Clinical and
Experimental
Immunology

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Journal:	*Clinical and Experimental Immunology*
Manuscript ID:	CEI-2008-1384.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
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Key Words:	Dendritic cells (myeloid, plasmacytoid, monocyte-derived), Cytokines/interleukins, Immunodeficiency-Primary, Autoimmune polyendocrine syndromes, INFECTIONS

REVISED VERSION

Impaired Dendritic Cell Maturation and Cytokine Production in Patients with Chronic Mucocutanous Candidiasis with or without APECED

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Running title: Dendritic cells in CMC patients

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KEYWORDS

APECED, AIRE, Chronic Mucocutaneous Candidiasis, Human, Dendritic Cells

WORD COUNT

4081 (w/o references and figure legends)

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ABBREVIATIONS

AAbs, autoantibodies;

AIRE, Autoimmune Regulator;

APECED, Autoimmune Polyendocrinopathy Candidiasis Ectodermal Dystrophy;

CH, *Candida* hyphae;

CTRLS, Controls;

EAE, Experimental Autoimmune Encephalomyelitis

F, female;

M, male;

moDCs, monocyte-derived dendritic cells;

OMIM, Online Mendelian Inheritance in Man;

PRRs, Pattern Recognition Receptors;

STAT3, Signal Transducer and Activation of Transcription 3,

SUMMARY

Patients with Chronic Mucocutaneous Candidiasis (CMC) suffer persistent infections with the yeast *Candida*. CMC includes patients with *AIRE* gene mutations who have Autoimmune PolyEndocrinopathy Candidiasis Ectodermal Dystrophy (APECED), and patients without known mutations. CMC patients have dysregulated cytokine production and dendritic cells (DCs) as central orchestrators, may underlie pathogenic disease mechanisms. In 29 patients with CMC (13 with APECED) and controls, we generated monocyte-derived DCs (moDCs), stimulated them with *Candida albicans*, Toll-like receptor 2/6 ligand and lipopolysaccharide, to assess cytokine production (IL-12p70, IL-23, IFNγ, IL-2, TNFα, IL-6, TGFβ, IL-10, IL-5, IL-13) and cell-surface maturation marker expression (CD83, CD86, HLA-DR). In both APECED and non-APECED CMC patients, we demonstrate impairment of DC function as evidenced by altered cytokine expression profiles and DC maturation/activation: 1) both groups over-produce IL-2, IFNγ, TNFα, IL-13 and demonstrate impaired DC maturation. 2) Only non-APECED patients showed markedly decreased *Candida*-stimulated production of IL-23 and markedly increased production of IL-6, suggesting impairment of the IL-6/IL-23/Th17 axis. 3) In contrast, only APECED patients showed DC hyper-activation, which may underlie altered T-cell responsiveness, autoimmunity and impaired response to *Candida*. We demonstrate different pathogenic mechanisms on the same immune response pathway underlying increased susceptibility to *Candida* infection in these patients.

WORD COUNT: 199

INTRODUCTION

Candida is an opportunistic yeast, colonising the skin and mucosa of most healthy humans without causing tissue damage [1], but quickly establishes disease in a variety of permissive circumstances, often on a background of impaired immune function. Protective immunity to *Candida* involves both the innate and adaptive immune system [2]. Defects in cell-mediated immunity predispose to mucocutaneous candidiasis, as well as to a wide range of other infectious agents [3]. As opposed to this, there are very rare patients with a selective susceptibility to mucocutaneous infections with *Candida*, who suffer from recurring or persistent, often severe, debilitating infections with this yeast [4]. The diagnosis of this disease, coined Chronic Mucocutaneous Candidiasis (CMC) is clinical and encompasses a heterogeneous group of conditions [5]. The APECED syndrome (Autoimmune PolyEndocrinopathy Candidiasis Ectodermal Dystrophy), also known as APS1 (Autoimmune PolyEndocrinopathy Type 1) identifies patients with CMC who have associated organ-specific autoimmune involvement of endocrine glands and other organs, and an underlying mutation of the *AIRE* gene (Online Mendelian Inheritance in Man - OMIM 240300) (reviewed in [6], [7]. There is surprisingly little data addressing the link between *AIRE* mutations, the associated autoimmunity and the immune defect seen in APECED patients which underlies susceptibility to *Candida* infections [8], [9]. Other subgroups of CMC have also been clinically defined and include patients with associated thyroid disease (OMIM 606415), isolated CMC with various modes of inheritance (OMIM 11458, OMIM 212050) and sporadic CMC [4]. In these CMC patients the diagnosis remains clinical, given that a genetic or biochemical marker is not yet available.

In recent years a wealth of new knowledge has emerged elucidating the mechanisms involved in protective responses against *Candida* in both mouse and human models [2], [10]. Cytokines produced by the innate immune system, in particular IL-12 secreted by dendritic cells (DCs), are crucial for generating a protective Th1 response in mice. However, in striking

contrast, patients with inborn errors of the IL-12/IFNγ pathway do not show increased susceptibility to *Candida* or other fungal infections [11], strongly suggesting that our current understanding of immune mechanisms involved in protection against fungi may need reassessment. A newly identified Th-17 pathway, involving IL-6 in the initiation phase and IL-23 in the perpetuation of IL-17 secreting T cells [12], was recently shown to be crucially involved in both human [13], [14] and murine [15] immune responses to *Candida*, and was not identical in mice and men [16].

