Cholesterol crystals enhance TLR2-and TLR4-mediated pro-inflammatory cytokine responses of monocytes to the proatherogenic oral bacterium Porphyromonas gingivalis

Køllgaard, Tania Maria Simonsen; Enevold, Christian; Bendtzen, Klaus; Hansen, Peter R.; Givskov, Michael; Holmstrup, Palle; Nielsen, Claus H.

Published in:
P L o S One

DOI:
10.1371/journal.pone.0172773

Publication date:
2017

Document version
Publisher's PDF, also known as Version of record

Document license:
CC BY

Citation for published version (APA):
Køllgaard, T. M. S., Enevold, C., Bendtzen, K., Hansen, P. R., Givskov, M., Holmstrup, P., & Nielsen, C. H. (2017). Cholesterol crystals enhance TLR2-and TLR4-mediated pro-inflammatory cytokine responses of monocytes to the proatherogenic oral bacterium Porphyromonas gingivalis. P L o S One, 12(2), [0172773]. https://doi.org/10.1371/journal.pone.0172773
Cholesterol crystals enhance TLR2- and TLR4-mediated pro-inflammatory cytokine responses of monocytes to the proatherogenic oral bacterium Porphyromonas gingivalis

Tania Køllgaard¹, Christian Enevold¹, Klaus Bendtzen¹, Peter R. Hansen², Michael Givskov³, Palle Holmstrup⁴, Claus H. Nielsen¹*¹

¹ Institute for Inflammation Research, Center for Rheumatology and Spine Disease, Rigshospitalet, Copenhagen University Hospital, Copenhagen, Denmark, ² Department of Cardiology, Copenhagen University Hospital Gentofte, Copenhagen, Denmark, ³ Costerton Biofilm Center, Department of International Health, Immunology and Microbiology University of Copenhagen, Copenhagen, Denmark, ⁴ Section for Periodontology, Microbiology and Community Dentistry, Department of Odontology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

* clauhnieelsen@hotmail.com

Abstract

Cholesterol deposits and pro-inflammatory cytokines play an essential role in the pathogenesis of atherosclerosis, a predominant cause of cardiovascular disease (CVD). Epidemiological evidence has linked periodontal disease (PD) with atherosclerotic CVD. Accordingly, viable periodontal pathogens, including Porphyromonas gingivalis, have been found in atherosclerotic plaques in humans and mice. We aimed to determine whether cholesterol crystals (CHCs) and oral bacteria synergestize in the stimulation of human monocytes. Incubation of human monocytes with CHCs induced secretion of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, and IL-8. Moreover, CHCs markedly enhanced secretion of IL-1β by monocytes stimulated with the toll-like receptor (TLR) 4 agonist Escherichia coli lipopolysaccharide (LPS), and the TLR2 agonist Staphylococcus aureus lipoteichoic acid. Notably, CHCs also enhanced IL-1β secretion induced by Porphyromonas gingivalis LPS and IL-1β secretion induced by whole P. gingivalis bacteria. This enhancement was abrogated by the NLRP3 inflammasome inhibitors Z-YVAD-FMK and glibenclamide. CHCs had no effect on cytokine production induced by P. gingivalis gingipains. Taken together, our findings support that CHCs, via stimulation of NLRP3 inflammasomes, act in synergy with the periodontal pathogen Porphyromonas gingivalis to promote monocyte secretion of pro-atherogenic cytokines.

Introduction

Growing evidence suggests that periodontal disease (PD) is a risk factor for atherosclerotic cardiovascular disease (CVD) [1,2]. Atherosclerosis is generally accepted to be a chronic...
inflammatory disease in which the pro-inflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α play an important role [3,4]. Accumulation of cholesterol crystals (CHCs) is thought to play an important role in atherosclerotic plaque destabilization and consequent atherosclerotic disease manifestations, e.g. acute myocardial infarction and stroke [5,6].

The major source of IL-1β and TNF-α in atherosclerotic lesions is macrophages, derived from blood monocytes infiltrating the subintima [7]. Secretion of biologically active IL-1β from monocytes and macrophages requires two activation signals. Signal 1 is delivered through toll-like receptors (TLRs) and leads to transcription of pro-IL-1β, pro-IL-18 [8] and pro-IL-33 [9]. Signal 2 is delivered by danger molecules, e.g. reactive oxygen species, and fungal, bacterial and viral pathogens, which bind to nucleotide-binding oligomerization domain-like receptors (NLRs) and cause assembly of multiprotein oligomers known as inflammasomes. Inflammasomes, in turn, activate caspase-1, which cleaves the precursor forms of the cytokines into active IL-1β and IL-18 [8,10]. CHCs have been shown to trigger inflammation by activation of the NLR family pyrin domain-containing 3 (NLRP3) inflammasome, leading to secretion of active IL-1β and TNF-α from monocytes and macrophages primed with TLR ligands, e.g. lipopolysaccharide (LPS) from Escherichia coli, cell wall components of Gram-positive bacteria, and oxidized low-density lipoprotein [11–13].

