SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

MECHANISM OF HOST DEFENCE AGAINST CANDIDA IN PATIENTS WITH AUTOIMMUNE POLYENDOCRINE SYNDROME TYPE I.

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The Exmination takes place at Department of Infectious Diseases and Pediatric Immunology (PR room), Faculty of Medicine, University of Debrecen
at 11:00 AM, on September 3, 2014

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen
at 1:00 PM, on September 3, 2014

Introduction

The term „chronic mucocutaneous candidiasis (CMC)" was introduced in the late 1960s, and defines a heterogenous collective group of inherited conditions characterized by chronic, recurrent or persistent superficial mucocutaneous infections, caused by members of the *Candida* genus, mostly the commensal *Candida albicans*, an ubiquitous, opportunistic yeast; affecting the skin, nails and mucosal surfaces. *Candida* change from commensal to pathogen, only if the homeostasis between the virulence of the fungus and the resistance of the host immune system is damaged. CMC frequently occurs in early childhood and newborn age, and is also common in patients with broad and profound, acquired or inherited T-cell deficiencies. Principally, the most common manifestation is recurrent candidiasis of oral and eosophageal epithelial surfaces, and in case of CMC, patients rarely develop disseminated or systemic invasive *Candida* infections. Severe oropharyngeal candidiasis remains common in HIV-infected patients and was the most frequent opportunistic fungal infection in these patients before the introduction of effective antiretroviral treatments. It is also seen in patients with immunosuppressive, antibiotic or steroid treatments. The first sporadic CMC cases were described in the 1960s but its genetic causes remained unknown until recently. The first familial cases, typically with inheritence as an autosomal dominant trait or, rarely, as an autosomal recessive trait in some consanguineous families, were reported in the 1970s. Over the next 40 years, other sporadic and familial cases were reported, suggesting that CMC results from gene lesions in at least some patients. Invasive candidiasis, dermatophytosis, bacterial infections of the respiratory tract, staphylococcal diseases of the skin and autoimmune components have been reported as additional symptomes in CMC patients. The further genetic and immunological dissection of thepathogenesis of CMC should delineate the function of various genes, controlling and mediating the IL-17 signalisation and Th17 cell development. Predisposition to mucosal and skin candidiasis is a hallmark of several primary immunodeficiency disorders (PIDs), especially associated with impaired CD4+IL-17+ T cell immunity. Nevertheless, CMC may be the only or the principal disease manifestation in patients with some immundeficiencies, such as AD IL-17F and AR IL-17RA deficiencies, and gain-of-function (GOF) mutations of STAT1. This rare condition of essentially isolated CMC (about 1/100 000 individuals) nowadays is often referred to as „CMC-disease" (CMCD). The complex group of CMC syndromes can be subclassified according to spreading and distribution and by the underlying pathomechanism. Above all, CMC is diagnosed clinically, and by *in vitro* isolation and cultivation of *Candida* from smear, and the molecular diagnosis

can be revealed by functional and mutational analysis, based on the known underlying signal transduction pathways and genetic defects.

Autoimmune polyendocrine syndromes (APS) are rare disorders, characterized by the coexistence of at least two endocrine gland insufficiencies mediated by autoimmune machanisms. Additionally, nonendocrine immune components may be present. According to the Neufeld and Blizzard Classification of 1980, there are four main types of APS. The first description of the assotiation between hypoparathyroidism and candidiasis was reported by Thorpe and Handley in 1929, the condition was commonly referred to as Schmidt's syndrome, after the German pathologist who studied autoimmunity to more than one endocrine organ. APS associated with CMC is a rare autosomal recessive disease and is also referred to as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy - (APECED) syndrome. Research into APS 1 has shed new light on normal self-tolerance induction in the thymus and how its breakdown leads to autoimmunity. APECED is caused by a loss-of-function mutation of the autoimmune regulator gene (*AIRE*), resulting the production of a mutant AIRE protein, which allows autoreactive T cells to escape negative selection in the thymus, leading to autoimmunity to various endocrine organs and the production of autoantibodies against self-antigens, enzymes, which have important role in synthesis of hormons and neurotransmitters, and various cytokines. APS 1 is classically defined by the association of at least two of three major disease components: chronic mucocutaneous candidiasis (CMC), primary hypoparathyroidism and autoimmune adrenal insufficinecy, which was not related to the syndrome until 1946.

