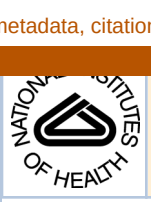

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Naturally occurring mutation affecting the MyD88-binding site of *TNFRSF13B* impairs triggering of class switch recombination

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Abstract

Mutations in the transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI) were previously found to be associated with hypogammaglobulinemia in humans. It has been shown that proliferation inducing ligand (APRIL) elicits class switch recombination (CSR) by inducing recruitment of MyD88 to a TACI highly conserved cytoplasmic domain (THC). We have identified a patient with hypogammaglobulinemia carrying a missense mutation (S231R) predicted to affect the THC. Aiming to evaluate the relevance of this novel mutation of TACI in CSR induction, we tested the ability of TACI, TLR9, or/and CD40 ligands to trigger CSR in naive B cells and B-cell lines carrying S231R. IgG secretion was impaired when triggered by TACI or/ and TLR9 ligands on S231R-naive B cells. Likewise, these stimuli induced less expression of activation-induced cytidine deaminase, *I*(*γ*)*1-C*(μ), and *I*(*γ*)*1-C*(μ), while induction by optimal CD40 stimulation was indistinguishable from controls. These cells also showed an impaired cooperation between TACI and TLR9 pathways, as well as a lack of APRIL-mediated enhancement of CD40 activation in suboptimal conditions. Finally, after APRIL ligation, S231R-mutated TACI failed to colocalize with MyD88. Collectively, these results highlight the requirement of an intact MyD88-binding site in TACI to trigger CSR.

Keywords

Class-switch-recombination; Common-variable-immunodeficiency; MyD88; TACI

Introduction

Antibody diversification is critical for the generation of immune protection [1]. B cells emerging from the bone marrow mature and pursue diversification of their Ig gene repertoire through two processes, known as somatic hypermutation and class switch recombination (CSR). Somatic hypermutation inserts point mutations into recombined $V_H(D)J_H$ and V_LJ_L exons, thus enabling the selection of high affinity Ig variants by antigen, whereas CSR replaces the heavy chain constant region (C_H) of IgM with that of IgG, IgA, or IgE and thereby provides new effector functions to the Ig, but preserves antigen specificity [2, 3]. A

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hallmark of CSR is the fact that actively transcribed S regions of the Ig gene become substrates of activation-induced cytidine deaminase (AID) [4].

In general, CSR has been shown to be triggered by a primary signal derived from the interaction between a receptor belonging to the superfamily members of TNF receptors (TNF-R) (e.g. CD40) expressed on the surface of B cells, with a cosignal from cytokines.

Transmembrane activator and calcium-modulating cyclophilin ligand interactor (TACI) is a TNF-R expressed on a fraction of B cells and binds to the TNF superfamily members B-cell activating factor (BAFF) and a proliferation inducing ligand (APRIL). BAFF is expressed primarily by neutrophils, monocytes, and DCs and promotes B-lymphocyte survival and maturation, while APRIL is expressed by monocytes, macrophages, DCs, and activated T cells [5,6]. TACI mediates isotype switching through binding of APRIL and synergizes with CD40 and TLR9 to increase Ig production and generation of plasma cells in vitro [7, 8].

Physiologically, the T cell-dependent (TD) pathway by which B cells become activated requires a long period of time to control rapidly replicating pathogens. To overcome this restriction, a T cell-independent (TI) pathway involving BAFF and APRIL in extrafollicular B cells is able to rapidly produce IgM, IgG, and IgA [9]. Innate immune cells release BAFF and APRIL due to microbial signals arising from TLRs and then activate NF-κB by recruiting TNF receptor-associated factors (TRAFs) through the receptor TACI [5, 10, 11].

Recently, it has been shown that BAFF and APRIL triggered CSR after recruitment of MyD88 to the THC domain of TACI; which was identified through the generation of E228R, T229R, S231R, and C233G substitutions by site-directed mutagenesis [12]. The toll-IL-1 receptor (TIR) domain of TLRs and the IL-1 receptor (IL-1R) activate transcription factors such as NF-κB through MyD88, thereby regulating innate immunity. However, BAFF and APRIL recruit MyD88 to a THC of TACI different from the TIR domain of TLRs. Impaired germline C_H gene transcription and AID activation after TACI stimulation were observed in both mice and humans lacking MyD88 or IL-1R-associated kinase 4 (IRAK-4), a signal transducer that binds MyD88 [12].

Mutations in *TNFRSF13B*, the gene encoding TACI, have been identified in 5 to 10% of patients with common variable immunodeficiency (CVID) [13, 14]. CVID is a heterogeneous primary immunodeficiency disease diagnosed on the basis of an impaired ability to produce specific antibodies after vaccination or antigen exposure, markedly reduced serum levels of IgG, IgA, and frequently IgM and exclusion of other defined causes of antibody deficiency (e.g. Hyper IgM syndromes, X-linked lymphoproliferative disorder) [15, 16].

In the present study, we identified and evaluated a missense mutation (S231R) predicted to affect the highly conserved segment of the THC domain of TACI at the MyD88-binding site, in a patient with childhood onset of CVID [17]. This patient constitutes the first CVID case associated with a mutation in this region of TACI, prompting us to evaluate its relevance through expression and functional studies.

