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ORIGINAL ARTICLE

Topical curcumin can inhibit deleterious effects of upper respiratory tract bacteria on human oropharyngeal cells in vitro: potential role for patients with cancer therapy induced mucositis?

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Abstract
Purpose Curcumin exerts its anti-inflammatory activity via inhibition of nuclear factor κB. Oropharyngeal epithelia and residing bacteria closely interact in inflammation and infection. This in vitro model investigated the effects of curcumin on bacterial survival, adherence to, and invasion of upper respiratory tract epithelia, and studied its anti-inflammatory effect. We aimed to establish a model, which could offer insights into the host–pathogen interaction in cancer therapy induced mucositis.
Methods *Moraxella catarrhalis* (*Mcat*) and the oropharyngeal epithelial cell line Detroit 562 were used. Time–kill curves assessed the inhibition of bacterial growth and adherence assays and gentamicin protection assays determined the effect of curcumin-preincubated cells on bacterial adherence and invasion. Curcumin-mediated inhibition of pro-inflammatory activation by *Mcat* was determined via interleukin-8 concentrations in the supernatants. The synergistic role of secretory IgA (sIgA) on adherence was investigated.
Results Curcumin was bactericidal at concentrations >50 µM. Preincubation of Detroit cells for 60 min demonstrated that concentrations >100 µM inhibited bacterial adherence. Together with sIgA, curcumin inhibited adherence at concentrations ≥50 µM. Both 100 and 200 µM curcumin significantly inhibited *Mcat* cell invasion. Finally, curcumin inhibited *Mcat*-induced pro-inflammatory activation by strongly suppressing IL-8 release. At a concentration of 200 µM, 10 min of curcumin exposure inhibited IL-8 release significantly, and complete suppression required a pre-exposure time of ≥45 min.
Conclusion Curcumin, in clinically relevant concentrations for topical use, displayed strong antibacterial effect against a facultative upper respiratory tract pathogen by inhibiting bacterial growth, adherence, invasion, and pro-inflammatory activation of upper respiratory tract epithelial cells in vitro.

Keywords Curcumin · Bacterial adherence · Bacterial invasion · Interleukin-8 · Otitis media · Mucositis

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Introduction

Upper respiratory tract mucosae are physiologically colonized by numerous microorganisms, with which they live in mutual, non-inflammatory coexistence. Cancer therapy-induced mucosal surface injury deranges this delicate balance and leads to inflammation, local tissue damage, and ultimately, microbial invasion. In this study, we tested topical curcumin for its capacity to inhibit bacteria-induced deleterious effects on human upper respiratory tract epithelial cells in vitro. As curcumin (diferuloylmethane), the yellow pigment of the curry spice and major component of turmeric (*Curcuma longa* Linn.), has been used in traditional medicine for centuries, its anti-inflammatory properties have evoked major scientific interest in the recent past as light was shed on its molecular actions, e.g., as a potent inhibitor of nuclear factor κB (NF-κB). We used *Moraxella catarrhalis (Mcat)*, a Gram-negative aerobic

bacterium, which is a major, exclusively human, facultative pathogen occupying the oro- and nasopharyneal niche. *Mcat* accounts for common infections throughout the entire life: acute otitis media and sinusitis in childhood [21, 26], as well as bronchitis and exacerbations of chronic obstructive pulmonary diseases (COPD) in the elderly [20]. Cumulative nasopharyngeal colonization rates with *Mcat* reach up to 80% in early childhood [9], and *Mcat* carriage may even be underestimated due to its ability to invade respiratory cells and submucosal tissue and thus escape detection by respiratory surface swabbing [13, 30].

