AIRE-Deficient Patients Harbor Unique High-Affinity Disease-Ameliorating Autoantibodies

Highlights

- Each AIRE-deficient patient has a private repertoire of autoantibody reactivities
- Loss of B cell tolerance occurs during T cell-dependent somatic hypermutation
- Patient autoantibodies have unprecedented affinities for conformational epitopes
- Patient autoantibodies can display disease-ameliorating properties in vivo

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In Brief

Self-reactive antibodies specific for type I interferons are associated with protection against type I diabetes in patients with an autoimmune syndrome caused by mutations in AIRE.
AIRE-Deficient Patients Harbor Unique High-Affinity Disease-Ameliorating Autoantibodies

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SUMMARY

APS1/APECED patients are defined by defects in the autoimmune regulator (AIRE) that mediates central T cell tolerance to many self-antigens. AIRE deficiency also affects B cell tolerance, but this is incompletely understood. Here we show that most APS1/APECED patients displayed B cell autoreactivity toward unique sets of approximately 100 self-proteins. Therefore, autoantibodies from 81 patients collectively detected many thousands of human proteins. The loss of B cell tolerance seemingly occurred during antibody affinity maturation, an obligatorily T cell-dependent step. Consistent with this, many APS1/APECED patients harbored extremely high-affinity, neutralizing autoantibodies, particularly against specific cytokines. Such antibodies were biologically active in vitro and in vivo, and those neutralizing type I interferons (IFNs) showed a striking inverse correlation with type I diabetes, not shown by other anti-cytokine antibodies. Thus, naturally occurring human autoantibodies may actively limit disease and be of therapeutic utility.

INTRODUCTION

T lymphocyte tolerance is essential for limiting autoimmune disease. Tolerance occurs “centrally” when developing thymocytes with strongly self-reactive T cell receptors (TCRs) are deleted following engagement of self-antigen-derived peptides presented by major histocompatibility complex (MHC) antigens. The expression of thousands of tissue-specific self-antigens (TSAa) by medullary thymic epithelial cells (mTEC) is directly promoted by AIRE, a poorly understood transcriptional regulator (Mathis and Benoist, 2009; Klein et al., 2014). Reflecting its importance, AIRE deficiency is defined by the APS1/APECED syndrome for which autoimmune polyendocrinopathy and chronic mucocutaneous candidiasis are pathognomonic (Nagamine et al., 1997).

There are also several mechanisms of peripheral T cell tolerance, including requirements for co-stimulatory signals for the activation of naïve T cells; the expression of molecular “brakes” (e.g., CTLA-4, PD-1) by activated T cells; and the suppression of effector T cells in trans by FOXP3-expressing T-regulatory (T-reg) cells. Reflecting its importance, FOXP3 deficiency is defined by early-onset, life-threatening autoimmunity (Bennett et al., 2001; Wildin et al., 2001).

Central and peripheral tolerance mechanisms have likewise been hypothesized to shape the B cell compartment. Thus, self-reactive B cells developing in the bone marrow may be censored by clonal deletion, clonal anergy, or B cell receptor (BCR) editing in which secondary gene rearrangements replace the initial BCR with a new specificity (Goodnow et al., 2010; Pillai et al., 2011; Übelhart and Jumaa, 2015). Peripheral B cell tolerance is less well characterized, although some checkpoints have been inferred. For example, immature transitional B cells recently emigrated from the bone marrow contain many autoreactive and polyreactive cells, whereas there are relatively few among mature naïve B cells, strongly suggesting that tolerance is imposed as transitional B cells differentiate into naïve B cells (Wardemann et al., 2003).

Interestingly, this B cell checkpoint is T cell dependent, as reflected by its impairment in patients with T-reg deficiencies (Kinnunen et al., 2013). Likewise, CD40L and MHC class II deficiencies that each impair T-B interactions also display more autoreactive B cells (Meffre and Wardemann, 2008). These
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considerations raise the possibility that B cell tolerance is largely governed by the state of T cell tolerance.

Certainly, any autoreactive B cell that might progress through to the naive B cell compartment of a healthy individual should lack cognate autoreactive T cells to help it mature. Likewise, T cell help is required in the germinal center (GC) reaction in which B cells undergo somatic hyper-mutation (SHM) of the immunoglobulin (Ig) variable (V) region genes, thereby driving T cell-dependent selective expansion of clones with increased antigen affinity (Brink, 2014). The question that then arises is whether major defects in central T cell tolerance provoke wide-ranging losses of B cell tolerance at either or both of these stages.

An approach to assessing this is to examine B cell reactivities in AIRE-deficient APS1/APECED patients whose under-expression of TSAs in the thymus is predicted to lead to increased numbers of peripheral autoreactive T cells. Thus, there are reports of APS1/APECED patients carrying autoantibodies against twenty-five TSAs, with prevalence ranging from 6% to 69% (Kisand and Peterson, 2015). Their specificities include steroidogenic enzymes, consistent with the patients’ polyendocrinopathies (Krohn et al., 1992; Uibo et al., 1994; Winqvist et al., 1993). In addition, most patients display autoantibodies toward type I IFNs and T helper (Th)-17-related cytokines, antibodies to which limit resistance to Candida infection (Kisand et al., 2010; Meager et al., 2006; Puel et al., 2010).

