120				
Corrected Total	570361.99	119				
a. R Squared = .330 (Adjusted R Squared = .275)						
MIP-2						
Source	Type III Sum of	df	Mean Square	F	Sig.	Partial Eta
Corrected Model	1516999.412[a]	9	168555.490	54.953	<.001	.818
Intercept	2696576.963	1	2696576.963	879.152	<.001	.889
LPS	1434227.702	1	1434227.702	467.594	<.001	.810
PRR	41048.325	4	10262.081	3.346	.013	.108
LPS * PRR	23176.701	4	5794.175	1.889	.117	.064
Error	337397.158	110	3067.247			
Total	5875212.463	120				
Corrected Total	1854396.570	119				
a. R Squared = .818 (Adjusted R Squared = .803)						
IL-6						
Source	Type III Sum of	df	Mean Square	F	Sig.	Partial Eta
Corrected Model	62224.02[a]	9	6913.78	2.88	.004	.190
Intercept	616962.20	1	616962.20	256.66	<.001	.700
LPS	14936.23	1	14936.23	6.21	.014	.053
PRR	26526.82	4	6631.71	2.759	.031	.091
LPS * PRR	11256.24	4	2814.06	1.171	.328	.041
Error	264422.37	110	2403.84			
Total	1036932.62	120				
Corrected Total	326646.39	119				
a. R Squared = .190 (Adjusted R Squared = .124)						
IL-10						
Source	Type III Sum of	df	Mean Square	F	Sig.	Partial Eta
Corrected Model	2089.38[a]	9	232.15	1.67	.105	
Intercept	33253.80	1	33253.80	238.80	<.001	
LPS	935.60	1	935.60	6.72	.011	
PRR	682.54	4	170.64	1.23	.304	
LPS * PRR	285.59	4	71.40	.51	.726	
Error	15317.97	110	139.25			
Total	55915.29	120				
Corrected Total	17407.35	119				
a. R Squared = .120 (Adjusted R Squared = .048)						

Table 4. Results of Bonferroni post hoc tests

MIP-1α					95% Confidence interval	
(I) Laser	(J) Laser	Mean Difference (I-J)	Std. Error	Sig.	Lower bound	Upper bound
Control	20 Hz	44.25*	12.46	.006	8.56	79.94
	40 Hz	51.35*	12.46	<.001	15.66	87.03
TNF-α					95% Confidence interval	
(I) Laser	(J) Laser	Mean Difference (I-J)	Std. Error	Sig.	Lower bound	Upper bound
Control	20 Hz	9.46*	2.97	.019	.96	17.97
	40 Hz	10.77*	2.97	.004	2.26	19.28
	60 Hz	11.14*	2.97	.003	2.63	19.65
MCP-1					95% Confidence interval	
(I) Laser	(J) Laser	Mean Difference (I-J)	Std. Error	Sig.	Lower bound	Upper bound
Control	20 Hz	92.10*	17.01	<.001	43.36	140.84
	30 Hz	49.64*	17.01	.043	.90	98.38
	40 Hz	87.47*	17.01	<.001	38.73	136.21
	60 Hz	94.45*	17.01	<.001	45.71	143.19
MIP-2					95% Confidence interval	
(I) Laser	(J) Laser	Mean Difference (I-J)	Std. Error	Sig.	Lower bound	Upper bound
Control	20 Hz	58.96*	15.99	.004	13.16	104.76
	40 Hz	59.30*	15.99	.003	13.55	105.15
	60 Hz	48.07*	15.99	.033	2.27	93.87
IL-6					95% Confidence interval	
(I) Laser	(J) Laser	Mean Difference (I-J)	Std. Error	Sig.	Lower bound	Upper bound
Control	40 Hz	43.76*	14.15	.025	3.21	84.30

*Mean difference is significant at the .05 level.