Much research has focused on pro-inflammatory conditions that facilitate the development of atherosclerotic disease. One such condition is PD, which affects up to 50% of the adult population over 50 years of age in industrialized countries [14,15]. Viable periodontal bacteria including Porphyromonas gingivalis have been found in atherosclerotic plaques in humans and in mice [16–18]. A considerable body of evidence has linked this bacterium to the pathogenesis of both PD and atherosclerotic CVD, but the underlying mechanisms remain unclear [19–21]. Oral infection with P. gingivalis and other bacteria induces secretion of IL-1β and TNF-α, as revealed in the crevicular fluid [22,23]. This promotes local tissue damage due to hyperinflammation, which may also cause systemic low-grade inflammation, and thereby increase the risk of atherosclerotic CVD [14]. Moreover, inflammation also causes ulceration in the periodontal pockets, thereby facilitating access of periodontal bacteria to the bloodstream, where they may spread suspended in plasma or attached to red blood cells (as is the case for P. gingivalis), thereby evading elimination by phagocytes [15,24], and subsequently gain access to the arterial wall and contribute to atherogenesis.

Recently, it has been shown that CHCs enhance the production of IL-1β by human macrophages primed with P. gingivalis LPS (Pg-LPS) [25]. However, results obtained with purified Pg-LPS may not apply to whole bacteria. Thus, gingipains, a family of cysteine proteases, constitute another major virulence factor of P. gingivalis, and are key players in the stimulation of bone matrix destruction and modulation of host responses in PD [26]. For example, the arginine (Arg) or lysine (Lys) specific gingipains of P. gingivalis may cleave CD14, a receptor for LPS [27], leading to LPS hyporesponsiveness [27]. While other studies have suggested that TLR2 is more important [28,29], recently Pg-LPS was shown to activate TLR4 which led to induction of pro-inflammatory cytokines in human gingival fibroblasts [30]. Regardless of the relative binding contribution of these TLRs, cleavage of CD14 by P. gingivalis gingipains may compromise signaling through both. Of note, gingipains per se stimulate the human monocyte cell line THP-1 for production of IL-8, IL-6, and monocyte chemotactic peptide (MCP)-1, in a process involving protease-activated receptors (PAR)-1, -2 and -3 [31], and human macrophages produce TNF-α after stimulation with both Arg- and Lys-gingipains [32].

In this study, we examined the ability of CHCs to stimulate unprimed and primed monocytes for production of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6, the anti-
inflammatory IL-10, and the chemokine IL-8. We also investigated the potential synergy between CHCs on the one hand, and Pg-LPS, Arg-gingipain and whole \textit{P. gingivalis} bacteria on the other, in stimulation of these responses. In addition, we examined the role of inflammasomes in mediating \textit{P. gingivalis}- and CHC-induced pro-inflammatory cytokine production.

**Materials and methods**

**Cells**

Blood from anonymous, healthy blood donors attending the Blood Bank at Copenhagen University Hospital, Rigshospitalet, Denmark was used. Peripheral blood mononuclear cells (PBMCs) were isolated using Lymphoprep (Axis-shield, Oslo, Norway) gradient centrifugation. PBMCs were resuspended in RPMI 1640 with HEPES (Biological Industries, Haemek, Israel), L-glutamine and gentamicin (Fischer Scientific, Slangerup, Denmark), and monocytes were isolated using EasySep\textsuperscript{TM} human CD14+ positive selection kit (STEMCELL Technologies, Grenoble, France) according to the manufacturer’s instructions. Purity was shown to be >90% when tested by flow cytometry.

**Cholesterol crystal preparation**

Cholesterol ≥99% (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 95% ethanol (12.5 g/L), heated to 60°C, filtered, and left at room temperature to allow crystallization in 15 mL polypropylene tubes (Nunc\textsuperscript{TM}, Fisher Scientific, Slangerup, Denmark). CHCs were collected by filtering and grinding using a sterile mortar. CHCs were stored in plastic tubes at -20°C until use. Any LPS contamination of CHCs was found to be below the detection limit of the \textit{Limulus} amebocyte lysate assay QCL-1000 kit (Lonza, Walkersville, MD, USA).

**Stimulation of monocytes with LPS from \textit{P. gingivalis} and \textit{E. coli}, lipoteichoic acid, and Arg-gingipain**

Freshly isolated CD14+ monocytes were cultured overnight in Nunclon Delta microwell plates (Thermo Fischer Scientific, Roskilde, Denmark) using 3x10^5 cells/well with 200 \(\mu\)L RPMI 1640 with no serum. The cells were incubated with 10.0 \(\mu\)g/mL Ultrapure LPS from \textit{P. gingivalis} (Pg-LPS) (Invivogen, San Diego, CA, USA), 0.01 and 1.0 \(\mu\)g/mL LPS from \textit{E. coli} (Ec-LPS: O55:B5 endotoxin; Lonza, Walkersville, MN, USA), and 0.1 and 1.0 \(\mu\)g/mL the TLR2 agonist lipoteichoic acid from \textit{Staphylococcus aureus} (Sa-LTA) (Invivogen, San Diego, CA, USA), in the presence or absence of 2 mg/mL CHCs. Furthermore, isolated monocytes were stimulated with Arg-gingipain (210 nM) (Hölzel Diagnostika Handels GmbH, Köln, Germany). Before use, Arg-gingipain was activated in RPMI 1640 and 10 mM cysteine for 10 minutes at 37°C/5% CO\(_2\), and then diluted in media. After 20 hours at 37°C/5% CO\(_2\), supernatants were harvested and analyzed for the presence of cytokines. No endotoxin was detected in the Arg-gingipain preparation.

