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Neutrophil extracellular traps involvement in corneal fungal infection

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Purpose: Neutrophils release neutrophil extracellular traps (NETs) when defending against invading microorganisms. We investigated the existence of NETs in fungal keratitis.

Methods: Fourteen patients with unilateral fungal keratitis were included. Detailed information about each patient was recorded, including (1) patient history (onset of symptoms and previous therapy), (2) ocular examination findings by slit-lamp biomicroscopy, (3) laboratory findings from direct smear examination and culture of corneal scrapings, (4) NET formation, and (5) treatment strategy and prognosis. Immunofluorescence staining was used to evaluate the existence of NETs on corneal scrapings. The relationship between the quantification of NETs and the clinical character of the fungal keratitis was identified.

Results: NETs were identified in all 14 patients. Patients with a higher grade of NET formation and fewer fungal hyphae always showed a good treatment response and a short course of infection. NETs were consistently found mixed with fungal hyphae in the corneal scrapings from infected patients. No statistical significance was found between the grade of NETs formed and the course of infection before presentation, and no relationship between the quantification of NETs and the size of the ulcer was found.

Conclusions: The results suggest that NETs are involved in fungal keratitis. The number of NETs in infected corneas may provide a tool for evaluating the prognosis for fungal keratitis.

Fungal keratitis is a sight-threatening eye disease that requires timely and appropriate treatment. The predominant causes of fungal keratitis are *Aspergillus*, *Acremonium*, *Penicillium*, and *Fusarium solani*, which are all non-pigmented fungi [1]. Although *Aspergillus* and *Fusarium* clearly dominate the list, causative agents vary in different geographic areas. Xie et al. [2] reported that *Fusarium* species accounted for 73.3% of isolated fungal keratitis with *Fusarium solani* as the most common in northern China. Moharram et al. [3] reported that *Fusarium* species accounted for 13.8% of isolated fungal keratitis with *Fusarium solani* as the most common in Upper Egypt. Moreover, since June 2005, a global increase in the incidence of keratomycosis from *Fusarium* species has appeared in contact lens wearers who use Bausch & Lomb (Rochester, New York, NY) ReNu® with MoistureLoc or ReNu® Multiplus [4]. To avoid permanent vision loss and other related complications, antifungal treatment must be promptly started based on clinical findings and microbial examination [5]. Neutrophils are one of the first lines of defense against invading pathogenic microorganisms and play an important role in host defense against fungal pathogens. Neutrophils are shown to defend against the invasion of microorganisms by phagocytosis, degranulation, production of reactive oxygen species (ROS) [6-8], production of antimicrobial peptides [9], and extrusion of neutrophil extracellular traps (NETs) [10]. Neutrophils are capable of phagocytic clearance of unicellular microbes but are also reported to be challenged by fungal hyphae that are too large to be ingested. NETs are a newly discovered extracellular structure composed of the chromatin associated mainly with histones and many granular antimicrobial proteins, which trap, disarm, and destroy bacteria [11], as well as fungi [12]. However, the relationship between the existence of NETs and fungal keratitis is still unknown.

The early neutrophil response to fungal invasion is poorly understood but is critically important in corneal infection, which could be highly related to the prognosis for keratitis. In the present study, we investigated the existence of NETs in fungal keratitis and explored the relationship between the quantification of NETs and prognosis. Our results indicated that NETs are involved in the immune response following fungal infection and the number of NETs was highly related to the prognosis for this disease.

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METHODS

Patients: Fourteen patients with unilateral fungal keratitis who presented at the Eye Center of the 2nd Affiliated Hospital, School of Medicine, Zhejiang University in China were included in this study, which took place between June 2013 and October 2015. The study was approved by the Ethics Committee of the 2nd Affiliated Hospital, School of Medicine, Zhejiang University in China and complied with the tenets of the Declaration of Helsinki for Research Involving Human Tissue and adhered to the ARVO statement on human subjects. Written informed consent was obtained from all patients after they received an explanation of the nature and possible consequences of the procedures. Detailed information from each patient was recorded, including (1) patient history (onset of symptoms and previous therapy), (2) ocular examination findings by slit-lamp biomicroscopy, (3) laboratory findings from direct smear examination and culture of corneal scrapings, (4) NET formation, and (5) treatment strategy and prognosis.

