

# Cytokines Elicited by HSP60 in Periodontitis with and without Coronary Heart Disease

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## ABSTRACT

The human 60 kDa and microbial 65 kDa heat shock proteins (HSP) have been implicated in the pathogenesis of chronic periodontitis (CP) and coronary heart disease (CHD). We have studied 100 subjects: Group (a) consisted of patients with gingivitis (n = 25), group (b) were patients with CP (n = 25), group (c) patients with CHD and gingivitis (n = 25) and group (d) patients with CHD and CP (n = 25). PBMCs separated from peripheral blood were stimulated with medium, PMA/ionomycin, human HSP60, microbial HSP65, or no stimulus for 18 hours before intracellular IL-2, IFN- $\gamma$ , TNF- $\alpha$ , IL-4, IL-5, or IL-17 were detected by flow cytometry. The mean fluorescence intensity (MFI) for intracellular TNF- $\alpha$  was significantly increased when PBMC were stimulated with human HSP60 amongst the four groups (p = 0.001, ANOVA); pairwise comparisons revealed significant differences in MFI between the gingivitis group and the CP (p = 0.017); between gingivitis and ging/CHD (p = 0.001) as well; but no significant difference between the CP and CP/CHD (p = 0.442). There was no significant difference in intracellular expression of IL-17, or any of the other cytokines tested; and the MFI for HSP-stimulated were comparable to unstimulated cultures. When heat-labile human HSP60 was heated, intracellular cellular TNF- $\alpha$  expression was abrogated. In contrast, heat-stable LPS elicited TNF- $\alpha$  expression from monocytes in bulk cultures in all groups. These results suggest that the cytokine expression was dependent on human HSP60 and not LPS. Serum CRP was significantly associated with MFI of intracellular TNF- $\alpha$  in CP patients ( $r_s = 0.665$ , p = 0.026) and CP/CHD ( $r_s = 0.699$ , p = 0.011). We conclude that human HSP60 elicits increased monocytic expression of TNF- $\alpha$  in patients with CP, CP/CHD or ging/CHD compared to patients with gingivitis. Since the marker of inflammation, namely CRP correlates with CP with or without CHD and not with mild chronic gingivitis or ging/CHD, this suggests that human HSP60-induced production of TNF- $\alpha$  is associated with CP and not CHD. There was no significant difference in intracellular expression of IL-17.

## KEYWORDS

Heat Shock Proteins; Periodontitis; Cardiovascular Disease; Cytokines; IL-17

## 1. Introduction

Chronic periodontitis (CP) is an inflammatory disease characterised by connective tissue destruction and bone resorption, affecting 10% - 15% of the developed world population and is the major cause of tooth loss in adults [1]. There is evidence from cross-sectional studies implicating a multitude of organisms [2,3]. HSPs are highly conserved proteins [4,5], expressed in all eukaryotic and prokaryotic cells and involved in both innate and adap-

tive immune responses, with innate responses helping to focus the cellular immune responses [6]. The high degree of homology between microbial and human HSPs has led to the hypothesis that tissue damage can occur as a result of cross-reactivity between bacterial and human HSPs. Humoral and T cell responses to HSP60/65 have been demonstrated in CP [4,5,7-9].

In CP, there is controversy as to whether cells with a TH1 or TH2 cytokine profile are associated with disease progression, as both TH1 and TH2 cytokines are pro-

duced [10]. TH1 cells produce IL-2 and IFN- $\gamma$  and facilitate cell mediated immune responses and are pro-inflammatory. In contrast, TH2 cells produce IL-4, IL-5 and IL-10 [11] simultaneously inhibiting TH1 responses and promoting humoral immunity. The histological picture of stable periodontitis lesions resembles a type IV delayed hypersensitivity response and in progressive lesions changes to lesions dominated by B cells and plasma cells [12]. These findings have been previously interpreted as meaning the stable lesion is mediated by TH1 cells and the progressive lesion by TH2 cells [12]. Animal studies suggest that Th1 are involved in the progression of chronic periodontitis [13-16]. However, several studies using human cells have reported a predominance of TH2 responses in patients with CP compared to control patients [17-19]. These conflicting results may be due in part to differences in materials examined and methodology used.

