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Anticommensal Responses Are Associated with Regulatory T Cell Defect in Autoimmune Polyendocrinopathy–Candidiasis–Ectodermal Dystrophy Patients

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Autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) is a monogenic autoimmune disease caused by mutations in the AIRE gene. Although mainly an endocrine disease, a substantial fraction of patients have gastrointestinal manifestations. In this study, we have examined the role of anticommensal responses and their regulation. APECED patients had increased levels of Abs against *Saccharomyces cerevisiae* ($p < 0.0001$) and against several species of commensal gut bacteria, but not against species predominantly associated with other locations. The anticommensal Ab levels did not correlate with gastrointestinal autoantibodies, neutralizing anti–IL-17 or –IL-22 Abs, or gastrointestinal symptoms, although scarcity of the available clinical data suggests that further study is required. However, the anti–*S. cerevisiae* Ab levels showed a significant inverse correlation with FOXP3 expression levels in regulatory T cells (Treg), previously shown to be dysfunctional in APECED. The correlation was strongest in the activated CD45RO$^+$ population ($\rho = -0.706$; $p < 0.01$). APECED patients also had decreased numbers of FOXP3$^+$ cells in gut biopsies. These results show that APECED patients develop early and sustained responses to gut microbial Ags in a pattern reminiscent of Crohn's disease. This abnormal immune recognition of gut commensals is linked to a systemic Treg defect, which is also reflected as a local decrease of gut-associated Treg. To our knowledge, these data are the first to show dysregulated responses to non-self commensal Ags in APECED and indicate that AIRE contributes to the regulation of gut homeostasis, at least indirectly. The data also raise the possibility of persistent microbial stimulation as a contributing factor in the pathogenesis of APECED. ***The Journal of Immunology*****, 2016, 196: 2955–2964.**

Autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED), also called autoimmune polyendocrine syndrome type 1, is a rare, recessively inherited disease caused by mutations in the autoimmune regulator (AIRE) gene (1, 2). AIRE is expressed in medullary thymic epithelial cells (1, 2), where it promotes the expression of tissue-restricted Ags. In the periphery AIRE is expressed at a lower level in lymphoid tissues and dendritic cells (3–5). A hematopoietic Aire$^+$ population has been identified in murine lymphoid organs and shown in a transgenic model to be able to induce deletional tolerance (6). Patients lacking functional AIRE develop a multiorgan autoimmune disease, affecting in particular endocrine organs (1, 7).

AIRE's role in promoting ectopic transcription of tissue-restricted Ags indicates that it contributes to thymic negative selection, but it is clear that it has other functions, as well. Several studies have reported defects in the number or function of regulatory T cells (Treg) (8–11), and dendritic cells have been shown to be abnormal (12, 13). Practically all patients have neutralizing Abs against type I IFNs (14), which are often the earliest manifestation of the disease, and autoantibodies targeting other cytokines are also found, of which anti–IL-17 and –IL-22 Abs have been linked to impaired defense against *Candida* (2, 15, 16). A more recent study reported that AIRE interacts with Dectin-1, an inflammasome-activating pathway linked to antifungal immunity (17). A substantial fraction of the patients also exhibit gastrointestinal manifestations, including chronic obstipation, diarrhea, gastritis, and hepatitis (1, 7, 18).

Gastrointestinal diseases, especially inflammatory bowel diseases (IBDs), have been linked to mishandling of commensal flora. This is best characterized in Crohn's disease (CD), in which Abs against several species of gut normal flora have been used as a diagnostic aid and in the prediction of disease progression (19, 20). Although the pathogenetic significance of these serological markers in CD remains controversial, large-scale genetic association studies have identified variants of innate pattern recognition receptors as risk factors (21), supporting the role of antimicrobial

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Abbreviations used in this article: AIRE, autoimmune regulator; APECED, autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy; ASCA, anti–*Saccharomyces cerevisiae* Ab; CD, Crohn's disease; EIA, enzyme immunoassay; IBD, inflammatory bowel disease; MFI, mean fluorescence intensity; TPH, tryptophan hydroxylase; Treg, regulatory T cell.

responses in the pathogenesis. It has been suggested that alterations in the innate immune system may lead to increased mucosal permeability and decreased microbial clearance, and, as a consequence, adaptive responses against commensals are induced (22). Also, beyond the local effects, the commensal microbiota is emerging as an important modulator of the systemic immune system, and its effects on autoimmunity in other organs are also gaining increased attention.

Given the high incidence of gastrointestinal manifestations in APECED, we studied whether the patients have abnormal immune responses to commensal organisms. Our results reveal a CD-like pattern of antimicrobial Abs, and they also show that this pattern is associated with defects in Treg.

Materials and Methods

Subjects

The study was conducted according to the Declaration of Helsinki, was approved by the Ethics Committee of Helsinki University Hospital, and written informed consent was obtained. The main study group consisted of 12 APECED patients (7 women; see Table I) and 44 healthy controls (mean age, 34.8 y; range, 21–75 y; 24 women). Eleven of the patients were sampled at three time points: at the mean age of 13.6 (range, 5–39), 27.4 (15–50), and 38.0 y (20–62). From one patient only one sample was obtained, at the third sampling. Eleven of the patients were homozygous for the Finn major mutation R257X, and one had the Finn major mutation in heterozygous combination with the 1085–1097 deletion. The most common disease components were chronic mucocutaneus candidiasis (12 of 12 patients), Addison's disease (10 of 12), hypoparathyroidism (9 of 12), diabetes, ovarian atrophy, severe chronic constipation (4 of 12), hypothyroidism, vitiligo and alopecia (3 of 12). The gastrointestinal manifestations included the following: gastritis, hepatitis, obstipation, and chronic diarrhea. At the third sampling the patients did not receive immunosuppressive treatment, had no acute infections, and none of them was pregnant. Similar information was not available for the earlier samplings.

Archival biopsy material from the upper gastrointestinal tract from APECED patients was provided by the pathology departments of each university or central hospital. Control duodenum samples were obtained from subjects examined to exclude celiac or other inflammatory disease, with negative biopsy results. Plasma samples were obtained from 37 patients with CD (15 women) aged 24–63 y (average, 39.2 y). The activity of the disease varied among patients [CD activity index (23), 20–367; average, 110.7). Most of them were in remission (28 of 37; CD activity index < 150).