Aims of the study

1. To analyse the clinical data of autoimmune poliendocrine syndrome type I patients and their family members by focusing on disease manifestations, laboratory findings and organ specific autoantibody profiles. We studied how early their various manifestations and autoantbodies appeared, and analyzed the correlation between organ specific autoantibody production and organ functions.

2. To investigate the genetic heterogenity and genotype-fenotype correlations of 19 autoimmune poliendocrine syndrome type I patients from 7 different countries.

3. To measure anti-cytokine autoantibody levels to interleukin-17A, interleukin-17F, interleukin-22 and type I interferons in sera of autoimmne poliendocrine syndrome type I patients, and to analyze the correlation between anti-cytokine autoantibody levels and the clinical manifestations, especially chronic mucocutaneous candidiasis. We planned to show chronological relationship between the production of autoantibodies and the development of clinical signs of autoimme polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome.

4. To study anti-*Candida* cytokine responses of autoimmune polyendocrine syndrome type I patients, by measuring interleukin-17 and interleukin-22 secretion by *Candida*-exposed peripheral blood mononuclear cells.

5. To examine in vitro differentiation of CD4+ interleukin-17+ /interleukin-22+ T helper cells.

6. To follow up a patients from 7 week of age who carried R257X/R257X mutation of autoimmune regulator in order to detect the very first clinical and laboratory manifestations of autoimmune polyendocrine syndrome type I.

Materials and methods

Patients

We tested 19 patients from seven countries: six patients from Hungary (P1-P6), three patients from the USA (P7-8, P13), three patients from Finland (P9-10, 14), two patients from Sardinia (P11, P19) two patients from Norway (P15, P18) and two patients from Russia (P16-17) and one patient from Azerbaijan (P12). With informed consent and ethics committee approval, sera were collected at diagnosis of APS I or soon afterwards, or beforehand in three, at first sampling-time, as yet unaffected sieblings of known patients.

Serum samples

Blood was taken under aseptic conditions and serum was isolated by centrifuging blood at 2,500 rpm (550×g) for 10 min at room temperature. Aliquots of the samples from the patients

and healthy controls were frozen at −20 °C until use. Routine Laboratory Assays Anti-endocrine organ antibody levels, clinical chemistry and immunology parameters were determined by routine laboratory assays with freshly isolated serum.

Human blood cells

Peripheral blood mononuclear cells (PBMCs) were isolated from the patients and controls. After density gradient centrifugation and removal of the mononuclear cell layer, the cellswere washed several times in Krebs-Ringer Phosphate Puffer. The cells were resuspended in Dulbecco's modified Eagle's Medium (DMEM; Sigma-Aldrich, St. Louis, MO). PBMCs (5×10^5 cells/well) were incubated either alone or in the presence of heat-killed C. albicans, at 37 °C, in 96-well round-bottomed culture plates 5 days. After the incubation, the plates were centrifugedand the supernatants were removed and stored at −20 °C for the determination of cytokine concentrations.

Candida

C. albicans (ATCC 10231) was maintained on Sabouraud dextrose agar at 4 °C and stationary-phase cultures were prepared by inoculating 2 ml of DMEM. Heat-killed *Candida* was prepared by inoculating 5 ml SalSol (TEVA, Debrecen, Hungary) with *Candida* and incubating the culture at 56 °C for 60 min. We checked that the heat inactivation was effective by transferring various dilutions of *Candida* suspension onto Sabouraud dextrose agar and incubating for 48 h.The *Candida* suspension was then centrifuged at4,000×g for 10 min and the cell pellet was resuspended in Krebs-Ringer phosphate buffer with dextrose (KRPD)/ in DMEM. The density of heat-inactivated *Candida* was adjusted to 10^6/ml with a McFarland densitometer.

Routine laboratory assays

Anti-endicrine organ antibody levels, clinical chemistry and immunology parameters were determined by routine laboratory assays with freshly isolated serum.

Analysis of autoimmune regulator gene mutations

Genomic DNA was sequenced by amplifying the exons and flanking intron regions of *AIRE*by PCR. Amplicons were sequenced with the Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and analyzed with an ABI PRISM 3130 capillary sequencer (Applied Biosystems). Sequence variations were described with respectto

a reference sequence (GenBank accession No. ENST00000291582) for AIRE cDNA, in which the c.1 position corresponds to the A of the ATG translation initiation codon. Mutations are designated as recommended by den Dunnen and Antonarakis.

