JOURNAL OF ORAL MICROBIOLOGY
2024, VOL. 16, 2301199
https://doi.org/10.1080/20002297.2023.2301199

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RESEARCH ARTICLE

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Detection of Epstein-Barr virus infection in primary junctional epithelial cell cultures

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ABSTRACT

Background: Junctional epithelium (JE) provides the front-line defense against pathogens invading periodontium. The breakdown of the JE barrier is the hallmark of periodontitis. Recent studies have implicated the Epstein-Barr virus (EBV) as one of the etiopathogenetic factors of periodontitis. EBV exhibits tropism for two target cells *in vivo*: B cells, where it primarily remains latent, and epithelial cells, where viral replication occurs.

Objective: Our knowledge of junctional epithelial cell (JEC) infection with EBV has been limited by the difficulty of generating cell cultures and the inability to infect JECs *in vitro* readily.

Design: To study EBV infection in JECs, we developed human JEC cultures derived from a periodontitis patient. Furthermore, we established a successful contact-free co-culture infection model between the EBV-donor B95–8 cell line and the EBV-permissive JEC culture. JECs and EBV infection of JECs were detected using immunofluorescent staining of cytokeratin 19 and EBNA1, respectively. In addition, EBV infection was confirmed by RT-qPCR for EBNA1, LMP1, and BZLF1 expression.

Results and conclusions: Our results suggest that the infection of JECs with EBV can occur in an *in vitro* experimental model. These outcomes have the potential to enhance our understanding of EBV's involvement in periodontitis and advance periodontal research.

ARTICLE HISTORY
Received 27 June 2023
Revised 20 December 2023
Accepted 28 December 2023

KEYWORDS
Periodontitis; junctional epithelial cell culture; Epstein-Barr virus; EBV infection; *in vitro* models

Introduction

Epstein-Barr virus (EBV), a ubiquitous human herpesvirus, is one of the most common human viruses, with estimates suggesting that more than 95% of adults worldwide are infected with the virus at some point in their lives [1]. EBV preferentially infects B cells (BCs), where it establishes a latent reservoir with sporadic reactivation and viral production by differentiated BCs, namely plasma cells (PCs). Occasionally, EBV infects other cell types, especially epithelial cells (ECs) of the oropharynx, predominantly resulting in a lytic infection that may contribute to EBV transmission from saliva [2]. EBV is best known as the cause of infectious mononucleosis, but it is also associated with several types of lymphoid and epithelial malignancies, affecting the two primary cell types targeted by the virus. Moreover, EBV has been associated with several autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, Sjogren's syndrome, systemic scleroderma [3], and multiple sclerosis, for which EBV has recently been identified as a leading cause [4]. In the past decade, EBV has also been implicated in the pathogenesis of destructive periodontitis [5].

Periodontitis is a serious gum disease characterized by inflammation and infection of the gums and the supporting structures of the teeth, including the alveolar bone, periodontal ligament, and cementum. It can cause the destruction of the supporting structures of the teeth, leading to loose teeth and tooth loss. Periodontitis is believed to be a multifactorial disease caused by a complex interplay of bacterial, genetic, environmental, and systemic factors. The most accepted cause of periodontitis is the accumulation of plaque, a film of bacteria that forms on the teeth and gums, and the body's immune response to the bacteria [6]. However, a new theory has emerged, the 'vicious circle hypothesis', which suggests a link between herpesviruses, such as EBV, and chronic periodontitis [7,8]. Indeed, mounting evidence supports that EBV may directly contribute to the pathogenesis of periodontitis [9]. EBV has been abundantly detected in periodontal lesions, ranging from hundreds to millions of copy counts, depending on the severity of periodontitis [10]. Furthermore, we have shown active EBV infection in the ECs of the junctional epithelia (JE) [11] and in the PCs that had infiltrated the periodontal lesions [12]. According to this 'vicious circle hypothesis', an infection with

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/20002297.2023.2301199.

a herpesvirus like EBV may lead to a chronic inflammatory response in the gums, which in turn may promote the overgrowth of bacteria associated with periodontitis. This chronic inflammation then leads to a further increase in the viral load, creating a 'vicious circle' of inflammation and infection. The hypothesis suggests that the herpesvirus may establish a latent infection in the gingival tissue, and that reactivations of the virus may trigger the inflammatory response.

During periodontitis, the JE, which is the layer of cells that lines the tooth-gingival margin, degrades and migrates apically (towards the tooth), forming a periodontal pocket [13]. This pocket is a space between the tooth and gums that becomes filled with infectious agents and inflammation, leading to the destruction of the supporting structures of the tooth. The JE is critical in maintaining the integrity of periodontal tissue and represents a crucial site with respect to the initiation and development of periodontal disease [14]. Thus, to clarify the involvement of EBV in periodontitis, it is important to investigate the EBV infection of junctional epithelial cells (JECs). So far, the JE has mostly been investigated by histological and anatomical analysis, while *in vitro* investigations have not been attainable due to the difficulty in setting up a primary JEC culture and the lack of established JEC cultures [15]. Such shortcomings may hamper research in understanding the role that EBV plays in periodontitis. This prompted us to exploit a successful *in vitro* infection system for investigating the ability of EBV to infect JECs.

