Original article

The C76R transmembrane activator and calcium modulator cyclophilin ligand interactor mutation disrupts antibody production and B-cell homeostasis in heterozygous and homozygous mice

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Background: Mutations in *TNFRSF13B*, the gene encoding transmembrane activator and calcium modulator cyclophilin ligand interactor (TACI), are found in 10% of patients with common variable immunodeficiency. However, the most commonly detected mutation is the heterozygous change C104R, which is also found in 0.5% to 1% of healthy subjects. The contribution of the C104R mutation to the B-cell defects observed in patients with common variable immunodeficiency therefore remains unclear.

Objective: We sought to define the functional consequences of the C104R mutation on B-cell function.

Methods: We performed *in vitro* studies of TACI C104R expression and signaling. A knock-in mouse with the equivalent mutation murine TACI (mTACI) C76R was generated as a physiologically relevant model of human disease. We examined homozygous and heterozygous C76R mutant mice alongside wild-type littermates and studied specific B-cell lineages and antibody responses to T cell–independent and T cell–dependent challenge.

Results: C104R expression and ligand binding are significantly diminished when the mutant protein is expressed in 293T cells or in patients' cell lines. This leads to defective nuclear factor κB activation, which is proportionally restored by reintroduction of wild-type TACI. Mice heterozygous and homozygous for mTACI C76R exhibit significant B-cell dysfunction with splenomegaly, marginal zone B-cell expansion, diminished immunoglobulin production and serological responses to T cell–independent antigen, and abnormal immunoglobulin synthesis.

Conclusions: These data show that the C104R mutation and its murine equivalent, C76R, can significantly disrupt TACI function, probably through haploinsufficiency. Furthermore, the heterozygous C76R mutation alone is sufficient to disturb B-cell function with lymphoproliferation and immunoglobulin production defects. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: *Hypogammaglobulinemia, B cell, common variable immunodeficiency, primary immunodeficiency, transmembrane activator and calcium modulator cyclophilin ligand, a proliferation-inducing ligand, lymphoproliferation*

Common variable immunodeficiency (CVID) has a prevalence of approximately 1:25,000 in Europeans and is characterized by low serum immunoglobulin levels, defective specific antibody responses, and increased susceptibility to bacterial infections. Twenty percent of cases are complicated by autoimmune manifestations and lymphoproliferation, with splenomegaly seen in one third.[1] Immunologic abnormalities in patients with CVID include defects of B-cell survival, decreased frequency of circulating $CD27^+$ memory B cells, failure of isotype switching to IgA and IgG, and defective B-cell activation.[2] Further studies highlight the inability to mount responses to polysaccharide antigens, which might be related to abnormalities of marginal zone (MZ) B cells.[3] Mutations in *TNFRSF13B*, the gene encoding transmembrane activator and calcium modulator cyclophilin ligand interactor (TACI), are found in approximately 10% of patients with CVID.[4,5]

TACI is a TNF receptor family member found on the surfaces of B cells and acts as a receptor for B cell–activating factor of the TNF family (BAFF) and a proliferation-inducing ligand (APRIL), both of which are expressed on multiple cell types.[6] The extracellular domain of TACI contains 2 cysteine-rich domains (CRDs; CRD-1 and CRD-2),[7] and after ligand binding, the intracellular domain of TACI binds TNF receptor–associated factors 2, 5, and 6, resulting in activation of nuclear factor κB (NF-κB) and Jun amino terminal kinases.[8]

In patients with CVID, the most common TACI mutation is C104R. Compound heterozygotes and homozygotes have been

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Abbreviations used
APRIL: A proliferation-inducing ligand
BAFF: B cell–activating factor of the TNF family
CRD: Cysteine-rich domain
CVID: Common variable immunodeficiency
hTACI: Human TACI
mTACI: Murine TACI
MZ: Marginal zone
MZP: Marginal zone progenitor
NF-κB: Nuclear factor κB
TACI: Transmembrane activator and calcium modulator cyclophilin ligand interactor
TD: T cell dependent
TI: T cell independent
WT: Wild type

identified, but in the majority of cases, C104R mutations have been found as simple heterozygous variants.[9] However, studies also report incomplete familial penetrance[9,10] and the presence of C104R heterozygous changes in approximately 0.5% to 1% of reportedly healthy donors.[9,11] These observations have led to the suggestion that the C104R mutations are not pathogenic by themselves but might act as risk factors or disease modifiers in combination with other genetic variants or environmental factors, although the mechanisms of disease susceptibility are not well understood. Controversy also exists as to whether the heterozygous C104R mutation interferes with TACI function through a dominant-negative effect or through haploinsufficiency.

The C104R mutation is highly disruptive in that C104 is necessary for the formation of a disulfide bond with C93 in the CRD2 domain of TACI,[7] and the nature of the amino acid change might lead to loss of a protein integrity.[9] The positions of residues C104/C76, Y79, and I87 are shown in the online material (see Fig E1 in this article's Online Repository at www.jacionline.org). To understand in greater detail whether the C104R mutation alone significantly disrupts B-cell function and contributes to the CVID phenotype, we studied *in vitro* receptor signal transduction and also generated mice with the murine equivalent of the C104R mutation. We demonstrate that the C104R mutation in isolation impairs TACI protein expression and its ability to initiate NF-κB signaling and that mice carrying the equivalent C76R mutation in both the homozygous and heterozygous states show dysregulated B-cell homeostasis and defective immunoglobulin production.

METHODS

Plasmids and transfections

Site-directed mutagenesis was performed on full-length wild-type (WT) hTACI cDNA plasmid in a pCR3 vector backbone to generate mutant hTACI expression plasmids in 293T cells, as described previously.[9] For NF-κB reporter assays, 293T cells were transfected with 2 ng of either full-length WT hTACI or mutant hTACI plasmids, 100 ng of NF-κB luciferase reporter plasmid, 2 ng of *Renilla* luciferase plasmid together with 50 ng of full-length human Flag-APRIL cDNA, or full-length human BAFF cDNA and adjusted to a total of 200 ng of plasmid with mock empty PCR3 plasmid by using Lipofectamine 2000 (Invitrogen, Carlsbad, Calif), according to the manufacturer's instructions. Luciferase activity was measured 20 hours later by using the Dual-Luciferase reporter assay (Promega, Madison, Wis). NF-κB luciferase induction was normalized to *Renilla* intensity, and then fold activation ratios were calculated compared with empty vector.

Generation of C76R TACI knock-in mice

Heterozygous C76R TACI knock-in mice were generated by InGenious-Targeting Laboratory, Inc (Stony Brook, NY), on a C57BL/6 background by using homologous recombination in embryonic stem cells to replace endogenous mTACI (NM_021349.1) with the transgene carrying C76R (c.226T>C; see Fig E2, *B*, in this article's Online Repository at www.jacionline.org). See the Methods section in this article's Online Repository at www.jacionline.org for detailed generation methods. Animals were housed and bred in a sterile animal facility. All surgical procedures were performed according to guidelines of the Animal Act of 1986. Age- and sex-matched WT littermates were used as control animals for all the experiments.

Naive immune phenotyping and immunizations

Groups of 8- to 12-week-old naive mice were culled, and blood, spleen, and lymph nodes were collected for analysis of naive serum and B-cell compartments. Peripheral blood was collected into heparin for flow cytometry or without heparin for immunoglobulin analysis. Immunoglobulin levels were measured in naive serum by means of ELISA. Spleens were weighed, and then splenocytes were extracted in RBC lysis buffer (eBioscience, San Diego, Calif), washed, and resuspended at 5×10^6 cells/mL for staining or 10×10^7 cells/mL for MACS purification. Separate groups of age- and sex-matched 8- to 40-week-old mice were immunized intraperitoneally with 50 μg of TNP-FICOLL (Biosearch Technologies, Novato, Calif) emulsified with 50 μL of complete Freund adjuvant (Sigma-Aldrich, St Louis, Mo) in PBS or 50 μg of NP-CGG (Biosearch Technologies) adsorbed to aluminum hydroxide (Serva, Heidelberg, Germany) in PBS. Sera were sampled at days 3, 10, and 21 after immunization and frozen for subsequent analysis. Serial dilutions of sera were analyzed for TNP- or NP-specific antibodies by means of ELISA. See the Methods section in this article's Online Repository for ELISA details.

