Exposure of a Corneal Epithelial Cell Line (hTCEpi) to *Demodex*-Associated *Bacillus* Proteins Results in an Inflammatory Response

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PURPOSE. A role for a bacterium, *Bacillus oleronius*, originally isolated from a *Demodex* mite, in the induction of ocular rosacea has been proposed. The aim of this work was to characterize the response of a corneal epithelial cell line to *Bacillus* proteins, as this might give an insight into how such proteins contribute to the symptoms of ocular rosacea in vivo.

METHODS. The effect of exposing *Bacillus* protein preparation on human telomerase-immortalized corneal epithelial cells (hTCEpi) was measured by monitoring changes in cell proliferation and the expression of a number of genes associated with inflammation. The production of inflammatory cytokines was measured and the expression and activity of MMP-9 was quantified.

RESULTS. Exposure of hTCEpi cells to 2 or 6 μg/mL *Bacillus* protein resulted in a dose-dependent reduction in cell proliferation. Exposure of cells to 6 μg/mL *Bacillus* protein did not induce apoptosis, but there was an increase in the expression of genes coding for IL-6 (13.8-fold), IL-1β (4.0-fold), IL-8 (11.1-fold), and TNF-α (4.1-fold). Increased expression of genes coding for the defensins, CCL20 (4.5-fold) and S100A7 (6.8-fold) also was observed. Elevated production of IL-6 and IL-8 was evident from cells exposed to 2 and 6 μg/mL *Bacillus* protein. The hTCEpi cells demonstrated increased MMP-9 expression (3.2-fold, $P = 0.003$) and activity (2.2-fold, $P = 0.0186$) after 48 hours of exposure to 6 μg/mL *Bacillus* protein preparation.

CONCLUSIONS. The results suggest that interaction of *Demodex*-associated *Bacillus* proteins with the corneal surface could lead to tissue degradation and inflammation, possibly leading to corneal scarring.

Keywords: *Bacillus*, corneal surface, corneal scarring, *Demodex*, ocular rosacea, sterile ulcers

Rosacea is a chronic inflammatory dermatological condition that predominantly affects the skin of the face and the eyes.[1,2] It is estimated that the condition affects approximately 3% of the population and there are at least 14 million sufferers in the United States. A number of forms of rosacea are recognized, including papulopustular, erythematotelangiectatic, ocular, and phymatous.[3] Ocular rosacea affects the eyes and the eyelids, causing symptoms such as blepharitis and keratitis, and up to half of those diagnosed with facial rosacea also manifest ocular symptoms.[4,5] Corneal (sterile) ulcers and corneal scarring are associated with severe cases of ocular rosacea and can lead to a deterioration in vision and potentially blindness in affected eyes.[6] Although the classification of rosacea is now well established, there is no agreement on its etiology. A number of factors have been implicated in the induction and persistence of rosacea, including alterations in the immune response, increased dermal vascularization, and the presence of reactive oxygen species in the skin.[7]

Patients affected with rosacea display a higher density of facial mites, *Demodex folliculorum*, than unaffected controls,[8–11] although the significance of this observation has never been fully explained. Elevated numbers of *Demodex* mites also are present on the eyelashes of patients with ocular rosacea.[12]

A potential role for bacteria in the induction of rosacea has been suggested, because antibiotics (e.g., erythromycin, metronidazole) can be used to treat the condition, although these may also exhibit anti-inflammatory properties.[2,13] *Staphylococcus epidermidis* isolated from rosacea patients were consistently β-hemolytic and grew better at 37°C than 30°C, suggesting a potential link between mesophilic hydrolytic bacteria and rosacea.[14] This bacterium also has been isolated from the pustules of patients with papulopustular rosacea and from the eyelids of patients with ocular rosacea, suggesting a possible role for this bacterium in the induction of these conditions.[15] A bacterium (*Bacillus oleronius*), originally isolated from a *Demodex folliculorum* mite from a patient with papulopustular rosacea, produced proteins that induced an inflammatory immune response in 72% of rosacea patients but only 29% of controls ($P = 0.01$).[16] This bacterium also has been isolated from the eyelashes of patients with blepharitis[17] and is sensitive to the antibiotics used to treat rosacea.[16] A strong correlation has been established between ocular *Demodex* inflammation and serum reactivity to these bacterial

proteins in patients with ocular rosacea.[12] In addition, the presence of eyelid margin inflammation ($P = 0.04$) and facial rosacea ($P = 0.009$) correlated with reactivity to these proteins.[12] Neutrophils exposed to *Bacillus* proteins demonstrated increased migration and elevated release of MMP-9, an enzyme known to degrade collagen, and cathelicidin, an antimicrobial peptide. Exposure of neutrophils to the bacterial proteins resulted in elevated production of IL-8 and TNF-α.[18] Increased production of IL-8 is a trigger for neutrophil recruitment to the site of infection, and TNF-α is indicative of an inflammatory response. These studies suggest a possible role for bacterial proteins in the etiology of rosacea.[12,16,18]

Human telomerase-immortalized corneal epithelial cells (hTCEpi) are an excellent model for studying the response of the corneal surface to pathogens or pathogen-derived materials (e.g., toxins, antigens) and may give an insight into the response of the cornea to bacterial interactions in ocular rosacea. Human telomerase-immortalized corneal epithelial cells have previously been used to study the interaction of flagellin from pathogenic and nonpathogenic bacteria,[19] and in understanding the response of the corneal surface to the combined effect of contact lens and *Pseudomonas aeruginosa*.[20]

In a previous study, we characterized the response of the hTCEpi cells to *Bacillus* proteins in terms of alterations in cell migration and invasiveness.[21] The results indicated increased cell migration and invasiveness after exposure of the cells to the *Bacillus* proteins. Cells exposed to *Bacillus* proteins showed a dose-dependent increased expression of genes coding for matrix metalloproteinase-3 (MMP-3) (61-fold) and MMP-9 (300-fold). This dose-dependent increase in gene expression also was reflected in elevated levels of MMP-9 protein and increased MMP activity in the culture supernatant. These results suggest a possible link between exposure to *Bacillus* proteins and the development of corneal damage in ocular rosacea patients. The aim of present work was to characterize whether exposure of hTCEpi cells to the *Bacillus* proteins induced an inflammatory response, as this might give an indication as to how these bacterial proteins induce the erythema that is a feature of ocular rosacea.[4,5] The complete elucidation of the role of these proteins in inducing the symptoms of ocular rosacea could open the possibility of developing new therapeutic strategies for the control of this disfiguring condition.

