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TNFRSF13B hemizygosity reveals TACI haploinsufficiency at later stages of B-cell development

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Abstract

Background—Heterozygous C104R or A181E *TNFRSF13B* mutations impair the removal of autoreactive B cells, weaken B-cell activation and convey to common variable immune deficiency (CVID) patients an increased risk for autoimmunity. How mutant TACI influences wildtype TACI function is unclear; different models suggest either a dominant-negative effect or haploinsufficiency.

Objective—We investigated potential TACI haploinsufficiency by analyzing antibody-deficient Smith-Magenis Syndrome (SMS) patients, who possess only one *TNFRSF13B* allele and antibody-deficient patients carrying one c.204insA *TNFRSF13B* null mutation.

Methods—We tested the reactivity of antibodies isolated from single B cells from SMS patients and patients with a c.204insA *TNFRSF13B* mutation and compared them with counterparts from CVID patients with heterozygous C104R or A181E *TNFRSF13B* missense mutations. We also assessed if loss of a *TNFRSF13B* allele induced haploinsufficiency in naïve and memory B cells recapitulate abnormal immunological features typical of CVID patients with heterozygous *TNFRSF13B* missense mutations.

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Results—We found loss of a *TNFRSF13B* allele does not impact TACI expression, activation responses, or establishment of central B-cell tolerance in naïve B cells. Additionally, SMS patients and patients with a c.204insA *TNFRSF13B* mutation display normal Treg function and peripheral B-cell tolerance. The lack of a *TNFRSF13B* allele did result in decreased TACI expression on memory B cells, resulting in impaired activation and antibody secretion.

Conclusion—*TNFRSF13B* hemizygosity does not recapitulate autoimmune features of CVID-associated C104R and A181E *TNFRSF13B* mutations, which likely encode dominant-negative products, but instead reveals selective TACI haploinsufficiency at later stages of B-cell development.

Keywords

TNFRSF13B; TACI; B-cell tolerance; Common variable immune deficiency; Smith-Magenis Syndrome

INTRODUCTION

Common variable immune deficiency (CVID) is a heterogeneous group of primary antibody deficiency disorders (1). Approximately 20% of all CVID cases are further complicated by autoimmune manifestations, a rate that is further increased (36-55%) in patients carrying a mutation in the CVID risk-gene *TNFRSF13B* (2-4). *TNFRSF13B* encodes TACI, a trimeric transmembrane receptor that plays an essential role during the counterselection of early B cells expressing self-reactive B-cell receptors (BCRs) in the bone marrow (5). At later stages of B-cell development, TACI can support class-switch recombination, plasma cell differentiation, and antibody secretion (6-9). The extracellular domain of TACI binds two ligands: a proliferation inducing ligand (APRIL) and B-cell activation factor (BAFF) (10). Intracellular TACI domains interact with several signaling molecules including MYD88 as well as activated endosomal Toll-like Receptors (TLRs) seven and nine (5, 11). 90% of all CVID associated *TNFRSF13B* mutations consist of either a C104R mutation, which alters ligand binding, or the A181E mutation, which affects transmembrane function (12-15). The mechanism by which C104R or A181E mutated TACI molecules exert their influence over wild type TACI is unclear. Evidence generated from one transgenic mouse model suggests a role for haploinsufficiency (12) while another mouse model and experiments with transfected cell lines indicate that mutant proteins may function as a dominant-negative products (13, 14).

We investigated TACI haploinsufficiency in humans by analyzing several conditions that reflect hemizygosity i.e. lack of an allele at the *TNFRSF13B* locus. CVID patients with one 204insA frameshift *TNFRSF13B* mutation have been reported; this functionally null allele yields a severely truncated gene product that lacks ligand-binding, transmembrane and intracellular signaling domains (2, 16).

Smith-Magenis Syndrome (SMS) is a complex neurodevelopmental disorder that results from a heterozygous 3.5Mb deletion of chromosome 17p11.2, a region encompassing the entire *TNFRSF13B* locus (17). Although the most overt neurological aspects of this syndrome stem from heterozygous loss of non-immunologic gene(s), SMS patients routinely

experience chronic otitis and vaccine failure suggesting an underlying humoral immune deficiency (17, 18).

We report herein that *TNFRSF13B* hemizygosity in SMS patients and patients with a 204insA frameshift *TNFRSF13B* mutation does not result in defective naïve B-cell activation or antibody repertoire selection that are associated with the C104R and A181E *TNFRSF13B* mutations. This suggests that these mutated *TNFRSF13B alleles* do not encode functionally inert products but rather dominant negative molecules favoring the development of autoimmunity (2, 5). The loss of one *TNFRSF13B* allele reveals TACI haploinsufficiency in later stages of B-cell development when its expression should normally be upregulated; the failure to enhance TACI expression in memory B cells of SMS patients and patients with a 204insA frameshift *TNFRSF13B* mutation correlates with activation defects and clinical antibody deficiency.

METHODS

Patients

SMS patients with a documented 17p11.2 deletion were recruited for the study (Table 1). Healthy donors with and without *TNFRSF13B* mutations, CVID patients with a C104R or A181E *TNFRSF13B* mutation, and antibody-deficient patients with a c.204insA *TNFRSF13B* mutation were previously described (5, 16). All participants provided informed consent prior to participation in this study. All aspects of the study were approved by the Yale University School of Medicine Human Investigation Committee, New Haven, Connecticut, USA.

Cell staining and sorting, cDNA, RT-PCR, antibody production, ELISAs and indirect fluorescent assays

Single $CD19^{+}CD21^{lo}CD10^{++}IgM^{hi}CD27^{-}$ new emigrant/transitional and $CD19^{+}CD21^{+}CD10^{-}IgM^{+}CD27^{-}$ mature naïve B cells from patients and healthy donors were sorted on a FACSAria flow cytometer (Becton Dickinson, Mountain View, Calif) into 96-well PCR plates, and antibody reactivity was tested as previously described (19). For indirect immunofluorescence assays, HEp-2 cell coated slides (Bion Enterprises, LTD) were incubated in a moist chamber at room temperature with recombinant IgG antibodies at a standardized concentration of 100μg/mL or patient plasma samples at 1:80 and 1:320 dilutions in PBS. FITC-conjugated goat anti-human IgG was used as detection reagent for fluorescent microscopy. Serum BAFF concentrations were determined by ELISA according to the manufacturers instructions (R&D Systems, Minneapolis, Minn).

B-cell activation

B cells were enriched from the blood of research subjects either by positive selection using CD20 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) or by negative selection using the Naïve B Cell Isolation Kit II (Miltenyi) and plated at 150,000-200,000 cells per well in a 96 well plate in RPMI 10% FBS and either 2.5 μg/mL polyclonal $F(ab)'_2$ rabbit anti-human IgM or BCR (Jackson ImmunoResearch, West Grove, Pa), 1.0 μg/mL Gardiquimod™ (TLR7 agonist; InvivoGen, San Diego, Calif) or 0.5 μg/mL CpG (TLR9

agonist; Invitrogen, San Diego, Calif) for 48 hours. Induction of surface CD86 and CD69 was detected on gated $CD19^+CD21^+CD27^-$ naïve or $CD19^+CD21^+CD27^+$ memory B cell populations using an LSRII flow cytometer (Becton Dickinson) and analyzed with FloJo software (FloJo, Ashland, Oreg).

