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Supernatants from oral epithelial cells and gingival fibroblasts modulate Human Immunodeficiency Virus type 1 promoter activation induced by periodontopathogens in monocytes/macrophages

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SUMMARY

Bacterial and host cell products during coinfections of Human Immunodeficiency Virus type 1-positive (HIV-1$^+$) patients regulate HIV-1 recrudescence in latently infected cells (e.g. T cells, monocytes/macrophages), impacting highly active antiretroviral therapy (HAART) failure and progression of acquired immunodeficiency syndrome. A high frequency of oral opportunistic infections (e.g. periodontitis) in HIV-1$^+$ patients has been demonstrated; however, their potential to impact HIV-1 exacerbation is unclear. We sought to determine the ability of supernatants derived from oral epithelial cells (OKF4) and human gingival fibroblasts (Gin-4) challenged with periodontal pathogens, to modulate the HIV-1 promoter activation in monocytes/macrophages. BF24 monocytes/macrophages transfected with the HIV-1 promoter driving the expression of chloramphenicol acetyltransferase (CAT) were stimulated with *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, or *Treponema denticola* in the presence of supernatants from OKF4 or Gin4 cells either unstimulated or previously pulsed with bacteria. CAT levels were determined by enzyme-linked immunosorbent assay and cytokine production was evaluated by Luminex beadlyte assays. OKF4 and Gin4 supernatants enhanced HIV-1 promoter activation particularly related to *F. nucleatum* challenge. An additive effect was observed in HIV-1 promoter activation when monocytes/macrophages were simultaneously stimulated with gingival cell supernatants and bacterial extracts. OKF4 cells produced higher levels of granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukins -6 and -8 in response to *F. nucleatum* and *P. gingivalis*. Preincubation of OKF4 supernatants with anti-GM-CSF reduced the additive effect in periodontopathogen-induced HIV-1 promoter activation. These results suggest that soluble mediators produced by gingival resident cells in response to periodontopathogens could contribute to HIV-1 promoter activation in monocytes/macrophages, albeit this effect is most notable following direct stimulation of the cells with oral gram-negative bacteria.

INTRODUCTION

The infectious disease caused by the Human Immunodeficiency Virus (HIV) remains incurable, albeit as a

more chronic disease. The disease has evolved with the advent of therapeutic approaches, using a combination of different highly active anti-retroviral therapies (HAART), which has allowed HIV-1$^+$ patients to survive longer. Nevertheless, a complete eradication of HIV has not been possible, as it remains latently infecting host cell reservoirs, such as CD4$^+$ T cells, macrophages, and dendritic cells (Kulkosky & Bray, 2006). The success of HAART has been associated with maintenance of low or undetectable levels of viral load in plasma, which at the same time promotes the preservation of a functional immune response in these patients (Flynn *et al.*, 2004). It has been hypothesized that a chronic immune activation in HIV-1$^+$ patients induced by common and/or opportunistic pathogens is a cofactor influencing viral exacerbation and faster progression of the disease as well as HAART failure (Lawn, 2004; Brenchley *et al.*, 2006). Indeed, several studies addressing this question have showed that different microorganisms commonly found in HIV-1$^+$ patients, such as *Mycobacterium tuberculosis*, *Neisseria gonorrhoea*, *Leishmania donovani*, *Toxoplasma gondii*, *Plasmodium falciparum*, and herpesviruses (Bafica *et al.*, 2004), as well as some proinflammatory mediators (e.g. cytokines/chemokines) produced by immune and non-immune cells in response to infectious challenge, have the capacity to regulate HIV-1 recrudescence from latently infected cells (Devadas *et al.*, 2004).

As such, immunosuppression in HIV-1$^+$ patients has been associated with the onset of opportunistic infections, which also appears to be correlated with an increase of virus reactivation within latently infected cells (Wahl *et al.*, 2003). Such reciprocal interactions appear to be associated with phenotypic and genotypic viral changes, including the generation of drug-resistant viral strains (Devadas *et al.*, 2004). Therefore, the identification of infectious cofactors in HIV disease is an important issue related to HAART and acquired immune deficiency syndrome (AIDS) progression.

Although there is no direct demonstration that the oral cavity is a viral reservoir in HIV-1$^+$ patients, several findings suggest that it could be a potential site for HIV-1 pathogenesis because both HIV-1 RNA and DNA proviral forms are present in saliva as well as in gingival crevicular fluid (Maticic *et al.*, 2000; Shugars *et al.*, 2001) and oral epithelial cells are susceptible to either cell-free or cell-associated HIV-1 infection through galactosylceramide receptor and the chemokine receptor CXCR4 (coreceptor), both *in vitro* and *in vivo* (Liu *et al.*, 2003; Rodriguez-Inigo *et al.*, 2005). Normally, the oral mucosa is constantly exposed to a high density and diversity of gram-positive and gram-negative bacteria, including commensal bacteria and opportunistic pathogens; however, certain changes in the oral ecology leading to overgrowth of particular gram-negative anaerobes (e.g. *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, *Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans*) have been associated with the initiation and progression of local inflammatory responses (e.g. periodontal disease) (Socransky *et al.*, 1998). Most of the microbiological studies in HIV-1$^+$ patients have demonstrated a similar distribution of periodontal pathogens between HIV-infected and HIV-uninfected subjects (Patel *et al.*, 2003; Goncalves Lde *et al.*, 2004). However, recent findings suggest that other opportunistic bacterial species may contribute to the oral ecology in HIV-1-associated periodontal diseases (Aas *et al.*, 2007). Although the advent of HAART appears to reduce the frequency of HIV-associated oral pathology including periodontal diseases, evidence only supports a significant reduction for oral candidiasis in patients taking antiretroviral therapy (Patton *et al.*, 2000; Greenspan *et al.*, 2004). Beyond the local microbial challenge in the oral cavity, there is substantial evidence that non-immune cells in gingival tissues (e.g. keratinocytes and gingival fibroblasts) play an active role in chronic local inflammatory responses to bacterial biofilms (Hasegawa *et al.*, 2007). Some of these cytokines/chemokines produced by these resident cells have the ability to modulate HIV-1 reactivation in latently infected immune cells (Lane *et al.*, 2001). These particular gingival inflammatory events associated with periodontitis would be predicted to impact HIV-1 cell reservoirs in the oral mucosa; however, to date this remains undetermined. Recent evidence suggests a positive correlation between HIV-1 viral load and periodontal disease related to levels of *F. nucleatum* and *Prevotella intermedia*, as well as interferon-γ (IFN-γ) concentrations in gingival crevicular fluid (Alpagot *et al.*, 2003, 2004).