Organ specific antibody assays

Organ-specific autoantibodies against 21-hydroxylase (21OH), side-chain cleavage enzyme (SCC), aromatic L-amino acid decarboxylase (AADC), NACHT leucine-rich-repeat protein 5 (NALP-5), tryptophan hydroxylase (TPH) and glutamic acid decarboxylase (GAD-65) were assayed by radio-immunoassays, as for cytokine autoantibodies (Wolff et al. 2010). In RIA, levels of autoantibodies are estimated based on a positive control (index 1000) and a negative control (index 0). A threshold value is created based on the mean of a pool of healthy blood donors (N=50–150). This assay has the drawback of giving fairly high background signals, but is a very effective semi-quantitative autoantibody assay tool which demands little time and volume of sample. Positive results usually range from the threshold value, which differs between the different autoAbs (between 48 and 200), to about 1500. Some patient sera were assayed for ovary, adrenal and islet cell antibodies by routine indirect immunofluorescence tests.

Anti-cytokine antibody assays

Levels of serum autoantibodies binding to IL-17A, IL-17F, IL-22, IFN-α and IFN-ω were determined by ELISA. Briefly, cytokine samples (IL-17A, IL-17F and IL-22 from Peprotech, EC, London; INF-α and IFN-ω from Rocky Hill, NJ, USA) were diluted in PBS (0.1µg/ml). We then coated Nunc MaxiSorp immunosorbent 96-well flat-bottomed plates with 100µl of cytokine suspension/well and incubated the plates overnight at 4 °C. The supernatant was removed and the nonspecific binding sites were blocked by incubation for 2 h at room temperature with 100 µl of blocking buffer (0.1 % Tween-20, 3 % bovine serum albumin in PBS) per well. The plates were then washed four times with washing buffer (0.1 % Tween-20 in PBS). Serum samples were diluted 1:1000 in blocking buffer, and 100 µl of the dilutions was added to wells and incubated for 1 h at room temperature. The plates were washed four more times. Alkaline phosphatase-conjugated goat anti-human IgG was diluted 1:10000 in antibody buffer, and 100 µl was added to each well. The plates were then incubated for 1 h at room temperature. The plates were washed five times with washing buffer and antibody binding was visualized by adding a solution of the substrate, p-nitrophenol, according to the

manufacturer's protocol and determining absorbance at 405 nm, with a Multiskan EX photometer (Thermo Scientific,Shanghai, China), after incubation in the dark for 4.5 min.

IgG antibody subclass assay

Autoantibody subclasses were determined as described previously by Kärner et al. In brief, streptavidin agarose resin (Invitrogen, 1µl per well) was incubated for 1 h with biotin-conjugated anti-human subclass-specific antibodies (from BD Pharmingen against IgG1, IgG2 and IgG4, and from Invitrogen against IgG3) in 96-well MultiScreen filter HTS plates (Millipore, Bedford, MA, USA), and washed. At the same time, APS-I or control sera (1:25) were incubated for 1 h with 10^5 luminescence units of luciferase-linked IL-22 or IFN-α 2a; any immune complexes were then captured onto the coated beads. After washing, their luminescence intensities were measured in a 1450 MicroBeta TriLux Liquid Scintillation Counter & Luminometer (Perkin Elmer Life Sciences, Waltham, MA, USA).

Measurement of cytokine concentrations

The concentrations of IL-17A, IL-17F, IL-22 and TNF-α in the supernatants of Candida - exposed PBMCs were determined by sandwich enzyme-linked immunosorbent assays (Quantikine, R&D Systems, MN, USA). All experiments were performed in duplicate or triplicate.

Differentiation and flow cytometry analysis of IL-17 and IL-22 producing T cells

PBMCs from patient and healthy control were purified by gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare). PBMCs were washed 3 times with 1X Phosphate buffer saline (PBS; Sigma-Aldrich) and suspended in RPMI-1640 supplemented with 10% foetal bovine serum (FBS; Sigma-Aldrich). Adherent cells were removed by incubation at 37°C for 4 hours in RPMI. Non-adherent leukocytes were stimulated with 2 µg/ml anti-CD3 (Miltenyi Biotec, Bergisch Gladbach, Germany) and treated with a cocktail of different cytokines: recombinant human interleukin (IL)-23 (20ng/ml), IL-1β (10 ng/ml), IL-6 (50 ng/ml), and human transforming growth factor (TGF)-β (5 ng/ml); all from PeproTech. After two days, RPMI medium containing with cytokines listed above and plus IL-2 (22 ng/ml) were added into the cells and another two days we added again RPMI medium with cytokines. The following day, the cells were treated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 10^{-5} M ionomycin (IMC; Sigma-Aldrich) in the presence of 1 µg/ml GolgiPlug (Sigma-Aldrich) for 6 hours at 37°C. The cells were then washed with washing buffer (2% FBS-