Corneal scrapings: Corneal scrapings were obtained aseptically from the base and edges of each ulcer using a disposable microblade. The operated eyes were anesthetized topically with 0.5% proparacaine hydrochloride. A portion of each scraping was examined for the presence of fungi and bacteria using 10% potassium hydroxide wet mounts, smears stained with Gram stain, and saline wet mounts. Another portion of each scraping was inoculated onto Sabouraud glucose agar, brain–heart infusion broth, and chocolate agar and cultured for potential growth of fungi and bacteria. Sabouraud glucose agar slants were incubated at 28 °C, with others at 37 °C. All media were cultured for 14 days and observed daily. The third portion of each scraping was used to detect the NETs with immunofluorescence staining.

Microbial analysis was performed for cultures of all 14 infected corneas by a bacteriologist at the 2nd Affiliated Hospital, School of Medicine, Zhejiang University in China. The diagnosis of infectious keratitis was made when at least one of the following was confirmed: 1) The corneal scraping examination revealed fungal or bacterial presence in smears. 2) The same fungal or bacterial pathogens grew in culture media. Confocal microscopy was used to assist diagnosis in some patients but was not taken as singular proof of final diagnosis.

Treatment protocol: The patients diagnosed with infectious keratitis were treated following the protocol used in previous studies [13-15]. All patients with fungal keratitis were treated hourly with 5% natamycin, alone or combined with 0.25% amphotericin B. All patients also received 400 mg of itraconazole orally per day. Patients suspected of having a bacterial ulcer received topical antibiotic treatment with 0.5% Cravit (Santen Pharmaceutical (China) Co, Ltd, Suzhou, China) every hour while awake for the first 48 h, while the culture results were pending.

NET analysis: NETs were identified as previously described [16-18]. Corneal scrapings on Poly D-lysine-coated slides were briefly washed with saline, followed by immediate fixation with 4% paraformaldehyde (Merck, Hohenbrunn, Germany) in PBS (pH7.2~7.4; NaCl 137 mmol/l,KCl 2.7mmol/l,Na_2HPO_4 10 mmol/l, KH_2PO_4 2 mmol/l). The fixed samples were washed with PBS (pH 7.4) and blocked (10% normal donkey serum, 1% bovine serum albumin, and 0.5% Triton X-100 in PBS). Subsequently, the samples were incubated with the goat primary antibody anti-human neutrophil elastase (Santa Cruz Biotechnology, Santa Cruz, CA), as recommended by the manufacturer. A gentle washing step in PBS was performed following incubation. The primary antibody was detected using a secondary antibody coupled to Alexa Fluor® 555 (Life Technology, Carlsbad, CA). Finally, DNA was stained with the molecular probe Hoechst 33,258 (Life Technology). The specimens were analyzed with inverted fluorescence. The DNA released by neutrophils colocalized with the granule protein elastase within the NETs. Positive cells were counted in ten consecutive fields (400X magnification) of each specimen. According to the proportion of DNA-releasing neutrophils, the NET numbers were rated as +: <10%, ++: 10% to 30%, and +++: 31% to 50%.

Clinical assessments: A slit-lamp biomicroscope was used to assess the size of the ulcer or infiltrate and epithelial defect at the study visits. The response to the clinical therapy was assessed by the condition of the corneal ulcer or infiltrate, the hypopyon of the anterior chamber, and the patients' symptoms. Re-epithelialization was defined as the absence of an epithelial defect following the administration of fluorescein.

Statistical analysis: The statistical significance of correlations was determined with Kendall tau_b correlation test using SPSS (Version 22 for Mac). Differences were considered to be statistically significant when the p value was less than 0.05.

RESULTS

All patients agreed to the treatment before the smear examination. Patient information is shown in Table 1. The results of the microbial tests and clinical therapy confirmed that 14 patients had fungal keratitis. Thirteen cases were identified from the microbial culture, and one fungal keratitis case was identified with confocal microscopy.

Table1. Clinical and smear examination data.

