Pattern recognition receptor expression is not impaired in patients with chronic mucocutaneous candidiasis with or without autoimmune polyendocrinopathy candidiasis ectodermal dystrophy


*Institute for Cellular Medicine, Faculty of Medical Sciences, 9Bioinformatics Support Unit, and 8School of Mathematics and Statistics, Newcastle University; 7Department of Paediatric Immunology, and 9Regional Immunology Department, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne, 7Booth Hall Children’s Hospital, University of Manchester, 6Education and Research Centre, Wythenshawe Hospital, Manchester, 9‘Northern Ireland Regional Genetics Service, Belfast City Hospital, Belfast, Northern Ireland, UK, and 9Our Lady’s Hospital for Sick Children, Dublin, Republic of Ireland

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Correspondence: D. Lilic, Musculoskeletal Research Group (MRG), Institute for Cellular Medicine, Faculty of Medical Sciences, Newcastle University, Newcastle upon Tyne NE2 4HH, UK.
E-mail: desa.lilic@ncl.ac.uk

Present addresses: 1Nanjing University of Chinese Medicine, Nanjing, China; 2Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT, USA.

Introduction

Pattern recognition receptors (PRRs) on cells of the innate immune system recognize core structures specific to microorganisms (pathogen-associated molecular patterns: PAMPs) that are alien to mammals [1,2]. Toll-like receptors (TLR), particularly TLR-1, -2, – and –6, and C-type lectin receptors such as Dectin-1 and -2, mannose receptor and dendritic cell (DC)-specific intercellular adhesion molecule-3-grabbing non-integrin, are known to be important PRRs in fungal recognition, initiating immune responses to these pathogens [3]. More recently, complex interactions and collaboration of these receptors through formation of heterodimers have been demonstrated to modulate immune responses to different fungi [4].

In mice, activation of TLR-4 initiates interleukin (IL)-12 production by DCs generating a protective Thelper type 1 (Th1) response. In contrast, activation of TLR-2 mediates a

Summary

Patients with chronic mucocutaneous candidiasis (CMC) have an unknown primary immune defect and are unable to clear 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, suggesting that defective expression of pattern recognition receptors (PRRs) may underlie disease pathogenesis. In 29 patients with CMC (13 with APECED) and controls, we assessed dendritic cell (DC) subsets and monocyte Toll-like receptor (TLR) expression in blood. We generated and stimulated monocyte-derived (mo)DCs with Candida albicans, TLR-2/6 ligand and lipopolysaccharide and assessed PRR mRNA expression by polymerase chain reaction [TLR-1–10, Dectin-1 and -2, spleen tyrosine kinase (Syk) and caspase recruitment domain (CARD) 9] in immature and mature moDCs. We demonstrate for the first time that CMC patients, with or without APECED, have normal blood levels of plasmocytoid and myeloid DCs and monocyte TLR-2/TLR-6 expression. We showed that in immature moDCs, expression levels of all PRRs involved in antifungal responses (TLR-1, -2, -4, -6, Dectin-1, Syk, CARD9) were comparable to controls, implying that defects in PRR expression are not responsible for the increased susceptibility to Candida infections seen in CMC patients. However, as opposed to healthy controls, both groups of CMC patients failed to down-regulate PRR mRNA expression in response to Candida, consistent with defective DC maturation, as we reported recently. Thus, impaired DC maturation and consequent altered regulation of PRR signalling pathways rather than defects in PRR expression may be responsible for inadequate Candida handling in CMC patients.

Keywords: APECED, Candida, DCs, PRRs, TLR
non-protective Th2 response via IL-10 production and generation of T regulatory cells [5]. TLR-1 and TLR-6 form heterodimers with TLR-2, modulating the Th1/Th2 balance in response to fungi [6], while Dectin-1 synergizes with TLR-2 and TLR-4 for cytokine production [7]. However, humans with inborn errors of the IL-12/interferon (IFN)-γ pathway do not show increased susceptibility to Candida or other fungal infections [8], suggesting that this pathway might not have the same importance in fungal defences in humans as in mice. Recent evidence suggests that the Th17 pathway is involved crucially in immune response to fungi [9]. Dectin-1 signalling through a non-TLR signalling pathway, that involves the spleen tyrosine kinase (Syk) and adaptor caspase recruitment domain (CARD) 9 [10], primes DCs to ‘instruct’ differentiation of CD4+ IL-17 producing effector T cells, demonstrating a direct link between the innate and adaptive immune system [11] in generating protective fungal responses.

Chronic mucocutaneous candidiasis (CMC) is a primary immunodeficiency disorder (PID) with selective susceptibility to recurring and/or persistent debilitating infections with the yeast Candida [12], and includes several subtypes. The APECED syndrome (autoimmune polyendocrinopathy candidiasis ectodermal dystrophy), also known as APS1 (autoimmune polyendocrinopathy type 1), is associated with organ-specific autoimmunity of particularly the endocrine glands and an underlying mutation of the AIRE gene (online mendelian inheritance in man – OMIM 240300). Other subgroups of CMC include patients with associated thyroid disease but no other autoimmunity (OMIM 606415), isolated CMC with various modes of inheritance (OMIM 11458, OMIM 212050) and sporadic CMC. In these CMC patients the diagnosis remains clinical, given that a genetic or biochemical marker is not yet available.

