· Original article ·

Combined deficiency of T and B cells impact on the initiation of *Aspergillus* or *Candida* keratitis in murine models

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Abstract

• AIM: To explore whether adaptive immunity is involved in initiation of fungal keratitis (FK).

• METHODS: Balb/c mice and severe combined T/B-null immunodeficiency (SCID) mice on same background were subjected to keratitis induction *via* intrastromal injection of 1×10^5 *Candida albican* or *Aspergillus fumigatus* spores. The disease was monitored using slit-lamp and scored according to area and density of opacity and surface irregularity of the affected corneas. At desired time points, murine eyes were enucleated for histological examination by hematoxylin-eosin and Periodic acid Schiff staining. The number of live-fungi in corneal tissue was detected with colony forming assay, and the levels of IL17, IFNγ and IL10 in plasma and corneal tissue homogenate were evaluated by ELISA.

• RESULTS: Both Balb/c mice and SCID mice developed typical keratitis when subjected to FK induction. Gross clinical presentation and disease scoring, as well as histology and pathogen load assay showed no significant difference between Balb/c mice and SCID mice with either pathogen. With the cytokine levels in either plasma or cornea, neither IL17 nor IFNγ showed significant difference between these two mouse strains, but IL10 was undetectable in any SCID mice though it could be detected in Balb/c mice, especially at early time points after FK induction.

• CONCLUSION: The presence or absence of adaptive immunity compartment, at least in the studied Balb/c and SCID mice pairing, does not alter the course of primary FK, supporting the hypothesis that innate immunity component is more relevant with the pathogenesis of FK.

• KEYWORDS: *Aspergillus fumigatus*; *Candida albican*; fungal keratitis; SCID mice; IL17; adaptive immunity; innate immunity

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INTRODUCTION

Fungal keratitis (FK) is a highly blinding disease caused by infections to the corneas by various fungal pathogens. Reported predisposing factors include trauma to the cornea, extended wearing of corneal contact lenses, deficiency or suppression of host immunity, and other conditions[1]. In recent years, with the progress made in management of other infectious keratitis like viral or bacterial ones, the incidence and harm of FK has been growing more significant in this country. The spectrum of causative fungi also shifts with time or geographic areas[2,3]. However, the pathogenesis of FK is poorly understood, thus the development of protocols or therapeutics for clinical FK is hindered. In specific, the relative contribution of innate and adaptive immunity compartments in each stage of pathogenesis remains undetermined. Using a murine *Candida albican* model, we previously showed that the FK induced typical immunological memory that gives the host enhanced resistance to secondary infection, implying that adaptive immunity might be involved in fighting against FK at late stage of murine FK model[4,5]. In such a murine model, however, the affected animals usually start recovery around day 7-10, at which time the specific immunological effectors like T lymphocytes or immunoglobulins have just been effectively activated. Thus it is reasonable to speculate that it was the components of innate immunity compartment play main roles, either beneficial or detrimental, in the first week of the model. This study was performed to check this possibility. By comparing the whole course of disease progression in immunocompetent Balb/c and severe combined immunodeficient (SCID) mice [6], we got the impressive that presence or absence of adaptive immunity has no effect on the overall presentation and outcome of FK, and this was true with both *Candida albican* and *Aspergillus fumigatus*.

MATERIALS AND METHODS

Materials Balb/c mice and SCID mice, 8-10 weeks old, male, were purchased from the Company of Vital River (Beijing, China). Use of animals was approved by institutional review board and handling of animals was in line with the ARVO and national regulations. Only the left corneas were used for experiment, with the right eyes used as untreated controls.

Fungal keratitis models Two leading pathogens genesis, namely *Candida albicans* (strain MYA-2876, ATCC, Manassas, Virginia, USA) and *Aspergillus fumigatus* (strain No. 3.0772, China General Microbiological Culture Collection Center, Beijing, China) were cultured on plates routinely following Shandong Eye Institution Biosafety Code at 28℃ for 5 days. The spores were collected into phosphate buffered saline (PBS) and adjusted to a density of 1×10^8/mL. The FK models, namely *Aspergillus fumigatus* keratitis (AfK) and *Candida albicans* keratitis (CaK) were established by injecting 1μL (1×10^5/cornea) of the spore suspension into the stroma of corneas of mice as detailed before[5]. Briefly, the mice were anesthetized with 0.02mL/g intraperitoneal injection of hydrochloric acid ketamine/chlorpromazine hydrochloride/normal saline (4:1:45 v/v) mixture. Under a surgical microscope, the mouse cornea was pierced to the depth of basement membrane with a 30G needle. A 33-gauge needle (Hamilton, Reno, NV) was used to inject one microliter of spore suspension into the center of the cornea. In the sham groups, the same volume of saline buffer was substituted for the fungal suspension. The corneas were observed with a slit lamp at desired time points and pictures of the eyes were taken with a camera mounted on the slit lamp microscope. A12-point scoring system based on corneal opacity, density of opacity, and surface regularity was adopted to evaluate the severity of the corneal diseases[7].