MATERIALS AND METHODS

Preparation of Bacterial Proteins

Cultures of *B. oleronius* were grown at 30°C in nutrient broth (Oxoid, Ltd.; Thermo Fisher Scientific, Inc., Waltham, MA, USA) under aerobic conditions to stationary phase of growth before protein extractions were performed as described.[16] This protein fraction was termed "crude *B. oleronius* protein" and was diluted to a working concentration of 200 µg/mL in PBS (pH 7.4). The *B. oleronius* protein extract was purified by anion exchange separation using fast protein liquid chromatography (FPLC) using an ÄKTA Purifier 100 system (ÄKTA FPLC; GE Healthcare, Little Chalfont, UK) as described.[18] The "crude *B. oleronius* protein" extract was incubated at 4°C overnight in Q-Sepharose beads (Q Sepharose High Performance; GE Healthcare) and binding buffer (20 mM piperazine, 10 mM NaCl in dH$_2$O, pH 4.6). Fractionation by Q-Sepharose charge separation of the crude *B. oleronius* protein extract to the semipurified *B. oleronius* protein preparation was performed by increasing the concentration of a salt gradient using elution buffer (20 mM piperazine, 1 M NaCl in dH$_2$O, pH

4.6). The fractions separated from the ÄKTA FPLC column of interest were pooled and precipitated. The semipurified protein preparation was visualized by 1D SDS-PAGE staining with Coomassie blue as a quality assurance of the Q-Sepharose ÄKTA FPLC separation process and maintained at a concentration of 200 µg/mL in PBS (termed as "pure" protein in Figs. 1–8).

Cell Culture

Human telomerase-immortalized corneal epithelial cells were maintained in keratinocyte growth medium (KGM)-2 supplemented with KGM-2 SingleQuot Kit Supplements and Growth Factors (Lonza, Basel, Switzerland), in a 5% CO$_2$ humidified atmosphere incubator at 37°C, and passaged every 3 to 5 days. Cells were left to adhere overnight before stimulants were added to the media: crude *B. oleronius* protein preparation (2 µg/mL) or semipurified *B. oleronius* protein preparation (2 µg/mL and 6 µg/mL).

Corneal Cell Proliferation

Human telomerase-immortalized corneal epithelial cells were seeded into a 6-well culture plate (Corning Life Sciences, Corning, NY, USA) at a density of 5×10^5 cells per well, allowed to attach, and exposed to semipurified *B. oleronius* proteins at concentrations of 2 and 6 µg/mL. Cell counts were performed on days 0, 1, 2, 3, and 6 using a hemocytometer (Neubauer improved Hemocytometer; Marienfeld-Superior Ltd., Lauda-Königshofen, Germany) and cell viability was measured using the trypan blue exclusion method. Human telomerase-immortalized corneal epithelial cells were co-cultured with the *Bacillus* proteins for the duration of the assay.

Cell Cycle Analysis

Human telomerase-immortalized corneal epithelial cells were seeded into 6-well plates (Corning) at a density of 1×10^4 cells per well. Cells attached during a 2-hour incubation and were then treated using KGM-Gold media (Lonza) alone or KGM-Gold media supplemented with 6 µg/mL "pure" *B. oleronius* proteins. After 48 hours of exposure, cells were harvested using 0.25% trypsin/0.022% EDTA, washed with PBS, and fixed with ice-cold 90% methanol (Sigma-Aldrich Corp., St. Louis, MO, USA). Samples were stained using propidium iodide (Biotium, Inc., Hayward, CA, USA) according to the manufacturer's instructions and analyzed using a Guava easyCyte HT flow cytometer (Millipore, Billerica, MA, USA). The percentages of cells in Gap-1 (G$_1$), Synthesis (S), and Gap-2 (G$_2$) phases of the cell cycle were calculated using the ModFit LT 3.2 DNA cell-cycle analysis software (Verity Software House, Topsham, ME, USA).

Caspase-3 and Caspase-7 Immunofluorescence Assay

Human telomerase-immortalized corneal epithelial cells were cultured for 3 days in culture dishes (CELLVIEW, cell culture dish with glass bottom, TC, 35 mm, 1 compartment; Greiner Bio-one GMbH, Frickenhausen, Germany) (2×10^5 cells per well) under the following conditions: untreated, dimethyl sulfoxide (DMSO) (1%, vol/vol), and purified *B. oleronius* protein (6 µg/mL) before fixing and staining for caspase-3/7 activity and assessed following the guidelines in the Image-iT LIVE Red Caspase-3 and -7 Detection Kit (Invitrogen, Dún Laoghaire Co., Dublin, Ireland). Cells were stained with fluorescent-labeled inhibitor of caspases (FLICA) reagent that
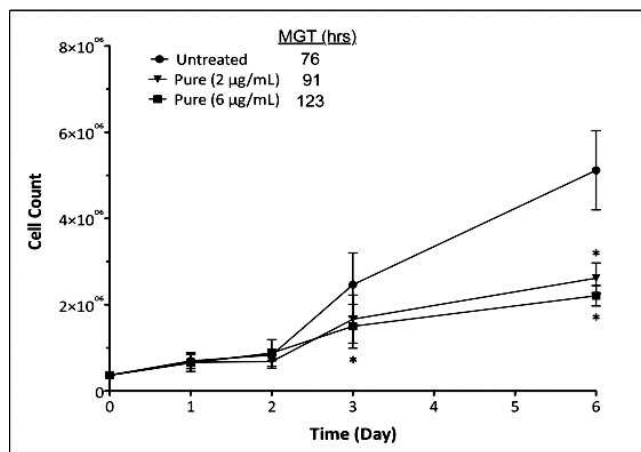
FIGURE 1. Exposure of *B. oleronius* proteins reduces the rate of hTCEpi cell proliferation. The MGT for hTCEpi cells during the log phase of growth between day 2 and day 3 of exposure to *Bacillus* proteins at 2 and 6 µg/mL was calculated to be 91 hours and 123 hours, respectively, compared with 76 hours for untreated cells. The growth of hTCEpi cells was reduced in the presence of *B. oleronius* in a dose-dependent manner. *$P < 0.05$.

becomes covalently and irreversibly coupled to active caspase-3/7 (excitation/emission at 550/595 nm), and Hoescht 33342 (a nuclear stain with excitation/emission at 350/461 nm) was used to visualize hTCEpi cells positive and negative for caspase-3/7. Results represent experiments performed in triplicate and determined for five fields of view per treatment for each repeat of the assay. Images were captured on a Nikon TiE microscope (Nikon UK Limited, Kinston upon Thames, Surrey, UK) controlled and analyzed using MetaMorph software (Molecular Devices, Marlow, Buckinghamshire, UK).