Very little is known about the immune defect underlying increased susceptibility to Candida infections in CMC patients. Previously we have demonstrated dysregulated cytokine production in response to Candida [12], suggesting that the immune defect might be at the level of orchestrating appropriate cytokine responses, rather than the effector T cell level itself.

Our assumption that defects of PRRs, which can recognize moieties from many different organisms, could specifically cause an immune defect against Candida is based on the recent recognition of similar scenarios underlying non-conventional primary immune deficiencies (PIDs), defined as a selective susceptibility to a single weakly pathogenic or opportunistic organism. Surprisingly, in these PIDs a predisposition to a single type of infection is caused by immune defects affecting pathways central to the immune response. Examples include disorders of the IL-12/IFN-γ circuit, resulting in selective susceptibility to infections with mycobacteria and Salmonella; defects in the TLR-3 pathway resulting in selective predisposition to Herpes simplex infection; MyD88 deficiency and susceptibility to pyogenic bacterial infections. The infectious phenotype that the above disorders confer in humans is much narrower than those of corresponding mutant mice, suggesting that there is much redundancy in human host defence in nature [13,14]. In analogy, given that DCs are central initiators of immune responses, while PRRs and TLRs are known to be involved in production of cytokines in protective Candida immunity, we hypothesized that reduced DC numbers and/or a defect of PRRs involved in immune responses to Candida might underlie a selective susceptibility to this microorganism in patients with CMC.

In CMC patients and age- and gender-matched healthy controls we investigated the proportion of DCs and DC subsets in peripheral blood, as well as TLR receptor expression on blood monocytes; we then isolated monocytes from peripheral blood and cultivated them in vitro into monocyte-derived DCs (moDCs), which we stimulated/matured with Candida and other relevant antigens to assess expression of TLR-1–10, Dectin-1 and 2, CARD9 and Syk. We demonstrate for the first time that CMC patients, with or without APECED, have normal blood levels of plasmacytoid and myeloid DCs and monocyte TLR-2/TLR-6 expression. In immature moDCs, expression levels of all PRRs involved in anti-Candida responses (TLR-1, -2, -4, -6, Dectin-1, Syk, CARD9) were comparable to controls. However, as opposed to healthy controls, both groups of CMC patients failed to down-regulate PRR mRNA expression in response to Candida, suggesting that impaired DC maturation and consequently altered regulation of PRR signalling pathways rather than defects in PRR themselves, may be responsible for inadequate Candida handling in CMC patients.

These results complement our previous report of impaired DC maturation, presented in a separate publication [15], where activation and function of these same DCs was investigated in parallel through cytokine production and cell surface marker expression.

Materials and methods

General conditions

The moDCs 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 not deemed acceptable 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 [16]. With the aim of investigating putative impaired Candida binding to DCs, we assessed moDC stimulation with a TLR ligand 2/6 (MALP-2) that engages selectively the same TLRs that are known to bind Candida and other yeasts [17]. Lipopolysaccharide (LPS) was used as
a ‘positive’ non-\textit{Candida} control, in order to assess moDC functionality in response to other potent stimuli. Repeat assessments and inclusion of additional stimuli were limited by the quantity of blood we could draw from each patient, particularly children.

\subsection*{Subjects}

\textit{Patients.} We studied 29 patients with CMC, 13 APECED with a confirmed \textit{AIRE} gene mutation and 16 non-APECED without. All patients were screened for the two most common \textit{AIRE} gene mutations: p.R257X (a non-sense mutation in exon 6) and c.964del13 (a 13 base pairs deletion in exon 8) (Huch-Laboratory Diagnostics, Helsinki University Hospital, Finland and Northern Molecular Genetics Service, Institute of Human Genetics, Newcastle Upon Tyne, UK). Thirteen patients were found to have an \textit{AIRE} gene mutation and the APECED syndrome, of whom nine had the c.964del13 deletion and four had the p.R257X mutation. In the remaining 16 non-APECED patients an \textit{AIRE} mutation was not detected. None of the patients without the \textit{AIRE} gene mutation had any clinical signs suggestive of APECED that would justify investigations of additional \textit{AIRE} gene mutations. All patients were screened for autoantibodies to type 1 IFNs, which were shown recently to be highly specific for APECED patients [18]; these autoantibodies were present in all APECED patients and none of the non-APECED patients and controls.