Analysis of antimicrobial Abs

The microbes used in the study were stool, skin, or blood isolates from persons not belonging to study group. *Candida albicans* was obtained from American Type Culture Collection (strain 28366). They were cultured and identified using standard bacteriological methods. Abs were measured using enzyme immunoassay (EIA). Ninety-six–well MaxiSorp plates (Nunc, Thermo Fisher Scientific, Waltham, MA) were coated with microbes. Optimal dilution for plasma was titrated (1:100–1:300 for IgG and 1:30 for IgA) and plasma was added for 1 h at room temperature. Plates were incubated for 1 h at room temperature with secondary Ab rabbit anti-human IgG/HRP (Dako, Agilent Technologies, Santa Clara, CA) or rabbit anti-human IgA/HRP (Dako) and developed with *o*-phenylediamine. Re-

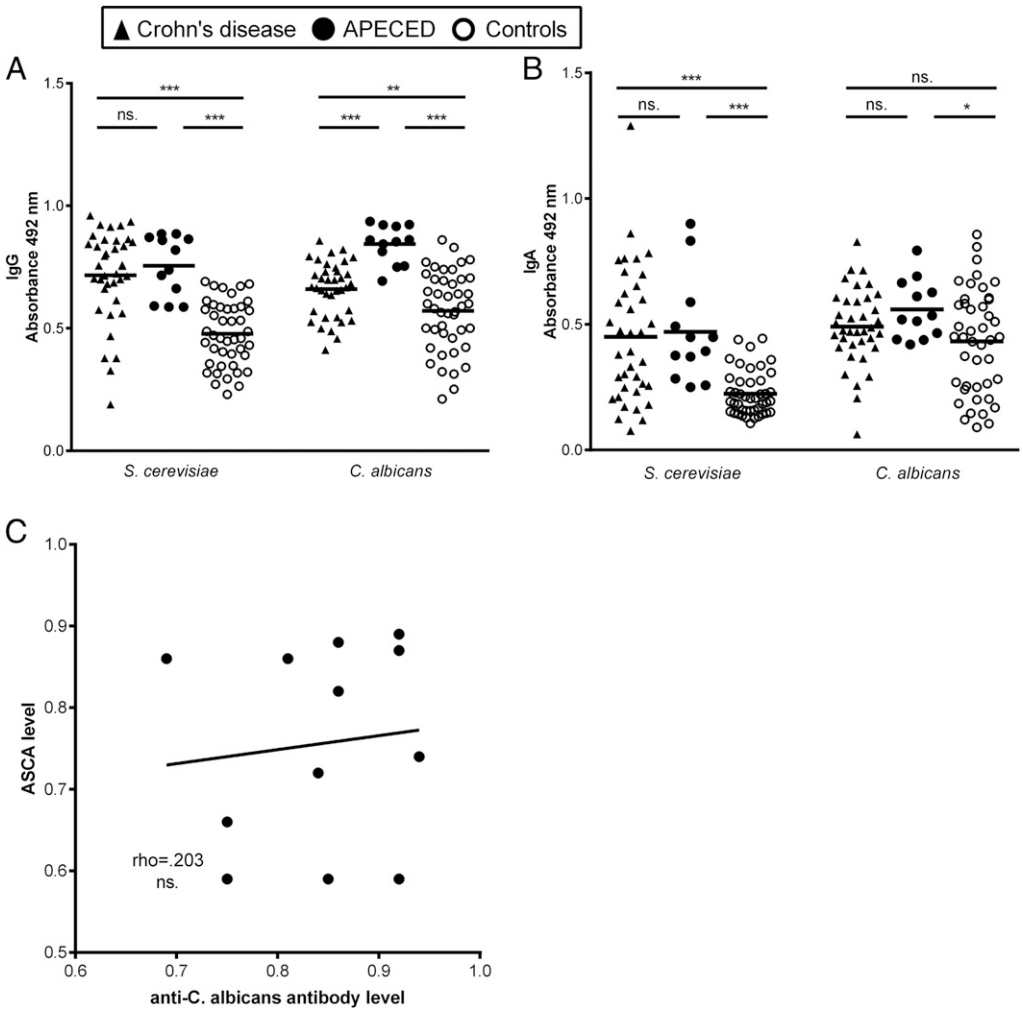

FIGURE 1. Antifungal Ab levels in patients with CD, APECED patients, and healthy controls. (**A**) ASCA and *C. albicans* IgG (Student *t* test) and (**B**) IgA levels (Mann–Whitney *U* test). (**C**) Comparison of ASCA and anti–*C. albicans* IgG levels in APECED patients (Spearman ρ). The Ab levels were measured using EIA and are shown as absorbance values. $^{**}p < 0.01$, $^{***}p < 0.001$.

action was stopped with 0.5 M H_2SO_4 and the absorbances were read at 492 nm using the iEMS reader MF (Labsystems, Helsinki, Finland). Commercial anti–*Saccharomyces cerevisiae* Ab (ASCA) determination was done using clinically validated diagnostic enzyme-linked assays at Fimlab Laboratories (Tampere, Finland). Serum total IgG and IgA levels were determined at the HUSLAB laboratory (Helsinki, Finland) using a clinically validated, accredited nephelometric method.

Anti–tryptophan hydroxylase RIA

The construction of plasmids containing cDNA encoding the Ags has been described previously (24). Radioactive Ags were expressed by an in vitro transcription and translation TNT coupled rabbit reticulocyte lysate system (Promega, Fitchburg, WI) with [^{35}S]methionine, and autoantibodies against tryptophan hydroxylase (TPH)-1 and TPH-2 were analyzed in a fluid-phase RIA as described previously (25).

Anti-cytokine Ab measurements

Anti–IL-17 Abs were measured using EIA, as their titer has been shown to correlate closely with neutralizing activity (15). Wells were coated with IL-17A or IL-17F (BioLegend, San Diego, CA; 2 μg/ml in PBS, pH 7.0) and blocked with 3% human serum albumin. Plasma (diluted 1:10) was added for 2 h at 22°C before washing and development with anti-human IgG–alkaline phosphatase conjugate (Sigma-Aldrich, St. Louis, MO). *p*-Nitrophenyl phosphate substrate and then 3 M NaOH were added, and the absorbances were read at 405 nm.

Neutralizing anti–IL-22 Abs were measured by using a cell-based bioassay. Colo205 cells were seeded at 3×10^4 cells/well in which IL-22 (2 ng/ml, R&D Systems, Minneapolis, MN) had been preincubated with serially diluted patient sera for 2 h. Supernatants were collected after incubation at 37°C for 24–30 h and analyzed for IL-10 secretion by EIA (R&D Systems). Neutralizing titer was calculated from EIA graphs as the serum titer that halved (ED_{50}) the IL-22 activity of the positive control sample.

