MyD88 Regulation of *Fusarium* Keratitis Is Dependent on TLR4 and IL-1R1 but Not TLR2

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Fusarium species achieved notoriety as fungal pathogens after a recent outbreak of corneal infection related to a contact lens care solution in which the Centers for Disease Control and Prevention reported 318 cases of Fusarium keratitis in the United States in 2005 and 2006 (1), and appears to be recognized as an important cause of microbial keratitis unrelated to contact lens wear, with high prevalence in warm, humid areas of the United States (3) and in southern and southeastern Asia, especially India and China (4–6). Members of the F. solani and Fusarium oxysporum species complex are ubiquitous in the environment, where they survive as plant pathogens and saprophytes (7), and Fusarium species and other filamentous fungi are an increasing cause of invasive human mycoses in immune-compromised or neutropenic individuals, where they cause systemic fusariosis and endophthalmitis (8–11). However, the most common manifestation of Fusarium infection is keratitis, which occurs in otherwise healthy individuals engaged in agricultural work (12, 13). For example, recent studies on the prevalence of noncontact lens-associated microbial keratitis in India and China showed that Fusarium is a major cause of disease and that agricultural work is an important risk factor (4, 14).

Despite the frequency, chronicity, and severity of the disease, very little is known about the host response to Fusarium. Epidemiological studies indicate that the pathogenesis of this disease involves traumatic injury incurred during farm work facilitating entry of Fusarium conidia into the corneal stroma. Once present at this site, germination, toxin secretion, and hyphal invasion of the tissues either initiates a host response that controls infection at the expense of causing extensive tissue damage, or the host is unable to control Fusarium growth, and keratitis results from unregulated microbial growth and resulting tissue destruction. Animal models of Fusarium keratitis are characterized by a profound neutrophil infiltrate and systemic steroid treatment causes unregulated fungal growth and subsequent destruction of the cornea (15–19), indicating an essential role for the host response in controlling fungal growth and development of keratitis. In the current study, we examined the host innate immune response to Fusarium using a murine model of keratitis in which Fusarium conidia are injected directly into the corneal stroma.

Our findings clearly demonstrate that development of Fusarium keratitis involves: 1) an essential role for MyD88 in Fusarium keratitis, since neutrophil recruitment to MyD88−/− corneal stroma is delayed and ineffective, resulting in unimpaired fungal growth in the stroma and anterior chamber, and corneal perforation; 2) no role for TLR2, because TLR2−/− mice have an identical phenotype as C57BL/6 mice; 3) a role for TLR4 in fungal killing, because TLR4−/− and TLR2/4−/− mice have delayed fungal clearance; and 4) an essential role for IL-1R1, which also signals through MyD88, because IL-1R1−/− mice have the same phenotype as MyD88−/− mice. These observations demonstrate a definitive role for the IL-1R1/MyD88 pathway in regulating Fusarium growth and severity of corneal disease and suggests a putative role for TLR4 in neutrophil-mediated antifungal activity.
Fusarium strain and growth conditions

F. oxysporum species complex strain MRL8996 (NRRL 47514) was obtained by corneal scraping from a patient with a severe case of fungal keratitis at the Cleveland Clinic Foundation (Cleveland, OH) as described (2). Organisms were stored at −80°C at the Center for Medical Mycology, University Hospitals Case Medical Center (Cleveland, OH), and a 50-μl stock was used to inoculate 50 ml of Sabouraud dextrose broth (Difco). After 48 h of growth under shaking incubator conditions at 30°C, a second culture was then inoculated and incubated under identical conditions for 40 h. To collect conidia, hyphae were removed by passing the culture suspension through sterile PBS-soaked gauze positioned at the tip of a 30-ml syringe. Conidia were centrifuged at 3000 rpm for 5 min., washed three times with sterile PBS, and adjusted to 5 × 10^6 conidia/ml. Endotoxin-free water (MilliQ; Millipore) was used in all stages of fungal growth and isolation.