In the APECED group, 10 patients had affected siblings and nine patients in the non-APECED group, who are all included in this study. Three patients from the latter group had hypothyroidism, two with thyroid peroxidase antibodies. No more than three patients from any one family were studied. At the time of sampling, patients did not have any other serious infections, were not on systemic antibiotic treatment or receiving steroids. All patients suffered from recurrent mucocutaneous \textit{Candida} infection (mouth, nails, skin, oesophagus and perineum), but no other fungal infections including dermatophyte (e.g. tinea corporis) infections. Patients were screened for systemic autoantibodies including anti-nuclear factor (NF), smooth muscle, liver–kidney microsomal, mitochondrial and gastric parietal cell antibodies. Systemic autoantibodies were found in four of 13 APECED patients and five of 16 non-APECED patients. Organ-specific autoantibodies and/or endocrinopathy were observed in one or more of the following organs: parathyroid, thyroid, adrenal cortex, gonads and pancreas. Autoantibodies were evaluated in patients’ sera using indirect immunofluorescence on commercial rodent tissue (Euroimmun, Lubeck, Germany) for systemic autoantibodies and monkey organ tissue (The Binding Site, Birmingham, UK) for organ-specific autoantibodies. Endocrinopathy was diagnosed if/when there was clinical and laboratory evidence of glandular hypofunction.

\subsection*{Controls.}

A total of 25 age- and sex-matched controls was recruited for the study. Adults were healthy laboratory volunteers, while control children were undergoing general anaesthesia for surgery to treat non-infectious causes (eye squints, circumcision, hernia, etc.).

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.

\subsection*{Generation of moDCs from patient blood}

Peripheral blood mononuclear cells were isolated from patient blood by density centrifugation over a layer of lymphophrep (Axis-Shield, Oslo, Norway). CD14-positive cells were then purified by magnetic separation on an LS column following labelling of the cells with anti-CD14-coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), which regularly yielded a purity of 94–98% CD14+ cells, excluding T cell contamination. Purified monocytes were then seeded into a 24-well plate at 0·75 × 10^4 monocytes per well in 1 ml total volume of RF10 media. RF10 media consists of RPMI-1640 (BioWhittaker, Lonza, Wokingham, UK) media supplemented with 10% fetal calf serum (FCS) (PAA Laboratories, Pasching, Austria), 2 mM L-glutamine (Sigma Aldrich, St Louis, MO, USA), and 1% penicillin–streptomycin (Gibco, Carlsbad, CA, USA). Fifty ng/ml IL-4 and granulocyte–macrophage colony-stimulating factor (GM-CSF) (Immunotools, Friesoythe, Germany) were added to each well. Cells were incubated at 37°C with 5% CO\textsubscript{2}. On day 3, the addition of 50 ng/ml IL-4 and GM-CSF to the wells was repeated.

\subsection*{Dendritic cell maturation}

On day 6 of the DC culture, immature DCs were divided into four groups and activated as follows: no treatment (unstimulated); addition of 1:10 000 final dilution of heat-killed CH \{American Type Culture Collection (ATCC) \#18804, Manassas, VA, USA\}; 1 mg/ml of LPS (Invivogen, San Diego, CA, USA); and 10 ng/ml of the purified TLR-2/6 ligand, MALP-2 (Aptech, Epalinges, Switzerland). Cells or cytokines were harvested for analysis after 24 h on day 7.

\subsection*{Flow cytometry}

To assess TLR expression on monocytes and quantitate DC subsets in the blood, antibodies to various cell surface molecules and appropriate isotype controls were added directly to 40 μl aliquots of patient blood. After a 15-min incubation at room temperature, red blood cells were lysed by the addition of 1·5 ml of BD FACS Lysing Buffer (BD Biosciences, San Jose, CA, USA) for 10 min. Samples were then washed 2× in 1 ml fluorescence activated cell sorter (FACS) wash (phosphate-buffered saline + 0·1% bovine serum albumin)
and fixed in 1% paraformaldehyde (Sigma-Aldrich). The following antibodies were used to stain cells in whole blood: CD11c-fluorescein isothiocyanate (FITC), CD3-phycocerythrin (PE), CD14-FITC, CD14-PE (ImmunoTools, Friesoythe, Germany), CD19-PE, human leucocyte antigen D-related (HLA-DR)-peridinin chlorophyll (BD Biosciences), CD123-allophycocyanin (Miltenyi Biotec), TLR-1-PE, TLR-2-PE, TLR-4-PE (eBioscience, San Diego, CA, USA), TLR-6-FITC (Imgenex, San Diego, CA, USA), immunoglobulin (Ig)G1-FITC, IgG1-PE, IgG2a-PE (BD Pharmingen, San Jose, CA, USA) and appropriate isotype controls (BD Biosciences). DCs were identified as HLA-DR/CD11c (myDCs) or CD123 (pDCs) positive, CD3, CD14, CD19 negative cells. CD14+ cells were stained for TLR expression.