Immunohistochemistry

Formalin-fixed, paraffin-embedded duodenal biopsy specimens from 7 APECED patients and 13 healthy controls were cut into 3-μm sections and deparaffinized. Controls underwent gastroscopy due to suspected celiac disease but with normal findings. Endogenous peroxidase activity was quenched using 1:10 hydrogen peroxide and blocked with normal horse serum 2.5% (Vector Laboratories, Burlingame, CA). Ag retrieval was performed by heating slides in citrate buffer at pH 6 for 10 min. Slides were incubated overnight at 37°C with mAbs for human FOXP3 (dilution 1:50, rabbit mAb, M3972; Spring Bioscience, Pleasanton, CA). As a detection system, a commercially available set was used (ImmPRESS universal Ab peroxidase polymer detection kit and 3-amino-9-ethylcarbazole SK-4200; Vector Laboratories). Quantitation of $FOXP3^+$ cells was done by calculating their number in an area bounded by 50 crypts in well-oriented duodenal samples. When the sample contained <50 crypts the number was normalized to 50 crypts. The data are shown as the ratio of $FOXP3^+$ cells/crypt.

Skin blister analysis

Blisters were induced by separating the epidermis from the dermis (26). A negative pressure of 40-50 kPa below atmospheric pressure was applied through suction cups (Dermovac, Ventipress, Lappeenranta, Finland), using a clinical suction pump (Itkavac WS-85, Instrumentarium Itka, Lahti, Finland), until the blisters were visible, a period of 30 to 90 min. To allow infiltration of skin-resident cells, the contents of the blisters were harvested 23 h later with a hypodermic needle, followed by a gentle lavage with PBS. The cells were freshly analyzed by flow cytometry.

Flow cytometry

Flow cytometric analysis of the patient cohort was done at the third sampling, and the phenotypic analysis of Treg and $CD8^+$ T cells has been previously published (9, 27); analysis of $CD4^+$ cells has not been published. Briefly, PBMCs were isolated using Ficoll-Paque (GE Life Sciences, Little Chalfont, U.K.) gradient centrifugation and stained in a single step with directly conjugated mAb: CD4-allophycocyanin-Cy5, CD5-PerCP-Cy5.5, CD45RO-allophycocyanin (BD Biosciences, Franklin Lakes, NJ), CD8–Pacific Blue, CD25-PE-Cy7, CD31-PE–Texas Red, and CD127-allophycocyanin-Cy7 (eBioscience, San Diego, CA), although not all these markers are presented in the present study. The cells were permeabilized using the FOXP3 permeabilization kit (eBioscience) and

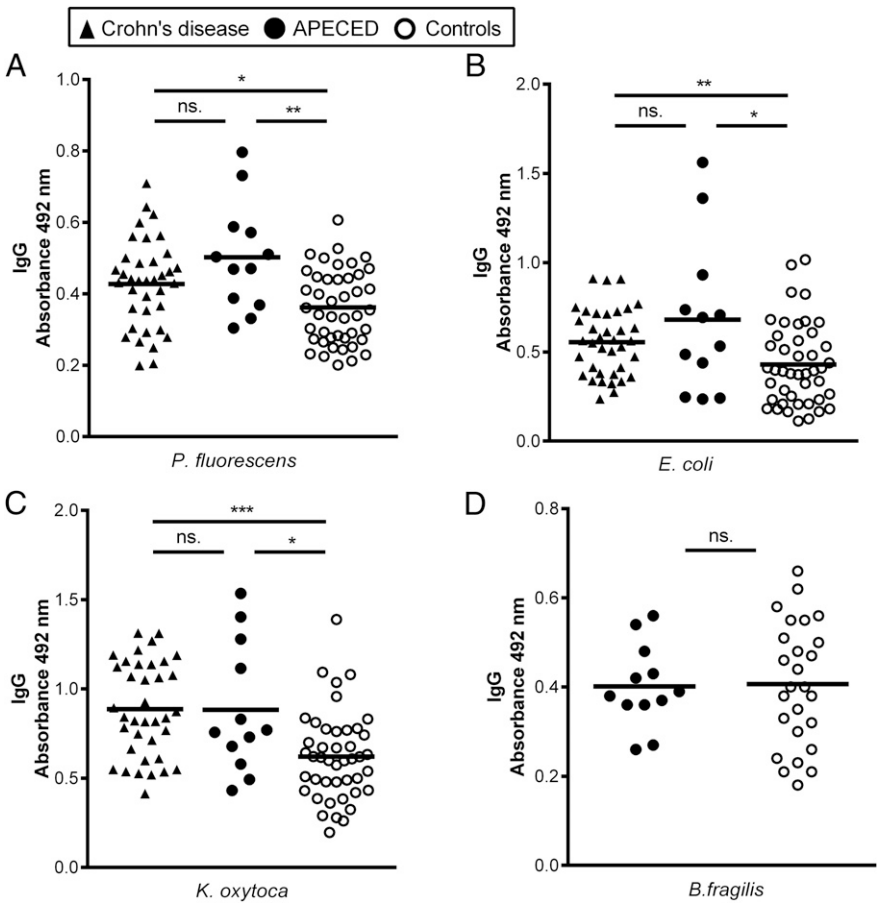

FIGURE 2. Ab levels against gut commensal bacteria. (**A**) *E. coli*–, (**B**) *P. fluorescens*–, (**C**) *K. oxytoca*–, and (**D**) *Bacteroides fragilis*–specific IgG levels were measured using EIA and are shown as absorbance values. *$p < 0.05$, **$p < 0.01$ by Mann–Whitney *U* test.

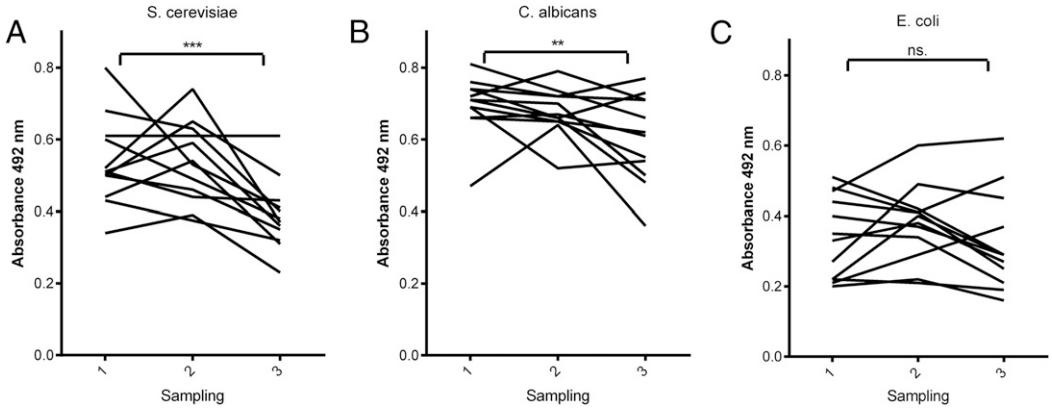

FIGURE 3. Persistence of anticommensal Abs. Abs against (**A**) *S. cerevisiae*, (**B**) *E. coli*, and (**C**) *C. albicans* were measured at three sampling points from 11 patients. The mean age of patients during the first sampling was 13.6 y (range, 5–39 y), during the second 27.4 y (15–50 y), and during the third 39.6 y (27–62 y). The data are shown as EIA absorbance values. The *p* values were calculated using a paired Student *t* test comparison of the first and third sampling. $^{**}p < 0.01$, $^{***}p < 0.001$.

