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Decreased T-cell response against latent cytomegalovirus infection does not correlate with anti-IFN autoantibodies in patients with APECED

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Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) is an inborn error of immunity affecting both multiple endocrine organs and susceptibility to candidiasis, each with an autoimmune basis. Recently, high titer neutralizing anti-type I interferon (IFN) autoantibodies have been linked with increased severity of SARS-CoV-2 and *varicella zoster* virus infections in APECED patients. Examining immunity against cytomegalovirus (CMV), we found a higher prevalence of anti-CMV IgG antibodies in patients with APECED (N = 19) than in 44 healthy controls (90% vs 64%, p = 0.04); the similar difference in their IgG levels did not achieve significance (95 ± 74 vs 64 ± 35 IU/mL, ns.). In contrast, the frequency of CMV-specific T cells was lower (804 ± 718/million vs 1591 ± 972/million PBMC p = 0.03). We saw no correlations between levels of anti-CMV IgG and anti-IFN antibodies in APECED patients or in a separate cohort of patients with thymoma (n = 70), over 60% of whom also had anti-IFN antibodies. Our results suggest a dysregulated response to CMV in APECED patients and highlight immunodeficiency to viral infections as part of the disease spectrum.

Key words: AIRE; autoimmune polyglandular syndrome type 1; type I interferons; inborn errors of immunity; herpes.

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Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) is a rare, recessively inherited autoimmune disease caused by bi-allelic mutations in the *AIRE* gene. Due to its well-defined genetic background, it has been extensively studied as a model of autoimmunity, but recent studies have highlighted that the disease has an immunodeficiency component as well (1). In addition to the pathognomonic chronic candidiasis, the patients are also more susceptible to severe viral infections (1–3). The patients also exhibit increased levels of antibodies against commensal bacteria, their gastrointestinal microbiome is altered, and they have an increased burden of gynecological infections (4–7). Moreover, the overall mortality from infectious diseases is increased in these patients (8).

At least part of their susceptibility to infections is thought to be explained by anti-cytokine antibodies. Often before any clinical symptoms of autoimmunity (9), almost all APECED patients develop high levels of antibodies neutralizing all interferon-α subtypes (IFN-α) (10), key mediators of early defense against many viruses including cytomegalovirus (CMV) (2, 3, 11, 12). These antibodies are also reported in the general population and predispose to severe COVID-19 pneumonia (2). We recently observed that, in patients with

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APECED, they are associated with severe sequelae of *herpes simplex* and *varicella zoster* virus infections – a finding that has since been replicated in patients with systemic lupus erythematosus or with inborn errors in the alternative NF-κB pathway (3, 13, 14). In patients with severe COVID-19, anti-IFN antibodies were also associated with concomitant CMV reactivation (12). In APECED, susceptibility to *Candida* spp. has been attributed to other antibodies neutralizing IL-17 and IL-22, to immunopathology promoting type 1 inflammation, or a combination of both (1, 15, 16).

Human herpesvirus-5 or CMV is a complex double-stranded DNA virus and a potent modifier of the immune system. In some healthy adults, up to 30% of memory $CD8^+$ T cells may be CMV-specific, and twin studies indicate that CMV infection is among the strongest non-heritable factors shaping the immune system (17–19). Primary infection is usually mild or even subclinical and latency is asymptomatic. Chronic CMV is, however, linked to oligoclonal $CD8^+$ T-cell expansions, chronic background inflammation, immunosenescence and excess mortality (17, 19). APECED patients have an abnormal accumulation of highly differentiated $CD8^+$ T cells with a phenotype very similar to the terminally differentiated cytotoxic T cells specific to latent CMV. These cells show decreased expression of the IL-7Rα chain CD127, express cytotoxic effector molecules such as perforin, and have reverted to the $CD45RA^+$ phenotype (20). Here we have examined the role of CMV immunity in APECED patients and compared them to a cohort of patients with acquired thymomas, who show several intriguing parallels; loss of AIRE expression in these thymopoietic tumors, development of anti-IFN antibodies and similar oligoclonal $CD8^+$ T-cell expansions (10, 15, 21, 22).

