Potential Role of Macrophages in Experimental Keratomycosis

Jianzhang Hu, Ye Wang, and Lixin Xie

PURPOSE. To investigate the potential role of macrophages in keratomycosis.

METHODS. Macrophage elimination was achieved by repeated subconjunctival injection of liposomes containing dichloromethylene diphosphonate (Cl₂MDP-LIP) in BALB/c mice with Fusarium solani and Candida albicans keratitis, respectively. Controls received liposomes containing phosphate-buffered saline (PBS-LIP). Infected corneas were homogenized for colony counts. Immunohistochemistry was used to determine the efficiency (PBS-LIP). Infected corneas were homogenized for colony counts. Immunohistochemistry was used to determine the efficiency of macrophage depletion. The cytokine production of Th1 cells, expressed by IFN-γ and IL-12, and of Th2 cells, expressed by IL-4 and IL-10, in corneas was analyzed by enzyme-linked immunosorbent assay. Cytokine mRNA levels were detected by real-time polymerase chain reaction. Toll-like receptor 4 (TLR4) expression and mRNA levels were also examined. Immunoblotting and immunofluorescence staining were performed to evaluate the correlation between macrophages and TLR4 distribution.

RESULTS. Histopathologically, more severe keratomycosis developed in the mice treated with Cl₂MDP-LIP than those treated with PBS-LIP 5 and 7 days after inoculation. In the Cl₂MDP-LIP–treated group, little expression of macrophages could be detected in the conjunctiva and cornea. Numerous colonies were observed. There was a marked decrease of Th1 and Th2 cytokine production and mRNA expression, and TLR4 was significantly upregulated. Immunofluorescence staining demonstrated that partial F4/80+ cells were TLR4+ The positive correlation between TLR4 mRNA and IFN-γ mRNA expression.

CONCLUSIONS. Local depletion of macrophages may downregulate the immune response and aggravate fungal keratitis. Macrophages appear to play an important role in host defense against corneal infection of F. solani and C. albicans. (Invest Ophthalmol Vis Sci. 2009;50:2087–2094) DOI:10.1167/iovs.07-1237

Fungal keratitis is a common blinding disease, and its incidence has increased in many agricultural countries in past decades. The predominant filamentous fungal pathogens are Fusarium species. 1-4 Candida albicans is also a prevalent cause of keratomycosis in the United States. 5,6 Poor knowledge of the mechanism and pathologic characteristics of this disease makes it difficult to deliver effective treatment. Immunosuppressants may increase disease severity and delay fungal clearance. 7,8 There is an inverse correlation in the degree and distribution of inflammatory cells and fungal filaments, respectively. 9 Thus, virulence factors (adherence and proteases) 10 and host immune response may play critical roles in the pathophysiology of fungal keratitis.

Macrophages are first-line defender cells of the immune system. 11-17 As active participants in host resistance to fungal infection of lung, kidney, and skin, 14-16 they have been reported to readily kill spores and hyphae of Aspergillus fumigatus in vitro 17 and to limit Candida neoformans infection in the rat lung. 18 Although macrophages usually could not be easily found in corneas, they became obvious in the corneal limbus and center once the cornea was injured and infected with microorganisms. 19-25 Macrophages in the subepithelial tissue of the conjunctiva could help in nonspecific or specific resistance to a wide variety of protozoal and viral infections. 20-25 Although little is known about the immune effector mechanism against fungus in the corneal infection, macrophages were found during the development of fungal keratitis by histopathologic studies, 24-25 which may affect the clinical course of the disease.

Toll-like receptor (TLR) 4, a classic member of the TLR superfamily expressed in the cells of the innate immune system such as macrophages and T cells, can detect microbial products and trigger antimicrobial host responses. 26,27 It was reported that mononuclear macrophages were involved in the innate immune response to invasive fungal pathogens, and TLR4 may participate in the signaling of Aspergillus hyphae. 28 In this study, we investigated the potential role of macrophages in keratomycosis using a mouse model infected with Fusarium solani and C. albicans.

MATERIALS AND METHODS

Animals

Six- to 8-week-old inbred BALB/c mice of either sex, weighing 18 to 20 g each, were used in this study. They were housed at 20°C with a cycle of 12-hour light and 12-hour darkness. Food and water were available ad libitum. No disease was found in these animals by slit lamp examination and indirect fundoscopy. All mice were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Corneal infections were induced in right eyes.