RNA Extraction and Gene Expression Analysis

Human telomerase-immortalized corneal epithelial cells (6×10^4/well) were seeded in 6-well culture dishes and exposed to semipurified *B. oleronius* proteins (0, 2, or 6 µg/mL) for 5 days, with feeding and treatment on alternate days. Following PBS washes ($\times 3$), tri-reagent (1 mL/well) was applied and RNA extracted according to manufacturer's instructions (Sigma-Aldrich). RNA concentration was determined using a Nano-Drop spectrophotometer (ND-1000; Labtech International, East Sussex, England) and cDNA synthesized using a high-capacity RNA to cDNA kit (Applied Biosystems, Carlsbad, CA, USA). Amplification of chemokine ligand-20 (CCL20), IL-1β, IL-6, IL-8, S100A7, and TNF targets was performed using TaqMan Gene Expression Assays (Hs01011368_m1, Hs01555410_m1, Hs00985639_m1, Hs00174103_m1, Hs00161488_m1, Hs00174128_m1, respectively) in conjunction with an ABI 7500 Fast Real-time PCR thermal cycler (Applied Biosystems, Foster City, CA, USA).

Electrophoresis and Western Blots

Cell supernatants were collected from hTCEpi cells exposed to semipurified *B. oleronius* protein (2 and 6 µg/mL) for 24, 48, or 72 hours in culture conditions described above. Collected samples were concentrated by centrifugation using 3000 MCWO Vivaspin-20 (Vivaproducts, Littleton, MA, USA) filter columns, and protein (20 µg) was loaded onto a 10% acrylamide gel and separated under reducing conditions. After electrophoresis, resolved proteins were transferred onto a nitrocellulose membrane (Fisher Scientific, Ballycoolin, Dublin,

Ireland), using a transfer rig in transfer buffer (25 mM Tris, 192 mM glycine, methanol [20%, vol/vol] in dH$_2$O, pH 8.3).

Membranes were blocked using 1% (wt/vol) BSA in Tris-buffered saline (TBS) (pH 7.6) with 0.05% (vol/vol) Tween-20 (Sigma-Aldrich) for 1 hour at room temperature. Primary antibodies were diluted as follows: anti-CCL20 (raised in rabbit [Abnova, Taipei City, Taiwan]) 1/500; anti-S100A7 (raised in rabbit [Sigma-Aldrich]) 1/600; anti-MMP-9 (raised in rabbit [Sigma-Aldrich]) 1/500; and incubated at 4°C overnight.

An anti-human horseradish peroxidase (HRP)-linked secondary antibody (HRP-linked rabbit antisera at a dilution of 1/1000) was applied and incubated at room temperature for 2 hours. Immunoreactive protein bands were visualized by incubating the membranes for 10 minutes in 10 mg diaminobenzidine tetrahydrochloride in 15 mL of 100 mmol/L Tris-HCl (pH 7.6) containing 15 µL hydrogen peroxide (Sigma-Aldrich) before washing in distilled water and drying. Immuno-bands were quantified by densitometry using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

Enzyme-Linked Immunosorbent Assay

Human telomerase-immortalized corneal epithelial cells were exposed to crude *B. oleronius* protein (2 µg/mL), or semipurified *B. oleronius* protein (2 µg/mL) for 24 hours or 72 hours and the supernatants were collected. Secretion levels of IL-6 and IL-8 were measured using commercial ELISA kits (mini-development ELISA kit; PeproTech, Rocky Hill, NJ, USA) according to the manufacturer's guidelines.

Gelatinase Zymograms for MMP-9 Activity

Cell supernatants collected from cell proliferation assays at days 3 and 6 after exposure of hTCEpi cells to *B. oleronius* semipurified protein extract at 2 and 6 µg/mL were concentrated by centrifugation using 3000 MCWO Vivaspin-20 (Vivaproducts) filter columns and loaded onto Novex 10% zymogram (gelatin) gels (Invitrogen). Following electrophoresis, gels were incubated in zymogram-renaturing buffer (Invitrogen) for 30 minutes at room temperature, left at 37°C overnight in developing buffer (Invitrogen), and stained with Colloidal Coomassie (Brilliant Blue G-Colloidal Concentrate, Electrophoresis Reagent; Sigma-Aldrich) for 2 hours before destaining of gels in distilled water and recording of gel images.

Statistical Methods

Resultant cycle threshold (Ct) values from real-time PCR expression analyses, were normalized against human glyceraldehyde 3-phosphate dehydrogenase endogenous control expression (Hs99999905_m1) and changes in expression calculated using the relative quantification (RQ) = $2-\Delta\Delta Ct$ equation. All experiments were performed on three independent occasions and results are presented as the mean $\pm$ SE. Statistical significance was assessed by the Student's *t*-test using GraphPad Prism version 5.00 for Mac OS X (GraphPad Software, San Diego, CA, USA; www.graphpad.com). *P* values less than 0.05 (*), less than 0.01 (**), or less than 0.001 (***) were deemed statistically significant.

RESULTS

Effect of *Bacillus* Proteins on hTCEpi Cell Proliferation

Exposure of hTCEpi cells to 2 or 6 µg/mL *Bacillus* protein reduced the growth rate of cells over a 6-day period (Fig. 1). Cell proliferation was reduced by 34% and 42% in those cultures that
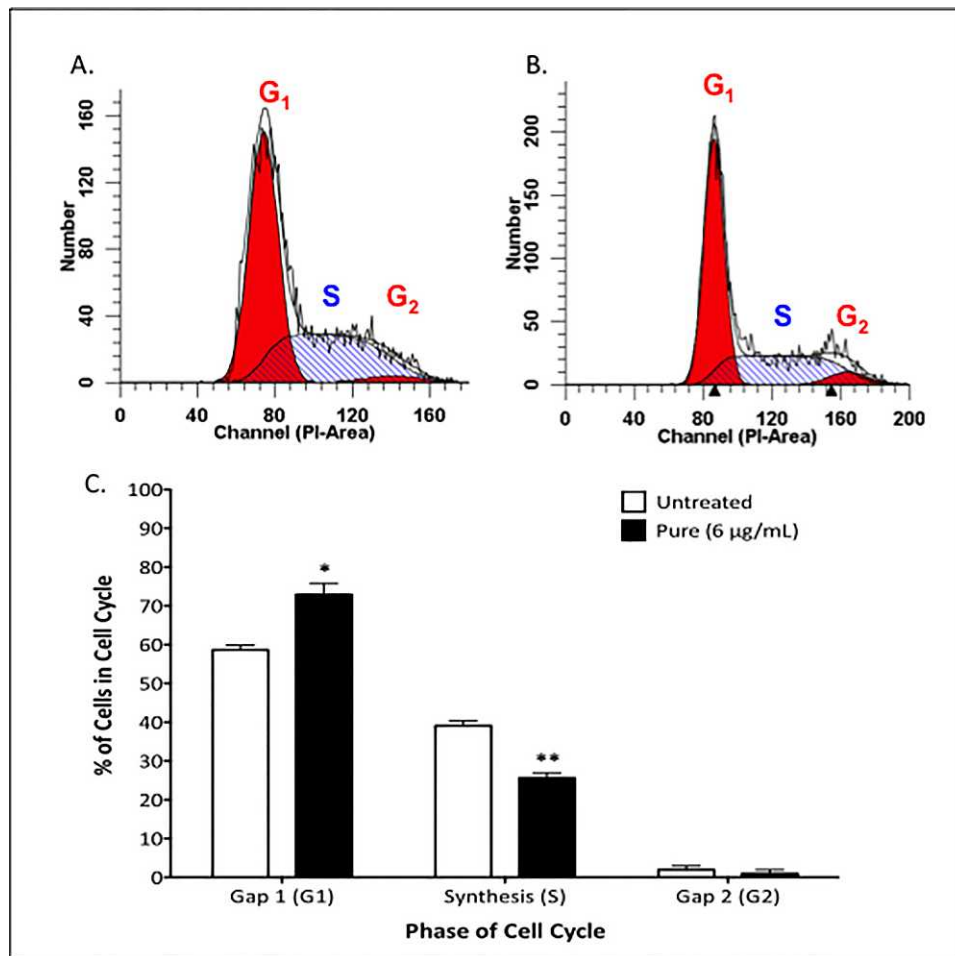
FIGURE 2. Effect of *B. oleronius* protein on hTCEpi cell cycle. Cell cycle characteristics of both untreated (**A**) and semipurified *B. oleronius* protein (6 µg/mL)-treated (**B**) hTCEpi cells were assessed by DNA content analysis after 48 hours of incubation. *B. oleronius* semipurified protein-treated cells demonstrate an increased proportion of cells in G_1 phase and a decreased proportion of cells in the S phase relative to untreated controls (**C**) indicating that exposure to semipurified *B. oleronius* protein inhibits transition from the G_1 growth phase into the S phase. *$P < 0.05$, **$P < 0.001$.