bovine serum albumin (BSA)-PBS). For the surface labelling the cells were stimulated with allophycocianin (APC)-conjugated mouse anti-hCD4 IgG_1 mAb (BD, San Jose, CA, USA) or Peridinin chlorophyll (PerCP)-conjugated mouse anti-hCD3 IgG_1 mAb (BD) for 30 min at 4°C. They were incubated with Fixation Medium A (Invitrogen, Camarillo, CA, USA) for 15 min at 4°C. The cells were then washed with washing buffer and stained in Perm Medium B (Invitrogen) with phycoerythrin (PE)-conjugated mouse anti-human IL-17A IgG_1 mAb (R&D Sytems, Minneapolis, MN, USA) and fluorescein isothiocyanate (FITC)-conjugated anti-human IL-22 IgG_1 mAb (R&D Sytems) for 30 min at 4°C and after washing steps resuspended in 1% paraformaldehyde to analyse with AccuriC6 cytometer (BD).

Results

Clinical presentation

Sample were tested from 19 APS I patients. Among these 19 subjects, we have noted mild or severe CMC in 15, HP in 14 and 11 had AD. The mean onset-ages of the major or minor manifestations were 45,7 months (range 12 months–11years). The mean onset-ages for CMC were 61,6 months (range 12 months-21years), for HP were 63,7months (range 18 months-11 years), and for AD were 110,8 months (range 48 months-25,4 years). 7 of the 19 patients presented the classical diagnostic triad of APS I, Before 7 years of age, 15 of the 19 subjects fulfilled the traditional diagnostic criteria for APS-I, having at least two of the CMC, HP and AD triad; in addition, one patient had CMC plus an affected sibling at onset. In 18 subjects, mutations were detected in both AIRE alleles, but in a case of one patient only in one allele, he only had HP. In an other case, despite homozygosity for the R257X AIRE mutation, still only has dental enamel dysplasia, alopecia universalis and neail dystrophyby age 10, whereas his sister had developed both CMC and HP till the age 18 months.

Genetic data

Eight patients were found to be homozygous for the c.769C>T nonsense mutation of the AIRE gene, which replaces the arginine codon in position 257 with a stop codon (R257X/R257X). The available, tested parents of some patients were heterozygous for the mutant allele, consistent with autosomal recessive inheritance for this trait. Heterozygosity

was also detected in the available siblings of patients. Five patients carry the c.769C>T/c.1344delC or c.1344delC/c.769C>T compound heterozygous mutations in the *AIRE* gene. One patient were found to be homozygous for the c.1344delC, one for the C302Y mutation, one for the R139X and one for the 520X mutations. One patient carry the c.1344delC/R92W compound heterozygous mutation in the *AIRE* gene (Table 2.).

Autoantibodies against IL-17A, IL-17F and IL-22

OD values for autoantibodies against IL-22 were higher for all patients than for healthy controls. In Patient 1, anti-IL-22 values increased gradually between the ages of 81 and 102 months. OD values for anti-IL-22 antibodies were slightly higher in the adult patients than in children. By contrast, the levels of anti-IL-17A autoantibodies differed considerably between patients. These levels were higher in Patient 2 than in the other patients. Patient 2, who had presented CMC since the age of 18 months, had persistently high anti-IL-17A levels. Patient 2 was referred to our center at the age of 4 years, and unfortunately no serum samples were available for antibody testing before this age. Her brother, who had no signs of CMC (Patient 1), had low or undetectable levels of anti-IL-17A antibodies over the period of observation. Patient 3, with no signs of mucosal or skin candidiasis, also had low or undetectable anti-IL-17A antibody levels over a period of 4 years. Similarly, OD values for anti-IL-17A antibodies in Patients P4, P5, and P6, who had candidiasis of a single fingernail beginning at the age of 17 years (P5), angular cheilitis of unknown etiology since the age of 21 years (P6) or three short episodes of mucosal candidial disease during the first 3 years of life (P6), were negligible, as shown by comparison with control values and with those for Patient 2, who had prolonged, severe CMC. OD values for IL-17F were intermediate, between those obtained for anti-IL-17A and anti-IL-22 antibodies, and were not correlated with the severity of CMC. Experiments were performed to measure OD values of anti-IL-17A and anti-IL-22 in sera of Patients 1, 2 and 3 by using varying serum concentrations. Data of these experiments confirmed the differences in OD values found with a single dilution of sera.