Very little is known about the immune defect underlying increased susceptibility to *Candida* infections in CMC patients. These patients have different clinical diseases and (known and unknown) genetic defects, but they all demonstrate the same selective susceptibility to mucocutaneous *Candida* infections, which suggests that they either harbour the same underlying immune defect or, more likely, have different defects on the same immune response pathway necessary for protection against *Candida*. Earlier studies both *in vivo* and *in vitro* demonstrated defects in cell-mediated immunity, generally interpreted as disorders of effector T-cell function [4, 17]. More recently, we [18], [19] and others [20] demonstrated dysregulated cytokine production in response to *Candida*, suggesting that the immune defect might be at the level of orchestrating appropriate Th1 (or other?) cytokine responses, rather than the effector T cell level itself.

In the current study we investigated DC function in response to *Candida* and non-*Candida* stimuli, to assess if impairment of these central orchestrators of cytokine production (28) could underlie pathogenic disease mechanisms in CMC. Our results demonstrate that DCs from both APECED and non-APECED patients show hyper-responsive cytokine expression profiles following stimulation with LPS, with over-production of IFNγ, IL-2, TNFα, IL-5 and IL-13, as well as impaired DC maturation. Only non-APECED patients showed markedly decreased production of IL-23 and markedly increased production of IL-6 specifically in response to *Candida*, suggesting that impairment of the IL-6/IL-23/Th17 axis

may underlie defective clearance and susceptibility to *Candida* infections. Thus, both APECED and non-APECED CMC patients have impaired/altered DC function, albeit with different defects, suggesting different pathogenic mechanisms on the same immune response pathway underlying increased susceptibility to *Candida* infection in these patients.

MATERIALS AND METHODS

Our study included 29 CMC patients, 13 APECED patients with the *AIRE* gene mutation, 16 non-APECED patients without a detectable AIRE gene mutation and 25 age-and-sex-matched healthy controls (Table 1). In unstimulated (immature) and stimulated (mature) monocyte-derived DC (moDC) cultures, we assessed supernatants for secreted cytokines (IL-12p70, IL-23, IFNγ, IL-2, TNFα, IL-6, TGFβ, IL-10, IL-5 and IL-13), as well as moDCs cell-surface maturation and activation markers (CD83, CD86 and HLA-DR). Toll-like (TLR) 1-10 and other receptor expression was also studied (data not shown, manuscript in preparation). Monocyte-derived DCs were used as representatives of skin and mucosal myeloid-DCs involved in *Candida* recognition, because obtaining skin biopsies from CMC patients for purely research purposes was unacceptable for ethical reasons.

We stimulated moDCs with *Candida albicans* hyphae (CH) rather than yeasts, as several studies suggest that hyphae are the invasive morphotype of *Candida* in clinical infections [21]. With the aim of investigating putative impaired *Candida* binding to DCs, we assessed moDC stimulation with a Toll-like receptor (TLR) ligand 2/6 (MALP2) that selectively engages the same TLRs that are known to bind *Candida* and other yeasts [32]. Lipopolysaccharide (LPS) was used as a "positive" non-*Candida* control, in order to assess moDC functionality in response to other potent stimuli. Assessment of additional stimuli was limited by the quantity of blood we could draw from each patient, particularly children.

Generation of monocyte-derived dendritic cells from patient blood

moDC were generated from peripheral blood CD14-positive cells in the presence of IL-4 and GM-CSF. Peripheral blood mononuclear cells were isolated by density centrifugation (LymphoPrep, Axis-Shield, Oslo, Norway) and CD14-positive cells purified by magnetic separation on an LS column following labelling with anti-CD14-coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes were seeded into 24-well plates at $0.75x10^6$ per well in 1ml total volume of RF10 culture media (RPMI-1640 media

(BioWhittaker, Lonza Wokingham, UK), supplemented with 10% fetal calf serum (PAA Laboratories, Pasching, Austria), 2mM L-glutamine (Sigma Aldrich, St. Louis, MO) and 1% Penicillin-Streptomycin (Gibco, Carlsbad, CA). 50ng/ml IL-4 and GM-CSF (Immunotools, Friesoythe, Germany) were added to each well on days 0 and 3. Cells were incubated at 37^{o}C with 5% CO_2.

Dendritic cell maturation

On day 6 of the DC culture, immature dendritic cells were activated as follows: no treatment (unstimulated); addition of 1:10,000 final dilution (10mg/L protein content or 25x10^{6} cells) of heat-killed *Candida albicans* hyphae (ATCC #18804, Manassas, VA); 10ng/ml of the purified TLR2/6 ligand, MALP-2 (Apotech, Epalinges, Switzerland); 1µg/ml of lipopolysaccharide (LPS) (Invivogen, San Diego, CA). Cells or cytokines were harvested after 24h on day 7.

Cytokine analysis

Culture supernatants were harvested 24 hours after activation, and stored at -20^{o}C. Cytokine levels were assessed either by sandwich ELISA or the electrochemiluminescence-based MSD (Meso Scale Discovery, Gaithersburg, MD) immunoassay. IL-12p70, IFNγ, IL-2, IL-5, IL-10 and IL-13 were part of an MSD multiplex Th1/Th2 plate (detection limits for IL-12p70, IFNγ, and IL-10 were 4pg/ml, for IL-2, IL-5 and IL-13, 2pg/ml); IL-6 and TNFα were MSD duplex custom made plates (extra-sensitive, detection limit 3pg/ml for both cytokines); TGFβ duoset kit was purchased from R&D Systems (Minneapolis, MN, detection limit 20pg/ml) and IL-23 Ready-Set-Go kit from eBioscience (San Diego, CA, detection limit 15pg/ml). Cytokine levels were calculated using manufacturer software, given in pg/ml and presented as medians with interquartile ranges (IQR).