Results

B cells expressing mutated TACI-S231R protein have impaired CSR processes

The mutation S231R has been reported in silico to affect the cytoplasmic conserved motif of TACI, crucial for the induction of CSR triggered by MyD88 engagement [12]. We identified a patient carrying this mutation and presenting CVID [17]. Blood $CD19^+$ B-cell counts were normal in the patient, while $CD27^+$ switched memory B cells were very low (2% versus 14

± 7% in age-matched controls). Virtually all $CD19^+$ $CD27^+$ B cells were IgM^+ IgD^+, pointing to a defective in vivo CSR. These results are in accordance with the serum Ig levels determined at onset and were typical of a CSR deficiency, with normal serum IgM levels and profoundly low serum IgG levels with an absence of IgA. To further investigate the in vivo mechanism of CSR in this patient, individual Sμ–Sα junctions were amplified from genomic DNA using the previously described nested-PCR assay [18]. Despite the extremely low level of serum IgA in the patient, the fragments could be amplified, even if the intensity of the fragments was much lower as compared with that of a healthy donor (Fig. 1A). However, these changes are modest compared with the few and faint bands amplified from a DNA ligase IV gene (LIG4)-deficient patient cells, where the major double-strand break DNA repair pathway in mammals (the non-homologous end joining machinery) is compromised (Fig. 1A). Residual IgA switching appears to occur in our S231R patient, although subsequent cloning and sequencing of these fragments (26 unique Sμ–Sα sequences) showed a majority of CSR junctions representing direct switching from IgM to IgA1, whereas in controls both IgM to IgA1 and IgM to IgA2 were observed (data not shown). When we compared the sequences from the patient with 54 unique Sμ–Sα junctions from a group of six healthy young individuals a slight preference of microhomology was observed (Fig. 1A). However, no significant difference was demonstrated by the two-tailed Student's *t*-test (perfectly matched sequence homology, on average 4.44 ± 3.80 nt in the patient versus 2.52 ± 4.45 nt in controls; $p = 0.0616$). This pattern of in vivo generated CSR junctions rules out that a significant defect in the DNA repair pathway could account for the low number of switched $CD27^+$ B cells.

In order to test the ability to trigger TACI-dependent class switching in the patient's B cells, we first investigated TACI expression and APRIL-binding capacity of TACI-S231R cells. TACI was similarly expressed in these cells than in controls (Fig. 1B and 1C) and its ability to bind APRIL seems to be within the range observed in healthy donors (Fig. 1D). Note that as previously shown, TACI can be expressed as a full-length protein or an alternatively spliced form lacking exon 2, whose physiological role remains undefined [19]. To evaluate CSR via TACI in vitro, we cultured naive B cells from the patient in the presence or absence of a cross-linking agonistic antibody to TACI (anti-TACI) combined with IL-4 or IL-10, which provides necessary CSR-inducing cosignals. The induction of CSR was evaluated by analyzing the secretion of IgG by ELISA (Fig. 2A). We observed that while in healthy donors' cells anti-TACI or anti-TACI plus cytokines augmented the secretion of IgG after 7 days of activation, a significantly attenuated increase was shown in TACI-S231R cells. It is interesting to note that the mother of the patient, a carrier of the same mutation [17], also showed a reduced secretion of IgG (Fig. 2A).

Given that the engagement of TLR9 by CpG DNA has been shown to enhance the secretion of IgG in B cells exposed to anti-TACI in the presence of activating cytokines [8, 11], we sought to investigate its effect in these naive B cells carrying the S231R mutation. After 7 days of exposure to anti-TACI, IL-4, and CpG DNA, B cells with the S231R mutation showed a pronounced impairment in the production of IgG (Fig. 2A). As previously shown in some patients with CVID [20, 21], TACI-S231R exhibited an impaired response to TLR9 on enhancing B-cell Ig production. This failure to respond to TLR9, which was not rescued by TACI ligation (Fig. 2A), raises the possibility of cross-talk between both signaling pathways.

In an attempt to localize the precise step of the CSR defect, we checked for the occurrence of CSR-related events by quantitative RT-PCR analysis of *Iγ1-Cγ1*, *Iγ1-Cμ*, and activation-induced cytidine deaminase *(AICDA*) transcripts in lymphoblastoid B cells obtained from the patient and her mother (Fig. 2B–D). Compared with control cells, these cells induced less *Iγ1-Cγ1* transcripts, an early marker of IgG1 CSR, in response to IL-4

and cognate TACI ligands such as APRIL (Fig. 2B). Switched *Iγ1-Cμ* transcripts and *AICDA* transcripts encoding AID, two hallmarks of ongoing IgG1 CSR, also showed little or no induction after 2 days of stimulation (Fig. 2C and 2D). Similar results were obtained through TACI engagement by APRIL or anti-TACI (Fig. 2). *AICDA* impairment was also detected in patients heterozygous for either of the two most common mutations in TACI found in CVID, C104R, and A181E (Fig. 2C). The C104R abolishes ligand binding and, consequently, signaling [13, 14]. The A181E mutation, which is in the transmembrane domain, does not affect ligand binding but severely impairs ligand signaling and TACI function in vitro and in vivo [22].

