AIRE expression controls the peripheral selection of autoreactive B cells

Joel Sng[1], **Burcu Ayoglu**[2], **Jeff W. Chen**[1], **Jean-Nicolas Schickel**[1], **Elise M. N. Ferre**[3], **Salomé Glauzy**[1], **Neil Romberg**[4,5], **Manfred Hoenig**[6], **Charlotte Cunningham-Rundles**[7], **Paul J. Utz**[2,8], **Michail S. Lionakis**[3], **Eric Meffre**[1,*]

[1]Department of Immunobiology, Yale University School of Medicine, New Haven, CT 06511, USA.

[2]School of Medicine, Division of Immunology and Rheumatology, Stanford University, Stanford, CA 94305, USA.

[3]Laboratory of Clinical Immunology and Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD USA.

[4]Division of Immunology and Allergy, Children's Hospital of Philadelphia, Philadelphia, PA, USA.

[5]Department of Pediatrics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA.

[6]Department of Pediatrics, University Medical Centre Ulm, Ulm, Germany.

[7]Division of Allergy and Immunology, Department of Medicine, Icahn School of Medicine, Mount Sinai, New York, NY 10029, USA.

[8]Institute for Immunity, Transplantation, and Infection (ITI), Stanford University, Stanford, CA 94305, USA.

Abstract

Autoimmune regulator (AIRE) mutations result in autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) syndrome characterized by defective central T cell tolerance and the production of many auto-antibodies targeting tissue-specific antigens and cytokines. By studying CD3- and AIRE-deficient patients, we found that lack of either T cells or AIRE function resulted in the peripheral accumulation of autoreactive mature naïve B cells. Proteomic arrays and Biacore affinity measurements revealed that unmutated antibodies expressed by these autoreactive naïve B cells recognized soluble molecules and cytokines including insulin, IL-17A, and IL-17F, which are AIRE-dependent thymic peripheral tissue antigens targeted by autoimmune responses in APECED. AIRE-deficient patients also displayed decreased frequencies of regulatory T cells

(T$_{regs}$) that lacked common TCRβ clones found instead in their conventional T cell compartment, thereby suggesting holes in the T$_{reg}$ TCR repertoire of these patients. Hence, AIRE-mediated T cell/T$_{reg}$ selection normally prevents the expansion of auto-reactive naïve B cells recognizing peripheral self-antigens.

INTRODUCTION

Patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) also named autoimmune polyglandular syndrome type 1 (APS-1) commonly suffer from a classic triad of condition and symptoms, including chronic mucocutaneous candidiasis, hypoparathyroidism, and adrenal insufficiency caused by mutations in the autoimmune regulator (*AIRE*) gene (1, 2). Similarly, AIRE-deficient mice also exhibit APECED-like features, such as multi-organ lymphocytic infiltrations and often circulating autoantibodies against tissue antigens (3–5). AIRE ensures immunologic tolerance by controlling the selection of the T cell receptor (TCR) repertoire through the ectopic expression of peripheral tissue antigens (PTAs) in medullary thymic epithelial cells (mTECs) (3, 6), the regulation of genes associated with antigen processing and presentation (7), and the expression of chemokines that mediate the accumulation of dendritic cells in the thymic medulla (8). AIRE is therefore responsible for the deletion of autoreactive thymocytes and for directing autoreactive T cell specificities into the regulatory T cell (T$_{reg}$) lineage (7, 9–11).

Although the absence of functional AIRE results in abnormal T cell compartments with autoreactive clones found in conventional T cells (T$_{conv}$) instead of the T$_{reg}$ compartment, B cells also play a critical role in the initiation of autoimmunity in *Aire*$^{-/-}$ mice because *Aire*$^{-/-}$ B cell-deficient mice are protected from multi-organ auto-inflammation and lack lymphocytic infiltrates in some of the organs that are targeted by Aire-less autoimmunity (12, 13). The depletion of B cells with anti-hCD20 antibody in both the nonobese diabetic (NOD) and NOD *Aire*$^{-/-}$ mice delays the onset of diabetes, inhibits inflammation, and induces T$_{reg}$ expansion, suggesting that B cells may play an important early role in promoting T cell activation (12,14). In addition, B cell tolerance is broken in patients with APECED as illustrated by the wide variety of autoantibodies found in the sera of these individuals and that target tissue-specific antigens such as glutamic acid decarboxylase 1 (GAD1) and GAD2 or CYP21A2 as in patients with type 1 diabetes or Addison's disease, respectively (15,16). AIRE deficiency in humans is also associated with the development of a distinct signature of anticytokine antibodies against interferon-ω (IFN-ω), IFN-α, and interleukin-17A (IL-17A), IL-17F, and IL-22, whereas only anti-IL-17A antibodies have been reported in Aire$^{-/-}$ BALB/c mice (4, 17–19). These autoantibodies are often present in young AIRE-deficient individuals even before they develop other typical APECED symptoms, suggesting that the break in B cell tolerance is a primary defect of the disease and not a secondary consequence of inflammation or therapy in patients with APECED (20). Although AIRE deficiency is associated with the emergence of auto-reactive B cells and autoantibody production, the stage at which B cell tolerance is breached has not yet been investigated.

Developing B cells with autoreactive B cell receptors (BCRs) are eliminated at two discrete early B cell tolerance checkpoints. A central checkpoint in the bone marrow removes most clones expressing polyreactive and antinuclear antibodies (ANAs), whereas the peripheral B cell tolerance checkpoint prevents the accumulation of autoreactive mature naïve B cells (21). This peripheral selection step appears to be controlled by B cell extrinsic factors such as T_{regs} because specific defects in this checkpoint has been associated with either decreased T_{reg} frequencies or impaired T_{reg} suppressive function in patients with adenosine deaminase (ADA), CD40L, DOCK8, major histocompatibility complex (MHC) class II, and Wiskott-Aldrich syndrome protein (WASP) deficiency (22–25). In addition, FOXP3-deficient, immune dysregulation, polyendocrinopathy, enteropathy, and X-linked (immune dysregulation, polyendocrinopathy, enteropathy, X-linked; IPEX) patients who lack functional T_{regs} also display a dysfunctional peripheral B cell tolerance checkpoint (26). The abnormal selection of T cells when AIRE functions are abrogated may therefore affect the removal of autoreactive B cells in the periphery.

We report here that either the absence of T cells in CD3-deficient patients or impaired AIRE expression in patients with APECED results in increased frequencies of circulating, autoreactive, mature naïve B cells in these individuals. The dysfunctional peripheral B cell tolerance checkpoint in AIRE-deficient patients is associated with decreased numbers of T_{regs} lacking TCRβ sequences found instead in their T_{conv} compartment that is abnormally selected in the absence of functional AIRE.

RESULTS

Central B cell tolerance is functional in CD3- and AIRE-deficient patients

Central B cell tolerance, which mediates the removal of developing B cells expressing highly polyreactive and ANAs in the bone marrow, appears to be regulated by intrinsic B cell pathways and is not affected by decreased T_{reg} frequencies or suppressive function (22–28). To determine whether this checkpoint is established independently of T cells, we tested the reactivity by enzyme-linked immunosorbent assay (ELISA) of recombinant antibodies cloned from single $CD19^+CD27^-$ $CD10^+IgM^{hi}CD21^{lo}$ new emigrant/transitional B cells from two CD3-deficient patients lacking T cells with either homozygous *CD3D* or homozygous *CD3E* gene mutations (fig. S1A) (21). In addition, we also assessed whether impaired expression of AIRE, which regulates T cell selection through the expression of peripheral tissue antigens in the thymus, may affect central B cell tolerance by studying four AIRE-deficient patients and three asymptomatic relatives carrying a heterozygous *AIRE* gene mutation (table S1). Most B cell subpopulations from CD3- and AIRE-deficient and heterozygous individuals were present within normal ranges of healthy donors (HDs), except $CD19^+CD27^-CD21^{-/lo}$ B cells that were expanded in AIRE-deficient patients like in other patients with autoimmune conditions (figs. S1B and S2) (29, 30). Immunoglobulin heavy chain gene segment usage, third complementarity-determining region (CDR3) length, and positively charged amino acid content in antibodies expressed by new emigrant/transitional B cells from CD3- and AIRE-deficient patients and AIRE heterozygous carriers were similar to HD counterparts, suggesting that mutations in *CD3D*, *CD3E*, or *AIRE* may not affect B cell development (fig. S3). In agreement with this hypothesis, the proportions of new

emigrant/transitional polyreactive and anti-nuclear B cells in both CD3-deficient patients were low and comparable with those in HDs, which demonstrates that central B cell tolerance does not require CD3$^+$ T cells to be properly established (Fig. 1, A to C; fig. S4; and table S2). Similarly, AIRE-deficient patients and heterozygous relatives displayed normal low frequencies of polyreactive and antinuclear new emigrant/transitional B cells, revealing an efficient removal of developing autoreactive B cells in the bone marrow of these individuals (Fig. 1, A to C; fig. S4; and table S2). Together, these findings show that human central B cell tolerance is established independently of T cells and their AIRE-dependent selection.