In the present study, we attempted to isolate and culture human JECs from periodontal tissues. We examined the specific phenotype of these cells based on cytokeratin 19 (CK19) immunostaining. Further, we developed a successful *in vitro* model for EBV infection of JECs and investigated the infected cells by immunostaining for Epstein – Barr virus nuclear antigen 1 (EBNA1) and by reverse transcription quantitative PCR (RT-qPCR) analysis of EBV latent and lytic gene expression. Our data revealed that the cultured JECs were infectable *in vitro* by contact-free co-culture with EBV-producing cells, indicating that EBV is able to infect JECs.

Materials and methods

Processing of the tissue samples

Tissue samples were obtained from periodontitis-impacted gingiva attached to the teeth. The teeth were extracted for periodontal therapy reasons, and the gingival biopsies were taken as surgical waste. This study is classified as non-interventional research involving acts devoid of risks for the patients (category 3 in the context of the French 'Jardé Law'). The patient was informed of his/her right to oppose the use of his/her specimens and data for research purposes (authorized biomedical collection N°DC-2022–5040, French Research Ministry).

JE tissues that were tightly attached to the teeth neck were scraped off from the teeth surface, as previously described [14]. Excised tissues were extensively rinsed with Dulbecco's PBS supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (2.5 µg/ml), and caspofungin (0.5 µg/ml) to remove contaminating debris and blood. They were then aseptically dissected into small pieces.

Preparation of primary cell cultures

Several methods were evaluated for the generation of primary cell cultures to determine the most appropriate conditions for JECs. These included outgrowth from tissue explants, tri-step enzymatic digestion, and enzymatic digestion coupled with mechanical dissociation.

Outgrowth from tissue explants

Small 1–2 mm^3 fragments of the tissues were placed in a tissue culture plate that had been precoated with fetal bovine serum (FBS) for 30 min at room temperature. One drop of cell culture medium was added to the tissues and kept in a semi-drying condition to facilitate the adhesion of the fragments to the bottom of the plate. After the explants adhered to the plate surface, the fragments were fed with complete defined keratinocyte serum-free medium (defined K-SFM supplemented with 25 µg/ml bovine pituitary extract, 10 ng/ml epidermal growth factor, 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B, and 0.5 µg/ml caspofungin) to promote EC growth and attachment. The culture medium was changed every 2–3 days, and the cell outgrowth from the explants was regularly monitored with a microscope. All cells were maintained under standard conditions with 5% CO_2 at 37°C in a humidified atmosphere.

Tri-step enzymatic digestion

Small pieces of JE tissue were digested in 3 steps. First, they were incubated with dispase II (4 mg/ml) at 4°C overnight. Next, they were incubated with trypsin/EDTA (0.25%, 0.1%) for 2 × 15 min at 37°C, and then with collagenase I/dispase II (3 mg/ml, 4 mg/ml) for 2 × 15 min at 37°C. The digestion was stopped by adding defined K-SFM containing 10% FBS, followed by filtration using a 100-µm mesh cell strainer, and centrifugation. The resulting pellet was mixed in complete defined K-SFM to form a single cell suspension. To culture the generated primary JECs, cells were seeded at a concentration of 10^5 cells/ml in FBS-precoated 6-well plates.

Enzymatic digestion coupled with mechanical dissociation using a GentleMACS™ dissociator in a C tube

Small pieces of JE tissue were incubated in the digestion solution containing liberase TL (0.1 mg/ml) and DNase I (0.1 mg/ml) for 30 min at 37°C. The digestate was then mechanically dissociated using a GentleMACS™ dissociator and C tube (Miltenyi Biotec). The following steps corresponded to the procedure with the tri-step enzymatic digestion.

In all cases, for passaging of the cultures, cells were passaged at 80% confluency by adding trypsin/EDTA at 37°C for 5 min and re-cultured in complete defined K-SFM. Harvested cells were cryopreserved in the freezing medium consisting of defined K-SFM supplemented with 50% FBS and 10% dimethyl sulfoxide at – 80°C. Cells were subsequently used for downstream infection assays from passages 1 to 2.

Primary cells and cell lines used in the infection assay

Primary JECs were derived from the periodontal tissues of a single patient generated by the tri-step enzymatic digestion method. To prepare for the infection assay, JECs were seeded at a concentration of 10^5 cells/ml in a 12-well plate and were grown in complete defined K-SFM medium for 3 days. On day 3, the media was replaced with fresh K-SFM, and the cells were grown for another 4 days.

B95–8 cells are EBV-secreting BC lines from cotton-top tamarin monkeys that are commonly used to obtain infectious virus and are known to spontaneously replicate producing viral particles. B95–8 cell line was a generous gift from Pr. Cliona Rooney, Cell and Gene Therapy Center, Baylor College, Houston. Although the morphology of the B95–8 cell line is predominantly a suspension of singular cells or clusters of small clumps, roughly 10% of the cells remain adherent. To prepare for the infection assay, B95–8 cells were seeded in the inserts of a 12-transwell plate (Corning) at a concentration of 10^5 cells/ml and were maintained in complete RPMI medium (supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 10% FBS) for 4 days to settle and attach.

