Human cytomegalovirus and Epstein–Barr virus inhibit oral bacteria-induced macrophage activation and phagocytosis


Introduction: Periodontal disease is an inflammatory condition caused by periodontal microorganisms. Viruses such as human cytomegalovirus (HCMV) and Epstein–Barr virus (EBV) are associated with certain types of periodontal disease, but their roles in promoting the disease are unclear. Because both viruses infect human macrophages, cells which play key roles in the clearance of pathogenic bacteria, it is likely that the viruses alter the functional capacity of macrophages by inhibiting their defense mechanisms against invading pathogens.

Methods: Macrophages preinfected with HCMV or EBV were evaluated following stimulation by selected oral bacteria. Bacteria-induced macrophage activation was assayed by measuring the levels of tumor necrosis factor-α (TNF-α) produced in the media, and phagocytic activity was analysed by a phagocytosis assay with fluorescein isothiocyanate-labeled bacteria. The virus-infected macrophages were also subjected to semi-quantitative polymerase chain reaction to measure the expression of toll-like receptor 9, which is involved in the activation of phagocytosis-related pathways.

Results: Both HCMV and EBV significantly diminished the TNF-α production typically induced by oral bacteria, inhibited the phagocytic activity of macrophages, and downregulated the expression of toll-like receptor 9.

Conclusion: Infection by HCMV or EBV inhibits the functional ability of macrophages to respond to bacterial challenge, thereby suggesting their pathogenic role in the development of periodontal disease.

Key words: cytomegalovirus; Epstein-Barr virus; macrophages; phagocytosis

Mengtao Li, Center for Oral Health Research, Dental Science Building, MN310, University of Kentucky College of Dentistry, 800 Rose Street, Lexington, KY 40536, USA. Tel.: +1 859 323 0011; fax: +1 859 257 6566; e-mail: mli4@email.uky.edu
Accepted for publication December 2, 2008
244 Lin & Li

review Board of the University of Kentucky. The HCMV AD169 strain was obtained from the National Institute of Health AIDS reference reagent program and amplified in MRC-5 cells. EBV was collected from the medium of B95.8 cells. The virus titers were determined by real-time PCR. Oral bacteria P. gingivalis, F. nucleatum, A. actinomyctetomcomitans, and Streptococcus gordonii were grown in an anaerobic chamber with appropriate media.

**TNF-α induction**

THP-1 cells were induced to differentiate into macrophages by adding 12-O-tetradecanoylphorbol-13-acetate (TPA, 20 ng/ml) to the cultures for 24 h. The cells were then maintained in regular medium for 4 h. The virus infection was carried out by inoculation with HCMV or EBV for 2 h at a multiplicity of infection (MOI) of one. The virus-infected cells were incubated overnight before stimulation with oral bacteria: P. gingivalis, F. nucleatum, A. actinomyctetomcomitans, or S. gordonii. Culture media were collected for enzyme-linked immunosorbent assay (ELISA) analysis of TNF-α concentration after 5 h of stimulation. ELISA was performed with a TNF-α detection kit (eBioscience, San Diego, CA) according to the manufacturer’s instructions. For the primary macrophages, the isolated cells were incubated in complete RPMI-1640 medium for 24 h, after which virus infection and bacterial stimulation were performed similarly as described for the THP-1 cells.

**Semi-quantitative PCR**

Total RNA from virus-infected and non-infected THP-1-derived macrophages were prepared using Trizol reagent (Invitrogen, Carlsbad, CA), and first-strand complementary DNA (cDNA) was synthesized with the transcriptor high fidelity cDNA synthesis kit (Roche, Indianapolis, IN). Toll-like receptor 9 (TLR9) message was amplified by PCR with the forward primer 5'-CAA AAG CCT CAC TGT GGT GC-3' and the reverse primer 5'-GAG TGA GCG GAA GAA GAT GC-3' in 31 cycles. PCR amplification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to show equivalent cDNA input.

