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HtpG, the *Porphyromonas gingivalis* HSP-90 homologue, induces the chemokine CXCL8 in human monocytic and microvascular vein endothelial cells

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Summary

CXCL8 (interlukin 8, IL-8) has a diverse spectrum of biological activities including T cell, neutrophil and basophil chemotactic properties. It is produced by a wide variety of cell types and plays a significant role in the initiation of the acute inflammatory response. During inflammation, CXCL8 attracts and activates leukocytes at the site of infection leading to leukocyte infiltration, which can lead to tissue damage. *Porphyromonas gingivalis*, an aetiological agent of periodontitis, induces production of CXCL8 from several types of cells via its LPS and outer membrane proteins. Bacterial chaperones elicit a strong pro-inflammatory response in cells of the innate immune system. In *P. gingivalis* the *htpG* gene codes for the homologue of human Hsp90, a chaperone that associates with transcription factors, hormone receptors and protein kinases, affecting signal transduction pathways. CXCL8 mRNA and CXCL8 protein production was induced in monocytic/human microvascular vein endothelial cells treated with *P. gingivalis* cells or rHtpG protein. Blocking of receptors CD91 and TLR4 reduced the production of CXCL8 by rHtpG either using receptor-specific antibody or by siRNA silencing. Pre-incubation of *P. gingivalis* rHtpG preparations with human anti-HtpG significantly inhibited CXCL8 production. A *P. gingivalis HtpG* disruption mutant also induced less CXCL8 mRNA and protein. These results suggest that *P. gingivalis* HtpG might be involved in CXCL8-mediated immunopathogenesis.

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Introduction

Chronic infection with *Porphyromonas gingivalis* is strongly correlated to periodontitis and is an important risk factor for the maintenance and progression of the disease (Socransky and Haffajee, 1992; 1997). It is also frequently found in atherosclerotic plaques, presumably via minor bacteremias associated with personal or professional oral hygiene (Coulter *et al.*, 1990; McLaughlin *et al.*, 1996; Kozarov *et al.*, 2006). This chronic infection associates adaptive lymphocyte immunity with innate immunity to produce local infiltration of neutrophils, macrophages and T and B cells specific for a plethora of *P. gingivalis* antigens (Cutler *et al.*, 1991; 1999). Chronic inflammation is initiated and maintained by chemokines and cytokines that are secreted by gingival epithelial cells and macrophages. CXCL8 is one of the principal mediators of this inflammatory response.

CXCL8 is a member of the C-X-C family of chemokines that has a diverse spectrum of biological activities including T cell, neutrophil and basophil chemotactic properties (Gangur *et al.*, 2002; Robertson, 2002; Gear and Camerini, 2003). It can be produced by a wide variety of cell types and is believed to play a significant role in the initiation of the acute inflammatory response (Balasubramanian *et al.*, 2005; Barker *et al.*, 2005; Lin *et al.*, 2005; Skjolaas *et al.*, 2006). In the course of inflammation, CXCL8 attracts leukocytes to the site of infection leading to neutrophil infiltration, which may subsequently culminate in epithelial cell damage (Baggiolini, 1998). Therefore, its downregulation is vital in the prevention of chronic inflammation. A number of bacterial surface molecules such as LPS and heat-shock proteins (Hsps) are closely associated with inflammation. LPS-induced production of inflammatory molecules via toll-like receptors (TLR) on a number of host epithelial cells (Bainbridge *et al.*, 2002; Darveau *et al.*, 2002) has been reported, including human microvascular vein endothelial cells (HMVEC) which may suggest a role for *P. gingivalis* in the initiation of cardiovascular plaque (Coats *et al.*, 2003).

Heat-shock proteins are a group of evolutionary conserved proteins ranging in size from 8 kDa to 150 kDa that are synthesized rapidly by most cells responding to stress-related events (Kaufmann, 1990). They act as

Table 1. Selected increase in cytokine mRNA in THP-1 cells treated with rHtpG.

