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Cell mediated immune response dominates in experimental mammary gland *Candida krusei* infection in immuno-competent and immuno - compromised *(nu/nu)* mice

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Summary.
Experimental local mammary gland *Candida krusei* infection in immuno-competent and in congenic athymic nude BALB/c mice over a period of 21 days demonstrated that the immuno-competent mice readily cleared the infection whereas the infection persisted in the athymic mice. The fungal burden, however, was similar in the nude mice and in the immuno-competent mice until day 18 after infection. The ratio of interferon-γ/interleukin-4 (IFN-γ/IL-4) concentrations in supernatants from ConA stimulated splenocytes of the infected mice indicated a predominant Th1 response to the *C. krusei* infection. Stimulated splenocytes of the infected immuno-competent mice synthesised significantly higher concentrations of the two cytokines than did the splenocytes from the infected nude mice. The present study indicates that local *C. krusei* infection is associated with a predominant IFN-γ - Th1 response although a gradual activation of the Th2 -arm (IL-4) of the immune system may be indicated late in the course of infection.

Introduction
Fungal infections have increased in frequency in recent decades because of the growing number of immuno-compromised patients who survive longer than in the past, the widespread use of immunosuppressive drugs, a large aging population with increased numbers of malignancies and the spread of AIDS (*Chimelli & Mahler-Araujo, 1997*). *Candida* species are the most commonly pathogenic fungi isolated from immuno - compromised individuals and they constitute a leading blood stream isolate in hospitalised patients (*Muller et al., 1999*). Mucosal candidosis is the most prevalent opportunistic infection in HIV-infected patients and in both invasive and superficial infections non-*Candida albicans* species are on the increase (*Muller et al., 1999*). While *C. albicans* is the most notorious *candida* pathogen, non-*albicans* species, perhaps in particular *Candida krusei* (*Hazen, 1995*), are emerging as pathogens of concern, especially in immuno-compromised hosts (*Samaranayake, 1997*). *C. krusei* has been identified as a pathogen of immuno-compromised patients, causing invasive candidosis but it is also a cause of mycotic mastitis in livestock (*Aalbaek et al., 1994*).
Animal models for studies of fungal infections often rely on studies of animals with experimental systemic infection or studies of local infections in immunosuppressed animals (*Jensen, 1994; Guhad & Hau 1999*). We have developed a model for studies of fungal infections that remain localised to a single *organ throughout infection, namely the mammary gland of lactating intact untreated mice (Guhad et al., 1995, 1998a, 1998b, 1999, 2000)*. The model has been found useful for studies of *Candida* virulence factors (*Guhad et al., 1998ab*), studies of efficacy of antifungal treatment (*Guhad et al., 1999*) and studies of the relationship between severity of infection and activation of the complement system (*Guhad et al., 2000*).
The aim of the present study was (a) to determine the course of a localised infection of *C. krusei* in immuno-competent and congenic immuno-compromised mice and (b) to assess the splenocytic interferon-γ (IFN-γ) and interleukin-4 (IL-4) cytokine response of cytokines indicative of

Th1 and Th2 T-cell activity, respectively.

Materials and Methods

Mice

Twenty-four female BALB/cJ and 22 female BALB/cABom *nu (nude)* mice (M & B, Bomholtgård, Ry, Denmark), 7 weeks of age were housed with male BALB/c mice (three females and one male) in Macrolone type III cages (Scanbur, Köge, Denmark). The mice were separated into individual cages (Macrolone type II) once pregnancy was confirmed. The temperature was maintained at 20 °C +/- 1 °C. The mice had free access to a pelleted diet (R36, Lactamin, Stockholm, Sweden) and tap water. Light/dark ratio and humidity were maintained at 12h:12h and at 45-60%, respectively.

Preparation of C. krusei inoculum

A number of pure colonies from a culture plate of *C. krusei* S10B3 were mixed in sterile saline in a cryovial to make a 1 ml suspension. The mixture was mixed well by vortex and ten-fold serial dilutions made up to 10^{-9} by adding 100 µl of suspension into 900 µl of sterile saline. The suspensions were then cultured on YPD plates (State Veterinary Institute, Uppsala, Sweden) with 100 µl of each dilution and the original suspension. The plates were incubated for 48 hours and the colonies counted to determine the number of Colony Forming Units/ml (CFU/ml).