Autoantibodies against type I interferons

OD values for antibodies against IFN-α and IFN-ωwere measured in three patients, and were high and similar in Patients 1 and 3. In Patient 2, with CMC, the low anti IFN-ω antibody level contrasted with the high level of anti-IFN-α antibodies. In Patient 3, who was followed up from early infancy, we observed a gradual increase in the levels of IFN-α and IFN-ω

antibodies until the age of 14 months. Autoantibody production began at the age of 7 months and sustained increases in the levels of these antibodies resulted in high OD values later on.

IgG subclass analysis of IFN-α2 and IL-22 antibodies

Kärner et al have recently reported high proportions of IgG4, in addition to IgG1, among the autoantibodies against IFN-α2 and IL-22 in APS-I patients. IgG1 was again prevalent in all the autoantibodies detected in 6 of the present subjects. Surprisingly, against IL-22, there were already substantial proportions of IgG4 at 7 months in P3 (with traces of IgG2 and IgG3); also as early as 42 months in P13, where they exceeded those of IgG1. Against IFN-α2, IgG4 again constituted a substantial proportion in P3 (at 30 months) and P10 (30 months), but not in the two other seropositive patients.

Organ-specific autoantibodies

We detected high levels of autoantibodies against adrenal cortex or steroid hydroxylases in 13 subjects—including all of the 11 with AD; they preceded its onset by 2–3 years in P15 and P13 and by over 18 years in P11; they were also strongly positive in 3 of the other subjects who do not yet have AD. Antibodies to NALP-5 were found in 3 of the 6 subjects who had HP (among a total of 10 tested); in P8, they preceded its onset by 6 months. Antibodies to ovaries, or specifically to SCC, were detected in 3 of the 12 subjects tested (weakly in one), including P11 who has clinical ovarian failure (OF). Autoantibodies against pancreatic islets or GAD-65, and thyroid tissue or antigens, were also tested in all subjects. They were detected against islets in P11, over 31 years before onset of diabetes; also against GAD-65 in 4 others who do not yet have diabetes. TPO and Tg antibodies were found in one of two patients with thyroid disease (P14) 5 years before its onset. TPH antibodies were detected in 2 patients, AADC in 5 and TH in 3, but they are too few to correlate with manifestations. Overall, endocrine autoantibodies were found surprisingly early in P19, P13 and P2 (at 6, 24 and 48 months respectively), but only later in P10, P14 and P15.

Release of cytokines by *Candida*-exposed blood cells

We measured the release of IL-17A, IL-17F, IL-22 and TNF-α by PBMCs after 5 days of stimulation with heat-killed *Candida*. PBMCs from patients released negligible or small amounts of anti-cytokine antibodies on exposure to *Canadida*, compared to results of healthy controls (C on Figure 7, means values of controls), who presented high levels of IL-17A, IL-

17F and IL-22, and higher levels of TNF-α. These data suggest an impaired Th17-type response to Candida in patients with APS. To prove the possibility of a broader defect of cytokine release by Candida-exposed PBMCs, we measured concentrations of TNF-α in supernatants as positive control. The data of these control experiments showed that release of TNF-α by cells from patients and controls were comparable. These result suggest an isolated impairment of Th17 cell functions and cytokine secretion.

IL-17+/IL-22+ T helper cell differetiation of autoimmune poliendocrine syndrome type I patients

PBMCs of APS I patients and healthy controls were isolated and treated with IL-17-inducing cytokines as described above. Flow cytometry was used to determine the percentages of CD3+ T cells and CD4+ T cells, producing cytoplasmic IL-17 or IL-22. Before marker analysis, the cells were treated with phorbol-12-myristate 13-acetat (PMA) -an activating factor of protein kinase C-, ionomycin (IMC) -an ionophore- and Brefeldin A –"Golgi-plug", which inhibits protein transport from the endoplasmic reticulum to the Golgi apparatus- for 6 h. Consistent with the cytokine-release data, APS I patient displayed a mild impaired development of IL-17-producing and IL-22 - producing T helper cells, compared with healthy controls. Within Brefeldin A treated, unstimulated cell population; IL-17 and IL-22 secreting cell percents were similarly low in patients and healthy controls. After PMA stimulation, we found moderately decreased cell persents in APS I patients, involved the CD3+IL-17+, CD4+IL-17+ and CD4+IL-22+ cells, compared with healty controls.