There is an association between CP and atherosclerosis (cardiovascular disease) and an increased risk of developing cardiovascular disease in patients who have CP [20]. TH1 cytokines are found to be predominantly involved in the pathogenesis of atherosclerosis [21,22]. The TH1 subpopulation of CD4 + T cells predominates among atheroma plaque infiltrating lymphocytes [21,22] and becomes activated to secrete IFN- $\gamma$ , IL-2, TNF- $\alpha$ , TNF- $\beta$  [21-23], and IL-18 [23,24]. Upregulation of IFN- $\gamma$ , IL-2, TNF- $\alpha$ , and TNF- $\beta$  results in the activation of macrophages and vascular cells, promoting inflammation and cellular immunity [25]. IL-18 and its receptor act in concert to induce the production of IFN- $\gamma$ , by smooth muscle cells [23]. Most human atherosclerotic lesions produce little TH2 type cytokines such as IL-4, IL-5 and IL-10 [21,22], except for IL-6 [26]. Furthermore, IL-6 correlates with CRP and plaque size in patients with acute myocardial infarction [27].

Various studies have demonstrated that HSP60/65 can activate monocytes/macrophages to produce pro-inflammatory cytokines such as TNF- $\alpha$  [28-31], IL-6 [32,33] and IL-8 [32]. In addition, human HSP60 has been shown to induce PBMC to produce IL-4, IL-10 [34] and IFN- $\gamma$  [19,34] in patients with rheumatoid arthritis and chronic periodontitis [35].

Cytokines produced by T-cells cannot always be easily categorised as either TH1 or TH2. TH17 cells, which produce IL-17 and have been implicated in autoimmune and chronic inflammatory conditions such as rheumatoid arthritis, may offer an alternative to the TH1/TH2 paradigm, however, their role in atherosclerosis is similarly unclear [36]. We have previously found autoimmune or cross-reactive CD4+ class II-restricted T cell responses to the human HSP60 in CP and CHD [8]. Given the potential for innate immunity to focus adaptive immune responses, the objective of this study was to investigate the role of HSP-mediated induction of intracellular TNF-

$\alpha$ , IFN- $\gamma$ , and IL-2, IL-4 and IL-5, and IL-17 from PBMC of patients with chronic periodontitis and coronary heart disease, and to determine if these responses are related to inflammation, as reflected by CRP levels.

## 2. Material and Methods

### 2.1. Subjects

Patients with CG and CP were recruited from the Unit of Periodontology at Guy's and St Thomas' Foundation Trust and the Coronary Care Unit at St Thomas' Hospital (**Table 1**). All subjects were aged between 38 and 58 years of age and had at least 15 standing teeth. Subjects were excluded from the study if they had systemic disease including diabetes mellitus, recurrent aphthous stomatitis, autoimmune disease, a history of malignancies, previous treatment for periodontitis, or a history of antibiotic therapy within the past six months prior to recruitment. Subjects were allocated into 4 groups: gingivitis, CP, Ging/CHD or CP/CHD. In each subject, probing depths and recession of all teeth were determined using a William's probe. Recession was measured as the distance from the cemento-enamel junction to the gingival margin. Measurements were taken from 6 sites; probing attachment loss was then calculated as the sum of probing pocket depth and recession. Periodontitis was defined as probing attachment loss  $\geq 4$  mm in at least 4 teeth and CG group as probing attachment loss  $< 2$  mm in all teeth. Ethical committee approval was obtained (code no 98/12/04) and subject consent obtained. 40 ml of venous blood were withdrawn from each subject.

The gingivitis subjects had minimal gingival inflammation and bleeding on probing (percentage of bleeding sites, mean  $\pm$  s.d.,  $1.8 \pm 0.8$ ), compared with the diseased groups, with bleeding sites in CP ( $48.9 \pm 11.7$ ), coronary heart disease with gingivitis (Ging/CHD;  $1.9 \pm 0.7$ ) and CP/CHD ( $45.9 \pm 8.7$ ). All Ging/CHD patients had CHD as determined by greater than 60% diameter stenosis in at least two major epicardial coronary arteries, and conversely only eight of the healthy control subjects were confirmed to be free of CHD based on angiography, the remaining members of the group denying any chest pain symptoms.

### 2.2. HSP

Recombinant HSP65 derived from *Mycobacterium bovis* was prepared at the National Institute of Public Health and Environmental Protection, Bilthoven, the Netherlands and used at a predetermined optimal concentration of 10  $\mu\text{g/ml}$ . Human HSP60 was purchased from Stressgen (Victoria, Canada). The two HSPs were detoxified using Detoxi-gel columns (Pierce, Oxford, UK) and the endotoxin level was determined by Limulus Amoebocyte Lysate assay (Sigma-Aldrich, Poole, Dorset, UK). The concentration of endotoxin was  $< 0.007$  U/ $\mu\text{g}$  or 7 pg endotoxin/ $\mu\text{g}$  for both HSPs.