Infection of JECs with EBV

The infection was carried out by establishing a contact-free co-culture model. In this model, EBV-producing B95–8 cells were cultured in transwell inserts, while adherent primary JECs were cultured in the well underneath. Transwells have a pore size of 0.4 µm, which obstructs the passage of B95–8 cells, yet allows the EBV viral particles to pass through and migrate to the well underneath.

On the day of infection, RPMI and K-SFM media were gently removed from the transwell inserts housing B95–8 cells and the 12-well plate housing the JECs, respectively. The inserts were then placed on top of the JECs. JECs were covered with 2 ml of fresh K-SFM, and the B95–8 cells in inserts were covered with 1 ml fresh K-SFM. JECs (in the lower compartment) and B95–8 cells (in the upper compartment) were kept in co-culture for 1 day. After completion of the infection, the inserts and K-SFM media were removed, and the JECs were gently but thoroughly washed twice with K-SFM to remove virus donor cells in suspension and free-floating virus particles. They were then regrown in K-SFM for another 3 days to allow JECs to express EBV genes. A schematic representation of the contact-free co-culture setup is presented in Figure 1. JECs in 3 ml K-SFM were included as control uninfected JECs. Uninfected and infected JECs were in 4 replicates.

The infection of JECs was also tested using a cell-free culture supernatant of EBV-producing B95–8 cells. To prepare the cell-free viral supernatant, a B95–8 cell suspension (in RPMI medium) at a concentration of 3.5×10^6 cells/ml was centrifuged (5 min at $800 \times g$), and the supernatant was filtered through a 0.45-µm pore-size filter. For cell-free infection, 1 ml of virus supernatant was added to the cultured JECs in a 12-well plate in 2 ml of K-SFM medium.

Dual immunfluorescent (IF) staining

Dual IF was used to simultaneously detect the presence of CK19 (a JEC marker [14,16]) and EBNA1 (an EBV infection marker). To perform the IF, JECs were grown on FBS-precoated coverslips, which were placed in a 12-well plate (the lower compartment of the infection setup described above). Note that the infection and non-infection procedures were performed in the contact-free co-culture setup (Figure 1), with coverslips (used for IF, $n = 2$ each) and without coverslips (used for RT-qPCR, $n = 4$ each). The IF staining of JECs grown on coverslips was performed using the following steps: fixation with 3.7% formaldehyde for 15 min, neutralization with 50 mM NH_4Cl in PBS for 30 min, permeabilization in 0.1% Triton-X 100 in PBS for 5 min, non-specific antigen blocking using 1% bovine serum albumin (BSA) for 60 min, and co-incubation with CK19 and EBNA1 specific primary antibodies (diluted 1/100 in 1% BSA) in a humid chamber overnight at 4°C. The primary antibodies used were rabbit anti-CK19 (Abcam; ab52625) and mouse anti-EBNA1 (Santa Cruz Biotechnology; sc57719). After 4 wash steps in PBS, the JECs on coverslips were incubated