Flow cytometry

To assess DC maturation, DCs were harvested 24h after activation and stained for 20 minutes on ice with the following antibodies: CD86-FITC, CD83-PE, and HLA-DR-PerCP

and appropriate isotype controls (BD Biosciences, San Jose, CA). Stained cells were washed in FACS Wash (1x PBS + 0.1% BSA) and fixed in 1% paraformaldehyde (Sigma Aldrich, St. Louis, MO). All stained cells were acquired using a FACScan (BD Biosciences, San Jose, CA) equipped with a 488nm laser and a 633nm laser upgrade. Acquired events were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR). Monocyte transformation into DCs was confirmed by absence of CD14 staining in cultures. Results of DC activation marker analysis are presented as percentage of positive cells and median fluorescence intensity (MFI).

Candida albicans

Freeze-dried *Candida albicans* was purchased from American Type Culture Collection (#18804, Manassas, VA) and rehydrated according to the supplier's instructions. To culture the hyphal form, *Candida albicans* was grown in autoclaved 1x broth (67g/L Yeast Nitrogen Base and 10% D-Glucose, Becton-Dickinson, Sparks, MD) at 30^{o}C, heat-killed (pressure cooker for 30 minutes at 120^{o}C), pelleted at 400g for 10 minutes and used in cell cultures at a final concentration of 1:10000, defined as optimal in previous titrations.

Subjects

We investigated 29 CMC patients of which 13 APECED patients with the *AIRE* gene mutation, 16 non-APECED patients without a detectable AIRE gene mutation and 25 age-and-sex-matched healthy controls (Table 1).

Patients: we studied 29 patients with Chronic Mucocutaneous Candidiasis (CMC), who were all screened for the two most common *AIRE* gene mutations: p.R257X (nonsense mutation in exon 6) and c.964del13 (13bp deletion in exon 8) either in Huch-Laboratory Diagnostics, Helsinki University Hospital, Finland or Northern Molecular Genetics Service, Institute of Human Genetics, Newcastle Upon Tyne, UK. Thirteen patients (children 3-15 years of age, adults 17 – 38) had an *AIRE* gene mutation and the APECED syndrome, of

which 9 had the c.964del13 deletion. In the remaining 16 non-APECED patients (children 2-15 years of age, adults 19-47) an AIRE mutation was not detected. All patients were also screened for auto-antibodies to Type 1 interferons (IFNs), shown to be highly specific for APECED patients [33]; these autoantibodies were present in all APECED patients and none of the non-APECED patients and controls.

Ten patients in the APECED group, and 9 in the non-APECED group had affected siblings which are all included in this study (maximum 3 patients from any one family). Three non-APECED patients had hypothyroidism, 2 with thyroid peroxidase antibodies. At the time of sampling, patients did not have other serious infections, were not on systemic antibiotic treatment or receiving steroids. All patients suffered with recurrent mucocutaneous *Candida* infection (mouth, nails, skin, oesophagus and perineum). Patients were screened for systemic autoantibodies including antinuclear factor, smooth muscle, liver-kidney microsomal, mitochondrial and gastric parietal cell antibodies. Organ-specific autoantibodies and/or endocrinopathy affected parathyroid, thyroid, adrenal cortex, gonads and pancreas. Autoantibodies were evaluated in patients sera using indirect immunofluorescence on commercial rodent tissue (Euroimmune, Lubeck, Germany) for systemic autoantibodies and monkey organ tissue (The Binding Sire, Birmingham, UK) for organ-specific autoantibodes. Endocrinopathy was diagnosed if/when there was clinical and laboratory evidence of glandular hypofunction.

Controls: 25 age-and-sex matched controls were recruited for the study. Adults (19 - 55 years of age) were healthy laboratory volunteers, while control children (2 - 16 years of age) were undergoing general anaesthesia for surgery to treat non-infectious causes (eye squints, circumcision, hernia, etc).

The number of patients and controls in each experiment may vary, due to the limitation of blood available. Both patients and healthy controls – parents on behalf of children - received verbal and written explanations of the study and signed informed consent

forms. Ethical approval was obtained from the Newcastle and North Tyneside Local Research Ethics Committee 1.

Statistical analysis

Statistical analysis and graphic presentations were performed using the GraphPad PRISM software package. Average values are presented as medians with inter-quartile ranges (IQR). P values were calculated using the two-tailed, 95% confidence intervals Mann-Whitney rank sum test for independent, non-parametric data. The level of significance was set at $p<0.05$.

RESULTS

Cytokine production by moDCs

IL-6/IL-23/Th17 axis cytokine production

IL-6: one of the most important findings in this study was the selectively increased IL-6 production in non-APECED CMC patients, who demonstrated significantly higher unstimulated IL-6 production compared to both APECED patients and controls, where IL-6 levels were mostly undetectable or very low levels (Fig 1a). Importantly, non-APECED patients produced significantly more IL-6 in response to CH and TLR2/6 ligand stimulation compared to both APECED patients and controls (Fig 1a). LPS stimulation resulted in high levels of IL-6 produced in both patient groups, which were on average higher albeit not significantly different compared to controls (Fig 1a). There was no major difference between levels produced by adults and children in any of the groups (data not shown).