**Table 1. Demographic data of patients showing median, interquartile range, mean and standard error of mean (SEM) of age, smoking status, smoking pack years and probing attachment level.**

Group (Number of patients)		Gingivitis n = 25	CP n = 25	Gingivitis/CHD n = 25	CP/CHD n = 25
Age (years) ANOVA p = 0.239	Median (IQ range)	48.00 (44 - 58)	49.00 (43 - 53)	52.00 (48 - 56)	54.00 (50 - 55)
	Mean (SEM)	50.20 (1.8)	48.70 (1.7)	51.90 (1.3)	52.10 (1.0)
	NS	13	7	7	4
Smoking status	ES	7	13	13	15
	PS	5	5	5	6
Smoking Pack years ANOVA # < 0.001 *0.016 **0.004 ***0.097	Median (IQ range)	0.00 (0 - 4)	3.50 (0 - 25.5)	7.50 (0 - 30)	22.50 (5.0 - 37.5)
	Mean (SEM)	2.40 (1.7)	15.00 (4.0)	15.30 (3.4)	22.30 (3.7)
Probing attachment level (mm)	Median (IQ range)	0.90 (0.8 - 1.0)	3.20 (2.8 - 4.2)	1.00 (0.8 - 1.2)	3.20 (2.4 - 4.8)
	Mean (SEM)	0.90 (0.05)	3.70 (0.26)	1.00 (0.06)	4.00 (0.4)

#significant p value for between group analysis of variance by Kruskal-Wallis test. \*p value for pairwise comparison by Bonferroni between gingivitis and chronic periodontitis group. \*\*p value for pairwise comparison by Bonferroni between gingivitis and gingivitis/CHD group. \*\*\*p value for pairwise comparison by Bonferroni between chronic periodontitis and chronic periodontitis/CHD group. NS = never smokers ES = ex-smokers PS = present smokers IQ range = interquartile range.

### 2.3. Separation of PBMC

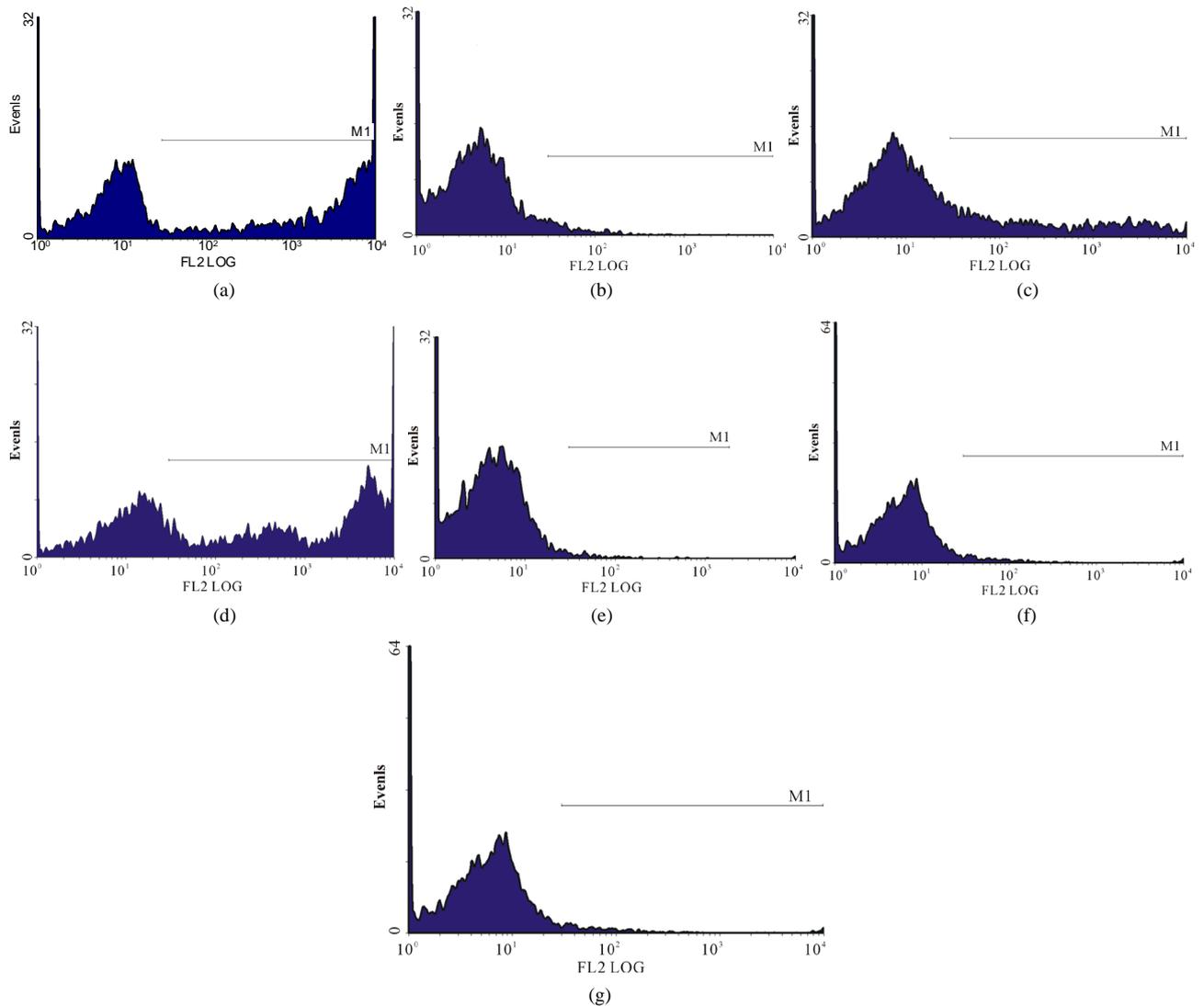
Peripheral blood mononuclear cells (PBMC) were separated from defibrinated blood samples by density gradient centrifugation (Nycomed Pharma As, Oslo, Norway). Blood was diluted 1:1 with tissue culture medium (RPMI 1640, Sigma-aldrich, UK) containing penicillin 100 µg/ml, streptomycin 100 U/ml (Gibco, Paisley, Scotland, UK), 2 mM L-glutamine (Sigma-aldrich, Irvine UK) at room temperature. This mixture was then layered onto an equal volume of lymphoprep (Ficoll-Paque TM PLUS, Amersham Biosciences, Uppsala Sweden) in 50 ml plastic tubes (Bibby Sterilin Staffordshire UK) and centrifuged at 600 g for 30 minutes at 20°C. Mononuclear cells were removed from the interface, and washed twice by spinning at 200 g for 10 minutes. The serum was retained and diluted with medium containing penicillin 100 µg/ml, streptomycin 100 U/ml (Gibco, Paisley, Scotland, UK), 2 mM L-glutamine (Sigma-aldrich, UK) to 10% and used in cell cultures.