These findings notwithstanding, there has been no large-scale analysis of the scope and nature of autoantibodies in APS1/APECED patients, thereby resolving how T cell tolerance impacts upon B cell tolerance in humans. By analyzing 81 APS1/APECED patients, we found that each was much more likely than a healthy relative or an unrelated control to harbor strong serum reactivities toward ~100 human proteins. About 10 of those, including type I IFNs and interleukin-22 (IL22), were recognized by almost all patients, whereas others were mostly “private specificities.” Hence, 81 patients collectively harbored antibodies toward >3,700 human proteins.

Focusing on antibodies to type I IFNs, IL22, and IL17, we found unexpectedly that most were reactive to conformational determinants and included highly mutated antibodies of subpimocorial affinity. Because their germline counterparts were not self-reactive, B cell autoreactivity was most probably driven by self-reactive T cells in the GC reaction. The autoantibodies commonly neutralized their targets in vivo, and APS1/APECED patients with signature type 1 diabetes (T1D)-associated antibodies (e.g., anti-GAD65) commonly failed to develop a disease-defining antibody repertoire.

RESULTS

High-Titer Autoreactivities in APS1/APECED

Sera from 81 APS1/APECED patients from discrete Finnish, Norwegian, Slovenian, and Sardinian cohorts were directed against a ProtoArray displaying ~9,000 immobilized recombinant human proteins or protein fragments. Because some patients were sampled longitudinally, 97 sera were assayed in total. Control sera were from healthy first-degree relatives (n = 9) and healthy unrelated volunteers (n = 12) across the same age range. Data readouts for the binding of individual sera were normalized by applying robust linear modeling (Sboner et al., 2009), whereafter each signal was assigned a Z score denoting the number of standard deviations (SD) above or below the mean of the combined healthy relatives and controls.

Most patients and the combined controls displayed Z scores of 1–2 for ~200 proteins (Figure 1A). However, when the convention was employed of defining Z ≥ 3 as bona fide positives, the patients segregated from the two control cohorts, considered either jointly or separately. Thus, each control serum displayed reactivities of Z ≥ 3 toward an average of ≤20 proteins, with most recognizing < 10 (Figures 1A, 1B, and S1A). Given that there was inter-individual variation, the 21 control sera collectively displayed Z ≥ 3 reactivities toward 406 distinct proteins, i.e., ~5% of those displayed on the array (Figure 1B). For only 2 proteins was Z ≥ 4, and for none was Z ≥ 5 (Figures 1A and 1B). Hence, as expected, the control cohorts largely lacked high-titer serum autoantibodies.

Conversely, most patients at any one time displayed Z ≥ 3 autoreactivities toward ≥80 proteins (Figures 1A, 1B, and S1A). These data were re-analyzed with stringent procedures to minimize false-positives, including exclusion of any signals that might have arisen from cross-sample print contamination. With this achieved, the patients’ “private” autoantibody repertoires collectively detected 3,731 distinct targets (Figure 1B). Furthermore, almost all patients displayed Z ≥ 4 scores for at least 10 proteins (mean of ~30), collectively recognizing > 1,500 proteins, and >50% of patients displayed Z ≥ 5 scores for ≥10 proteins (mean of >12), collectively recognizing 636 proteins (Figures 1A and 1B). Hence, high-level reactivity toward multiple self-proteins was a disease-defining property. This was further illustrated by the qualitative difference in Z score distribution curves for patients versus controls, which cannot simply be explained by there being 5-fold more patient sera (Figure 1C). Thus, whereas sampling greater numbers would likely have increased the protein species detected by control cohorts at Z ≥ 4, it would not bridge the 1,000-fold gap between two proteins detected by 21 control sera versus >1,500 proteins detected by 97 patient sera (Figure 1B).

Figure 1. Immune Response Profiling of APS1/APECED

(A) Distributions of hits between patients and controls at different Z scores.

(B) Z scores for all samples against all protein features and mean hits for each group calculated for Z ≥ 3, Z ≥ 4, and Z ≥ 5. The number of distinct proteins targeted in each group (P, n = 97; C, n = 21) at Z scores denoted. The complexity factor was calculated by dividing the number of distinct proteins by average number of hits per patient.

(C) The max Z score distribution of all proteins in patient and control groups.

(D) Fraction of patients recognizing each of 3,731 proteins at Z ≥ 3. Red dots depict 126 proteins shared between patients and controls.
In sum, 81 different patients collectively displayed strong reactivities to >40% of human proteins arrayed. For most proteins (blue dots 13–3731, Figure 1D), reactivities were spread across the cohort, reflecting high inter-patient variation, whereas ~12 proteins (blue dots 1–12, Figure 1D), including several type I IFNs, were recognized by > 60% of patients, as reported (Meager et al., 2006). However, the “public specificities” were not enriched among the 126 reactivities shared between patients and controls at z > 3 (red dots, Figure 1D), emphasizing that their common autoantigenicity is unique to the patients. Patient auto-reactivity frequencies were largely comparable across geographical locations, albeit somewhat less in Norway and Slovenia, and age ranges (Figures S1B and S1C). Indeed, most anti-IFN auto-antibodies of APECED patients were reported to increase in early life and remain stable thereafter (Meager et al., 2006; Wolff et al., 2013).

