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Distinct roles for interleukin-12p40 and tumour necrosis factor in resistance to oral candidiasis defined by gene-targeting

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Cell-mediated immunity is important for anti-*Candida* host defence in mucosal tissues. In this study we used cytokine-specific gene knockout mice to investigate the requirement for T helper type 1 (Th1) and Th2 cytokines in recovery from oral candidiasis. Knockout mice used in this study included interleukin-4 (IL-4), IL-10, IL-12p40, interferon-γ (IFN-γ), and tumour necrosis factor (TNF). The mice were challenged either orally or systemically with *Candida albicans* yeasts, and levels of colonization were determined. IL-12p40 knockout mice developed chronic oropharyngeal candidiasis, but were not more susceptible to systemic challenge. On the other hand, TNF knockout mice displayed increased susceptibility to both oral and systemic challenge, but only in the acute stages of infection. TNF apparently has a protective effect in the acute stages of both oral and systemic candidiasis, whereas IL-12p40 is essential for recovery from oral but not systemic candidiasis. The role of IL-12p40, and its relation to T-cell-mediated responses remain to be determined.

Key words: oral candidiasis; cytokine; knockout mice

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Diseases associated with *Candida albicans* infection occur in two discrete forms, mucosal and systemic, and these infections present a significant and increasing clinical problem. Clinical observations and data from both human and animal experimental models demonstrate that cell-mediated immunity is essential for anti-*Candida* host defence in mucosal tissues (11). Individuals with congenital or acquired defects in cell-mediated immunity are particularly susceptible to mucocutaneous, but not systemic, candidiasis, whereas systemic candidiasis is more commonly associated with myeloperoxidase deficiencies and neutropenia (2, 5, 11).

Oropharyngeal candidiasis is prevalent in acquired immune deficiency syndrome (5) and human immunodeficiency virus-seropositive individuals, patients undergoing radiation and chemotherapy (11), and transplantation recipients (11). Reduction of cell-mediated immunity in peripheral circulation has also been shown to lead to an increased incidence of chronic mucocutaneous candidiasis (12). Patients with chronic mucocutaneous candidiasis show an impaired production of T helper type 1 (Th1) cytokines, particularly interleukin-12 (IL-12)/IL-23 and interferon-γ (IFN-γ), resulting in an inability to mount protective cell-mediated responses, and failure to clear the yeast (16). These patients also produce unusually high levels of Th2 cytokines, such as IL-10, in response to *Candida* (16).

We have previously established a mouse model of cell-mediated immunity in oropharyngeal candidiasis (8). Nude mice lacking functional T cells demonstrated significantly increased levels of oral colonization compared to heterozygous littermates, resulting in the development of a chronic infection that persisted for more than 3 months. $CD4^+$, but not $CD8^+$, T cells were capable of reducing the fungal burden in the oral tissues of chronically infected nude mice, resulting in eventual

clearance of the infection and full recovery (8). Reconstituted mice produced IL-12 and IFN-γ in the draining lymph nodes, but no detectable IL-4 or IL-10 (8), and expressed tumour necrosis factor (TNF) in the oral tissues (9). Irradiation to the head and neck of mice also led to an increased susceptibility to oral infection, and recovery was again associated with the production of high levels of IL-12 and IFN-γ by cells from the draining lymph nodes (10).

The present experiments used cytokine-specific gene knockout mice to evaluate the requirements for cytokines representative of the Th1 and Th2 subsets in recovery from oral candidiasis, and to contrast this with systemic infection.

Materials and methods

Mice

Specific pathogen-free, 6- to 8-week-old, female cytokine knockout mice and their respective controls were used in this experiment. Mice were obtained from various sources and bred at the Herston Medical Research Centre, Brisbane Australia, with the genotypes checked regularly by polymerase chain reaction. These mice undergo routine microbiological screening and do not harbour *C. albicans* in the gut. Animal experiments were approved by the Animal Experimentation Ethics Committee of the University of Queensland, and carried out in accordance with the National Health and Medical Research Council's Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 1997. Mice were housed in filter-top cages in a Physical Containment Level 2 (PC2) facility, and provided with food and water *ad libitum*. Knockout (−/−) mice used in this study included IL-10, IL-12p40, IFN-γ, and TNF on the C57BL/6J background, and IL-4 on the BALB/c background.