MATERIALS AND METHODS

Study subjects

The study was conducted according to the Declaration of Helsinki and approved by the Ethics Committees of Helsinki and Oxford University Hospitals; written informed consent was obtained. The main study group consisted of 19 Finnish APECED patients and 44 healthy controls. We also tested archival samples from 70 patients with thymoma, many of whom have been described previously (23). Age and sex of study subjects is displayed in Table 1.

Table 1. Study subject characteristics.

	APECED	Thymoma	Controls
Number	19	70	44
Age mean (range; years)	43 (21–65)	33 (24–75)	35 (21–75)
Female (%)	12 (63%)	36 (51%)	24 (55%)

Samples

Blood was drawn into EDTA Vacutainer tubes (BD Biosciences, Franklin Lakes, New Jersey, USA) and plasma was separated by centrifugation. PBMCs were isolated using Ficoll-Paque (GE Lifesciences, Chicago, Illinois, USA) gradient centrifugation and frozen fresh after isolation with CTL-Cryo ABC (CTL, Shaker Heights, Ohio, USA) freezing kit and stored at −140 °C before analysis.

ELISpot

The ELISpot assay was performed with Human IFN-γ ELISpot kit (Mabtech, Stockholm, Sweden). In brief, the frozen cells were thawed, stained with trypan blue and counted. 200 000 cells per well in serum-free CTL Test media (Cellular Technology) were stimulated with PepMix HCMVA (pp65) peptide pool of 138 peptides spanning the whole pp65 protein (JPT, Berlin, Germany) at a concentration of 2 μg/mL. After overnight incubation at 37 °C in a cell incubator the samples were removed, secondary antibody added, and spots developed as instructed by the manufacturer. The results were read with an AID Elispot Reader.

Quantification of anti-viral antibodies

The antiviral antibodies were measured by Vidas CMV IgG (Biomérieux, Marcy-l'Éloite, France) as instructed by manufacturer. The results were read with Vidas 30-analyzer (Biomérieux).

Quantification of viral DNA load

Viral DNA was extracted from plasma samples by PureLink Viral RNA/DNA kit (Life Technologies, Waltham, Massachusetts, USA) as instructed by the manufacturer. A qPCR reaction was performed in duplicate with human Herpes virus-5 Taqman assay (Pa03453400_s1, Life Technologies) and commercial Taqman MasterMix using the iCycler-IQ real-time PCR detection system (Bio-Rad, Hercules, California, USA). A positive control with known high-plasma CMV DNA levels was kindly donated by Helsinki University Hospital Laboratory (HUSLAB, Helsinki, Finland).

Flow cytometry

The following antibodies and reagents were used to stain unstimulated thawed PBMCs for flow cytometry: MHC Dextramer A*0201 NLVPMVATV (Immudex, Copenhagen, Denmark), CD45RA-FITC (ImmunoTools, Friesoythe, Germany), CD8-PeCy7 (BD Bioscience), CD27-APC-Cy7, PD-1-PE (BioLegend, San Diego, California, USA), CCR7-PE-CF594 (BD), and CD 127-PE (BD). The samples were run and analyzed on Cyan flow cytometer (Beckman Coulter, Brea, California, USA).

HLA-A2 positivity was quantified by staining PBMC with anti-HLA-A2-FITC (BD Biosciences).

Quantification of anti-interferon antibodies

The presence and titer of anti-IFN antibodies in patients with APECED was measured using a luciferase immunoprecipitation assay as reported (24). Human IFNA cDNAs were cloned in fusion with NanoLuc luciferase (Nluc). Cell culture medium of transfected HEK293 cells containing Nluc-fusion proteins was collected after 48 h. Sera were incubated with the fusion protein overnight, then incubated with Protein G agarose beads in 96-well microfilter plates (Merck Millipore, Burlington, Massachusetts, USA) to capture immune complexes to the beads. After washing, a luciferase substrate (furimazine) was added and luminescence intensity (LU) was measured in Victor X Multilabel Plate Reader (PerkinElmer Life Sciences, Shelton, Connecticut, USA). Results were expressed as relative units LU for the sample/average LU of healthy control samples. The positive/negative discrimination level was set to the mean plus three SDs of the healthy control samples.