B-cell proliferation and antibody secretion

B cells were enriched from peripheral blood of research subjects by negative selection using the EasySep® Human B cell Enrichment Kit (STEMCELL Technologies, Vancouver, British Columbia, Canada) and then further separated into $CD27^+$ memory and $CD27^-$ naïve B cell fractions using an APC conjugated anti-CD27 IgG followed by anti-APC conjugated magnetic beads (Miltenyi). Each fraction was labeled with CellTrace® CFSE (Life Technologies, Grand Island, N.Y.) at 0.05 μM and cultured at 100,000 cells/well in a 384 well plate in RPMI 10% FBS, 500 ng/ml APRIL (R&D Systems) with and without 2.5 μg/mL polyclonal $F(ab)'_2$ rabbit anti-human BCR (Jackson), 0.5 μg/mL CpG (Invitrogen). After 6 days in culture cells were stained with the LIVE/DEAD® kit (Life Technologies) to measure viability. B-cell proliferation was analyzed by CFSE dilution by flow cytometry. IgM concentrations in culture supernatants were measured via ELISA.

In vitro Treg suppression assay

$CD4^+$ T cells were enriched using the EasySep® Human $CD4^+$T cell enrichment kit (STEMCELL). $CD4^+CD25^{hi}CD127^{lo/-}$ Tregs were sorted by flow cytometry whereas $CD3^+CD4^+CD25^-$ Tresp cells were obtained after the depletion of $CD25^+$ cells with anti-human CD25 microbeads (Miltenyi) and then labeled with CFSE at 5 μM. Treg and Tresp cells were co-cultured at a 1:1 ratio in the presence of beads loaded with anti-CD2, anti-CD3 and anti-CD28 (Treg suppression inspector human, Miltenyi). At 3.5-4.5 days, co-cultures were stained for viability with the LIVE/DEAD kit and proliferation of the viable Tresp was analyzed by CFSE dilution.

Flow cytometry

The following antibodies were used for flow cytometric stainings anti-TACI PE (clone 1A1), anti-CD19 APC-Cy7, anti-CD27 PerCP-Cy5.5 or APC, anti-CD10 PE-Cy7, anti-IgM FITC, anti-CD21 APC, anti-CD69 PE-Cy7, anti-CD86 PE, anti-CD4 APC-Cy7, anti-CD25 PECy7, anti-CD127 PerCP-Cy5.5, anti-CD45RO Pacific Blue, anti-CXCR5 PerCP-Cy5.5, anti-PD-1 PE-Cy7, anti-CD25 PE, anti-CD25 PE-Cy7 (all from BioLegend, San Diego, Calif), anti-CD3 eFluor 605NC, anti-CD21 BD Horizon V450 (Becton Dickinson) and goat polyclonal anti-TACI biotin (R&D Systems). Intracellular staining with anti-Foxp3 Alexa Fluor 488 and anti-BCL6 PE (eBioscience, San Diego, Calif) was performed using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience), in accordance with the manufacturer's instructions.

Statistics

Differences between patients and healthy donors were analyzed for statistical significance with unpaired Student *t* tests or chi-squared tests using GraphPad Prism software (GraphPad Software, La Jolla, Calif). P-values of less than 0.05 were considered statistically significant.

RESULTS

A single *TNFRSF13B* allele is sufficient to establish central B-cell tolerance

We previously reported that *TNFRSF13B* mutations interfere with the establishment of central B-cell tolerance, a checkpoint in the bone marrow normally responsible for the removal of most polyreactive and anti-nuclear clones before they enter the new emigrant/transitional B-cell compartment (5, 19). To determine whether *TNFRSF13B* hemizygosity may impact central B-cell tolerance, we tested the reactivity of recombinant antibodies cloned from single $CD19^{+}CD10^{++}CD21^{lo}IgM^{hi}CD27^{-}$ new emigrant/transitional B cells from three SMS patients and two patients with a heterozygous 204insA *TNFRSF13B* mutation both meeting clinical criteria for diagnosis of CVID (20) (Table 1, and see Tables E1-E10 in this article's Online Repository at www.jacionline.org). For comparison, we represented previously reported reactivity data for recombinant antibodies cloned from new emigrant/transitional B cells from 11 healthy donors without *TNFRSF13B* mutations, and eight individuals heterozygous for the C104R or A181E *TNFRSF13B* missense mutations; four were CVID patients and four were immune competent carriers of a mutated *TNFRSF13B* allele (5).

We found that new emigrant/transitional B cells from SMS patients and patients with a 204insA *TNFRSF13B* mutation expressed low frequencies of polyreactive antibodies, which represented 6.6-9.1% and 7.4-9.5% of the clones, respectively, and were similar to those of healthy donors without *TNFRSF13B* mutation (5-11.5%) (Fig 1, A and B). New emigrant/transitional B cells from SMS patients and patients with a 204insA *TNFRSF13B* mutation were also normally purged from anti-nuclear clones since none were detected in B cells from these patients (see Fig E1). In contrast, subjects harboring one C104R or a A181E *TNFRSF13B* mutation displayed a defective central B-cell tolerance checkpoint characterized by elevated frequencies of new emigrant/transitional B cells expressing polyreactive and anti-nuclear antibodies (Fig 1, A and B, see Fig E1). Hence, *TNFRSF13B* hemizygosity does not affect the establishment of central B-cell tolerance in humans.

TNFRSF13B hemizygosity decreases TACI expression and activation responses in memory but not naïve B cells

Impaired central counterselection of polyreactive and anti-nuclear B cells has been associated with defects in BCR- and TLR-mediated B-cell activation (5, 21-23). In agreement with this hypothesis, we previously reported that C104R or A181E *TNFRSF13B* mutations altered the induction of the B-cell activation marker CD86 and CD69 on naïve B-cells after stimulation with $F(ab')_2$ anti-IgM, TLR7, or TLR9 agonists (5). In contrast, we found that naïve B cells from SMS patients or patients carrying a 204insA *TNFRSF13B* mutation were able to normally upregulate CD86 after stimulation through the BCR, TLR7 (gardiquimod stimulation) or TLR9 (CpG stimulation) (Fig 2, A). Other activation markers including CD69 were poorly induced on naïve B cells from CVID patients after stimulation with BCR or TLR agonists were also normally upregulated in B cells with only one productive *TNFRSF13B* allele (Fig 2, B). We also measured induction of CD86 and CD69 on activated $CD27^{+}$ memory B cells. In contrast to their naïve counterparts, memory B cells from SMS patients and patients carrying a 204insA *TNFRSF13B* mutation showed impaired

CD86 induction after BCR, TLR7 and TLR9 stimulation and decreased CD69 expression after BCR stimulation (Fig 2, A and B).