Impaired peripheral B cell tolerance checkpoint in CD3- and AIRE-deficient patients

T$_{regs}$ have been suggested to prevent the accumulation of autoreactive clones in the mature naïve B cell compartment (22–26). To determine whether T cells control the peripheral selection of B cells, we analyzed the reactivity of recombinant antibodies cloned from single CD19$^+$CD27$^-$CD10$^-$IgM$^+$CD21$^+$ B cells, which are mostly mature naïve B cells but may also include some late transitional T3 B cells and marginal zone B cell precursors (31, 32) from CD3-deficient patients who lack T cells (21). The studied CD3-deficient patients were pretransplanted and did not show any signs of infections or inflammation when blood samples were collected. Because CD3 is solely expressed in T cells, its loss does not affect the function of all other cell types. Although immunoglobulin heavy chain repertoire analysis did not reveal significant differences between patients and controls, analysis of CD3-deficient patients showed increased frequencies of HEp-2 reactive mature naïve B cells, which represented $46.1 \pm 5.6\%$ compared with $20.5 \pm 3.3\%$ in HD counterparts. This result reveals that the absence of T cells interferes with the establishment of the peripheral B cell tolerance checkpoint ($P = 0.019$; Fig. 2, A and B; figs. S5 and S6A; and table S2). Peripheral B cell selection defects in CD3 deficiency were further evidenced by the elevated proportions of mature naïve B cells that expressed polyreactive antibodies compared with HDs, whereas frequencies of clones producing ANAs remained low, similar to healthy controls (Fig. 2C and fig. S6, B and C).

We also assessed whether the lack of functional AIRE, which alters T cell selection, affects B cell selection in the periphery by analyzing the frequencies of autoreactive mature naïve B cells in AIRE-deficient patients and heterozygous relatives. We found that mature naïve B cells from both AIRE-deficient patients and heterozygous carriers often expressed HEp-2-reactive antibodies compared with control B cells, demonstrating that proper AIRE expression is required to inhibit self-reactive B cell accumulation in the periphery (AIRE-deficient: $38.9 \pm 3.9\%$, $P = 0.0008$; AIRE-heterozygotes: $47.2 \pm 2.8\%$, $P = 0.0036$; Fig. 2, A and B; fig. S6A; and table S2). AIRE deficiency was also associated with the reemergence of polyreactive clones during the transition between the new emigrant/transitional and mature naïve B cell stages, whereas AIRE$^{+/-}$ individuals exhibited low frequencies of polyreactive mature naïve B cells that were similar to those in HDs. Hence, the lack of AIRE function induces more severe defects in autoreactive B cell selection than putative partial loss in AIRE expression associated with a single functional allele (Fig. 2, C to E, and fig. S6B). These alterations in B cell reactivity in individuals with *AIRE* mutation(s) were not associated with obvious immunoglobulin heavy chain repertoire differences nor with the

identification of clones expressing ANAs, which are also not found in the sera of patients with APECED (figs. S5 and S6C). We conclude that T cells and their AIRE-dependent selection are required to prevent the expansion of autoreactive B cells in the periphery.

Mature naïve B cells from AIRE-deficient patients show increased homeostatic expansion associated with elevated systemic cytokine concentrations

To investigate the mechanisms by which autoreactive clones were favored in the mature naïve B cell compartment in individuals with AIRE mutation(s), we first analyzed the phenotype of B cells from patients with APECED and heterozygous carriers. Reminiscent of B cells from IPEX patients, mature naïve B cells from individuals with one or two mutated *AIRE* alleles showed a significantly up-regulated expression of CD69, an activation marker that retards the egress of activated B cells from lymph nodes but lacked CD80 and CD86 costimulatory molecules (Fig. 3, A and B) (26). We then assessed the proliferative history of B cell subsets from patients and controls by measuring k-deleting recombination excision circles (KRECs), which provide an estimate of the number of divisions a B cell population has undergone (33). Similar to HDs, there was no expansion of transitional B cells that recently emigrated from the bone marrow in individuals with either one or two mutated *AIRE* alleles, further attesting to the unaltered early B cell development in these individuals (Fig. 3C). In contrast, mature naïve B cells from AIRE-deficient patients and, to a lesser extent, heterozygous carriers displayed increased homeostatic proliferation with an average of 3.5 and 2.4 divisions, respectively, compared with about 2.0 divisions in HD counterparts (Fig. 3C). The enhanced proliferation of mature naïve B cells in patients with APECED was correlated with the expansion of polyreactive clones and was associated with a specific increase in serum B cell activating factor (BAFF), an important B cell survival factor that regulates the number of peripheral B cells and may favor autoreactive B cell survival (Fig. 3D) (34, 35). In addition, patients with APECED also displayed elevated systemic plasma concentrations of IFN-γ, which promotes BAFF production (35) and IL-2, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-4, IL-5, and IL-10, T_H1, and T_H2 cytokines that favor B cell differentiation into plasma cells previously also identified in other patients harboring an impaired peripheral B cell tolerance checkpoint (Fig. 3E and fig. S7) (26, 36–38). Hence, the elevated plasma concentrations of cytokines favoring B cell survival and activation in patients with APECED likely promote the expansion of their autoreactive mature naïve B cells.

Characterization of the antibody reactivity of naïve B cells from individuals with AIRE mutations

To further study the specificity of antibodies cloned from new emigrant/transitional and mature naïve B cells from these AIRE-deficient patients, we performed a FLEXMAP 3D Luminex multiplexing antibody assay, containing a wide array of cytokines and other self-antigens. Although central B cell tolerance was functional in HDs and individuals with *AIRE* mutation(s), about 10% of new emigrant/transitional B cells from these individuals recognized a large cadre of self-antigens that included actin, IFN-ω, IL-11, IL-17A, IL-17B, IL-17E, IL-17F, IL-20, IL-21, IL-28A, kallikrein-related peptidase 5 (KLK5), macrophage-derived chemokine (MDC/CCL22), matrix metallopeptidase 3 (MMP-3), threonyl-transfer RNA synthetase (PL-7), thyroid peroxidase (TPO), and tumor necrosis factor superfamily

member 12/tumor necrosis factor-like weak inducer of apoptosis (TNFSF12/TWEAK) (fig. S8). These autoreactive clones often recognized more than one self-antigen on the protein array, and several were identified as polyreactive by ELISAs (Fig. 1 and figs. S4 and S9A). These data reveal that central B cell tolerance does not appear to be efficient at silencing clones, which express antibodies reacting with several soluble enzymes and cytokines that are likely only present at very low concentrations in the bone marrow (fig. S8). However, most of the clones that escaped central B cell tolerance were no longer identified in the mature naïve B cell compartment of HDs and asymptomatic AIRE$^{+/-}$ heterozygous carriers (Fig. 4). Hence, the peripheral B cell selection step is responsible for the counterselection of soluble self-antigens including many cytokines. In contrast, 16.7% of mature naïve B cells from AIRE-deficient patients continued to express anticytokine autoantibodies that targeted IFN-α2A, IFN-ω, IL-10, IL-11, IL-13, IL-17A, IL-17B, IL-17E, IL-17F, IL-20, IL-21, IL-28A/IFN-λ2 as well as actin, bone morphogenic protein 4 (BMP4), type VIII and X collagen, KLK5, MDC/CCL22, MMP-3, PL-7, TPO, and TNFSF12/TWEAK, further revealing an impaired peripheral B cell tolerance checkpoint in patients with APECED (Fig. 4). Notably, actin, BMP4, type VIII and X collagen, IL-17A, IL-17B, IL-17F, IL-21, KLK5, MDC/CCL22, MMP-3, and TPO are all AIRE-dependent PTAs expressed by mTECs in the thymus (table S3) (3, 39). Moreover, most of these autoreactive clones were previously identified as HEp-2 reactive and/or polyreactive by ELISAs, thereby demonstrating that traditional assays can effectively probe the functionality of the peripheral B cell tolerance checkpoint (fig. S9B). Thus, the peripheral naïve B cell selection normally prevents the accumulation of clones expressing antibodies that recognize many cytokine and other soluble molecules expressed in mTECs under the control of AIRE, further revealing that thymic T cell education plays an important role in the regulation of this peripheral naïve B cell tolerance checkpoint.

Mature naïve B cells from AIRE-deficient patients often express unmutated autoantibodies with measurable affinity for PTAs

Candidate antibodies identified by either the autoantigen array profiling or the polyreactive and HEp-2 ELISAs were then analyzed by surface plasmon resonance (SPR) using Biacore T100 optical biosensor to evaluate their affinity to both AIRE-dependent (insulin, IL-17A, IL-17F, fibrinogen, collagen, MDC/CCL22, and KLK5) and AIRE-independent (IFN-α, IFN-ω, and TWEAK) self-antigens (39). The analysis of new emigrant/transitional B cells identified few clones that had low affinity, measurable in the micromolar range, for both AIRE-dependent (insulin, IL-17A, IL-17F, and MDC/CCL22) and AIRE-independent (TWEAK) self-antigens in patients and controls, confirming that developing autoreactive B cells targeting soluble self-antigens present at low concentrations in the bone marrow may escape central tolerance (Fig. 5, A and B; fig. S10; and tables S3 and S4) (40,41). In contrast, we identified a higher proportion of antibodies with a measurable equilibrium dissociation constant (K_D) against the AIRE-dependent antigens insulin, IL-17A, and IL-17F, ranging from 10^{-5} to 10^{-7} M, cloned from the mature naïve B cells of AIRE-deficient patients compared with either HDs or asymptomatic AIRE$^{+/-}$ heterozygous relatives (Fig. 5, C and D, and table S4) (39). Although anti-type I IFN clones were identified by protein array in mature naïve B cells from patients with APECED, only one recombinant antibody had measurable affinity for IFN-α and IFN-ω by SPR (fig. S11B and

table S4). One anti-fibrinogen clone was found in the mature naïve B cell compartment of an AIRE$^{+/-}$ heterozygous carrier and may reflect the altered peripheral B cell tolerance checkpoint in this individual (fig. S11A). Unexpectedly, we also identified several high-affinity anti-TWEAK antibodies, with a κ_d ranging from 10^{-5} to 10^{-11} M expressed by mature naïve B cells from CD3- and AIRE-deficient patients (figs. S11B and S10 and table S4). Although the expression of TWEAK has not been previously linked with AIRE function, our observation suggests that the peripheral removal of TWEAK-reactive B cells could be affected by defects in the AIRE-mediated selection of T cells. We conclude that AIRE deficiency induces the accumulation of self-reactive B cells with enhanced affinity for AIRE-dependent antigens, which are normally counterselected by the peripheral B cell tolerance checkpoint.