Cytokine	Fold increase in mRNA by THP-1 cells after treatment with rHTPG (μg ml^{-1}) for 30 min			
	0	1	10	50
IL-1α	1.0	0.7	0.7	1.2
IL-1β	1.0	0.7	1.1	4.9
CXCL8	1.0	0.9	2.5	14.9
TNFα	1.0	0.9	1.0	4.3

chaperones through protein–protein interaction to protect proteins from denaturation during synthesis or during noxious processes including heat stress, infection and inflammation. The chaperones of several bacteria including *Campylobacter rectus* (Hinode *et al*., 1998), *Heliobacter pylori* (Lin *et al*., 2005) and *Chlamydia pneumoniae* (Da Costa *et al*., 2004) have been reported to induce the production of CXCL8 in a number of cells including human gingival fibroblasts, endothelial cells and monocytic cells (Bulut *et al*., 2002). Therefore, we speculated that *P. gingivalis* HtpG might also be involved in the modulation of CXCL8 in monocytic or HMVEC cells.

To investigate the mechanisms of monocyte and HMVEC activation associated with HtpG, we cloned whole HtpG and produced a disruption mutant of the gene (Sweier *et al*., 2003). We report here that CXCL8 was secreted from monocytic cells (THP-1) and HMVEC following treatment with rHtpG and that CXCL8 induction was inhibited by pre-treatment of the protein with human antibody to rHtpG. We also describe reduced CXCL8 mRNA and CXCL8 protein production in monocytic cells treated with the *htpG* disruption mutant compared with those treated with the parent strain. Finally, we show that rHtpG induces CXCL8 by interaction with at least two types of surface molecules, TLR and scavenger receptors.

Results

Porphyromonas gingivalis *HtpG induces CXCL8 mRNA and protein in human monocytic cells*

We examined the CXCL8-specific mRNA and protein response to treatment with rHtpG by culturing the cells with dilutions of rHtpG. Determination of a pro-inflammatory cytokine, TNFα, was included in some experiments for comparison.

Fold increases up to 14 for CXCL8 and TNFα mRNA were observed by reverse transcription polymerase chain reaction (RT-PCR) of THP-1 cells treated with 1, 10 and 50 μg ml^{-1} concentrations of rHtpG as measured by the RT-PCR array (Table 1). Similar results were observed using Affymetrix microarray Hu133a and single-tube RT-PCR assays using different primers for the same cytokines (data not shown). When we measured CXCL8 and TNFα protein production in the THP-1 cells there was also dose–response to rHtpG (Fig. 1). As expected, we also found a dose–response production of CXCL8 and TNFα when THP-1 cells were treated with *P. gingivalis* LPS.

Porphyromonas gingivalis htpG *disruption mutant induces less CXCL8 protein than the parent strain*

We examined production of CXCL8 protein in THP-1 cells treated with *P. gingivalis* W83 or the disruption mutant W83Δ*htpG* (Sweier *et al*., 2003) using a protein microarray (Table 2). We were able to detect increases in the production of IL-1α, IL-1β, CXCL8 and TNFα when the W83 parent or mutant were combined with THP-1 cells at a ratio of 100:1 as compared with untreated THP-1 cells. There was no change in the production of INFγ or GMSF in the treated cells compared with the untreated cells. However, there was a significant ($P = 0.01$) reduction in the levels of IL-1α, IL-1β, CXCL8 and TNFα when the W83Δ*htpG* cells were compared with the parent strain.

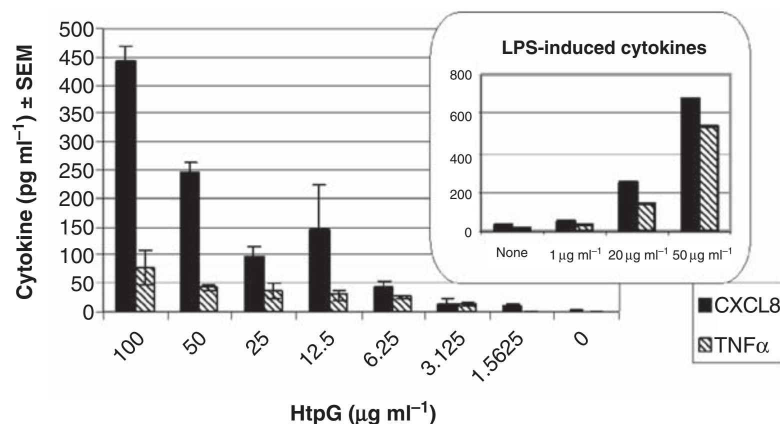

Fig. 1. Cytokine production in THP-1 cells treated with rHtpG. Cells were combined with rHtpG at the indicated concentration for 8 h. CXCL8 production was determined by ELSIA and is expressed as mean ± SEM. Inset: CXCL8 production in cells treated with *P. gingivalis* LPS at indicated concentrations.

