Blocking Proinflammatory Cytokine Release Modulates Peripheral Blood Mononuclear Cell Response to *Porphyromonas gingivalis*

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Background: Chronic periodontitis (CP) is an inflammatory disease in which cytokines play a major role in the progression of disease. Anti-inflammatory cytokines (interleukin 4 [IL-4] and IL-10) were reported to be absent or reduced in diseased periodontal tissues, suggesting an imbalance between the proinflammatory and anti-inflammatory mediators. This study tests the hypothesis that there is cellular crosstalk mediated by proinflammatory and anti-inflammatory cytokines and that blocking proinflammatory cytokine (tumor necrosis factor-α [TNF-α] and IL-1) production will enhance anti-inflammatory cytokine (IL-4 and IL-10) production from peripheral blood mononuclear cells (PBMCs) in response to *Porphyromonas gingivalis*.

Methods: PBMCs were isolated from individuals diagnosed with CP or healthy individuals and cultured for 24 hours. Concanavalin A (ConA) was used as an activator of lymphocyte function. Live and heat-killed *P. gingivalis* or lipopolysaccharide from *P. gingivalis* were used as the bacterial stimulants. TNF-α and IL-1 production was neutralized by specific antibodies against TNF-α and IL-1α or IL-β. Culture supernatants were evaluated by enzyme-linked immunosorbent assay for TNF-α, IL-1β, IL-4, and IL-10 production.

Results: Live *P. gingivalis* did not result in any significant IL-10 or IL-4 release, whereas heat-killed *P. gingivalis* led to a significant increase in IL-10 levels compared with unstimulated or live *P. gingivalis*–stimulated cells from both healthy individuals or those with CP. Overall, PBMCs from patients with CP produced significantly lower IL-10 in response to ConA and *P. gingivalis*, suggesting chronic suppression of the anti-inflammatory cytokine production. Blocking the proinflammatory cytokine response did not result in any substantial change in IL-10 or IL-4 response to live *P. gingivalis*. Blocking the proinflammatory cytokine response restored IL-10 production by cells from CP in response to *P. gingivalis* lipopolysaccharide.

Conclusions: These findings suggest that PBMCs from patients with CP have suppressed anti-inflammatory cytokine production that can, in part, be restored by neutralizing proinflammatory cytokines. Monocytes are an important source of IL-10 production, and monocyte-derived IL-10 might play a regulatory role in the pathogenesis of CP. *J Periodontol 2013;84:1337-1345*.

KEY WORDS

Interleukin-4; interleukin-10; monocytes; periodontitis; *Porphyromonas gingivalis*.

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Chronic inflammatory periodontal diseases are associated with *Porphyromonas gingivalis* as a major pathogen with a large array of virulence factors.[1-4] Complex immune responses to *P. gingivalis* play an important role in the progression of tissue breakdown in chronic periodontitis (CP).[4-7] Lymphocytes (B and T cells) as well as mononuclear phagocytes are present in diseased tissues and participate in host defense by actively producing cytokines.[7,8] Cytokine balance is considered to play an important role in the initiation and progression and host modulation of periodontal disease.[9,10] T cells can be categorized into various subgroups with different functions.[11] T-helper 1 (Th1) clones produce interleukin-2 (IL-2), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α), whereas Th2 clones produce IL-4, IL-5, IL-6, and IL-13.[11] IL-10 was originally described as a product of Th2 clones, but it is now known that Th1 cells and activated monocytes/macrophages also secrete IL-10 in humans, suggesting a critical role for IL-10-mediated regulation of the inflammatory response.[12] Various studies have reported decreased Th1 and increased Th2 responses in CP, with gingival mononuclear cells producing higher levels of IL-5 and IL-6 but not IL-2.[13] Memory T cells from the peripheral blood of patients with CP stimulated in vitro with *P. gingivalis* were shown to produce significantly more IL-4 than cells from healthy individuals.[14] However, it is not clear how the interactions between T-cell clones and monocytes/macrophages might modulate disease activity and chronicity and at what stage IL-10 is involved.

