Cytokine 115 (2019) 64–75

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

Tea polyphenols protect gingival keratinocytes against TNF-α-induced tight junction barrier dysfunction and attenuate the inflammatory response of monocytes/macrophages

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ARTICLE INFO

Keywords:
TNF-α
Keratinocytes
Macrophages
Epithelial barrier
Periodontal disease

ABSTRACT

Tea, an aromatic beverage prepared with the leaves of the *Camellia sinensis* plant, is well known to contain bioactive polyphenols. Green tea contains mainly catechins such as epigallocatechin-3-gallate (EGCG), while black tea is characterized by the presence of theaflavins. TNF-α, which is a pro-inflammatory cytokine that activates the endogenous inflammatory cascade, plays a key role in periodontitis. In the present study, we investigated the ability of tea compounds to attenuate TNF-α-mediated activation of the host inflammatory response in monocytes/macrophages as well as the protective effect of green and black tea polyphenols on gingival keratinocyte barrier dysfunction induced by TNF-α. Tea compounds inhibited both the activation of NF-κB and caspase-1 as well as IL-1β secretion by monocytes/macrophages. TNF-α time-dependently damaged keratinocyte tight junction barrier integrity, as determined by changes in transepithelial electrical resistance and FITC-dextran transport. Green tea extract, EGCG, theaflavins, and to a lesser extent, black tea extract protected keratinocytes against the TNF-α-mediated breakdown of barrier integrity. The treatment of keratinocytes with tea polyphenols markedly mitigated the morphological changes of tight junction proteins such as zonula occludens-1 and occludin compared to cells exposed only to TNF-α, as determined by immunofluorescence. Tea polyphenols also time-dependently decreased the paracellular flux of TNF-α-treated keratinocytes. In conclusion, the ability of tea polyphenols to exert an anti-inflammatory effect and to attenuate the gingival epithelial barrier dysfunction induced by TNF-α supports their potential for the prevention and treatment of periodontal disease.

1. Introduction

Periodontitis is a chronic and degenerative inflammatory disease involving the overproduction of cytokines and matrix metalloproteinases, which modulates periodontal tissue destruction. It is initiated by the dysbiosis of the commensal oral microbiota, which results in the overgrowth of a specific and limited group of Gram-negative bacteria. These bacteria interact with mucosal and immune cells, leading to the production of inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) [1]. The chronic inflammation induces osteoclast differentiation and activation, leading to the progressive destruction of alveolar bone. TNF-α is a multi-function cytokine and is believed to play a pivotal role in periodontitis progression and severity [2,3]. The level of this cytokine is significantly higher in gingival crevicular fluid from patients with chronic periodontitis than in healthy individuals, and decreases following periodontal treatments [4,5].

TNF-α up-regulates the production of other key pro-inflammatory cytokines such as IL-1β [6]. IL-1β is the signature innate cytokine and has been associated with inflammatory cell migration and osteoclastogenesis [7,8]. Macrophages, which secrete large amounts of IL-1β, are found in high numbers in diseased periodontal sites [9]. These cells detect and respond to bacterial pathogens, and mediate both inflammation and its resolution [10]. Cytokines from the innate response, including TNF-α and IL-1β, are the first to initiate cell communication in disease pathogenesis [11]. These mediators interact with each other, amplify signals, modulate cell surface receptor expression, and have synergistic or antagonistic interactions with host cell functions [12].

The gingival epithelium protects the underlying connective tissue from the external environment and thus plays an active role in maintaining periodontal health [13]. More specifically, the epithelial barrier is made of closely opposed cells attached to each other by intercellular tight junctions (TJ) [14]. Previous studies have shown that TNF-α can induce disruption of TJ in intestinal epithelial cells and cause the

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https://doi.org/10.1016/j.cyto.2018.12.009
Received 2 May 2018; Received in revised form 6 December 2018; Accepted 10 December 2018
Available online 11 January 2019
1043-4666/

breakdown of the epithelial barrier [15,16]. To the best of our knowledge, such effect on oral epithelial cells has not been reported.

Conventional periodontal treatments include mechanical scaling and root planing as well as the debridement of infected and inflamed tissues. Finding molecules that can module the host response is considered a valid adjunctive therapy in the treatment of periodontitis [17,18]. In this regard, plant polyphenols are promising bioactive molecules given their ability to attenuate the host inflammatory response [19]. Tea, an aromatic beverage prepared with the leaves of the *Camellia sinensis* plant, has a high polyphenol content [20]. Green tea (non-fermented) is particularly rich in catechins and their derivatives, the most important being epigallocatechin-3-gallate (EGCG), while black tea (fermented) contains mainly theaflavins and their derivatives [21,22]. A number of studies have shown that tea polyphenols exert beneficial impacts against several diseases, including cardiovascular disease, cancers, and inflammatory bowel disease [21]. In the present study, we investigated (i) the ability of tea polyphenols to reduce the TNF-α-mediated activation of the NF-κB and caspase-1 signaling pathways and the secretion of the pro-inflammatory cytokine IL-1β by monocytes/macrophages and (ii) the protective effect of green and black tea polyphenols on gingival epithelial barrier dysfunction induced by TNF-α.

2. Material and methods

2.1. Tea compounds

The commercial green and black tea extracts were purchased from Hangszhou Gosun Biotechnologies Co. (Hangzhou, China). These extracts contain 98.4% and 92% of polyphenols, respectively. Stock solutions were prepared in distilled water by dissolving green tea extract and black tea extract at final concentrations of 20 mg mL^{-1} and 10 mg mL^{-1}, respectively. Tea extract solutions were sterilized by filtration (0.22-μm-pore-size membrane filter). EGCG (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada), that represents the main catechin in the green tea extract (47.9%), was prepared similarly at a concentration of 10 mg mL^{-1}. The theaflavin fraction, obtained from BeHe Biotechnology (Jiangsu, China) is a mixture of theaflavin, theaflavin-3-gallate, theaflavin-3′-gallate, and theaflavin-3,3′-digallate (80% purity). A stock solution (20 mg mL^{-1}) was prepared in 95% ethanol.

2.2. IL-1β secretion by macrophages

U937 human monocytes (CRL-1593.2; American Type Culture Collection, Manassas, VA, USA) were cultivated in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 μg mL^{-1} of penicillin G/streptomycin at 37 °C in a 5% CO_2 atmosphere. The monocytes (2.5×10^5 cells mL^{-1}) were incubated in RPMI-10% FBS containing 100 ng mL^{-1} of phorbol myristic acid (PMA; Sigma-Aldrich Canada Ltd.) for 48-h to induce differentiation into adherent macrophage-like cells [23]. Adherent macrophage-like cells were detached by scraping and were harvested by centrifugation at 1200*g* for 5 min. The cells were washed, suspended in RPMI-1% FBS at a concentration of 1×10^6 cells mL^{-1}, seeded into the wells of a 12-well microplate (1×10^6 cells/well), and incubated overnight at 37 °C in a 5% CO_2 atmosphere. The macrophage-like cells were then pre-treated for 2 h with either non-cytotoxic concentrations of green tea extract, black tea extract, EGCG, or theaflavins in RPMI-1% FBS prior to being stimulated with 10 or 100 ng mL^{-1} of recombinant human TNF-α (AnaSpec, Fremont, USA). An assay using a commercial inhibitor (BAY-11-7082; 5 μg mL^{-1}; EMD Millipore, Mississauga, ON, Canada) was used as a positive control for the inhibition of IL-1β secretion. Cells incubated in culture medium with or without tea polyphenols and stimulated or not with TNF-α were used as controls. After a 24-h incubation at 37 °C in a 5% CO_2 atmosphere, the culture supernatants were collected and stored at −20 °C until used. The cells were lysed with 0.1% Triton X-100 (60-min incubation) to ensure the maximum release of intracellular cytokines. An enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems Inc., Minneapolis, MN, USA) was used to quantify the levels of intracellular and released IL-1β.

2.3. Caspase-1 activation and quantification

Adherent macrophage-like cells (2×10^6) were seeded in the wells of a 6-well microplate and were treated with TNF-α (100 ng mL^{-1}) in the presence or absence of tea polyphenols at different concentrations at 37 °C in a 5% CO_2 atmosphere. After 24-h, the macrophage-like cells were washed twice with ice-cold phosphate-buffered saline (PBS; pH 7.2) and were detached by adding Accutase® (1 mL; Sigma-Aldrich Canada Ltd.) at 37 °C in a 5% CO_2 atmosphere for 7 min. To assess caspase activation, the macrophage-like cells were stained with FAM-VAD-FMK reagent (Thermo Fisher Scientific, Waltham, MA, USA), a fluorochrome inhibitor of caspases (FLICA), for 1 h according to the manufacturer's protocol. FAM-VAD-FMK is a cell-permeable carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor of caspase-1 that binds covalently to the reactive cysteine residue in the active center of the enzyme. Unbound reagent was removed by washing the cells. A total of 20,000 cells were analyzed using a Cytomics FC 500 flow cytometer (Beckman Coulter Canada, Mississauga, ON, Canada). Data were analyzed using CXP software. To confirm caspase-1 activation, the Caspase-Glo® 1 inflammasome assay (Promega Corporation, Madison, WI, USA) was used in accordance with the manufacturer's protocol; this bioluminescent assay directly measures inflammasome activity. Luminescence was monitored with a Synergy 2 microplate reader (BioTek Instruments, Winooski, VT, USA). The amounts of caspase-1 secreted into the culture medium or retained in the macrophages were determined by ELISA (R&D Systems Inc.).

2.4. Activation of the NF-κB signaling pathway

The human monoblastic leukemia cell line U937-3xκ B-LUC, a subclone of the U937 cell line stably transfected with a luciferase gene coupled to a promoter with three NF-κB binding sites, was kindly provided by R. Blomhoff (University of Oslo, Norway) [24]. The cells were cultivated in Roswell Park Memorial Institute 1640 medium (RPMI-1640; Life Technologies Inc., Burlington, ON, Canada) supplemented with 10% heat-inactivated FBS, 100 μg mL^{-1} of penicillin G/streptomycin, and 75 μg mL^{-1} of hygromycin B at 37 °C in a 5% CO_2 atmosphere. To investigate the effect of tea polyphenols on TNF-α-induced NF-κB activation, U937-3xκ B-LUC cells (10^6 cells mL^{-1}) were pre-incubated with the compounds (non-cytotoxic concentrations; in RPMI-1% FBS) for 30 min in the wells of a black wall, black bottom 96-well microplate (Greiner Bio-One North America Inc., Monroe, NC, USA). The cells were then stimulated for 6-h with recombinant TNF-α at a concentration of 10 ng mL^{-1}. Wells with no TNF-α or no compounds were used as controls. An assay using the commercial inhibitor BAY-11–7082 (5 μg mL^{-1}) was used as a positive control for the inhibition of the NF-κB signaling pathway. NF-κB activation was determined by measuring luciferase activity following the addition of Bright-Glo reagent (Promega Corporation) in accordance with the manufacturer's protocol. Luminescence was monitored using a Synergy 2 microplate reader (BioTek Instruments).

