**ANALYSIS OF THE CANDIDA ALBICANS - SPECIFIC T-CELL RESPONSE AND OROPHARYNGEAL CANDIDA COLONIZATION IN A COHORT OF HIV-1-INFECTED PATIENTS**

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**Abstract**

To investigate *Candida* epidemiology and immunologic correlates of protection in HIV-1 infected patients, we analyzed oral *Candida* colonization in correlation to the Candida-specific T-cell response measured by g-IFN ELISPOT using different *Candida* (*C.*) albicans strains. In 16/46 patients (13 asymptomatic, 3 with oral thrush), but in 0/28 controls, *Candida* (13 *C*. albicans, 1 *C*. lusitaniae, 1 *C*. krusei, 1 *C*. parapsilosis) was isolated. *Candida* specific T-cells were detected more frequently in controls (20/28) than in HIV-1+ subjects (16/46, p= 0.03). We observed a significant association of higher CD4 cell numbers with both detection of *Candida* specific T-cells and lack of oral *Candida* colonization, but there was no significant correlation of oral *Candida* colonization to the detection of *Candida* specific T-cells, viral load or antiretroviral therapy. Thus, local mucosal immunity seems to be more important in the pathogenesis of *Candida* colonization than circulating *Candida* specific T-cells. The pathogenic *C*. albicans strain K24122 was less frequently recognized by patients (6/46) than the laboratory adapted strain SC5314 (14/46, p= 0.03), whereas a similar recognition of both strains was observed in healthy controls. This indicates an impaired *Candida*-specific T-cell repertoire in HIV+ patients that could increase the risk of immune evasion by *C*. albicans.

**Key words:** HIV, *Candida albicans*, non-albicans *Candida*, *Candida* specific T-cells

**INTRODUCTION**

An infection with *Candida* (*C.*) albicans is one of the most frequently observed opportunistic infections in HIV-1 positives [1]. Clinical evidence and experimental data have indicated that both the innate and adaptive immune systems regulate the control of *Candida* infections [2]. However, T-cell mediated immunity (CMI) is considered to be the predominant host defense mechanism against mucosal candidiasis, as evidenced not only by the high incidence of OPC in HIV-1 positive patients with reduced CMI [1, 3-5], but also by a similar prevalence in other conditions of T-cell immunosuppression such as transplantation or chemotherapy [6-8]. Despite a strong correlation between reduced blood CD4 T-cells and the incidence of oropharyngeal candidiasis (OPC) [1, 4, 6, 9, 10], a distinct population of HIV-1 positive patients with a clearly reduced CD4 T-cell count seems not to be susceptible to OPC. In contrast, other HIV-1 infected patients with CD4 T-cells within the normal range show active candidiasis despite assumed immunocompetence. Potential explanations for the variation in susceptibility to OPC include differences with regard to the *Candida* specific immune response, even in patients with similar CD4 counts, or colonization by *Candida* variants that exhibit differences in fungal virulence factors. Some of these virulence factors like the specific abilities of *C. albicans* to alter its phenotype and cell shape by producing filaments, expressing specific adhesion molecules, and secreting enzymes like aspartyl-proteinases and phospholipases have been well characterized [11]. *C. albicans* reversibly converts unicellular yeast cells to either pseudohyphal or hyphal growth and the ability to reversibly switch between its different forms is thought to be important for *Candida* virulence [11-19].

To elucidate the interaction between the different fungal forms and adaptive immune system in men, we focused on the specific T-cell recognition of *C. albicans* depending on three *C. albicans* strains with different virulence associated properties. Hypothesizing the predominance of Th1-type mediated host defense and the importance of the cytokine profile, we investigated the frequency of gamma-interferon (g-IFN) producing *C. albicans* specific T-cells in peripheral blood in correlation to oral *Candida* colonization. Additionally, we wanted to gain information about the epidemiology of oral *Candida* carriage in HIV-1 infected patients, as little data are known about the incidence of *Candida* species other than *C. albicans* in Europe. The increasing emergence of non-albicans *Candida* (NAC) such as *C*. krusei, *C*. lusitaniae, *C*. glabrata, *C*. tropicalis, or *C*. parapsilosis is reported and many of these fungi show a decreased susceptibility to currently available antifungal drugs [20-24].