were exposed to 2 and 6 µg/mL semipurified *Bacillus* protein, respectively, compared with the total cell number in the untreated controls after a 72-hour incubation period. The mean generation time (MGT) for cells during the log phase of growth between day 2 and day 3 of exposure to *Bacillus* proteins at 2 and 6 µg/mL was calculated to be 91 hours and 123 hours, respectively, whereas it was 76 hours in the control. A significant decrease in corneal epithelial cell growth was observed at day 3 following exposure to semipurified *Bacillus* protein (6 µg/mL) ($P = 0.0185$) and reduced growth of hTCEpi cells following 6 days of exposure to 2 or 6 µg/mL semipurified *Bacillus* protein relative to control hTCEpi cells was significant ($P = 0.0111$ and $P = 0.0133$, respectively). During the course of a 6-day exposure to the semipurified *Bacillus* protein at 2 and 6 µg/mL, a statistical difference in total cell number between the two doses at each time point was not observed, but a dose-dependent increase in the MGT at day 3 between the semipurified *Bacillus* protein treatments at 2 and 6 µg/mL was found to be statistically significant ($P = 0.0427$).

Effect of *B. oleronius* Protein Exposure on Cell Cycle

Cell-cycle analysis was performed to determine the effect of semipurified *B. oleronius* proteins (6 µg/mL) on hTCEpi progression through the phases of the cell cycle. By comparing the DNA content of untreated hTCEpi cells (Fig. 2A) and *B. oleronius* protein-treated hTCEpi cells (Fig. 2B), it was observed that exposure to *B. oleronius* proteins induced significant G_1 phase arrest ($P = 0.0015$) by increasing the percentage of hTCEpi cells in the G_1 phase from 58.66% to 72.91% (Fig. 2C). Accordingly, the percentage of cells in the S phase also decreased (from 39.13% to 25.61%), concurrent with *B. oleronius* protein treatment ($P = 0.002$) (Fig. 2C), suggesting that *B. oleronius* protein exposure may reduce hTCEpi proliferation by impeding progression from the G_1 growth phase to the S (synthesis) phase of the cell cycle.

Exposure to *Bacillus* Proteins Does Not Induce Apoptosis in hTCEpi Cells

The cleavage of caspases, a family of cysteine–aspartic acid–specific proteases, represent a distinctive feature of early apoptosis. The exposure of hTCEpi cells to 6 µg/mL semipurified *B. oleronius* protein did not induce the increased production of caspase-3 and -7. After a 72-hour treatment period, the semipurified *Bacillus* protein preparation showed that 3.09% of corneal cells were positive for the apoptotic markers caspase-3 and -7, compared with 3.16% of control cells. In contrast, 61.34% of corneal cells exposed to DMSO (1%, vol/vol) were positive for these markers ($P < 0.0001$) (Fig. 3). The noninduction of caspases
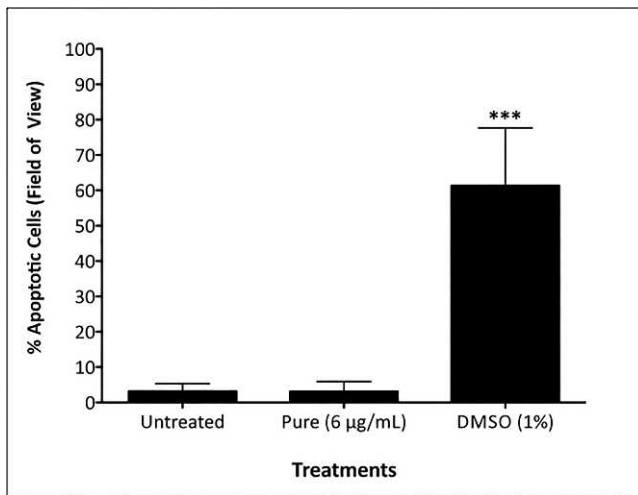
FIGURE 3. The hTCEpi cells exposed to *B. oleronius* protein preparation do not exhibit caspase activation. The hTCEpi cells were exposed to semipurified *Bacillus* proteins (6 μg/mL), DMSO (1%, vol/vol), or untreated (PBS) for 3 days. Caspase-3 and -7 activity was assessed by capturing immunofluorescence images followed by analysis through MetaMorph software. Results represent experiments performed in triplicate and determined using five fields of view per treatment for each repeat of the assay. ***$P < 0.0001$.
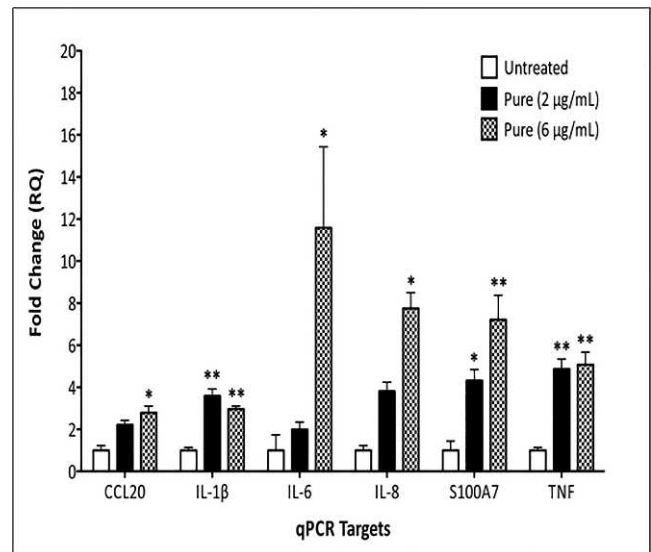