Evidence suggests that stimulation of peripheral blood mononuclear cells (PBMCs) from individuals with CP and gingivitis results in upregulation of IFN-γ and IL-13, whereas IL-4 and IL-10 are downregulated.[15] An imbalance of cytokine production may induce bone and collagen breakdown in periodontal disease, as demonstrated by cell infiltration and elevated levels of proinflammatory cytokines (IL-1, TNF, and IL-6) associated with active tissue breakdown in CP and other chronic inflammatory diseases such as rheumatoid arthritis.[16-19] One theory suggests that a lack of, or insufficient, response in anti-inflammatory cytokines is associated with the upregulation of proinflammatory cytokines.[20,21] Therefore, it is hypothesized in this study that the release of anti-inflammatory cytokines will be restored when proinflammatory cytokines are neutralized after triggering the host response with *P. gingivalis*. The purpose of this study is to analyze *P. gingivalis*–mediated IL-4 and IL-10 production from PBMCs after blocking of TNF-α and IL-1 in the presence and absence of donor periodontitis.

MATERIALS AND METHODS

Selection of Participants

Twelve individuals were included in the study. Six of these participants (four males and two females) were diagnosed with moderate-to-severe generalized CP as defined by the accepted criteria,[22] whereas six healthy donors (three males and three females) were used as matched controls with respect to age, sex, and race. The study was approved by the Institutional Review Board at Boston University Medical Center, and written informed consent was obtained from all individuals before evaluation. The study was conducted from 2004 to 2005. Nine of 12 of the individuals were nonsmokers. Mean age was 39.3 ± 9.8 years. None of the individuals had any known systemic disorders or used antibiotics or anti-inflammatory medications within 3 months of the experiment. The following were exclusion criteria: 1) participants with active infectious diseases, such as hepatitis, human immunodeficiency virus, and tuberculosis; 2) participants chronically treated with medications (phenytoin, cyclosporin A, or calcium channel blockers); and 3) females who were lactating or pregnant.

P. gingivalis *Culture and Lipopolysaccharide Production*

P. gingivalis strain A7436 was cultured as previously described.[23,24] After 24 hours of anaerobic growth in Schaedler broth,[‡] bacteria were harvested by centrifugation, washed with sterile pyrogen-free saline, and adjusted to an OD_{660} of 1.0 (approximately 1×10^9 colony forming units/mL). Bacterial cell counts were determined in all bacterial cultures to confirm *P. gingivalis* viability prior to cell culture experiments. A Gram stain kit[§] was used for assessing the purity of bacterial cell cultures. Three different *P. gingivalis* preparations were used; live *P. gingivalis* was prepared as described above and used at a multiplicity of infection (MOI) of 100. Heat-killed *P. gingivalis* was used after adjusting the bacterial cell counts and incubating the *P. gingivalis* colonies in water bath heated to 60°C for 20 minutes. MOI for heat-killed bacterial cultures was also set at 100. Lipopolysaccharide (LPS) from *P. gingivalis* A7436 was isolated by the technique described by Westphal and Jann.[25] Briefly, after 48 to 72 hours of growth, bacteria were washed, pelleted, and resuspended in distilled water. Phenol was melted and slowly

‡ Difco, Detroit, MI.
§ Thermo Fisher Scientific, Waltham, MA.

added to an equal volume of bacterial suspension at 68°C. The emulsion was then chilled on ice for 5 minutes, and phases were separated by centrifugation at 10,000 revolutions per minute for 30 minutes at 4°C. The aqueous phase (containing the LPS) was removed and dialyzed against distilled water for 72 hours at 4°C. Phenol–water LPS extract was then lyophilized, purified on cesium chloride isopycnic gradient, and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. For cell stimulation, 100 ng/mL *P. gingivalis* LPS was used.

Cell Isolation and Culture

Ninety milliliters of peripheral venous blood were obtained from each individual into heparinized (10 U/mL) tubes, and mononuclear cells (PBMCs) were isolated using a discontinuous gradient system as described previously.[26,27] Briefly, peripheral blood was layered on a mixture of resolving medium[‖] and density gradient cell separation medium,[¶] and the tubes were centrifuged at 500 × g for 30 minutes. The PBMC-rich layer was collected and washed twice with phosphate buffered saline (pH 7.4), counted, and suspended in cell culture medium[#] with 5% AB (blood group) serum. PBMCs (1×10^6 cells/mL) were cultured for 24 hours at 37°C in a 5% CO_2 atmosphere. After incubation, the cell supernatants were aspirated and saved at –80°C for cytokine analysis. Viability of cells was assessed by blue dye exclusion[**] under a light microscope.