Inocula

The strains of F. solani and C. albicans were CGMCC 3.1829 (China General Microbiologic Culture Collection Center, Beijing, China) and ATCC MYA-2876 (American Type Culture Collection, Rockville, MD), originally isolated from human skin and a patient with generalized candidiasis, respectively. These isolates were cultured on potato glucose agar at 28°C for 5 days, and spores were harvested into 1 mL sterile saline solution and then diluted with sterile saline to yield 10⁶ U/mL (cultrable).

Macrophage Depletion

Multilamellar liposomes (LIPS) containing dichloromethylene diphosphonate (Cl₂MDP, Sigma, St. Louis, MO) and phosphate-buffered saline

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(PBS), respectively, were prepared as described. To eliminate conjunctival macrophages in vivo, mice were injected subconjunctivally with 50 μL Cl2-MDP-LIP, containing approximately 0.25 mg clodronate, on days 7 and 2 before infection. After phagocytosis and disruption of the phospholipid bilayers, the Cl2-MDP can be released into the cytoplasm of the cell, which leads to macrophage apoptosis. Controls were pretreated with PBS-LIP.

**Infection of the Cornea**

The fungal keratitis model was prepared according to a previous report with a minor modification. Briefly, the mice were anesthetized intraperitoneally with ketamine (37.5 mg/mL) and xylazine (1.9 mg/mL). Proparacaine hydrochloride (0.5%) was used topically for corneal anesthesia. The central corneal epithelium was removed in a diameter of 2 mm before a full-thickness rat corneal button was placed on the mouse cornea. Five microliters of inoculum (spores, 10^6 U [culturable]) of F. solani and C. albicans were injected into the space between the two corneas, respectively. Then tarsorrhaphy was performed with two interrupted 10–0 nylon sutures to secure the rat corneal button, and another 5 μL inoculum was injected into the conjunctival sac. Immediately after surgery, 0.3% olofoxacin eye ointment (Tarivid; Samen, Osaka, Japan) was administered. All rat corneal buttons were removed 24 hours after inoculation. At 1, 3, 5, and 7 days after surgery, mice were photographed and scored clinically before they were humanely killed. Eyes were enucleated and processed for histologic examination, quantitative microbial culture, polymerase chain reaction (PCR) analysis, and enzyme-linked immunosorbent assay (ELISA).

**Clinical Scoring**

Inoculated eyes were monitored daily, and the severity of keratomycosis was scored with the aid of a dissecting microscope at 1, 3, 5, and 7 days. A grade of 0 to 4 was assigned to the area of opacity, density of opacity, and surface regularity. Scores from these three categories were tallied for each eye to yield a total score ranging from 0 to 12 (1–5, mild eye disease; 5–9, moderate disease; >9, severe disease).

**Histology**

At every time point, corneal samples were dehydrated with ethanol, formalin fixed, and paraffin embedded. Continuous 4-μm sections were stained with hematoxylin and eosin. The presence of inflammation, type of cells, and degree of inflammation were observed by light microscopy.

**Quantitative Isolate Recovery**

Infected corneas were trephined in a diameter of 2 mm at 1, 3, 5, and 7 days after inoculation and were processed for microbial culture. The removed corneas were individually ground in a frosted glass grinder, and 0.5 mL homogenate aliquot was serially diluted eightfold. The CFU was assayed with 5% dry milk in Tris-buffered saline containing 0.1% Tween 20.

