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Research Article **Autoimmunity**

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Decreased somatic hypermutation induces an impaired peripheral B cell tolerance checkpoint

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Patients with mutations in *AICDA*, which encodes activation-induced cytidine deaminase (AID), display an impaired peripheral B cell tolerance. AID mediates class-switch recombination (CSR) and somatic hypermutation (SHM) in B cells, but the mechanism by which AID prevents the accumulation of autoreactive B cells in blood is unclear. Here, we analyzed B cell tolerance in AID-deficient patients, patients with autosomal dominant AID mutations (AD-AID), asymptomatic *AICDA* heterozygotes ($AID^{+/-}$), and patients with uracil *N*-glycosylase (UNG) deficiency, which impairs CSR but not SHM. The low frequency of autoreactive mature naive B cells in UNG-deficient patients resembled that of healthy subjects, revealing that impaired CSR does not interfere with the peripheral B cell tolerance checkpoint. In contrast, we observed decreased frequencies of SHM in memory B cells from AD-AID patients and $AID^{+/-}$ subjects, who were unable to prevent the accumulation of autoreactive mature naive B cells. In addition, the individuals with *AICDA* mutations, but not UNG-deficient patients, displayed Tregs with defective suppressive capacity that correlated with increases in circulating T follicular helper cells and enhanced cytokine production. We conclude that SHM, but not CSR, regulates peripheral B cell tolerance through the production of mutated antibodies that clear antigens and prevent sustained interleukin secretions that interfere with Treg function.

Introduction

Developing B cells with autoreactive B cell receptors (BCRs) generated by random V(D)J recombination are removed at 2 discrete early B cell tolerance checkpoints; a central checkpoint in the bone marrow removes most clones expressing polyreactive and antinuclear autoantibodies, whereas a peripheral B cell tolerance checkpoint prevents the accumulation of autoreactive mature naive B cells (1). This second selection step appears to be regulated by B cell–extrinsic factors such as Tregs, since FOXP3-deficient patients who lack functional Tregs display a defective peripheral B cell tolerance checkpoint (2). In agreement with this observation, decreased Treg numbers in patients with class-switch recombination deficiency (CSR-D) caused by mutations in *CD40L/TNFRSF5* or in the *AICDA* gene encoding activation-induced cytidine deaminase (AID), which mediates CSR and somatic hypermutation (SHM), also correlate with an impaired peripheral B cell tolerance checkpoint (3–9). However, the mechanisms by which AID may affect Treg homeostasis or function remain unknown.

To assess the individual contribution of CSR and SHM to the establishment of peripheral B cell tolerance, we analyzed the frequency of autoreactive mature naive B cells and Treg function in rare uracil *N*-glycosylase–deficient (UNG-deficient) patients, CSR-D patients with autosomal dominant (AD) *AICDA* mutations, and healthy asymptomatic individuals carrying a single autosomal recessive *AICDA* mutation ($AID^{+/-}$ heterozygotes). Patients lacking UNG, an enzyme that excises from DNA uracils resulting from enzymatic deamination of cytosines by AID, have impaired CSR but functional SHM processes, although with a skewed pattern (3). Patients with the V186X or R190X heterozygous AD mutation in *AICDA*, which preserves AID enzymatic activity, also have

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impaired CSR (10). The V186X or R190X stop mutations result in the truncation of the AID C-terminal nuclear export signal and cause an accumulation in the nucleus of mutated AID proteins that are unable to catalyze CSR, whereas unmutated AID produced by the normal allele can be shuttled out of the nucleus (11).

We report here that autoreactive B cells are normally counterselected in UNG-deficient patients, demonstrating that CSR and the generation of isotype-switched B cells are not involved in the regulation of the peripheral B cell tolerance checkpoint. In contrast, altered SHM in AID-deficient patients, AD-AID individuals, and $AID^{+/-}$ heterozygotes resulted in increased numbers of autoreactive mature naive B cells in the periphery and correlated with impaired Treg function that was likely due to elevated cytokine production.

Results

Abnormal peripheral B cell tolerance checkpoint in subjects carrying heterozygous AICDA mutation(s). We have previously reported that AID-deficient patients display an impaired peripheral B cell tolerance checkpoint (8). However, it is unclear whether this altered selection step results from defective CSR or SHM, since both are catalyzed by AID. In an attempt to discern the single contribution of CSR or SHM to preventing the accumulation of autoreactive B cells in the periphery, we analyzed the Ig repertoire and the reactivity of recombinant antibodies cloned from mature naive B cells from CSR-D patients including 3 UNG-deficient and 4 AD-AID patients with impaired CSR. We also studied 5 asymptomatic $AID^{+/-}$ subjects with a single recessive *AICDA* mutation and 2 additional AID-deficient patients (8). Repertoire analysis in mature naive B cells from UNG-deficient patients revealed normal frequencies of the $V_{H/4\text{-}34}$ gene (Figure 1A and Supplemental Tables 3–16; supplemental material available online with this article; doi:10.1172/JCI84645DS1), which is known to encode intrinsically self-reactive cold agglutinin antibodies (12, 13). In contrast, we found that $V_{H/4\text{-}34}$ gene segment usage was increased in mature naive B cells from AID-deficient patients, AD-AID patients, and $AID^{+/-}$ heterozygotes, suggesting an abnormal peripheral B cell tolerance checkpoint in subjects carrying *AICDA* mutation(s) (Figure 1A). We performed ELISA on HEp-2 cell lysates to test the reactivity of recombinant antibodies cloned from mature naive B cells to determine the functionality of the peripheral B cell tolerance checkpoint (1, 14). The analysis of 2 additional AID-deficient patients confirmed our previous observation of increased frequencies of HEp-2–reactive clones, which represented 52.1% ± 7.1% of the mature naive B cells compared with 20.4% ± 3.6% in healthy donor (HD) counterparts ($P < 0.0001$; Figure 1, B and C, and Supplemental Figure 1) (8). In agreement with abnormal $V_{H/4\text{-}34}$ gene segment usage, the frequency of HEp-2–reactive clones was also increased in $AID^{+/-}$ heterozygotes (36.8% ± 6.0%) and in AD-AID patients (42.7% ± 10.0%), revealing an impaired peripheral B cell tolerance checkpoint (Figure 1, B and C, and Supplemental Figure 1). Peripheral B cell tolerance checkpoint defects were further evidenced in all subjects carrying *AICDA* mutation(s) by the elevated frequencies of polyreactive clones compared with frequencies in HDs (Figure 1D and Supplemental Figure 2). In addition, the frequencies of antinuclear B cells were also elevated in AID-deficient patients (13.1% ± 5.4% in AID-deficient patients compared with 3.3% ± 2.2% in HDs, $P < 0.001$) (Figure 1E). Various patterns of HEp-2–reactive antibodies that recognized nuclear or cytoplasmic structures are shown in Figure 1F. Of note, the increased self-reactivity in $AID^{+/-}$ B cells was less severe than in $AID^{-/-}$ B cells, suggesting a gene dosage effect of *AICDA* on this peripheral B cell selection step (Figure 1, B and C, and Supplemental Figure 1). In contrast, UNG-deficient patients displayed normal frequencies of HEp-2–reactive (23.8% ± 2.0%), polyreactive (10.9% ± 5.3%), and antinuclear (1.7% ± 3.0%) mature naive B cells, demonstrating that impaired CSR and the absence of isotype-switched memory B cells do not affect the establishment of peripheral B cell tolerance (Figure 1, B–E). We conclude that *AICDA* mutations induce defects in the peripheral B cell tolerance checkpoint independently of CSR impediments.

Elevated systemic cytokine concentrations correlate with increased homeostatic B cell expansion of autoreactive mature naive B cells. We have previously observed that defects in peripheral B cell tolerance may be associated with increased cytokine production in patients with common variable immunodeficiency (CVID) and in those with SLAM-associated protein (SAP) deficiency (15, 16). Serum concentrations of IL-2, IL-6, IL-10, IL-17, IFN-γ, and IL-21 were increased in AID-deficient patients, while some $AID^{+/-}$ subjects and AD-AID patients displayed increased serum IL-6 and IL-10 or IL-12 and IL-21 concentrations, respectively, although differences did not reach statistical significance for the latter as determined by ANOVA with Dunnett's correction for multiple comparisons (Figure 2A). Although we could only test 2 UNG-deficient patients, they did not show any difference in proinflammatory cytokines compared with HDs, thus correlating with the normal peripheral B cell tolerance checkpoint observed in these UNG-deficient patients (Figure 2A).