Pat. No	M/F	At diagnosis		Diagnostic biopsy				clinical cure course after NETs detective
		Age (years)	Courses (days)	Pretreatment	Culture	direct smear	NETs	
1	F	58	18	antibacterial	Candida albicans	(+)	(+)	29
2	M	63	22	Antifungal	Fusarium solani	(+)	(++)	21
3	F	42	3	antibacterial	Aspergillus flavus	(+)	(+++)	10
4	M	51	7	Antifungal + antibacterial	Fusarium oxysporum	(+)	(++)	18
5	F	67	5	antibacterial	Aspergillus fumigatus	(+)	(++)	18
6	F	58	14	antifungal	Fusarium oxysporum	(+)	(+++)	10
7	M	50	10	Antifungal + antibacterial	Fusarium oxysporum	(-)	(+++)	9
8	F	35	9	Antivirus + steroids	Candida albicans	(+)	(++)	15
9	M	54	10	Antifungal + antibacterial	Aspergillus fumigatus	(+)	(+)	20
10	F	58	2	antibacterial	Aspergillus fumigatus	(+)	(+++)	10
11	M	61	7	antifungal	Alternaria sp	(+)	(+)	21
12	M	71	15	Antifungal + antibacterial	Fusarium oxysporum	(-)	(++)	18
13	M	49	10	antibacterial	(-)	(+)	(+)	20
14	M	57	18	Antifungal + antibacterial	Fusarium solani	(+)	(+)	21

NETs were identified in all 14 patients with fungal keratitis (Figure 1). The NETs and fungal hyphae (12/14) were often found in corneal scrapings of fungal keratitis with immunofluorescence staining (Figure 2 and Figure 3). The existence of NETs was detected at different stages of infection from day 2 to day 22, suggesting that the formation of NETs may have a crucial role during the entire stage of infectious keratitis. NETs were counted in ten consecutive fields (400X magnification) of each specimen. The number of NETs according to the proportion of DNA-releasing neutrophils is shown in Figure 2. We found that the relationship between the number of NETs and the size of the ulcer is variable (Figure 3). The patients' responses to antifungal therapy showed a close relationship to the number of NETs formed ($r = -0.813$, $p<0.001$). Patients with a higher grade of NET formation and fewer fungal hyphae always showed a good treatment response and a short course of infection (Table 1 and Figure 4A–C). Different NET formation and clinical courses among fungal species are shown in Figure 5A and Figure 5B. However, statistical analysis was not performed among the fungal species as the sample was too small. No statistical significance was found between the course of treatment and the course of fungal keratitis before presentation ($r = 0.479$, $p = 0.051$). No statistical significance was found between the course of fungal keratitis before presentation and the grade of NET formation ($r = -0.321$, $p = 0.157$).

DISCUSSION

The present study showed that neutrophils are activated in fungal keratitis to produce NETs, thus pointing to a possible role of NETs in fungal keratitis. Previously, most studies in infectious keratitis have been limited to studying the role of T-lymphocytes, macrophages, nature kill cells, and dendritic cells. Less attention has been given to the role of neutrophils, especially NETs, which serve as the first line of defense against infectious microorganisms.

NETs are web-like structures released extracellularly from activated neutrophils during infection and are composed of DNA, histones, and neutrophil elastase [7,8]. In the context of infectious disease, the formation of NETs has been shown to play a more important role in antifungal defense [11,12]. Different from bacteria, pathogenic fungi are dimorphic and switch between conidia and hyphal forms [16]. The conidia can be cleared by phagocytosis and intracellular degranulation, but the hyphal forms are too large to be internalized by this classic mechanism of antimicrobial host defense [11]. Instead, released NETs provide the host with an effective extracellular antifungal defense. These large extracellular web-like structures provide a physical barrier that prevents microbial dissemination and increases the local concentration of antimicrobial substances [10,11,17]. Our results show that the existence of NETs can be detected at different stages of infection from day 2 to day 22, suggesting that the formation of NETs may have a crucial role during the whole