stained with FOXP3-PE (eBioscience) and Ki-67–FITC (BD Biosciences) mAb. Compensations for spectral overlap were done using single-stained cells as well as BD CompBeads (BD Biosciences). The samples were run on a FACSAria instrument and analyzed using the FACSDiva software (BD Biosciences).

Suppression assay

Functional suppression assays were performed as previously described (8). Briefly, $CD4^+CD25^{high}$ cells were isolated using the Dynal Treg isolation kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The Treg were mixed with unselected PBMCs at a ratio of 1:3 and cultured for 5 d, stimulated by mitogenic anti-CD3 mAb immobilized to the bottom of the culture wells. The PBMCs were pulsed for the last 8 h with [^{3}H]thymidine (1 μCi/well; PerkinElmer, Waltham, MA), harvested with a Skatron harvester, and analyzed with a liquid scintillation counter. The amount of suppression was calculated as cpm (PBMCs) − cpm (PBMCs + Treg)/cpm (PBMCs) and compared with anticommensal Ab levels.

Statistical analysis

The normal distribution of all values was confirmed by a Shapiro–Wilk test. For data with normal distribution, *p* values for differences were calculated using a two-tailed Student *t* test, and correlations were calculated as Pearson correlation coefficient. For data significantly differing from normal distribution, a nonparametric Mann–Whitney *U* test and a Spearman rank correlation coefficient were used. The *p* value for the absence or presence of $FOXP3^+$ cells in duodenal biopsies was analyzed using a Fisher exact test. The limit for statistical significance was 0.05.

Results

Anticommensal Abs associated with IBD are found in APECED patients

One of the best established biomarkers for CD activity are ASCA, with higher ASCA levels associated with a more severe disease (19). Compared with healthy controls, our cohort of 12 adult APECED patients had significantly higher ASCA IgG and IgA levels (Fig. 1). On the average, the ASCA levels were as high as in patients with CD (n = 37, Fig. 1); this was also true when only those CD patients with an active disease (CD activity index > 150, n = 9) were analyzed. To further quantify the ASCA in APECED patients, samples were analyzed using a clinically validated diagnostic assay at a commercial laboratory. When using the clinical cut-off point (<20 IU/l), 8 of 12 APECED patients had positive ASCA IgG levels (range, 15–61 IU/l; mean, 28.6; data not shown), which closely matches data from CD patients (29–69% positive in different studies) (19). Five of the 12 patients also had increased ASCA IgA levels (data not shown), but these did not correlate with the IgG levels.

In accordance with earlier studies (28), the patients also had increased amounts of anti–*C. albicans* IgG, but anti-*Candida* IgA levels were not higher than in the controls (Fig. 1). Because anti–*C. albicans* Abs can cross-react with ASCA, we next determined whether the ASCA levels correlated with anti-*Candida* Abs (Fig. 1C), but found no significant correlation in either IgG or IgA Abs. To further ascertain the presence of serological markers of IBD, we measured the IgG Abs against four bacterial species reported to be associated with CD (19, 29, 30) (Fig. 2). The patients had significantly increased IgG levels against three of the four species, *Escherichia coli, Pseudomonas fluorescens,* and *Klebsiella oxytoca*; anti–*E. coli* IgG also showed a positive correlation with anti-*Klebsiella* IgG. The other anticommensal responses did not show a significant correlation, and the highest responders among the patients were different for different commensals. Again, these responses were similar to those found in CD patients, in whom the reported prevalence of anti–*E. coli* Abs is

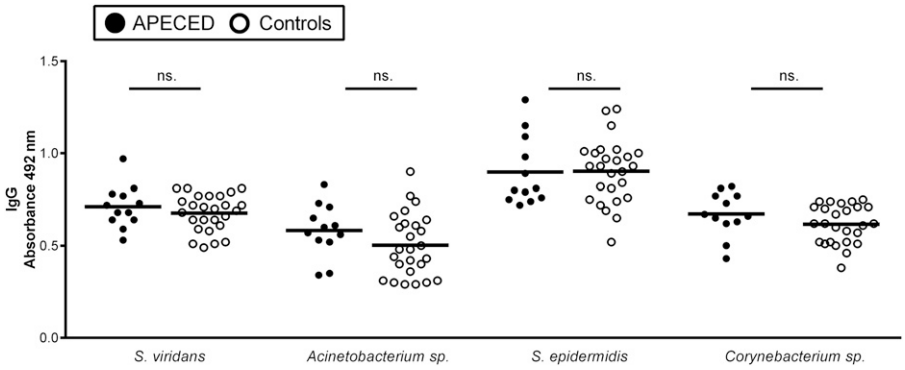

FIGURE 4. Ab levels against commensals not predominantly associated with gut. (**A**) *S. viridans*–, (**B**) *Acinetobacterium* sp.–, (**C**) *S. epidermidis*–, and (**D**) *Corynebacterium* sp.–specific IgG levels were measured using EIA and are shown as absorbance values. Mann–Whitney *U* test was used to determine significance.

27% (31). Using +2 SD of the healthy control values as a cut-off point for positive Ab levels, 3 of 12 (25%) and 4 of 12 (33%) of the APECED patients were positive for anti–*E. coli* and anti–*K. oxytoca* Abs, respectively. There was no significant difference in anti-*Bacteroides* Abs. IgA responses against *P. fluorescens* were significantly higher in the patients (not shown); no significant differences were found in IgA responses against the other bacteria.