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Table 2. Cytokine production induced by *P. gingivalis* W83 or W83ΔHTPG.

	Cytokine concentration (pg ml^{-1}) after treatment of THP-1 cells with		
Cytokine	No treatment	W83 (moi 100)	W83-ΔHTPG (moi 100)
IL-1α	6.2	144.6	72.5*
IL-1β	2.6	609.7	149.6*
CXCL8	292.5	2019.1	1169.4*
GMCSF	38.2	33.4	29.5
IFNγ	248.5	218.1	225.5
TNFα	17.5	240.9	67.7*

*$P = 0.01$ compared with W83 (*t*-test).

Porphyromonas gingivalis *rHtpG induces CXCL8 in endothelial cells but* P. gingivalis *LPS does not*

In HMVEC cells we detected CXCL8 production in response to rHtpG, but not in response to LPS (Fig. 2). There was also very little or no production of TNFα in HMVEC treated with rHtpG (Fig. 3).

Antibodies to cell surface receptors block production of CXCL8 by rHtpG

It has been suggested that chaperones interact with cell surface receptors to induce signalling pathways, so we examined the effect of blocking two such cell surface receptors, TLR4 and CD91. We found that in HUVEC blocking of CD91 reduced CXCL8 production by 33%; blocking of TLR4 reduced CXCL8 production by 60%. When we simultaneously blocked both receptors the reduction was 96% (Fig. 4).

siRNA silencing of THP-1 receptor genes reduces induction of CXCL8 by P. gingivalis *HtpG*

In order to further support our notion that HtpG induced CXCL8 production by binding to CD91 and TLR4 receptors, we investigated the chemokine production by THP-1 cells in which the receptors had been substantially eliminated by siRNA gene silencing. The production of CXCL8 protein and mRNA were compared in cells electroporated with siRNA and subsequently either treated with 50 μg ml^{-1} HtpG or left untreated. siRNA treatment of THP-1 cells reduced CXCL8 production by 80% when

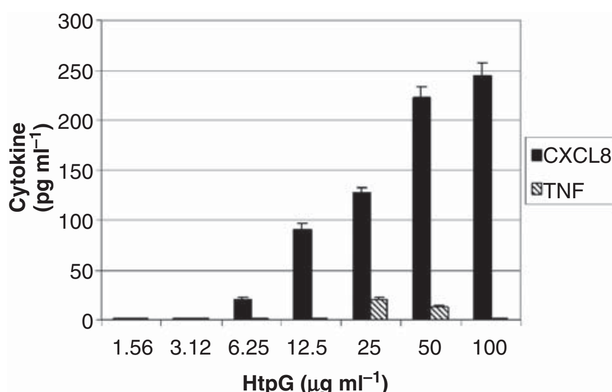

Fig. 2. CXCL8 and TNF production in HMVEC by rHtpG. Cells were combined with rHtpG or *P. gingivalis* LPS at the indicated concentration for 8 h. CXCL8 and TNFα production was determined by ELISA and is expressed as mean ± SEM.

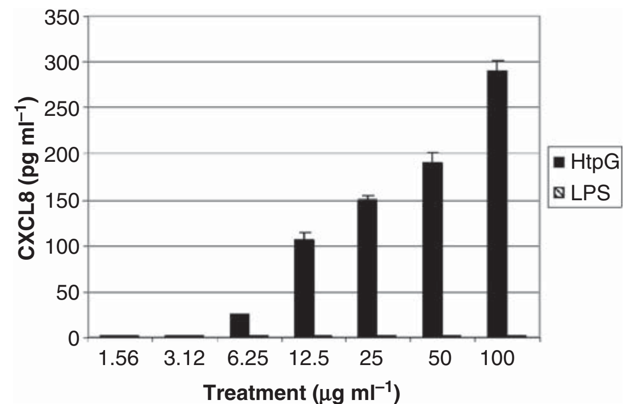

Fig. 3. CXCL8 production in HMVEC cells induced by HtpG or LPS. Cells were combined with rHtpG or *P. gingivalis* LPS at the indicated concentration for 8 h. CXCL8 production was determined by ELISA and is expressed as mean ± SEM.