Synergy between TACI and CD40 signaling fails in TACI-S231R B cells

It has been suggested that in addition to its key role in the antibody response to TI antigens, TACI might be important for the antibody response to TD antigens [7, 8, 12]. In agreement with this, TACI engagement enhanced transcription of *AICDA* by healthy donor cells stimulated with suboptimal concentrations of anti-CD40 and IL-10 (Fig. 3A). In contrast, in lymphoblastoid B cells from TACI-S231R patient, APRIL ligation had no detectable effect on CD40 stimulation (Fig. 3A). It is important to note that CD40 engagement with optimal conditions for driving CSR was preserved in patient's cells (Fig. 3B–D), suggesting that S231R mutation on TACI specifically impaired the cooperation between TACI and CD40 signaling to induce CSR. Germline *IgE* transcripts were also normally expressed after optimal activation with CD40L and IL-4 (Fig. 3E).

Together, our results show that cells carrying the unique S231R change presented an impaired induction of CSR by TACI and TLR9 pathways and a lack of APRIL enhancement on CD40-driven suboptimal B-cell activation. These findings point to a defective in vivo CSR, as in vitro activation with cognate ligands of TACI in the presence of activating cytokines led to a poorly efficient CSR toward IgG, suggesting a defect-located upstream of the transcription step, because even IgG germline transcripts were greatly affected (Fig. 2B and Fig. 3B). Indeed, hallmarks of ongoing IgG1 synthesis, such as the expression of *Iγ1-Cγ1*, *Iγ1-Cμ*, and *AICDA* transcripts, were induced via CD40, ruling out an intrinsic deficiency in the CSR machinery (Fig. 3B–D). Since TACI was clearly expressed by TACI-S231R cells (Fig. 1B and C), these results appear to be specifically linked to a failure in the triggering of TACI signaling pathway.

B cells carrying mutated S231R-TACI protein are unable to recruit MyD88 protein

So far, our results suggest that B cells from TACI-S231R exposed to TACI and TLR9 or TACI and CD40 ligands fail to integrate signals from these receptors to achieve optimal antibody production. In all cases, intracellular signaling leads to the activation of NF-κB members [23], critical for *AICDA* induction [24]. It has been shown that TACI induces NF-κB activation by recruiting MyD88 to a THC domain of TACI [12]. Since the mutation S231R resides in the THC site, we evaluated the ability of APRIL to induce recruitment of MyD88 to TACI in unstimulated naive B cells carrying the S231R mutation, knowing that these cells express comparable levels of MyD88 with those found in healthy donor cells (Fig. 4A). After exposure to APRIL, confocal microscopy showed that healthy donor B cells developed large and polarized membrane patches of colocalized TACI and MyD88 (Fig. 4B). It is important to note that the control cells chosen in this experiment did not differ significantly from the patient's cells in the capacity to bind APRIL (Fig. 1D). As previously shown TRAF2, required to activate downstream signaling events, colocalizes with TACI after stimulation of donor cells with APRIL [12] (Fig. 4B). Although in B-cell lines a constitutive occupation of TACI by autocrine ligands complicate the demonstration of APRIL-induced recruitment of MyD88 to the receptor, the same outcome was observed by using lymphoblastoid B cells (Fig. 4B). However, in TACI-S231R naive B cells and

lymphoblastoid B cells from both the patient as well as from her mother, APRIL failed to induce such patches (Fig. 4B and data not shown). The impairment was specific of this mutation affecting the THC domain, since in B cells from a patient heterozygous for A181E the ability of APRIL to recruit MyD88 to TACI seems to be preserved (Fig. 4B). Previous results on this mutant have indicated that besides a failure in signaling, TACI-A181E properly binds to the ligand and engages in homotypic interaction [22, 25], now our findings also exclude an impaired MyD88 clustering underlying the lack of *AICDA* induction in response to TACI ligation (Fig. 2C and Fig. 4B).