The collective targets of patient antibodies included intracellular, trans-membrane, and secreted proteins. Because many proteins displayed on the ProtoArray may be denatured, there may be false-negatives that underestimate patient reactivities to conformational determinants. Although a detailed analysis of the types of proteins targeted will be presented, it is evident that the proteins most commonly detected by patient sera included numerous cytokines, particularly type I IFNs, for which reason this study focuses on the nature of those autoreactivities.

**Strong, Selective Anti-Cytokine Reactivities**

Human type I IFN genes include 13 IFNα genes, 1 IFNβ gene, and 1 IFNω gene. There is also a type II IFNγ gene and three type III IFN genes. IFNγ is largely limited to lymphocytes, whereas type I and type III IFNs are broadly expressed, with their functional uniqueness and/or redundancy unresolved (Ivashkiv and Donlin, 2014). As assessed by ProtoArray, patient sera showed significantly stronger reactivities than controls toward all IFNα subtypes, albeit the reactivities to some (e.g., α1/13, α5, and α14) were higher than those to others (e.g., α2, α16, and α21) (Figure 2A). The differential between patients versus controls was emphasized by luciferase-based immunoprecipitation (LIPS) in which many target proteins were recognized in their native conformations (Figure 2B). Many patients showed strong reactivities to IFNω but rarely toward IFNβ (Figure 2B) and never toward IFNκ and IFNω, two phylogenetically distant type I IFNs (data not shown). By contrast, patient sera harbored reactivities significantly above controls toward IL1α, IL5, IL6, IL17A, IL17F, IL20, IL22, IL28A (IFNα2), IL28B (IFNα3), and IL29 (IFNλ1) (Figure 2C). Whereas reactivities toward some targets (e.g., IL17F, IL22) were common to most patients, reactivities toward others (e.g., IL20, IL28, IL6) were not (Table S1), and with the exception of IL5, patient sera mostly did not detect either Th2 cytokines (e.g., IL4 and IL13) or IL21, a Thf (T follicular helper) cell cytokine that drives high-affinity antibody maturation. There were also no reactivities toward G-CSF and GM-CSF (Table S1), which drive the development of myeloid cells associated with the patients’ inflammatory endocrinopathies.

Cytokine reactivities were largely validated by ELISA, which confirmed that IFNγ was only rarely and weakly recognized by patient sera (Figure 2D; Table S1) and that there was no reactivity toward TNFα (data not shown). By contrast, ELISA revealed auto-antibodies toward IL32α and IL32γ, two poorly characterized proinflammatory cytokines (Figure 2D; Table S1). In sum, 81 APS1/APECED patient sera collectively displayed strong reactivities to a very selective subset of human cytokines.

**Very High-Affinity Human Antibodies**

To understand the nature of patient serum reactivities, nine IFNκ-specific monoclonal antibodies (mAbs) were derived by limit-dilution cloning from memory B cells of four patients. Two were characterized in detail (26B9 and 19D11), whereas a more limited analysis of the others strongly argued that the properties of 26B9 and 19D11 were generally representative of patients’ cytokine-specific antibodies. First, their Vκ and Vλ sequences were highly mutated relative to their germline counterparts, with non-conservative replacements enriched in complementarity-determining regions (CDRs), as expected (white; Figure 3A). The antibodies bore no obvious resemblance to each other in V-gene segment or CDR3 usage. Conversely, a third anti-IFNκ antibody, 50E11, shared with 19D11 the same Vκ (IGHV1-69) and junctional (IGHJ4) segments and a very similar light chain (IGKV3-11 versus V3-20) (Figure S2A). However, there were very different template-independent nucleotide insertions in the Vκ CDR3s of 19D11 and 50E11, and the somatic mutation patterns were different: whereas 19D11 and 26B9 showed high mutation frequencies in Vκ, CDR2 and Vκ CDR1, 50E11 did not (Figures 3A and S2A).

The recombinant antibodies 26B9 and 19D11 harvested from transfected CHO cells were immobilized on surface plasmon resonance (SPR) chips over which were run recombinant human IFNκ2b, IFNκ4, IFNκ14, and IFNω, the latter being recognized by 26B9 but not by 19D11 (Figure 3B). These experiments revealed very slow off-rates reflecting extremely high affinities of the antibodies for their targets, ranging from $K_D = 3.28 \times 10^{-14} \text{M}$ for 26B9 toward IFNκ14 to $K_D = 2.09 \times 10^{-11} \text{M}$ for 26B9 toward IFNκ2b (Figures 3B and 3C). Sub-picomolar/near-femtomolar dissociation constants were likewise shown by 19D11 (Figures 3B and 3C). Thus, APS1/APECED patients harbor some of the strongest affinity antibodies described.

