Toll-like Receptors Involved in the Pathogenesis of Experimental *Candida albicans* Keratitis

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PURPOSE. To investigate the expression and function of toll-like receptors (TLRs) during experimental keratomycosis.

METHODS. Scarified corneas of BALB/c mice were topically inoculated with *Candida albicans* and compared with control corneas by a murine gene microarray and immunostaining. Real-time reverse transcription polymerase chain reaction (RT-PCR) determined relative *TLR* gene expression in murine and human donor corneas. The scarified corneas of *TLR2*^{-/-} mice, *TLR4*^{-/-} mice, and C57BL/6J control mice were also inoculated with *C. albicans*, to determine relative severity, fungal load, and cytokine transcript levels.

RESULTS. *TLR1*, -2, -4, -6, and -13 were significantly upregulated (5- to 10-fold; $P < 0.01$) by microarray, and *TLR1*, -2, -4, and -13 were significantly increased (4- to 11-fold; $P < 0.05$) by real-time RT-PCR in BALB/c murine corneas. Similarly, *TLR2*, -6, and -13 were significantly upregulated (5- to 16-fold; $P \leq 0.001$) by real-time RT-PCR in C57BL/6J murine corneas the day after inoculation, and *TLR2* and -13 remained significantly ($P < 0.05$) increased after 1 week. *TLR2* transcript was also upregulated twofold ($P = 0.04$) in *C. albicans*-inoculated explanted human corneas. Although murine keratitis severity scores were similar, significantly more fungi were recovered from *TLR2*^{-/-} mouse corneas ($P = 0.04$) than from *TLR4*^{-/-} mouse corneas ($P = 0.9$). Tumor necrosis factor- α , interleukin 23, chemokine C-C ligands 3 and 4, and dectin-1 were significantly ($P < 0.05$) downregulated in *C. albicans*-infected corneas of *TLR2*^{-/-} mice.

CONCLUSIONS. *TLR2* signals proinflammatory cytokines that control fungal growth during *C. albicans* keratitis. *TLR13* may have an additional role in the innate immune response of murine corneal candidiasis. (*Invest Ophthalmol Vis Sci.* 2010; 51:2094-2100) DOI:10.1167/iovs.09-4330

The eye encounters invasive microorganisms through cell-surface molecules such as toll-like receptors (TLRs), structural homologues of Toll proteins discovered in *Drosophila melanogaster*.¹ Most vertebrates have at least one TLR representative from six major clades. For example, *TLR1* to -9 and *TLR11* to -13 are expressed in BALB/c mice and, at lower levels, C57BL/6J mice.²

Membrane-bound TLRs are capable of recognizing a discrete set of microbial ligands. This interaction leads to changes

of an internal domain that, in association with adaptor proteins such as myeloid differentiation marker 88 (MyD88), initiates a molecular cascade that generates proinflammatory cytokines and antimicrobial peptides.^{3,4}

TLRs and other pathogen recognition receptors expressed by the cornea serve as antimicrobial defenses of the ocular surface, and specific TLRs mediate the eye's inflammatory reaction to infectious pathogens.⁵⁻⁷ In bacterial keratitis *TLR2* engages *Staphylococcus aureus*,^{8,9} and *TLR4* promotes corneal responses to *Pseudomonas aeruginosa*.^{10,11} *TLR2* and -4 on corneal epithelial cells, keratocytes, and leukocytes also recognize fungi such as *Aspergillus fumigatus* and *Fusarium solani*.¹²⁻¹⁷

TLR4 is upregulated during *F. solani* keratitis in BALB/c mice¹⁸ and is involved in controlling fungal infection during *Fusarium oxysporum* keratitis in C57BL/6J mice.¹⁹ MyD88^{-/-} and *TLR4*^{-/-} mice, but not *TLR2*^{-/-} mice, have an initially increased *F. oxysporum* burden because of reduced fungal clearance in the cornea.¹⁹ A human pathologic analysis found that *TLR2*, -4, and -9 levels are increased in the cornea during *F. solani* keratitis.²⁰ These studies suggest that TLR-mediated responses are involved in the pathogenesis of fungal keratitis, but the relative roles of TLRs for different fungal species remain to be more clearly determined.