Discussion

We have presented evidence in this study that B cells carrying the substitution S231R at the MyD88-binding site of TACI abrogated TACI-driven CSR induction, as well as IgG secretion by CpG stimulation. Furthermore, a lack of cooperation between the latter pathways was also evidenced beside a failure of APRIL-mediated enhancement of CD40 activation in suboptimal conditions. CD40 engagement initiates TD CSR, whereas dual TLR–BCR, TACI–BCR, or TLR–TACI engagement induces TI CSR, but also has an important role in the early stages of TD antibody responses [3]. Whether in a TD or TI manner, AID is required for CSR induction, and is differentially expressed depending on the B-cell lineage and the differentiation stage. AID is clearly expressed in B cells undergoing CSR but almost undetectable in resting mature B cells. Transcription of the *AICDA* gene depends on NF-κB, and translocation to the nucleus of members of the NF-κB family is a common event triggered by TLR9 and TACI engagement via MyD88 dimerization by TIR domain-dependent or -independent induction, respectively [12]. In this report, we showed that mutation S231R affecting the MyD88-binding site could prevent the association of this adaptor molecule with TACI, and this in a specific manner since the mutation A181E lying outside the THC domain retained the ability to interact with MyD88. Consistent with this interpretation, ectopic expression of TACI-S231R in 293T cells decreased the binding of MyD88 and attenuated TACI signaling via both NF-κB and AP-1 compared with that of WT-TACI [12]. Furthermore, some patients with MyD88 or with its downstream partner, IRAK4, deficiency showed lower IgG responses to TI antigens, and a poor induction of *AICDA* and IgG CSR in B cells stimulated with APRIL or CpG DNA [12, 26]. However, MyD88 and IRAK4 were not critical for the control of circulating IgG and IgA under steady-state conditions [26], introducing the possibility that TACI signals through TRAF2 to compensate for the lack of MyD88 or IRAK4. A canonical TRAF2-binding site lies immediately downstream to the MyD88-binding site in the cytoplasmic domain of TACI. It has been suggested that both adaptor molecules function in a cooperative manner, since only the deletion of both MyD88- and TRAF2-binding sites completely abrogated TACI signaling [12]. Here, we showed that naive B cells carrying TACI-S231R failed to colocalize TACI and TRAF2 after stimulation with APRIL, suggesting that the mutation could also prevent the association with TRAF2, probably due to the close proximity with its binding site (spanning amino acids 240–244).

TRAF2 is also a mediator in the signaling pathway of CD40 receptor. A synergy between TACI and CD40 ligation has been shown previously [7,8], while our data indicates that B cells with TACI-S231R failed to integrate signals from these receptors to achieve an enhancement of *AICDA* transcription as observed in B cells from healthy donors. Since activation of CD40 with adequate concentrations of ligand showed an intact cascade of CSR-related events, we can hypothesize that in a context of a suboptimal activation of CD40 it is necessary the cooperation with a TI pathway signaling such as TACI, to achieve an optimal level of TRAF2 activation and subsequent CSR. This cooperation could be impaired in our patient as we noted a failure of association between TACI and TRAF2.

The lack of APRIL enhancement on CD40-driven B-cell activation and the clear reduction of switched-memory B cells in our patient, allows us to speculate that this kind of cooperation could account for the generation of $CD27^+$ B cells in germinal centers. Follicular activated $CD4^+$ T cells and DCs are well-established sources of CD40 and TACI ligands, respectively, in germinal centers [27]. Thus, it would be expected that TACI carrying the S231R mutation would have an impaired cooperation between TACI and CD40 in a TD response.

One can also speculate that the mutation S231R rather than simply preventing an association with the intracellular adaptor proteins, directly affects the tertiary structure of the protein such that it impairs intracellular receptor association. This lack of association would therefore prevent fully functional signaling of TACI. No significant difference in TACI surface expression compared with controls was shown on peripheral B cells of the patient [17]. TACI activation by APRIL requires ligand multimerization, suggesting a requirement for receptor clustering or for a certain signaling threshold to be reached [28]. Activated TACI recruits TRAFs molecules intracellularly [19, 29], which are trimeric intermediates in the signaling pathway of numerous TNF-R family members [30]. Oligomerization of trimeric TRAF2 is required to activate downstream signaling events [31] being necessary a multimeric ligand to bring together more than three receptors, which will recruit to the complex the intracellular TRAF molecules. In this regard preliminary results, as part of an attempt to determine the structure of the cytoplasmic domain of TACI show a difference in the apparent molecular weight of the mutated protein versus the wild type, while MS results only evidences the gap due to the amino acid change (M. Almejun, unpublished data).

The S231R-patient evaluated in the current study is the first CVID case carrying a substitution affecting the THC domain of TACI at the MyD88-binding site. Following the identification of TACI as the defective gene in approximately 10% of patients with CVID, a number of studies have questioned its causative role in the pathogenesis of the observed humoral defects [13,14,32]. Among these, He et al. [12] have shown that TACI-ligands elicited CSR by inducing recruitment of MyD88 to the THC cytoplasmic domain of TACI.

Our data extend these results showing that S231R mutation attenuates TACI signaling and recruitment of MyD88, by providing cellular and biochemical evidence of an impaired CSR in B cells obtained from a patient carrying this mutation and presenting CVID with pediatric onset. This patient did not overtly have more severe symptoms than other patients described with other mutations in TACI, although the clinical manifestation of such mutations is broad and a missense mutation affecting the THC domain as observed here has not been previously reported. In this regard, it is striking that the impairment in triggering CSR via TACI was similarly observed in B cells from the mother of the patient, a carrier of S231R mutation but without clinical manifestations of CVID [17]. Heterozygous *TNFRSF13B* mutations have been previously associated with incomplete penetrance, therefore these heterozygous variants could increase the risk, but would not be sufficient to cause CVID [32]. A second event could be necessary for expressing the disease, as observed in patients with autoimmune lymphoproliferative syndrome who carry a somatic mutation in addition to that inherited, in another member of the TNF-R superfamily, *TNFRSF6* [33]. In conclusion, one of the greatest challenges in understanding the incomplete penetrance observed in these families would be achieved by exposing the additional environmental or/and genetic factors that act in concert with a heterozygous gene alteration on *TNFRSF13B* for the onset of CVID.