### 2.4. Detection of Intracellular Cytokines by Flow Cytometry

PBMC from 100 patients were cultured ( $5 \times 10^5$  cells/well) in 24 well flat bottomed plates (Corning 25,820). Cytokine secretion by PBMC was blocked by adding monensin 4 µg/ml (Sigma-aldrich, Irvine UK) and brefeldin A 10 µg/ml (Sigma-aldrich, Irvine UK). PBMC were then activated with phorbol myristate acetate (PMA) 10 ng/ml plus ionomycin 1 µg/ml (Sigma-aldrich, Irvine UK), human HSP60 10 µg/ml (NSP-540 Stressgen UK), microbial HSP 65 10 µg/ml (NSP581 stressgen UK) or medium alone and incubated at previously pre-

determined optimal time of 18 hours (Figure 1) in a humidified atmosphere (5% CO<sub>2</sub> at 37°C). To investigate the role of LPS contamination some of the patients PBMC were further incubated with heat treated LPS or HSP by boiling 100°C for 30 minutes. After 18 hours of incubation, cells were transferred into 5 ml round-bottom tubes (Falcon 352,058). Cells were stained using CD4 fluorescein isothiocyanate (FITC) 5 µl (BD Pharmingen, San Diego), CD14 and CD8 peridinin chlorophyll-protein (PerCP) 5 µl (BD Pharmingen, San Diego), then incubated in the dark at room temperature for 30 minutes. Cells were then centrifuged at 500 g for 5 minutes and washed once in 2 mls of PBS/azide ( $\times 1$  PBS, 0.5% BSA, 0.1% sodium azide). Cells were fixed with 1 ml FACS lysing solution containing <50% Diethylene glycol and < 15% formaldehyde (BD Pharmingen 349,202) diluted 1:10 with PBS/azide and incubated at room temperature for 10 minutes. The cells were washed in PBS/azide cells and then permeabilised with 500 µl of FACS permeabilising solution (BD pharmingen 340,973) containing 0.02% saponin, diluted 1:10 with deionised water. Cells were incubated for 10 minutes at room temperature and then centrifuged at 500 g for 5 minutes, washed once and resuspended in PBS/azide.

