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Proinflammatory chemokines during *Candida albicans* keratitis

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ABSTRACT

Chemotactic cytokines mediate the recruitment of leukocytes into infected tissues. This study investigated the profile of chemokines during experimental *Candida albicans* keratitis and determined the effects of chemokine inhibition on leukocyte infiltration and fungal growth during murine keratomycosis. Scarified corneas of BALB/c mice were topically inoculated with *C. albicans* and monitored daily over one week for fungal keratitis. After a gene microarray for murine chemokines compared infected corneas to controls, real-time reverse transcription polymerase chain reaction (RT-PCR) and immunostaining assessed chemokine expression in infected and mock-inoculated corneas. An anti-chemokine antibody was then administered subconjunctivally and evaluated for effects on clinical severity, corneal inflammation, fungal recovery, and cytokine expression. Of 33 chemokine genes examined by microarray, 6 CC chemokines and 6 CXC chemokines were significantly ($P < 0.05$) upregulated more than two-fold. Chemokine (CC-motif) ligand 3 (CCL3) was upregulated 108-fold ($P = 0.03$) by real-time RT-PCR within one day after fungal inoculation and remained increased 28-fold ($P = 0.02$) at one week, and its *in situ* expression increased in the epithelium and stroma of infected corneas. Compared to the control antibody-treated group, eyes treated with anti-CCL3 antibody showed reduced clinical severity ($P < 0.05$), less corneal neovascularization ($P = 0.02$), and fewer inflammatory cells infiltrating corneal tissue, but the amount of recoverable fungi was not significantly ($P = 0.4$) affected. Anti-CCL3 treatment significantly ($P = 0.01$) reduced the expression of tumor necrosis factor and interleukin-1β in infected corneas. These results indicate that chemokines, especially the CC chemokine CCL3, play important roles in the acute inflammatory response to *C. albicans* corneal infection.

1. Introduction

Candida albicans, a component of the normal flora, is a fungal opportunist for the eye (Kercher et al., 2001). Trauma and disease predispose to corneal infection (Sun et al., 2007). During *C. albicans* keratitis yeasts transition into filamentary forms that breach the eye's defenses and penetrate the stroma (Jackson et al., 2007). Hyphal invasion elicits host responses that bring about corneal inflammation and ulceration (Yuan et al., 2009).

Innate immunity and acute inflammation actively participate in the pathophysiology of fungal keratitis. The cornea detects the presence of invasive *C. albicans* by toll-like receptors and other pathogen-recognition molecules (Yuan and Wilhelmus, 2010). Corneal epithelial cells, keratocytes, and phagocytes are involved in distinguishing pathogen-associated molecular patterns, and this interaction activates innate responses. Neutrophils, key effector cells for controlling fungal growth, afford a first line of defense during mucosal *C. albicans* infection.

Leukocytes are drawn into the cornea by the local production of chemotactic cytokines during the initial stages of fungal keratitis (Zhong et al., 2009). Chemokines are released at an early stage of fungal infection and bind to receptors that trigger the synthesis of interleukins and other cytokines that promote fungal clearance (Traynor and Huffnagle, 2001). Members of the CC chemokine subfamily such as CCL2 and CCL3 have potent chemotactic and activating properties for neutrophils and are rapidly induced in bacterial keratitis (Xue et al., 2007).

This study examined the profile of chemokines after the onset of experimental *C. albicans* keratitis and followed the relative expression of selected CC chemokines during the first week of fungal keratitis in mice. We also studied the effect of an anti-CCL3 antibody on inflammatory severity, fungal growth, and cytokine expression during *C. albicans* keratitis. Our findings suggest that chemokines such as CCL3 facilitate innate immune responses in the pathogenesis of fungal keratitis.

Abbreviations: CCL2, chemokine (CC-motif) ligand 2; CCL3, chemokine (CC-motif) ligand 3; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α.

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2. Methods

2.1. Fungi and media

C. albicans strain SC5314, a clinical isolate capable of producing experimental keratomycosis, was cultured on Sabouraud dextrose agar (Difco, Detroit, MI) for 3 days at 25 °C. Colonies were harvested after 3 days of inoculation and diluted in sterile phosphate-buffered saline (PBS) to yield 2×10^5 colony-forming units (CFU)/μL based on the optical density (OD) at 600 nm, using an OD_{600} conversion factor of 3×10^7 CFU/mL.

2.2. Animal model

Animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research under protocols approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. Female BALB/c mice 6–8 weeks of age (Harlan Sprague–Dawley, Houston, TX) were anesthetized with an intraperitoneal injection of ketamine, xylazine, and acepromazine. The corneas of right eyes were superficially scarified with a 22-gauge needle. A 5-μL inoculum of either *C. albicans* containing 1×10^6 CFU or sterilized PBS was topically applied to eyes of infected and control groups, respectively. Mice were monitored daily for 7 days postinoculation (p.i.) using a dissecting microscope to categorize corneal inflammation and angiogenesis. The severity of keratitis was graded by a scoring system that consisted of the relative area of corneal infiltrate, density of corneal opacity, and surface regularity (Wu et al., 2003). The amount of corneal vascularization was assessed by a neovascularization scoring system that assigned grades of 0–4 for the number, density, and length of visible corneal blood vessels (Yuan and Wilhelmus, 2009). Corneal photographs with the eye positioned *en face* or in lateral profile were captured with a Zeiss photo slit-lamp and Nikon digital camera.