Methods

Histology Eyeballs were collected at desired times after infection and fixed in 4% paraformaldehyde in neutralized PBS. Routine procedure was employed and 4μm thick serial sections were prepared. Neighboring sections were subjected to hematoxylin-eosin (HE) and Periodic acid Schiff (PAS) staining respectively. The area and severity of the disease could be qualitatively reflected by cellular infiltration, hyphae distribution and regularity of the corneal structure under light microscope, but quantitative evaluation was not attempted in this study.

Pathogen burden assay After enucleation of eyeballs from euthanized mice, the corneas were cut by careful removal of all other parts. The corneas were put into the sterile 1.5mL tube containing 500μL buffer (0.1mol/L Tris-HCl pH 8.0, 0.02mol/L EDTA in distilled water), cut it into pieces and homogenized with a Tissue Tearor (Biospec Products, Bartleville, OK, USA). After centrifugation at 5000r/min for 10 minutes, the 400μL supernatant was collected and 100μL subnatant serially diluted at 1:10, 1:100 and 1:1000. 100mL aliquots of each dilute or original homogenate were spread on 90mm Sabouraud's dextrose agar plates in triplicates. After incubated at 37℃ for 48 hours, the colonies were counted and converted to a pathogen load in a whole cornea.

ELISA for cytokines The cytokines in the animal circulation or corneas were measured with ELISA method. In brief, at desired times after FK induction, blood samples were collected from individual mouse orbital sinus and plasma were prepared. Corneal homogenates were prepared as above. All samples were diluted according to predetermined scale and subjected to ELISA measurement using the Mouse ELISA MAX™ Deluxe Sets for IL17A, IFNγ or IL10 respectively according to the manufacturer's instructions (BioLegend, San Diego, CA, USA). Standard curves were run at the same time and the optical density values of all samples were converted to cytokine amount in each cornea.

Statistical Analysis When necessary, statistical significance was determined by the Student's t-test, and by applying a 95% confidence interval ($P<0.05$) to judge significance.

RESULTS

Similar clinical presentation of FK in Balb/c and SCID mice Many pathological processes, especially those immunological ones, show strict dependence on the immunogenetic background of host. We previously showed that Balb/c ($H2^d$) and C57BL/6 ($H2^b$) mice manifested differential sensitivity and molecular responses to a same challenge of CaK[8]. However, when Balb/c and SCID mice were similarly subjected to FK induction, no significant difference was observed in either AfK or CaK (Figure 1). With both pathogens, and in both strains of mice, corneal opacity started on the same day of inoculation and the disease reached maximum by around day 5. But few perforation was seen in any groups. Starting from day 7, the corneas gradually regained their normal appearance, and only localized opacity was present in some corneas by day 14 (Figure 1).

Similar histological changes of FK in Balb/c and SCID mice In the situation of FK, both fungal growth or expansion and leukocyte infiltration contribute to histological changes. By conventional pathology, it was found that the changes occurred in corneal tissues in Balb/c and SCID mice were basically similar (Figure 2). In general, by day 3 post infection, a large number of fungal hyphae could be detected. At the same time, inflammatory infiltration of leukocytes, mainly neutrophils, also reached maximum. Though the exact sequential or causative relationship was not attempted, it is noteworthy that the congressing of fungal hyphae and leukocyte infiltration were complementary to each other in the cornea. In another word, hyphae growth was more significant in areas showing less leukocyte, and vise versa (Figure 2). This may suggest that leukocytes are effective in fungicidal, either by killing the pathogen or by inhibiting their growth. By day 7, much less fungal hyphae were present, but inflammation and edema of the corneas were still obvious. At 14 days, the corneal structure was close to normal except for scattered small infiltration focus or iris synechia. Iris synechia might be caused by hypopyon during the critical period (day 3-7).