We used a murine model of posttraumatic *Candida albicans* keratitis to examine the corneal TLR profile during the onset and progression of experimental keratomycosis in BALB/c and C57BL/6J mice. In this system, fungi adhere to the injured corneal surface and invade the stroma, triggering an influx of neutrophils and macrophages.^{18,21} We also compared corneal inflammatory severity and fungal recovery from *TLR2*^{-/-} and *TLR4*^{-/-} knockout mice and investigated the relative production of downstream mediators of innate immunity during *C. albicans* corneal infection. In addition, we assessed the profile of *TLR* gene expression in human donor corneas after ex vivo *C. albicans* exposure.

MATERIALS AND METHODS

Fungi

C. albicans strain SC5314 is a clinical isolate capable of producing experimental keratomycosis.²¹ Yeasts cultured on Sabouraud dextrose agar (Difco, Detroit, MI) for 3 days at 25°C were harvested and diluted in sterile phosphate-buffered saline (PBS). Inocula of 2×10^6 , 2×10^5 , or 10^4 colony-forming units (CFU), based on an optical density (OD) of 600 nm with a conversion factor of 1 OD₆₀₀ unit equal to 3×10^7 CFU/mL, were prepared for BALB/c mice, C57BL/6J mice, or donor corneas, respectively.

Animals

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 and C57BL/6J mice were

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used at 6 to 8 weeks of age (Harlan Sprague-Dawley, Houston, TX). TLR2^{-/-} and TLR4^{-/-} mice, backcrossed to C57BL/6J mice, were provided by the Research Institute for Microbial Disease (Osaka, Japan).^{22,23} Genotypes were confirmed by polymerase chain reaction (PCR) amplification of tail DNA. After intraperitoneal anesthesia with ketamine, xylazine, and acepromazine, the corneas of right eyes were superficially scarified.²¹ A 5-μL inoculum of either *C. albicans* (1 × 10⁶ CFU or 1 × 10⁵ CFU) or sterilized PBS buffer was topically applied to eyes of infected and control groups, respectively. Mice were monitored daily by dissecting microscope for 7 days postinoculation (pi), to categorize disease severity according to the area of corneal infiltrate, density of corneal opacity, and surface regularity, as previously reported.²¹ Corneal photographs were captured with a photo slit lamp (Carl Zeiss Meditec, Dublin, CA) and digital camera (Nikon, Tokyo, Japan).

Ex Vivo Human Cornea Model

Six human corneas were provided by the Lions Eye Bank of Texas, Houston, after informed consent for research use was obtained from the decedent donors' next of kin. The corneas were managed in accordance with the guidelines in the Declaration of Helsinki. An ex vivo human corneal model of keratomycosis followed our previously reported method.²⁴ In brief, donor corneas held in an artificial anterior chamber (Refractive Technologies, Cleveland, OH) were superficially scarified with a 22-gauge needle to produce a 15 × 15 cross-hatch pattern. Scarification was followed by topical application of 10 μL of 1 × 10⁴ CFU *C. albicans* or 10 μL of PBS per cornea. Inoculated corneas were put epithelial side up into a six-well culture dish (Corning, Corning, NY), with the sclera immersed in modified supplemented hormonal epithelial medium so that the central cornea vaulted upward. The tissues were incubated at 34°C in 5% CO₂ with 95% humidity. After 24 hours, corneas were processed for RNA extraction.

Corneal Fungal Recovery

Five infected corneas from each group of TLR2^{-/-}, TLR4^{-/-}, and C57BL/6J control mice were excised at days 1 and 3 pi and ground up

in PBS. Serial dilutions were inoculated onto Sabouraud dextrose agar plates to estimate fungal recovery. Colony counts, converted into CFU per cornea, were compared between knockout mouse strains and controls by Student's *t*-test. *P* < 0.05 was considered statistically significant.

Corneal RNA Extraction

Mice were euthanatized 1, 3, and 7 days pi, and eyes were enucleated for analysis. The corneas were dissected by removing adjacent conjunctiva and uvea. Pools of five corneas were prepared in triplicate from *C. albicans*-infected and control groups of BALB/c mice at days 1, 3, and 7 pi and from TLR2^{-/-}, TLR4^{-/-}, and C57BL/6J mice at day 1 pi. RNA was extracted and isolated (RNeasy MicroKit columns; Qiagen, Valencia, CA).²⁵ The corneal epithelium was separated from the stroma by debridement 24 hours after inoculation from three BALB/c murine eyes, and RNA was separately extracted from the epithelial and stromal layers. All samples were treated with DNase (Qiagen) to avoid DNA contamination and were stored at -80°C until use.