In vitro immunoglobulin production

Naive B cells were purified from murine splenocytes by using the MACS negative selection kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were cultured at 1×10^6 cells/mL in RPMI containing 10% FCS, penicillin, streptomycin, and 50 μmol/L 2-mercaptoethanol in 96-well plates with or without 1 μg/mL megaAPRIL (Alexis Biochemicals, San Diego, Calif) or 1 μg/mL Flag-APRIL (produced as previously described[12]) or 0.1 μg/mL anti-murine IgM $F'Ab_2$ fragment (Jackson Immunoresearch, West Grove, Pa) and/or 20 ng/mL IL-4 (Peprotech, Rocky Hill, NJ) for 6 days at 37°C in a 5% CO_2 atmosphere. IgM, IgG, and IgA were assayed in one-quarter dilutions of supernatants by means of ELISA.

Flow cytometry

For lymphocyte quantification, single-cell suspensions were labeled with anti-murine CD3, CD4, CD8, B220, IgD, CD21, and CD23 (BD PharMingen, San Jose, Calif) and CD24 and IgM (MI/69, 11/41; eBioscience) antibodies and analyzed on an LSRII flow cytometer (BD Biosciences). Cell populations were gated as follows: $B220^+CD21^{low}CD24^{high}$, T1 cell; $B220^+CD21^{int}CD24^{high}$, T2 cells; $B220^+CD21^{int}CD24^{int}$, follicular mature cells; $B220^+CD21^{high}CD24^{high}CD23^{high}$, marginal zone progenitors (MZPs); $B220^+CD21^{high}CD24^{high}$, marginal zone (MZ) cells; and $B220^+IgM^+IgD^+$, mature B cells. For TACI expression, murine splenocytes were labeled with rat anti-mouse CD267 (8F10, BD PharMingen) in combination with B220 (RA3-6BT; BioLegend, San Diego, Calif), CD21, and CD23 (7G6, B3B4; BD PharMingen), and cell populations were gated as follows: $B220^+$, total B cells; $B220^+CD21^{high}CD23^{int}$, MZ cells; and $B220^+CD21^{int}CD23^{high}$, follicular mature cells.

Data analysis

Flow cytometric data were analyzed with FlowJo 7.6 (TreeStar, Inc, Ashland, Ore). Statistical analysis was performed with a Mann-Whitney *U* test in Prism 4.0 software (GraphPad Software, Inc, La Jolla, Calif). *P* values are represented in figures as follows: *less than or equal to .05, **less than or equal to .01, and ***less than or equal to .001.

RESULTS

NF-κB activation is quantitatively dependent on WT TACI receptor expression

The C104R TACI mutation is known to affect cell-surface expression of the mutant receptor and also disrupts ligand binding to both APRIL and BAFF (see Fig E3 in this article's Online Repository at www.jacionline.org).[5,9,13] 293T cells were transfected with WT or mutant human TACI (hTACI) or empty vectors as a control and NF-κB luciferase reporter plasmids to determine the functional effect of TACI C104R and other defined CVID mutations on downstream NF-κB activation. Cells were also transfected with either APRIL or BAFF plasmid constructs to mimic receptor activation by native ligand (Fig 1, *A*). 293T cells transfected with WT TACI alone without stimulation increased NF-κB activity 2.5-fold above the level stimulated by control empty vector. Furthermore, NF-κB activation was seen after cotransfection with APRIL and further still with BAFF cotransfection. NF-κB activity was almost entirely abrogated after receptor transfection in the C104R, c.571insG, Y79C, and I87N mutants, either alone or with ligand. Y79C and I87N are relatively well expressed in 293T cells, and this lack of functional activity might be a direct consequence of the severely impaired ligand-binding ability of these mutants (see Fig E3). The low levels of receptor expression and ligand binding might both contribute to the lack of signaling in the C104R mutant. Signaling is retained in R202, which is a known polymorphism, and A181E, a missense mutation in the transmembrane domain. The lack of NF-κB activation in the c.571insG mutation is expected because this mutation truncates a major part of the intracellular domain, eliminating the TNF receptor–associated factor–binding sites.

To investigate the effect of the C104R mutation further, we titrated increasing amounts of C104R mutant plasmid with WT plasmid and measured NF-κB activity (Fig 1, *B*). Upregulation of NF-κB activity was seen with WT TACI alone, but as the WT TACI/C104R TACI ratio was decreased, there was a proportional decrease in NF-κB luciferase activity. A 1:1 ratio of WT to C104R, which is analogous to the heterozygous form, produced approximately half the signaling activity of the WT-only transfectants. Signaling activity after transfection of APRIL and the C104R mutant only (the homozygous state) was less than one quarter that of WT only. These data suggest that signaling through C104R TACI is severely diminished and that signaling activity is quantitatively dependent on the number of WT TACI receptors expressed.

TACI C76R knock-in mice have reduced cell-surface TACI expression

To study in detail the effect of the human C104R mutation on B-cell function in both homozygous and heterozygous forms, we generated TACI knock-in mice on a C57BL/6 background using homologous recombination in embryonic stem cells to replace endogenous mTACI with a transgene carrying the corresponding C76R (c.226T>C) point mutation (see Figs E2 and E4 in this article's Online Repository at www.jacionline.org). The knock-in strategy ensures that the mutant gene is expressed under the control of its native TACI promoter. Because TACI gene expression is highly regulated *in vivo*, this strategy is more representative of the human mutation than the use of transgenic models, where TACI mutants are expressed by a B cell–specific promoter on a TACI-deficient background.[13,14] TACI protein expression is reduced

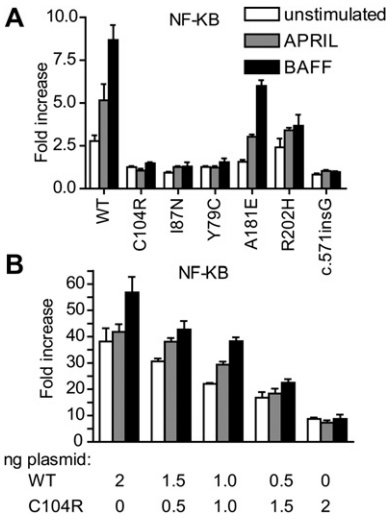


FIG 1. Dual-Luciferase reporter assays for detection of NF-κB–specific transcriptional activity in 293T cells. **A**, Cells were cotransfected with either WT or mutant TACI plasmids together with full-length APRIL *(gray bars)* or BAFF *(black bars)* plasmids or without stimulating ligand *(white bars)* and along with NF-κB luciferase reporter plasmid and *Renilla* plasmid. **B**, Activation of NF-κB transcription by titration of WT and C104R mutant hTACI performed as in Fig 1, *A*, except that cells were transfected with a total of 2 ng of plasmids at the indicated ratio. WT and C104R data displayed are representative of 3 experiments, and 187N, Y79C, A181E, and R202H data are representative of 2 experiments. Triplicate data are shown as means ± SDs.

in MZ B cells of both heterozygous and homozygous C76R mutant mice (Fig 2). Because the level of TACI expression is greater in MZ B cells, the difference in expression levels are more pronounced when examining this subset compared with follicular B cells or total $B220^+$ cells (see Fig E5 in this article's Online Repository at www.jacionline.org). The approximately 50% reduction of TACI expression in heterozygotes compared with WT mice (Fig 2) supports a haploinsufficiency effect as opposed to a dominant-negative effect on receptor trimerization.