**Fluorescein isothiocyanate-labeling of Escherichia coli and phagocytosis assay**

Fluorescein isothiocyanate (FITC) was purchased from Sigma-Aldrich Chemicals (St Louis, MO). Escherichia coli were washed once with phosphate-buffered saline (PBS), resuspended at $1 \times 10^{10}$ bacteria in 1 ml FITC/NaHCO$_3$ buffer (0.1 mg/ml FITC in 0.1 M NaHCO$_3$, pH 9.0), and incubated at room temperature for 1 h. The unconjugated FITC was then removed by washing the bacteria five times with PBS. For the phagocytosis assays, FITC-labeled E. coli were added to THP-1-derived macrophages at MOI 100 and incubated together for 30 min in antibiotic-free medium. The cells were then fixed with formalin for 30 min. Ethidium bromide (5 μg/ml in PBS) was added to the sample to quench the FITC signals of the extracellular bacteria. Digital pictures were taken immediately using a fluorescence microscope. Eight randomly selected fields were photographed for each sample, and FITC-positive bacteria as well as the total cell number were enumerated using NIH IMAGE J software (National Institutes of Health, Bethesda, MD).

**Statistic analysis**

The results were obtained from three independent experiments performed in quadruplicate. A standard Student’s two-tailed t-test was performed to compare two mean values, and data were presented as mean values ± standard deviation (SD). The results were considered statistically significant when $P < 0.05$.

**Results**

**Inhibition of oral bacteria-induced TNF-α production**

Widely used to study macrophage differentiation and functions, THP-1 cells are also used as a model for HCMV latent infection. Using THP-1-derived macrophages, we first determined whether or not HCMV infection could alter their activation in response to stimulation by oral bacteria. Measuring TNF-α as an indicator of macrophage activation, we tested the effects of four oral bacteria, P. gingivalis, F. nucleatum, A. actinomyctetomcomitans, and S. gordonii. THP-1 cells were first induced to differentiate into macrophages with TPA and then infected with HCMV. As shown in Fig. 1A, all four oral bacteria tested the effects of oral bacteria.
for *P. gingivalis* (ratio of 100 : 1) and *S. gordonii* (ratio of 1000 : 1), HCMV by itself did not induce TNF-α production in the macrophages, but the viral infection significantly inhibited bacteria-induced TNF-α production for each type of bacteria tested. Similar outcomes were obtained when using EBV as the infectious agent (Fig. 1B). These results indicate that HCMV and EBV infections inhibit the activation of macrophages.

To confirm that HCMV and EBV can inhibit bacterially associated TNF-α production in macrophages, we repeated the experiments using human primary macrophages. Human primary macrophages were isolated from Ficoll-purified PBMC by attachment to tissue culture plates. Twenty-four hours after attachment, the macrophages were infected with HCMV or EBV for 2 h. The virus-infected cells were then incubated in regular culture media overnight for recovery before stimulation by oral bacteria. As shown in Fig. 2, *F. nucleatum* and *A. actinomycetemcomitans* induced TNF-α production in the primary macrophages, while *P. gingivalis* and *S. gordonii* were unable to do so. In addition, the levels of *F. nucleatum*-induced and *A. actinomycetemcomitans*-induced TNF-α in these cells were significantly lower than that in THP-1-derived macrophages. Furthermore, *F. nucleatum*-induced TNF-α production was only modestly inhibited by HCMV or EBV, whereas *A. actinomycetemcomitans*-induced TNF-α production in the primary macrophages was significantly inhibited by the viruses.

**Inhibition of phagocytic activity of macrophages**

To determine whether or not HCMV and EBV infections could alter the phagocytic activity of macrophages, we performed phagocytosis assays in which THP-1-derived macrophages were incubated with FITC-labeled *E. coli*. Non-specific FITC signals from extracellular (non-phagocyted) *E. coli* were quenched by ethidium bromide, while fluorescence signals from internalized bacteria remained unaffected. Consequently, the FITC-positive bacteria and total cell number in each microscopic field were enumerated. As shown in Fig. 3, HCMV and EBV infections significantly inhibited the phagocytic activity of the macrophages.

**Downregulation of TLR9**

Toll-like receptors function as biological sensors that recognize a wide variety of microbes, including bacteria, viruses, and fungi. TLR9 was recently found to play an important role in triggering the signals for phagocytosis in macrophages (8). In this study, we investigated whether or not the expression of TLR9 was altered after HCMV and EBV infection. We performed semi-quantitative PCR to compare the expression levels of TLR9 between non-infected macrophages, HCMV-infected cells, and EBV-infected cells. The *GAPDH* gene was used as an internal control to verify equivalent cDNA input between samples. As shown in Fig. 4, TLR9 expression was downregulated in both HCMV-infected and EBV-infected macrophages.