In parallel experiments, PBMCs were isolated from the same individuals using a discontinuous gradient system (0.25×10^6/mL). Contaminating non-monocytic cells were removed, and monocyte populations were purified by magnetic cell sorting (MACS) column in the magnetic field of a separator with a monocyte isolation kit using an indirect magnetic labeling system for the isolation of untouched monocytes from human PBMCs.[††] Contaminating non-monocytes, i.e. T cells, NK cells, B cells, dendritic cells, and basophils, were magnetically labeled using a cocktail of biotin-conjugated antibodies against CD3, CD7, CD16, CD19, CD56, CD123, and glycophorin A and antibiotin microbeads and depleted by retaining them on a MACS column in the magnetic field of a MACS separator, while the unlabeled monocytes passed through the column. Pure monocyte preparations were washed and cultured for 24, 48, or 72 hours in the same way as PBMCs as outlined above. Cells were stimulated with various agents. In addition to live and heat-killed *P. gingivalis*, concanavalin A (ConA)[‡‡] (500 μg/mL) was used to stimulate T-lymphocyte-mediated cytokine release in PBMC cultures. *Escherichia coli* LPS (100 ng/mL; strain O55:B5[§§]) was used to activate monocytes.

Neutralization of Proinflammatory Cytokines (TNF-α, IL-1α, and IL-1β)

Activity of TNF-α, IL-1α, and IL-1β secreted by monocytes in cultures was blocked by monospecific antibodies.[‖‖] Neutralization bioactivity doses (ND_{50}) of antihuman TNF-α, IL-1α, and IL-1β antibodies were 0.02 to 0.04, 0.05 to 0.15, and 0.001 to 0.003 μg/mL, respectively. A dose–response experiment performed to determine the optimal concentrations for neutralizing cytokines indicated that 0.25 μg/mL (anti-TNF-α), 0.31 μg/mL (anti-IL-1α), and 0.031 μg/mL (anti-IL-1β) were needed to block the cytokine activity from 1×10^6 cells. The inhibition ranged from 87% to 94% for TNF-α, 62% to 93% for IL-1α, and 93% to 94% for IL-1β production. TNF-α, IL-1α, and IL-1β are not produced in significant amounts from resting, ConA-stimulated or live *P. gingivalis*–stimulated cell cultures. Neutralization achieved at 24 hours stayed at the same level throughout 48 and 72 hours of culture; therefore, the PBMC data are presented over 24 hours. No major variation between different periods in terms of inhibition was noted (data not shown); antibodies were used in combination to ensure complete neutralization of TNF-α, IL-1α, and IL-1β.

Enzyme-Linked Immunosorbent Assay

The levels of TNF-α, IL-1α, IL-1β, IL-4, and IL-10 production were measured by commercially available enzyme-linked immunosorbent assay (ELISA). The assays were conducted according to the instructions of the manufacturer. For IL-4 and IL-10 assays, high-sensitivity kits[¶¶] were used to detect low levels, whereas ELISA development systems[##] were used to monitor TNF-α, IL-1α, and IL-1β levels. Briefly, diluted standards and standard cytokine dilutions were added to 96-well microplates coated with mouse antihuman antibodies. Biotinylated antihuman antibodies were used as the detection antibody, and streptavidin–horseradish peroxidase was added as the conjugate. Hydrogen peroxide and tetramethylbenzidine were used as the substrate solution, and the reaction was stopped by adding 2N sulfuric acid. All samples and

‖ MonoPoly, Flow Laboratories, McLean, VA.
¶ Histopaque 1119 and Histopaque 1077, Sigma-Aldrich, St. Louis, MO.
# RPMI1640, American Type Culture Collection, Manassas, VA.
** Trypan blue, Invitrogen, Carlsbad, CA.
†† Monocyte Isolation Kit II, Miltenyi Biotec, Auburn, CA.
‡‡ Sigma-Aldrich.
§§ Sigma-Aldrich.
‖‖ R&D Systems, Minneapolis, MN.
¶¶ R&D Systems.
DuoSet ELISA development system, R&D Systems.