Gene Microarray

Microarray analysis was performed at the Microarray Core Facility of Baylor College of Medicine.²⁵ After quality assurance of RNA samples, two cycles of amplification (Affymetrix GeneChip 430.2; Affymetrix, Santa Clara, CA) was applied to qualified samples of three five-cornea pools from infected and mock-infected groups (five corneas per group) of BALB/c mice at 1 pi. Images and quality-control metrics were recorded with the microarray software (GCOS software version 1.4; Affymetrix). Raw signal-intensity data were adjusted and analyzed with R-based software (Bioconductor, Seattle, WA). Criteria for significance of differentially regulated genes were established as a more than two-fold change and adjusted *P* < 0.05.

Quantitative Polymerase Chain Reaction

Total RNA isolated from the groups of murine corneas or from cultured human corneas was quantified by optical absorbance at 260 nm. The

TABLE 1. Primers Used for Real-Time RT-PCR

Gene Name	Gene Symbol	GenBank Accession No.	Assay ID	Amplicon Length (bp)
Toll-like receptor 1	<i>TLR1</i>	NM_003263.3	Hs00413978_ml	72
Toll-like receptor 2	<i>TLR2</i>	NM_003264.3	Hs00610101_ml	80
Toll-like receptor 3	<i>TLR3</i>	NM_003265.2	Hs01551078_ml	132
Toll-like receptor 4	<i>TLR4</i>	NM_138554.3	Hs01060206_ml	125
Toll-like receptor 5	<i>TLR5</i>	NM_003268.4	Hs01920773_ml	89
Toll-like receptor 6	<i>TLR6</i>	NM_006068.2	Hs00271977_s1	122
Toll-like receptor 7	<i>TLR7</i>	NM_016562.3	Hs00152971_ml	125
Toll-like receptor 8	<i>TLR8</i>	NM_138636.3	Hs00607866_mH	106
Toll-like receptor 9	<i>TLR9</i>	NM_017442.2	Hs00152973_ml	139
Toll-like receptor 10	<i>TLR10</i>	NM_030956.2	Hs01675179_ml	121
Toll-like receptor 1	<i>TLR1</i>	NM_030682.1	Mm00446095_ml	82
Toll-like receptor 2	<i>TLR2</i>	NM_011905.3	Mm00442346_ml	69
Toll-like receptor 4	<i>TLR4</i>	NM_021297.2	Mm00445274_ml	117
Toll-like receptor 6	<i>TLR6</i>	NM_011604.3	Mm02529782_ml	96
Toll-like receptor 13	<i>TLR13</i>	NM_205820.1	Mm01233819_ml	66
Tumor necrosis factor-α	<i>TNFα</i>	NM_013693.2	Mm99999068_ml	63
Interleukin 1-β	<i>IL1β</i>	NM_008361.3	Mm00434228_ml	90
Interleukin 6	<i>IL6</i>	NM_031168.1	Mm00446191_ml	124
Interleukin 23	<i>IL23</i>	NM_031252.2	Mm00518984_ml	61
Chemokine (C-C motif) ligand 3	<i>CCL3</i>	NM_013652.2	Mm00443111_ml	70
Chemokine (C-C motif) ligand 4	<i>CCL4</i>	NM_011337.2	Mm99999057_ml	81
Matrix metalloproteinase 8	<i>MMP8</i>	NM_008611.4	Mm00772335_ml	127
Matrix metalloproteinase 9	<i>MMP9</i>	NM_013599.2	Mm00442991_ml	76
Matrix metalloproteinase 13	<i>MMP13</i>	NM_008607.1	Mm00439491_ml	65
Dectin-1	<i>CLEC7A</i>	NM_020008.2	Mm01183349_ml	77
Glyceraldehyde-3-phosphate dehydrogenase (murine)	<i>GAPDH</i>	NM_008084.2	Mm99999915_g1	107
Glyceraldehyde-3-phosphate dehydrogenase (human)	<i>GAPDH</i>	NM_002046.3	Hs99999905_ml	122

Assay ID is from Applied Biosystems, Inc., Foster City, CA.