Correlating with memory B-cell activation defects in these patients, TACI cell surface expression levels on freshly isolated memory B cells from SMS patients and patients with a 204insA *TNFRSF13B* mutation were decreased by 39-57% compared to memory B cells from healthy donors, revealing TACI haploinsufficiency at this late stage of B cell development ($P<0.0001$; Fig 2, C). In contrast, TACI expression on freshly isolated naïve B cells from SMS patients and patients with a 204insA *TNFRSF13B* mutation was similar to that observed on healthy donor naïve B cells (Fig 2, C). Hence, TACI expression can be sustained by a single functional *TNFRSF13B* allele on naïve but not memory B cells. Of note, TACI expression was not quantitatively diminished on naïve or memory B cells harboring one missense C104R *TNFRSF13B* mutation; the C104R mutant TACI protein, which is not detected by the 1A1 monoclonal anti-TACI antibody, represented about half of all TACI cell surface molecules (Fig E2) (2, 24). Induction of TACI by the TLR9 agonist CpG, which typically increases its expression on naïve and memory B cells from healthy donors, was significantly diminished (2.0-4.2 fold induction, $P<0.0001$) on memory B cells from SMS patients (Fig 2, D). Induction of TACI expression by the TLR7 agonist gardiquimod was similarly decreased in SMS memory B cells compared to healthy donor B-cell counterparts (see Fig E3). In contrast, TACI induction was not significantly affected in naïve B cells from SMS patients and correlated with the normal activation of these B cells after BCR and TLR stimulations (Fig 2, D).

We further assessed the impact of the lower expression and impaired induction of TACI in SMS memory B cells by testing their proliferation and antibody secretion ability *in vitro* after CpG and BCR stimulation for 6 days in the presence or absence of TACI ligand APRIL. We found that memory B cells from SMS patients failed to properly become activated, evidenced by a profound decrease in blasting cells after 6 days of stimulation. Naïve B cells from the same patients blasted normally after BCR and TLR9 triggering (Fig 3, A). The decrease in blasting memory B cells in SMS patients was associated with reduced cell viability, regardless of the stimulations, which included CpG and BCR with or without APRIL (Fig 3, B). In addition, memory B cell activation impairments in SMS patients were further evidenced by the decreased proportion of SMS memory B cells undergoing ≥ 2 divisions, a fraction reduced by 30-52% compared to healthy donor B cells, and also by their low IgM secretion (Fig 3, C and D, see Fig E4). In contrast, naïve B cells from SMS patients displayed normal proliferation and antibody secretion responses (Fig 3, B-D). We conclude that *TNFRSF13B* hemizygosity induces abnormal memory B cell responses and that a single *TNFRSF13B* allele cannot therefore compensate for the loss of the other allele in memory B cells, where TACI expression is normally enhanced.

TNFRSF13B hemizygosity affects isotype-switched memory B cells *in vivo*

To determine if *TNFRSF13B* hemizygosity may affect memory B cells *in vivo*, we analyzed the peripheral B-cell compartments of patients lacking a productive *TNFRSF13B* allele. We found that the frequency of $IgM^{-}CD27^{+}$ isotype-switched memory B cells was significantly decreased in both SMS patients and patients carrying a 204insA *TNFRSF13B* mutation in

which they represented only 1.7-14.6% and 5.1-5.5 % of peripheral B cells, respectively, compared to 7.9-37% in age-matched healthy controls (Fig 4, A and B). These low frequencies of isotype-switched memory B cells were similar to those of CVID patients with missense C104R/A181E *TNFRSF13B* mutations (Fig 4, A and B). In contrast, null *TNFRSF13B* mutations did not impact frequencies of IgM^+CD27^+ memory B cells (Fig 4, C) or those of new emigrant/transitional and $CD21^{lo}$ B cells, which are elevated in CVID patients with missense *TNFRSF13B* mutations (Fig E5) (25). Hence, TACI plays an important role in the production or maintenance of isotype-switched memory B cells in SMS patients and patients carrying a 204insA *TNFRSF13B* mutation.

A normal peripheral B-cell tolerance checkpoint and normal Treg function in subjects with one productive *TNFRSF13B* allele

In healthy humans autoreactive new emigrant/transitional B cells capable of recognizing peripheral self-antigens are further eliminated at a second, more peripheral B-cell tolerance checkpoint before they enter the mature naïve B-cell compartment (19). We previously reported that a general feature of CVID is a defective peripheral B-cell tolerance checkpoint that occurs independently of *TNFRSF13B* mutations (5). Notably, peripheral B-cell tolerance is also defective in other primary immune deficiency diseases affecting antibody production including CD40 Ligand, MHC class II, Activated Cytidine-Deaminase (AID) and Dedicator of Cytokinesis 8 (DOCK8) deficiencies (26-28) To determine if the peripheral B-cell tolerance checkpoint was dysfunctional in SMS patients and patients with a 204insA *TNFRSF13B* mutation who display various antibody production defects (Table 1), we tested the reactivity of recombinant antibodies from their $CD10^-CD21^+IgM^+CD27^-CD20^+$ mature naïve B-cells against HEp-2 cell lysates by ELISA (see Tables E6-E10). As previously reported 38.5-47.6% of mature naïve B cells from CVID patients with heterozygote C104R or A181E *TNFRSF13B* mutation expressed HEp-2 reactive antibodies and 30.8-34.6% expressed polyreactive antibodies (5). In contrast, we found a much lower frequency of mature naïve B cells expressing HEp-2 reactive antibodies in SMS patients (18.8-21.4%, $P<0.0004$) and patients with a 204insA *TNFRSF13B* mutation (13.6-17.4%, $P<0.0008$) that were comparable to frequencies reported in healthy donors with and without *TNFRSF13B* mutations (16-26.3%) (Fig 5, A and B). The frequencies of polyreactive mature naïve B cells in patients with loss of a *TNFRSF13B* allele and healthy donors with two unmutated alleles were also similarly low (3.5-13.6%) (Fig E6). In addition, anti-nuclear clones, which were found increased (3.8-14%) in mature naïve B cells from CVID patients with heterozygote C104R or A181E *TNFRSF13B* mutation, were not identified in patients with only one productive *TNFRSF13B* allele (Fig 5, C). Thus, SMS patients and patients with a 204insA *TNFRSF13B* mutation display an intact peripheral B-cell tolerance checkpoint.

Defects in peripheral B-cell tolerance have been correlated with altered T regulatory cell (Treg) frequency and function as well as elevated plasma BAFF concentrations: both features are commonly associated with CVID (5, 29-31). To further investigate the impact of a loss of a *TNFRSF13B* allele, we analyzed the frequency and function of Tregs in SMS patients and patients with a 204insA *TNFRSF13B* mutation and compared them with CVID patients. Previously we reported low frequencies of $CD4^+CD25^{hi}CD127^{lo}FOXP3^+$ Tregs in

CVID patients with heterozygote C104R or A181E *TNFRSF13B* mutation (0.7-4% of $CD4^+$ T cells) with defective suppressive function (5). In contrast, we found that Treg frequencies in SMS patients and patients with a 204insA *TNFRSF13B* mutation were similar to those of healthy donors with or without *TNFRSF13B* mutations (Fig 6, A and see Fig E7). Direct assessments of Treg suppressive function *in vitro* demonstrated that unlike the Tregs from most CVID patients, Tregs from SMS patients and patients with 204insA frameshift *TNFRSF13B* mutation were capable of inhibiting the proliferation of CFSE-labeled $CD3^+CD4^+CD25^-$ autologous and heterologous T responder cells (Fig 6, B, and see Fig E8) (5). Measurement of plasma BAFF in SMS patients and patients with a 204insA *TNFRSF13B* mutation revealed normal BAFF concentrations that were similar to those in healthy donors and significantly lower than plasma BAFF concentrations in CVID patients (Fig E9) (5, 30). We conclude that *TNFRSF13B* hemizygosity does not affect Treg frequency or function and does not significantly increase plasma BAFF concentrations.