2.5. Gingival keratinocyte tight junction integrity

The previously characterized gingival keratinocyte cell line B11 [25], which was kindly provided by S. Groeger (Justus-Liebig-University Giessen, Germany), was used to evaluate the effect of tea polyphenols on the TNF-α-induced disruption of tight junction integrity. Keratinocyte barrier integrity was determined by measuring transepithelial electrical resistance (TER) [26]. Keratinocytes were cultured in keratinocyte serum-free medium (K-SFM; Life Technologies

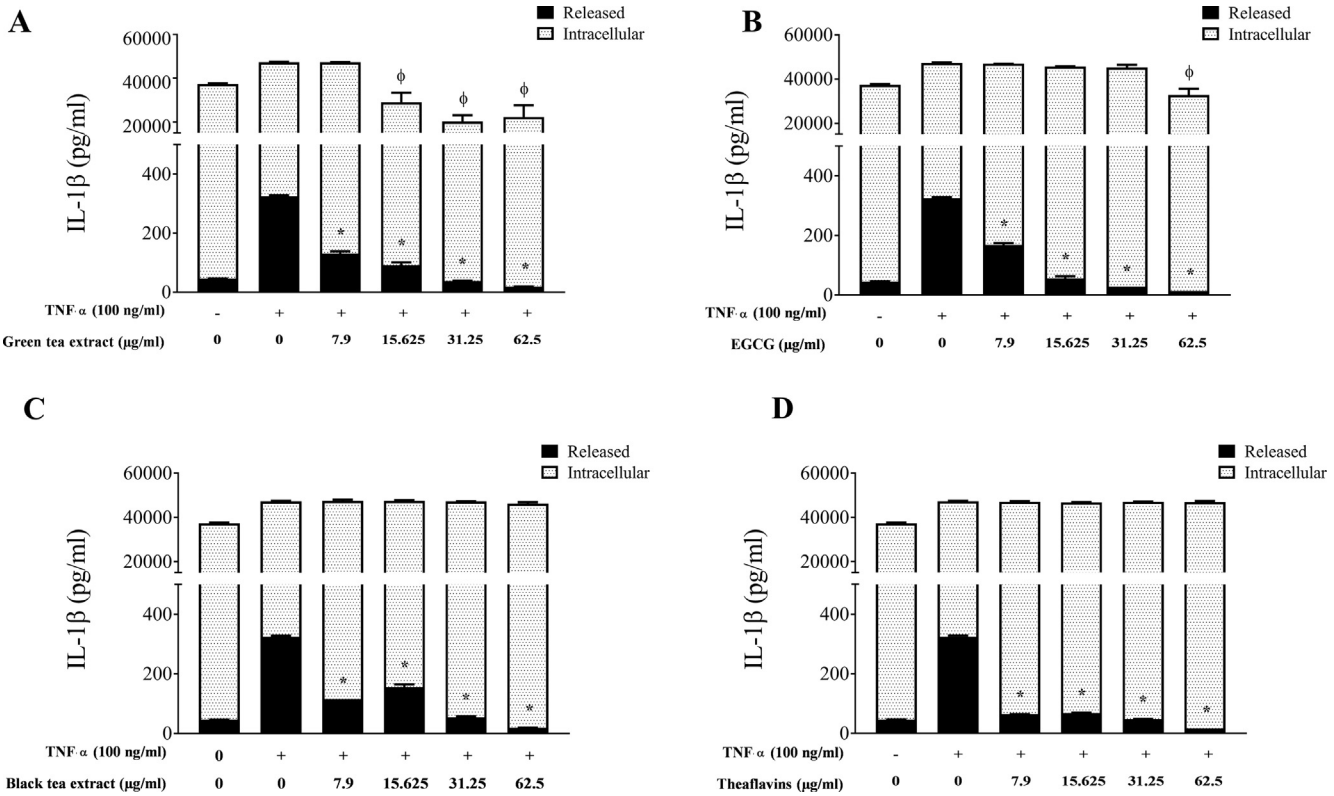

Fig. 1. Effects of tea polyphenols on the TNF-α-induced secretion of IL-1β by macrophages. Stimulation in the presence of the green tea extract (Panel A), EGCG (Panel B), black tea extract (Panel C), and theaflavins (Panel D). Results are expressed as the means ± SD of triplicate assays from three independent experiments. (φ) Significant decrease ($p < 0.001$) of intracellular IL-1β compared to TNF-α-stimulated cells not treated with tea compounds. (*) Significant decrease ($p < 0.001$) of released IL-1β compared to TNF-α-stimulated cells.

Inc.) supplemented with growth factors (50 μg mL^{-1} of bovine pituitary extract and 5 ng mL^{-1} of human epidermal growth factor) and 100 μg mL^{-1} of penicillin G-streptomycin at 37 °C in a 5% CO_2 atmosphere. Keratinocytes (3×10^5 cells per insert) were seeded in Costar™ Transwell™ clear polyester membrane inserts (6.5 mm in diameter, 0.4 μm pore size; Corning Co., Cambridge, MA, USA). The basolateral and apical compartments were filled with 0.6 mL and 0.1 mL of culture medium, respectively. Following a 72-h incubation, the conditioned medium was replaced with antibiotic-free K-SFM, and the keratinocytes were incubated for a further 16-h prior treating the cells. TNF-α (100 ng mL^{-1}) and tea compounds were added to the apical compartment. The concentrations of tea compounds used were based on preliminary assays showing that green tea extract, EGCG, and theaflavins at ≤62.5 μg mL^{-1} and black tea extract at ≤125 μg mL^{-1} had no effect on keratinocyte viability as determined using an MTT (3-[4,5-diethylthiazol-2-yl]-2,5diphenyltetrazolium bromide) colorimetric assay (Roche Diagnostics, Laval, QC,Canada). The TER values were determined using an ohm/voltmeter (EVOM^2; World Precision Instruments, Sarasota, FL, USA) after 0, 6, 12, 24, and 48 h of incubation. Resistance values were calculated in Ohms (Ω)/cm^2 by multiplying the resistance values by the surface area of the membrane filter. Results are expressed as the percentage of the basal control value measured at time 0-h (100% value).

2.6. Immunofluorescence microscopy

For the immunofluorescence analysis, keratinocytes were grown on a poly-D-lysine-coated glass multiwell culture slide (8-wells) (Thermo Fisher Scientific) for 3 days. The cells were then treated for 12-h with 100 ng mL^{-1} of TNF-α with or without tea polyphenols at concentrations ranging from 125 to 31.25 μg mL^{-1}. Thereafter, the keratinocytes were washed with PBS, fixed for 20 min with 4% paraformaldhehyde, permeabilized for 10 min with 0.1% Triton X-100, and blocked for 40 min in 3% nonfat milk in 20 mM Tris hydrochloride (pH 8) containing 150 mM NaCl and 0.5% Tween 20. The cells were labeled with 2.5 μg mL^{-1} of anti-occludin antibody Alexa Fluor® 488 conjugate or anti-ZO-1 antibody Alexa Fluor® 594 conjugate (Thermo Fisher Scientific) in blocking buffer overnight at 4 °C. The slides were prepared with the ProLong® Diamond Antifade Mountant (Life Technologies Inc.), sealed using nail polish, and kept in the dark at 4 °C. The distribution of tight junction proteins in the keratinocytes was visualized using an inverted Olympus FSX100 fluorescent microscope (Olympus, Tokyo, Japan).

2.7. Fluorescein isothiocyanate-conjugated dextran (FD-4) transport

Gingival keratinocytes were cultured on Transwell™ filters as described above. Immediately after adding FD-4 (MW 4.4 kDa, 1 mg mL^{-1} in culture medium) to the apical compartment, tea compounds (125 to 15.625 μg mL^{-1}) were added in presence of TNF-α (100 ng mL^{-1}). Fluorescence in the basolateral compartment was recorded after 0, 6, 12, 24, and 48 h of incubation using a Synergy 2 microplate reader (BioTek Instruments).

2.8. IL-8 secretion by oral epithelial cells

The human oral epithelial cell line GMSM-K [27], kindly provided by V. Murrah (University of North Carolina, Chapel Hill, NC, USA), was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated FBS and 100 μg mL^{-1} of penicillin G-streptomycin in a 5% CO_2 atmosphere at 37 °C. Oral epithelial cells were seeded in a 12-well plate (4×10^5 cells/well) and were cultured overnight to allow cell adhesion. The epithelial cells were pre-treated with increasing concentrations of tea polyphenols (0–125 μg mL^{-1})