Individuals with *AIRE* mutation(s) display decreased frequencies of T$_{regs}$ with altered phenotype

Because alterations in T$_{reg}$ frequencies or suppressive function have been associated with specific defects of the peripheral B cell tolerance checkpoint in many patients with primary immunodeficiencies (23–26), we analyzed the T$_{reg}$ compartments of individuals with *AIRE* mutation(s). Circulating CD3$^+$CD4$^+$CD25^{hi}CD127$^{-/lo}$FOXP3$^+$ T$_{reg}$ frequencies were significantly decreased in AIRE$^{+/-}$ heterozygous relatives ($3.79 \pm 1.23\%$, $P = 0.0016$) and even lower in AIRE-deficient patients ($3.26 \pm 0.82\%$, $P = 0.000014$) compared with healthy controls ($5.12 \pm 1.47\%$) (Fig. 6, A and B). Similar observations were obtained with a CD3$^+$CD4$^+$FOXP3$^+$Helios$^+$ T$_{reg}$ gating strategy that has been useful to identify T$_{reg}$ when CD25 expression was altered (Fig. 6, A to C) (26). The reduction in T$_{reg}$ frequency was less severe in AIRE$^{+/-}$ individuals than in patients with APECED, suggesting an *AIRE* gene dosage effect on thymic T$_{reg}$ development potentially through differential PTA expression by mTECs (3, 42). Although decreased circulating T$_{regs}$ may result in elevated frequencies of T follicular helper (T$_{FH}$)-like cells (26, 36, 38), we found that T$_{FH}$-like cell proportions were similar between HDs and individuals with AIRE mutation(s) (fig. S12), an observation that may correlate with normal suppressive function in T$_{regs}$ both from AIRE$^{+/-}$ heterozygous carriers and AIRE-deficient patients (fig. S13). However, frequencies of highly suppressive human leukocyte antigen-DR$^+$ (HLA-DR$^+$) T$_{regs}$ were decreased in the blood of AIRE-deficient patients compared with HDs and AIRE$^{+/-}$ heterozygous relatives (Fig. 6, D and E) (43). Additional phenotypic analysis of T cell subpopulations both from AIRE-deficient and heterozygous individuals revealed elevated proportions of circulating CD3$^+$CD4$^+$CD8$^-$CD25^{lo}CD45RO$^+$CCR6$^+$CXCR3$^-$ T cells, which includes both T$_H$17 and T$_H$22 subsets; there was also a reduction in circulating CD3$^+$CD4$^+$CD8$^-$CD25^{lo}CD45RO$^+$CXCR3$^+$CCR6$^-$ T cells, which are enriched for the T$_H$1 subset, in these individuals compared with HDs (fig. S14). The activation of peripheral blood mononuclear cells (PBMCs) in vitro with phorbol 12-myristate 13-acetate (PMA)-ionomycin revealed significantly decreased frequencies in patients with APECED of naïve CD4$^+$ T cells that produced IFN-γ, tumor necrosis factor-α (TNF-α), and IL-2 and expressed T-box transcription factor TBX21 (T-bet), further suggesting that T$_H$1 differentiation is disfavored in AIRE deficiency (figs. S15 to S17). Of note, decreased IFN-γ, TNF-α, and T-bet expression was also observed in naïve CD8$^+$ and TCR$\gamma\delta^+$ T cells (figs. S15 and S16).

Hence, AIRE-deficient patients display an altered T cell compartment characterized by decreased T_{reg} and T_H1 precursor frequencies.

Common T_{reg} TCRBV rearrangements are diverted into the CD4+ T_{conv} cell compartment of AIRE-deficient patients

The absence of *Aire* in mice resulted in the diversion of autoreactive TCRs from the CD4+ T_{reg} to the T_{conv} compartment (10). To study the impact of AIRE-dependent processes on the selection of CD4+ T cells, we analyzed the TCRβ chain [T cell receptor b variable (TCRBV)] repertoires of CD4+CD127+ T_{conv} and CD4+CD25^{hi}CD127$^{-/lo}$ T_{regs} isolated from the blood of five AIRE-deficient patients and five healthy controls using the ImmunoSEQ platform at Adaptive Biotechnologies. We obtained a total of 11.10^4 AIRE-deficient and $13.4.10^4$ HD distinct in-frame TCRBV sequences for control T_{conv} (fig. S18A) and $5.65.10^4$ and $9.56.10^4$ T_{reg} sequences from patients with APECED and HD, respectively (Fig. 7A), allowing us to survey the impact of AIRE expression on T cell repertoires. T_{conv} and T_{regs} from AIRE-deficient individuals displayed TCRBV gene segment usage, CDR3 length, and TCR diversity similar to HD counterparts (Fig. 7B and figs. S18B and S19).

Comparative analysis of Treg TCR representation revealed a total of 3657 T_{reg} TCR sequences that were shared by two or more individuals. Although 2162 (59.1%) of these TCR sequences were found in T_{regs} both from HD and AIRE-deficient patients, we found that 1155 (31.6%) were only shared among HD T_{regs}, whereas only 340 (9.3%) were specific to T_{regs} from patients with APECED (Fig. 7C). This suggests that commonly shared T_{reg}-biased TCRBV rearrangements are less efficiently selected in the T_{reg} compartment in the absence of functional AIRE. In addition, out of a total of 1155 T_{reg} TCRβ chains shared among HD, we identified 354 (30.6%) of these TCR sequences in the T_{conv} compartment of at least one AIRE-deficient patient, which reveals that AIRE-dependent T_{reg} precursors expressing these TCRBV rearrangements may be diverted into the CD4+ T_{conv} subset of AIRE-deficient patients (Fig. 7C). Of note, 36.6% of the commonly shared Treg TCR sequences were also detected in T_{conv} from HDs, potentially due to alternative TCRα chains (Fig. 7C). These shared AIRE-dependent T_{reg} clones favored TCRBV V05–01 gene segment usage, whereas TCRBV V10–03 and V15–01 were less used and displayed shorter CDR3s than those of distinct T_{reg} sequences (Fig. 7D and fig. S20). This likely reflects the bias of identical sequences to be found within VDJ rearrangements with small nontemplate nucleotide insertions that increase CDR3 length. TCRBV sequences shared by only T_{regs} from patients with APECED also harbored short CDR3s and slightly different TCRBV gene usage and were found in HD T_{conv}, further illustrating altered TCR selection in the absence of functional AIRE (Fig. 7, C and E, and fig. S20). Together, defective AIRE-mediated T cell selection modifies the TCR repertoire of T_{regs} from AIRE-deficient patients.

DISCUSSION

We report that lack of T cells in severe combined immunodeficiency patients with mutations in *CD3D* or *CD3E* genes and impaired AIRE expression in AIRE-deficient patients result in the accumulation of autoreactive clones in the mature naïve B cell compartment, whereas central B cell tolerance remains functional. This T-independent regulation of central B cell

tolerance was previously suggested by the analysis of $Rag1^{-/-}$ mice expressing a transgenic autoreactive BCRs and patients with diverse primary immunodeficiencies with altered T cell compartment or T_{reg} suppressive function, all displaying normal low frequencies of autoreactive clones exiting the bone marrow (23, 24,26, 44). In contrast, mutations in molecules regulating BCR and potentially Toll-like receptor pathways such as Bruton's tyrosine kinase, ADA, WASP, myeloid differentiation primary response 88, interleukin 1 receptor associated kinase 4, TNF receptor superfamily member 13B, and activation-induced cytidine deaminase (AID) affected central B cell tolerance, thereby revealing a B cell-intrinsic regulation of this checkpoint (22, 27, 28,36,45,46). Protein microarray and plasmon resonance analyses examining the reactivity of recombinant antibodies cloned from transitional B cells that recently emigrated from the bone marrow from control HDs, AIRE$^{+/-}$ heterozygous asymptomatic carriers, and AIRE-deficient patients revealed that self-reactive clones recognizing soluble enzymes and cytokines can escape central tolerance, which is therefore incomplete. However, anti-insulin and anticytokine clones were virtually absent from the mature naïve B cell compartment of HDs and asymptomatic AIRE$^{+/-}$ heterozygous carriers, revealing that the peripheral naïve B cell selection step is responsible for their elimination. Of note, few very-low-affinity anti-IL-17A mature naïve B cells were identified by Biacore in HDs, which suggests that, similarly to central tolerance, the peripheral counterselection of autoreactive B cells is also not absolute. In contrast, many mature naïve B cells isolated from AIRE-deficient patients were found to express antibodies that bound IFN-α2A, IFN-ω, IL-10, IL-11, IL-13, IL-17A, IL-17B, IL-17E, IL-17F, IL-20, IL-21, IL-28A/IFN-λ2 as well as actin, BMP4, type VIII and X collagen, KLK5, MDC/CCL22, MMP-3, PL-7, TPO, TNFSF12/TWEAK, and insulin. Because AIRE controls the expression of insulin, actin, BMP4, type VIII and X collagen, IL-17A, IL-17B, IL-17F, IL-21, KLK5, MDC/CCL22 MMP-3, and TPO in mTECs (39), PTA thymic expression may be necessary to prevent the accumulation of autoreactive naïve B cells that recognize these self-antigens in the periphery. In addition, mature naïve B cells from AIRE-deficient patients also included clones that expressed antibodies, which bound AIRE-independent self-antigens such as type I and type III IFNs, IL-10, IL-11, IL-13, IL-20, PL-7, and TWEAK. Although the mechanisms that normally prevent the accumulation of these clones are unclear, T cells are required because CD3-deficient patients displayed mature naïve B cells that expressed anti-TWEAK antibodies. Additional investigations are warranted to determine the significance of these anti-TWEAK clones and whether they neutralize or prolong the half-life of TWEAK, which in other studies, was shown to favor the production of proinflammatory cytokines including IL-17, and the development of autoimmune conditions (47).

