

Anti-cytokine autoantibodies suggest pathogenetic links with autoimmune regulator deficiency in humans and mice

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Summary

Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) is a recessive disorder resulting from mutations in the autoimmune regulator (AIRE). The patients' autoantibodies recognize not only multiple organ-specific targets, but also many type I interferons (IFNs) and most T helper type 17 (Th17) cell-associated cytokines, whose biological actions they neutralize *in vitro*. These anti-cytokine autoantibodies are highly disease-specific: otherwise, they have been found only in patients with thymomas, tumours of thymic epithelial cells that fail to express AIRE. Moreover, autoantibodies against Th17 cell-associated cytokines correlate with chronic mucocutaneous candidiasis in both syndromes. Here, we demonstrate that the immunoglobulin (Ig)Gs but not the IgAs in APECED sera are responsible for neutralizing IFN- ω , IFN- α 2a, interleukin (IL)-17A and IL-22. Their dominant subclasses proved to be IgG1 and, surprisingly, IgG4 without IgE, possibly implicating regulatory T cell responses and/or epithelia in their initiation in these AIRE-deficiency states. The epitopes on IL-22 and IFN- α 2a appeared mainly conformational. We also found mainly IgG1 neutralizing autoantibodies to IL-17A in aged AIRE-deficient BALB/c mice – the first report of any target shared by these human and murine AIRE-deficiency states. We conclude that autoimmunization against cytokines in AIRE deficiency is not simply a mere side effect of chronic mucosal *Candida* infection, but appears to be related more closely to disease initiation.

Keywords: AIRE, APECED, IgG4, IL-22, thymoma, type I interferons

Introduction

The autoimmune regulator (AIRE) protein promotes ectopic expression of peripheral tissue autoantigens in medullary thymic epithelial cells, where they ensure the negative selection of potentially autoaggressive T cells, which is fundamental to self-tolerance induction [1,2]. Mutations in the *AIRE* gene cause autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy (APECED) [3,4]. Its most common features are chronic mucocutaneous candidiasis (CMC), hypoparathyroidism and adrenal insufficiency, but other endocrine and ectodermal cell types are also targeted frequently, although even more variably [5]. The autoimmune damage is often accompanied by high-titre autoantibodies against affected organs [5,6]. Even in mice with the two most common Nordic *AIRE* gene mutations, the disease phenotype differs from that in APECED

in many respects, with milder signs, no endocrine organ involvement, different autoantibody profiles and strong dependence on the genetic background [7–12].

Besides organ-specific autoantibodies, almost 100% of APECED sera have high neutralizing titres against certain cytokines. Those against type I interferons (IFNs) (especially IFN- ω and all 12 IFN- α s) appear early in infancy, even before any clinical signs [13,14]. As they also persist for decades, they are useful diagnostic markers of APECED [15]. While they down-regulate expression of IFN-stimulated genes by blood mononuclear cells, APECED patients do not show the expected susceptibility to common viral infections [16]. However, IFN-neutralizing autoantibodies might have contributed to the viral encephalitis they rarely develop [17]. Patients with thymomas – which also generate new T cells in the absence of AIRE – may have strikingly similar autoantibodies [6,18,19], and are also

prone to unexplained infections, occasionally including CMC [19–21].

Variable titres of autoantibodies against the T helper type 17 (Th17) cell-associated interleukin (IL)-17A, IL-17F and IL-22 are also found in 40–90% of APECED patients and in rare thymoma patients [19,22]. Remarkably, they correlate with loss of Th17/Th22 cells and with these patients' chronic mucocutaneous *Candida albicans* infections, in agreement with the known importance of these particular cytokines in normal protection against certain fungal infections on epithelial surfaces, rather than systemically [6,19,23]. We assumed that the cytokine neutralization by patients' sera was due solely to their respective autoantibodies, as it generally correlated well with their binding capacity detected with anti-immunoglobulin (Ig)G secondary antibodies [13,19]. Against IL-22, however, correlations were sometimes much worse [19], so we still need to exclude possible effects of other serum constituents.

Furthermore, the site(s) and cause(s) of autoimmunization against IFNs and cytokines in APECED and thymoma patients remain unclear. In chronically inflamed mucosal sites, *Candida* infection might overstimulate secretion of certain cytokines and so lead to autoimmunization against them. If so, many of these autoantibodies might be IgAs. Indeed, mucosal surfaces are important sites of protection by type I IFNs against viruses, by IL-17 against *Staphylococci* and by IL-22 against *Candida* [19,24–28]. Hence, IgA autoantibodies produced at sites of pathogen entry could interfere in these local protective mechanisms even more effectively than circulating IgG autoantibodies.

In this study, we demonstrate that APECED patient sera neutralize cytokines via IgG and not IgA autoantibodies, and that IgG1 and, unexpectedly, IgG4 are their predominant subclasses in both APECED and thymoma patients. We also show that the immunodominant epitopes of IL-22 and IFN- α 2a are conformational or located close to their C-termini. Finally, we report IL-17A-neutralizing autoantibodies, which are mainly IgG1, in AIRE-deficient mice, and discuss the implications of these novel findings for autoimmune pathogenesis in these AIRE-deficiency states.