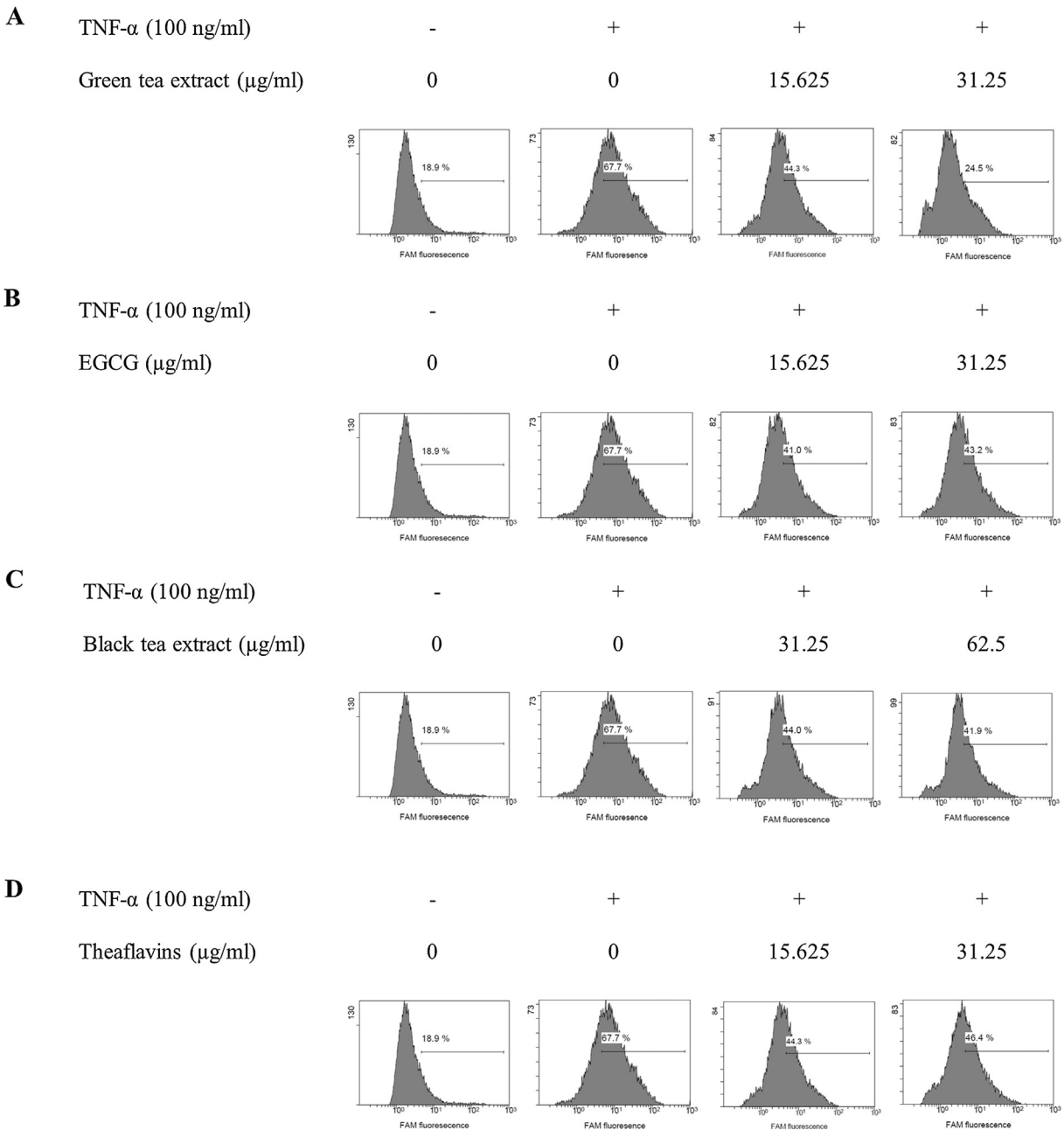

Fig. 2. Effects of TNF-α on the activation of caspase-1 in macrophages using the caspase-1 inhibitor probe FAM-VAD-FMK to detect caspase activation. Stimulation in the presence of the green tea extract (Panel A), EGCG (Panel B), black tea extract (Panel C), and theaflavins (Panel D).

prior to being stimulated with recombinant TNF-α (100 ng mL^{-1}). After a 24-h incubation, cell-free supernatants were collected and stored at –20 °C until used. A commercial enzyme-linked immunosorbent assay (ELISA) kit (eBioscience Inc.) was used to quantify interleukin-8 (IL-8) according to the manufacturer's protocol.

2.9. Statistical analysis

Unless indicated otherwise, all experiments were performed in triplicate in three independent experiments. The data are expressed as means ± standard deviations (SD). Statistical analyses were performed using a one-way analysis of variance with a post hoc Bonferroni multiple comparison test (GraphPad Software Inc.; La Jolla, CA, USA). All results were considered statistically significant at $p < 0.01$ or $p < 0.001$.

3. Results

The stimulation of macrophage-like cells with recombinant TNF-α increased IL-1β secretion in a dose-dependent manner (Supplementary Fig. 1). When used at 100 ng mL^{-1}, TNF-α increased IL-1β secretion 7.1-fold compared to the control macrophage-like cells. All the tea compounds tested caused a significant inhibition of IL-1β secretion. More specifically, at 62.5 μg mL^{-1}, the green tea extract, EGCG, black tea extract, and theaflavins inhibited the secretion of IL-1β by 94.1%, 95.6%, 64.5%, and 94.8%, respectively (Fig. 1A-D). The reduced secretion of IL-1β was not related to a cytotoxic effect of the tea compounds on the macrophage-like cells, as indicated by the results of an MTT assay (data not shown). Moreover, in the presence of TNF-α at 100 ng mL^{-1}, the intracellular level of IL-1β increased by 1.26-fold compared to the control macrophage-like cells. Only the green tea polyphenols reduced the levels of intracellular IL-1β; a reduction of

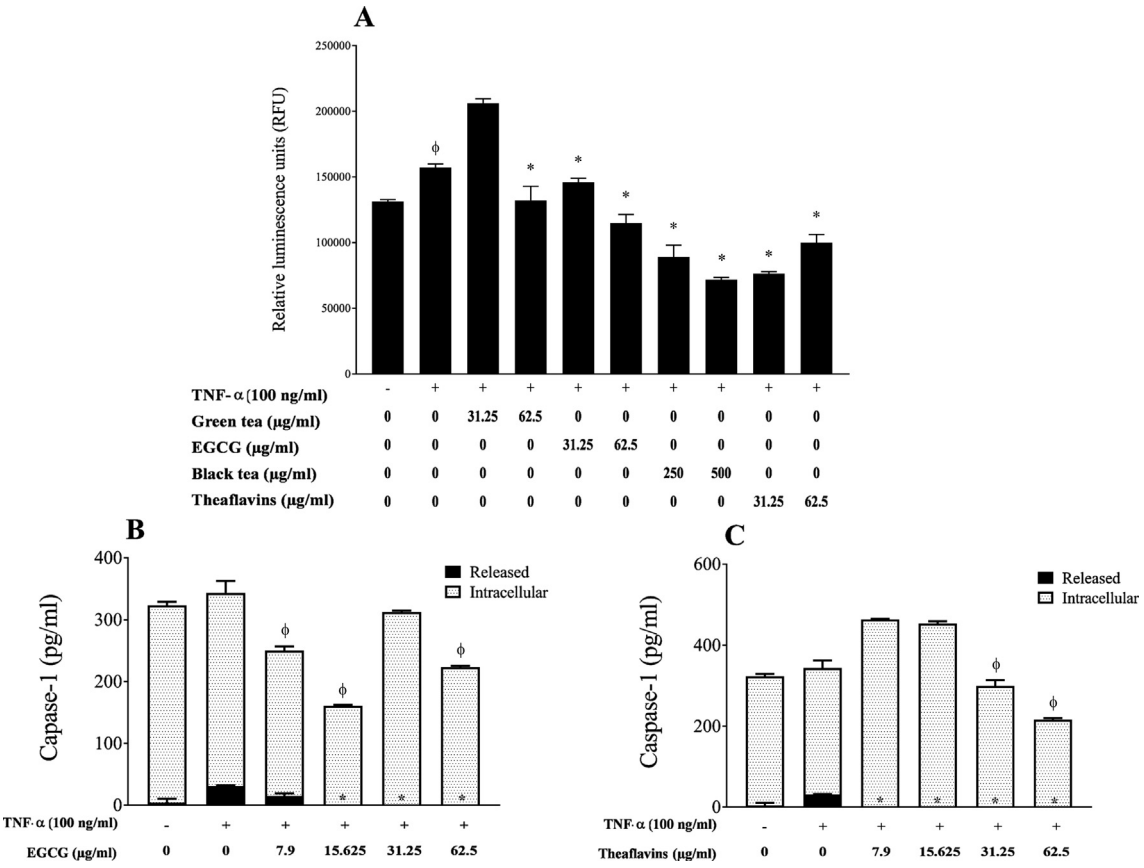

Fig. 3. Effects of TNF-α on the activation of inflammasome in macrophages using caspase-Glo 1 inflammasome assay to detect intracellular and released caspase-1 activation. Stimulation in the presence of the green tea extract, EGCG, black tea extract, and theaflavins (Panel A). Effect of the green tea extract (Panel A), EGCG (Panel B), black tea extract (Panel C), and theaflavins (Panel D) on intracellular and released caspase-1 from TNF-α-treated macrophages. Φ: significant increase in intracellular caspase-1 ($p < 0.001$) relative to control macrophages treated with TNF-α alone. *: significant decrease in released caspase-1 ($p < 0.001$) relative to control macrophages treated with TNF-α alone.

52.61% and 30.35% was observed in the presence of green tea extract and EGCG, respectively (Fig. 1A-D).

We hypothesized that the increase in the secretion of IL-1β induced by TNF-α may be related to the ability of TNF-α to modulate the activation of caspase-1 in macrophage-like cells. We assessed the activation of caspase-1 by flow cytometry using the fluorescent reagent FAM-VAD-FMK FLICA and found that there is a significant shift in the population of caspase-1-positive cells following a treatment with 100 ng mL^{-1} of TNF-α (Fig. 2). Further analyses were performed to link the inhibitory effects of tea compounds on IL-1β secretion with their ability to prevent caspase-1 activation in macrophage-like cells. The dose-dependent decrease in fluorescence following the treatment of the macrophage-like cells with the tea compounds indicated that caspase-1 activation was attenuated by the compounds (Fig. 2). More specifically, following a 24-h stimulation with 100 ng mL^{-1} of TNF-α, caspase activity increased by 48.8%, while in the presence of 31.25 µg mL^{-1} of the green tea extract, EGCG, black tea extract, or theaflavins, caspase activity was reduced by 43.2%, 24.5%, 23.7%, and 21.3%, respectively (Fig. 2). To confirm caspase-1 activation, we used the Caspase-Glo® 1 inflammasome assay that directly measures inflammasome activity. We showed that TNF-α increases inflammasome activation by 36.33% (Fig. 3), while in the presence of 62.5 µg mL^{-1} of the green tea extract, EGCG or theaflavins, caspase activation was reduced by 35.95%, 44.3% and 51.58%, respectively (Fig. 3). A higher concentration of the black tea extract was required to reduce inflammasome activation; a reduction of 65.19% was obtained at 500 µg mL^{-1}. The stimulation of macrophage-like cells with recombinant TNF-α increased caspase-1 secretion in a dose-dependent manner (Fig. 3). When used at 100 ng mL^{-1}, TNF-α increased caspase-1 secretion by 7.1-fold compared to the control macrophage-like cells. EGCG and theaflavins caused a significant inhibition of caspase-1 secretion. More specifically, at 62.5 µg mL^{-1}, EGCG and theaflavins completely inhibited caspase-1 secretion (Fig. 3). In the presence of TNF-α at 100 ng mL^{-1}, the intracellular level of caspase-1 was not modified compared to non-stimulated macrophage-like cells. At 62.5 µg mL^{-1}, EGCG and theaflavins reduced the intracellular caspase-1 levels by 28.4% and 30.8%, respectively.