IL-23: impaired production of IL-23 in non-APECED patients was another important finding in our study (Fig 1b). In response to *Candida* stimulation, non-APECED patients produced significantly less IL-23 than controls, whereas this was not the case with APECED patients. The difference was most marked in non-APECED adults ($p=0.035$ compared to controls, data not shown). TLR2/6 stimulation resulted in modest and comparable IL-23

increases in all groups (Fig 1b). Interestingly, the non-*Candida* stimulant LPS resulted in huge increases of IL-23 levels in all groups, with both APECED and non-APECED patients producing significantly more IL-23 compared to controls.

Th1 cytokine production

IL-12p70: production of IL-12p70 in unstimulated cultures was barely detectable in all groups. Stimulation with *Candida* and TLR2/6 ligand resulted in a low but clearly detectable and similar response in all groups (data not shown). The response to LPS was impressively and significantly higher in non-APECED patients (particularly children, data not shown) compared to levels produced by APECED patients and controls, which were almost identical (Table 2).

IFNγ: background levels of IFNγ in unstimulated cultures were detectable in all groups and neither CH nor TLR2/6 increased IFNγ production above unstimulated levels. However, LPS induced IFNγ production in all groups, although significantly more in CMC patients than in controls (Table 2). Healthy children produced less IFNγ than healthy adults, although this was not statistically significant (data not shown).

IL-2: this cytokine was produced only with LPS stimulation and non-APECED patients interestingly produced significantly more IL-2 than controls (Table 2).

Inflammatory cytokine production

TNFα: high unstimulated production was seen in both APECED and non-APECED patients, compared to controls where it was mostly undetectable or low. Following CH and TLR2/6 ligand stimulation, all groups responded to a similar degree, but in response to LPS, APECED and non-APECED patients produced significantly more TNFα compared to controls (Table 2).

Anti-inflammatory cytokine production

TGFβ: production of this cytokine was not enhanced and remained close to baseline in both patients and controls (data not shown). Assessment of mRNA levels yielded similar results (unpublished data).

Th2 and Th2-inducing cytokine production

IL-10, IL-5 and IL-13: CH and TLR2/6 did not stimulate production of these cytokines by moDCs. LPS increased production of IL-10 to low and comparable levels in all groups, while LPS-stimulated IL-5 and IL-13 production was markedly higher in APECED and non-APECED patients than in controls (Table 2).

Cytokine plasma levels

IL-6, TNFα and TGFβ plasma levels: levels of IL-6 and TNFα in plasma for all groups studied were mostly low or undetectable, while TGFβ levels were detectable and significantly higher in APECED and non-APECED patients than in controls (data not shown).

DC activation markers

CD83

For CD83, we present only findings in children, where the differences were most marked. Percentages of CD83+ cells in unstimulated cultures were comparable between patients and controls (Fig 2A). In response to CH and TLR2/6 ligand stimulation, APECED and non-APECED children had markedly lower percentages of CD83+ cells than controls (Fig 2A). Following LPS stimulation, the trend was the same but did not reach statistical significance (Fig 2A). In adults, the percentages of CD83+ cells were not significantly different between the groups for any of the stimuli used (data not shown).

MFI levels of CD83 in unstimulated DC cultures were low and comparable in all groups (Fig 2B). In response to stimulation with CH and TLR2/6 ligand, APECED and non-APECED children up-regulated CD83 to a significantly lesser degree than controls. LPS increased CD83 expression in all groups; this was again to a lesser degree in APECED and non-APECED children than in controls, albeit not statistically significant (Fig 2B). CD83 expression in adults did not differ between groups (data not shown).

CD86

Percentages of CD86+ cells in unstimulated cultures were significantly higher in APECED patients compared to controls (Fig 3A). Following stimulation with CH and TLR2/6 ligand, the percentage of CD86+ cells in APECED patients increased significantly compared to both controls and non-APECED patients. In contrast, percentages of CD86+ cells in non-APECED patients remained similar to control levels throughout. LPS stimulation resulted in almost all cells expressing CD86 in all individuals (Fig 3A).

MFI levels of CD86 in unstimulated cultures were significantly higher in APECED patients compared to controls. Following stimulation with CH and TLR2/6 ligand, CD86 MFI levels in APECED patients were significantly higher compared to controls, whereas MFI levels in non-APECED patients also increased but not significantly (Fig 3B). Stimulation with LPS resulted in higher CD86 MFI in both APECED and non-APECED compared to controls (Fig 3B).

HLA-DR

Percentages and MFI of HLA-DR+ cells after stimulation by CH, TLR2/6 and LPS were not statistically different between groups (data not shown)

DISCUSSION

We demonstrate for the first time that both APECED and non-APECED CMC patients have impaired DC function, implying that this may be the pathogenic mechanism underlying increased susceptibility to *Candida* infection in these patients. Importantly, although some DC defects were common for both patient groups, other defects were unique, suggesting different pathogenic mechanisms on the same immune response pathway, resulting in a similar phenotype of increased susceptibility to *Candida* infection.