**MATERIALS AND METHODS**

**STUDY POPULATION**

46 HIV-1 infected patients (12 females and 34 males) were recruited from the Immunodeficiency Center,
Department of Medicine III, University Hospital Erlangen. 28 healthy HIV-1/2 negative subjects served as controls. Informed consent was obtained and all procedures were done in accordance to the local Institutional Review Board. Number of circulating CD4 T-cells and CD8 T-cells was measured by flow cytometry, and HIV-1 viremia was measured by the Bayer bDNA assay 3.0 (limit of detection at 50 HIV-1-RNA copies/ml).

29 (63 %) patients were treated with active antiretroviral therapy (ART) including protease-inhibitors (PI) as part of their therapy in 25 (54 %) cases. 17 (37 %) patients were therapy naïve. Oral cavity lavages (Candida isolation). After rinsing the mouth with a cup of tap water, 20 ml of sterile 0.9 % NaCl-solution were used to collect oral lavages, which were subsequently spun down and yeast species were grown on Sabouraud agar plates and differentiated with a commercial test kit (API 32C, bioMerieux, France).

ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC)

PBMC were obtained from heparinized whole blood by Ficoll-Hypaque® density gradient centrifugation (Pharmacia, Uppsala, Sweden). Prior to ELISPOT analysis the cells were cultured overnight in R5AB medium consisting of RPMI 1640 medium containing 5% (vol/vol) heat-inactivated human AB serum (Sigma-Aldrich, Steinheim, Germany), supplemented with L-glutamine (4 mM), penicillin (50 U/ml), streptomycin (50 µg/ml) and Hepes (10 mM).

C. ALBICANS STRAINS AND CULTURE CONDITIONS

For ELISPOT-analysis, three different strains of C. albicans were used: SC5314, a common, laboratory strain, originally isolated from a patient with disseminated candidiasis [25]; HLC52, an isogenic germination-deficient mutant efg1/efg1 cph1/cph1, which is defective in germ tube formation and in its interaction with macrophages that shows reduced virulence [17]; and K24122, isolated from a local HIV-1 positive patient with severe uncontrollable fluconazole resistant esophagitis. Standard growth of the yeast form in liquid culture was performed in yeast extract-peptone-dextrose (YPD) medium at 30°C. To induce germ tube formation, cells were diluted 10-fold from overnight cultures grown in YPD into Lee’s medium and incubated for 3 hours at 37°C. Before the use in the ELISPOT assay, yeasts were inactivated by UV-light, washed in PBS (Phosphate Buffer Saline) and kept frozen at -20°C.

γ-IFN - ELISPOT ANALYSIS

Ninety-six well nitrocellulose filter backed microtiter plates (MAHA-S-4510, Millipore, Molsheim, Germany) were coated with 50 µl of IFN-γ antibody 1-D1K (Hoelzel, Cologne, Germany) at a concentration of 10 µg/ml. After four washes with PBS and blocking with R5AB (RPMI 1640 medium with 5% human AB serum and supplements), 1 x 10^5 PBMC were added in 100 µl R5AB to the precoated wells. Antigens were added to final concentrations as previously established optimal for T-cell stimulation in the ELISPOT assay: either 1 x 10^4 inactivated C. albicans cells/well, or tetanus-toxoid at 75 µg/ml. As positive control, Phytohemagglutinin (PHA; Sigma-Aldrich, Seelze, Germany) stimulation was performed at a final concentration of 10 µg/ml. The plates were incubated as duplicates for 10 hours at 37°C / 5% CO2. After 6 washes with PBS containing 0.05% Tween 20 (PBS/T 0.05%) 100 µl of biotinylated IFN-γ-mAB 7-B6-1 (Hoelzel, Cologne, Germany) were incubated at a concentration of 2 µg/ml final for two hours at 37°C / 5% CO2. After washing with PBS/T 0.05% 100 µl of avidin/peroxidase solution (Vectastain ABC-Kit, Vector Laboratories, CA, USA) were added to each well. After incubation for one hour at room temperature and after three washes with PBS / T 0.05% and three washes with PBS, 100µl 3-Amino-9-Ethylcarbazole (Sigma-Aldrich, Seelze, Germany) containing 0.01% H2O2 were added as chromogenic and spots developed within four minutes. A spot count ≥ 5/10^5 PBMC after subtraction of the negative control was considered as positive reaction. In preliminary tests, HLA class I or class II restriction was analyzed in the ELISPOT assay using PBMC depleted of CD4+ or CD8+ T-cells, respectively, by magnetic beads (DynaBeads, DynALN). As there was a predominantly CD4+ associated T cell response, all below-mentioned results apply to assays performed with CD8+ depleted cell populations.