All stained cells were acquired using a FACScan (BD Biosciences) equipped with a 488 nm laser and a 633 nm laser upgrade. Acquired events were analysed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Real-time polymerase chain reaction

RNA was purified using the RNaseasy Mini kit, including an on-column DNase I digestion (Qiagen, Crawley, UK). cDNA was synthesized from 0.4 μg of total RNA, using Superscript II reverse transcriptase and random hexamers in a total volume of 20 μl according to the manufacturer’s instructions (Invitrogen). cDNA was stored at −20°C until used in downstream real-time polymerase chain reaction (PCR). Oligonucleotide primers were designed using Primer3 as part of the Universal Probelibrary package (Roche; http://www.roche-applied-science.com).

Primers for PRRs were as follows: TLR-1 forward 5′-CCTAGGAGTTATCCACAAGGCTCAA-3′, reverse 5′-TCTTTCCTGGGCACT TC-3′; TLR-2 forward 5′-CGTTC TCTCAGGGTACTGCTC-3′, reverse 5′-CCCTTG GATCC TGCTTGC-3′; TLR-3 forward 5′-AGTTGCTCATGAAAT CAATTTAAA GAG-3′, reverse 5′-AATTTCCAATGGG TGA AAA-3′; TLR-5 forward 5′-GA CCAATCTCGGCT GACTG-3′, reverse 5′-GCCAGGAACATGAAATC CAA-3′; TLR-6 forward 5′-TGAGGT TTAGCGCTGCCAGTTAG-3′, reverse 5′-GCATT TACT CAAAAGAGACTGTTTCA-3′; TLR-7 forward 5′-GCTAGACTGTCTC AAAAA ACAAA AA-3′, reverse 5′-GCCCA AACACTCAATCTGCAC-3′; TLR-8 forward 5′-GGGAGGAATGAGGACTCTTT TTT-3′, reverse 5′-TCAGCATTCAGCAGCTGA GA-3′; TLR-9 forward 5′-TTG TTCAGATCTC TCTCC GCAG-3′; TLR-10 forward 5′-TCTCAGCCA TCTTGG ATT-3′, reverse 5′-TTGATTTCTTCCGCAT TTA-3′; Syk forward 5′-CTTT CTGGGCCCCGA CA-3′, reverse 5′-TTGGGTACCGTTGGTCTA GA-3′; Dectin-1 forward 5′-CCTTCTGGGC CCA-3′, reverse 5′-AGGGAGGACTTCTTCTTCG TT-3′; Dectin-2 forward 5′-TGAA GAGACTTCTTCAATC-3′, reverse 5′-TCAAGGAA GCTTGCAGAA-3′.

Relative quantitation of genes was performed using the ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA). TLR-1, -2, -3, -5-10, Dectin-1, Syk and Dectin-2 expressions were determined using SYBR Green (Tokara Bio Inc., Shiga, Japan), in accordance with the manufacturer’s suggested protocol. PCR mixtures contained 50% SYBR-Green PCR mix (Tokara Bio Inc.); 50 nM (except 500 nM for TLR-3 and -10) of each primer and 3.2 ng cDNA in a total volume of 20 μl. Conditions for PCR were as follows: 10 s at 95°C, then 40 cycles each consisting of 5 s at 95°C and 30 s at 60°C, followed by a dissociation plot. To confirm that the amplification product was a single amplicon, products were analysed by agarose gel electrophoresis.

The TLR-4 and CARD9 expression was determined using TaqMan gene expression assays. PCR mixtures contained 50% TaqMan mastermix reagents (Sigma-Aldrich), 1 μl of primer and probe mixture (assay ID Hs00152939_m1 for TLR-4, Hs00364485_m1 for CARD9; Applied Biosystems), 1 μl H2O and 3.2 ng cDNA in a total volume of 20 μl. Conditions for PCR were as follows: 2 min at 50°C, 10 min at 95°C, then a following 40 cycles, each consisting of 15 s at 95°C and 1 min at 60°C.

The GAPDH gene was used as an endogenous control to normalize for differences in the amount of total RNA present in each sample; GAPDH TaqMan primers and probe were purchased from Applied Biosystems. TaqMan mastermix reagents (Sigma-Aldrich) were used according to the manufacturer’s protocol. Where data are presented, the 2−(ΔΔCT) calculation was used as an approximate measure of expression to allow comparison of expression levels between genes.

The C. albicans

Freeze-dried C. albicans was purchased from ATCC (#18804) and rehydrated according to the supplier’s instructions. C. albicans was grown in autoclaved 1× broth [67 g/l yeast nitrogen base (YNB) and 10% D-glucose] at 30°C. Candida yeasts were propagated into hyphal forms in RPMI-1640 with 10% FCS at 37°C and heat-killed before use in DC cultures. The concentration of Candida yeasts in YNB was determined to be 24·5×106 by counting in a haemocytometer (10 mg/ml protein content). Transformation of >98% yeasts into hyphal forms was confirmed by visual inspection under the microscope. Dense flasks of hyphae were decanted into newly purchased sterile glass bottles and heated in a pressure cooker for 30 min at 120°C, based on previous titration experiment. Heat-killed C. albicans was centrifuged at 400 g for 10 min, and the supernatant was removed without disrupting the pellet. The pellet material was used in cell cultures at a final concentration of 1:10 000.

