Altered, but not diminished specific T cell response in chronic mucocutaneous candidiasis patients

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Abstract Patients suffering from chronic mucocutaneous infections with the yeast *Candida albicans* (CMC) are discussed to have an underlying primary cellular immunodeficiency. In order to characterise cellular immunity in CMC patients, we analysed chemotaxis and myeloperoxidase (MPO) releases of neutrophils and T cell proliferation and cytokine production to *Candida albicans*. Patients with chronic mucocutaneous candidiasis ($n = 4$) and healthy volunteers of same sex and similar age ($n = 14$) were enrolled into the study. Neutrophil chemotaxis was assessed by transwell migration assay, and MPO release by ELISA. T cell proliferation capacity was investigated by thymidine incorporation and cytokine secretion in supernatants by ELISA. Neither neutrophil migration nor MPO release differed between CMC patients and healthy controls. The relative lymphocyte stimulation index (SI Candida/SI PHA) was heterogenous, but overall it was higher in CMC patients compared to controls. However, Candida-specific IFN-γ production was significantly reduced in CMC patients. Notably, Candida-specific T cell IL-10 production was markedly higher in CMC patients. The inability to clear the yeast *Candida albicans* in our CMC patients does not seem to be due to an impaired neutrophil function or reduced antigen specific proliferation of lymphocytes. In fact, our patients tended to proliferate stronger to Candida antigen relative to PHA than healthy controls. However, the impaired Th1 cytokine production with an enhanced IL-10 production could play an important role in the pathogenesis of chronic mucocutaneous Candida infections.

Keywords Chronic mucocutaneous candidiasis · *Candida albicans* · MPO release · Neutrophil migration · T cell proliferation · Cytokines · Th1–Th2 balance

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Introduction

Candida albicans is an ubiquitous opportunistic yeast, colonizing membranes of human skin and mucosal surfaces. The yeast causes infections (candidiasis) only, if the homeostasis between virulence of the microbe and resistance of the host immune system is disturbed. A broad spectrum of candidiasis syndromes is known ranging from "simple" persistent paronychia and onychodystrophy over a complex group of disorders characterised by persistent or recurrent infections of the skin, nails and mucosal tissues (chronic mucocutaneous candidiasis—CMC) [16], to a recently described syndrome with concomitant polyendocrinopathy and autosomal recessive inheritance (autoimmune polyendocrinopathy candidiasis ectodermal dystrophy syndrome—APECED) [11, 29]. The underlying defect of candidiasis syndromes may be a primary immune defect. This is the case in APECED, where a monogenic defect in the autoimmune regulator gene (AIRE) has been described [11], or in a form of dominant inherited CMC [18] with malfunction of the thyroid gland, where the underlying genetic defect has been mapped on chromosome 2*p* [4]. However, the link between these mutations and the immune defect(s) remains unclear.

Most of the studies in patients with chronic infection with candida conducted to date demonstrated an intact innate immunity [23]. Nevertheless, there are some studies suggesting a weakness in natural killer (NK) cells in CMC as either decreased [27, 28] or functionally impaired [12]. A subtle impairment of macrophage or monocyte [35] activation and migration due to an altered cytokine production has also been reported in some CMC patients [3, 16]. Concerning neutrophil function, controversial studies have been published, describing a serum-dependent defect of neutrophil function [34] or a normal function [7].

Most of the CMC patients show normal humoral immune responses with normal serum concentrations of immunoglobulins and high titres of specific antibodies against Candida species [16, 20]. However, within the heterogeneous group of CMC patients, a small subgroup seems to suffer from recurrent respiratory infections accompanied by deficiency of the IgG subclasses 2 and 4 [5].

Protection against mucocutaneous Candida infections seems to rely mainly on cell-mediated immunity [6], particularly in T cells. Patients lacking T cells due to a severe combined immunodeficiency often suffer from this form of Candidiasis [15]. However, the ability of T cells to proliferate to Candida antigen is discussed controversially: while some studies describe a diminished proliferation both to Candida and to mitogens [12], other authors report subgroups of CMC patients with a Candida-specific defect or a normal response of T cells [14, 16, 35]. Recently, the cytokine production of T cells has been the focus in numerous studies [12, 17, 20, 22, 23, 33], describing an altered cytokine production with a diminished production of type-1 cytokines such as IFN-γ, IL-12 and IL-2 and an increased secretion of IL-10 or IL-4 [17].