To investigate the possible impairment of secretion mechanisms, we performed the population analysis without secretion inhibitor (brefeldin A), to. We measured significant difference between results of the brefeldin A-inhibited and the untreated cells of healthy controls. Without secretion inhibitior, healthy controls presented lower IL-17+ and IL-22+ cell persents after PMA stimulation, as a normal consequence of the lower intracellular cytokine concentrations without inhibitor. In contrast, we found similarly percents of CD3+IL-17+ (Figure 8A-E), CD4+IL-17+ (Figure 9A-E) and CD4+IL-22 (Figure 10A-E) cells, with and without secretion inhibitor in patients with APS I. This results suggest that the decreased cytokine secretion of Candida-exposed blood cells may based on a functional impairment of the secretion mechanisms of Th17 cells, confirming the possibility of a broader defect of the anti-Candida response in APS I patients.

Discussion

CMC is the most common infectious manifestation in APS I, which is one of three diagnostic criteria of the syndrome. In this study, we found CMC with variable severity (mild angular cheilitis, isolated nail candidiasis, recurrent severe oropharyngeal candidiasis) in almost 80 % of the 19 patients tested. Hypoparathyreosis, the second most common manifestation and diagnostic criteria was present in 73,6% of patients, consistent with the published literature. Close to sixty percent of our patients presented with adrenal insufficiency, and 78,9% of patients fulfilled the classical diagnostic criteria i.e. at least two of the CMC, HP and AD, moreover one patient had CMC and an affected member in the family. The most common mutation, called "Finnish major mutation" were found in 42,1% of patients, includong the two Finnish patient. In this study, the second most common genetic condition was the compound heterosigosity for R257/13 bp deletion. The 13 bp deletion is the second most common *AIRE* mutation worldwide. The classical diagnostic triad were present in 36,8%, especially in patients with the Finnish major mutation and the R257X/13 bp deletion. Both Sardinian patients presented with autoimmune hepatitis and carried the R139X mutation. P1, despite homozygosity for the R257X AIRE mutation, has had only dental enamel dysplasia, alopecia universalis and neail dystrophy at age 10, whereas his sister (P2) had developed both CMC and HP by the age of 18 months. P3, with the R257X mutation is till symptomless now, despite the severe clinical phenotype of his sister. These results confirmed the clinical and genetical heterogeneity of APS I, in accordance with data from others.

We provide the first description of the possible effect of IVIG as a treatment option in patients with severe CMC in APECED patients, and the transient improvement of mucosal candidiasis following monthly IVIG infusions in Patient 2 was intriguing. IVIG replacement therapy is widely used to prevent infectious complications in patients with primary immunodeficiency disorders. In addition to prophylaxis, IVIG concentrates may also be helpful for the treatment of various inflammatory conditions. However, the mode of action of this therapy in autoimmune diseases remains poorly understood. IVIG contains anti-idiotype antibodies against disease-associated autoantibodies. These anti-idiotype antibodies may be able to bind and neutralize the pathogenic anticytokine antibodies typically produced in patients with APECED, consistent with the decrease in autoantibody levels observed in other conditions following IVIG therapy. We believe that IVIG treatment may increase the elimination of Candida by opsonophagocytosis, given the clinical observation that patients with X-linked agammaglobulinemia characterized by an almost complete lack of serum immunoglobulins

typically do not suffer from CMC or invasive candidiasis. We observed no detectable decrease in anti-IL-17A and anti-IL-22 antibody levels during IVIG therapy in Patient 2 (data not shown), but we believe that further studies of the possible therapeutic effects of IVIG in APS I patients with severe CMC are warranted, based on our observation.