Mature naïve B cells in AIRE-deficient patients that express un-mutated antibodies recognizing type I IFNs, IL-17A, and IL-17F likely contribute to the production of high-affinity anti–IFN-ω, anti–IFN-α, anti-IL-17A, and anti-IL-17F autoantibodies frequently detected in the serum of these individuals (17–19, 48). Because AIRE deficiency is often accompanied by fungal infections associated with increased IL-17 production by γS T cells from patients with APECED (17–19, 49), IL-17A secretion may therefore promote the activation of naïve anti-IL-17A B cells and lead to the production of high-affinity anti–IL-17A autoantibodies with the help of IL-17A–reactive T_{conv} cells (17–19, 48). By

extension, production of type I IFN linked to autoimmune processes may also favor the activation of anti-IFN-α and anti-IFN-ω clones present in the mature naïve B cell compartment of AIRE-deficient patients. In addition, anti-cytokine autoantibodies are frequently found not only in patients with APECED but also in individual with hypomorphic *RAG* gene mutations that dampen the generation of thymocytes and TCR repertoire diversity (50). Because the decreased colonization of the thymus by thymocytes in patients with hypomorphic *RAG* mutation results in decreased AIRE expression by mTECs, the selection of the rare T_{regs} that develop in these patients may be altered similarly to AIRE deficiency and induce peripheral autoreactive B cell selection defects and the accumulation of naïve B cells expressing BCR with anticytokine reactivity (51). Hence, defects in early B cell tolerance checkpoints may be responsible for the production of autoreactive clones that may be activated by T cells and increase the specificity of their autoantibodies for self-antigens by acquiring somatic mutations.

How can AIRE expression normally control the peripheral selection of autoreactive naïve B cells? Previous studies of other patients with primary immunodeficiencies revealed that loss of function in molecules involved in T_{reg} development or function interfered with the establishment of the peripheral B cell tolerance checkpoint. We previously reported that the mature naïve B cell compartment of FOXP3-deficient IPEX patients who lack functional T_{regs} is specifically enriched in autoreactive clones (26). Similarly, high frequencies of autoreactive mature naïve B cells were also observed in CD40L-, MHC class II-, and AID-deficient patients with decreased T_{reg} numbers (23, 38,45); in DOCK8- and WASp-deficient patients who displayed defective T_{reg} suppressive function (24–26); and in signaling lymphocytic activation molecule (SLAM)-associated protein-and AID-deficient patients who showed elevated serum cytokines that inhibited T_{reg} function (37, 38). Our current analysis of CD3D- and CD3E-deficient patients, who lack virtually all T cells and showed an accumulation of autoreactive clones in their mature naïve B cell compartment, provides further evidence that T cells are key regulators of this peripheral B cell selection step. We and others also observed that *AIRE* mutations result in decreased T_{reg} frequencies in patients with APECED and abrogate their peripheral B cell tolerance checkpoint, further suggesting an involvement of T_{regs} in the regulation of this selection step (11, 52). AIRE enforces immune tolerance via two complimentary mechanisms, i.e., eliminating self-reactive clonotypes from the T_{conv} lineage and diverting these cells into the FOXP3$^+$ T_{reg} compartment (7, 9,10,53,54). Our data studying AIRE-deficient patients are in agreement with this model because TCR sequences commonly found in the T_{reg} compartment of HDs are missing from the T_{reg} TCR repertoire selected in the absence of functional AIRE and are instead identified in the CD4$^+$ T_{conv} subset of patients with APECED. Although these observations are currently limited to the analysis of non-HLA-matched control donors and patients, they may correspond to TCRβ sequences expressed by rare clones selected on common shared MHC II features among individuals. In addition, the absence of insulin, IL-17A, IL-17B, IL-17F, KLK5, and MDC/CCL22 PTA expression by mTECs in patients with APECED likely prevents the education of developing thymocytes into T_{regs} that produce TCRs specific for these molecules and result in the accumulation of mature naïve B cells expressing antibodies that bind these AIRE-dependent PTAs. Lack of AIRE in thymic B cells might also contribute to incomplete PTA expression in the thymus and abnormal

T_{reg}/T_{conv} differentiation (14, 55). Hence, decreased numbers and incomplete TCR repertoire of T_{regs} from AIRE-deficient patients may contribute to the escape of developing autoreactive mature naïve B cells with AIRE-dependent PTA reactivities. However, we cannot exclude that peripheral AIRE expression in extrathymic Aire-expressing cells and/or indirect effects of cytokine alterations may also play a role in the BCR repertoire selection of mature naïve B cells (56). It is unclear at this point whether Tregs may control the peripheral B cell tolerance checkpoint via direct interactions with autoreactive B cells as suggested (57–60) and potentially involving self-peptide presentation by MHC class II to their TCRs or whether they suppress other cell types that favor autoreactive B cell survival and which remain to be identified.

In summary, we showed that T cells and their AIRE-dependent selection normally control the peripheral selection of autoreactive B cells. However, defects in AIRE expression divert a subset of T_{reg}-biased sequences into the T_{conv} compartment, creating holes in the T_{reg} TCR repertoire of AIRE-deficient patients that are associated with the accumulation of mature naïve B cells reactive for AIRE-dependent PTAs. These autoreactive B cells may promote peripheral organ autoimmune manifestations in patients with APECED and produce autoantibodies after activation by diverted T_{conv} cells expressing autoreactive TCRs in the absence of specific T_{reg} suppression.

MATERIALS AND METHODS

Patients and HD controls

We obtained peripheral blood or frozen PBMC from one CD3D-deficient patient with homozygous *CD3D:* c.[202C>T];[202C>T]; p.[Arg68X];[Arg68X] mutation [patient 5; (61)], one CD3E-deficient patient with homozygous *CD3E:* c.[173 del T];[173 del T]; p. [L58HfsX9];[L58HfsX9] mutations, 15 AIRE-deficient patients with homozygous or compound heterozygous autosomal recessive *AIRE* gene mutations, and 17 AIRE heterozygous relatives with a single autosomal recessive *AIRE* mutated allele (table S1). In addition, we collected additional samples from healthy unrelated donors. Frequencies of polyreactive and HEp-2–reactive and anti-nuclear new emigrant and mature naïve B cells from an HD were previously reported (26, 36, 37, 46) except for HD-Y541 who is a 59-year-old non-Hispanic Caucasian female (table S2).

Study design

The four AIRE-deficient patients enrolled at the time of the study for the analysis of their B cell reactivity did not receive any steroids, which could have interfered with our experiment readouts, as they did not suffer from adrenal insufficiency often associated with the disease. All samples were collected in accordance with institutional review board-reviewed protocols.

B cell staining and sorting

Peripheral B cells were purified from the blood of patients and control donors by positive selection using CD20 magnetic beads (Miltenyi Biotec). For sorting, enriched B cells were stained with FITC-anti-IgM, PE-anti-IgG, PE-Cy7-anti-CD10, APC-anti-CD21, Pacific

Blue-anti-CD19, and PercP-Cy5.5-anti-CD27 (all from BioLegend) (gating strategy is shown in fig. S21). Single- or batch-sorted $CD19^+CD21^{lo}CD10^+IgM^{hi}CD27^-$ new emigrant and $CD19^+CD21^+CD10^-IgM^+CD27^-$ mature naïve B cells from patients, heterozygous relatives, and HDs were sorted on a FACSAria flow cytometer (Becton Dickinson, Mountain View, CA) into 96-well polymerase chain reaction (PCR) plates or 5-ml round-bottom polystyrene test tubes, respectively. For phenotyping, enriched B cells were stained with FITC-anti-IgM, PE-anti-CD69, PE-Cy7-anti-CD10, APC-anti-CD86, PercP-Cy5.5-anti-CD27, APC-Cy7-anti-CD19 (all from BioLegend), and V450-anti-CD21 (BD Bioscience) (gating strategy is shown in fig. S22).

Complementary DNA, RT-PCR, antibody production, and purification

RNA from single cells was reverse-transcribed in the original 96-well plate in 12.5 ml reactions containing 100 U of Superscript II RT (Gibco BRL) for 45 min at 42°C. Reverse transcription (RT)–PCR reactions, primer sequences, cloning strategy, expression vectors, antibody expression, and purification were as described (21).

BCR repertoire analysis

Ig sequences and mutation status were analyzed by IgBLAST comparison with GenBank using the National Center for Biotechnology Information's IgBLAST server (www.ncbi.nlm.nih.gov/igblast/). Heavy chain complementarity-determining region 3 was defined as the interval between amino acids at position 94 in the variable region of immunoglobulin heavy chain framework 3 and the conserved tryptophan at position 103 in JH segments. Sequences are shown in table S2.