Source of mice

C57BL/6 mice (6–12 wk old) were purchased from The Jackson Laboratory. TLR2-deficient, TLR4-deficient, and MyD88-deficient mice were provided by Shizuo Akira (Research Institute for Microbial Disease, Osaka University, Osaka, Japan). IL-1R-deficient mice on a C57BL/6 background were obtained from S. Mohr (Department of Medicine, Case Western Reserve University, Cleveland, OH). All animals were bred under specific pathogen-free conditions and maintained according to institutional guidelines.

Development of a mouse model of Fusarium keratitis

Based on preliminary dose-response studies, we found that 1 × 10^4 inoculum/2 μl (5 × 10^6 conidia/ml) in sterile PBS was optimal to induce keratitis and to recover organisms from immunocompetent C57BL/6 mice. An intrastromal corneal injection was performed by first abrading the epithelial layer using a 30-gauge needle, then injecting a 1 × 10^7 conidia in 2 μl using a 33-gauge Hamilton syringe. Mice were examined daily under a stereomicroscope for corneal opacification, ulceration, and perforation, and evaluated in a masked fashion by an experienced technician using a stereomicroscope for corneal opacification, ulceration, and perforation.

In vivo confocal microscopy

In vivo analysis of the corneal infiltration was evaluated using the ConfoScan3 microscope system (Nidek Technologies) as described in our previous studies (22, 23). Transparent gel (Gentel; Novartis Ophthalmics) was used between the corneal surface and objective, and corneas were examined using a ×40 objective. The software (NAVIS; Lucent Technologies) was used to capture sequential images of the entire cornea.

Quantitative fungal growth

Whole eyes were homogenized under sterile conditions using the Mixer Mill MM300 (Retsch) at 33 Hz for 4 min. Serial log dilutions were performed and plated onto Sabouraud dextrose agar (Difco). Plates were incubated at 30°C for 40 h and the number of CFU was determined by direct counting.

Histology and immunohistochemistry

Periodic acid-Schiff-hematoxylin staining of paraffin sections was performed following standard techniques. Briefly, after deparaffinization, slides were placed in periodic acid solution (Sigma-Aldrich) for 10 min and placed in Schiff’s solution (Sigma-Aldrich) for 20 min. The slides were counterstained with hematoxylin, then dehydrated and mounted under Permount (Fisher Scientific).

H&E staining was performed using Gill’s hematoxylin for 3–5 min following deparaffinization. The slides were dipped into glacial acetic acid solution, placed in Shandon Bluing Solution (ThermoShandon) for 1 min and counterstained with alcoholic Eosin Y solution (ThermoShandon). The slides were then dehydrated and mounted under Permount medium (Vector Laboratories).

To detect neutrophils, 5-μm paraffin sections were deparaffinized in Citrisolv (Fisher Scientific) and hydrated by serially dipping into 100, 95, and 80% ethanol, distilled water, and then PBS. Slides were blocked with 1.5% normal rabbit serum in PBS for 2 h at room temperature. The slides were then washed in PBS plus 0.05% Tween 20 and incubated with goat anti-rat-biotin (Southern Biotechnology Associates) diluted 1/200 in PBS for 30 min. The slides were washed in PBS/Tween 20 and streptavidin/alkaline phosphatase (BioGenex) and incubated for 30 min. Vector Red Solution (Vector Laboratories) was prepared in Tris buffer adjusted to pH 8.3 and was added to the slides and incubated in the dark for 10 min. The slides were counterstained in Gill’s hematoxylin (Vector Laboratories), then treated with 2% glacial acetic acid and 0.5% lithium carbonate. The slides were then dehydrated and placed again in Citrisolv, then mounted with Permount medium and allowed to dry overnight.

Cytokine assays

Infected corneas were dissected and stored overnight in 5% Natamycin Solution (Alcon). (Sterility was ensured by inoculating samples onto Sabouraud dextrose agar.) Corneal samples were then homogenized using the Retsch MM 300 ball miller at 33 Hz for 4 min. Half-well cytokine assays were performed using Duoset ELISA kits (R&D Systems) according to the manufacturer’s directions.

Statistics

Statistical analysis was performed using GraphPad Prism software. To determine statistical significance, we used nonparametric one-way ANOVA with post hoc Tukey’s multiple comparison. Differences were considered significant at p < 0.05.