Heterozygous and homozygous C76R TACI mutant mice show accumulation of MZ and mature B cells and low serum IgM and IgG subclass immunoglobulin levels

We examined B-cell compartments in bone marrow, secondary lymphoid organs, and the periphery. Consistent with previous studies in $TACI^{-/-}$ mice,[15,16] analysis of bone marrow showed no changes in pro–B-cell ($B220^+CD43^+$), pre–B-cell ($B220^+CD43^-HSA^+$), or immature B-cell ($B220^{low}IgM^+$) compartments in either homozygous or heterozygous C76R TACI mutant mice (data not shown). C76R TACI mutant mice had normal percentages of $B220^+$, $CD4^+$, and $CD8^+$ cells in peripheral blood (see Fig E6 in this article's Online Repository at www.jacionline.org). However, the spleens of homozygous C76R TACI knock-in mice were visibly enlarged and significantly heavier than those of age-matched WT littermates (Fig 3). The heterozygous group also had a marked increase in spleen weight compared with the WT group. The total number of $B220^+$ splenocytes was significantly increased in the enlarged spleens of heterozygous and homozygous C76R TACI mice compared with that seen in WT mice (see Table E1 in this article's Online Repository at www.jacionline.org).

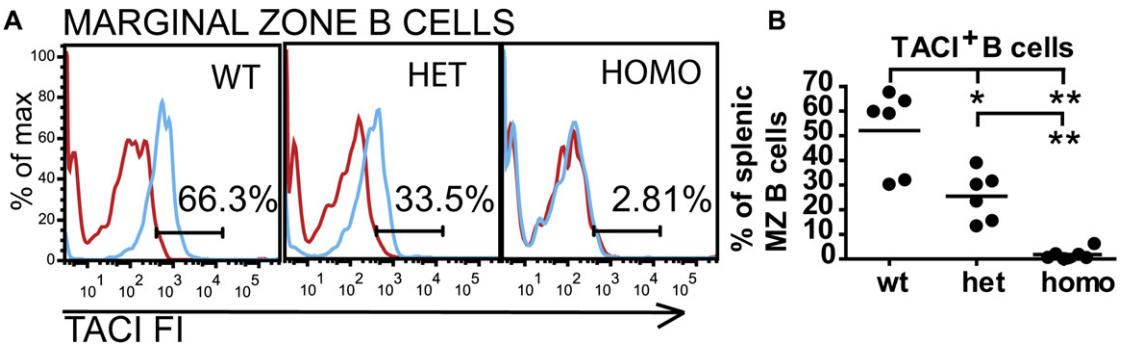


FIG 2. TACI expression on MZ B cells from C76R TACI homozygous *(HOMO)*, heterozygous *(HET)*, and WT mice. **A**, Representative histogram plots. The *blue line* indicates labeling with anti-mouse TACI antibody, and the *red line* indicates isotype control staining. **B**, Data shown are means ± SEMs (n = 6). *FI*, fluorescence intensity.

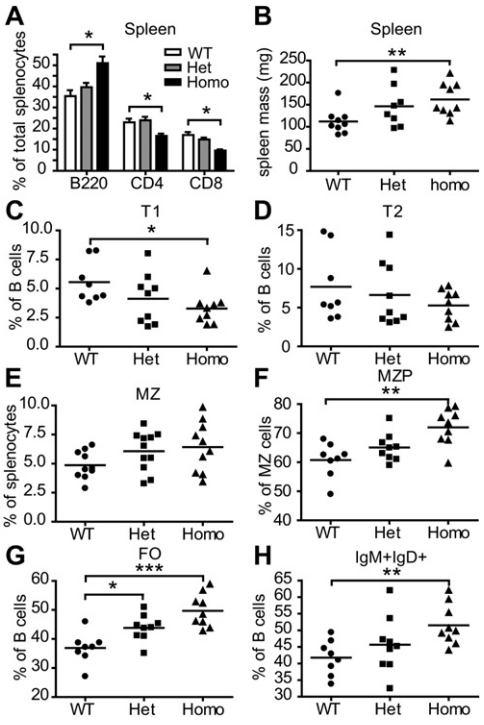


FIG 3. Immunologic phenotyping. **A**, Percentage of $B220^+$, $CD4^+$, and $CD8^+$ lymphocytes in peripheral blood. **B**, Spleen mass. **C-H**, Quantification of splenic B-cell populations. Fig 3, *A*, Mean ± SEM (n = 6-7). Fig 3, *B-H*, Individual values are shown; *bars* represents means. *Het*, Heterozygous, *Homo*, homozygous.

Analysis of cellular profile within the spleen revealed a marked increase in heterozygotes and a significant increase in homozygotes in the percentage of $B220^+$ cells compared with that seen in WT mice (Fig 3). $CD4^+$ and $CD8^+$ T-cell percentages were consequently lower in spleens of heterozygous mice and significantly lower in homozygous mice compared with WT littermates (Fig 3). Similarly, in cervical and facial lymph nodes the percentages of $B220^+$ cells were increased in C76R mutants compared with those seen in WT mice, and there was a corresponding significant reduction in the percentage of $CD8^+$ cells in both heterozygous and homozygous mice (see Fig E6).

Analysis of specific B-cell subsets showed a trend toward decreased numbers of transitional T1 and T2 B cells in C76R mutants but increased numbers of B cells in other splenic B-cellsubpopulations from both heterozygous and homozygous C76R; mice, with significant increases seen in MZPs ($CD21^{high}CD24^{high}CD23^{high}$) and follicular mature ($B220^+$ $CD21^{int}CD24^{int}$) and mature ($B220^+IgM^+IgD^+$) B-cell populations in homozygous C76R mice (Fig 3) and in follicular mature B cells from heterozygous C76R mice.

Heterozygous and homozygous TACI C76R mutant mice have reduced naive immunoglobulin levels

Comparison of serum immunoglobulin levels from naive heterozygous and homozygous C76R TACI mice with those in WT mice revealed a significant reduction in IgM, IgG1, and IgG2b levels in homozygous mice (Fig 4). Heterozygous C76R TACI mice had significantly reduced naive levels of IgM and IgG1 and a marked reduction in IgG2b levels compared with those seen in WT mice. In contrast with reports of TACI-null and A141E-transgenic mice, the C76R mutation did not significantly reduce naive IgA or total IgG levels compared with those in WT mice (Fig 4 and see Fig E7 in this article's Online Repository at www.jacionline.org).

Reduced humoral responses to immunization with T cell–independent antigens

TACI has been shown in mice and in patients with CVID to be critical for the generation of specific antibody after challenge with T cell–independent (TI) antigens. TI type II antigen TNP-FICOLL–immunized homozygous C76R mutant mice generated significantly less total IgG anti-TNP antibody than WT mice (Fig 5). This IgG defect was significant specifically in the IgG2a subclass, but consistent reductions were also detected in IgG1 and IgG2b subclasses (Fig 5). Similar to C76R-transgenic heterozygote mice,[13] the heterozygous C76R mutant mice showed a consistent trend toward reductions in serum anti-TNP IgG1, IgG2a, IgG2b, and IgG3 levels that approached the significant reductions seen in homozygous mice, especially at early time points after vaccination. The C76R mutation does not appear to affect the generation of IgM or IgA antibody to TI antigen, at least at the later time point of D21, but might reduce the levels produced at day 10 (Fig 5). The C76R TACI mutation had almost no effect on T cell–dependent (TD) antibody production after NP-CGG immunization with the exception of IgG2a, levels of which

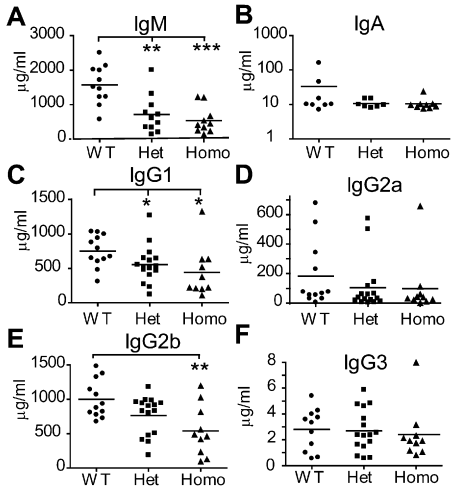


FIG 4. C76R TACI mutant mice have reduced naive serum immunoglobulin levels. Levels of immunoglobulin subtypes in C76R homozygous *(Homo)*, heterozygous *(Het)*, and WT naive serum measured by means of ELISA. Individual values are shown; *bars* indicates means.