Cells were then stained for intracellular cytokines, using 1 µg/ml of the following monoclonal antibodies conjugated to phycoerythrin (PE): anti-human IL-2 phycoerythrin (PE) conjugate (554,566 BD Pharmingen, San Diego), anti-human IL-4PE conjugate (554,516 BD Pharmingen, San Diego), anti-human IL-6PE conjugate (554,545 BD Pharmingen, San Diego), anti-human IFN- $\gamma$  PE conjugate (554,701 BD Pharmingen, San Diego), anti-human TNF- $\alpha$  PE conjugate (554,513 BD Pharmingen, San Diego).



**Figure 1.** (a)-(g): Histogram of flow cytometric intracellular cytokine expression in monocytes stained with PE-labelled anti-cytokine monoclonal in a patient with chronic periodontitis antibody showing events versus mean fluorescence intensity. (a) TNF- $\alpha$  expression following stimulation with LPS; (b) TNF- $\alpha$  expression following stimulation with microbial HSP65; (c) TNF- $\alpha$  expression following stimulation with Human HSP60; (d) TNF- $\alpha$  expression following heat treatment with LPS; (e) TNF- $\alpha$  expression following stimulation with heat treated microbial HSP65; (f) TNF- $\alpha$  expression following stimulation with heat treated human HSP60; (g) TNF- $\alpha$  expression following incubation with medium only. (a) LPS TNF- $\alpha$ ; (b) HSP65 TNF- $\alpha$ ; (c) HSP60 TNF- $\alpha$ ; (d) LPS TNF- $\alpha$  following heat treatment; (e) Heat denatured microbial HSP65 TNF- $\alpha$ ; (f) Heat denatured human HSP60 TNF- $\alpha$ ; (g) Medium TNF- $\alpha$ .

gen, San Diego), anti-human IL-17PE conjugate (560,438 BD Pharmingen) or CD64PE conjugate (Serotec Oxford). After 30 minutes cells were washed twice and resuspended in PBS/Azide. Samples were analysed in a Coulter Epics FACS machine, at least 10,000 events were analysed. Results were expressed as the mean fluorescence intensity (MFI).

## 2.5. Statistical Method

Data recorded on dental charts and data obtained from the different experiments were transcribed onto computer

records and analysed using SPSS-11.0 for windows (2001). All continuous variables were examined to establish whether the data conformed to a normal distribution with the Lilliefors (Kolmogorov-Smirnov) test for normality. Skewness and kurtosis were also examined. Analysis revealed non-normal distribution for all variables tested which were not amenable to log or square root transformation. Therefore the non-parametric Kruskal-Wallis ANOVA test was used to assess differences between the groups established. The significance level was set at  $p < 0.05$ . Post ANOVA pairwise comparisons be-

tween the gingivitis group, CP group, ging/CHD group and CP with CP/CHD group were carried out with Mann-Whitney U test, using the Bonferroni correction. Interrelations between two variables were tested using the Spearman ranked correlation coefficients ( $r_s$ ); when the p value was  $<0.05$ , correction for smoking pack years was carried out. Non-parametric data are displayed in tables as median (1st and 3rd quartile). Data were also displayed as mean  $\pm$  standard error of mean (SEM) to facilitate comparison with previously reported findings in the literature.

## 2.6. Detection of C-Reactive Protein by ELISA

Pre-diluted standard (50  $\mu$ L) and blank were added to a 96-well plate pre-coated with anti-serum CRP IgG (Kalon Biological Ltd). Serum samples were diluted 1:1000 with assay diluent (Kalon Biological Ltd.) and dispensed in duplicates to designated wells in CRP precoated plates. The plate was then incubated at room temperature for 60 minutes. Plates were washed 4 times with wash buffer (Kalon Biological Ltd); 100  $\mu$ L of CRP tracer (affinity purified sheep anti-CRP labelled with alkaline phosphatase, Kalon Biological Ltd UK) were then dispensed to each well and incubated uncovered for 30 minutes at room temperature. Plates were washed again 4 times with washing buffer (Kalon Biological Ltd, UK).