As human respiratory tract epithelia form a large surface, they are constantly in contact with a variety of (facultative) pathogens. Intact mucosal surfaces and defense mechanisms essentially contribute to pathogen control and facilitate non-inflammatory coexistence. Rapid growth, adherence to, and penetration of epithelial cells, on the other hand, may herald the initiation of bacterial infection [22, 24] as bacteria are capable of triggering inflammatory cell responses in respiratory epithelia via, e.g., NF-κB activation and subsequent interleukin-8 (IL-8) release [28, 29, 33]. IL-8 plays a critical role in neutrophil chemotaxis toward sites of infection as well as recruitment to chronic inflammatory sites in COPD patients [15].Not only in infection but also in the onset of mucosal barrier injury, which precedes oral mucositis, the NF-κB pathway of cytokine release plays a critical role. Thus, the large patient group receiving anti-cancer treatment comes into consideration: cytotoxic chemotherapy and irradiation cause DNA damage, which itself can activate NF-κB [6, 31] and the subsequent release of pro-inflammatory cytokines, adhesion molecules, and cyclooxygenase-2 [7, 18]. Damaged mucosal barriers cannot protect against the resident flora, and (systemic) infection may ensue. For instance, the risk of infection in chemotherapy-induced neutropenia was shown to be more than doubled in patients with mucositis, with the risk of infection being proportional to the severity of mucositis [8].

Inhibition of NF-κB thus could offer a tool to reduce bacteria-induced pro-inflammatory epithelial cell responses and, at the same time, provide local protection against NF-κB mediated mucosal barrier injury in the early stages [18] of mucositis in anti-cancer treatment. The curcumin modulates various cell signaling pathways and is a potent inhibitor of cytoplasmic NF-κB activation and subsequent cytokine release [16, 27, 35]. Inactive NF-κB is bound to an inhibitory molecule, IκBα, and is restrained to the cytoplasm. As a response to extracellular stimuli, IκB kinase (IKK) complex phosphorylates IκBα. Phosphorylated IκBα is degraded and consecutively releases active NF-κB, which can then translate to the nucleus, where it binds to specific DNA sequences resulting in transcriptional activation of several genes [4]. This signal cascade induces inflammatory cell responses, which can be blocked by curcumin [16, 35]. It has also been demonstrated that NF-κB stimulates CEACAM-1 expression in epithelial cells [19], a molecule used by *Mcat* and other pharyngeal pathogens to adhere to and invade these cells.

We hypothesized that the inhibition of NF-κB by curcumin may reduce the adherence of bacteria to human respiratory tract epithelia. Thus, the current study addressed the effect of curcumin on growth, adherence, and invasion of bacteria to human upper respiratory tract cells in an in vitro model with *Mcat* as a representative of the human pharyngeal flora.

Methods

Bacterial strains, cell lines, and culture conditions The *Mcat* strain 25238 was purchased from the American Type Culture Collection (ATCC). The laboratory strain O35E is a middle ear isolate from a child with otitis media [14]. Bacteria were cultured on brain–heart infusion (BHI) agar plates (Difco, Detroit, MI, USA) at 37°C in an 5% CO_2 atmosphere or in BHI broth at 37°C and 200 rpm. In some experiments, bacteria were heat-inactivated by re-suspension of live bacteria in phosphate-buffered saline (PBS) and incubation at 60°C for 60 min. The human pharyngeal cell line Detroit 562 (ATCC CCL 138) was maintained in Eagle's minimal essential medium (MEM; Invitrogen, Basel, Switzerland) supplemented with 10% of heat-inactivated fetal calf serum (FCS), 2 mM of L-glutamine, 1 mM sodium pyruvate (Sigma, St. Louis, MO, USA), 1× nonessential amino acids (Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2.

Reagents Curcumin (diferuloylmethane) was purchased from Sigma (no. C1386). For use in experimental procedures, curcumin was solubilized in dimethyl sulfoxide (DMSO) (stock solution, 73.678 mg/ml, i.e., 200 mM) and added to cell culture medium.

Autoagglutination assay Bacteria were cultured overnight on BHI plates, scraped from the plates, and re-suspended in 10 ml of PBS or PBS with 100 or 200 μM curcumin, respectively, to reach an OD_{600} of 2.0. The suspensions were left in glass tubes at room temperature or at 37°C. The OD_{600} was measured from 1-ml aliquots taken from the top of the suspensions after 0, 15, 30, 45, and 60 min.

Time–kill analysis of Mcat exposed to curcumin Bacteria were grown in BHI broth to an OD_{600} of 0.4 (~5×10^7 cfu/ml), aliquoted, and subsequently grown in BHI supplemented with 0, 20, 50, or 100 μM curcumin, respectively. Growth in DMSO was used as control. Quantitative

cultures were obtained by serial plating of 100-μl aliquots at 0, 60, 120, 180, and 240 min, respectively.