**Cytokine measurements**

IL-1β, IL-6, IL-10, and TNF-\(\alpha\) were measured in supernatants from cell cultures using the BD Cytometric Bead Array Human Inflammation Kit (BD Bioscience, San Jose, CA, USA) as described previously [33]. Data acquisition was done with a FACScalibur flow cytometer (BD Bioscience), and data were analyzed using the FCAPArray Software (Softflow, Burnsville, MN, USA). IL-8 in supernatants was measured using the Luminex100 detection system (Luminex Corporation, Austin, TX, USA) according to the manufacturer’s instructions.
Blockade of TLR-2, TLR-4, the inflammasome, and IL-1 signaling

For blockade of TLR2 and TLR4, isolated monocytes were preincubated with 1 μg/mL monoclonal anti-TLR2 IgA (α-TLR2-IgA) (Invivogen, San Diego, CA, USA) or 2.5 μg/mL of the TLR4 antagonist LPS from Rhodobacter sphaeroides (Rs-LPS) (Invivogen, San Diego, CA, USA) for 30 minutes at 37˚C/5% CO2 before stimulation with whole Pg bacteria. For blockade of inflammasome activity, 25 μM of the pan-caspase inhibitor Z-VAD-FMK (sc-3067, Santa Cruz Biotechnology, Santa Cruz), various concentrations of the caspase-1/NLRP3 inhibitor, Z-YVAD-FMK (Abcam, Cambridge, UK) (1, 10, and 100 μM), and the ATP-sensitive potassium channel inhibitor/NLRP3 inhibitor, glibenclamide (Invivogen, San Diego, CA, USA) (5, 25, and 50 μg/mL) were added. For blockade of IL-1 signaling, 2 ng/mL of the IL-1 receptor antagonist (IL-1RA) (Novus Biologicals, Cambridge, UK) was added.

Stimulation of monocytes with whole bacteria

P. gingivalis (ATCC 33277) was cultured on trypticase soy blood agar plates containing 5 mg/mL hemin and 50 μg/L Vitamin K for 4 days at 37˚C/10% CO2/10% H2O/80% N2. Subsequently, the bacteria were frozen in phosphate-buffered saline, pH 7.4, and kept at -80˚C. Isolated CD14+ monocytes were stimulated overnight (3x10⁵ cells/well) with 4x10⁵ thawed whole bacteria in RPMI 1640 with no serum added. After 20 hours at 37˚C/5% CO2, supernatants were harvested and analyzed for the presence of cytokines.

Analyses of cell viability using flow cytometry

Purified CD14+ cells were stained with anti-CD14-antibody (BD Bioscience, San Jose, CA) and 7-Amino Actinomycin D (7-AAD) and incubated on ice for 30 minutes. Cells were analyzed using a FACS canto II flow cytometer (BD Bioscience, San Jose, CA) and data were analyzed using FlowJo v.X, (TreeStar, Inc. Ashland, OR).

Statistical analyses

Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, CA, USA). Data are presented as means±SDs. Cytokine secretion upon stimulation with and without CHCs was compared using repeated measures ANOVA. Differences between paired data were assessed using paired sample t-tests. The Bonferroni correction was used where independent comparisons were made. Data were log10-transformed to obtain normal distribution when indicated. P<0.05 was considered significant.

Results

Monocyte cytokine responses to CHCs

While examining the ability of CHCs to stimulate production of pro-inflammatory cytokines by unprimed human monocytes, we observed significant secretion of IL-1β, TNF-α, IL-6 (Fig 1A–1C), and a statistical trend towards IL-8 secretion (Fig 1E). The CHC concentration used (2 mg/mL) was based on preliminary titration studies (S1 Fig).

Influence of CHCs on TLR4-mediated cytokine responses

We next examined the influence of CHCs on monocyte cytokine responses induced by LPS from E. coli (Ec-LPS), a classic TLR4 agonist (Fig 1). The CHCs enhanced IL-1β responses to Ec-LPS at two concentrations (0.01 and 1 μg/mL) (Fig 1F) without affecting the production of TNF-α, IL-6, IL-10, and IL-8 (Fig 1G–1J).
Influence of CHCs on TLR2-mediated cytokine responses

Further we examined the effect of CHCs on TLR2-induced cytokine responses by stimulating monocytes with the TLR2 agonist Sa-LTA. Dose-dependent secretion of IL-1β, IL-6, IL-10, and IL-8 was observed (Fig 2). Again, IL-1β secretion was enhanced by co-incubation with CHCs at 0.1 and 1.0 μg/mL of Sa-LTA (Fig 2A), while CHCs had no effect on secretion of the other cytokines (Fig 2B–2E).

Influence of CHCs on cytokine responses to *P. gingivalis* LPS

Examination of the monocyte cytokine responses to LPS from *P. gingivalis* confirmed previous findings that TLR2 contributed to cytokine production induced by Pg-LPS [28,29] using a TLR2-blocking antibody (anti-TLR2, clone B4H2), which inhibited the production of IL-1β by 29±42%, TNF-α by 55±27%, IL-6 by 60±46%, and IL-10 by 54±45% (S2A–S2D Fig). Secretion of IL-8 was not affected by anti-TLR2 (S2E Fig).
Pg-LPS-induced secretion of IL-1β was greatly enhanced in a dose-dependent manner (Fig 3A) by co-stimulation with CHCs. In contrast, CHCs mediated a dose-dependent lowering of Pg-LPS-induced secretion of TNF-α, IL-6 and IL-10 (Fig 3B, 3C and 3D). Addition of CHCs did not affect the Pg-LPS induced secretion of IL-8 (Fig 3E).