The presence of anti-IFN antibodies in patients with thymoma was assayed by a radioimmunoassay (RIA) against ^{35}S-labeled recombinant human autoantigens expressed by *in vitro* transcription and translation essentially as described previously (25).

Statistical analyses

Statistical analyses were performed using SPSS version 23 (IBM, Armonk, New York, USA). Fisher's exact t-test, Mann–Whitney *U*-test, as well as Spearman's signed rank were used with the limit for statistical significance set to 0.05.

RESULTS

Higher seroprevalence and antibody responses against cytomegalovirus in APECED patients

Given that APECED patients have aberrant immune responses against *herpes simplex* and *varicella zoster* virus, and expansion of highly differentiated CD8$^+$ T cells of unknown specificity, we tested whether their responses to the highly prevalent latent virus CMV might also be abnormal. In a cohort of 19 adult APECED patients and 44 healthy controls, we first measured CMV-specific IgG levels using an enzyme-linked fluorescence assay validated for clinical use (VIDAS). In the healthy controls, the seroprevalence rate was similar to that in the general Finnish population (28 of 44; 63.6%) (26, 27), but it was significantly higher in the APECED patients (17 of 19; 89.5%; p = 0.04). The CMV-specific IgG levels were comparable in seropositive APECED patients and healthy controls (95 ± 74 vs 64 ± 35 IU/mL, ns., Fig. 1A).

Decreased T-cell responses to cytomegalovirus

In immunocompetent humans, anti-CMV antibody titers do not correlate with protective immunity against reactivation in times of critical illness, and passive antibody therapy has not proved effective, highlighting the importance of cell-mediated responses in controlling CMV infections (11). We used an IFN-γ Elispot assay to measure the frequency of CMV-specific T cells in those

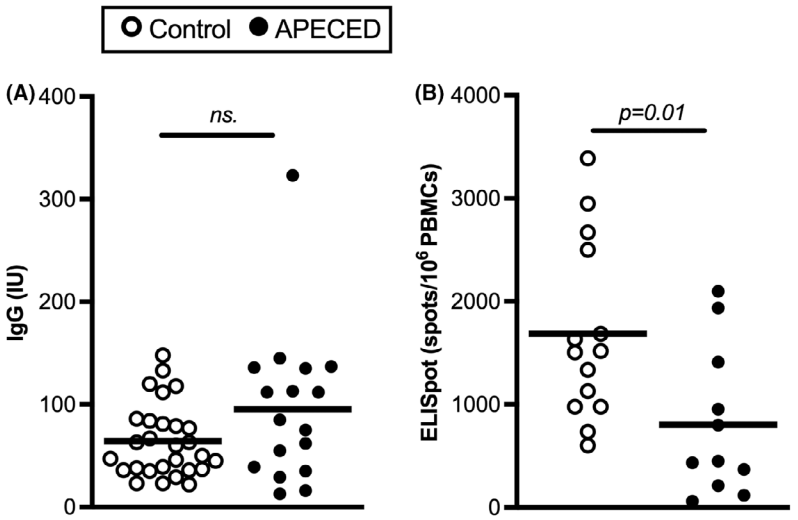

Fig. 1. Adaptive immune responses to CMV in controls and patients with APECED. (A) Anti-CMV IgG levels and (B) ELISpot responses against CMV peptide pp65 in in seropositive controls and patients with APECED. The bars indicate means. ns.: nonsignificant.

seropositive subjects from whom cells were available (11 patients and 14 controls). As stimulating antigen we selected the virion tegument protein pp65, a major constituent of virions and an immunodominant antigen for both $CD4^+$ and $CD8^+$ T cells. Its pooled peptides elicited significantly lower cellular responses in the patients than the controls. The frequency of CMV pp65-specific cells in the patients was 804 ± 718/million PBMC compared with 1591 ± 972/million PBMC in the controls ($p = 0.01$, Fig. 1B).

These data suggest that, although both cellular and humoral responses to CMV were activated, the CMV-specific adaptive response in the patients was skewed in favor of humoral immunity. To test whether this could be associated with subclinical CMV reactivation, we used quantitative PCR to detect CMV genomes in the plasma of 12 patients taken when asymptomatic for any viral symptoms, with the gene UL132 as the target. No CMV DNA was detected (data not shown) at that one time point; obviously, we cannot not exclude transient abnormal reactivation at other times.