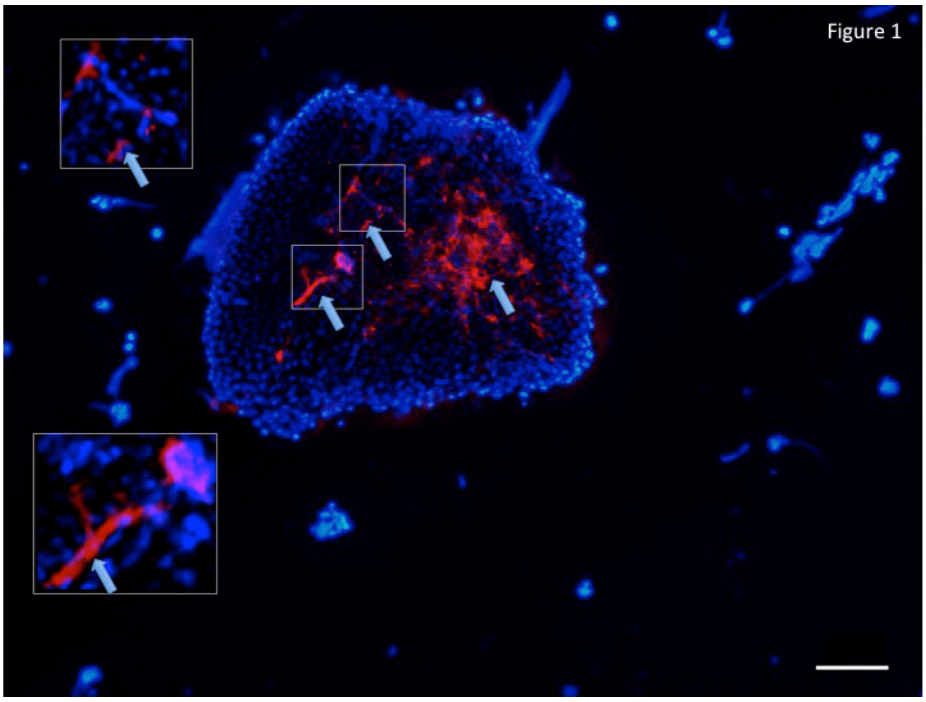

Figure 1. NETs (arrows) were identified in corneal scrapings of fungal keratitis. DNA (extracellular and nuclear DNA of epithelial cells and neutrophils) was stained with 4',6-diamidino-2-phenylindole (blue), and neutrophil elastase was stained red. Bar: 100 µm. The colocalization analysis shows r = 0.412 (Pearson correlation) and r = 0.987 (overlap) by Image J. Arrows point to the formation of the neutrophil extracellular traps (NETs) in immunofluorescence staining.

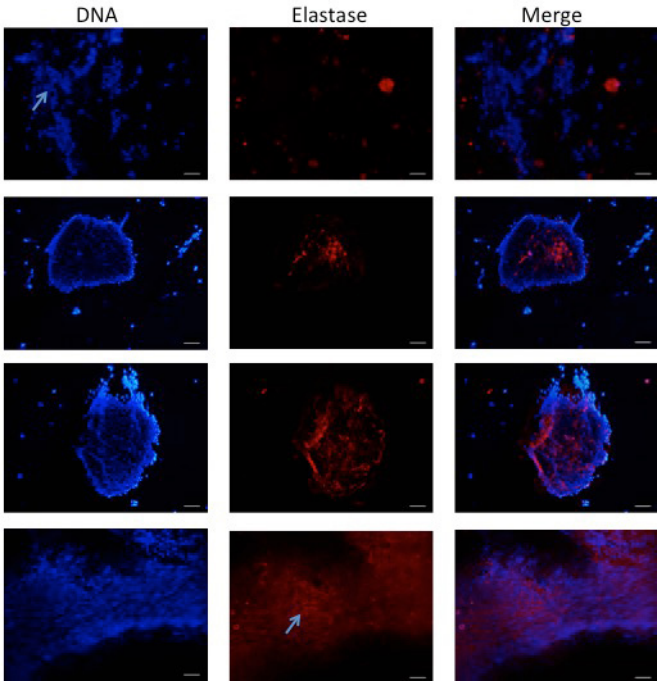

Figure 2. Example of quantification of NETs in different specimens. Row 1: none, Row 2: +, Row 3: ++, Row 4: +++, respectively, representing the number of DNA-releasing neutrophils as +: <10%, ++: 10% to 30%, and +++: 31% to 50%. The negative control was taken from a negative field of a positive sample. The structure of the multilobular nuclei of neutrophils can be seen in the left top image indicated with an arrow. Morphology of fungal hyphae was also determined in the Row 4 images (arrowhead). DNA (left panel), elastase (middle panel), Merge (right panel). Bar: 100 µm.

stage of fungal keratitis. In contrast to most other tissues, the initial immune response to infectious pathogens may be critically important, as the cornea is avascular. Therefore, neutrophil-derived NETs could be an important mechanism for defending against fungal infection in the cornea.