Yeast

C. albicans isolates 3630 and 3683 were obtained from the Mycology Reference Laboratory at the Royal North Shore Hospital, Sydney, Australia, and stored at −70°C in Sabouraud's broth/15% (v/v) glycerol. Strain 3630 was from the nail of a patient with cutaneous candidiasis, and 3683 was an oral isolate. For use, yeasts were grown in Sabouraud's broth for 48 h at room temperature with continuous agitation on a magnetic stirrer. Blastospores were washed in phosphate-buffered saline (PBS) and adjusted to the appropriate concentration for inoculation.

Oral infection

Mice were inoculated orally with 10^8 live *C. albicans* in 20 µl sterile PBS. The infection was monitored by swabbing the oral cavity on days 1, 4, 8 and 14 and then weekly thereafter, with sterile cotton swabs moistened with sterile PBS, and directly plating on Sabouraud's agar plates. Agar plates were incubated for 48 h at 37°C. All inoculation and sampling procedures were conducted under halothane anaesthesia using an inhalation apparatus and a scavenging system. Colony-forming units (CFU) were counted on Sabouraud's agar plates and the counts were assigned into five groups correlating with the level of recoverable yeast from the oral cavity, as previously described (8). This provided a semi-quantitative measure of the level of floridity of the infection. The scoring system used was as follows: 0, no detectable yeast; 1, 1–10 CFU/plate; 2, 11–100 CFU/plate; 3, 101–1000 CFU/plate; 4, ≥1000 CFU/plate.

Systemic infection

Mice were injected with 3×10^5 *C. albicans* 3630 in 200 µl PBS via the tail vein. Mice were sacrificed on day 5 after infection. The brains and kidneys were harvested, weighed, suspended in 1 ml sterile PBS, and homogenized. Then, 100 µl of suspension was titrated in duplicate on Sabouraud's agar plates, incubated at 37°C for 48 h, and colonies were counted. The results were expressed as $\log_{10}$ CFU/g tissue.

Histopathology

Mice were sacrificed at various time-points throughout the course of the experiment for histopathological examination of oral tissues. Skulls were fixed in 10% neutral buffered formalin (pH 7.0), decalcified in a 5% formic acid and sodium formate mixture, dehydrated, and embedded in wax. Frontal sections of the skulls were taken at approximately 3-mm intervals; consecutive sections were stained with haematoxylin & eosin and according to the Periodic acid-Schiff technique, and were examined by light microscopy (Olympus, Tokyo, Japan).

Statistics

Quantitative data were analysed using the statistical features of GRAPHPAD PRISM Version 2.01 (GraphPad Inc., San Diego, CA, USA). Student's *t*-test and one-way analysis of variance were used with $P < 0.05$, unless otherwise indicated.

Results

Oral infection

There were no differences in the severity or duration of the oral infection in IL-4, IL-10, or IFN-γ knockout mice compared to their respective controls (Fig. 1). There was however, a significant increase in the severity, but not the duration, of the oral infection in $TNF^{-/-}$ mice, but this was only seen in the early stages of infection (Fig. 1). IL-$12p40^{-/-}$ mice were much more susceptible to oral infection with *C. albicans* 3630 than control mice, and developed a chronic oropharyngeal infection that could not be cleared, and that lasted for more than 3 months (Fig. 2A). To demonstrate that the effect seen in the IL-$12p40^{-/-}$ mice was the result of an inherent defect in the mouse, and not related to the strain of *Candida* used, these mice were infected with *Candida* strain 3683 using the same protocol. The IL-$12p40^{-/-}$ mice were unable also to clear the oral infection established with *C. albicans* strain 3683 (Fig. 2B). The severities and durations of infections with *Candida* strains 3630 and 3683 were comparable. Extensive hyphal penetration of the keratinized layer of the oral epithelium was observed in IL-$12p40^{-/-}$ mice throughout the course of the infection, and this was still evident 3 months post-challenge. There were no inflammatory cells noted in the vicinity of the hyphae at this time (Fig. 3). There was no evidence of pathological alterations following infection of the oral tissues of the other knockout mice, or of wild-type controls.