B cell–activating factor (BAFF) is another molecule that controls the number of peripheral B cells and has been associated with peripheral B cell tolerance dysregulation (17). However, the impact of BAFF on the accumulation of autoreactive mature naive B cells remains unclear. While increased serum BAFF concentrations in CD40L- and AID-deficient patients as well as in CVID patients correlated with an altered peripheral B cell tolerance checkpoint, FOXP3-, SAP-, and DOCK8-deficient patients showed normal serum BAFF concentrations, despite increased frequencies of autoreactive mature naive B cells (2, 7, 8, 15, 16, 18). We found that all CSR-D patients, who have no switched memory B cells, had a 2-fold increase in serum BAFF concentrations (Figure 2B). Since the peripheral B cell tolerance checkpoint is functional in UNG-deficient patients, we can conclude that a 2-fold increase in the serum BAFF concentration does not alter this peripheral B cell selection step. In addition, $AID^{+/-}$ subjects who display a defective peripheral B cell tolerance checkpoint showed normal serum BAFF concentrations, further revealing a lack of correlation between serum BAFF concentrations and the accumulation of autoreactive mature naive B cells (Figure 2B). Increased serum BAFF concentrations may be associated with increased homeostatic B cell expansion, as previously observed in other patients with primary immunodeficiency (16). Using a quantitative PCR–based assay, we measured the κ-deletion recombination excision circles (KRECs) to obtain an estimate of the number of divisions undergone by a B cell population, and found an increase in the number of divisions in mature naive B cells from AID-deficient (2.7 ± 0.9, $P < 0.01$) and AD-AID (2.9 ± 0.8, $P < 0.05$) patients but not in those from UNG-deficient patients (2.2 ± 0.4) or $AID^{+/-}$ subjects (1.6 ± 0.4), thereby revealing

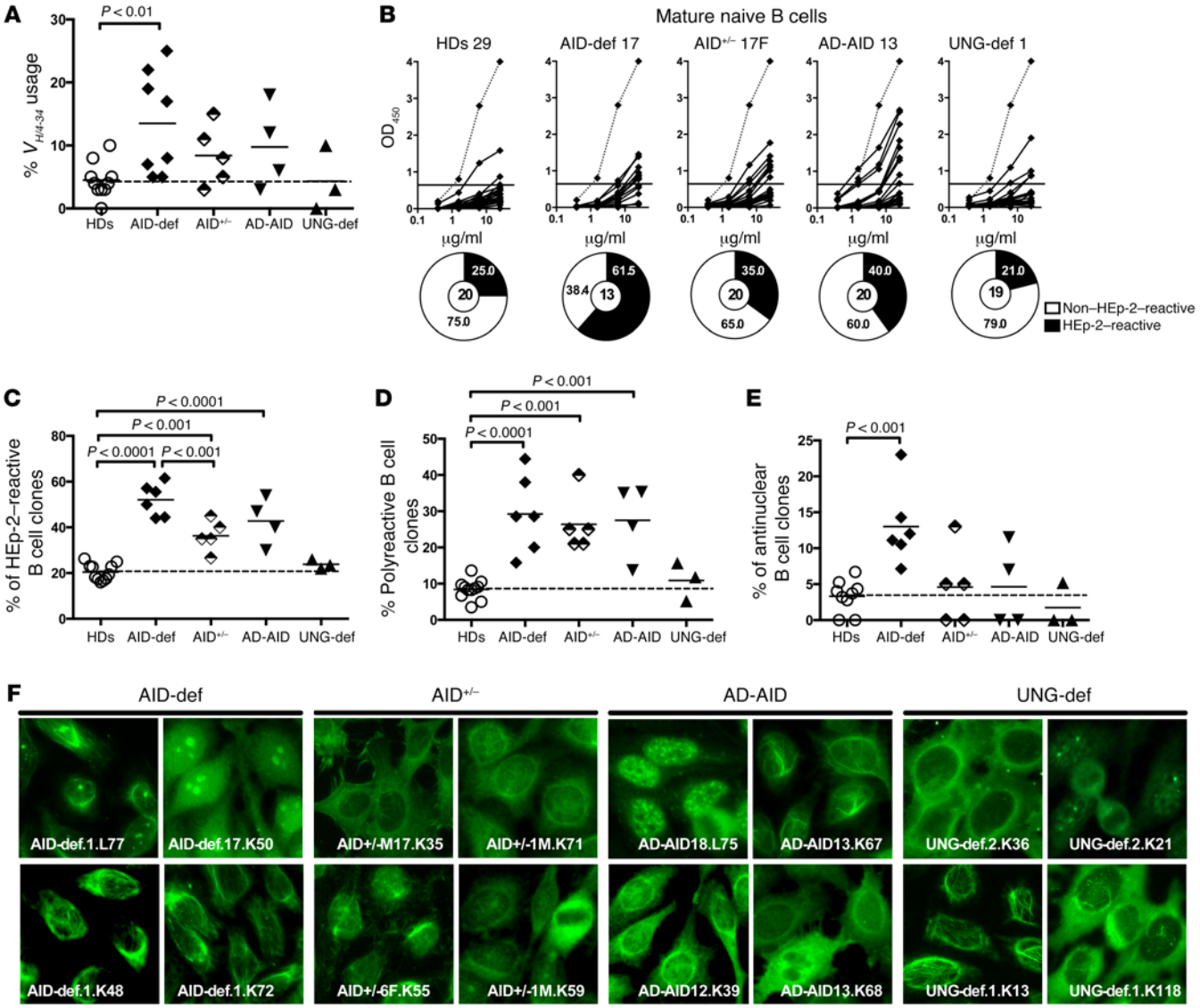


Figure 1. Defective peripheral tolerance checkpoint in patients with *AICDA* gene mutations. (**A**) Increased frequency of $V_{H/4\text{-}34}$ gene usage in AID-deficient (AID-def) patients (n = 8), asymptomatic healthy heterozygotes (AID$^{+/-}$) (n = 5), and AD-AID patients (n = 4) compared with that of HDs (n = 11) or UNG-deficient (UNG-def) patients (n = 3). Bars indicate the mean ± SD; dashed line indicates the mean value for the HDs. (**B**) Antibodies from mature naive B cells were tested by ELISA for anti–HEp-2 cell reactivity. Dotted lines show ED38-positive control, and solid lines show binding for each cloned recombinant antibody. Horizontal lines define the cutoff OD_{405} for positive reactivity. For each individual, the frequency of autoreactive (black area) and nonautoreactive (white area) clones is summarized in pie charts, with the total number of clones tested indicated in the centers. Summaries of the frequencies of HEp-2–reactive (**C**), polyreactive (**D**), and antinuclear (**E**) mature naive B cells in HDs (n = 11) and AID-deficient (n = 6), AID$^{+/-}$ (n = 5), AD-AID (n = 4), and UNG-deficient (n = 3) patients. Bars indicate the mean, and dashed lines indicate the mean value for HDs as a comparison. (**F**) Diverse staining patterns of antibodies expressed by mature naive B cells obtained by IFA on HEp-2 cells. Original magnification, ×40. P values were determined by ANOVA with Dunnett's correction for multiple comparisons.

an incomplete correlation between serum BAFF concentration and B cell homeostatic expansion (Figure 2C). The dysregulated B cell homeostasis was further evidenced in AID-deficient patients by increased expression of the Ki-67 proliferation marker in mature naive B cells (Supplemental Figure 3, A and B). In addition, in AID-deficient and AD-AID patients, we observed an expansion of unusual CD19$^+$CD27$^-$CD10$^-$CD21$^{-/lo}$ B cells that have been reported to contain autoreactive clones and that correlated with increased serum concentrations of IL-21 (Figure 2A and Supplemental Figure 3C) (19). Hence, increased serum BAFF concentrations are associated with the absence of class-switched B cells and do not account for the alteration of the peripheral B cell tolerance checkpoint.

Subjects with AICDA mutation(s) display an altered Treg phenotype and function. Tregs play an important role in preventing the accumulation of autoreactive mature naive B cells, which is revealed by specific defects in the peripheral B cell tolerance checkpoint in FOXP3-deficient, dedicator of cytokinesis 8–deficient (DOCK8-deficient), and Wiskott-Aldrich patients who have nonfunctional Tregs (2, 18, 20). In addition, both CD40L- and AID-deficient patients showed decreased peripheral blood Treg