The objectives of these studies were to evaluate the ability of supernatants from oral cell cultures (oral epithelial cells and human gingival fibroblasts) to induce HIV-1 promoter activation in a

BF24 monocyte/macrophage model as previously shown (Hoshino *et al.*, 2002; Tanaka *et al.*, 2005). We also assessed the potential synergism between bacteria and host cell factor stimulation in this model system. This information should provide some biological basis for the relationship between oral infections and HIV-1 reactivation, particularly as related to understanding the variation in AIDS progression and failure of HAART.

MATERIALS AND METHODS

Tissue cultures

Primary human gingival fibroblasts (Gin-4) were cultured in 75-cm^2 flasks with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The oral epithelial cell line OKF4 was grown until one-third of confluent in 75-cm^2 flasks with keratinocyte–serum-free medium (Ker-SFM; Gibco, Carlsbad, CA) supplemented with 0.2 ng ml^{-1} epidermal growth factor and 25 μg ml^{-1} bovine pituitary extract. The BF24 cell line used in this study is a subclone of the monocytic leukemia cell line THP-1 which was obtained through the National Institutes of Health (NIH) AIDS Research and Reference Program, Division AIDS, NIAID, NIH reagents program (cat# 1296) (http://www.aidsreagent.org) from Dr. Barbara K. Felber and Dr. George N. Pavlakis. These cells are transfected with the HIV-1 long terminal repeat (LTR) promoter driving the chloramphenicol acetyltransferase (CAT) reporter gene expression. All cell types were maintained in a 5% CO_2 atmosphere at 37°C.

Bacterial strains and growth conditions

The bacterial strains used in this study were *P. gingivalis* ATCC 33277, *Treponema denticola* ATCC 35404, and *F. nucleatum* ATCC 25586. *P. gingivalis* was grown in anaerobe broth (Difco-Becton Dickinson, Sparks, MD) supplemented with 5 μg ml^{-1} hemin and 1 μg ml^{-1} menadione, *F. nucleatum* in trypticase soy broth supplemented with 0.6% yeast extract (Difco-Becton Dickinson), and *T. denticola* in GM-1 broth (Kesavalu *et al.*, 1999). All bacterial cultures were grown at 37°C under anaerobic conditions (85% N_2, 10% H_2, and 5% CO_2) using a Coy anaerobic chamber. Bacterial extracts were obtained starting with five colonies grown in blood agar plates, which were placed into 25 ml of the corresponding broth and incubated for 24 h. Then, bacterial cultures were transferred into 500 ml of broth, and incubated in the same conditions for 24 h. The bacterial suspension was washed three times with sterile phosphate-buffered saline (PBS) at 10,000 ***g*** for 20 min at 4°C. The pellet was resuspended in 15 ml PBS with complete ethylenediamine tetraacetic acid-free protease inhibitor cocktail (Roche, Mannheim, Germany), and bacteria were sonicated using an ultrasonic disrupter (Branson Digital Sonifier model 450, Danbury, CT). The crude extract after sonication was centrifuged at 13,000 ***g*** for 10 min at 4°C and protein concentration of supernatants was determined by bicinchoninic acid assay (Pierce, Rockford, IL).

Stimulation of BF24 macrophages with bacteria-pulsed gingival resident cells supernatants and recombinant cytokines/chemokines

OKF4 cells were cultured in 24-well plates at a density of 1×10^5 cells $well^{-1}$ with 1 ml Ker-SFM overnight to allow adherence. The Ker-SFM was removed and the epithelial monolayers were washed and incubated with 1 ml $well^{-1}$ fresh RPMI-1640 supplemented with 2% fetal bovine serum alone (non-pulsed) or with the extract from each bacterium (pulsed). The terms pulsed and non-pulsed emphasize the transient nature of the bacterial challenge of the cells for 1 h at 37°C. This process then enabled the bacterial stimuli to be removed, the wells washed with fresh medium several times to remove remaining bacteria, and the OKF4 cells were then incubated with 1 ml of the same medium for a further 24 h. The media were harvested and centrifuged at 13,000 ***g*** for 10 min at 4°C and then supernatants were used to evaluate their ability to activate HIV-1 promoter in BF24 monocytes/macrophages using a 1 : 1 volume. The same protocol was followed for Gin-4 cells using a cell density of 5×10^4 cells $well^{-1}$. Supernatants from bacterial-pulsed OKF4 cells harvested at several time-points until 24 h were maintained at −20°C until used for stimulation of BF24 cells. In addition, HIV-1/CAT activity was measured in BF24 cells incubated overnight with recombinant forms of interleukin-6 (IL-6), IL-8 and granulocyte–macrophage colony-stimulating factor (GM-CSF) (eBioscience, San Diego,

CA) alone, using different combinations as well as in the presence of bacterial extracts. For neutralization experiments, BF24 cells were challenged with bacteria and supernatants from OKF4 cells either preincubated or not with 10 μg/ml of a monoclonal rat anti-human GM-CSF (BD Pharmingen™, San Diego, CA) or its correspondent isotype control (eBioscience) for 1 h at 4°C.

CAT enzyme-linked immunosorbent assay

BF24 cells were placed into 24-well plates at a cell density of 2.5×10^5 cells well^{-1} in 500 μl RPMI-1640 medium supplemented with 2% fetal bovine serum. The BF24 cells were treated with 500 μl of either unstimulated gingival cell supernatants or bacterial-pulsed gingival cell supernatants in either the presence or absence of individual bacterial extract. Cells were incubated overnight (16 h) and HIV-1 promoter activation was measured by quantifying CAT levels using a CAT enzyme-linked immunosorbent assay kit (Roche). Briefly, BF24 cells were harvested and washed twice with 1× PBS at 3000 ***g*** for 15 min. The pellets were resuspended in lysis buffer for 30 min at room temperature. The extracts from lysed cells (210 μl) were added into 96-well plates and CAT detection was performed following the manufacturer's instructions. The absorbance was measured using an MRX 4000 plate reader (Dynatech Laboratories, Chantilly, VA) at 405 nm.