Absence of plasma autoantibody production in subjects with one productive *TNFRSF13B* allele

Despite an impaired production of immune protective antibodies, IgG anti-nuclear antibodies (ANAs) were detected in the plasma of a large majority of CVID patients and healthy donors with heterozygote C104R or A181E *TNFRSF13B* mutation, revealing that TACI plays an important role in preventing the secretion of autoantibodies (5). To assess if *TNFRSF13B* hemizygosity also leads to autoantibody production, we measured plasma ANA titers in seven SMS patients and two patients with a 204insA *TNFRSF13B* mutation. We found that plasma of SMS patients and patients with 204insA frameshift *TNFRSF13B* mutation were devoid of IgG ANAs (Fig 6, C and D). High-titer plasma ANAs in CVID patients correlated with increased frequencies of peripherally circulating $CXCR5^+PD\text{-}1^{hi}CD4^+$ T follicular helper-like (Tfh-like) cells, which favor late B-cell development and antibody secretion (5, 32). Frequencies of circulating Tfh-like cells in the blood of SMS patients and patients with 204insA frameshift *TNFRSF13B* mutation were low and similar to those in healthy donors (Fig 6, E and see Fig E10). In addition, Tfh-like cells from SMS patients and patients with a 204insA *TNFRSF13B* mutation did not express high amounts of the B-cell co-activation molecule Inducible T-cell Co-stimulator (ICOS) or the intracellular transcription factor B-cell lymphoma protein six (BCL6), whereas both of these molecules were expressed in classical Tfh cells from germinal centers (33) and circulating Tfh from CVID patients (see Fig E10) (5). Hence, contrasting with common C104R and A181E heterozygote *TNFRSF13B* mutations, *TNFRSF13B* hemizygosity does not lead to a breach in peripheral B-cell tolerance or autoantibody production.

DISCUSSION

We reported that *TNFRSF13B* hemizygosity does not recapitulate the naïve B-cell selection and activation defects caused by the presence of one C104R or A181E mutant *TNFRSF13B* allele. TACI is essential for the establishment of central B-cell tolerance since all subjects carrying missense *TNFRSF13B* mutations suffered from an inability to remove developing autoreactive B cells in the bone marrow (5). Here, using two different human genetic models of *TNFRSF13B* hemizygosity, we demonstrate that a single unmutated *TNFRSF13B* allele is

sufficient for normal surface expression of TACI on naïve B cells and to maintain TACI-dependent central B-cell tolerance. Thus, the impairment of central B-cell tolerance observed when a normal *TNFRSF13B* allele is paired with a C104R and A181E mutated product reveals that these two most common *TNFRSF13B* mutations do not exert their effects through haploinsufficiency but more likely through negative interference. In agreement with this hypothesis is the normal activation of naïve B cells from SMS patients and patients with a 204insA *TNFRSF13B* mutation who display a single productive *TNFRSF13B* allele, whereas naïve B cells carrying one C104R or A181E *TNFRSF13B* allele fail to properly respond to BCR, TLR7 and TLR9 (5). Mutant TACI molecules may therefore limit the ability of wild type TACI to enhance BCR- and TLR-mediated B-cell activation. Since we previously reported that TACI binds TLR7 and TLR9 (5), mutated TACI variants may associate with these TLRs and therefore decrease non-mutated TACI-TLR complexes required to mediate TLR7 and TLR9 responses. Other B-cell intrinsic defects disrupting TLR signaling and function, such as IRAK4 and MYD88 deficiencies, also interfere with the establishment of central B-cell tolerance, suggesting an important role for TLRs in the removal of developing autoreactive B cells, especially those cells expressing anti-nuclear antibodies which may recognize nucleic acids bound by TLR7 and TLR9 (5, 22).

The peripheral B-cell tolerance checkpoint operates independently of the central tolerance checkpoint and appears to rely upon B-cell extrinsic factors (34). Indeed, FOXP3-deficient patients who lack functional Tregs displayed a defective peripheral B cell tolerance checkpoint whereas central B-cell tolerance was established normally in these patients (26). Interestingly, healthy donors carrying a C104R or an A181E *TNFRSF13B* mutation who showed normal Treg frequencies and function were able to prevent the accumulation of autoreactive clones in their mature naïve B cell compartment despite their impaired central B-cell tolerance checkpoint, demonstrating a dominant role for Tregs in ensuring tolerance (5, 35). The numerically and functionally replete Treg pool with corresponding intact peripheral B-cell tolerance checkpoints we observe in SMS patients and patients with a 204insA *TNFRSF13B* mutation further supports this hypothesis.

The C104R and A181E *TNFRSF13B* mutations are also associated with a more distal breach in B-cell tolerance characterized by the production of autoreactive antibodies, increased circulating Tfh cells and often the development of autoimmune manifestations (2, 5). All these features are unrelated to the loss of a *TNFRSF13B* allele since there is no evidence of autoimmunity in SMS patients or the two patients with a 204insA *TNFRSF13B* mutation reported here. In addition, we could not detect ANAs in the plasma of these patients, who also displayed normal low frequencies of Tfh-like cells in their blood. Hence, C104R and A181E *TNFRSF13B* mutations likely generate dominant negative products that also interfere with TACI's tolerogenic functions in the periphery. However, similar to CVID patients that harbor a single C104R or A181E *TNFRSF13B* mutation and mice with only a single *TNFRSF13B* allele (12), *TNFRSF13B* hemizygosity in humans decreases circulating isotype-switched memory B cells, production of serum immunogloblulins and vaccine responses. Both patients with a 204insA *TNFRSF13B* mutation (16) and at least two of the seven SMS patients we studied fulfill the accepted diagnostic criteria for CVID (Table 1).

The remaining five SMS patients experienced recurrent sinopulmonary infections or failed to properly respond to vaccines, illustrating abnormal antibody responses when one *TNFRSF13B* allele is lost (Table 1). The decrease in TACI expression, which is restricted to memory B cells from SMS patients and patients with a 204insA *TNFRSF13B* mutation, reveals that the loss of a *TNFRSF13B* allele results in haploinsufficiency at this stage of B-cell development, which has also been observed in EBV-transformed B cells from these patients (18). In light of B cells from patients carrying two non-functional *TNFRSF13B* alleles, which are virtually irresponsive to many stimulations (5), decreased TACI expression on memory B cells is likely responsible for their diminished responses to BCR, TLR7 and TLR9 stimulation and alters antibody responses by affecting B-cell proliferation as well as plasma cell differentiation and survival (11, 36, 37). Further exploration will be necessary to determine the mechanisms by which steps of terminal B-cell differentiation require two functional *TNFRSF13B* alleles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

ANA	Anti-nuclear antibody
APRIL	a proliferation inducing ligand
BAFF	B-cell activation factor
BCL6	B-cell lymphoma protein six
BCR	B-cell receptor
CVID	Common variable immune deficiency
ICOS	Inducible T-cell Co-stimulator
SMS	Smith-Magenis Syndrome
TACI	Transmembrane Activator and CAML Interactor
TNFRSF13B	Tumor necrosis factor receptor superfamily member 13b
Treg	T regulatory cell
Tfh	T follicular helper

TLR Toll-like receptor

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Key Messages

- Unlike heterozygous C104R and A181E *TNFRSF13B* mutations, complete loss of one *TNFRSF13B* allele does not affect B-cell functions at early stages of B cell development.
- Two functional *TNFRSF13B* alleles are required at later stages of B-cell development when TACI expression is upregulated.