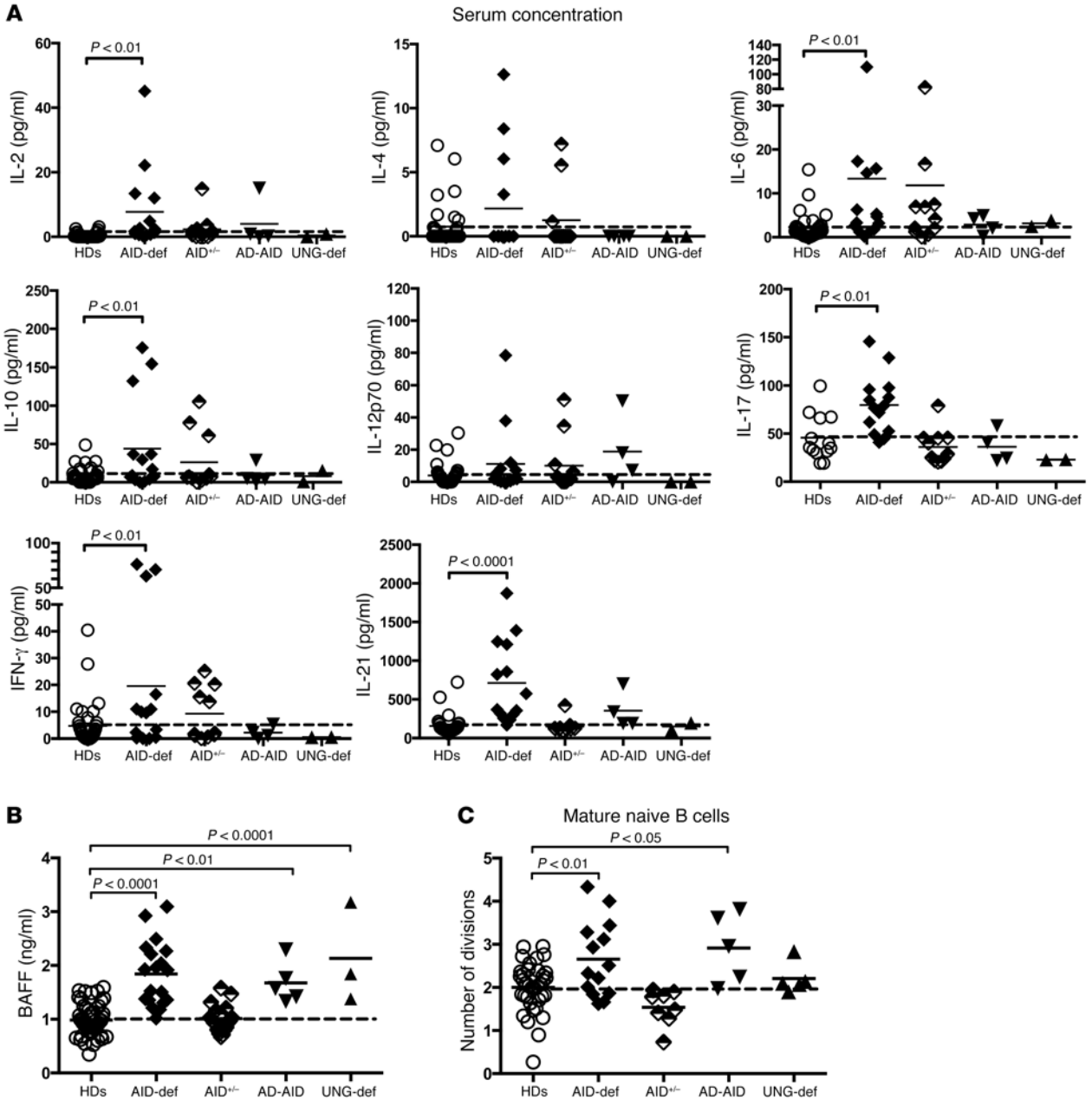


Figure 2. Increased serum cytokine and BAFF concentrations and B cell proliferation in AID-deficient and AD-AID patients. (**A**) Serum concentrations of various cytokines measured by Luminex or ELISA (pg/ml) in HDs (n = 36), AID-deficient patients (n = 14), asymptomatic healthy heterozygotes ($AID^{+/-}$) (n = 12), AD-AID patients (n = 4), and UNG-deficient patients (n = 2). (**B**) Serum BAFF concentrations (pg/ml) in HDs (n = 34), AID-deficient patients (n = 17), asymptomatic healthy heterozygotes ($AID^{+/-}$) (n = 8), and AD-AID (n = 5) and UNG-deficient (n = 3) patients were measured by ELISA and found to be elevated in all patients with hyper-immunoglobulin M (HIGM) syndrome. (**C**) Evaluation of the number of cell divisions undergone in vivo by KREC analysis of mature naive B cells from HDs (n = 37) and AID-deficient (n = 14), $AID^{+/-}$ (n = 8), AD-AID (n = 5), and UNG-deficient (n = 5) patients. An increased proliferative history was observed in both AID-deficient and AD-AID patients. Bars represent the mean, and dashed lines indicate the mean for HDs as a comparison. P values were determined by ANOVA with Dunnett's correction for multiple comparisons.

frequencies, correlating with an increase in autoreactive mature naive B cells (7, 8). The analysis of additional AID-deficient patients further confirmed that the frequencies of $CD4^+CD25^{hi}CD127^{lo}FOXP3^+$ Tregs (3.5% ± 1.7%) were decreased in these subjects compared with frequencies detected in HDs (5.1% ± 1.7%, $P < 0.01$) (Figure 3, A and B). In contrast, $AID^{+/-}$ individuals, AD-AID patients, and UNG-deficient patients displayed normal Treg frequencies in their blood (Figure 3, A and B). In addition, Tregs from AID-deficient patients exhibited an abnormal phenotype evidenced by a significant increase in the Ki-67 proliferative marker, suggesting defective suppressive activity (Figure 3C) (20). A direct assessment of the in vitro suppressive capacity of Tregs showed that Tregs from AID-deficient patients, $AID^{+/-}$ heterozygotes, and AD-AID patients had an impaired ability to inhibit the proliferation of CFSE-labeled $CD3^+CD4^+CD25^-$ autologous and heterologous T responder (Tresp) cells (Figure 4, A–C). In con-

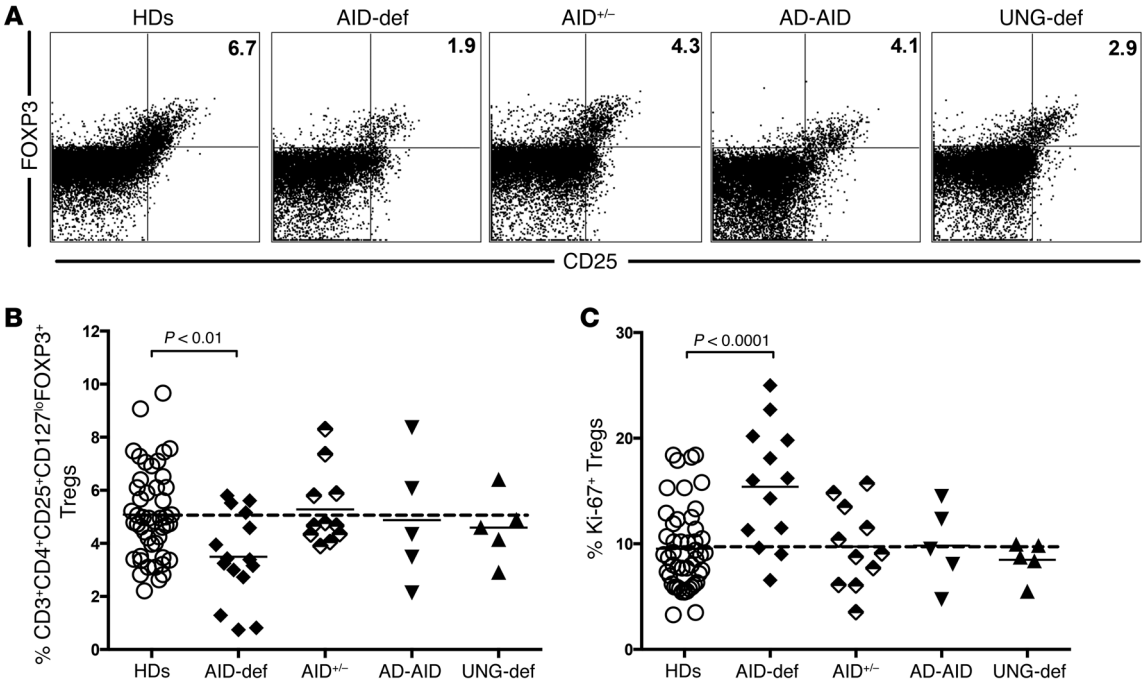


Figure 3. Altered Treg phenotype is restricted to AID-deficient patients. (**A**) Representative CD25 and FOXP3 staining on $CD3^+CD4^+$ cells. (**B** and **C**) Summary of the data shows lower frequencies of $CD3^+CD4^+CD25^+CD127^{lo}FOXP3^+$ Tregs but a higher proportion of Ki-67$^+$ Tregs in AID-deficient patients (n = 13) compared with frequencies detected in HDs (n = 48) and $AID^{+/-}$ (n = 11), AD-AID (n = 5), and UNG-deficient (n = 5) patients. Bars represent the mean, and dashed lines indicate the mean for HDs as a comparison. P values were determined by ANOVA with Dunnett's correction for multiple comparisons.

trast, Tregs from UNG-deficient patients showed normal in vitro suppression, which correlated with their low frequencies of autoreactive mature naive B cells (Figure 4, A–C). In addition, Tresps from AID-deficient patients were refractory to suppression by HD Tregs, whereas the proliferation of Tresps from asymptomatic healthy heterozygotes as well as AD-AID and UNG-deficient patients could be suppressed by Tregs from HDs (Figure 4D). In conclusion, impaired Treg suppressive function correlates with peripheral B cell tolerance checkpoint defects in $AID^{+/-}$ individuals as well as AD-AID and AID-deficient patients who also have T cells that are refractory to Treg suppression.

Defective Treg suppressive function is associated with increased cytokine production of Tresps. We have previously reported that increased Tresp proliferation associated with decreased Treg suppression could result from the dysregulated production of specific cytokines (15). We therefore analyzed cytokine production ex vivo by $CD4^+$ T cells freshly isolated from AID-deficient patients or $AID^{+/-}$ heterozygotes. We could not assess cytokine production in AD-AID or UNG-deficient patients, because the results are severely altered when frozen peripheral blood mononuclear cells (PBMCs) are used. We observed an increase in IL-4 production by $CD4^+$ T cells from subjects with *AICDA* mutation(s), which have previously been reported to interfere with proper Treg function (15, 21), whereas IL-10 production did not appear to be dysregulated (Figure 5, A and B). We also found an increase in the production of IL-2, IL-21, and IFN-γ by $CD4^+$ T cells from AID-deficient patients, further attesting to the hyperresponsiveness of these cells (Figure 5, A and B). Quantitative PCR analyses validated the increased production of IL-2, IL-4, and IL-21 by freshly isolated $CD4^+$ T cells from AID-deficient patients and in some from $AID^{+/-}$ subjects (Supplemental Figure 4). Several of these cytokines induce an inability of Treg cells to suppress Tresps, similar to what we observed in cocultures of Tregs and Tresps isolated from AID-deficient patients (Supplemental Figure 5, A and B) (15). IL-12, which we found to be increased in the serum of some AD-AID patients (Figure 2A), has also been shown to interfere with proper Treg function (22). In addition, we directly tested the effect of cytokines solely on Treg function by preincubating HD Tregs for 4 days with various ILs and then assessed their suppressive ability on fresh Tresps in the absence of any additional cytokines. We found that Tregs preincubated with IL-4 and IL-12 were substantially less effective at inhibiting Tresp proliferation than were nontreated Tregs, whereas preincubation with IL-10 and IL-21 did not affect Treg function (Figure 5, C and D). In contrast, Tregs cultured with elevated IL-2 concentrations showed an increased capacity to inhibit Tresp proliferation as reported previously (23) (Figure 5D). When HD Tregs were preincubated with all the cytokines (referred to as "mix" in the figure) found to be elevated in AID-deficient patients, we observed an inability of these Tregs to suppress Tresp proliferation (Figure 5D). Altogether, increased cytokine production in patients with *AICDA* mutation(s) may contribute to the poor Treg suppressive function and a defective peripheral B cell tolerance checkpoint in these individuals.