Statistical analysis

Results were analysed separately for groups of patients with a confirmed AIRE gene mutation (APECED), patients
myDCs 0·54 0·46 0·52

Monocytes, identified as CD14+ cells, showed significantly higher percentages in APECED and non-APECED patients compared with controls (Table 1). There was no significant difference in percentages of myeloid and plasmacytoid DCs between controls, APECED and non-APECED patients (Fig. 1).

Expression of TLR-2 and TLR-6 on monocytes ex vivo

The percentage and intensity of TLR-2 and TLR-6 expression was assessed on peripheral blood monocytes, defined as CD14+ cells. The percentage of TLR-2+ CD14+ cells did not differ between patient groups and controls, but median fluorescence intensity (MFI) was significantly higher in APECED patients compared with controls and non-APECED patients. Interestingly, non-APECED patients had a significantly higher frequency of TLR-6+ CD14+ monocytes compared with both APECED patients and controls, although the MFI was comparable (Table 1).

The moDC mRNA expression of TLR-1, -4 and -6 (involved in protection against Candida)

All groups had similar TLR-1 mRNA expression in immature DCs. Healthy controls significantly down-regulated TLR-1 expression in response to all stimuli, in particular to LPS (Fig. 2a). As opposed to this, APECED and non-APECED CMC patients failed to down-regulate TLR-1 mRNA expression following stimulation with either CH or TLR-2/6 ligand, although they did respond to LPS (Fig. 2a). APECED and non-APECED patients expressed lower levels of TLR-4 mRNA in immature DCs compared with controls, albeit not significantly. As opposed to controls where TLR-4 was down-regulated vigorously to all stimuli used, non-APECED patients did not respond to CH, while APECED patients did not respond to either CH or TLR-2/6 ligand. They did respond, however, to LPS (Fig. 2b). An identical pattern to TLR-1 was seen for TLR-6 (Fig. 2c), i.e. controls down-regulated mRNA expression in response to all stimuli used without a confirmed AIRE gene mutation (non-APECED) and healthy controls. Statistical analyses were performed using the freely available software package R [19]. For each gene, we fitted a random effects model with treatment type, subgroups, age and patient as covariates. Interactions between treatment type and subgroups were also considered. Where necessary we transformed the expression level via a log or a square root transformation. Variables that were not statistically significant at the 0·05 level (based on the ANOVA results) were removed and the model was refitted. At each point of model fitting the residuals were examined to check that our assumptions were valid. To explore individual differences among subgroups and treatments we calculated adjusted P-values to control the family-wise error rate. Average values are presented as means ± standard errors of the mean (s.e.m.); level of significance was set at P < 0·05.

The graphic network in Fig. 4 was calculated using graphic Gaussian models and shows partial correlations which took into account all available variables. Inclusion and exclusion of edges was determined using the method described by Drton and Perlman [20].

Expression analysis and clustering were assessed with GeneSpring, Agilent Technologies software. The data analysis for the clusters involved importing the data into GeneSpring and genes were clustered using a 5-cluster k-means with 100 iterations using a Pearson correlation for the similarity measure.

Results

Percentages of monocytes, plasmacytoid and myeloid DC subsets in peripheral blood (ex vivo) from patients with CMC and controls

In peripheral blood, monocytes, identified as CD14+ cells, showed significantly higher percentages in APECED and non-APECED patients compared with controls (Table 1). There was no significant difference in percentages of myeloid and plasmacytoid DCs between controls, APECED and non-APECED patients (Fig. 1).

Table 1. Whole blood myeloid (my) and plasmacytoid (p) dendritic cell (DC) percentages and expression of Toll-like receptor (TLR)-2 and TLR-6 on CD14+ monocytes.

<table>
<thead>
<tr>
<th></th>
<th>APECED</th>
<th>Non-APECED</th>
<th>Controls</th>
<th>APECED</th>
<th>Non-APECED</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14+ Percentage(%)</td>
<td>7·6** (±0·4)</td>
<td>8·0** (±0·7)</td>
<td>6·3 (±0·6)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>TLR-2</td>
<td>97 (±1·5)</td>
<td>95 (±1·3)</td>
<td>93 (±2·4)</td>
<td>193* (±21)</td>
<td>160 (±9)</td>
<td>142 (±19)</td>
</tr>
<tr>
<td>TLR-6</td>
<td>83 (±6)</td>
<td>96** (±1)</td>
<td>74 (±8)</td>
<td>27 (±8)</td>
<td>16 (±2)</td>
<td>14 (±2)</td>
</tr>
<tr>
<td>myDCs</td>
<td>0·54 (±0·01)</td>
<td>0·46 (±0·01)</td>
<td>0·52 (±0·01)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>pDCs</td>
<td>0·10 (±0·02)</td>
<td>0·07 (±0·01)</td>
<td>0·10 (±0·01)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

*P = 0·03; **P = 0·02. DCs in whole blood were identified as human leucocyte antigen D-related (HLA-DR) positive, CD11c (myDCs) or CD123 (pDCs) positive, CD3, CD14, CD19 negative cells. Monocytes, identified as CD14+ cells, were stained for TLR expression. Numbers are means ± standard error of the mean; n.a., not applicable. APECED, autoimmune polyendocrinopathy candidiasis ectodermal dystrophy.
while APECED and non-APECED patients responded only to LPS.