Capsule Summary

Lack of a *TNFRSF13B* allele only affects later stages of B-cell development and memory B-cell responses. In contrast, a heterozygous C104R or A181E *TNFRSF13B* mutation alters naïve B-cell responses and the establishment of central tolerance, suggesting that these alleles encode dominant-negative products.

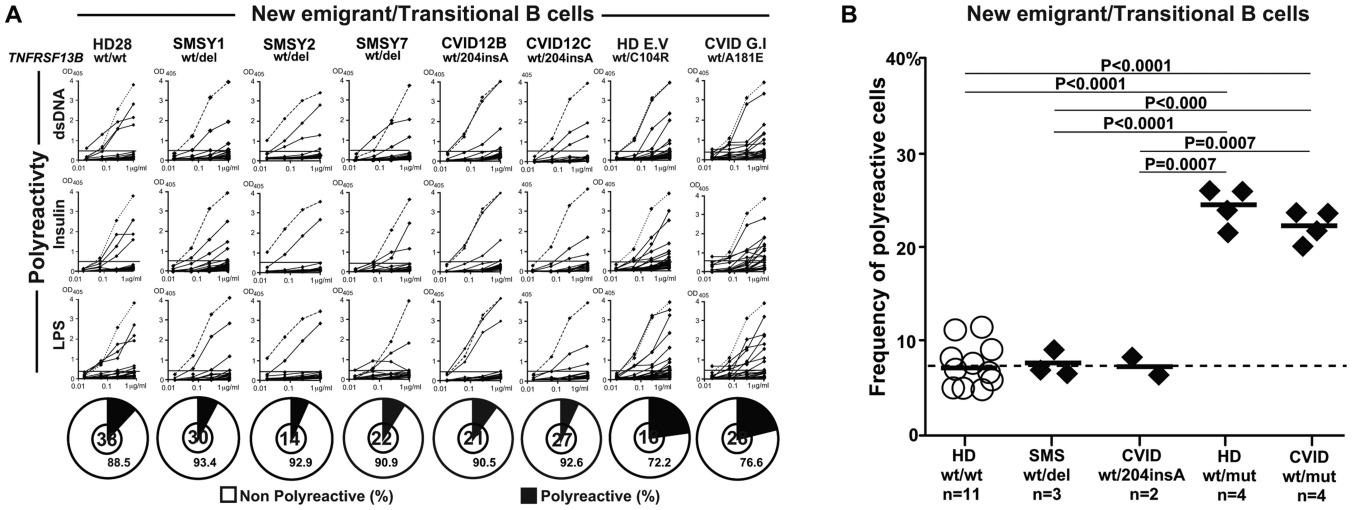

Figure 1. Central B-cell tolerance is functional in SMS patients and patients with a 204insA *TNFRSF13B* mutation

(A) Recombinant antibodies cloned from new emigrant/transitional B cells were tested by ELISA for reactivity against dsDNA, insulin, and lipopolysaccharide (LPS) (19). Antibodies were considered polyreactive if reacting against all three antigens. Dashed lines show ED38 antibody-positive control and solid lines show binding for each cloned recombinant antibody. Horizontal solid lines define the cutoff OD^{405} for positive reactivity. For each individual, the frequency of polyreactive and non-polyreactive clones is summarized in pie charts, with the total number of antibodies tested indicated in the center. (B) The frequency (black bars) of polyreactive new emigrant/transitional B cells is increased in all individuals carrying a C104R or an A181E *TNFRSF13B* mutation (*TNFRSF13B* wt/mut) but not in SMS patients or patients with a 204insA *TNFRSF13B* mutation. Statistical significance by unpaired student t-test is indicated.