Increased circulating Tfh cells in individuals with AICDA gene mutation(s). To assess whether peripheral tolerance was further breached in individuals with *AICDA* or *UNG* gene mutations, we analyzed the presence of autoantibodies and antinuclear antibodies (ANAs) in the plasma or serum of these CSR-D patients and

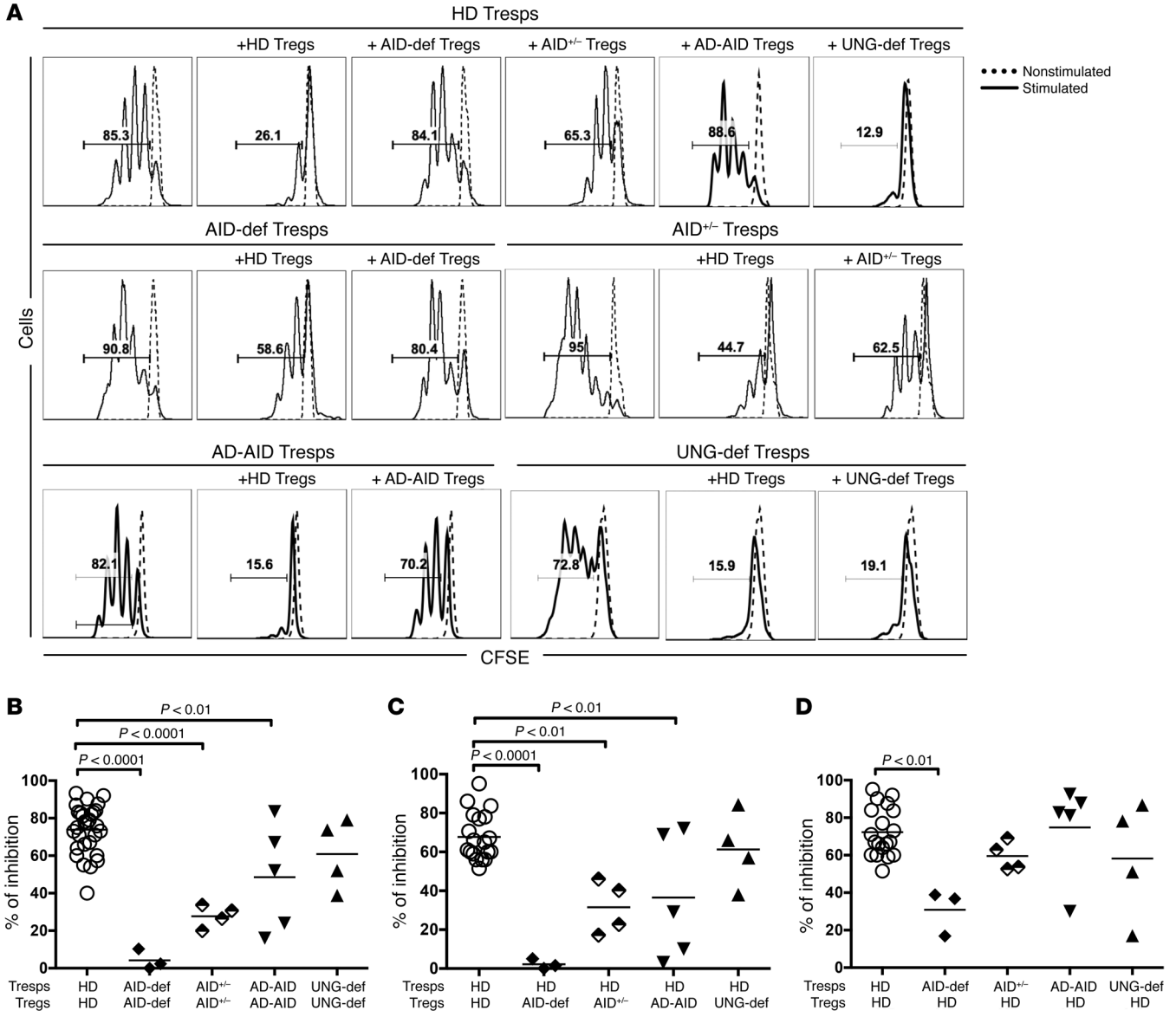


Figure 4. Peripheral B cell tolerance defects in patients carrying *AICDA* mutations correlate with impaired Treg function. (**A**) Representative histograms of Treg-mediated suppression of autologous and heterologous CFSE-labeled Tresps on day 3.5 from 1 AID-deficient patient, 1 asymptomatic healthy carrier, 1 AD-AID patient, and 1 UNG-deficient patient compared with those from a HD. Dashed line shows nonstimulated Tresps. (**B**) Autologous suppressive capacity of Tregs from HDs (n = 19), AID-deficient patients (n = 3), AID$^{+/-}$ subjects (n = 4), AD-AID patients (n = 5), and UNG-deficient patients (n = 4). Bars indicate the mean, whereas **C** and **D** display the heterologous suppressive capacity of mixed Treg/Tresp cocultures. P values were determined by ANOVA with Dunnett's correction for multiple comparisons.

AID$^{+/-}$ individuals. We first confirmed, by analyzing additional AID-deficient patients, that all 14 of these patients had high titers (1:320) of IgM autoantibodies and ANAs in their plasma/serum (Figure 6A) (8). In contrast, no serum IgM autoantibodies could be detected in asymptomatic AID$^{+/-}$ heterozygotes or in AD-AID or UNG-deficient patients (Figure 6A). Since T cell help is important for ANA production, we analyzed circulating CXCR5$^+$PD-1$^+$ T follicular helper (Tfh) cells that are efficient at inducing antibody secretion (16, 24, 25). We found the highest frequencies of CD3$^+$CD4$^+$CXCR5$^+$PD-1$^+$ Tfh cells in AID-deficient patients (7.2% ± 2.6%, $P < 0.0001$) and an increase in Tfh cells in AD-AID patients (4.8% ± 1.5%, $P < 0.01$) compared with HDs (1.8% ± 1.4%) (Figure 6, B and C). Asymptomatic healthy heterozygotes also showed a modest increase in Tfh cells (3.1% ± 1.6%), although differences with HDs did not reach statistical significance when analyzed by ANOVA with Dunnett's correction for multiple comparisons (Figure 6, B and C). In contrast, UNG-deficient patients showed no increase in Tfh cells (2.2% ± 0.7%) (Figure 6, B and C). Circulating Tfh cells in AID-deficient patients resembled tonsillar counterparts because of increased ICOS expression (Figure 6, B and C). Tfh cells were most prevalent in the blood of patients with the lowest frequencies of Tregs (Figure 6D), an inverse correlation previously observed in CVID patients (16). Hence, *AICDA* mutations correlate with elevated frequencies of circulating Tfh cells that