Our findings of NETs in all 14 fungal keratitis patients suggested a higher sensitivity of NETs than in other routine microbial investigations. The detection of NETs in the fungi-infected cornea may represent a promising appending method to standard microbial testing for rapid detection of causative organisms, particularly in cases where standard microbial test results are negative. Unfortunately, the discrimination of NET formation in fungal keratitis from that in other types of infectious keratitis, such as bacterial keratitis, is difficult. Until now, the different characteristics of NETs among different types of infectious keratitis have not been exploited, a topic that merits more research.

Shan et al. analyzed the contribution of NETs to the mediation of protection during ocular keratitis. Their results suggest that a balance between NET production and NET

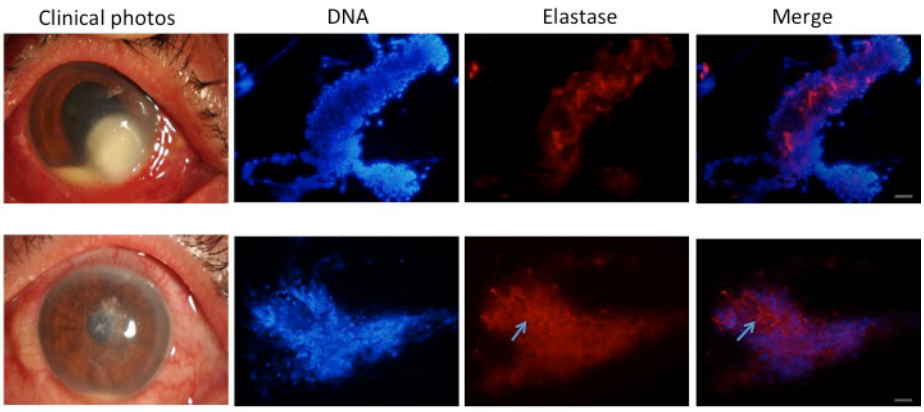

Figure 3. Images of corneal ulcer with slit-lamp microscopy and NET formation by immunofluorescence. In both specimens from the large (upper) and small (lower) corneal ulcers, a similar number of neutrophil extracellular traps (NETs) were detected. The arrow points to the fungal hyphae that exist in immunofluorescence staining. Blue = DNA; red = elastase. Bar: 100 µm.