Considering that NF-κB signaling pathway plays a major role in second inflammatory processes by orchestrating the secretion of pro-inflammatory cytokines, we assessed the ability of the tea compounds to prevent TNF-α-induced NF-κB activation using the U937-3xκB-LUC cell line. In general, all the tea compounds tested exhibited a comparable dose-dependent inhibitory effect on TNF-α-induced NF-κB activation (Fig. 4). More particularly, 62.5 µg mL^{-1} of the green tea extract, EGCG, black tea extract, or theaflavins inhibited NF-κB activation induced by 10 ng mL^{-1} of TNF-α by 70.7%, (Fig. 4B), 71.9% (Fig. 4C), 30.6% (Fig. 4D), and 46.7% (Fig. 4E), respectively.

In the second part of the study, we investigated whether TNF-α may alter the keratinocyte barrier integrity as determined by monitoring TER. A treatment of the gingival keratinocytes with 100 ng mL^{-1} of TNF-α decreased the TER values 1.4-fold, 1.3-fold, and 1.9-fold at 12 h, 24 h, and 48 h of incubation, respectively (Fig. 5). A 24-h treatment of the keratinocytes with 62.5 µg mL^{-1} of green tea extract, EGCG, or theaflavins significantly attenuated the ability of TNF-α to decrease the TER by 2.2-fold (Fig. 5A), 2.1-fold (Fig. 5B) and 2.6-fold (Fig. 5D), respectively. At this concentration and for a 24-h treatment, the black tea extract did not prevent the decrease in TER induced by TNF-α,

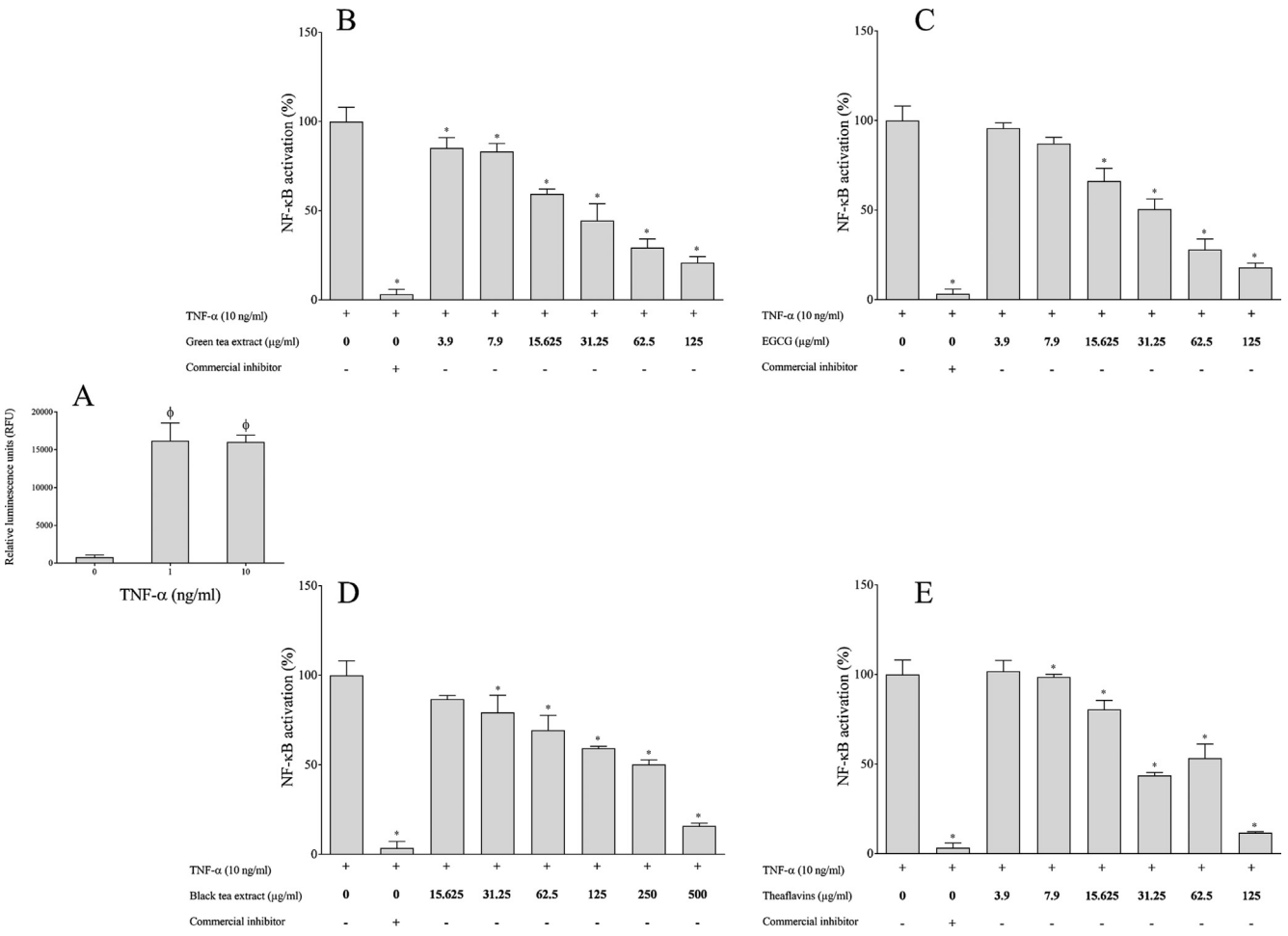

Fig. 4. Effects of TNF-α on the activation of the NF-κB pathway using the U937-3xκB cell model. Stimulation in the absence of tea polyphenols (Panel A). Stimulation in the presence of the green tea extract (Panel B), EGCG (Panel C), black tea extract (Panel D), and theaflavins (Panel E). A value of 100% was assigned to the activation obtained with TNF-α (10 ng mL^{-1}) in the absence of tea polyphenols (Panel B-E). The commercial inhibitor BAY-11-7082 (5 μg mL^{-1}) was used as a positive control. Results are expressed as the means ± SD of triplicate assays from three independent experiments. (φ) Significant increase ($p < 0.001$) compared to cells not stimulated with TNF-α. (*) Significant decrease ($p < 0.001$) compared to TNF-α-stimulated cells.

while a 12-h treatment with 62.5 μg mL^{-1} of the black tea extract prevented TNF-α-induced damage 1.4-fold (Fig. 5C).

Immunofluorescence staining showed that the TNF-α treatment induced discontinuities in the distribution of occludin and ZO-1 in keratinocytes. A treatment with the green tea extract, EGCG, black tea extract, or theaflavins prevented these morphological changes to occludin and ZO-1 compared to control cells treated only with TNF-α (Fig. 6).

Thereafter, we showed that the tea compounds decreased the TNF-α-induced paracellular flux of FD-4 through the keratinocyte monolayer. A 48-h treatment with 62.5 μg mL^{-1} of green tea extract, EGCG, black tea extract, or theaflavins, decreased TNF-α-induced permeability by 1.2-fold, 1.9-fold, 1.8-fold, and 2.1-fold, respectively (Fig. 7D).

Given that the chemokine IL-8 is involved in the migration of immune cells such as macrophages in the epithelial tissue, we investigated the effect of TNF-α on the secretion of IL-8 by oral epithelial cells. The stimulation of epithelial cells with TNF-α significantly increased the secretion of IL-8 by 38.3-fold (10 ng mL^{-1} of TNF-α) and 125.6-fold (100 ng mL^{-1} of TNF-α) (Fig. 8A). At a concentration of 62.5 μg mL^{-1}, the green tea extract, EGCG, and theaflavins reduced the secretion of IL-8 by 93.1% (Fig. 8B), 98.8% (Fig. 8C), and 70.8% (Fig. 8E), respectively. A much higher concentration of black tea extract (250 μg mL^{-1}) was required to reduce the secretion of IL-8 (78%) (Fig. 8D).

4. Discussion

The initiation, progression and severity of periodontal disease is associated with complex interactions between periodontopathogenic bacteria and mucosal and immune host cells. The pro-inflammatory cytokine TNF-α is a critical component involved in the pathogenesis of periodontal disease [2,28], and plays a key role in orchestrating the cytokine cascade in a number of inflammatory diseases [29]. TNF-α is a master regulator and has been proposed as a therapeutic target for a number of inflammatory diseases [30]. In this regard, several studies have shown that TNF-α antagonists may potentially reduce tissue destruction associated with periodontal disease [31–34]. Given their anti-inflammatory properties, tea polyphenols may be of interest as immune regulatory molecules for controlling periodontal disease. In this study, we investigated the effect of green and black tea polyphenols on the secretion of pro-inflammatory mediators by monocytes/macrophages induced by TNF-α via the caspase-1 and NF-κB signaling pathways. We also determined the impact of tea polyphenols on the disruption of gingival keratinocyte permeability and tight junction integrity by TNF-α.