18-mer peptides spanning IFNκ2b and IFNω were used to map linear epitopes recognized by 26B9 and 19D11. However, no specific reactivities were detected (data not shown), consistent with the antibodies binding conformational determinants shared by several type I IFNs. Also, the antibodies reacted poorly or not at all to mouse IFNs (Table S2).

To investigate the origins of the high-affinity, conformation-specific antibodies, germline counterparts for 19D11, 26B9, and 50E11, albeit with the same CDR3-VDJ sequences, were expressed and tested by LIPS against recombinant human IFNκ2b, IFNκ4, and IFNω, and IFNω, the latter being recognized by 26B9 but not by 19D11 (Figure 3B). These experiments revealed very slow off-rates reflecting extremely high affinities of the antibodies for their targets, ranging from $K_D = 3.28 \times 10^{-14} \text{M}$ for 26B9 toward IFNκ14 to $K_D = 2.09 \times 10^{-11} \text{M}$ for 26B9 toward IFNκ2b (Figures 3B and 3C). Sub-picomolar/near-femtomolar dissociation constants were likewise shown by 19D11 (Figures 3B and 3C). Thus, APS1/APECED patients harbor some of the strongest affinity antibodies described.

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The high affinities of 26B9 and 19D11 were not unique. Thus, a patient-derived IgGκ mAb (20A10) specific for IL20 (which is not a target detected by most patients; Figure 2C; Table S1)
Figure 2. Serology of APS1/APECED to IFNs and Other Cytokines
Seroreactivity of APS1/APECED patients (blue) and controls (red) toward selected interferons and cytokines as measured in ProtoArray (A), LIPS (B and C), and ELISA (D).
displayed a $K_D$ of 9.1e-14M, (Table S3). Likewise one IgGκ mAb (17E3) and one IgGλ mAb (24D3), each specific for IL17F, displayed dissociation constants of <10 pM, and one IgGκ antibody (30G1) and one IgGλ antibody (35G11) specific for IL22 displayed dissociation constants of 37 pM and 39 pM, respectively. As a comparison, a CHO cell-expressed form of fezakinumab, a humanized anti-IL22 mAb tested in the clinic, displayed a $K_D$ of 54 pM (Table S3). The only exception to this pattern was 2C2, an IgGκ mAb specific for IL32γ (for which no human antibody has been reported), which displayed nanomolar dissociation (Table S3).

Similar to IFNα antibodies, most cytokine-specific antibodies did not detect linear peptides from relevant target proteins, strongly suggestive of complex conformational determinants (data not shown). The one exception was 20A10, which bound to an IL20 peptide and within which key amino acids were identified by mutagenesis (Figures S2C and S2D).

The antibody sequences of IL17F-reactive 17E3 and 9A2 and of IL22-reactive 30G1 and 35G11 displayed myriad non-conservative mutations in the CDRs. Again their germline counterparts did not detect the respective targets (Figures 3D, S2E, and S2F). Moreover, neither patient-derived antibodies nor their germline counterparts showed any general autoreactivity (judged by immunofluorescent staining of tissue sections or HEp-2 cells) or reactivity to Candida albicans, thus arguring against candida infection being the trigger for autoantibody generation (data not shown).

The highly mutated CDRs of all studied antibodies suggested that they derived from GC reactions that partially rely on Tfh cells. Aberrant generation and/or activation of Tfh cells has been described in several autoimmune diseases (Jeno et al., 2019), but when four pediatric and four adult APS1/APECED were compared to controls, we found no differences in the percentages of circulating CXCR5⁺ Tfh cells, or their activation state, as judged by ICOS (inducible costimulator) and CCR7 levels (Figure S3).

### Biologically Active Human Antibodies

To test the biological activities of 19D11 and 26B9, HEK293 cells transfected with type I IFN-stimulated response elements (ISRE) fused to firefly luciferase were treated with recombinant forms of each of 12 IFNα subtypes and IFNλ in the presence or absence of increasing concentrations of 19D11 or 26B9. Following treatment, firefly luciferase values were normalized to those of co-transfected Renilla luciferase, so as to control for variations in transfection efficiency. Both antibodies strongly inhibited the IFN-dependent response, with median $IC_{50}$ values of 2.83 ng/ml for 26B9 and 0.9 ng/ml for 19D11 (Figure 4A; Table S4). By comparison, median $IC_{50}$ values of 76.24 ng/ml and 10.86 ng/ml, respectively, were displayed by in-house-generated recombinant sifalimumab and rontalizumab, two anti-IFNα mAbs used in clinical trials for systemic lupus erythematosus patients (Table S4).

Predictably, the antibodies varied in their inhibition of IFN-stimulated responses. Thus, 26B9 neutralized IFNα5, but not IFNα16, and only poorly inhibited IFNα8 (Figure 4A; Table S4). Likewise, in the same assay, rontalizumab failed to efficiently neutralize IFNα6, IFNα7 and IFNα10, whereas sifalimumab neutralized several IFNα subtypes only weakly. By contrast, 19D11 neutralized all 12 IFNα subtypes tested (Table S4).