FIGURE 1. Clinical evaluation of *C. albicans* keratitis in C57BL/6J, TLR2^{-/-}, and TLR4^{-/-} mice. Each point represents the mean severity score with SD.

first-strand cDNA was synthesized from 0.4 μ g of total RNA for murine tissues or from 1.0 μ g total RNA for human tissues (Ready-To-Go You-Prime First-Strand Beads; GE Healthcare, Princeton, NJ) and random hexamers (Applied Biosystems, Inc. [ABI], Foster City, CA). Real-time PCR was performed (TaqMan Gene Expression Master Mix and Assays; ABI). Primers were used to quantify gene expression levels (Table 1). The threshold cycle (C_T) for each target mRNA was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and averaged. Two-group comparisons were performed by Student's *t*-test and three-group comparisons by one-way analysis of variance (ANOVA). For longitudinal analysis of transcriptional levels, mean results were compared by ANOVA, using the Tukey-Kramer method. $P < 0.05$ was considered statistically significant.

Immunofluorescent Staining

Eyes removed at 1 and 3 days pi were embedded in OCT compound (Sakura Finetek, Torrance, CA), snap-frozen in liquid nitrogen, and sectioned on a cryostat at 16- μ m thickness. Sections were thawed, dehydrated, fixed in cold acetone, and blocked with 10% normal donkey serum (Jackson ImmunoResearch Laboratories, Philadelphia, PA). Goat anti-TLR2 antibody (sc-12504; Santa Cruz Biotechnology, Santa Cruz, CA) and goat anti-TLR4 antibody (sc-16240; Santa Cruz Biotechnology) were diluted 1:100 and applied to blocked sections that were incubated overnight at 4°C. Secondary Alexa-Fluor 488-conjugated donkey anti-goat antibody (Invitrogen, Carlsbad, CA) was applied to sections incubated in a dark chamber for 1 hour. After counterstaining with propidium iodide (Invitrogen) in mounting medium (Gel/Mount; Biomedex, Foster City, CA), the sections were observed with a laser-scanning confocal microscope (LSM 510; Carl Zeiss Meditec) with 488- and 543-nm excitation and emission filters. Images were acquired with a 40 $\times$ oil-immersion objective and processed (LSM-PC software; Carl Zeiss Meditec).

RESULTS

Keratitis Severity and Fungal Load

All fungus-inoculated mice showed clinical signs of fungal keratitis. Lower severity was found ($P = 0.02$) in TLR2^{-/-} mice (8.2 ± 0.8) than in wild-type C57BL/6J mice (8.9 ± 0.3) 1 day pi, although no difference was found between TLR4^{-/-} mice and C57BL/6J mice ($P = 0.48$). On subsequent days, daily severity scores were similar in TLR2^{-/-} or TLR4^{-/-} mice compared with wild-type control animals ($P > 0.05$ for each comparison at days 2–7; Fig. 1). Counts of fungal cultures in

infected corneas from TLR2^{-/-} and TLR4^{-/-} mice were similar to those in control mice at 1 day pi. At 3 days pi, the number of colonies recovered from TLR2^{-/-} mouse corneas was significantly higher than in TLR4^{-/-} mice or control mice (both $P = 0.04$; Table 2).

TLR Gene Expression Profile

Comparison of gene arrays for *C. albicans*-infected corneas and mock-inoculated control corneas of BALB/c mice showed that ratios of expression levels at 1 day pi for *TLR1* (8.4-fold, $P = 0.007$), *-2* (8.2-fold, $P = 0.004$), *-4* (4.7-fold, $P = 0.008$), *-6* (11.3-fold, $P = 0.003$), and *-13* (20.1-fold, $P = 0.005$) were significantly upregulated, whereas *TLR3*, *-5*, *-7*, *-8*, *-9*, and *-12* did not differ significantly between infected and control corneas (Table 3). Transcript levels detected by quantitative real-time RT-PCR of BALB/c mice were consistent with microarray findings (Table 3). Compared with normal eyes, scarified and mock-inoculated control eyes were not significantly different in expression levels of *TLR1*, *-2*, *-4*, *-6*, or *-13*. Similar results were found in C57BL/6J mice, which had significantly upregulated expression of *TLR2* (5.4-fold, $P = 0.001$), *-6* (16.2-fold, $P = 0.0003$), and *-13* (10.5-fold, $P = 0.0002$), whereas *TLR1* (2.1-fold, $P = 0.13$) and *-4* (1.3-fold, $P = 0.28$) did not change significantly during *C. albicans* keratitis compared with the control animals (Table 4). For upregulated TLR genes, real-time RT-PCR was performed on RNA extracted separately from epithelium and stroma, and no significant difference was found in *TLR2* ($P = 0.45$), *-4* ($P = 0.42$), *-6* ($P = 0.83$), and *-13* ($P = 0.36$) in BALB/c mouse corneas at 1 day pi. In human corneas ex vivo challenged by *C. albicans*, *TLR2* was significantly upregulated (2.2-fold, $P = 0.037$; Table 5).