Our most important finding was the *Candida*-specific decreased IL-23 and increased IL-6 production seen only in non-APECED patients, implicating impairment of the IL-6/IL-23/IL-17 axis as the mechanism underlying defective clearance of *Candida* in these patients. In contrast, only APECED patients showed DC hyper-activation, which may underlie altered T-cell responsiveness leading to autoimmunity as well as impaired cell-mediated responses to *Candida*. Both APECED and non-APECED patients demonstrated LPS-induced cytokine hyper-production (IFNγ, IL-2, TNFα, IL-5, IL-13) and impaired DC maturation.

It is currently believed that protective immunity to *Candida* in both mice and humans is highly dependent on the IL-12 initiation of a protective Th1 response, and there is ample evidence supporting the role of DCs as master orchestrators of this scenario [10]. DCs phagocytose *Candida* yeasts leading to production of Th1 cytokines (IL-12), while the β-glucan receptor Dectin-1 and Toll-like receptor (TLR) 2 collaborate to trigger phagocytosis and secretion of IL-12 and TNFα. In contrast, *Candida* hyphae stimulate production of Th2 cytokines (IL-4 and IL-10) and are poor stimulators of IL-12 production. In our study, the low IL-12 produced in both patients and controls, was likely due to stimulation with the hyphal rather than the yeast *Candida* morphotype. This is consistent with our previous work [19] where we also found low/undetectable IL-12 production in CMC patients. A recent study demonstrated that rather than IL-12, hyphae induce IL-23 (see below), which plays a key role in the differentiation of Th17 cells [22]. Fungal triggering of TLRs on DCs is now known to be of paramount importance, with TLR4 implicated in triggering Th1-inducing cytokines (IL-

12) and TLR2 in initiating the Th2 cytokine cascade (IL-4, IL-5) [2]. Our own results (in preparation), demonstrate that stimulated mRNA expression of TLR1-10 and Dectin-1 in the same moDCs as used in this study, differs compared to healthy controls. The high IL-12 levels produced by non-APECED patients in response to LPS in this study, suggest poor control i.e. dysregulation of IL-12 production. Notably, under the same circumstances APECED patients did not show any abnormalities of IL-12 production.

The current belief that the IL-12/IFNγ axis is central for generating protective immunity to fungi is crucially questioned by the fact that patients with inborn errors of this pathway do not demonstrate increased susceptibility to *Candida* or other fungal infections [11], suggesting that other cytokine pathways may be the main players (see below). Indeed, the role of IFNγ in murine models of candidiasis has been controversial [10], while in CMC patients, the effects of IFNγ treatment have been disappointing [23].

A new and significant finding in our study, was that non-APECED CMC patients produced markedly less IL-23 in response to *Candida* stimulation compared to controls, which was not seen in APECED patients. The importance of IL-23 in the generation of interleukin 17-producing T helper (Th-17) cells, initiated by the effect of IL-6 and IL-1 on newly primed CD4 T cells in humans (IL-6 and TGFβ in mice), with IL-23 required for further Th-17 expansion was recently recognised [12]. IL-23 receptor-ligand interaction activates the human signal transducer and activator of transcription 3 (*STAT3*) gene, resulting in binding of IL-17A and IL-17F promoters [12]. Th-17 cells were reported to be preferentially induced by fungal binding and signalling through Dectin-1 [15]. It was also demonstrated that in humans *Candida albicans* specific T memory cells have a Th-17 phenotype and chemokine receptor expression pattern indicative of mucosal homing [13, 14]. In light of these findings, the low levels of IL-23 produced by non-APECED CMC patients in response to *Candida* stimulation could translate into an inability to mount and sustain an anti-fungal Th17 response resulting in impaired clearance of *Candida* in vivo, which is currently

under investigation by our group. Interestingly, stimulation of IL-23 production by TLR2/6 ligand and LPS was comparable in all groups, suggesting that the defect in IL-23 production in non-APECED CMC patients is *Candida*-specific and may be at the level of *Candida* recognition, which is known to simultaneously involve multiple receptors and signalling pathways activated by *Candida*, but not engaged by other stimuli (e.g. TLRs, Dectin-1 and 2, complement receptors, mannose receptor etc) [13].

A crucial finding in our study is the markedly increased production of IL-6 by non-APECED CMC patients, specifically in response to *Candida* and *Candida*-like ligands, but not to LPS. It has been reported that mice deficient in IL-6 demonstrate increased susceptibility to systemic candidiasis [24], while newer studies demonstrate a crucial role for IL-6, together with TGFβ or IL-1 in the initiation of Th17 cells [12]. Recently [25], in a model of experimental autoimmune encephalomyelitis (EAE) it was demonstrated that stimulation of myelin-reactive Th17 cells by IL-6 and TGFb alone leads to IL-17 production but abrogates their pathogenic role, due to co-expression of IL-10. In contrast, stimulation of Th17 by IL-23 leads to IL-17 production in the absence of IL-10, leading to inflammation. In our CMC patients, there is clearly a defect in IL-23 production together with elevated IL-6. It seems this could similarly lead to IL-17 and IL-10 production by T cells, which might suppress the ability to clear the pathogen. Indeed, although moDCs in this study did not produce high IL-10 levels, our previous studies demonstrated very high IL-10 production by *Candida* stimulated whole-blood cultures in CMC patients [18].