Expression of outer membrane proteins (OMP) by bacteria exposed to curcumin OMP preparations of the strains 25238 and O35E exposed to 0 or 20 μM curcumin were prepared by the EDTA buffer method [3], resolved by SDS-PAGE (7.5% polyacrylamide), and electrotransferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore Corporation, Bedford, MA, USA). Immunoblot analysis was performed using the monoclonal antibodies 24B5 (specific for UspA1) [2] and 10F3 (specific for CopB) [14], diluted 1:4, as respective primary antibodies, and a 1:4,000 diluted goat anti-mouse IgG labeled with horseradish peroxidase (Sigma). SuperSignal West Pico chemiluminescent substrate (Pierce Chemical Co., Rockford, IL, USA) was used for detection of antibody binding.

Epithelial cell adherence assays The ability of *Mcat* to adhere to human epithelial cells in vitro was measured as previously described [1] with the following modifications. Epithelial cells (~2.5×10^5 per well) grown to a confluent monolayer in 24-well tissue culture plates were exposed to various concentrations of curcumin (0–200 μM) for 60 min in MEM supplemented with 10% FCS followed by washing three times in MEM. Bacteria were grown overnight and adjusted to the appropriate multiplicities of infection (MOI). In some experiments, bacteria were also incubated with human colostrum secretory IgA (sIgA) (Sigma), incubated for 20 min at 37°C, centrifuged at 150 rpm for 5 min, and resuspended in MEM. Bacteria were added to tissue culture wells in tissue culture medium without antibiotics, centrifuged for 5 min at 1500 rpm, and subsequently incubated for 30 min at 37°C. Wells were then washed five times in MEM and trypsinized, and the suspensions were cultured quantitatively to determine the number of adherent bacteria. Data were expressed as the proportion of bacteria of the original inoculum adhering to the epithelial cells. Each strain was analyzed in triplicate, and at least three experiments were performed. Cell viability was ascertained morphologically and by trypan blue exclusion. Curcumin and/or bacteria-induced apoptosis was ruled out by fixation and staining of cells post-treatment with the cleaved caspase 3 antibody Asp 175 (Cell Signalling Technology, Inc., Danvers, MA, USA), followed by secondary staining with goat-anti-rabbit Cy3 (Dianova GmbH, Hamburg, Germany) and examination using fluorescent microscopy. To investigate whether pre-incubation of cells with curcumin is essential to achieve an inhibition of adherence, bacteria were re-suspended in MEM containing 0, 25, or 50 μM curcumin with or without sIgA (100 μg/ml) and were then directly used to infect cellular monolayers and perform adherence assays as described above.

Epithelial cell invasion assays Bacterial invasion was estimated using a gentamicin protection assay as previously described [32] with the following modifications. Cells were prepared in medium without antibiotics and subsequently exposed to curcumin as described for the adherence assays. After washing, bacteria were added at the indicated MOI, centrifuged for 5 min at 1500 rpm, and incubated for 3 h at 37°C in 5% CO_2. To determine the number of intracellular bacteria, the infected monolayer was washed three times in PBS and treated with gentamicin sulfate (200 μg/ml) for 2 h at 37°C. After washing, cells were detached from the plastic surface by treatment with 0.25% trypsin-EDTA, lysed by the addition of 1% saponin, and serially diluted in PBS for quantitative bacterial culture. Invasion ratios were calculated by dividing the number of colony-forming units recovered after gentamicin exposure by the number of colony-forming units inoculated.

Determination of interleukin-8 (IL-8) secretion by Detroit cells stimulated with Mcat Monolayers were prepared, pre-incubated with curcumin at various concentrations, and infected at the indicated MOI as described. For 4-h experiments, live bacteria were used. For 16-h experiments, bacteria were heat-inactivated. Negative controls consisted of medium without bacteria. Ten microgram per milliliter of lipopolysaccharide from *Salmonella enteritidis* (Sigma) was used as positive control. Supernatants were collected, centrifuged, and stored at −80°C. IL-8 was determined using a commercially available ELISA kit according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). In an experiment designed to investigate the effect of pre-incubation of bacteria with sIgA on IL-8 release, heat-inactivated bacteria were pre-incubated in MEM containing 10% FCS with or without sIgA (100 μg/ml) for 20 min at 37°C, centrifuged at 150 rpm for 5 min, and resuspended in MEM before infection of monolayers. IL-8 release was determined after 4 h as described.