In summary, healthy controls down-regulated TLR-1, -4 and -6 mRNA expression significantly in response to all stimuli used. In contrast, APECED and non-APECED CMC patients failed to down-regulate TLR-1, -4 and -6 expression following stimulation with CH and/or TLR-2/6 ligand, although the response to LPS stimulation was similar to controls.

The moDC mRNA expression of TLR-2 (with controversial role in protection against Candida)

The TLR-2 mRNA in immature moDCs was readily detectable and comparable in both patient groups and controls. Interestingly, upon stimulation/maturation, healthy controls down-regulated TLR-2 expression only in response to LPS. CMC patients, both APECED and non-APECED failed to down-regulate TLR-2 in response to any of the stimuli (data not shown).

The moDC mRNA expression of TLR-3, -5 and -10 (not known to have a role in protection against Candida)

The TLR-3 and TLR-10 mRNA were detectable, but did not change in response to any of the stimuli used (data not shown). An unusual finding was the down-regulation of TLR-5 mRNA expression in healthy controls in response to all stimuli – CH, TLR-2/6 ligand and LPS. Non-APECED patients also responded to all three stimuli while APECED patients did not respond to any (data not shown).

Dectin-1, Syk and CARD9 moDC mRNA expression

Immature moDCs expressed readily detectable and comparable levels of mRNA in patients and controls. Dectin-1 mRNA levels in moDCs did not change in either healthy controls or CMC patients (both APECED and non-APECED) in response to CH or TLR-2/6-ligand stimulation, but decreased significantly following LPS stimulation (Fig. 3a). In contrast, healthy controls responded by
Fig. 2. Messenger RNA (mRNA) expression of Toll-like receptors (TLR)-1, -4 and -6 in monocyte-derived DCs (moDCs) from chronic mucocutaneous candidiasis (CMC) patients with or without autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) and controls following stimulation with Candida albicans hyphae (CH), TLR-2/6 ligand and lipopolysaccharide (LPS) (means ± standard error of the mean). Immature moDCs from patients and controls were either left unstimulated or treated with CH, a specific TLR-2/6 ligand or LPS. Immature moDCs from CMC patients demonstrate normal mRNA expression, whereas down-regulation after stimulation with CH and TLR-2/6 is impaired.

Fig. 3. mRNA expression of Dectin-1, Syk and CARD9 in monocyte-derived dendritic cells (moDCs) from chronic mucocutaneous candidiasis (CMC) patients with or without autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) and controls following stimulation with Candida albicans hyphae (CH), Toll-like receptor (TLR)-2/6 ligand and lipopolysaccharide (LPS) (means ± standard error of the mean). Immature moDCs from patients and controls were either left unstimulated or treated with CH, a specific TLR-2/6 ligand or LPS. Immature moDCs from both CMC patients and controls have detectable mRNA expression, whereas down-regulation after stimulation with CH and TLR-2/6 is seen only with Syk.
decreasing Syk mRNA expression in response to all three stimuli. Further, APECED patients responded only to LPS stimulation but not to either CH or TLR-2/6-ligand. Non-APECED patients did not respond to TLR-2/6-ligand stimulation but decreased their mRNA expression following stimulation with both CH and LPS (Fig. 3b). Interestingly, healthy controls did not alter CARD9 expression except in response to LPS, as was also the case with APECED and non-APECED patients (Fig. 3c). An additional finding was that in healthy controls, the levels of Dectin-1 mRNA increased significantly with age, which was not seen in either APECED or non-APECED CMC patients (data not shown).

The moDCs TLR-7, -8, -9 and Dectin-2 mRNA expression

mRNA levels of TLR-7, -8, -9 and Dectin-2 were undetectable or borderline low in both immature and mature moDCs with all stimuli used.

A summary of changes in mRNA PRR expression following stimulation in APECED, non-APECED patients and controls is given in Table 2.

Based on the above results and data on cytokine production by these same moDCs published previously [15], we present a graphic dependency network (Fig. 4) demonstrating the interactions between cytokine production and PRR expression by stimulated moDCs from CMC patients and healthy controls. The network demonstrates the interdependence of cytokines and PRRs relevant for protective immunity to Candida, and highlights differences between CMC patients and healthy controls. The network also suggests that the immune defect in CMC patients may lie in the interaction of TLR-1, -4, -6 and Th1 cytokine production, which may be compensated by the Dectin-Syk-CARD9 pathway in CMC patients.