Materials and methods

Patient and controls

The patient was diagnosed with probable CVID according to the European Society for Immunodeficiencies (ESID) criteria at the age of 16.1 years. Clinical and immunological characteristics of the patient was recently published by our group [17]. Briefly, the onset with a clinical manifestation was reported at 4.9 years of age, showing autoimmune thrombocytopenia. This was followed by the development of recurrent upper respiratory tract infections (sinusitis) as well as recurrent pneumonia and, later on splenomegaly and autoimmune hemolytic anemia. At diagnosis, prior to IVIG substitution, decreased levels of IgG and IgA with elevated IgM were reported in circulation (IgG, 33 mg/dL; IgA, <7 mg/dL; IgM, 129 mg/dL). In order to discard hyper IgM (HIGM) syndrome, we evaluated *AICDA*, *CD40*, *uracil DNA glycosylase*, and *CD40L* genes and no mutations were found. The patient presented 15% (630 cells/mm^3) of $CD19^+$ B cells in total blood lymphocytes, while the percentage of $CD27^-IgD^+$ naïve B cells was 80%, within the normal range (74 ± 10%). The proportion of $CD27^+IgD^-$ switched memory B cells was highly decreased compared to healthy donors (2% of total B cells; normal range: 14 ± 7%).

Informed written consent was obtained from the patient prior to participation, in accordance with the Declaration of Helsinki. The research protocol was approved by the internal ethics review board of the Hospital Garrahan.

Age-matched healthy donors included in the study provided written consent under a separate ethics protocol for healthy donors.

Human cell isolation

PBMCs were isolated by density centrifugation over a Ficoll–Hypaque Plus (Amersham) gradient.

Total preswitched naïve IgD+ B cells were further purified from and enriched PBMC population as previously reported [5].

Cell cultures and reagents

Lymphoblastoid B-cell lines were generated by incubating PBMCs from the CVID patient or healthy donors in 1 mL B95/8 supernatant for 2 h at 37°C, then adjusting to a concentration of at least 1×10^6 cells/mL in medium containing 2 μg/mL cyclosporin A. Cultures were performed in complete RPMI medium supplemented with 10% v/v FBS (GIBCO). Cells were incubated with CD40L (PeproTech), 500 ng/mL; APRIL MegaLigand (Alexis), 500 ng/mL; IL-4 (Schering-Plough), 200 U/mL, and/or IL-10 (Peprotech), 50 ng/mL. TACI was cross-linked with 1 μg/mL mouse biotin-conjugated 11H3 mAb (eBioscience) and anti-biotin-microbeads (Miltenyi). A similar strategy was followed to cross-link CD40 with 1 μg/mL mouse 89 mAb (Schering-Plough). Mouse IgG1 mAb with irrelevant-binding activity (Santa Cruz Biotechnology) was used as control. TLR9 was activated with 5 μg/mL phosphorothioate-modified 5′-tcgtcgttttgtcgttttgtcgtt-3′oligodeoxynucleotide-2006 (Operon Technologies).

Characterization of switch recombination junctions

Genomic DNA was purified from peripheral blood cells from patients and healthy donors using standard methods. Amplification of Sμ–Sα from in vivo switched cells was performed using a previously described nested PCR assay [18]. The PCR-amplified switch fragments were gel purified (GE Healthcare), cloned into a pGEM-T vector (Invitrogen), and sequence analysis was performed with DNA Sequencing Analysis software (PE Applied Biosystems)

on ABI 3130 (Applied Biosystems). The CSR junctions were determined by aligning the switch fragment sequences with the Sμ (X54713) and Sα1 (L191219) or Sα2 (AF030305). Analysis of microhomology usage and mutation pattern at the CSR junctions was performed as described previously [18, 34].

Immunoblotting

Proteins separated by SDS–PAGE were transferred to PVDF membranes (Millipore), incubated with monoclonal mouse antibodies against TACI (eBiosciense), MyD88 (Millipore AB16527), or actin (Santa Cruz), and developed using secondary HRPO-conjugated antibodies from Southern Biotech and visualized with ECL substrate (GE Healthcare).

Ligand APRIL binding

Lymphoblastoid B-cell lines were cultured at 37°C in RPMI 1640 medium with L-glutamine and 10% heat-inactivated FCS in triplicates. To assess binding of the ligand APRIL, B cells were incubated with 250 ng/mL FLAG-tagged megaAPRIL (Axxora, San Diego, Calif) on ice in the presence of heparin (1000 U/mL). A 1 mg/mL biotinanti FLAG monoclonal M2 antibody (Sigma, St Louis, Mo) was further added. The cells were washed and incubated with streptavidin-PE. Resulting cultures were applied to a FACSCalibur (Becton Dickinson, Mountain View, Calif) and results analyzed with FlowJo.