STATISTICAL ANALYSIS

We carried out statistical analysis using SPSS version 6.0. Statistical significances (p values) of results were calculated by χ² analysis (if n ≥ 5) or Fisher’s exact test (if n < 5), Mann-Whitney-U- and McNemar-test. Statistical adjustment for multiple end-points was performed by Bonferroni-Holm procedure.

RESULTS

LYMPHOCYTE COUNT AND VIRAL LOAD

At the time point of first study visit the HIV-1 positive patients presented with a median CD4 count of 353 cells/µl (range 12 to 1043 cells/µl ) and a median viral load of 65 copies/ml (range <50 to 500,000 copies/ml). 22 patients (21 on ART, one without ART) had a viral load below the limit of detection at 50 copies HIV-1 RNA per ml. A plasma viremia could be detected in 24 (52 %) patients (15 therapy naïve, 10 on ART) with a median viral load of 19,500 copies/ml (range 60 to 500,000 copies/ml). 14 (30 %) patients had a CD4 T-cell count between 0 and 199 cells/µl, 17 (37 %) had a CD4 T-cell count between 200 and 499 cells/µl, and 15 (33 %) had a CD4 T-cell count above 500 cells/µl.

CANDIDA COLONIZATION

Candida species could be cultured from oral lavages in none of 28 healthy donors, but in 16 out of 46 (35%) patients (p= 0.04, χ² test). 13 of these 16 patients (81%) were asymptomatic and three (9%) suffered...
from an oral thrush. 13 of 16 (81%) isolates were identified as C. albicans, one of 16 (6%) isolates was identified as C. lusitaniae, one as C. krusei and one as C. parapsilosis. From six patients on fluconazole treatment, C. albicans was grown from two patients and C. parapsilosis was grown from one, suggesting reduced fluconazole susceptibility. Patients with oral yeast carriage had significantly lower CD4 counts (median 333/µl, range 12-574) than patients without oral Candida colonization (median 498/µl, range 66-1043; p = 0.02, Mann-Whitney-U test; the six patients with or without Candida carriage on fluconazole were excluded from this analysis). Only three out of 15 (20%) patients with a CD4 cell count ≥ 500/µl showed an oral Candida colonization compared to seven out of 16 (44%) in the group with 200-499/µl and three out of 9 (33%) in the advanced group with CD4 cells below 200/µl. Yeast carriage did not correlate significantly to viral load, CD8 counts or ART despite higher median HIV-1 viremia or lower CD8 counts in patients with oral Candida colonization.

**ANALYSIS OF CANDIDA- AND TETANUS-SPECIFIC T-CELLS BY IFN-γ ELISPOT**

The C. albicans specific T-cell response in freshly isolated PBMC was investigated by IFN-γ ELISPOT analysis of T-cell recognition of three different C. albicans strains. To get further information about the patients T-cell reactivity, we analyzed the T-cell response to Tetanus-toxoid (TT), as all observed patients and controls were vaccinated against Tetanus in the past. A TT specific T-cell reactivity could be found only in 14 (30%) patients, but in 24 (86%) healthy controls (p = 0.000, χ² test; Table 1). The frequency of TT - specific T-cells in the patients correlated significantly with CD4 T-cell counts (p = 0.011, Mann-Whitney-U test) and viral load (p = 0.019, Mann-Whitney-U test) but

<p>| Table 1. Recognition pattern of C. albicans strains and tetanus toxoid in the ELISPOT (in relation to viral load, CD4- and CD8-counts in HIV-1+ patients). |
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<th>TT HLC52</th>
<th>SC5314</th>
<th>K24122</th>
<th>at least one strain</th>
<th>HLC52 and SC5314</th>
<th>K24122 and HLC52</th>
<th>SC5314 and K24122</th>
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<tr>
<td><strong>controls</strong></td>
<td>n=28</td>
<td>24 (86%)¹</td>
<td>17 (66%)²</td>
<td>15 (54%)³</td>
<td>16 (57%)⁴</td>
<td>20 (71%)⁵</td>
<td>12 (43%)⁶</td>
<td>14 (50%)⁶</td>
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<td>median sfc (range)</td>
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<td>15 (5-46)</td>
<td>7 (6-31)</td>
<td>7 (5-49)</td>
<td>9 (5-29)</td>
<td>14 (50%)⁶</td>
<td>13 (46%)⁷</td>
<td>12 (43%)⁸</td>
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<td><strong>HIV-1+ patients</strong></td>
<td>n=46</td>
<td>14 (30%)¹</td>
<td>10 (22%)²</td>
<td>14 (30%)³</td>
<td>6 (13%)⁴</td>
<td>16 (35%)⁵</td>
<td>10 (22%)⁶</td>
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<td>median sfc (range)</td>
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<td>11 (6-35)</td>
<td>9 (7-38)</td>
<td>15 (5-44)</td>
<td>8 (5-18)</td>
<td>14 (30%)³</td>
<td>14 (30%)³</td>
<td>10 (22%)⁶</td>
<td>2 (4%)⁶</td>
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<td><strong>TT+</strong></td>
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<td><strong>TT-</strong></td>
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<td>41</td>
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<td><strong>CD4 in patients (cells/µl)</strong></td>
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<td><strong>viral load (copies/ml)</strong></td>
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<td>13%</td>
<td>25%</td>
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Sfc: Spot forming cells/100.000 PBMC
TT+/TT -: Detection or lack of detection of Tetanus toxoid recognition by ELISPOT
Values with significant differences between controls and HIV-1 infected patients are indicated in bold letters.