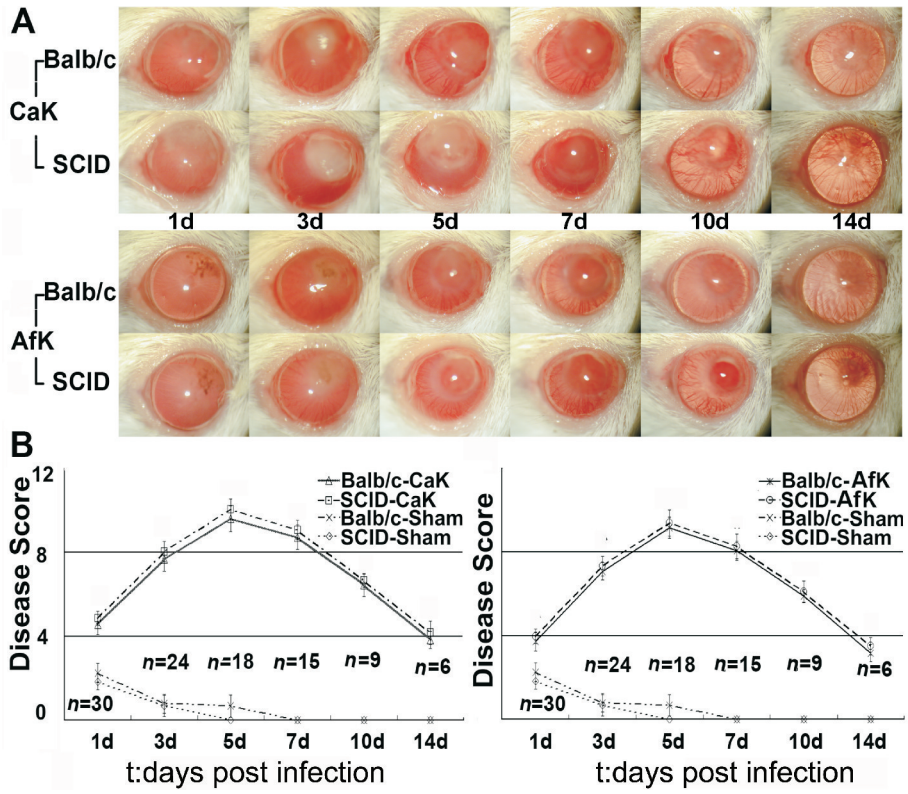

Figure 1 Presentation of *Candida albican* keratitis (CaK) and *Aspergillus fumigatus* keratitis (AfK) in Balb/c and SCID mice on the same immunogenetic background A: After intrastromal injection of 1×10^5 live *Candida albican* or *Aspergillus fumigatus* spores or same volume of saline buffer (sham groups), the corneas were monitored under a slit lamp and pictures were taken; B: The disease of the cornea was evaluated with a 12-score system. The pictures for sham groups were not shown in (A) but their scores were given in (B). "*n*=" in (B) indicates the number of eyes evaluated in each group at that time point. Shown was one of two experiments with similar results.