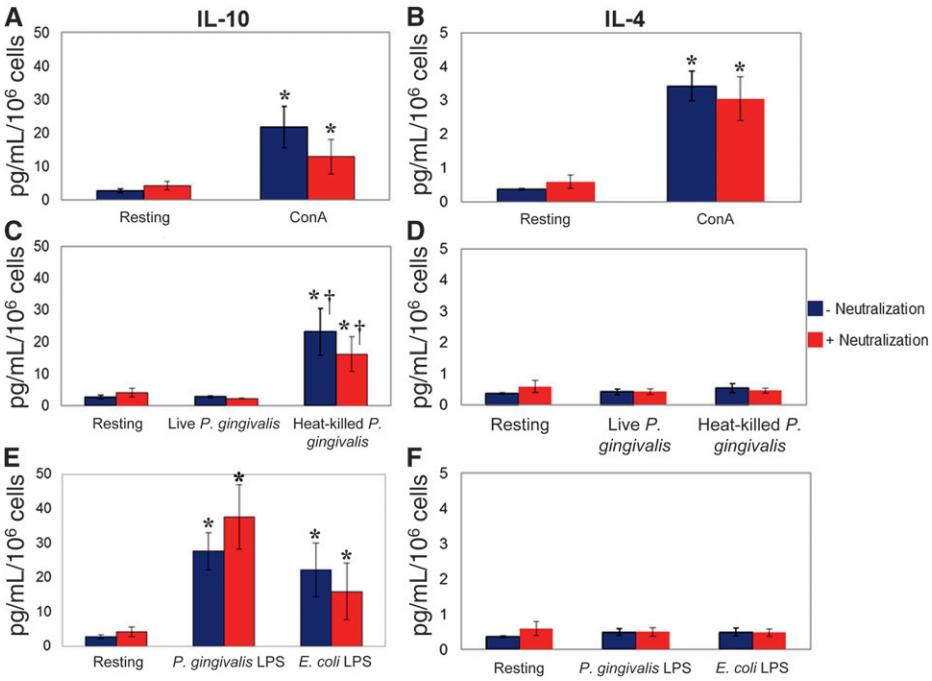

Figure 1.
IL-10 and IL-4 release by PBMCs from healthy donors. PBMCs were stimulated in the presence and absence of a combination of neutralizing antibodies to the proinflammatory cytokines TNF-α (0.25 μg/mL), IL-1α (0.31 μg/mL), and IL-1β (0.031 μg/mL). Each experiment was repeated in duplicate; results represent means ± SDs. ***A*** *and* ***B)*** *A specific T-cell activator (ConA; 500 μg/mL) significantly increased cytokine release compared with unstimulated (resting) cells (*P <0.05). *Blocking proinflammatory cytokine release did not significantly change the ConA-induced IL-10 and IL-4 release by healthy cells (P >0.05).* ***C*** *and* ***D)*** *Live* P. gingivalis *did not significantly induce IL-10 or IL-4 production by the PBMCs (P >0.05); heat-killed* P. gingivalis *led to a significant increase in IL-10 levels compared with unstimulated cells (*P <0.05) *and live* P. gingivalis*–stimulated cells (†P <0.05). Blocking proinflammatory cytokine response did not result in any substantial change in IL-10 or IL-4 response (P >0.05).* ***E*** *and* ***F)*** P. gingivalis *LPS (100 ng/mL) induced a significant increase in IL-10 (*P <0.05), *with no impact on IL-4 levels (P >0.05).* E. coli *LPS (100 ng/mL) induced significant IL-10 production (*P <0.05), *with no substantial change in IL-4 levels (P >0.05).*

unstimulated (resting) cells (21.69 ± 6.20 versus 2.70 ± 0.53 pg/mL/10^6 cells IL-10 and 3.42 ± 0.44 versus 0.37 ± 0.02 pg/mL/10^6 cells IL-4; *P* <0.05; Figs. 1A and 1B). Blocking proinflammatory cytokine release did not significantly change ConA-induced IL-10 and IL-4 release by healthy cells. Live *P. gingivalis* did not result in any significant production of IL-10 or IL-4 (2.80 ± 0.24 pg/mL/10^6 cells IL-10 and 0.42 ± 0.09 pg/mL/10^6 cells IL-4), whereas heat-killed *P. gingivalis* led to a significant increase in IL-10 levels compared with unstimulated or live *P. gingivalis*-stimulated cells (23.11 ± 7.27 pg/mL/10^6 cells; *P* <0.05; Figs. 1C and 1D). Blocking the proinflammatory cytokine response did not result in any substantial change in IL-10 or IL-4 response in healthy cells. *P. gingivalis* LPS induced a significant increase in IL-10 (27.66 ± 5.37 pg/mL/10^6 cells) by healthy PBMCs with no effect on IL-4 levels. This response was comparable to LPS from *E. coli* (22.16 ± 7.77 pg/mL/10^6 cells; Figs. 1E and 1F).

standards were run in duplicate, and optical density was determined with a microplate reader*** at a 450-nm wavelength. Samples above the standard determination range for optical density readings were assayed again and read at an appropriate dilution to ensure that the levels were within the linear slope of the standard curve.

Statistical Analyses and Data Presentation

Each experiment was repeated in triplicate, and data are expressed as the mean ± SD of three separate values for each individual. Data analysis was performed by Mann-Whitney *U* test, and significance was set at *P* <0.05.