**Discussion**

In recent years, accumulated data have shown that HCMV and EBV are associated with the development of certain types of periodontitis. These viruses presumably play a role in promoting the disease. It is not well understood how periodontal HCMV and EBV influence host immune
responses to the challenge of oral pathogens. Both viruses are able to infect monocytes/macrophages, indicating their ability to potentially limit the inherent function of macrophages to eradicate invading microbial pathogens.

In this study, we first investigated whether or not HCMV and EBV can inhibit the activation of macrophages when challenged by four different strains of oral bacteria, *P. gingivalis*, *F. nucleatum*, *A. actinomycetemcomitans*, and *S. gordonii*, some of which typically cause periodontal disease. Using TNF-α as a marker for macrophage activation, our data showed that the tested oral bacteria differentially activated THP-1-derived macrophages. Periodontal pathogens *P. gingivalis*, *F. nucleatum* and *A. actinomycetemcomitans* were able to induce high levels of TNF-α production at low or medium bacteria : cell ratios. The non-pathogenic *S. gordonii* induced TNF-α only when a much higher bacteria : cell ratio was used, suggesting that macrophages were relatively unresponsive to commensal bacteria. Both HCMV and EBV downregulated bacteria-induced TNF-α production, indicating that this could be a mechanism used by the viruses to inhibit the activation of macrophages when challenged by microorganisms.

When the experiment was performed with primary macrophages, we observed reduced TNF-α induction by the bacteria. We found that *F. nucleatum* and *A. actinomycetemcomitans*, but not *P. gingivalis* and *S. gordonii*, were able to induce TNF-α. In addition, the levels of the induced TNF-α were several-fold lower than what was observed in THP-1 cells. The discrepancy between THP-1-derived macrophages and the primary macrophages was probably the result of the heterogeneous population of the primary macrophages responding to oral bacteria differently.

In this study, we showed that HCMV and EBV could inhibit the phagocytic ability of macrophages. In assessing the feasibility of using FITC-labeled oral bacteria for the phagocytosis assay, we found that the labeling condition was not optimal for *P. gingivalis* and *F. nucleatum*: poor labeling for *P. gingivalis* and aggregation for *F. nucleatum* (data not shown). However, given that *P. gingivalis*, *F. nucleatum*, and *A. actinomycetemcomitans* are able to actively invade cells, the definitive cause for internalization of labeled bacteria – phagocytosis or invasion – may need to be resolved using a combination of alternative labeling conditions and killed bacteria.

The inhibition of phagocytosis by HCMV and EBV may be involved in multiple ways. It has been reported that HCMV downregulates complement receptors (CRs) that mediate phagocytosis. Because HCMV suppresses the expression of CR3 and CR4, it inhibits CR-mediated phagocytosis in macrophages (10). In addition, HCMV inhibits cytokine-induced macrophage differentiation (12). EBV inhibits the activation of monocytes by suppressing the biosynthesis of prostaglandin E2 (20). Furthermore, EBV infects monocytes and reduces the phagocytic ability of these cells by blocking protein kinase C activity (19, 25). In this study, we found that HCMV and EBV downregulated the expression of TLR9. TLR9 is part of the innate immune system and functions as a sensor for microbial DNA sequences containing unmethylated CpG dinucleotides. A recent study showed that activation of the TLR9 signaling pathway not only promotes phagocytosis, but also induces the expression of genes involved in the phagocytic program (8). Consequently, downregulation of TLR9 expres-

---

![Graph](image-url)
Viral infection in various ways. For example, TLR9 expression by the viruses. The reduced function of macrophages caused by HCMV and EBV inhibited macrophage functions, each affecting macrophage functions, each affected by viral infection, as indicated by the current study. While there are overlapping abilities among viruses in affecting macrophage functions, each virus exhibits its own unique property as well. Our data indicate that HCMV and EBV may contribute to the development of certain types of periodontal disease by inhibiting phagocytic clearance of pathogenic bacteria and by reducing host immune response via TNF-α production.

**Acknowledgments**

This project was supported by Grant RFA-RR-03-014 from the National Center for Research Resources (NCRR), a component of National Institutes of Health (NIH), and its contents are solely the responsibility of the authors and do not necessarily represent the official views of NCRR or NIH.

**References**