The aim of this study was to elucidate possible dysfunctions in cellular immune reactions in patients suffering from chronic Candida infections, focusing on neutrophil functions on the one, and T cell proliferation and cytokine production on the other hand, with a special emphasis on the Th1/Th2 balance in cellular immune response.

Materials and methods

Patients

Four patients were included into the study and compared with 19 healthy volunteers matched for sex and age. All patients were characterised both clinically and by several laboratory markers (Table 1). Before blood was taken, each participant gave his informed consent. The study was approved by the ethical committee of the Technical University Munich, following the guidelines of the Helsinki declaration [1].

Neutrophil migration and phagocytosis capacity

In three patients (CMC1, CMC3, CMC4) human polymorphonuclear neutrophils (PMN) were isolated from total

Table 1 Clinical characteristics of patients enrolled in the study

Patient	CMC1	CMC2	CMC3	CMC4
Age (years)	37	27	48	8
Sex	♀	♀	♀	♂
Clinical symptoms	Chronic oral, esophageal, vaginal and cutaneous candidiasis, paronychia with nail dystrophy			Oral candidiasis, paronychia with nail dystrophy
Onset (at age)	Early (4)	Early (3)	Early (birth)	Early (3)
Endocrinology	Normal	Normal	Normal	Morbus Addison, polyendocrinopathy syndrome
Immunology	Squamous cell carcinoma, ANA+	Normal	Squamous cell carcinoma, ANA+	Normal
Standard laboratory markers	CRP constantly elevated (3.5 mg/dl)	Iron deficiency anemia	Normal	Normal
Electrophoresis	Albumin 56.9%↓, γ-globulin 22.0%↑	Albumin 50.9%↓, γ-globulin 22.8%↑	Albumin 55.9%↓, γ-globulin 20.4%↑	Normal
Candida-Abs serum	IgG <40 U/ml, IgM <60 U/ml	IgG 258 U/ml, IgM 1,172 U/ml	IgG 170 U/ml, IgM <60 U/ml	IgG 47 U/ml, IgM 96 U/ml
Immunoglobulins serum	Normal	Normal, IgG 40.20 g/l↓	IgG 1635 mg/dl↑, IgA 940 mg/dl↑	Normal
Phenotyping of T cells	Lymphopenia	Normal	Lymphopenia	Lymphopenia

blood by gradient centrifugation using Histopaque 1119 (Sigma, Deisenhofen, Germany) as previously described [32]. PMN were resuspended in RPMI complete medium [RPMI 1,640 medium (Invitrogen, Paisley, Scotland) supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1 mM sodium pyruvate (Invitrogen, Paisley, Scotland) and 0.05 mM 2-mercapto ethanol (Sigma, Deisenhofen, Germany)] with 5% bovine serum albumine (BSA; Sigma, Deisenhofen, Germany). Neutrophil chemotactic capacity was investigated by measuring migration through a 5 µm pore polycarbonate filter in 24 well chambers as previously reported [31, 32]. Briefly, PMN cells were added into the top chamber at a concentration of 1×10^6 ml^{-1}. LTB_4 at a concentration of 1×10^{-9} M or IL-8 at 300 µg/ml were added to the bottom chamber. After 60 min, cell free supernatants were collected and immediately stored at −70°C until further analysis by ELISA. PMN that had transmigrated into the bottom chamber were counted by flow cytometry (FACScalibur, Becton Dickinson, Heidelberg, Germany). Results are shown as migration index, defined as ratio of migrated cells in the presence of a chemokine and migrated cells to medium alone.

Quantification of MPO in neutrophil supernatants was performed by ELISA according to manufacturer's instructions (Assay Designs, Ann Arbor, MI, USA).