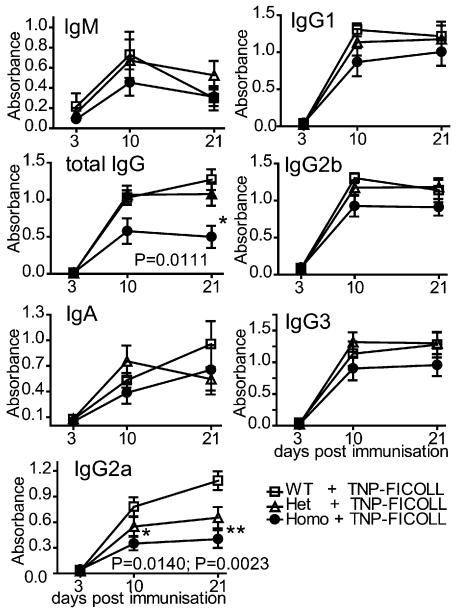


FIG 5. Defective TI type II humoral immune responses. The time course of TNP-specific immunoglobulin responses is shown. IgM and IgG levels were measured at 1:8100 dilution of sera, and IgA levels were measured at 1:900 dilution of sera. IgG1, IgG2a, IgG2b, and IgG3 IgG subclass antibodies were measured at 1:80, 1:160, 1:160, and 1:1250 dilutions, respectively. Data shown are means ± SEMs from 2 independent experiments (n = 7). *P* values are given for comparisons with WT mice.

were unexpectedly reduced (see Fig E8 in this article's Online Repository at www.jacionline.org).

Impaired *in vitro* immunoglobulin production to both APRIL and anti-CD40 stimulation

Naive B cells from CD40$^{-/-}$ mice have been used to demonstrate that TACI ligands alone can induce immunoglobulin synthesis in the absence of anti-IgM costimulation.[17] We investigated the ability of APRIL to stimulate immunoglobulin secretion in naive C76R TACI mutant and WT B cells in

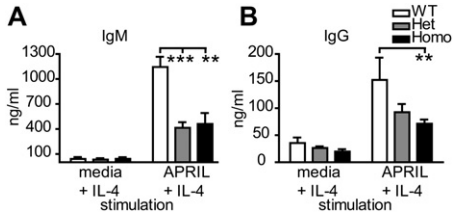


FIG 6. *In vitro* immunoglobulin production. Purified murine splenic B cells were cultured with APRIL in the presence of IL-4. IgM and IgG levels were measured in culture supernatants by using ELISA. Data shown are means ± SEMs of triplicate means from 3 independent experiments. *Het*, Heterozygous; *Homo*, homozygous.

comparison with stimulation with an anti-CD40 antibody, which mimics T-cell help. We were unable to detect any significant IgA production (data not shown). APRIL plus IL-4 stimulated a 20-fold increase in IgM levels detected in the culture supernatants from WT naive B cells, which were significantly reduced in both homozygous and heterozygous C76R TACI mutants (Fig 6). APRIL also stimulated a 6-fold increase in IgG levels in culture supernatant from naive WT B cells, which were halved in C76R TACI homozygous mutant cells and reduced by one third in heterozygous mutant cells (Fig 6). These data suggest the presence of a defect in TI type II IgM and IgG class switching or antibody secretion. Anti-CD40–stimulated IgM production was not significantly reduced in cells from heterozygous or homozygous C76R mutants (see Fig E9 in this article's Online Repository at www.jacionline.org). In contrast, the production of IgG after stimulation with anti-CD40 antibody was significantly reduced in heterozygous B cells and similarly reduced in homozygous B cells (see Fig E9). The defect in IgG secretion after both TI and TD stimulation is consistent with the defective humoral response to immunization demonstrated in Fig 5.

DISCUSSION

Following of 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. Most controversial of all is the relevance of the heterozygous C104R allele, which does not segregate with the phenotype in some familial cases[9,10] and is found in the simple heterozygous state at low frequency (<1%) in reportedly healthy subjects.[11,18] Therefore there is a need to understand whether this particular mutation affects B-cell function in isolation. We show in this report that the C104R mutation on its own has significant effects on receptor signaling and that its murine equivalent, C76R, significantly disrupts B-cell homeostasis, naive immunoglobulin production, and antigen-stimulated humoral responses.

TACI$^{-/-}$ mice have been described and show splenomegaly, an increase in splenic B cells, and decreased serum IgA, IgM and IgG levels.[15,16,19] However, a complete TACI knock-out does not accurately represent the genotypes identified in patients with CVID. TACI expression is also highly regulated and varies considerably among human B-cell subpopulations.[20] C76R-transgenic mice might go some way to representing the human mutation, but the recently described model lacks physiologically regulated TACI expression.[13] For these reasons, we generated a knock-in mouse harboring the murine equivalent mutation C76R. In our model the C76R mutation was inserted into its

native locus through homologous recombination to keep mutant TACI transcription under the control of its endogenous promoter, thereby allowing physiological expression at appropriate stages of B-cell development. This model is therefore a more accurate representation of the human C104R TACI mutation in human subjects than existing models.[13,14]

The C104R TACI mutation was shown previously to be normally expressed yet to affect ligand-induced NF-κB activation and signaling after association with WT receptors, thereby suggesting a dominant-negative effect.[21] Our data offer strong evidence to the converse. C104R, when expressed either transiently in heterologous 293T cells or natively in lymphoblast cell lines from patients with CVID, shows markedly decreased expression. In splenic B-cell subsets of C76R homozygotes, we have observed a profound reduction in extracellular TACI expression. Furthermore, the direct comparison of WT, heterozygote, and homozygote littermates, possible only in our study and not in the C76R-transgenic model because of the intensity of TACI expression on transgenic C76R/TACI$^{+/-}$ mice being greater than that of TACI$^{+/+}$ mice, demonstrates for the first time that the C76R mutation quantitatively reduces extracellular TACI expression on splenic B cells.

Functionally, the reduced NF-κB activation on titration of WT and C104R mutant plasmids in the NF-κB luciferase reporter assay indicates that NF-κB activation is quantitatively dependent on the amount of WT receptor expressed. It is unlikely that the decrease in NF-κB activation would occur in a concentration-dependent manner if the C104R mutation was to act through a dominant-negative mechanism. Together, the human and murine data suggest that the C104R mutation leads to significantly decreased protein expression, and therefore ligand binding and signaling are virtually abolished in homozygotes. In the heterozygous form receptor binding and TACI signaling are dependent on the number of WT receptors, and this therefore argues that the observed functional defects occur through haploinsufficiency.

Detailed analysis of B-cell subpopulations demonstrates that the C76R mutant leads to significant abnormalities in B-cell development in secondary lymphoid organs, with significant expansion of total splenic B cells and specifically of MZPs and follicular and mature B cells. This dysregulation of B-cell development and proliferation is similar to that previously described in TACI$^{-/-}$ models.[19] The C76R transgene model, which lacks the regulated TACI expression of our knock-in model, shows a less severe phenotype with no abnormalities of B-cell homeostasis and less pronounced immunoglobulin production defects.[13] The C76R defects are also very different to the splenic B-cell phenotype reported in the murine A144E-transgenic model, where no B-cell expansions were seen.[13,14] It is likely that the C76R mutation (like the human C104R counterpart) acts functionally in a manner that is similar to a null mutant and leads to more severe disruption of TACI receptor function than the A144E variant.

The mechanism of B-cell expansion can be attributed to the different signals delivered by TACI and BAFF receptor. BAFF/BAFF receptor signaling promotes B-cell survival because deletion of either of these genes results in a significant reduction in mature B cells in the circulation and lymphoid organs.[22-24] TACI is thought to regulate B-cell homeostasis by delivering a balancing proapoptotic signal, and therefore severe abrogation of TACI signaling might lead to unchecked BAFF/BAFF receptor–mediated survival.