Experimental design

At day 5 post partum the pups were removed and the mother was inoculated with 50 µl suspension containing $1X10^6$ CFU/ml of *C. krusei* into each of two mammary glands (the left fourth and fifth glands: L_4 and L_5) as described previously (*Guhad et al., 1995*) during intaperitoneal anaesthesia with a mixture of 50 mg ketamine hydrochloride (Ketalar, Park-Davis Scandinavia AB, Solna, Sweden), 5 mg xylazine (Rompun vet., Bayer AG, Leverkusen, Germany) and 4.75 ml water (Pharmacia, Uppsala, Sweden) at a dose of 0.1 ml/10 g bodyweight. As a control, the fifth right mammary gland (R_5) was inoculated with 50µl of sterile saline.

Sampling at necropsy

At days 3, 6, 9, 12, 15, 18 and 21 post infection three mice of each of the two groups were anaesthetised using 0.1 ml/10g of the Ketamine-Xylazine mixture. This was followed by exsanguinations via the heart. Blood was collected for production of serum. The second most posterior mammary gland L4 was removed together with adjacent lymph nodes and kept in 0.5 ml sterile water. The spleen was removed in toto and placed in RPMI medium.

From six animals on days 3, 9, and 18 following challenge of both groups (BALB/c and *nu/nu* mice, respectively) the brain, heart, lungs, liver, pancreas, kidneys, mammary glands R5 and L5 were removed and fixed in 10 % neutral buffered formalin for 3 days.

Pathology and immunohistochemistry

The formalin fixed organs were processed through graded concentrations of alcohols to xylene and embedded in paraffin wax. Haematoxylin and eosin (HE) and periodic acid Schiff (PAS) staining were performed on 4-5 µm tissue sections. For immunohistochemistry, tissue sections were mounted on SuperFrost Plus slides, heated to 65 °C for 10 min and taken to 70% ethanol for 10 min. *C. krusei* was visualized immunohistochemically by the peroxidase antiperoxidase technique (PAP) as described previously (*Guhad et al., 1995*).

Disintegration of mammary tissue

Using a homogeniser (Tissue-Tearor model 985-370, Biospec Products Inc., Bartlesville, Oklahoma, USA) the mammary gland L4 was made into a thick solution, which was then made up to 1 ml with sterile water. This was then serially diluted ten fold in sterile water and cultured in YPD plates for 48 hrs at 37 °C. The colonies in all dilutions were counted and the concentration of *C. krusei* recorded.

Splenocyte cultures

The spleen was homogenized by squashing through a metal sieve and suspended twice and centrifuged in incomplete RPMI-1640 medium. The splenocytes were then re-suspended in complete RPMI-1640 medium (incomplete RPMI-

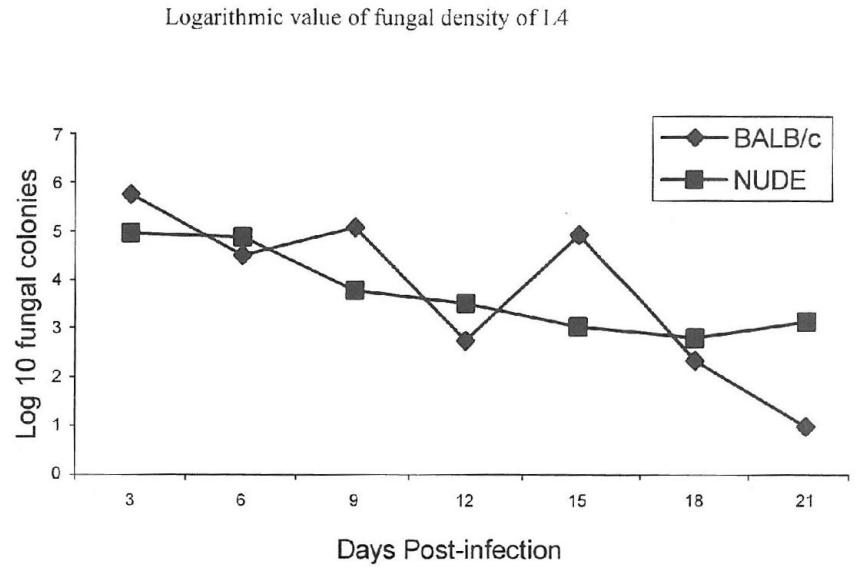

Figure 1. Density of *C. krusei* colony forming units in infected milk glands (mean of three values (animals) per time point).