**Immunoblotting and Immunohistochemistry**

The specificity of TLR4 was shown by immunoblotting. Infected corneas were excised within the 2-mm central area and stored at −80°C until assayed. Samples were weighed, thawed, minced, homogenized with a glass pestle in 1.5 mL PBS, sonicated for 30 seconds, and clarified by centrifugation at 12,000g for 10 minutes. A 100-μL aliquot of the supernatant was assayed with SDS-polyacrylamide gels (Mini-Protean II system; Bio-Rad Laboratories, Mississauga, ON, Canada) and blotted onto polyvinylidene difluoride membranes, followed by blocking with 5% dry milk in Tris-buffered saline containing 0.1% Tween 20. Membranes were then incubated overnight with primary antibodies (2 μg/mL in blocking buffer, 4°C), washed for 60 minutes, and stained with horseradish peroxidase-conjugated secondary antibodies (1:5,000). Immunodetection was performed with ECL reagents (Amersham Biosciences, Piscataway, NJ). The sections were mounted on poly-lysine–precoated slides (Polysciences, Warrington, PA), air dried, and fixed in cold acetone for 10 minutes. After blocking with normal goat serum, neighboring sections were incubated with 100 μL of the appropriately diluted primary antibody at 4°C overnight and then with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:200 dilution) at room temperature for 2 hours. Finally, the sections were covered with mounting media and examined by fluorescence microscopy (Eclipse E800; Nikon, Tokyo, Japan). The primary antibody used in this study was either either F4/80 (specific for murine macrophages; Serotec, Oxford, UK; clone CI A3–1) or anti-TLR4 (BD Pharmingen, San Diego, CA; clone MTS510). To find the correlation of macrophages and TLR4 distribution, primary antibodies of anti-F4/80 and anti-TLR4 and FITC-conjugated and rhodamine red-X (RRX)-conjugated secondary antibodies were incubated during the procedures described.

**Real-Time PCR**

The mRNA was isolated from each excised cornea (NucleoSpin RNA II System; Macherey-Nagel, Düren, Germany) according to the manufacturer’s protocol. Total RNA was quantified by ultraviolet spectrophotometer measurement of OD260 and OD280. To eliminate contamination with genomic DNA, the RNA samples (1 μg) were treated with DNaseI and subjected to reverse transcription at 42°C for 60 minutes in a 40-μL reaction mixture using the first-strand cDNA synthesis kit (BBI, Toronto, ON, Canada). Reagents (TaqMan; Applied Biosystems, Foster City, CA) and a sequence detection system (ABI Prism 7500 System; Applied Biosystems) were used in real-time PCR, as recommended by the manufacturer. Each sample was assayed in duplicate (TaqMan Universal PCR Master Mix; Applied Biosystems). Primers and oligonucleotide probes used are listed in Table 1. Cycling conditions were as follows: 10 minutes at 95°C and 40 cycles of amplification for 15 seconds at 95°C and 1 minute at 60°C. The expected fragment length was between 150 and 300 bp. Quantification data were analyzed with SDS system software (7500 System; Applied Biosystems). After PCR baseline subtraction was performed by the software, the loglinear portion of the fluorescence versus cycle plot was extended to determine a fractional cycle number at which a threshold fluorescence was obtained (threshold cycle [Ct]) as a reference for each analyzed gene and GAPDH.

We checked the slope of real-time PCR amplification and verified the amplification efficiency of each primer/probe system by amplifying the respective gene and the GAPDH endogenous control of serial 2 dilutions of a cDNA sample. Different ΔCt values between the target gene and the endogenous control of each dilution were plotted compared with log quantity of the cDNA (1, 0.5, 0.25, 0.125, and so on). Amplification efficiency was acceptable if the slope of the resultant graph was between −0.01 and +0.01; that is, ΔCt did not show a clear trend over a series of at least five dilutions, and the comparative Ct method was available. The 1-day group was used as a calibrator, and the data were presented as the fold change in gene expression relative to the calibrator.

**Enzyme-Linked Immunosorbent Assay of Cytokines**

Protein levels of the cytokine production of Th1 cells expressed by IFN-γ and IL-12 and Th2 cells expressed by IL-4 and IL-10 in corneas were quantitated using an ELISA kit (R&D Systems). Infected corneas were excised within the 2-mm central area and stored at −80°C until
assayed. Samples were weighed, thawed, minced, and homogenized with a glass pestle in 1.5 mL PBS, sonicated for 30 seconds, and clarified by centrifugation at 12,000g for 10 minutes. A 100-μL aliquot of the supernatant was assayed according to the manufacturer’s instruction. The reported sensitivity of these assays was 7 pg/mL for IL-4, 24 pg/mL for IL-12, 7 pg/mL for IFN-γ, and ≥24 pg/mL for IL-10.