Ab responses against gut flora are long lasting but do not reflect general increase in antimicrobial Abs

To study the duration of the anticommensal responses, we analyzed archived samples taken earlier from the same patients (n = 11), starting in one case from 5 y of age. Two earlier samples were chosen, taken on the average 12 and 26 y before the current sampling, and ASCA, anti–*E. coli*, and anti–*C. albicans* IgG levels were determined (Fig. 3). The Ab levels showed individual variation, but overall the levels were at least as high in the early samples as in the current sampling. The antifungal Abs declined with age, whereas anti–*E. coli* Abs showed no significant change.

To test whether the elevated and persistent Abs against gut flora might reflect generally increased antimicrobial Ab levels in the patients, we measured IgG levels against four other commensal bacteria, *Streptococcus viridans*, *Acinetobacter* sp., *Staphylococcus epidermidis*, and *Corynebacterium* sp., associated predominantly with tissues other than the gut. No significant differences in the Ab levels were observed between the patients and controls (Fig. 4).

Anticommensal responses are not associated with intestinal or anticytokine autoimmunity

Autoimmune damage to gut might lead to abnormal exposure and loss of tolerance to commensal Ags. To test this possibility we measured IgG Abs against TPH-1 and TPH-2, which are associated with gastrointestinal components in APECED (24, 32). Eight of the 12 patients had Abs against both forms of TPH, compared with 0 of 18 healthy controls tested (Table I). The medical records available indicated that 6 of the 12 patients had gastrointestinal manifestations, and five of them had anti-TPH Abs. However, no significant correlation between ASCA and anti-TPH Abs or clinical gastrointestinal manifestations was observed (data not shown).

Another possibility was that neutralizing Abs against IL-17 and IL-22, both important cytokines in the maintenance of gut homeostasis (33, 34), could cause abnormal antimicrobial responses. Five of our patients had autoantibodies against IL-17A, 11 against IL-17F, and all 12 against IL-22 (Table I). None showed any significant correlation with ASCA levels (Fig. 5).

*Anti–*S. cerevisiae *Abs correlate with the regulatory T cell defect*

Regulatory T cells are essential to intestinal tolerance, but in APECED patients they are dysfunctional (8) and express significantly decreased levels of FOXP3 (8, 10, 11). Only 8 of the 12 patients were available for isolation of live cells and functional suppressive testing. The degree to which isolated $CD4^+$ $CD25^{high}$ cells were able to suppress T cell proliferation triggered by anti-CD3 mAb showed no significant correlation with any of the anticommensal Ab levels (data not shown).

However, the complete cohort described in the present study has been analyzed earlier in detail by flow cytometry, and the decreased expression of FOXP3 and other abnormalities in the Treg population was confirmed. The phenotypic analysis has been previously published (9). Treg were defined as $CD4^+FOXP3^+$ $CD127^-$ cells. Using FOXP3 mean fluorescence intensity (MFI)

Table I. APECED patient characteristics

	Patients												Patient Mean ± SD (n = 12)	Control Mean ± SD (9) (n = 12)	p Value
	1	2	3	4	5	6	7	8	9	10	11	12			
Age (y)	43	34	23	31	47	50	41	44	30	65	35	45	40.7 ± 11.1	31.9 ± 7.8	
Sex	F	F	M	F	F	F	F	M	M	M	M	F	na	na	
GI symptoms	+	–	–	+	–	+	+	–	+	+	–	–	na	na	
ASCA rank	2	1	10	7	4	3	12	11	8	6	9	5	na	na	
Anti-TPH	–	–	+	+	–	+	+	+	+	+	+	–	na	na	
Anti–IL-17A abs.	—	3.6	—	1.7	—	—	0.9	3.7	0.9	—	—	—	na	na	
Anti–IL-17F abs.	2.8	3.9	—	3.8	2.5	0.9	3.2	2.6	3.9	2.6	3.8	0.6	na	na	
Anti–IL-22 titer	500	1500	20,000	1400	12,500	7500	40,000	1000	15,000	500	2500	2000	na	na	
Serum IgG	13.9	14.8	11.4	10.3	9.5	16.4	10.2	15.1	14.2	8.8	12.8	13.0	12.5 ± 2.5	6.8–15.0[a]	
Serum IgA	1.7	2.2	1.2	1.5	1.0	2.3	1.7	2.8	3.9	2.7	4.5	1.3	2.3 ± 1.1	0.5–4.8[a]	
% $CD4^+$ cells	60.8	36.3	35.4	18.0	54.9	28.5	41.2	28.5	46.7	39.7	21.1	55.6	38.9 ± 13.7	41.3 ± 6.8	ns
% $CD8^+$ cells	21.1	31.4	27.3	42.2	15.2	25.9	28.9	25.8	22.0	32.5	37.3	30.1	28.3 ± 7.3	26.0 ± 4.4	ns
% Treg	3.4	2.4	1.7	1.4	4.1	2.6	2.3	2.2	3.9	3.9	2.6	2.3	2.7 ± 0.8	2.6 ± 0.7	ns
FOXP3 MFI in Ref. 9															
Treg	2736	1924	3577	2815	3083	3347	3457	4205	3008	2306	3211	2175	2987 ± 646	3983 ± 1271	<0.05
$CD45RO^+$ Treg	2327	2519	3912	3089	3250	3463	4169	5276	3288	2357	3771	2221	3304 ± 901	4688 ± 1301	<0.01
$CD45RO^-$ Treg	2021	1628	2542	2010	2373	2396	2039	2527	2654	1885	2554	1765	2199 ± 248	2659 ± 733	ns

[a]Range in normal donors (HUSLAB, Helsinki, Finland), g/l.
abs., absorbance in EIA; GI (gastrointestinal) symptoms, any or several of B12 malabsorption, chronic diarrhea, steatorrhea, or chronic constipation; na, not applicable/not analyzed.