ELISAs and IFAs

Antibody concentrations and reactivity were measured as described (21). Highly polyreactive ED38 was used as positive control in HEp-2 reactivity and polyreactivity ELISAs. Antibodies were considered polyreactive when they recognized all three analyzed antigens, which included double-stranded DNA (dsDNA), insulin, and lipopolysaccha-ride (LPS). For indirect immunofluorescence assays (IFAs), HEp-2 cell-coated slides (Bion Enterprises Ltd.) were incubated in a moist chamber at room temperature with purified recombinant antibodies at 50 to 100 μg/ml and detected with FITC-conjugated goat anti-human IgG.

KREC assay

The ratio of KREC joints (signal joint) to the Jκ-Cκ recombination genomic joints (coding joint) was determined as previously described (33). The number of cell divisions was calculated by subtracting the threshold cycle (C_T) of the PCR detecting the coding joint from that detecting the signal joint.

Antigen arrays and analysis of cloned antibodies on the antigen arrays

Antigen arrays were generated, and assays on the arrays were performed as described previously (62, 63). The list of protein micro-array antigens and procedures are provided in table S5, data file S1, and Supplementary Materials and Methods.

Plasmon resonance analysis

Binding studies were performed at 25°C using a Biacore T100 optical biosensor (GE Healthcare, Biacore, Piscataway, NJ). The list of antigens and their immobilized on a CM5 research-grade sensor chip using amine coupling is provided in the Supplementary Materials and Methods. Antibodies were injected from threefold dilution series starting with either 3 or 1 μM stock using a single-cycle approach (64). The surfaces were regenerated by 30-s injections of 3 M $MgCl_2$. The binding responses were double-referenced against the nonspecific binding to dextran alone and injections of buffer alone. Binding affinity was determined by either fitting the amplitudes observed during the steady-state phase, or kinetics of the binding reaction, to a simple 1:1 binding model using BioEvaluation software (GE Healthcare, Biacore, Piscataway, NJ).

T_{reg} phenotype and in vitro suppression assay

Peripheral $CD4^+$ T cells were enriched with the EasySep Human $CD4^+$T cells kit (STEMCELL Technologies) from the blood of patients, heterozygous relatives, and control donors. Enriched $CD4^+$ T cells were stained with the following antibodies: APC-Cy7-anti-CD4, PE-anti-CD25, PerCP-Cy5.5-anti-CD127, AlexaFluor700-anti-HLA-DR (all from BioLegend) and with AlexaFluor647-anti-Helios (BioLegend), Alexa Fluor 488-anti-FOXP3, and eV605-anti-CD3 (both from eBioscience) after fixation and permeabilization of T cells (gating strategy is shown in figs. S23 and S24). After staining enriched $CD4^+$ T cells with APC-Cy7-anti-CD4, PE-anti-CD25, and Alexa Fluor 647-anti-CD127 (BioLegend), $CD4^+CD127^+T_{conv}$ cells and $CD4^+CD25^{hi}CD127^{-/lo}$ T_{reg} cells from patients, heterozygous relatives, and HDs were sorted using a FACSAria flow cytometer (Becton Dickinson, Mountain View, CA). $CD4^+CD25^+CD127^+$ Tconv cells were labeled with CellTrace carboxyfluorescein diacetate succinimidyl ester (CFSE) (InVivogen). Cocultures of T_{reg} and T_{conv} at 1:1, 1:4, 1:8, and 1:16 ratios were stimulated with the T_{reg} Suppression Inspector Human kit (Miltenyi Biotec) at a 1 bead/1 cell ratio. Proliferation of the viable T_{conv} was analyzed by CFSE dilution at day 3.5.

T_{conv} and T_{reg} TCRBV sequencing

Both $CD4^+CD127^+$ T_{conv} cells and $CD4^+CD25^{hi}CD127^{lo/-}$ T_{reg} cells from patients and HDs were sorted on a FACSAria flow cytometer (Becton Dickinson, Mountain View, CA) into 5-ml round-bottom polystyrene test tubes. Dry pellets were then frozen at –80°C and sent for TCRBV sequencing at Adaptive Biotechnologies (https://clients.adaptivebiotech.com/pub/sng-2019-sciimmunol; DOI 10.21417/B7V926).

Cytokine measurements

Serum BAFF concentrations were determined by ELISA according to the manufacturer's instruction (R&D Systems). Cytokines (GM-CSF, TNF-a, IFN-γ, IL-1B, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, and IL-13) in plasma were measured with the High Sensitivity Human Cytokine Magnetic Bead kit (Millipore), using a Luminex 200, according to the manufacturer's instruction.

Statistics

Statistical analysis was performed using GraphPad Prism (version 7.03; GraphPad, San Diego, CA). Differences between groups of research individuals were analyzed for statistical significance with the Mann-Whitney U test. Multiple group comparisons were corrected for statistical significance with the Bonferroni-Dunn method. A P value of 0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

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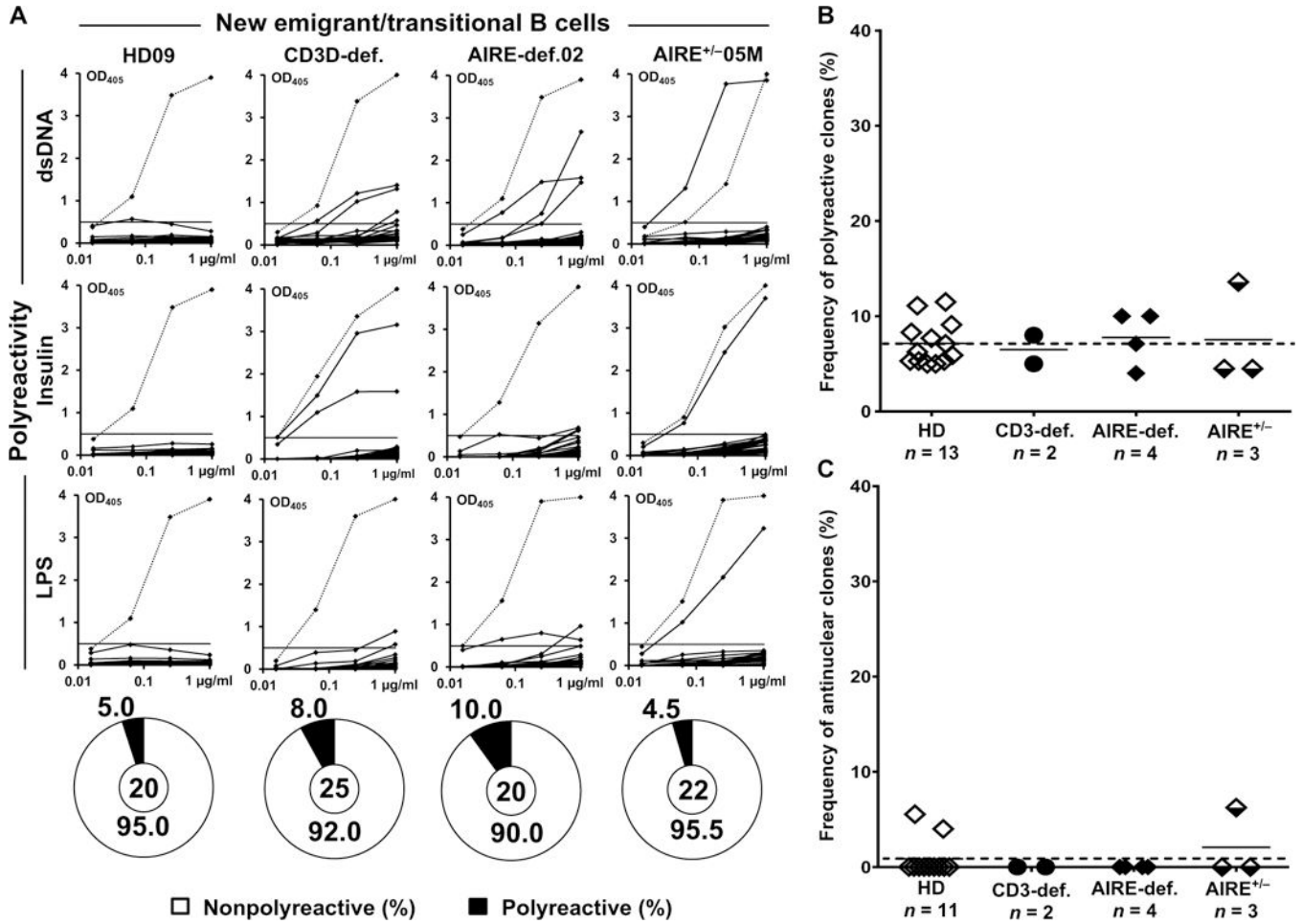
Fig. 1. Central B cell tolerance is functional in CD3- and AIRE-deficient patients.
(**A**) Antibodies from new emigrant B cells from HDs ($n = 12$), CD3-deficient patients (CD3-def., $n = 2$), AIRE-deficient patients (AIRE-def., $n = 4$), and AIRE$^{+/-}$ heterozygous relatives (AIRE$^{+/-}$, $n = 3$) were tested by ELISA for reactivity against dsDNA, insulin, and LPS. Antibodies were considered polyreactive when they recognized all three analyzed antigens. Dotted lines show ED38-positive control. Horizontal lines show cut-off OD$_{405}$ for positive reactivity. For each individual, the frequency of nonpolyreactive (open area) and polyreactive (filled area) clones is summarized in pie charts, with the total number of clones tested indicated in the centers. The frequencies of polyreactive and antinuclear new emigrant/transitional B cells are summarized in (**B**) and (**C**), respectively. Each symbol represents an individual. Solid lines show the mean, and dashed lines indicate the averaged mean value for HDs.
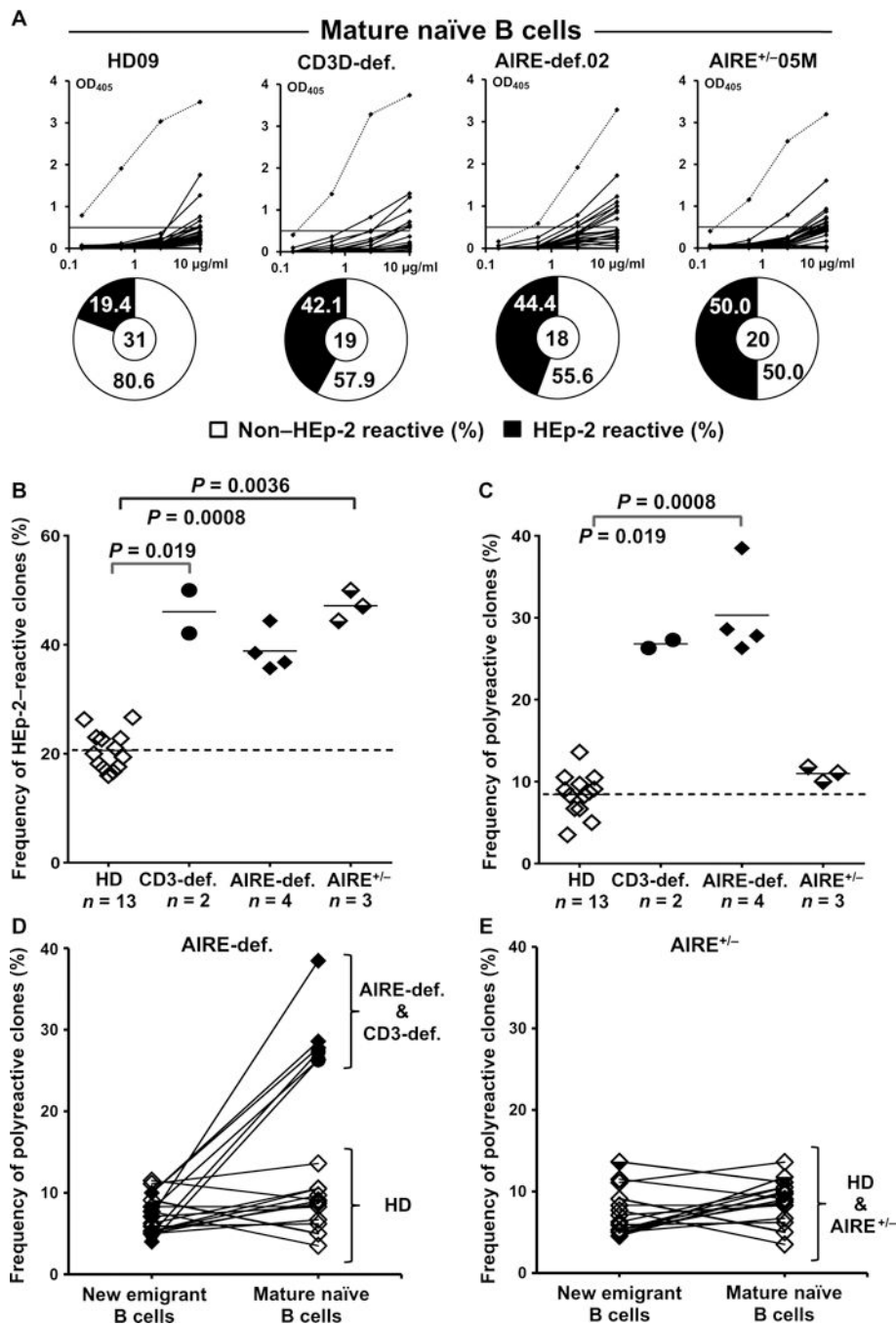
Fig. 2. Defective peripheral B cell tolerance checkpoint in CD3- and AIRE-deficient patients.
(**A**) Antibodies from mature naïve B cells from HDs ($n = 12$), CD3-deficient patients ($n = 2$), AIRE-deficient patients ($n = 4$), and AIRE$^{+/-}$ heterozygous relatives ($n = 3$) were tested by ELISA for anti-HEp-2 cell reactivity. Dotted lines show ED38-positive control. Horizontal lines show cut-off OD$_{405}$ for positive reactivity. For each individual, the frequency of non-HEp-2 reactive (open area) and HEp-2-reactive (filled area) clones is summarized in pie charts, with the total number of clones tested indicated in the centers. The frequencies of HEp-2-reactive and polyreactive mature naïve B cells are summarized in (**B**) and (**C**),