Materials and methods

Patients and controls

We used sera from 19 APECED patients of Norwegian and Slovenian origin and from age- and nationality matched healthy controls described previously [16,19,29]. They were collected and stored in parallel at -20°C until used. Immunoglobulins were isolated from nine patient and seven control sera. The APECED diagnosis was confirmed by mutation analysis of *AIRE* genes and by the presence of autoantibodies to IFN- ω . We also studied sera from 40 UK thymoma patients [6,18,19]. The study was conducted in

accordance with the Helsinki Declaration, with informed consent and local Ethics Committees approval.

Mice

We used *AIRE*-mutant (967–979 del13) mice, originally on the C57BL/6 background [12]; one subline was back-crossed onto the BALB/c background for at least 10 generations. Homozygous *AIRE*-mutant, heterozygous and wild-type littermates were bred and maintained at the mouse facility of the Institute of Molecular and Cell Biology (Tartu University, Tartu, Estonia). Test sera were collected from mice aged either 6–8 weeks or 1.5–2 years, in accordance with the European Communities Directive (86/609/EEC).

Purification of immunoglobulins

Total IgG fractions were separated with fast protein liquid chromatography using Protein G Sepharose 4 Fast Flow (GE Healthcare, Biosciences, Little Chalfont, UK), concentrated and buffer-exchanged for phosphate-buffered saline (PBS) with iConTM concentrator 7 ml/20 K tubes (Pierce Biotechnology, Inc., Rockford, IL, USA). Total IgA was separated using agarose-bound Jacalin lectin (Vector Laboratories Inc., Burlingame, CA, USA) and dialysed against $1 \times$ PBS using Spectra/Por Dialysis Membrane (MWCO 12–14 000) (Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA). Protein concentrations were determined with the Bio-Rad Protein Assay, based on Bradford's method and using bovine gamma globulin as standard (Bio-Rad Laboratories Inc., Hercules, CA, USA).

The purities of the isolated IgGs and IgAs were assessed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The identity of the IgH bands was confirmed with specific antibodies (data not shown). The mean [$\pm$ standard deviation (s.d.)] protein concentration of IgG samples was $3.581 \mu\text{g/ml} \pm 2.570$ (range 514.5–10 158 $\mu\text{g/ml}$) and for IgA $533 \mu\text{g/ml} \pm 278$ (range 177.5–1 012 $\mu\text{g/ml}$). We detected $\leq 3\%$ IgG contamination in isolated IgA preparations by immune-turbidimetry with Cobas Integra 400 Plus (Hoffmann-La Roche, Basel, Switzerland). Binding enzyme-linked immunosorbent assays (ELISAs) for detecting type I IFN-specific IgG in purified fractions were performed as described previously [13]. IgG preparations were tested at 25 $\mu\text{g/ml}$.

Cell-based cytokine-neutralization assays

Anti-viral neutralization assay (AVINA)

AVINA was performed as described previously [13]. Briefly, the human glioblastoma cell line 2D9 was preincubated with dilute IFN- α 2a (Hoffmann-La Roche) or IFN- ω (Bender and Co., Vienna, Austria) preparations (10

laboratory units per ml) that had been preincubated for 2 h with serial dilutions of IgG or IgA samples (starting from 12.5 µg/ml). The cells were then challenged with encephalomyocarditis virus for 24 h before staining with 0.05% amido black, fixed with 4% formaldehyde in acetic acid buffer and washed with 0.15 ml of 0.05 M sodium hydroxide solution before absorbance was read at 620 nm.

Human IL-17A, IL-17F and IL-22 neutralization assays

The assays were carried out as described previously [19]. Briefly, for IL-17A, HFB4 human foreskin fibroblast cells (Schering-Plough Corporation, Reno, NV, USA) were seeded at 1×10^4 cells/well in which IL-17A (2 ng/ml; R&D Systems Inc., Minneapolis, MN, USA) had been preincubated with serially diluted IgG or IgA for 2 h. For IL-17F, NCTC 2544 keratinocytes (Interlab Cell Line Collection, Genova, Italy) preincubated with 0.1 ng/ml tumour necrosis factor (TNF) (Biolegend, San Diego, CA, USA) were used instead of HFB4. After incubation at 37°C for 16–20 h, supernatants were collected and assayed by ELISA (R&D Systems) for CXCL1. For human IL-22, we used the cell line Colo205. Cells were seeded at 3×10^4 cells per well in which IL-22 (2 ng/ml; R&D Systems) had been preincubated with serially diluted patient sera or IgGs or IgAs for 2 h. After incubation at 37°C for 24–30 h, supernatants were collected and analysed for IL-10 by ELISA (R&D Systems Inc.). Results from all the cytokine neutralization assays were estimated from graphs of ELISA absorbances as the ED50s – the concentration of Ig needed to halve the cytokine activity of the test sample, and represented graphically as cytokine neutralization units (NU) per µg of protein.

To test the neutralization capacity of mouse sera we used Cop5 mouse fibroblast cells for IL-17A and Colo205 cells for IL-22. Different (1:10, 1:100) dilutions of sera were preincubated with recombinant mouse cytokines from Biolegend: IL-17A (final concentration 1 ng/ml or IL-22 (0.3 ng/ml) for 2 h before Cop5 (1×10^4 cells/well) or Colo205 (3×10^4 cells/well) cells were added and incubated at 37°C for 24 h. Supernatants were assayed for IL-6 or IL-10, respectively, by ELISA (R&D Systems). The results are presented as the percentage inhibition of cytokine activity.