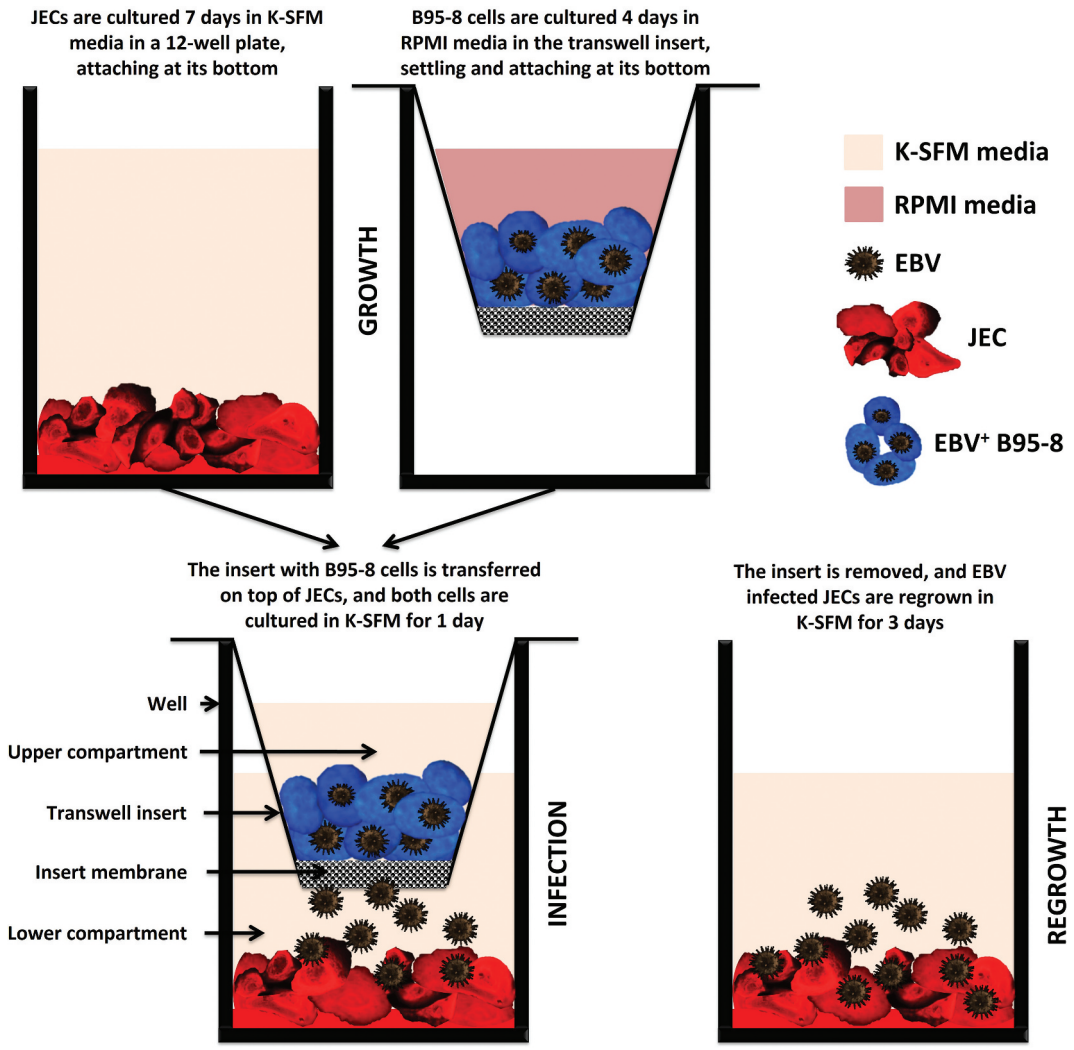

Figure 1. Schematic representation of the procedure used to set up a contact-free co-culture model of infection.

with secondary antibodies for 30 min at room temperature. The secondary antibodies were donkey anti-rabbit IgG, Alexa Fluor 594 (for CK19; Invitrogen; A-21207) and donkey anti-mouse IgG, Alexa Fluor 488 (for EBNA1; Invitrogen; A-21202), diluted 1/500 in 1% BSA. Cell nuclei were identified by DAPI (Thermo Scientific; 62248) co-staining with specific secondary antibodies. After 3 wash steps, the specimens were visualized under a fluorescent microscope and photographed.

RNA extraction and RT-qPCR

Total RNA was extracted from the JECs using the RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. The concentration and purity of the extracted RNA were determined using a SimpliNano spectrophotometer (Biochrom). The purity evaluation included measuring the A260/A280 and A260/A230 ratios to check for DNA, protein, and other potential contaminants, which remained within acceptable limits. After extraction, the RNA was stored at – 20°C until further use.

Complementary DNA (cDNA) was generated by reverse transcription (RT) using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems). The RT reaction was carried out at 37°C for 60 min in a total volume of 40 µl, which was then heated at 95°C for 5 min to stop the reaction. cDNA synthesized from 1.2 µg of total RNA was used for each PCR. The cDNA was mixed with Power SYBR Green PCR Master Mix (Applied Biosystems) and EBV-specific gene primer sets [EBNA1, LMP1 (latent membrane protein 1), BZLF1 (BamHI Z fragment leftward open reading frame 1); Table 1] previously described [11,17]. It is worth mentioning that the employed primers were not specifically designed to span exon-exon junctions for exclusive cDNA amplification [18]; rather, they were designed as within-exon primers. While this design theoretically opens the possibility of amplifying genomic DNA (gDNA) in the presence of gDNA within RNA samples, the risk of gDNA contamination is low. This is attributed to the RNeasy Mini Kit's effective DNA removal capabilities, eliminating the need for DNase digestion due to the advanced technology of its silica membrane. PCR was performed using the QuantStudio 5 System

Table 1. List of genes and primers used in RT-qPCR.

Gene	Description	Primer sequence (5′ → 3′)	Coordinates of primers	Coordinates of exons
36B4	Acidic ribosomal phosphoprotein	F: TGCATCAGTACCCCATTCTATCAT R: AGGCAGATGGATCAGCCAAGA	F: 839.862 R: 940.960	789.929, 930.1165
EBNA1	Epstein-Barr nuclear antigen 1	F: TACAGGACCTGGAAATGGCC R: TCTTTGAGGTCCACTGCCG	F: 95682.95701 R: 95742.95760	50134.97654
LMP1	Latent membrane protein 1	F: CAGTCAGGCAAGCCTATGA R: CTGGTTCCGGTGGAGATGA	F: 167655 … 167673 R: 167745 … 167763	166483.168507
BZLF1	BamHI Z fragment leftward open reading frame 1	F: AAATTTAAGAGATCCTCGTGTAAAACATC R: CGCCTCCTGTTGAAGCAGAT	F: 89926 … 89954 R: 89998 … 90017	89838.90053

(Applied Biosystems). The amplification conditions consisted of an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing and elongation at 60°C for 1 min. Each sample was run in technical duplicates. The housekeeping gene acidic ribosomal phosphoprotein *36B4* was used as a reference gene. The relative levels of mRNA expression were calculated according to the $2^{-\Delta\Delta CT}$ method by normalizing to the expression of the *36B4* reference gene. Uninfected JECs were used as a reference sample. DNA-free water served as a negative control in each PCR run.

