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Patients with Chronic Mucocutaneous Candidiasis Exhibit Reduced Production of Th17-Associated Cytokines IL-17 and IL-22

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Chronic mucocutaneous candidiasis (CMC) constitutes a selective inability to clear infection with the yeast *Candida*, resulting in persistent debilitating inflammation of skin, nails, and mucous membranes. The underlying defect is unknown. Only recently, IL-17-producing T cells have been reported to be involved in clearing *Candida* infections. In order to characterize T cellular immune response to *Candida*, we analyzed T-cell cytokine secretion to *Candida* antigen and mitogenic stimuli in CMC patients, immunocompetent patients suffering from acute *Candida* infection, and healthy volunteers. Peripheral blood mononuclear cells (PBMCs) from CMC patients produced significantly lower amounts of IL-17 and IL-22 mRNA and protein when stimulated with *Candida albicans* or mitogen *in vitro* compared with that in matched healthy individuals. Additionally, PBMCs from immunocompetent *Candida*-infected patients secreted more IL-17 and IL-22 than those of both CMC patients and healthy, non-infected controls. Flow cytometry revealed a decreased number of CCR6+ IL-17-producing T cells in CMC patients, whereas the amount of CCR6+/CCR4+ cells was not altered. Levels of differentiating cytokines for human Th17 cells, IL-1β and IL-6, tended to be higher in CMC patients. The inability to clear *C. albicans* in CMC patients could be due to a defect in the immune response of IL-17-producing T cells.

INTRODUCTION

Chronic mucocutaneous candidiasis (CMC) is a rare, complex, and heterogeneous group of syndromes characterized by persistent or recurrent infections of skin, nails, and mucosal tissues with the ubiquitous, opportunistic yeast *Candida albicans* (Kirkpatrick, 2001). The underlying defect may be a primary immune defect, as it is the case in the recently described subgroup ''Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy'' (APECED) (Collins *et al.*, 2006), or in a form of dominant inherited CMC with malfunction of the thyroid gland (Atkinson *et al.*, 2001). However, the link between these mutations and the immune defect(s) remains unclear.

Even though a coordinated contribution of both innate and adaptive immunity is required in order to mount an effective host response against *Candida*, it seems that primarily a defect of the adaptive immune system leads to enhanced *Candida* infection. Patients lacking T cells due to a severe combined immunodeficiency often undergo chronic mucocutaneous candidiasis (IUIS scientific group, 1999). T-cell-knockout mice suffer from severe systemic candidiasis (Ashman *et al.*, 1999); impaired T helper-1 (Th1) immune response leads to increased susceptibility to severe *Candida* infections (Mencacci *et al.*, 2001), whereas reduction of IL-10 increases resistance against these infections (Tavares *et al.*, 2000). The recently described T-cell subtype Th17, characterized by production of IL-17 and expression of CCR6 (Harrington, 2006; Singh *et al.*, 2008), has been found, aside its involvement in autoimmune diseases and immunosurveillance, to be involved in host defense against *Candida* in mice and humans (Huang *et al.*, 2004; Acosta-Rodriguez *et al.*, 2007b; Bettelli *et al.*, 2007).

Concerning CMC, we, and others, reported a heterogeneous, but not generally diminished, proliferative capacity of T cells from CMC patients (De Morales-Vasconcelos *et al.*, 2001; Eyerich *et al.*, 2007). A more critical parameter in the pathogenesis of CMC could be the cytokine secretion of T-cell subtypes rather than proliferation: recently, T-cell cytokine secretion has been the focus of numerous studies (Kobrynski *et al.*, 1996; Lilic *et al.*, 2003; Van der Graaf *et al.*, 2003; Eyerich *et al.*, 2007), describing an altered cytokine

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Abbreviations: CMC, chronic mucocutaneous candidiasis; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; Th, T helper

production with a reduced production of type-1 cytokines such as IFN-γ, IL-12, and IL-2, and increased secretion of IL-10 or IL-4.