Statistics The GraphPad Prism® version 5.02 statistics tools (GraphPad Software, La Jolla, CA, USA) were used for analysis of variance (ANOVA) and Student's *t* tests as indicated.

Results

Curcumin inhibits growth of Mcat in vitro Four-hour time–kill analyses of the strains 25238 and 035E, respectively (Fig. 1a, b) demonstrated that concentrations of curcumin >50 μM were bactericidal and that O35E was more susceptible than 25238. Curcumin at concentrations up to 200 μM did not affect the capacity of *Mcat* to autoag-

glutinate (data not shown). This was an important finding with respect to the reproducibility of quantitative cultures. Growth of both strains in a medium containing 20 μM curcumin did not appear to affect the organism's outer membrane profile and its expression of the UspA1 adhesin and the iron-regulated CopB protein (Fig. 1c).

Curcumin inhibits the adherence of Mcat to human pharyngeal cells Adherence assays [1] were performed to assess whether pre-incubation of Detroit cells with various concentrations of curcumin for 60 min preceding the assay had an effect on the adherence of *Mcat* to the cellular surface. Concentrations ≤50 μM had no effect and are not shown. However, at 100 and 200 μM, respectively, adherence of both strain 25238 (Fig. 2a) and O35E (Fig. 2b) was significantly reduced. In this setting, curcumin was washed off from the monolayers before infection with live bacteria. Thus, the quantitative bacterial cultures used to determine the proportion of adherent bacteria was not affected by the bactericidal effect of curcumin.

Curcumin enhances the inhibitory effect of sIgA on bacterial adherence In an experimental setup more closely mimicking the pharyngeal surface environment and the potential therapeutic use of curcumin, bacteria, sIgA (100 μg/ml) and curcumin (50 μM in order to maintain bacterial viability) were pre-incubated in tissue culture