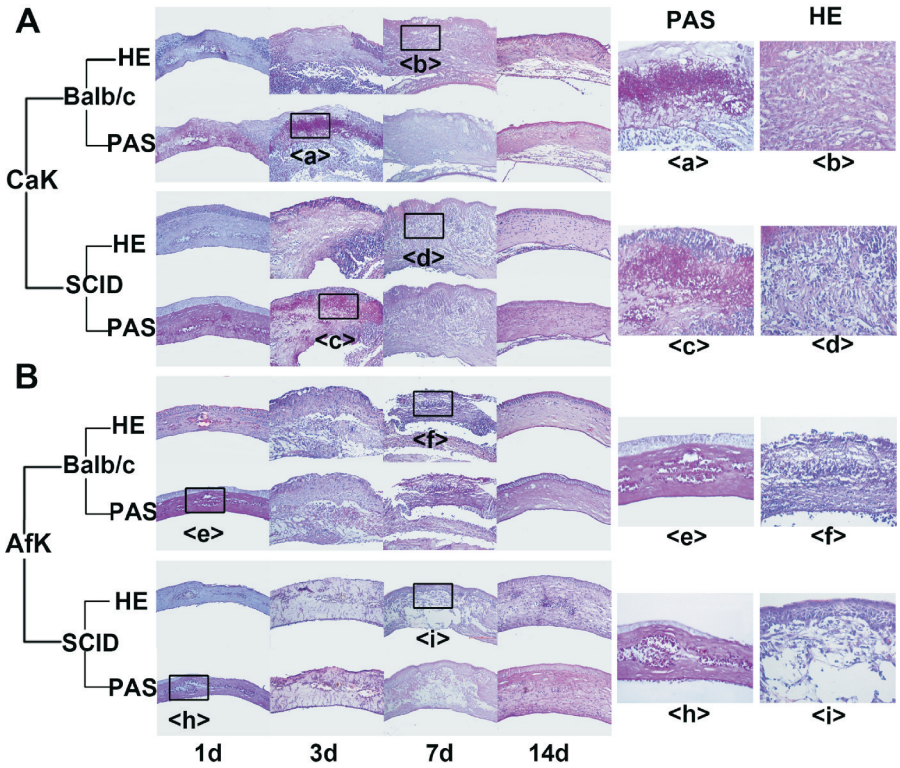

Figure 2 Histology of corneal sections at different time points of mice with CaK (A) and AfK (B). The corneas were stained with HE for regular structure examination and with PAS for better revelation of fungal components. Higher magnification of inlets was listed in the right two columns. Bar, 50μm. Shown were representatives of samples from two experiments with similar results.

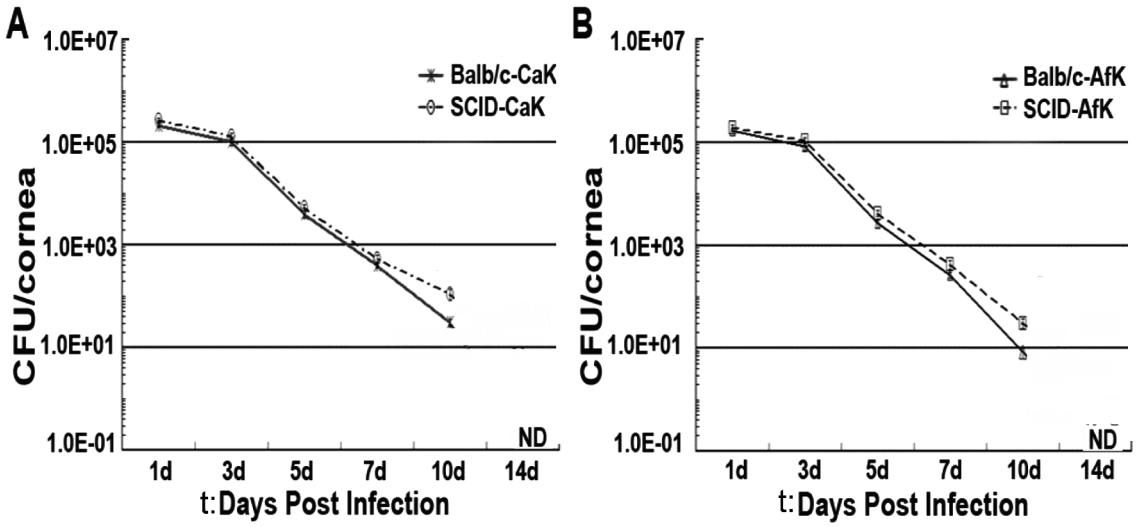

Figure 3 Pathogen loads as measured in corneas with CaK or AfK after the colony forming assay with corneal homogenate at different dilution, the numbers of colonies were converted to total pathogen load in each cornea. Three corneas were included for each time point and triplicates were set for each dilution of each cornea. Given was mean±standard error of three samples in each group(n=3).

Consistent with similar pathology result, the colony forming assay also revealed similar pathogen burdens in corneas of Balb/c mice and SCID mice, and this was true with both AfK and CaK models (Figure 3).

Similar IL17A/IFNγ but differential IL10 levels in FK models in Balb/c and SCID mice We previously showed that IL17A and IFNγ were involved in the initiation of CaK as well as in the later recovering stage in immunocompetent mice[5,8]. Continuing studies in this lab (submitted for publication) as well as other labs[9,10] suggested that neutrophils might be main source of IL17A in the FK situation, and IL17 likely plays a detrimental role in various infectious keratitis [11-13]. Since the SCID mice, though deficient in T and B cells, effectively recruited presumably neutrophils into the corneas after fungal inoculation as shown above (Figure 2), we assumed that SCID and Balb/c mice may produce matching amount of IL17A. ELISA measurement confirmed that in both the peripheral circulation and the infected corneas, IL17A reached comparable levels in Balb/c and SCID mice, and this was true when either *Aspergillus fumigatus* or *Candida albican* was used (Figure 4A). Similar parallel changes were observed for IFNγ levels (Figure 4B) but not for IL10 levels (Figure 4C). While Balb/c mice presented significant amount of IL10 at early days in serum and cornea, SCID mice did not produce any detectable IL10 in either serum or cornea, at any time points, with either CaK or AfK models (Figure 4C).