TLR2 and -4 Protein Expression Pattern

The in situ pattern determined by immunofluorescent staining showed moderately increased epithelial staining for TLR2 among infected corneas of both BALB/c and C57BL/6J mice at 1 day pi (Fig. 2). TLR4 expression in epithelial and stromal layers during early postscarification infection was not detectably different from that of scarified control eyes.

TLR Kinetic Analysis

Real-time RT-PCR on total RNA extracted from pooled corneas showed differences in selected TLRs between *C. albicans* keratitis and scarified controls in BALB/c mouse corneas at 1, 3, and 7 days pi (Fig. 3). Despite relatively lower levels on the third day pi, *TLR13* and, to a lesser extent, *TLR1*, *-2*, and *-4* remained significantly ($P = 0.01$) upregulated at 1 week pi. Compared with normal corneas, scarification alone did not significantly alter TLR expression levels at 1, 3, and 7 days of follow-up (data not shown).

Cytokines, Chemokines, and Metalloproteinases by Real-Time RT-PCR

At 1 day pi, transcript levels significantly downregulated during *C. albicans* keratitis in TLR2^{-/-} mouse corneas compared to

TABLE 2. Recovery of Viable *C. albicans* from Infected Murine Corneas

Genotype	n	Day 1			Day 3		
		CFU/Cornea	P		CFU/Cornea	P	
C57BL/6J	5	2355.0 $\pm$ 1855.4	—	10	111.5 $\pm$ 137.4	—	
TLR2 ^{-/-}	5	2385.0 $\pm$ 1475.6	0.98	10	421.0 $\pm$ 431.9	0.045	
TLR4 ^{-/-}	5	2014.0 $\pm$ 1694.3	0.77	10	103.5 $\pm$ 111.1	0.89	

Data are the mean $\pm$ SD. CFU, colony-forming units.

TABLE 3. Microarray Analysis and Real-Time RT-PCR of BALB/c Mice Cornea, Confirming *TLR* Gene Expression Ratios Comparing *C. albicans* Keratitis to Mock-Infected Control

Molecule	GenBank Accession No.	Mean Signal Intensity Ratio by Microarray	<i>P</i> *	Mean Fold Change by Real-Time RT-PCR	<i>P</i> *
<i>TLR1</i>	AAG35062	8.4	0.007	4.61	0.03
<i>TLR2</i>	AAO21125	8.2	0.004	4.21	0.01
<i>TLR3</i>	BAE38611	-1.5	0.34	ND	—
<i>TLR4</i>	AAD29272	4.7	0.008	3.85	0.01
<i>TLR5</i>	CAD61977	1.2	0.57	ND	—
<i>TLR6</i>	AAH55366	11.3	0.003	4.21	0.06
<i>TLR7</i>	AAL73192	1.4	0.17	ND	—
<i>TLR8</i>	AAK62677	-1.0	0.83	ND	—
<i>TLR9</i>	AAK29625	1.0	0.99	ND	—
<i>TLR12</i>	AAS37673	1.0	0.89	ND	—
<i>TLR13</i>	AAS37674	20.1	0.005	10.73	0.01

GenBank is provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD (www.ncbi.nlm.nih.gov). ND, not determined.

* Comparing TLR signals between *C. albicans*-infected and mock-infected groups. Three pools per group, with five corneas per pool.