Determination of cytokine/chemokine production by OKF4 cells

OKF4 supernatants harvested at 24 h after bacterial challenge were tested with the presence of IL-1β, tumor necrosis factor-α (TNF-α), IFN-γ, GM-CSF, IL-6, and IL-8 using a Luminex multiplex bead (Upstate, St. Charles, MO) according to the manufacturer's directions. Briefly, 50 μl well^{-1} of OKF4 supernatants was incubated with 25 μl well^{-1} of beads coated with primary specific antibodies for each cytokine and incubated 24 h at 4°C. After washing, 25 μl/well of secondary antibodies was added and incubated at room temperature for 1.5 h. The reaction was revealed by adding 25 μl well^{-1} of streptavidin–phycoerythrin for 30 min at room temperature. Plates were read using the Luminex 100 hardware and concentrations of the analytes were determined using Upstate BeadView software (Version 1.0) compared with a standard curve for each assessment. All samples were tested in quadruplicate.

Statistical analyses

Statistical analyses were performed using a Mann–Whitney *U*-test or Kruskal–Wallis analysis of variance on ranks (SigmaStat 3.5; Systat Software, Point Richmond, CA). An alpha value of $P < 0.05$ was accepted as statistically significant when comparing the test conditions with media derived from untreated cells.

RESULTS

Supernatants from oral epithelial cells modulate HIV-1 promoter activation in monocytes/macrophages

In addition to bacterial challenge, oral epithelial cells are also considered active players during periodontal disease through production of cytokines/chemokines and soluble mediators in response to periodontopathogens. Because some of the soluble factors produced by these gingival resident cells can also reactivate HIV-1 in latently infected cells, we evaluated the ability of supernatants from oral epithelial cells (OKF4), either unstimulated or stimulated with periodontopathogens, to induce HIV-1 promoter activation, using a BF24 monocytic cell line model that mimics a chronic latent HIV-1 infection. BF24 cells incubated with supernatants from non-pulsed OKF4 cells (OKF4) showed an increase in HIV-1/CAT activity compared with cells incubated only with media (Fig. 1). This response was significantly up-regulated by supernatants from OKF4 cells pulsed with *F. nucleatum* extract (Fn-OKF4), but not by supernatants from cells pulsed with either *P. gingivalis* (Pg-OKF4) or *T. denticola* (Td-OKF4) (Fig. 1A). Kinetic analysis of HIV-1 promoter activation showed that Fn-OKF4 supernatants harvested as early as 4 h and through to 24 h after bacterial stimulation generated a significant increase in HIV-1 promoter activation compared with non-pulsed OKF4 supernatants (Fig. 1B).

The ability of OKF4 cells to respond to the periodontopathogen extracts was evaluated by determination of production of a group of cytokines/chemokines whose capacity to modulate HIV-1

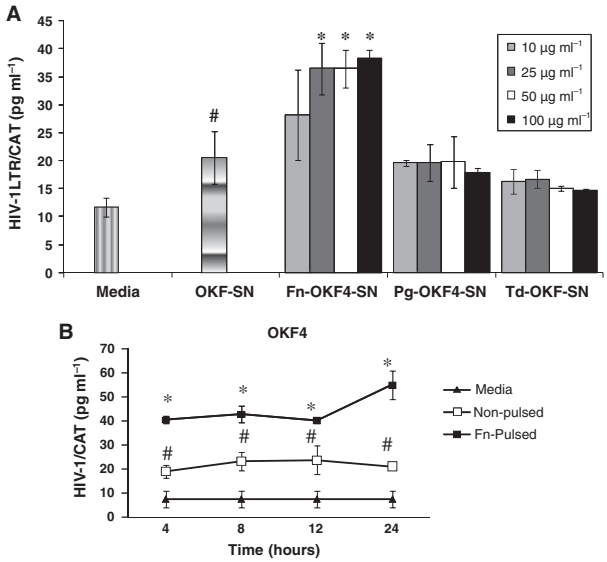

Figure 1 Effect of supernatants from oral epithelial cells (OKF4) stimulated with periodontopathogens in Human Immunodeficiency Virus type 1 (HIV-1) long terminal repeat/chloramphenicol acetyltransferase (LTR/CAT) activation of BF24 monocytes/macrophages. (A) BF24 cells were incubated with media or supernatants harvested at 24 h from oral epithelial cell cultures either non-pulsed or pulsed with different concentrations of *Fusobacterium nucleatum* (Fn), *Porphyromonas gingivalis* (Pg), or *Treponema denticola* (Td) extracts as described in the Materials and methods. (B) Effect of either non-pulsed or Fn-pulsed OKF4 supernatants harvested at several time-points after bacterial challenge is shown. Data are expressed as mean values ± standard errors of the means from triplicates per condition. Values that were significantly different ($P < 0.05$) from media vs. non-pulsed (OKF4) as well as non-pulsed vs. pulsed cells supernatants are indicated by number signs (#) and asterisks (*), respectively.

promoter activation has also been previously suggested (Poli & Fauci, 1992; Devadas *et al.*, 2004). Although IL-1β, TNF-α, and IFN-γ were not detectable, OKF4 cells pulsed with different concentrations of *F. nucleatum* extracts, showed a significant increase in GM-CSF, IL-6, and IL-8 production compared with unstimulated OKF4 cells. This response was doses-dependent with higher *F. nucleatum* extract concentrations (Fig. 2). OKF4 cells pulsed with only the highest dose of *P. gingivalis* extract showed significant increases in IL-6 and IL-8 production compared with non-pulsed OKF4 cells; however, these values were lower compared with the cytokine production induced by the same concentration of the *F. nucleatum* extract (Fig. 2). *T. denticola* extracts did not induce significant changes compared with the levels produced by unstimulated cells.