Statistical analysis

The expression of EBV genes between infected and uninfected cells was compared using multiple unpaired t-tests. The statistical significance of differences in EBV gene expression within infected cells was determined using a two-way ANOVA and Tukey's multiple comparison test. Results with p-values less than 0.05 were considered statistically significant.

Results

Establishment and characterization of primary junctional epithelial cell cultures

Establishing JEC culture is challenging due to the small volume of JE tissue, which is difficult to collect [14], especially from periodontitis-impacted tissues. However, after multiple efforts, we succeeded in establishing an *in vitro* JEC culture system. Initial trials comparing 3 different tissue culturing methods (detailed in the Materials and Methods section) resulted in the generation of monolayers of primary cell cultures with similar levels of success (Figures 2a, S1, and S3). In all cases, the cells derived from periodontitis biopsies were able to grow on FBS-precoated plastic wells, which optimized the attachment rate. It is noteworthy that in our experiments, the freezing/unfreezing steps for subculturing were the limiting factor. This was evident from the fact that previously adherent, epithelial-like cells originated from tissue explants in passage 1 did not continue to exhibit the epithelial-like morphology (Figure S2). We found that the tri-step enzymatic digestion method was the most effective in generating viable cell cultures and therefore adopted it for our primary cell culture production and subculturing. After enzymatic digestion, on day 2 of inoculation, a large number of single cells were obtained (Figure 2a). Due to the epithelial culture conditions favoring the proliferation of mainly ECs, the number of cells decreased by day 10. Generally, non-adherent cells were lost during medium changes. By 3 weeks of culturing, attached starter cells gave rise to a monolayer of closely packed polygonal epithelial-like cells arranged in a paving stone pattern. In general, the primary ECs remained viable in the culture for the duration of 4 weeks.

During subculturing, the cells exhibited typical epithelial polygonal morphology and cobblestone growth similar to the morphology of primary cells derived from tissues. These ECs were also examined with IF staining at the second passage. CK19 has been reported as a specific marker of JE. Cells from the EC culture were intensely labeled with anti-CK19, indicating that the majority of the ECs were JECs (Figure 2b).

Establishment of in vitro infection model

Initial trials to establish an efficient infection model showed that contact-free co-culture with B95–8 EBV-producing cells, but not incubation with cell-free viral supernatant, resulted in efficient infection of JECs (Figure S4). The EBV-positive B95–8 cell line was used as a virus donor for the infection of JECs. The contact-free co-culture model of EBV^+ B95–8 cells and JECs isolated from periodontal tissues was established in transwell plates (Figure 1). EBV^+ B95–8 cells were seeded in the upper compartment (transwell insert), where they were cultured for 4 days. The JECs were seeded in the lower compartment (12-well plate), forming an adherent epithelial layer within 7 days. EBV infection was carried out for 1 day when the upper and lower compartments were placed together. The separation by the membrane of the transwell insert should prevent direct contact of the two cell cultures. On the day of infection, the cultured JECs had an epithelial-like appearance under the light microscope.