RESULTS

IL-10 and IL-4 Release by PBMCs From Healthy Donors

ConA induced reproducibly significant IL-10 and IL-4 production in healthy cells compared with

IL-10 and IL-4 Release by PBMCs From Patients With CP

PBMCs obtained from patients with CP failed to produce significant levels of IL-10 in response to ConA compared with those from healthy donors, whereas IL-4 production was also significantly less (4.17 ± 1.65 versus 1.81 ± 0.38 pg/mL/10^6 cells IL-10 and 1.36 ± 0.39 versus 0.42 ± 0.06 pg/mL/10^6 cells IL-4; *P* <0.05; Figs. 2A and 2B). Likewise, there was a significant loss of IL-10 production in response to heat-killed *P. gingivalis* (12.36 ± 4.25 pg/mL/10^6 cells) and LPS from *P. gingivalis* (4.25 ± 1.98 pg/mL/10^6 cells) compared with healthy cells, with no impact on IL-4 production (Figs. 2C and 2D). *E. coli* LPS generated comparable levels of IL-10 by PBMCs from patients with CP (17.90 ± 6.30 pg/mL/10^6 cells). Blocking the proinflammatory cytokine response

*** Molecular Devices, Sunnyvale, CA.

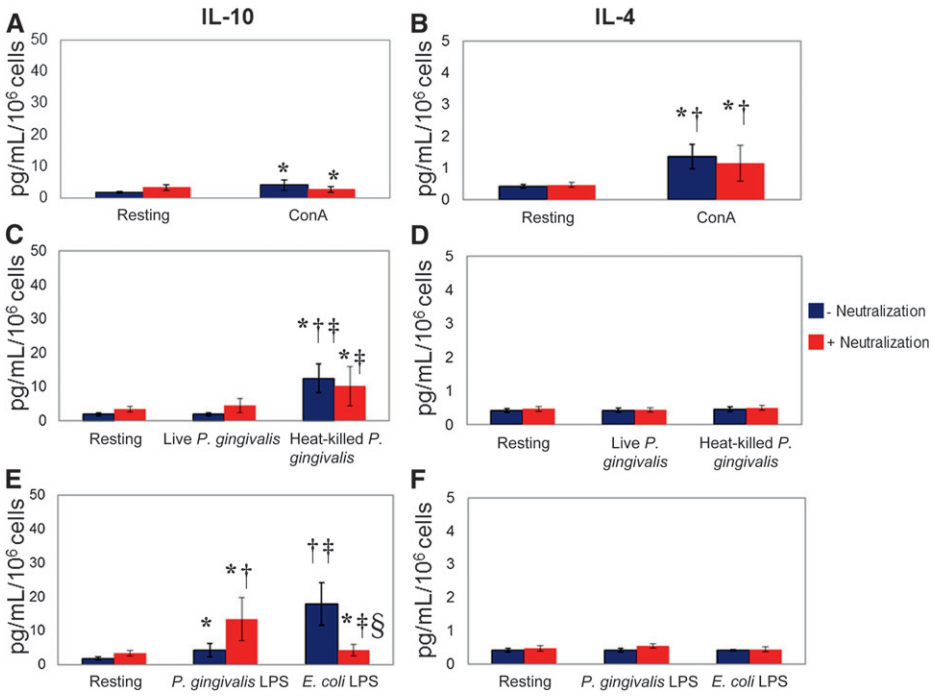

Figure 2.