The aim of this study was to elucidate a possible role for IL-17-producing T cells in the pathogenesis of CMC.

RESULTS

Candida- and PHA-stimulated PBMCs from CMC patients transcribe and secrete less IL-17 and IL-22 compared with those in healthy controls

PBMCs of two CMC patients and two age- and sex-matched controls were stimulated with *C. albicans*. After 24 hours, total mRNA was isolated, reverse transcribed, and used for real-time PCR. In healthy (non-*Candida* infected) controls, IL-17F and IL-22 were significantly induced after 24 hours of stimulation with *Candida* or phytohemagglutinin (PHA), whereas CMC patients failed to upregulate the expression of these cytokines on mRNA level (Figure 1a), irrespective of the stimulus. IL-17A was not upregulated in CMC patients and healthy controls in the monitored time course (data not shown).

The low transcription of IL-17 and IL-22 mRNA in CMC patients was reflected by the scarce and significantly lower secretion of IL-17 and IL-22 as compared with that in healthy

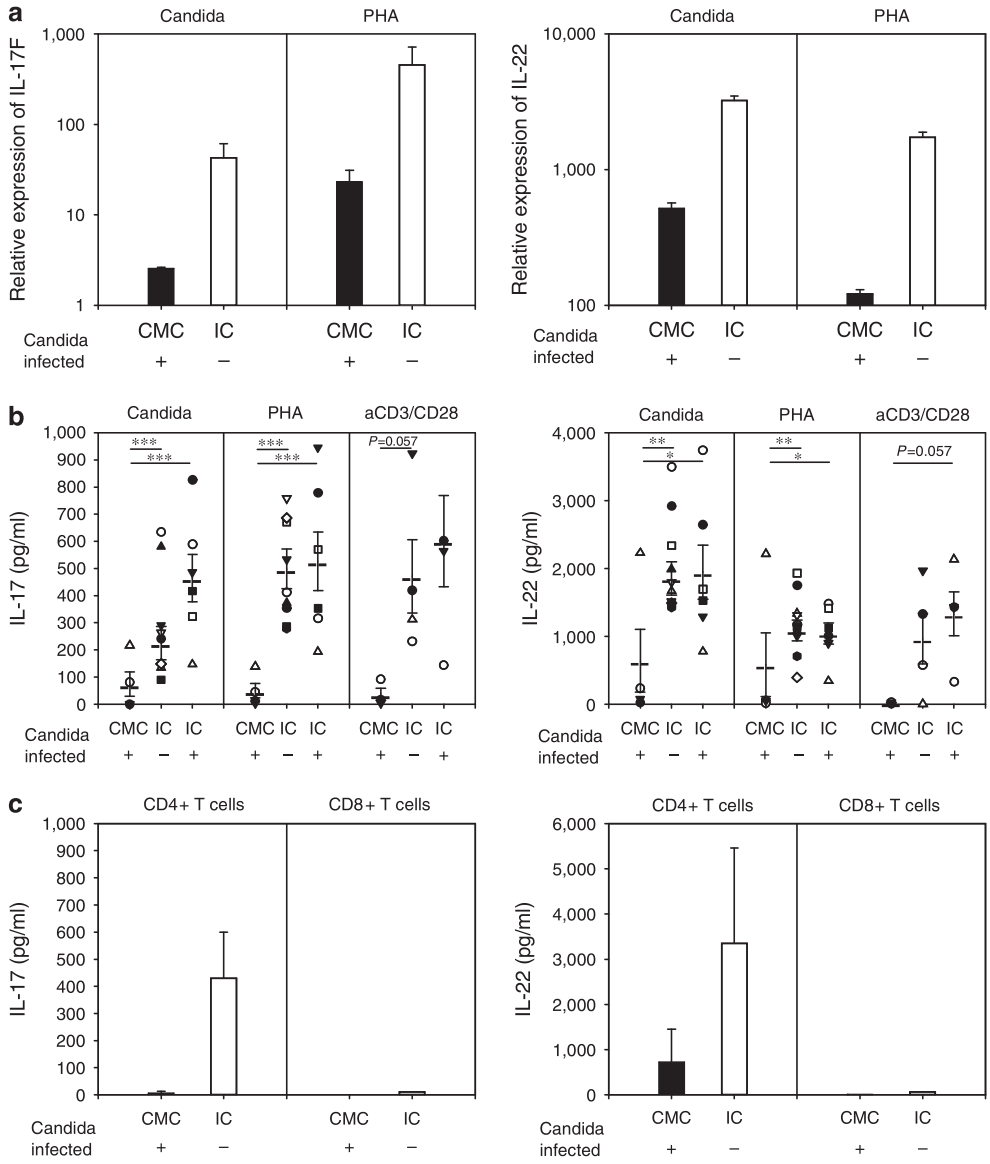

Figure 1. PBMCs of CMC patients exhibit significantly reduced expression and release of IL-17 and IL-22 upon stimulation with *Candida* or PHA. Total mRNA of PBMCs was isolated 24 hours after stimulation with *C. albicans* or PHA, reverse transcribed, and analyzed by real-time