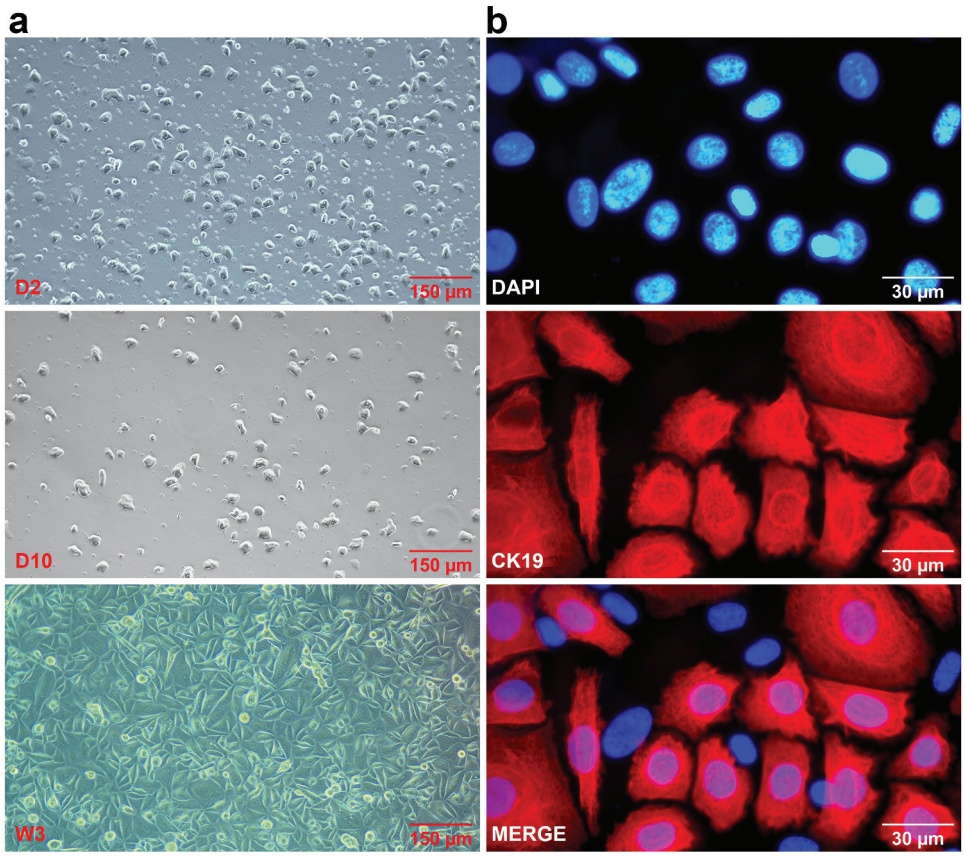

Figure 2. (a) JEC cultures generated from periodontitis tissues by tri-enzymatic digestion. Micrographs on day 2 (D2), day 10 (D10), and week 3 (W3) are presented. Magnification, 100×. After 3 weeks of culture, cells grew to over 90% confluency and exhibited epithelial-like morphology. (b) Immunofluorescent staining of CK19 (junctional epithelial cell marker; red fluorescence) in JEC monolayers at passage 2 (P2). Cell nuclei are stained with DAPI. Magnification, 1000×. Most of the cells in the P2 subculture were JECs.

Detection of EBV infection of JECs

After 1 day of exposure to EBV, JECs were examined by simultaneous IF staining for CK19 and EBNA1 (Figure 3). The micrographs from IF staining indicated that most of the cultured cells were JECs (according to CK19 expression) in uninfected and infected specimens. The micrographs from uninfected cells showed no fluorescence signal from EBNA1, while infected JECs were positive for EBNA1, indicating possible EBV infection of JECs.

EBV gene expression profile in JECs

The expression of 2 latent (EBNA1, LMP1) and 1 immediate-early lytic (BZLF1) EBV genes in JECs was examined by RT-qPCR. Figure 4a shows that there was no expression of EBV genes in uninfected JECs (detection threshold), while latent and lytic EBV transcripts were detected in EBV-infected JECs. EBV genes were expressed in infected JECs in the following decreasing order: LMP1 > BZLF1 > EBNA1 (Figure 4b), suggesting a simultaneous latent and lytic infection status. In the infected cells, LMP1 and BZLF1 were expressed significantly higher than EBNA1 (p-value < 0.0001 and p-value = 0.0043, respectively), while there was no significant difference between LMP1 and BZLF1 expression (p-value = 0.1166). Apparently, the contact-free JEC-B95-B co-culture model allows for successful EBV infection of recipient JECs.

Discussion

The JE is an epithelial component located at a strategically important interface between the gingival sulcus, populated with pathogens, and the periodontal soft and mineralized connective tissues that need protection from these pathogens [19]. It plays a critical role in the host defense against pathogen invasion in periodontal disease. Periodontal tissue breakdown begins in JE; therefore, JE is involved in the pathogenic mechanism of periodontitis. EBV is one of the frequent resident pathogens in the oral microenvironment. As EBV infects oral ECs, it is logical to assume that EBV infection of JECs is implicated in the pathogenesis of periodontitis. However, the possibility that JECs can be infected with EBV has never been considered before. Also, the ability of EBV to infect JECs has remained unclear owing to the lack and difficulty to establish primary *in vitro* culture