Clustering analysis of PRRs and cytokine levels, which were reported previously [15], showed associations for TLR-1, -4, -6, Dectin-1, Syk, CARD9 (set 1), TLR-2, -5, -10, IL-2, -10, -13 (set 2) and TLR-7, 8, IL-6, IL-12, IL-23, IL-27 (set 3), which did not differ significantly between healthy controls and patients (data not shown).

Discussion

Candida infections range from benign, commensal colonizations of skin and mucous membranes in normal individuals to persistent, debilitating mucocutaneous infections (as seen in CMC patients) or life-threatening systemic candidaemias with a significant mortality of more than 30% in immunocompromised patients [16]. The role of the innate immune system in protection against Candida has long been recognized, but was thought to be limited to first-line phagocytosis and killing. More recently, it has become clear that the innate immune system not only distinguishes between various microorganisms, but through interactions with DCs initiates and guides subsequent adaptive immune responses [21]. The crucial task of recognizing invading pathogens and activating host responses is delivered by PRRs, which recognize PAMPs on the microbe’s cell wall [3].

Most components of the fungal cell wall are not found in mammals and are thus ideal PAMPs for recognition of non-self. The core of the C. albicans cell wall is made of polysaccharide fibrils composed of β-glucan and chitin. The outer layer is made of proteins that are glycosylated with mannose – coined ‘mannan’ or ‘mannoprotein’. These structures differ between fungi and are the major structures recognized by PRRs such as TLRs and CTLs [22].
The role of TLRs in defence was first recognized in the Drosophila model of fungal infection [23]. Subsequent studies demonstrated that ligand recognition by TLRs induces differential activation of signalling cascades and leads to cytokine and chemokine production [24]. TLR-2 and TLR-6 were the first TLRs shown to be involved in recognition of a fungal structure – zymosan, derived from Saccharomyces cervisiae – and TLR-6 were the first TLRs shown to be involved in triggering cytokine production by C. albicans. Recognition of Candida at the cellular level is mediated by TLRs and CTL receptors [25]. TLR-4 induces mainly proinflammatory signals through the MyD88-dependent pathway, leading to activation of NF-κB, mitogen-activated protein-kinases and interferon-releasing factor 3 (IRF-3), stimulating Th1 responses through production of IL-12, IFN-γ, tumour necrosis factor (TNF-α) and type 1 IFNs, resulting in efficient elimination of Candida infection. In contrast, binding of Candida to TLR-2 leads to production of transforming growth factor (TGF)-β and IL-10, leading to tolerance, immunosuppression and generation of T regulatory cells [26]. However, TLR-2 is also able to associate with Dectin-1 in the induction of proinflammatory responses [27]. In CMC patients, we demonstrated previously low IL-12 and very high IL-10 and IL-6 production by peripheral blood mononuclear cells [28], with low IL-23 and high IL-6 responses by moDCs [15]. The results in our current study show normal expression of TLRs and CTLs on immature moDCs but inadequate down-regulation of TLR-1, -4 and -6, suggesting defects in signalling pathways downstream of these receptors. More recent data suggest that TLR-9 can recognize fungal DNA, but the importance of this in fungal defence is still not clear [3]. Whether any of these pathways is involved directly or impacts upon other signalling pathways such as Dectin-1 is unclear, as would be suggested by our ongoing studies which have demonstrated markedly reduced IL-17 production in non-APECED (but not APECED) patients [29]. AIRE gene mutations have also been shown to down-regulate immunological pathways critically in DCs from APECED patients [30], suggesting that this mechanism may be involved in impaired immune responses to Candida. However, AIRE gene mutations may affect DC function in alternative ways, such as increasing antigen-presentation to T cells [31] or interfering with T regulatory cell induction/function, as has been demonstrated [32,33].

Recently, patients with MyD88 deficiency were reported to suffer with pyogenic infections, including invasive pneumococcal diseases, but were otherwise healthy. Our patients with CMC did not suffer with severe, life-threatening infections caused by pyogenic bacteria, although a previous report in children with CMC demonstrated a high incidence of bacterial infection and mortality from non-Candida infections [34].

Mannose-binding lectin (MBL) is a soluble C-type lectin receptor that binds Candida and other microorganisms, promoting complement deposition. Low levels of MBL in humans predispose to infections, although MBL-deficient mice do not show increased susceptibility to infections with Candida [35]. There is some evidence for a role of MBL in recurrent vulvovaginal candidiasis [36], but its importance – if any – in CMC is not clear.