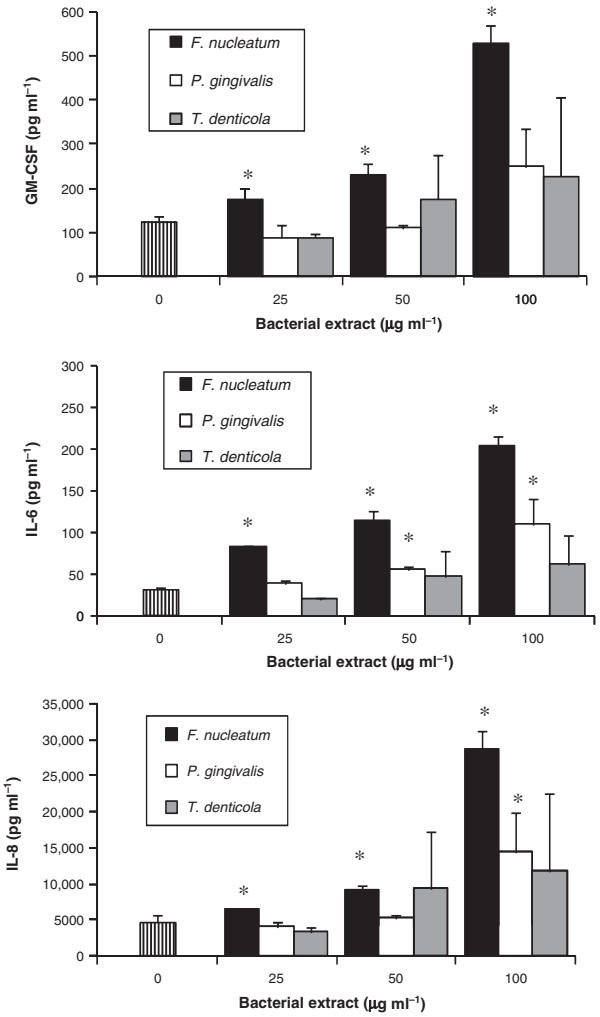

Figure 2 Cytokine/chemokine production by oral epithelial cells (OKF4) in response to bacterial extracts. OKF4 cells were pulsed with different concentrations (0–100 μg ml^{-1}) of *Fusobacterium nucleatum*, *Porphyromonas gingivalis* or *Treponema denticola* extracts. Bacterial extracts were removed 1 h later and cells were washed and incubated for a further 24 h with fresh media. Supernatants were harvested and evaluated for cytokine levels as described in the methods. Data are represented as mean values ± standard errors of the means from quadruplicates. Values that were significantly different ($P < 0.05$) from non-pulsed cells vs. pulsed cells are indicated by asterisks.

Gingival resident cell supernatants and bacterial extracts from periodontopathogens do not promote synergistic HIV-1 promoter activation in monocytes/macrophages

It has been shown that several opportunistic microorganisms or their structural components [e.g. lipopolysaccharide (LPS), lipoteichoic acid, and DNA] have the ability to elicit HIV-1 promoter activation in

latently infected monocytes/macrophages (Goletti *et al.*, 1996; Equils *et al.*, 2003). However, undermining the oral epithelial barrier that occurs with periodontitis could create an environment with both soluble factors produced by gingival resident cells and bacterial components interacting simultaneously with bystander HIV-1 latently infected monocytes/macrophages. For this reason, we evaluated a potential synergism in HIV-1 promoter activation in BF24 monocytes/macrophages between OKF4 or Gin4 supernatants and bacterial extracts. Incubation of BF24 monocytes/macrophages with media containing increasing concentrations of *F. nucleatum*, *P. gingivalis*, and *T. denticola* extracts induced a significant activation of the HIV-1 promoter with regard to cells incubated only with media (Figs 3 and 4). Consistently, Fn-OKF4 supernatants in the absence of bacterial extract induced higher HIV-1/CAT activation compared with BF24 cells incubated only with OKF4 supernatants or media (Fig. 3). The presence of OKF4 supernatants from non-pulsed cells increased HIV-1 promoter activity induced by all three bacterial extracts compared with the effect elicited by bacterial extract alone. Although supernatants from OKF4 cells previously pulsed with *F. nucleatum* and *P. gingivalis* increased significantly the HIV-1/CAT response induced by each bacterial extract, it was similar to the response elicited by non-pulsed OKF4 supernatants (Fig. 3). The additive effect between OKF4 supernatants and bacterial extracts was not observed using higher concentrations of either *F. nucleatum* or *T. denticola* nor in the presence of Td-pulsed OKF4 supernatants.

A similar set of experiments was performed with gingival fibroblasts, which also have been suggested to be active players during advanced states of periodontitis. Supernatants from gingival fibroblasts previously pulsed with *F. nucleatum* and *P. gingivalis* induced a significant increase of HIV-1 promoter activation in the absence of bacterial extract compared with supernatants from non-pulsed gingival fibroblasts (Fig. 4). A significant increase of bacteria-induced HIV-1 promoter activation was related to the presence of supernatants from Gin4 cells previously pulsed with *F. nucleatum* and *P. gingivalis* but not supernatants from *T. denticola*-pulsed Gin4 cells. Supernatants from un-stimulated Gin4 as well as cells previously pulsed with *T. denticola* did not increase HIV-1/CAT activity (Fig. 4).