Substrate solution (100  $\mu$ l of 4-nitrophenylphosphate in substrate buffer Kalon Biological Ltd.) was then dispensed to each well and incubated at room temperature for 30 minutes. The reaction was stopped with 100  $\mu$ l of (120 g/L) sodium hydroxide. Optical densities were read at 405 nm with microplate reader (Anthos 2001, Anthos labtec instruments UK). A standard curve was constructed with standard points and curve fitted with four para-

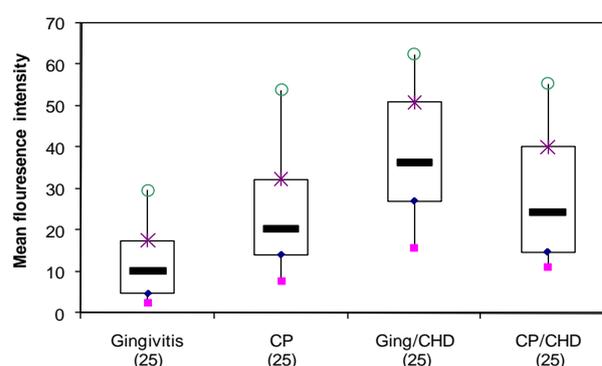
meter logistic curve fitting software. Test serum values were then read off the standard curve.

## 3. Results

### 3.1. Detection of Intracellular Cytokines

Unstimulated cells incubated in medium showed relatively low mean fluorescence intensity (MFLI) (Figure 2(g)) in all intracellular cytokines studied; no statistically significant difference was found amongst the 4 groups (ANOVA,  $p = 0.803$ , Table 2).

There was a statistically significant difference amongst the 4 groups in MFLI for intracellular TNF- $\alpha$  by ANOVA ( $p = 0.001$ ) (Table 2, Figures 2 and 3); also between the gingivitis group compared to the CP group ( $p = 0.017$ ) and between the gingivitis group and the ging/CHD



**Figure 2.** Detection of intracellular TNF- $\alpha$  in monocytes stimulated with human HSP60 showing median, interquartile range, mean and standard error of mean (SEM) of MFLI for each of 4 groups of patients (ANOVA  $p = 0.001$ ) o maximum;  $\times$  75<sup>th</sup> percentile;  $\blacklozenge$  minimum;  $\blacksquare$  25<sup>th</sup> percentile;  $\blacksquare$  median.

**Table 2.** Detection of intracellular TNF- $\alpha$  in monocytes stimulated with phorbol myristate acetate(PMA)/ionomycin, human HSP60, microbial HSP65 or no stimulus showing median, interquartile range, mean and standard error of mean (SEM) of MFLI.

Intracellular TNF- $\alpha$		Gingivitis (25)	CP (25)	Ging/CHD (25)	CP/CHD (25)
Medium ANOVA $p = 0.061$	Median (IQ range)	3.06 (2.5 - 8.2)	4.82 (4.4 - 6.8)	8.10 (5.6 - 10.0)	4.21 (3.7 - 6.1)
	Mean (SEM)	6.70 (2.1)	7.40 (1.3)	8.80 (2.4)	5.30 (0.9)
PMA/ionomycin ANOVA $p = 0.803$	Median (IQ range)	40.40 (34.3 - 65.4)	58.60 (37.3 - 68.0)	56.50 (45.3 - 87.0)	49.40 (37.3 - 67.9)
	Mean (SEM)	68.30 (18.7)	55.60 (20.8)	78.20 (19.1)	57.00 (37.3 - 67.9)
Human HSP60 ANOVA $\#p = 0.001$ $^*0.017$ $^{**}0.001$ $^{***}0.442$	Median (IQ range)	10.20 (4.8 - 17.4)	20.40 (14.1 - 32.3)	36.40 (27.0 - 51.0)	24.30 (14.8 - 40.1)
	Mean (SEM)	12.30 (2.9)	24.20 (2.3)	38.90 (8.1)	28.70 (4.6)
Microbial HSP65 ANOVA $p = 0.329$	Median (IQ range)	9.040 (4.2 - 22.6)	14.00 (10.0 - 32.6)	22.50 (13.9 - 24.7)	16.50 (13.1 - 32.0)
	Mean (SEM)	13.10 (3.1)	22.00 (2.2)	21.20 (13.9-24.7)	23.20 (4.6)

$\#$ significant p value for between group analysis of variance by Kruskal-Wallis test.  $^*$ p value for pairwise comparison by Bonferroni between gingivitis and chronic periodontitis group.  $^{**}$ p value for pairwise comparison by Bonferroni between gingivitis and gingivitis/CHD group.  $^{***}$ p value for pairwise comparison by Bonferroni between chronic periodontitis and chronic periodontitis/CHD group.