PCR for IL-17F and IL-22 transcription (CMC patients, $n=2$; IC, non-*Candida*-infected control, $n=2$) (**a**). The culture supernatants of PBMCs stimulated for 72 hours with *C. albicans*, PHA or anti-CD3/anti-CD28 were analyzed by ELISA for protein content of IL-17 and IL-22 (CMC patients, $n=4$; IC, non-*Candida*-infected control, $n=9$; IC, *Candida*-infected control, $n=6$) (**b**). CD4+ or CD8+ T cells were incubated with autologous monocytes and *Candida* for 72 hours, and supernatants were analyzed for IL-17 and IL-22 by ELISA (CMC patients, $n=2$; IC, non-*Candida*-infected control, $n=4$) (**c**). Error bars indicate mean ± SD (IC, immunocompetent control; $*P\leqslant 0.05$; $**P\leqslant 0.01$; $***P\leqslant 0.001$).

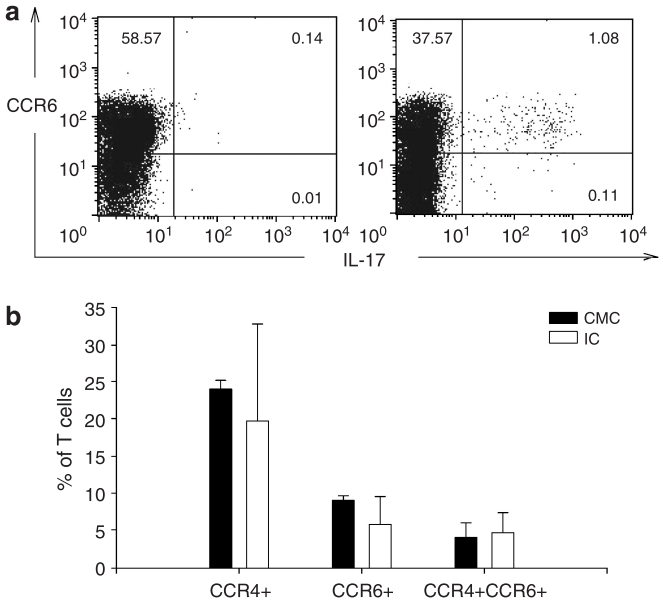

Figure 2. The CCR6+ IL-17A+ cell population is strongly decreased in CMC patients, while the total number of CCR4+CCR6+ cells is not diminished. PBMCs of CMC patients and healthy controls were stained intracellularly after phorbol 12-myristate 13-acetate/ionomycin stimulation for IL-17 (**a**) (one representative experiment of three is shown) and analyzed for expression of the surface markers CCR4 and CCR6 (**b**) by flow cytometry (CMC patients, $n=3$ and healthy controls, $n=4$). Error bars indicate mean ± SD (IC, immunocompetent non-*Candida*-infected control).