Patient-derived IFN-specific mAbs were also assessed for their capacity to inhibit STAT1 phosphorylation in cells treated with each of 12 IFNα subtypes, IFNλ, IFNβ, or IFNγ (Figures 4B, 4C, and 4D). As predicted from the luciferase assay, 19D11 inhibited STAT1 phosphorylation levels (normalized to total STAT1 or tubulin) driven by all IFNα subtypes but did not affect responses to IFNλ, IFNβ, or IFNγ. By contrast, 25C3, an additional patient-derived mAb (Table S2), was highly selective for discrete IFNα subtypes, whereas other antibodies tested, including 26B9, showed neutralization profiles between those of 19D11 and 25C3 (Figures 4B–4D). Only 26B9 and 31B4 neutralized IFNλ, and none neutralized IFNβ or IFNγ. By comparison, sifalimumab, rontalizumab, and AGS-009 (another IFNα-targeting mAb in clinical development) showed variable and less uniform inhibition of STAT1 phosphorylation induced by different IFNα subtypes (Figure S4A).

The striking biological activities of patient mAbs were not limited to those specific for IFNs in that potent functional target neutralization was shown by mAbs targeting IL17F, IL22, IL32γ, and IL20, respectively (Figure S4B).

### Biologically Active Human Antibodies In Vivo

We next asked whether patient autoantibodies could functionally neutralize targets in vivo. To test this, mice were treated intraperitoneally (i.p.) with a single aliquot of antibodies 26B9, 19D11, or sifalimumab, and their ears inoculated intradermally (i.d.) on days 1, 3, 6, and 8 with recombinant human IFNα14 (data not shown). Relative to repeated inoculation with vehicle/PBS, the cytokines induced ear swelling, reflecting an inflammatory response that includes rapid TNFα and IFNγ induction (Figures S5A and S5B). This ear swelling was significantly inhibited by single injections of antibodies (Figure 5B). Again, neutralization varied toward the effector IFNα subtype: 26B9 and 19D11, but not sifalimumab, largely ablated the IFNα5 response, whereas all three partially yet significantly limited swelling induced by IFNα14 (Figure 5B).

Specific, antibody-mediated neutralization in vivo was likewise seen when the same assay was applied to human IL17F...
or IL32γ (Figures 6A and 6B). For IL17 neutralization, the data are clearly consistent with the known capacity of APS1/APECED patients’ antibodies to neutralize Th17-family cytokines (Kisand et al., 2010; Puel et al., 2010), thereby predisposing to Candida infection.

Additionally, the detection of mouse IL22 by antibody 30G1 offered an opportunity to measure its bio-activity toward endogenous IL22, a primary effector of imiquimod (IMQ)-induced dermatitis used to model psoriasis (van der Fits et al., 2009). IMQ-induced pathology measured by modified PASI scoring was significantly ameliorated by 30G1 relative to IgG control, particularly following an initial inflammatory response (Figures 6C and S6). Again, 30G1 was at least as effective as an in-house-expressed anti-IL22 antibody, fezakinumab (see above) (Figure 6C). Collectively these data establish the capacity of patient anti-cytokine antibodies to limit pathologies induced by their targets in vivo.

Clinical Correlates of Neutralizing Antibodies

Given the results from animal models, it was appropriate to consider the potential impact of APS1/APECED antibodies in the patients themselves. Because circulating IFNα levels are extremely low in human peripheral blood, even following vaccination (Sobolev et al., 2016), circulating IFN levels do not offer robust biomarkers of anti-IFNα antibodies. Neither does measurement of interferon-stimulated genes (ISGs) because many, e.g., CXCL10, can be upregulated by type II IFNs (Welcher et al., 2015). By contrast, antibody activities may be reliably reflected in discrete pathologies, as in the correlation of anti-IL22 with candidiasis.

In this regard, many datasets, particularly in mouse models, suggest that type I IFN contributes to type 1 diabetes (T1D) (Carrero et al., 2013; Downes et al., 2010; Foulis et al., 1987; Huang et al., 1995; Li et al., 2008). Although APECED/APS1 patients by definition suffer from polyendocrinopathy, T1D affects only 10%–20% of patients and manifests primarily in adulthood (Husebye et al., 2009; Kisand and Peterson, 2015). This is despite the fact that radioimmunoassays have revealed that many APS1/APECED patients carry GAD65-reactive autoantibodies, a clinically applied biomarker for likely onset of T1D (Ziegler et al., 2013). Consistent with this, ProtoArray and LIPS data showed that many patients carried GAD65- and/or GAD67-reactive antibodies, but among them relatively few presented with T1D (red dots, Figures 7A and 7B). Collectively, these many observations suggest that patients at risk of T1D, as judged by anti-GAD65/GAD67, might fail to develop T1D if they harbored powerfully neutralizing anti-IFNα antibodies. Indeed, we reported a seemingly exceptional APS1/APECED patient, completely lacking IFNα-neutralizing antibodies and presenting with T1D (Kisand et al., 2008).