Statistical Analysis

Commercial software (SPSS 11.5; SPSS, Chicago, IL) was used for statistical analysis. One-way analysis of variance (ANOVA) and Student’s t-test were used to determine any significant differences between the mice treated with Cl2MDP-LIP and PBS-LIP. P < 0.05 was considered significant. All experiments were repeated at least once to ensure reproducibility.

RESULTS

Course of Fungal Keratitis

Two hundred twenty-five eyes received inoculation, 96.4% (217/225) of which contracted invasive fungal keratitis. Clinical features of the mice infected with F. solani and C. albicans were similar. Features were manifested most evidently 1 day after inoculation and varied significantly between eyes treated with Cl2MDP-LIP and those treated with PBS-LIP (Fig. 1; some data not shown). In the Cl2MDP-LIP–treated group, the inflammatory reaction was mild in the peripheral area but severe in the local center, with marked corneal edema, ulceration, necrosis, and perforation at 7 days after inoculation. By contrast, keratitis in the Cl2MDP-LIP–treated mice displayed an acute inflammatory cell infiltration, extensive stromal edema and occasional PMN infiltration in the local central corneas. A small number of fibrous exudates were present in the anterior chamber. 5 and 7 days, with a decrease of PMN infiltration, significant neovascularization, uneven thickening, and many fibrocytes were seen in the corneal stroma of the mice that received PBS-LIP treatment. The ulcer tended to heal by fibrosis. By contrast, keratitis in the Cl2MDP-LIP–treated group displayed an increase of inflammatory cell infiltration, marked edema, ulceration, necrosis, and crater or severe descemetocele formation.

Histology

Histopathologic features were different between the groups treated with Cl2MDP-LIP and those treated with PBS-LIP (Fig. 3). At 1 and 3 days after inoculation, corneas had marked dense inflammatory cell infiltration, extensive stromal collagen destruction, edema, and complete obliteration of keratocyte nuclei in the PBS-LIP-treated eyes. Most inflammatory cells were polymorphonuclear leukocytes (PMNs). Many PMNs and fibrous exudates were also visible in the anterior chamber. However, these changes in the Cl2MDP-LIP–treated group were mild and showed slight stromal edema and occasional PMN infiltration in the local central corneas. A small number of fibrous exudates were present in the anterior chamber. At 5 and 7 days, with a decrease of PMN infiltration, significant neovascularization, uneven thickening, and many fibrocytes were seen in the corneal stroma of the mice that received PBS-LIP treatment. The ulcer tended to heal by fibrosis. By contrast, keratitis in the Cl2MDP-LIP–treated mice displayed an increase of inflammatory cell infiltration, marked edema, ulceration, necrosis, and crater or severe descemetocele formation.

Colonies Counts

To determine whether the depletion of local macrophages influenced the immune responses against fungal growth in corneas, the corneal samples obtained at different time points were ground and cultured for colony growth. Eyes treated with Cl2MDP-LIP and PBS-LIP had similar colony counts 1 day after inoculation (P = 0.18; Fig. 4) and showed a downtrend in both groups thereafter. More colonies were observed in the Cl2MDP-LIP–treated group than in the PBS-LIP–treated group at 3, 5, and 7 days (P = 0.016, P < 0.001, respectively). Fewer colonies were observed at 5 days and none at 7 days in the eyes treated with PBS-LIP.

Effect of Cl2MDP-LIP on Local Macrophage Content

F4/80 antibody is a monoclonal antibody directed specifically against the mouse macrophage. Immunofluorescence study showed no F4/80+ cells in the uninoculated normal corneas. After inoculation, many F4/80+ cells were detected in the subconjunctival, limbal, and central corneas of the PBS-LIP–treated mice, but fewer were detected in the Cl2MDP-LIP–treated group at each time point (Fig. 5; some data not shown).

Levels of Th1 and Th2 Cytokines

The expression of cytokines normalized to GAPDH RNA and the fold change in gene expression related to the calibrator of the 1-day group are shown (Figs. 6A–D). There were statistically significant differences in IFN-γ, IL-12, IL-4, and IL-10 expression between the Cl2MDP-LIP– and PBS-LIP–treated groups except IL-10 expression at 7 days. All cytokines presented a downtrend in the eyes with PBS-LIP treatment; in those with Cl2MDP-LIP treatment, there were an increase in...
the expression of IFN-γ and a mild downtrend in the other cytokine mRNAs.

mRNA data were confirmed by ELISA (Fig. 6E), which also showed that the protein levels of Th1 and Th2 cytokines downregulated more significantly in the corneas of the Cl2MDP-LIP group than of the PBS-LIP group (P < 0.001). Th2 cytokine levels were lower than Th1 cytokine levels (P < 0.001). During disease development, a shift occurred from the Th1 response to the Th2 type because of the depletion of local macrophages.