T cell proliferation and cytokine secretion

Peripheral blood mononuclear cells (PBMC) were separated by centrifugation over Ficoll-Hyperpaque (Lymphoprep, Progen biotech, Heidelberg, Germany). PBMC were cultured in RPMI complete medium (see above) with 5% human serum (Sigma, Deisenhofen, Germany). PBMC were stimulated for 60 h either with 1% phytohemagglutinin (PHA) as positive control or with 10 mg/ml *Candida albicans* antigen (Allergopharma, Hamburg, Germany). Complete medium served as negative control. After 60 h, 100 µl/well supernatant was obtained and stored at −70°C until further analysis by ELISA. Quantification of cytokines (IFN-γ, IL-10 and IL-4) in supernatants was performed by ELISA according to the manufacturer's instructions (BD, Heidelberg, Germany).

Cell proliferation was measured by [^{3}H]thymidine incorporation. PBMC were incubated with 10 µCi/ml [^{3}H]thymidine for 6 h and then harvested on a filter. Counts per minute (cpm) were found by a β-counter (Perkin Elmer, Rodgau, Germany). A stimulation index (SI) was calculated as ratio of cpm of stimulus (Candida or PHA) to cpm of medium control. A relative stimulation index (rSI) was calculated as ratio of SI Candida and SI PHA.

In another experimental set-up, PBMC of two CMC patients (CMC1 and CMC4) were left to adhere on petri dishes for 2 h at 37°C at a concentration of 6×10^6 ml^{-1}. The adherent cells (monocytes) were carefully detached and served as antigen presenting cells. The nonadherent fraction was separated into CD4+ and CD8+ T cells by positive selection using immunomagnetic beads coated with CD4 or CD8 antibody (Miltenyi, Bergisch Gladbach, Germany), respectively. Monocytes (8×10^5 ml^{-1}) and T cells (1×10^6 ml^{-1}) were incubated with PHA or *Candida albicans* antigen for 60 h. Afterwards, proliferation and cytokine production were analysed as described above.

Statistical analysis

Statistical analysis was performed by the software programme SPSS 14.0. All results were analysed using a two-sided students unpaired *t*-test. Statistically significant differences between CMC patients and controls were defined as $p < 0.05$.

Results

Chemotactic and killing capacity of PMN in CMC patients

No significant difference to the matched controls was observed in neutrophil migration potency neither to LTB_4 nor to IL-8 as migration indices (MI) of PMN of CMC patients were comparable to MI of matched controls (Fig. 1).

Concerning the MPO release from PMN of CMC patients, no significant differences to the matched controls were detected (Fig. 1).

T cell proliferation

Proliferative capacity of CMC PBMC, as determined by the stimulation index (SI) to PHA, did not differ significantly between CMC patients and their matched controls (Fig. 2a). The relative stimulation index (SI Candida/PI PHA) was heterogenous in CMC patients (Fig. 2b). However, the difference between CMC patients and their controls was significant with a higher rSI of CMC patients ($p = 0.04$). Investigating T-cell subpopulations, a higher relative proliferation to *Candida albicans* was observed also for CD4+ lymphocytes in the two tested patients (CMC1 and CMC4) with monocytes serving as antigen presenting cells, while CD8+ cells did not show a significant difference in the relative proliferation capacity (data not shown).

T cell cytokine secretion

In contrast to the proliferative capacity of T cells, which showed a trend to higher values, CMC patients showed a decreased secretion of the Th1 cytokine IFN-γ (Fig. 3a)