¹, ², ³, ⁴, ⁵, ⁶, ⁷, ⁸p = 0.000; ³p = 0.048; ⁴p = 0.001; ⁵p = 0.001; ⁶p = 0.000; ⁷p = 0.002
not to CD8 T-cell counts. Recognition of at least one C. albicans strain (SC5314, HLC52, or K24122) was detected in 16 out of 46 (35%) patients. SC5314 was recognized by 14 (30%) patients, HLC52 was recognized by 10 (22%) patients, and K24122 was recognized by six (13%) patients. Only two patients recognized all three strains (4%; Table 1). There was a significant correlation between Candida recognition and CD4 T-cell count for HLC52 only ($p=0.014, \chi^2$ test), but not to CD8 T-cell counts or viral load. Probably due to the small number of cases, no significant correlation of viral load, CD4 and CD8 counts was found regarding the parallel recognition of at least two or all three Candida strains. In the healthy control group, Candida specific T-cells were detected at a significantly higher degree compared to the HIV-1 infected patients. At least one strain was recognized by 20 healthy controls (71%) while all three strains were recognized by 12 (43%; $p=0.000$ and $p=0.002, \chi^2$ test, respectively, compared to the HIV-1 infected group). HLC52 was recognized by 17 (61%; $p=0.001$), SC5314 by 15 (54%, $p>0.05$) and K24122 by 16 (57%; $p=0.001$) of the controls (all $\chi^2$ test).

Statistical analysis of the occurrence of T-cells recognizing the individual Candida strains showed a significantly lower recognition of K24122 in the patients group in comparison to SC5314 ($p=0.033, \text{McNemar test}$), whereas in the control group there was no difference with regard to the recognition of these Candida strains. Simultaneous recognition of HLC52 and K24122, as well as simultaneous recognition of SC5314 and K24122 was significantly lower in HIV-1 positives than in healthy controls ($p=0.000$ for both; $p>0.05$ for recognition of HLC52 and SC5314, McNemar test). 60% of the healthy controls with a positive Candida specific T-cell response recognized all three strains, whereas recognition of all three strains was seen only in two patients (12.5% of patients with a positive Candida T-cell reaction). Both patients were Candida negative in the oral lavage, had a viral load <50 copies/ml and CD4 T-cell counts of 352/µl and 985/µl, respectively. We found invariably significantly higher numbers of SFC in healthy controls than in HIV-1 positive patients (TT: $p=0.000$; HLC52: $p=0.004$; SC5314: $p=0.027$; K24122: $p=0.000$, Mann-Whitney-U test).

**DISCUSSION**

In this study, we could confirm the correlation of mucosal Candida colonization with reduced CD4 counts [1, 6, 10, 26, 27]. However, despite this significant association there was a wide range of CD4 counts in patients with a positive Candida culture and the proportion of patients with detection of oral Candida colonization was about the same in the groups of patients with CD4 counts of 200 - 499/µl and with CD4 counts <200/µl, respectively. This is indicating that other factors in addition to CD4 cells must be important for the development of candidiasis. HIV-1 viremia itself does not seem to be a major factor for OPC as the colonization by Candida did not correlate to viral load.