Laser-scanning confocal microscopy

Cells were resuspended in Cell Adhesive Solution as instructed by the manufacturer (Crystalgen), then were applied to slides (Gold Seal Products), fixed with 1.6% v/v paraformaldehyde and made permeable with 0.2% v/v Triton X-100 in PBS. Cells were stained with the following unconjugated or biotin-conjugated primary polyclonal antibodies: goat anti-MyD88 (N-19), rabbit anti-TRAF2 (C-20), mouse anti-TACI (11H3; eBioscience). Negative controls were primary antibodies with irrelevant-binding activity and included biotin-conjugated F(ab′)2 polyclonal antibody and unconjugated goat, mouse or rabbit polyclonal antibodies (Santa Cruz Biotechnology). Secondary reagents included streptavidin, Alexa Fluor 488–conjugated anti-mouse (A21202), Alexa Fluor 546–conjugated anti-rabbit (A10040) or Alexa Fluor 647–conjugated anti-goat (A21447; all polyclonal antibodies from Molecular Probes). Nuclei were visualized with DAPI 4′,6-diamidine-2′-phenylindole dihydrocloride (Boehringer Mannheim). Slides were sealed with coverslides and FluorSave reagent (Calbiochem). Fluorescent images were generated with a Leica SP5 DMI upright confocal microscope (Wetzlar) through the acquisition of at least three different xy planes with a 63×objective lens with numerical aperture of 1.4 (Carl Zeiss) and with optimal *z* spacing (~0.016 μm). Views were constructed with maximum projection and were exported as 30–40 tagged image file format images. AutoQuant X2 AutoDeblur software (Media Cybernetics) was used for deconvolution of all images and restoration of detail to data sets. Adobe Photoshop software CS3 for Macintosh, version 10 (Adobe Systems) was used for further processing.

RT-PCR and quantitative RT-PCR

Quantification of human germline *Iγ1-Cγ1* transcripts, switch *Iγ1-Iγ2–Cμ* circle transcripts, *AICDA* transcript and *ACTB*, was performed by total RNA extraction from 2 or 4-days cultures with TRIzol (Invitrogen). cDNA was generated by reverse transcription with Superscript II RT (Invitrogen). PCR primers and conditions used for standard or quantitative RT-PCR analysis were done as reported [10, 35]. Results are normalized to *ACTB* mRNA and are presented as relative expression (RE) compared with that of B cells incubated with control antibody (Control).

Quantification of *TACI* and *DCLRE1C* were performed by total RNA extration from PBMCs, and cDNA was prepared using a first-strand cDNAs synthesis kit (Amersham Biosciences, UK) according to the manufacturer's instructions. PCR amplification of *TACI* was performed using primers and conditions as previously described [36]. *DCLRE1C* transcript was amplified using ART.cDNA-F 5′TCGAGGGGCAGATGGCCGAGTATC 3′and ART.cDNA-R 5′GACAGAGGTAGCTCAAGAAATCT 3′in a total volume of 40 μL and PCR conditions were: 5 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 60°C, 1 min at 72°C; the reaction was finished with a final elongation at 72°C for 10 min.

ELISA

Human IgG was measured by ELISA as described [10].

Statistical analyis

The statistical analysis of results was performed with one or two tailed Student's *t*-tests using the PRISM software (GraphPad Software Inc.).

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Abbreviations

AICDA	activation-induced cytidine deaminase
AID	activation-induced cytidine deaminase protein
APRIL	a proliferation inducing ligand
BAFF	B-cell activating factor
CSR	class switch recombination
CVID	common variable immunodeficiency
IRAK-4	IL-1R-associated kinase 4
TACI	transmembrane activator and calcium-modulating cyclophilin ligand interactor
TD	T cell-dependent
THC	TACI highly conserved domain
TI	T cell-independent
TIR	toll-interleukin-1 receptor
TNF-R	TNF receptor
TRAF	TNF receptor-associated factor

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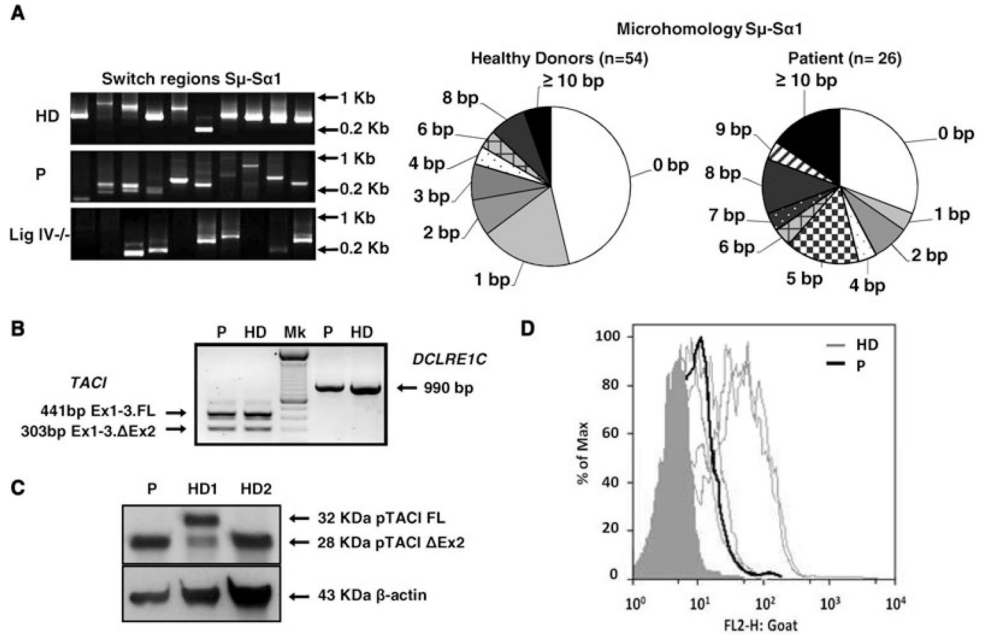

Figure 1.