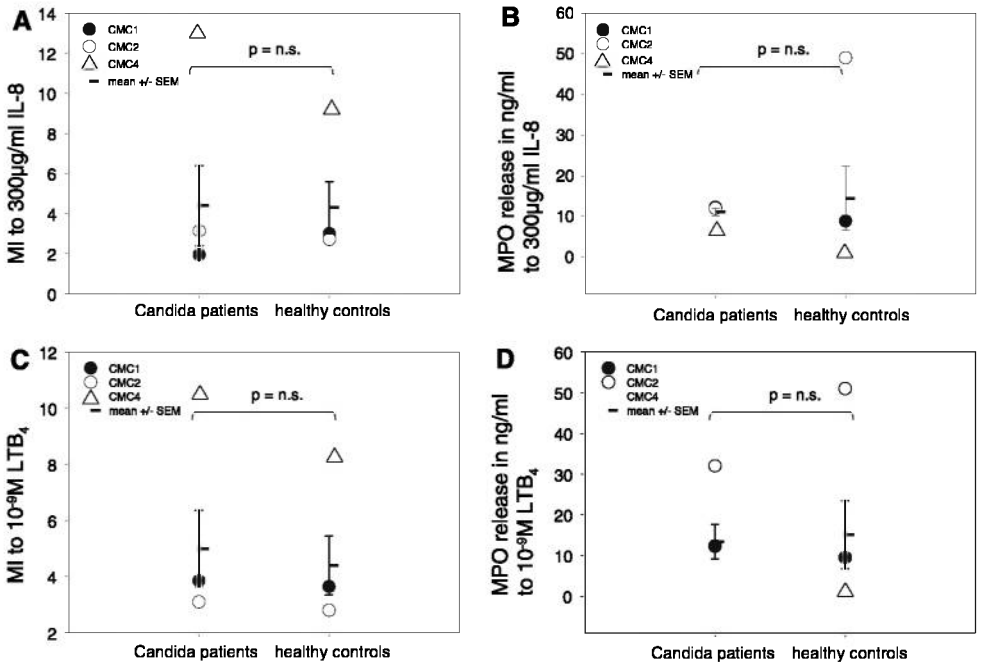

Fig. 1 PMN effector functions of chronic mucocutaneous candidiasis (CMC) patients do not show significant differences to polymorphonuclear neutrophils (PMN) of matched controls. **a**, **c** Migration index (MI) and **b**, **d** myeloperoxidase (MPO) secretion (ng/ml) by PMN stimulated with LTB_4 (1×10^{-9} M) or IL-8 (300 µg/ml) of CMC patients and their corresponding controls (mean MI and mean MPO release ± SEM)

after mitogen stimulation and to a higher degree after specific stimulation with Candida antigen. T cells from CMC patients produced significantly lower amounts of IFN-γ ($p = 0{,}01$) compared to their matched controls. Secretion of the Th2 cytokine IL-10 showed a distruct feature (Fig. 3B): while, after mitogen stimulation with PHA no difference was observed, concentrations of IL-10 were elevated in three out of four CMC patients after specific stimulation with Candida ($p = 0{,}56$). IL-4 secretion was under the detection limit of the ELISA. The difference in both IFN-γ and IL-10 production was also observed when isolated CD4+ or CD8+ lymphocytes were stimulated with *Candida albicans* antigen with monocytes serving as antigen presenting cells (data not shown).

Discussion

The underlying deficiencies of cellular immune responses in patients chronically infected with *Candida albicans* are controversially discussed. A complex heterogeneity of immune defect(s), probably characteristic for various subgroups, seems to be involved. In this study, we investigated neutrophil and T cellular function of four CMC patients. We found normal PMN effector functions, while the specific stimulation of T cells showed higher proliferation indices albeit impaired IFN-γ secretion and a trend to a higher secretion of the inhibitory cytokine IL-10.

Several studies report a defect of the innate immunity leading to CMC [12, 27, 28, 35]. Therefore we investigated neutrophil effector functions in three out of four CMC patients. However, no significant difference was observed in migration capacity to LTB_4 and to IL-8, suggesting that no general migration inability of the first line defence—the neutrophils—is substantial at least in our CMC patients. Notably, this kind of defect has been shown in two case reports of CMC patients with autoimmune or endocrinopathy dysfunctions [19, 25]. However, both reports documented a migration inability which was reversible by anti-fungal therapy. Our experiments were performed before and after anti-fungal therapy without revealing any differences in the migration indices. Further effector functions of PMN were analysed by measuring MPO release into the supernatants of the migration experiments. Again, no significant differences were detected in CMC patients compared to controls. Notably, the interindividual difference in MPO release by neutrophils was enormous. Thus, it seems very difficult to estimate "abnormal" values or differences between CMC patients and controls in single case reports which might help to explain the controversial reports of increased [7] or decreased [19, 25] candidicidal capacity of neutrophils in CMC patients. Another hypothesis was put forward by a study describing a defect of neutrophil function dependent on autologous serum in CMC patients which was reversible by sufficient anti-fungal therapy, suggesting that soluble factors may inhibit neutrophil functions [34].