We focused on the detection and quantification of IFN-γ secreting T-cells in the peripheral blood as there had been reports that cellular immunity and IFN-γ play an important role in the control of C. albicans [1, 5]. Both Candida specific and tetanus specific T-cells were found significantly less frequently in HIV-1 infected patients than in healthy controls and the lack of Candida specific T-cells correlated with lower CD4 counts. There was no significant association between the observed colonization by Candida strains and the detection of circulating C. albicans specific T-cells. Leigh et al. found a similar result without correlation between reductions in delayed skin test reactivity to Candida antigen and OPC [28]. This is suggesting that colonization by Candida is less dependent on the systemic Candida specific T-cell response than on other defence mechanisms. Quinti et al used a manno-protein fraction from C. albicans cell wall (GMP) to analyze non-MHC-restricted cytotoxic responses of PBMC. In contrast to our results, they showed a clearly decreased response with CDC stage [29]. Furthermore, a growing number of antimicrobial molecules has been discovered within the mucosa and saliva that exhibit anti-fungal effect such as protegrin [30], beta-defensins [31], lactoferrin [32] and mannose binding proteins. Furthermore, some studies show an inhibition of C. albicans by oral and vaginal epithelial cells in vitro, suggesting a potential role for epithelial cells in the innate host defence [33, 34]. The regulation of the anti-fungal proteins and peptides is not well understood yet, but polymorphisms in the genes of mannos-binding proteins and beta-defensins seem to increase the risk of mucosal candidiasis [35, 36].

To study potential immune evasive mechanisms of C. albicans we investigated the recognition of the pathogenic K24122 strain in comparison to a laboratory adapted Candida strain (SC5314) and an attenuated germination-deficient mutant Candida (HLC52). Only in the patients group, but not in the healthy controls, we observed a lower recognition in the ELISPOT assay of K24122 compared to SC5314. The reasons, why this strain might be recognized less frequently are not yet clear. To date, the yeast-to-hyphal transition is the only known inducible transcriptional program that contributes to virulence in vivo. Other infection-related programs may exist e.g. by modifying its transcriptional profile as recent findings suggest [37, 38]. C. albicans or its constituents have been shown to directly modulate the response of accessory cells of innate immunity [39-43]. Thus, K24122 could exert a direct immunosuppressive effect in vitro in the ELISPOT assay. However, the fact, that in those subjects recognizing K24122, the frequency and cytokine secretion capacity of specific cells as assessed by the spot size was not lower in comparison to T-cells recognizing the other C. albicans strains (data not shown) argues against an direct immune suppressive effect, for example by inhibition of antigen presentation by macrophages or by inhibiting the IFN-γ secretion. Because of the heterogeneous recognition pattern of the three Candida strains with some patients even recognizing only K24122 we would hypothesize that variation within T-cell epitopes could account for the observed differences of T-cell recognition of the three Candida strains. As there is almost no information available about Candida-specific T-cell epitopes, we
only can speculate whether the lower recognition of K24122 by patients could be caused by a higher rate of immune escape mutations in T-cell epitopes in comparison to the other two strains that are long-term cultured laboratory strains. However, as the healthy controls recognized K24122 as well as the other two strains, mutational escape within dominant epitopes of K24122 seems to be less likely for the lower recognition by patients unless patients and controls would target different T-cell epitopes by differences in the distribution of HLA alleles. Therefore, a more likely hypothesis is a decrease of the breadth of the Candida specific T-cell repertoire in the HIV-1 infected patients in comparison to healthy controls. It is known, that the decrease of CD4 cells in HIV-1 infected individuals is accompanied by growing perturbations of the T-cell receptor (TCR) repertoire [44]. These TCR perturbations are not corrected, when CD4 cells start to rise again after initiation of antiretroviral therapy [45]. The heterogenous recognition pattern of the Candida specific T-cell response in this study indicates at least an oligoclonal T-cell response against this pathogen. Thus, a narrower Candida specific TCR repertoire in HIV-1 infected patients could impair the targeting of fungal variants and facilitate fungal immune escape.

The results of our analysis of Candida species colonizing HIV-1 infected patients confirm previously published data regarding the rising incidence of NAC species in systemic Candida infections [24]. Although the majority of Candida isolates could be identified as C. albicans, we observed oral carriage of C. krusei, C. Insaetianiae and C. parapsilosis in 19% of patients with positive Candida cultures. Three out of six patients on treatment with fluconazole showed manifest Candida colonization (two with C. albicans, one C. parapsilosis) as a possible sign of reduced drug susceptibility. This is suggesting that wide spread use of fluconazole prophylaxis and treatment in HIV-1 infected patients will increasingly select fluconazole resistant Candida.

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