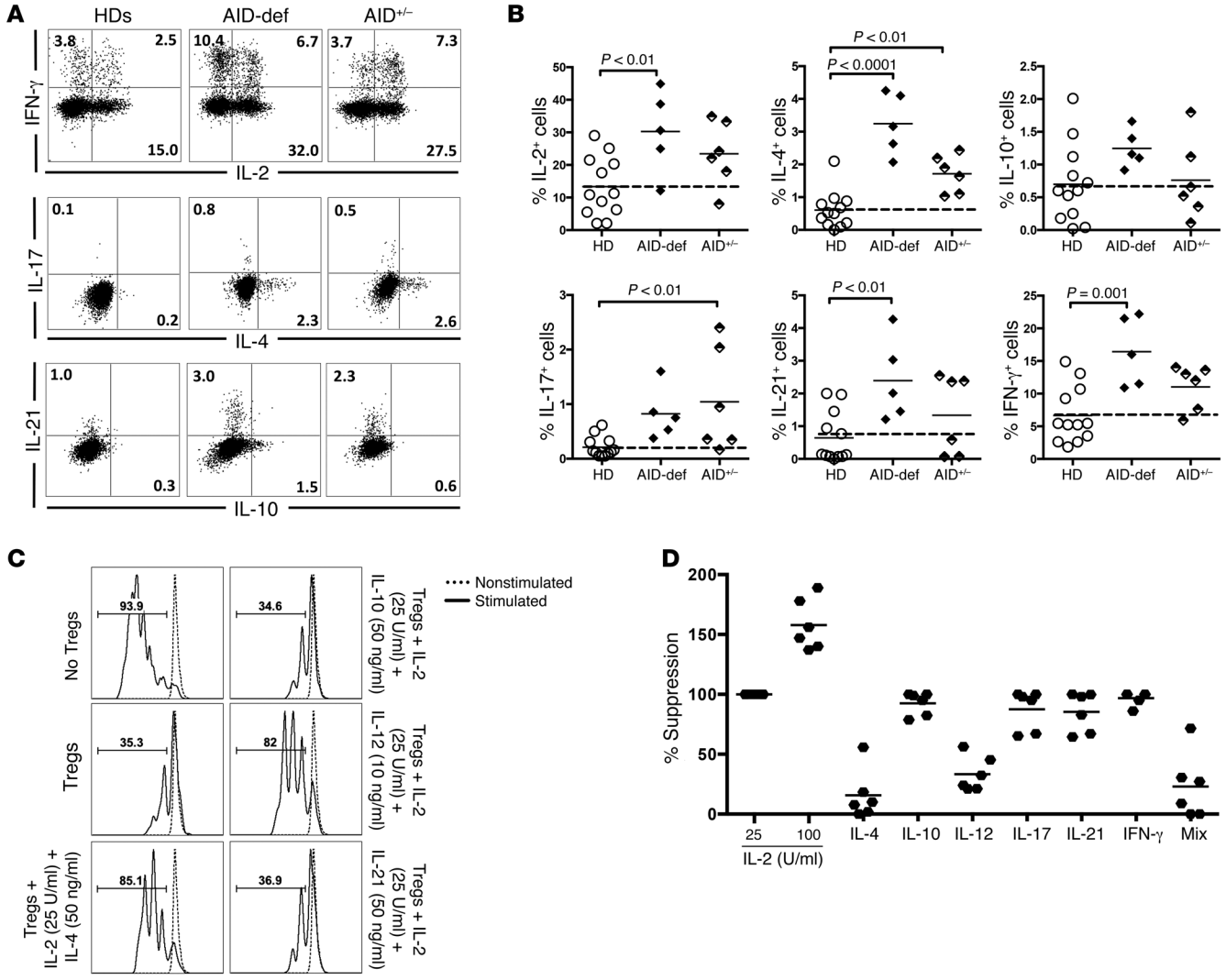


Figure 5. Increased cytokine production by Tresps interferes with proper Treg suppression in all individuals carrying *AICDA* mutations. (**A**) Representative dot plots of IL-2$^+$, IL-4$^+$, IL-10$^+$, IL-17$^+$, IL-21$^+$, and IFN-γ$^+$ CD4$^+$ T cells directly ex vivo after 4 hours of PMA/ionomycin stimulation of CD4$^+$ T cells from HDs (n = 12), AID-deficient patients (n = 5), and AID$^{+/-}$ patients (n = 6). Data are summarized in **B**, where bars indicate the mean and dashed lines the mean for HDs. P values in **B** were determined by ANOVA with Dunnett's correction for multiple comparisons. (**C**) Representative histograms of Tregs preincubated 4 days with or without the addition of IL-2, IL-4, IL-10, IL-12, or IL-21 prior to coculture with freshly isolated autologous, CFSE-labeled Tresps. Dashed lines show nonstimulated Tresps. Data are summarized in **D** (n = 6).

express ICOS and may lead to a breach in B cell tolerance and the production of serum IgM autoantibodies in AID-deficient patients who display the highest elevation in Tfh cell numbers.

Altered SHM in subjects with AICDA mutation(s) correlates with their defective peripheral B cell tolerance checkpoint. The lack of CSR involvement in establishing the peripheral B cell tolerance checkpoint suggested a potential role for SHM in the regulation of this selection step. However, examination of IgM $V_{H/5\text{-}51}$ in total CD27$^+$ B cells and $V_{H/3\text{-}23}$ rearrangements in purified IgM$^+$CD27$^+$ memory B cells revealed normal SHM frequencies in asymptomatic AID$^{+/-}$ individuals and AD-AID patients (10). This observation contrasted with the decreased induction of SHM in B cells from AID$^{+/-}$ mice, in which AID expression was decreased compared with that observed in their WT counterparts (26, 27). Since we recently observed a similar decrease in expression of AID protein in EBV cell lines and activated B cells from AID$^{+/-}$ individuals (28), we assessed the incidence of SHM in AD-AID patients and AID$^{+/-}$ subjects by analyzing heavy chain (*IGH*) gene sequences amplified from single CD27$^+$IgM$^+$ and CD27$^+$IgG$^+$ memory B cells and compared them with the sequences amplified from B cell counterparts from HDs as well as AID-deficient and UNG-deficient patients. We found that CD27$^+$IgM$^+$ B cells from HDs showed, on average, 10.8 ± 6.8 mutations in their *IGH* genes, as previously reported for different donor controls (Figure 7A) (29). CD27$^+$IgM$^+$ B cells from AID$^{+/-}$ individuals and a UNG-deficient patient harbored mutation numbers that were similar to those in HDs, whereas CD27$^+$IgM$^+$ B cells from AID-deficient patients were devoid of SHM, as expected (Figure 7A). In contrast, CD27$^+$IgM$^+$ B cells from AD-AID patients showed decreased SHM frequencies, with only 3.9 ± 4.3 mutations, on average, in *IGH* genes (Figure 7A). CD27$^+$IgG$^+$ memory B cells from AID$^{+/-}$ subjects displayed significantly decreased SHM in their *IgH* genes, with the average only reaching 16.5 ± 7.8 mutations com-

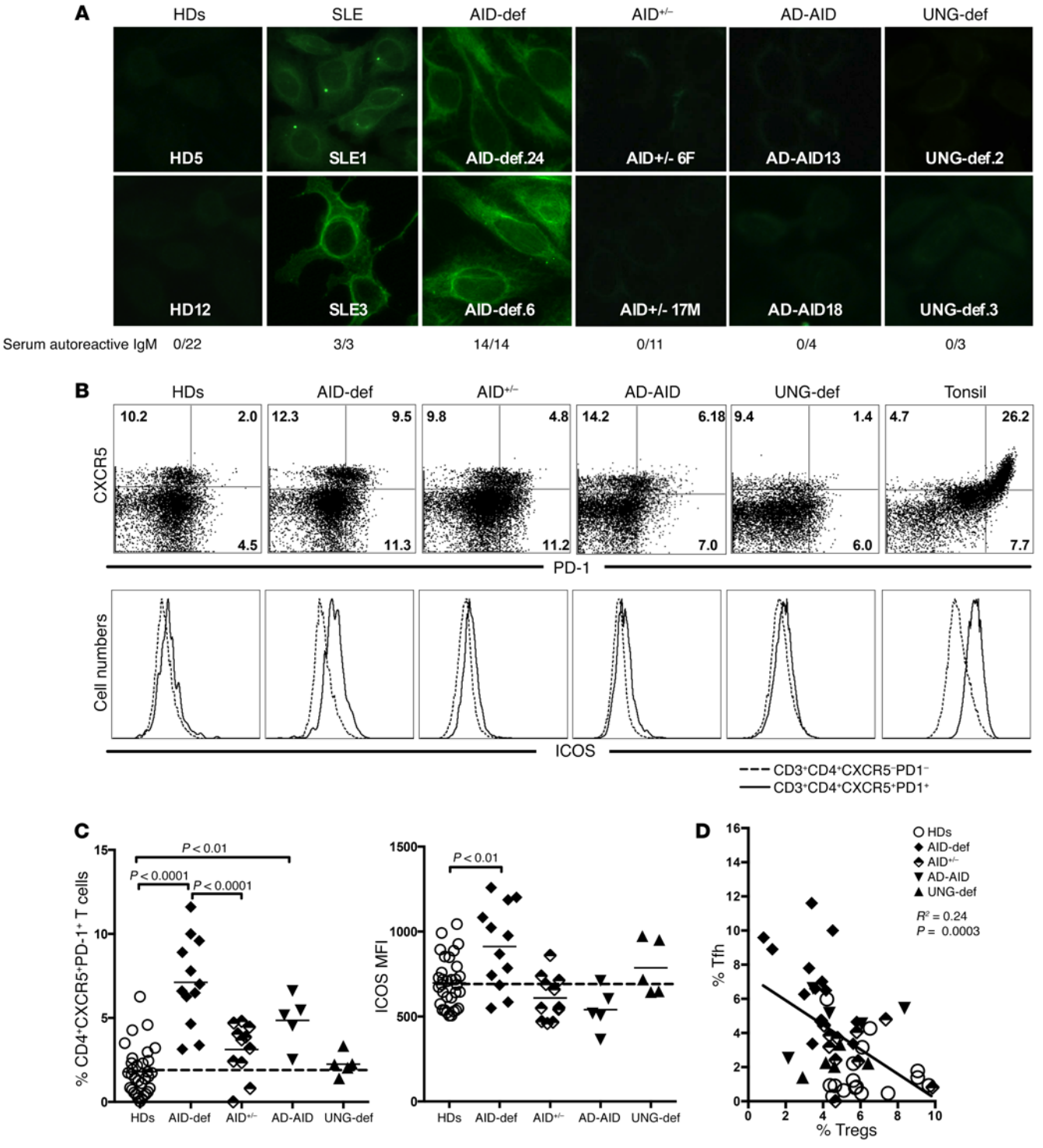


Figure 6. B cell tolerance is further breached in AID-deficient patients and correlates with the presence of circulating Tfh cells. (**A**) IgM autoantibodies and ANAs with diverse staining patterns were only present in SLE and AID-deficient patients. Serum dilution: 1:80. (**B**) Representative dot plots of PD-1 and CXCR5 staining on $CD3^+CD4^+$ cells and histograms of ICOS expression on $CD3^+CD4^+CXCR5^-PD\text{-}1^-$ T cells (dashed line) compared with $CD3^+CD4^+CXCR5^+PD\text{-}1^+$ T cells (solid line) in all groups. (**C**) Summary of data comparing the frequency of circulating $CD3^+CD4^+CXCR5^+PD\text{-}1^+$ cells and the mean fluorescence intensity (MFI) of ICOS in HDs (n = 32) versus AID-deficient patients (n = 11), $AID^{+/-}$ subjects (n = 10), AD-AID patients (n = 5), and UNG-deficient patients (n = 5). Bars indicate the mean; dashed line indicates the mean for HDs as a comparison. (**D**) The frequency of circulating Tfh cells inversely correlated with the frequency of circulating Tregs in all subjects. Line was obtained by linear regression analysis. P values were determined by ANOVA with Dunnett's correction for multiple comparisons.