C57BL/6J wild-type corneas included genes encoding tumor necrosis factor- α , interleukin 23, chemokine C-C ligand (CCL)3, and CCL4 (Table 6). No significant changes were found in *TLR4*^{-/-} mouse corneas for these genes nor in genes coding for MMP8, -9, or -13. The gene encoding dectin-1 was downregulated significantly in infected *TLR2*^{-/-} (-3.3-fold, *P* = 0.02) and *TLR4*^{-/-} (-2.8-fold, *P* = 0.04) mice compared with wild-type C57/6J mice at day 1 pi (Table 6). Interrelationships of TLR expression in *TLR2*^{-/-} or *TLR4*^{-/-} mouse corneas are shown in Table 7.

DISCUSSION

C. albicans, part of the eye's normal microflora, is a corneal opportunist and ocular pathogen.^{26,27} The pathogenesis of *C. albicans* keratitis involves fungal virulence factors that induce yeasts to generate filamentous forms capable of invading the injured cornea.²⁸ The dynamic equilibrium between commensalism and infection also depends on a stable immune response.²⁹ Altered local defenses and immunosuppression upset the eye-fungus confrontation to increase susceptibility toward keratomycosis.^{21,30}

Innate immunity is a pivotal host response during candidal infection.³¹ Pathogen recognition receptors form a critical arm in the immune recognition of invasive fungi.³² Toll proteins were found to control resistance to fungal infection in *Drosophila*,³³ and TLRs have emerged as important intermediates during human mycoses.³⁴ TLRs detect molecular patterns on fungi^{35,36} that may include components of the *C. albicans* cell wall.³⁷ Which receptors are activated most likely depends on the fungal species or morphotype and on the site of infection.^{38,39}

In the present study we evaluated TLR ligand effects using *C. albicans* in models of keratomycosis. In BALB/c mice, *TLR1*,

-2, -4, -6, and -13 were upregulated within 1 day after the onset of *C. albicans* keratitis, with *TLR1*, -2, -4, and -13 remaining increased more than twofold 1 week later. C57BL/6J mice were comparatively more susceptible to experimental fungal keratitis and demonstrated enhanced expression of *TLR2*, -6, and -13 during early infection. Furthermore, ex vivo *C. albicans* infection of explanted human donor corneas resulted in significant upregulation of *TLR2* the day after fungal inoculation.

TLR-knockout mice helped to characterize the relative influence of *TLR2* and -4 in experimental *C. albicans* keratitis. Although the severity of fungal keratitis in murine mutant strains was similar to that in wild-type control mice, more fungi were recovered after 3 days of infection from *TLR2*^{-/-} than from *TLR4*^{-/-} mouse corneas. Our study supports the hypothesis that *TLR2* is a fungal recognition receptor during *C. albicans* infection of the cornea.⁴⁰

TLRs have been implicated in the mediation of host immune responses during systemic candidiasis.^{41,42} *TLR2* and -4 may play complementary roles,⁴³ and explanations of the specific TLR responses to *C. albicans* have been set forth. One scheme proposes that *TLR2*, in cooperation with lectinlike or protease-activated receptors or as a heterodimer with *TLR1* or -6, detects *C. albicans* hyphae and contributes to local tissue destruction whereas the recognition of yeasts by *TLR2* and -4 brings about the release of chemokines and recruitment of leukocytes.^{44,45} The switch from yeasts to filamentous forms could be responsible for eluding recognition by *TLR4* but not *TLR2*, enabling *C. albicans* to invade.³⁹ An alternative proposal infers that *TLR2* recognizes fungal cell wall glycolipids and instigates the production of inflammatory cytokines.^{46,47} Our

TABLE 4. Real-Time RT-PCR Analysis of *TLR* Gene Expression Ratios Comparing *C. albicans* Keratitis to Mock-Infected Controls in C57BL/6J Mice

Gene	Mean Ratio	<i>P</i>
<i>TLR1</i>	2.11	0.13
<i>TLR2</i>	5.35	0.001
<i>TLR4</i>	1.27	0.28
<i>TLR6</i>	16.19	0.0003
<i>TLR13</i>	10.51	0.0002

Three pools per group, with five corneas per pool.

TABLE 5. Real-Time RT-PCR Analysis of *TLR* Gene Expression Ratios Comparing *C. albicans*-Challenged Donor Corneas to Mock-Inoculated Corneas

Gene	Mean Ratio	<i>P</i>
<i>TLR1</i>	1.30	0.53
<i>TLR2</i>	2.23	0.037
<i>TLR3</i>	1.81	0.25
<i>TLR4</i>	2.44	0.19
<i>TLR5</i>	-1.34	0.25
<i>TLR6</i>	-1.68	0.62
<i>TLR7</i>	-2.23	0.18
<i>TLR8</i>	-3.87	0.11
<i>TLR9</i>	-1.95	0.45

Three corneas per group.