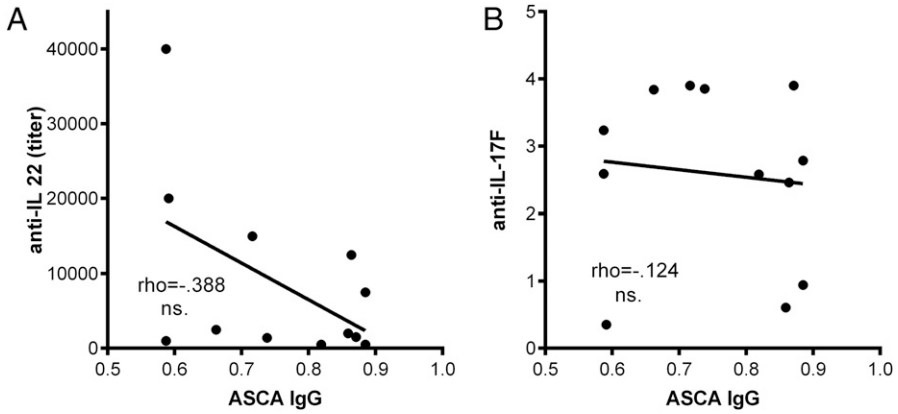

FIGURE 5. Correlation between neutralizing anticytokine Abs and ASCA. (**A**) Anti–IL-22 Abs were measured using a cell-based bioassay and are shown as titer. (**B**) Anti–IL-17F Abs were measured using EIA and are shown as absorbance. A Spearman correlation coefficient and the corresponding *p* value are shown.

as a surrogate marker for suppressive phenotype (8, 9), we found a negative correlation between ASCA and FOXP3 MFI in circulating Treg (Fig. 6A). Abs against bacterial commensals also showed an inverse correlation with FOXP3 MFI, but these correlations were not statistically significant. There was no significant difference between the patients and controls in the frequency of Treg (Table I), and Treg frequency showed no significant correlation with either ASCA or antibacterial Abs.

We then compared ASCA levels separately with the two main subsets of $FOXP3^+$ Treg, the $CD45RO^-$ cells, which form the resting reservoir with limited suppressive capability, and the activated $CD45RO^+$ cells responsible for the actual suppressive function (35). A strong inverse correlation between ASCA and FOXP3 MFI was found in the activated $CD45RO^+$ subset (Fig. 6B), but not in the resting Treg (data not shown). Notably, no correlations were observed in healthy controls (n = 12, Fig. 6C, 6D), indicating that the findings were linked to the disease. *C. albicans*–specific Abs or *S. cerevisiae*–specific IgA levels showed no significant correlations with Treg phenotype (data not shown).

APECED patients have a local defect of duodenal Treg

To further explore the link between the Treg dysfunction and ASCA, we analyzed archival gut biopsies, available from 7 APECED patients, 4 of whom were also included in our analysis of blood samples, and 13 healthy controls. Immunohistochemical analysis showed the presence of $FOXP3^+$ cells in the duodenum of all 13 healthy controls tested, whereas of the 7 APECED patients only 2 had detectable $FOXP3^+$ cells (Fig. 7A, 7B; Fisher exact test p = 0.001). For both of these patients serological analysis was also available; they did not have noticeably low anticommensal responses compared with other patients.

To test whether a similar apparent absence of $FOXP3^+$ cells was found in other epithelial locations, too, we used a method in which suction is used to induce skin blisters, and cells are then harvested from the blister fluid (26). Flow cytometric analysis (Fig. 7C, 7D) of skin-resident cells from four patients and six healthy controls showed that all of the patients had detectable $CD4^+FOXP3^+$ cells in their skin, although the previously reported decreased FOXP3 MFI (9) was seen also in the skin-resident cells. The systemic Treg defect in APECED patients may thus manifest in skin, as well, but $FOXP3^+$ cells are still present.

Discussion

Increasing numbers of observations suggest that gastrointestinal involvement in APECED has been underestimated, and recent data, although very limited, suggest that the composition of the intestinal flora may be altered in the patients (36). The data reported in the present study indicate that AIRE is an important regulator of intestinal homeostasis, although it is likely that the regulation is indirect. Whereas in healthy individuals the normal interaction between the immune system and gut normal flora is characterized by limited local IgA responses and systemic ignorance, in APECED patients a clear shift to IgG-dominated responses has taken place. Notably, the difference in ASCA IgG levels between patients and controls was comparable to the difference in anti-*Candida* IgG, and the latter is known to reflect an important facet of APECED, chronic candidiasis (1, 2). The data also indicate that the ASCA reflect generally dysregulated responses to gut-associated commensals and are not simply due to cross-reaction with *Candida* Ags. The levels of ASCA showed no significant correlation with anti-*Candida* Abs, and the results were confirmed by a clinically validated ASCA assay. Moreover, in addition to ASCA, responses against bacterial commensals were clearly increased in the APECED patients, and indeed as high as in CD.

A similar shift to systemic IgG responses is observed in IBDs (22, 37), and indeed not only the magnitude but also the pattern of anticommensal responses found in the present study in APECED was remarkably similar to that in the cohort of 37 patients with CD. The best characterized of the serological markers of CD is ASCA, which can be used for differential diagnosis between CD and ulcerative colitis, and also for the prediction of disease course (19, 20). Abs against several other commensals linked to CD were also found in APECED patients. Importantly, no increase in Abs against various other bacterial species was found; this was especially true for species preferentially associated with nonintestinal locations. Therefore, the ASCA and other anticommensal responses were not due to generally increased antimicrobial Ab levels.

Many of the complex immunological perturbations found in APECED could potentially contribute to mishandling of normal flora, including autoimmune damage to the gut or the neutralization of IL-22, a key mucosal effector cytokine. Anti-TPH is currently the best seromarker for intestinal autoimmunity (32), but neither it nor autoantibodies against Th17 cytokines correlated with ASCA. The present results thus do not support either of these potential mechanisms. Note, however, that all patients had high titers of neutralizing anti–IL-22 Abs, so the lack of correlation with ASCA does not necessarily exclude the role of IL-22 neutralization. Moreover, beyond the neutralizing anti–IL-22 Abs, the IL-22–producing cells themselves have been reported to be defective in APECED patients (15), which may manifest locally at the intestinal interphase.

It is also possible that the lack of AIRE affects the gut through other local pathways not examined in the present study. Extrathymic Aire-expressing cells have been shown to contribute to immune tolerance in secondary lymphoid organs (6) and both dendritic and NKT cell abnormalities have been reported in