Characterization of in vivo CSR junctions, TACI protein expression, and APRIL binding in TACI-S231R patient cells. (A) PCR amplification of Sμ–Sα fragments is shown (left). Ten PCR reactions were run in parallel using DNA from each individual. The microhomology usage at Sμ–Sα junctions in the patient ($n = 26$) and controls ($n = 54$) is shown (right). The proportion of switch junctions with a given size of perfectly matched short homology is indicated by the size of the slices. (B) PCR amplification of *TACI* Ex1–3 transcripts and *DCLRE1C* (ARTEMIS, loading control) from patient and healthy donor is shown. (C) Immunoblot analysis of TACI protein and actin (loading control) from patient and healthy donors is shown. (D) APRIL binding was assessed by flow cytometry. HD = Healthy donors, P = Patient TACI-S231R. Ex1–3. FL = cDNA *TACI* exon 1 to 3 full lenght. Ex1–3. ΔEx2 = cDNA *TACI* lacking exon 2. pTACI FL = TACI full-length protein; pTACI ΔEx2 = TACI protein lacking exon 2. Mk = molecular weight marker. Data shown are representative of four experiments performed.

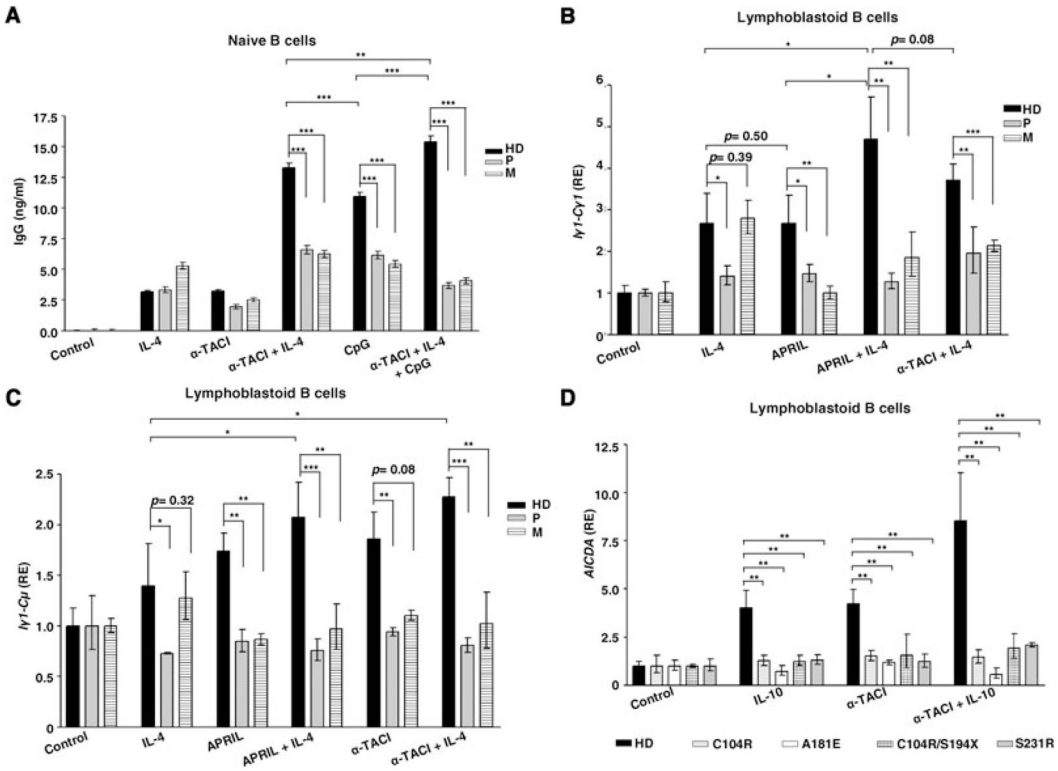

Figure 2.

Evaluation of CSR induced by specific stimuli in B cells. (A) IgG secreted from primary naive B cells incubated for 7 days with isotype-matched control antibody (Control), anti-TACI, IL-4, and/or CpG DNA was evaluated by ELISA. (B and C) qRT-PCR analysis of (B) *I*γ1-*C*γ1 and (C) *I*γ1-*C*μ in cells cultured with or without anti-TACI, APRIL, and/or IL-4 is shown. (D) qRT-PCR analysis of *AICDA* in controls and TACI-deficient cells cultured with or without anti-TACI and/or IL-10 is shown. Data are shown as the mean ± SD of three independent experiments each performed with five samples. The statistical analysis was performed using one-tailed Student's *t*-test; ***$p < 0.001$; **$p < 0.005$; *$p < 0.05$. HD: Healthy donors ($n = 4$), P = Patient, M = Mother of the patient.