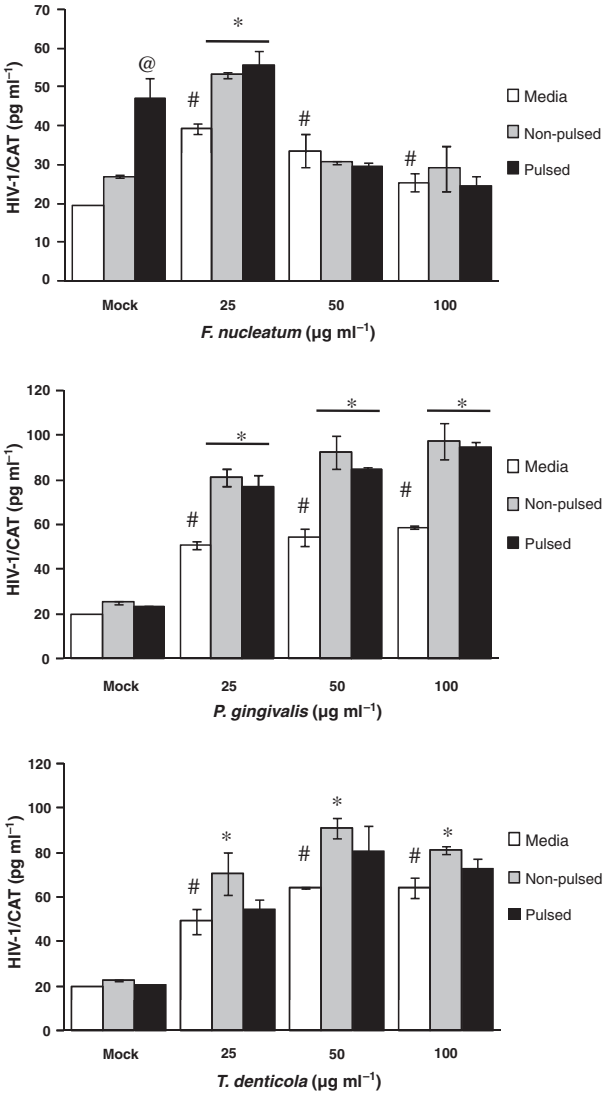

Figure 3 Human Immunodeficiency Virus type 1 long terminal repeat/chloramphenicol acetyltransferase (HIV-1 LTR/CAT) activation in BF24 monocytes/macrophages induced by supernatants from oral epithelial cells (OKF4) and bacterial extracts. BF24 cells were challenged or not (mock) with different concentrations of bacterial extracts from *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, or *Treponema denticola*, in the presence of medium alone (white bar) or 24 h supernatants from either non-pulsed (gray bar) or previously pulsed (black bar) OKF4 cells with the same bacterial extract as described in the Materials and methods. Data are expressed as mean values ± standard errors of the means from triplicates per condition. Values that were significantly different ($P < 0.05$) from non-pulsed vs. pulsed OKF4 supernatants in the absence of bacterial extract (Mock) are indicated by an @ sign, from unstimulated BF24 cells vs. bacteria-stimulated cells in media are indicated by a # sign, as well as from bacteria-stimulated BF24 cells in the presence of medium vs. non-pulsed or pulsed OKF4 supernatants are indicated by asterisks.

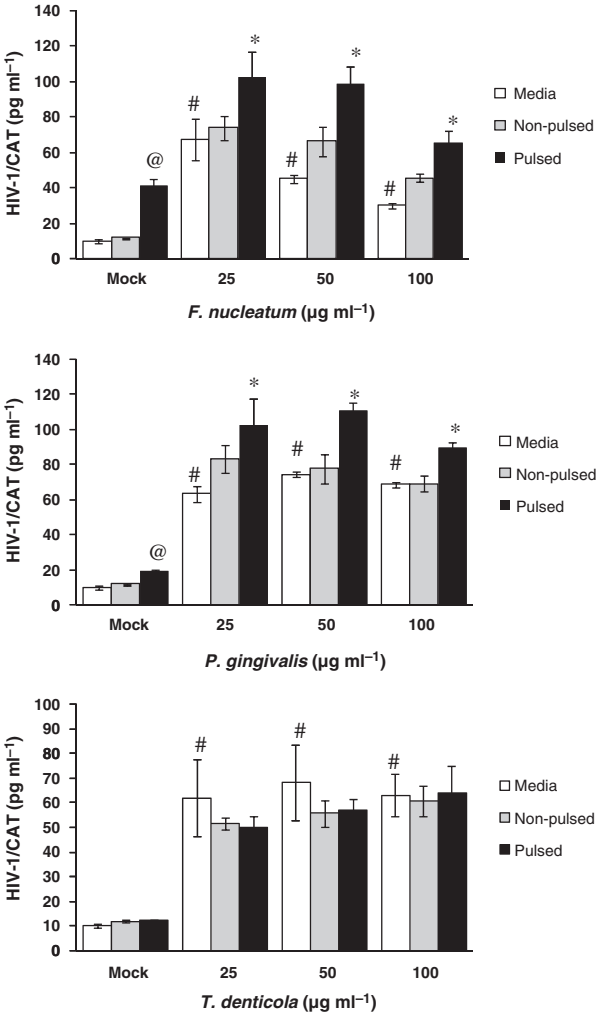

Figure 4 Effect of supernatants from gingival fibroblasts (Gin4) in Human Immunodeficiency Virus type 1 long terminal repeat/chloramphenicol acetyltransferase (HIV-1 LTR/CAT) activation of BF24 monocytes/macrophages. Supernatants from Gin-4 cells either non-pulsed or previously pulsed with *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, or *Treponema denticola* were tested for their ability to induce HIV-1/CAT activation in BF24 cells similarly, as described for oral epithelial cells in Fig. 3. Data are expressed as mean values ± standard errors of the means from triplicates per condition. Values that were significantly different ($P < 0.05$) from non-pulsed vs. pulsed Gin-4 supernatants in the absence of bacteria extract (Mock) are indicated by an @ sign, from unstimulated BF24 cells vs. bacteria-stimulated cells in media are indicated by a # sign, as well as from bacteria-stimulated BF24 cells in the presence of medium vs. non-pulsed or pulsed Gin4-supernatants are indicated by asterisks.

Since our primary focus in this study was the responses of the epithelial cells and HIV-1 reactivation, we did not directly measure the cytokine profiles of the Gin-4 cells. However, our previous results showed that using these bacterial species and fibroblast line, IL-6, IL-8, and GM-CSF were also produced, although IL-6 levels were higher than IL-8 and GM-CSF (unpublished data, M.J. Steffen, J.L. Ebersole). This suggests a substantial variation in cytokine profiles produced by these resident cell types in response to a similar bacterial challenge.

Effect of recombinant GM-CSF and IL-8 in bacteria-induced HIV-1 promoter activation

The OKF4 cells produced higher amounts of GM-CSF, IL-6, and IL-8 in response to *F. nucleatum* and *P. gingivalis* and evidence suggests that these and other cytokines/chemokines have been suggested to be positive or negative regulators of HIV-1 replication in latently infected cells, so the potential for recombinant forms of these factors to induce HIV-1 promoter activation in BF24 monocytes/macrophages was evaluated. None of these recombinant factors, either individually or in different combinations, induced HIV-1 promoter activation using a broad range of concentrations (0.01–100 ng ml^{-1}) (data not shown). However, when BF24 cells were stimulated with *F. nucleatum* or *P. gingivalis* in presence of 100 pg ml^{-1} recombinant (r) GM-CSF, HIV-1 promoter activation was significantly increased with regard to cells stimulated only with bacterial extract (Fig. 5A). In contrast, the presence of rIL-8 did not affect bacteria-induced HIV-1 promoter activity. The bacteria-induced HIV-1 promoter activation observed with 100 pg ml^{-1} rGM-CSF, was similar when higher concentrations of rGM-CSF (500 and 1000 pg ml^{-1}) were used (Fig. 5B). Finally, the presence of neutralizing anti-GM-CSF, but not of its correspondent isotype control, reduced the ability of Fn-OKF4 supernatants to increase HIV-1 promoter activation induced by *F. nucleatum* in BF24 cells (Fig. 5C).