Results

MyD88−/− mice develop delayed, but nonresolving corneal opacification

MyD88 has an important role in regulating the host response to systemic and pulmonary Aspergillus fumigatus infections and to systemic infection with Cryptococcus neoformans and Candida albicans (24–26). To determine the role of MyD88 in Fusarium keratitis, corneas of C57BL/6 and MyD88−/− mice were inoculated with 1 × 10^4 conidia in 2 μl. The severity of keratitis was...
examined by slit-lamp microscopy, and clinical scores were evaluated in a masked fashion as described previously (21).

As shown in Fig. 1, C57BL/6 mice developed severe corneal opacification within 24 h, which remained high for 7 days and gradually resolved over 14 days. In marked contrast, MyD88−/− mice had moderate corneal opacification after 24 h compared with C57BL/6 mice (Fig. 1). However, by 48 h, MyD88−/− corneas showed increased corneal opacification scores and in contrast to C57BL/6 mice, corneal disease in MyD88−/− progressed rapidly to perforation after 72 h. Clinical stromal disease scores for day 1 are shown in Fig. 2. Together, these findings demonstrate that MyD88 regulates the outcome of Fusarium keratitis.

Impaired neutrophil infiltration and fungal clearance in MyD88−/− corneas

To examine the early response of C57BL/6 and MyD88−/− mice to Fusarium challenge, before detectable corneal opacification, corneas were examined by in vivo confocal microscopy, which provides a series of images throughout the cornea and can clearly identify cellular infiltrates in the corneal stroma (22, 23). In vivo confocal microscopy images of the central corneal stroma 6 h after injection of conidia revealed extensive growth of hyphal elements in the central corneal stroma in C57BL/6 and in MyD88−/− mice (Fig. 3, a and c), indicating that conidia had germinated in both strains of mice by this time point.

Because the mammalian cornea is normally avascular, cellular infiltration occurs from peripheral limbal vessels. As shown in Fig. 3, b and d, cellular infiltration in the peripheral corneal stroma was clearly detected in C57BL/6 mice, but was absent in MyD88−/− mice. This observation demonstrates a critical role for MyD88 in cellular infiltration to the corneal stroma.

To characterize the cellular infiltrate, Fusarium-infected C57BL/6 and MyD88−/− corneas were examined by histology and immunohistochemical analyses 24 and 48 h after intrastromal injection of conidia. Fig. 4 shows corneal sections stained with periodic acid-Schiff for the presence of fungi and counterstained with hematoxylin. Neutrophils were identified by immunostaining using mAb NIMP-H16 (24).

As shown in Fig. 4 (upper panels), hyphae were present throughout the MyD88−/− corneal stroma 24 h after intrastromal injection and in the anterior chamber, indicating that Fusarium hyphal elements had penetrated Descemet’s (basement) membrane. By 48 h, hyphae had replicated throughout the corneal stroma and anterior chamber. In contrast to MyD88−/− corneas,
48 h, eyes were dissected, homogenized, and plated for CFU determination. As shown in Fig. 6, although similar numbers of organisms were recovered from C57BL/6 and MyD88−/− mice at 6 h, after 24 and 48 h, the number of CFU diminished in C57BL/6 mice until no colonies were detected after 72 h. In contrast, the number of CFU isolated from MyD88−/− mice increased over 48 h, which is consistent with the presence of hyphae in the cornea and anterior chamber. Together, these findings demonstrate an essential role for MyD88 in regulating fungal growth and replication, and support the notion that neutrophil infiltration is important in this process.

**TLR4 mediates antifungal activity, but not neutrophil infiltration to the corneal stroma or development of keratitis**

Since all TLRs except TLR3 signal through MyD88 (27) and TLR2 or TLR4 regulate the outcome of infections with *A. fumigatus* (28–31) and *C. albicans* (32–34), we next examined whether the central role for MyD88 in *Fusarium* keratitis is based on activation of these pathogen recognition receptors.