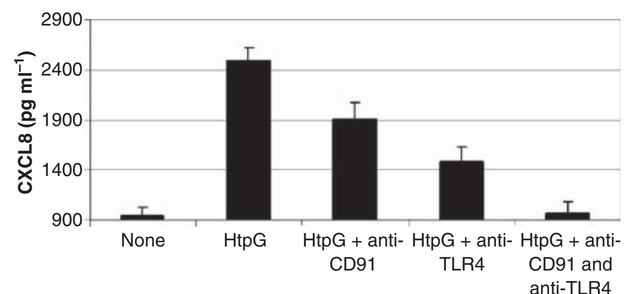

Fig. 4. Effect of anti-CD91 and anti-TLR4 on HtpG induction of CXCL8 in HUVEC. Cells were untreated (None); treated with 50 μg ml^{-1} rHTPG (HtpG); treated with 50 μg ml^{-1} rHTPG after pre-treatment with 1 μg ml^{-1} anti-CD91 (HtpG + anti-CD91) or 1 μg ml^{-1} anti-TLR4 (HtpG + anti-TLR4) or 1 μg ml^{-1} of both anti-CD91 and anti-TLR4 (HtpG + anti-CD91 and anti-TLR4). Results are mean CXCL8 production (pg ml^{-1}) ± SEM.

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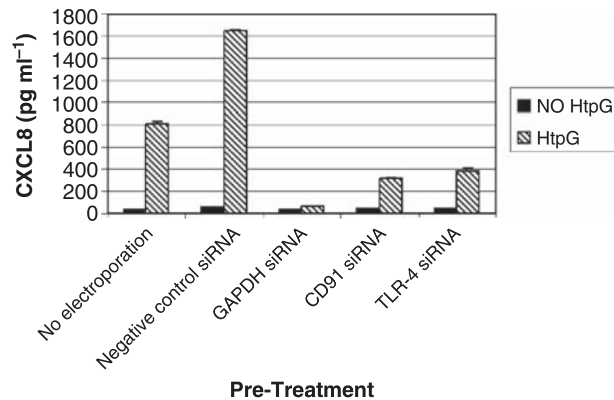

Fig. 5. Effect of siRNA silencing on rHTPG induction of CXCL8 in THP-1 cells. Cells were electroporated with siRNA for either CD91 or TLR4, allowed to recover for 48 h and then incubated for 8 h with 50 μg ml^{-1} rHTPG. CXCL8 production was determined by ELISA and is expressed as mean ± SEM.

CD91 was silenced and 77% when TLR4 was silenced (Fig. 5). Quantitative RT-PCR confirmed that mRNA for both receptors was reduced simultaneously (data not shown).

Serum antibody to HtpG reduces CXCL8 production in vitro

We measured the antibody levels to rHtpG in total IgG purified from 10 subjects participating in a geriatric oral science programme at the University of Michigan (Loesche *et al*., 1995) and found that eight of the 10 selected serum samples had measurable titres of IgG antibody to rHtpG (Table 3). When we treated the THP-1 cells with a mixture of rHtpG and IgG there was a reduction in the production of CXCL8. IgG from serum samples without HtpG antibody did not reduce the production of CXCL8 by the target cells. We made dilutions of IgG from one serum sample and found that there was a dose–response between the IgG and reduction of CXCL8 production. There was also a trend for IgG from serum samples with higher titres of anti-HtpG to demonstrate greater reductions in CXCL8 production.