DISCUSSION

In a parallel study we found that contrary to Balb/c mice, nude mice on a same immunogenetic background did not develop CaK when subject to a same intrastromal inoculation of *Candida albican* spores (submitted for publication). To explain the differential response of Balb/c and nude mice to CaK induction, we proposed that nude mice were deficient in producing chemokines like CCL2 that normally mediate neutrophil infiltration, and such deficiency should be related with the absence of functional T lymphocytes. Thus the fact that SCID mice are sensitive to FK induction implied that SCID mice were equipped with the innate ability to produce chemoattractants that mediate neutrophil infiltration. Taking all three mice strains' sensibility to FK induction into account, it is tempting to speculate that B cells, the only group of immune cells that were present in both Balb/c and nude mice but absent in SCID mice, play a suppressor role in the initiation of FK, but this hypothesis warrant further experimental support. Contrary to the sensitivity of SCID mice to FK, earlier reports demonstrated that neither nude mice[14] nor SCID mice[15] infected via the cornea with herpes simple virus fail to develop typical herpes simple keratitis as Balb/c mice did. We proposed that the pathogenesis of viral or fungal keratitis involve quite different compartments thus the immunodeficiency status in SCID mice leads to differential effects on the host presentation when subjected to viral or fungal inoculation. However, it seems that the difference between *Candida albican* and *Aspergillus fumigatus* had no effect on the host response, since in all measured indexes, CaK and AfK gave similar results (Figures 1-4).

It is understandable that the SCID mice respond to pathogen challenge mainly via the innate immunity, thus the detected IL17 and IFNγ in SCID mice must be produced by non-T and non-B cells. So far we have no idea how much the local cells or cells of the immune system contribute to the overall pool of each cytokine. It is noteworthy that IL10 was produced in Balb/c mice with CaK or AfK but not in SCID mice with similar diseases. The presence or absence of IL10 seemed not affect the FK progress much, though IL10 has long been known as an immunomodulator [16] and proposed to suppress host inflammatory response to recurrent viral keratitis [17]. Absolute deficiency of IL10 production in SCID mice with FK suggested that IL10 in Balb/c mice with FK might be produced