IL-10 and IL-4 release by PBMCs from patients with CP. PBMCs were stimulated in the presence and absence of a combination of neutralizing antibodies to the proinflammatory cytokines TNF-α (0.25 μg/mL), IL-1α (0.31 μg/mL), and IL-1β (0.031 μg/mL). Each experiment was repeated in duplicate; results represent means ± SDs. ***A*** *and* ***B)*** *ConA (500 μg/mL) stimulation did not produce significant IL-10 in PBMCs from patients with CP compared with unstimulated cells (P >0.05). The difference was significant compared with cells from healthy donors (*P <0.05). IL-4 production was significantly increased compared with unstimulated cells (†P <0.05) but significantly less than healthy cells (*P <0.05).* ***C*** *and* ***D)*** *Live P. gingivalis did not significantly induce IL-10 or IL-4 production (P >0.05); heat-killed P. gingivalis led to a significant increase in IL-10 levels compared with unstimulated (†P <0.05) and live P. gingivalis-stimulated cells (‡P <0.05). Blocking the proinflammatory cytokine response did not result in any substantial change in IL-10 or IL-4 response (P >0.05). IL-10 production by PBMCs from patients with CP in response to heat-killed P. gingivalis was significantly less than the cells from healthy donors (*P <0.05).* ***E*** *and* ***F)*** *P. gingivalis LPS (100 ng/mL) did not induce a significant increase in IL-10 or IL-4 levels (P >0.05) compared with unstimulated cells. When IL-1 and TNF-α were blocked, P. gingivalis LPS–induced IL-10 production was significantly higher than unstimulated cells (†P <0.05), with no change in IL-4 release. P. gingivalis LPS–induced IL-10 production by the PBMCs from patients with CP was significantly less than the healthy cells with or without neutralization of proinflammatory cytokines (*P <0.05). E. coli (100 ng/mL) LPS–stimulated IL-10 by the PBMCs significantly compared with resting (†P <0.05) and P. gingivalis LPS–stimulated (‡P <0.05) cells. When the TNF-α and IL-1 responses were blocked, E. coli LPS–induced IL-10 production was significantly reduced (§P <0.05), significantly lower than the P. gingivalis LPS (‡P <0.05) or healthy cells (*P <0.05).*

in part restored the IL-10 production by cells from CP in response to *P. gingivalis* LPS (13.38 ± 6.29 pg/mL/10^6 cells) but reduced *E. coli* LPS–induced IL-10 production (4.27 ± 1.76 pg/mL/10^6 cells) (Figs. 2E and 2F).

IL-10 Production From Monocytes in Response to P. gingivalis

To identify the contribution of mononuclear phagocytes (monocytes) to overall IL-10 production by the PBMCs, pure monocyte cultures were next obtained by negative selection through MACS and treated the same way as the PBMCs. Figure 3 demonstrates the time course of IL-10 production in response to *P. gingivalis* over 72 hours. As in the case of PBMCs, monocytes failed to produce any detectable IL-10 in response to live *P. gingivalis*, whereas heat-killed *P. gingivalis* led to a statistically significant increase in IL-10 production (Fig. 3A); blocking modestly restored this response (Fig. 3B). Cells were then challenged with LPS from *E. coli* or *P. gingivalis* (Fig. 3C). Both LPS preparations caused significant and parallel IL-10 production from monocytes (P <0.05). Blocking proinflammatory cytokines did not lead to any significant change in IL-10 production in response to *E. coli* LPS, whereas the IL-10 response to LPS from *P. gingivalis* was significantly restored after 48 hours of neutralization (Fig. 3D).

DISCUSSION

P. gingivalis can modulate the innate immune response, rendering the host susceptible to disease by its virulence factors, such as LPS, capsule, and gingipains.[1,2,28-31] *P. gingivalis* can perturb the cytokine network not only by stimulating the release of cytokines from host cells but also by removing cytokines from the local environment in periodontal lesions.[29-34] "Proinflammatory" cytokines initiate a profound immune response, whereas IL-4 and IL-10 act as "anti-inflammatory" cytokines and regulate the immune response by controlling the proinflammatory cytokine response.[35-39] The net outcome of this crosstalk between the proinflammatory and anti-inflammatory arms of the immune response is the downregulation of the excessive and harmful inflammation in infections preventing host-mediated tissue breakdown and controlling the resolution of inflammation. Based on the assumption that CP is attributable to an