Influence of CHCs on cytokine responses to *P. gingivalis* gingipains

The cytokine production induced by another constituent of *P. gingivalis*, the cysteine protease Arg-gingipain activated by cysteine in vitro and added to monocyte cultures showed that Arg-gingipain induced secretion of IL-1β and TNF-α (Fig 3F and 3G), but not of IL-6, IL-10 (Fig 3H and 3I), and IL-8 (data not shown). Furthermore, CHCs did not influence IL-1β or TNF-α secretion (Fig 3F and 3G).

Role of inflammasomes in cytokine responses to Pg-LPS and CHCs

To investigate the role of the inflammasome in the synergistic effect of Pg-LPS and CHCs on monocyte responses, the pan-caspase inhibitor Z-VAD-FMK was included in the experiments at a concentration of 25 μM, which was chosen on the basis of titration studies (S3A Fig). Z-VAD-FMK abolished secretion of IL-1β by monocytes stimulated with CHCs alone, confirming that CHCs induced IL-1β production by monocytes in an inflammasome-dependent manner (S3B Fig). Importantly, incubation of monocytes for 20 hours with Z-VAD-FMK at concentrations ranging from 5–25 μM did not cause significant cell death (S3C Fig). The enhancing effect of CHCs on Pg-LPS-induced IL-1β secretion by monocytes was abrogated by addition of Z-VAD-FMK (S3D Fig). In addition, production of TNF-α, IL-6, IL-10, and IL-8 after stimulation with Pg-LPS and CHCs was inhibited by Z-VAD-FMK (S3E–S3H Fig).

To further investigate the role of the NLRP3 inflammasome, we included the caspase-1/NLRP3 inhibitor, Z-YVAD-FMK, and the ATP-sensitive potassium channel/NLRP3 inhibitor,
glibenclamide in the experiments. Both Z-YVAD-FMK and glibenclamide caused a dose-dependent decrease in IL-1β secretion by monocytes stimulated with a combination of Pg-LPS and CHCs, confirming that NLRP3 was critically involved in the induction of IL-1β secretion (Fig 4A and 4F). In addition, secretion of TNF-α was lowered by both Z-YVAD-FMK and glibenclamide (Fig 4B and 4G), while the two NLRP3 inhibitors had no effect on the production of IL-6, or IL-8 (Fig 4C, 4D, 4E, 4H, 4I and 4J). The data on IL-10 were ambiguous (Fig 4D and 4I).

Addition of each inhibitor separately did not completely abolish secretion of IL-1β. However, addition of the inhibitors in combination at the highest concentration resulted in complete inhibition of IL-1β production (data not shown).

To examine the role of IL-1β for induction of TNF-α, IL-1 receptor antagonist (IL-1RA) was included in the experiments. IL-1RA did not affect secretion of IL-1β significantly (Fig 5A), but inhibited the production of TNF-α (Fig 5B), indicating a role for IL-1β in induction of TNF-α, as also observed by others [34]. In addition, IL-6 production was nonsignificantly lowered after addition of IL-1RA (Fig 5C). Secretion of IL-10 and IL-8 was not affected by addition of IL-1RA (Fig 5D and 5E).

The notion that secretion of TNF-α was a consequence of stimulation with IL-1β was supported by the finding that IL-1β secretion production preceded TNF-α secretion by approximately 3 hours (Fig 5F).

**Influence of CHCs on cytokine responses to whole bacteria**

Incubation of monocyte cultures with *P. gingivalis* as whole bacteria led to production of IL-1β, TNF-α, IL-6, IL-10, and IL-8 (Fig 6A–6E). Addition of anti-TLR2 and Rs-LPS, separately or in combination, lowered secretion of all cytokines (Fig 6A–6E), demonstrating the involvement of both TLR2 and TLR4 in their induction. Anti-TLR2 and Rs-LPS inhibited the production of IL-1β by 65±18% and 44±17%, respectively, with no additive effect of their combined use (data not shown).

![Fig 4. Role of the NLRP3 inflammasome in induction of cytokine responses to cholesterol crystals (CHCs) and *P. gingivalis* lipopolysaccharide (Pg-LPS).](image-url)

(A–E) Freshly isolated monocytes were cultured in presence of Pg-LPS (10 μg/mL) and CHCs, and the caspase-1 inhibitor Z-YVAD-FMK was added in concentrations of 5, 25 and 50 μg/mL. Concentrations of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6, IL-10, and IL-8 in supernatants after 20 hours of incubation were measured, and data were normalized to *P. gingivalis*+CHCs. Data are shown as means±SD for experiments using 3 healthy donors. (F–J) Freshly isolated monocytes were cultured in presence of *P. gingivalis* lipopolysaccharide (Pg-LPS) (10 μg/mL) and CHCs (2 mg/mL), and the ATP-sensitive potassium channel inhibitor glibenclamide was added at concentrations of 1, 10 and 100 μM. Concentrations of IL-1β, TNF-α, IL-6, IL-10, and IL-8 in supernatants after 20 hours of incubation were measured, and data were normalized to *P. gingivalis*+CHCs. Data are shown as means±SD for experiments using 5 healthy donors.

doi:10.1371/journal.pone.0172773.g004
The pan-caspase inhibitor Z-VAD-FMK completely abolished secretion of IL-1β, TNF-α, IL-6, IL-10, and IL-8 (Fig 6A–6E), demonstrating a critical role for inflammasomes in induction of all these cytokines by whole bacteria. As observed for Pg-LPS (Fig 3A), CHCs markedly enhanced the secretion of IL-1β induced by whole bacteria (Fig 6A). In contrast, CHCs lowered the secretion of TNF-α, IL-6, IL-10, and IL-8 (Fig 6B–6E). Secretion of IL-1β, TNF-α, IL-6, IL-10, and IL-8 induced by P. gingivalis and CHCs was abrogated by Z-VAD-FMK, demonstrating the critical role of inflammasomes, also under these conditions (Fig 6A–6E).