FIGURE 4. Effect of *B. oleronius* protein extract on gene expression in hTCEpi cells. The effect of semipurified *B. oleronius* protein exposure on wound-associated gene expression was assessed by quantitative RT-PCR. *$P < 0.05$, **$P < 0.001$.

after exposure to semipurified *B. oleronius* protein preparation suggests that the effect on growth of hTCEpi cells is antiproliferative rather than via the induction of apoptosis.

Gene Expression Changes Induced by *B. oleronius* Antigen Exposure

To assess the effect of *B. oleronius* protein exposure on hTCEpi gene expression, the expression of a number of genes that are known to participate in proinflammatory, immune-modulated, and defense-associated activities during wound healing was assessed (Fig. 4). Immediately after barrier disruption in a healthy wound, proinflammatory cytokines, including IL-6, IL-8, IL-1β, and TNF, are released. These cytokines function in the acute phase to stimulate inflammation and attract immune cells to the site of injury; however, if proinflammatory cytokines continue to be secreted at elevated concentrations after the acute phase, complications may result. The hTCEpi cells exposed to both low (2 μg/mL) and high (6 μg/mL) concentrations of *B. oleronius* protein resulted in significant overexpression of proinflammatory cytokines IL-1β (3.6-fold, $P = 0.0027$, and 3.0-fold, $P = 0.0083$, respectively) and TNF (4.9-fold, $P = 0.0019$, and 5.1-fold, $P = 0.0014$, respectively) relative to untreated cells (Fig. 4). Interleukins are regulators of the corneal response to injury, and IL-1β acts as a proinflammatory mediator, stimulating stromal fibroblasts to secrete chemokines and amplify inflammatory responses,[22] whereas TNF also plays important roles in corneal inflammation and wound healing.[23] The highest relative upregulation of cytokine expressions assessed were those of IL-6 (11.6-fold, $P = 0.0337$) and IL-8 (7.8-fold, $P = 0.0378$) after exposure of cells to 6 μg/mL *B. oleronius*. Secreted IL-6 is known to be elevated in chronic wounds and overproduction of IL-6 protein may lead to the development of autoimmune inflammatory diseases.[24,25] Elevated IL-8 has been observed in wounds that display retarded healing and IL-8 may directly contribute to this effect by inhibition of keratinocyte replication.[26] In the cornea, IL-8 overexpression also has

been associated with ulcer formation via chemoattraction of macrophages and subsequent destruction of tissue.[27] Although not itself a cytokine, overexpression of CCL20 (2.8-fold, $P = 0.0355$) also was observed in cells exposed to *B. oleronius* antigen (6 μg/mL). Chemokine ligand-20 affects corneal wound healing indirectly by attraction of specialized IL-22–secreting γδ T cells, which induce an inflammatory response.[28] The antimicrobial peptide Psoriasin-coding gene S100A7 displayed overexpression at both 2 μg/mL and 6 μg/mL *B. oleronius* exposure (4.3-fold, $P = 0.0124$, and 5.1-fold, $P = 0.0022$, respectively). This protein was first discovered due to its oversecretion in inflammatory skin disease psoriasis and is known to be present at the ocular surface in both tear films and corneal epithelium; however, associations with corneal disease are as yet undescribed.[29]