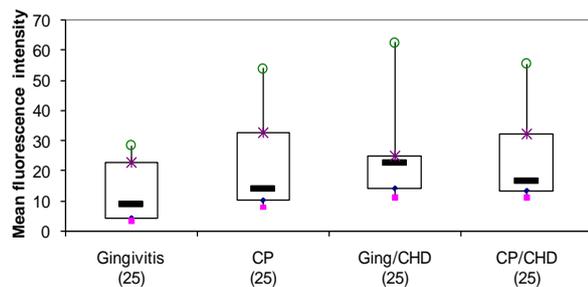
group ( $p = 0.001$ ) (Table 2). However, there was no statistical difference between the MFLI for the CP group and the CP/CHD group ( $p = 0.442$ ) (Table 2, Figures 2 and 3).

There were no statistically significant differences amongst the groups in the MFLI for any of the remaining intracellular cytokines (IFN- $\gamma$ , IL-2, IL-4, IL-5, IL-6, IL-17) following stimulation with human HSP60 or microbial HSP65.

There were no significant differences in intracellular IFN- $\gamma$ , IL-2, IL-4, or IL-6 amongst the 4 groups when monocytes were stimulated with either human HSP60 or microbial HSP65.

### 3.2. CRP

Serum CRP was significantly associated with MFLI of intracellular TNF- $\alpha$  in CP patients ( $r_s = 0.665$ ,  $p = 0.026$ ) and CP/CHD ( $r_s = 0.699$ ,  $p = 0.011$ ) even after correction for smoking pack years in chronic periodontitis ( $r_s = 0.649$ ,  $p = 0.002$ ) and chronic periodontitis/CHD ( $r_s = 0.603$ ,  $p = 0.011$ ) (Table 3). However, serum CRP levels were not significantly associated with MFLI of intracellular TNF- $\alpha$  production in the gingivitis group ( $r_s = 0.596$ ,  $p = 0.053$ ) and the gingivitis/CHD group ( $r_s = 0.515$ ,  $p = 0.128$ ). There was no direct association be-



**Figure 3.** Detection of intracellular TNF- $\alpha$  in monocytes following stimulation with microbial HSP65 in 4 groups of patients (ANOVA  $p = 0.329$ ).

**Table 3.** Association of serum CRP and smoking pack years to human HSP60-induced monocytic production of intracellular TNF- $\alpha$  in 4 groups of patients.

Intracellular TNF- $\alpha$	CRP		Smoking pack years	
	$r_s$	p value	$r_s$	p value
Gingivitis n = 25	0.596	0.053	0.253	0.452
CP n = 25	#0.665	*0.026	0.296	0.377
Ging/CHD n = 25	0.515	0.128	0.325	0.359
CP/CHD n = 25	#0.699	*0.011	0.526	0.079

Correlation coefficient between CRP and TNF- $\alpha$  in the chronic periodontitis ( $r = 0.649$ ,  $p = 0.002$ ) and chronic periodontitis/CHD ( $r_s = 0.603$ ,  $p = 0.011$ ) group remained significant even after correction for smoking pack years.  $r_s$  = Spearman rank correlation coefficient; p = probability value; #moderate correlation coefficient; \*significant p value.

tween smoking pack years to human HSP60 induced intracellular TNF- $\alpha$  production in the control ( $r_s = 0.253$ ,  $p = 0.452$ ), CP ( $r_s = 0.296$ ,  $p = 0.377$ ), ging/CHD ( $r_s = 0.325$ ,  $p = 0.359$ ) and CP/CHD ( $r_s = 0.526$ ,  $p = 0.079$ ) (Table 3).

### 3.3. Excluding Any Activity Due to Contamination of HSP with LPS

To exclude the possibility of LPS contamination affecting the production of intracellular cytokine, the microbial HSP65 and human HSP60 was heat-treated as previously described [8]. HSP is heat labile whereas, LPS is heat stable [8]. Stimulation of cultures with heat-treated microbial HSP65 (Figure 1(e)) and human HSP60 (Figure 1(f)) abolished the production of intracellular TNF- $\alpha$ . However, LPS stimulation of PBMC producing TNF- $\alpha$  (Figure 1(a)) was not affected by heat treatment (Figure 1(d)). The abrogation of TNF- $\alpha$  production makes it unlikely that LPS contamination was responsible for the intracellular cytokine expression.