controls as measured by ELISA after 72 hours of stimulation with PHA or *Candida* (Figure 1b). Notably, IL-17 and IL-22 release were also reduced in CMC patients when PBMCs were stimulated with anti-CD3/anti-CD28, pointing to a defect in the T-cell compartment rather than antigen-presentation or absent antigen-presenting cell-derived signals. Furthermore, PBMCs from immunocompetent patients with acute *Candida* infection exhibited not only significantly higher release of IL-17 upon stimulation with *Candida* compared with CMC patients, but also compared with healthy controls. This underlines the importance of IL-17 in defense against *Candida*.

Incubation of purified CD4+ and CD8+ T cells with autologous monocytes as antigen-presenting cells and *Candida* revealed that IL-17 and IL-22 are predominantly derived from CD4+ T cells in healthy controls, and conclusively, the disability of CMC patients to produce IL-17 and IL-22 is mainly attributed to CD4+ T cells (Figure 1c).

CMC patients exhibit reduced total number of IL-17-producing T cells, but normal amounts of CCR6+/CCR4+ T cells

The surface phenotype of IL-17-producing T cells is characterized by CCR4 and CCR6. Here we confirm these data showing that the majority of IL-17-producing cells expressed CCR6 (Figure 2a). In line with our data from ELISA, CMC patients showed an evanescent number of IL-17-producing (CCR6+) T cells after phorbol 12-myristate 13-acetate/ionomycin stimulation. However, no significant differences in the number of CCR4+, CCR6+ and CCR4/CCR6 double positive cells between CMC patients and healthy controls were observed (Figure 2b).

PBMCs of CMC patients are able to secrete Th17-differentiating and Th17-maintaining cytokines

IL-1β and IL-6 are important for differentiation of human IL-17-producing T cells. We analyzed the expression of IL-1β and IL-6 at the transcriptional level by real-time PCR (Figure 3a). PBMCs of CMC patients stimulated with *C. albicans* for 24 hours tended to show higher mRNA expression of IL-1β and IL-6 compared those of with healthy controls. Transcriptional upregulation of these genes in CMC patients resulted in only slightly higher amounts of corresponding protein measured by ELISA in culture supernatants of *Candida*- or PHA-stimulated PBMCs after 72 hours (Figure 3b). IL-23, a cytokine important for maintenance of Th17 cells, was analyzed on mRNA level, revealing no differences in expression between patients and controls (data not shown).

DISCUSSION

Recent data suggest that the inability to clear *C. albicans* in CMC patients is based upon a complex heterogeneity of immune defect(s), probably characteristic for various disease subgroups. In this study, we present four CMC patients deficient in the production of Th17-associated cytokines IL-17 and IL-22, whereas levels of the mediators important for differentiation (IL-6 and IL-1β) and maintenance (IL-23) of the Th17 lineage were enhanced or not altered.

IL-17-producing T cells represent a recently described T-cell subset characterized by production of IL-17 and IL-22 (Liang *et al.*, 2006; Bettelli *et al.*, 2007). They are proinflammatory linkers between myeloid and lymphoid host defense that are able to help B cells, show low cytotoxicity, and poor regulative susceptibility to T regulatory cells (Annunziato *et al.*, 2007).

First evidence for the importance of IL-17 in clearing *Candida* infections has been provided in the mouse system: IL-17A-receptor-knockout mice showed a dose-dependent, substantially reduced survival in a murine model of systemic candidiasis (Huang *et al.*, 2004). Recent data revealed that infection with *C. albicans* leads to induction of murine IL-17-producing T cells (LeibundGut-Landmann *et al.*, 2007). Furthermore, the hyphal form of *C. albicans* triggers IL-17 production of freshly isolated human CD4+ T cells of healthy donors *in vitro* (Acosta-Rodriguez *et al.*, 2007b).