2.3. RNA extraction

Mice were sacrificed 1 day p.i. After enucleation corneas were excised and dissected from surrounding conjunctiva and uvea. Pools of 5 corneas were prepared in triplicate from *C. albicans*-infected and control groups at day 1 p.i. RNA was extracted by a previously reported procedure (Yuan et al., 2009) and was isolated with RNeasy MicroKit columns (Qiagen, Valencia, CA). Samples were treated with DNase (Qiagen, Valencia, CA) and stored at −80 °C.

2.4. Gene microarray

Genetic analysis was performed at the Microarray Core Facility, Baylor College of Medicine, as reported (Yuan et al., 2009). After checking RNA samples for quality assurance, Genechip (Affymetrix, Santa Clara, CA) microarray protocols were applied to qualified samples of 3 five-cornea pools from each group for two cycles of amplification. Images and quality control metrics were recorded using Affymetrix GCOS software version 1.4, and signal-intensity data were adjusted and analyzed with BioConductor software. The criterion for significance of differentially regulated genes was established as >2-fold change with adjusted $P < 0.05$.

2.5. Quantitative polymerase chain reaction

Total RNA isolated from corneas at 1, 3, and 7 days p.i. was quantified by absorbance at 260 nm. The first-strand cDNA was synthesized from 0.4 μg RNA with Ready-To-Go You-Prime First-Strand Beads (GE Healthcare, Princeton, NJ) and random hexamers (Applied Biosystems, Foster City, CA). Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed using TaqMan Gene Expression Master Mix and Assays (Applied Biosystems). Primers specific for *CCL3, CCL2, CXCL1, TNF, IL-1β, IL-6, VEGF-A, MMP8, MMP13,* and *CRAMP* (Applied Biosystems) were used to quantify gene expression levels. The threshold cycle (C_T) for each target mRNA was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and averaged. Three five-cornea pools were processed for each group. Two-group comparisons were done using the Student *t*-test, and three-group comparisons used one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

2.6. Immunofluorescence

Eyes obtained 1 day p.i. were embedded in OCT compound (Sakura Finetek, Torrance, CA), snap-frozen in liquid nitrogen, and sectioned at 15-μm thickness. Sections were thawed, dehydrated, and fixed in 2% paraformaldehyde then blocked with 10% normal donkey serum (Jackson ImmunoResearch Laboratories, Philadelphia, PA). Immunofluorescent staining was performed as reported (Yuan et al., 2009). Goat serum antibody to the N-terminus of murine CCL3 (R&D Systems, Minneapolis, MN) was diluted 1:100, and applied to blocked sections that were incubated overnight at 4 °C. Secondary Alexa-Fluor 488-conjugated donkey anti-goat immunoglobulin (Invitrogen, Carlsbad, CA) was applied to sections that were then incubated in a dark chamber for 1 h and counterstained with propidium iodine (Invitrogen) in Gel/Mount (Biomeda, Foster City, CA). Sections were observed with a laser-scanning confocal microscope (LSM 510, Zeiss, Thornwood, NY) with 488- and 543-nm excitation and emission filters. Images were acquired with a 40× oil-immersion objective and processed using Zeiss LSM-PC software.

2.7. Anti-CCL3 treatment

Goat anti-mouse CCL3 and control goat immunoglobulin were obtained (R&D Systems). Five mice were allocated to treatment and control groups, respectively. Each mouse was administered 10 μg of antibodies subconjunctivally 30 minutes before superficial corneal scarification. Scarified corneas of the treated group were topically inoculated with *C. albicans* at a dosage of 1×10^6 CFU/5 μL. Control mice received 5-μL of normal goat IgG antibody. Eyes were observed daily with a dissecting microscope to grade the severity of keratitis. Ten additional mice were treated subconjunctivally with either anti-CCL3 antibody or goat IgG 30 min before fungal inoculation and then sacrificed one day p.i. for fungal recovery.

2.8. Quantitative fungal recovery

Quantitative fungal cultures were performed on excised corneas by a previously reported method (Yuan and Wilhelmus, 2009). In brief, corneas were homogenized in a frosted-glass grinder with 500 μL PBS, and the homogenated aliquot was diluted 10-fold with PBS. The entire aliquot was then inoculated onto Sabouraud dextrose agar that was incubated for 4 days at 25 °C. Visible colonies were counted, and the number of CFUs per cornea was compared between treated and control groups with the Student *t*-test.