Constructs encoding luciferase (LUC) fused to full-length or truncated recombinant IFN-α2a and IL-22

An overview of all full-length and truncated human IFN-α2a and IL-22 proteins and the primer sequences we used are given in Table 1. Human IFN-α2a and IL-22 coding sequences were amplified by polymerase chain reaction (PCR) without the signal sequences. The PCR products were ligated into the *Bam*HI/*Not*I site of pPK-CMV-F4 (PromoCell GmbH, Heidelberg, Germany) mammalian expression vector using T4 ligase (Invitrogen, Carlsbad, CA, USA). All plasmids containing correct inserts (as confirmed by DNA sequencing) were propagated in *Escherichia coli* NOVA XG cells, amplified, extracted and purified using conventional methods. Finally, human embryonic kidney (HEK) 293 cells were transfected with the plasmids; after 48 h the crude protein extracts were prepared using 1× passive lysis buffer (Promega, Madison, WI, USA).

Table 1. Primer sequences for recombinant antigen cloning.

Antigen	Fragments		Primer sequences
Interleukin 22	34–76aa	F1	5'-TTTGGGATCCTCGCGCCCATCAGCTCCCCTGCA-3'
		R1	5'-TTTGCGGCCGCTCACCC AAT GAG ACG AAC GTC TGT-3'
	74–114aa	F2	5'-TTTGGGATCCTCC TCATTGGGGAGAACTGTTCC-3'
		R2	5'-TTTGCGGCCGCTCAATA AGG CTG GAA CCT ATC AGA-3'
	113–179aa	F3	5'-TTTGGGATCCTC CCTTATATGCAGGAGGTGGT-3'
		R3	5'-TTTGCGGCCGCTCAAATGCAGGCATTCTCAGA-3'
	34–114aa	F1	5'-TTTGGGATCCTCGCGCCCATCAGCTCCCCTGCA-3'
		R2	5'-TTTGCGGCCGCTCAATA AGG CTG GAA CCT ATC AGA-3'
	74–179aa	F2	5'-TTTGGGATCCTCC TCATTGGGGAGAACTGTTCC-3'
		R3	5'-TTTGCGGCCGCTCAAATGCAGGCATTCTCAGA-3'
Interferon-α2a	24–69aa	F1	5'-TTTGGGATCCTCTGTGATCTGCCTCAAACCCACA-3'
		R1	5'-TTTGCGGCCGCTCACTG GTT GCC AAA CTC CTC CTG-3'
	67–124aa	F2	5'-TTTGGGATCCTCGGCAACCAGTTCCAAAAGGCT-3'
		R2	5'-TTTGCGGCCGCTCACTG TAT CAC ACA GGC TTC CAG-3'
	123–188aa	F3	5'-TTTGGGATCCTCATAAGGGGTGGGGGTGACA-3'
		R3	5'-TTTGCGGCCGCTTACTTCTTAACTTTCTTGCA-3'
	24–124aa	F1	5'-TTTGGGATCCTCTGTGATCTGCCTCAAACCCACA-3'
		R2	5'-TTTGCGGCCGCTCACTG TAT CAC ACA GGC TTC CAG-3'
	67–188aa	F2	5'-TTTGGGATCCTCGGCAACCAGTTCCAAAAGGCT-3'
		F3	5'-TTTGCGGCCGCTTACTTCTTAACTTTCTTGCA-3'

Luciferase immunoprecipitation system (LIPS) assay

LIPS assays were conducted according to Burbelo *et al.* [30] in 96-well MultiScreen filter HTS plates (Millipore, Bedford, MA, USA) at room temperature using buffer A (50 mM Tris, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 1% Triton X-100) for all dilutions. Igs from test sera (diluted 1:25, tested in two parallels) were captured onto Protein G Sepharose 4 Fast Flow beads (25 µl of 4% suspension; GE Healthcare), which were then incubated with extracts containing IL-22, IFN-α2a or their fragments [10⁵ luminescence units (LU)]. After 1 h, washing and incubation with luciferase substrate (Promega), luminescence intensity was measured in a 1450 MicroBeta TriLux Liquid Scintillation Counter and Luminometer (PerkinElmer Life Sciences, Waltham, MA, USA). For autoantibody subtype detection, beads (1 µl of streptavidin agarose resin; Invitrogen) were incubated with biotin-conjugated human subclass-specific antibodies (anti-IgG1, anti-IgG2, anti-IgG4, anti-IgE were purchased from BD Pharmingen, anti-IgG3 from Invitrogen). At the same time, APECED or control sera (1:25) were incubated with antigen preparations (10⁵ LU of luciferase-linked IL-22 or IFN-α2a) and finally the immune complexes were captured onto the coated beads or anti-human IgA Agarose (Sigma) before LU reading as above.

ELISA

Serum autoantibodies binding to mouse cytokines were assayed by ELISA. Microtitre wells were coated with carrier-free recombinant mouse IL-17A, IL-17F or IL-22 (Biolegend) or IFN-α4 (PBL InterferonSource, Piscataway, NJ, USA) at 1–2 µg protein/ml (PBS, pH 7.0), overnight at 4°C. After blocking, mouse sera diluted 1:10 were incubated overnight at 4°C, before washing and development with either anti-mouse IgG (γ-chain-specific)-alkaline phosphatase conjugate (Sigma-Aldrich Corporation, Missouri, MI, USA) or anti-mouse IgG subclass-specific (IgG1, IgG2b, IgG2b, IgG3) biotinylated antibodies (Biolegend) followed by streptavidin-conjugated horseradish peroxidase (HRP)

and appropriate enzyme substrate and optical density (OD) reading. We considered as positive any values over 2 standard deviations above the mean of the control group (wild-type and heterozygous mice).