Our study affirms the importance of IL-17 producing-T cells in clearing *Candida* infections, because PBMCs of immunocompetent patients suffering from *Candida* infection showed significantly higher secretion of IL-17 and IL-22 after *in vitro* stimulation with *C. albicans* as compared with healthy (non-*Candida*-infected) donors. In concordance with previous reports (Annunziato *et al.*, 2007; LeibundGut-Landmann *et al.*, 2007; Singh *et al.*, 2008), our data show that the source of IL-17 in PBMCs is almost exclusively limited to CD4+ CCR-6+ T cells. T-cell-receptor-specific

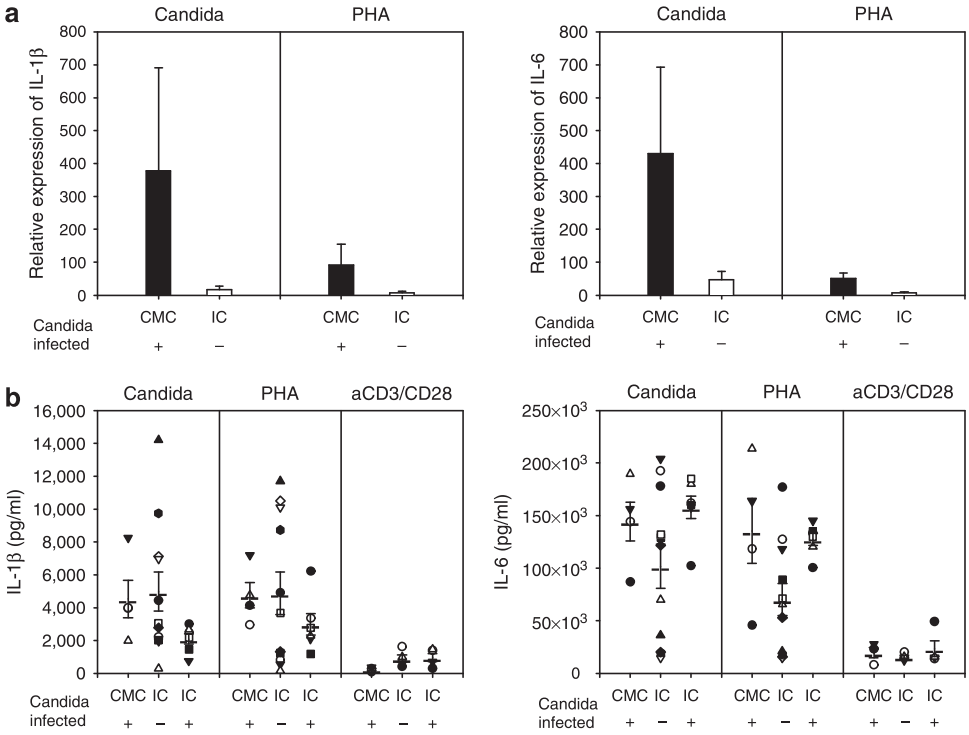

Figure 3. No significant differences in Th17-differentiating cytokine secretion between CMC patients and healthy volunteers. Total mRNA was isolated from *Candida*-stimulated PBMCs (CMC patients, $n=2$; IC, non-*Candida*-infected control, $n=2$) after 24 hours and analyzed for expression of IL-1β and IL-6 by real-time PCR (**a**). The supernatants were analyzed after 72 hours by ELISA (CMC patients, $n=4$; IC, non-*Candida*-infected control, $n=9$; IC, *Candida*-infected control, $n=6$) (**b**). There were no significant differences in secretion of IL-1β and IL-6 between patients and controls. Error bars indicate mean ± SD (IC, immunocompetent control).

stimulation of PBMCs and stimulation of isolated CD4+ T cells with autologous monocytes showed comparable amounts of secreted IL-17, indicating that IL-17 was predominantly derived from CD4+ T cells. Flow cytometry analysis of PBMCs revealed nearly all cells positive for IL-17 in intracellular staining to be positive also for CCR-6.