The importance of the IL-17 family cytokines in immunity to *Candida* is well established. Previous studies suggested that anti-cytokine autoantibodies are involved in the pathogenesis of CMC in APECED patients. It has been suggested that autoantibodies against cytokines, including IL-17A, IL-17F and IL-22, may underlie CMC in patients with APECED. Recent findings have suggested that the presence of autoantibodies against IL-22 may be a useful predictor of CMC. It has also been suggested that IL-17 plays a key role in protection against *Candida* infection, and some patients with CMC disease have smaller than normal proportions of IL-17-producing T cells, and produce low levels of IL-17. In this study, Patient 2, with severe and prolonged CMC, presented with high levels of autoantibodies against IL-17A. The other five patients with the same genotype or heterozygous AIRE mutation had low or undetectable anti-IL-17A antibody levels and were asymptomatic or presented only mild and transient signs of mucocutaneous candidiasis. Previous studies have suggested that IL-22 plays an important role in the protection of skin and epithelial surfaces against *Candida*. It has been reported that IL-22-producing cells help to protect mucosal surfaces against fungal infection in conditions of defective adaptive immunity, through a primitive antifungal effector mechanism. Furthermore, IL-22 has been reported to play a role in candidiasis, by controlling fungal invasion and epithelial homeostasis. All six patients included in this study had high levels of anti-IL-22 autoantibodies, but only Patient 2, with high levels of anti-IL-17A antibodies, displayed a predisposition to severe CMC. Furthermore, OD values for anti-IL-17F antibodies were high in all patients, but those in Patient 2 they were no higher than those in the other patients. These findings argue against a primary role of anti-IL-17F and anti-IL-22 antibodies in the susceptibility of APS I patients to CMC, instead favoring the concept that anti-IL17A antibodies are the primary components of impaired anti-candidial immunity in APS I patients. Measuring functional activity of these antibodies could have confirmed further our findings but such assays are not currently available in our lab.

The role of anti-IFN antibodies in the pathogenesis of disease signs in APECED patients is not fully understood. Recent studies have shown that high levels of autoantibodies against type I IFNs, including IFN-α and IFN-ω in particular, are found in most patients with APECED, suggesting that anti-IFN autoantibodies are hallmarks of the disease. These studies

showed that autoantibodies against IFN-ω were clearly present in all patients but their levels were not clearly correlated with the number or duration of disease manifestations. We found high levels of autoantibodies against IFN-α in the sera of four patients studied, and of autoantibodies against IFN-ω in Patients 1 and 3. The levels of anti-IFN-ω autoantibodies were low in Patient 2, 4 and 5. These data confirm the lack of relationship between severity of APECED and anti-IFN autoantibody level. The role of the variable levels of anti-IFN-ω antibodies in this multiorgan primary immunodeficiency disorder therefore remains to be elucidated. Moreover, the almost 100% presence of this autoantibodies in APS I patients propose the role of this antibodies as a new diagnostic criterion. In sibling P3, the antibodies against IFN-ω appeared before those against IL17 type cytokines. It would be interesting to test more pre-symptomatic infants in APS-I families serially, starting with cord blood, both for autoantibodies and for Th17 and Th22 cell function such long term studies may provide evidence on the precise timing of the various responses and Th-cell deficiencies and on their prognostic value. Our results show strikingly high neutralizing autoantibody titers against IFN-ω, IFN-α2 and/or IL-22 already at 6-7 months of age -well before any sign of APS-I- in an unaffected AIRE-mutant patient (P3). We also found endocrine organ-specific autoantibodies, especially to adrenal cortex, surprisingly early in 2 in two cases. Since several weeks, at least, are needed to generate an antibody response, even in adults- and still longer for organ damage sufficient to cause disease - the process must have begun soon after birth in some of the infants studied here, or even earlier. The well-known delays, sometimes for several years, between appearance of tissue-specific autoantibodies and the corresponding autoimmune feature are often taken to implicate T-cells as the pathogenic effectors, whereas the autoantibodies behave as useful diagnostic markers. Autoimmunity in APS-I appears to result primarily from a failure to tolerize T-cells developing in the absence of AIRE, leading stochastically to autoimmunization against a wide range of AIRE-regulated targets. That may be compounded by loss of tolerogenic AIRE-expressing cells in peripheral lymphoid tissues. However, both the highly precocious, sharply focused autoantibody responses shown here and the consistent early onset of such unusual autoimmune features as CMC, HP and AD argue for more selective autoimmunization in AIRE-deficient thymus.