C57BL/6, TLR2−/−, TLR4−/−, and TLR2/4−/− mice were inoculated intrastromally with 1 × 10^4 conidia, and corneal opacification, neutrophil infiltration, and fungal killing was measured as described above. Clinical evaluation of keratitis in TLR2−/−, TLR4−/−, and TLR2/4−/− mice compared with C57BL/6 mice showed no differences in corneal opacification among any of the strains after 24 or 48 h (Fig. 7). Consistent with this observation, the neutrophil infiltrate in TLR2−/−, TLR4−/−, and TLR2/4−/− mice was similar to that in C57BL/6 mice, indicating that there is no

**FIGURE 5.** Neutrophils as a major component of the cellular infiltrate. C57BL/6 mice were inoculated intrastromally with *Fusarium* conidia as described in the legend to Fig. 3, eyes were dissected 24 h after inoculation, and 5-μm paraffin sections were immunostained with NIMPR-14, which specifically identifies murine neutrophils. Representative corneal sections are shown by light (A) and fluorescence (B) microscopy. Note that neutrophils are a major component of the cellular infiltrate. Sections are representative of four repeat experiments with five mice per group. Original magnification, ×400.

**FIGURE 6.** CFU from C57BL/6 and MyD88−/− eyes. *Fusarium* conidia (1 × 10^4) were inoculated into the corneal stoma of C57BL/6 and MyD88−/− mice. After 6, 24, and 48 h, mice were sacrificed, whole eyes (including cornea and anterior chamber) were homogenized, diluted, and the number of CFU was determined by direct counting. Graphs are mean ± SEM for five mice per group. ANOVA comparing CFU from C57BL/6 and MyD88−/− eyes was p < 0.001 at 24 and 48 h. This experiment was repeated three times with similar results.

**FIGURE 7.** *Fusarium*-induced corneal opacification in TLR2−/−, TLR4−/−, and TLR2/4−/− mice. *Fusarium* conidia (1 × 10^4) were inoculated into the corneal stoma of C57BL/6, TLR2−/−, TLR4−/−, and TLR2/4−/− mice. After 24 and 48 h, corneas were examined by slit-lamp biomicroscopy and scored as described above. A. Representative eyes of each group of mice. B. Clinical scores ± SEM for five mice per group. Note that there was no difference in corneal opacification among these mouse strains. This experiment was repeated three times with similar results.
role for TLR2 or TLR4 in modulating cellular infiltration to the corneal stroma.

Closer examination of histological sections revealed *Fusarium* hyphae in TLR4/H11002/H11002 and TLR2/4/H11002/H11002 corneas (Fig. 8, A and B), but not in TLR2/H11002/H11002 or C57BL/6 corneas (data not shown). To quantify the effect of TLR4 on *Fusarium* survival in the cornea and anterior chamber, eyes were homogenized and analyzed by CFU evaluation. As shown in Fig. 8C, ~500 CFU were recovered from TLR2/−/− and C57BL per six eyes after 24 h, whereas ~2500 CFU were recovered from TLR4/−/− and TLR2/4/−/− eyes. No differences were detected between C57BL/6 mice and TLR2 mice or between TLR4/−/− and TLR2/4/−/− mice, indicating that TLR2 does not contribute to protection against *Fusarium* infections. After 48 h, CFU were significantly reduced in all strains and fungi were not detected after 72 h.

**FIGURE 8.** *Fusarium* survival in TLR4/−/− and TLR2/4/−/− mice compared with C57BL/6 and TLR2/−/− mice. *F. oxysporum* conidia (1 × 10⁴) were inoculated into the corneal stoma of C57BL/6, TLR2/−/−, TLR4/−/−, and TLR2/4/−/− mice. After 24 h, 5-μm corneal sections were examined after periodic acid-Schiff/hematoxylin staining. A, Representative corneal section from a TLR2/4/−/− mouse showing intense cellular infiltrate. B, *Fusarium* hyphae can be detected in the posterior corneal stroma. Original magnification, ×400. C, CFU in eyes dissected from C57BL/6, TLR2/−/−, TLR4/−/−, and TLR2/4/−/− mice 24 h after inoculation. Note that TLR4/−/− and TLR2/4/−/− mice show significantly higher CFU compared with C57BL/6 and TLR2/−/− mice. Data points represent individual eyes. The experiment was repeated three times with similar results.