Discussion

CXCL8 is released from cells following stimulation with LPS of *P. gingivalis* and other bacteria and its effects include neutrophil and T-lymphocyte chemotaxis, neutrophil activation and enhanced expression of neutrophil adhesion molecules. CXCL8 was induced following both stimulation by *P. gingivalis* cells and highly purified *P. gingivalis* rHtpG, similar to reports that chaperone molecules from a number of bacteria, including *H. pylori* and *C. pneumoniae*, induce pro-inflammatory cytokines which may be related to chronic diseases (Da Costa *et al*., 2004; Lin *et al*., 2005). CXCL8 is found at significantly high levels in leukocyte tissue infiltrates from gingivitis and chronic periodontitis subjects (Lappin *et al*., 2003) and evidence supports the notion that these leukocytes are recruited from nearby lymphoid organ/tissue (Kinane *et al*., 1999; Koulouri *et al*., 1999), not derived from precursor cells *in situ*. Chemoattraction of plasma cells and neutrophils (the predominant infiltrating cell type in the gingival space and tissue) is an important step in the immune response to periodontal infection, a step facilitated by CXCL8.

In this study rHtpG induced CXCL8 production by signalling through a non-TLR receptor, CD91, and TLR4. The HUVEC cells do not usually respond to *P. gingivalis* LPS because these cells do not have the TLR2 receptor that *P. gingivalis* LPS has been shown to signal through (Coats *et al*., 2003). CD91 (also known as the α-macroglobulin receptor or LRP-1) has been shown to be a receptor for Hsp90 the human homologue of HtpG

Table 3. Human serum antibodies inhibit induction of CXCL8 secretion by rHtpG in THP-1 cells.

Subject	Anti-HtpG (net RFU)	Treatment serum (total IgG μg ml^{-1})	CXCL8 (pg ml^{-1}) production	Treatment serum (total IgG μg ml^{-1})	CXCL8 (pg ml^{-1}) production
1	28 651	0	2017	100	410*
1	28 651	0	2133	10	388*
1	28 561	0	1997	1	962*
1	28 561	0	2091	0.1	1987
1	28 561	0	2045	0.01	2173
2	19 873	0	2175	10	727*
3	25 500	0	1983	10	206*
4	17 855	0	2001	10	1121*
5	18 462	0	1920	10	989*
6	0	0	2175	10	2105
7	0	0	1999	10	1983
8	15 743	0	2084	10	1720
9	11 029	0	1963	10	807*
10	14 449	0	1844	10	639*

*Significant mean difference between IgG treated and untreated cells $P = 0.05$ (*t*-test).

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(Basu *et al.*, 2001). When CD91 was blocked there was a significant reduction of CXCL8 production and nearly total reduction of CXCL8 production when both CD91 and TLR4 were blocked by antibody or silenced using siRNA. Finally, we were able to demonstrate that serum IgG from subjects with titres to rHtpG were able to interfere with CXCL8 production in THP-1 cells. This, combined with our findings that elevated titres to HtpG appear protective in periodontitis (Lopatin *et al.*, 1999), suggests a mechanism whereby antibodies to HtpG (which might cross-react to human Hsp90) could effect CXCL8 production in both antigen presenting and non-antigen presenting cells. This also suggests that a generalized, but as yet uncharacterized control of immune response to chaperones may exist.

CD91 is one of several receptors that endocytose polyanionic ligands including low-density lipoproteins and have important roles in the formation of foam cells and atherosclerotic lesions (Lupu *et al.*, 1994; van Berkel *et al.*, 2005). Recently it has become increasingly apparent that they also have important roles in innate immunity (Peiser *et al.*, 2002a,b). Bacterial chaperones have been shown to enhance both MHC I- and MHC II-mediated responses to chaperoned proteins. Of particular interest here, the response to mycobacterium hsp70 has shown CD91 to be involved in uptake of exogenous HSP:peptide complexes for alternate MHC-I processing and presentation. In addition, CD91 has been shown to bind mammalian gp96, HSP90, HSP70 and calreticulin (Basu *et al.*, 2001). Chylamidial hsp60 activates macrophages and endothelial cells through TLR4 (Bulut *et al.*, 2002) and microbial HSP70 binds CD40 and stimulates CC-chemokines production (Wang *et al.*, 2001). *P. gingivalis* GroEL has recently been shown to signal through TLR2 and TLR4 in THP-1 cells (Argueta *et al.*, 2006). However, this is the first report that microbial HtpG molecules can have similar activity.

The fact that both CD91 and TLR4 are receptors for HtpG may explain our earlier observation that antibodies to HtpG appear protective. In periodontitis the tissue damage that is the hallmark of the disease is the result of ongoing inflammation mediated by infiltration of the subgingival space by leukocytes, a process mediated by CXCL8 chemoattraction that could result in such a persistent inflammation.