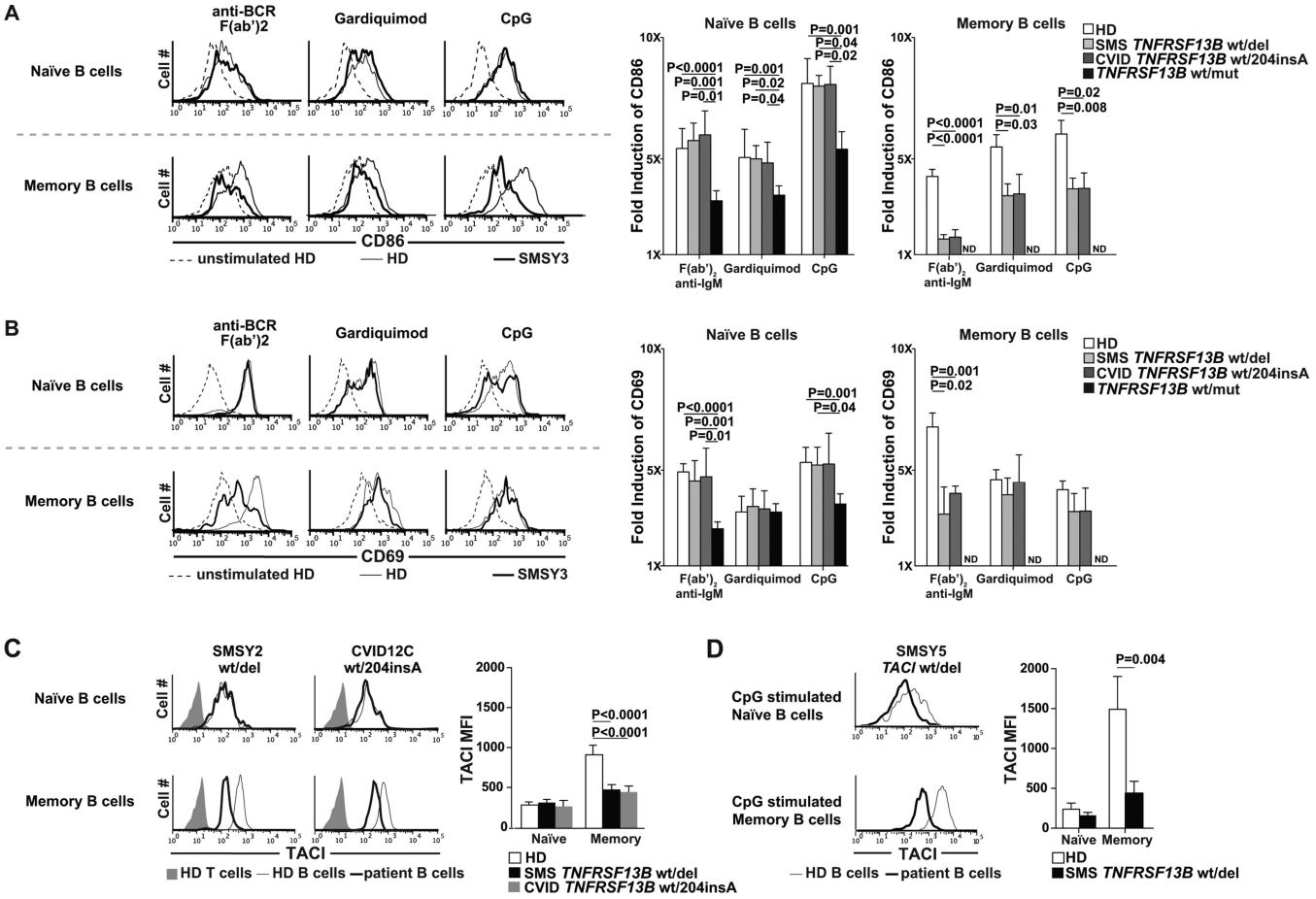

Figure 2. *TNFRSF13B* hemizygosity disturbs memory but not naïve B-cell activation by selectively altering TACI expression on memory B cells

Histograms display (A) CD86 and (B) CD69 induction after stimulation for 48 hours with either F(ab'$_2$) anti-BCR (2.5 μg/ml), the TLR7 agonist gardiquimod (1 μg/ml), or the TLR9 agonist CpG (0.5 μg/ml), which is normal in CD19$^+$CD21$^+$CD27$^-$ naïve B cells (upper panels) but defective in CD19$^+$CD21$^+$CD27$^+$ memory B cells (lower panels) from a representative SMS patient. Bar graphs on the right represent CD86 and CD69 induction on naïve and memory B cells from SMS patients with a heterozygous deletion of 17p11.2 (n=6), CVID patients with 204insA (n=2) or a missense (C104R or A181E) *TNFRSF13B* mutation (n=15) relative to same-day healthy donor controls (n=8-17). (C) The mean florescent intensity (MFI) of cell surface TACI is significantly lower on memory B cells from SMS patients (n=3) and patients with a 204insA *TNFRSF13B* mutation (n=2) than on counterparts from healthy donor controls (n=8), whereas TACI expression on naïve B cells is not altered by the lack of a *TNFRSF13B* allele. (D) CpG-mediated (0.5 μg/ml) TACI induction is decreased on SMS memory B cells (n=3) compared to healthy donor memory B cells (n=6) at 48 hours, whereas TACI induction on naïve B cells from SMS patients appears unaffected. Error bars represent the mean ± SEM. Statistical significance by unpaired student t-test is indicated. ND indicates experiments not performed.

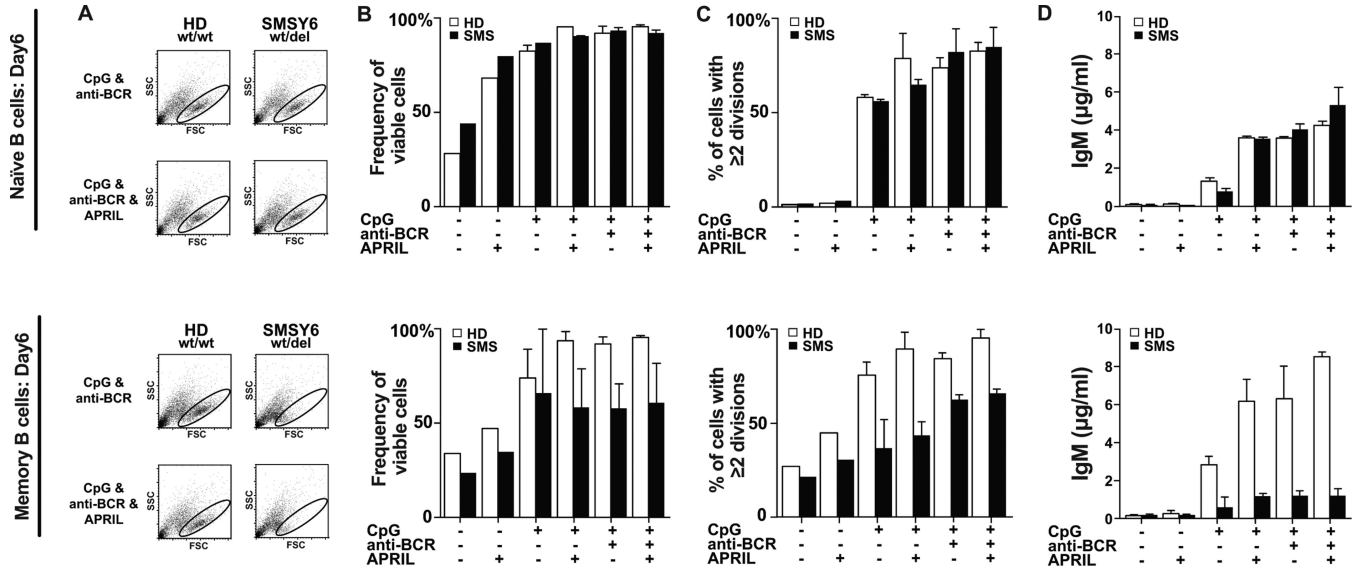

Figure 3. *TNFRSF13B* hemizygosity results in impaired memory B-cell responses *in vitro*

(A) Representative dot plots display forward (FCS) and side (SSC) scatter of naïve and memory B cells from a representative healthy donor and SMS patient after CpG and BCR stimulation with or without APRIL for 6 day. The absence of blasting B cells in memory but not naïve B cells from SMS patients reveals defective memory responses in these patients. Bar graphs display the proportion of (B) viable cells, (C) cells undergoing multiple (≥ 2) divisions and (D) the production of IgM secreted into culture supernatants in SMS patients (n=2) and healthy donors (n=2) on day 6. Various culture conditions are indicated. Error bars represent the mean ± SEM. Whereas responses of naïve B cells from SMS patients and healthy donors were similar, the lack of a *TNFRSF13B* allele resulted in abnormal memory B cell proliferation, survival and antibody production.