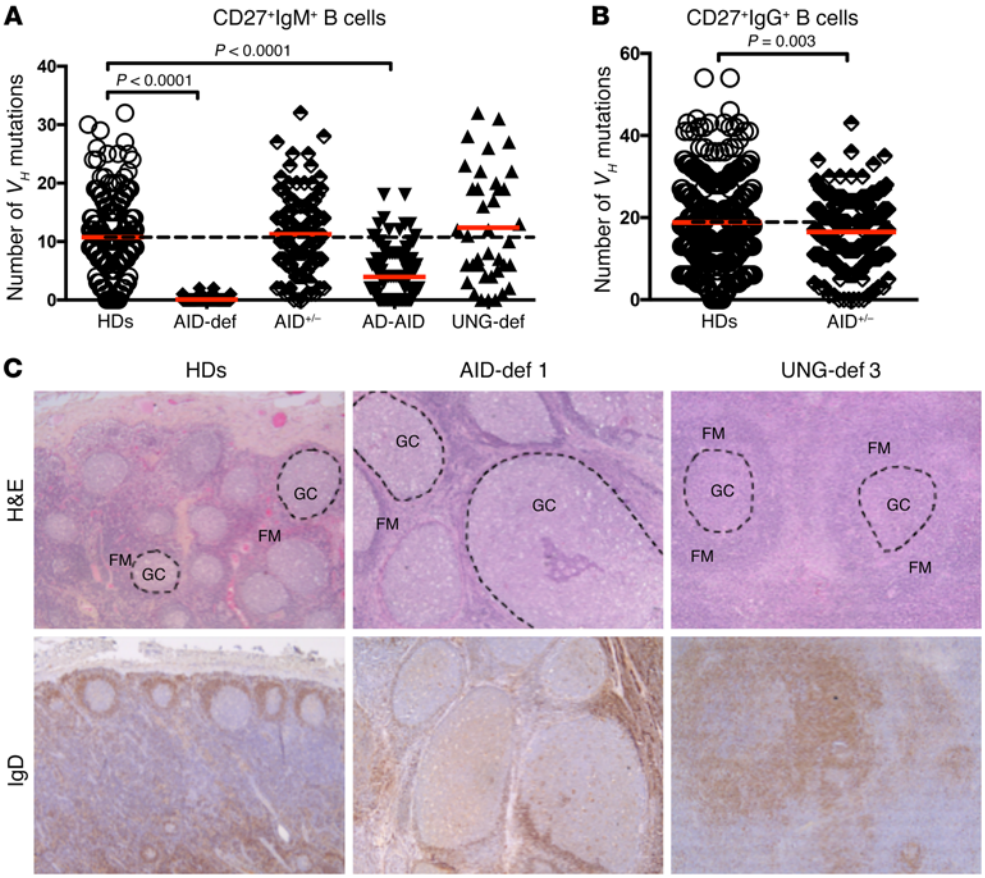


Figure 7. Decreased SHM frequencies in memory B cells from subjects with *AICDA* gene mutation(s). (**A**) Decreased SHM in IgM$^+$ memory B cells from AD-AID patients. The number of mutations evaluated in V_H sequences derived from single CD27$^+$IgM$^+$ B cells from HDs (n = 16), AID-deficient patients (n = 5), AID$^{+/-}$ subjects (n = 4), AD-AID patients (n = 4), and 1 UNG-deficient patient is shown. P values were determined by ANOVA with Dunnett's correction for multiple comparisons. (**B**) Decreased SHM in IgG$^+$ memory B cells from AID$^{+/-}$ subjects. The number of mutations in V_H sequences from single CD27$^+$IgG$^+$ B cells from HDs (n = 15) and AID$^{+/-}$ healthy subjects (n = 7) is shown. Each sequence is depicted by a symbol, and bars represent the mean. P values were determined by an unpaired, 2-tailed Student's t test. (**C**) Left: Control reactive cervical lymph node with lymphoid follicles composed of normal-sized GCs and a well-defined follicular mantle (FM). Middle: cervical lymph node biopsies from AID-deficient patient 1 showing lymphoid follicular hyperplasia with giant GCs and a characteristic reduced FM. Right: cervical lymph node biopsy from UNG-deficient patient 3 with lymphoid follicle hyperplasia with ill-defined GC separation from the normal-sized mantle zone. Original magnification, ×50.

pared with 18.8 ± 9.8 mutations in the *IgH* genes of HDs (P = 0.003, Figure 7B) (30). Surprisingly, IgG antibodies encoded by $V_{H/3\text{-}23}$ genes did not follow this trend and displayed similar SHM averages between HDs and AID$^{+/-}$ heterozygotes, as previously reported for mutated IgM sequences in these subjects (Supplemental Figure 6) (10). Finally, lack of SHM in AID-deficient patients was found to be associated with enlarged germinal centers (GCs) (Figure 7C) (31). We could not assess GCs from asymptomatic AID$^{+/-}$ subjects or AD-AID patients, but we managed to obtain a lymph node biopsy from a UNG-deficient patient that revealed normal-sized GCs, a finding consistent with the normal SHM responses identified in the CD27$^+$IgM$^+$ memory B cells (Figure 7A). However, the morphology of GCs from the UNG-deficient patient was not completely normal; GCs showed ill-defined borders and appeared to include some IgD$^+$ B cells (Figure 7C and data not shown). We conclude that both AID$^{+/-}$ individuals and AD-AID patients show decreased SHM in CD27$^+$IgG$^+$ and CD27$^+$IgM$^+$ B cells, respectively, suggesting that SHM defects are associated with an impaired peripheral B cell tolerance checkpoint.

Discussion

We report here that impaired SHM resulting from various *AICDA* mutations interferes with the establishment of the peripheral B cell tolerance checkpoint and correlates with defective Treg suppression, increased numbers of circulating Tfh cells, and enhanced cytokine secretion. Indeed, SHM-defective, CSR-defective patients with *AICDA* gene mutation(s) had elevated frequencies of autoreactive mature naive B cells, whereas SHM-competent, CSR-defective UNG-deficient patients showed a functional peripheral B cell tolerance checkpoint, revealing that SHM is the AID-mediated mechanism that appears to prevent the accumulation of autoreactive mature naive B cells.

Could an impaired counterselection of autoreactive immature B cells in the bone marrow be responsible for the defective peripheral B cell tolerance checkpoint in individuals with *AICDA* mutation(s)? Indeed, *Aicda*-KO mice and AID-deficient patients as well as asymptomatic AID$^{+/-}$ individuals display an impaired central B cell tolerance, resulting in the production of large numbers of autoreactive B cells that migrate from the bone marrow into the periphery (8, 28, 32). However, we previously reported that autoreactive clones can be prevented from colonizing the mature naive B cell compartment when Tregs are functional, despite the impaired removal of developing autoreactive immature B cells in the bone marrow (16). In addition, AD-AID patients show only a defective peripheral B cell tolerance checkpoint, since their central B cell tolerance was found to be functional, thereby excluding a contribution from the bone marrow to peripheral selection impediments in subjects with *AICDA* gene mutation(s) (28). For those reasons, we sought to determine which factors control the peripheral B cell tolerance checkpoint that is defective when SHM is impaired.