HtpG may well be available as a non-cell-associated protein. Approximately 17% of subjects participating in a geriatric oral clinical research programme at the University of Michigan (Loesche *et al.*, 1995) were found to have circulating *P. gingivalis* HtpG (C.E. Shelburne, M.D. Coopamah, D.G. Sweier and D.E. Lopatin, unpubl. obs.). In some individuals HtpG might function as a 'danger signal' as has been described by a number of laboratories for other chaperone molecules (Moseley, 2000). The same availability could also support a role for HtpG in formation of atherosclerotic plaques (Hajishengallis *et al.*, 2002). The bacteria may enter the blood stream during tooth brushing or dental procedures (Coulter *et al.*, 1990) and this laboratory (Kozarov *et al.*, 2006) and others (Haraszthy *et al.*, 2000; Cairo *et al.*, 2004) have reported the presence of *P. gingivalis* in such plaques from individuals both with and without periodontitis. HtpG, like most chaperones tested, induces a strong humoral response. That response may have consequences in the pathogenesis of chronic diseases like periodontitis (Lopatin *et al.*, 1999) or atherosclerosis. It has also been suggested that chaperones can play roles in both sterile and septic inflammatory responses, and probably have a role in control of the commensal microbial flora (Stewart and Young, 2004). All of these functions are important in the establishment and perpetuation of chronic inflammatory diseases like periodontitis.

Overproduction of CXCL8 has been shown to be an important factor in the pathology of gastric ulcers and possibly gastric carcinoma (Yuan *et al.*, 2004) and inflammatory bowel disease (Daig *et al.*, 1996). Induction of CXCL8 by the *H. pylori* chaperone Hsp60 (Lin *et al.*, 2005) is thought to be an important part of the disease process of that organism. Passive immunization of mice with anti-CXCL8 has been shown to significantly reduce the growth of transplanted human cancer cells from a variety of tumours (Huang *et al.*, 2002; Mian *et al.*, 2003). Inhibition of CXCL8 production by human serum anti-HtpG as demonstrated here, when considered in light of the CD91 and TLR4 receptor interactions, suggests a novel mechanism by which anti-HtpG is 'protective' (Lopatin *et al.*, 1999) and which can be tested in periodontitis subjects. Validation of such a mechanism could have practical application in controlling that disease. If in addition the mechanism can be generalized it might have significance in several chronic bacterial infections.

Experimental procedures

Bacterial strains and culture conditions

Porphyromonas gingivalis W83 were maintained by weekly transfer in an anaerobe chamber (Coy Manufacturing, Grass Lake, MI) at 37°C on PRAS Brucella agar plates (Anaerobe Systems, Morgan Hill, CA) in 5% hydrogen, 10% carbon dioxide and 85% nitrogen atmosphere. Broth cultures (BHTS) were grown in modified Brucella Broth (BBL) supplemented with 5 mg l^{-1} haemin and 5 μg l^{-1} Vitamin K. *P. gingivalis* W83Δ*HtpG* cells were maintained as previously described (Sweier *et al.*, 2003). *Escherichia coli* cells for protein expression were grown aerobically in LB broth, supplemented with 100 μg ml^{-1} ampicillin and 34 μg ml^{-1} chloramphenicol.

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Monocyte/HMVEC/HUVEC culture conditions

THP-1 cells (ATCC TIB-202) were maintained by twice weekly transfer in complete RPMI 1640 medium modified to contain 2 mM L-glutamine, 10 mM Hepes, 1 mM sodium pyruvate, 4.5 g l^{-1} glucose, 1.5 g l^{-1} sodium bicarbonate, and supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 0.05 mM 2-mercaptoethanol. Cell viabilities were routinely above 95% as determined by Trypan blue exclusion. Primary human microvascular vein cells (passage 5–7) were a gift from Drs Jacques E. Nör and Peter Polverini and maintained in complete BM-2 medium (Cambrex, Walkersville, MD) as were human umbilical vein cells (Cambrex).