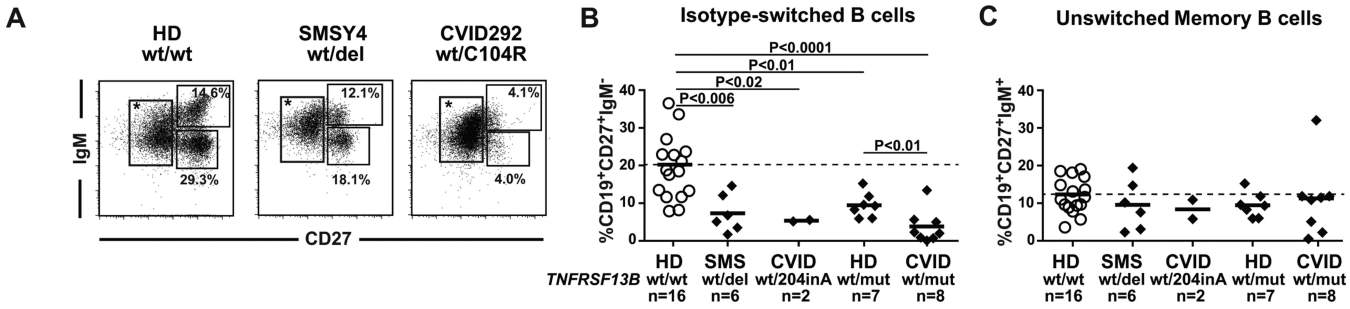

Figure 4. *TNFRSF13B* hemizygosity correlates with decreased class-switched memory B cell frequencies

(A) Dot plots for CD27 and IgM expression on CD19-gated B cells in representative subjects and frequencies of (B) $CD27^{+}IgM^{-}$ isotype-switched and (C) unswitched $CD27^{+}IgM^{+}$ memory B cells in enrolled individuals reveals decreased isotype-switched memory B cells in SMS patients (mean age = 14.3 yrs) and in patients with one 204insA *TNFRSF13B* mutation (mean age = 15 ys) compared to age-matched healthy donor controls (mean age= 19.9 yrs). Error bars represent the mean ± SEM. Statistical significance by unpaired student t-test is indicated.

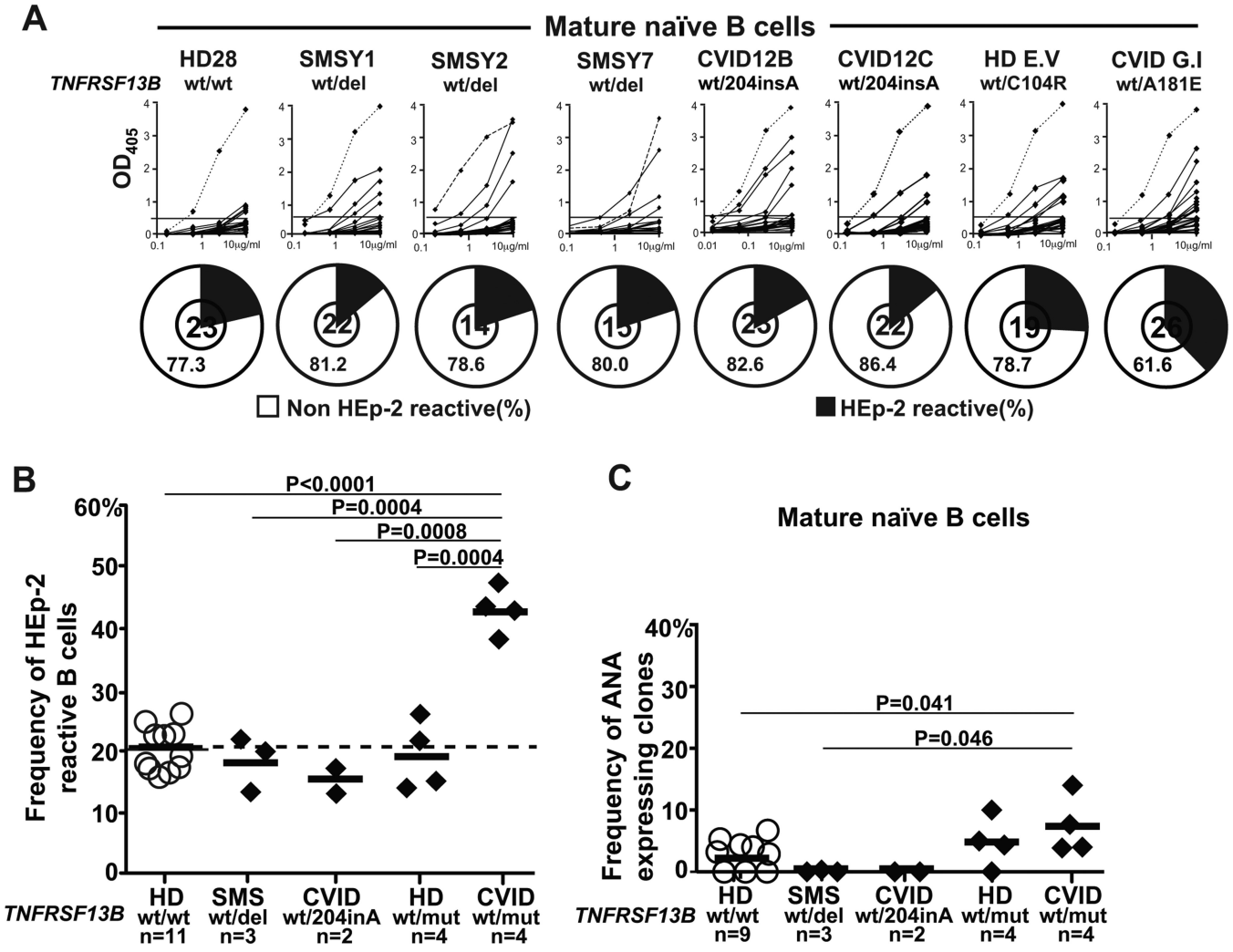

Figure 5. *TNFRSF13B* hemizygosity is compatible with a normal peripheral B-cell tolerance checkpoint

(A) Recombinant antibodies cloned from mature naïve B cells were tested by ELISA for anti-HEp-2 cell reactivity. Dashed lines show a ED38 positive control (19). Horizontal lines define cutoff OD405 for positive reactivity. For each individual, the frequencies of HEp-2 reactive and non HEp-2 reactive clones are summarized in pie charts, with the number of antibodies tested indicated in the center. (B) The mean frequency of HEp-2 reactive mature naïve B cells (black bars) is normal in patients with a single productive *TNFRSF13B* allele, whereas it is increased in CVID patients carrying a heterozygote C104R or A181E *TNFRSF13B* mutation (CVID wt/mut). (C) Mature naïve B cells from SMS patients and patients with a 204insA *TNFRSF13B* mutation are virtually devoid of anti-nuclear clones. Statistical significance by unpaired student t-testing is indicated.