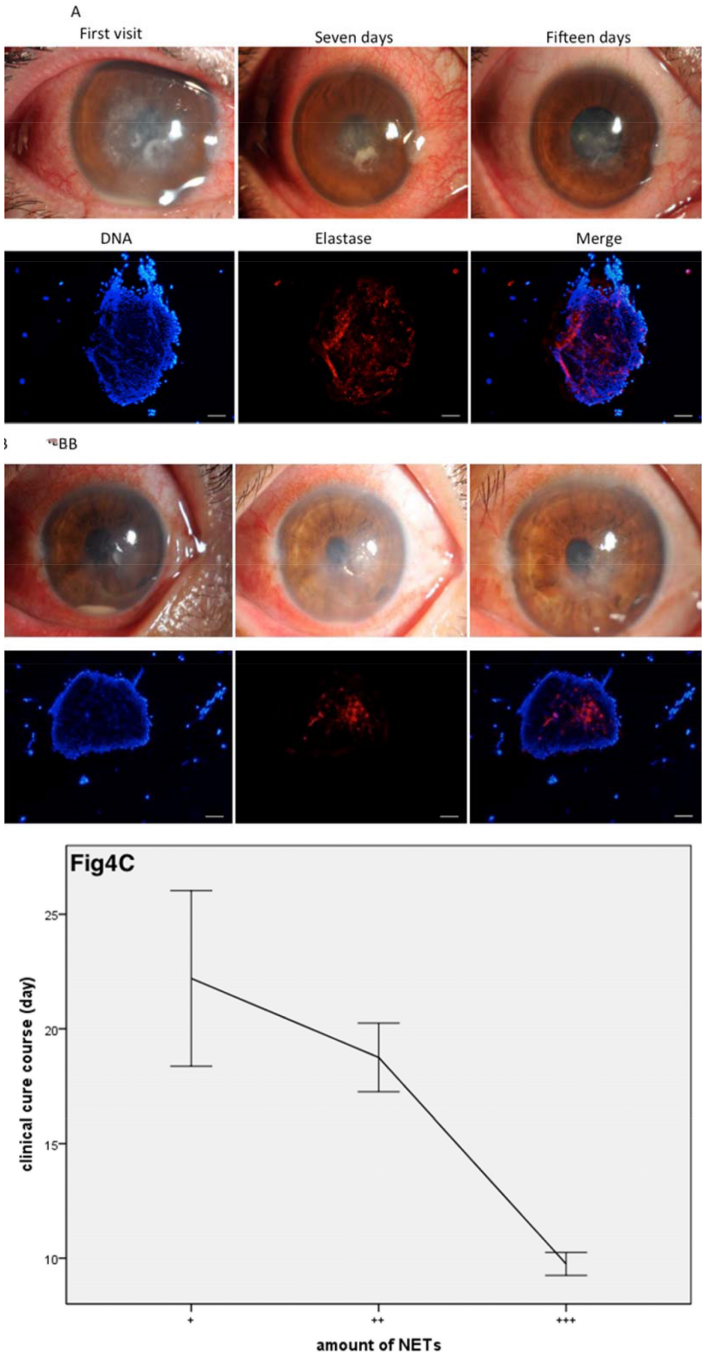

Figure 4. Relationship between fungal keratitis and NET formation. **A**: Slit-lamp microscopy observation of the cornea and the anterior chamber shows that the corneal ulcer healed after a cure course of a 15-day treatment in the patient infected with *Candida albicans* in whom the number of neutrophil extracellular traps (NETs) was rated "++" with immunofluorescence. **B**: A longer cure course of treatment of 21 days was needed for another patient infected with *Fusarium solani* in whom the number of NETs rated "+." **C**: A higher number of NETs is closely related to a better treatment response and a shorter course of infection. Data represent mean ± standard deviation (SD), r = –0.891, p<0.001 by Kendall tau_b correlation test. Bar: 100 µm.