Macrophages are the main source of IL-1β in inflamed periodontal tissue [8]. We showed that the green and black tea extracts, EGCG and theaflavins significantly and dose-dependently reduce IL-1β secretion by TNF-α-treated macrophages. Given that the pro-inflammatory cytokine IL-1β is involved in bone resorption, tea polyphenols, through

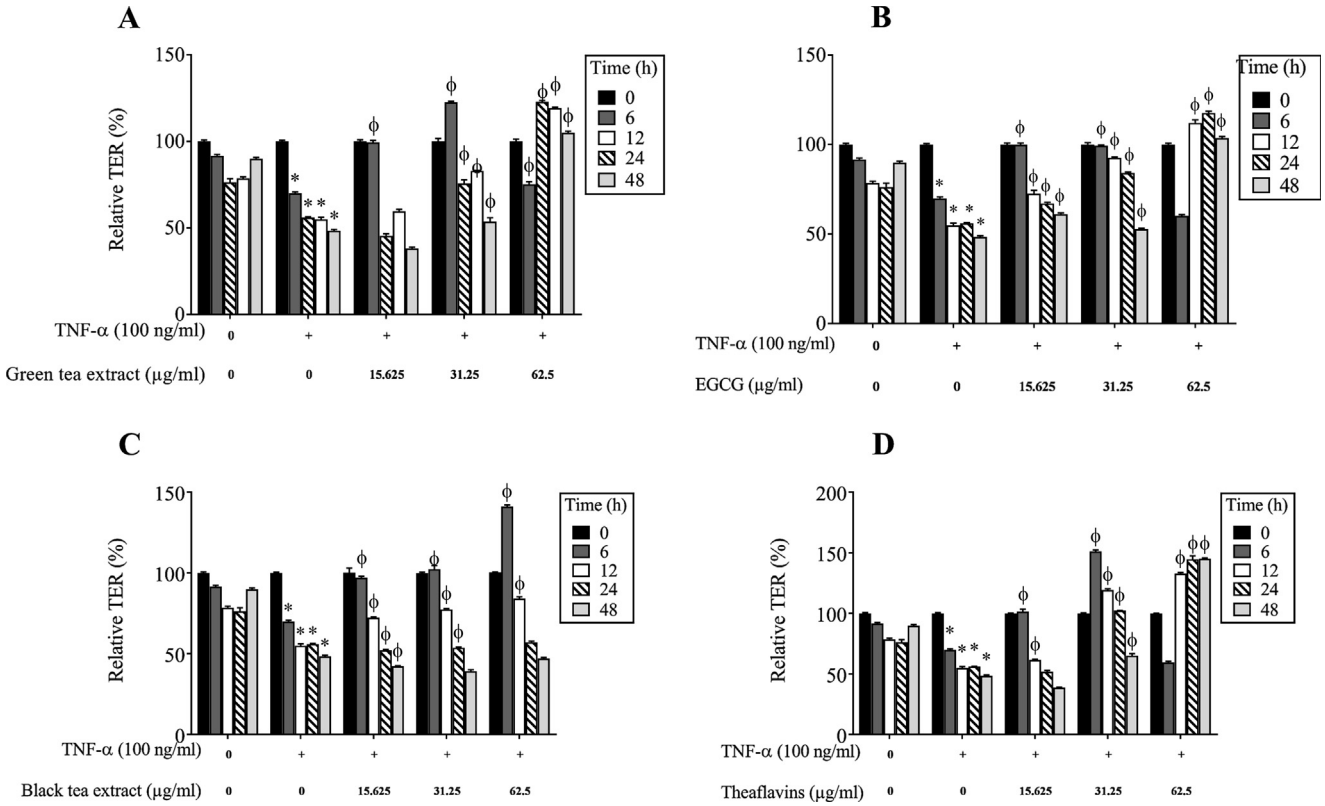

Fig. 5. Effects of TNF-α in the absence and presence of the green tea extract (Panel A), EGCG (Panel B), black tea extract (Panel C), and theaflavins (Panel D) on gingival keratinocyte tight junction integrity determined by monitoring TER. A 100% value was assigned to the TER value at time 0. Results are expressed as the means ± SD of triplicate assays from three independent experiments. (φ) Significant increase ($p < 0.001$) compared to TNF-α-stimulated cells not treated with tea compounds. (*) Significant decrease ($p < 0.001$) compared to TNF-α-stimulated cells.

their ability to attenuate IL-1β secretion, show great potential as agents for preventing and treating periodontal disease. More specifically, IL-1β may be a promising therapeutic target because it plays a critical role in the initiation of inflammatory immune reactions and is produced for extended periods during inflammatory processes [35].

IL-1β is a major pro-inflammatory cytokine whose local and circulating levels are strictly regulated to avoid uncontrolled activation of pathways that can lead to chronic inflammatory disease [36]. IL-1β accumulates as a biologically inactive 33-kDa precursor (pro-IL-1β) in the cytoplasm of monocytes and macrophages. The biologically active 17-kDa form is produced following a proteolytic maturation by caspase-1 [37,38], which itself is regulated by an assembly of multiprotein complexes called inflammasomes [39,40]. We thus evaluated the effect of tea polyphenols on caspase-1 activation in macrophages. We used flow cytometry to show that the green and black tea extracts as well as their major polyphenols (EGCG and theaflavins) significantly and dose-dependently reduce the activation of caspase-1. Caspase-1 activation induces inflammation through pro-inflammatory cytokine production and recruitment of neutrophils [41]. In addition to its well-known role in the maturation of IL-1β and IL-18, caspase-1 is also capable of activating NF-κB [42].

TNF-α regulates a number of inflammatory signaling pathways in macrophages, including the NF-κB pathway. We used the U937-3xκ B-LUC cell line to show that TNF-α activates the NF-κB signaling pathway, which is a key driver of the pro-inflammatory response. The green and black tea extracts as well as EGCG and theaflavins almost completely prevented TNF-α-induced NF-κB activation. NF-κB is one of the main pathways by which host cells respond to stress and microbial challenges [43]. NF-κB is considered as the central mediator of the immune response and plays an essential role in numerous aspects of human health [44]. In a previous study, we reported that tea polyphenols prevent the activation of NF-κB induced by the periodontopathogen *Fusobacterium nucleatum* [45]. Although there are drawbacks to using an anti-cytokine therapy to reduce inflammation especially an anti-TNF-α therapy [33,46], attenuating the host response may down-regulate chronic inflammation and facilitate the restoration of periodontal health [46]. Such a strategy has been proposed for the treatment of tissue destructive inflammatory diseases such as rheumatoid arthritis [31].

Host-pathogen interactions in the gingival sulcus and periodontal pocket are characterized by an infiltration of immune cells. This creates a pro-inflammatory environment in which cytokines such as TNF-α contribute to the installation of chronic inflammation and tissue destruction. The overproduction of TNF-α may cause damages to the integrity of the epithelial TJ and promote invasion by periodontopathogens. Previous studies have showed that TNF-α plays a key role in the morphological disruption and delocalization of tight junction proteins in intestinal epithelial cells [15,16,47,48]. The TJ barrier includes two functionally pathways: a high-capacity, charge-selective pore pathway that permits the passage of small ions and uncharged molecules, and a low-capacity leak pathway that allows flux of larger molecules, regardless of their charge [49]. In this regard, it was of interest to examine the effect of TNF-α on gingival epithelial TJ integrity by measuring TER, which determines the flux of ions across the epithelium. This is typically done by applying a transepithelial current, measuring the potential generated, and using Ohm's law to calculate the resistance to current flux. These ions do not discriminate between pore and leak pathways and, as such, TER values cannot be used to determine tight junction size or charge selectively. An increase in the permeability of either pathway reduces TER. Using gingival keratinocytes, TNF-α decreased TER while the green tea extract, EGCG, black tea extract, and theaflavins protected the epithelial barrier against this TNF-α-mediated disruption. In intestinal epithelial cells, TNF-α-

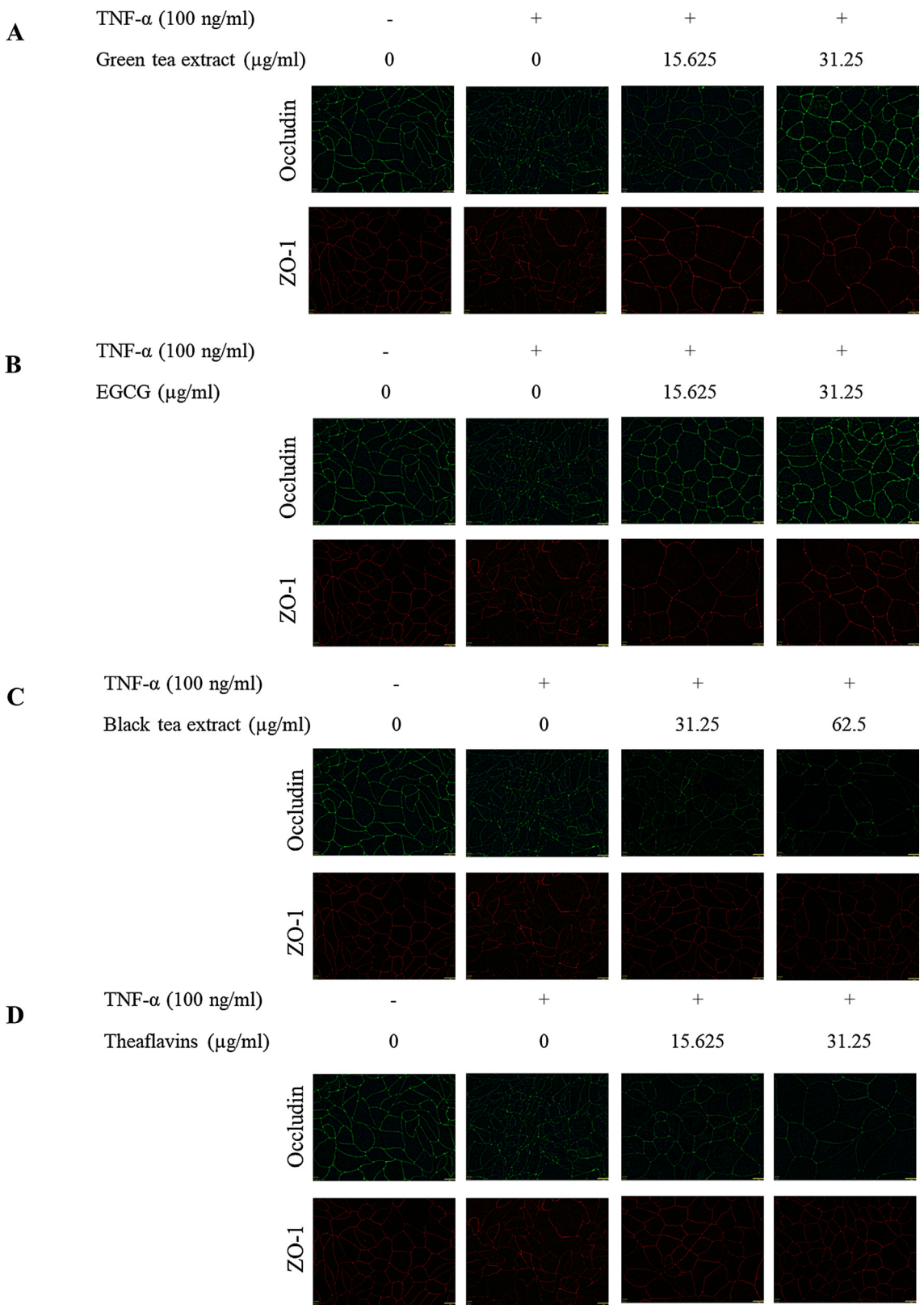

Fig. 6. Immunofluorescence staining of the tight junction proteins occludin and zonula occludens-1 (ZO-1) of gingival keratinocytes treated for 12 h with 100 ng mL^{-1} of TNF-α in the absence and presence of the green tea extract (Panel A), EGCG (Panel B), black tea extract (Panel C), and theaflavins (Panel D).