Effect of *Bacillus* Proteins on Expression of CCL20 and S100A7 in Cells

Human telomerase-immortalized corneal epithelial cells were exposed to semipurified *B. oleronius* proteins (2 μg/mL and 6 μg/mL) for 24, 48, and 72 hours, as described, and Western blots were performed to assess the effect on the expression of the defensins, CCL20 and S100A7 (Figs. 5A, 5C, respectively). The results revealed increased expression of CCL20 in corneal cells exposed to semipurified *B. oleronius* proteins at 2 μg/mL and 6 μg/mL, with a 2.1 ($P = 0.0323$) and 2.9-fold increase, respectively, relative to the control at 24 hours. An increase of CCL20 expression was observed at 48 hours, with a 2.6 ($P = 0.0057$) and 3.9-fold ($P = 0.0178$) increase of CCL20 expression after exposure to the 2 μg/mL and 6 μg/mL semipurified *B. oleronius* protein preparations, respectively. At 72 hours, CCL20 expression in corneal cells treated with 2 μg/mL and 6 μg/mL semipurified *Bacillus* proteins showed a 2.5-fold ($P = 0.0024$) and 4.1-fold ($P = 0.0129$) increase, respectively, compared with the control. A significant dose-dependent response between the 2-μg/mL and 6-μg/mL semipurified *Bacillus* protein concentrations for the expression of CCL20 was not observed at 24, 48, or 72 hours after stimulation (Fig. 5B). Similarly, an increase in the expression of S100A7 was observed in hTCEpi cells exposed to semipurified *B. oleronius*
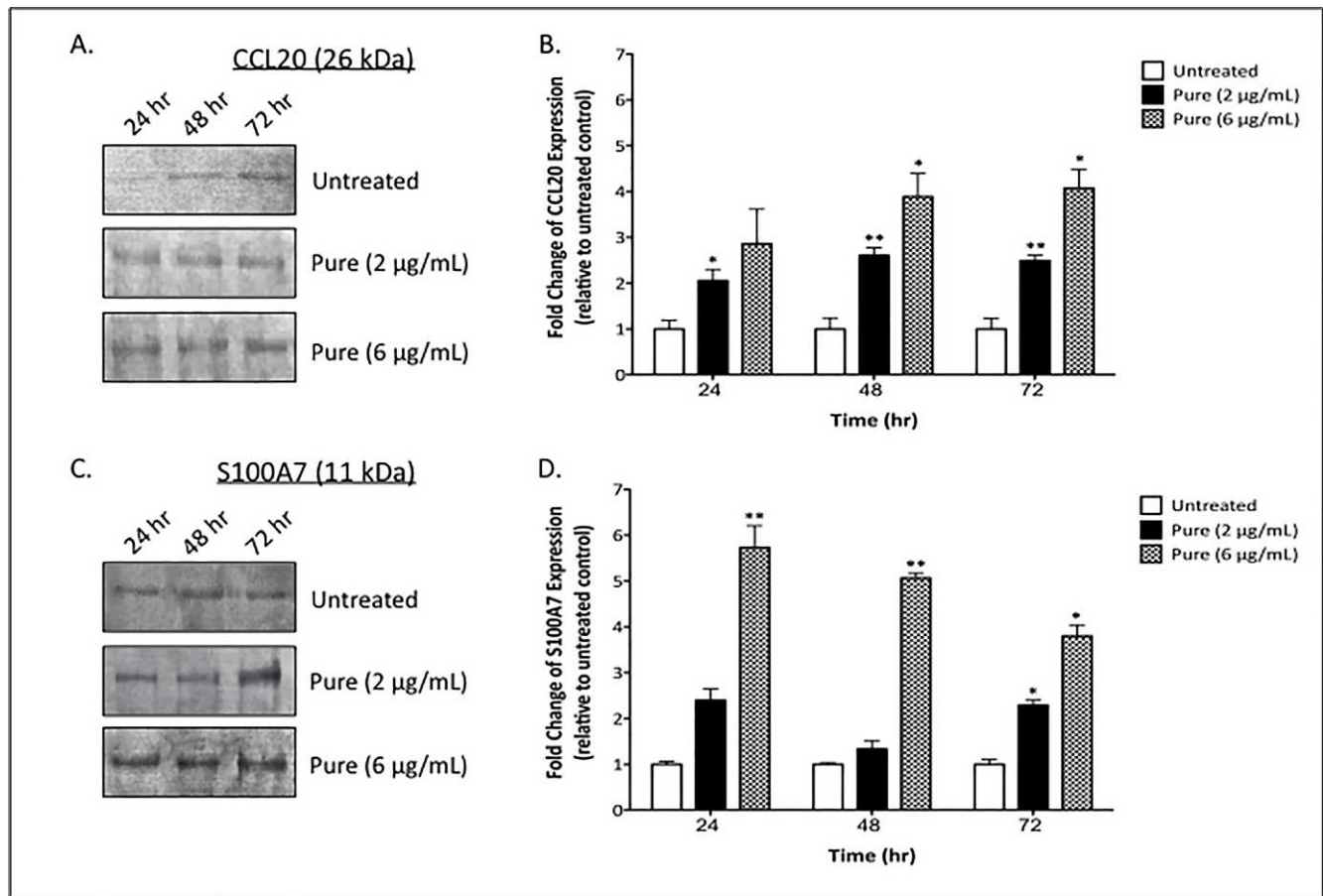
FIGURE 5. Effect of *B. oleronius* protein preparation on CCL20 and S100A7 expression in hTCEpi cells. The expression of CCL20 and S100A7 by hTCEpi cells following exposure to semipurified *B. oleronius* proteins (pure 2 and 6 μg/mL) was assessed by Western blot at 24, 48, and 72 hours after stimulation (**A**, **C**), and the expression of CCL20 and S100A7 was assessed by densitometric analysis (**B**, **D**). *$P < 0.05$, **$P < 0.001$.

proteins (2 and 6 μg/mL). At 24 hours, a 2.4- and 5.7-fold ($P = 0.005$) increase in expression of S100A7 compared with the untreated control was recorded. At 48 hours, the 6-μg/mL dose of semipurified *B. oleronius* protein extract induced a 5.1-fold increase in the expression of S100A7 ($P = 0.0012$). An increase of S100A7 expression was evident at 72 hours after exposure to the 2-μg/mL and 6-μg/mL semipurified *Bacillus* protein preparations with a 2.3-fold ($P = 0.0163$) and 3.8-fold ($P = 0.0103$) increase observed for each dose, respectively. A significant dose-dependent difference between the 2-μg/mL and 6-μg/mL semipurified *Bacillus* protein treatments for the expression of S100A7 was recorded after 24- ($P = 0.0005$), 48- ($P < 0.0001$), and 72-hour ($P = 0.0002$) exposure of corneal cells (Fig. 5D).

Cytokine Production in hTCEpi Cells Following Exposure to *Bacillus* Proteins

Analysis of the production of cytokines by hTCEpi cells exposed to crude and semipurified *B. oleronius* proteins (2 μg/mL) for 24 and 72 hours revealed increased production of IL-6 and IL-8 (Figs. 6A, 6B) but not of IL-1β and TNF-α (data not presented). There was a statistically significant increase in the secretion of IL-6 in cells exposed to semipurified *B. oleronius* protein preparation at 24 and 72 hours ($P < 0.0001$ and $P = 0.0014$, respectively). A significant increase in IL-6 secretion also was observed in cells exposed to crude *B. oleronius* protein preparation at 24 hours ($P = 0.0255$). The increased production of IL-6

after exposure of the hTCEpi cells to the semipurified *B. oleronius* protein (2 μg/mL) compared with the crude *B. oleronius* protein (2 μg/mL) was statistically significant at 24 hours ($P = 0.0034$) but not at 72 hours (Fig. 6A). Interleukin-8 secretion reached the highest level in cells exposed to crude *Bacillus* protein preparation at 24 hours (230 pg/mL) ($P = 0.0003$). Semipurified *B. oleronius* protein preparation also stimulated IL-8 secretion at 24 and 72 hours. The secretion of IL-8 by corneal cells stimulated by crude *B. oleronius* protein preparation (2 μg/mL) compared with that by corneal cells stimulated by pure *B. oleronius* protein (2 μg/mL) at 24 hours was statistically significant ($P = 0.0485$). At 72 hours of exposure, secretion of IL-8 by cells exposed to the semipurified *B. oleronius* protein was deemed to be significant ($P = 0.0003$) compared with that by cells exposed to the crude *B. oleronius* protein (Fig. 6B).