FIGURE 2. Protein expression patterns in situ in corneas with *C. albicans* keratitis (Infected) or mock-inoculated control corneas (Mock), comparing negative control corneas lacking primary antibody (Control) and those processed with anti-TLR2 or -TLR4 antibodies. Immunofluorescence showed TLR2 in the healed corneal epithelium of mock-infected eyes and of infected eyes 1 day after the onset of experimental *C. albicans* keratitis. TLR4 was present in the epithelium and stroma. Original magnification, $\times 400$. Scale bar, 20 μm .

results support the premise that TLR2, possibly in collaboration with TLR1 or -6, detects pathogen-associated molecular patterns on *C. albicans* during corneal infection.

Our findings also suggest that TLR13 may mediate responses to fungal infection. Although its microbial ligands are as yet unclear, TLR13 acts via MyD88 and increases during viral and parasitic infections in mice.^{48,49} TLR13 may be an intra-

FIGURE 3. Differential gene expression ratios of TLRs detected by real-time RT-PCR throughout the first week in BALB/c mouse corneas with posttraumatic *C. albicans* keratitis compared to those in scarified, mock-infected control corneas. On day 3 pi, TLR13 was increased 5.1-fold ($P = 0.01$). By day 7 pi, TLR2 was upregulated 2.8-fold ($P = 0.01$), whereas TLR13 had increased 18.1-fold ($P = 0.01$) compared with levels in scarified control corneas.

cellular TLR with transcription that is controlled by the activated signaling of TLR2 or -4.⁵⁰ TLR13 is downregulated after exposure to components of Gram-positive or -negative bacteria.⁵⁰ Thus, our results are novel in indicating TLR13 upregulation by cells in the corneal epithelium and stroma during fungal infection. Further studies on TLR functionality are needed to understand the role of TLR13, a highly and persistently expressed corneal TLR in murine keratomycosis.

Our results also indicate that other receptors may be involved in the recognition of *C. albicans* corneal infection. The expression of dectin-1, a lectin-like transmembrane receptor that recognizes β -glucan of *C. albicans*, increases soon after the onset of experimental *C. albicans* keratitis in wild-type mice but not in TLR2- or TLR4-knockout mice. Other studies have also shown that dectin-1 collaborates with TLRs to amplify TLR2-dependent phagocytosis and production of proinflammatory cytokines during fungal infection.^{51,52} We hypothesize that TLR2 and dectin-1 participate in the recognition of *C. albicans* by the cornea and in the activation of specific immune responses.⁵³

Molecular mediators of inflammation increase at the onset of experimental *C. albicans* keratitis.⁵⁴ Cytokines brought

TABLE 6. Molecular Changes in TLR2^{-/-}, TLR4^{-/-}, and C57BL/6J Mice 1 Day after *C. albicans* Inoculation

Gene	C57BL/6J Mice	TLR2 ^{-/-} Mice	Change in TLR2 ^{-/-} Mice	P	TLR4 ^{-/-} Mice	Change in TLR4 ^{-/-} Mice	P
<i>TNFα</i>	5.25 $\pm$ 0.66	6.68 $\pm$ 0.28	-2.69	0.03	6.67 $\pm$ 0.79	-2.66	0.08
<i>IL1β</i>	1.90 $\pm$ 0.63	2.96 $\pm$ 0.53	-2.08	0.09	2.70 $\pm$ 0.72	-1.74	0.22
<i>IL6</i>	4.35 $\pm$ 1.73	6.01 $\pm$ 0.52	-3.16	0.19	5.69 $\pm$ 0.36	-2.53	0.26
<i>IL23</i>	7.41 $\pm$ 0.61	8.73 $\pm$ 0.50	-2.51	0.04	7.37 $\pm$ 0.30	1.02	0.94
<i>CCL3</i>	5.21 $\pm$ 0.89	7.54 $\pm$ 0.88	-5.02	0.03	7.16 $\pm$ 1.04	-3.87	0.07
<i>CCL4</i>	3.56 $\pm$ 0.82	5.44 $\pm$ 0.21	-3.67	0.02	4.45 $\pm$ 0.68	-1.85	0.22
<i>TNFα</i>	5.25 $\pm$ 0.66	6.68 $\pm$ 0.28	-2.69	0.03	6.67 $\pm$ 0.79	-2.66	0.08
<i>MMP8</i>	7.78 $\pm$ 0.33	8.17 $\pm$ 0.20	-1.31	0.15	8.57 $\pm$ 0.44	-1.73	0.07
<i>MMP9</i>	5.49 $\pm$ 0.58	5.80 $\pm$ 0.20	-1.23	0.44	6.98 $\pm$ 1.04	-2.80	0.10
<i>MMP13</i>	3.12 $\pm$ 0.99	4.08 $\pm$ 0.51	-1.95	0.21	4.55 $\pm$ 1.50	-2.70	0.24
<i>CLEC7A</i>	8.38 $\pm$ 0.73	10.09 $\pm$ 0.24	-3.26	0.02	9.87 $\pm$ 0.44	-2.80	0.04