An alternative way that IL-6 could influence susceptibility to *Candida* infections in CMC patients would be through its effect on T regulatory cells. It was reported that TLR activation of DCs abrogated the suppressive effects of CD4+CD25+ T regulatory cells partially due to the effect of DC-produced IL-6 on responder T cells [26]. Recently, a direct blocking effect of IL-6 on the *de novo* induction of adaptive T regulatory cells in mice has also been reported [22]. High IL-6 levels seen in non-APECED patients could abrogate the

suppressive effects of T regulatory cells, which could explain the cytokine hyper-production in response to LPS (IL-12, IL-23, IFNγ, IL-2, TNFα, IL-5, IL-13). As opposed to this, in APECED patients, the hyper-production in response to LPS (IFNγ, IL-2, TNFα, IL-13) could also be due to impaired T regulatory cell numbers and/or function, albeit not involving IL-6 as was indeed reported by us [27] and confirmed by others [28]. The resulting dysregulation of cytokine production could undermine an efficient immune response needed to clear the *Candida* infection in CMC patients.

An important and novel finding in this study was impaired moDC maturation in young APECED and non-APECED patients, as evidenced by low CD83 percentages and MFI expression on moDCs. CD83 is a well-recognised marker of DC maturation, and is upregulated on moDCs together with co-stimulatory molecules CD80 and CD86 [29], implying that expression of CD83 is crucial in regulating the development of cellular immunity. It is tempting to hypothesise that this may be of major importance in understanding impaired immunity to *Candida* in CMC patients, and is the first evidence of a DC maturational defect in CMC patients. Our data on defective TLR mRNA downregulation in the same moDCs also suggests impaired DC maturation (in preparation). It is intriguing that this defect is seen only in children and not adults with CMC, suggesting that the immune system gradually overcomes or compensates this defect. Interestingly, APECED (but not non-APECED) patients demonstrated a higher percentage and MFI of CD86+ moDCs after stimulation with CH and TLR2/6, suggesting more of their DCs become activated when exposed to *Candida* antigens. This may be linked to the ongoing *Candida* infections *in vivo* as *Candida* hyphae have generally been reported to increase expression of co-stimulatory molecules and MHC class II on DCs [21], [10]. Activation of DCs without maturation may render these cells efficient in phagocytosing *Candida* antigens but less inefficient in antigen presentation and activation of T cell responses. Altered DC activation in APECED patients

may underlie aberrant T-cell responsiveness leading both to autoimmunity as well as to impaired cell-mediated responses, resulting in increased susceptibility to *Candida* infections.

Taken together, our findings strongly suggest that both APECED and non-APECED CMC patients have impaired/altered DC function, albeit with different defects on the same immune response pathway necessary for protection against *Candida*, and that this impairment may be the pathogenic mechanism underlying increased susceptibility to *Candida* infection in these patients. In APECED patients, we demonstrate impaired DC maturation and hyper-activation, which may underlie increased T-cell responsiveness (also previously demonstrated by others [9]), leading to autoimmunity and impaired handling of *Candida*. As opposed to this, in non-APECED patients, the DC defect is likely at the level of DC cytokine secretion, with inadequate production of IL-23 and an overproduction of IL-6, which together leads to an inefficient IL-17 response. In non-APECED patients the gene defect is not known, but the degree of cytokine dysregulation is reminiscent of findings in the "classical" hyper-IgE syndrome, where it was recently reported that the underlying defect is a mutation in the human *STAT3* gene, which is activated in response to IL-23 (see above) as well as to a wide variety of cytokines and growth factors, resulting in dysregulation of multiple cytokines [30]. Elucidating the immune defect(s) in APECED and non-APECED CMC patients is of paramount significance not only because of the obvious implications for patient management, but because these conditions are prime examples of recently defined "non-conventional" primary immune deficiencies [31], which are characterised by a narrow spectrum of specific infections usually limited to one microbe (in this case *Candida*) as a consequence of inborn errors of immunity. Dissection of underlying mechanisms in diseases such as CMC contributes to the understanding of fundamental pathways in immunity.

ACKNOWLEDGEMENTS

This work was supported by The Primary Immunodeficiency Association (PIA), UK, Registered Charity No # 803217 and The UK / China Scholarships for Excellence Programme. The authors do not have any conflicts of interests to disclose.

We are very grateful to patients, healthy volunteers and their families for supporting and participating in this research. We thank Dr Idse Harrema, Dr Mike Clarke and the Medical and Nursing staff in the Departments of Paediatric Surgery and Paediatric Ophtalmology, Royal Victoria Infirmary, NUTH NHS Foundation Trust for enabling and supporting collection of blood samples from healthy children. We thank Anne Curtis, Head of the Molecular Genetic Diagnostics Laboratory, NUTH NHS Foundation Trust for screening for *AIRE* gene mutations. We are grateful to Daniel Swan, Bioinformatics Department, Newcastle University, for help with data analysis.

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FIGURE LEGENDS

FIGURE 1. Non-APECED patients produce very high IL-6 but low IL-23 levels in response to *Candida*-specific stimuli. Immature moDCs from patients and controls were either left unstimulated or treated with *Candida albicans* hyphae, a specific TLR2/6 ligand or LPS. Culture supernatants were collected at 24h and measured by MSD multiplex assay for detection of IL-6 or ELISA for detection of IL-23. Note log scale for LPS. The level of significance was set at $p<0.05$. Average values are presented as medians with inter-quartile ranges (IQR). **(A) Increased production of IL-6.** Detection limit (----) was 3pg/ml. **(B) Decreased production of IL-23.** Detection limit (----) was 15pg/ml.