![Figure 8](http://www.jimmunol.org/)

**FIGURE 9.** *Fusarium* keratitis in IL-1R1/−/− mice. *Fusarium* conidia (1 × 10⁴) were inoculated into the corneal stoma of C57BL/6 and IL-1R1/−/− mice. After 24 and 48 h, mice were examined by slit-lamp biomicroscopy and eyes were processed for histology or CFU as described above. A, Representative IL-1R1/−/− and C57BL/6 corneas 24 h after intrastromal injection. B, Clinical scores at 24 h. C, Representative histological sections of IL-1R1/−/− and C57BL/6 corneas. Original magnification, ×400. D, CFU in eyes from C57BL/6 and IL-1R1/−/− mice recovered 24, 48, and 72 h after intrastromal injection. CFU are mean ± SEM of 5–10 mice/group. ANOVA comparing CFU from C57BL/6 and IL-1R1/−/− eyes revealed p < 0.05 at 24 h and p < 0.01 at 48 h. The experiment was repeated three times with similar results. Epi, Corneal epithelium; a/c, anterior chamber; Dm, Descemet’s (basement) membrane.

![Figure 9](http://www.jimmunol.org/)
tological analysis showed that IL-1R1 increased at 48 h, and eventually perforated (data not shown). His-
phils, but numerous Fusarium/H11006 mean C
(Fig. 8 mice, development of corneal opacification was impaired in sever-
cytokine receptor in adaptor molecule (27); therefore, we next examined the role of this
itivity, possibly when expressed on neutrophils.
opacification; and 2) TLR4 has a significant role in antifungal ac-
do not regulate cellular infiltration and development of corneal
Taken together, these findings indicate that: 1) TLR2 and TLR4
do not regulate cellular infiltration and development of corneal
regulation of oxidative pathways (28, 34). Production of ROS by neutrophils
is important in regulating fungal hyphae that cannot be ingested,
CXCL1/KC production in the cornea is dependent on IL-1R1
Since neutrophil recruitment to the corneal stroma is associated
with clearance of the organisms, and our previous studies showed
an essential role for CXCL1/KC in neutrophil recruitment to the
corneal stroma (35), we next determined whether IL-1R1 mediates
production of this chemokine in keratitis. C57BL/6 and IL-1R1−/−
mice were inoculated intrastromally with 1 × 10^5 conidia as
before, corneas were dissected after 4 h (before neutrophil infil-
), homogenized, and CXCL1/KC and IL-1α in supernatants was measured by ELISA. Data are
mean ± SEM of five mice per group and are representative of two repeat
experiments.

Taken together, these findings indicate that: 1) TLR2 and TLR4
do not regulate cellular infiltration and development of corneal
tissue; and 2) TLR4 has a significant role in antifungal ac-
tivity, possibly when expressed on neutrophils.

**IL-1R1 is essential for fungal clearance, neutrophil infiltration
to the corneal stroma, and development of keratitis**

In addition to TLRs, IL-1R1 signals through the MyD88 common
adaptor molecule (27); therefore, we next examined the role of this
cytokine receptor in Fusarium keratitis. Corneas of C57BL/6 and
IL-1R1−/− mice were inoculated intrastromally with Fusarium
conidia as before and corneal opacification was assessed by slit-
lamp examination. Fig. 9, A and B, shows that as with MyD88−/−
mice, development of corneal opacification was impaired in severity
in IL-1R1−/− mice compared with C57BL/6 mice after 24 h,
increased at 48 h, and eventually perforated (data not shown). His-
tological analysis showed that IL-1R1−/− mice had few neutro-
phils, but numerous Fusarium hyphae were observed in the cor-
neal stroma and anterior chamber compared with C57BL/6 mice
(Fig. 8C). Consistent with these findings, CFU analysis showed
that hyphae continued to replicate in IL-1R1−/− eyes in contrast
to C57BL/6 mice where CFU were reduced by 90% after 72 h
(Fig. 9D).