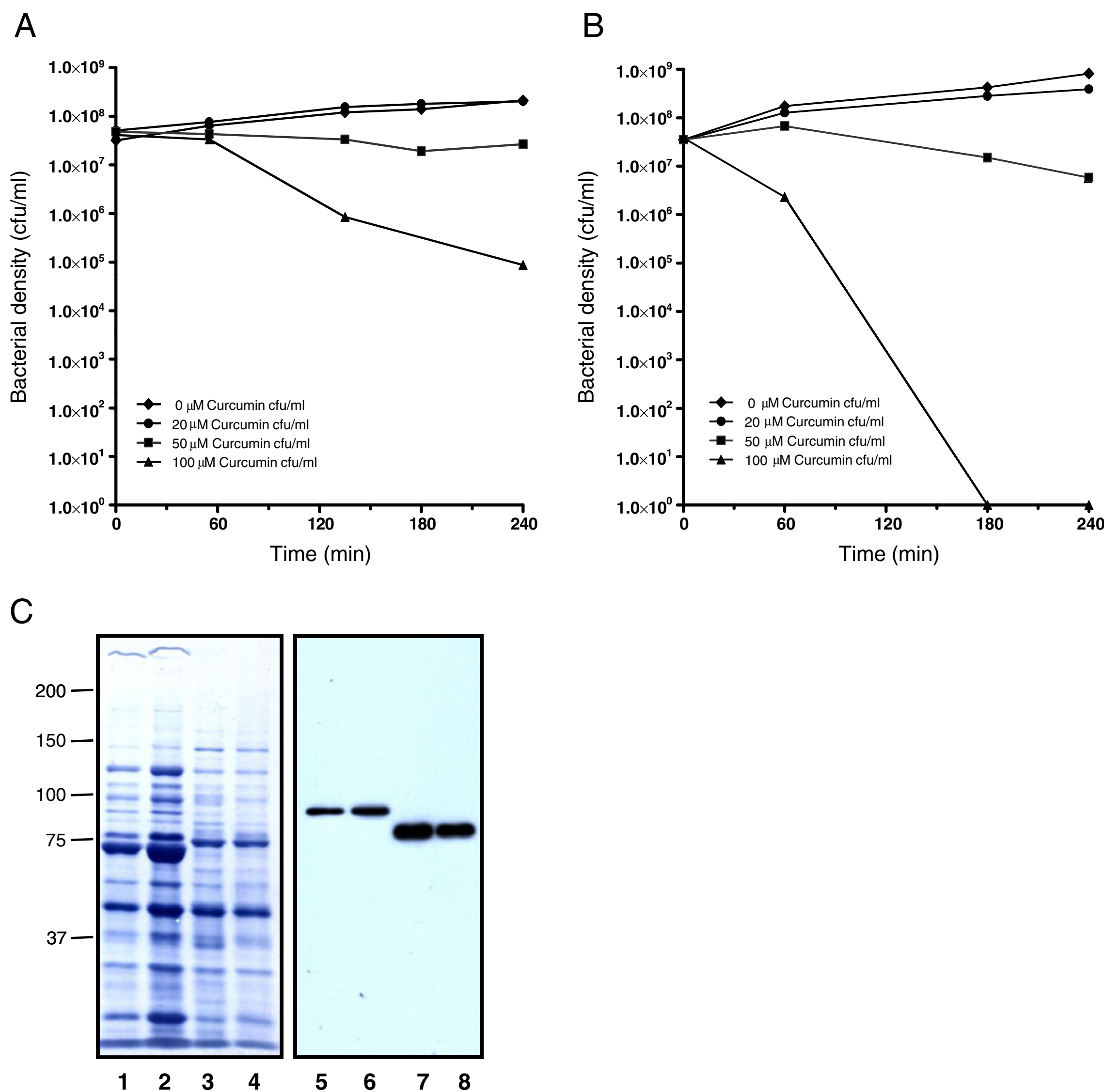

Fig. 1 Four-hour time–kill analysis of strains 25238 (**a**) and O35E (**b**), respectively, exposed to various concentrations of curcumin (*diamond* 0 μM; *circle* 20 μM; *square* 50 μM; *triangle* 100 μM). Subpanel **c** (*left*) demonstrates Commassie blue-stained OMP profiles of strains O35E and 25238 grown in BHI (*lanes 1* and *3*) or in BHI supplemented with 20 μM curcumin (*lanes 2* and *4*). Immunoblots with mAb 24B5 (035E, *lane 5*; 25238, *lane 6*) and mAb 10F3 (035E, *lane 7*; 25238, *lane 8*) are shown on the right

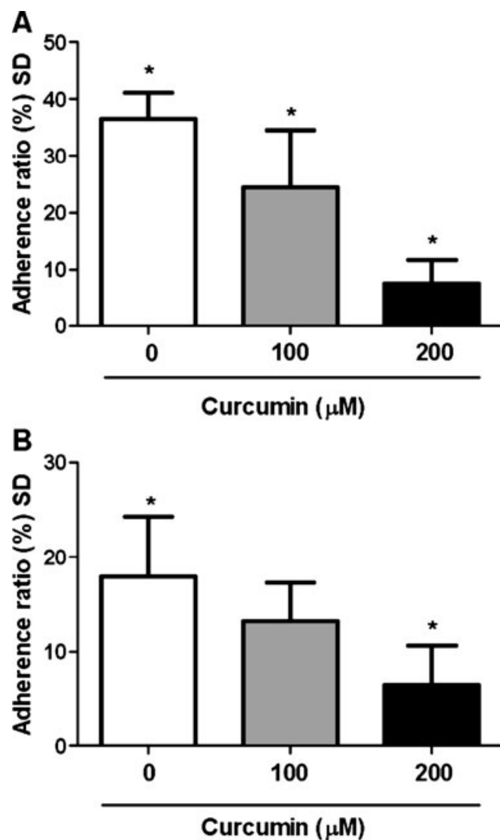

Fig. 2 *M. catarrhalis* adherence assay. After pre-incubation of Detroit cells for 60 min with curcumin at the indicated concentrations, bacteria (**a**, strain 25238; **b**, strain O35E) were inoculated at an MOI of 30 and incubated for 30 min at 37°C. Non-adherent bacteria were removed, and adherent cells were determined by quantitative culture of trypsinized cells. Statistics: **a** overall one-way ANOVA $p<0.0001$, *$p<0.05$ using Bonferroni's multiple comparisons test. **b** Overall one-way ANOVA $p=0.0002$, *$p<0.05$. Mean adherence ratios +1 SD of three independent experiments, each run in triplicate, are shown

medium for 30 min. Subsequently, bacteria (strain 25238) were centrifuged and resuspended at an MOI of 30, and a standard adherence assay was performed. Figure 3 demonstrates that sIgA significantly inhibited adherence when combined with 50 μM curcumin, while both sIgA and 50 μM curcumin alone were less effective and did not reduce adherence significantly. Thus, low-dose curcumin added to the inhibitory effect of sIgA.