**Discussion**

Accumulation of CHCs plays a crucial role in atherogenesis, possibly via induction of pro-inflammatory cytokine responses through inflammasomes [5,6,11,12,25]. Infection with P. gingivalis which is thought to be a keystone pathogen in PD [20], has also been linked to the pathogenesis of atherosclerotic CVD [14,15,19–21]. We therefore assessed the ability of CHCs to stimulate human monocytes for secretion of pro-inflammatory cytokines and to enhance pro-inflammatory cytokine responses to P. gingivalis, and we examined the role of inflammasomes and TLRs in these processes.
CHCs alone were capable of inducing monocyte secretion of IL-1β, an important inflammatory mediator in CVD and PD [3,35,36]. Similar observations were made by Rajamäki et al., who used human adherent mononuclear cells and monocyte-derived macrophages [37]. Two other studies, however, found that CHCs alone did not induce IL-1β secretion by human PBMCs [12] or monocyte-derived macrophages [25]. These discrepancies may be explained by differences in incubation time (20 hours in our study versus 6 hours [12] and 6 days [25]), and the fact that we did not include GM-CSF or M-CSF [25]. We further observed that CHCs (2 mg/mL) alone induced secretion of TNF-α, IL-6, and IL-8 by monocytes. We recently found that matrix-bound cholesterol failed to stimulate monocyte-derived spindle-shaped cells for cytokine production [13], and this discrepancy may be due to differences in cholesterol formulation and differentiation stage of the cell cultures.

We show here that CHCs markedly enhance secretion of IL-1β by monocytes stimulated with the TLR4-ligand LPS from *E. coli*, supporting similar findings by Rajamäki et al. [37]. The Ec-LPS-induced production of TNF-α was not affected, however. The reverse pattern was found in our previous study using matrix-bound cholesterol [13]. CHCs also enhanced the secretion of IL-1β by monocytes stimulated with the TLR2-ligand lipoteichoic acid from *S. aureus*.

A main focus of this study was to examine whether *P. gingivalis* and CHCs acted synergistically in stimulation of pro-inflammatory cytokine production by monocytes, a molecular
mechanism that could contribute to the association between PD and atherosclerosis. Indeed, CHCs markedly enhanced secretion of IL-1β induced by LPS isolated from *P. gingivalis*, in accordance with results of a recent study of human macrophages [25]. However, CHCs did not enhance the Pg-LPS-induced production of TNF-α or IL-6. Another component of *P. gingivalis*, Arg-gingipain, which is considered a key virulence factor, induced secretion of IL-1β and TNF-α, but not IL-6, IL-8 and IL-10. Similarly, previous studies showed that gingipains induced IL-8 and TNF-α production by human macrophages [32] and IL-1β and IL-10 production by the human monocytic cell line THP-1 [38]. Notably, CHCs did not enhance Arg-gingipain-mediated secretion of IL-1β by monocytes.

To our knowledge, this is the first study to assess the combined effect of CHCs and whole *P. gingivalis* bacteria on cytokine production by monocytes. *P. gingivalis* alone was a potent inducer of IL-1β, TNF-α-, IL-6-, and IL-10 secretion, as also described previously [39,40], and of IL-8 secretion, respectively, and TLR2 and TLR4 both contributed to *P. gingivalis*-mediated induction of cytokine responses. Notably, CHCs enhanced *P. gingivalis*-induced secretion of IL-1β by monocytes, but lowered the concomitant secretion of TNF-α, IL-6, IL-8, and IL-10. The underlying mechanisms and the consequences of these responses with respect to atherosclerotic plaque development and stability remain to be studied.

The pan-caspase inhibitor Z-VAD-FMK abolished the ability of CHCs to induce IL-1β secretion by monocytes and neutralized the CHC-mediated enhancement of IL-1β production induced by Pg-LPS and whole *P. gingivalis*. This implied that CHCs exerted their effects on IL-1β production via caspase activation, and thereby through inflammasomes. It has previously been demonstrated that CHCs activate the NLRP3 inflammasome in Ec-LPS stimulated macrophages [37] and human PBMC [12]. Here, we investigated the role of the NLRP3 inflammasome in the enhancement of IL-1β secretion induced by Pg-LPS and CHCs by use of the NLRP3 inhibitors, Z-YVAD-FMK and glibenclamide. We demonstrated that the NLRP3 inflammasome is involved in the CHC-mediated enhancement of IL-1β production by monocytes stimulated with LPS from the periodontal bacterium *P. gingivalis*.

In line with the findings of others [34], our experiments including IL-1RA showed that IL-1β enhanced the secretion of TNF-α, IL-6 and IL-10, but not IL-8. Also, IL-1β secretion began approximately 3 hours before TNF-α secretion.