Effect of *Bacillus* Proteins on Expression of MMP-9 hTCEpi in Cells

Human telomerase-immortalized corneal epithelial cells were exposed to semipurified *B. oleronius* proteins (2 μg/mL and 6 μg/mL) for 24, 48, and 72 hours. Cell culture supernatants were collected, filter-concentrated, and resolved as described, and Western blots were performed to assess the effect of *Bacillus* proteins on the expression of MMP-9. The results revealed increased expression of MMP-9 in corneal cells exposed to semipurified *B. oleronius* proteins at 2 μg/mL and 6 μg/mL, with a 2.2-fold ($P < 0.0001$) and 3.5-fold ($P =$
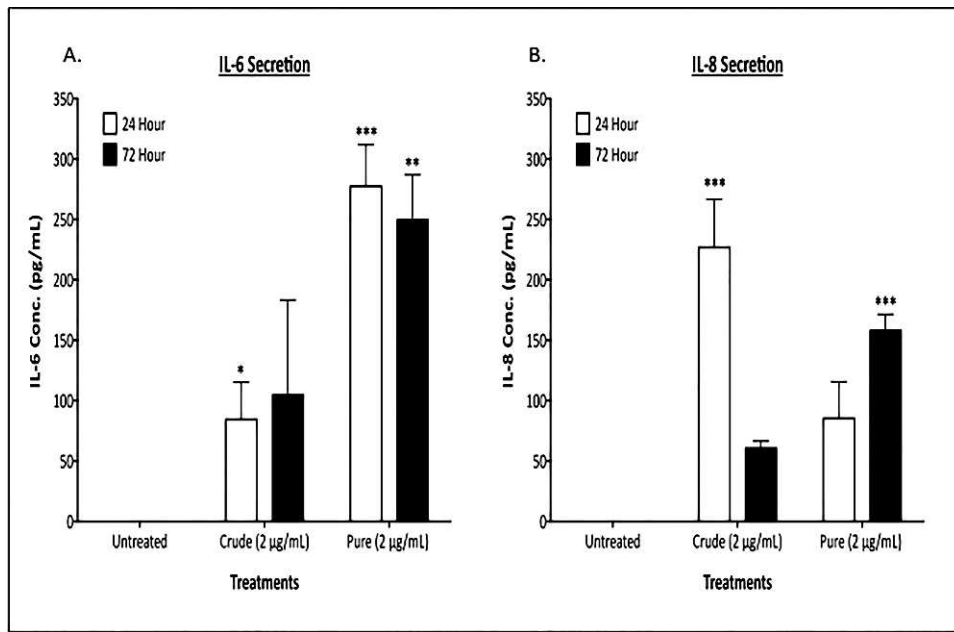
FIGURE 6. Interleukin-6 and IL-8 secretion by hTCEpi cells exposed to *B. oleronius* protein preparation. Interleukin-6 and IL-8 secreted by hTCEpi cells in response to crude (crude 2 μg/mL) and semipurified *Bacillus* (pure 2 μg/mL) protein preparations were assessed 24 hours and 72 hours after stimulation by ELISA analysis (**A, B**). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

0.0096) increase, respectively, relative to the control at 24 hours. A 1.5-fold ($P = 0.0355$) and 3.2-fold ($P = 0.003$) increase of MMP-9 expression was observed at 48 hours, after exposure to the 2- and 6-μg/mL semipurified *B. oleronius* protein preparations, respectively. At 72 hours, MMP-9 expression in corneal cells treated with 2- and 6-μg/mL semipurified *Bacillus* proteins showed a 1.3- and 2.2-fold increase, respectively, compared with the control. A significant dose-dependent
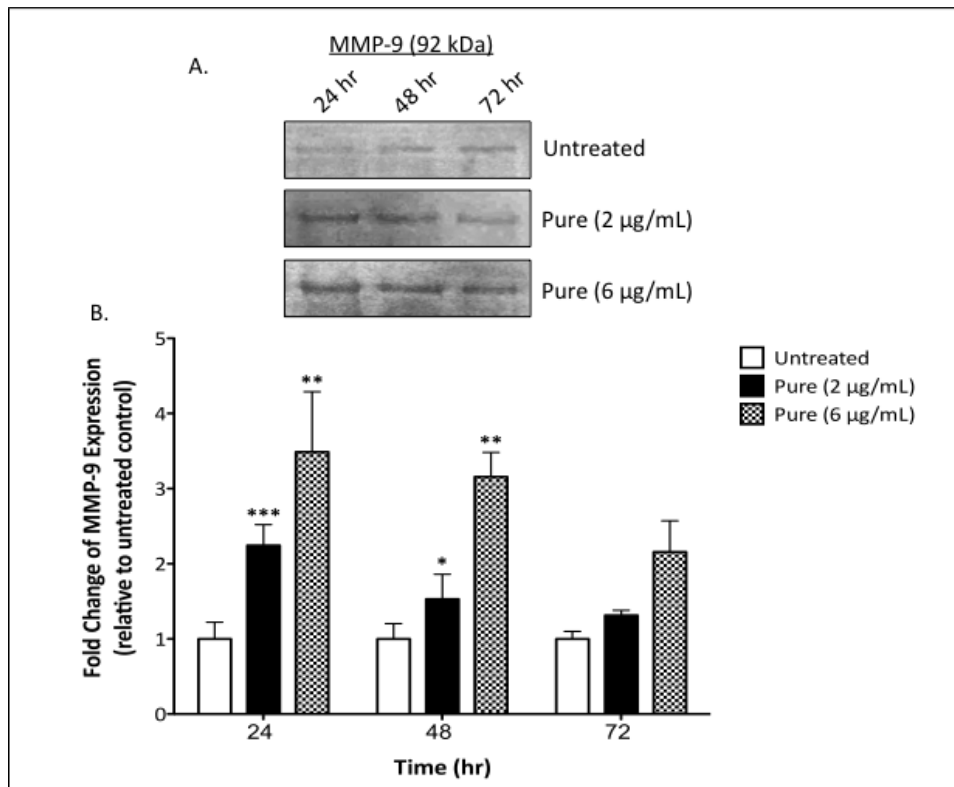

FIGURE 7. Matrix metalloproteinase-9 expression in hTCEpi cells exposed to *B. oleronius* protein preparation. The expression of MMP-9 in supernatants of hTCEpi cells exposed to semipurified *B. oleronius* protein preparations (2 and 6 μg/mL) was analyzed through Western blotting 24, 48, and 72 hours after stimulation (**A**), and MMP-9 expression was assessed by densitometric analysis (**B**). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
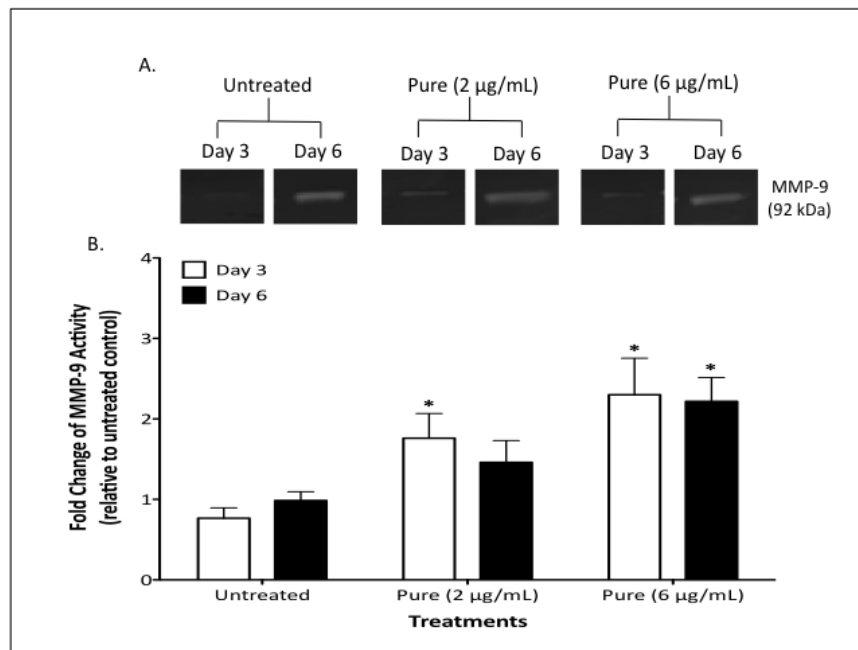
FIGURE 8. Matrix metalloproteinase-9 activity in hTCEpi cells exposed to *B. oleronius* protein preparation. Increased enzymatic activity of MMP-9 in hTCEpi cells exposed to semipurified *B. oleronius* proteins (2 and 6 μg/mL), 3 days and 6 days after stimulation was observed compared with the untreated control (**A**). Densitometry was calculated using ImageJ software and images from corresponding zymograms show that activity of MMP-9 increases after stimulation of hTCEpi cells to the *B. oleronius* protein extracts, and a significant increase of MMP-9 activity was recorded at day 6 after exposure to the higher concentration of protein (6 μg/mL) ($P = 0.0186$) (**B**). *$P < 0.05$.