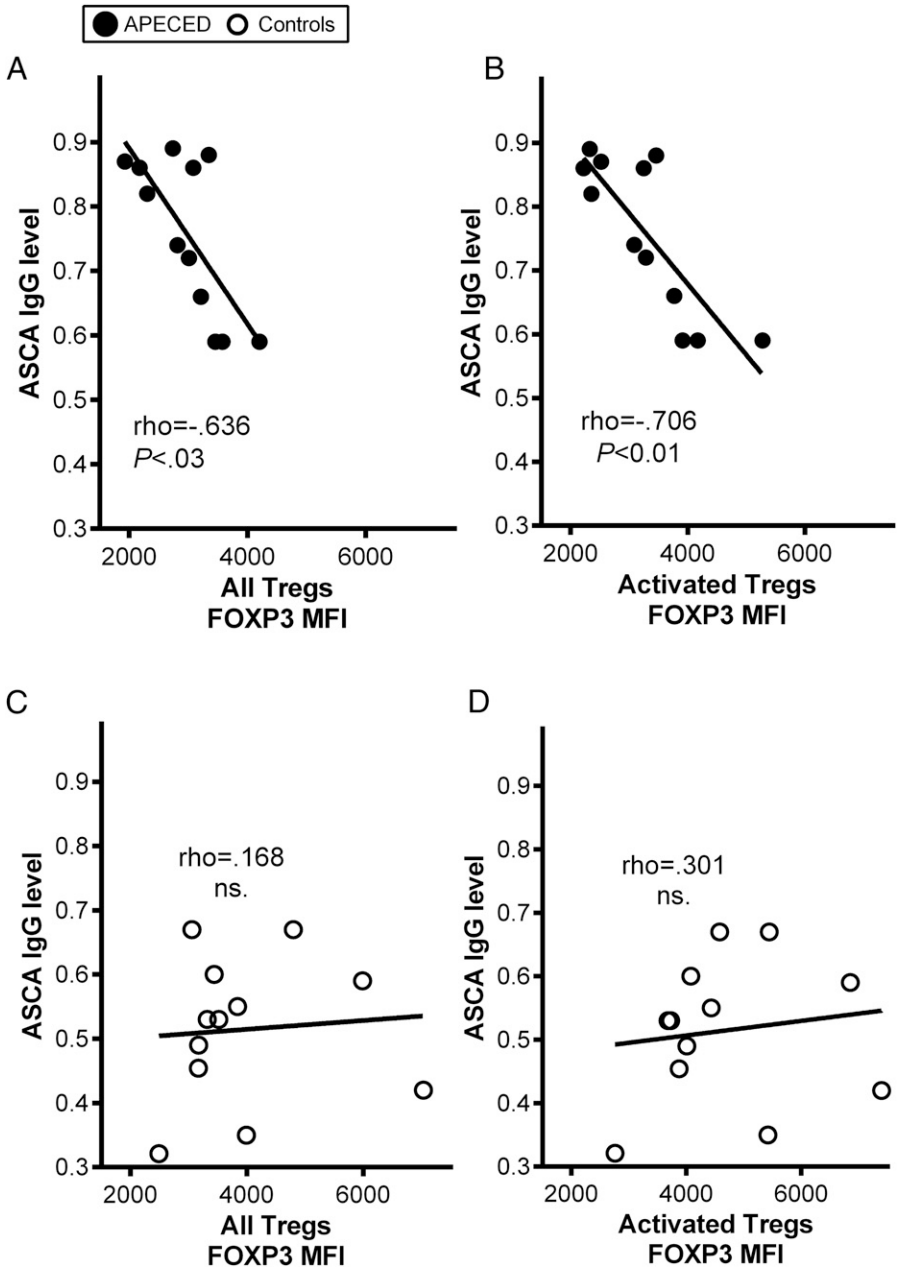

FIGURE 6. Correlation between ASCA and FOXP3 expression levels in Treg. Anti–*S. cerevisiae* IgG levels were compared with FOXP3 MFI in (**A**) all Treg and (**B**) activated Treg of the patients. Corresponding correlations for the healthy controls are shown in (**C**) and (**D**). The Spearman correlation coefficient and the corresponding *p* value are shown.

APECED (6, 12, 13, 38). All of these cells have also been implicated in the regulation of intestinal immunity, so disruption of their function might promote loss of tolerance to commensal Ags. However, given the localized nature of their function, it is difficult to assess their significance in the gut by analyzing peripheral blood cells in the patients. Finally, the increased anticommensal responses might be a reflection of changes in the composition of the intestinal microbiota. There are currently no data on the microbiome of APECED patients, but several studies have shown that in CD the microbial diversity is decreased, and some species, for example, *E. coli*, are overrepresented (39). The serological parallels between APECED and CD may suggest similarities also in the microbiota.

However, the strongest correlation that emerged from our data was the inverse relation between FOXP3 expression in Treg and ASCA. Although suppressive function showed no significant correlations, the functional analysis was done on only a subset of the patient cohort, and the current suppression assays are susceptible to considerable experimental variation. Moreover, they do not reveal heterogeneity within the Treg population, and in this study the inverse correlation was found predominantly in a subset of Treg. Extensive literature indicates that FOXP3 expression level correlates closely with suppressive capability (8, 9, 40–43), and as it is measured directly ex vivo, it is less likely than suppression assay to be affected by experimental artifacts. Indeed, in a recent analysis of human Treg the correlation was shown to be very strong ($p < 0.001$) (41). Thus, FOXP3 MFI on a single-cell level allows more accurate quantitative analysis than do the current suppression assays of bulk populations.

The inverse correlation was found particularly in the activated $CD45RO^+$ Treg subset, which is responsible for the actual suppressive function. Moreover, histological analysis of gut biopsies revealed a local deficiency in $FOXP3^+$ cells in the patients, whereas Treg were found in skin blister aspirates. Although the biopsies were archival samples and thus not taken at the same time as the blood samples, the temporal difference is unlikely to affect the results. As shown in Fig. 3, the anticommensal responses are a persistent and, at least in some patients, an early phenomenon, and therefore were present already when the biopsies were taken.