Curcumin inhibits invasion of Mcat into Detroit cells Mcat is capable to invade epithelial cells [30, 32]. Here, we demonstrate (Fig. 4) that pre-incubation of Detroit cells with both 100 or 200 μM curcumin for 60 min resulted in a significant inhibition of cellular invasion as determined using a standard gentamicin protection assay [32].

Curcumin inhibits IL-8 secretion by Detroit cells Preliminary data demonstrated that pre-incubation of Detroit cells with ≥ 100 μM curcumin for 60 min inhibited IL-8 secretion

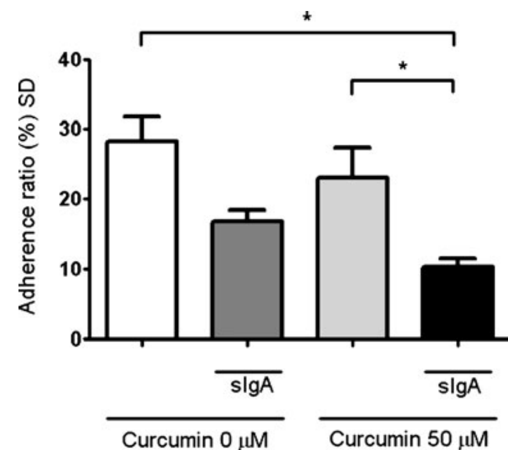

Fig. 3 *M. catarrhalis* adherence assay. Before infection of Detroit, strain 25238 was pre-incubated with sIgA (100 μg/ml) and/or 50 μM curcumin for 20 min at 37°C. Pre-treated bacteria and controls were then used to infect the monolayer, and a standard adherence assay was performed. Statistical analysis was performed using one-way ANOVA, *$p<0.05$. Mean adherence ratios +1 SD of three independent experiments, each run in triplicate, are shown

induced by subsequent infection of cells with live strain 25238 for 4 h (data not shown). Next, we determined the duration of pre-incubation time needed for curcumin to achieve maximum inhibition of IL-8 secretion. While 10–60 min of exposure to 100 μM curcumin resulted in an approximately 50% reduction of IL-8 secretion (Fig. 5), 200 μM curcumin was associated with complete inhibition. A period as short 10 min resulted in a significant reduction of IL-8 secretion ($p=0.002$, two-tailed t test), but complete inhibition required an exposure time of 45–60 min.

Duration of inhibition of IL-8 secretion induced by curcumin Subsequently, we examined the duration of

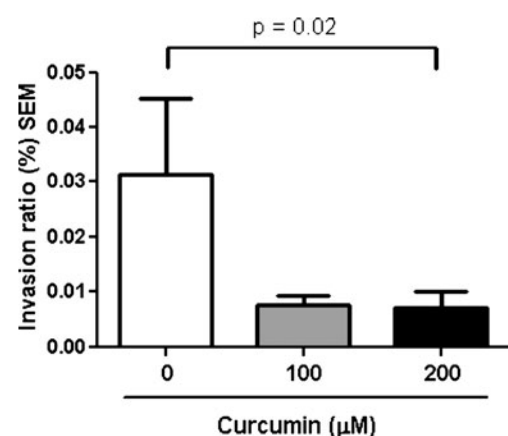

Fig. 4 Determination of Detroit cell invasion by strain 25238 using a gentamicin protection assay. Cells were pre-treated with 0, 100, or 200 μM of curcumin for 1 h. Following 3 h of infection, extracellular bacteria were killed by exposure to gentamicin for 2 h. Following cell lysis, quantitative bacterial cultures identified viable intracellular bacteria