To investigate this, the 8 patients presenting with T1D (red dots, Figure 7B; mean age ± SD, 48 ± 11 years) were compared with an available cohort of 13 patients without
T1D but with strong GAD65 reactivity (relative luciferase units > 5) (blue dots, Figure 7B; mean age ± SD, 31 ± 12 years). Consistent with T1D developing in adult APS1/APECED patients, GAD65 reactivities mostly arose post-adolescence, and hence the patient cohorts comprised 20 adults and one 8 year old.

As expected, all 21 patients harbored antibodies to IFNα and IFNγ (see Figure 2B), but when tested for IFNα and IFNγ neutralization, the antibodies showed a striking segregation with clinical status (Figures 7C, 7D, and S7A): patients without T1D showed only low or negligible neutralization. Particularly strong differences were seen vis-a-vis IFNα with T1D collectively neutralizing all IFNα subtypes, whereas those with T1D showed only low or negligible neutralization. Particularly strong differences were seen vis-a-vis IFNα, IFNγ, IFNα2, IFNα5, IFNα8, IFNα14, and IFNα17 neutralization (Figure 7C). The two subgroups of the 21 patients also showed statistically significant differences in neutralizing IFNα, but the difference was weaker than for IFNα (Figure S7A). Interestingly, the two GAD65-reactive non-diabetics who displayed relatively low IFNα neutralization were young adults who may be en route to developing T1D.

In a small subcohort of GAD65/67-reactive patients for whom longitudinal samples were available, the three T1D patients (red bars) again showed lower IFNα neutralization relative to the two patients without T1D. Moreover, one patient was able to neutralize IFNα4 in 1978 but by 2012 could no longer do so and presented with T1D (Figure S7B).

Such striking correlations with T1D (Figure 7D) were not evident for any other naturally arising anti-cytokine antibodies, supporting the view that IFNα may contribute critically to the natural progression of T1D. Moreover, although the data do not prove that active anti-IFNα antibodies underpin selective protection from T1D, they provide a firm foundation for exploring the potentials of APS1/APECED-derived autoantibodies to ameliorate other major diseases that are rarely if ever present in APS1/APECED patients.

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DISCUSSION

This analysis of the impact of AIRE deficiency on human B cells has revealed a signature pattern of humoral autoreactivity with general implications for our understanding of autoimmunity. First, the autoantibodies studied were mostly extremely high affinity and specific for native conformational epitopes. These properties were shared by antibodies specific for cytokines targeted by most patients (e.g., IFNα, IL17, IL22) and by antibodies specific for IL20 to which few patients displayed reactivity. Because such properties are very rare among antibodies raised by immunization, when B cells are primed de novo to antigen for short periods of time, it seems inappropriate to continue to model one type of mAb on the other.

Second, essentially all 81 APS1/APECED patients studied showed strong reactivities toward a common set of 10–15 proteins, coupled with patient-specific reactivity profiles toward 80–90 additional proteins. This limited frequency (< 1% of proteins displayed on the array) is consistent with a recent report that B cell tolerance was not globally disrupted in 51 APS1/APECED patients sampled (Landegren et al., 2016). Nonetheless, the patient-to-patient variation in reactivity profiles meant that the 97 sera analyzed in our study collectively harbored antibodies toward over 3,500 proteins.

The patient-to-patient variation argues that B cell autoimmunity resulting from AIRE deficiency is not simply an amplification of sporadic, low-level autoreactivities seen in healthy controls but has distinct origins. By this perspective, defects in central T cell tolerance may underpin other autoimmune and autoinflammatory pathologies attributed to high-affinity autoantibodies. Whereas this contrasts with the widely held view that autoimmune diseases mostly reflect peripheral tolerance defects, it aligns with data that central tolerance defects contribute to the NOD mouse model of T1D (Geng et al., 1998; Zucchelli et al., 2005). Moreover, wherever autoantibodies reflect central T cell tolerance defects, donor-to-donor variation is to be expected, as individuals will generate distinct TCR repertoires via quasirandom gene rearrangements, will be exposed to different physiologic and environmental triggers that promote the selective outgrowth of autoreactive T cell clones, and will differ in immune response modifier genes (e.g., HLA) that regulate the magnitude of antigen-specific responses.
Autoantibodies to some non-tissue-restricted antigens, including multiple type I IFN subtypes, are displayed by almost all patients, sometimes early post-partum (Wolff et al., 2013). Most likely, the immunogenicity of these proteins arises by mechanisms distinct from those shaping patient-specific autoantibody repertoires. Possibly the public autoantibodies arise from a direct impact of AIRE deficiency on B cell tolerance, for example, via the dysregulation of AIRE-expressing thymic B cells that resemble GC B cells by several criteria (Yamano et al., 2015). Arguing against this, however, autoantibodies to type I IFNs, Th17 cytokines, and additional self-proteins are found in thymoma patients with AIRE-sufficient B cells (Kisand et al., 2011; Meager et al., 1997; Wolff et al., 2014). This likewise argues against autoantibodies to type I IFNs and Th17 cytokines originating from defects in lymph node AIRE+ cells termed eTACs (Gardner et al., 2008). Although studies in mice have suggested tolerizing roles of eTACs, the functions of their rare human counterparts are unknown (Poliani et al., 2010).