However, even though they had been chronically exposed to *C. albicans*, PBMCs from CMC patients secreted significantly lower amounts of IL-17 and IL-22 than PBMCs from healthy donors and patients with current *Candida* infection, after stimulation with *C. albicans in vitro*—both on the mRNA and on protein level. The underlying immune defect was not specific for the *Candida* stimulus, as mitogen stimulation (PHA) and T-cell-receptor-specific stimulation (anti-CD3/anti-CD28) also resulted in reduced secretion of IL-17 and IL-22 in CMC patients. This decrease was due to a strongly diminished total number of IL-17 producing T cells, as detected by surface CCR-6 and intracellular IL-17 staining of PBMCs by flow cytometry. The weaker secretion of IL-22 was less pronounced than that of IL-17, as compared with immunocompetent patients either infected or not infected with *Candida*. This could be explained by the fact that IL-22 production is not limited to Th17 cells, but is also produced by other activated T-cell subtypes (Xie *et al.*, 2000; Conti *et al.*, 2003).

The so far identified differentiation factors for human IL-17-producing T cells are IL-1β and IL-6 (Acosta-Rodriguez *et al.*, 2007a), whereas IL-23 seems to be important for maintaining production of IL-17 and IL-22 in mouse (Chen *et al.*, 2006; Veldhoen *et al.*, 2006; Kreymborg *et al.*, 2007), but to a lesser degree in human IL-17-producing T cells (Acosta-Rodriguez *et al.*, 2007a, b). In concordance with these studies, we observed strong enhancement of IL-1β and IL-6, but not of IL-23, after stimulation with *Candida*. The secretion of these cytokines in PBMCs of patients suffering from CMC was not diminished. In contrast, mRNA expression of IL-1β and IL-6 was induced much stronger in CMC patients, resulting in slightly higher release of proteins after 72 hours. This could indicate a defect in differentiation or survival of IL-17-producing T cells downstream of IL-1β and IL-6.

Concerning the mechanism of the candidicidal effects of IL-17-producing T cells, two possible pathways could be involved in clearing infection: on the one hand, there is the strong neutrophil-recruiting capacity of IL-17 via induction of IL-8 in human keratinocytes (Albanesi *et al.*, 1999). On the other hand, IL-17 and IL-22 synergistically induce β-defensins in human keratinocytes (Liang *et al.*, 2006) that are able to kill *C. albicans* (Feng *et al.*, 2005; Vylkova *et al.*, 2007). Taken together, a decrease in the absolute number of IL-17-producing T cells and the resulting diminished stimulation of epithelial cells could explain why candidiasis is limited to the skin and mucosal membranes in CMC patients, and help to understand why such patients do not suffer from systemic candidiasis.

In summary, this study underlines the importance of IL-17-producing T cells for clearance of *Candida* infections. Furthermore, our data suggest that an impaired IL-17 and IL-22 response seems to be, at least in part, responsible for the pathogenesis of CMC. With its limited number of CMC patients, the power of this study is not high enough to draw general conclusions. Therefore, further studies with greater numbers of CMC patients will be required to investigate the immune response of IL-17-producing T cells in order to elucidate the pathomechanisms of CMC.

MATERIALS AND METHODS

Patients

Four CMC patients (three female, one male; age 8–50 years, mean 31 years; all suffering from chronic *Candida* infections of the skin and esophagous since early childhood) were included into the study and compared with nine healthy, age and sex-matched volunteers. Furthermore, six patients suffering from an (at the time of investigation) untreated *Candida* infection (two paronychia, two oral, and two genital candidiasis) without any immune suppression were enrolled into the study. Before drawing blood, each participant provided informed consent. The study was approved by the ethical committee of the Technical University Munich and followed the Declaration of Helsinki Principles (41st World Medical Assembly, 1997).