induced barrier dysfunction can be attributed to a variety of factors, including changes in expression and localization of TJ components such as occludin and ZO-1 [15,50,51]. We thus examined the organization of TJ proteins in gingival keratinocytes stimulated with TNF-α in the presence and absence of tea polyphenols. An immunofluorescence analysis of TJ proteins showed that TNF-α alters the localization of occludin and ZO-1 in gingival keratinocytes. In contrast, the treatment with tea polyphenols largely prevented this TNF-α induced change in

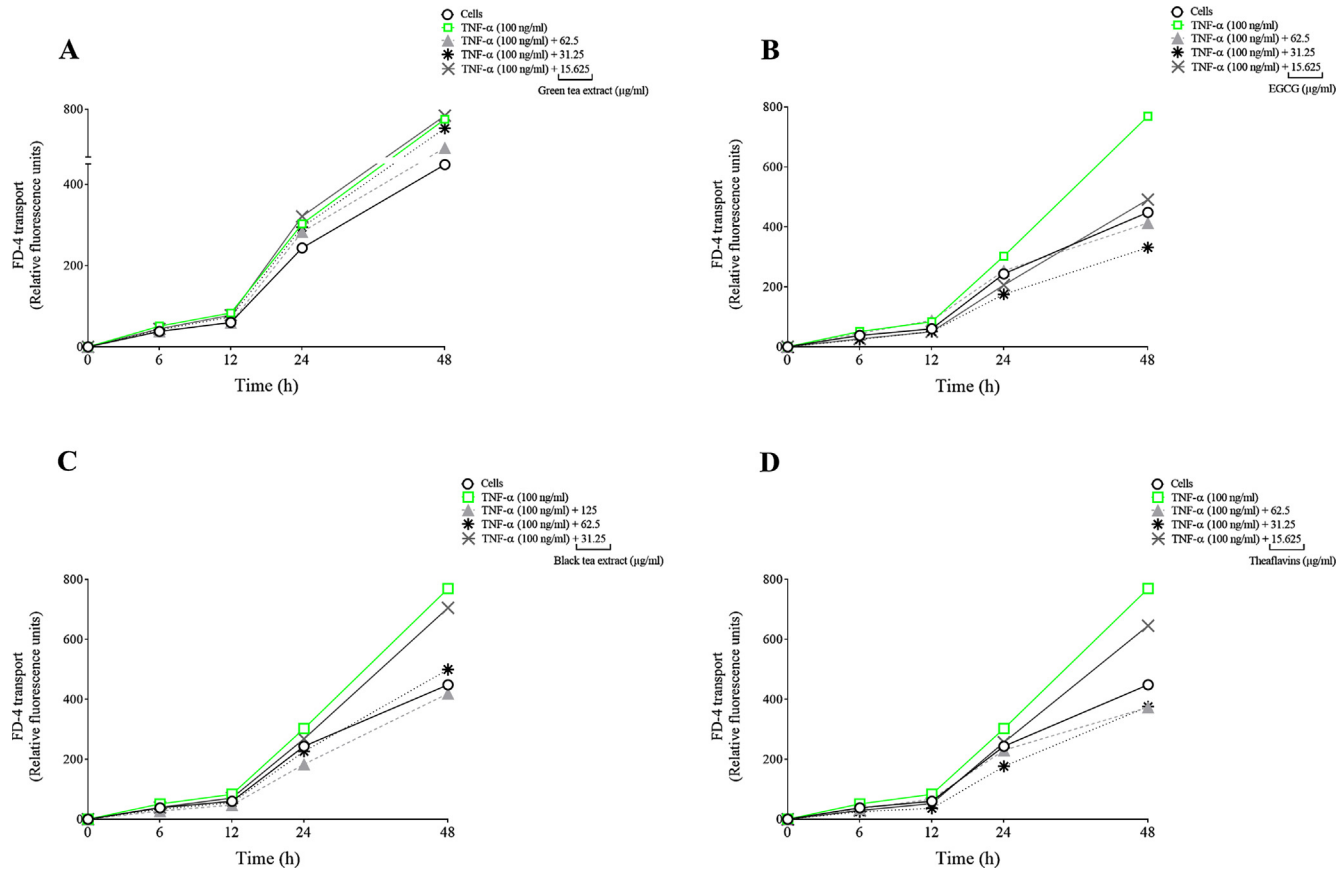

Fig. 7. Effects of the green tea extract (Panel A), EGCG (Panel B), black tea extract (Panel C), and theaflavins (Panel D) on the paracellular permeability of gingival keratinocytes treated with 100 ng mL^{-1} of TNF-α determined by measuring the transport of FITC-dextran 4 (FD-4). Results are expressed as the means ± SD of triplicate assays from three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

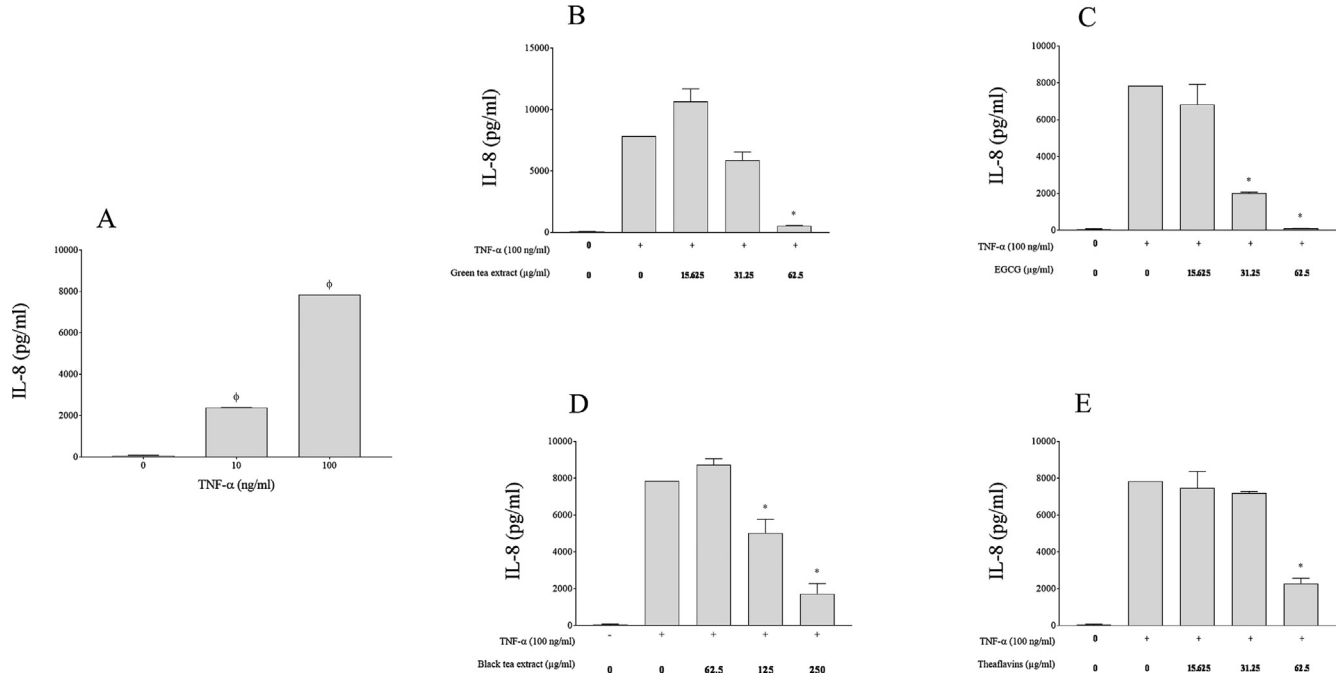

Fig. 8. Effects of TNF-α on the secretion of IL-8 by oral epithelial cells. Stimulation in the absence of tea polyphenols (Panel A). Stimulation in the presence of the green tea extract (Panel B), EGCG (Panel C), black tea extract (Panel D), and theaflavins (Panel E). Results are expressed as the means ± SD of triplicate assays from three independent experiments. (φ) Significant increase ($p < 0.001$) compared to cells not stimulated with TNF-α. (*) Significant decrease ($p < 0.001$) compared to TNF-α-stimulated cells.