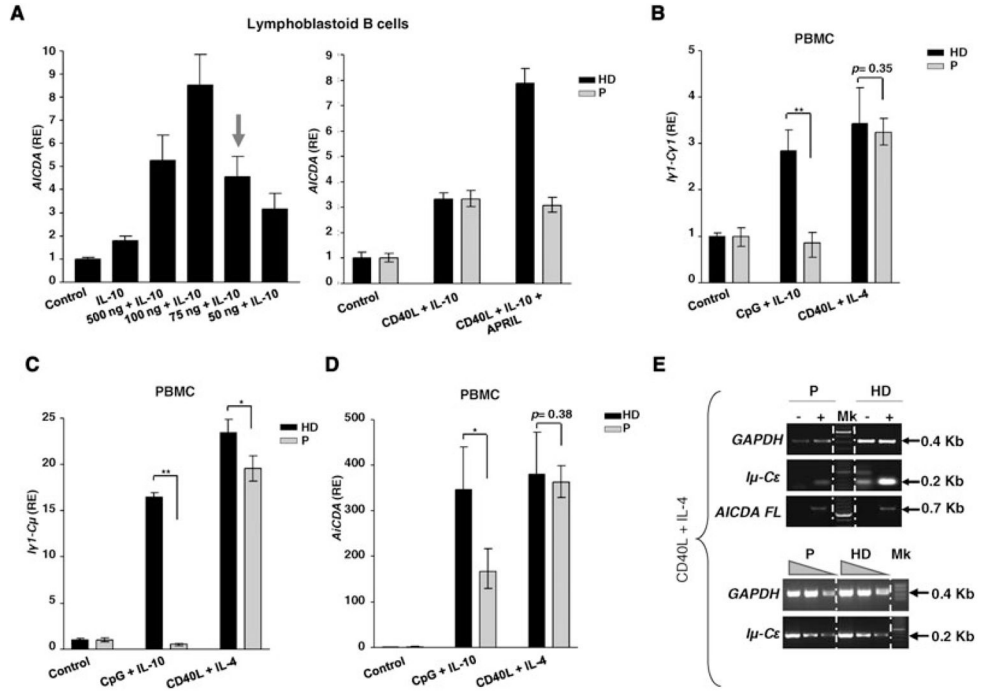

Figure 3.

APRIL-mediated enhancement of TI and TD response. (A) *AICDA* expression was evaluated after stimulation of healthy donor cells with IL-10 and different concentrations of anti-CD40 (left). qRT-PCR analysis of *AICDA* in cells cultured for 2 days with APRIL and suboptimal concentration of anti-CD40 (75 ng/mL) was also performed (right). (B–D) qRT-PCR analysis of (B) *Iγ1-Cγ1*, (C) *Iγ1-Cμ*, and (D) *AICDA* in cells cultured for 2 or 4 days with or without CpG and IL-10 or anti-CD40 (optimal concentration) and IL-4 is shown. Data are shown as the mean ± SD of three independent experiments each performed with two samples. The statistical analysis was performed using one-tailed Student's *t*-test; ***$p <$ 0.001; **$p <$ 0.005; *$p <$ 0.05. P = Patient, HD = Healthy donors (n = 3). (E) The expression of *Iμ-Cε*, *AICDA* mRNA was measured by RT-PCR in B cells stimulated for 2 days with CD40L (optimal concentration) and IL-4 (top). Semiquantitative RT-PCR analysis of the expression of *Iμ-Cε* after 2 days of stimulation was also performed (bottom). Data shown are representative of three experiments performed. P = patient, HD = healthy donors, Mk = molecular weight marker.

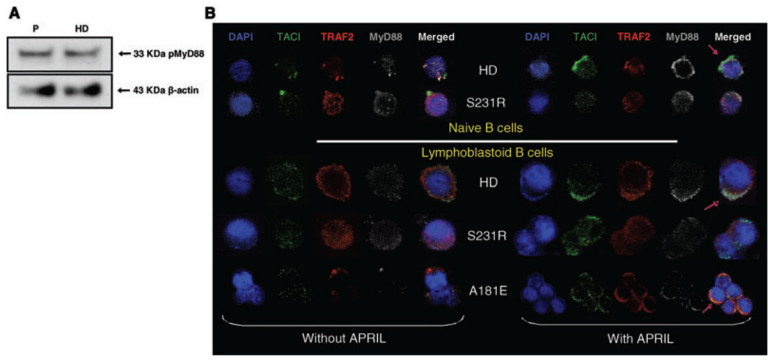

Figure 4.

Recruitment of MyD88 and TRAF2 after APRIL ligation. (A) Immunoblot analysis of MyD88 protein and actin (loading control) from patient and healthy donor is shown. (B) Confocal microscopy of cells exposed for 15 min to medium or APRIL with staining for TACI (green), TRAF2 (red), and MyD88 (gray) is shown. Original magnification 63×. Arrowheads indicate colocalization of TACI, MyD88, and TRAF2 proteins. Data shown are representative of three independent experiments performed for each sample. P = patient, HD = healthy donors, S231R = cells from patient carrying TACI-S231R mutation, A181E = cells from patient carrying TACI-A181E mutation.