respectively. Each symbol represents an individual. Solid lines show the mean, and dashed line indicates the averaged mean value for HDs. The frequency of polyreactive B cells and their evolution between the new emigrant/ transitional and mature naïve B cell stages in (**D**) CD3- and AIRE-deficient patients and (**E**) AIRE$^{+/-}$ heterozygous relatives are shown in comparison with HDs.
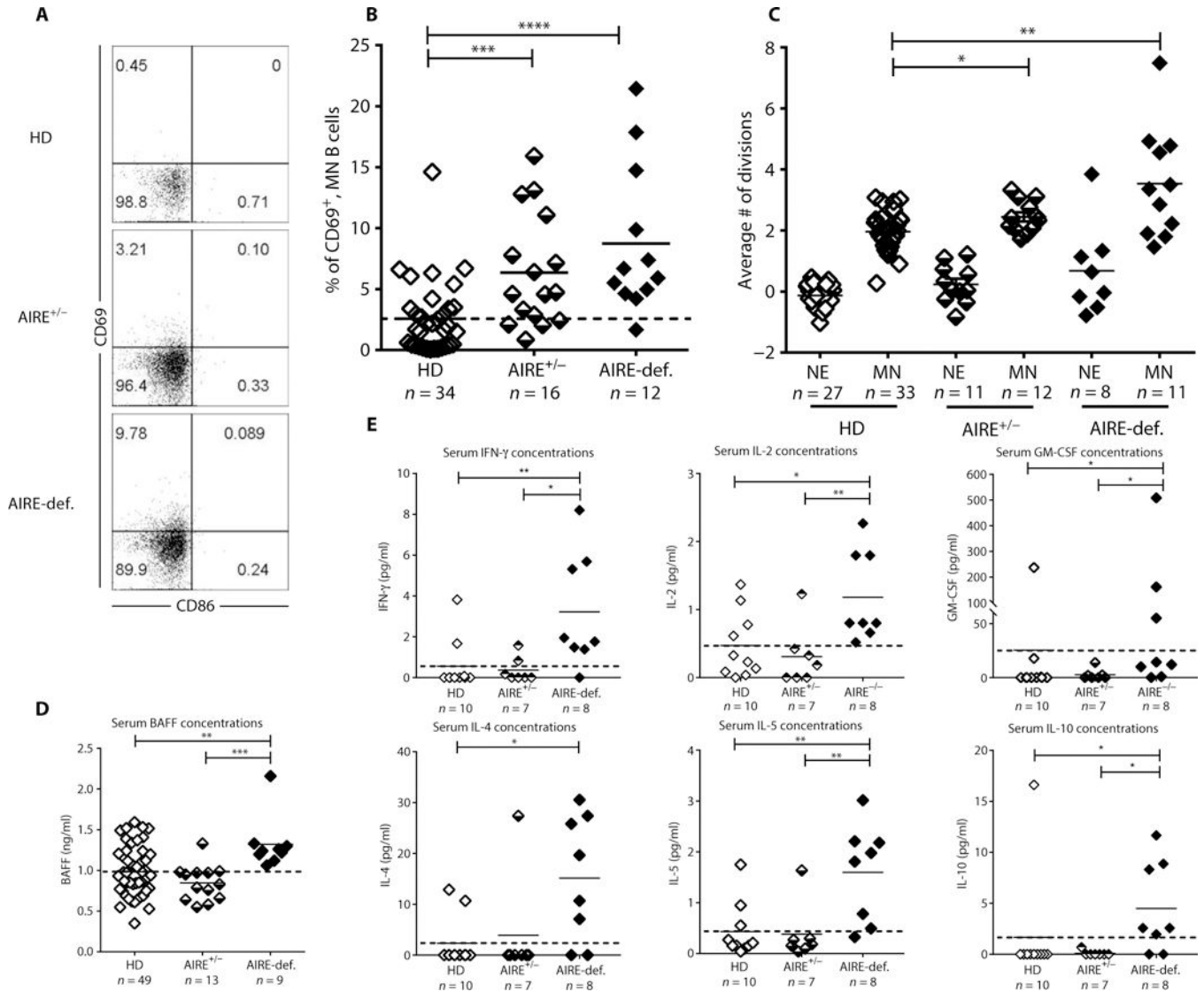
Fig. 3. Mature naïve B cells from AIRE-deficient patients show increased activation and homeostatic expansion associated with elevated serum BAFF and systemic cytokine concentrations. (A)