Among the limitations of the study, it should be noted that we isolated monocytes based on their expression of CD14, which is also expressed by macrophages. The cell cultures may therefore have contained macrophages at low frequencies.

In summary, we demonstrated an inflammasome-dependent role for CHCs in enhancement of the pro-inflammatory cytokine responses of human monocytes exposed to the pro-atherogenic periodontal bacterium *P. gingivalis*. This interaction may contribute to the association between PD and atherosclerotic CVD.

**Supporting information**

S1 Fig. **Dose-titration of cholesterol crystals (CHCs).** Isolated monocytes were cultured with no stimulation and CHCs at different concentrations (0.1, 0.5, 1, 2 and 4 mg/mL as indicated by numbers after CHCs). The content of interleukin (IL)-1β in the supernatants after 20 hours is shown as means±SD for experiments using two healthy donors.

(TIF)

S2 Fig. **The role of toll-like receptors (TLR) 4 and TLR2 in cytokine responses upon stimulation with *E. coli* (Ec)-lipopolysaccharide (LPS) and *P. gingivalis* (Pg)-LPS.** (A–E) Isolated monocytes were cultured with no stimulation, anti-TLR2, Pg-LPS) alone and Pg-LPS in combination with anti-TLR2 antibody. The content of IL-1β (*n* = 11), TNF-α (*n* = 11), IL-6
(n = 11), IL-10 (n = 10) and IL-8 (n = 7) in the supernatants after 20 hours is shown as means ± SD. P-values were calculated from log10-transformed data using paired t-test.

S3 Fig. Dose titration of the pan-caspase inhibitor Z-VAD-FMK. (A) Isolated monocytes were cultured with no stimulation, P. gingivalis (P.g)-lipopolysaccharide (LPS) and cholesterol crystals (CHCs), and Pg-LPS and CHCs in combination with different concentrations of Z-VAD-FMK (5, 10 and 25μM). Concentrations of interleukin (IL)-1β in the supernatants after 20 hours are shown as means±SD for results of experiments using two healthy donors. (B) Isolated monocytes were cultured with no stimulation, CHCs (2mg/mL) alone and in combination with Z-VAD-FMK (25μM). Concentrations of IL-1β in the supernatants after 20 hours are shown as means±SD for experiments using 10 healthy donors. (C) Isolated monocytes were cultured in presence of different concentrations of Z-VAD-FMK (5, 10, 25μM), Pg-LPS (10 μg/mL), and Pg-LPS in combination with Z-VAD-FMK (25 μM). After 20 hours, cells were stained with the dead cell marker 7-AAD and analyzed by flow cytometry. Frequencies of living CD14+ cells are shown for experiments using two healthy donors. (D-H) Freshly isolated monocytes were cultured in presence of Pg-LPS (10 μg/mL), Pg-LPS in combination with CHCs, or Pg-LPS in combination with both CHCs and the pan-caspase inflammasome-inhibitor Z-VAD-FMK. Concentrations of IL-1β, tumor necrosis factor (TNF)-α, IL-6, IL-10, and IL-8 in supernatants after 20 hours are shown as mean±SD for experiments using seven healthy donors.

Acknowledgments
The authors thank Winnie Hansen, Pia Grothe Meincke and Ole Christensen (Institute for Inflammation Research, Copenhagen University Hospital, Rigshospitalet) for their excellent technical assistance and help in the laboratory; Christian Damgaard (Section for Periodontology, Microbiology and Community Dentistry, Department of Odontology, University of Copenhagen) for provision of whole P. gingivalis bacteria; and Alistair Reeves for editing the manuscript.

Author Contributions
Conceptualization: TK CHN CE PRH PH.
Data curation: TK.
Formal analysis: TK CE CHN.
Funding acquisition: TK CHN PRH PH.
Investigation: TK CE.
Methodology: TK CE KB.
Project administration: TK CHN.
Resources: KB MG CHN.
Software: TK CE.
Supervision: TK CHN.
Validation: TK.
Visualization: TK CE CHN.
Writing – original draft: TK CHN.
Writing – review & editing: TK CE KB PRH MG PH CHN.