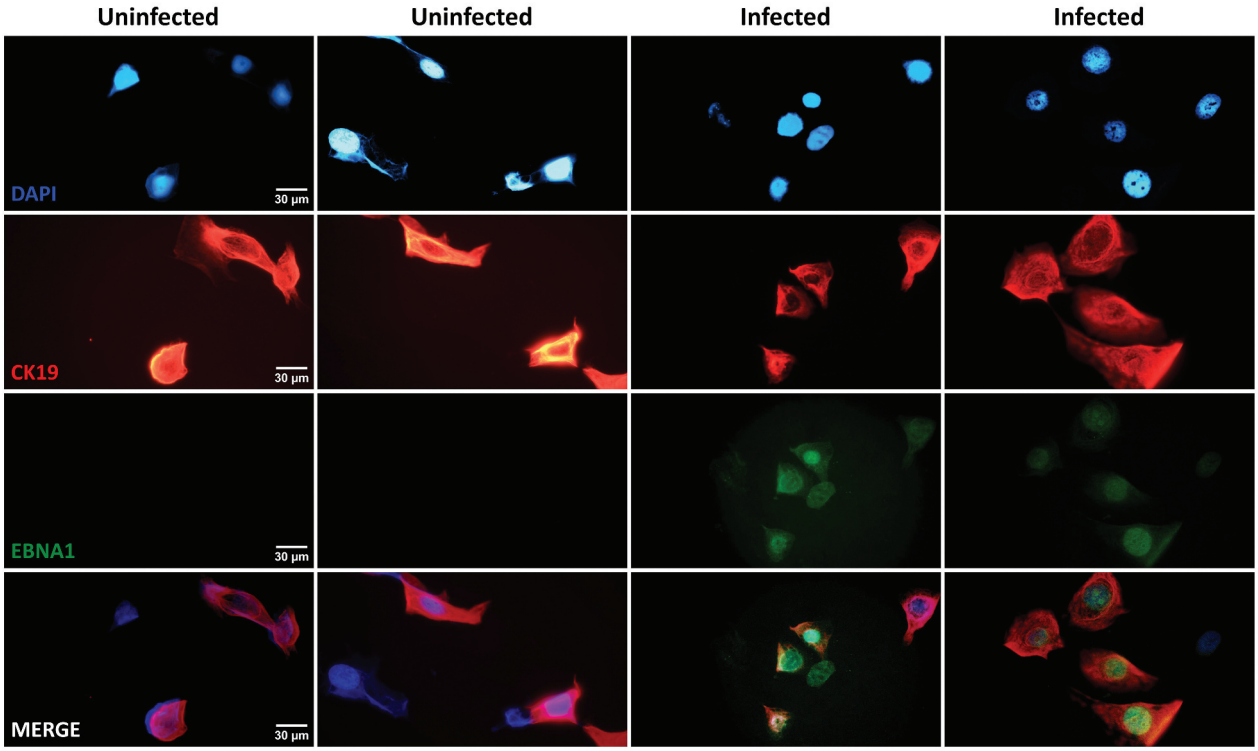

Figure 3. Dual immunofluorescent staining of CK19 (junctional epithelial cell marker; red fluorescence) and EBNA1 (EBV infection marker; green fluorescence) in uninfected and infected cell cultures generated from a single patient. After the completion of 1-day infection, JECs were cultured for an additional 3 days. Micrographs from duplicate experiments are shown. Cell nuclei are stained with DAPI. Magnification, 630×. Positive ENBA1 signal indicates possible EBV infection of JECs.

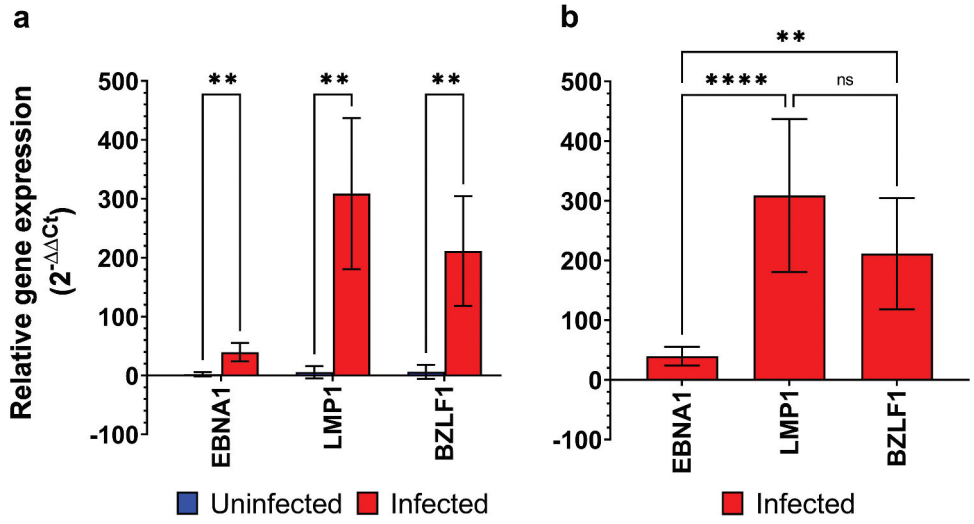

Figure 4. Real-time RT-qPCR analysis of EBV infection of JECs. (a) The comparison of the expressions of EBV genes (EBNA1, LMP1, and BZLF1) between uninfected and infected JECs. (b) The expression levels of EBV genes (EBNA1, LMP1, and BZLF1) within infected JECs. After the completion of 1-day infection, JECs were cultured for an additional 3 days. The relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method, with the *36B4* gene serving as the reference gene and uninfected JECs as the reference sample. The data are expressed as mean ± SD (n = 4); the asterisks indicate significant differences (****p-value < 0.0001; **p-value ≤ 0.005; ns – not significant).

systems and infection models. The reason for the lack of JEC cultures is attributed to the small volume of JE and the difficulty in collecting it. Nevertheless, Jiang and colleagues successfully generated a primary JEC culture using double enzymatic digestion (dispase and trypsin/EDTA) and serum-free medium (defined K-SFM), which were successfully passaged for 5 times [14]. They observed that these cultured JECs were strongly positive for CK19. While Dabija-Wolter and colleagues isolated primary gingival ECs through a combination of enzymatic digestion and mechanical separation of cells, and culturing in serum-free medium [20]. Consequently, these gingival ECs were used to reconstruct an organotypic model of JE by growing