Humoral responses in the C76R knock-in model are very abnormal and resemble the immunologic phenotype of patients with CVID. There is a reduction in naive immunoglobulin levels, with decreases in IgM, IgG1, and IgG2b levels in both homozygous and heterozygous mice. When challenged with a TI type II antigen, there is a clear defect in IgG and IgG2a class-switched responses in homozygotes. These defects mirror the inability of patients with CVID to mount both IgM and IgG responses to TI antigens, such as encapsulated bacteria.[25] Heterozygous mice also showed a decreased antibody response, again suggesting that the mutant receptor is nonfunctional and exerts its effect through haploinsufficiency. These defects might be related to the dysregulated splenic B-cell profile because it is thought that TI responses mostly derive from MZ populations.[26]

Previous *in vitro* studies have identified a role for TACI in IgA class switching.[4] However, we did not observe any significant abnormalities in IgA production after vaccination or after *in vitro* stimulation, although in the former the serum IgA level varied widely, and in the latter the total amount of IgA produced was insufficient to form any definite conclusions. However, the data on IgA production in TACI-deficient murine models is inconsistent, with no abnormalities of IgA production after TI type II vaccination in 3 of the 4 models thus far reported.[14,15] Therefore our data might be in keeping with an overall picture that, despite the *in vitro* evidence, the role of TACI in mediating IgA class switching *in vivo* is negligible. However, in all models reported, including our C76R model, there is an unambiguous defect in IgG production after TI type II antigen challenge.

The T cell–dependent response is mediated primarily by follicular B and T cells in the germinal center through interaction of costimulatory molecules, such as CD40 and CD40 ligand. In our *in vivo* and *in vitro* studies we observed a reduction in IgG2a and IgG production in response to CD40 stimulation and TD vaccination, respectively, an observation not seen in the C76R-transgenic model.[13] The mechanism by which this defect arises is unclear but might relate to a generalized dysregulation of follicular B-cell function initiated by the C76R TACI mutation rather than by a specific mutant TACI receptor interaction.

The C76R knock-in model reported here is genetically highly representative of the most common mutation seen in patients with CVID. The mutant gene is transcribed in a physiologically relevant manner, and we have been able to study both the homozygous and heterozygous forms. The evidence arising from this model argues strongly for a significant B-cell dysfunction in both homozygous and heterozygous mice. Defects in secondary lymphoid organ B-cell development and proliferation are observed, as well as abnormalities of naive and TI type II antigen–stimulated class switching to IgG. These are supported by *in vitro* studies of the human C104R mutation that show defects in receptor signal transduction. Together, we show that the C104R mutation alone has significant effects on B-cell function and can lead to the humoral defects typical of CVID. Therefore the presence of apparently healthy subjects with heterozygous C104R mutations must be related to genetic or environmental influences that modify the effect of C104R.

We thank Laure Willen (University of Lausanne) for excellent technical assistance.

Key messages

- **Insertion in the correct gene locus ensures the C76R mutation is expressed under the control of the native TACI gene promoter, allowing physiological levels of expression of the mutant protein.**
- **Homozygous expression of the mutated amino acid sequence under the control of the native TACI gene promoter reveals that a reduced level of mutant TACI receptor reaches the cell surface.**
- **The immunoglobulin defects and disrupted B-cell homeostasis show that C104R mutation can have severe effects on B-cell function and might result in the humoral defects of CVID.**

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METHODS

EBV-transformed patient lymphoblast cell lines

Seventeen Epstein-Barr virus (EBV)-transformed B-cell lines were created by using standard techniques[E1] from patients selected from an initial cohort of 533 patients with hypogammaglobulinemia and given a diagnosis of CVID according to the European Society for Immunodeficiencies criteria. WT cell lines were created from a selection of 675 unrelated healthy control subjects. Patients and healthy volunteers originated from the United Kingdom, Germany, the United States, Sweden, Norway, Italy, Brazil, and Columbia. These participants provided consent under ethics review board–approved research protocols registered at University Hospital Freiburg, Freiburg, Germany, and Great Ormond Street Hospital and Royal Free Hospital, London, United Kingdom. Site-directed mutagenesis was performed on full-length WT hTACI cDNA plasmids in a pCR3 vector backbone to generate the mutant hTACI expression plasmids Y79C, I87N, c.571insG C104R, R202H, and A181E. Human embryonic kidney 293T cells were transfected with lipofectamine or by using the calcium phosphate method.

TACI expression

Staining of EBV-transformed B cells and 293T-transfected cell lines for expression of hTACI was performed as previously described.[E2] Briefly, 10^6 cells were stained with either monoclonal rat anti-human TACI antibody (1A1; Abcam, Cambridge, United Kingdom) or a biotinylated polyclonal goat anti-human TACI antibody (500-P166GBt, PeproTech) and detected with goat anti-rat IgG phycoerythrin or streptavidin phycoerythrin-Cy5 (554062, BD PharMingen). The appropriate isotype control was used in each staining.

Binding of Flag-APRIL and Flag-BAFF

Staining of transfected 293T cell lines and EBV cell lines from patients with CVID carrying *TNFRSF13B* mutations was performed, as previously described.[E2] Briefly, 10^6 cells were stained with 10 to 50 ng of Flag-ACRP-hAPRIL or Flag-ACRP-hBAFF in the presence of 0.1 μL of heparin to block nonspecific binding of APRIL to proteoglycans. Flag-APRIL and Flag-BAFF were detected with monoclonal mouse anti-Flag antibody M2 (F1804, Sigma) and goat anti-mouse IgG1 phycoerythrin-Cy5 (15-5090, eBioscience). In some experiments staining was performed with Fc-hBAFF and Fc-hAPRIL.[E3] Flow cytometry was performed on a CyAn ADP cell sorter (Beckman Coulter, Fullerton, Calif) and analyzed with Summit software (DAKO, Glostrup, Denmark).

Generation of C76R TACI knock-in mice

Heterozygous C76R TACI knock-in mice were generated by InGenious-Targeting Laboratory on a C57BL/6 background by using homologous recombination in embryonic stem cells to replace endogenous mTACI (NM_021349.1) with the transgene carrying C76R (c.226T>C). It has been reported that a putative splice site leading to a mTACI slice variant lacking exon 3, where the cysteine residue 76 is located, was present in 1 EST (GenBank accession no. BY212677).[E4] The cDNA originates from an activated murine spleen, and it is expressed in bone marrow, the thymus, and the spleen among other organs. We therefore introduced a second synonymous nucleotide change (c.207G>A) that does not change arginine residue 49 to delete the putative slice site recognition sequence and retain exon 3 and the CRD2 domain (Fig 3, *B*). Four mutagenesis primers, PT1 and 2 and PT3 and 4 (sequences available on request), were designed to amplify the PCR product with the length of approximately 0.2 kb, including exon 3. The mutations were engineered into primers PT2 and PT3. The PCR products carrying the mutations were then used to replace the WT sequence by using conventional ligation methods. A LoxP/FRT floxed Neo cassette was then inserted downstream of *TNFRSF13B* exon 3, including the 2 point mutations (Fig E2). Derived heterozygous C76R TACI mice were crossed with WT mice on the same C57BL/6 background. F2 heterozygous mice were back-crossed to generate WT, heterozygous, and homozygous C76R TACI mice. Animals were genotyped by means of PCR of exon 3 followed by standard Sanger Sequence with the primers m*TNFRSF13B* _1F (5′-CCCCAAAGATCAGTACTGGGAC-3′) and m*TNFRSF13B* _1R (5′-GCAGTAGAGAGTCAGCTGGTCG-3′). Murine DNA was extracted from the animals' ear notches by using the QIAGEN DNAeasy Blood and Tissue Kit (catalog no. 69504), according to the manufacturer's instructions. Sequence reactions were run on a capillary MegaBACE 1000 DNA Analyser (GE Healthcare, Fairfield, Conn), and sequence analysis was performed with Sequencher version 3.4.1 (Gene Codes Corporation, Ann Arbor, Mich) and BioEdit (Ibis Biosciences, Carlsbad, Calif; Fig E4).

Flow cytometry

Single-cell suspensions from the spleen, blood, or lymph nodes were washed and resuspended in PBS containing 0.1% FCS and 0.01% sodium azide. Nonspecific binding sites were blocked with 2% normal rat serum, and then cells were stained with fluorescently conjugated antibodies to murine antigens CD3, CD4, CD8, CD21, CD23, CD24, B220, IgD, and IgM purchased from BD PharMingen. Excess antibody was washed, cells were fixed with cell fix (BD Biosciences), and cell populations were analyzed on a BD LSRII flow cytometer (BD Biosciences).