FIGURE 10. CXCL1/KC and IL-1α production by C57BL/6 and IL-
1R1−/− corneas. Fusarium conidia (1 × 10^5) were injected into the corneal
stroma of C57BL/6 and IL-1R1−/− mice. After 4 h, corneas were dissected,
homogenized, and insoluble material was removed by centrifugation. CXCL1/KC and IL-1α in supernatants was measured by ELISA. Data are
mean ± SEM of five mice per group and are representative of two repeat
experiments.

**Discussion**

The normal mammalian cornea maintains its transparent nature
because of the barrier function of the external corneal epithelium,
the highly organized, antiparallel arrangement of collagen fibrils,
and the single layer of corneal endothelial cells lining the anterior
chamber, which maintains the essential hydration level. Other fac-
tors also contribute to corneal transparency, including the translu-
cent nature of the resident stromal keratocytes and the absence
of blood vessels and resident inflammatory cells such as neutrophils.
During microbial infection, the cornea becomes opaque after re-
lease of cytotoxic mediators from microbes and from activated
neutrophils that infiltrate the corneal stroma.

Our previous studies showed that activation of TLR2 and TLR4
through MyD88 in the cornea rapidly induces production of proin-
flammatory and chemotactic cytokines, which lead to neutrophil
recruitment to the corneal stroma and loss of corneal clarity (22,
23). Results from the current study show that although MyD88 is
essential for neutrophil recruitment in response to Fusarium, TLR2
and TLR4 have no effect on this stage of the host response. Instead,
we found a role for TLR4 in fungal killing, as the number of
Fusarium colonies recovered from whole eyes (including cornea
and anterior chamber) was significantly higher in TLR4−/− and
TLR2/4−/− mice compared with C57BL/6 and TLR2−/− mice.
The observation that TLR4 is important in fungal killing but not
neutrophil recruitment suggests that it is TLR4 expression on neu-
phils that mediates antifungal activity, possibly by production
of reactive oxygen species (ROS). Although not shown in the cur-
rent study, it is likely that MyD88 is also involved in TLR4 activa-
tion of neutrophils. TLR2 and TLR4 are important in the host
response to other filamentous fungi, such as A. fumigatus (28, 31),
and to the pathogenic yeasts C. albicans and Cryptococcus neo-
formans (32, 36). In addition, hyphal and macroconidia forms of
the organisms stimulate different TLRs, including TLR4 activation
of oxidative pathways (28, 34). Production of ROS by neutrophils
is important in regulating fungal hyphae that cannot be ingested,

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**Abbreviation used in this paper:** ROS, reactive oxygen species.
Since mice that are defective in ROS production are highly susceptible to infection with fungal pathogens such as *C. albicans* (37, 38), and this mechanism is likely to be involved in limiting *Fusarium* growth in the cornea and anterior chamber.

Given that neutrophil recruitment to the corneal stroma is MyD88 dependent, but independent of TLR2 and TLR4, we examined the role of IL-1R1, which also signals through MyD88. We found that IL-1R1 is required for production of CXCL1/KC and neutrophil recruitment, consistent with the role of IL-1 and CXC chemokines in LPS and *Pseudomonas* keratitis (36, 39). However, at least two observations indicate that other pathogen recognition molecules are involved in the pathogenesis of *Fusarium* keratitis: 1) IL-1R1 is required for CXC chemokine, but not IL-1α production, indicating that an unidentified receptor is needed for IL-1 production in the cornea; and 2) although TLR4 has an important role in fungal killing, the organisms are eventually cleared in TLR4−/− and TLR2/−/− mice, indicating that other receptors are also involved. Future studies will examine the role of C-type lectins including Dectin-1, which is expressed on macrophages and is also involved. Future studies will examine the role of C-type lectins including Dectin-1, which is expressed on macrophages and is also involved. Future studies will examine the role of C-type lectins including Dectin-1, which is expressed on macrophages and is also involved. Future studies will examine the role of C-type lectins including Dectin-1, which is expressed on macrophages and is also involved.