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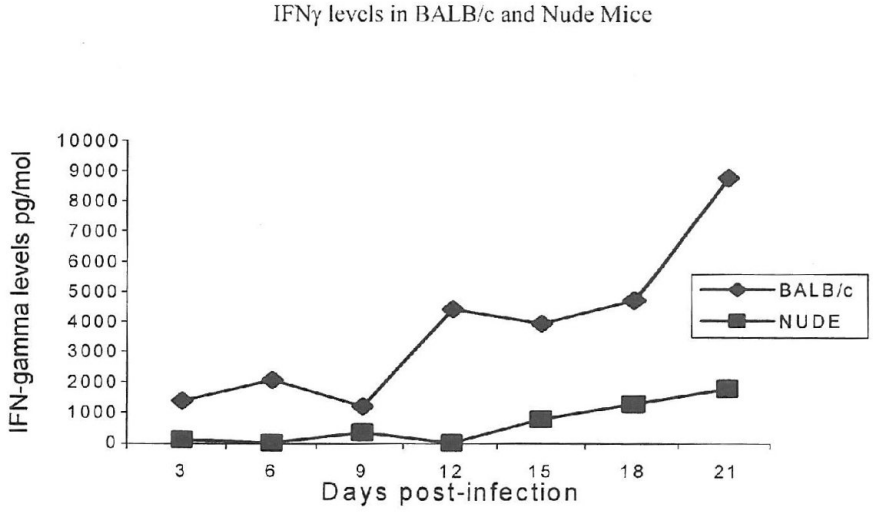

Figure 2: IFNγ levels in supernatants from conA stimulated splenocytes at different time points after infection (means of three values (mice) per time point).

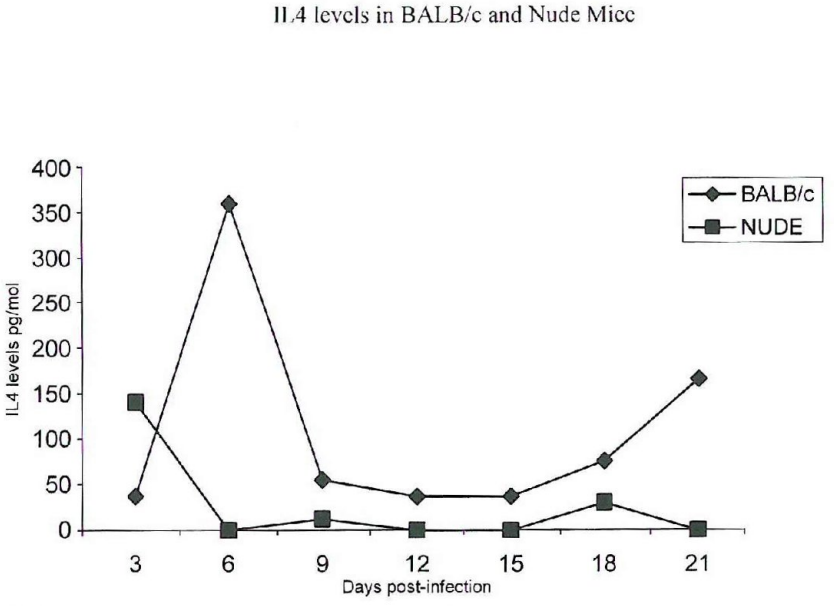

Figure 3: IL4 levels in supernatants from conA stimulated splenocytes at different time points after infection (means of three values (mice) per time point).

1640 medium and 10% foetal calf serum) and cultured at a concentration of $3X10^6$/ml, for cytokine production in 48-well tissue culture plates in the presence of concanavalin A (conA) or complete RPMI-1640 (control). The cultures were incubated at 37 °C in a humidified atmosphere with 5% CO_2. Supernatants were harvested after 24 hours (IL-4), and 96 hours (IFN-γ) for the quantification of IL-4 and IFN-γ.

Cytokine assays

Levels of IFN-γ and IL-4 were assayed using commercial sandwich ELISA kits (Duo set™ ELISA Development Systems, R&D Systems, Abingdon, Oxford, UK). In summary, 96-well microplates were coated overnight at room temperature with 4 μg/ml of rat anti-mouse IFN-γ or IL4. The plates were then blocked using 1 % bovine serum albumin (BSA) and 5 % sucrose in phosphate buffered saline (PBS) and incubated at room temperature for 1 hour. The samples (in duplicate) and standards (recombinant IFN-γ or IL-4) were then added and the plates incubated for 2 hours at room temperature. The detection antibody consisting of biotinylated goat anti-mouse IFN-γ (72 μg/ml) or IL-4 (36 μg/ml) was then added to the plates after which they were incubated for 2 hours at room temperature. Streptavidin conjugated to horseradish-peroxidase was then added to the plates and these were incubated for 20 min at room temperature. Following this, substrate solution containing tetramethylbenzidine (TMB, H_2O_2; R&D Systems, Abingdon, Oxford, UK) was added and the plates incubated for 20 min at room temperature. The colour reaction was stopped using 2N H_2SO_4 and absorbance at 450 nm was measured. The plates were washed three times (with PBS containing 0.05 % Tween 20) between successive steps except after the addition of TMB and H_2SO_4.