ELISA

ELISAs were performed by using a standard method in 96-well plates. Total IgA, IgG, IgM, and IgG1, IgG2a, IgG2b, and IgG3 levels were measured in sera or culture supernatants by using paired antibodies and standards from BD PharMingen. Total IgG levels were measured with anti-mouse IgG antibody (Zymed, South San Francisco, Calif) and anti-mouse IgG-HRP (DAKO). Plates were coated with 10 μg/mL TNP-BSA or NP-BSA (Biosearch Technologies) and biotinylated isotype specific anti-murine immunoglobulin secondary antibodies used as above to measure anti-TNP– or anti-NP–specific antibodies. Absorbance was measured with a FLUOstar Optima colourimeter (BMG Labtech, Offenburg, Germany). Total immunoglobulin levels were calculated for naive serum samples and supernatants by using a standard curve. TNP- and NP-specific immunoglobulin data are presented as absorbance values.

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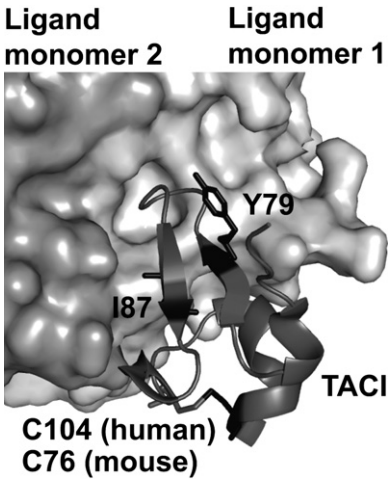


FIG E1. Position of residues C104, C76, Y79, and I87 in the TACI domain structure. TACI is shown as a ribbon representation (dark gray). Y79, I87 and C104 are shown in black. In mTACI the residue corresponding to C104 is C76. The surface of the 2 adjacent subunits of the ligand are shown in 2 different shades of light gray. The illustration was drawn with PyMol by using atomic coordinates of pdb entry 1xu1.

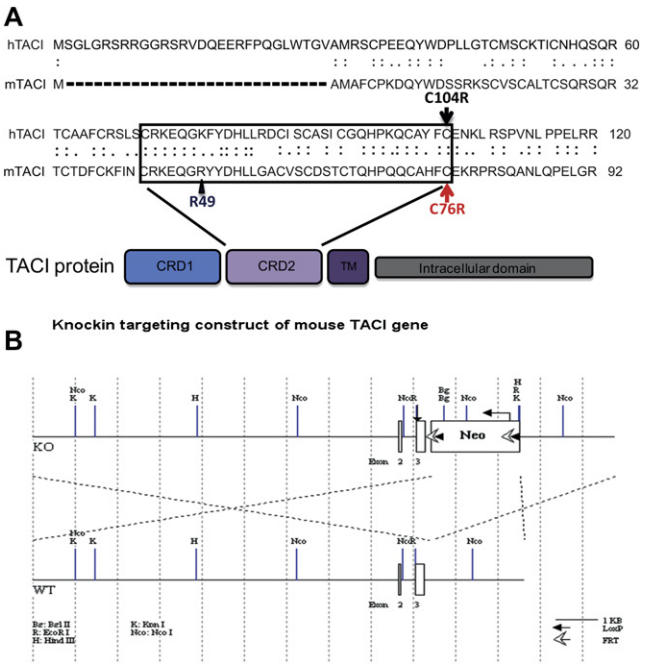


FIG E2. Generation of C76R TACI mutant mice. **A,** Amino acid sequence homology between hTACI and mTACI. *Colons* indicate identical amino acid residues, and single dots indicate similar residues. The *black box* highlights the CRD2 domain. *Arrows* indicate the position of the murine C76R point mutation *(red arrow)* and its corresponding human mutation, C104R *(black arrow)*, in the CRD2 domain. The *blue bold triangle* indicates the arginine 49 residue. A schematic diagram of TACI protein structure is also shown. *TM*, Transmembrane domain. **B**, Generation of C76R TACI mutant mice by means of homologous recombination.

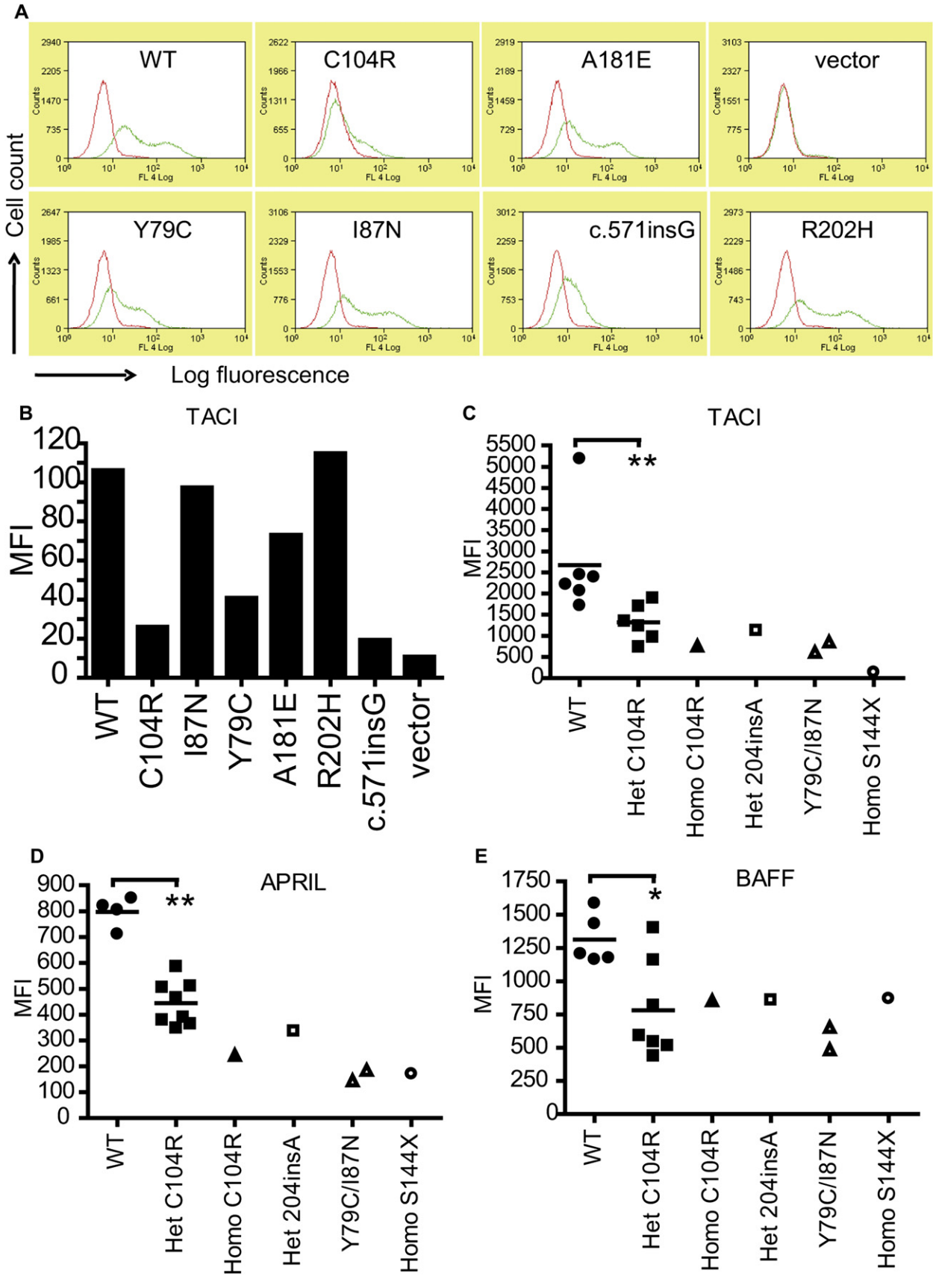

A
WT
C104R
A181E
vector
Y79C
I87N
c.571insG
R202H
Counts
FL 4 Log
Cell count
Log fluorescence
B
TACI
MFI
WT
C104R
I87N
Y79C
A181E
R202H
c.571insG
vector
C
TACI
MFI
**
WT
Het C104R
Homo C104R
Het 204insA
Y79C/I87N
Homo S144X
D
APRIL
MFI
**
WT
Het C104R
Homo C104R
Het 204insA
Y79C/I87N
Homo S144X
E
BAFF
MFI
*
WT
Het C104R
Homo C104R
Het 204insA
Y79C/I87N
Homo S144X