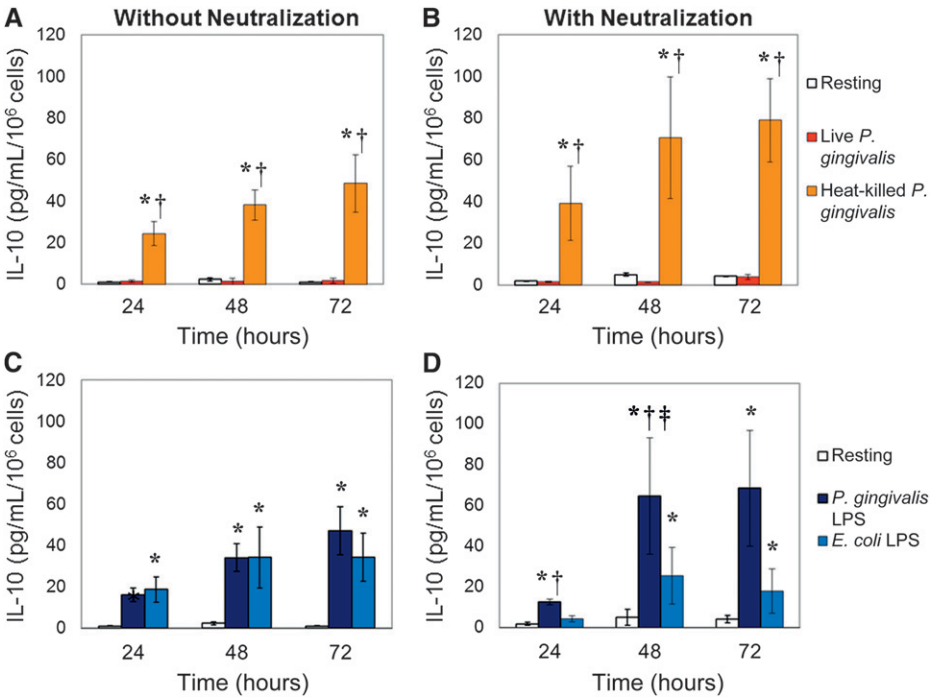

Figure 3.

IL-10 production from monocytes in response to P. gingivalis. *Each experiment was repeated in duplicate; results represent means ± SDs.* ***A*** *and* ***B)*** *Live* P. gingivalis *did not generate any significant IL-10 production by the monocytes; heat-killed* P. gingivalis *led to a statistically significant increase in IL-10 production compared with resting (*P <0.05) *or live bacteria-stimulated (†*P <0.05) *cells. Blocking the proinflammatory cytokine activity did not result in any significant change compared with cells that were not exposed to neutralization but were statistically significant compared with resting (*P <0.05) *or live bacteria–stimulated (†*P <0.05) *cells with neutralization.* ***C*** *and* ***D)*** *LPS (100 ng/mL) from both* E. coli *and* P. gingivalis *caused significant IL-10 production from monocytes compared with resting cells (*P <0.05). *Blocking proinflammatory cytokines did not lead to any significant change in IL-10 production in response to* E. coli *LPS (*P >0.05), *but there was a significant increase in IL-10 response to LPS from* P. gingivalis *after 48 hours of neutralization (‡*P <0.05). *At both 24 and 48 hours,* P. gingivalis *LPS–stimulated IL-10 production by the monocytes was significantly higher than the* E. coli *LPS–treated cells under neutralization (†*P <0.05).

imbalance between proinflammatory and anti-inflammatory cytokines,[8,9,15,16,18,19,40] this study addresses the regulation of anti-inflammatory cytokines in *P. gingivalis*–stimulated PBMC cultures and the role of proinflammatory cytokines in this process in healthy people and patients with CP. The results demonstrate that heat-killed *P. gingivalis* had a profound impact on IL-10 production in PBMCs with reduced production by the cells from donors with CP, suggesting an aberrant IL-10-mediated host response. There was also differential regulation of cytokine release when cells were stimulated with LPS from *P. gingivalis* compared with *E. coli*, suggesting different Toll-like receptor (TLR) pathways. Blocking proinflammatory cytokine discharge restored *P. gingivalis* LPS–elicited IL-10 production, suggesting that the host response is, at least in part, directed to resolution in the absence of proinflammatory stimuli. The lack of response to live *P. gingivalis* is likely attributable to in vitro degradation of mediators by proteases as reported in similar systems. Overall, these findings indicate that the resolution pathways mediated by IL-10 are downregulated in chronic periodontal inflammation and can only be partially restored when acute proinflammatory cytokine release has been blocked.

P. gingivalis synthesizes and secretes high levels of proteolytic enzymes to degrade peptides to essential amino acids as a food source. These bacterial proteases (gingipains) have been shown to cleave and inactivate released cytokines and, as a consequence, are thought to impair the inflammatory response.[34,41] Gingipains cleave monocyte CD14, result in attenuation of the cellular recognition of bacteria, and sustain chronic inflammation.[32] In this study, both live *P. gingivalis* and heat-killed *P. gingivalis* are tested. Live cultures did not result in significant IL-10 and IL-4 from PBMCs, whereas heat-killed bacterial preparations led to a substantial anti-inflammatory response, indicating that *P. gingivalis* surface molecules affect the immune mechanisms of the host when the bacterium is not viable. The inhibition of the production of IL-4 and IL-10 by PBMCs from healthy individuals and patients with CP elicited by live *P. gingivalis* is interpreted as an in vitro artifact of high gingipain concentration in the absence of serum protease inhibitors. Within the limits of this study, it is not clear whether the inhibition is attributable to an active impact on the transcription or the secretion of IL-4 and IL-10 or post-release proteolytic degradation. The most likely mechanism is proteolytic degradation, which is supported by the previous findings for IL-4 release that demonstrate that gingipain complexes inactivated the Th2 cytokines IL-4 and IL-5.[42] It is possible that the same action is being observed for IL-10 release, accounting for broader immunosuppression.