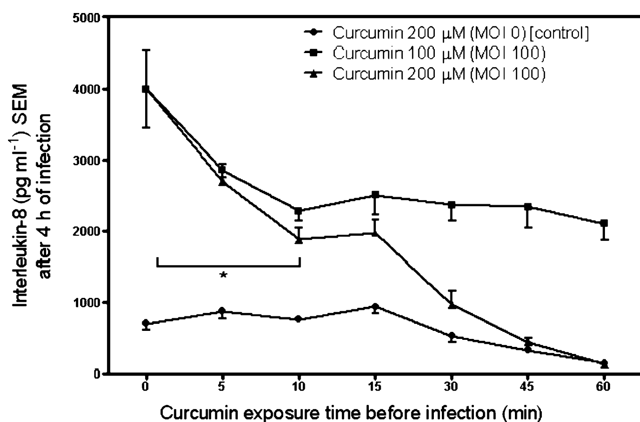

Fig. 5 IL-8 secretion following a 4-h infection with live strain 25238 (MOI 100) as a function of the duration of pre-incubation of Detroit cells with 100 (*square*) or 200 μM (*triangle*) curcumin, respectively. Uninfected cells pre-incubated with 200 μM curcumin (*circle*) are included as negative control. Statistics, *p=0.002 (two-tailed t test between 0 and 10 min of exposure to 200 μM curcumin)

curcumin-induced inhibition of IL-8 secretion by Detroit cells. This approach required the use of heat-killed bacteria because *Mcat* proliferates in MEM. Figure 6 indicates that, as expected, pre-incubation with 200 μM curcumin not only resulted in more profound inhibition of IL-8 release after 4 h but that it also translated in prolonged IL-8 inhibition (12 h) in comparison with pre-infection exposure to 100 μM curcumin (4 h). Pre-incubation in this experiment was carried out for 60 min. Pre-incubation of strain 25238 with sIgA did not affect IL-8 secretion (data not shown).

Discussion

Curcumin is a powerful anti-inflammatory agent, which exerts its main effect by inhibiting activation and nuclear translocation of NF-κB [34]. Pharmacologically, its major disadvantage is the extremely poor oral bioavailability. Its topical use as an anti-inflammatory agent on mucosal surfaces, obviating the need for systemic absorption, is thus an attractive approach to its eventual use as a therapeutic agent [25].

The in vitro data presented here demonstrate that curcumin is capable of inhibiting the growth of the highly prevalent facultative pathogen *Mcat* by exhibiting concentration-dependent bacteriostatic and bactericidal activity (Fig. 1). If applicable to the entire oral flora [23], this antibacterial effect may contribute to the beneficial effects of curcumin because the bacterial density on the mucosal surface affects the severity of cancer chemotherapy- and radiation-induced mucositis [5] and increases the risk of systemic infections [11]. Furthermore, curcumin strongly inhibited epithelial cell adherence and cellular invasion of *Mcat* (Figs. 2 and 4, respectively), both being considered key early events in mucosal infection [22]. Clearly, the use of one single bacterial species oversimplifies the complexity of the oropharyngeal flora and its interaction with the mucosa. In addition, the fact that we used prolonged curcumin exposure times (30–60 min) before infecting the monolayer with bacteria may not closely mimic the in vivo use of curcumin mouth washes, which may result in shorter exposure times. Nevertheless, we consider our *Mcat* model useful because this upper respiratory tract organism commonly switches from commensal to pathogen and vice versa under appropriate conditions and because its pathogenicity appears to be density-dependent [24]. In addition, as discussed below, exposure times as short as 10 min resulted in significant anti-inflammatory responses. It is noteworthy that curcumin-inhibited adherence has also been described for the related organisms *Neisseria meningitidis* (on A549 lung cells [12]) and *Neisseria gonorrhoeae* (on HeLa cells [35]), respectively. Detroit cells were used because this epithelial cell line originates from the human upper respiratory tract, where *Mcat* typically resides and where cancer therapy-induced oral lesions frequently occur. This cell line has previously been investigated for both its susceptibility to curcumin-induced NF-κB inhibi-