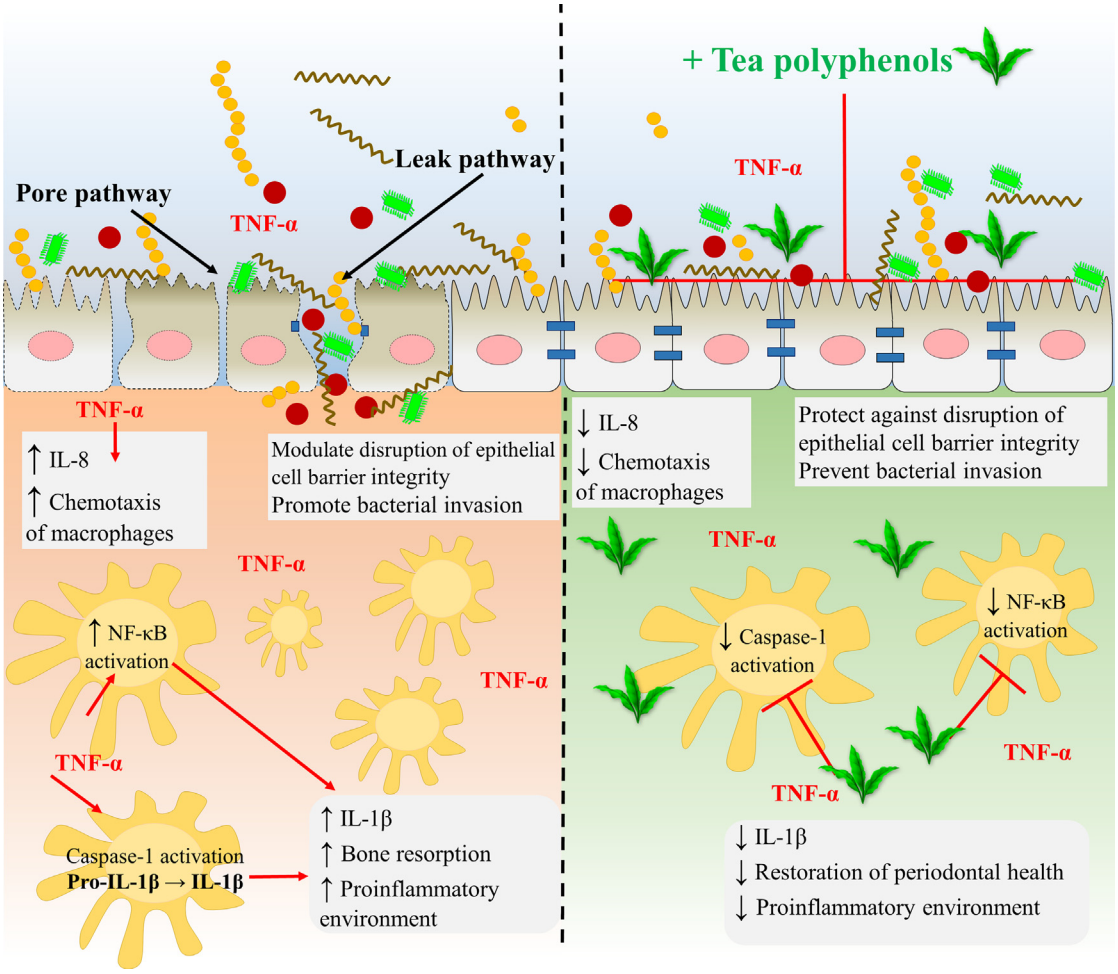

Fig. 9. Proposed protective roles of tea polyphenols in the pathogenesis of periodontal disease associated with an uncontrolled secretion of TNF-α. Tea polyphenols protect against the disruption of epithelial cell barrier induced by TNF-α. This physical protection prevents invasion by periodontopathogens such as *P. gingivalis*, a keystone bacterium largely involved in dysbiosis of the commensal oral microbiota. Moreover, in the presence of TNF-α, tea polyphenols reduce the activation of two major pro-inflammatory signaling pathway NF-κB and caspase-1. This anti-inflammatory effect leads an attenuation of the pro-inflammatory microenvironment and may promote periodontal health.

the morphological distribution of these TJ proteins. On the one hand, ZO-1 is responsible for the link between transmembrane proteins and the cytoskeleton. On the other hand, occludin is a membrane protein with two extracellular loops that interact with ZO-1. These proteins play a key role in the maintenance of the epithelial barrier. Moreover, occludin internalization has been reported to be the main alteration observed following TNF-induced barrier loss in intestinal epithelial cells [52,53]. These two proteins are essential for creating cell-to-cell contacts and in maintaining the epithelial barrier function and the permeability of the paracellular pathway.

We also showed that TNF-α increases the transport of FITC-conjugated 4-kDa dextran in a double-chamber system that mimics paracellular flux. The transport of macromolecular tracers such as dextran across the epithelium can be assessed by measuring the leak pathway. Because of its size, the migration of dextran reflects the leak pathway but not the pore pathway. It has been suggested that ZO-1 and occludin are involved in the maintenance and regulation of the barrier to leak pathway flux [54–56].

In gingival tissue from patients with aggressive periodontitis, high levels of IL-8 expression have been detected in sites with high numbers of PMN cells [57]. The fact that tea polyphenols inhibit IL-8 secretion by epithelial cells suggests that they have the potential to reduce both the accumulation of inflammatory cells in diseased sites and the severity of TNF-α-induced inflammatory processes. Chemokines play a key role in the recruitment of phagocytic cells to sites of infection [58,59]. It has been proposed that IL-8-mediated chemotactic and activation effects on neutrophils largely modulate periodontal tissue destruction [60]. In the case of periodontal disease, attenuation of cytokine secretion may have a major indirect impact given that anti-inflammatory modalities can indirectly exert antimicrobial effects. Indeed, periodontal dysbiosis may be associated with an inflammatory environment since inflammatory tissue breakdown products may serve as nutrients for periodontopathogens [61].

5. Conclusion

Periodontal disease is a current public health problem that compromises a patient's quality of life. According to the World Health Organization, periodontitis affects 10–15% of adult populations worldwide [62]. We provide evidence that TNF-α may exert deleterious effects through the amplification of the inflammatory process and the disruption of the keratinocyte barrier (Fig. 8). We also show that tea polyphenols attenuate the gingival epithelial barrier dysfunction caused by TNF-α and modulate the inflammatory host response (Fig. 9). Given that pathological inflammation, such as that associated with periodontitis, involves a loss of tolerance and/or of regulatory processes, the ability of tea polyphenols to attenuate inflammatory processes suggests that they may be promising preventive or therapeutic agents for use in

anti-cytokine therapies.

Acknowledgements

A. Ben Lagha is a recipient of a scholarship from the Réseau de recherche en santé buccodentaire et osseuse (Fonds de recherche du Québec - Santé). This study was supported by the Laboratoire de Contrôle Microbiologique de l'Université Laval (2018-05-02).

The authors declare that there is no conflict of interest related to this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cyto.2018.12.009.