AIRE deficiency may, however, act indirectly on thymic B cells, for example by hyperactivity of functionally competent thymic γδ T cells (Ribot et al., 2009) that may likewise be dysregulated in thymoma. Such cells may create an intra-thymic milieu favoring priming rather than tolerance of thymic B cells toward proteins highly expressed in the thymus (Dudakov et al., 2012; Meager et al., 2006).

Notwithstanding this possibility, our findings suggest that high-affinity autoantibodies in APS1/APECED patients probably reflect dysregulated GC reactions, wherein autoreactive T cells, e.g., Tfh cells, that were not tolerized in the thymus promote the competitive outgrowth and affinity maturation of B cells that were initially primed to exogenous antigen(s) but whose mutated IgGs bind to self-proteins. Consistent with this, autoantibodies targeting thyroid-stimulating hormone receptor in Graves’ disease cross-react to Yersinia enterocolitica antigens (Brink, 2014; Hargreaves et al., 2013), and activated peripheral blood Tfh cells correlate positively with serum autoantibodies and disease activity/severity in multiple autoimmune diseases (Ueno et al., 2015). Although our analysis of four adult and four pediatric APS1/APECED patients revealed no alterations in Tfh cell numbers relative to age-matched healthy controls, this did not exclude Tfh cells being enriched in autoreactive specificities. Moreover, no patients displayed neutralizing autoantibodies to IL21, a major mediator of Tfh cells in the GC.

This etiology of APS1/APECED B cell autoimmunity is strikingly similar to proposed origins of highly mutated anti-desmoglein-3 antibodies in autoimmune pemphigus (Di Zenzo et al., 2012) and of anti-GM-CSF antibodies pathognomonic in pulmonary alveolar proteinosis (Piccoli et al., 2015). In those studies, as in this, the closest germline counterparts (“unmutated common ancestors” [UCAs]) showed no reactivity toward the targets of the affinity-matured autoantibodies. By contrast, germline versions of antiviral antibodies showed only slightly reduced binding to target viral antigens (Corti et al., 2011, 2013). Moreover, it is not the case that UCAs intrinsically lack autoreactivity, as germline counterparts of some autoantibodies with few replacement mutations showed autoantigen reactivity in pemphigus patients (Cho et al., 2014). The underlying defect(s) in T cell tolerance that dysregulate affinity maturation in pemphigus, pulmonary alveolar proteinosis, and other organ-specific autoimmune diseases may be limited to few antigens, by contrast to broad-spectrum defects in APS1/APECED.
That almost all APS1/APECED-derived mAbs were biologically active in vivo against a range of cytokine targets has profound implications for patients. Clearly, immune-effector responses may be reduced, as in the association of anti-IL22 with susceptibility to Candidiasis (Kisand et al., 2010). Likewise, gut barrier integrity may be compromised, leading to increased levels of anti-commensal antibodies (Hetemäki et al., 2016). Conversely, despite the common neutralization of IFNα and IFNβ, APS1/APECED patients do not show severe viral infections, as were recently reported for a child genetically impaired in type I IFN (Ciancanelli et al., 2015). Possibly preserved IFNβ function mediates anti-viral protection in APS1/APECED patients.

On the other hand, some autoantibodies may target key mediators of immunopathologies, thereby ameliorating disease. Thus, a unique correlation was observed between antibody-mediated neutralization of IFNα and failure to develop T1D, providing a novel strand of support for animal studies arguing that targeting type I IFNs could be effective in T1D. The concept that naturally arising autoantibodies may be beneficial is not widely considered, despite its underpinning the widespread use of therapeutic mAbs. In this regard, it is striking that despite their severe flaws in central T cell tolerance, APS1/APECED patients do not present with systemic sclerosis, Sjögren’s syndrome, MS, or SLE. These pathologies are considered to involve interplays of IL17/Th17 and type I IFNs—two main targets of APS1/APECED autoantibodies (Ambrosi et al., 2012). Likewise, Th17-driven psoriasis was diagnosed in only two of our patients, each of whom lacked autoantibodies to IL17A, IL17F, and IL22 (our unpublished data). Furthermore, atopy/allergy is seemingly rare among APS1/APECED patients, although whether anti-IL5 antibodies underpin this requires more study.

For now, the data presented by this study strongly suggest that antibodies recovered from APS1/APECED patients include ones with profound therapeutic and diagnostic potential.

**EXPERIMENTAL PROCEDURES**

More details are available in the Supplemental Experimental Procedures.