Cloning and purification of P. gingivalis *rHtpG*

The full-length sequence of *P. gingivalis htpG* was obtained from The Institute for Genomic Research Comprehensive Microbial Resource database (http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi). PCR primers were designed to produce full-length products that were subsequently purified from agarose gels and inserted into pCR®T7 TOPO® cloning vector following the manufacturer's instructions (Invitrogen, Carlsbad, CA). Clones of One Shot® Chemically Competent *E. coli* transformed with the vector were ampicillin selected and then screened by PCR using the initial primers. Inserts that produced amplicons of the correct size were sequenced to verify the full-length insert (Biomedical Core, University of Michigan, Ann Arbor, MI). Plasmids with in-frame inserts were used to transform TOP10F′ BL21(DE3)pLysS *E. coli* cells that were subsequently induced with 100 μM ITPG for 4 h. The induced fusion protein contains a segment with six consecutive histidine (6xhis) residues preceding the N-terminal of the cloned proteins. The protein was purified by Ni-agarose chromatography (Ni-NTA Agarose, Qiagen, Valencia, CA). The 6xhis component of the fusion protein was removed by enzymatic cleavage of the enterokinase site between the protein and the 6xhis and then dialysed into PBS. *E. coli* LPS was removed by chromatography over a Detoxi-Gel™ Endotoxin Removing Column (Pierce Chemical, Rockford, IL) and subsequent filtration through a sterile Acrodisc Unit [Mustang E (LPS binding) membrane, 0.2 μM, Pall, Ann Arbor, MI]. There was no detectable LPS in the final preparation as determined by QCL-1000 LAL Assay (sensitivity 0.01 EU ml^{-1}, Cambrex Bio Science, Rockland, ME).

Purification of P. gingivalis *LPS*

LPS was purified from *P. gingivalis* W83 using the method of Darveau and Hancock (1983). Briefly, pellets of *P. gingivalis* W83 were extracted three times with 0.275 M $MgCl_2$ and the partially purified LPS precipitated with cold ethanol. Finally, the LPS was pelleted by ultracentrifugation, re-suspended in sterile water and lyophilized. Purity was confirmed by double staining (protein and silver stain) of SDS gels by the method of Tsai (1986).

Monocyte exposure to P. gingivalis *rHtpG and LPS*

THP-1 cells were harvested and re-suspended in complete RPMI 1640 medium supplemented with 10% FBS and 0.05 mM 2-mercaptoethanol (ATCC, Gaithersburg, MD) to a final concentration of 10^6 cells ml^{-1}. The cell suspension was dispensed into wells (1 ml) of a 24-well plate and recombinant rHtpG added to a final concentration of 1, 10 or 50 μg ml^{-1}. Cells were treated with *P. gingivalis* W83 LPS at a final concentration of 50 μg ml^{-1}. Each treatment was carried out in triplicate. The plate was then placed at 37°C in an incubator containing a 5% CO_2 in air atmosphere. Triplicate samples were collected and pooled after 30 min to 8 h of incubation.

HMVEC/HUVEC exposure to P. gingivalis *rHtpG and LPS*

HMVEC or HUVEC cells were seeded into 24-well plates and allowed to grow to confluence. rHtpG or LPS was added at concentrations of 1.125–100 μg ml^{-1} (rHtpG) or 1–100 ng ml^{-1} (LPS) and incubated for 8 h. The supernatants were harvested and assayed for CXCL8 production by ELISA (R&D Systems, Minneapolis, MN).

For receptor blocking studies HUVEC (10^6 ml^{-1} $well^{-1}$) were aliquoted into a 24-well plate. Purified anti-human CD91 or TLR4 (BD Biosciences, San Jose, CA) was then added to a final concentration of 1.0 μg ml^{-1}. Both the antibodies were also added in combination at the same concentration for one set of experiments. The cells were incubated for 1 h at 37°C, after which rHtpG was added to a final concentration of 50 μg ml^{-1}. Incubation was carried out for 8 h at 37°C. Supernatants were harvested and analysed for CXCL8 using a commercial ELISA kit (R&D Systems).

Monocyte exposure to P. gingivalis *cells*

Porphyromonas gingivalis and the *P. gingivalis* Δ*htpG* mutant were grown to mid-log phase, centrifuged at 5000 *g* for 10 min, and re-suspended in antibiotic-free RPMI 1640 media containing 1% serum. Bacterial cell density was estimated by determining the A_{600} in a 1 cm cuvette. Mammalian cells were washed and re-suspended in RPMI 1640 antibiotic-free media containing 1% serum. The bacteria were added to the washed cells at a ratio of 100 bacteria for each mammalian cell (100:1), unless otherwise stated, and incubated for 8 h. Triplicate samples were collected and pooled after 30 min to 8 h of incubation.