Decreased frequency but unaltered phenotype of CMV-specific T cells

Next we wanted to examine whether diminished IFN-γ secretion in response to CMV stimulation reflected differences in frequencies or responsiveness of CMV-reactive T cells. We used CMV pp65/HLA-A2 dextramers to identify CMV-specific $CD8^+$ T cells *ex vivo* in the 5 patients and 11 controls who were HLA-A2-positive. In the patients, the frequency of pp65-specific $CD8^+$ T cells was very low (0.1 ± 0.02% of all $CD8^+$ T cells), whereas in the controls it was 0.8 ± 0.5%. Despite the small number of samples and high variation in the controls, this difference, combined with the Elispot results, suggests decreased frequency of pp65-specific T cells in the patients (Fig. 2A). Analysis of the phenotype of the pp65-specific $CD8^+$ T cells showed that in the patients the effector memory $CD45RA^-CD27^-$ subset was decreased (Fig. 2B). The distribution of other subsets within the pp65-reactive population was similar in patients and controls, including the $CCR7^+$ central memory subset. The expression of CD127 associated with memory phenotype in CMV specific cells was also similar (mean fluorescence intensity 11.3 ± 1.8 vs 9.7 ± 1.9; Fig. 2C), nor was there any difference in the expression of the exhaustion marker PD-1 (42.71 ± 9.6% vs 51.97 ± 6.6%; Fig. 2B). The patients thus appeared to differ in numbers of circulating CMV-specific T cells rather than in their phenotypic or function.

No correlation between anti-IFN antibodies and CMV immunity

As with the vast majority of patients with APECED (9, 10, 15, 23), all the APECED patients tested had high neutralizing titers against IFN-α2 (11 of 11) and IFN-ω (12 of 12). We next checked for possible effects on CMV immunity, but found no significant correlation between anti-type I IFN antibody levels and CMV IgG levels (Fig. S2A). As

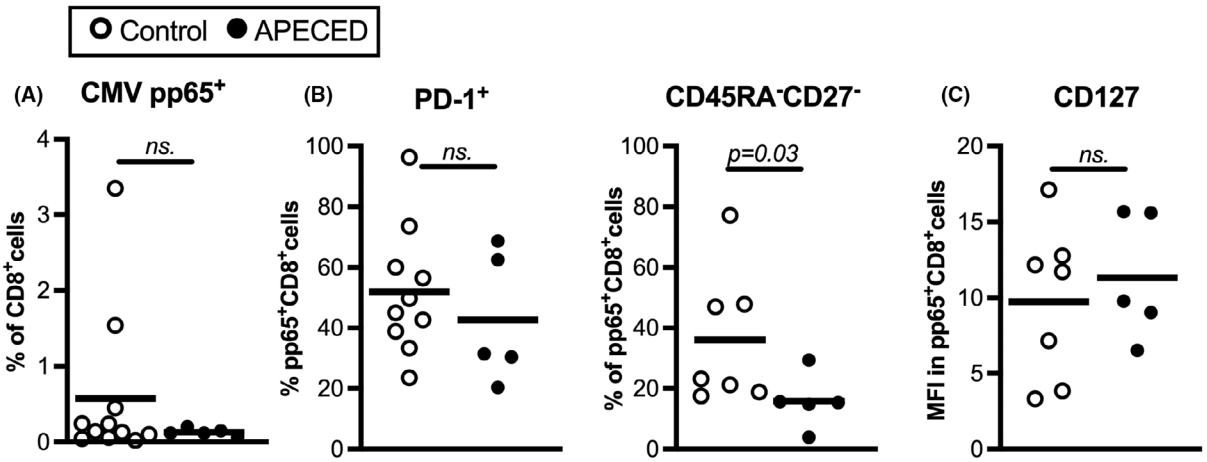

Fig. 2. Phenotype and frequency of T cells specific for CMV pp65 antigen in HLA-$A2^+$ APECED patients and controls. Fraction of $CD8^+$ T cells staining positive for CMV pp65 dextramer in healthy controls and patients with APECED. (B) Fractions of CMV pp65 dextramer-specific $CD8^+$ T cells that are PD-1^+ or $CD45RA^-CD27^-$, respectively and (C) CD127 mean fluorescence intensity in CMV pp65-specific $CD8^+$ T cells. The bars indicate means.