## 4. Discussion

Unstimulated cells incubated in medium showed relatively low mean fluorescence intensity (Table 2). This is not surprising as unstimulated cells usually produce little or no measurable cytokines [37] except in patients with progressive malignancies [38]. PMA/ionomycin stimulation of cells resulted in an increase in the mean fluorescence intensity of TH1 cytokines in both controls and patients with CP and CHD (Table 2). Although this increased mean fluorescence intensity was not statistically significant, there was a trend towards higher mean fluorescence intensities for TNF- $\alpha$  and IFN- $\gamma$  in the patient groups compared to the control group (Table 2). There was little or no increase in the mean fluorescence intensity for IL-4 and IL-5 following stimulation with PMA/ionomycin in the patient groups. Perhaps the stimulation protocol employed in this study was not sensitive enough to detect IL-4 and IL-5 [39]; as PMA/ionomycin stimulation of IL-4 and IL-10 was insufficient for their intracellular detection, often requiring secondary culture with cytokines followed by re-stimulation with mitogen [40]. However, some studies have shown an induction of intracellular IL-4 with only primary stimulation with PMA/ionomycin [38].

Stimulation of cell cultures with human HSP60 [38,41] resulted in a statistically significant increase in mean fluorescence intensity for intracellular TNF- $\alpha$  in the CP and CHD patients compared with gingivitis group (Table 2, Figure 2), but no significant differences were seen amongst the 4 groups for intracellular IFN- $\gamma$ , IL-2, IL-4, IL-5 and IL-17. In order to ensure HSP60 and not LPS was eliciting the cytokine response, we denatured HSP60

before addition to the culture. Denaturing HSP completely abolished the cytokine response (**Figure 1(f)**). Since LPS is heat stable, this suggests that HSP is eliciting the cytokine production, and not LPS. Either CD14 or TLR4 or both receptors have been implicated in HSP recognition and are thought to mediate activation of monocytes/macrophages in response to human HSP60-stimulation [6,42]. As HSP60 has been implicated in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis [43] and atherosclerosis [44], the abundant expression of HSP and TLR-4 within periodontitis lesions [42,45] together with HSP's role in eliciting TNF- $\alpha$  production, suggests that HSP60 is also likely to be playing a role in chronic inflammatory periodontal disease. The highest intracellular TNF- $\alpha$  levels were in patients with CHD with or without periodontal disease and CP, suggesting that the cytokine levels are related to disease severity, however, the smoking status was not the same across the groups potentially confounding the results.

Although microbial HSP65 cultures showed an increase in mean fluorescence intensity for intracellular TNF- $\alpha$  compared to unstimulated samples, there was no statistically significant difference amongst the 4 groups (**Table 2, Figure 2**). Additionally, no significant differences were seen amongst the groups in the mean fluorescence intensity of intracellular IFN- $\alpha$ , IL-2, IL-4 and IL-5 (data not shown).

These results are consistent with previous studies showing that TNF- $\alpha$  can be induced by HSP60 in monocyte/macrophages [28-30]. The lack of any significant difference in the mean fluorescence intensity of intracellular TNF- $\alpha$  amongst groups with periodontal disease with/without coronary artery disease following stimulation with microbial HSP65 is consistent with the lymphoproliferative responses which showed no difference between the 4 groups. Most subjects respond to microbial HSP65 and this may be a result of the ability of microbial HSP65 to stimulate both naïve and primed cells [8,31,46].

Smoking has been a confounding variable in studies of the association of CP and CHD [47]. Exposure to smoking has been associated with reduced release of TNF- $\alpha$  in human alveolar macrophages [48,49]. In this study there was significant difference in the smoking pack years amongst the patient group compared to the gingivitis group (**Table 2**). There was no correlation of smoking pack years to human HSP60-induced intracellular TNF- $\alpha$  production in this study (**Table 2**).

CRP a marker of inflammation was significantly associated with human HSP 60 induced intracellular TNF- $\alpha$  production in the CP group with or without CHD, but not in the gingivitis group or the ging/CHD (**Table 2**). The lack of significant association between serum CRP and

TNF- $\alpha$  in the gingivitis and ging/CHD groups may simply reflect the complexity of inflammation and its many pathways and HSP60-induced TNF- $\alpha$  may be an indirect association with CRP and that CP induced inflammation drives TNF- $\alpha$  production. There is evidence for this from previous studies showing that CRP and TNF- $\alpha$  are both increased in patients with CP [48-50] and reduced following anti-microbial and non-surgical treatment of chronic periodontitis [50].

## 5. Conclusion

This study suggests that human HSP60 but not microbial HSP65 induces significant production of intracellular TNF- $\alpha$  in patients with CP and CHD. Since the marker of inflammation, namely CRP correlates with CP with or without CHD and not with mild chronic gingivitis or ging/CHD, this suggests that human HSP60-induced production of TNF- $\alpha$  is associated with CP and not CHD. There was no direct link between smoking pack years and the production of human HSP60 induction of TNF- $\alpha$ , however this merits further investigation.

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