Systemic infection

There were no differences in the fungal burden in either the brain or the kidneys of IL-4, IL-10, IL-12p40, or IFN-γ knockout mice, compared to their respective controls (data not shown). $TNF^{-/-}$ mice showed a twofold increase in the fungal burden in the kidneys ($P < 0.001$), but not in the brain, compared to C57BL/6J control mice (6.65 vs. 3.21 CFU/g $\log_{10}$).

Discussion

It is generally accepted that extracellular infections are cleared by the actions of phagocytic cells, the microbicidal properties of which are enhanced by cytokines, typically IFN-γ and TNF, produced by Th1 lymphocytes. In the current study, IFN-γ knockout mice were found not to be susceptible to either oral or systemic infection. These results are somewhat

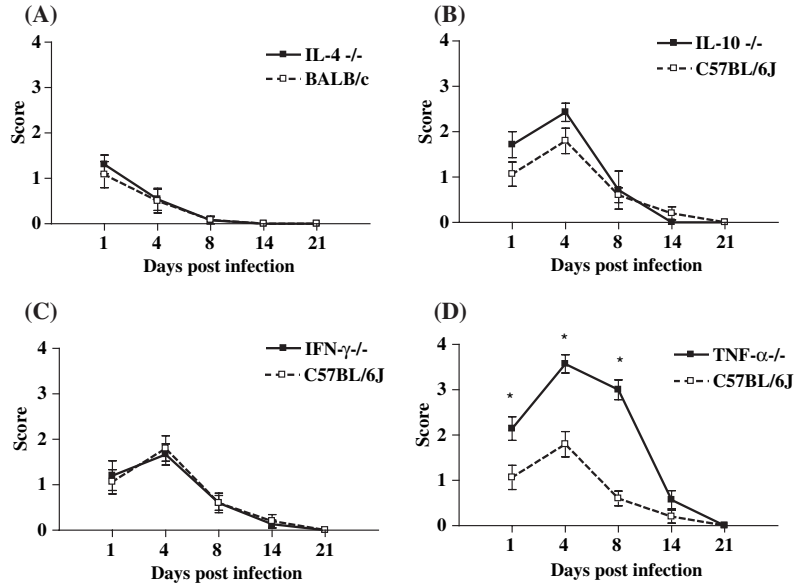

Fig. 1. Oral infection with *Candida albicans* 3630 in IL-4 (A), IL-10 (B), IFN-γ (C) and TNF (D) knockout mice. Data points represent the score (mean ± SEM) for a minimum 10 mice/time-point in each group. All experiments were repeated at least twice. There is a significant difference between $TNF^{-/-}$ and control mice at specified time points; $*P < 0.05$.

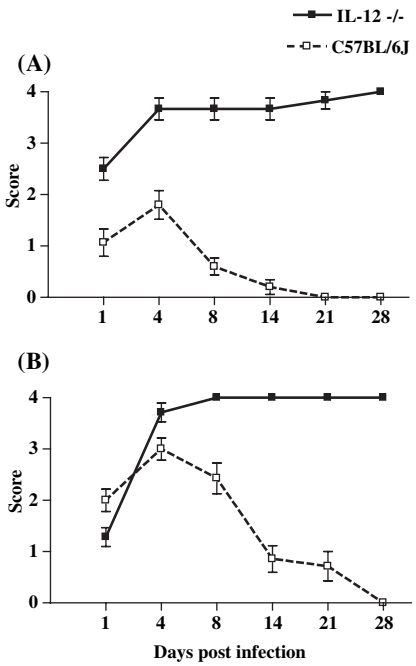

Fig. 2. Oral infection with strains 3630 (A) and 3683 (B) in IL-12p40$^{-/-}$ mice. Data points represent the score (mean ± SEM) for a minimum of 10 mice/group for each time point. All experiments were repeated at least twice. There is a significant difference ($P < 0.01$) between knockout and control mice at all time-points but there is no significant difference in the severity or duration of the infection between the *Candida* strains 3630 and 3683.