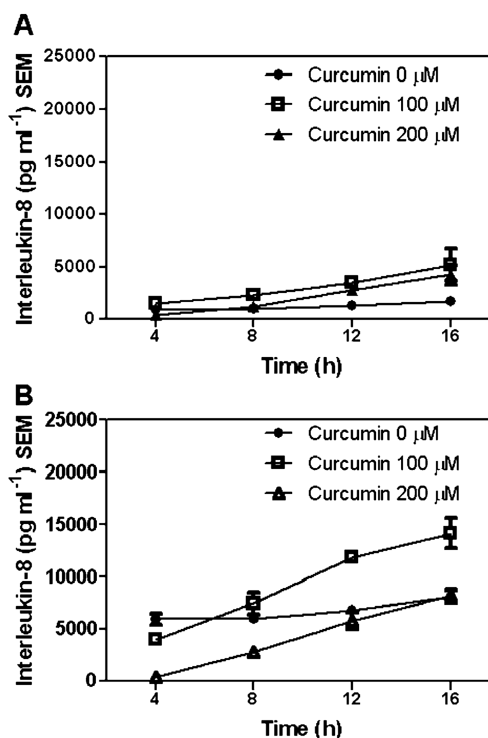

Fig. 6 IL-8 secretion following a 16-h exposure of Detroit cells with heat-inactivated strain 25238 at a MOI 0 (uninfected, **a**) or 100 (**b**), respectively. Sixty-minute pre-incubation before exposure with bacteria was carried out with 0 (*circle*), 100 μM (*square*), or 200 μM (*triangle*) curcumin, respectively

tion and induction of apoptosis [17]. While activation of NF-κB was inhibited after 16 h of exposure to up to 100 μM curcumin, caspase-3 mediated apoptosis was not observed before 24 h of continuous exposure to curcumin. This finding is in line with our observation that an exposure time of 60 min did not induce apoptosis of Detroit cells [17].

Our findings are novel in several respects. First, we established for the first time dose–response curves for the antibacterial activity of curcumin for a major upper respiratory tract and otitis media pathogen (Fig. 1). This is an important finding because, as mentioned above, the risk of development of infection, e.g., otitis media caused by *Mcat*, *Haemophilus influenzae*, and *Streptococcus pneumoniae*, correlates with the mucosal surface density of the organism at the time of florid otitis media [24]. Second, dose–response curves established that the inhibitory effect of curcumin on epithelial cell adherence and invasion clearly is limited to concentrations of curcumin >50 μM (Figs. 2 and 4, respectively). While other epithelial cell types may differ in their minimum concentrations needed, the data maintain that cellular penetration of curcumin requires a certain minimum extracellular concentration, when it is to be used as a topical agent on mucosal surfaces. Third, our time analysis (Fig. 5) indicates that the duration of contact between curcumin and the epithelial cell layer has a profound effect on its capacity to inhibit *Mcat*-induced pro-inflammatory activation, the occurrence of which has been firmly established in the literature [10, 28, 33]. Our data indicate that curcumin at a concentration of 100 μM only moderately inhibits IL-8 release and that maximum inhibition of this key chemokine requires exposure of Detroit cells to relatively high concentrations (200 μM) for at least 45 min. Fourth, not surprisingly, the concentration of curcumin also affected the duration of IL-8 suppression, in that 200 μM was effective approximately three times longer (12 h) than 100 μM (4 h; Fig. 6). Of potential concern was the observation that short-term suppression by 100 μM resulted in an excessive "rebound" release of IL-8 after 12 and 16 h, respectively, which may be an undesirable effect, e.g., in the treatment of cancer therapy-induced mucositis. We are not aware that such a rebound phenomenon has previously been described.

On the other hand, we established that co-exposure of epithelial cells with both salivary IgA and low-dose curcumin (50 μM) displayed a synergistic or at least additive effect on the reduction of *Mcat*-induced inflammation. Since the buccal mucosa is constantly bathed in IgA-containing saliva, the rebound phenomenon mentioned above may be mitigated by the presence of saliva in patients who receive curcumin intermittently and/or at low doses only.

In conclusion, these data from our in vitro model demonstrate that curcumin, applied topically to oropharyngeal surface epithelia, may have a beneficial effect on local infection and also on cancer therapy-induced mucositis by reducing the bacterial load, inhibiting bacterial adherence and cellular penetration, as well as preventing pro-inflammatory cytokine release. Additional studies will be needed because our data also indicate that both the duration and concentration of curcumin exposure are likely to affect the potential therapeutic benefit.

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Conflict of interest None declared.

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