Decreased Treg numbers or function and elevated BAFF concentrations have both been reported to be associated with a dysregulated peripheral B cell tolerance checkpoint (2, 14, 16, 18, 20). Elevated serum BAFF concentrations can potentially impact the peripheral checkpoint due to the ability of BAFF to rescue autoreactive B cells from cell death in mice (33, 34). However, the

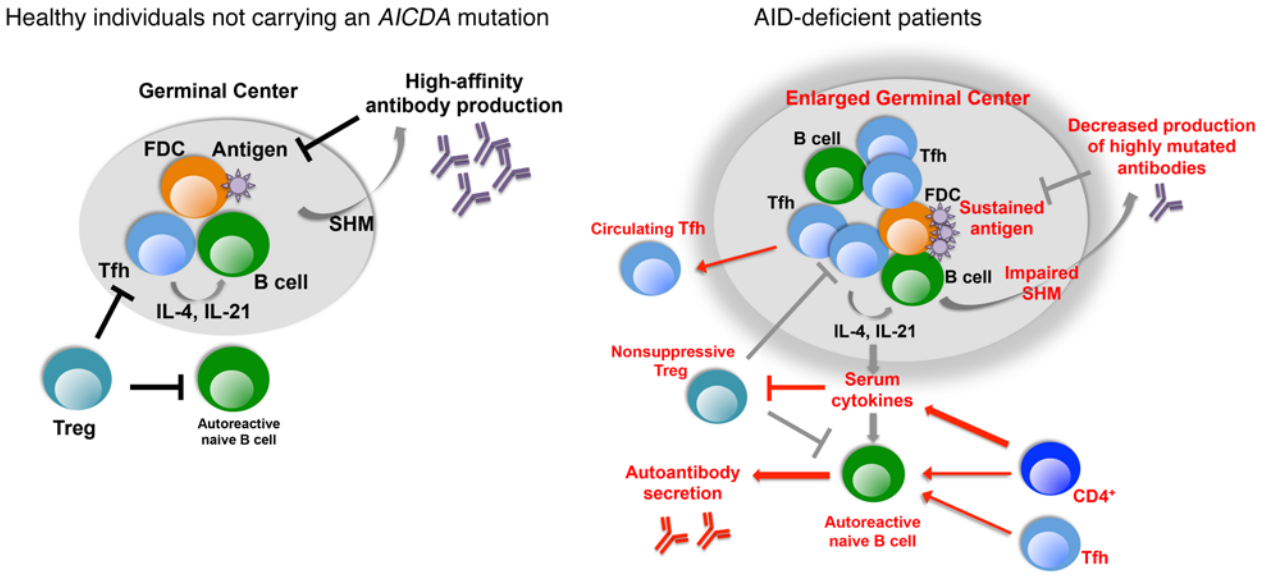


Figure 8. Disruption of the peripheral B cell tolerance checkpoint by untamed GC reactions. Decreased SHM processes result in a reduced efficiency at generating highly mutated specific antibodies, potentially leading to enhanced GC reactions, increased Tfh cell production, and cytokine secretion. These cytokines interfere with Treg suppressive function and potentially result in a defective peripheral B cell tolerance checkpoint. This impaired selection step allows the accumulation of large numbers of autoreactive B cells in the periphery that could be activated by Tfh cells and systemic cytokines that support not only autoantibody secretion but potentially the development of autoimmune manifestations.

impaired peripheral B cell tolerance checkpoint in asymptomatic AID$^{+/-}$ individuals as well as in FOXP3-, SAP-, and DOCK8-deficient patients, all of whom display normal serum BAFF concentrations, suggests that the regulation of this checkpoint is independent of BAFF (2, 15). A functional peripheral B cell tolerance checkpoint in UNG-deficient patients further reveals that a 2- to 3-fold elevation in serum BAFF concentration does not induce defects in peripheral B cell selection. Hence, anti-BAFF therapy, which has shown some efficacy in systemic lupus erythematosus (SLE), may not improve the condition of these patients by restoring their impaired counterselection of autoreactive B cells (35–37). The elevated serum BAFF concentrations in patients with CSR-D as well as in many CVID patients correlate, rather, with decreased memory B cell numbers (16, 38, 39). Interestingly, many patients with autoimmune diseases such as SLE or rheumatoid arthritis who show elevated serum BAFF concentrations have been reported to have decreased circulating memory B cells in their blood (40–43). Compared with naive B cells, memory B cells that express increased cell-surface transmembrane activator and CAML interactor (TACI), another BAFF receptor, may constitute a sink for serum BAFF in humans. In agreement with this hypothesis is the absence of elevated serum BAFF concentrations in *Aicda*-KO mice, which correlates with the very low proportion of memory B cells among total B cells in mice compared with humans (32, 44).

Circulating mature naive B cells contain many autoreactive clones in FOXP3-deficient patients who lack functional Tregs, suggesting a role for Tregs in controlling the peripheral B cell tolerance checkpoint (2). Moreover, the accumulation of autoreactive mature naive B cells in DOCK8-deficient, Wiskott-Aldrich, and CVID patients correlates with abnormal Treg suppressive function (2, 16, 18, 20). Our current finding of impaired Treg suppressive function in individuals with *AICDA* mutation(s) who show defective peripheral B cell selection further supports a major role for Tregs in regulating this checkpoint. How can *AICDA* mutations affect Treg function? Since T cells do not express AID, the defective suppressive ability of Tregs from AID-deficient patients and asymptomatic AID$^{+/-}$ subjects should be the consequence of extrinsic dysregulated factors. In line with this hypothesis is the overexpression of many cytokines such as IL-4 and IL-21 by CD4$^+$ T cells from subjects with *AICDA* mutation(s) that inhibit Treg suppressive function (15). Other cytokines such as IL-6 and IL-12 produced by other cell types and known to alter Treg suppression were also elevated in the serum of individuals carrying recessive and dominant *AICDA* mutations, respectively (15, 22). In addition, we discovered that, of the cytokines suspected to alter Treg suppression, IL-12 and IL-4, which are key cytokines for Th1 and Th2 responses, respectively, had inhibitory effects on Treg function upon pre-exposure. We hypothesize that IL-12 and IL-4 secretions during Th1, Th2, and Tfh responses not only polarize T cell phenotype and function but also inhibit Tregs, thereby allowing autoimmune responses to proceed. Moreover, CD4$^+$ T cells from AID-deficient patients were refractory to Treg suppression, a feature also identified in SAP-deficient patients who also had hyperactive CD4$^+$ T cells secreting many cytokines and a defective peripheral B cell tolerance checkpoint (15).

The altered CD4$^+$ T cell compartment in subjects with *AICDA* mutation(s) also included an increase in circulating Tfh cells; Tfh cells, in conjunction with elevated serum IL-21 and other cytokines, in AID-deficient patients may allow the activation of autoreactive B cells, leading to the secretion of unmutated ANAs (45, 46). A similar scenario was observed in many CVID patients who had increased frequencies of Tfh cells, together with enhanced

production of IL-4 production and serum ANAs, all of which correlated with decreased Treg frequencies, an impaired peripheral B cell tolerance checkpoint, and autoimmune manifestations (16). In contrast, AID$^{+/-}$ heterozygotes and AD-AID patients who show some AID activity had a milder phenotype than did AID-deficient patients, as illustrated by an absence of serum ANAs, suggesting that peripheral tolerance is not breached, despite an abnormal peripheral B cell tolerance checkpoint. These observations are not paradoxical, because it is unlikely that autoreactive mature naive B cells from AID$^{+/-}$ heterozygotes and AD-AID patients secrete the antibodies expressed on their cell surface in the absence of T cell help, since these individuals only showed a modest increase in Tfh cells and serum cytokines that may not be sufficient to overrule other more downstream checkpoints preventing the activation of these self-reactive clones and production of autoantibodies. In line with this hypothesis, none of these individuals has been reported to suffer from autoimmune conditions. Hence, elevated Tfh cells with increased ICOS expression and serum cytokines, in combination with decreased Treg frequency and function, may favor autoreactive B cell activation and ANA production.

How do *AICDA* mutation(s) result in increased circulating Tfh cells? It has previously been reported that AID deficiency results in enlarged GCs in both mice and humans (5, 6). Tfh cells are normally found in GCs, where they activate B cells through cell-cell interactions and production of cytokines including IL-4 and IL-21 (47). Tfh cells induce AID expression in GC B cells, leading to the initiation of SHM that ultimately increases antibody affinity through antigen selection during immune responses (48–50). The production of mutated high-affinity antibodies was recently shown to downregulate GC reactions, probably by eliminating specific antigens on follicular DCs (FDCs) (51). The presence of high-affinity IgG$^+$ memory B cells that can rapidly differentiate into plasma cells secreting large amounts of specific antibodies also inhibits GC formation, further suggesting that high-affinity antibodies generated by SHM play an important role in controlling GC development (52). A failure to induce SHM, and probably to generate high-affinity–specific antibodies in AID-deficient patients, may therefore lead to sustained antigen expression on FDCs in GCs, leading to prolonged B and T cell activation and proliferation as well as to increased production of Tfh cells and secretion of their cytokines, namely IL-4, IL-10, and IL-21, which can all be found in the blood of AID-deficient patients (24, 25) (Figure 8). In addition, increased resistance to apoptosis in AID-deficient B cells may further support an enhanced GC reaction (32, 53). This model is corroborated by observations in mice carrying a knockin point mutation in *AICDA*, which decreases the SHM activity of AID without altering CSR function (54). GC B cell hyperplasia in gut lymphoid tissue was observed in these mice and disappeared after antibiotic treatment, similar to what was observed in *Aicda*$^{-/-}$ mice (55). Hence, loss of SHM independently of CSR defects leads to increased GC formation associated with the inability to control microbiota. This scenario may also apply to both AID$^{+/-}$ subjects and AD-AID patients, because these subjects showed decreased SHM in their IgG$^+$ and IgM$^+$ memory B cells, respectively. Altered SHM processes in these individuals had initially been missed because of the small numbers of mutated sequences obtained from the amplification of $V_{H/5\text{-}51}$ and $V_{H/3\text{-}23}$ genes (10). However, our more global and unbiased approach revealed lower SHM frequencies in single IgG$^+$ memory B cells isolated from asymptomatic AID$^{+/-}$ subjects, a feature correlating with decreased AID expression in their B cells upon activation (28). These data are also in agreement with the identification of increased microhomology sequences in switched regions of AID$^{+/-}$ memory B cells, a characteristic associated with less efficient CSR through decreased AID activity (56). Dominant *AICDA* mutations encoding hyperactive AID variants that cannot catalyze CSR also resulted in decreased SHM frequencies in IgM$^+$ memory/marginal zone B cells (57). This paradoxical observation associating hyperactive AID enzyme activity with lower SHM may be reconciled with the possibility that hyperactive AID activity may induce exacerbated DNA lesions in GC B cells, causing B cells that attempt many rounds of antibody gene mutations to undergo apoptosis and be eliminated. Finally, 1 UNG-deficient patient who lacked CSR but had normal SHM frequency displayed GCs similar in size to those in the HD counterparts, further suggesting that SHM regulates GC responses.