Western blot

Human IFN-α2 (PBL InterferonSource) was boiled for 5 min in reducing sample buffer [3% SDS, 10% glycerol, 0.1 M dithiothreitol (DTT), 0.02% bromophenol blue and 6.25 mM Tris-HCl, pH 6.8], run in 12% SDS-PAGE and blotted onto polyvinylidene difluoride (PVDF) filters. After blocking, strips of the filter were incubated with patient or control sera (1:100) or mouse anti-IFN-α2b antibody (1:1000; Abcam, Cambridge, UK) followed by secondary antibodies (anti-human HRP 1:100 000 and anti-mouse-HRP 1:30 000; Jackson ImmunoResearch, West Grove, PA, USA, Inc.). Reaction was visualized by enhanced chemiluminescence (ECL) using the manufacturer's protocol (GE Healthcare).

Statistical analysis

Mann-Whitney *U*- or Kruskal-Wallis tests were used to compare the median values between groups with GraphPad Software (San Diego, CA, USA). *P* < 0.01 was considered statistically significant.

Results

The cytokine-neutralizing autoantibodies are mainly IgG

We aimed first to identify the major Ig subclasses neutralizing the cytokines in APECED, and therefore isolated IgG and IgA from patient and control sera. As expected, ELISA detected autoantibodies against IFN-α2a in APECED, but not healthy control, IgG samples (Fig. 1a). Purified IgGs and IgAs were next assayed for neutralization of IFN-α2a, IFN-ω and Th17 cell-associated cytokines (Fig. 1, Table 2).

Table 2. The presence of neutralizing autoantibodies in nine APECED sera, and immunoglobulin (Ig)Gs and IgAs purified from them, against T helper type 17 (Th17).

Sample	Serum antibodies against			IgG neutralizing activity ED ₅₀ (ng/ml) against			IgA neutralizing activity ED ₅₀ (ng/ml) against		
	IL-22	IL-17A	IL-17F	IL-22	IL-17A	IL-17F	IL-22	IL-17A	IL-17F
A1	P	N	P	43	> 25000	5540	2500	> 25000	> 25000
A2	P	P	P	200	2200	11890	10000	> 25000	> 25000
A3	P	P	P	1900	1000	> 25000	> 16700	> 25000	> 25000
A4	P	N	N	105	> 25000	> 25000	> 16700	> 25000	> 25000
A5	P	N	P	900	> 25000	> 25000	> 16700	> 25000	> 25000
A6	P	N	N	2600	> 25000	> 25000	> 16700	> 25000	> 25000
A7	N	N	N	> 16670	> 25000	> 25000	> 16700	> 25000	> 25000
A8	N	N	N	> 16670	> 25000	> 25000	> 16700	> 25000	> 25000
A9	N	N	N	> 16670	> 25000	> 25000	> 16700	> 25000	> 25000

Cytokines (P: positive, N: negative). ED₅₀: the concentration of Ig needed to halve the cytokine activity of the test sample.

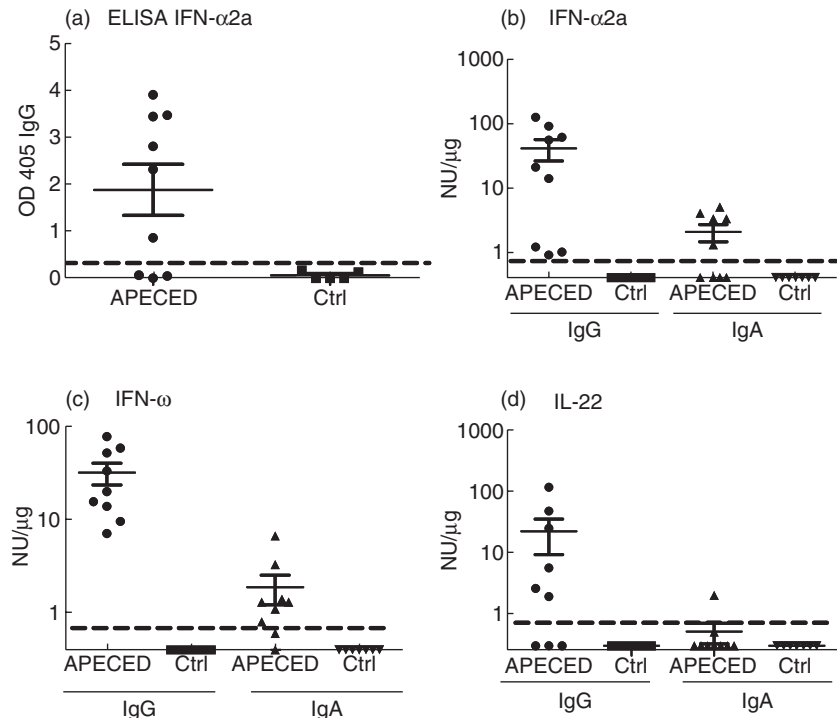


Fig. 1. Binding and neutralizing activities of immunoglobulin (Ig) isolated from autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) and control sera. (a) Enzyme-linked immunosorbent assay (ELISA) was used to test for anti-interferon (IFN)- α 2a autoantibodies in patient and control (ctrl) IgG fractions. The neutralizing activity of the isolated IgG and IgA fractions towards IFN- α 2a, (b), IFN- ω (c) and interleukin (IL)-22 (d) in cell-based assays. Cytokine neutralizing units (NU) per μ g of protein are shown. Nine patient and seven control samples were tested.