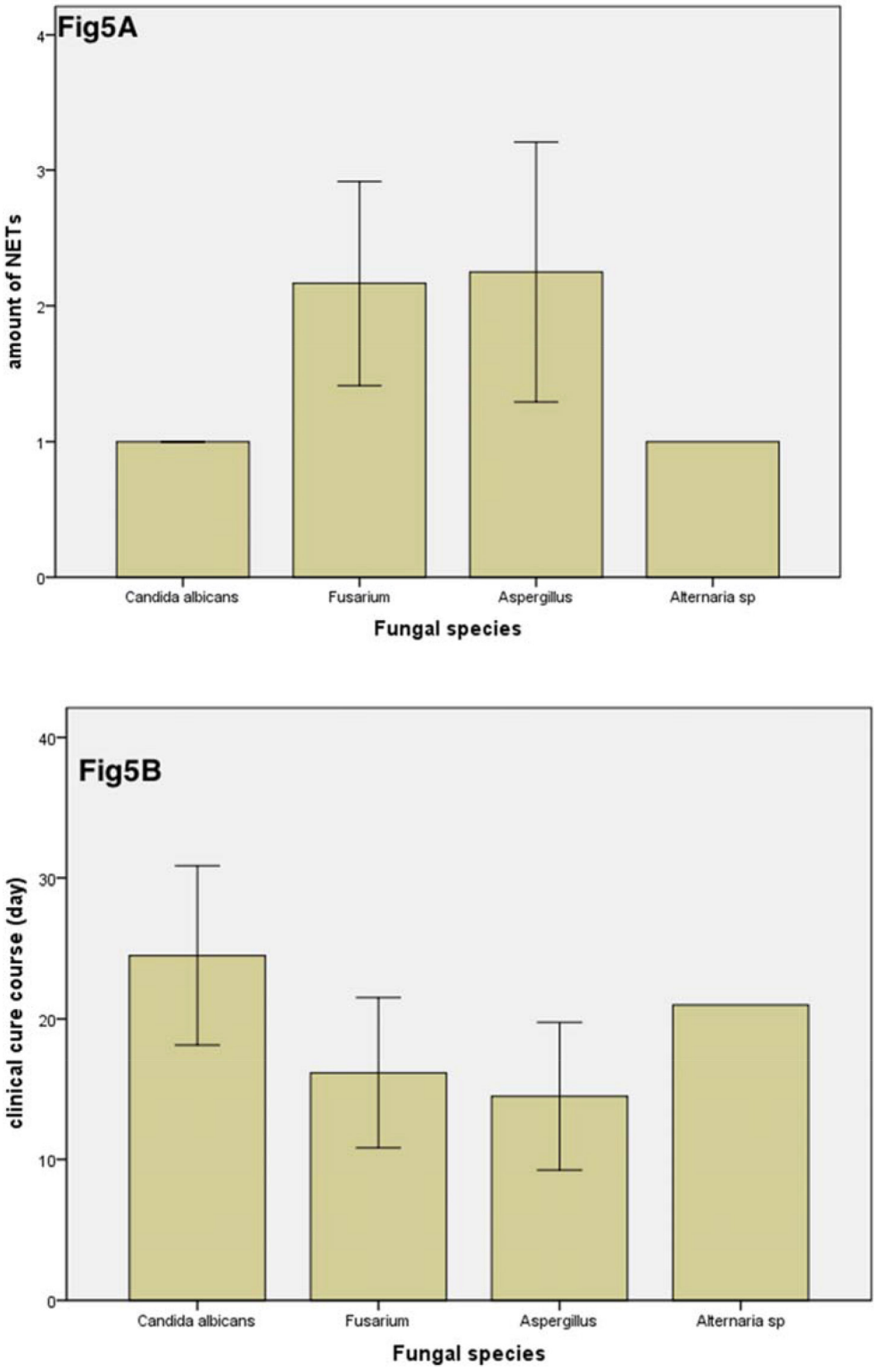

Figure 5. Different numbers of NETs and clinical courses among fungal species. The number of neutrophil extracellular traps (NETs; **A**) formed for *Candida albicans* and *Alternaria* sp. is higher than for *Fusarium* and *Aspergillus*, but for the clinical cure course (**B**) of treatment, the former is shorter than the latter. Data represent mean ± standard deviation (SD).

degradation is needed to achieve maximal NET immunity [18]. The course of fungal keratitis is always longer than 20 days [19]; therefore, NETs may play a crucial role in combatting fungal infection of the cornea. Previously, the prognosis for fungal keratitis was difficult to evaluate clinically, and the therapy could be modified only in accordance with the clinical response [20]. Previous reports presented that phagocytosis is the main mechanism for clearing microbes during the first 60 to 120 min following infection after which neutrophils' phagocytic capacity is restricted [19]. Afterward, active neutrophils display a high ratio of coiling versus conventional phagocytosis [21], which leads to nuclear and granular components mingling in the cytoplasm and then rapidly being released as NETs when the neutrophils finally die. NETs can then bind, trap, and kill microbes by providing a high local concentration of antimicrobial peptides. In this vein, the NETs act as soldiers to protect the cornea from fungi invasion, and a higher number of NETs may lead to a better prognosis. In the present study, we explored the relationship between the number of NETs and prognosis. Although the number of NETs was not related to the course of the infection or the size of the ulcer, NETs were related to the prognosis for fungal keratitis. A higher number of NETs was closely related to a better treatment response and a shorter course of infection. These data suggest that measuring host immune markers of NET formation could prove useful for in vivo monitoring of treatment responses. However, the discrimination of NET formation in different types of fungus is difficult. In the present study, four fungal species were examined in the experiment, and in particular, for *Alternaria* sp., there was only one case; thus, as the total sample was small, statistical analysis could not be performed. Nonetheless, we still observed that the number of NETs of *Candida albicans* and *Alternaria* sp. was higher than that of *Fusarium* and *Aspergillus*, and the clinical course of treatment for the former was shorter than that for the latter. More samples and further studies are needed to elucidate the different NET formation and clinical courses among various fungal species.

In conclusion, this study provided in vivo data on the release of NETs during fungal keratitis. We presumed that neutrophils first performed phagocytosis of the fungi and then released NETs to defend against fungal infection. The number of NETs may be used to evaluate the prognosis for infectious keratitis. Further studies to analyze other functional roles of NETs in infectious keratitis are warranted.

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