DISCUSSION

Activation of the immune system in HIV-1^+ patients by non-HIV-1 coinfecting pathogens and their products, which translocate from mucosal surfaces to the systemic circulation, seems to be a critical factor in AIDS progression (Lawn, 2004; Brenchley *et al.*, 2006; Jiang *et al.*, 2009). Approximately 40–50% of HIV-1^+ persons have oral fungal, bacterial, or viral

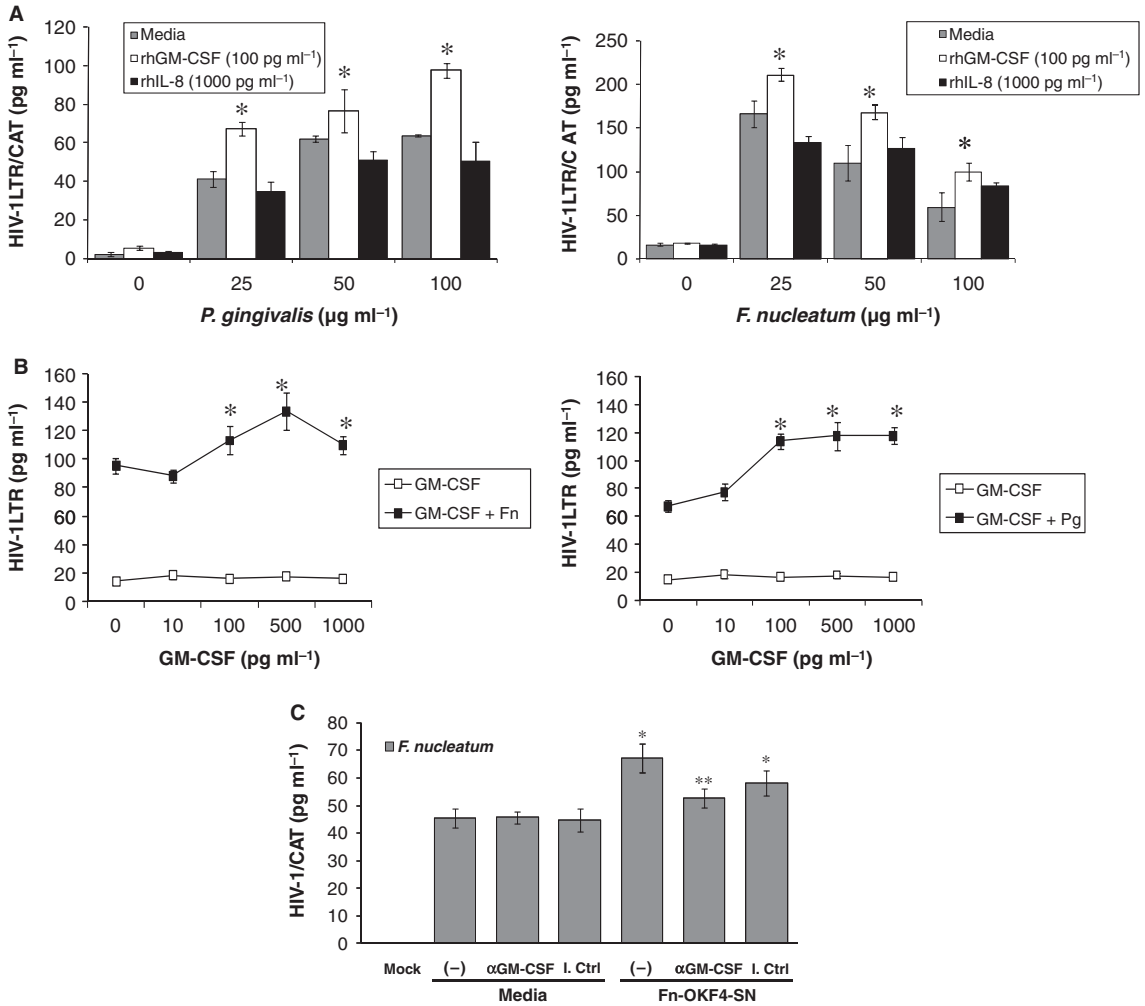

Figure 5 The effect of recombinant granulocyte–macrophage colony-stimulating factor (rGM-CSF) and recombinant interleukin-8 (rIL-8) in bacteria-induced HIV-1 promoter activation. (A) Human Immunodeficiency Virus type 1 (HIV-1) promoter activation in BF24 cells challenged with different concentrations of *Fusobacterium nucleatum* (Fn) or *Porphyromonas gingivalis*, either in the presence or absence of defined rGM-CSF and rIL-8 concentrations; (B) HIV-1 promoter activation induced by 25 μg ml^{-1} *F. nucleatum* or 100 μg ml^{-1} *P. gingivalis* in the presence of growing concentrations of rGM-CSF. (C) HIV-1/chloramphenicol acetyltransferase (CAT) activity induced by *F. nucleatum* in the presence of OKF4 supernatants either preincubated or not with 10 μg ml^{-1} of neutralizing anti-GM-CSF or its correspondent isotype control as described in Materials and methods. Data are expressed as mean values ± standard errors of the means from triplicates per condition. Values that were significantly different ($P < 0.05$) from bacteria-stimulated BF24 cells in absence vs. presence of recombinant cytokines/chemokines, Fn-challenged BF24 cells in presence of medium vs. Fn-OKF4-SN are indicated by single asterisks, and Fn-challenged BF24 cells incubated with Fn-OKF4-SN in the presence of anti-GM-CSF vs. isotype control are indicated by two asterisks.

infections that occur early in the course of the disease generating oral pathology (e.g. oral candidiasis, oral hairy leukoplakia, gingivitis and periodontitis, Kaposi's sarcoma) (Coogan *et al.*, 2005). Emerging evidence supports the concept of a potential relationship between oral disease and an increased HIV-1 viral load. For instance, the presence of oral candidiasis correlates with a higher HIV viral load (Patton, 2000), and most recently the same positive association was described for periodontal disease in HIV-1^{+} patients (Alpagot *et al.*, 2003, 2004). A biological explanation for this association remains undetermined. Here we show that soluble factors produced *in vitro* by oral gingival cells contributed to HIV-1 promoter activation in monocytes/macrophages; however, these host cell products did not synergize with oral bacteria to reactivate the HIV-1 promoter in BF24 monocyte/macrophages but

appear to prime HIV-1 latently infected monocytes/macrophages to increase bacteria-induced HIV-1 promoter reactivation.