In summary, our analysis of individuals with *AICDA* mutations revealed that decreased SHM processes lead to enhanced GC reactions and increased Tfh cell production and cytokine secretion, all of which likely result from the reduced efficiency and delayed kinetics of generating highly mutated antibodies that eliminate antigens from FDCs (Figure 8). These cytokines affect the polarization and composition of T cells in these patients and alter Treg function, which further results in a defective peripheral B cell tolerance checkpoint. This impaired selection step allows the accumulation of large numbers of autoreactive naive B cells, which are then activated by abundant Tfh cells and systemic cytokines, not only supporting ANA secretion but also the development of autoimmune manifestations. Hence, antibody replacement therapy in immunodeficient patients may be beneficial by providing mutated antibodies that dampen these abnormal immune features.

Methods

Patients and healthy donor controls. We obtained peripheral blood or frozen PBMCs from 21 AID-deficient patients with homozygous or compound heterozygous autosomal recessive *AICDA* mutations, 11 of whom were described previously (8). In addition, we collected samples from 16 healthy subjects related to these patients, who carried 1 mutated *AICDA* allele, as well as from 5 AD-AID patients and 5 UNG-deficient patients. Because of the lack of fresh PBMCs and the limited amount of frozen PBMCs or serum from these very rare patients, some experiments could not be performed on all samples. All patients' information is included in Supplemental Table 1. Age-matched HDs were previously reported (2, 8, 15, 16, 19, 20, 40).

Cell staining and sorting. Peripheral B cells from the blood of patients and control donors were purified by positive selection using CD20-magentic beads (Miltenyi Biotec). Enriched B cells were stained with the following antibodies: anti–IgM-FITC (G20-127), anti–IgG-PE (G18-145), and anti–CD21-APC (clone B-ly4) from BD Biosciences; and anti–CD19–Pacific Blue (clone HIB19), anti–CD27-PercP-Cy5.5 (clone M-T271), and anti–CD10-PE-Cy7 (clone HI10a) from BioLegend. Single CD19$^+$CD21^{lo}CD10$^+$IgM^{hi}CD27$^-$ new emigrant/transitional and CD19$^+$CD21$^+$CD10$^-$IgM$^+$CD27$^-$ mature naive B cells were sorted on a FACSAria Flow Cytometer (BD) into 96-well PCR plates and immediately frozen on dry ice. The following antibodies were used for T cell phenotyping: anti–CD4 APC-Cy7 (clone OKT4), anti–CD25

PE-Cy7 (clone BC96), anti–CD127 PerCP-Cy5.5 (clone A019D5), anti–CXCR5 PerCP-Cy5.5 (clone J252D4), anti–PD-1 PE-Cy7 (clone EH12.2H7), and anti–ICOS APC (clone C398.4A) (all from BioLegend); anti–CD3 eFluor 605NC (clone OKT3; eBioscience); and anti–HLA-DR APC (G46-6; BD Biosciences). Intracellular staining with anti–FOXP3 Alexa Fluor 488 (clone PCH101; eBioscience) and anti–Ki-67 PE (clone Ki-67; BioLegend) was performed using the FOXP3/Transcription Factor Staining Buffer Set in accordance with the manufacturer's instructions (eBioscience).

cDNA, RT-PCR, antibody production and purification, ELISAs, and indirect immunofluorescence assays. Reverse transcription-PCR reactions, primer sequences, cloning strategy, expression vectors, recombinant antibody expression, purification, and reactivity determination were described previously (1). For indirect immunofluorescence assays (IFAs), HEp-2 cell–coated slides (Bion Enterprises Ltd.) were incubated in a moist chamber at room temperature with 1:80 and 1:320 diluted sera. FITC-conjugated monoclonal mouse anti-human IgM (Pharmingen, BD) was used as a detection reagent. For detection of cytokine transcripts, RNA was extracted from sorted $CD4^+$ T cells using the Absolutely RNA Microprep Kit (Agilent Technologies), and 150 ng RNA was reverse transcribed using random hexamers (Applied Biosystems) and a SuperScript III Reverse Transcriptase Kit (Invitrogen, Thermo Fisher Scientific). The resulting cDNA was amplified in duplicate using Brilliant SYBR Green QPCR Master Mix (Agilent Technologies) and the Stratagene MX3005 real time detection system and normalized to CD4 for each sample before comparisons. Primer sets are summarized in Supplemental Table 2. Values are represented as the difference in Ct values normalized to *HPRT1* for each sample.

Repertoire analysis. Ig sequences and mutation status were analyzed by Ig BLAST comparison with GenBank using the NCBI's IgBLAST server (http://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 V_H framework 3 and the conserved tryptophan at position 103 in JH segments. Sequences are shown in Supplemental Tables 3–16.

KREC assay. The ratio of KREC joints (signal joint) to the Jκ-Cκ recombination genomic joints (coding joint) was determined as previously described (58). In brief, genomic DNA was isolated from sorted B cell fractions by lysing cell pellets in 10 mM Tris-HCl, pH 8.0, containing 100 μg/ml proteinase K (Roche), incubating for 1 hour at 56°C, and heat inactivating the enzyme at 95°C for 10 minutes. Two separate RT-PCR reactions were performed, one reaction to amplify the signal joint and another to amplify the coding joint, as previously detailed (58). The number of cell divisions was calculated by subtracting the Ct of the PCR detecting the coding joint from that detecting the signal joint.

In vitro Treg suppression assay. $CD4^+$ T cells were enriched using the EasySep Human $CD4^+$ T Cell Enrichment Kit (STEMCELL Technologies). $CD4^+CD25^{hi}CD127^{lo/-}$ Tregs were sorted by flow cytometry, whereas $CD3^+CD4^+CD25^-$ Tresps were obtained after the depletion of $CD25^+$ cells with anti-human CD25 microbeads (Miltenyi Biotec) and then labeled with CellTrace CFSE (InvivoGen) at 5 μM. Tregs and Tresps were cocultured at a 1:1 ratio in the presence of beads loaded with anti-CD2, anti-CD3, and anti-CD28 (Treg Suppression Inspector, human; Miltenyi Biotec) at a 1 bead/1 cell ratio, with or without the addition of cytokines at 50 ng/ml concentration (Peprotech). On days 3.5 to 4.5, cocultures were stained for viability with the LIVE/DEAD Kit (Invitrogen, Thermo Fisher Scientific), and proliferation of viable Tresps was analyzed by CFSE dilution. For cytokine preincubation experiments, Treg populations were stimulated with 1 μg/ml plate-bound anti-CD3, 1 μg/ml soluble anti-CD28, and 25 U/ml IL-2 in the presence or absence of 50 ng/ml IL-4, IL-10, IL-17, IL-21, and IFN-γ, 10 ng/ml IL-12, or 100 U/ml IL-2 for 4 days, washed twice, counted, and added to freshly isolated CFSE-labeled Tresps.

Cytokine detection. Plasma or serum BAFF concentrations were determined by ELISA according to the manufacturer's instructions (R&D Systems). Cytokine plasma concentrations were measured with the High-Sensitivity Human Cytokine Magnetic Bead Kit (EMD Millipore) using a Luminex 200. IL-21 was measured by ELISA with the Human IL-21 ELISA Ready-Set-Go Kit (eBioscience). IL-17A was measured with the Duoset ELISA Kit (R&D Systems). For intracellular cytokine detection, PBMCs were stimulated with 30 nM PMA and 200 nM ionomycin for 4 hours in the presence of GolgiStop (BD Biosciences), and intracellular staining of cytokines was performed using FOXP3 staining buffers (eBioscience) and the following antibodies: anti–IFN-γ PE (clone 4S.B3; eBioscience); anti–IL-10 PE (clone JES319F1; BD Biosciences); anti–IL-4 PE (clone MP4-25D2), anti–IL-2 Alexa Fluor 647 (clone MQ17H12), anti–IL-17A Alexa Fluor 647 (clone BL168), and anti–IL-21 APC (clone 3A3N2) from BioLegend.

Statistics. Statistical analysis was performed using GraphPad Prism (version 5.0; GraphPad). Data are reported as the mean ± SD. Differences between groups of research subjects were analyzed for statistical significance using either ANOVA with Dunnett's correction for testing multiple comparisons or unpaired, 2-tailed Student's *t* tests. A P value of 0.05 or less was considered significant.

Study approval. The human studies were reviewed and approved by the IRB of Yale University. All subjects provided informed consent prior to their participation in the study.

Author contributions

TC, JNS, TK, LM, and EM designed the experiments and discussed the data with AL, JEW, LDN, WAH, SSK, HDO, CCR, MVDB, TWK, SK, HK, YS, SN, and AD, who provided patients' samples and valuable research suggestions. TC, JNS, JMB, YSN, CM, FRD, NY, SG, NC, TK, LM, and JB performed the experiments, with help from EM for cell isolation and sorting. TC and EM wrote the manuscript.

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