References
1. Mattila KJ, Nieminen MS, Valtonen V V, Rasi VP, Kesäniemi Y a, Syrjälä SL, et al. Association between dental health and acute myocardial infarction. BMJ. 1989; 298: 779–81. PMID: 2496855
2. Lockhart PB, Bolger AF, Papapanou PN, Osinbowa O, Trevisan M, Levison ME, et al. Periodontal disease and atherosclerotic vascular disease: does the evidence support an independent association?: a scientific statement from the American Heart Association. Circulation. 2012; 125: 2520–44. doi: 10.1161/CIR.0b013e31825719f3 PMID: 22514251
3. Dinarello CA. A clinical perspective of IL-1β as the gatekeeper of inflammation. Eur J Immunol. 2011; 41: 1203–1217. doi: 10.1002/eji.201141550 PMID: 21523780
4. Moreira DM, da Silva RL, Vieira JL, Fattah T, Lueneberg ME, Gottschall CAM. Role of vascular inflammation in plaques of atherosclerotic mice using hyperspectral CARS imaging. J Lipid Res. 2011; 52: 2177–86. doi: 10.1194/jlr.M018077 PMID: 21949051
5. Small DM. George Lyman Duff memorial lecture. Progression and regression of atherosclerotic lesions. Insights from lipid physical biochemistry. Arteriosclerosis. 1988; 8: 103–129. PMID: 3348756
6. Lim RS, Suhalim JL, Miyazaki-Anzai S, Miyazaki M, Potma EO, et al. Identification of cholesterol crystals in plaques of atherosclerotic mice using hyperspectral CARS imaging. J Lipid Res. 2011; 52: 2177–86. doi: 10.1194/jlr.M018077 PMID: 21949051
7. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. Nat Rev Immunol. 2007; 7: 678–89. doi: 10.1038/nri2156 PMID: 17717539
8. Schroder K, Tschopp J. The inflammasomes. Cell. 2010; 140: 821–32. doi: 10.1016/j.cell.2010.01.040 PMID: 20303873
9. Zhang L, Lu R, Zhao G, C SP, Li D-Q. TLR-mediated Induction of Pro-allergic Cytokine IL-33 in Ocular Mucosal Epithelium. 2011; 43: 1383–1391. doi: 10.1016/j.biocel.2011.06.003 PMID: 21684348
10. Dehghan A, Yang Q, Peters A. Association of novel genetic loci with circulating fibrinogen levels a genome-wide association study in 6 population-based cohorts. Circulation. 2009; 2: 125–33. doi: 10.1161/CIR.0b013e3181f57a5a PMID: 19168601
11. Rajamäki K, Lappalainen J, Rni KO, Valimaki E, Matikainen S, Kovanen PT, et al. Cholesterol crystals activate the NLRP3 inflammasome in human macrophages: a novel link between cholesterol metabolism and inflammation. Unutmaz D, editor. PLoS One. Public Library of Science; 2010; 5: 9.
12. Duewell P, Kono H, Rayner K, Sirois C. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. Nature. 2010; 464: 1357–61. doi: 10.1038/nature08938 PMID: 20428172
13. Bendtzen K, Christensen O, Nielsen CH, Holmstrup P. A matrix of cholesterol crystals, but not cholesterol alone, primes human monocytes/macrophages for excessive endotoxin-induced production of tumor necrosis factor-alpha. Role in atherosclerotic inflammation? Discov Med. 2014; 17: 309–12. PMID: 24979250
14. Belstrøm D, Damaa C, Nielsen CH, Holmstrup P. Does a causal relation between cardiovascular disease and periodontitis exist? Microbes Infect. Elsevier Masson SAS; 2012; 14: 411–418. doi: 10.1016/micinf.2011.12.004 PMID: 22200601
15. Pihlstrom BL, Michalowicz BS, Johnson NW. Periodontal diseases. Lancet. 2005; 366: 1809–20. doi: 10.1016/S0140-6736(05)67728-8 PMID: 16298220
16. Velsko IM, Chukkapalli SS, Rivera MF, Lee J-Y, Chen H, Zheng D, et al. Active Invasion of Oral and Aortic Tissues by Porphyromonas gingivalis in Mice Causally Links Periodontitis and Atherosclerosis. Glogauer M, editor. PLoS One. 2014; 9: e97811. doi: 10.1371/journal.pone.0097811 PMID: 24836175
17. Kozarov E, Dorn B, Shelburne CE, William A Dunn J, Progulske-Fox A. Human atherosclerotic plaque contains viable invasive Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis. Arter Thromb Vasc Biol. 2005; 22: 17–18.
18. Gaetti-Jardim E, Marcelino SL, Feltos ACR, Romito GA, Avila-Campos MJ. Quantitative detection of periodontopathic bacteria in atherosclerotic plaques from coronary arteries. J Med Microbiol. 2009; 58: 1568–1575. doi: 10.1099/jmm.0.013983-0 PMID: 19679682
19. Fiehn N-E, Larsen Tove, Christiansen N, Holmstrup P, Schroeder T V. Identification of periodontal pathogens in atherosclerotic vessels. J Periodontol. 2005; 76: 731–6. doi: 10.1902/jop.2005.76.5.731 PMID: 15898933

20. Hajishengallis G, Darveau R, Curtis M. The keystone pathogen hypothesis. Nat Rev Microbiol. 2012; 10: 717–725. doi: 10.1038/nrmicro2873 PMID: 22941505

21. Hussain M, Stover CM, Dupont A. P. gingivalis in periodontal disease and atherosclerosis—scenes of action for antimicrobial peptides and complement. Front Immunol. 2015; 6: 1–5.

22. Hirose K, E I, H M, I U. Levels of Porphyromonas gingivalis Fimbriae and inflammatory cytokines in gingival crevicular fluid from adult human subjects. Microbiol Immunol. 1997; 41: 21–6. PMID: 9087964

23. Stathopoulos PG, Benbakanecka MR, Galicia JC, Kinane DF. The host cytokine response to Porphyromonas gingivalis is modified by gingipains. Oral Microbiol Immunol. 2009; 24: 11–17. doi: 10.1111/j.1399-302X.2008.00467.x PMID: 19121064

24. Belstrøm D, Holmstrup P, Damgaard C, Borch TS, Skjødt MO, Bendtzen K, et al. The atherogenic bacteria Porphyromonas gingivalis evades circulating phagocytes by adhering to erythrocytes. Infect Immun. 2011; 79: 1559–1565. doi: 10.1128/IAI.01036-10 PMID: 21245264