Concerning the maximum T cell proliferation capacity tested by PHA-stimulation, no significant difference was observed between CMC patients and their matched controls with a huge interindividual difference in maximum stimulation indices (SI). In order to be able to compare SI of differ-

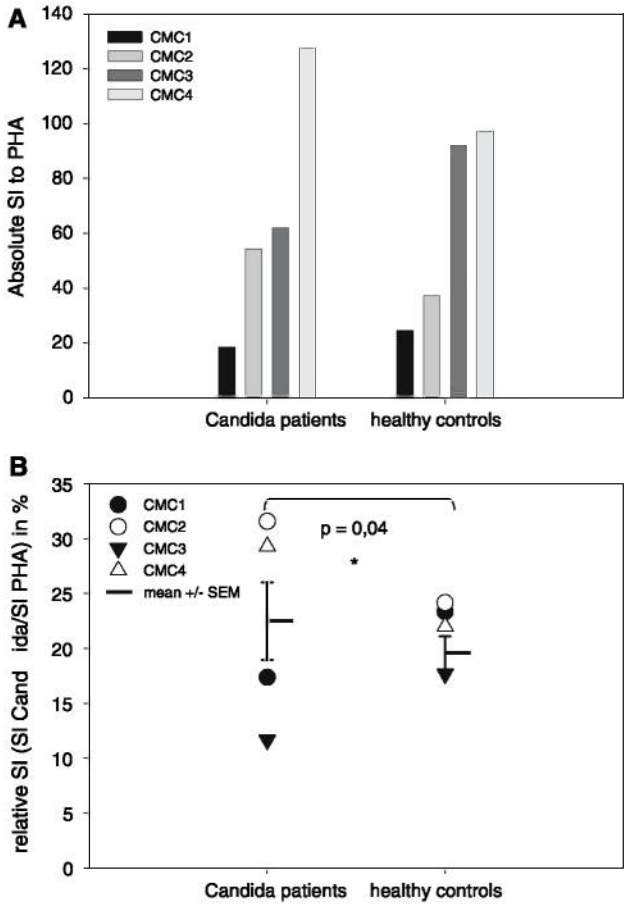

Fig. 2 Mucocutaneous candidiasis (CMC) patients do not generally suffer from a reduced T cell proliferation capacity. **a** The relative stimulation index (SI Candida/ SI PHA) in % is shown for each CMC patient compared to the matched healthy controls. Mean rSI ± SEM was higher in CMC patients. **b** Mean stimulation index to PHA (proliferation to PHA/proliferation to medium alone) of CMC patients was similar to controls