Epithelial cells act as sensors during microbial infections and offer an interactive interface for subgingival plaque by generating and transmitting signals between bacteria and the underlying immune cells of periodontal tissues (e.g. dendritic cells, macrophages, and $CD4^+$ T cells) (Kagnoff & Eckmann, 1997; Dale, 2002). As a wide array of soluble factors produced by oral epithelial cells in response to periodontopathogens has been described, including cytokines, chemokines, and anti-microbial peptides (Hasegawa *et al.*, 2007), which have also shown the ability to modulate HIV-1 reactivation in latently infected cells (Devadas *et al.*, 2004), we hypothesized that bacteria-pulsed OKF4 supernatants would increase HIV-1/CAT activity. Indeed, oral epithelial cell supernatants showed a capacity to induce HIV-1 LTR activation in a microorganism-dependent manner. Incubation of BF24 monocytes/macrophages with supernatants from OKF4 cells pulsed with *F. nucleatum* extract resulted in significant increases in HIV-1 promoter activation; a similar result was not observed following pulsing with *P. gingivalis* or *T. denticola*. This finding was not unexpected, because each of these microorganisms has unique structural components that could contribute to the ability to promote particular responses, as has been shown using microarray analyses of oral epithelial cells exposed to different oral microbial species (Hasegawa *et al.*, 2007). As an example, the LPS from *P. gingivalis* shows a low endotoxin activity compared with LPS from other oral gram-negative bacteria (Darveau *et al.*, 2004). It has also been suggested by our group and others that human gingival fibroblasts appear to play an active role in local inflammatory processes during periodontal disease through production of cytokines/chemokines such as IL-6, IL-8, and monocyte chemoattractant protein-1 in response to bacteria (Dongari-Bagtzoglou & Ebersole, 1998; Steffen *et al.*, 2000; Uehara & Takada, 2007). Supernatants from gingival fibroblasts pulsed with the same bacterial extracts demonstrated a similar ability to what was observed in oral epithelial cells to activate the HIV-1 promoter in the BF24 cell line. We did not rule out the potential effect of LPS traces in supernatants from gingival cells pulsed with bacterial extracts on HIV-1 promoter activity. However, in agreement with Nordone *et al.* (2007), we could not demonstrate HIV-1 promoter activation in BF24 monocytes/macrophages incubated with a broad range of LPS concentrations (10–100 ng ml^{-1}) from oral and enteric bacteria (data not shown).

Further, we evaluated any synergism of gingival cell culture supernatants combined with bacterial extracts for HIV-1 promoter activation. Supernatants from non-pulsed oral epithelial cells coincubated with all three bacterial extracts showed a significant increase in HIV-1 promoter activation compared with the response induced by bacteria alone; nevertheless rather than synergistic it was more an additive effect. A similar effect was induced by Gin-4 supernatants from cells previously pulsed with *F. nucleatum* and *P. gingivalis*. These results suggest that soluble factors produced by gingival cells, at basal levels or in response to some periodontopathogens, when acting simultaneously with bacterial products on HIV-1 latent cell reservoirs could heighten HIV-1 viral reactivation in monocytes/macrophages. A great variety of receptors [e.g. Toll-like receptors (TLR), cytokine receptors, Fc receptors, integrins] decorate the surface of monocytes/macrophages, it has been suggested that during an infectious process, signaling through all of these receptors may be involved in the final net cellular outcome (Hu *et al.*, 2007). Studies with other opportunistic pathogens commonly found coinfecting $HIV\text{-}1^+$ patients have shown that cytokine-mediated activation of the HIV-1 promoter is thought to be a major mechanism by which concurrent infectious agents may enhance proviral transcription, which may be contributing also to TLR-dependent triggering (Lawn, 2004). Gingival resident cells are able to produce a great variety of soluble mediators after oral bacterial challenge, which include recognized HIV-1 stimulators (IL-1β, TNF-α, IL-6, IL-8, IFN-γ, etc.) (Hasegawa *et al.*, 2007; Uehara & Takada, 2007). A particular group of these soluble factors produced by either epithelial cells or gingival fibroblasts after bacterial challenge could therefore be playing a role in the HIV-1 promoter activation observed in BF24 monocytes/macrophages. OKF4 cells produced higher levels of GM-CSF, IL-6, and IL-8 in response to *F. nucleatum* and *P. gingivalis*. However, treatment of BF24 cells with recombinant forms of these cytokines/chemokines alone or in different combinations did not induce significant changes in HIV-1 promoter activity, which suggests that the HIV-1 promoter

activation induced by OKF4 supernatants in the absence of bacteria may involve other soluble factors that remain to be determined. Based on the biological properties of these factors, rather than having a direct effect, we speculate that they could indirectly modulate the capacity of the bacteria to induce HIV-1 reactivation in latently infected cells. A recent study suggests that GM-CSF and LPS synergize to stimulate HIV-1 production in monocytes, a response that seems to involve an increased activation of Sp1 transcription factor (Osiecki *et al.*, 2005). Although HIV-1 promoter activation (as used in these studies) has been suggested as a critical event for viral replication (Cullen, 1991; Sgarbanti *et al.*, 2008), additional basic studies using models of HIV-1 latency in cells infected with the whole HIV-1 virus are logical extensions of these findings. Ongoing studies in our laboratory have initiated similar studies employing the THP89 cell line, which is a monocyte/macrophage cell line transfected with the entire HIV-1 genome linked to a green fluorescent protein (GFP) reporter gene (Kutsch *et al.*, 2002). Initial results generated using this cell line with bacterial stimulants evaluated in these experiments showed similar results (data not shown). Here we show that OKF4 cells produce significant increases in GM-CSF in response to *F. nucleatum* extracts. Presence of rGM-CSF but not rIL-8 significantly increased HIV-1/CAT promoter activation induced by *F. nucleatum* and *P. gingivalis*. This effect was similar in magnitude for all rGM-CSF concentrations greater than 100 pg/ml. These results are consistent with the response induced by *F. nucleatum* and *P. gingivalis* in the presence of supernatants from either non-pulsed or bacteria-pulsed OKF4 supernatants, where differences were not observed. Although GM-CSF levels are substantially increased after *F. nucleatum* challenge, only lower levels (100 pg ml^{-1}) appear to be sufficient to enhance bacteria-induced HIV-1 promoter activity in a similar manner to that observed with unstimulated oral epithelial cell supernatants. In addition, the presence of neutralizing anti-GM-CSF blocked the effect of OKF4 supernatants in bacteria-induced HIV-1 reactivation. These results suggest that basal levels of GM-CSF produced by OKF4 cells appear to be sufficient to prime BF24 monocytes/macrophages to increase the HIV-1 promoter activation induced by bacteria. It has been shown that GM-CSF increases the expression of TLR2 and TLR4 in monocytes (Osiecki *et al.*, 2005; O'Mahony *et al.*, 2008), and *F. nucleatum* and *P. gingivalis* can activate these receptors (Kikkert *et al.*, 2007), which could explain the response observed in this study. Of note, increased levels of GM-CSF in gingival crevicular fluid have been associated with chronic periodontitis (Gamonal *et al.*, 2003).