The neutralizing activity proved to be in the IgG fractions purified from APECED sera, as concentrations as low as 40 ng/ml blocked the actions of the cytokines tested.

Some purified IgA samples from several APECED sera showed weak neutralizing activity against IFN- α 2a and IFN- ω (Fig. 1b,c). Per μ g of protein, however, it was only ~3% (for IFN- α 2a) and ~7% (for IFN- ω) of that measured in the respective IgGs, so it could be attributed largely to the small amount of IgG (~3%) contaminating these IgA preparations. For IL-22, neutralization by the IgAs was very weak and was seen only with two patient samples (Fig. 1d, Table 2).

Neutralizing autoantibodies were detected in only two of the nine patients tested against IL-17A and 4 against IL-17F *versus* 6 against IL-22 (Table 2). Neutralizing activity was restricted even more clearly to the IgG and not the IgA fraction (Table 2).

IgG1 and IgG4 are the dominant subclasses against IFN- α 2a and IL-22

Seeking clues to autoimmunizing environments, we next determined the prevailing IgG subclasses among the anti-cytokine autoantibodies. Although we found significantly more positives for each subclass among the APECED than the control samples (except for IgG3 against anti-IL-22), the dominant subclasses against IFN- α 2a and IL-22 proved to be IgG1 and IgG4 (Fig. 2a). Signals for IgG1 were mainly higher than for IgG4, but two exceptional APECED patients had only IgG4 autoantibodies *versus* IFN- α 2a and IL-22. Interestingly, these two neutralized as well as the others in

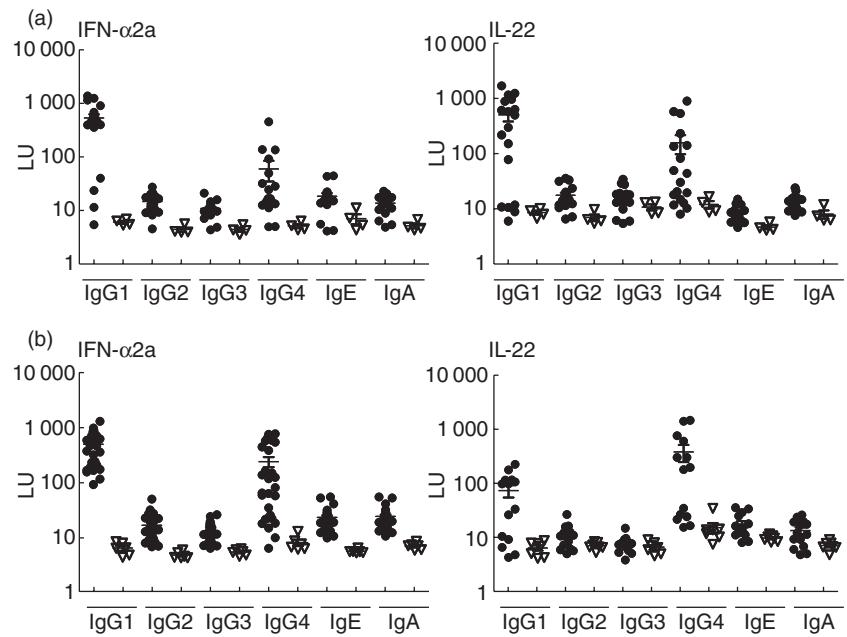
bioassays. Although IgE type antibodies often co-occur with IgG4 in allergic diseases, they were low in every APECED patient, exceeding the controls significantly only against IL-22 (Fig. 2a).

As autoantibodies against type I IFNs and Th17 cell-associated cytokines are also found in thymoma patients, although at lower frequencies [6,18,19], we also assessed their subclass distribution. Notably, the autoantibody positive sera showed even stronger IgG4 dominance, especially against IL-22 (Fig. 2b), where binding was almost exclusively by IgG4 in four of 15 sera; however, both subclasses were detected in the antibodies against their IFN- α 2a in every positive serum. Again, IgE autoantibodies were low and differed significantly from controls only against IFN- α 2a. IgA-type autoantibody levels were low in both syndromes, corroborating the previous results with isolated Ig isotypes. Collectively, the similar subclass/isotype distributions of their anti-cytokine autoantibodies suggest Th2 and/or regulatory T cell involvement in their induction.

Immunodominant epitopes of IFN- α 2a and IL-22 are conformational or C-terminal

To understand more clearly the neutralizing capacity of anti-cytokine autoantibodies, we cloned several cDNA fragments of IFN- α 2a and IL-22 to map their immunodominant epitopes. The three shorter truncated polypeptides from IFN- α 2a (24–69aa; 67–124aa, 123–188aa) or IL-22 (34–76aa, 74–114aa, 113–179aa) were not bound by any of the autoantibody positive sera tested (data not shown): any specific epitopes had apparently been lost from these

Fig. 2. Immunoglobulin subclasses against interferon (IFN)- α 2a and interleukin (IL)-22 in autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) (a) and thymoma (b) patients. Antibody levels are shown in luminescence units (LU). Mean values with standard error of the mean are indicated. Thymoma sera were selected by prior testing for autoantibody positivity ($n = 30$ for anti-IFN- α 2a and $n = 15$ for IL-22). All the sera were tested at least twice in three different experiments. Patient samples are indicated with filled circles, control samples with open triangles.