Recent studies suggested that impairment of Th17 cell population and development, or IL-17F and IL-22 secretion by PBMC's is CMC patients may be an important factor in predisposition to candidiasis. The role of IL-17A secretion seems somewhat controversial and poorly defined in studies in APS I patients. Reposrt on increased numbers of *C. albicans*-induced IL-17A-

producing cells in PBMC from APS I patients, measured by flow cytometry by intracellular staining of IL-17A, contrasting other studies, which consistently found impaired IL-17A responses in APECED patients. Candida-exposed PBMCs from our patients released small amounts of IL-17A and much smaller, or negligible amounts of IL-17F and IL-22 compared to cells from healthy controls. Recent studies suggest that in patients with APS I, the pools of CD16(+) monocytes and regulatory T cells (Tregs) were also lower compared with healthy individuals, and propose that this difference may influence the inflammatory processes in APS-I patients. Th17 cell proliferation and IL-17 production were normal unless exposed to APS I plasma, which inhibited both functions in both APS I and normal PBMCs. *Candida* species–stimulated IL-22 production was impaired in all patients with CMC, including APS I patients, whereas IL-6 and IL-23 responses were also normal. We found impaired secretion of IL-17A, IL-17F and IL-22 by mononuclear cells from APS patients, with, or without severe candidiasis, in contrast to release of TNF-α. Moreover, patients with APS I had a decreased percentages of CD3+IL-17+, CD4+IL-17+ and CD4+IL-22+, Il-17 and IL-22 producing T cells, after in vitro differentiation, and stimulation by phorbole myrystate-acetate, compared with healthy controls. The percentages of the IL-17 and IL-22 producing T cells are nearly constant with and without the inhibition of cytokine secretion in APS I, in contrast with the results of healthy controls. Recent findings suggest, that the wild type (WT) *AIRE* gene product has the ability to be targeted into the nucleus, where it is found associated with distinct speckled domains in the nucleoplasm. These findings showed with immunocytofluorescence detection, that the normal AIRE protein co-lokalizes with the cytoskeletal filaments, especially with the vimenti, and the truncated AIRE protein showed altered cytoskeletal interactions and subcellular localization. We suggest that these differences between the wild type and the mutant subcellular distribution, may effect as a disturbing factor the subcellular processes of the mechanisms of cytokine secretion and transport.

Summary

In summary, we report here a correlation between anti-IL-17A autoantibody level and predisposition to chronic mucocutaneous candidiasis in APS I patients. We suggest that anti-IL-17A autoantibodies may play a more important role than anti-IL-22 and anti-IL-17F antibodies in rendering APS I patients susceptible to candidiasis. We suggest that high levels of anti-IL-22 and anti-IL-17F autoantibodies may not be correlated with the occurrence of candidiasis in APECED patients. We present data suggesting that the release of IL-17A, IL-17F and IL-22 by peripheral blood mononuclear cells after *Candida* stimulation is impaired in

APS I patients, and this impairment affects only IL-17 and IL-22 secretion, and does not influence secretion of other cytokines. This result is confirmed by the normal secretion of TNF-α of APS I patients, after *Candida* stimulation. We found, that patients with APS I have a decreased persent of CD3+IL-17+, CD4+IL-17+ and CD4+IL-22+, Il-17 and IL-22 producing T cells, after in vitro differentiation, compared with healthy controls. Moreover, the percents of the IL-17 and IL-22 producing T cells are nearly constant with and without the inhibition of cytokine secretion in APS I, in contrast with the results of healthy controls. Based on this results, we suggest, that the development of IL-17+, IL-22+ T helper cells is impaired in APS I patients, nevertheless, secretion mechanisms of these cell are also affected.

We propose that the predisposition to candidiasis of APS I patients is based on a complicated conjuction of diversified factors, included the antifungal cytokine-neutralization by autoantibodies, the impaired development of IL-17 and IL-22 producing T cells, and decreased secretion capacity of this cells.

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2. Wolff, A.S.B., **Sarkadi, A. K.**, Maródi, L., Kärner, J., Orlova, E., Oftedal, B.E.V., Kisand, K., Oláh, É., Meloni, A., Myhre, A.G., Husebye, E.S., Motaghedi, R., Perheentupa, J., Peterson, P., Willcox, N., Meager, A.: Anti-cytokine autoantibodies preceding onset of autoimmune polyendocrine syndrome type I features in early childhood.
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List of other publications

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4. Soltész, B., Tóth, B., Shabashova, N., Bondarenko, A., Okada, S., Cypowyj, S., Abhyankar, A., Csorba, G., Taskó, S., **Sarkadi, A. K.**, Méhes, L., Rozsíval, P., Neumann, D., Chernyshova, L., Tulassay, Z., Puel, A., Casanova, J., Sediva, A., Litzman, J., Maródi, L.: New and recurrent gain-of-function STAT1 mutations in patients with chronic mucocutaneous candidiasis from Eastern and Central Europe.
   J. Med. Genet. 50 (9), 567-578, 2013.
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