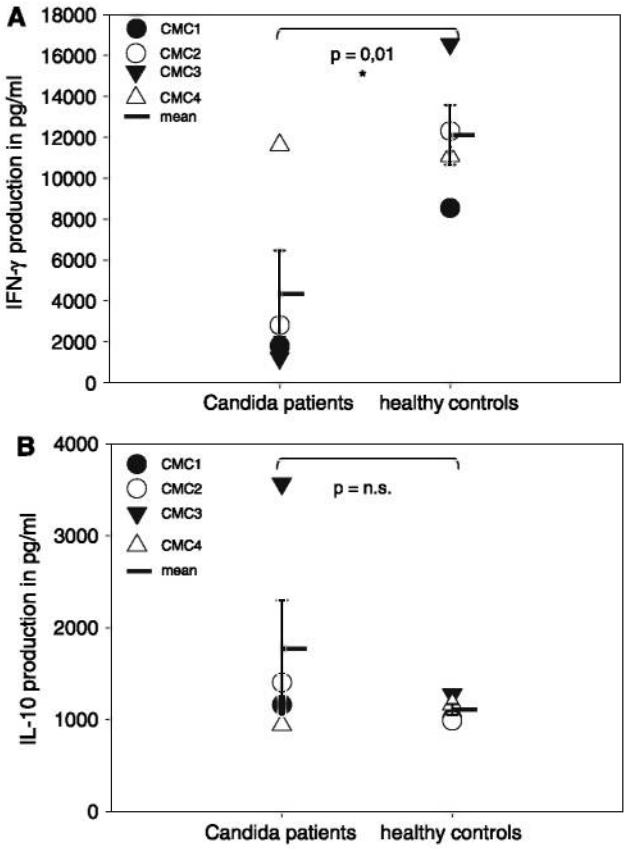

Fig. 3 Impaired Th1 cytokine secretion in mucocutaneous candidiasis (CMC) patients after *Candida albicans* stimulation. Concentrations are shown in pg/ml, mean secretion ± SEM. **a** Interferon-γ was significantly reduced in the supernatant of PBMC of CMC patients compared to controls ($p < 0,05$). **b** Mean IL-10 secretion from PBMC stimulated with *Candida* was higher in CMC patients compared to controls ($p > 0,05$)

ent individuals, we assumed the proliferation to PHA as maximum proliferation capacity of PBMC of the very patient and regarded the proliferation to the specific antigen—*Candida*—relative to this proliferation. This allowed a more confident comparison of the SI between individuals. Therefore we applied the relative stimulation index as the ratio of the stimulation index to PHA and the response to Candida antigen. This rSI was higher in the CMC group compared to the corresponding controls. A possible explanation for this higher immune response could be the boostering of the adaptive immune system when confronted with a chronic stimulus (in this case *Candida albicans*) [2]. This boostering effect is probably based on CD4+/CD29+/CD45RO+ memory T cells, a cell population reported to be slightly decreased in CMC patients in one study [17]. However, our study reveals a higher relative T cell proliferation to Candida. Thus, a general decrease of specific T cell function does not seem to be responsible for the chronic infections with Candida in our patients. An even more likely explanation for the inability to clear *Candida albicans* seems to be an altered rather than a diminished immune response of T cells. A dysregulated T cell cytokine production has been described for other chronic infections, for example with human immunodeficiency virus [8–10], *Leishmania braziliensis* [30] and atypical mycobacteria [26].

In this study we have shown that CMC patients react to Candida, but not to PHA, with a lowered Th1 response and with a higher production of the inhibitory cytokine IL-10. This observation is in concordance with several previous studies [13, 17, 21, 24, 36, 37]. However, the reason for this deviant cytokine production after *Candida albicans* stimulation remains to be elucidated. In our study this phenomenon of an impaired Th1 response occurred also when antigen-specific proliferation assays were performed with purified CD4+ or CD8+ lymphocytes and monocytes as Antigen presenting cell (APC) in the presence of heterolo-

gous human serum. This observation does not exclude a role of NK cells, B cells or cells of the innate immune system in the immune-impotency towards candida albeit making it more implausible to play a role in our patients. More likely, the higher specific immune response to *Candida albicans* observed in this study could ignite negative feedback mechanisms (e.g. via T regulatory cells) leading to an increased IL-10 production.

In summary, our data reveal normal neutrophil migration and MPO-releasing capacity in CMC patients. Relative proliferation of T cells was higher to Candida, but showed an impaired Th1 immune response and a higher production of the inhibitory cytokine IL-10. Whether this observation is a primary T cell dysfunction or the consequence of an abnormality at the antigen presenting level in APC is open for discussion. With four CMC patients, the power of this study is, as in most studies concerning CMC, not high enough to draw general conclusions. Therefore, further studies with larger number of patients will be required for understanding the underlying mechanisms of this impaired Th1 immune response in CMC patients.

Acknowledgments The authors wish to thank Gaby Pleyl-Wisgickl and Simone Knabl for their excellent technical support.

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