References

[1] T. Suda, N. Takahashi, N. Udagawa, E. Jimi, M.T. Gillespie, T.J. Martin, Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families, Endocr. Rev. 20 (3) (1999) 345–357.
[2] T. Tervahartiala, H. Koski, J.W. Xu, R. Hayrinen-Immonen, J. Hietanen, T. Sorsa, Y.T. Konttinen, Tumor necrosis factor-alpha and its receptors, p55 and p75, in gingiva of adult periodontitis, J. Dent. Res. 80 (6) (2001) 1535–1539.
[3] Y. Soga, F. Nishimura, H. Ohyama, H. Maeda, S. Takashiba, Y. Murayama, Tumor necrosis factor-alpha gene (TNF-alpha) -1031/-863, -857 single-nucleotide polymorphisms (SNPs) are associated with severe adult periodontitis in Japanese, J. Clin. Periodontol. 30 (6) (2003) 524–531.
[4] C.C. Turer, D. Durmus, U. Balli, B. Guven, Effect of non-surgical periodontal treatment on gingival crevicular fluid and serum endocan, vascular endothelial growth factor-A, and tumor necrosis Factor-alpha levels, J. Periodontol. 88 (5) (2017) 493–501.
[5] Q. Zhang, B. Chen, D. Zhu, F. Yan, Biomarker levels in gingival crevicular fluid of subjects with different periodontal conditions: A cross-sectional study, Arch. Oral Biol. 72 (2016) 92–98.
[6] H. Okada, S. Murakami, Cytokine expression in periodontal health and disease, Crit. Rev. Oral Biol. Med. 9 (3) (1998) 248–266.
[7] V. Bloemen, T. Schoenmaker, T.J. de Vries, V. Everts, IL-1beta favors osteoclastogenesis via supporting human periodontal ligament fibroblasts, J. Cell. Biochem. 112 (7) (2011) 1890–1897.
[8] Y.J. Lo, C.M. Liu, M.Y. Wong, L.T. Hou, W.K. Chang, Interleukin 1beta-secreting cells in inflamed gingival tissue of adult periodontitis patients, Cytokine 11 (8) (1999) 626–633.
[9] U. Zappa, M. Reinking-Zappa, H. Graf, M. Espeland, Cell populations and episodic periodontal attachment loss in humans, J. Clin. Periodontol. 18 (7) (1991) 508–515.
[10] K.V. Anderson, Toll signaling pathways in the innate immune response, Curr. Opin. Immunol. 12 (1) (2000) 13–19.
[11] G.P. Garlet, Destructive and protective roles of cytokines in periodontitis: a re-appraisal from host defense and tissue destruction viewpoints, J. Dent. Res. 89 (12) (2010) 1349–1363.
[12] H. Hasturk, A. Kantarci, T.E. Van Dyke, Oral inflammatory diseases and systemic inflammation: role of the macrophage, Front. Immunol. 3 (2012) 118.
[13] B.A. Dale, Periodontal epithelium: a newly recognized role in health and disease, Periodontol. 2000 (30) (2002) 70–78.
[14] S.E. Groeger, J. Meyle, Epithelial barrier and oral bacterial infection, Periodontology 2000 69 (1) (2015) 46–67.
[15] W. Cui, L.X. Li, C.M. Sun, Y. Wen, Y. Zhou, Y.L. Dong, P. Liu, Tumor necrosis factor alpha increases epithelial barrier permeability by disrupting tight junctions in Caco-2 cells, Braz. J. Med. Biol. Res. 43 (4) (2010) 330–337.
[16] F. He, J. Peng, X.L. Deng, L.F. Yang, A.D. Camara, A. Omran, G.L. Wang, L.W. Wu, C.L. Zhang, F. Yin, Mechanisms of tumor necrosis factor-alpha-induced leaks in intestine epithelial barrier, Cytokine 59 (2) (2012) 264–272.
[17] W.V. Giannobile, Host-response therapeutics for periodontal diseases, J. Periodontol. 79 (8 Suppl.) (2008) 1592–1600.
[18] P.M. Preshaw, Host response modulation in periodontics, Periodontology 2000 (48) (2008) 92–110.
[19] H. El Gharras, Polyphenols: food sources, properties and applications a review, Int. J. Food Sci. Tech. 44 (12) (2009) 2512–2518.
[20] H.N. Graham, Green tea composition, consumption, and polyphenol chemistry, Prev. Med. 21 (3) (1992) 334–350.
[21] J.V. Higdon, B. Frei, Tea catechins and polyphenols: health effects, metabolism, and antioxidant functions, Crit. Rev. Food Sci. Nutr. 43 (1) (2003) 89–143.
[22] H. Chen, K. Shurlknight, T. Leung, S. Sang, Structural identification of theaflavin trigallate and tetragallate from black tea using liquid chromatography/electrospray ionization tandem mass spectrometry, J. Agric. Food Chem. 60 (43) (2012) 10850–10857.
[23] G. Rovera, D. Santoli, C. Damsky, Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with a phorbol diester, Proc. Natl. Acad. Sci. USA 76 (6) (1979) 2779–2783.
[24] H. Carlsen, J.O. Moskaug, S.H. Fromm, R. Blomhoff, In vivo imaging of NF-kappa B activity, J. Immunol. 168 (3) (2002) 1441–1446.
[25] S. Groger, J. Michel, J. Meyle, Establishment and characterization of immortalized human gingival keratinocyte cell lines, J. Periodontal. Res. 43 (6) (2008) 604–614.
[26] B. Gumbiner, K. Simons, A functional assay for proteins involved in establishing an epithelial occluding barrier: identification of a uvomorulin-like polypeptide, J. Cell Biol. 102 (2) (1986) 457–468.
[27] E.P. Gilchrist, M.P. Moyer, E.J. Shillitoe, N. Clare, V.A. Murrah, Establishment of a human polyclonal oral epithelial cell line, Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 90 (3) (2000) 340–347.
[28] Z.L. Jiang, Y.Q. Cui, R. Gao, Y. Li, Z.C. Fu, B. Zhang, C.C. Guan, Study of TNF-alpha, IL-1beta and LPS levels in the gingival crevicular fluid of a rat model of diabetes mellitus and periodontitis, Dis. Markers 34 (5) (2013) 295–304.
[29] N. Parameswaran, S. Patial, Tumor necrosis factor-alpha signaling in macrophages, Crit. Rev. Eukaryot. Gene Expr. 20 (2) (2010) 87–103.
[30] Y. Mayer, A. Balbir-Gurman, E.E. Machtei, Anti-tumor necrosis factor-alpha therapy and periodontal parameters in patients with rheumatoid arthritis, J. Periodontol. 80 (9) (2009) 1414–1420.
[31] R.N. Maini, M. Feldmann, Cytokine therapy in rheumatoid arthritis, Lancet 348 (9030) (1996) 824–825.
[32] C.Y. Chiang, G. Kyritsis, D.T. Graves, S. Amar, Interleukin-1 and tumor necrosis factor activities partially account for calvarial bone resorption induced by local injection of lipopolysaccharide, Infect. Immun. 67 (8) (1999) 4231–4236.
[33] A.J. Delima, T. Oates, R. Assuma, Z. Schwartz, D. Cochran, S. Amar, D.T. Graves, Soluble antagonists to interleukin-1 (IL-1) and tumor necrosis factor (TNF) inhibits loss of tissue attachment in experimental periodontitis, J. Clin. Periodontol. 28 (3) (2001) 233–240.
[34] D.T. Graves, A.J. Delima, R. Assuma, S. Amar, T. Oates, D. Cochran, Interleukin-1 and tumor necrosis factor antagonists inhibit the progression of inflammatory cell infiltration toward alveolar bone in experimental periodontitis, J. Periodontol. 69 (12) (1998) 1419–1425.
[35] S. Takashiba, K. Naruishi, Y. Murayama, Perspective of cytokine regulation for periodontal treatment: fibroblast biology, J. Periodontol. 74 (1) (2003) 103–110.
[36] C.A. Dinarello, Biologic basis for interleukin-1 in disease, Blood 87 (6) (1996) 2095–2147.
[37] D.K. Miller, J.M. Ayala, L.A. Egger, S.M. Raju, T.T. Yamin, G.J. Ding, E.P. Gaffney, A.D. Howard, O.C. Palyha, A.M. Rolando, et al., Purification and characterization of active human interleukin-1 beta-converting enzyme from THP.1 monocytic cells, J. Biol. Chem. 268 (24) (1993) 18062–18069.
[38] P. Li, H. Allen, S. Banerjee, S. Franklin, L. Herzog, C. Johnston, J. McDowell, M. Paskind, L. Rodman, J. Salfeld, et al., Mice deficient in IL-1 beta-converting enzyme are defective in production of mature IL-1 beta and resistant to endotoxic shock, Cell 80 (3) (1995) 401–411.
[39] F. Martinon, K. Burns, J. Tschopp, The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta, Mol. Cell 10 (2) (2002) 417–426.
[40] Y. Ogura, F.S. Sutterwala, R.A. Flavell, The inflammasome: first line of the immune response to cell stress, Cell 126 (4) (2006) 659–662.
[41] S. Faubel, E.C. Lewis, L. Reznikov, D. Ljubanovic, T.S. Hoke, H. Somerset, D.J. Oh, L. Lu, C.L. Klein, C.A. Dinarello, C.L. Edelstein, Cisplatin-induced acute renal failure is associated with an increase in the cytokines interleukin (IL)-1beta, IL-18, IL-6, and neutrophil infiltration in the kidney, J. Pharmacol. Exp. Ther. 322 (1) (2007) 8–15.
[42] M. Lamkanfi, M. Kalai, X. Saelens, W. Declercq, P. Vandenabeele, Caspase-1 activates nuclear factor of the kappa-enhancer in B cells independently of its enzymatic activity, J. Biol. Chem. 279 (23) (2004) 24785–24793.
[43] M.M. Rahman, G. McFadden, Modulation of NF-kappaB signalling by microbial pathogens, Nat. Rev. Microbiol. 9 (4) (2011) 291–306.
[44] S.C. Gupta, C. Sundaram, S. Reuter, B.B. Aggarwal, Inhibiting NF-kappaB activation by small molecules as a therapeutic strategy, Biochim. Biophys. Acta 1799 (10–12) (2010) 775–787.
[45] A.B. Lagha, D. Grenier, Tea polyphenols inhibit the activation of NF-kappaB and the secretion of cytokines and matrix metalloproteinases by macrophages stimulated with *Fusobacterium nucleatum*, Sci. Rep. 6 (2016) 34520.
[46] R. Assuma, T. Oates, D. Cochran, S. Amar, D.T. Graves, IL-1 and TNF antagonists inhibit the inflammatory response and bone loss in experimental periodontitis, J. Immunol. 160 (1) (1998) 403–409.
[47] T.Y. Ma, M.A. Boivin, D. Ye, A. Pedram, H.M. Said, Mechanism of TNF-{alpha} modulation of Caco-2 intestinal epithelial tight junction barrier: role of myosin light-chain kinase protein expression, Am. J. Physiol. Gastrointest. Liver Physiol. 288 (3) (2005) G422–G430.
[48] D. Ye, I. Ma, T.Y. Ma, Molecular mechanism of tumor necrosis factor-alpha modulation of intestinal epithelial tight junction barrier, Am. J. Physiol. Gastrointest. Liver Physiol. 290 (3) (2006) G496–G504.
[49] L. Shen, C.R. Weber, D.R. Raleigh, D. Yu, J.R. Turner, Tight junction pore and leak pathways: a dynamic duo, Annu. Rev. Physiol. 73 (2011) 283–309.
[50] M. Amasheh, A. Fromm, S.M. Krug, S. Amasheh, S. Andres, M. Zeitz, M. Fromm, J.D. Schulzke, TNFalpha-induced and berberine-antagonized tight junction barrier impairment via tyrosine kinase, Akt and NFkappaB signaling, J. Cell Sci. 123 (Pt 23) (2010) 4145–4155.
[51] T.Y. Ma, G.K. Iwamoto, N.T. Hoa, V. Akotia, A. Pedram, M.A. Boivin, H.M. Said, TNF-alpha-induced increase in intestinal epithelial tight junction permeability requires NF-kappa B activation, Am. J. Physiol Gastrointest. Liver Physiol. 286 (3) (2004) G367–G376.
[52] D.R. Clayburgh, T.A. Barrett, Y. Tang, J.B. Meddings, L.J. Van Eldik, D.M. Watterson, L.L. Clarke, R.J. Mrsny, J.R. Turner, Epithelial myosin light chain

kinase-dependent barrier dysfunction mediates T cell activation-induced diarrhea in vivo, J. Clin. Invest. 115 (10) (2005) 2702–2715.
[53] D.R. Clayburgh, M.W. Musch, M. Leitges, Y.X. Fu, J.R. Turner, Coordinated epithelial NHE3 inhibition and barrier dysfunction are required for TNF-mediated diarrhea in vivo, J. Clin. Invest. 116 (10) (2006) 2682–2694.
[54] A.S. Yu, K.M. McCarthy, S.A. Francis, J.M. McCormack, J. Lai, R.A. Rogers, R.D. Lynch, E.E. Schneeberger, Knockdown of occludin expression leads to diverse phenotypic alterations in epithelial cells, Am. J. Physiol. Cell Physiol. 288 (6) (2005) C1231–C1241.
[55] C.M. Van Itallie, A.S. Fanning, J. Holmes, J.M. Anderson, Occludin is required for cytokine-induced regulation of tight junction barriers, J. Cell Sci. 123 (Pt 16) (2010) 2844–2852.
[56] A.M. Marchiando, L. Shen, W.V. Graham, C.R. Weber, B.T. Schwarz, J.R. Austin 2nd, D.R. Raleigh, Y. Guan, A.J. Watson, M.H. Montrose, J.R. Turner, Caveolin-1-dependent occludin endocytosis is required for TNF-induced tight junction regulation in vivo, J. Cell Biol. 189 (1) (2010) 111–126.
[57] R.K. Liu, C.F. Cao, H.X. Meng, Y. Gao, Polymorphonuclear neutrophils and their mediators in gingival tissues from generalized aggressive periodontitis, J. Periodontol. 72 (11) (2001) 1545–1553.
[58] S.A. Luther, J.G. Cyster, Chemokines as regulators of T cell differentiation, Nat. Immunol. 2 (2) (2001) 102–107.
[59] C. Gerard, B.J. Rollins, Chemokines and disease, Nat. Immunol. 2 (2) (2001) 108–115.
[60] D.A. Scott, J. Krauss, Neutrophils in periodontal inflammation, Front. Oral Biol. 15 (2012) 56–83.
[61] G. Hajishengallis, Immunomicrobial pathogenesis of periodontitis: keystones, pathobionts, and host response, Trends Immunol. 35 (1) (2014) 3–11.
[62] P.E. Petersen, H. Ogawa, The global burden of periodontal disease: towards integration with chronic disease prevention and control, Periodontology 60 (1) (2000 2012,) 15–39.