change in expression of MMP-9 between the 2 μg/mL and 6 μg/mL semipurified *Bacillus* protein treatments was not observed at 24 or 72 hours, but a significant difference in dose-response was recorded at 48 hours ($P = 0.0105$) (Fig. 7).

Exposure of Cells to *Bacillus* Proteins Induces Increased MMP-9 Activity

Human telomerase-immortalized corneal epithelial cells were exposed to 2- or 6-μg/mL *Bacillus* protein preparations for up to 6 days, and MMP-9 activity was measured as described. The results revealed a significant dose-dependent increase in MMP-9 activity over the course of the experiment and a 1.8-fold ($P = 0.0134$) and 2.3-fold ($P = 0.0215$) increase in MMP-9 activity was shown after stimulation of hTCEpi cells with 2- and 6-μg/mL semipurified *B. oleronius* protein after 3 days of exposure. At day 6, a 1.5-fold increase in MMP-9 activity was recorded after exposure of hTCEpi cells to the semipurified *B. oleronius* protein (2 μg/mL), and a statistically significant increase in MMP-9 activity was observed in cells exposed to the higher dose of semipurified *B. oleronius* protein extract (6 μg/mL) ($P = 0.0186$); a 2.2-fold increase was observed at day 6 compared with untreated hTCEpi cells (Fig. 8). Matrix metalloproteinase-9 plays a role in tissue degradation, but is also a trigger for neovascularization in vivo. The growth of new blood vessels is a feature of corneal damage in ocular rosacea.[5,6]

DISCUSSION

The results presented here demonstrate that exposure of a corneal epithelial cell line to *Bacillus* proteins results in a reduction in cell proliferation and the induction of an inflammatory response as indicated by the increased expression of genes coding for IL-6, IL-1β, IL-8, S100A7, and TNF-α, and elevated MMP-9 production and activity. A dose-response effect was observed when hTCEpi cells were exposed to

different concentrations of *Bacillus* protein (i.e., 2 μg/mL and 6 μg/mL), and this effect is particularly evident in changes in cell proliferation (MGT at day 3; $P = 0.0427$) (Fig. 1), S100A7 expression (at 24 [$P = 0.0005$], 48 [$P < 0.0001$], and 72 hours [$P = 0.0002$]) (Figs. 5C, 5D), and MMP-9 expression (at 48 hours; $P = 0.0105$) (Fig. 7). Corneal damage is one of the features of ocular rosacea and leads to the degradation of the corneal surface and the formation of sterile ulcers and corneal scaring.[5,6] We previously demonstrated the ability of *Bacillus* proteins to induce an aberrant wound-healing response in cultured hTCEpi cells.[21]

Exposure of hTCEpi cells to the *Bacillus* protein preparation reduced the rate of proliferation and the antiproliferative effect was demonstrated to be due to G₁ phase arrest. Exposure to the *Bacillus* proteins did not induce apoptosis, consequently the reduction in cell proliferation was not due to increased levels of cell death. The elevated expression of the cytokines IL-1β, IL-6, IL-8, and TNF-α has been shown in a number of inflammatory cornea pathologies.[30,31] The elevation of these cytokines results in angiogenesis, inflammation, and sterile ulcer formation due to their upregulation of MMP-9.[31-33] However, a recent study demonstrated no increase in these cytokines in the tear fluid of rosacea patients and an upregulation of these cytokines in ocular rosacea patients was not observed.[34] The lack of expression may be due to the ocular rosacea patients possessing only mild symptoms and thus alterations in expression were too low to be detected. Chemokine ligand-20/MIP-3α is similar functionally to the β-defensins in its role as an antibacterial agent and the recruitment of cells into the cornea after wounding.[35-37] It has been shown to be upregulated by the cytokines IL-1β and TNF-α in cornea.[38]

The exposure of hTCEpi cells to *Bacillus* proteins induced the increased expression and activity of MMP-9. Matrix metalloproteinase-9 is expressed during cell migration and matrix remodeling in cornea wound repair by the cornea

epithelium.[39] It also controls timing of inflammation response around cornea repair.[40] However, its expression is finely balanced and elevated levels of MMP-9 have been demonstrated to have a negative role in epithelial cell replication and barrier function in the cornea.[41] In this context, elevation of MMP-9 has been linked to a number of corneal pathologies, including sterile ulcer formation and ocular rosacea.[42–44] The results presented here show the effect of *Bacillus* proteins on transformed corneal cells over a short time frame and demonstrate that the increased expression and activity of MMP-9 corresponds with an increased mean generation time and decreased corneal cell proliferation at day 3, a G_1/S transition block in the cell cycle at day 2, increased expression of S100A7 and CCL20/MIP-3α, and increased production of IL-6 and IL-8.

Elevated densities of *Demodex* mites on the eyelashes are a feature of ocular rosacea,[5,6] but their role in the etiology of the condition is unclear.[7,10,11] We have previously demonstrated a positive correlation between serum reactivity to *Bacillus* proteins and ocular rosacea, thus suggesting a role for this bacterium in the condition.[12] One possible scenario linking the elevated density of *Demodex* mites and the induction of corneal damage involves the release of bacterial cells/antigens from dead *Demodex* mites on the eyelashes. These may fall onto the corneal surface and induce an aberrant wound-healing response.[21] In addition, the results presented here indicate the induction of an inflammatory response that would contribute to the degradation of corneal tissue and the induction of sterile ulcers or scarring in vivo. Understanding the interaction of *Demodex*-associated *Bacillus* proteins with the corneal surface may aid in the design of specific therapies to reduce the severity of symptoms arising from this interaction.

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