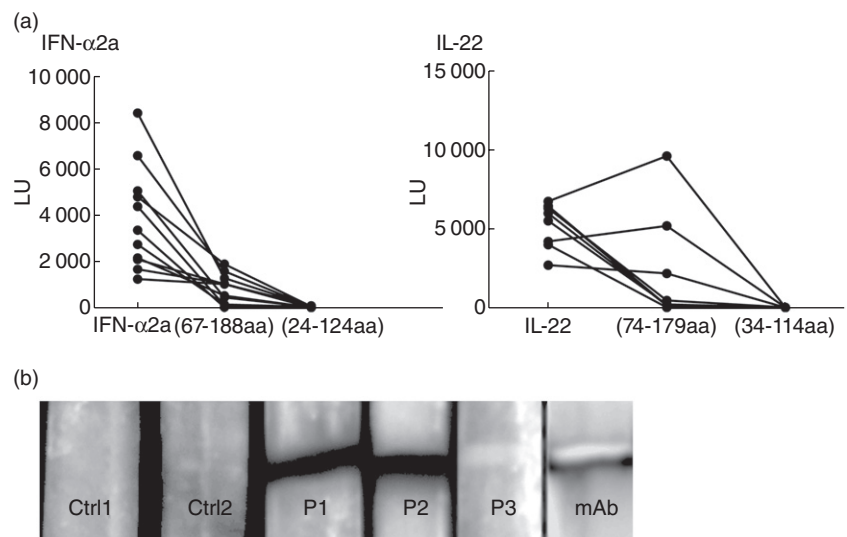
constructs. However, we did detect some binding of the longer C-terminal fragments of IFN- α 2a (67–188aa) and IL-22 (74–179aa), but not their more N-terminal fragments (24–124aa and 34–114aa; Fig. 3). Nevertheless, reactivity was much stronger – or exclusive – to the intact cytokines, suggesting that their major epitopes are conformational. These results were corroborated further using recombinant human IFN- α 2 that was denatured in reducing conditions before SDS-PAGE. We found that the APECED sera that bound exclusively to the full-length construct in LIPS assays (e.g. P3 in Fig. 3b) did not recognize denatured IFN- α 2 in Western blots, unlike other sera which had additional binding to the C-terminal construct (P1 and P2 in Fig. 3b).

AIRE-deficient mice develop binding and neutralizing autoantibodies against Th17-related cytokines: strain- and age-dependent responses

Previous studies in AIRE-deficient mice have not detected APECED-type organ-specific [10] or anti-IFN autoantibodies [12] (Meager *et al.*, unpublished observations), on either BALB/c or C57BL/6 backgrounds. Now we confirm the latter results once more with sera from 1.5–2-year-old BALB/c mice (Fig. 4a).

We next tested for autoantibodies to Th17 cell-associated cytokines at ages 6–8 weeks and 1.5–2 years. Although C57BL/6 AIRE-deficient mice show multiple tissue

Fig. 3. Epitope mapping of interferon (IFN)- α 2a and interleukin (IL)-22. (a) Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) sera were tested against full-length and N- and C-terminal fragments of IFN- α 2a and IL-22 using luciferase-conjugated polypeptides as antigens. (b) Control (ctrl) or APECED sera having exclusively conformation (P3)- or in addition C-terminus-specific (P1 and P2) autoantibodies to IFN- α 2 were tested on Western blot against denatured human IFN- α 2. Monoclonal antibody to IFN- α 2 was used as a positive control.