another check for potential effects of these anti-IFN-I autoantibodies, we next tested a separate cohort of 70 patients with thymomas (23). Of these, 53% (n = 37) had antibodies against IFN-ω, 64% (45) against IFN-α2, and 66% (46) against IFN-α8. Fifty-three percent (n = 37) of the thymoma patients were CMV-seropositive. Their CMV-specific IgG levels were very similar (A) to those in the healthy controls (Fig. S2B) and (B) in the thymoma patients with and without anti-IFN antibodies (Fig. S2C).

DISCUSSION

Our data show that, in APECED patients, the prevalence of anti-CMV antibodies was higher than in healthy controls, but lower for CMV specific T cells. We detected no link here between antibodies to CMV and the anti-IFN-α or IFN-ω antibodies in APECED or thymoma patients. That contrasts with other settings where anti-IFN antibodies affect antiviral immunity, their titer appearing to be an important determinant (3, 28–30). The high anti-IFN seroprevalence in APECED makes it difficult to exclude a binary difference between patients with or without anti-IFN antibodies, but that was clearly not observed in the thymoma patients. It should, however, be noted that the relative timing between the appearance of anti-IFN antibodies and CMV infection is likely to be very different in these two syndromes. Almost 100% of APECED patients have high titers of anti-IFN antibodies even in infancy (9, 10) – whereas only one-third of children are CMV seropositive by ages 6–11, and only ~70% in adulthood (26, 27, 31). Only then are the tumors – and anti-IFN antibodies – acquired in thymoma patients (21).

Anti-type I IFN antibodies are associated with susceptibility to HSV and VZV infections (3, 13, 14). So are genetic defects of type I IFN signaling but there is surprising lack of CMV disease in these patients and it has been hypothesized that the CMV virus has evolved mechanisms to overcome the type I IFN-mediated antiviral defense (32). Instead, susceptibility to CMV infection or reactivation in patients with primary or acquired immunodeficiency is mostly related to impaired T-cell function (11). In healthy elderly subjects, the response to latent CMV infection biases the T-cell repertoire and can be a driver for immune aging and dysfunction (19). APECED patients display a complex phenotype of endocrine autoimmunity, chronic inflammation, anti-cytokine antibodies, abnormalities in T-cell subset distribution and function, and signs of accelerated lymphocyte differentiation and senescence (1, 20, 33). As the patients had a reduced frequency of CMV-specific T cells with seemingly normal phenotype, it is likely that the large population of terminally differentiated $CD8^+$ T cells in the patients is not explained by the accumulation of CMV-specific clones. Their specificity thus remains unknown, but it is possible that at least some fraction of them represents autoreactive T cells. The altered immune response against CMV observed in patients with APECED may affect their ability to control the latent infection, which may in turn promote a proinflammatory milieu. Nonetheless, the immune response appears to be sufficiently robust to prevent clinical CMV disease.

Our findings highlight the complex interplay between immune system and microbes in APECED.

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AUTHOR CONTRIBUTIONS

IH, NH and NW collected samples, IH, NH, PP and ASBW performed experiments, IH analyzed and interpreted the data, IH and TPA conceived and designed the study, HJ and TPA supervised the study, IH and TPA wrote the first draft of the paper, all authors contributed to the editing of the manuscript and the approved the final version.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ETHICS STATEMENT

The study was conducted according to the Declaration of Helsinki and approved by the Ethics Committees of Helsinki and Oxford University Hospitals; written informed consent was obtained.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available upon request.

ABBREVIATIONS

APECED: autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Gating strategy to identify CMV specific $CD8^+$ $pp65^+$ lymphocytes, their $CD27^-$ $CD45RA^-$ fraction and their $PD1^+$ expression.
Fig. S2. Correlation of anti-CMV igG levels with neutralising anti-IFN α2 and anti-IFNω antibody titers in patients with APECED.