**TLR4 Expression**

Immunoblotting and immunofluorescence showed that TLR4 was not expressed in the uninfected corneas but was distributed in the limbus, epithelium, and stroma of the infected corneas after inoculation. However, the TLR4+ cell population decreased more significantly in the Cl2MDP-LIP–treated group than in the PBS-LIP–treated group at each time point (Figs. 7A, 7B). Real-time PCR for TLR4 showed that TLR4 mRNA levels were significantly lower in the corneas of the Cl2MDP-LIP group than in the PBS-LIP group at 1, 3, 5, and 7 days (P = 0.001, P = 0.011, P = 0.023, P = 0.038, respectively; Fig. 7C). Immunofluorescence staining demonstrated that partial F4/80+ cells were TLR4+ and confirmed that TLR4 was one of the receptors of macrophages (Fig. 7D). Moreover, TLR4 expression was also seen on cells in addition to macrophages. A significant correlation was observed between TLR4 mRNA and IFN-γ mRNA expression in the F. solani- and C. albicans–inoculated corneas (Pearson correlation coefficient, r = 0.612 and 0.647; P < 0.001 for both; data not shown).
DISCUSSION

Macrophages play a critical role in the acute defense system against microbial growth and spread in the early phase of invasion. Acquired immunity in murine aspergillosis has been mediated by macrophages. In our study, selective elimination of the conjunctival-corneal macrophages through repeated subconjunctival injections of Cl2MDP-LIP before infection had a profound effect on the course of fungal keratitis. Although the score in the early stage was low, the infection gradually became worse and resulted in perforation in most eyes. In the PBS-LIP–treated group, however, moderate keratitis seldom produced corneal perforations. Depletion of local macrophages led to severe infections by preventing the generation of a nonspecific and fungal-specific immune response. Normal conjunctival-corneal macrophages cannot inhibit corneal infections, but they can limit the severity and chronicity of corneal disease. This effect may be exerted shortly after the fungal spores bind to the corneal epithelial basement membrane. Macrophages can inhibit spore germination and kill fungal spores and hyphae in vivo and in vitro.

In this study, the velocity of fungal clearance was higher in the PBS-LIP–treated group than in the Cl2MDP-LIP–treated group, indicating that the depletion of local macrophages can impair and delay fungal clearance in corneas and can induce corneal destruction and perforation. The number of colonies was similar between the two groups at 1 day after inoculation, but there was a sharper reduction in the PBS-LIP–treated eyes at 3, 5, and 7 days.

**FIGURE 4.** Mean colony counts of *F. solani* isolated from infected mouse corneas. The colony number was similar between the eyes treated with Cl2MDP-LIP and with PBS-LIP at 1 day after inoculation, but there was a sharper reduction in the PBS-LIP–treated eyes at 3, 5, and 7 days.

Innate immunity can generate proinflammatory cytokines and chemokines, which are chemoattractants for PMN infiltration and can prolong infiltration. PMNs induce the expression of antimicrobial peptides that may contribute to microbe clearance. In this study, more infiltration of PMNs, most of which were neutrophils, was observed in the corneas of the PBS-LIP group than in the Cl2MDP-LIP group, indicating that the depletion of local macrophages may reduce PMN infiltration and cause disruption of a potentially protective innate immune response, leading to an exacerbation of keratitis in the Cl2MDP-LIP–treated eyes. According to Bonnett et al., early PMN recruitment and formation of oxidative-active aggregates were essential in preventing the germination of *A. fumigatus* conidia, in part explaining the delayed fungal clearance in the Cl2MDP-LIP–treated mice.