FIGURE 2. Impaired upregulation of CD83 in both APECED and non-APECED children in response to *Candida albicans*. Immature moDCs were cultured overnight with either the hyphal form of *Candida albicans*, a specific TLR2/6 ligand, LPS or were left untreated. After 24h, moDCs were harvested, stained with anti-CD83-PE, and analyzed by flow cytometry for CD83 expression. The level of significance was set at $p<0.05$. Average values are presented as medians with inter-quartile ranges (IQR). **(A) Percentage of cells. (B) Median fluorescence intensity (MFI).**

FIGURE 3. moDCs from APECED patients express higher CD86 levels than controls. Immature monocyte-derived DCs were cultured overnight with either the hyphal form of *Candida albicans*, a specific TLR2/6 ligand, LPS or were left untreated. After 24h, DCs were harvested, stained with anti-CD86-FITC, and analyzed by flow cytometry for CD86 expression. The level of significance was set at $p<0.05$. Average values are presented as medians with inter-quartile ranges (IQR). **(A) Percentage of cells. (B) Median fluorescence intensity (MFI). Note log scale.**

Table 1. Patients and Controls

	Total	Adults	Children	Autoantibodies		Endocrinopathy
				Organ-specific	Systemic	
PATIENTS						
APECED	13	5 (1M 4F)	8 (6M 2F)	7	4	10
Non-APECED	16	7 (2M 5F)	9 (5M 4F)	2	5	3
Total	29	12	17	9	9	13
CONTROLS	25	12 (4M 8F)	11 (5M 6F)			

Numbers in boxes denote numbers of patients and age-matched controls in specified groups. Autoantibodies were evaluated in patients sera using indirect immunofluorescence on commercial rodent tissue for systemic autoantibodies and monkey organ tissue for organ-specific autoantibodes. A diagnosis of endocrinopathy was based on routine clinical and laboratory criteria. M = male, F = female

Table 2. LPS-stimulated cytokine hyper-production in APECED and non-APECED patients

pg/ml	APECED	Non-APECED	CTRLS#	APECED	Non-APECED	CTRLS
	UNSTIMULATED			LPS		
IL-12p70	7▲ (6-8)	7 (4-8)	4 (4-5)	291 (73-1583)	1112** (570-2623)	292 (13-861)
IFNγ	93 (84-114)	97 (98-115)	87 (82-101)	130* (101-146)	141* (115-165)	102 (83-118)
IL-2	27 (25-31)	26 (25-29)	25 (23-28)	32* (29-43)	37** (33-44)	29 (23-33)
TNFα	47* (14-89)	56** (32-84)	1 (1-22)	6555* (5233-9692)	5456* (4019-9596)	3568 (928-6618)
IL-10	38 (35-41)	39 (35-42)	37 (32-44)	73 (49-98)	78 (61-148)	65 (50-100)
IL-5	11 (9-12)	11 (10-12)	9 (8-13)	15 (12-20)	17** (13-21)	12 (11-15)
IL-13	3 (3-4)	3 (3-4)	2 (2-3)	5 (4-7)	6 (3-7)	3 (2-4)

Immature moDC were stimulated with LPS or left unstimulated. After 24h, cytokine levels in culture supernatants were analyzed by MSD multiplex assay. Detection limits for each cytokine are given in section "Materials and Methods". #CTRLS - healthy controls; ▲ = medians (interquartile ranges); *p<0.05, **p<0.01 compared to controls.