FIG E3. Transfected 293T cells and EBV-transformed patient B-lymphoblastic cell lines expressing mutant forms of TACI show reduced TACI expression and APRIL binding. **A** and **B**, 293T cells were transfected with plasmids expressing WT and mutant forms of hTACI. **C-E**, EBV-transformed patient B-lymphoblastic cell lines expressing homozygous *(Homo)*, heterozygous *(Het)*, and compound heterozygous *TNFRSF13B* mutations. TACI expression was measured by staining with a polyclonal anti-human TACI antibody (P166) directed against the extracellular domain of hTACI. Fig E3, *A*, Representative plots showing TACI expression (green) and isotype control antibody (red) of 293T cells. Fig E3, *B*, Mean fluorescence intensity *(MFI)* of TACI expression of 293T cells. Fig E3, *C*, Level of TACI expression on patients' lymphoblastic cell lines. APRIL (Fig E3, *D*) and BAFF (Fig E3, *E*) binding to lymphoblastic cell lines or transfected 293T cells were determined by means of incubation of cells with Flag-tagged recombinant APRIL or BAFF and detection with anti-Flag mAb. Fig E3, *A* and *B*, Data displayed are representative of at least 3 independent experiments. Fig E3, *C*, *E*, and *F*, *Symbols* represent MFI of cell lines from individual patients, and *bars* indicate means of group. *WT*, Healthy control expressing WT TACI. $*P \leq .05$. $**P \leq .01$.

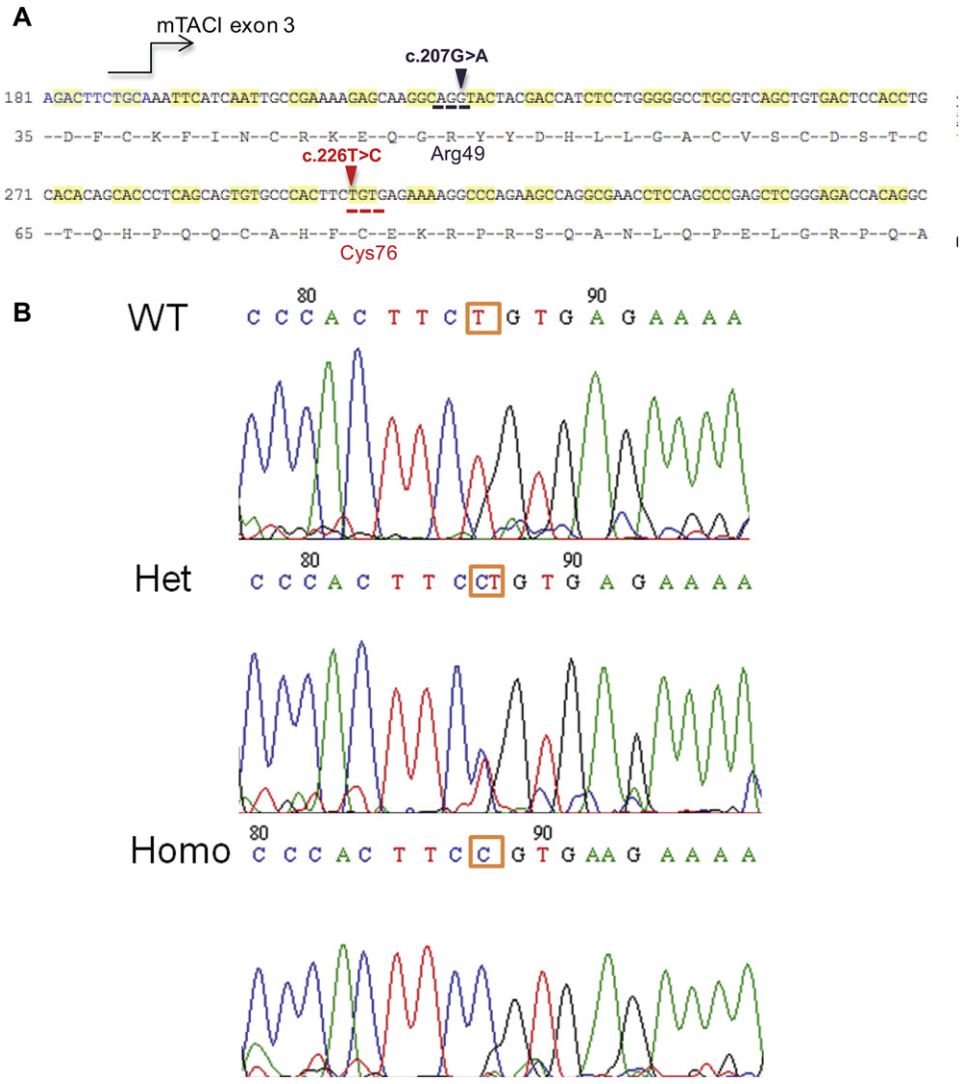


FIG E4. Genotyping of C76R knock-in mice and WT littermates. **A**, Partial cDNA sequence of *TNFRSF13B* exon 3 (NM_021349.1). Point mutation c.226T>C (C76R) and synonymous change c.207G>A (R49R) are indicated by *red* and *black arrows*, respectively. **B**, Fluorescence-based DNA sequencing of generated knock-in and littermate mice was performed by means of capillary electrophoresis. Representative electropherograms are shown for DNA from WT, heterozygous *(Het)*, and homozygous *(Homo)* mice. The *orange box* indicates the position of the point mutation c.226T>C (C76R).