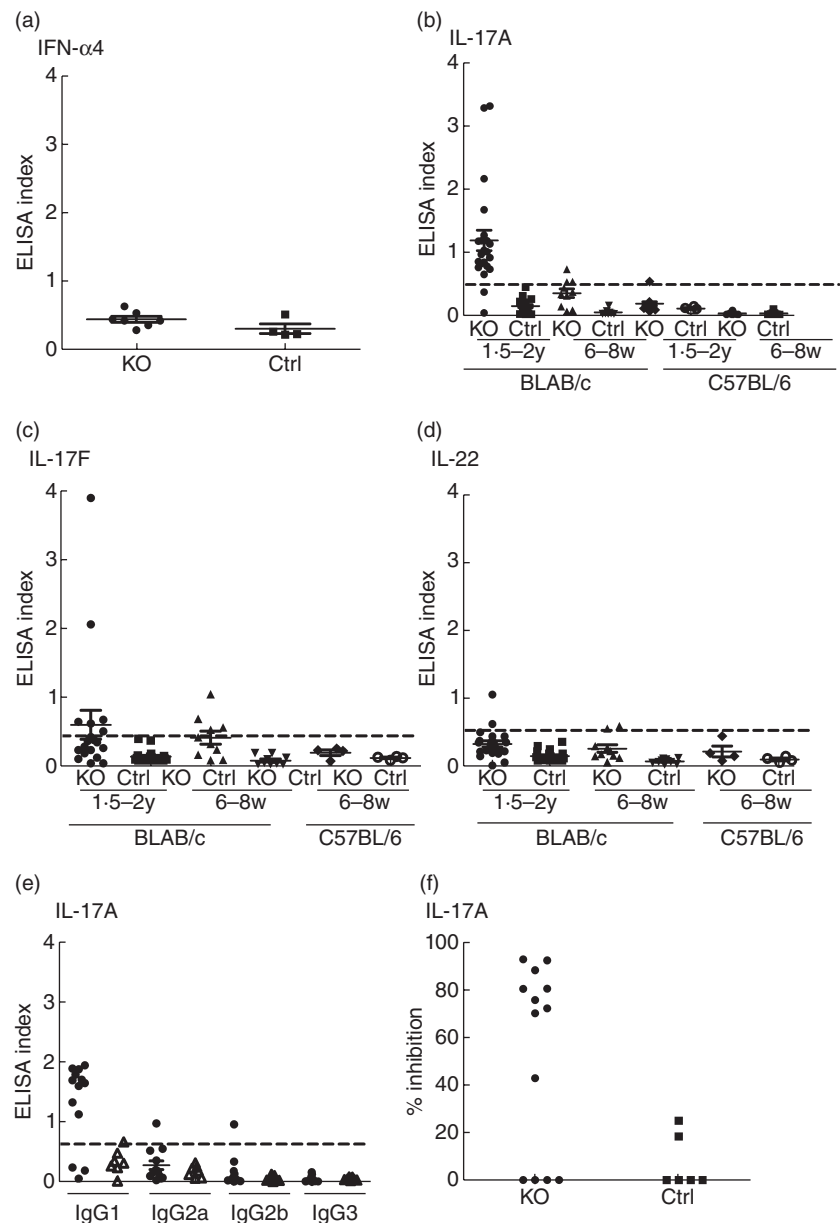


Fig. 4. Autoantibodies in autoimmune regulator (AIRE)-deficient mice against helper type 17 (Th17) cell-associated cytokines. Autoantibodies were assayed in mouse sera by enzyme-linked immunosorbent assay (ELISA) against murine interferon (IFN)- α 4 (a), interleukin (IL)-17A (b), IL-17F (c) and IL-22 (d) and by cell-based neutralizing assay against IL-17A (f). Autoantibody subclasses *versus* IL-17A are shown in (e). KO: AIRE-deficient mice; ctrl: combined AIRE wild-type and heterozygotes. Mean values with standard error of the mean are indicated. Horizontal lines indicate positive–negative discrimination levels drawn according to the old BALB/c group (mean + 2 standard deviations).

infiltrates by ages 5 months [9], 7 months [12] or 2 years [31], their life expectancies are unchanged and function is unaffected in most of the infiltrated organs, apart from reduced fertility in most mice, and blindness in a minority. On the BALB/c background more tissues are infiltrated [9] and 100% are blind by age 1.5 years.

Notably, sera from the AIRE-deficient BALB/c mice bound IL-17A significantly from 22 of the 24 aged animals (92%) *versus* only three of the eight young mice (38%; all very weak, Fig. 4b). In sharp contrast, we found weak binding in only one of the 12 (8%) aged AIRE-deficient C57BL/6 mice *versus* none of their young counterparts (Fig. 4b). Furthermore, nine of the 13 available aged BALB/c sera neutralized IL-17A bioactivity (Fig. 4f) *versus* none of those from young BALB/c or old C57BL/6 mice

(data not shown). Their neutralizing activity correlated broadly with the ELISA binding values.

We also found autoantibodies against IL-17F in seven of the 19 old (37%) and five of the 10 young (50%) BALB/c mouse sera (Fig. 4c). Signals were mainly weaker than against IL-17A, and almost all sera from the C57BL/6 mice were negative (Fig. 4c). Very few BALB/c or C57BL/6 sera showed even moderate binding of IL-22; it was detected mainly in the mice positive against IL-17F, but age differences were again less obvious (Fig. 4d). In addition, their sera did not neutralize IL-22 detectably (data not shown).

Finally, we tested the subclasses of the mouse autoantibodies to IL-17A. Interestingly, they were mainly IgG1 (Fig. 4e), implying a Th2-bias even more clearly than in the human syndromes.

Thus, like young APECED patients, ageing AIRE-deficient mice have neutralizing and/or binding autoantibodies to Th17 cell-associated cytokines. They are much more prevalent on the more severely affected BALB/c background and in older mice, possibly reflecting the progression of the pathological changes.

Discussion

For the first time, we show in this study that the IFN- α 2a, IL-17 and IL-22-neutralizing autoantibodies in both APECED and thymoma sera are IgG, rather than IgA, with a surprising bias towards IgG4 in many, and that there are similar IL-17A-neutralizing IgG1 autoantibodies in ageing/more severely affected AIRE-deficient mice on the BALB/c background. These results argue very strongly against the hypothesis that autoimmunization is a mere side effect of chronic mucosal *Candida* infection, in either APECED or its mouse 'model'.

One might expect IgA autoantibodies to be especially potent neutralizers of cytokines involved in mucosal protection against *C. albicans*. Although most typical human autoantibodies are primarily IgG, those characteristic of some gastrointestinal autoimmune diseases often include a high proportion of IgA. For instance, in coeliac disease, IgA anti-tissue transglutaminase antibodies are extremely useful and specific disease markers [32,33]. Similarly, in primary biliary cirrhosis, IgA autoantibodies against the major autoantigen, pyruvate dehydrogenase, are almost as prevalent as IgGs, and both isotypes inhibit its enzymatic activity [34]. In the event, our results argue very strongly against mucosal autoimmunization against cytokines, as almost all the neutralizing activity was recovered in the IgG but not the IgA fractions.