Representative CD69 and CD86 expression on $CD19^+CD27^-CD21^+CD10^-$ mature naïve B cells from HDs, $AIRE^{+/-}$ heterozygous relatives, and AIRE-deficient patients. (**B**) Frequencies of $CD69^+$ mature naïve B cells in indicated individuals. (**C**) Evaluation of the number of cell divisions undergone in vivo by KREC analysis of new emigrant (NE)/ transitional and mature naïve (MN) B cells from HDs, $AIRE^{+/-}$ carriers, and AIRE-deficient patients. Serum concentrations of (**D**) BAFF and (**E**) IFN-γ, IL-2, GM-CSF, IL-4, IL-5, and IL-10 measured by ELISA and Luminex, respectively, in various individuals' groups. Each symbol represents an individual in (B), (C), and (D). Solid lines show the mean, and dashed line indicates the averaged mean value for HDs. Statistically significant differences are indicated. $*P$ 0.05, $**P$ 0.01, $***P$ 0.001, $****P$ 0.0001.
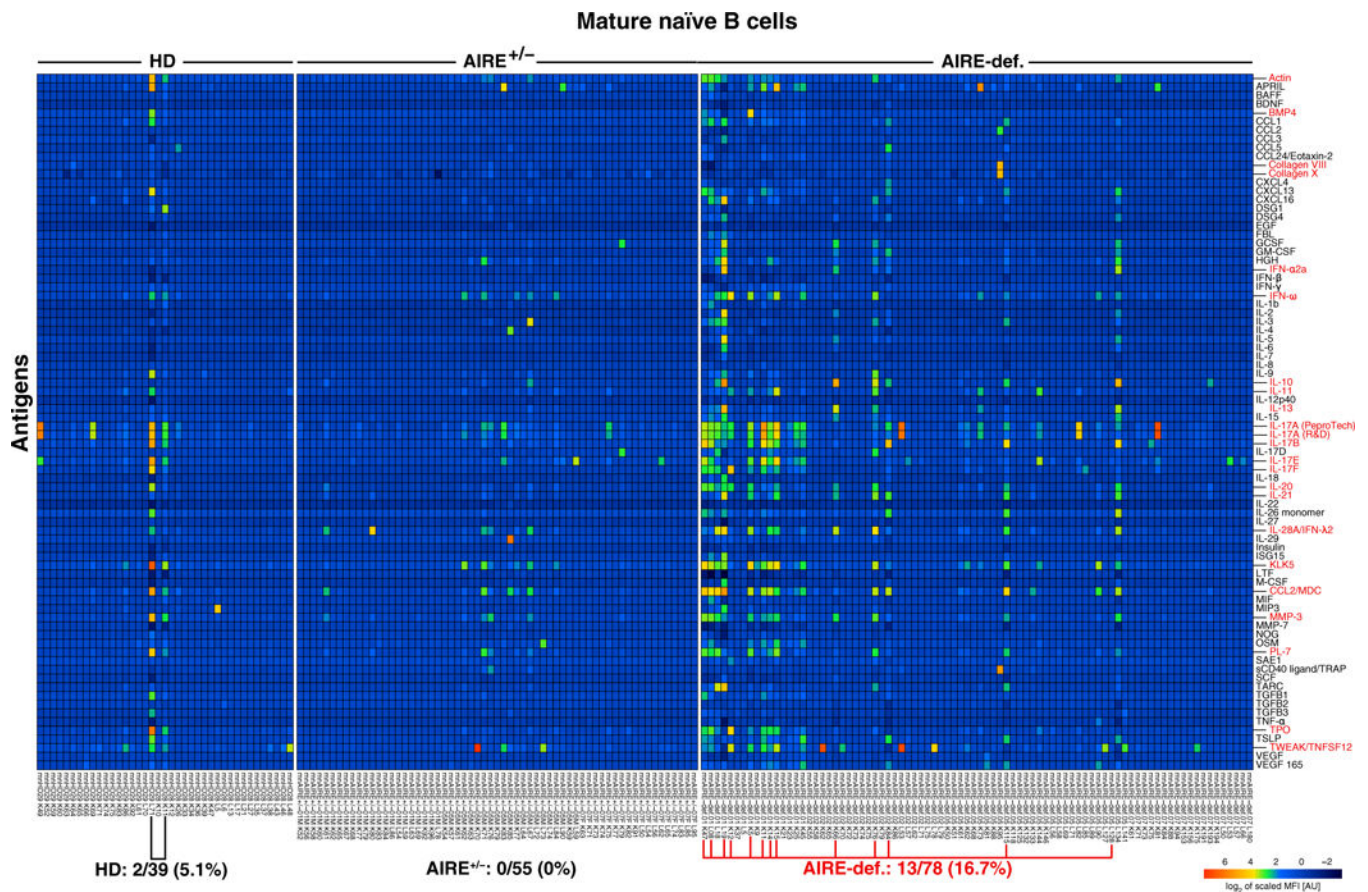
Fig. 4. Autoantigen array profiling of recombinant antibodies cloned from single-cell sorted mature naïve B cells from HDs, AIRE heterozygotes, and AIRE-deficient patients.
Heatmap displaying reactivity of individual mature naïve B cells from HDs ($n = 2$), AIRE$^{+/-}$ heterozygous individuals ($n = 3$), and AIRE-deficient patients ($n = 4$) against selected cytokines and other self-antigens. The frequency of self-reactive mature naïve B cells is elevated in AIRE-deficient patients compared with HDs and AIRE$^{+/-}$ heterozygous individuals, reflecting the accumulation of anticytokine and other autoreactive clones. MFI, mean fluorescent intensity; AU, arbitrary units.
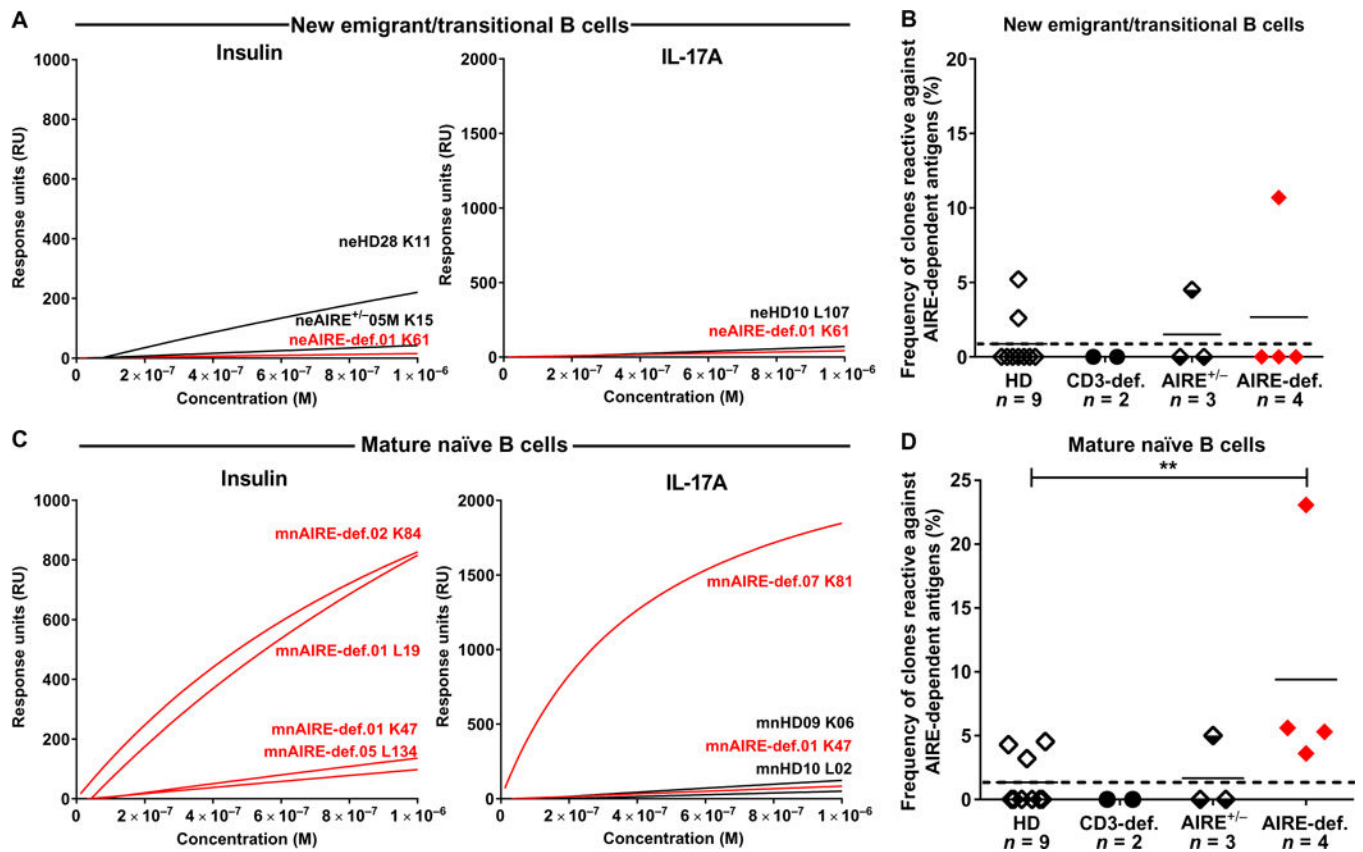
Fig. 5. Elevated frequency of AIRE-dependent antigen-reactive mature naïve B cells in AIRE-deficient patients.