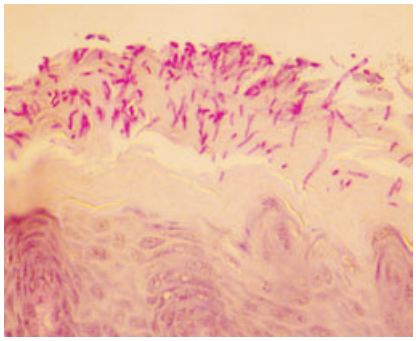

Fig. 3. Histopathological section of oral mucosa from IL-12p40$^{-/-}$ mouse infected with 3630.

surprising, but other studies have been equivocal. One found that IFN-γ knockout mice demonstrated increased susceptibility to both gastric and systemic candidiasis (4), a second reported increased mortality of the knockout mice, although the increased susceptibility did not correlate with the extent of organ colonization (15), while a third found no effect on either form of the disease (22). We have verified that IFN-γ was inactivated in these mice (7), and although the contradictions remain to be resolved, they may be related either to the experimental model, or to differences between the isolates of yeast used for challenge (14).

TNF mediates pro-inflammatory activities in the early phases of the host response, and $TNF^{-/-}$ mice have been found to demonstrate increased susceptibility to systemic *C. albicans* infection (18). Deletion of both TNF and lymphotoxin results in substantially increased growth of *Candida* in organs; attributed to delayed recruitment of neutrophils and a reduced phagocytic capacity of these cells, although killing of yeasts was unaffected in these mice (21). Comparable experiments (20) demonstrated an increased susceptibility to both systemic and gastrointestinal infection, again attributed to the impaired effector function of neutrophils, although in this case, the effect correlated with the induction of non-protective Th2, rather than protective Th1 responses. In our study, $TNF^{-/-}$ mice showed a significant increase in the severity, but not the duration, of the infection after oral inoculation. This strongly suggests that TNF is involved in constraining the initial proliferation of the yeast, whereas other TNF-independent mechanisms, centred on IL-12p40, are responsible for its clearance from infected tissues. Other findings of interest in the current experiments were kidney-specific effects following systemic infection in $TNF^{-/-}$ mice. In recent years, the use of TNF antagonists for the treatment of rheumatoid arthritis and other autoimmune diseases has been associated with an increase in the incidence of opportunistic *C. albicans* infections (13). Because the use of these TNF antagonists is expected to increase in the future, understanding the role that TNF plays and the effect of its antagonism on host defences against *C. albicans* infections is critical for reducing associated morbidity and mortality (13).

IL-12p40$^{-/-}$ mice developed a chronic oropharyngeal infection that did not resolve regardless of the strain of *Candida* used for inoculation, while the severity of systemic infection in these mice was unaltered, suggesting that IL-12p40 is important in promoting the development of the immune responses required for recovery from mucosal infection, but that the pathways of innate immunity may dominate in systemic infection (3). If it is accepted that innate immunity is the primary mechanism responsible for clearance of yeasts in systemic candidiasis, as exemplified by the resistance of immunodeficient mice (6, 19, 23), and that recovery from oral infection is absolutely dependent on $CD4^+$ T-cell function (8), then it can be inferred that susceptibility to oral candidiasis in IL-12p40$^{-/-}$ mice is linked to an IL-12-dependent defect in the generation of an adaptive immune response. It is important to remember, however, that IL-12p40$^{-/-}$ mice are also deficient in IFN-γ production (1, 17), and

it may be that the presence of both of these cytokines is essential for effective host resistance against oral candidiasis.

In conclusion, it has been reported that IFN-γ, IL-12p40 and IL-10 interact in the generation of protective T-cell responses against mucosal (oral, orogastric, and gastrointestinal) infections, but they are less clearly implicated in the resolution of primary systemic infections. TNF is a crucial cytokine that exerts a protective role in the acute stages of both oral and systemic candidiasis, whereas IL-12p40 plays a dominant role in oral candidiasis. Current work is defining the pathways involved in the immune response of IL-12p40 knockout mice to *C. albicans*.

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