Although innate defense mechanisms, primarily mediated by granulocytes and macrophages, are regarded as the initial means for fungal infections to be contained, clearance of infection and long-term immunity appear to be determined by T cell-mediated immune responses. In the present study, Th1 and Th2 cytokines could be detected in the infected corneas, and Th2 cytokine levels were lower than Th1 cytokine levels. Systemic mycosis is a T cell–mediated disease that is common in humans and can be induced in mice. Intact cell-mediated immunity is essential for resistance to this infection. It was reported that the protection is associated with the expression of a Th1 response and the production of IFN-γ and IL-12, whereas nonprotective immunity is correlated with a Th2 response and the production of IL-4 and IL-10 in a model of *C. albicans* infection. Our data also provide evidence that the Th1 response is predominant in the resistance response to fungal keratitis. Moreover, the typical Th1 and Th2 cytokine concentrations were significantly reduced in the corneas of macrophage-depleted mice. We conclude that macrophage depletion impairs Th1 and Th2 responses in the fungus-infected corneas, breaks the balance between Th1 and Th2 responses, and results in the downregulation of the immune response specific to the fungal keratitis, with which corneal necrosis and perforation may occur.

**FIGURE 5.** Effects of Cl2MDP-LIP and PBS-LIP on corneal macrophage content. With the use of immunofluorescence, the F4/80⁺ cell population in the central cornea of the PBS-LIP–treated mouse (A) was greater than that in the Cl2MDP-LIP–treated cornea (B) at 5 days after inoculation. Original magnification, ×200.
Bioactivity of IL-12, which is produced primarily by macrophages, can modulate infectious disease progression by enhancing the cytotoxicity of natural killer cells and T cells, modulating T-cell proliferation, and potentiating Th1 differentiation. IFN-γ is helpful in generating protective immunity and killing microbes by macrophages and neutrophils. Endogenous production of IL-12 and IFN-γ might be related to protective immunity in mice with candidiasis. In this study, the reduction of IL-12 was more significant than that of IFN-γ in Cl2MDP-LIP–treated mice. We may speculate that macrophage depletion induces a shift from a Th1 cell–dominated population to the Th2 direction in the T-cell response, leading to downregulation of the immune response. Decreased antigen presentation and cytokine production may result, with a loss of T-cell activation or a reduction in the effect of growth factors liberated by macrophages on T-cell functions.

We also investigated the possible mechanisms involved in the signal pathway of initiating immunity and recognizing fungus by macrophages. As the first-defined pathogen recognition receptors, TLRs can initiate innate immunity through the recognition of microbial products and the activation of stress-activated protein kinases and transcription factors and can generate the expression of immune and proinflammatory genes. In the present study, TLR4 was barely expressed in the uninfected corneas. After inoculation, corneal mRNA levels were significantly upregulated. TLR4 signaling was activated and involved in inflammation, consistent with the findings of an investigation on bacterial keratitis. The function of macrophages in fungal keratitis may be closely related to TLR4. Moreover, we found that almost all F4/80+ cells were TLR4+. In addition, on the surfaces of mononuclear macrophages, TLR4 expression was seen on the other cells, which indicates...
that many kinds of cells might express TLR4 in the corneas infected with fungal pathogens. TLR4 may be one of the major receptors of macrophages for the recognition of fungus that promotes proliferation and recruits and activates immunocytes such as neutrophils and T cells.

A positive correlation was observed between TLR4 mRNA and IFN-γ mRNA in the present study. IFN-γ can regulate the antimicrobial potential of macrophages by upregulating major histocompatibility complex class I and II protein expression, enhancing the production of macrophage-derived mediators such as IL-12, and downregulating the synthesis of anti-inflammatory mediators such as IL-10. We speculate that the depletion of local macrophages accompanied by TLR4 deficiency may reduce the production of corneal proinflammatory cytokines such as IFN-γ and may result in detrimental side effects related to fungal clearance delay and corneal destruction.

In summary, macrophages play a critical role in host resistance to fungal infection in corneas. Depletion of local macrophages can aggravate fungal keratitis by impairing fungus killing and stasis, decreasing PMN infiltration, and reducing proinflammatory cytokine expression. TLR4 may be involved in the recognition of fungi by macrophages and may be related to the inflammation of fungal keratitis. The pathophysiology of fungal keratitis should be further investigated to provide a better therapeutic approach for this disease.

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