25. Champaiboon C, Poolgesorn M, Wisirasameewong, Sa-Ard-lam N, Rerkyen P, Mahanonda R. Differential inflammasome activation by Porphyromonas gingivalis and cholesterol crystals in human macrophages and coronary artery endothelial cells. Atherosclerosis. Elsevier Ltd; 2014; 235: 38–44. doi: 10.1016/j.atherosclerosis.2014.04.007 PMID: 24814648

26. Veillard F, Potempa B, Poreba M, Drag M, Potempa J. Gingipain aminopeptidase activities in Porphyromonas gingivalis gingipains. Biol Chem. 2012; 393: 1471–6. doi: 10.1515/hsz-2012-0222 PMID: 23667904

27. Sugawara S, Nemoto E, Tada H, Miyake K, Imamura T, Takada H. Proteolysis of Human Monocyte Tumor Necrosis Factor (TNF) in Human Peripheral Blood Mononuclear Cells In Vitro and a Circulating TNF-like Activity in Rabbits. 2016;162:215–223.

28. Jain S, Coats SR, Chang AM, Darveau RP. A novel class of lipoprotein lipase-sensitive molecules mediates Toll-like receptor 2 activation by Porphyromonas gingivalis. Infect Immun. 2013; 81: 1277–86. doi: 10.1128/IAI.01036-12 PMID: 23381996

29. Ogawa T, Uchida H. Differential induction of IL-1b and IL-6 production by the nontoxic lipid A from Porphyromonas gingivalis in comparison with synthetic Escherichia coli lipid A in human peripheral blood mononuclear cells. FEMS Immunol Med Microbiol. 1996; 14: 1–13. PMID: 8804970

30. Herath TDK, Darveau RP, Seneviratne CJ, Wang C-Y, Wang Y, Jin L. Tetra- and Penta-Acylated Lipid A Structures of Porphyromonas gingivalis LPS Differentially Activate TLR4-Mediated NF-κB Signal Transduction Cascade and Immuno-Inflammatory Response in Human Gingival Fibroblasts. Ojcius DM, editor. PLoS One. 2013; 8: e58496. doi: 10.1371/journal.pone.0058496 PMID: 23554896

31. Uehara a, Imamura T, Potempa J, Travis J, Takada H. Gingipains from Porphyromonas gingivalis synergistically induce the production of proinflammatory cytokines through protease-activated receptors with Toll-like receptor and NOD1/2 ligands in human monocytic cells. Cell Microbiol. 2008; 10: 717–725. doi: 10.1038/nrmicro2873 PMID: 15898933

32. Grenier D, Tanabe S-I. Porphyromonas gingivalis gingipains trigger a proinflammatory response in human macrophage-derived macrophages through the p38 mitogen-activated protein kinase signal transduction pathway. Toxins (Basel). 2010; 2: 341–52.

33. Nielsen CH, Hegedüs L, Rieneck K, Moeller a C, Leslie RGQ, Bendtzen K. The atherogenic bac-

34. Ikejima T, Okusawa S, Ghezzi P, Meer JWV Van Der, The S, Diseases I, et al. Interleukin-1 Induces Tumor Necrosis Factor (TNF) in Human Peripheral Blood Mononuclear Cells in Vitro and a Circulating TNF-like Activity in Rabbits Charles A. Dinarello Published by: Oxford University Press Press Stable URL: http://www.jstor.org/stable/30127865 Interleukin-1 Induces Tumor Necrosis Factor (TNF) in Human Peripheral Blood Mononuclear Cells in Vitro and a Circulating TNF-like Activity in Rabbits. 2016;162:215–223.

35. Vahabi S, Sattari M, Taheriaslani M, Bagheban AA. Correlation between Interleukin-1β, Interleukin-6 and Tumor Necrosis Factor-α and Clinical Parameters in Chronic and Aggressive Periodontal Disease. J Periodontol Implant Dent. 2011; 3: 51–56.

36. Fearon WF, Fearon DT. Inflammation and cardiovascular disease: role of the interleukin-1 receptor antagonist. Circulation. 2008; 117: 2577–2579. doi: 10.1161/CIRCULATIONAHA.108.772491 PMID: 18490534

37. Rajamäki K, Lappalainen J, Öömi K. Cholesterol crystals activate the NLRP3 inflammasome in human macrophages: a novel link between cholesterol metabolism and inflammation. PLoS One. 2010; 5: e11765. doi: 10.1371/journal.pone.0011765 PMID: 20668705
38. Fitzpatrick RE, Aprico A, Wijeyewickrema LC, Charles N, Wong DM, Potempa J, et al. High molecular weight gingipains from Porphyromonas gingivalis induce cytokine responses from human macrophage-like cells via a non-proteolytic mechanism. 2011; 1: 109–117.

39. Borch TS, Holmstrup P, Bendtzen K, Nielsen CH. In vitro cytokine responses to periodontal pathogens: Generalized aggressive periodontitis is associated with increased IL-6 response to Porphyromonas gingivalis. Scand J Immunol. 2010; 71: 440–446. doi: 10.1111/j.1365-3083.2010.02390.x PMID: 20500696

40. Park E, Na HS, Song YR, Shin SY, Kim YM, Chung J. Activation of NLRP3 and AIM2 inflammasomes by Porphyromonas gingivalis infection. Infect Immun. 2014; 82: 112–123. doi: 10.1128/IAI.00862-13 PMID: 24126516