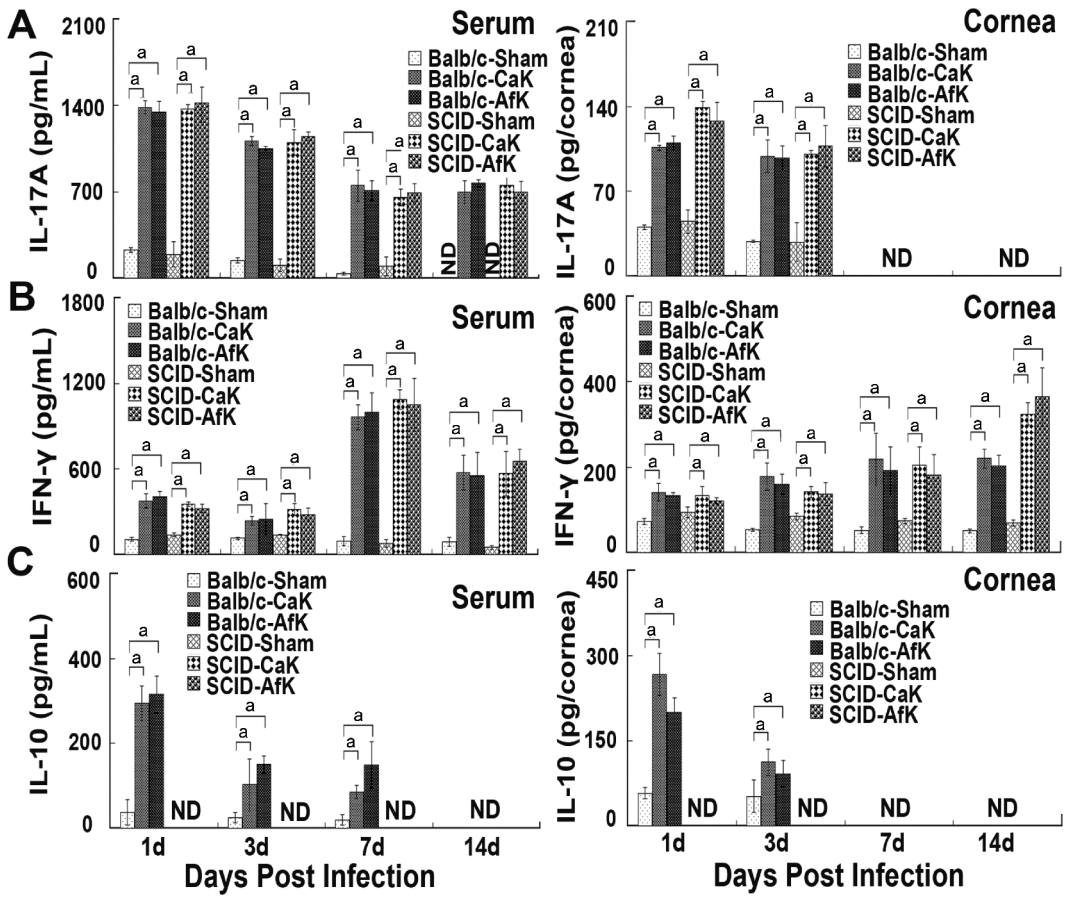

Figure 4 The levels of cytokines in corneas and serum at different times. The measured amounts of cytokines in corneal homogenates were converted to total amounts in each cornea. ND, undetectable for those groups. [a]$P<0.05$ for comparison between two mouse strains or between different processing groups.

mainly by T or B cells, but this possibility deserve further investigation.

In summary, we observed that either *Candida albican* or *Aspergillus fumigatus* induced same pathological changes and clinical presentation to the corneas of immunocompetent Balb/c and SCID mice, clearly demonstrating that the adaptive immunity compartment does not play key roles in the primary fungal keratitis. However, the potential significance of this finding in clinical practice in human FK patients is unclear, since in later condition the infection usually starts with unknown numbers of unidentified pathogens via unknown routes. Still, further investigation along the innate immunity components in FK models will result in better view of the whole process of FK, thus providing reference for search of therapeutics or interfering protocol for translational purpose.

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T 和 B 细胞缺陷对真菌性角膜炎发病过程的影响

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摘要

目的:探讨特异性免疫应答是否参与真菌性角膜炎(fungal keratitis, FK)发病早期的病理过程。

方法:通过角膜基质内注射 1×10^5 白色念珠菌孢子或烟曲霉菌孢子的方法在相同免疫遗传背景的 Balb/c 小鼠和重症联合免疫缺陷小鼠(severe combined T/B-null immuno-deficiency, SCID)诱导 FK。用裂隙灯监测疾病临床变化特征并依据角膜病灶面积和病变深度以及角膜表面规则性进行临床评分。在病变显著的时间点,摘除小鼠眼球用 HE 和 PAS 染色观察小鼠角膜组织的病理变化特征。用菌落形成实验检测角膜组织中活菌数量的变化,用 ELISA 检测小鼠血浆和角膜组织匀浆液中细胞因子 IL17、IFNγ 和 IL10 的动态变化。

结果:Balb/c 小鼠和 SCID 小鼠都可诱导出典型的 FK。在白念菌性角膜炎或烟曲霉菌性角膜炎中,无论是大体临床表现和疾病分数,抑或组织病理变化和真菌负荷分析,在两种小鼠之间均无显著性差异;两种小鼠间在血浆或角膜组织中 IL17 及 IFNγ 的水平等方面也没有显着差异。但诱导两种 FK 的 SCID 小鼠中,无论角膜匀浆液抑或外周血浆,在任何时间点均检测不到 IL10 的表达,而同样发生角膜炎的 Balb/c 小鼠则在 FK 发病早期检测到 IL10 的表达。

结论:在免疫遗传背景相同的条件下,小鼠中获得性免疫组分的存在与否并不影响 FK 的发病过程,提示小鼠中初次 FK 的发病与固有性免疫组分相关。

关键词:烟曲霉菌;白色念珠菌;角膜炎;SCID 小鼠;IL17;特异性免疫;非特异性免疫