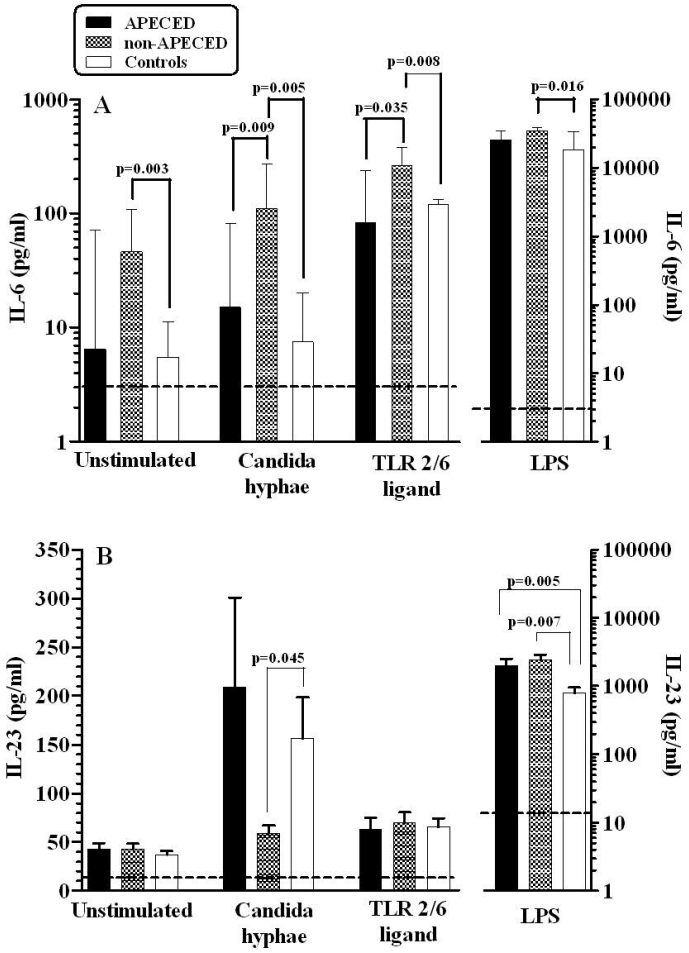

Fig 1

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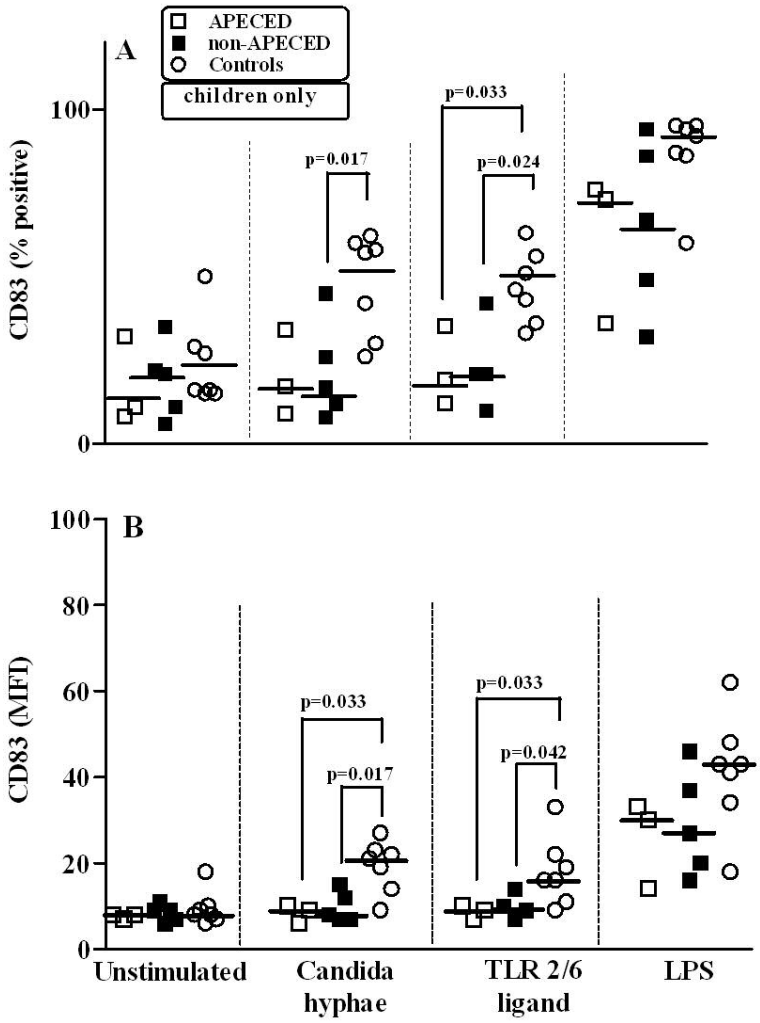

Fig 2

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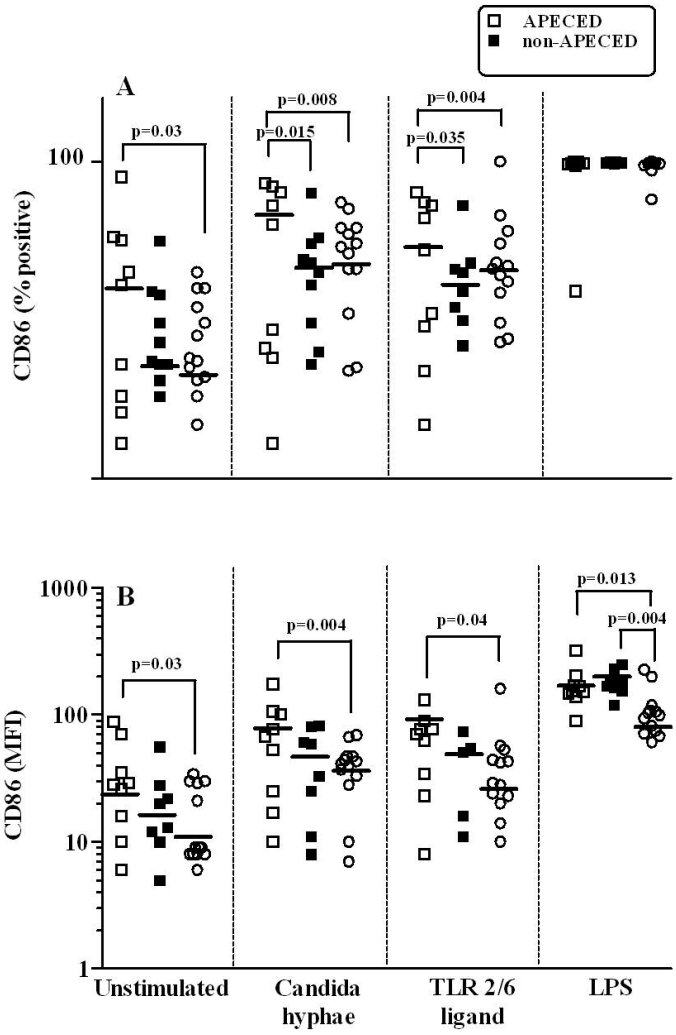

Fig 3

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