them on top of collagen matrices populated with periodontal ligament fibroblasts. The JE in their model was identified by the presence of odontogenic ameloblast-associated protein, follicular dendritic cell-secreted protein, and CK19. Earlier, Papaioannou *et al.* sampled gingival pocket epithelium from periodontitis patients to harvest the JE, from which ECs were dissociated by digestion with pronase and incubation on a tumble mill [21]. In the second passage, this monolayer cell culture was regrown in a 2-compartment system using a clear polyester transwell membrane as a culture surface and K-SFM +10% fetal calf serum as a culture medium. Eventually, the authors were able to establish a multi-layered epithelium that expressed CK19, a marker for JE. For the generation of our cultures, we used a tri-step enzymatic digestion (dispase, trypsin/EDTA, and collagenase/dispase) and culturing in serum-free medium. We achieved maintaining the subcultures during 2 passages. Considering that CK is a consistent marker for JE [16], we confirmed the junctional epithelial nature of our cultured cells using IF staining of CK19. Indeed, most of the cultured cells exhibited CK19 immunoreaction (Figure 2b). In our experiments, the limiting factor for JEC cultures was the subculturing after cryopreservation (Figure S2). It is worth noting that we succeeded in producing monolayers of JECs using the explant tissue culturing method (Figure S1) and single cell suspensions prepared by enzymatic digestion combined with GentleMACS™ mechanical tissue processing (Figure S3). However, as mentioned above, these primary cells did not survive the detachment, cryopreservation, and subculturing procedures. In general, though this study identifies certain culture conditions to generate human JECs *in vitro*, in future experiments, these cells should be immortalized to be used as established JE cell lines.

Though JECs were cultured *in vitro* [14], and JEs were reconstructed *in vitro* [20,21], the infection of these systems with EBV was not previously reported. On the other hand, there have been numerous reports of developing *in vitro* EC models for EBV infection including, monolayers of unpolarized and polarized ECs, stratified epithelium, using oropharyngeal or tonsillar ECs, employing explant culturing method or single cell suspensions generated through enzymatic and/or mechanical digestion, implementing direct cell-free, cell-to-cell, and transfer infection modes which generated a great deal of useful data [22–30]. In our study, we implemented a contact-free co-culture infection model (Figure 1). In this 3D co-culture model, there is physical separation between the EBV-donor and EBV-recipient cells in the form of a transwell system, using a semi-permeable membrane that only allows the infiltration of viral particles from the apical to the basal compartment. Prior to establishing this working *in vitro* model for infection, we conducted a trial with the goal of determining optimal conditions for successful infection. This trial showed that the contact-free co-culture model, but not infection using cell-free viral supernatant, resulted in efficient infection (Figure S4). This is consistent with literature that cell-free virus supernatant is usually incapable of or inefficient at infecting most ECs *in vitro* [22].

EBNA1 and CK19 IF co-staining established that EBV infection occurred in CK19-positive cultured JECs (Figure 3). However, IF co-staining is impractical for quantitative use. To confirm this qualitative result, we performed RT-qPCR experiments for detection of EBNA1 (expressed during all latency programs), LMP1 (expressed during latency III, II), and BZLF1 (immediate-early lytic gene) transcripts and compared their expression between uninfected and infected JECs. EBV-specific latent and lytic transcripts were detected only in infected JECs (Figure 4a). According to the gene expression profile, EBV-infected JECs were likely in a state of latent infection. However, lytic BZLF1 was also expressed at a significant level. To escape from latency and produce infectious virions, lytic stimuli (such as the BZLF1 protein itself) induces the expression of the lytic master regulator gene BZLF1 [31]. As surprising as it may seem, many other groups have also detected BZLF1 transcripts at early times after infection, when lytic reactivation would be detrimental to the establishment of latency [31]. Additionally, the possibility of transient lytic bursts in EBV-infected cells for viral amplification and spread cannot be excluded. However, these remarks need further and deeper investigations. Furthermore, it is important to note that the primers utilized for EBV gene expression were designed within exons, which may lead to the unintended amplification of gDNA instead of selectively targeting exon-exon junctions to prevent gDNA amplification and ensure exclusive targeting of mRNA transcripts [18]. Therefore, to enhance the precision of data on viral gene expression, future studies should employ primer designs specifically tailored to target mRNA transcripts.

In summary, subsequent research efforts should be directed toward elucidating the mechanisms of EBV infection in JECs. Furthermore, possible *in vivo* infection of JE with EBV and its outcomes should be considered. In particular, the possibility that EBV-productive PCs in periodontal infiltrate may favor infection of ECs is of major interest. The EBV produced from PCs is more prone to infect ECs; hence, the lytic EBV infection of JE will result in the destruction of this tissue. Therefore, a model for EBV transmission in periodontal tissues should be developed.

Conclusions

We have developed an optimized approach for generating *in vitro* human JEC cultures from gingival tissues using a three-step enzymatic digestion process. These cultures have demonstrated cryopreservation endurance and can be utilized for subsequent downstream applications. Although at this point, we cannot rule out the establishment of latency or lytic activation, it is clear that in our contact-free co-culture infection model EBV was able to infect the cultured JECs. If this *in vitro* model of JEC infection reflects the real situation *in vivo*, then it could be one of the mechanisms by which EBV may be involved in the pathogenesis of periodontitis. In future experiments, this model can be further used to study the interactions between EBV, periodontal bacteria, and host responses to the pathogenic challenge.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by the European Union's Horizon 2020 research and innovation program under the Marie Skłodowska-Curie grant agreement No 896201.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

Informed consent statement

Donors were informed of their right to oppose the use of their biospecimen and data for research purposes.

Institutional review board statement

This study is non-interventional research involving acts devoid of risks for the patients (category 3 in the context of the French 'Jardé Law') and utilized the authorized biomedical collection N°DC-2022–5040 from the French Research Ministry.

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