**Human Samples**

Eighty-one APS1/APECED patients were diagnosed by mutational analysis of AIRE and by autoantibodies to type I IFNs. All provided informed consent, and many were analyzed previously (Kisand et al., 2011; Kluger et al., 2015; Meloni et al., 2012; Wolff et al., 2007). Approvals by local ethics committees are described in the Supplemental Experimental Procedures. Ages at serum sampling were 4–73 years; mean = 31.9. For protoarray there were 12 age-matched controls and 9 healthy first-degree relatives, and there were additional healthy controls for LIPS and ELISA.

**Immune Response Profiling by ProtoArray**

Sera of patients, healthy relatives, and controls were tested against > 9,000 human proteins displayed on the Human Protein Microarray v5.1 (ThermoFisher Scientific). Preprocessing methods were applied to account for technical variability. First, corresponding local background intensity was subtracted.
whereafter values were log-transformed and subjected to robust linear normalization (Sboner et al., 2009). Z scores were calculated as the number of standard deviations of the signal from the mean of the corresponding controls and healthy relatives; Z ≥ 3 was considered positive. After scoring, stringent quality assessment was undertaken, including high correlation coefficients of duplicate spots of printed proteins (average r = 0.92), reactivity toward known autoantibody targets, and perfect correlation of signals for proteins spotted in different locations. Printing contaminants were identified as proteins showing high correlation coefficients with known APECED antibody targets and were further verified by cross-reference to another protoArray (5.0) used for 23 patients and 7 controls. Thus, 31 suspect false-positives were identified and excluded from further consideration.

**Antibody Isolation and Cloning**

cloning, production, and purification of human mAbs were performed as described (patent application WO2013/098419). In brief, memory B cells (CD22+, IgD-, IgM-, CD3-, CD8-, and CD54+) were flow-sorted (MoFlo) from patient PBMC, incubated transiently with EBV-containing B95-8 supernatant (SN) for 3.5 hr at 37°C, and then incubated in Transferrin- and CpG-supplemented IMDM at 37°C, 5% CO2, at 10 cells/well in 96-well plates coated with irradiated PBMC feeders. Short-term, oligochlonal B cell culture SN were analyzed for IgG and antigen-specific antibodies detected by ELISA and/or LIPS. Positive wells were harvested, cells single-cell-sorted into reverse transcriptase (RT) buffer (Life Technologies), and RT-PCR performed using Superscript III (Life Technologies) and random hexamers. IgG V̄, Vδ, and Vε regions were amplified from cDNA by two-step nested PCR reaction using Advantage 2 cDNA polymerase (Clontech) and primer mixes specific for germline families (VBASE2 database). Nested primers attached restriction sites for V-region cloning into expression vectors providing IgG1, Igκ, or Igλ constant regions. Recombinant antibodies were produced in HEK293T cells and antigen specificity analyzed by ELISA. Corresponding closest germline region sequences were identified using the IMGT (Lefranc, 2003).

Complete Ig-V̄δ and Vδ regions described in US7741449 (Sifalimumab), US7087726 B2 (Rontalizumab), US8361463 (ACO-1), and US20070258982 A1 (Fezakinumab) were ordered as CHO-codon-optimized synthetic constructs (GenScript) and expressed as above.

**mAb Characterization In Vitro**

EC50 binding of mAbs was determined by ELISA. Neutralizing capacities of type 1 IFN-specific mAbs were studied using phospho-STAT1 quantitation in immunoblot and ISRE-luciferase reporter assay. IL17F, IL22, IL20, and IL32 neutralization assays were performed on respective responsive cell lines. mAb affinities were measured with a Biacore T200 (GE Healthcare). Epitope mapping used overlapping 18-mer peptides.

**mAb Characterization In Vivo**

CS768L8J (WT, from Charles River) mice were administered i.p. with mAbs (day 0) and inoculated i.d. on days 1, 3, 6, and 8 with cognate human cytokines, IFNα2a, IFNα2b, IFNα4, IFNα14, IL17F, and IL32γ, and their ear thicknesses measured with a micrometer. For IL22 mAbs cross-reactive to mouse, bioactivity was assessed in imiquimod-treated mice.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2016.06.024.

**CONSORTIA**

The members of the APECED patient collaborative are Antonella Meloni, Nicolas Kluger, Eystein S. Husebye, Katarina Trebusak Podkrajsek, Tadej Batelino, Nina Bratanic, and Aleksandar Peet.

**AUTHOR CONTRIBUTIONS**

S.M., A.M., and S.C.O. cloned monoclonal antibodies from patient samples, and K.J. and K.H. assisted. S.M., P.V., and A.M. characterized antibodies in vitro; M.W. and Y.H. did so in vivo. C.H. analyzed ProtoArray data and wrote and edited the paper. J.K. assayed neutralization by sera and Tfh subsets and performed LIPS. D.F. and H.P. analyzed ProtoArray data. K.M. and R.U. screened sera for T1D autoantibodies and tested germine antibody specificities. K. Krohn and A.R. developed the clinical database, sampled Finnish patients, and employed ELISA. A.S.B.W. sampled Norwegian patients, contributed to the clinical database, and assayed antibodies. APECED patient collaborative contributed to the clinical database and sampled respective patients. P.P., K. Kisand, and A.H. supervised research, reviewed data, and wrote and edited the paper.

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