SPR analysis of binding to AIRE-dependent antigens insulin and IL-17A by recombinant antibodies cloned from single (**A**) new emigrant/transitional and (**C**) mature naïve B cells from nine HDs, two CD3-deficient patients, three AIRE$^{+/-}$ heterozygous individuals, and four AIRE-deficient patients. Frequencies of new emigrant/transitional and mature naïve B cells from HDs, CD3-deficient patients, AIRE$^{+/-}$ heterozygous individuals, and AIRE-deficient patients reactive against AIRE-dependent antigens are represented in (**B**) and (**D**), respectively. Each symbol represents an individual. **P 0.01.
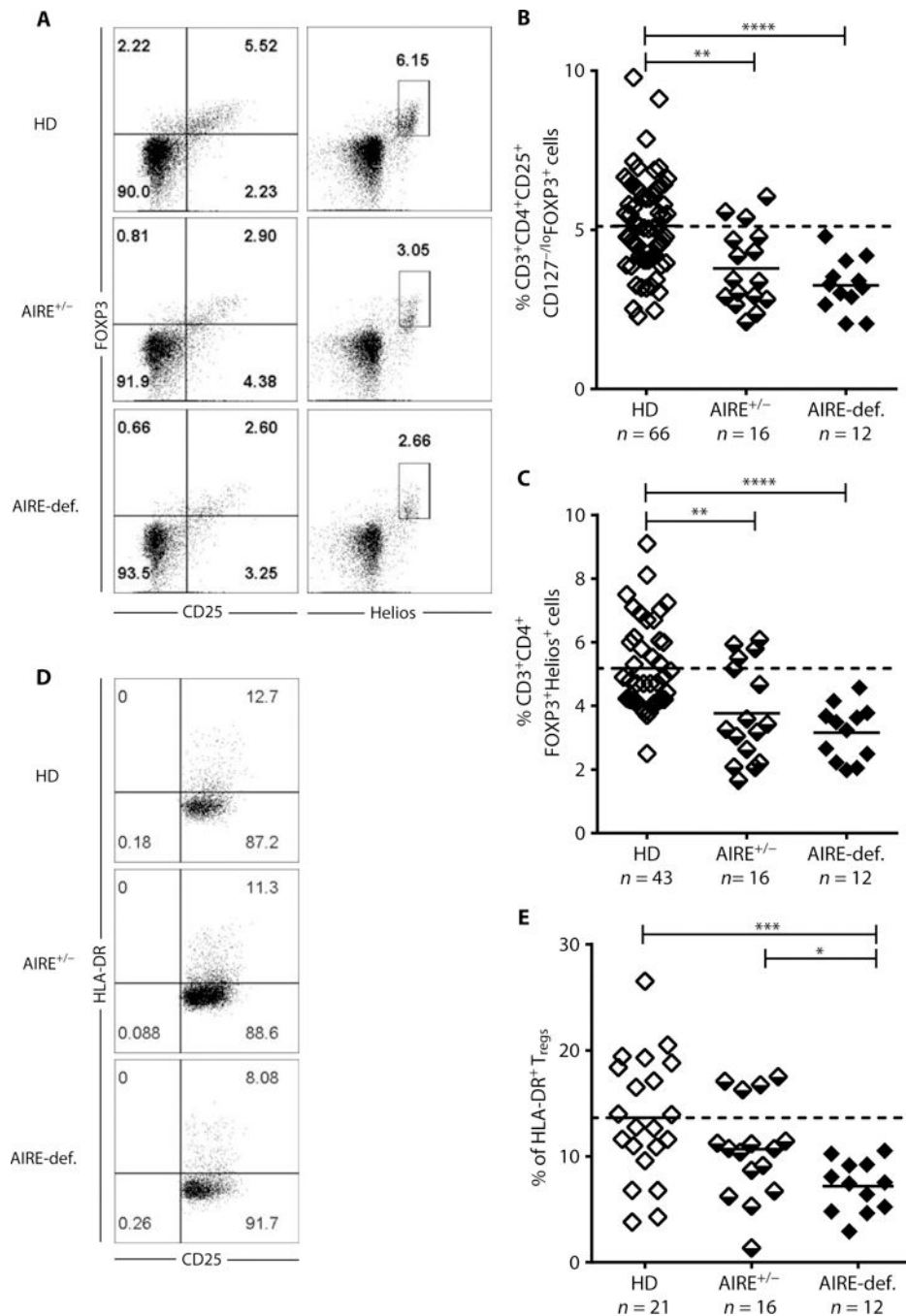
Fig. 6. Altered T_{reg} frequencies in AIRE-deficient patients and AIRE^{+/} heterozygous individuals.
(**A**) Representative CD25 and FOXP3 (left) and Helios and FOXP3 (right) staining on CD3$^+$CD4$^+$ cells from an HD, AIRE$^{+/-}$ heterozygous individual, and AIRE-deficient patient. (**B**) CD3$^+$CD4+CD25$^+$CD127$^{-/lo}$FOXP3$^+$ and (**C**) CD3$^+$CD4$^+$FOXP3$^+$Helios$^+$ T_{reg} frequencies in HDs, AIRE$^{+/-}$ heterozygous individuals, and AIRE-def. patients. (**D**) Representative CD25 and HLA-DR staining on CD3$^+$CD4$^+$CD25$^+$CD127$^{-/lo}$ T cells and (**E**) CD3$^+$CD4$^+$CD25$^+$CD127^{lo}HLA-DR$^+$ T cell frequencies in HDs, AIRE$^{+/-}$ heterozygous individuals, and AIRE-def. patients. Each symbol represents an individual and solid lines

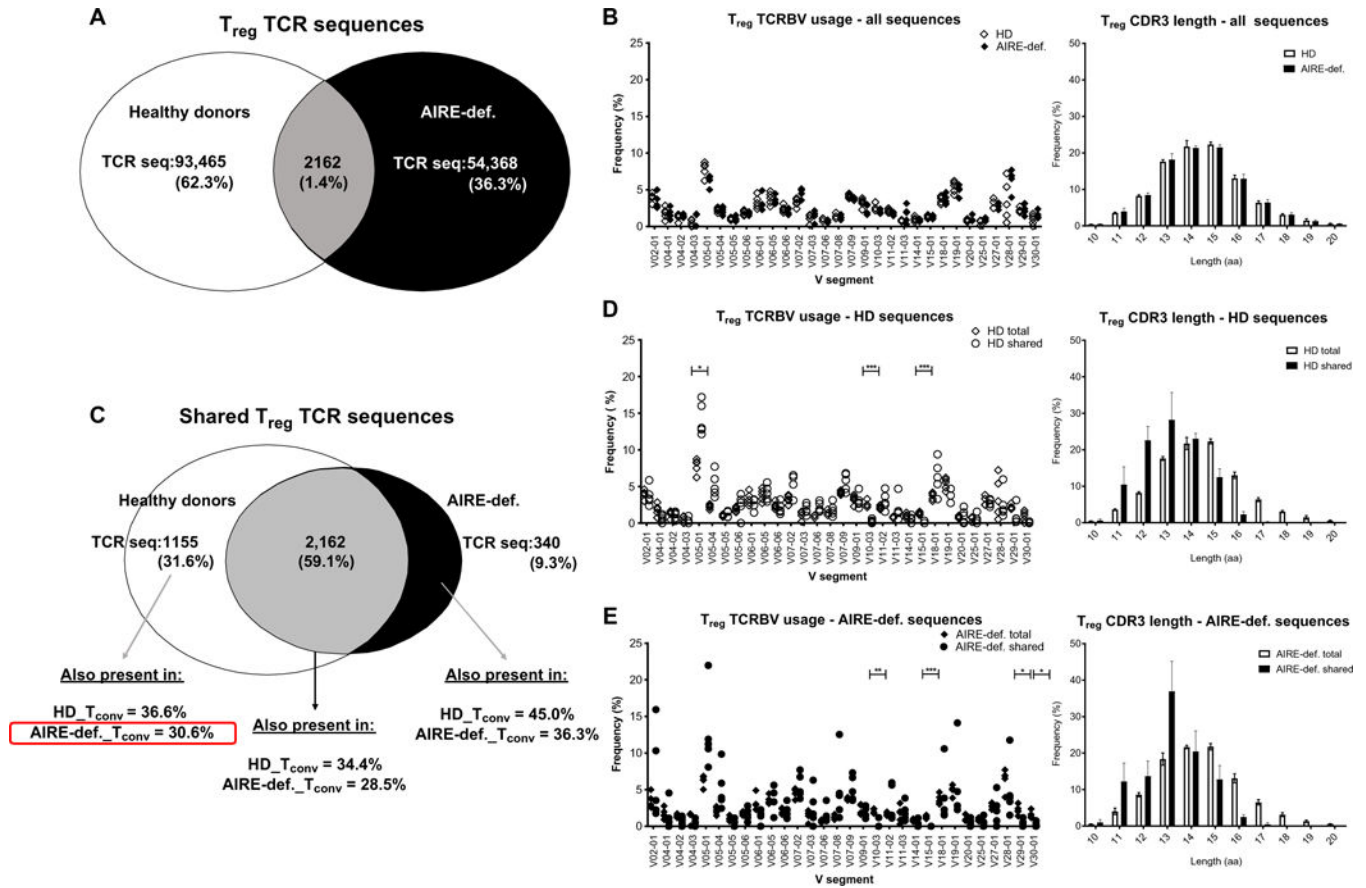show the mean; dashed line indicates the averaged mean value for HDs. *P 0.05, **P 0.01, ***P 0.001; **** P 0.0001.

Fig. 7. AIRE deficiency affects the peripheral CD4+ Treg and Tconv cell TCR repertoire.
CD4+CD25hiCD127−/lo Treg and CD4+CD127+ Tconv cells were purified by fluorescence-activated cell sorting from an HD (*n* = 5) controls and AIRE-deficient patients (*n* = 5) and subjected to TCRBV sequencing. (**A**) Summary chart of total distinct Treg CDR3 TCR sequences obtained from pooled HD controls and AIRE-deficient patients. (**B**) TCRBV V gene segment usage and CDR3 length in Tregs from HDs and AIRE-deficient patients. (**C**) Treg TCRBV sequences common to HDs but absent in patients with APECED can be identified in the Tconv compartment of AIRE-deficient patients and HDs. Comparison of TCRBV V gene segment usage and CDR3 length in total versus shared Treg sequences in (**D**) HDs and (**E**) AIRE-deficient patients. aa, amino acids. *P 0.05, **P 0.01, ***P 0.001.