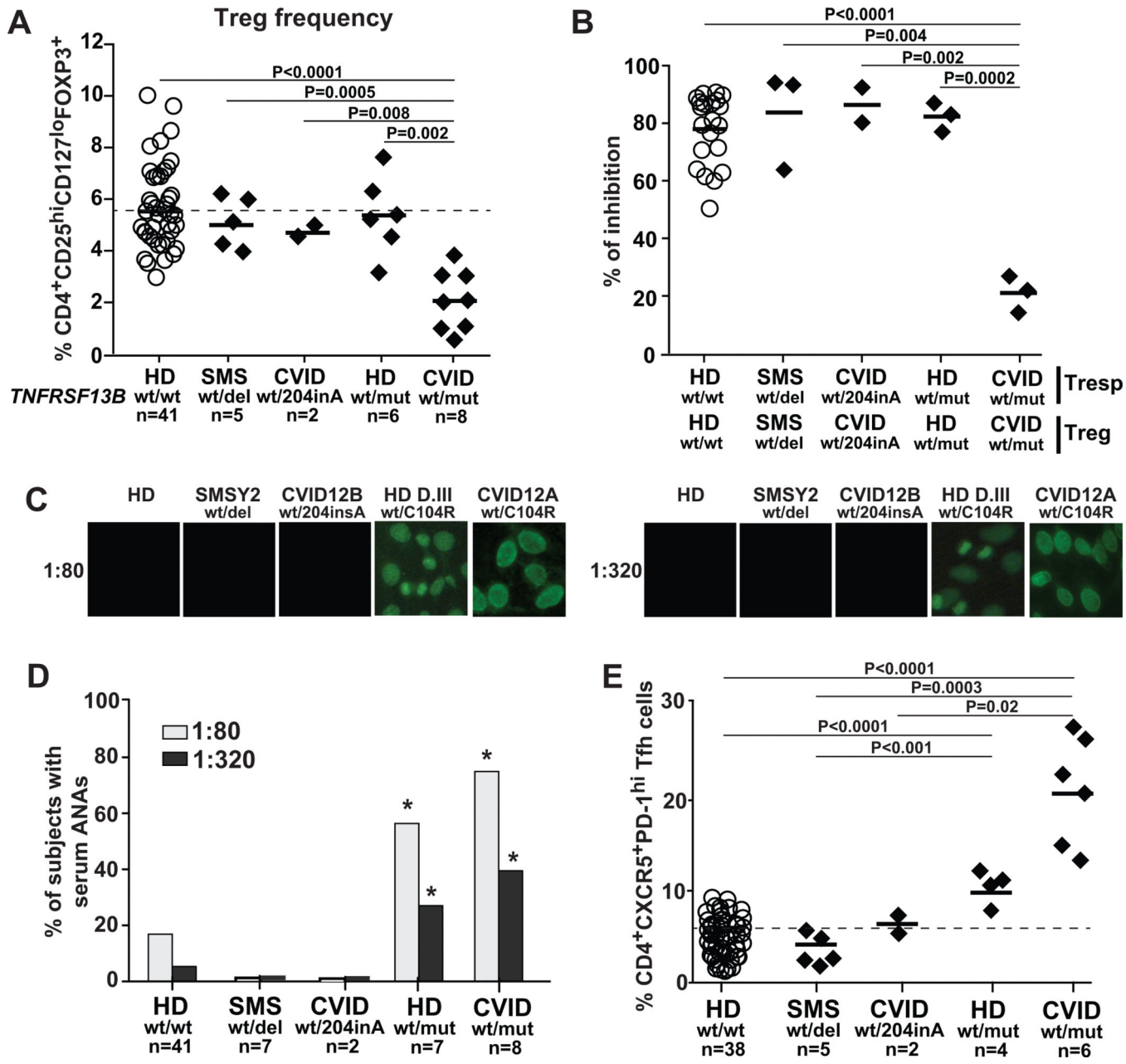

Figure 6. *TNFRSF13B* hemizygosity does not alter Treg function, autoantibody production or circulating T follicular helper (Tfh) cell frequency

Normal (A) Treg frequency and (B) Treg suppressive function in SMS patients and patients with a 204insA *TNFRSF13B* mutation whereas both are altered in CVID patients carrying one C104R or one A181E *TNFRSF13B* mutation (CVID wt/mut). Each symbol represents an individual and horizontal bars show the average. (C) Representative ANA staining patterns of 1:80 (right) and 1:320 (left) dilutions of plasma from SMS patients, patients with a 204insA *TNFRSF13B* mutation and subjects with a C104R or A181E *TNFRSF13B* mutation. (D) ANAs are absent in the plasma of SMS patients and patients with a 204insA *TNFRSF13B* mutation whereas they are commonly detected in individuals carrying a C104R or a A181E *TNFRSF13B* mutation (wt/mut). (E) Normal $CD4^+PD\text{-}1^{hi}CXCR5^+$ Tfh

frequencies in SMS patients and patients with a 204insA *TNFRSF13B* mutation whereas these cells are increased in CVID patients with a C104R or a A181E *TNFRSF13B* mutation (wt/mut). Each symbol represents an individual and horizontal bars show the average. Statistical significance is indicated.

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Table 1

Clinical characteristics of research subjects

Subject	Age (years)	Sex	*TNFRSF13B* mutation	Diagnosis	Laboratory abnormality	Recurrent infections	IVIG/SQIG	Autoimmune diseases
SMSY1	7	F	Del	SMS	hypogammaglobulinemia	PNA, OM, S	Yes	-
SMSY2	6	F	Del	SMS	vaccine failure	PNA, OM	No	-
SMSY3	12	F	Del	SMS	IgA def, vaccine failure	PNA, OM	No	-
SMSY4	26	F	Del	SMS	IgG2 def, vaccine failure	Warts	No	-
SMSY5	20	F	Del	SMS	vaccine failure	OM, GI	No	-
SMSY6	9	M	Del	SMS	hypogammaglobulinemia	OM	No	-
SMSY7	20	M	Del	SMS	IgA def and IgM def	OM, PNA	No	-
C12B[1]	17	F	204insA	CVID	hypogammaglobulinemia	AOM, S	Yes	-
C12C[1]	13	M	204insA	CVID	hypogammaglobulinemia	OM, PNA, S	Yes	-
HD G.II[2]	38	F	A181E	Healthy	ND	-	No	-
HD E.V[2]	80	F	C104R	Healthy	ND	-	No	-
HD D.II[2]	82	M	C104R	Healthy	ND	-	No	-
HD E.II[2]	79	M	C104R	Healthy	ND	-	No	-
HD D.III[2]	14	M	C104R	Healthy	ND	-	No	-
M.I.1[2]	78	M	C104R	Healthy	ND	-	No	-
M.I.2[2]	78	F	C104R	Healthy	ND	-	No	-
CVID321[2]	27	F	A181E	CVID	hypogammaglobulinemia	GI, PNA	Yes	ITP, arthritis, uveitis
CVIDY15[2]	40	M	C104R	CVID	hypogammaglobulinemia	PNA, S	Yes	-
CVIDY17[2]	48	F	C104R	CVID	hypogammaglobulinemia	PNA, S	Yes	AH
CVID B.I[2]	19	F	A181E	CVID	hypogammaglobulinemia	S	Yes	-
CVID292[2]	45	F	C104R	CVID	hypogammaglobulinemia	-	Yes	ITP
CVID G.I[2]	11	F	A181E	CVID	hypogammaglobulinemia	-	Yes	ITP, AIHA
CVID376[2]	45	M	C104R	CVID	hypogammaglobulinemia	S	Yes	ITP

Subject	Age (years)	Sex	*TNFRSF13B* mutation	Diagnosis	Laboratory abnormality	Recurrent infections	IVIG/SQIG	Autoimmune diseases
C12A[1]	12	M	C104R	CVID	hypogammaglobulinemia	OM, S	Yes	-

***AIHA*, Autoimmune hemolytic anemia; *AH*, autoimmune hepatitis; *CVID*, common variable immune deficiency; *Def*, deficient; *F*, female; *ITP*, idiopathic thrombocytopenia; *GI*, gastrointestinal infection; *M*, male; *ND*, not done; *OM*, otitis media; *PNA*, pneumonia; *S*, sinusitis**

[1] as described in Castigli et al. *Nat Genet.* (2005)

[2] as described in Romberg et al. *J Clin Invest.* (2013)