The subclasses of IgG antibodies can be indicators of the T helper subset that induced them originally. Previous studies have found a predominance of IgG1 among the APECED-typical autoantibodies against the steroidogenic enzymes 21-hydroxylase, 17 α -hydroxylase and cholesterol side-chain cleavage enzyme, implicating Th1-type immune responses in autoimmune destruction of adrenals and ovaries [35,36]. Surprisingly, we found that both IgG1 and IgG4 subclasses are prevalent among autoantibodies against IFN- α 2a and IL-22, both in APECED and thymoma patients although, in normal human serum, IgG1 is the most abundant and IgG4 the least. The production of IgG4 antibodies is thought to depend partly on Th2 cells/cytokines that favour allergic/IgE responses [37]. Interestingly, because of labile interheavy chain disulphide bonds, many IgG4 molecules are heterodimers (of two different heavy:light chain pairs) [38], and are thus suspected of blocking allergen recognition. Nevertheless, two sera containing only IgG4 autoantibodies neutralized IFN- α 2 and IL-22 just as strongly as others with abundant IgG1 autoantibodies.

The high IgG4 but minimal IgE autoantibody activity observed here suggests a primary role for IL-10, regulatory T cells and possibly also chronic antigen exposure in driving the IgG4 responses [39]. The main function of IgG4 is presumably to moderate immune inflammation or hypersensitivity induced by complement-fixing antibodies or IgE, but its role in APECED has yet to be defined. IgG4 antibodies are induced during hyposensitizing therapy (intradermal injection of small amounts of allergen) [40]. They are also prevalent or even predominant in certain autoimmune diseases that affect the epidermis, including pemphigus [41] or other epithelial tissues, e.g. IgG4-related sclerosing disease [42]. To us, they suggest autoimmunization in epithelial tissues, such as skin, or thymus, where AIRE-expressing medullary epithelial cells show maturation markers similar to those in epidermis [6,43]. This is supported by the strikingly similar IgG4 bias in patients with thymomas (Fig. 2b), where the key feature shared with APECED appears to be the generation of new T cells in the absence of AIRE [6,19]. Also, one subset of myasthenia gravis (MG) patients have mainly IgG4 autoantibodies that target the muscle-specific kinase (MuSK) [44]. Although IgG4s do not activate complement, their pathogenicity is generally accepted in pemphigus and has now gained ground in MuSK-MG [44], where it might result from interference in MuSK-dimerization or other interactions. However, the IgG4 bias in autoantibodies to cytokines in APECED and thymoma patients might reflect active regulatory mechanisms rather than tissue destruction, thus implying attempts to deviate from harmful Th1 responses.

To understand more clearly the neutralizing mechanisms of the anti-cytokine autoantibodies, we attempted to map their immunodominant epitopes in IFN- α 2a and IL-22. In many cases we found conformational epitopes, as none of the shorter truncated polypeptides or denatured IFN- α 2 was recognized by these patients' sera. Other sera were able to bind to their longer C- but not N-terminal polypeptides (encompassing approximately two-thirds of their lengths), as well as the denatured cytokine. These C-terminal regions contain amino acids that are more prone to make β -turns and are hydrophilic: two important qualities for evoking specific antibodies that recognize intact proteins [45]. As receptor-binding of both IL-22 and IFN- α 2a is via sites closer to their C-termini [46,47], any autoantibodies against these are likely to neutralize.

As yet, surprisingly few clinical or serological features of APECED have been reproduced in AIRE-deficient mice [10]. The absence of the APECED-characteristic autoantibodies against type I IFNs in AIRE-deficient mice is intriguing, and is also confirmed in Fig. 4d. Here we show that many of these mice instead develop autoantibodies against Th17 cell-associated cytokines. Like the autoantibodies in APECED, those we found against mouse IL-17A were able to neutralize it in functional assays. However, in the mice, IL-17A was recognized most often, IL-17F less frequently

and responses against IL-22 were only borderline, exactly reversing the order seen in APECED [19]. Moreover, human anti-cytokine autoantibodies appear in infancy or early childhood, even before APECED onset in the informative patients [14,22,48], and may decline slightly over subsequent decades [19], whereas those in the AIRE-deficient mice seem, rather, to rise in adult life. Importantly, IL-17A was clearly targeted more frequently in AIRE-deficient BALB/c mice, whose pathology is more severe, than on the C57BL/6 background where there are few signs of disease. The dominance of IgG1 autoantibodies in BALB/c is not surprising, as this mouse strain is Th2-biased. Variability in these autoantibodies between different strains might help to resolve some apparent contradictions between reports on *Candida* susceptibility in AIRE-deficient mice [12,49]. Moreover, their appearance, even in the absence of overt candidiasis, confirms further that they are not effects of CMC.

In conclusion, we have shown that the cytokine-neutralizing capacity of human APECED patient sera is due to its constituent IgG and not its IgA, arguing against autoimmunization provoked by chronic candidiasis at mucosal surfaces. The dominance of the IgG1 and IgG4 subclasses may implicate regulatory rather than Th1 cell-associated cytokines and cells in priming these autoimmune B cells. Our novel finding of IgG1 autoantibodies against Th17 cytokines in AIRE-deficient mice and their association with the disease severity supports further the pathogenetic significance of anti-cytokine immunoreactivity in AIRE deficiency. The specific sites and mechanisms leading to autoimmunization against these cytokines are currently under investigation.

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Disclosure

The authors declare no financial or commercial conflicts of interest.

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