ELISA for CXCL8 and TNF

A commercial 96-well microplate-based kit (R&D Systems) was used for most CXCL8 and TNFα determinations following the manufacturer's instructions. Initial survey experiments were performed using a protein array (EMD Biosciences, San Diego, CA) according to the manufacturer's instructions.

Real-time PCR for cytokine mRNA

Total RNA was isolated using either an RNAeasy kit (Qiagen) or Trizol™ reagent (Invitrogen). Samples were treated with DNAse before reverse transcription and the RNA concentration was determined by UV spectroscopy. SuperScript™ III (Invitrogen) was used to produce cDNA from 1.0 μg of total RNA using random 18-mer primers according to the manufacturer's instructions. GAPDH, 18S rRNA and β-actin were also used as internal con-

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trols for calculation of the fold change of mRNA. Cytokine mRNA determinations were made using Human Inflammatory Cytokines and Receptors RT^2 *Profiler*™ PCR Array according to the manufacturer's instructions (SuperArray Biosciences, Frederick, MD). Fold changes were calculated as $2^{-\Delta\Delta Ct}$.

Inhibition of CXCL8 production by anti-HtpG antibodies

IgG was isolated by Protein A chromatography from 0.5 ml of whole serum and dialysed into PBS. The IgG concentration was estimated by determination of the A_{280}/1.4. The IgG was treated with immobilized papain to produce F(ab) fragments. The F(ab) fragments (0.01–100 μg ml^{-1}) were combined with 50 μg ml^{-1} rHtpG and incubated overnight at 4°C. The treated rHtpG was then diluted in RPMI 1640 with 1% FBS as noted below and added to THP-1 cells for 8 h. The supernatants were collected, filtered sterile and assayed for CXCL8 as described.

Serum ELISA for anti-rHtpG

rHtpG with intact 6× fusion tags were combined with serum from subjects with diverse medical backgrounds (Loesche *et al*., 1995). After incubation for 4 h with shaking at room temperature the samples were transferred to a 96-well plate coated with Ni-NTA (HisSorb™ Strips, Qiagen), covered with transparent tape and incubated for another 4 h as before. The plates were washed four times with PBS + 0.05% Tween 20 and 100 μl of goat anti-human IgG (γ chain specific) labelled with alkaline phosphatase (100 ng ml^{-1} in PBS + 0.5% BSA) added. After 4 h of incubation at room temperature the plates were washed again and 100 μl of alkaline phosphatase substrate (4-MUP, 100 μg ml^{-1} in pH 9.5 carbonate buffer) was added. The relative fluorescence (RFU) for each well was determined using a Genios™ (Tecan, Durham, NC) microtitre plate reader. Positive serum samples were confirmed by Western blots using rHtpG diluted in non-reactive human serum. Serum samples were considered positive when the mean for all replicates was greater than 5 SD above the group mean and were positive on the Western blots using purified rHtpG.

siRNA silencing of THP-1 receptor genes

siRNA for CD91, TLR4 and GAPDH were obtained from Ambion (Austin, TX). THP-1 cells (10^6 ml^{-1}) were combined with 1.5–2.5 μg of each siRNA and electroporated (550 V for 100 microseconds) using a Bio-Rad Gene Pulser XCell. The cells were allowed to recover for 48 h in complete medium and then *P. gingivalis* HtpG (50 μg ml^{-1}) was added for 8 h. The supernatants from treated and control cultures were harvested and the total RNA was extracted from the cells as described above. Supernatants were assayed for the levels of CXCL8 by ELISA. Total RNA was used to produce cDNA using random primers and the levels of CD91, TLR4 and CXCL8 message were determined by quantitative RT-PCR using specific primer sets (TaqMan Gene Expression Assays, Applied Biosystems, Foster City, CA).

Statistical analysis

Results are expressed as means ± SEM. Differences of means calculated using the Student's *t*-test were considered significant for values of $P < 0.05$.

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