Changes (x-fold) and P are from the comparison between C57BL/6J mice and TLR2^{-/-} mice or TLR4^{-/-} mice; a change >2 -fold with $P < 0.05$ was considered a statistically significant difference.

TABLE 7. TLR Gene Expression Patterns in Normal and TLR-Knockout Mice 1 Day after Scarification and *C. albicans* Inoculation

Gene	C57BL/6J Mice	TLR2 ^{-/-} Mice	Change in TLR2 ^{-/-} Mice	P	TLR4 ^{-/-} Mice	Change in TLR4 ^{-/-} Mice	P
<i>TLR1</i>	10.10 ± 0.61	10.41 ± 0.54	-1.24	0.54	10.29 ± 0.23	-1.14	0.64
<i>TLR2</i>	6.27 ± 0.42	ND	—	—	6.07 ± 0.39	-1.26	0.36
<i>TLR4</i>	7.13 ± 0.26	7.23 ± 0.12	-1.16	0.23	ND	—	—
<i>TLR6</i>	9.02 ± 0.40	9.43 ± 0.29	-1.33	0.22	10.18 ± 0.76	-2.23	0.08
<i>TLR13</i>	6.55 ± 0.29	6.76 ± 0.39	-1.15	0.50	7.33 ± 0.24	-1.72	0.02

Changes (x-fold) and *P* are from the comparison between C57BL/6J mice and TLR2^{-/-} mice or TLR4^{-/-} mice. A change >2-fold with *P* < 0.05 was considered statistically significant. ND, not determined.

about by TLR2 signaling in response to *C. albicans* include tumor necrosis factor- α and interleukin, which in turn can upregulate matrix metalloproteinases, promote neovascularization, and interact with T cells.^{55,56} TLR2 is also involved in the production of CCL3 and -4, chemokines that attract leukocytes during microbial keratitis.⁵⁷ Thus, TLR2 signaling results in a coordinated interplay of effector molecules that contribute to inflammatory tissue responses during keratomycosis.

As in experimental filamentous fungal keratitis,¹⁹ interpretation of our results is restrained by murine models of *C. albicans* infection. Host susceptibility to candidiasis may depend on the microbial inoculum if some fungal forms are able to partially escape immune recognition.⁴¹ TLR-knockout mice are able to mount an inflammatory response to *C. albicans*, suggesting that additional pathogen recognition receptors may signal inflammatory responses during fungal infection of the cornea. Also, experimental assessment of the TLR profile depends on sampling times. In a model of oropharyngeal candidiasis *TLR2* and -4 are upregulated soon after fungal inoculation followed by a persistent increase of *TLR2* and gradual waning of *TLR4*.⁵⁸

Despite methodological limitations, this study indicates a role for TLR sensing during *C. albicans* keratitis. An interaction between *C. albicans* and a specific repertoire of TLRs results in the release of inflammatory mediators and other innate immune responses. Certain TLR polymorphisms may increase susceptibility to oculomycosis.⁵⁹ On the other hand, blocking microbial ligands from binding to one or more TLRs could alter the severity of early infection.⁶⁰ Exploiting the interaction between microorganisms and the eye's pathogen recognition receptors offers a strategic opportunity in the prevention and therapy of microbial keratitis.

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