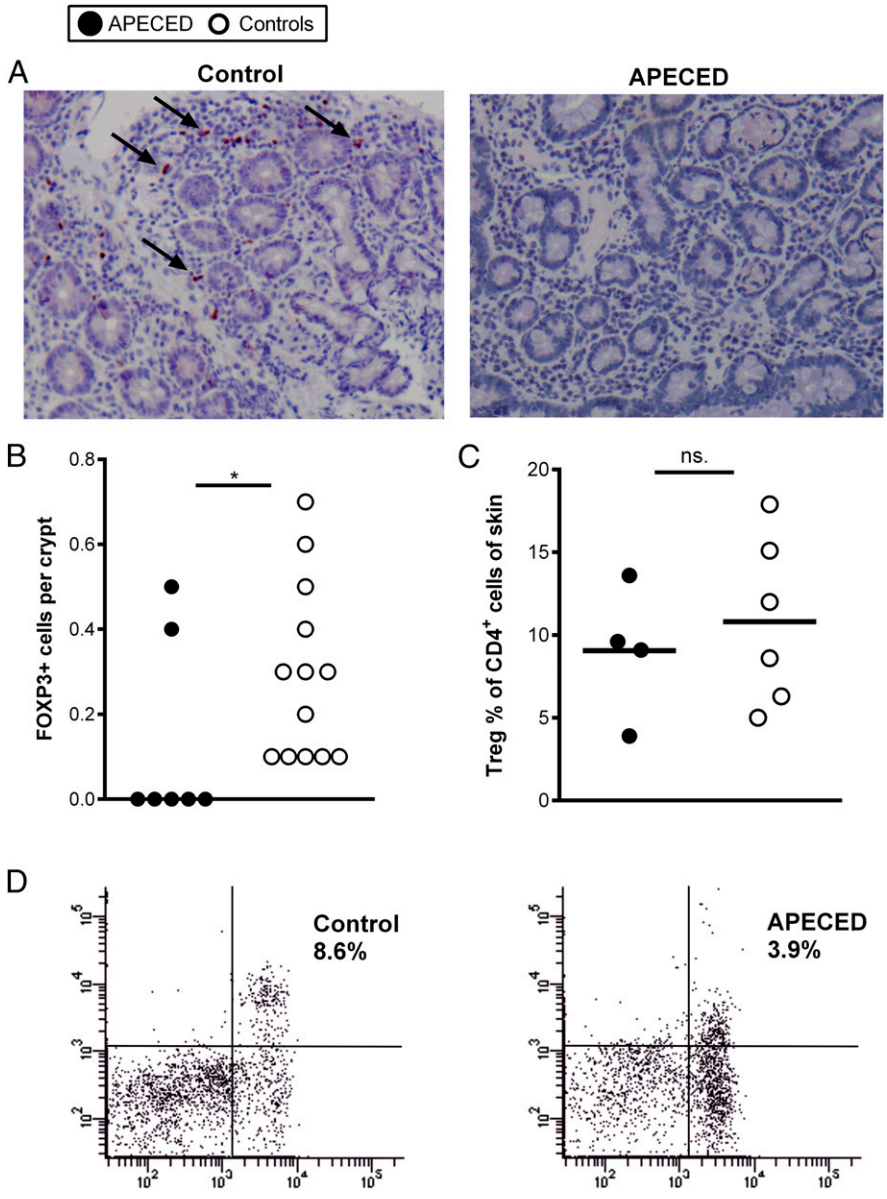

FIGURE 7. Presence of FOXP3$^+$ cells in epithelial locations. (**A**) Representative example of immunohistochemical analysis of FOXP3 expression in duodenal biopsies. FOXP3$^+$ cells are indicated by arrows. (**B**) Quantitation of FOXP3$^+$ cells in duodenal biopsies, shown as the number of FOXP3$^+$ cells per crypt. The p value was calculated using a Mann–Whitney U test. *$p < 0.05$. (**C**) Fraction of FOXP3$^+$ cells of all CD4$^+$ skin-resident cells, harvested from induced skin blisters. (**D**) Flow cytometric analysis of a patient and control showing the frequency of skin-resident FOXP3$^+$ T cells. The p values in (B) and (C) were calculated using a Mann–Whitney U test.

Our findings thus identify a putative mechanism to the emergence of the anticommensal IgG responses by showing an association with systemic impairment of the FOXP3$^+$ Treg population and identifying a local disruption of Treg in the gut. Treg are crucial for the maintenance of mucosal tolerance, so their decreased function might allow resident T and B cells to initiate and sustain responses against commensals, perhaps in conjunction with defects in Th17 cytokines. A parallel can be found in patients with IPEX, a rare autoimmune disease caused by mutations in FOXP3, in which the Treg defect gives rise to severe colitis (44, 45). Treg impairment has also been suggested to contribute to the pathogenesis of IBDs (46).

The alternative explanation for the correlation between ASCA and FOXP3 expression is that the mishandling of gut normal flora is the primary event and the Treg abnormalities a consequence, but this seems unlikely. First, the Treg defect in APECED manifests already in recent thymic emigrants (27), strongly suggesting that its origins lay at least partly in abnormal thymic development. This skewed maturation of Treg is difficult to explain by increased responses to gut commensals, if for no other reason than that much of the thymic development precedes the establishment of normal flora. Second, the decrease of FOXP3$^+$ cells in the gut is consistent with a local failure of Treg. We therefore propose as the most probable interpretation of our data that one manifestation of the underlying Treg dysfunction in APECED patients is that it allows the generation of abnormal responses against gut flora.

What, then, are the consequences of the enhanced anticommensal reactivity? The current view holds that in CD the anticommensal Abs are a marker of the pathological process and not pathogenic in themselves, and this is likely true of APECED, as well. However, in IBD the mishandling of normal flora is thought to be at least partly responsible for the local inflammation and tissue damage (22). A possible consequence of the responses found in APECED patients could thus be a similar local activation of immune mechanisms giving rise to intestinal inflammation. Our analysis of ASCA in relationship to clinical gastrointestinal manifestations or anti-TPH Abs provided no support for this outcome. Moreover, histological analysis of gut biopsies taken from APECED patients, although not from the same cohort as described in this study, showed in most cases no inflammation, abnormal lymphocyte infiltrates, or increased lymphocytic infiltration of the lamina propria (N. Kluger, M. Jokinen, A. Lintulahti, K. Krohn, and A. Ranki, manuscript in preparation) (36). However, the available clinical information and other data

are too scant to definitely exclude local effects, and the serological parallels with IBDs warrant a more detailed clinical examination. Also, a recent study reported the selective loss of enteroendocrine cells in the gastrointestinal tract of four APECED patients (47), another indication that the extent of intestinal pathology in APECED has not been fully characterized.

Another potential outcome is that the continuous stimulation by intestinal non–self Ags may be one of the factors driving the systemic immunopathology, and thereby contributes to organ-specific autoimmunity. This might be the result of direct stimulation by gut microbial Ags or structures recognized by innate receptors. The disturbed interaction between gut microbiota and the immune system may also result in increased local production of proinflammatory cytokines, with systemic effects. However, the small number of patients and lack of clinical follow-up in the present study does not allow us to correlate endocrine manifestations or disease progression with the anticommensal responses. The putative systemic effects thus remain to be studied.

Our data reveal a failure of intestinal tolerance to normal flora in APECED patients, and they suggest that it is linked to the functional Treg defect found in several studies. To our knowledge, this is the first demonstration of dysregulated responses against commensal non–self Ags in APECED, and thus reveals a novel aspect of the disease. Moreover, the findings suggest that AIRE regulates at least indirectly gut homeostasis, which may also have wider relevance regarding the impact of microbiota in autoimmunity.

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Disclosures

K.K., A.R., and P.P. are members of the Scientific Advisory Board of ImmunoQure AG (Martinsried, Germany) and are also minority shareholders. The other authors have no financial conflicts of interest.

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