Results

Up to 18 days after inoculation the *nu/nu* mice exhibited a fungal density similar to that of the immuno-competent BALB/c mice. The nude mice were unable to clear the infection by day 21 (Figure 1), whereas the immuno-competent BALB/c mice completely cleared the infection by day 21.

Cytokine Levels

The conA stimulated splenocytes from BALB/c mice synthesized higher levels of both IFN-γ and IL-4 than did those from *nu/nu* mice (Figures 2 and 3). The IFN-γ levels in both strains of mice were higher than the IL-4 levels. The nude mice had low but gradually increasing levels throughout infection. The immuno-competent mice had higher levels and also exhibited a gradual increase in IFN-γ levels. The IL-4 response in the nude mice was low during infection with the exception of the first samples obtained day 3 after inoculation. The immuno-competent mice exhibited high levels on day 6 after infection followed by a period of low IL-4 responses and a final gradual increase observed in the samples from days 18 and 21 after inoculation.

Non-conA stimulated splenocytes and conA stimulated splenocytes of uninfected control mice showed low, often undetectable levels of cytokine production at any time-point.

Pathology and Immunohistochemistry

No lesions were observed at gross inspection. Histopathologically, lesions and fungal elements were restricted to the inoculated mammary gland (L5) of all animals. The process of involution of the mammary gland tissue was evident in both the inoculated and in the non-inoculated glands of all animals, and the course of infection appeared similar in both groups of animals. On day 3 following inoculation, *C. krusei* cells were located within the lumen of alveoli and accompanied by a scant infiltration of inflammatory cells. On day 9 following infection, circumscribed micro abscesses with centrally located *C. krusei* cells and peripheral infiltrations of mononuclear cells dominated. In animals euthanised on day 18 fungal elements were not detected, and within the highly involuted mammary tissue only minute infiltrations by mononuclear cells were observed.

Discussion

This study was performed to assess the course of experimental local *C. krusei* infection in immuno-competent and congenic athymic mice and the cytokine response over a period of 21 days. The athymic immunodeficient mice (*nu/nu*) failed to clear intra-mammary *C. krusei* infections, while the immuno-competent mice readily cleared the infection after three weeks. Interestingly, the fungal burden in nude mice was not significantly higher than that of the immuno-competent BALB/c mice until days 15-18 after inoculation. This finding confirms phenomena, which have been observed in several other studies (*Cutler, 1976; Rogers et al., 1976;, Miyake et al., 1977; Fulurija et al., 1997*) in which systemic infections with *C. albicans* in nude mice were less severe than in their heterozygous littermates.

In systemic candidosis in mice a Th1 response seems to be associated with effective macrophage activation and enhanced resistance to reinfection, whereas milder mucosal infections have been reported not to show a similar clear-cut Th1 response but rather a mixed Th1-Th2 response (*Ashman & Papadimitriou, 1995; Romani 1999*). That cell-mediated immunity is important also in the defence against localised *C. krusei* infection was observed in the present study. A cytokine profile indicative of a dominating Th1 response seems to be important for protection against *C. krusei* as shown by the high ratio of IFN-γ/IL-4 concentration in both the immuno-competent BALB/c and immunodeficient nude mice. This agrees with a study, which indicated that a systemic Th1 type response is generated as a result of a localised mucosal (vaginal) *Candida* (*C. albicans*) infection (*Fidel & Sobel, 1999*).

In athymic nude mice T-cell precursors are unaffected and some extrathymic functional T-cells are produced in adults. Hence, the Th1 response seen in this study is not surprising although the level of response is obviously lower than that of immuno-competent BALB/c mice. In a recent study of *C. albicans* in the mycotic mastitis model we found that both treated and

control nude mice showed less tissue inflammation compared to BALB/cJ and SCID mice, and revealed insignificant activation of the complement system (*Guhad et al., 2000*). The present results support that nude mice show minor responses to *Candida* infection, but also that they have difficulties in clearing the infection compared to immuno-competent congenic animals.

The pathology and immunohistochemistry results add weight to the usability of the murine mastitis model for studies of localised infections (*Guhad et al., 1995*) and confirm that the fungi do not disseminate to other organs. The PAP staining further strengthens this fact because only the mammary glands stained positive for *C. krusei*.

In conclusion, the present study suggests that local *C. krusei* infection is associated with a predominant IFN-γ- Th1 response although a gradual activation of the Th2 -arm (IL-4) of the immune system may be indicated late in the course of the infection. These results may be of assistance in the many attempts to develop vaccines against *Candida* spp.

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