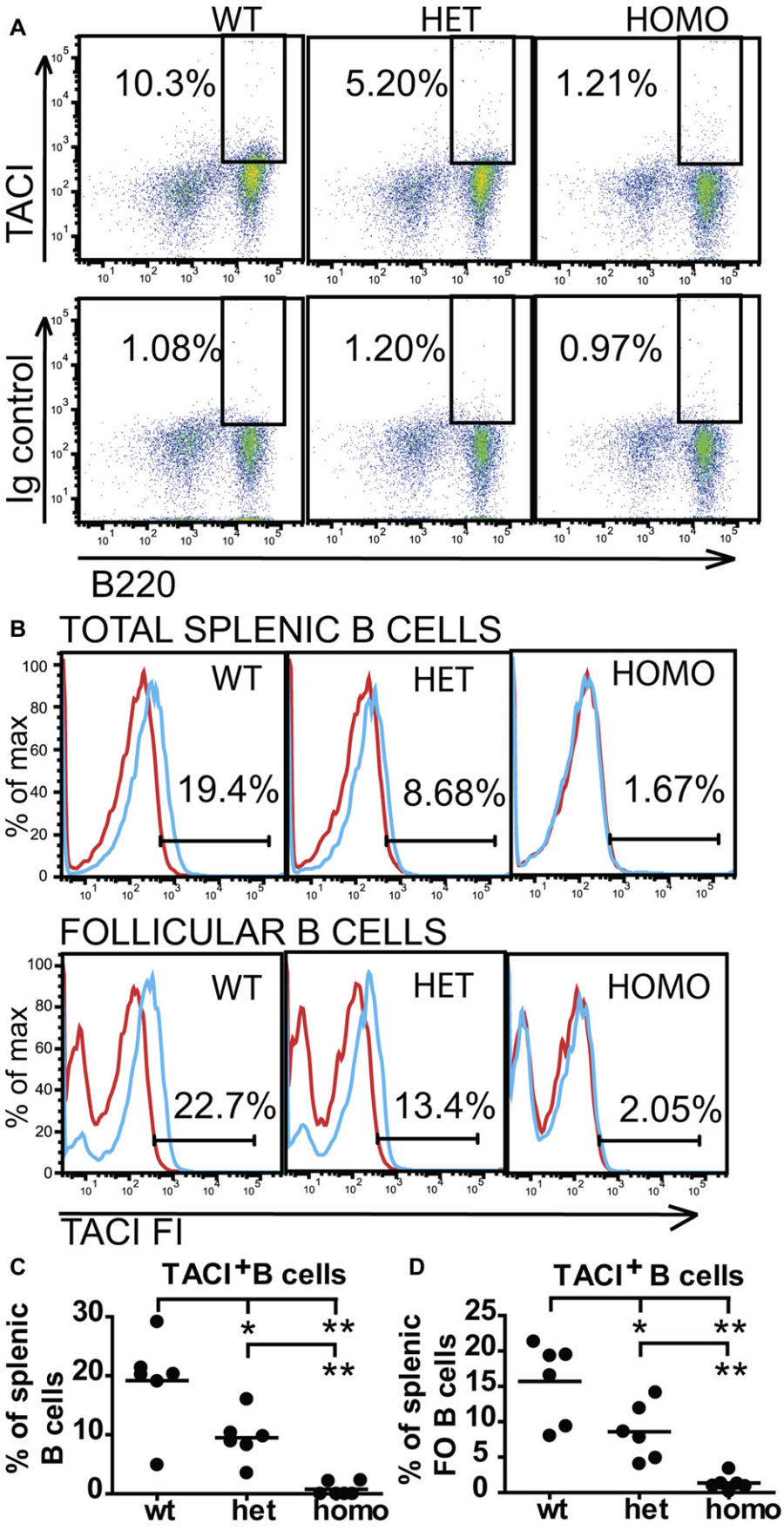

A
WT
HET
HOMO
10.3%
5.20%
1.21%
TACI
1.08%
1.20%
0.97%
Ig control
B220
B
TOTAL SPLENIC B CELLS
WT
HET
HOMO
% of max
19.4%
8.68%
1.67%
FOLLICULAR B CELLS
22.7%
13.4%
2.05%
TACI FI
C
TACI+B cells
% of splenic B cells
wt
het
homo
D
TACI+ B cells
% of splenic FO B cells

FIG E5. TACI expression on splenocytes from C76R TACI homozygous *(HOMO)*, heterozygous *(HET)*, and WT mice. **A** and **B**, Representative dot plots (Fig E5, *A*) and histograms (Fig E5, *B*) showing levels of TACI expression on splenic B cells. The *blue line* indicates labeling with anti-mouse TACI antibody, the *red line* indicates isotype control staining, and plots are representative of n = 6. **C** and **D**, TACI expression on total follicular B cells. Data are shown as means ± SEMs (n = 6).

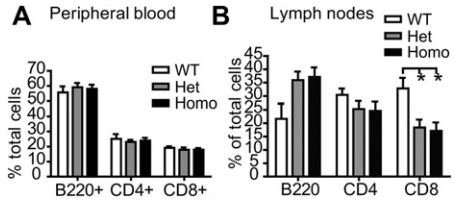


FIG E6. Immunologic phenotyping. Percentage of $B220^+$, $CD4^+$, and $CD8^+$ lymphocytes in peripheral blood **(A)** and facial and cervical lymph nodes **(B)**. Data are shown as means ± SEMs (n = 4-7).

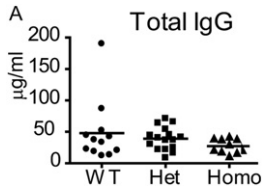


FIG E7. C76R TACI mutant mice have reduced naive serum immunoglobulin levels. Levels of total IgG in C76R homozygous *(Homo)*, heterozygous *(Het)*, and WT naive serum measured by means of ELISA. Individual values are shown, and *bars* indicates means.

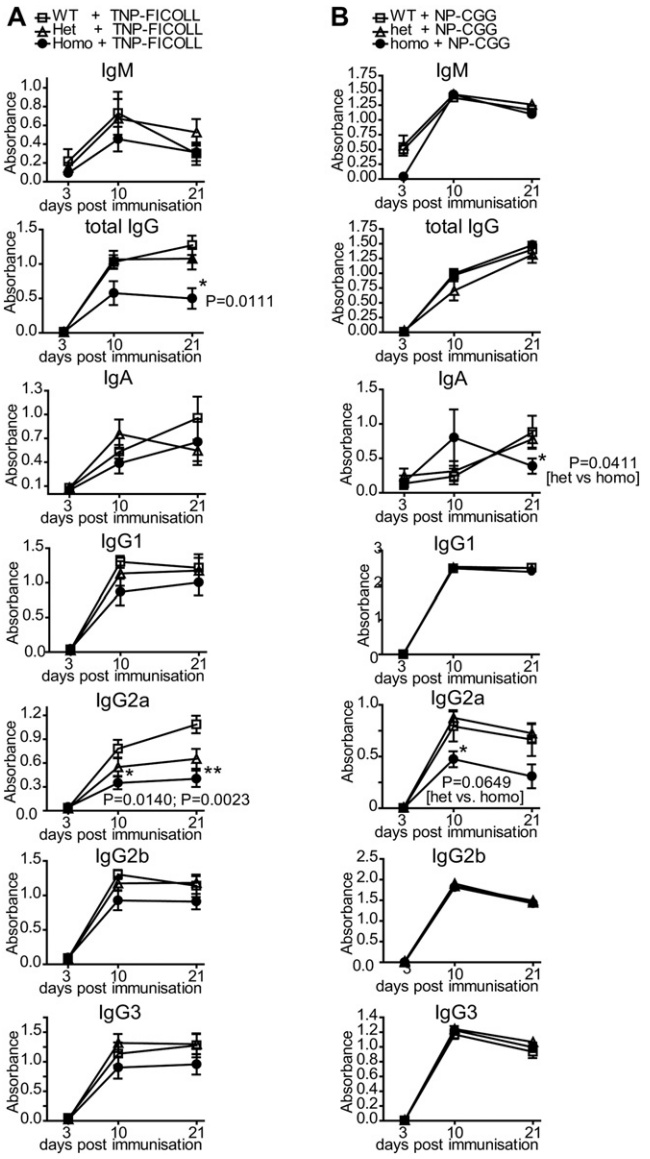


FIG E8. Defective T-independent type II humoral immune responses. Mice were immunized with TNP-FICOLL or NP-CGG. **A**, Time course of TNP-specific immunoglobulin response showing IgM and IgG levels measured at 1:8100 dilution and IgA at 1:900 dilution of sera. IgG1, IgG2a, IgG2b, and IgG3 IgG subclass antibody levels were measured at 1:80, 1:160, 1:160, and 1:1250 dilutions, respectively. **B**, Time course of NP-specific immunoglobulin response showing IgM and total IgG levels measured at 1:300 dilution. IgA, IgG1, IgG2a, IgG2b, and IgG3 levels were measured at 1:25, 1:160, 1:80, 1:640, and 1:1280 dilutions, respectively. Data shown are means ± SEMs from 2 independent experiments (Fig E8, *A*, n = 7; Fig E8, *B*, n = 6). *P* values given are for comparisons with WT, with the exception of IgA and IgG2a in Fig E8, *B*. *het*, Heterozygous; *homo*, homozygous.

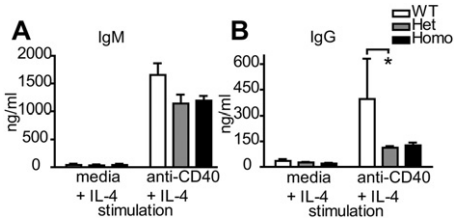


FIG E9. *In vitro* immunoglobulin production. Purified murine splenic B cells were cultured with anti-CD40 in the presence of IL-4. IgM and IgG levels were measured in culture supernatants by means of ELISA. Data are shown as means ± SEMs (n = 3).

TABLE E1. Total number of B cells in spleens of WT and TACI C76R mice

	Total $B220^+$ cells $\times 10^6$/spleen	No.	*P* value vs WT
WT	49.0 ± 4.6	7	NA
Heterozygous	68.7 ± 6.8	9	.0405
Homozygous	77.2 ± 11.4	7	.0407

NA, Not applicable.