LPS is among the possible virulence factors, which can still be highly active after the bacteria lose their viability. Therefore, LPS preparation from *P. gingivalis* was tested next. LPS from neither *P. gingivalis* nor *E. coli* stimulated the IL-4 response, suggesting the specificity of the LPS activation of monocytic cells. Conversely, IL-10 was produced by both monocytes and PBMCs in response to *P. gingivalis* LPS and *E. coli* LPS, supporting the notion that monocytes are an important source of IL-10. Blocking the proinflammatory cytokine response restored IL-10 production by cells from CP in response to *P. gingivalis* LPS but reduced *E. coli* LPS-induced IL-10 production, suggesting that different TLR-mediated responses are involved in LPS-mediated IL-10 production.

Monocytes are assumed to be initially in a quiescent state, and they are stimulated to produce IL-1 by an external stimulus, such as the LPS. This in turn invokes an autocrine IL-1 response and induces the production of the anti-inflammatory cytokine IL-10, which acts to downregulate IL-1 production. To identify the role of mononuclear phagocytes (monocytes) in overall IL-10 production, pure monocyte cultures were obtained by negative selection through MACS, and cells were treated in the same way as the PBMCs at 24, 48, and 72 hours. As in the case of PBMCs, pure monocytes failed to produce any detectable IL-10 in response to live *P. gingivalis*, whereas heat-killed *P. gingivalis* led to statistically significant increases in IL-10 production. Both LPS preparations caused significant and parallel IL-10 production from monocytes ($P < 0.05$). Blocking the proinflammatory cytokines did not lead to any significant change in IL-10 production in response to *E. coli* LPS, whereas IL-10 responses to LPS from *P. gingivalis* were significantly restored after 48 hours of neutralization. These findings suggest that blocking IL-1 and TNF-α may only provide a limited benefit for treatment of IL-10 deficiency in inflammatory periodontal lesions.

Crosstalk between proinflammatory and anti-inflammatory cytokines during different stages of inflammation may determine the shift to chronicity. To prevent an uncontrolled inflammatory response and rampant tissue breakdown, the activity of IL-1 and TNF-α must be regulated. This is done naturally by the elaboration of the anti-inflammatory cytokines or cytokine antagonists. When the soluble receptors to IL-1 or TNF-α are applied in vivo, pathologic processes can be inhibited in arthritis, septic shock, autoimmune diseases, and periodontitis.[43,44] Based on these observations, it is hypothesized that neutralization of proinflammatory cytokine release in cell cultures will restore anti-inflammatory cytokine production. More studies are needed to confirm these findings in larger cohorts. Individual variation and various other factors (e.g., smoking) known to play a role in the pathogenesis of periodontal disease can certainly impact the cell response. However, the statistically significant differences obtained in this study suggest that the imbalance between proinflammatory and anti-inflammatory regulatory molecules is impaired in CP, and *P. gingivalis*, as well as a crosstalk between cytokines, plays a major role in the process. IL-10 production is suppressed in monocytes from patients with CP, which raises the question of temporality on how the IL-10 release is associated with the colonization of *P. gingivalis*. This is a challenging question, and the answers to whether the changes in the immune response allow colonization or are the result of bacterial invasion has not been clear in vivo. It is possible that both can be true in which an initial decrease/insufficiency of IL-10 could predispose the *P. gingivalis* colonization, whereas CP may be associated with an extended IL-10 function. In primary cell cultures from both healthy and diseased tissues, the phenotypic characteristics of the cells do not change over the study period (24 to 72 hours). However, this report does not directly address whether depressed levels of IL-10 predispose to CP in humans in which *P. gingivalis* is a dominant pathogen or whether *P. gingivalis* directly suppresses the protective and anti-inflammatory arm of the immune response. The reported observations may imply that IL-10 has pleiotropic actions at different stages of infection and inflammation depending on the cellular source.

CONCLUSIONS

The present study suggests that, in CP, anti-inflammatory cytokine production is suppressed. The response can, in part, be restored by neutralizing proinflammatory cytokines. The data are also consistent with the suggestion that *P. gingivalis* can contribute to progression of CP by inducing high levels of inflammatory cytokines and by inhibition of regulator cytokines IL-10 and IL-4.

ACKNOWLEDGMENTS

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