In this study, we show for the first time that patients with CMC, both APECED and non-APECED, have normal frequencies of both myeloid and plasmacytoid DC subsets in peripheral blood, indicating that a major lack of these cells

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**Fig. 4.** Graphic dependency network describing interactions between cytokine production and PRR expression on monocyte-derived DCs (moDCs) from chronic mucocutaneous candidiasis (CMC) patients and healthy controls, relevant for protective immunity against Candida. The graphical network was calculated using graphical Gaussian models. Each line indicates significant partial correlations between genes. Black lines denote connections on networks for both CMC patients and healthy controls. Red lines denote connections on network for healthy controls only. Blue lines denote connections on network for CMC patients and healthy controls. Red lines denote connections on networks for both CMC patients and healthy controls. Blue lines denote connections on networks for healthy controls only. Black lines denote connections on networks for both CMC patients and healthy controls. Red lines denote connections on networks for both CMC patients and healthy controls. Black lines denote connections on networks for both CMC patients and healthy controls. Red lines denote connections on networks for both CMC patients and healthy controls.

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is not responsible for their increased susceptibility to *Candida* infections. The higher frequency of blood monocytes in CMC patients (both APECED and non-APECED) might be due to ongoing *Candida*-induced inflammation. Almost all monocytes in both patients and controls expressed TLR-2, which was up-regulated in APECED patients. Interestingly, TLR-6 was expressed on almost all monocytes in non-APECED patients but not in the other groups. These findings argue against an intrinsic defect in TLR-2 and TLR-6 expression on monocytes in CMC patients as an underlying cause of their increased susceptibility to *Candida* infections.

In our study, TLR-1, -4, -6, -2 and TLR-5 mRNA was expressed in immature moDCs in all patients and controls and the level of expression did not differ significantly between groups. This is the first study to report that expression of these TLRs in immature myeloid DCs of APECED and non-APECED CMC patients is normal.

Previous studies have demonstrated that when moDCs are induced to mature by stimulation with pathogens or pathogen PAMPs, levels of TLR mRNA decrease or disappear, as opposed to macrophages and monocytes [37] where TLR mRNA increases. In healthy individuals, decreased mRNA expression was already reported 2 h after stimulation, but was most obvious after 24 h [38], suggesting that decreased levels of TLR mRNA are consistent with normal DC maturation dynamics. In our study, we found that healthy controls down-regulated TLR-1, -4, -6 and -5 in response to all stimuli used (CH, TLR-2/6 ligand and LPS). In contrast, APECED patients responded only to LPS and did not down-regulate TLR-1, -4, -6 and -5 mRNA expression when stimulated with either CH or TLR-2/6 ligand. Non-APECED patients failed similarly to down-regulate TLR-1 and TLR-6, but they did down-regulate TLR-4 in response to TLR-2/6 ligand (but not CH) and down-regulated TLR-5 in response to all stimuli, similar to controls. On the other hand, TLR-2 expression in healthy controls changed only in response to LPS, but no change was seen following CH or TLR-2/6 ligand stimulation. In APECED and non-APECED patients, TLR-2 mRNA expression did not change in response to any of the stimuli used. In summary, when stimulated with *Candida* and *Candida*-like stimuli, APECED patients did not down-regulate any of the TLRs known to be involved in anti-*Candida* responses (TLR-1, -4, -6, -2), while non-APECED patients down-regulated only TLR-4 in response to TLR-2/6 ligand. These findings suggest that moDCs in both APECED and non-APECED patients have normal patterns of mRNA TLR expression in immature DCs, but that progression to maturation is altered, particularly in response to *Candida* and *Candida*-like stimuli, suggesting impaired maturation of DCs in these patients.

The results reported in this paper are part of a larger study, which includes assessment of a range of cytokines produced (IL-12p70, IL-23, IFN-γ, IL-2, TNF-α, IL-6, TGF-β, IL-10, IL-5, IL-13) as well as cell-surface matura-

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with other fungal (dermatophyte) infections as well [42]. The relevance of this finding for patients with classical CMC (which were investigated in this study) is under investigation, although our results demonstrate that both APECED and non-APECED CMC patients responded to Candida by down-regulating CARD9. Interestingly, the network connectivity analysis in our study (Fig. 4) actually suggests that the Dectin-1–Syk–CARD9 pathway was of greater relevance for Candida responses in our CMC patients than in healthy controls.

In summary, we have demonstrated that CMC patients, with or without APECED, do not lack plasmacytoid or myeloid DCs in blood, nor do they harbour defects of monocyte-TLR expression, relevant for anti-Candida responses. In immature moDCs, levels of mRNA expression for all PRRs involved in anti-Candida responses (TLR-1, -2, -4, -6, Dectin-1, Syk, CARD9) were comparable to normal controls, suggesting strongly that defects in PRR expression are unlikely to be responsible for the increased susceptibility to Candida infections in these patients. However, both groups of CMC patients (APECED patients in particular) fail to down-regulate PRR mRNA expression in response to Candida stimulation, implying defective DC maturation, possibly involving defective TLR and/or CTL signalling, which could be responsible for inadequate Candida handling seen in these patients. Further investigation of TLR and CTL signalling pathways will elucidate putative defects in CMC patients which may underlie their inability to clear Candida.

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