Evaluation of the cell distribution number in the periodontium with health or disease supports the theory that epithelial cells and fibroblasts continuously exceed the numbers of infiltrating inflammatory cells and so their contribution to the pro- or anti-inflammatory environment cannot be ignored. It is reasonable to propose that oral epithelial cells are an important source of GM-CSF in established lesions (i.e. periodontitis), and it could be speculated that at early stages of the disease (i.e. gingivitis) when epithelium integrity has not been disrupted, GM-CSF produced by epithelial cells upon sensing periodontopathogens in the subgingival sulcus could prime infiltrating latently infected immune cells, which would further increase bacteria-induced HIV-1 reactivation. Interestingly, reduced HIV-1 promoter activity with higher concentrations of *F. nucleatum* as well as the absence of an additive effect between Td-OKF4 supernatants and *T. denticola* extract were observed. These results suggest that the concentration of a particular microorganism as well as the ability to evade the immune response through hyporesponsiveness could determine the positive or negative modulation of HIV-1 promoter activity. In fact, evidence suggests that *F. nucleatum* is an efficient enhancer of antimicrobial peptide production by oral epithelial cells such as β-defensin 2 and the cathelicidin LL-37, which have been shown to negatively modulate HIV-1 replication and TLR signaling by blocking the nuclear translocation of nuclear factor-κB p50/p65 heterodimers induced by gram-negative bacteria (Maticic *et al.*, 2000; Hoshino *et al.*, 2002; Wahl *et al.*, 2003; Imai *et al.*, 2009). Consequently, the anti-inflammatory environment created by the antimicrobial peptides released from oral epithelial cells in response to a given bacterial count could be downregulating HIV-1LTR promoter activation in bystander HIV-1-infected cells. Moreover, *T. denticola* does not efficiently stimulate oral epithelial cells (Brissette *et al.*, 2008). The mechanisms by which several microorganisms or cytokines/chemokines induce HIV-1 reactivation in latently

infected cells remain not fully understood. However, it is known that signaling through several receptors including TLRs, Nucleotide-binding oligomerization domain-like receptors, and cytokine/chemokine receptors ends with the activation of similar transcription factors such as nuclear factor-κB, Sp1, cAMP response element binding protein, and controlled amino acid therapy/enhancer binding protein-β among others (Scheller *et al.*, 2004; Nordone *et al.*, 2007). These transcriptional pathways could be promoting a synergistic HIV-1 activation, because the viral promoter has several binding sequences for these transcription factors (Kingsman & Kingsman, 1996; Wahl *et al.*, 2003).

In general, we observed that soluble factors released by gingival resident cells at basal levels or upon stimulation with periodontopathogens exhibited the capacity to modulate HIV-1 promoter activation in monocytes/macrophages. Although statistically significant, this response was minimal compared with those responses induced directly by oral bacterial extracts. We suggest that soluble factors produced by gingival resident cells may function indirectly on HIV-1 promoter activation by priming the monocytes/macrophages before an encounter with bacterial components resulting in an additive effect in HIV-1 promoter reactivation. An alternative mechanism would be that the soluble factors produced by gingival resident cells engaged in reactivating HIV-1 promoter transcription are Tat-dependent. The transcriptional transactivator Tat is a critical HIV-1 protein that promotes a successful elongation during transcription of HIV-1 proviral forms. Interestingly, herein we demonstrated that oral gram-negative bacteria associated with periodontal disease have the ability to induce HIV-1 promoter reactivation in a Tat-independent manner, i.e. no Tat production in the BF24 cells. These findings support a mechanism of viral promoter activation irrespective of the presence of Tat that has been proposed by others (Sgarbanti *et al.*, 2008). A Tat-independent HIV-1 reactivation may be a mechanism by which HIV-1 provirus could become activated in chronic latently infected cells upon coinfection with oral gram-negative microorganisms even in the absence of active viral replication that guarantees production of Tat.

The study of potential cofactors associated with HIV-1 reactivation in latently infected cells has engendered substantial interest during the last years, because a potential modulation of viral transcription would be of great help in controlling the viral infection either purging HIV-1 viral reservoirs or inhibiting viral reactivation. This is the first report showing that soluble factors produced by gingival cells in response to periodontopathogens could indirectly contribute to regulating HIV-1 promoter activation in monocytes/macrophages, albeit primary reactivation occurs following direct interaction with oral gram-negative bacteria. Similarly, we have shown that oral periodontopathogens have also the ability to directly enhance HIV-1 promoter activation in T cells and dendritic cells (Huang *et al.*, 2009). The mechanisms by which oral bacteria induce HIV-1 promoter activation remain undetermined. Evidence suggests that histone acetylation is a critical factor in HIV-1 reactivation, so histone deacetylases inhibitors such as sodium butyrate have shown the ability to enhance HIV-1 reactivation in latently infected cells (Golub *et al.*, 1991). Because a diverse group of bacteria including periodontopathogens (such as *P. gingivalis*) produce short chain fatty acids, including butyric acid, as a basic part of their metabolic activities, this has been suggested as a potential indirect mechanism by which oral coinfections may impact HIV-1 reservoirs enhancing viral exacerbation (Imai *et al.*, 2009).

As importantly, the recent report of Imai *et al.* (2009) reinforced existing data that butyric acid had the ability to activate HIV-1, although their study suggested that this was related to *P. gingivalis*, in fact, a variety of microorganisms in the subgingival pathogenic biofilms produce this fatty acid (Niederman *et al.*, 2007). The current report documents more specific interactions between oral bacteria and these latently infected cells that could occur *in vivo*.

In general, these findings suggest a potential impact on the production of new virions associated with oral infectious pathologies such as periodontal disease, in which transcriptional regulation of the HIV-1 promoter could be triggered by activation of specific receptors for bacterial antigens (i.e. TLRs) or soluble factors (i.e. cytokine/chemokine receptors). Additional basic and clinical studies targeting HIV-1 recrudescence as associated with oral disease are required to clarify the potential of oral infections as risk modifiers for HIV-1 exacerbation and transmission as well as HAART failure and AIDS progression.

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