Quantitative mRNA analysis

PBMCs of two CMC patients and two healthy volunteers were stimulated with *Candida* antigen (100 μg ml^{-1}; Allergopharma, Hamburg, Germany), PHA (10 μg ml^{-1}), or anti-CD3/antiCD28 antibodies, respectively, for 6 hours. Total mRNA was extracted using PeqGold RNA extraction buffer (Peqlab, Erlangen, Germany). RNA was reverse transcribed using oligo(dT) primers and avian myeloblastosis virus reverse transcriptase (Roche, Mannheim, Germany). PCRs were performed with the following primers: IL-17A (forward 5′-CTCGATTTCACATGCCTTCA-3′; reverse 5′-GAGG GGCCTTAATCTCCAAA-3′), IL-17F (forward 5′-AGTTGGAGAAGG TGCTGGTG-3′; reverse 5′-CCATCCGTGCAGGTCTTATT-3′), IL-22 (forward 5′-GAGGAATGTGCAAAAGCTGA-3′; reverse 5′-GCTTT GGGGCATCTAATTGT-3′), IL-23A (forward 5′-CAGTTCTGCTTG CAAAGGAT-3′; reverse 5′-ATCTGCTGAGTCTCCCAGTG-3′), IL-1β (forward 5′-TTCGACACATGGGATAACGA-3′; reverse 5′-TCTTTCA ACACGCAGGACAG-3′), and IL-6 (forward 5′-ATGCAATAACCAC CCCTGAC-3′; reverse 5′-GAGGTGCCCATGCTACATTT-3′) (all from www.realtimeprimers.com) and SYBR green mastermix (Bio-Rad, Munich, Germany). PCRs were run on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using the following program: 10 minutes at 94 °C followed by 45 cycles of 15 seconds at 95 °C and 60 seconds at 58 °C. 18S RNA served as the housekeeping gene.

T-cell proliferation and cytokine secretion

PBMCs were separated as previously described (Eyerich *et al.*, 2007) and stimulated for 60 hours either with 10 μg ml^{-1} PHA as a positive control or with 100 μg ml^{-1} *C. albicans* antigen (Allergopharma). After 60 hours, 100 μl per well of supernatant was obtained and stored at −70 °C until further analysis by ELISA. Quantification of cytokines in the supernatants was performed by ELISA, according to the manufacturer's instructions (IL-17, IL-22; R&D Systems, Wiesbaden, Germany, and IL-1β, IL-6; BD Biosciences, Heidelberg, Germany).

Additionally, CD4+ and CD8+ T cells were isolated from PBMCs of one CMC patient and a matched control, as previously described, and stimulated with mitogen or *Candida* in the presence of monocytes that served as antigen-presenting cells.

Intracellular cytokine staining

PBMCs from CMC patients and healthy controls were stimulated with phorbol 12-myristate 13-acetate (20 ng ml^{-1}) and ionomycin (1 ng ml^{-1}) (both from Sigma-Aldrich, Munich, Germany) for 6 hours and examined for intracellular IL-17 accumulation.

To prevent cytokine secretion, the stimulation was performed in the presence of Monensin (from the beginning) and brefeldin-A (10 g ml^{-1}; Sigma-Aldrich) was added for the final 4 hours. T cells were fixed (2% paraformaldehyde), permeabilized (0.5% saponin), and stained with phycoerythrin-conjugated anti-human IL-17 (eBioscience, San Diego, CA) antibody or isotype-matched control antibody, and analyzed by flow cytometry.

Statistical analysis

Statistical analysis was performed with the software program SPSS 14.0. All results were analyzed by Mann—Whitney *U*-test. Statistically significant differences between CMC patients and controls were defined as $P<0.05$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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